

Universidade Estadual Paulista “Júlio de Mesquita Filho”

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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

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Dedicatória

Aos meus pais António e Marlí e meus irmãos Júlia e Renato, que sempre me deram amor, felicidade, apoio e muitas risadas em todos os momentos da minha vida.

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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 TSER3R/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 Vecchia *et al.*, 2003; Bingham e Riboli, 2004), já foram claramente associados ao aumento da incidência de câncer em seres humanos (Montesano e Hall, 2001). Ferguson (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 (Ferguson, 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 Ferguson, 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 Fujikuriyama, 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 Glutathione 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 hidrofília 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 *MspI* (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 significativo 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 MspI* 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, Carrière *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 Glutathione S-transferases (GSTs)

As enzimas glutathione S-transferases (GSTs) possuem a função básica de detoxificação, mediando a conjugação de um grande número de compostos eletrofílicos com glutathione 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 α (GSTA), μ (GSTM), π (GSTP) e 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 frequência 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 *et 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*. (Kocabaş *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 frequência 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 excessivamente 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 ácido 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 timidato 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).

Polimorfismos 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 1p36.3 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 sequência 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 *194trp* 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.

Referências Bibliográficas

1. Abdel-Rahman SZ, Soliman AS, Bondy ML, Omar S, El-Badawy SA, Khaled HM, et al. Inheritance of the 194Trp and the 399Gln variant alleles of the DNA repair gene XRCC1 are associated with increased risk of early-onset colorectal carcinoma in Egypt. *Cancer Lett.* 2000;159:79-86.
2. Ali-Osman F, Akande O, Antoun G, Mao JX, Buolamwini J. Molecular cloning, characterization, and expression in *Escherichia coli* of full-length cDNAs of three human glutathione S-transferase Pi gene variants: evidence for differential catalytic activity of the encoded proteins. *J Biol Chem.* 1997;272:10004-10012.
3. Ames BN, Wakimoto P. Are vitamin and mineral deficiencies a major cancer risk? *Nature Rev. Cancer.* 2002;2:694-704.
4. Ames BN. DNA damage from micronutrient deficiencies is likely to be a major cause of cancer. *Mut Res.* 2001;475:7-2001.
5. Amorim LMF, Rossini A, Mendonça G, Lotsch P, De Almeida Simão T, Gallo CVM, et al. CYP1A1, GSTM1 and GSTT1 polymorphisms and breast cancer risk in Brazilian women. *Cancer Lett.* 2002;181:179-186.
6. Arruda VR, Lima CS, Grignoli CR, de Melo MB, Lorand-Metze I, Alberto FL, et al. Increased risk for acute myeloid leukaemia in individuals with glutathione S-transferase mu 1 (GSTM1) and theta 1 (GSTT1) gene defects. *Eur J Haematol.* 2001;66:383-8.
7. Arvanitis DA, Goumenou AG, Matalliotakis IM, Koumantakis EE, Spandidos DA. Low penetrance genes are associated with increased susceptibility to endometriosis. *Fertil Steril.* 2001;76:1202-1206.
8. Ateş NA, Tamer L, Ateş C, Ercan B, Elipek T, Ocal K, et al. Glutathione S-transferase M1, T1, P1 genotypes and risk for development of colorectal cancer. *Biochem Genet.* 2005;43:149-63.
9. Bailey LB, Duhaney RL, Maneval DR, Kauwell GP, Quinlivan EP, Davis SR, et al. Vitamin B-12 status is inversely associated with plasma homocysteine in young women with C677T and/or A1298C methylenetetrahydrofolate reductase polymorphisms. *J Nutr.* 2002;132:1872-8.
10. Bailey LR, Roodi N, Verrier CS, Yee CJ, Dupont WD, Parl FF. Breast cancer and CYP1A1, GSTM1, and GSTT1 polymorphisms: evidence of a lack of association in Caucasians and African Americans. *Cancer Res.* 1998;58:65-70.
11. Berhane k, Widersten M, Engstrom A, Kozarich JW, Mannervik B. Detoxication of base propanals and other alpha, beta-unsaturated aldehyde products of radical reactions and lipid peroxidation by human glutathione transferases. *Proc Natl Acad Sci U S A.* 1994;91:1480-4.

12. Bingham S, Riboli E. Diet and Câncer – The European Prospective Investigation into Câncer and Nutrition. *Rev. Nature.* 2004;4:206-215.
13. Bingham SA, Pignatelli B, Pollock JR, Ellul A, Malaveille C, Gross G, et al. Does increased endogenous formation of N-nitroso compounds in the human colon explain the association between red meat and colon cancer? *Carcinogenesis.* 1996;17:515-23.
14. Blount BC, Ames BN. DNA damage in folate deficiency. *Bailleres Clin Haematol.* 1995;8:461-478.
15. Blount BC, Mack MM, Wehr CM, MacGregor JT, Hiatt RA, Wang G, et al. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. *Proc. Natl Acad Sci U S A.* 1997;94:3290-3295.
16. Board P, Coggan M, Johnston P, Ross V, Suzuki T, Webb G. Genetic heterogeneity of the human glutathione transferases: a complex of gene families. *Pharmacol Ther.* 1990;48:357-369.
17. Butkiewicz D, Grzybowska E, Phillips DH, Hemminki K, Chorazy M. Polymorphisms of the GSTP1 and GSTM1 genes and PAH-DNA adducts in human mononuclear white blood cells. *Environ Mol Mutagen.* 2000;35:99-105.
18. Cabral IR, Rossit AR, Hamel AR. Polimorfismos em genes de biometabolismo e prevalência do alelo CYP2E1*C2 em deficientes de G6PD assintomáticos: análises em uma população paraense. *Genet Mol Biol.* 1999;22:236-237.
19. Cai L, Zheng ZL, Zhang ZF. Cytochrome p450 2E1 polymorphisms and the risk of gastric cardia cancer. *World J Gastroenterol.* 2005;11:1867-71.
20. Canalle R, Burim RV, Tone LG, Takahashi CS. Genetic polymorphisms and susceptibility to childhood acute lymphoblastic leukemia. *Environ Mol Mutagen.* 2004;43:100-9.
21. Carrière V, Berthou F, Baird S, Belloc C, Beaune P, de Waziers I. Human cytochrome P450 2E1 (CYP2E1): from genotype to phenotype. *Pharmacogenetics.* 1996;6:203-11.
22. Cha IH, Park JY, Chung WY, Choi MA, Kim HJ, Park KK. Polymorphisms of CYP1A1 and GSTM1 genes and susceptibility to oral cancer. *Yonsei Med J.* 2007;48:233-9.
23. Chen J, Giovannucci EL, Hunter DJ. MTHFR polymorphism, methyl-replete diets and the risk of colorectal carcinoma and adenoma among U.S. men and women: an example of gene-environment interactions in colorectal tumorigenesis. *J Nutr.* 1999;129(2S Suppl):560S-564S.

24. Chen J, Hunter DJ, Stampfer MJ, Kyte C, Chan W, Wetmur JG, et al. Polymorphism in the thymidylate synthase promoter enhancer region modifies the risk and survival of colorectal cancer. *Cancer Epidemiol Biomarkers Prev.* 2003;12:958-62.
25. Chu J, Dolnick BJ. Natural antisense (rTSalpha) RNA induces site-specific cleavage of thymidylate synthase mRNA. *Biochim Biophys Acta.* 2002;1587:183-93.
26. Collins AR, Dusinska M, Gedik CM, Stetina R. Oxidative damage to DNA: do we have a reliable biomarker? *Environ Health Perspect.* 1996;104(Suppl 3):465-9.
27. Collins AR, Harrington V, Drew J, Melvin R. Nutritional modulation of DNA repair in a human intervention study. *Carcinogenesis.* 2003;24:511-515.
28. Crews H, Alink G, Andersen R, Braesco V, Holst B, Maiani G, et al. A critical assessment of some biomarker approaches linked with dietary intake. *Br J Nutr.* 2001;86 Suppl 1:S5-35.
29. Crofts F, Cosma GN, Currie D, Taioli E, Toniolo P, Garte SJA. novel CYP1A1 gene polymorphism in African-Americans. *Carcinogenesis.* 1993;14:2652.
30. Crott JW, Mashiyama ST, Ames BN, Fenech M. The effect of folic acid deficiency and MTHFR C677T polymorphism on chromosome damage in human lymphocytes in vitro. *Cancer Epidemiol Biomarkers Prev.* 2001;10:1089-96.
31. DeVos L, Chanson A, Liu Z, Ciappio ED, Parnell LD, Mason JB, et al. Associations between single nucleotide polymorphisms in folate uptake and metabolizing genes with blood folate, homocysteine, and DNA uracil concentrations. *Am J Clin Nutr.* 2008;88:1149-58.
32. D'Odorico A, Martines D, Kiechl S, Egger G, Oberhollenzer F, Bonvicini P, et al. High plasma levels of alpha- and beta-carotene are associated with a lower risk of atherosclerosis: results from the Bruneck study. *Atherosclerosis.* 2000;153:231-9.
33. Doll R, Peto R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst.* 1981; 66: 1191-308.
34. Dunning AM, Healey CS, Pharoah PDP, Teare MD, Ponder BAJ, Easton DF. A systematic review of genetic polymorphisms and breast cancer risk. *Cancer Epidemiol Biomark Prev.* 1999;8:843-854.
35. Duthie, S.J. Folic acid deficiency and cancer? Mechanisms of DNA instability. *British Med Bull.* 1999;55:578-592.

36. Egan KM, Cai Q, Shu X, Jin F, Zhu T, Dai Q, et al. Genetic polymorphisms in *GSTM1*, *GSTP1*, and *GSTT1* and the risk for breast cancer: results from the Shanghai breast cancer study and meta-analysis. *Cancer Epidemiol Biomark Prev.* 2004;13:197-204.
37. Esteller M, Garcia A, Martinez-Palones JM, Xercavins J, Reventos J. Germ line polymorphisms in cytochrome-P450 1A1 (C4887 CYP1A1) and methylenetetrahydrofolate reductase (MTHFR) genes and endometrial cancer susceptibility. *Carcinogenesis.* 1997;18:2307-11.
38. Eto I, Krumdieck CL. Role of vitamin B12 and folate deficiencies in carcinogenesis, In: Poirier LA, Newberne PM, Pariza MW. (Ed.), *Essential Nutrients en Carcinogenesis*, Plenum Press, 1986, pp. 313-331.
39. Fenech M, Aitken C, Rinaldi J. Folate, vitamin B12, homocysteine status and DNA damage in young Australian adults. *Carcinogenesis.* 1998;19:1163-71.
40. Fenech M, Ferguson LR. Vitamins/minerals and genomic stability in humans. *Mutat Res.* 2001;475:1-6.
41. Fenech M. The role of folic acid and Vitamin B12 in genomic stability of human cells. *Mutat Res.* 2001;475:57-67.
42. Fenech MF, Dreosti IE, Rinaldi JR. Folate, vitamin B12, homocysteine status and chromosome damage rate in lymphocytes of older men. *Carcinogenesis.* 1997;18:1329-36.
43. Ferguson LR. Natural and human-made mutagens and carcinogens in the human diet. *Toxicology.* 2002;181-182:79-82.
44. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet.* 1995;10:111-3.
45. Gelain DP, Moreira JC. Evidence of increased reactive species formation by retinol, but not retinoic acid, in PC12 cells. *Toxicol In Vitro.* 2008;22:553-8.
46. Gerster H. The potential role of lycopene for human health. *J Am Coll Nutr.* 1997;16:109-26.
47. Guengerich FP, Kim DH, Iwasaki M. Role of human P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem Res Toxicol.* 1991;4:168-179.
48. Hatagima A, Ramos VG, Latau-Guimarães MN, Cabello PH. Polimorfismos genéticos da glutationa-S-transferase Mu (*GSTM1*) e theta (*GSTT1*) em populações brasileiras. *Genet Mol Biol.* 1999;22:351.

49. Hayashi S, Watanabe J, Kawajiri K. Genetic polymorphisms in the 5'-flanking region change transcriptional regulation of the human cytochrome P450IIE1 gene. *J Biochem.* 1991;110:559-65.
50. Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol.* 1995;30:445-600.
51. Hecht SS. Approaches to cancer prevention based on an understanding of N-nitrosamine carcinogenesis. *Proc Soc Exp Biol Med.* 1997;216:181-91.
52. Hildebrand CE, Gonzalez FJ, McBride OW, Nebert DW. Assignment of the human 2,3,7,8-tetrachlorodibenzo-p-dioxin-inducible cytochrome P1-450 gene to chromosome 15. *Nucleic Acids Res.* 1985;13:2009-2016.
53. Hirvonen A, Husgafvel-Pursiainen K, Anttila S, Vainio H. The GSTM1 null genotype as a potential risk modifier for squamous cell carcinoma of the lung. *Carcinogenesis.* 1993;14:1479-81.
54. Honma HN, De Capitani EM, Perroud MW Jr, Barbeiro AS, Toro IF, Costa DB, et al. Influence of p53 codon 72 exon 4, GSTM1, GSTT1 and GSTP1*B polymorphisms in lung cancer risk in a Brazilian population. *Lung Cancer.* 2008;61:152-62.
55. Horie N, Aiba H, Oguro K, Hojo H, Takeishi K. Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5'-terminal regulatory region of the human gene for thymidylate synthase. *Cell Struct Funct.* 1995;20:191-7.
56. Horie N, Takeishi K. Identification of functional elements in the promoter region of the human gene for thymidylate synthase and nuclear factors that regulate the expression of the gene. *J Biol Chem.* 1997;272:18375-81.
57. Hung RJ, Hall J, Brennan P, Boffetta P. Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review. *Am J Epidemiol.* 2005;162:925-42.
58. hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1. *Cancer Sci.* 2004;95:1-6.
59. Kark JD, Selhub J, Adler B, Gofin J, Abramson JH, Friedman G, Rosenberg IH. Nonfasting plasma total homocysteine level and mortality in middle-aged and elderly men and women in Jerusalem. *Ann Intern Med.* 1999;131:321-30.
60. Kato S, Bowman ED, Harrington AM, Blomeke B, Shields PG. Human lung carcinogen-DNA adduct levels mediated by genetic polymorphisms in vivo. *J Natl Cancer Inst.* 1995;87:902-7. Erratum in: *J Natl Cancer Inst.* 1996;88:1595.

61. Kawajiri K, Nakachi K, Imai K, Yaoshii A, Shinoda N, Watanabe J. Identification of genetically high risk individuals to lung cancer by DNA polymorphisms of the cytochrome P-450IA1 gene. *FEBS Lett.* 1990;263:131-133.
62. Kawajiri K, Watanabe J, Gotoh O, Tagashira Y, Sogawa K, Fujiiikuriyama Y. Structure and drug inducibility of the human cytochrome P-450c gene. *Europ J Biochem.* 1996;159:219-225.
63. Keku T, Millikan R, Worley K, Winkel S, Eaton A, Biscocho L, et al. 5,10-Methylenetetrahydrofolate reductase codon 677 and 1298 polymorphisms and colon cancer in African Americans and whites. *Cancer Epidemiol Biomarkers Prev.* 2002;11:1611-21.
64. Ketterer B, Harris JM, Talaska G, Meyer DJ, Pemble SE, Taylor JB, et al. The human glutathione S-transferase supergene family, its polymorphism, and its effects on susceptibility to lung cancer. *Environ Health Perspect.* 1992;98:87-94.
65. kim RB, O' Shea D, Wilkinson GR. Relationship in healthy subjects between CYP2E1 genetic polymorphisms and the 6-hydroxylation of chlorzoxazone a putative measure of CYP2E1 activity. *Pharmacogenetics.* 1994;4:162-165.
66. Kim RB, Yamazaki H, Chiba K, O'Shea D, Mimura M, Guengerich FP, et al. In vivo and in vitro characterization of CYP2E1 activity in Japanese and Caucasians. *J Pharmacol Exp Ther.* 1996;279:4-11.
67. Kiyohara C, Takayama K, Nakanishi Y. Association of genetic polymorphisms in the base excision repair pathway with lung cancer risk: a meta-analysis. *Lung Cancer.* 2006;54:267-83.
68. Kluijtmans LA, Kastelein JJ, Lindemans J, Boers GH, Heil SG, Brusckhe AV, et al. Thermolabile methylenetetrahydrofolate reductase in coronary artery disease. *Circulation.* 1997;96:2573-7.
69. Kocabaş NA, Karahalil B, Karakaya AE, Sardaş S. Influence of GSTM1 genotype on comet assay and chromosome aberrations after induction by bleomycin in cultured human lymphocytes. *Mutat Res.* 2000;469:199-205.
70. Krajinovic M, Labuda D, Sinnott D. Glutathione S-transferase P1 genetic polymorphisms and susceptibility to childhood acute lymphoblastic leukaemia. *Pharmacogenetics.* 2002;12:655-8.
71. Krajinovic M, Lamothe S, Labuda D, Lemieux-Blanchard E, Theoret Y, Moghrabi A, et al. Role of MTHFR genetic polymorphisms in the susceptibility to childhood acute lymphoblastic leukemia. *Blood.* 2004;103:252-7.
72. Krinsky NI, Johnson EJ. Carotenoid actions and their relation to health and disease. *Mol Aspects Med.* 2005;26:459-516.

73. Krinsky NI. The antioxidant and biological properties of the carotenoids. *Ann N Y Acad Sci.* 1998;854:443-7.
74. La Vecchia C, Franceschi S, Levi F. Epidemiological research on cancer with a focus on Europe. *Eur J Cancer Prev.* 2003;12:5-14.
75. Landi S. Mammalian class theta GST and differential susceptibility to carcinogens: a review. *Mutat Res.* 2000;463:247-83.
76. Lasisi AO. The role of retinol in the etiology and outcome of suppurative otitis media. *Eur Arch Otorhinolaryngol.* In press 2008.
77. Le Marchand L, Donlon T, Seifried A, Wilkens LR. Red meat intake, CYP2E1 genetic polymorphisms, and colorectal cancer risk. *Cancer Epidemiol Biomarkers Prev.* 2002;11(10 Pt 1):1019-24.
78. Le Marchand L, Wilkinson GR, Wilkens LR. Genetic and dietary predictors of CYP2E1 activity: a phenotyping study in Hawaii Japanese using chlorzoxazone. *Cancer Epidemiol Biomarkers Prev.* 1999;8:495-500.
79. Levi F. Cancer prevention/ epidemiology and perspectives. *Eur J Cancer.* 1999;35:1046-1058.
80. Li WQ, Zhang L, Ma JL, Zhang Y, Li JY, Pan KF, et al. Association between Genetic Polymorphisms of DNA Base Excision Repair Genes and Evolution of Precancerous Gastric Lesions in a Chinese population. *Carcinogenesis.* In press 2009.
81. Liu YH, Taylor J, Linko P, Lucier GW, Thompson CL. Glutathione S-transferase mu in human lymphocyte and liver: role in modulating formation of carcinogen-derived DNA adducts. *Carcinogenesis.* 1991;12:2269-75.
82. Loft S, Høgh Danielsen P, Mikkelsen L, Risom L, Forchhammer L, Møller P. Biomarkers of oxidative damage to DNA and repair. *Biochem Soc Trans.* 2008;36(Pt 5):1071-6.
83. Louro ID, Llerena JR, J C, Melo MSV, Ashton-Prola P, Conforti-Froes N. *Genética Molecular do Câncer.* 2^a ed. São Paulo: MSG produção Editorial; 2002.
84. Lucas D, Ménez C, Girre C, Berthou F, Bodénez P, Joannet I, et al. Cytochrome P450 2E1 genotype and chlorzoxazone metabolism in healthy and alcoholic Caucasian subjects. *Pharmacogenetics.* 1995;5:298-304.
85. Ma J, Stampfer MJ, Giovannucci E, Artigas C, Hunter DJ, Fuchs C, et al. Methylentetrahydrofolate reductase polymorphism, dietary interactions, and risk of colorectal cancer. *Cancer Res.* 1997;57:1098-102.
86. Mandola MV, Stoehlmacher J, Zhang W, Groshen S, Yu MC, Iqbal S, et al. A 6 bp polymorphism in the thymidylate synthase gene causes message instability

- and is associated with decreased intratumoral TS mRNA levels. *Pharmacogenetics*. 2004;14:319-27.
87. Mangels AR, Holden JM, Beecher GR, Forman MR, Lanza E. Carotenoid contents of fruits and vegetables: an evaluation of analytical data. *J Am Diet Assoc*. 1993;93:284-96. Erratum in: *J Am Diet Assoc* 1993;93:527.
 88. Mannervik B, Awasthi YC, Board PG, Hayes JD, Di Ilio C, Ketterer B, et al. *Biochem J*. 1992;282(Pt 1):305-6.
 89. Mateuca RA, Roelants M, Iarmarcovai G, Aka PV, Godderis L, Tremp A, et al. hOGG1(326), XRCC1(399) and XRCC3(241) polymorphisms influence micronucleus frequencies in human lymphocytes in vivo. *Mutagenesis*. 2008;23:35-41.
 90. McCarver DG, Byun R, Hines RN, Hichme M, Wegenek W. A genetic polymorphism in the regulatory sequences of human CYP2E1: association with increased chlorzoxazone hydroxylation in the presence of obesity and ethanol intake. *Toxicol Appl Pharmacol*. 1998;152:276-81.
 91. Miao X, Xing D, Tan W, Qi J, Lu W, Lin D. Susceptibility to gastric cardia adenocarcinoma and genetic polymorphisms in methylenetetrahydrofolate reductase in an at-risk Chinese population. *Cancer Epidemiol Biomarkers Prev*. 2002;11:1454-8.
 92. Miller JA, Miller EC. Ultimate chemical carcinogen as reactive mutagenic electrophiles In: Hiatt HH, Watson JD, Winstein JA. (Eds). 1977. *Origins of human cancer*, Cold Spring Laboratory. New York: Cold Spring Harbor 605-628.
 93. Mittal RD, Mishra DK, Mandhani A. Evaluating polymorphic status of glutathione-S-transferase genes in blood and tissue samples of prostate cancer patients. *Asian Pac J Cancer Prev*. 2006;7:444-6.
 94. Mo Z, Gao Y, Cao Y, Gao F, Jian L. An updating meta-analysis of the GSTM1, GSTT1, and GSTP1 polymorphisms and prostate cancer: A HuGE Review. *Prostate*. In press 2009.
 95. Mohrenweiser HW, Carrano AV, Fertitta A, Perry B, Thompson LH, Tucker JD, et al. Refined mapping of the three DNA repair genes, ERCC1, ERCC2, and XRCC1, on human chromosome 19. *Cytogenet Cell Genet*. 1989;52:11-4.
 96. Moller P, Loft S. Oxidative DNA damage in human white blood cells in dietary antioxidant intervention studies. *Am J Clin Nutr*. 2002;76:303-310.
 97. Montesano R, Hall J. Environmental causes of cancer. *Eur J Cancer*. 2001;37:S67-S87.
 98. Morita M, Le Marchand L, Kono S, Yin G, Toyomura K, Nagano J, et al. Genetic polymorphisms of CYP2E1 and risk of colorectal cancer: the Fukuoka Colorectal Cancer Study. *Cancer Epidemiol Biomarkers Prev*. 2009;18:235-41.

99. Morita M, Tabata S, Tajima O, Yin G, Abe H, Kono S. Genetic polymorphisms of CYP2E1 and risk of colorectal adenomas in the Self Defense Forces Health Study. *Cancer Epidemiol Biomarkers Prev.* 2008;17:1800-7.
100. Moscow JA, Townsend AJ, Goldsmith ME, Whang-Peng J, Vickers PJ, Poisson R, et al. Isolation of the human anionic glutathione S-transferase cDNA and the relation of its gene expression to estrogen-receptor content in primary breast cancer. *Proc Natl Acad Sci U S A.* 1988;85:6518-6522.
101. Murtaugh MA, Curtin K, Sweeney C, Wolff RK, Holubkov R, Caan BJ, et al. Dietary intake of folate and co-factors in folate metabolism, MTHFR polymorphisms, and reduced rectal cancer. *Cancer Causes Control.* 2007;18:153-63.
102. Nakachi K, Imai K, Hayashi S, Watanabe J, Kawajiri K. Genetic susceptibility to squamous cell carcinoma of the lung in relation to cigarette smoking dose. *Cancer Res.* 1991;51:5177-5180.
103. Narayanan S, McConnell J, Little J, Sharp L, Piyathilake CJ, Powers H, et al. Associations between two common variants C677T and A1298C in the methylenetetrahydrofolate reductase gene and measures of folate metabolism and DNA stability (strand breaks, misincorporated uracil, and DNA methylation status) in human lymphocytes in vivo. *Cancer Epidemiol Biomarkers Prev.* 2004;13:1436-43.
104. Nebert DW, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levin W, Phillips IR, Sato R, Waterman MR. The P450 superfamily: recommended nomenclature. *DNA.* 1987; 6:1-11.
105. Nebert DW, Dalton TP. The role of cytochrome P450 enzymes in indigenous signalling pathways and environmental carcinogenesis. *Nat Rev Cancer.* 2006;6:947-60.
106. Nebert DW. Role of genetics and drug metabolism in human cancer risk. *Mutat Res.* 1991;247:267-281.
107. Norppa H. Cytogenetic biomarkers and genetic polymorphisms. *Toxicol Lett.* 2004;149:309-334.
108. Oikawa S, Murakami K, Kawanishi S. Oxidative damage to cellular and isolated DNA by homocysteine: implications for carcinogenesis. *Oncogene.* 2003;22:3530-8.
109. Paiva SA, Russell RM. Beta-carotene and other carotenoids as antioxidants. *Beta-carotene and other carotenoids as antioxidants. J Am Coll Nutr.* 1999;18:424-5.

110. Palli D, Masala G, Vineis P, Garte S, Seymour G, Saieva C, et al. Biomarkers of dietary intake of micronutrients modulate DNA adduct levels in healthy adults. *Carcinogenesis*. 2003;24:739-746.
111. Pandya U, Srivastava SK, Singhal SS, Pal A, Awasthi S, Zimniak P, et al. Activity of allelic variants of Pi class human glutathione S-transferase toward chlorambucil. *Biochem Biophys Res Commun*. 2000;278:258-262.
112. Pemble S, Schroeder KR, Spencer SR, Meyer DJ, Hallier E, Bolt HM, et al. Human glutathione S-transferase theta (GSTT1): cDNA cloning and the characterization of a genetic polymorphism. *Biochem J*. 1994;300:271-276.
113. Perera FP. Molecular epidemiology: insights into cancer susceptibility, risk assessment, and prevention. *J Natl Cancer Inst*. 1996;88:496-509.
114. Persson I, Johansson I, Bergling H, Marja-Liisa D, Seidegad J. Genetic polymorphism of cytochrome P450 2E1: regulation and toxicological significance. *Journal of Occupational and Environmental Medicine*. 1995;7:25-36.
115. Pickett CB, Lu AYH. Glutathione S-transferases: gene structure, regulation, and biological function. *Ann Rev Bioch*. 1989;58:743-764.
116. Powell H, Kitteringham NR, Pirmohamed M, Smith DA, Park BK. Expression of cytochrome P4502E1 in human liver: assessment by mRNA, genotype and phenotype. *Pharmacogenetics*. 1998;8:411-21.
117. Pullarkat ST, Stoehlmacher J, Ghaderi V, Xiong YP, Ingles SA, Sherrod A, et al. Thymidylate synthase gene polymorphism determines response and toxicity of 5-FU chemotherapy. *Pharmacogenomics J*. 2001;1:65-70.
118. Rao AV, Rao LG. Carotenoids and human health. *Pharmacol Res*. 2007;55:207-16.
119. Ribaya-Mercado JD, Solon FS, Solon MA, Cabal-Barza MA, Perfecto CS, Tang G, et al. Bioconversion of plant carotenoids to vitamin A in Filipino school-aged children varies inversely with vitamin A status. *Am J Clin Nutr*. 2000;72:455-65.
120. Rossit ARB. Investigação molecular de polimorfismos em genes relacionados ao metabolismo de xenobióticos e ao reparo do DNA em populações Brasileiras. [Tese]. São José do Rio Preto: Universidade Estadual Paulista; 2001.
121. Sadeghian S, Fallahi F, Salarifar M, Davoodi G, Mahmoodian M, Fallah N, et al. Homocysteine, vitamin B12 and folate levels in premature coronary artery disease. *BMC Cardiovasc Disord*. 2006;6:38.
122. Scarpato R, Hirvonen A, Migliore L, Falck G, Norppa H. Influence of GSTM1 and GSTT1 polymorphisms on the frequency of chromosome aberrations in lymphocytes of smokers and pesticide-exposed greenhouse workers. *Mutat Res*, 1997;389:227-235.

123. Schneider C. Chemistry and biology of vitamin E. *Mol Nutr Food Res.* 2005;49:7-30.
124. Schneider J, Bernges U, Philipp M, Woitowitz HJ. GSTM1, GSTT1, and GSTP1 polymorphism and lung cancer risk in relation to tobacco smoking. *Cancer Lett.* 2004;208:65-74.
125. Sei H. Vitamin A and sleep regulation. *J Med Invest.* 2008;55:1-8.
126. Selhub J, Miller JW. The pathogenesis of homocysteinemia: interruption of the coordinate regulation by S-adenosylmethionine of the remethylation and transsulfuration of homocysteine. *Am J Clin Nutr.* 1992;55:131-8.
127. Shen MR, Zdzienicka MZ, Mohrenweiser H, Thompson LH, Thelen MP. Mutations in hamster single-strand break repair gene XRCC1 causing defective DNA repair. *Nucleic Acids Res.* 1998;26:1032-7.
128. Shimada T, Fujii-Kuriyama Y. Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1. *Cancer Sci.* 2004;95:1-6.
129. Singh V, Rastogi N, Sinha A, Kumar A, Mathur N, Singh MP. A study on the association of cytochrome-P450 1A1 polymorphism and breast cancer risk in north Indian women. *Breast Cancer Res Treat.* 2007;101:73-81.
130. Skibola CF, Smith MT, Hubbard A, Shane B, Roberts AC, Law GR, et al. Polymorphisms in the thymidylate synthase and serine hydroxymethyltransferase genes and risk of adult acute lymphocytic leukemia. *Blood.* 2002;99:3786-91.
131. Skibola CF, Smith MT, Kane E, Roman E, Rollinson S, Cartwright RA, Morgan G. Polymorphisms in the methylenetetrahydrofolate reductase gene are associated with susceptibility to acute leukemia in adults. *Proc Natl Acad Sci U S A.* 1999;96:12810-5.
132. (b)Skjelbred CF, Saebø M, Wallin H, Nexø BA, Hagen PC, Lothe IM, et al. Polymorphisms of the XRCC1, XRCC3 and XPD genes and risk of colorectal adenoma and carcinoma, in a Norwegian cohort: a case control study. *BMC Cancer.* 2006;6:67.
133. (a)Skjelbred CF, Svendsen M, Haugan V, Eek AK, Clausen KO, Svendsen MV, et al. Influence of DNA repair gene polymorphisms of hOGG1, XRCC1, XRCC3, ERCC2 and the folate metabolism gene MTHFR on chromosomal aberration frequencies. *Mutat Res.* 2006;602:151-62. Erratum in: *Mutat Res.* 2007;624:133.
134. Sørensen M, Autrup H, Tjønneland A, Overvad K, Raaschou-Nielsen O. Glutathione S-transferase T1 null-genotype is associated with an increased risk of lung cancer. *Int J Cancer.* 2004;110:219-24.
135. Sørensen M, Raaschou-Nielsen O, Brasch-Andersen C, Tjønneland A, Overvad K, Autrup H. Interactions between GSTM1, GSTT1 and GSTP1 polymorphisms and smoking and intake of fruit and vegetables in relation to lung cancer. *Lung Cancer.* 2007;55:137-44.

136. Soya SS, Vinod T, Reddy KS, Gopalakrishnan S, Adithan C. Genetic polymorphisms of glutathione-S-transferase genes (GSTM1, GSTT1 and GSTP1) and upper aerodigestive tract cancer risk among smokers, tobacco chewers and alcoholics in an Indian population. *Eur J Cancer*. 2007;43:2698-706.
137. Stephens EA, Taylor JA, Kaplan N, Yang CH, Hsieh LL, Lucier GW, et al. Ethnic variation in the CYP2E1 gene, polymorphisms analysis of 695 African-Americans, European Americans and Taiwanese. *Pharmacogenetics*. 1994;4:185-192.
138. Suzuki K, Mitsuoka T. Increase in faecal nitrosamines in Japanese individuals given a Western diet. *Nature*. 1981;294:453-6.
139. Taningher M, Malacarne D, Issoti A, Ugloni D, Parodi S. Drug metabolism polymorphisms as modulators of cancer susceptibility. *Mut Res*. 1999;436:227-261.
140. Trichopoulou A, Costacou T, Barnia C, Trichopoulos D. Adherence to a mediterranean diet and survival in a greek population. *The New Engl J Med*. 2003;348:2599-2608.
141. Ulrich CM, Bigler J, Bostick R, Fosdick L, Potter JD. Thymidylate synthase promoter polymorphism, interaction with folate intake, and risk of colorectal adenomas. *Cancer Res*. 2002;62:3361-4.
142. Ulrich CM, Bigler J, Velicer CM, Greene EA, Farin FM, Potter JD. Searching expressed sequence tag databases: discovery and confirmation of a common polymorphism in the thymidylate synthase gene. *Cancer Epidemiol Biomarkers Prev*. 2000;9:1381-5.
143. Ulrich CM, Curtin K, Potter JD, Bigler J, Caan B, Slattery ML. Polymorphisms in the reduced folate carrier, thymidylate synthase, or methionine synthase and risk of colon cancer. *Cancer Epidemiol Biomarkers Prev*. 2005;14(11 Pt 1):2509-16.
144. van der Put NM, Gabreëls F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, et al. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? *Am J Hum Genet*. 1998;62:1044-51.
145. van Poppel G, de Vogel N, van Balderen PJ, Kok FJ. Increased cytogenetic damage in smokers deficient in glutathione S-transferase isozyme mu. *Carcinogenesis*. 1992;13:303-5.
146. Vodicka P, Kumar R, Stetina R, Sanyal S, Soucek P, Haufroid V, et al. Genetic polymorphisms in DNA repair genes and possible links with DNA repair rates, chromosomal aberrations and single-strand breaks in DNA. *Carcinogenesis*. 2004;25:757-763.
147. Warwick AP, Sarhanis P, Redman C, Pemble S, Taylor J B, Ketterer B, et al. Theta class glutathione transferase GSTT1 genotypes and susceptibility to cervical neoplasia: interactions with GSTM1, CYP2D6 and smoking. *Carcinogenesis*. 1997;18:967-973.
148. Watanabe J, Hayashi S, Kawajiri K. Different regulation and expression of the human CYP2E1 gene due to the RsaI polymorphism in the 5'-flanking region. *J Biochem*. 1994;116:321-6.

149. Watters JL, Satia JA, Kupper LL. Correlates of antioxidant nutrients and oxidative DNA damage differ by race in a cross-sectional study of healthy African American and white adults. *Nutr Res.* 2008;28:565-76.
150. Wiemels JL, Smith RN, Taylor GM, Eden OB, Alexander FE, Greaves MF; United Kingdom Childhood Cancer Study investigators. Methylenetetrahydrofolate reductase (MTHFR) polymorphisms and risk of molecularly defined subtypes of childhood acute leukemia. *Proc Natl Acad Sci U S A.* 2001;98:4004-9.
151. Wilms LC, Cloughton TA, de Kok TM, Kleinjans JC. GSTM1 and GSTT1 polymorphism influences protection against induced oxidative DNA damage by quercetin and ascorbic acid in human lymphocytes in vitro. *Food Chem Toxicol.* 2007;45:2592-6.
152. Wisberg I, Tran P, Christensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. *Mol Gen Metab.* 1998;64:169-172.
153. Wright ME, Lawson KA, Weinstein SJ, Pietinen P, Taylor PR, Virtamo J, et al. Higher baseline serum concentrations of vitamin E are associated with lower total and cause-specific mortality in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. *Am J Clin Nutr.* 2006;84:1200-7.
154. Xu X, Kelsey KT, Wiencke JK, Wain JC, Christiani DC. Cytochrome P450 *CYP1A1* MspI polymorphism and lung cancer susceptibility. *Cancer Epidemiol Biomarkers Prev.* 1996;5:687-692.
155. Yi P, Melnyk S, Pogribna M, Pogribny IP, Hine RJ, James SJ. Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation. *J Biol Chem.* 2000;275:29318-23.
156. Zhang Z, Shi Q, Sturgis EM, Spitz MR, Hong WK, Wei Q. Thymidylate synthase 5'- and 3'-untranslated region polymorphisms associated with risk and progression of squamous cell carcinoma of the head and neck. *Clin Cancer Res.* 2004;10:7903-10.
157. Zhu SM, Xia ZL, Wang AH, Ren XF, Jiao J, Zhao NQ, et al. Polymorphisms and haplotypes of DNA repair and xenobiotic metabolism genes and risk of DNA damage in Chinese vinyl chloride monomer (VCM)-exposed workers. *Toxicol Lett.* 2008;178:88-94.
158. Ziegler RG. Vegetables, fruits, and carotenoids and the risk of cancer. *Am J Clin Nutr.* 1991;53(1 Suppl):251S-259S.
159. Zingg JM, Jones PA. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. *Carcinogenesis.* 1997;18:869-82.

160. Zittoun J, Tonetti C, Bories D, Pignon JM, Tulliez M. Plasma homocysteine levels related to interactions between folate status and methylenetetrahydrofolate reductase: a study in 52 healthy subjects. *Metabolism*. 1998;47:1413-8.

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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Key words: Carotenoids, α - tocopherol, chronic diseases, serum concentration, diet, fruits and vegetables

Running title: Dietary pattern influence serum micronutrients

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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-suppressive 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 occurred maybe due to the imbalanced 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 essential 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 symptoms of deficiency 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 (xerophthalmia), 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 radicals and inhibit the oxidation of LDL cholesterol, what contribute with the prevention of atherosclerosis and carcinogenesis [30]. Moreover, it can act 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, enhancement 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 described 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 department 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 $35,6 \pm 11,4$, 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 ($r=0.5265$), 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 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 cyclooxygenase-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 difficulties 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. Moreover, 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 accessible 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 community 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 selected 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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Literature Cited

- 1- Mangels AR, Holden JM, Beecher GR, Forman MR, Lanza E. Carotenoid contents of fruits and vegetables: an evaluation of analytical data. *J Am Diet Assoc.* 1993;93:284-96. Erratum in: *J Am Diet Assoc* 1993;93:527.
- 2- Rao AV, Rao LG. Carotenoids and human health. *Pharmacol Res.* 2007;55:207-16.
- 3- Talegawkar SA, Johnson EJ, Carithers TC, Taylor HA, Bogle ML, Tucker KL. Carotenoid intakes, assessed by food-frequency questionnaires (FFQs), are associated with serum carotenoid concentrations in the Jackson Heart Study: validation of the Jackson Heart Study Delta NRI Adult FFQs. *Public Health Nutr.* 2008;11:989-97.
- 4- Olson JA, Krinsky NI. Introduction: the colorful, fascinating world of the carotenoids: important physiologic modulators. *FASEB J.* 1995;9:1547-50.
- 5- Crews H, Alink G, Andersen R, Braesco V, Holst B, Maiani G, Ovesen L, Scotter M, Solfrizzo M, van den Berg R, Verhagen H, Williamson G. A critical assessment of some biomarker approaches linked with dietary intake. *Br J Nutr.* 2001;86 Suppl 1:S5-35.
- 6- Krinsky NI, Johnson EJ. Carotenoid actions and their relation to health and disease. *Mol Aspects Med.* 2005;26:459-516.
- 7- Gerster H. The potential role of lycopene for human health. *J Am Coll Nutr.* 1997;16:109-26.
- 8- Ziegler RG. Vegetables, fruits, and carotenoids and the risk of cancer. *Am J Clin Nutr.* 1991;53(1 Suppl):251S-259S.
- 9- D'Odorico A, Martines D, Kiechl S, Egger G, Oberhollenzer F, Bonvicini P, Sturniolo GC, Naccarato R, Willeit J. High plasma levels of alpha- and beta-

- carotene are associated with a lower risk of atherosclerosis: results from the Bruneck study. *Atherosclerosis*. 2000;153:231-9.
- 10- Daviglus ML, Orenca AJ, Dyer AR, Liu K, Morris DK, Persky V, Chavez N, Goldberg J, Drum M, Shekelle RB, Stamler J. Dietary vitamin C, beta-carotene and 30-year risk of stroke: results from the Western Electric Study. *Neuroepidemiology*. 1997;16:69-77.
- 11-Miller NJ, Sampson J, Candeias LP, Bramley PM, Rice-Evans CA. Antioxidant activities of carotenes and xanthophylls. *FEBS Lett*. 1996;384:240-2.
- 12- Voutilainen S, Nurmi T, Mursu J, Rissanen TH. Carotenoids and cardiovascular health. *Am J Clin Nutr*. 2006;83:1265-71.
- 13- Tapiero H, Townsend DM, Tew KD. The role of carotenoids in the prevention of human pathologies. *Biomed Pharmacother*. 2004;58:100-10.
- 14- Paiva SA, Russell RM. Beta-carotene and other carotenoids as antioxidants. Beta-carotene and other carotenoids as antioxidants. *J Am Coll Nutr*. 1999;18:424-5.
- 15-El-Agamey A, Lowe GM, McGarvey DJ, Mortensen A, Phillip DM, Truscott TG, Young AJ. Carotenoid radical chemistry and antioxidant/pro-oxidant properties. *Arch Biochem Biophys*. 2004;430:37-48.
- 16-Haeghele AD, Gillette C, O'Neill C, Wolfe P, Heimendinger J, Sedlacek S, Thompson HJ. Plasma xanthophyll carotenoids correlate inversely with indices of oxidative DNA damage and lipid peroxidation. *Cancer Epidemiol Biomarkers Prev*. 2000;9:421-5.

- 17- The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N Engl J Med.* 1994;330:1029-1035.
- 18-Kiefer I, Prock P, Lawrence C, Wise J, Bieger W, Bayer P, Rathmanner T, Kunze M, Rieder A. Supplementation with mixed fruit and vegetable juice concentrates increased serum antioxidants and folate in healthy adults. *J Am Coll Nutr.* 2004;23:205-11.
- 19-Collins AR. Antioxidant intervention as a route to cancer prevention. *Eur J Cancer.* 2005;41:1923-30.
- 20-Sei H. Vitamin A and sleep regulation. *J Med Invest.* 2008;55:1-8.
- 21-Ribaya-Mercado JD, Solon FS, Solon MA, Cabal-Barza MA, Perfecto CS, Tang G, Solon JA, Fjeld CR, Russell RM. Bioconversion of plant carotenoids to vitamin A in Filipino school-aged children varies inversely with vitamin A status. *Am J Clin Nutr.* 2000;72:455-65.
- 22-Lasisi AO. The role of retinol in the etiology and outcome of suppurative otitis media. *Eur Arch Otorhinolaryngol.* In press 2008.
- 23-Gelain DP, Moreira JC. Evidence of increased reactive species formation by retinol, but not retinoic acid, in PC12 cells. *Toxicol In Vitro.* 2008;22:553-8.
- 24-Mills JP, Simon PW, Tanumihardjo SA. Biofortified carrot intake enhances liver antioxidant capacity and vitamin a status in mongolian gerbils. *J Nutr.* 2008;138:1692-8.
- 25-Ross DA. Recommendations for vitamin A supplementation. *J Nutr.* 2002;132(9 Suppl):2902S-2906S.

- 26-Sommer A. Vitamin a deficiency and clinical disease: an historical overview. *J Nutr.* 2008;138:1835-9.
- 27- Surles RL, Mills JP, Valentine AR, Tanumihardjo SA. One-time graded doses of vitamin A to weanling piglets enhance hepatic retinol but do not always prevent vitamin A deficiency. *Am J Clin Nutr.* 2007;86:1045-53.
- 28- Paiva AA, Rondó PC, Gonçalves CR, Illison VK, Pereira JA, Vaz-de-Lima LA, Oliveira AC, Ueda M, Bergamaschi DP. Prevalência de deficiência de vitamina A e fatores associados em pré-escolares de Teresina, Piauí, Brasil. *Cad Saúde Pública.* 2006;22:1979-1987.
- 29- Meydani M. Vitamin E. *Lancet.* 1995;345:170-5.
- 30- Wright ME, Lawson KA, Weinstein SJ, Pietinen P, Taylor PR, Virtamo J, Albanes D. Higher baseline serum concentrations of vitamin E are associated with lower total and cause-specific mortality in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. *Am J Clin Nutr.* 2006;84:1200-7.
- 31- Schneider C. Chemistry and biology of vitamin E. *Mol Nutr Food Res.* 2005;49:7-30.
- 32- Tasinato A, Boscoboinik D, Bartoli GM, Maroni P, Azzi A. d-alpha-tocopherol inhibition of vascular smooth muscle cell proliferation occurs at physiological concentrations, correlates with protein kinase C inhibition, and is independent of its antioxidant properties. *Proc Natl Acad Sci U S A.* 1995;92:12190-4.
- 33- Harris A, Devaraj S, Jialal I. Oxidative stress, alpha-tocopherol therapy, and atherosclerosis. *Curr Atheroscler Rep.* 2002;4:373-80.

- 34-Saldanha LG, Johnson MA. Introduction to and perspectives from the Symposium on Nutrient Disease Relationships: closing the scientific knowledge gap. *J Nutr.* 2005;135:338-9.
- 35-Cardoso MA, Stocco PR. Development of a dietary assessment method for people of Japanese descent living in São Paulo, Brazil. *Cad Saúde Pública, Rio de Janeiro.* 2000;16:107-114.
- 36-Yeum KJ, Lee-Kim YC, Yoon S, Lee KY, Park IS, Lee KS, Kim BS, Tang G, Russell RM, Krinsky NI. Similar metabolites formed from beta-carotene by human gastric mucosal homogenates, lipoxygenase, or linoleic acid hydroperoxide. *Arch Biochem Biophys.* 1995;321:167-74.
- 37-Granado F, Olmedilla B, Blanco I, Rojas-Hidalgo E. Major fruit and vegetable contributors to the main serum carotenoids in the Spanish diet. *Eur J Clin Nutr.* 1996;50:246-50.
- 38-Block G, Norkus E, Hudes M, Mandel S, Helzlsouer K. Which plasma antioxidants are most related to fruit and vegetable consumption? *Am J Epidemiol.* 2001;154:1113-8.
- 39-Tucker KL, Chen H, Vogel S, Wilson PW, Schaefer EJ, Lammi-Keefe CJ. Carotenoid intakes, assessed by dietary questionnaire, are associated with plasma carotenoid concentrations in an elderly population. *J Nutr.* 1999;129:438-45.
- 40-Talegawkar SA, Johnson EJ, Carithers TC, Taylor HA, Bogle ML, Tucker KL. Carotenoid intakes, assessed by food-frequency questionnaires (FFQs), are associated with serum carotenoid concentrations in the Jackson Heart Study: validation of the Jackson Heart Study Delta NRI Adult FFQs. *Public Health Nutr.* 2008;11:989-97.

- 41-Michaud DS, Giovannucci EL, Ascherio A, Rimm EB, Forman MR, Sampson L, Willett WC. Associations of plasma carotenoid concentrations and dietary intake of specific carotenoids in samples of two prospective cohort studies using a new carotenoid database. *Cancer Epidemiol Biomarkers Prev.* 1998;7:283-90.
- 42-Lin YJ, Chien YW, Yang SH, Cheng HH. Fruits and stir-fried vegetables increase plasma carotenoids in young adults. *Asia Pac J Clin Nutr.* 2007;16:616-23.
- 43-Campbell DR, Gross MD, Martini MC, Grandits GA, Slavin JL, Potter JD. Plasma carotenoids as biomarkers of vegetable and fruit intake. *Cancer Epidemiol Biomarkers Prev.* 1994;3:493-500.
- 44-Al-Delaimy WK, Ferrari P, Slimani N, Pala V, Johansson I, Nilsson S, Mattisson I, Wirfalt E, Galasso R, Palli D, Vineis P, Tumino R, Dorronsoro M, Pera G, Ocké MC, Bueno-de-Mesquita HB, Overvad K, Chirlaque M, Trichopoulou A, Naska A, Tjønneland A, Olsen A, Lund E, Alsaker EH, Barricarte A, Kesse E, Boutron-Ruault MC, Clavel-Chapelon F, Key TJ, Spencer E, Bingham S, Welch AA, Sanchez-Perez MJ, Nagel G, Linseisen J, Quirós JR, Peeters PH, van Gils CH, Boeing H, van Kappel AL, Steghens JP, Riboli E. Plasma carotenoids as biomarkers of intake of fruits and vegetables: individual-level correlations in the European Prospective Investigation into Cancer and Nutrition (EPIC). *Eur J Clin Nutr.* 2005;59:1387-96.
- 45-Jansen MC, Van Kappel AL, Ocké MC, Van 't Veer P, Boshuizen HC, Riboli E, Bueno-de-Mesquita HB. Plasma carotenoid levels in Dutch men

- and women, and the relation with vegetable and fruit consumption. *Eur J Clin Nutr.* 2004;58:1386-95.
- 46-John JH, Ziebland S, Yudkin P, Roe LS, Neil HA; Oxford Fruit and Vegetable Study Group. Effects of fruit and vegetable consumption on plasma antioxidant concentrations and blood pressure: a randomised controlled trial. *Lancet.* 2002;359:1969-74.
- 47-Stryker WS, Kaplan LA, Stein EA, Stampfer MJ, Sober A, Willett WC. The relation of diet, cigarette smoking, and alcohol consumption to plasma beta-carotene and alpha-tocopherol levels. *Am J Epidemiol.* 1988;127:283-96.
- 48-Ascherio A, Stampfer MJ, Colditz GA, Rimm EB, Litin L, Willett WC. Correlations of vitamin A and E intakes with the plasma concentrations of carotenoids and tocopherols among American men and women. *J Nutr.* 1992;122:1792-801.
- 49-Vogel S, Contois JH, Tucker KL, Wilson PW, Schaefer EJ, Lammi-Keefe CJ. Plasma retinol and plasma and lipoprotein tocopherol and carotenoid concentrations in healthy elderly participants of the Framingham Heart Study. *Am J Clin Nutr.* 1997;66:950-8.
- 50-El-Sohemy A, Baylin A, Ascherio A, Kabagambe E, Spiegelman D, Campos H. Population-based study of alpha- and gamma-tocopherol in plasma and adipose tissue as biomarkers of intake in Costa Rican adults. *Am J Clin Nutr.* 2001;74:356-63.
- 51-Miller ER 3rd, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann Intern Med.* 2005;142:37-46.

- 52-Handelman GJ, Epstein WL, Peerson J, Spiegelman D, Machlin LJ, Dratz EA. Human adipose alpha-tocopherol and gamma-tocopherol kinetics during and after 1 y of alpha-tocopherol supplementation. *Am J Clin Nutr.* 1994;59:1025-32.
- 53-Handelman GJ, Machlin LJ, Fitch K, Weiter JJ, Dratz EA. Oral alpha-tocopherol supplements decrease plasma gamma-tocopherol levels in humans. *J Nutr.* 1985;115:807-13.
- 54-Jiang Q, Christen S, Shigenaga MK, Ames BN. gamma-tocopherol, the major form of vitamin E in the US diet, deserves more attention. *Am J Clin Nutr.* 2001;74:714-22.
- 55-Singh U, Devaraj S, Jialal I. Vitamin E, oxidative stress, and inflammation. *Annu Rev Nutr.* 2005;25:151-74.
- 56-Ramalho RA, Flores H, Saunders C. Hipovitaminose A no Brasil: um problema de saúde pública. *Pan Am J Public Health.* 2002; (20)12.
- 57-Ramalho EA, Saunders C, Natalizi DA, Cardoso LO, Accioly E. Níveis Séricos de retinol em escolares de 7 a 17 anos no município do Rio de Janeiro. *Rev Nutr.* 2004;17:461-468.
- 58-Milagres RCRM, Nunes LC, Pinheiro-Sant'ana HM. Vitamin A deficiency among children in Brazil and worldwide. *Ciênc Saúde Coletiva.* 2007;12:1253-1266.

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 differ 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

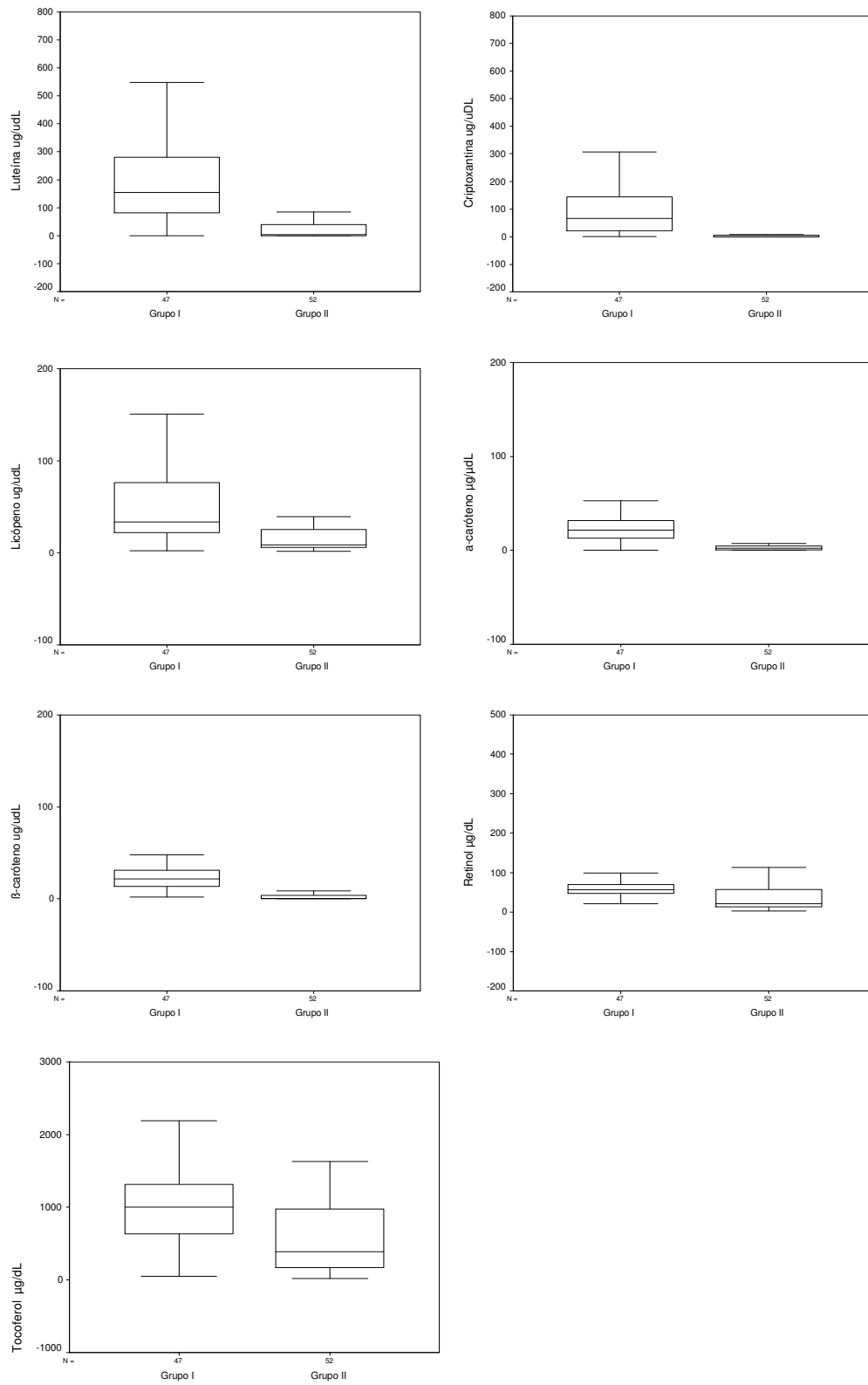


Figure 1.

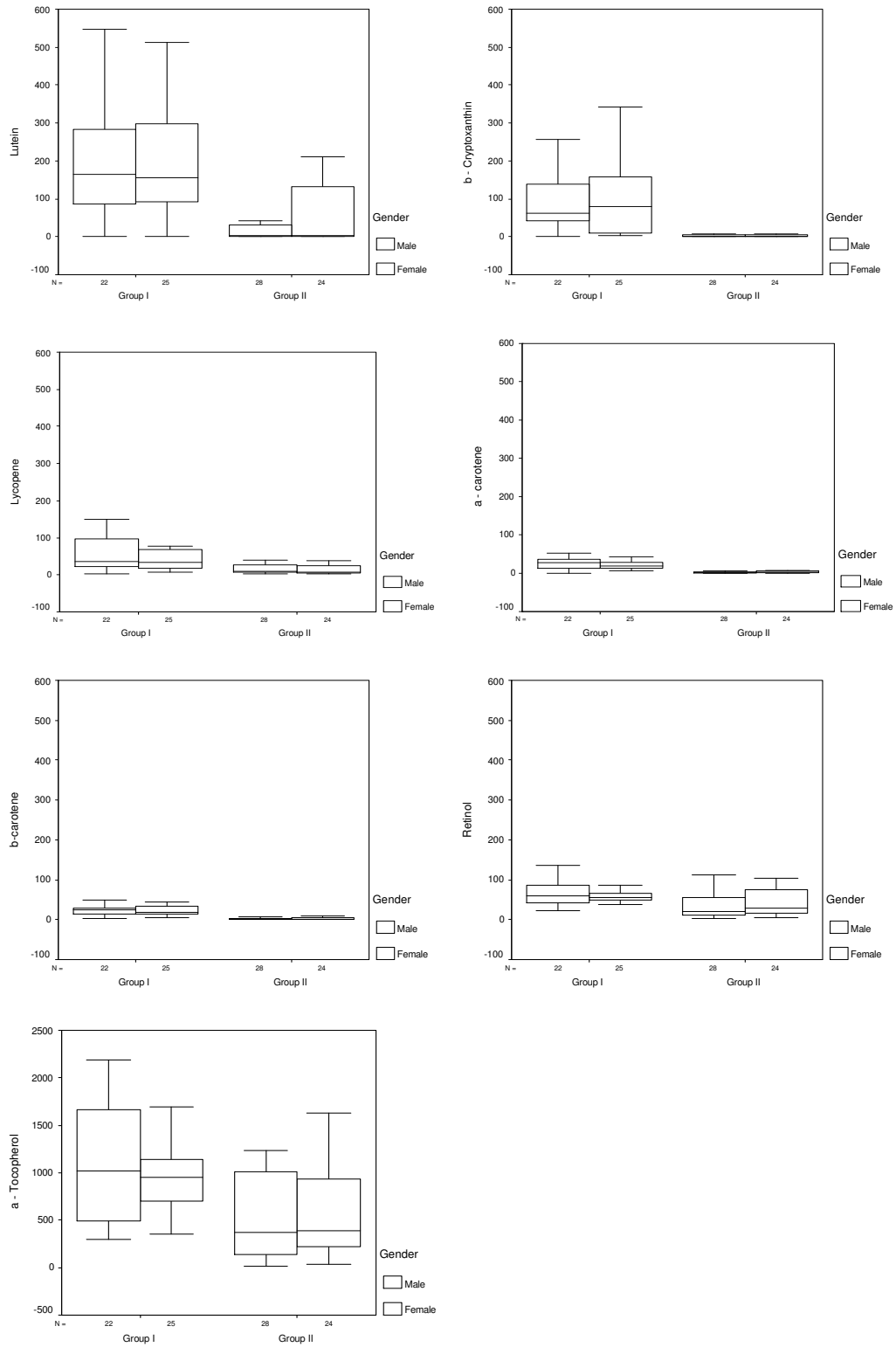


Figure 2.

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 β , 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 $35,6 \pm 11,4$, 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] ($\cong 2 \times 10^4$ 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°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 endonuclease 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 \cong 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 *194Trp* and the *399Gln* polymorphisms of the DNA repair gene *XRCC1* on the DNA repair capability, 200 μ l of the freshly isolated lymphocytes were treated with 100 μ l of H₂O₂ (100 μ M), for 30 minutes, in ice, and analysis of the level of damage was evaluated. Then, after two wash in PBS to remove of H₂O₂, 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 94°C (5 min) followed by 30 cycles of melting (94 °C, 30 s), annealing (62 °C, 1 min), extension (72°C, 45 s), and a final extension step (72 °C, 5 min). The PCR products were digested for 16 h at 37 °C 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 identified 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 *XRCC1* on levels of SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H₂O₂ and SBs H₂O₂R 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 H₂O₂ (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 H₂O₂, 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 H₂O₂ (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* genotype 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 myeloblastic 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, misincorporation 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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5. References

1. Goldman R, Shields PG. Food Mutagens J Nutr. 2003;133 Suppl 3:965S-973S.
2. Bull C, Fenech M. Genome-health nutrigenomics and nutrigenetics: nutritional requirements or 'nutriomes' for chromosomal stability and telomere maintenance at the individual level. Proc Nutr Soc. 2008;67:146-56.
3. Ames BN, Wakimoto P. Are vitamin and mineral deficiencies a major cancer risk? Nature Rev. Cancer. 2002;2:694-704.
4. Fenech M, Ferguson LR. Vitamins/minerals and genomic stability in humans. Mutat Res. 2001;475:1-6
5. Ames BN. DNA damage from micronutrient deficiencies is likely to be a major cause of cancer. Mutat Res. 2001;475:7-20.
6. Duthie SJ. Folic acid deficiency and cancer? Mechanisms of DNA instability. British Med Bull. 1999;55:578-92.
7. Blount BC, Ames BN. DNA damage in folate deficiency, Bailleres Clin Haematol. 1995;8:461-78.
8. Blount BC, Mack MM, Wehr CM, MacGregor JT, Hiatt RA, Wang G, Wickramasinghe SN, Everson RB, Ames BN. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. Proc Natl Acad Sci U S A. 1997;94:3290-95.
9. Abdel-Rahman SZ, Soliman AS, Bondy ML, Omar S, El-Badawy SA, Khaled HM, Seifeldin IA, Levin B. Inheritance of the 194Trp and the 399Gln variant alleles of the DNA repair gene XRCC1 are associated with increased risk of early-onset colorectal carcinoma in Egypt Cancer Lett. 2000;159:79-86.

10. Ali AA, Jukes RM, Pearl LH, Oliver AW. Specific recognition of a multiply phosphorylated motif in the DNA repair scaffold XRCC1 by the FHA domain of human PNK. *Nucleic Acids Res. In press* 2009.
11. Hung RJ, Hall J, Brennan P, Boffetta P. Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review. *Am J Epidemiol.* 2005;162:925-42. Epub 2005 Oct 12.
12. Lunn RM, Langlois RG, Hsieh LL, Thompson CL, Bell DA. XRCC1 polymorphisms: effects on aflatoxin B1-DNA adducts and glycoprotein A variant frequency. *Cancer Res.* 1999;59:2557-61.
13. Cornetta T, Festa F, Testa A, Cozzi R. DNA damage repair and genetic polymorphisms: assessment of individual sensitivity and repair capacity. *Int J Radiat Oncol Biol Phys.* 2006;66:537-45.
14. Tumiala J, Szekely G, Gundy S, Hirvonen A, Norppa H. Genetic polymorphisms of DNA repair and xenobiotic-metabolizing enzymes: role in mutagen sensitivity. *Carcinogenesis.* 2002;23:1003-8.
15. Zhang X, Miao X, Liang G, Hao B, Wang Y, Tan W, Li Y, Guo Y, He F, et al. Polymorphisms in DNA base excision repair genes ADPRT and XRCC1 and risk of lung cancer. *Cancer Res.* 2005;65:722-6.
16. Stern MC, Umbach DM, van Gils CH, Lunn RM, Taylor JA. DNA repair gene XRCC1 polymorphisms, smoking, and bladder cancer risk. *Cancer Epidemiol Biomarkers Prev.* 2001;10:125-31.
17. Shen H, Xu Y, Qian Y, Yu R, Qin Y, Zhou L, Wang X, Spitz MR, Wei Q. Polymorphisms of the DNA repair gene XRCC1 and risk of gastric cancer in a Chinese population. *Int J Cancer.* 2000;88:601-6.

18. Hao B, Wang H, Zhou K, Li Y, Chen X, Zhou G, Zhu Y, Miao X, Tan W, et al. Identification of genetic variants in base excision repair pathway and their associations with risk of esophageal squamous cell carcinoma. *Cancer Res.* 2004;64:4378-84.
19. Cardoso MA, Stocco PR. Development of a dietary assessment method for people of japanese descent living in São Paulo, Brazil. *Cad Saúde Pública, Rio de Janeiro.* 2000;16:107-114.
20. Singh NP, Mccoy MT, Tice RR, Schneider EL. A simple technique for quantification of low levels of DNA damage in individual cells. *Exp Cell Res.* 1988;175:184-91.
21. Collins AR, Raslová K, Somorovská M, Petrovská H, Ondrusová A, Vohnout B, Fábry R, Dusinská M. DNA damage in diabetes: correlation with a clinical marker. *Free Rad Biol Med.* 1998;25:373-7.
22. Giovannelli L, Saieva C, Masala G, Testa G, Salvini S, Pitozzi V, Riboli E, Dolara P, Palli D. Nutritional and lifestyle determinants of DNA oxidative damage? A study in a Mediterranean population. *Carcinogenesis.* 2002;23:1483-89.
23. Perera FP. Environment and cancer: who are susceptible? *Science.* 1997;278:1068-1073.
24. Shields PG & Harris CC. Cancer risk and low penetrance susceptibility genes in gene-environment interactions. *J Clin Oncol.* 2000;18:2309-2315.
25. Shen J, Gammon MD, Terry MB, Wang L, Wang Q, Zhang F, Teitelbaum SL, Eng M, Sagiv SK, et al. Polymorphisms in XRCC1 modify the association between polycyclic aromatic hydrocarbon-DNA adducts, cigarette smoking,

- dietary antioxidants, and breast cancer risk. *Cancer Epidemiol Biomarkers Prev.* 2005;14:336-42.
26. Norppa H. Cytogenetic markers of susceptibility: influence of polymorphic carcinogen-metabolizing enzymes. *Environ Health Perspect.* 1997;105:829-835.
 27. da Silva J, Moraes CR, Heuser VD, Andrade VM, Silva FR, Kvitko K, Emmel V, Rohr P, Bordin DL, et al. Evaluation of genetic damage in a Brazilian population occupationally exposed to pesticides and its correlation with polymorphisms in metabolizing genes. *Mutagenesis.* 2008;23:415-422.
 28. Godderis L, Aka P, Mateuca R, Kirsch-Volders M, Lison D, Veulemans H. Dose-dependent influence of genetic polymorphisms on DNA damage induced by styrene oxide, ethylene oxide and gamma-radiation. *Environ Mol Mutagen.* 2004;44:293-303.
 29. Weng Z, Lu Y, Weng H, Morimoto K. Effects of the XRCC1 Gene-Environment Interactions on DNA Damage in Healthy Japanese Workers. *Environ Mol Mutagen.* 2008;49:708-19.
 30. Matullo G, Guarrera S, Carturan S, Peluso M, Malaveille C, Davico L, Piazza A, Vineis P. DNA repair gene polymorphisms, bulky DNA adducts in white blood cells and bladder cancer: A case-control study. *Int J Cancer.* 2001;92:562-567.
 31. Cappelli E, Taylor R, Cevasco M, Abbondandolo A, Caldecott K, Frosina G. Involvement of XRCC1 and DNA ligase III gene products in DNA base excision repair. *J Biol Chem.* 1997;272:23970-23975.
 32. Satoh MS, Lindhal T. Role of poly(ADP-ribose) formation in DNA repair. *Nature.* 1992;356:356-358.

33. Dantzer F, de La Rubia G, Ménissier-De Murcia J, Hostomsky Z, de Murcia G, Schreiber V. Base excision repair is impaired in mammalian cells lacking poly(ADP-ribose) polymerase-1. *Biochemistry*. 2000;39:7559-7569.
34. Lévy N, Martz A, Bresson A, Spenlehauer C, de Murcia G, Ménissier-de Murcia J. XRCC1 is phosphorylated by DNA-dependent protein kinase in response to DNA damage. *Nuclei Acids Res*. 2006;34:32-41.
35. Gal TJ, Huang WY, Chen C, Hayes RB, Schwartz SM. DNA repair gene polymorphisms and risk of second primary neoplasms and mortality in oral cancer patients. *Laryngoscope*. 2005;115:2221-2231. Erratum in: 2006;116:507.
36. Seedhouse C, Bainton R, Lewis M, Harding A, Russell N, Das-Gupta E. The genotype distribution of the XRCC1 gene indicates a role for base excision repair in the development of therapy-related acute myeloblastic leukemia. *Blood*. 2002;100:3761-3766.
37. Nelson HH, Kelsey KT, Mott LA, Karagas MR. The XRCC1 Arg399Gln polymorphism, sunburn, and non-melanoma skin cancer: Evidence of gene-environment interaction. *Cancer Res*. 2002;62:152-155.
38. Stern MC, Umbach DM, van Gils CH, Lunn RM, Taylor JA. DNA repair gene XRCC1 polymorphisms, smoking, and bladder cancer risk. *Cancer Epidemiol Biomarkers Prev*. 2001;10:125-131.

Legends

Figure 1. Influence of *194Trp* polymorphisms of DNA repair gene *XRCC1* on DNA damage (Tail Intensity) expressed as SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H₂O₂ and SBs H₂O₂R 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 H₂O₂ = level of DNA damage after treatment with 100 µl of H₂O₂ (100 µM), for 30 minutes, in ice and SBs H₂O₂R = 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 *XRCC1* on DNA damage (Tail Intensity) expressed as SBs, SBs FPG, SBs Endo III, SBs UDG, SBs H₂O₂ and SBs H₂O₂R 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 H₂O₂ = level of DNA damage after treatment with 100 µl of H₂O₂ (100 µM), for 30 minutes, in ice and SBs H₂O₂R = 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.

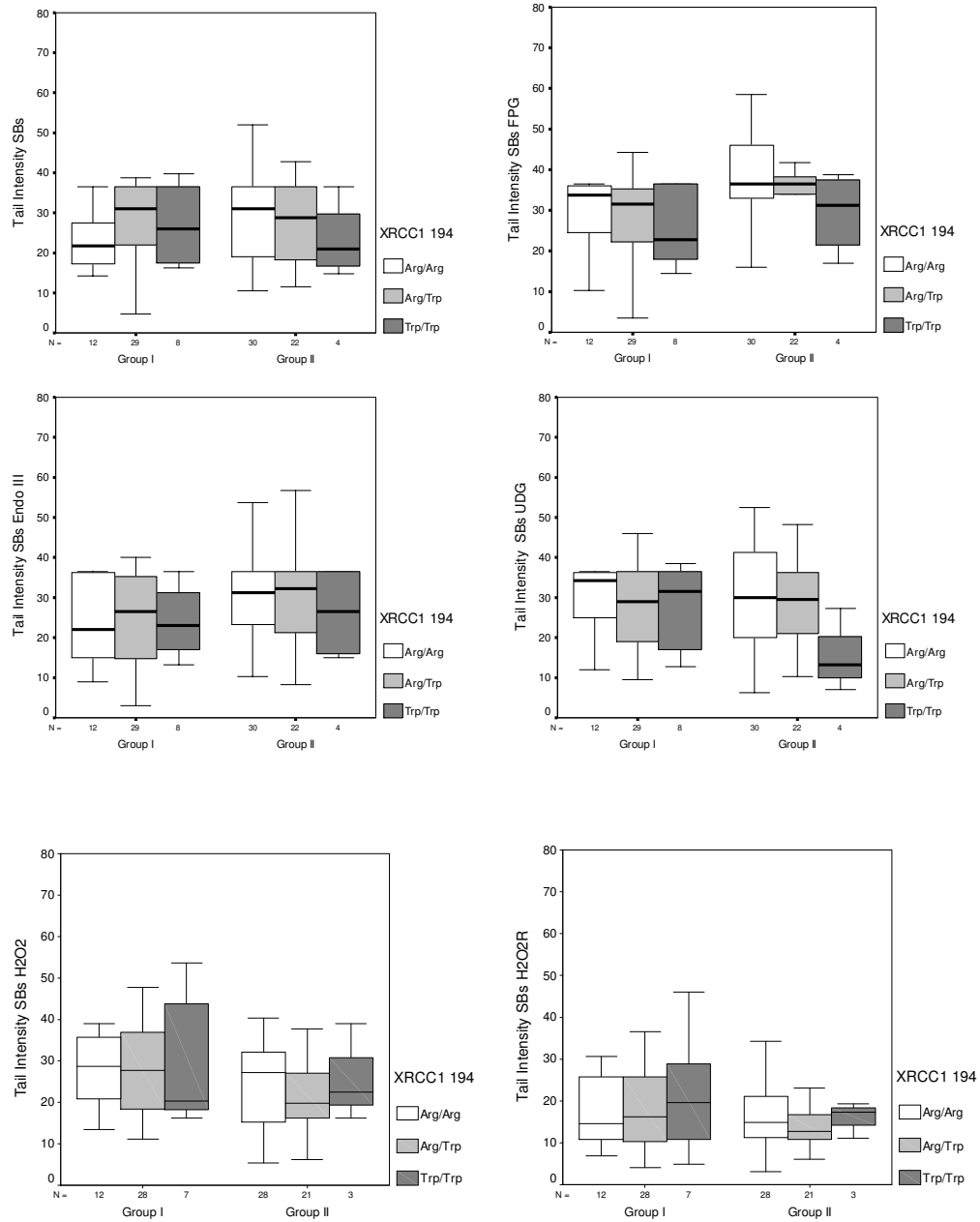


Figure 1.

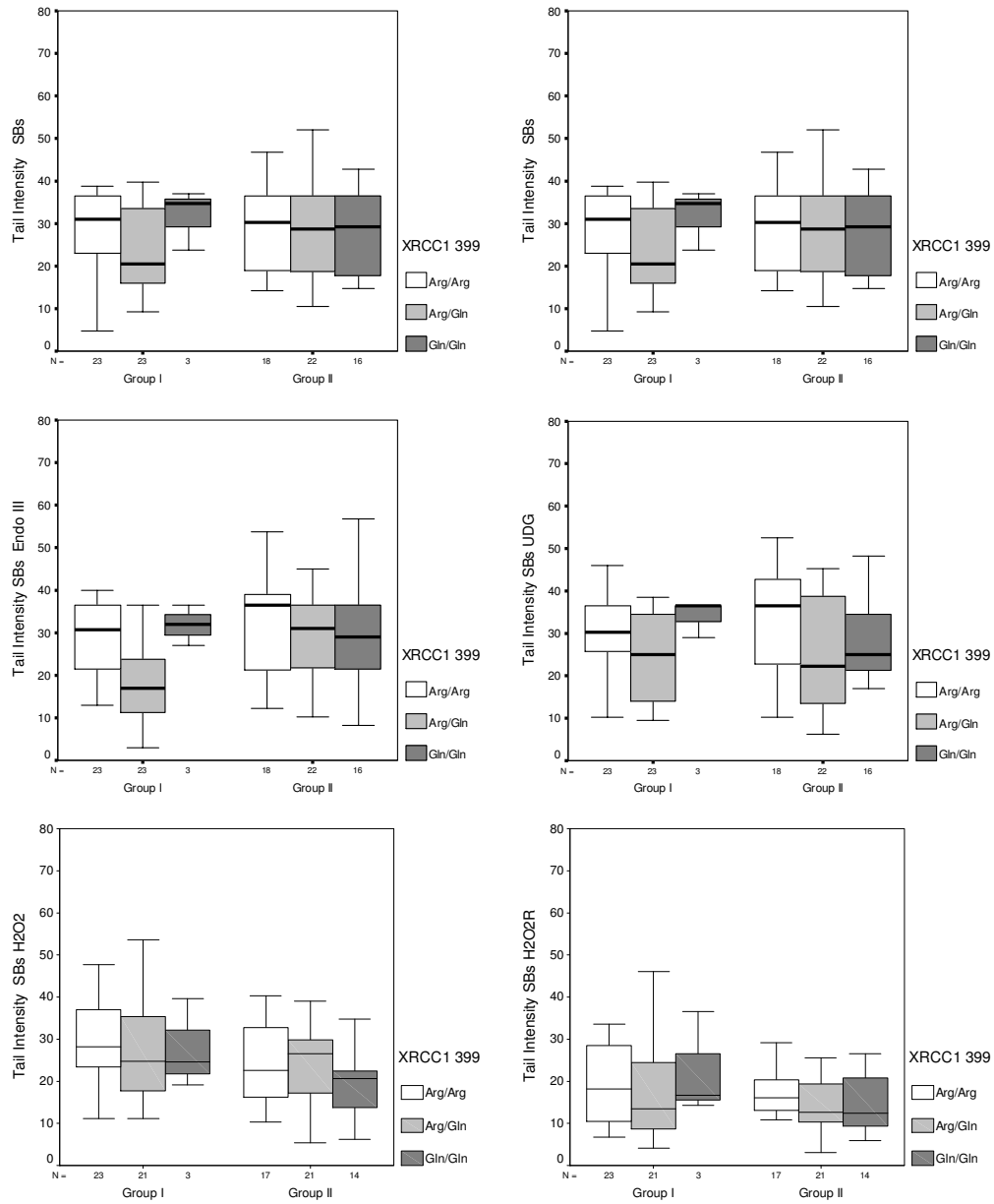


Figure 2.

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 μ M) 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].

Ferguson [3] showed that chemical additives used in meat preservation (N-nitros 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 $35,6 \pm 11,4$, 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 μ l 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°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 endonuclease 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 \cong 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 μ l of H₂O₂ (100 μ M), for 30 minutes, in ice, and analysis of the level of damage was evaluated. Then, after two wash in PBS to remove of H₂O₂, 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 CCTCCATCCAGAACTGGCG primers.

The PCR reaction was performed using 25mM MgCl₂, 100mM dNTPs, 10 μ M of each primer, 1.5U/ μ l Taq DNA polymerase (Invitrogen), and 100ng/ μ L DNA, in a volume of 27 μ l. Cycling conditions for each amplification were: 94°C for 30 seconds, followed by 94°C for 1 minute (step 2), 59°C for 1 minute, 72°C for 2 minutes, and the process returned to step 2 and was repeated 39 times; this was followed by a final extension cycle of 72°C for 10 minutes. Products with 445pb were obtained. A 15 μ l aliquot of PCR product was submitted to 1U BsmA I enzyme (Bio Labs-New England) and incubated at 55°C for 12 hours. The resulting products were visualized in 2.5% agarose gel and stained with ethidium bromide. The wild Ile 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 H₂O₂ and SBs H₂O₂R 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 H₂O₂ (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 lead to inhibition of enzyme activity, we hypothesized that this enzymatic deficiency 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 induced with treatment of H₂O₂ (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.

5. References

- 1- Bingham S, Riboli E. Diet and Cancer – The European Prospective Investigation into Cancer and Nutrition. *Rev Nature*. 2004;4:206-215.
- 2- Doll R, Peto R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J. Natl. Cancer Inst.* 1981;66:1191-308.
- 3- Ferguson LR. Natural and human-made mutagens and carcinogens in the human diet. *Toxicology*. 2002;181-182:79-82.
- 4- Ames BN. DNA damage from micronutrient deficiencies is likely to be a major cause of cancer. *Mutat Res*. 2001;475:7-20.
- 5- Eto I, Krumdieck CL. Role of vitamin B12 and folate deficiencies in carcinogenesis. *Adv Exp Med Biol*. 1986;206:313-30.
- 6- Andersen S, Heine T, Sneve R, König I, Krokan HE, Epe B, Nilsen H. Incorporation of dUMP into DNA is a major source of spontaneous DNA damage, while excision of uracil is not required for cytotoxicity of fluoropyrimidines in mouse embryonic fibroblasts. *Carcinogenesis*. 2005;26:547-555.
- 7- Ames BN, Wakimoto P. Are vitamin and mineral deficiencies a major cancer risk? *Nature Rev Cancer*. 2002;2:694-704.
- 8- Fenech M, Ferguson LR. Vitamins/minerals and genomic stability in humans. *Mutat Res*. 2001;475:1-6.
- 9- Louro ID, Llerena JR, Melo MSV, Ashton-Prola P, Conforti-Froes N. *Genética Molecular do Câncer*, 2^a ed. São Paulo, MSG produção Editorial, 2002.
- 10- Miller JA, Miller EC. Ultimate chemical carcinogen as reactive mutagenic electrophiles. In: Hiatt HH, Watson JD, Winstein JA. (Eds). *Origins of human*

- cancer, Cold Spring Laboratory. New York: Cold Spring Harbor 1977. p.605-628.
- 11- Guengerich FP. Catalytic selectivity of human cytochrome P450 enzymes: relevance to drug metabolism and toxicity. *Toxicol Lett.* 1994;70:133-138.
 - 12- Tjhuis MJ, Visker MH, Aarts JM, Peters WH, Roelofs HM, den Camp LO, Rietjens IM, Boerboom AM, Nagengast FM, et al. Glutathione s-transferase phenotypes in relation to genetic variation and fruit and vegetable consumption in an endoscopy-based population. *Carcinogenesis.* 2007;28:848-57.
 - 13- Strange RC, Fryer AA. The glutathione s-transferases: influence of polymorphisms on cancer susceptibility. In: VINEIS P, MALATS N, LANG M, et al. *Metabolic polymorphisms and susceptibility to cancer.* 1st ed. France: IARC, 1999. Cap. 19, p. 231-249.
 - 14- Harries LW, Stubbins MJ, Forman D, Howard GC, Wolf CR. Identification of genetic polymorphism at the glutathione S-transferase Pi locus and association with susceptibility to bladder, testicular and prostate cancer. *Carcinogenesis.* 1997;18:641-4.
 - 15- Leichsenring A, Losi-Guembarovski R, Maciel ME, Losi-Guembarovski A, Oliveira BW, Ramos G, Cavalcanti TC, Bicalho MG, Cavalli IJ, et al. CYP1A1 and GSTP1 polymorphisms in a oral cancer case-control study. *Braz J Med Biol Res.* 2006;39:1569-74.
 - 16- Cardoso MA, Stocco PR. Development of a dietary assessment method for people of japanese descent living in São Paulo, Brazil. *Cad Saúde Pública, Rio de Janeiro.* 2000;16:107-114.
 - 17- Singh NP, Mccoy MT, Tice RR, Schneider EL. A simple technique for quantification of low levels of DNA damage in individual cells. *Exp Cell Res.* 1988;175:184-191.

- 18- Collins AR, Raslová K, Smorovská MP, Petrovská H, Ondrusová A, Vohnout B, Fábry R, Dusinská M. DNA damage in diabetes: correlation with a clinical marker. *Free Rad Biol Med.* 1998;25:373-377.
- 19- Duthie SJ. Folic acid deficiency and cancer? Mechanisms of DNA instability. *British Med. Bull.* 1999;55:578-592.
- 20- Kirkland DJ, Hayashi M, Jacobson-Kram D, Kasper P, MacGregor JT, Müller L, Uno Y. Summary of major conclusions from the 4th IWGT, San Francisco, 9-10 September. *Mutat Res.* 2007;627:5-9.
- 21- Welfare M, Adeokun AM, Bassendine MF, Daly AK. Polymorphisms in GSTP1, GSTM1 and GSTT1 and susceptibility to colorectal cancer. *Cancer Epidemiol Biomarkers Prev.* 1999;8:289-292.
- 22- Perera FP. Environment and cancer: who are susceptible? *Science.* 1997;278:1068-1073.
- 23- Shields PG, Harris CC. Cancer risk and low penetrance susceptibility genes in gene-environment interactions. *J Clin Oncol.* 2000;18:2309-15.
- 24- Goldman R, Shields PG. Food Mutagens. *J Nutr.* 2003;133 Suppl 3:965S-973S.
- 25- Shen J, Gammon MD, Terry MB, Wang L, Wang Q, Zhang F, Teitelbaum SL, Eng M, Sagiv SK, et al. Polymorphisms in XRCC1 modify the association between polycyclic aromatic hydrocarbon-DNA adducts, cigarette smoking, dietary antioxidants, and breast cancer risk. *Cancer Epidemiol Biomarkers Prev.* 2005;14:336-42.
- 26- Norppa H. Cytogenetic markers of susceptibility: influence of polymorphic carcinogen-metabolizing enzymes. *Environ Health Perspect.* 1997;105:829-835.
- 27- da Silva J, Moraes CR, Heuser VD, Andrade VM, Silva FR, Kvitko K, Emmel V, Rohr P, Bordin DL, et al. Evaluation of genetic damage in a Brazilian

population occupationally exposed to pesticides and its correlation with polymorphisms in metabolizing genes. *Mutagenesis* 2008;23:415-422.

- 28- Dusinská M, Ficek A, Horská A, Raslová K, Petrovská H, Vallová B, Drlicková M, Wood SG, Stupáková A, et al. Glutathione s-transferase polymorphisms influence the level of oxidative DNA damage and antioxidant protection in humans. *Mutat Res.* 2001;482:47-55.
- 29- Naccarati A, Soucek P, Stetina R, Haufroid V, Kumar R, Vodickova L, Trtkova K, Dusinska M, Hemminki K, et al. Genetic polymorphisms and possible gene-gene interactions in metabolic and DNA repair genes: effects on DNA damage. *Mutat Res.* 2006;593:22-31.

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 H₂O₂ and SBs H₂O₂R 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 H₂O₂ = level of DNA damage after treatment with 100 µl of H₂O₂ (100 µM), for 30 minutes, in ice and SBs H₂O₂R = 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.

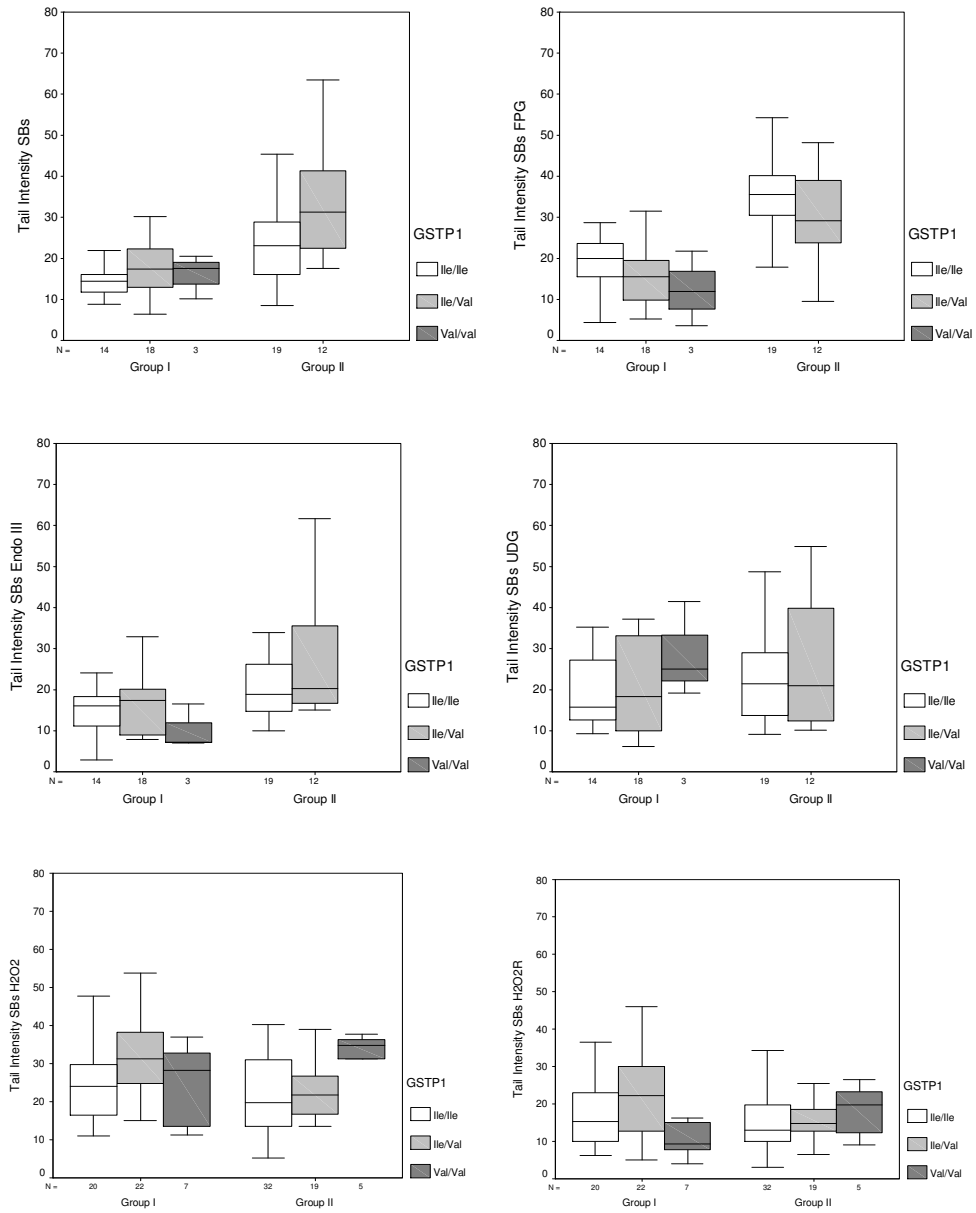


Figure 1.

Manuscrito IV

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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 genomic 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-methyletetrahydrofolate, 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-methylenetetrahydrofolate 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 Department, 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 $35,6 \pm 11,4$, 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 commercially 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'-CATGTCCACAGCATGGAG-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] ($\cong 2 \times 10^4$ 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°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 endonuclease 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 \cong 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 H₂O₂ (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 H₂O₂, 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 *I298CC* 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 *I298CC* genotype has be found to have 60% of the *I298AA* 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 *I298CC* 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* polymorphisms, 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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Literature Cited

- 1- Block G, Patterson B, Subar A. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr cancer*. 1992;18:1-29
- 2- Fenech M, Ferguson LR. Vitamins/minerals and genomic stability in humans. *Mutat Res*. 2001;475:1-6.
- 3- Ames BN. Micronutrients prevent cancer and delay aging. *Toxicol Lett*. 1998;102-103:5-18.
- 4- Glynn SA, Albanes D. Folate and cancer: a review of the literature. *Nutr Cancer*. 1994;22:101-19.
- 5- Glynn SA, Albanes D, Pietinen P, Brown CC, Rautalahti M, Tangrea JA, Gunter EW, Barrett MJ, Virtamo J, Taylor PR. Colorectal cancer and folate status: a nested case-control study among male smokers. *Cancer Epidemiol Biomarkers Prev*. 1996;5:487-94.
- 6- Giovannucci E. Epidemiologic studies of folate and colorectal neoplasia: a review. *J Nutr*. 2002;132(8 Suppl):2350S-2355S.
- 7- Fenech M. The role of folic acid and Vitamin B12 in genomic stability of human cells. *Mutat Res*. 2001;475:57-67.
- 8- Bailey LB, Duhaney RL, Maneval DR, Kauwell GP, Quinlivan EP, Davis SR, Cuadras A, Hutson AD, Gregory JF 3rd. Vitamin B-12 status is inversely associated with plasma homocysteine in young women with C677T and/or A1298C methylenetetrahydrofolate reductase polymorphisms. *J Nutr*. 2002;132:1872-8.

- 9- Selhub J, Miller JW. The pathogenesis of homocysteinemia: interruption of the coordinate regulation by S-adenosylmethionine of the remethylation and transsulfuration of homocysteine. *Am J Clin Nutr.* 1992;55:131-8.
- 10-Zingg JM, Jones PA. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. *Carcinogenesis.* 1997;18:869-82.
- 11-Yi P, Melnyk S, Pogribna M, Pogribny IP, Hine RJ, James SJ. Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation. *J Biol Chem.* 2000;275:29318-23.
- 12-Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature.* 1983;301:89-92.
- 13-Duthie SJ, Narayanan S, Sharp L, Little J, Basten G, Powers H. Folate, DNA stability and colo-rectal neoplasia. *Proc Nutr Soc.* 2004;63:571-8.
- 14-Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I. Hyperhomocysteinemia: an independent risk factor for vascular disease. *N Engl J Med.* 1991;324:1149-55.
- 15-Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. *BMJ.* 2002 Nov 23;325(7374):1202.
- 16-Prins ND, Den Heijer T, Hofman A, Koudstaal PJ, Jolles J, Clarke R, Breteler MM; Rotterdam Scan Study. Homocysteine and cognitive function in the elderly: the Rotterdam Scan Study. *Neurology.* 2002;59:1375-80.

- 17-Oikawa S, Murakami K, Kawanishi S. Oxidative damage to cellular and isolated DNA by homocysteine: implications for carcinogenesis. *Oncogene*. 2003;22:3530-8.
- 18-Blount BC, Mack MM, Wehr CM, MacGregor JT, Hiatt RA, Wang G, Wickramasinghe SN, Everson RB, Ames BN. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. *Proc Natl Acad Sci U S A*. 1997;94:3290-5.
- 19-Melnyk S, Pogribna M, Miller BJ, Basnakian AG, Pogribny IP, James SJ. Uracil misincorporation, DNA strand breaks, and gene amplification are associated with tumorigenic cell transformation in folate deficient/repleted Chinese hamster ovary cells. *Cancer Lett*. 1999;146:35-44.
- 20-Blount BC, Ames BN. DNA damage in folate deficiency. *Baillieres Clin Haematol*. 1995;8:461-78.
- 21-Sharp L, Little J. Polymorphisms in genes involved in folate metabolism and colorectal neoplasia: a HuGe review. *Am J Clin Epidemiol* 2004;159:423-43.
- 22-van der Put NM, Gabreëls F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, van den Heuvel LP, Blom HJ. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? *Am J Hum Genet*. 1998;62:1044-51.
- 23-Wisberg I, Tran P, Christensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. *Mol Gen Metab*. 1998;64:169-172.
- 24-Lievers KJ, Boers GH, Verhoef P, den Heijer M, Kluijtmans LA, van der Put NM, Trijbels FJ, Blom HJ. A second common variant in the methylenetetrahydrofolate reductase (MTHFR) gene and its relationship to

- MTHFR enzyme activity, homocysteine, and cardiovascular disease risk. *J Mol Med.* 2001;79:522-8.
- 25-Viel A, Dall'Agnes L, Simone F, Canzonieri V, Capozzi E, Visentin MC, Valle R, Boiocchi M. Loss of heterozygosity at the 5,10-methylenetetrahydrofolate reductase locus in human ovarian carcinomas. *Br J Cancer.* 1997;75:1105-10.
- 26-Skibola CF, Smith MT, Kane E, Roman E, Rollinson S, Cartwright RA, Morgan G. Polymorphisms in the methylenetetrahydrofolate reductase gene are associated with susceptibility to acute leukemia in adults. *Proc Natl Acad Sci U S A.* 1999;96:12810-5.
- 27-Wiemels JL, Smith RN, Taylor GM, Eden OB, Alexander FE, Greaves MF; United Kingdom Childhood Cancer Study investigators. Methylenetetrahydrofolate reductase (MTHFR) polymorphisms and risk of molecularly defined subtypes of childhood acute leukemia. *Proc Natl Acad Sci U S A.* 2001;98:4004-9.
- 28-Chango A, Boisson F, Barbé F, Quilliot D, Droesch S, Pfister M, Fillon-Emery N, Lambert D, Frémont S, Rosenblatt DS, Nicolas JP. The effect of 677C-->T and 1298A-->C mutations on plasma homocysteine and 5,10-methylenetetrahydrofolate reductase activity in healthy subjects. *Br J Nutr.* 2000;83:593-6.
- 29-Moyers S, Bailey LB. Fetal malformations and folate metabolism: review of recent evidence. *Nutr Rev.* 2001;59:215-24.
- 30-Eden A, Gaudet F, Waghmare A, Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science.* 2003;300:455.
- 31-Krajcinovic M, Lamothe S, Labuda D, Lemieux-Blanchard E, Theoret Y, Moghrabi A, Sinnett D. Role of MTHFR genetic polymorphisms in the

- susceptibility to childhood acute lymphoblastic leukemia. *Blood*. 2004;103:252-7.
Epub 2003 Sep 4.
- 32-Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet*. 1995;10:111-3.
- 33-Giovanelli L, Saieva C, Masala G, Testa G, Salvini S, Pitozzi V, Riboli E, Dolara P, Palli D. Nutritional and lifestyle determinants of DNA oxidative damage? A study in a Mediterranean population. *Carcinogenesis*. 2002;23:1483-89.
- 34-Chen J, Ma J, Stampfer MJ, Palomeque C, Selhub J, Hunter DJ. Linkage disequilibrium between the 677C>T and 1298A>C polymorphisms in human methylenetetrahydrofolate reductase gene and their contributions to risk of colorectal cancer. *Pharmacogenetics*. 2002;12:339-42.
- 35-Keku T, Millikan R, Worley K, Winkel S, Eaton A, Biscocho L, Martin C, Sandler R. 5,10-Methylenetetrahydrofolate reductase codon 677 and 1298 polymorphisms and colon cancer in African Americans and whites. *Cancer Epidemiol Biomarkers Prev*. 2002;11:1611-21.
- 36-Murtaugh MA, Curtin K, Sweeney C, Wolff RK, Holubkov R, Caan BJ, Slattery ML. Dietary intake of folate and co-factors in folate metabolism, MTHFR polymorphisms, and reduced rectal cancer. *Cancer Causes Control*. 2007;18:153-63. Epub 2007 Jan 23.
- 37-James SJ, Basnakian AG, Miller BJ. In vitro folate deficiency induces deoxynucleotide pool imbalance, apoptosis, and mutagenesis in Chinese hamster ovary cells. *Cancer Res*. 1994;54:5075-80.

- 38-Zittoun J, Tonetti C, Bories D, Pignon JM, Tulliez M. Plasma homocysteine levels related to interactions between folate status and methylenetetrahydrofolate reductase: a study in 52 healthy subjects. *Metabolism*. 1998;47:1413-8.
- 39-Wickramasinghe SN, Fida S. Bone marrow cells from vitamin B12- and folate-deficient patients misincorporate uracil into DNA. *Blood*. 1994;83:1656-61.
- 40-Mattson MP, Kruman II, Duan W. Folic acid and homocysteine in age-related disease. *Ageing Res Rev*. 2002;1:95-111.
- 41-Choi SW, Mason JB. Folate and carcinogenesis: an integrated scheme. *J Nutr*. 2000;130:129-32.
- 42-Shields DC, Kirke PN, Mills JL, Ramsbottom D, Molloy AM, Burke H, Weir DG, Scott JM, Whitehead AS. The "thermolabile" variant of methylenetetrahydrofolate reductase and neural tube defects: An evaluation of genetic risk and the relative importance of the genotypes of the embryo and the mother. *Am J Hum Genet*. 1999;64:1045-55.
- 43-Pogribny IP, Basnakian AG, Miller BJ, Lopatina NG, Poirier LA, James SJ. Breaks in genomic DNA and within the p53 gene are associated with hypomethylation in livers of folate/methyl-deficient rats. *Cancer Res*. 1995;55:1894-901. Erratum in: *Cancer Res* 1995;55:2711.
- 44-Wainfan E, Poirier LA. Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. *Cancer Res*. 1992;52(7 Suppl):2071s-2077s.
- 45-Foksinski M, Gackowski D, Rozalski R, Siomek A, Guz J, Szpila A, Dziaman T, Olinski R. Effects of basal level of antioxidants on oxidative DNA damage in humans. *Eur J Nutr*. 2007;46:174-80. Epub 2007 Jan 30.
- 46-Ames BN. DNA damage from micronutrient deficiencies is likely to be a major cause of cancer. *Mut. Res*. 2001;475:7-20.

- 47-Friso S, Girelli D, Trabetti E, Stranieri C, Olivieri O, Tinazzi E, Martinelli N, Faccini G, Pignatti PF, Corrocher R. A1298C methylenetetrahydrofolate reductase mutation and coronary artery disease: relationships with C677T polymorphism and homocysteine/folate metabolism. *Clin Exp Med.* 2002;2:7-12.
- 48-Gellekink H, den Heijer M, Heil SG, Blom HJ. Genetic determinants of plasma total homocysteine. *Semin Vasc Med.* 2005;5:98-109.
- 49-Friedman G, Goldschmidt N, Friedlander Y, Ben-Yehuda A, Selhub J, Babaey S, Mendel M, Kidron M, Bar-On H. A common mutation A1298C in human methylenetetrahydrofolate reductase gene: association with plasma total homocysteine and folate concentrations. *J Nutr.* 1999;129:1656-61.
- 50-Yin G, Kono S, Toyomura K, Hagiwara T, Nagano J, Mizoue T, Mibu R, Tanaka M, Kakeji Y, Maehara Y, Okamura T, Ikejiri K, Futami K, Yasunami Y, Maekawa T, Takenaka K, Ichimiya H, Imaizumi N. Methylenetetrahydrofolate reductase C677T and A1298C polymorphisms and colorectal cancer: the Fukuoka Colorectal Cancer Study. *Cancer Sci.* 2004;95:908-13.
- 51-Eaton AM, Sandler R, Carethers JM, Millikan RC, Galanko J, Keku TO. 5,10-methylenetetrahydrofolate reductase 677 and 1298 polymorphisms, folate intake, and microsatellite instability in colon cancer. *Cancer Epidemiol Biomarkers Prev.* 2005;14:2023-9.

Abbreviation 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 H₂O₂ and SBs H₂O₂R 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 H₂O₂ = level of DNA damage after treatment with 100 µl of H₂O₂ (100 µM), for 30 minutes, in ice and SBs H₂O₂R = 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).

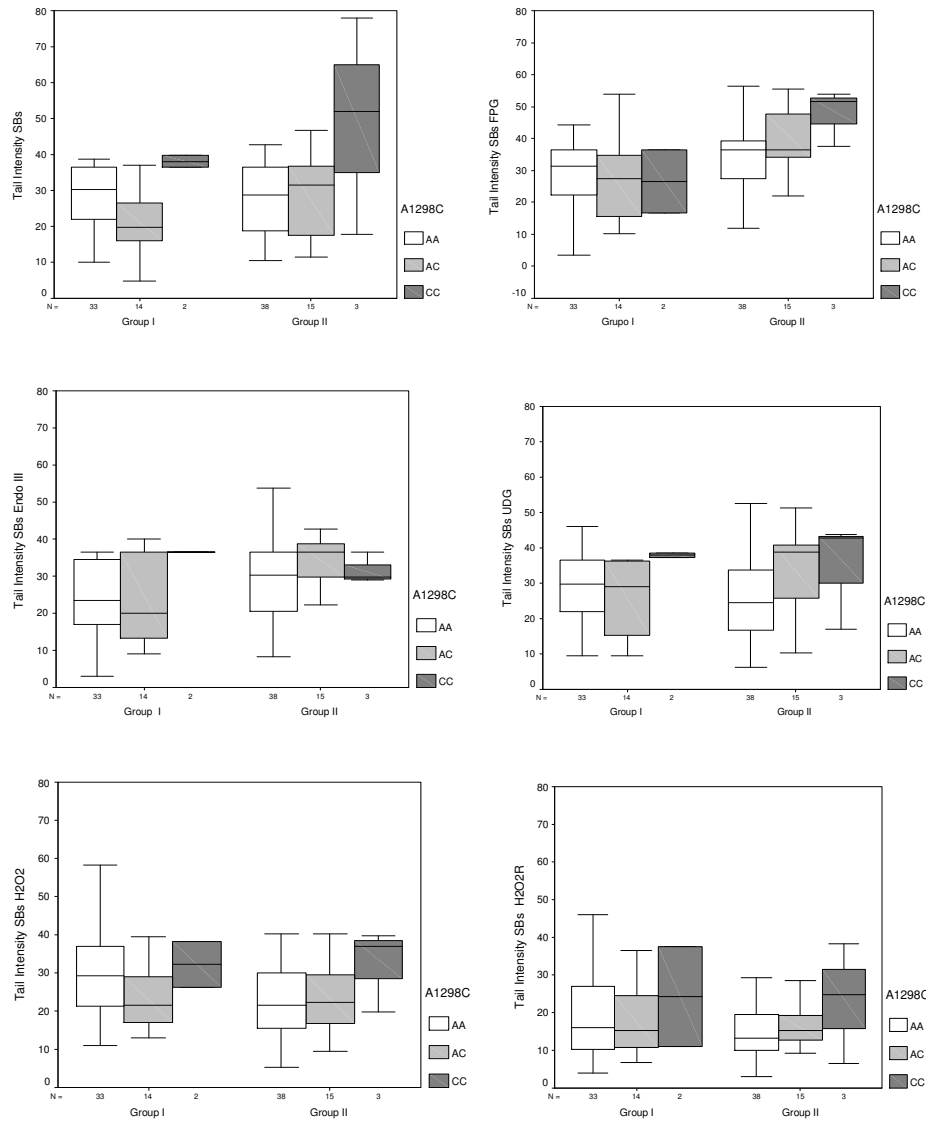
**Figure 1.**

Table 1.

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

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

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-methylenetetrahydrofolate 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 (*CTs*) and homozygous (*TTs*) 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 Department, 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 $35,6 \pm 11,4$, 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 commercially 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 *HinfI* digestion site, the amplified product derived from the mutant gene was cleaved into 175-bp and 23-bp fragments by *HinfI*, 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 μ l of the isolated lymphocytes suspension [35] ($\cong 2 \times 10^4$ 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°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 μ L of endonuclease III (Endo III - 1:1000; New England Biolabs Inc), 100 μ L of formamidopyrimidine-DNA glycosylase (FPG - 1:1000; New England Biolabs Inc), 100 μ L 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 \cong 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 C677T* on the DNA repair capability, 200 µl of the freshly isolated lymphocytes were treated with 100 µl of H₂O₂ (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 H₂O₂, 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 μ l of H₂O₂ (100 μ M), 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 H₂O₂. 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 (*CTs*) and homozygous (*TTs*) 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 H₂O₂, 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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Literature Cited

- 1- Block G, Patterson B, Subar A. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr Cancer*. 1992;18:1-29.
- 2- Fenech M, Ferguson LR. Vitamins/minerals and genomic stability in humans. *Mutat Res*. 2001;475:1-6.
- 3- Ames BN. Micronutrients prevent cancer and delay aging. *Toxicol Lett*. 1998;102-103:5-18.
- 4- Glynn SA, Albanes D. Folate and cancer: a review of the literature. *Nutr Cancer*. 1994;22:101-19.
- 5- Glynn SA, Albanes D, Pietinen P, Brown CC, Rautalahti M, Tangrea JA, Gunter EW, Barrett MJ, Virtamo J, Taylor PR. Colorectal cancer and folate status: a nested case-control study among male smokers. *Cancer Epidemiol Biomarkers Prev*. 1996;5:487-94.
- 6- Giovannucci E. Epidemiologic studies of folate and colorectal neoplasia: a review. *J Nutr*. 2002;132(8 Suppl):2350S-2355S.
- 7- Fenech M. The role of folic acid and Vitamin B12 in genomic stability of human cells. *Mutat Res*. 2001;475:57-67.
- 8- Bailey LB, Duhaney RL, Maneval DR, Kauwell GP, Quinlivan EP, Davis SR, Cuadras A, Hutson AD, Gregory JF 3rd. Vitamin B-12 status is inversely associated with plasma homocysteine in young women with C677T and/or A1298C methylenetetrahydrofolate reductase polymorphisms. *J Nutr*. 2002;132:1872-8.

- 9- Selhub J, Miller JW. The pathogenesis of homocysteinemia: interruption of the coordinate regulation by S-adenosylmethionine of the remethylation and transsulfuration of homocysteine. *Am J Clin Nutr.* 1992;55:131-8.
- 10- Zingg JM, Jones PA. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. *Carcinogenesis.* 1997;18:869-82.
- 11- Yi P, Melnyk S, Pogribna M, Pogribny IP, Hine RJ, James SJ. Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation. *J Biol Chem.* 2000;275:29318-23.
- 12- Blount BC, Mack MM, Wehr CM, MacGregor JT, Hiatt RA, Wang G, Wickramasinghe SN, Everson RB, Ames BN. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. *Proc Natl Acad Sci U S A.* 1997;94:3290-5.
- 13- Melnyk S, Pogribna M, Miller BJ, Basnakian AG, Pogribny IP, James SJ. Uracil misincorporation, DNA strand breaks, and gene amplification are associated with tumorigenic cell transformation in folate deficient/repleted Chinese hamster ovary cells. *Cancer Lett.* 1999;146:35-44.
- 14- Blount BC, Ames BN. DNA damage in folate deficiency. *Baillieres Clin Haematol.* 1995;8:461-78.
- 15- Duthie SJ, Narayanan S, Sharp L, Little J, Basten G, Powers H. Folate, DNA stability and colo-rectal neoplasia. *Proc Nutr Soc.* 2004;63:571-8.

- 16- Fenech MF, Dreosti IE, Rinaldi JR. Folate, vitamin B12, homocysteine status and chromosome damage rate in lymphocytes of older men. *Carcinogenesis*. 1997;18:1329-36.
- 17- Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet*. 1995;10:111-3.
- 18- Crott JW, Mashiyama ST, Ames BN, Fenech M. The effect of folic acid deficiency and MTHFR C677T polymorphism on chromosome damage in human lymphocytes in vitro. *Cancer Epidemiol Biomarkers Prev*. 2001;10:1089-96.
- 19- Zittoun J, Tonetti C, Bories D, Pignon JM, Tulliez M. Plasma homocysteine levels related to interactions between folate status and methylenetetrahydrofolate reductase: a study in 52 healthy subjects. *Metabolism*. 1998;47:1413-8.
- 20- Kluijtmans LA, Kastelein JJ, Lindemans J, Boers GH, Heil SG, Bruschke AV, Jukema JW, van den Heuvel LP, Trijbels FJ, Boerma GJ, Verheugt FW, Willems F, Blom HJ. Thermolabile methylenetetrahydrofolate reductase in coronary artery disease. *Circulation*. 1997;96:2573-7.
- 21- Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, Selhub J, Rozen R. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation*. 1996;93:7-9.
- 22- Kark JD, Selhub J, Adler B, Gofin J, Abramson JH, Friedman G, Rosenberg IH. Nonfasting plasma total homocysteine level and mortality

- in middle-aged and elderly men and women in Jerusalem. *Ann Intern Med.* 1999;131:321-30.
- 23- Fenech M, Aitken C, Rinaldi J. Folate, vitamin B12, homocysteine status and DNA damage in young Australian adults. *Carcinogenesis.* 1998;19:1163-71.
- 24- Oikawa S, Murakami K, Kawanishi S. Oxidative damage to cellular and isolated DNA by homocysteine: implications for carcinogenesis. *Oncogene.* 2003;22:3530-8.
- 25- Sadeghian S, Fallahi F, Salarifar M, Davoodi G, Mahmoodian M, Fallah N, Darvish S, Karimi A; Tehran Heart Center. Homocysteine, vitamin B12 and folate levels in premature coronary artery disease. *BMC Cardiovasc Disord.* 2006;6:38.
- 26- Esteller M, Garcia A, Martinez-Palones JM, Xercavins J, Reventos J. Germ line polymorphisms in cytochrome-P450 1A1 (C4887 CYP1A1) and methylenetetrahydrofolate reductase (MTHFR) genes and endometrial cancer susceptibility. *Carcinogenesis.* 1997;18:2307-11.
- 27- Chen J, Giovannucci EL, Hunter DJ. MTHFR polymorphism, methyl-replete diets and the risk of colorectal carcinoma and adenoma among U.S. men and women: an example of gene-environment interactions in colorectal tumorigenesis. *J Nutr.* 1999;129(2S Suppl):560S-564S.
- 28- Ma J, Stampfer MJ, Giovannucci E, Artigas C, Hunter DJ, Fuchs C, Willett WC, Selhub J, Hennekens CH, Rozen R. Methylenetetrahydrofolate reductase polymorphism, dietary interactions, and risk of colorectal cancer. *Cancer Res.* 1997;57:1098-102.

- 29- Skibola CF, Smith MT, Kane E, Roman E, Rollinson S, Cartwright RA, Morgan G. Polymorphisms in the methylenetetrahydrofolate reductase gene are associated with susceptibility to acute leukemia in adults. *Proc Natl Acad Sci U S A.* 1999;96:12810-5.
- 30- Wiemels JL, Smith RN, Taylor GM, Eden OB, Alexander FE, Greaves MF; United Kingdom Childhood Cancer Study investigators. Methylenetetrahydrofolate reductase (MTHFR) polymorphisms and risk of molecularly defined subtypes of childhood acute leukemia. *Proc Natl Acad Sci U S A.* 2001;98:4004-9.
- 31- Cardoso MA, Stocco PR. Development of a dietary assessment method for people of Japanese descent living in São Paulo, Brazil. *Cad Saúde Pública, Rio de Janeiro.* 2000;16:107-114.
- 32- Van Der Put NM, Steegers-Theunissen RP, Frosst P, Trijbels FJ, Eskes TK, Van Den Heuvel LP, Mariman EC, Den Heyer M, Rozen R, Blom HJ. Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. *Lancet.* 1995;346:1070-1.
- 33- Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantification of low levels of DNA damage in individual cells. *Exp Cell Res.* 1988;175:184-91.
- 34- Collins AR, Raslová K, Somorovská M, Petrovská H, Ondrusová A, Vohnout B, Fábry R, Dusinská M. DNA damage in diabetes: correlation with a clinical marker. *Free Rad Biol Med.* 1998;25:373-7.
- 35- Giovanelli L, Saieva C, Masala G, Testa G, Salvini S, Pitozzi V, Riboli E, Dolaro P, Palli D. Nutritional and lifestyle determinants of DNA oxidative

- damage? A study in a Mediterranean population. *Carcinogenesis*. 2002;23:1483-89.
- 36- Choi SW, Mason JB. Folate and carcinogenesis: an integrated scheme. *J Nutr*. 2000;130:129-32.
- 37- Goodman MT, McDuffie K, Hernandez B, Wilkens LR, Bertram CC, Killeen J, Le Marchand L, Selhub J, Murphy S, Donlon TA. Association of methylenetetrahydrofolate reductase polymorphism C677T and dietary folate with the risk of cervical dysplasia. *Cancer Epidemiol Biomarkers Prev*. 2001;10:1275-80.
- 38- Shen H, Xu Y, Zheng Y, Qian Y, Yu R, Qin Y, Wang X, Spitz MR, Wei Q. Polymorphisms of 5,10-methylenetetrahydrofolate reductase and risk of gastric cancer in a Chinese population: a case-control study. *Int J Cancer*. 2001;95:332-6.
- 39- Miao X, Xing D, Tan W, Qi J, Lu W, Lin D. Susceptibility to gastric cardia adenocarcinoma and genetic polymorphisms in methylenetetrahydrofolate reductase in an at-risk Chinese population. *Cancer Epidemiol Biomarkers Prev*. 2002;11:1454-8.
- 40- Crott JW, Mashiyama ST, Ames BN, Fenech MF. Methylenetetrahydrofolate reductase C677T polymorphism does not alter folic acid deficiency-induced uracil incorporation into primary human lymphocyte DNA in vitro. *Carcinogenesis*. 2001;22:1019-25.
- 41- Narayanan S, McConnell J, Little J, Sharp L, Piyathilake CJ, Powers H, Basten G, Duthie SJ. Associations between two common variants C677T and A1298C in the methylenetetrahydrofolate reductase gene and measures of folate metabolism and DNA stability (strand breaks,

misincorporated uracil, and DNA methylation status) in human lymphocytes in vivo. *Cancer Epidemiol Biomarkers Prev.* 2004;13:1436-43.

42- Zijno A, Andreoli C, Leopardi P, Marcon F, Rossi S, Caiola S, Verdina A, Galati R, Cafolla A, Crebelli R. Folate status, metabolic genotype, and biomarkers of genotoxicity in healthy subjects. *Carcinogenesis.* 2003;24:1097-103.

43- Botto N, Andreassi MG, Manfredi S, Masetti S, Cocci F, Colombo MG, Storti S, Rizza A, Biagini A. Genetic polymorphisms in folate and homocysteine metabolism as risk factors for DNA damage. *Eur J Hum Genet.* 2003;11:671-8.

Abbreviation 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 H₂O₂ and SBs H₂O₂R 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 H₂O₂ = level of DNA damage after treatment with 100 µl of H₂O₂ (100 µM), for 30 minutes, in ice and SBs H₂O₂R = 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	0,007
		2	3,9428	2,4299	
Fol. (mcg)	400	1	448,594	145,454	0,0001
		2	125,594	70,213	
KcaL		1	2203,936	544,601	0,232
		2	2201,318	447,206	
BMI		1	24,5427	4,7468	0,136
		2	24,5166	3,6638	

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	-0.021	-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	-0.077	-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

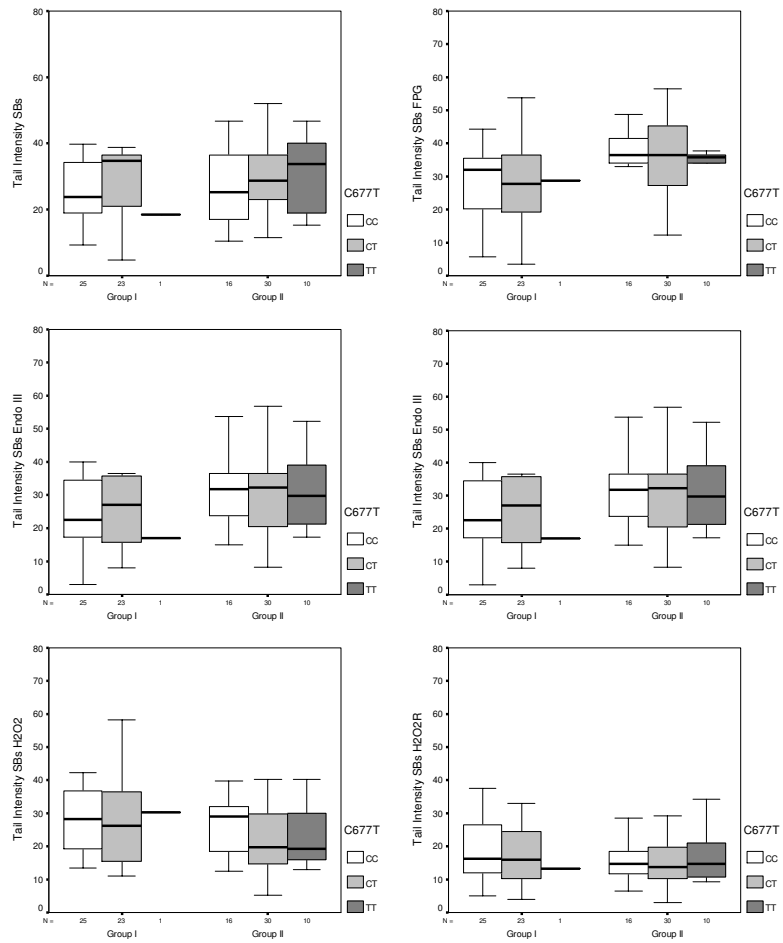


Figure 1.

Manuscrito VI

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 *TS3'UTR 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* polymorphisms 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 *TS3'UTR ins/ins* presented higher levels of purine and pyrimidines oxidized than individuals carrying *TS3'UTR 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 *TS3'UTR* 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 Department, 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 $35,6 \pm 11,4$, 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 commercially available kit (Invitrogen), according to the manufacture's instructions.

2.3.1. TS genotype analysis

Polymerase chain reaction (PCR) and PCR-based restriction fragment length polymorphism (PCR-RFLP) assays were used to identify the *TSER* and *TS3'UTR* polymorphisms, respectively. The primers of the *TSER* polymorphism were 5'-GTGGCTCCTGCGTTTCCCCC-3' (forward) [23] and 5'-GGCTCCGAGCCGCCACAGGCATGGCGCGG-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] ($\cong 2 \times 10^4$ 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°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 endonuclease 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 identify SBs. Subsequently the cells were exposed to alkali buffer (1mM EDTA and 300 mM NaOH, pH \cong 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 *TS3'UTR insertion/deletion* and *TSE2R/3R* polymorphisms of Thymidylate Synthase on the DNA repair capability, 200 μ l of the freshly isolated lymphocytes were treated with 100 μ l of H₂O₂ (100 μ M), for 30 minutes, in ice, and analysis of the level of damage was evaluated. Then, after two wash in PBS to remove of H₂O₂, 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 H₂O₂ and SBs H₂O₂R 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 H₂O₂ and SBs H₂O₂R 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 *TSE* 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 *TSE* 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 *TS*3'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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Literature Cited

- 1- Block G, Patterson B, Subar A. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr Cancer*. 1992;18:1-29.
- 2- Fenech M, Ferguson LR. Vitamins/minerals and genomic stability in humans. *Mutat Res*. 2001;475:1-6.
- 3- Ames BN. Micronutrients prevent cancer and delay aging. *Toxicol Lett*. 1998;102-103:5-18.
- 4- Glynn SA, Albanes D. Folate and cancer: a review of the literature. *Nutr Cancer*. 1994;22:101-19.
- 5- Glynn SA, Albanes D, Pietinen P, Brown CC, Rautalahti M, Tangrea JA, Gunter EW, Barrett MJ, Virtamo J, Taylor PR. Colorectal cancer and folate status: a nested case-control study among male smokers. *Cancer Epidemiol Biomarkers Prev*. 1996;5:487-94.
- 6- Giovannucci E. Epidemiologic studies of folate and colorectal neoplasia: a review. *J Nutr*. 2002;132(8 Suppl):2350S-2355S.
- 7- Ulrich CM, Curtin K, Potter JD, Bigler J, Caan B, Slattery ML. Polymorphisms in the reduced folate carrier, thymidylate synthase, or methionine synthase and risk of colon cancer. *Cancer Epidemiol Biomarkers Prev*. 2005;14(11 Pt 1):2509-16.
- 8- Duthie SJ, Narayanan S, Sharp L, Little J, Basten G, Powers H. Folate, DNA stability and colo-rectal neoplasia. *Proc Nutr Soc*. 2004;63:571-8.
- 9- Chen J, Giovannucci EL, Hunter DJ. MTHFR polymorphism, methyl-replete diets and the risk of colorectal carcinoma and adenoma among U.S. men and women: an example of gene-environment interactions in colorectal tumorigenesis. *J Nutr*. 1999;129(2S Suppl):560S-564S.

- 10- van den Donk M, Visker MH, Harryvan JL, Kok FJ, Kampman E. Dietary intake of B-vitamins, polymorphisms in thymidylate synthase and serine hydroxymethyltransferase 1, and colorectal adenoma risk: a Dutch case-control study. *Cancer Lett.* 2007;250:146-53. Epub 2006 Nov 17
- 11- Choi SW, Mason JB. Folate and carcinogenesis: an integrated scheme. *J Nutr.* 2000;130:129-32.
- 12- Duthie SJ. Folic acid deficiency and cancer: mechanisms of DNA instability. *Br Med Bull.* 1999;55:578-92.
- 13- Oikawa S, Murakami K, Kawanishi S. Oxidative damage to cellular and isolated DNA by homocysteine: implications for carcinogenesis. *Oncogene.* 2003;22:3530-8.
- 14- Zingg JM, Jones PA. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. *Carcinogenesis.* 1997;18:869-82.
- 15- Fenech M. The role of folic acid and Vitamin B12 in genomic stability of human cells. *Mutat Res.* 2001;475:57-67.
- 16- Radparvar S, Houghton PJ, Houghton JA. Characteristics of thymidylate synthase purified from a human colon adenocarcinoma. *Arch Biochem Biophys.* 1988 Jan;260:342-50.
- 17- Hori T, Ayusawa D, Shimizu K, Koyama H, Seno T. Chromosome breakage induced by thymidylate stress in thymidylate synthase-negative mutants of mouse FM3A cells. *Cancer Res.* 1984;44:703-9.
- 18- Hori T, Ayusawa D, Glover TW, Seno T. Expression of fragile site on the human X chromosome in somatic cell hybrids between human fragile X cells

- and thymidylate synthase-negative mouse mutant cells. *Jpn J Cancer Res.* 1985;76:977-83.
- 19- Brown KS, Kluijtmans LA, Young IS, McNulty H, Mitchell LE, Yarnell JW, Woodside JV, Boreham CA, McMaster D, Murray L, Strain JJ, Whitehead AS. The thymidylate synthase tandem repeat polymorphism is not associated with homocysteine concentrations in healthy young subjects. *Hum Genet.* 2004;114:182-5. Epub 2003 Oct 25.
- 20- Moertel CG. Chemotherapy for colorectal cancer. *N Engl J Med.* 1994;330:1136-42.
- 21- Tahara M, Ochiai A, Fujimoto J, Boku N, Yasui W, Ohtsu A, Tahara E, Yoshida S. Expression of thymidylate synthase, thymidine phosphorylase, dihydropyrimidine dehydrogenase, E2F-1, Bak, Bcl-X, and Bcl-2, and clinical outcomes for gastric cancer patients treated with bolus 5-fluorouracil. *Oncol Rep.* 2004;11:9-15.
- 22- Hu YC, Komorowski RA, Graewin S, Hostetter G, Kallioniemi OP, Pitt HA, Ahrendt SA. Thymidylate synthase expression predicts the response to 5-fluorouracil-based adjuvant therapy in pancreatic cancer. *Clin Cancer Res.* 2003;9:4165-71.
- 23- Horie N, Aiba H, Oguro K, Hojo H, Takeishi K. Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5'-terminal regulatory region of the human gene for thymidylate synthase. *Cell Struct Funct.* 1995;20:191-7.
- 24- Marsh S, Ameyaw MM, Githang'a J, Indalo A, Ofori-Adjei D, McLeod HL. Novel thymidylate synthase enhancer region alleles in African populations. *Hum Mutat.* 2000;16:528.

- 25-Luo HR, Lü XM, Yao YG, Horie N, Takeishi K, Jorde LB, Zhang YP. Length polymorphism of thymidylate synthase regulatory region in Chinese populations and evolution of the novel alleles. *Biochem Genet.* 2002;40:41-51.
- 26-Pullarkat ST, Stoehlmacher J, Ghaderi V, Xiong YP, Ingles SA, Sherrod A, Warren R, Tsao-Wei D, Groshen S, Lenz HJ. Thymidylate synthase gene polymorphism determines response and toxicity of 5-FU chemotherapy. *Pharmacogenomics J.* 2001;1:65-70.
- 27-Horie N, Takeishi K. Identification of functional elements in the promoter region of the human gene for thymidylate synthase and nuclear factors that regulate the expression of the gene. *J Biol Chem.* 1997;272:18375-81.
- 28-Ulrich CM, Bigler J, Bostick R, Fosdick L, Potter JD. Thymidylate synthase promoter polymorphism, interaction with folate intake, and risk of colorectal adenomas. *Cancer Res.* 2002;62:3361-4.
- 29-Villafranca E, Okruzhnov Y, Dominguez MA, García-Foncillas J, Azinovic I, Martínez E, Illarramendi JJ, Arias F, Martínez Monge R, Salgado E, Angeletti S, Brugarolas A. Polymorphisms of the repeated sequences in the enhancer region of the thymidylate synthase gene promoter may predict downstaging after preoperative chemoradiation in rectal cancer. *J Clin Oncol.* 2001 Mar 15;19(6):1779-86. Comment in: *J Clin Oncol.* 2001;19:3442.
- 30-Iacopetta B, Grieu F, Joseph D, Elsaleh H. A polymorphism in the enhancer region of the thymidylate synthase promoter influences the survival of colorectal cancer patients treated with 5-fluorouracil. *Br J Cancer.* 2001 Sep 14;85(6):827-30. Comment in: *Br J Cancer.* 2002;86:1365; author reply 1366.
- 31-Ulrich CM, Bigler J, Velicer CM, Greene EA, Farin FM, Potter JD. Searching expressed sequence tag databases: discovery and confirmation of a common

- polymorphism in the thymidylate synthase gene. *Cancer Epidemiol Biomarkers Prev.* 2000;9:1381-5.
- 32-Chu J, Dolnick BJ. Natural antisense (rTSalpha) RNA induces site-specific cleavage of thymidylate synthase mRNA. *Biochim Biophys Acta.* 2002;1587:183-93.
- 33-Mandola MV, Stoehlmacher J, Zhang W, Groshen S, Yu MC, Iqbal S, Lenz HJ, Ladner RD. A 6 bp polymorphism in the thymidylate synthase gene causes message instability and is associated with decreased intratumoral TS mRNA levels. *Pharmacogenetics.* 2004;14:319-27.
- 34-Chen J, Hunter DJ, Stampfer MJ, Kyte C, Chan W, Wetmur JG, Mosig R, Selhub J, Ma J. Polymorphism in the thymidylate synthase promoter enhancer region modifies the risk and survival of colorectal cancer. *Cancer Epidemiol Biomarkers Prev.* 2003;12:958-62.
- 35-Zhang J, Cui Y, Kuang G, Li Y, Wang N, Wang R, Guo W, Wen D, Wei L, Yu F, Wang S. Association of the thymidylate synthase polymorphisms with esophageal squamous cell carcinoma and gastric cardiac adenocarcinoma. *Carcinogenesis.* 2004;25:2479-85. Epub 2004 Jul 29.
- 36-Zhang Z, Shi Q, Sturgis EM, Spitz MR, Hong WK, Wei Q. Thymidylate synthase 5'- and 3'-untranslated region polymorphisms associated with risk and progression of squamous cell carcinoma of the head and neck. *Clin Cancer Res.* 2004;10:7903-10.
- 37-Shi Q, Zhang Z, Neumann AS, Li G, Spitz MR, Wei Q. Case-control analysis of thymidylate synthase polymorphisms and risk of lung cancer. *Carcinogenesis.* 2005;26:649-56. Epub 2004 Dec 3.

- 38-Cardoso MA, Stocco PR. Development of a dietary assessment method for people of japanese descent living in São Paulo, Brazil. *Cad Saúde Pública*, Rio de Janeiro. 2000;16:107-114.
- 39-Singh NP, Mccoy MT, Tice RR, Schneider EL. A simple technique for quantification of low levels of DNA damage in individual cells. *Exp Cell Res*. 1988;175:184-91.
- 40-Collins AR, Raslová K, Somorovská M, Petrovská H, Ondrusová A, Vohnout B, Fábry R, Dusinská M. DNA damage in diabetes: correlation with a clinical marker. *Free Rad Biol Med*. 1998;25:373-7.
- 41-Giovanelli L, Saieva C, Masala G, Testa G, Salvini S, Pitozzi V, Riboli E, Dolara P, Palli D. Nutritional and lifestyle determinants of DNA oxidative damage? A study in a Mediterranean population. *Carcinogenesis*. 2002;23:1483-89.
- 42-Bailey LB, Duhaney RL, Maneval DR, Kauwell GP, Quinlivan EP, Davis SR, Cuadras A, Hutson AD, Gregory JF 3rd. Vitamin B-12 status is inversely associated with plasma homocysteine in young women with C677T and/or A1298C methylenetetrahydrofolate reductase polymorphisms. *J Nutr*. 2002;132:1872-8.
- 43-Trinh BN, Ong CN, Coetzee GA, Yu MC, Laird PW. Thymidylate synthase: a novel genetic determinant of plasma homocysteine and folate levels. *Hum Genet*. 2002;111:299-302. Epub 2002 Jul 26.
- 44-Skibola CF, Smith MT, Hubbard A, Shane B, Roberts AC, Law GR, Rollinson S, Roman E, Cartwright RA, Morgan GJ. Polymorphisms in the thymidylate synthase and serine hydroxymethyltransferase genes and risk of adult acute lymphocytic leukemia. *Blood*. 2002;99:3786-91.

- 45- Hishida A, Matsuo K, Hamajima N, Ito H, Ogura M, Kagami Y, Taji H, Morishima Y, Emi N, Tajima K. Associations between polymorphisms in the thymidylate synthase and serine hydroxymethyltransferase genes and susceptibility to malignant lymphoma. *Haematologica*. 2003;88:159-66.
- 46- Kawakami K, Omura K, Kanehira E, Watanabe Y. Polymorphic tandem repeats in the thymidylate synthase gene is associated with its protein expression in human gastrointestinal cancers. *Anticancer Res*. 1999;19:3249-52.
- 47- Kealey C, Brown KS, Woodside JV, Young I, Murray L, Boreham CA, McNulty H, Strain JJ, McPartlin J, Scott JM, Whitehead AS. A common insertion/deletion polymorphism of the thymidylate synthase (TYMS) gene is a determinant of red blood cell folate and homocysteine concentrations. *Hum Genet*. 2005;116:347-53. Epub 2005 Jan 29.
- 48- Lindahl T, Nyberg B. Rate of depurination of native deoxyribonucleic acid. *Biochemistry*. 1972;11:3610-8.
- 49- Nakamura J, Swenberg JA. Endogenous apurinic/apyrimidinic sites in genomic DNA of mammalian tissues. *Cancer Res*. 1999;59:2522-6.
- 50- Hubner RA, Liu JF, Sellick GS, Logan RF, Houlston RS, Muir KR. Thymidylate synthase polymorphisms, folate and B-vitamin intake, and risk of colorectal adenoma. *Br J Cancer*. 2007;97:1449-56. Epub 2007 Oct 30.
- 51- Fenech MF, Dreosti IE, Rinaldi JR. Folate, vitamin B12, homocysteine status and chromosome damage rate in lymphocytes of older men. *Carcinogenesis*. 1997;18:1329-36.
- 52- Fenech M, Aitken C, Rinaldi J. Folate, vitamin B12, homocysteine status and DNA damage in young Australian adults. *Carcinogenesis*. 1998;19:1163-71.

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 H₂O₂ and SBs H₂O₂R 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 H₂O₂ = level of DNA damage after treatment with 100 µl of H₂O₂ (100 µM), for 30 minutes, in ice and SBs H₂O₂R = 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 H₂O₂ and SBs H₂O₂R 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 H₂O₂ = level of DNA damage after treatment with 100 µl of H₂O₂ (100 µM), for 30 minutes, in ice and SBs H₂O₂R = 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*.

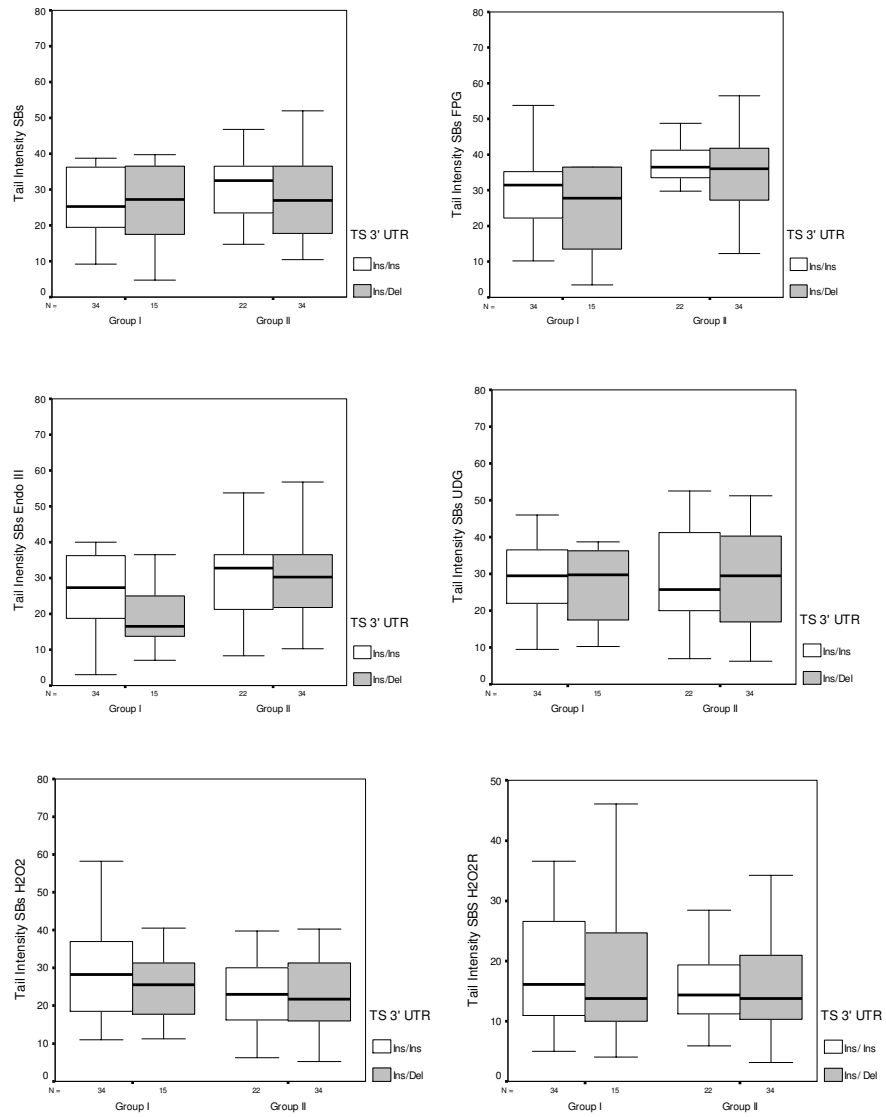
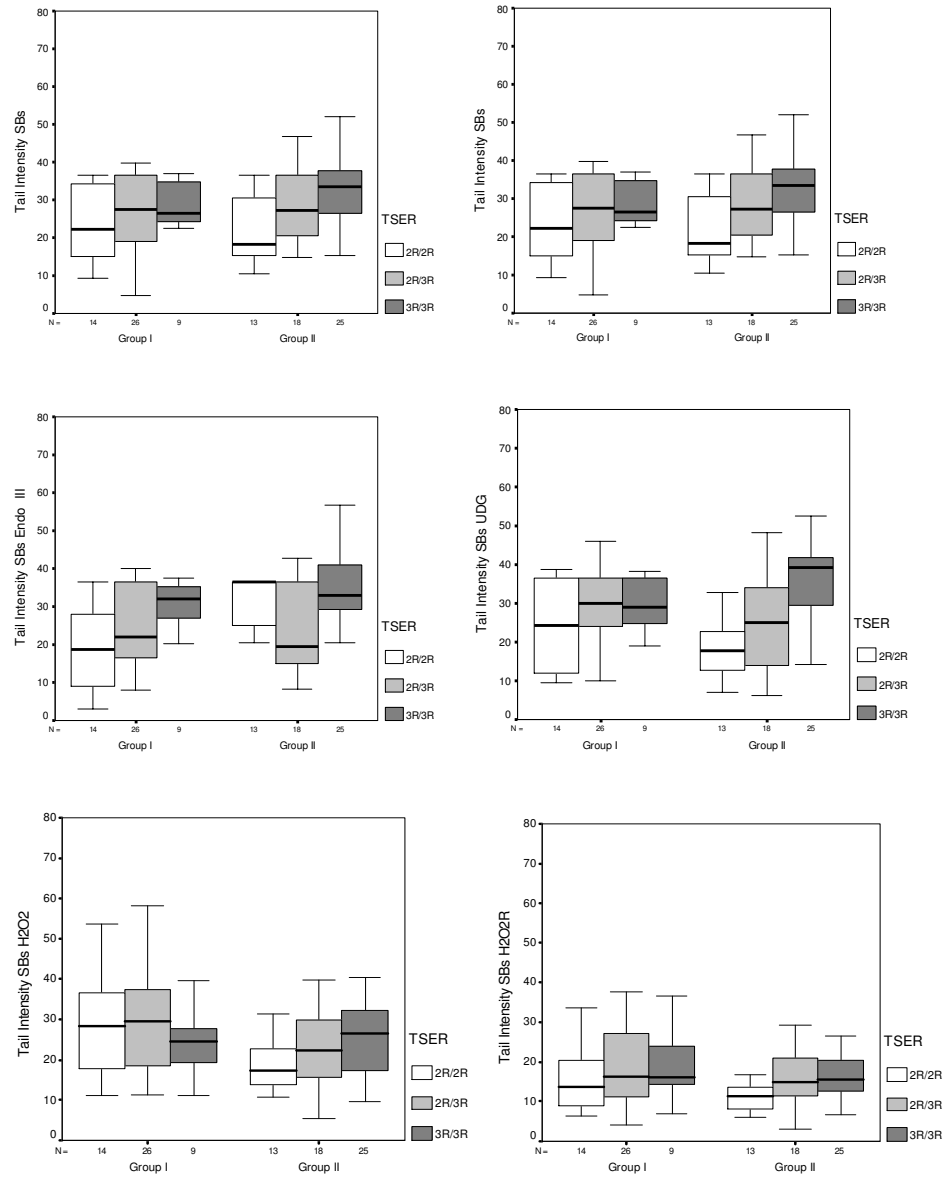


Figure 1.

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

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 μ M) 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].

Ferguson [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 $35,6 \pm 11,4$, 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 μ l 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°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 endonuclease 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 \cong 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 H₂O₂ (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 H₂O₂, 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 CCTCCATCCAGAACTGGCG primers.

The PCR reaction was performed using 25mM MgCl₂, 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: 94°C for 30 seconds, followed by 94°C for 1 minute (step 2), 59°C for 1 minute, 72°C for 2 minutes, and the process returned to step 2 and was repeated 39 times; this was followed by a final extension cycle of 72°C 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 55°C 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 H₂O₂ and SBs H₂O₂R 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 H₂O₂ (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 lead to inhibition of enzyme activity, we hypothesized that this enzymatic deficiency 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 induced with treatment of H₂O₂ (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.

5. References

- 1- Bingham S, Riboli E. Diet and Cancer – The European Prospective Investigation into Cancer and Nutrition. *Rev Nature*. 2004;4:206-215.
- 2- Doll R, Peto R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J. Natl. Cancer Inst.* 1981;66:1191-308.
- 3- Ferguson LR. Natural and human-made mutagens and carcinogens in the human diet. *Toxicology*. 2002;181-182:79-82.
- 4- Ames BN. DNA damage from micronutrient deficiencies is likely to be a major cause of cancer. *Mutat Res*. 2001;475:7-20.
- 5- Eto I, Krumdieck CL. Role of vitamin B12 and folate deficiencies in carcinogenesis. *Adv Exp Med Biol*. 1986;206:313-30.
- 6- Andersen S, Heine T, Sneve R, König I, Krokan HE, Epe B, Nilsen H. Incorporation of dUMP into DNA is a major source of spontaneous DNA damage, while excision of uracil is not required for cytotoxicity of fluoropyrimidines in mouse embryonic fibroblasts. *Carcinogenesis*. 2005;26:547-555.
- 7- Ames BN, Wakimoto P. Are vitamin and mineral deficiencies a major cancer risk? *Nature Rev Cancer*. 2002;2:694-704.
- 8- Fenech M, Ferguson LR. Vitamins/minerals and genomic stability in humans. *Mutat Res*. 2001;475:1-6.
- 9- Louro ID, Llerena JR, Melo MSV, Ashton-Prola P, Conforti-Froes N. *Genética Molecular do Câncer*, 2ª ed. São Paulo, MSG produção Editorial, 2002.
- 10- Miller JA, Miller EC. Ultimate chemical carcinogen as reactive mutagenic electrophiles. In: Hiatt HH, Watson JD, Winstein JA. (Eds). *Origins of human*

- cancer, Cold Spring Laboratory. New York: Cold Spring Harbor 1977. p.605-628.
- 11- Guengerich FP. Catalytic selectivity of human cytochrome P450 enzymes: relevance to drug metabolism and toxicity. *Toxicol Lett.* 1994;70:133-138.
 - 12- Tjhuis MJ, Visker MH, Aarts JM, Peters WH, Roelofs HM, den Camp LO, Rietjens IM, Boerboom AM, Nagengast FM, et al. Glutathione s-transferase phenotypes in relation to genetic variation and fruit and vegetable consumption in an endoscopy-based population. *Carcinogenesis.* 2007;28:848-57.
 - 13- Strange RC, Fryer AA. The glutathione s-transferases: influence of polymorphisms on cancer susceptibility. In: VINEIS P, MALATS N, LANG M, et al. *Metabolic polymorphisms and susceptibility to cancer.* 1st ed. France: IARC, 1999. Cap. 19, p. 231-249.
 - 14- Harries LW, Stubbins MJ, Forman D, Howard GC, Wolf CR. Identification of genetic polymorphism at the glutathione S-transferase Pi locus and association with susceptibility to bladder, testicular and prostate cancer. *Carcinogenesis.* 1997;18:641-4.
 - 15- Leichsenring A, Losi-Guembarovski R, Maciel ME, Losi-Guembarovski A, Oliveira BW, Ramos G, Cavalcanti TC, Bicalho MG, Cavalli IJ, et al. CYP1A1 and GSTP1 polymorphisms in a oral cancer case-control study. *Braz J Med Biol Res.* 2006;39:1569-74.
 - 16- Cardoso MA, Stocco PR. Development of a dietary assessment method for people of japanese descent living in São Paulo, Brazil. *Cad Saúde Pública, Rio de Janeiro.* 2000;16:107-114.

- 17- Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantification of low levels of DNA damage in individual cells. *Exp Cell Res.* 1988;175:184-191.
- 18- Collins AR, Raslová K, Smorovská MP, Petrovská H, Ondrusová A, Vohnout B, Fábry R, Dusinská M. DNA damage in diabetes: correlation with a clinical marker. *Free Rad Biol Med.* 1998;25:373-377.
- 19- Duthie SJ. Folic acid deficiency and cancer? Mechanisms of DNA instability. *British Med. Bull.* 1999;55:578-592.
- 20- Kirkland DJ, Hayashi M, Jacobson-Kram D, Kasper P, MacGregor JT, Müller L, Uno Y. Summary of major conclusions from the 4th IWGT, San Francisco, 9-10 September. *Mutat Res.* 2007;627:5-9.
- 21- Welfare M, Adeokun AM, Bassendine MF, Daly AK. Polymorphisms in GSTP1, GSTM1 and GSTT1 and susceptibility to colorectal cancer. *Cancer Epidemiol Biomarkers Prev.* 1999;8:289-292.
- 22- Perera FP. Environment and cancer: who are susceptible? *Science.* 1997;278:1068-1073.
- 23- Shields PG, Harris CC. Cancer risk and low penetrance susceptibility genes in gene-environment interactions. *J Clin Oncol.* 2000;18:2309-15.
- 24- Goldman R, Shields PG. Food Mutagens. *J Nutr.* 2003;133 Suppl 3:965S-973S.
- 25- Shen J, Gammon MD, Terry MB, Wang L, Wang Q, Zhang F, Teitelbaum SL, Eng M, Sagiv SK, et al. Polymorphisms in XRCC1 modify the association between polycyclic aromatic hydrocarbon-DNA adducts, cigarette smoking, dietary antioxidants, and breast cancer risk. *Cancer Epidemiol Biomarkers Prev.* 2005;14:336-42.

- 26- Norppa H. Cytogenetic markers of susceptibility: influence of polymorphic carcinogen-metabolizing enzymes. *Environ Health Perspect.* 1997;105:829-835.
- 27- da Silva J, Moraes CR, Heuser VD, Andrade VM, Silva FR, Kvitko K, Emmel V, Rohr P, Bordin DL, et al. Evaluation of genetic damage in a Brazilian population occupationally exposed to pesticides and its correlation with polymorphisms in metabolizing genes. *Mutagenesis* 2008;23:415-422.
- 28- Dusinská M, Ficek A, Horská A, Raslová K, Petrovská H, Vallová B, Drlicková M, Wood SG, Stupáková A, et al. Glutathione s-transferase polymorphisms influence the level of oxidative DNA damage and antioxidant protection in humans. *Mutat Res.* 2001;482:47-55.
- 29- Naccarati A, Soucek P, Stetina R, Haufroid V, Kumar R, Vodickova L, Trtkova K, Dusinska M, Hemminki K, et al. Genetic polymorphisms and possible gene-gene interactions in metabolic and DNA repair genes: effects on DNA damage. *Mutat Res.* 2006;593:22-31.

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 H₂O₂ and SBs H₂O₂R 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 H₂O₂ = level of DNA damage after treatment with 100 µl of H₂O₂ (100 µM), for 30 minutes, in ice and SBs H₂O₂R = 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.

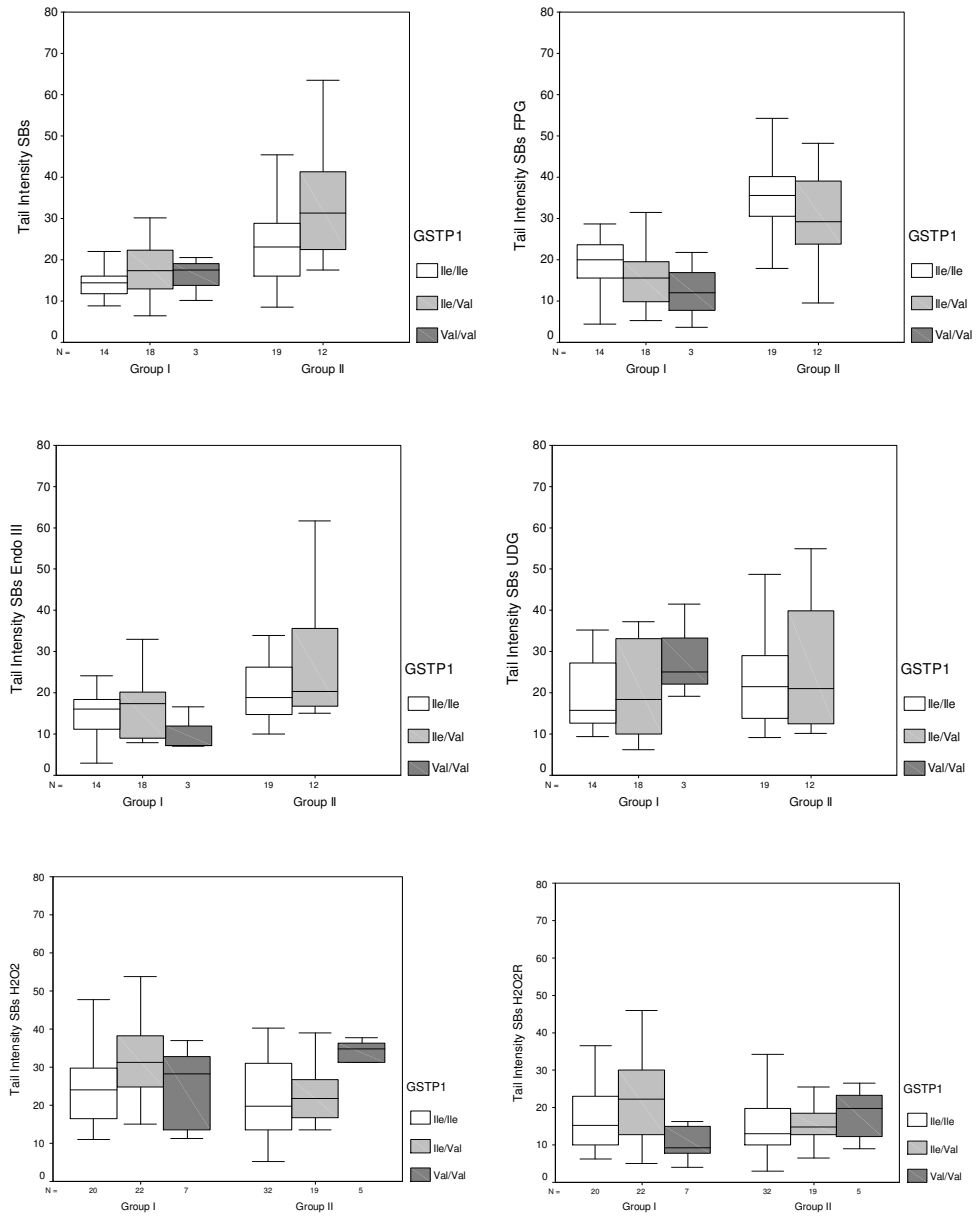


Figure 1.

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 *TSER3R/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 *XRCCI 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 Alzheimer, 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: <input type="checkbox"/> M <input type="checkbox"/> 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

Nome: Dr. Marcelo Sady Plácido Ladeira.

Endereço: Departamento de Clínica médica – Faculdade de Medicina de Botucatu, UNESP, Rubião Júnior S/N, Botucatu – SP.

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 frequência e estimar a quantidade consumida de alimentos fonte de vitaminas interferentes no processo oxidativo/ prevenção do dano oxidativo; (iii) conhecer a frequência e estimar a quantidade de alimentos fonte de carotenóides, retinol e vitamina E.

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 redução 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	Frequência

4. As questões seguintes relacionam-se ao seu hábito alimentar usual no período de um ano. Para cada quadro abaixo responda, por favor, a frequência 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 círculo da primeira coluna (N= nunca come). Não deixe itens em branco.

Grupo de alimentos	Com que frequência 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 10 O O	1	2	3	4	5	6	7	8	9	D S M A		1 concha média (150g)	P M G E O O O
Salgados fritos (pastel, coxinha, risólis, bolinho)	N 10 O O	1	2	3	4	5	6	7	8	9	D S M A		1 unidade grande (80g)	P M G E O O O
Salgados assados (esfiha, bauruzinho, torta)	N 10 O O	1	2	3	4	5	6	7	8	9	D S M A		2 unidades ou 2 pedaços médios (140g)	P M G E O O O
Macarrão com molho sem carne	N 10 O O	1	2	3	4	5	6	7	8	9	D S M A		1 prato raso (200g)	P M G E O O O
Macarrão com molho com carne, lasanha, nhoque	N 10 O O	1	2	3	4	5	6	7	8	9	D S M A		1 escumadeira ou 1 pedaço pequeno (110g)	P M G E O O O
Pizza, panqueca	N 10 O O	1	2	3	4	5	6	7	8	9	D S M A		2 fatias pequenas ou 2 unidades (180g)	P M G E O O O
Polenta cozida ou frita	N 10 O O	1	2	3	4	5	6	7	8	9	D S M A		2 colheres de sopa ou 2 fatias pequenas (70g)	P M G E O O O
Cereais integrais	Quantas vezes você come									Unidade			Porção média (M)	Sua porção
Aveia, quinoa, linhaça, trigo	N 10 O O	1	2	3	4	5	6	7	8	9	D S M A		3 colheres de sopa (40g)	P M G E O O O
arroz	N 10 O O	1	2	3	4	5	6	7	8	9	D S M A		2 escumadeiras médias (120g)	P M G E

	O		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 10 O O O O O O O O O O	1	2	3	4	5	6	7	8	9	D S M A O O O O	1 unidade ou 2 fatias (50g)	P M G E O O O O
Pão de forma integral	N 10 O O O O O O O O O O	1	2	3	4	5	6	7	8	9	D S M A O O O O	2 fatias (50g)	P M G E O O O O
Biscoito sem recheio	N 10 O O O O O O O O O O	1	2	3	4	5	6	7	8	9	D S M A O O O O	4 unidades (24g)	P M G E O O O O
Biscoito recheado, waffer, amanteigado	N 10 O O O O O O O O O O	1	2	3	4	5	6	7	8	9	D S M A O O O O	3 unidades (41g)	P M G E O O O O

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

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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 S M A O O O O	6 unidades (25g)	P M G E 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 S M A O O O O	1 unidade média (50g)	P M G E 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 S M A O O O O	1 unidade média (40g)	P M G E 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 S M A O O O O	10 gomos (80g)	P M G E 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 S M A O O O O	2 colheres de sopa (32g)	P M G E 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 S M A O O O O	1 colher de sopa cheia (30g)	P M G E 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 S M A O O O O	1 colher de sopa (30g)	P M G E 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 S M A O O O O	1 fatia média (160g)	P M G E 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 S M A O O O O	½ unidade média (160g)	P M G E 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 S M A O O O O	1 unidade média (120g)	P M G E 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 O	A 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 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 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 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 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 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 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 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 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 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 S M A O O O O	3 colheres de sopa (45g)	P M G E 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 S M A O O O O	1 colher de sopa cheia (30g)	P M G E 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 S M A O O O O	10 unidades (50g)	P M G E 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 S M A O O O O	1 unidade média (120g)	P M G E 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 S M A O O O O	1 unidade grande (225g)	P M G E 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 S M A O O O O	10 unidades (50g)	P M G E 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 S M A O O O O	1 fatia média (150g)	P M G E 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 S M A O O O O	7 unidades médias (84g)	P M G E O O O O		
Hortalças/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 S M A O O O O	1 colher de sopa cheia (30g)	P M G E 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 M G E 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 S M A O O O O	1 colher de sopa cheia (30g)	P M G E O O O O		
Uva	N 1 2 3 4 5 6 7 8 9 10	D S M A	10 gomos (70g)	P M G E		

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	<input type="radio"/> <input type="radio"/>	<input type="radio"/>		<input type="radio"/>

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 <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/>	D S M A <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/>	1 ramo (30g)	P M G E <input type="radio"/> <input type="radio"/> <input type="radio"/>
Acelga crua	N 1 2 3 4 5 6 7 8 9 10 <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/>	D S M A <input type="radio"/> <input type="radio"/> <input type="radio"/>	1 prato de sobremesa (38g)	P M G E <input type="radio"/> <input type="radio"/> <input type="radio"/>
Acelga cozida	N 1 2 3 4 5 6 7 8 9 10 <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/>	D S M A <input type="radio"/> <input type="radio"/> <input type="radio"/>	1 colher de servir (30g)	P M G E <input type="radio"/> <input type="radio"/> <input type="radio"/>
Repolho	N 1 2 3 4 5 6 7 8 9 10 <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/>	D S M A <input type="radio"/> <input type="radio"/> <input type="radio"/>	2 colheres de sopa (30g)	P M G E <input type="radio"/> <input type="radio"/> <input type="radio"/>
Melão	N 1 2 3 4 5 6 7 8 9 10 <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/>	D S M A <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/>	1 fatia média (150g)	P M G E <input type="radio"/> <input type="radio"/> <input type="radio"/>
Maçã	N 1 2 3 4 5 6 7 8 9 10 <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/>	D S M A <input type="radio"/> <input type="radio"/> <input type="radio"/>	1 unidade média (110g)	P M G E <input type="radio"/> <input type="radio"/> <input type="radio"/>
Banana nanica	N 1 2 3 4 5 6 7 8 9 10 <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/>	D S M A <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/>	1 unidade média (100g)	P M G E <input type="radio"/> <input type="radio"/> <input type="radio"/>
Banana prata	N 1 2 3 4 5 6 7 8 9 10 <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/>	D S M A <input type="radio"/> <input type="radio"/> <input type="radio"/>	1 unidade média (86g)	P M G E <input type="radio"/> <input type="radio"/> <input type="radio"/>
Banana maçã	N 1 2 3 4 5 6 7 8 9 10 <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/> <input type="radio"/>	D S M A <input type="radio"/> <input type="radio"/> <input type="radio"/>	1 unidade média (75g)	P M G E <input type="radio"/> <input type="radio"/> <input type="radio"/>
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, cuscuta, 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

	O O O O O O O O O O O	O O O O		O O O
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 M A O O O O	½ copo americano (80ml)	P M G E 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 M A O O O O	½ copo americano (80ml)	P M G E 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 M A O O O O	½ copo americano (80ml)	P M G E 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 M A O O O O	½ copo americano (80ml)	P M G E 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 M A O O O O O	½ copo americano (80ml)	P M G E 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 M A O O O O	½ copo americano (80ml)	P M G E 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 M A O O O O O	½ copo americano (80ml)	P M G E 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 M A O O O O	½ copo americano (80ml)	P M G E 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 M A O O O O	½ copo americano (80ml)	P M G E 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 M A O O O O	½ copo americano (80ml)	P M G E 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 M A O O O O	½ copo americano (80ml)	P M G E 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 M A O O O O	½ copo americano (80ml)	P M G E O O O O

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

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

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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 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

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

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

	O		O		O
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 peru	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 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 O	D S M A O O O O	1 fio (4ml)	P M G E O O O O	

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

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	O			O
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

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