UNIVERSIDADE ESTADUAL PAULISTA "JULIO DE MESQUITA FILHO"

FACULDADE DE MEDICINA – CAMPUS DE BOTUCATU

ALTERAÇÕES GENÉTICAS RELACIONADAS À OBESIDADE: DANOS NO DNA, PERFIL DE EXPRESSÃO E POLIMORFISMOS GÊNICOS

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DRA. DAISY MARIA FÁVERO SALVADORI

Tese apresentada ao programa de Pós-graduação em Patologia da Faculdade de Medicina de Botucatu, Universidade Estadual Paulista- UNESP, para obtenção do título de Doutora em Patologia.

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Índice

Resumo

Nas últimas décadas, a incidência de indivíduos com sobrepeso ou obesos vem aumentando exponencialmente. Hoje, a obesidade é considerada pela Organização Mundial da Saúde como uma epidemia mundial, com graves consequências que podem levar à morte. A obesidade é uma desordem multifatorial que envolve fatores hereditários, ambiente e estilo de vida, e suas consequências não são apenas sociais ou psicológicas, mas estão principalmente relacionadas à presença de co-morbidades como a hipertensão arterial, diabetes tipo II, doenças cardiovasculares e vários tipos de câncer. Portanto, o controle da obesidade é um desafio para a manutenção da saúde humana, atraindo o interesse de inúmeros pesquisadores que buscam o entendimento dos mecanismos associados ao seu desenvolvimento, bem como novos métodos terapêuticos e de prevenção. Com base nessas premissas, o presente estudo objetivou avaliar a associação entre alterações genéticas e a obesidade, com especial foco para a presença de danos no DNA e para o perfil de expressão e polimorfismos gênicos. A casuística do estudo incluiu 300 mulheres cadastradas na lista de espera para a realização de cirurgia bariátrica e 300 mulheres saudáveis, eutróficas, pareadas por idade. O teste do cometa foi utilizado para avaliação de danos primários no DNA de células sanguíneas; os polimorfismos dos genes da grelina (*GHRL*)e leptina (*LEP*)e dos seus receptores (*GHSR* e *LEPR*), da interleucina 6(*IL-6*) e da serotonina (*5-HT2C*) foram analisados pela técnica de RT-PCR; o perfil de expressão gênica em linfócitos foi avaliado pela metodologia do DNA *microarray* e a expressão dos genes adiponectina (*ADIPOQ*) e leptina (*LEP*) em adipócitos foi avaliada pela técnica de qRT-PCR. Os resultados mostraram que a frequência dos polimorfismos dos genes *GHRL*(rs26802), *GHRS*(rs572169), *LEP*(rs7799039), *LEPR*(rs 1137101), *IL-6* (rs1800796) e *5-HT2C* (rs 3813929) eram iguais entre o grupo de mulheres obesas e o controle. No entanto, as obesas apresentaram maiores níveis de danos no DNA (quebras de fita simples e dupla, sítios álcali-lábeis e bases oxidadas) que as eutróficas, independentemente do genótipo. Os dados mostraram, também, que a expressão de genes relacionados ao consumo alimentar, ao sistema imune, à reposta de defesa, ao sistema de reparo do DNA, entre muitos outros estava diferentemente modulada em leucócitos, assim como o gene *ADIPOQ* estava subexpresso em adipócitosde obesas. Concluindo, as mulheres com obesidade mórbida apresentaram maiores níveis de lesões primárias no DNA de

linfócitos do sangue periférico, fato que poderia ser explicado pelo estado inflamatório crônico característico dessa disfunção orgânico. Além disso, o perfil de expressão gênica foi bastante diferente entre os dois grupos, evidenciando a regulação gênica como importante fator para o desenvolvimento e controle da obesidade. Por outro lado, os resultados mostraram que as variantes estudadas dos genes *GHRL*, *GHRS*, *LEP*, *LEPR, IL-6* e *5-HT2C* não estavam associadas à obesidade mórbida, nem à genotoxicidade observada nas mulheres com essa disfunção. O volume de dados gerados com a análise da expressão gênica por *microarrays* permitirá, ainda, a construção de redes gênicas a fim de verificar a interação entre eles e identificar as principais vias relacionadas ao desenvolvimento e consequências da obesidade.

Abstract

In the last decades, the incidence of overweight and obesity has increased worldwide. Nowadays, obesity is considered by the World Health Organization as a global epidemic with severe consequences that can lead to death. Obesity is a multifactorial disorder which involves different factors such as genetic, environment and life style, and its consequences are not only social or psychological, but are also related to the presence of comorbidities such as hyperthension, type 2 diabetes, heart diseases and many types of cancer. Therefore, obesity control has become a challenge for the human health maintenance, catching the attention of researchers that are trying to understand the mechanisms associated to its development, as well as therapeutical and preventive methods. Based on the information above, the present study aimed to evaluate the association between genetic alterations and obesity, with special focus on the presence of DNA damage, gene expression profiling and genetic polymorphisms. Our study included 300 morbid obese women registered for the bariatric surgery and 300 healthy eutrophic women, matched by age. The comet assay was used to assess primary DNA damage in blood cells; the genetic polymorphisms of ghrelin (*GHRL*), leptin (*LEP*) and their receptors (*GHSR* and *LEPR*), interleukin-6 (*IL-6*) and serotonin receptor (*5-HT2C*) were evaluated by the RT-PCR; gene expression profiling in lymphocytes was assessed by DNA microarrays; and adiponectin (*ADIPOQ*) and leptin (*LEP*) gene expression in adipocytes were evaluated by qRT-PCR. Our results showed that the frequencies of *GHRL* (rs26802), *GHRS* (rs572169), *LEP* (rs7799039), *LEPR* (rs 1137101), *IL-6* (rs1800796) and *5-HT2C* (rs 3813929) polymorphisms were not different between obese and control groups. However, obese presented higher levels of primary DNA damage (single and double strand breaks, alkali-labile sites and oxidative damage) than eutrophic women, independent on the genotype. Our data also showed that the genes related to food intake, immune system, defense response and DNA repair, among others were differentially modulated in leukocytes, and *ADIPOQ* was downregulated in the adipocytes from obese women. In conclusion, morbid obese women presented higher levels of primary DNA lesions in lymphocytes, what could be explained by the chronic inflammatory state found in this dysfunction. Furthermore, the gene expression profiling was different between the two groups, making clear that gene regulation is an important factor for obesity development and control. On the other hand, our results showed that the *GHRL*, *GHRS*, *LEP*, *LEPR, IL-6* and *5-HT2C* gene variants were not associated to morbid obesity, neither with the genotoxicity observed in women with that

disorder. The amount of generated data will still allow building gene networks in order to assess the interaction between them and to identify the main pathways related to the obesity development and its consequences.

1. Revisão da Literatura

Apesar do acúmulo de conhecimentos sobre a obesidade e comorbidades associadas, a prevalência, em diversos países, atingiu níveis recordes (Chaput et al., 2012). Nos últimos anos, a obesidade e o sobrepeso chegaram a níveis epidêmicos, com cerca de 1 bilhão de pessoas com sobrepeso e 300 milhões consideradas obesas. Desse total, pelo menos 2,6 milhões vão a óbito todos os anos em decorrência das complicações oriundas do excesso de peso (WHO, 2005).

Sabe-se que a obesidade tem afetado não somente habitantes de países desenvolvidos e industrializados, mas, também, de países subdesenvolvidos e em desenvolvimento. Censo realizado no Brasil entre 2002 e 2003, com 95,5 milhões de brasileiros com idade acima de 20 anos, mostrou que 38,8 milhões (40,6%) apresentavam sobrepeso e 10,5 milhões (11%) eram obesos (IBGE, 2004). O número crescente de indivíduos obesos tem levado ao aumento do número de cirurgias bariátricas realizadas mundialmente, uma vez que parece ser esta a alternativa realmente efetiva para a perda significativa de peso e, consequente, para o controle da obesidade mórbida (Bushwald, 2004). Este tratamento cirúrgico tem como objetivo a diminuição do consumo energético, com consequente perda de peso, melhoria da qualidade de vida e das comorbidades associadas (Campos et al., 2008). No entanto, o resultado da cirurgia bariátrica, no que se refere à perda de peso, depende de diversos fatores nem sempre conhecidos, sendo que o indivíduo pode recuperar o peso, geralmente entre o 3º e o 5º ano após a cirurgia (Capella & Capella, 1996; Fobi, 2004; Kaplan, 2005; Brolin et al., 1994).

A obesidade é uma doença crônica de etiologia multifatorial que envolve a interação de fatores comportamentais, culturais, hereditários, fisiológicos e psicológicos. Esta doença está associada a alto índice de mortalidade prematura e a presença de comorbidades como a hipertensão arterial, diabetes tipo 2, doenças cardiovasculares, osteoartrite e a diferentes tipos de câncer (Calle et al., 2003; Bray, 2006). A obesidade pode ser classificada dentro de dois contextos: que ocorre por determinação genética ou por fatores endócrinos e metabólicos; e aquela influenciada por fatores externos, sejam eles de origem dietética, comportamental ou ambiental (Romero & Zanesco, 2006). A obesidade é resultado do acúmulo de tecido adiposo, causado pelo desequilíbrio entre o consumo de alimento e o gasto de energia, ambos regulados por mecanismos fisiológicos complexos que envolvem a interação de vários sinais periféricos sob coordenação do cérebro (Tataranni & Ravussin, 1997; den Hoed et al., 2008). Fisiologicamente, o tecido adiposo pode ser classificado em dois tecidos

distintos: o tecido adiposo branco e o tecido adiposo marrom. Nos mamíferos, o tipo predominante é o branco, constituído, principalmente, por adipócitos, pré-adipócitos (células ainda não contendo lipídeos), células endoteliais, fibroblastos e leucócitos, especialmente macrófagos (Tilg & Moschen, 2006). O excesso de energia resultado do desequilíbrio entre o consumo de alimento e o gasto de energia é armazenado nas células do tecido adiposo, que aumentam em tamanho e quantidade, levando a problemas clínicos não só pelo aumento de peso e de gordura corporal, mas, também, pelo aumento da produção de ácidos graxos livres e de peptídeos secretados pelas células do tecido(Bray, 2004).

Realmente, o tecido adiposo, além da estocagem de gordura, é reconhecido como importante órgão endócrino, com papel crucial na regulação de diversos processos patológicos (Tilg & Moschen, 2006; Federico et al., 2010). Nesse sentido, inúmeros estudos vêm sendo realizados com o objetivo de identificar e caracterizar a relação entre a obesidade e algumas doenças, especialmente aquelas relacionadas ao sistema imunológico. Ao que parece, essa relação seria orquestrada por complexa rede de mediadores derivados das células do sistema imune e dos adipócitos (Wellen & Hotamisligil, 2005). Dentre as substâncias secretadas pelo tecido adiposo estão as adipocitocinas (adiponectina, leptina, visfatina e resistina), que têm importante papel na obesidade com relação à resistência a insulina e desordens inflamatórias (Calle & Kaaks, 2004; Kusminski et al., 2005; Weisberg, 2006). Muitos outros produtos também secretados pelo tecido adiposo já foram identificados, incluindo o fator de necrose tumoral - α (TNF-α) -, as interleucinas 1 e 6 (IL-1 e IL-6) e mediadores envolvidos no processo de coagulação (Wellen & Hotamisligil, 2005; Calle & Kaaks, 2004).

Foi estabelecido que a obesidade, devido ao excesso de tecido adiposo, induz estado inflamatório crônico de baixa intensidade que, por meio da produção das espécies reativas de oxigênio, pode, entre outros efeitos, promover danos em estruturas celulares, proteínas, lipídeos e macromoléculas como o DNA (Tilg & Moschen, 2006; Lopes, 2007; Fernandez-Sanchez et al., 2011). Os primeiros estudos sobre o processo inflamatório induzido pela obesidade mostraram aumento nos níveis de TNF-α no tecido adiposo de camundongos obesos (Hotamisligil et al., 1995). Estudos subsequentes demonstraram que não somente o TNF-α, mas diversas outras citocinas, apresentam-se em níveis elevados em indivíduos obesos e que, embora sua produção seja predominante no tecido adiposo, níveis aumentados podem ser encontrados também no fígado, pâncreas, cérebro e músculo (Gregor & Hotamisligil, 2010). O

grande número de macrófagos presentes no tecido adiposo branco parece ser a principal fonte de TNF-α; por outro lado, os adipócitos contribuem com um terço da produção de IL-6 circulante em obesos (Weisberg et al., 2003; Xu et al., 2003; Fantuzzi, 2005).

Além do fato dessas citocinas serem potentes estimuladores da produção de espécies reativas de oxigênio (ERO) e de nitrogênio (ERN), os adipócitos possuem capacidade secretora de angiotensina II, a qual estimula a atividade da NADPH (nicotinamida adenina dinucleotídeo fosfatase), que é a principal via para a produção de ERO nos adipócitos (Fernandez-Sanchez et al., 2011). Em 2006, de La Maza et al. demonstraram que amostras de músculo esquelético de pacientes com sobrepeso apresentavam níveis aumentados de 8-OHdG, 4HNE e TNF-α (biomarcadores de dano oxidativo e inflamação), quando comparados a indivíduos com peso normal e estável. De forma semelhante, Al-Aubaidy & Jelinek (2011) descreveram que maiores níveis de 8-OHdG podiam ser observados em pacientes diabéticos e pré-diabéticos, e que estes estavam positivamente relacionados ao Índice de Massa Corporal (IMC), sugerindo que a obesidade poderia contribuir para o aumento de danos oxidativos no DNA. Embora muitos estudos já tenham relacionado diversas doenças à indução de estresse oxidativo e danos no DNA, são raras as avaliações sobre a frequência ou níveis de lesões genotóxicas em indivíduos com obesidade mórbida (Thavanati & Ortega, 2005).

Como se sabe, o consumo de alimento é basicamente uma interação físio-comportamental entre o indivíduo e o ambiente, com receptores hipotalâmicos que respodem à liberação de hormônios (an)orexigênicos como a leptina, peptídeo YY (PYY), grelina, GLP-1 e de hormônios neurotransmissores, como a serotonina (Kojima et al., 1999). O conceito de hormônio secretagogo do crescimento (GHS) foi inicialmente introduzido em 1978 e, após a descoberta da grelina por Kojima et al. (1999), constatou-se ser esta também um potente estimulador da liberação de hormônio do crescimento (GH) nas células somatotróficas da hipófise e do hipotálamo, atuando como ligante endógeno para o receptor secretagogo de GH (Kojima et al., 1999; Lim et al., 2010). A grelina é um peptídeo produzido no estômago e comumente denominado de "hormônio da fome", uma vez que seus níveis se elevam em estado de jejum e diminuem em estados pós-prandiais (Wren et al., 2000; Cummings et al., 2001). Estudos realizados em ratos mostram que a administração crônica de grelina leva ao aumento de peso, não somente pela indução da hiperfagia, mas, também, pelo aumento da expressão de proteínas que promovem o acúmulo de gordura no tecido adiposo (Tschop et al., 2000; Theander-Carrillo et al., 2006). Recentemente, Garin et al. (2013),

em um artigo de revisão, descreveram que a administração da grelina é capaz de estimular o apetite e aumentar a circulação de GH, ACTH (hormônio adrenocorticotrófico), cortisol, prolactina e glicose tanto em indivíduos saudáveis como em obesos, diabéticos, pacientes com câncer, com disfunção na glândula pituitária e com distúrbios alimentares.

O receptor da grelina, formado por meio de *splicing* alternativo do mRNA do gene do receptor do hormônio secretagogo do crescimento (*GHS-R*), também tem papel fundamental na biologia da obesidade. Duas variantes são geradas nesse *splicing*: o GHS-R tipo 1a, que atua como receptor da grelina, e o tipo 1b, que é uma forma farmacologicamente inativa do receptor. Em seres humanos, o mRNA do *GHS-R1a* já foi identificado em células da glândula pituitária, da tireóide, do pâncreas, baço, miocárdio e das glândulas adrenais (Depoortere, 2009).

O maior interesse pelos mecanismos de controle hormonal do balanço energético iniciou-se em 1994, com a descoberta da leptina. Esta, é uma proteína glicosilada com estrutura semelhante a da interleucina 2 e produzida principalmente no tecido adiposo, embora tenham sido detectados baixos níveis de leptina no hipotálamo, glândula pituitária, placenta, músculo esquelético e nos epitélios gástrico e mamário (Roseland et al., 2001; Moschos et al., 2002). A leptina está principalmente envolvida na energia de homeostase e saciedade, mas é também responsável pela regulação da função neuroendócrina e do metabolismo de glicose e de gorduras, além de participar de diversos processos fisiológicos (Friedman & Haalas, 1998). O total da massa gorda e o IMC são os indicadores mais fortemente associados às concentrações de leptina no sangue (Considini et al., 1996). Quando ocorre o aumento de tecido adiposo, os adipócitos produzem quantidades maiores de leptina, a qual é liberada no sangue ligando-se a proteínas, e transportada até o cérebro, onde estimula ou inibe a liberação de vários neurotransmissores. A leptina, que atravessa a barreira hematoencefálica por meio de processo de difusão facilitada, ligando-se a receptores de leptina existentes em múltiplos locais do hipotálamo (particularmente nos núcleos arqueados e paraventriculatres), promove a diminuição do apetite e aumento do gasto energético por meio de estimulação simpática (Brands et al., 2000; Paracchini et al., 2005; Romero & Zanesco, 2006). Vários mecanismos fisiológicos influenciam a síntese aguda de leptina levando a oscilações em suas concentrações. Sabe-se, por exemplo, que o jejum, o exercício físico e o frio levam à diminuição da expressão do gene da leptina com quedas eventuais nas concentrações plasmáticas da proteína (Hickey et al., 1997). Por outro

lado, a alimentação após o jejum, a insulina, os glicocorticóides e as citocinas pró-inflamatórias podem, também, estimular a expressão do gene e a produção do hormônio (Trayhurn, 2007). Os primeiros estudos sobre a função fisiológica da leptina foram realizados em camundongo e mostraram que animais com mutação no gene da leptina eram incapazes de produzir o hormônio de forma funcional e tornavam-se obesos, atingindo peso corporal quatro vezes maior que seu peso normal (Friedman & Haalas, 1998). Por outro lado, os benefícios terapêuticos do tratamento de indivíduos obesos com leptina exógena são ainda controversos. Em 1998, Friedman & Hallaas, observaram que a administração de leptina por quatro semanas consecutivas reduzia significativamente o peso de pacientes obesos e de eutróficos, porém a perda somente era observada nos indivíduos que não apresentavam hiperleptinemia.

O apetite e outros processos fisiológicos como o sono, o humor, a temperatura corporal e a secreção de outros hormônios são também regulados pela serotonina (5-HT)(Barsh & Shwartz, 2002; Rosmond et al., 2002). Enquanto em níveis normais desse neurotransmissor a saciedade é facilmente atingida, com maior controle sobre a ingestão de açúcares, baixos níveis estão relacionados ao aumento do desejo de ingerir doces e carboidratos, (Lam & Heisler, 2007; Neves & Paschoal, 2007; Lam et al., 2008). Assim sendo, o sistema serotoninérgico tem sido alvo terapêutico para o controle de peso. Diversos subtipos de receptores de serotonina foram identificados, dentre os quais o 5-HT1B e o 5-HT2C, que têm sido reconhecidos por induzir a saciedade (Lam et al., 2008; Ward et al., 2008). O receptor 5-HT2C parece ser o mais importante na relação entre a ingestão alimentar e o balanço energético, uma vez que foi observado que camundongos desprovidos do gene que codifica para esse receptor tornam-se obesos e epiléticos (Ward et al., 2008; Bickerdike, 2003).

A serotonina envolvida na modulação do comportamento alimentar tem sua atividade avaliada pelo nível de seu principal metabólito, o ácido 5-hidroixindolacético (5-HIAA). Foi observado que em pacientes com anorexia nervosa a concentração de 5-HT apresenta-se diminuída, e que essa redução poderia estar associada à menor ingestão de aminoácidos essenciais como o triptofano (precursor da serotonina), bem como à sensibilidade do receptor serotoninérgico 5-HT2C (Hermsdorff et al., 2006; Luras, 2009).

Alguns estudos têm indicado que populações geneticamente mais susceptíveis ao ganho de peso podem tornar-se excessivamente obesas devido ao estilo de vida e ambiente em que vivem (Friedman & Haalas, 1998). Segundo a hipótese de Neel, genes

que predispõem à obesidade teriam "vantagem seletiva" em populações nas quais seus ancestrais experimentaram períodos de fome (Neel, 1962). Portanto, os indivíduos que herdaram tais genes apresentariam superexpressão de elementos relacionados à fome, não apenas ganhando peso, mas se tornando extremamente obesos. Mais tarde, essa teoria foi comprovada por estudo realizado nos Estados Unidos, que demonstrou a desproporção entre afro-americanos e hispano-americanos obesos em relação a caucasianos (Crossrow & Falkner, 2004).

Nas últimas décadas foram inúmeros os estudos que buscaram identificar genes associados a doenças, dentre as quais à obesidade mórbida. Uma das grandes vantagens advindas do uso de técnicas de biologia molecular foi a possibilidade de se caracterizar variantes gênicas associadas à predisposição a determinada enfermidade. Em relação à obesidade, há pesquisas que mostram que polimorfismos de genes que codificam hormônios relacionados à fome e à saciedade podem ter papel fundamental para essa disfunção metabólica (Duarte et al., 2007). A maioria dos genes que predispõem à obesidade codifica componentes moleculares de sistemas fisiológicos que regulam o balanço energético (Duarte et al., 2007; Yurtcu et al., 2009). Genes como os dos receptores da leptina (*LEPR*) e da melanocortina-4 (*MC4R*) e as proteínas desacopladoras de prótons 2 e 3 (*UCP2* e *UCP3*) têm sido avaliados como potencialmente relacionados à fisiopatologia da obesidade e suas complicações (Jacobson et al., 2006). Além desses, o gene da grelina (*GHRL*), dos receptores da grelina (*GHSR*) e o da serotonina (*5-HT2C*) também parecem ter relevância tanto na gênese como nas complicações e consequências da obesidade (Leibel et al., 1995; Doucet et al., 2000; Rosmond et al., 2002 Garcia et al., 2008).

Em 2001, Ukkola et al. avaliando mulheres com obesidade mórbida e mulheres com peso normal, observaram que o polimorfismo *R51Q* do gene da grelina é mais frequentes em obesas. Foi descrito também, que variantes do gene *LEPR* resultam na produção errônea do receptor da leptina levando à obesidade e à diabetes em roedores e seres humanos (Lee et al., 1996; Liu et al., 2004). Muitos polimorfismos no gene da leptina foram identificados e investigados em populações com diferentes prevalências de obesidade. O polimorfismo Q223R foi avaliado em uma população da Tunísia e os resultados mostraram que este estava associado a baixos níveis do hormônio em indivíduos obesos (Ali et al., 2009). Polimorfismos de genes relacionados ao sistema serotoninérgico, como o do receptor *5-HT2C*, já foram identificados e também associados à obesidade (Pooley et al., 2004). Estudos farmacológicos e

comportamentais mostraram que o receptor *5-HT2C* está envolvido na regulação do apetite em seres humanos (Walsh et al., 1994; Sargent et al., 1997), e que o polimorfismo -759C/T da região promotora do gene tem forte associação com a obesidade (Yuan et al., 2000) e ganho de peso durante o início do tratamento com medicamentos antipsicóticos (Reynolds et al., 2002). Da mesma forma, Ramírez-López et al. (2012), observaram que adolescentes mexicanos com os polimorfismos 597G>A, -572G>C e -174G>C do gene da *IL-6* tinham risco aumentados para o desenvolvimento de obesidade e hiperglicemia.

Com o advento da tecnologia dos microarranjos de DNAfoi possível a análise do perfil de expressão gênica dos mais variados tecidos e organismos vivos. Estudos que combinam dados clínicos, genômica, transcriptômica e proteômica têm fornecido importantes contribuições para a elucidação da base genética, fisiopatológica e bioquímica de diversas doenças. No caso da obesidade, dados da análise da expressão gênica em tecido adiposo mostram expressão diferenciada especialmente de genes relacionados à resposta imune, angiogênese, transdução de sinais, ciclo celular e ao metabolismo de lipídeos e da glicose (Gómez-Ambrosi et al., 2004; Baranova et al., 2005). Estudo publicado em 2009demonstrou a relação positiva entre a expressão do gene associado à massa de gordura (*FTO*) e os níveis de leptina, perilipina e de visfatina, além da relação inversa entre este gene e os níveis de triglicerídeos (Zabena et al., 2009). Resultados importantes foram obtidos em células sanguíneas, confirmando que a análise da expressão gênica pode ser também relevante para o entendimento da etiologia e para a identificação de alvos terapêuticos na obesidade (Marteau et al., 2009)

2. Objetivos

Diante das informações apresentadas sobre a gênese e desenvolvimento da obesidade, este estudo propôs:

→determinar a prevalência dos polimorfismos dos genes da grelina (*GHRL*) e da leptina (*LEP*), assim como de seus receptores (*GHSR* e *LEPR*), do receptor da serotonina (*5-HT2C*) e da interleucina 6 (*IL-6*) em uma população de mulheres com obesidade mórbida (IMC < 40,0);

→avaliar os níveis de danos no DNA de linfócitos das mulheres obesas;

→avaliar a relação entre os polimorfismos gênicos e os níveis de danos no DNA;

→avaliar o perfil de expressão gênica em células de sangue periférico de mulheres obesas e eutróficas;

→avaliar a expressão dos genes *ADIPOQ* e *LEP* em adipócitos de mulheres obesas e eutróficas.

3. Artigo 1

GHRL, *GHSR*, *LEP*, *LEPR*, *5-HT2C* and *IL-6* gene polymorphisms and DNA damage in morbid obese women

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Abstract

Obesity is characterized by increased adipose tissue mass with low-grade systemic inflammation, resulting from a chronic energy imbalance between energy intake and expenditure. Food intake regulation is a behavioral-physiological interaction between hypothalamic receptors and released hormones. Herein, we aimed to evaluate if ghrelin (*GHRL* – rs26802), ghrelin receptor (*GHSR* – rs572169), leptin (*LEP* – rs7799039), leptin receptor (*LEPR* – rs 1137101), serotonin receptor (*5-HT2C*- rs 3813929) and interleukine-6 (*IL-6* – rs1800796) gene polymorphisms, and also oxidative DNA damage are related to obesity. The relationship between the gene variants and genotoxicty (detected by the comet assay) was also investigated. A total of 300 morbid obese and 300 healthy weight (controls) women was recruited for this study. The results demonstrated no statistically significant difference in the frequencies of *GHRL, GHSR, LEP, LEPR, 5-HT2C* and *IL-6* gene polymorphisms ($p>0.05$) between obese and control groups. In addition, data showed higher ($p<0.05$) amount of genetic damage (DNA strand breaks and oxidative lesions) in morbid obese women than in controls. Nevertheless, no relationship was detected between the genotypes and the amount of DNA damage. In conclusion, independent on the genotype, obesity was associated with increased DNA damage in lymphocytes, what may be caused by the inflammatory state related to this metabolic dysfunction.

Key words: DNA damage, gene polymorphisms, metabolic syndrome,obesity

1. Introduction

The etiology of obesity is associated to a complex interaction of genetic, diet, metabolism and physical activity, leading to a chronic imbalance between energy intake and energy expenditure (Tatarani & Ravussin, 2002). The genetic approach considers obesity as an inherited disease caused by mutations and/or gene polymorphisms. On the other hand, food intake regulation is mainly a behavioral-physiological interaction between individual and environment, with hypothalamic receptors responding to peripherally released (an)orexigenic hormones, such as peptide YY (PYY), glucagon-like peptide 1 (GLP-1), ghrelin and leptin, the latter two being key mediators for body weight regulation (den Hoed et al., 2008; Nakazato et al., 2001; Halaas et al., 1995; Zhang et al., 1994).

Ghrelin was discovered at the end of 1999 as an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (Kojima et al., 1999). It is produced predominantly in the stomach, and its concentration in human plasma rises during fasting and falls post-prandially, making this hormone known as "hunger hormone" (Cummings et al., 2001; Cummings, 2006). In 2001, Ukkola et al. analyzing Swedish morbid obese and lean subjects have observed that the -501A>C polymorphism of the ghrelin genewas more frequent in obese. Latter,Gueorguiev et al. (2009) genotyped 10 common *GHSR* SNPs in 1275 obese subjects and in 1059 subjects from the general population, and have observed that the SNP rs572169 was associated to obesity. These same authors have also detected associations between the ghrelin variant g.A265T (rs4684677) and obesity, between ghrelin variant gA-604G (rs27647) and insulin levels at 2-h post-oral glucose tolerant test (OGTT), and between the eating behavior "overeating" and the *GHSR* SNP rs2232169 in obese subjects.Leptin, a 167-amino acid protein coded by the *LEP*gene and produced in the white adipose tissue, is an important hormone for the regulation of adiposity and body weight. It acts by inhibiting food intake and stimulating energy expenditure (Paracchini et al., 2005). Similarly, leptin receptor, coded by the *LEPR*gene, is also important in the pathophysiology of human obesity (Yiannakouris et al., 2001). Functional mutations in the *LEPR* gene, resulting in a truncated receptor, have been associated to obesity and diabetes in rodents and humans (Lee et al., 1996; Takaya et al., 1996; Clément et al., 1998).

The neurotransmitter serotonin (5-hydroxy-tryptamine,5-HT), also plays an important role on energy balance, since depletion of brain serotonin promotes hyperphagia and obesity. Drugs increasing serotonergic transmission and serotonin

itself, can reduce food intake, decrease body weight and enhance energy expenditure (Lam & Heisler, 2007). It has been demonstrated that intracerebroventricular (ICV) injections of *p*-chlorophenylalanine, which inhibits tryptophan hydroxylase and thereby reduces brain serotonin, induces hyperphagia and weight gain in rats (Breisch et al., 1976). Among the serotonergic most investigated genes are tryptophan hydroxylase 1 and 2 (*TPH 1* and *TPH 2*; responsible by serotonin synthesis), serotonin receptors 2A and 2C (*5-HT2A* and *5-HT2C*; post-synaptic receptors) and serotonin transporter (*5-HTT;* serotonin re-uptake) (Walter & Bader, 2003). Many pharmacological and behavioral studies have showed that *5-HT2C* is involved in appetite regulation in humans (Reynolds et al., 2002; Reynolds et al., 2003). Furthermore, literature has also reported that the SNP -759C/T in the promoter region of this gene has strong association with obesity (Walsh et al., 1994) and weight gain in the beginning of antipsychotic treatment (Sargent et al., 1997; Yuan et al., 2000).

Other peptides, such as anti- and pro-inflamatory adipokines and adiponectin,have been also linked to adiposity (Guerre Millo, 2008). Interleukin-6 (IL-6), produced by fibroblasts, macrophages, endothelial cells and adipocytes, for instance, can inhibit the lipoprotein lipase activity and stimulates glucose and fatty-acid oxidation and glucagon and cortisol liberation (Mohamed-Ali et al., 1998). Obesity itself is associated with a chronic low-grade inflammatory state, able to produce free radicals which can interact with DNA, causing genotoxicity. In fact, experimental and clinical observations have demonstrated oxidative stress as an important mechanism in obesity-associated metabolic syndrome (Furukawa et al., 2004; Grattagliano et al., 1998).

Therefore, based on these previous comments, in this study we aimed to evaluate the frequencies of ghrelin, ghrelin receptor, leptin, leptin receptor, serotonin receptor and interleukine-6 genepolymorphisms, and also the level of oxidative DNA damage (detected by the comet assay) in lymphocytes of morbid obese and healthy weight women. The relationship between the gene variants and DNA damage was also investigated.

2. Material and Methods

Subjects

The Ethics Committee for Human Research of the Botucatu Medical School – UNESP approved this study protocol (Document n° 3361-2009). Signed informed consent was obtained from all subjects recruited.

Two groups of women were included in this study: 1) morbid obese women (BMI > 40 Kg/m^2; n = 300), age range 20-50 years old; 2) health weight women (BMI ≤ 24.9 Kg/m^2;control group;n = 300), matched for age and smoking habit. In the obese group were included those women who were registered for bariatric surgery at Center of Gastroenterology and Bariatric Surgery (Hospital dos Fornecedores de Cana), Piracicaba – SP, Brazil. The exclude criteria were alcohol consumption > 40g alcohol/day, presence of genetic syndromes associated to obesity, Cushing Syndrome, hypothyroidism, kidney or liver diseases, neoplasias, HIV, use of corticoids and estrogen replacement. Women for the control group should not be in use of any medicine, should not have exercised at least 24 hours before blood collection and should not have diabetes, hypercholesterolemia, hypertension or obesity, neither family history for these diseases.

Blood sampling

Peripheral blood sample (10mL) was collected through venipuncture in EDTA tubes. Part of this sample (4 mL) was immediately used for lymphocytes isolation and 6 mL were stored in a freezer (-80°C) for later DNA extraction.

Alkaline comet assay (DNA damage)

Firstly, lymphocytes were isolated using Ficoll-Paque® gradient. Then, the comet assay was performed according to themethodology described by Singh et al. (1988) and Tice et al. (1991), with some modifications. Briefly, fresh lymphocytes(10 µL/sample) were added to 120 µL of 0.5% low-melting-point agarose at 37°C. The mixture was layered onto 1.5% normal-agaroseprecoated slides, covered with a coverslip and left at 4◦C for 5 min, to allow agarose solidification. Then, coverlips were removed, and slides immersed into a cold lysis buffer (2.5M NaCl, 100 mM EDTA, 10mMTris at pH 10, 1% Triton X-100 and 10% DMSO) for, at least, 2 h. To evaluate oxidative DNA damage, slides were washed in PBS for 5 min and washed again (3 X 5 min each) in a buffer X 1 (40 mM HEPES, 100 mM KCl, 0.2

mg/ml bovine serum albumin and 0.5 mM EDTA at pH 8). Slides were incubated at 37ºC for 30 min in a moist and dry chamber, with 100 µl of fpg and endo III (1:1000) enzymes which recognize oxidised purine and pyrimidine, respectively. Subsequently, slides were left for 15 min at 4° C and then the coverslips were removed (Braz et al., 2011). All the slides were placed into a horizontal electrophoresis tank filled with freshly prepared alkaline buffer (1mM EDTA and 300 mM NaOH at pH >13). After a 40-min DNA unwinding period, electrophoresis was conducted at 25 V and 300 mA for 30 min. After 15 min neutralization with 0.4 M Tris (pH 7.5), the slides were fixed with absolute ethanol and stored at 4◦C. Slides were stained with 70µL of 2:10000 Sybr Gold solution (Invitrogen, USA) and immediately analyzed in fluorescent microscope, at 400 X magnification. Images from100 nucleoids (two replicateslides)per subject were scored using semi automated image analysis system (Comet Assay IV, Perceptive Instruments, UK).Tail intensity (% DNA in the tail) was used to estimate the extent of DNA damage.

DNA extraction

Genomic DNA was extracted from whole blood cells using Illustra Blood genomic prep mini spin kit (GE, Sweden), according to manufacturer's protocol. DNA concentration was evaluated in spectrophotometer (NanoVue - GE, Sweden), and each sample was assessed for purity by absorbance at 260 and 280nm (260/280 = 1.9 – 2.1). DNA integrity was assessed by 1.5% agarose gel, using Tris/borate/EDTA (TBE). DNA was stored at -20°C until amplification.

Genotyping

Identification of single nucleotide polymorphisms (SNPs) from *GHRL* (-501A>C, rs 26802), *GHSR* (477G>A, rs 572169), *LEP* (-2548G>A, rs 7799039), *LEPR* (668A>G, rs 1137101), *5-HT2C* (-759C/T, rs 3813929) and *IL-6* (-572G>C, rs 1800796) genes were carried out using TaqMan SNP Genotyping Assays (Applied Biosystems, USA). For Polymerase Chain Reaction (PCR) it was used 3 µL of extracted DNA , 1.75 µL of ultrapure H_2O, 5 µL of Master mix (TaqMan® SNP Genotyping Assays – Applied Biosystems, USA) and 0.25 µL of primers (TaqMan® SNP Genotyping Assays – Applied Biosystems, USA). Fluorescence was measured using an ABI 7500 Applied Biosystems. To assess genotyping reproducibility, 10% randomly selected samples were re-genotyped. 100% concordance was obtained.

Statistical analysis

Data obtained in the comet assay were analyzed by a general linear model with gamma distribution. For demographic data, it was used the Student's t test, followed by the Tukey test; X^2 was used to compare the genotype and allelic frequencies. $P < 0.05$ was used for statistical significance.

3. Results

Table 1 shows demographic characteristics (mean age, weight, height and BMI) of the obese and healthy weight groups. Metabolic syndrome was detected in 99/300 (33%) morbid obese women.

Table 1. Demographic characteristics of obese and healthy weight (control) groups

Group	Age (years)	Weight (Kg)	Height (m)	BMI
Control (n=300)	27.93 ± 5.89	59.20 ± 6.71	1.64 ± 0.06	21.96 ± 1.85
Obese (n=300)	33.41 ± 6.51	119.05 ±15.84	1.61 ± 0.06	45.69 ± 5.80

BMI – body mass index (Kg/m^2)

Genotype and allele frequencies of *GHRL*, *GHSR*, *LEP*, *LEPR*, *5-HT2C* and *IL-6* genes are presented in Table 2. Both obese and healthy weight populations were in Hardy-Weinberg Equilibrium. No statistically significant difference was detected between the two groups (control *vs* obese). *CC-GHRL, AA-GHSR*, *GG-LEP*, *AA-LEPR*, *TT-5-HT2C* and *CC-IL-6* were the less frequent genotypes in both obese and control groups. No relationship was found between body weight or BMI and the gene variants (data not shown).

Table 3 shows the amount of DNA damage in both control and obese populations. Obese women presented higher level of DNA single and double strand breaks, and alkali-labile sites than women of the control group ($p< 0.01$). Similarly, increased amount of oxidized purines and pyrimidines were detected in lymphocytes of obese women ($p< 0.01$).

Table 2. Genotype and allelic frequencies in control and obese sampled populations

Gene		Control	Obese
	Genotype		
	AA	0.473^{a}	0.445^{A}
	AC	0.449^{a}	0.448^{A}
GHRL	**CC**	0.078^{b}	0.106^{B}
	Allele		
	A	0.696^{a}	0.669^{A}
	C	0.303^{b}	0.330^{B}
GHSR	**Genotype**		
	GG	0.581^{a}	0.578^{A}
	AG	0.338^{b}	0.344^{B}
	AA	0.079^{c}	0.077^{C}
	Allele		
	G	0750^{a}	0.750^{A}
	A	0.250^{b}	0.250^{B}
LEP	**Genotype**		
	AA	0.379^{a}	0.376^{A}
	AG	0.418^{a}	0.474^{B}
	GG	0.202^{b}	0.1749^{C}
	Allele		
	A	0.588^{a}	0.613^{A}
	G	0.411^{b}	0.386^{B}
LEPR	**Genotype**		
	AA	0.207^{a}	0.219^{A}
	AG	0.483^{b}	0.472^{B}
	GG	0.309^{c}	0.300^{C}
	Allele		
	A	0.449^{a}	0.455^{A}
	G	0.550^{b}	0.544^{B}
5-HT2C	**Genotype**		
	CC	0.731^{a}	0.827^{A}
	CT+TT	0.268^{b}	0.172^{B}
	Allele		
	C	0.859^{a}	0.910^{A}
	T	0.140^{b}	0.089^{B}
IL-6	**Genotype**		
	GG	0.798^{a}	0.854^{A}
	GC+CC	0.202^{b}	0.141^{B}
	Allele		
	G	0.889^{a}	0.925^{A}
	C	0.107^{b}	0.074^{B}

No statistically significant difference was detected between obese and control; different letters mean statistically significant difference ($p<0.01$) between genotypes and between alleles in control (small letter) and obese (capital letter) groups.

Table 3. DNA damage in lymphocytes of morbid obese and healthy weight (control) women

Group	Strand breaks[1]	Oxidized purines	Oxidized pyrimidines
Control (n=70)	16.94 ± 23.51	33.29 ± 33.46	29.79 ± 29.81
Obese (n=70)	28.96 ± 33.12*	46.5 ± 37.06*	44.74 ± 35.5*

[1]- DNA single and double strand breaks, and alkali-labile sites; * $p<0.01$ (obese *vs* control)

When the obese women were distributed according to the presence or absence of metabolic syndrome, no statistically significant difference in the mean of DNA damage (single and double strand breaks, alkai-labile sites, and oxidized purines and pyrimidines) was detected between the two subgroups (Table 4).

Table 4. DNA damage according to the presence or absence of metabolic syndrome (MS) in the obese group

Group	Strand breaks [1]	Oxidized purines	Oxidized pyrimidines
Without MS	39.04 ± 33.67	50.30 ± 34.62	47.02 ± 34.22
With MS	38.58 ± 34.96	52.01 ± 34.34	46.61 ± 32.77

[1]- DNA single and double strand breaks, and alkali-labile sites

DNA damage according to the gene polymorphisms are showed in Table 5. For all genes investigated, morbid obese women always had higher level of DNA damage (strand breaks, oxidized purines and pyrimidines) than those from the control group. *GHRL*-CC, *LEP*-GG and *IL-6*-GC + CC genotypes presented the higher amount of strand breaks in both control and obese groups. Differently, this not happened for the *GHSR*, *LEPR* and *5-HT2C*. Regarding to oxidized DNA damage, only GG-*GHSR* had similar result (higher amount of oxidized purines and pyrimidines) in both groups.

Table 5. DNA damage in obese and healthy weight women according to gene polymorphisms

Gene	Genotype	Group	Strand breaks[1]	Oxidized purines	Oxidized pyrimidines
GHRL	**AA**	**Control**	17.68 ± 24.22^{a}	34.7± 33.91^{a}	28.75 ± 28.40^{a}
		Obese	30.22 ± 32.74$^{\#A}$	47.71 ± 37.44$^{\#A}$	46.79 ± 37.43$^{\#A}$
	AC	**Control**	16.71 ± 22.37^{a}	31.39 ± 32.05^{b}	27.99 ± 28.45^{a}
		Obese	31.66 ± 34.70$^{\#A}$	45.85 ± 36.07$^{\#A}$	43.69 ± 34.00$^{\#B}$
	CC	**Control**	22.13 ± 23.57^{b}	24.55 ± 24.98^{c}	30.11 ± 28.49^{a}
		Obese	44.33 ± 35.83$^{\#B}$	52.70 ± 36.15$^{\#B}$	48.13 ± 34.10$^{\#A}$
GHSR	**AA**	**Control**	18.59 ± 18.84^{a}	23.70 ± 21.05^{a}	25.54 ± 25.36^{a}
		Obese	33.27 ± 29.68$^{\#AB}$	41.43 ± 32.53$^{\#A}$	44.02 ± 34.17$^{\#A}$
	AG	**Control**	14.62 ± 21.22^{b}	32.72 ± 33.29^{b}	25.63 ± 27.13^{a}
		Obese	35.18 ± 33.87$^{\#A}$	48.03 ± 35.30$^{\#B}$	47.85 ± 34.94$^{\#B}$
	GG	**Control**	22.35 ± 26.79^{c}	35.12 ± 34.04^{bc}	32.63 ± 30.46^{b}
		Obese	32.59 ± 33.25$^{\#B}$	50.10 ± 35.66$^{\#BC}$	48.13 ± 34.27$^{\#BC}$
LEP	**AA**	**Control**	16.56 ± 21.79^{a}	28.16 ± 29.39^{a}	29.85 ± 30.79^{a}
		Obese	30.75 ± 32.40$^{\#A}$	41.16 ± 34.54$^{\#A}$	43.63 ± 33.41$^{\#A}$
	AG	**Control**	19.08 ± 24.72^{b}	35.74 ± 33.76^{b}	27.95 ± 27.21^{b}
		Obese	34.30 ± 33.74$^{\#B}$	51.02 ± 35.29$^{\#B}$	48.34 ± 34.84$^{\#B}$
	GG	**Control**	21.10 ± 22.55^{c}	31.94 ± 28.32^{c}	27.80 ± 25.13^{bc}
		Obese	48.14 ± 33.67$^{\#C}$	55.45 ± 34.63$^{\#BC}$	53.53 ± 33.21$^{\#C}$
LEPR	**AA**	**Control**	16.81 ± 21.37^{a}	31.00 ± 30.37^{a}	28.88 ± 28.05^{ab}
		Obese	43.04 ± 38.34$^{\#A}$	49.60 ± 35.06$^{\#A}$	49.52 ± 33.34$^{\#A}$
	AG	**Control**	20.07 ± 25.37^{b}	37.01 ± 34.18^{b}	30.66 ± 29.40^{a}
		Obese	34.39 ± 32.36$^{\#B}$	52.29 ± 34.40$^{\#B}$	51.07 ± 33.83$^{\#AB}$
	GG	**Control**	18.19 ± 23.47^{c}	31.21 ± 31.31^{ac}	27.68 ± 28.77^{b}
		Obese	44.33 ± 33.94$^{\#AC}$	57.27 ± 34.43$^{\#C}$	52.63 ± 33.80$^{\#B}$

Gene	Genotype	Group	Strand breaks[1]	Oxidized purines	Oxidized pyrimidines
5-HT2C	**CC**	**Control**	17.79 ± 23.36^{a}	39.39 ± 35.19^{a}	31.28 ± 29.54^{a}
		Obese	$33.02 \pm 33.69^{\#A}$	$54.38 \pm 36.20^{\#A}$	$51.59 \pm 35.10^{\#A}$
	CT+TT	**Control**	19.66 ± 25.31^{b}	30.92 ± 31.26^{b}	32.09 ± 30.46^{a}
		Obese	$27.16 \pm 32.82^{\#B}$	$41.96 \pm 37.00^{\#B}$	$44.93 \pm 36.09^{\#B}$
IL-6	**GG**	**Control**	18.25 ± 23.55^{a}	35.10 ± 33.53^{a}	29.46 ± 28.82^{a}
		Obese	$28.58 \pm 32.07^{\#A}$	$46.54 \pm 36.97^{\#A}$	$46.85 \pm 34.83^{\#A}$
	GC+CC	**Control**	19.75 ± 24.52^{b}	30.89 ± 31.66^{b}	30.51 ± 28.59^{a}
		Obese	$32.86 \pm 33.59^{\#B}$	$53.08 \pm 36.53^{\#B}$	$50.78 \pm 35.23^{\#B}$

[1]- DNA single and double strand breaks and alkali-labile sites; # $p<0.05$ (obese *vs* control with the same genotype); different letters (small for control and capital for obese group) indicate statistically significant difference ($p<0.05$) between the genotypes in a same group.

4. Discussion

The prevalence of obesity has increased over the past decades in all over the world (Heymsfield et al., 2004). Obesity is a multifactorial disorder that reflects complex interactions of genes, environment and lifestyle. Considering obesity epidemic from a genomic perspective, which takes into account the interactions between genome and environment, studies on molecular epidemiology may have the potential to improve the effectiveness of intervention strategies and obesity prevention (Newell et al., 2007).Based on these premises, we investigated whetherthe *GHRL*, *GHSR*, *LEP*, *LEPR*, *5-HT2C* and *IL-6* genotypes were different between obese and healthy weight subjects. Additionally, we also evaluated if some of these gene variants were related or favoring an increase of DNA damage in peripheral lymphocytes. In fact, the relationship between genotoxic events and some diseases has been extensively reported in literature. However, little is known regarding to obesity.

Overall, our data showed higher amount of DNA strand breaks and oxidized purines and pyrimidines in obese than in eutrophic women. However, we did not detect in the obese women any difference between those with and without metabolic syndrome associated. Since obesity is characterized by a low grade inflammatory state, the reactive oxygen species (ROS) generated due to this condition might have been responsible for DNA damage (Sakata et al., 2002; Lopes, 2007; Fernández-Sánchez et al., 2011). Recently, Karbownik-Levinska et al. (2012) performed a study where they evaluated the levels of lipid peroxidation (LPO) and 8-oxodG in obese adults patients. They observed that both LPO and 8-oxodG are positively correlated to BMI, blood pressure, waist/rip circumference and C-reactive protein. Al-Aubaidy & Jelinek (2011) have observed that increased amount of 8-OHdG (a biomarker for oxidative damage) in diabetic and pre diabetic patients were positively related to the BMI. Similarly, high level of 8-OHdG was detected in skeletal muscle of overweight patients. These what can suggest obesity as a contributing status for increasing oxidative stress. (de La Maza et al., 2006).

Regarding to the frequencies of *GHRL*, *GHSR*, *LEP*, *LEPR*, *5-HT2C* and *IL-6*polymorphisms, we did not observe any difference between the two studied populations. In fact, millions of gene polymorphisms have been identified, however, only few of them have shown functional consequences (Sandrin & Tanus-Santos, 2008). Literature has shown that SPNs in different regions of *GHRL*(including the rs572169) might be associated to early obesity, by mechanisms related to a deficient activation of

ghrelin precursor molecules (Ukkola et al., 2001;Baessler et al., 2005). Positive association between*GHRL* variants and mean BMIhas been also reported (Vartiainen et al., 2006). However, no association between fasting plasma total ghrelin concentrations and the SNPs was detected, suggesting that the gene variants analyzed do not play a major role in the overall determination of fasting ghrelin levels in plasma. According to Gueorguiev et al. (2009), *GHRL* (rs4864677) and *GHSR* (rs572169) polymorphisms were positively associated to obesity in French but not in a German population. No association among five SNPs of *GHRL*(including the rs26802) and body fat and serum lipid levels have been also detected in a Canadian population (Ukkola et al., 2001; Martin et al., 2008).

Leptin is another important hormone produced in the adipose tissueand related to body weight control (Roseland et al., 2001).Our resultsshowed that SNPs -2548G>A and 668A>G from leptin and leptin receptor genes, respectively, were not associated to the BMI. However, we detected for both SNPs higher amount of DNA damage in women who presented the GG than in those with AA and AG genotypes. Previous studies have demonstrated that SNPs in the promoter region (-2548G>A) of *LEP* gene are associated with increased levels of leptin in obese girls (Le Stunff et al., 2000) and in overweight European and Taiwanese aborigines (Wang et al., 2006). Stratigopoulos et al. (2009) have observed that some SNPs in *LEPR* are related to conservative alterations in the distal part of the membrane leptin receptor, in the extracellular domain, changing its affinity to leptin. Since leptin is a proinflammatory cytokine, its increased availability in obese may stimulates inflammatory process and, consequently, the generation of ROS (Heber & Carpenter, 2001), increasing damage in macromolecules, such as DNA.

It is from general knowledge that depression and serotonin are also associated with BMI, although their interactions are not completely understood (Feummeler et al., 2009). Serotonin is a neurotransmitter with an important role in the regulation of physiological processes, and its depletion in brain seems to promote hyperphagia and obesity (Lam & Heisler, 2007). Some authors have shown that several polymorphisms in the promoter region of the *5-HT2C* gene are associated with increased risk for diabetes and obesity in patients without psychiatric disorders (Yuan et al., 2000). In addition, Mulder et al., (2007) have reported thatthe presence of the *5-HT2C*-C allele genotype raises the risk for obesity. Interestingly, although we did not found differences

in the frequencies of *5-HT2C*polymorphism between obese and control subjects, our data showed that CC was the most frequent genotype in both groups.

We also evaluated whether some of those gene variants were associated to the amount of DNA damage.First of all, it is important to take in mind that, even when distributed according to genotypes obese women always presented increased amount of genetic lesions. Our data demonstrated that the *GHRL-CC* (-501A>C SNP; rs26802), *LEP-GG* (-2548G>A, rs 7799039), *LEPR-GG* (668A>G, rs 1137101),*5-HT2C-CC* (759C/T) and *IL-6-GG* and *GC*(-572G>C, rs 1800796)genotypes presented the higher level of DNA damage. Literature does not have enough data to confirm and explain possible associations between *GHRL* and genotoxicity. However, some of its SNPs might be related to a deficient activation of ghrelin precursor molecules (Ukkola et al., 2001) and changes in the protein function. This modification might lead to adiposity and higher inflammatory status and, consequently, to an increased amount of DNA damage. To help the assessment of possible associations among ghrelin activity, obesity and genotoxicity, we also evaluated the ghrelin receptor gene (*GHSR*). The studied 477G>A (rs572169)*GHSR* variant is located in the coding region of the gene and, therefore, it can cause changes in the whole amino acid, leading to structural alterations in the ghrelin receptor, affecting its interaction with the ligands (Nakayama et al., 1999). Although our findings did not show any association between the *GHSR* genotypes and body weight or BMI, obese women with at least one A allele presented increased amount of DNA strand breaks, while those with at least one G allele showed increased level of oxidized DNA purines and pyrimidines. Similarly to the *GHRL*-CC genotype, these findings might be explained by the enhancement of inflammatory condition and chemical bonding of different generated reactive radicals to DNA.

According to our results, women with the *5-HT2C-CC* (759C/T) genotype also presented higher amount of damage (DNA strand breaks and oxidized purines and pyrimidines). Since serotonin acts like neuromodulator, and it is able to control immune and inflammatory processes, changes in its receptor might similarly affect their interaction, causing cytotoxicity and genetic instability.Adipose tissue has important role not only as a storage tissue, but also as an endocrine organ, and its influence on the immune system is not restricted. There are strong evidences showing that overnutrition can increase susceptibility to inflammatory and autoimmune diseases, and even cancer. Since the level of proinflammatory cytokines (including IL-6) are increased in serum of obese subjects (Tataranni & Ortega, 2005), it would be possible to suppose an increase

of ROS generation and, consequently, an oxidative stress environment. Actually, we found higher amount of DNA damage in obese women with the *IL*-6 GG and *GC* genotypes, probably because the G allele favored the induction of ROS generation and genetic lesions.

Concluding, our data showed that not only one, but a set genes or gene variants, were related to the increased amount of DNA damage in obese women. Nevertheless, since those gene polymorphisms were also associated to increased damage in eutrophic women, we might suggest that large amount of genetic damage in morbid obese was not exclusively related to the protein isoforms coded by those genes.

In summary, our results indicated that the *GHRL* (-501A>C, rs 26802), *GHSR* (477G>A, rs 572169), *LEP* (-2548G>A, rs 7799039), *LEPR* (668A>G, rs 1137101), *5-HT2C* (-759C/T, rs 3813929) and *IL-6* (-572G>C, rs 1800796)gene polymorphisms were not closely associated with morbid obesity and they are not good biomarkers for predict risk susceptibility. Additionally, the data showed that, independent on the genotypes,obese womenpresented higher amount of DNA strand breaks and oxidative DNA lesions in peripheral lymphocytes than eutrophic women. ROS generation by the obese inflammatory status seemed to be the main cause of the genetic instability.

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4. Artigo 2

Different gene expression profiling between morbid obese and eutrophic women

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Abstract

Now-a-days, obesity represents one of the major public health issues. It is known that obesity is accompanied by complex changes in gene expression across various functional categories, making them suitable candidates for a large-scale gene expression analysis. In the present study, using DNA microarrays, we analyzed the gene expression profile in peripheral blood leukocytes of morbid obese and eutrophic women. Additionally, we also evaluated the expression of leptin (*LEP*) and adiponectin (*ADIPOQ*) genes in adipocytes by using the real-time qPCR. Our data showed that genes related to food intake, immune system, defense response, DNA repair and too many other different mechanisms were modulated,confirming the multifactorial and multigenic characteristics of obesity. *ADIPOQ,* but not *LEP,* was downregulated in adipocytes. Gene networks will be build in order to check relationships among them.

Key Words: Obesity,gene expression, microarray, qRT-PCR, adiponectin, leptin

1. Introduction

Obesity can be defined as excessive accumulation of adipose tissue caused by chronic energy imbalance between energy intake and energy expenditure (Tataranni & Ravoussin, 1997). Adipose tissue (AT) has been recognized as an important tissue, not only for energy storage, but also for its endocrine function.This role has emerged in recent years with the increased identification of adipocyte-secreted proteins and their broad effects on whole-body homeostasis (Kim & Moustaid-Moussa, 2001; Milan et al., 2002). In mammals, there are two types of AT: white and brown. Most AT in mammals is white and this is thought to be the site of energy storage. By contrast, brown AT is found mainly in human neonates, and it is important for regulating temperature. In addition to adipocytes, which are the most abundant cells in the white AT, pre-adipocytes (adipocyte that have not yet been loaded with lipids), endothelial cells, fibroblasts, leukocytes and, most importantly, macrophages, can be also found.These macrophages are bone marrow derived and their number in the white AT correlates directly with obesity (Tilg & Moschen, 2006). The expansion of AT in obesity is accompanied by increased secretion of hormones such as cytokines, resistin and adiponectin which affect insulin sensitivity, and angiotensin, that regulates blood pressure (Jones et al., 1997; Kim & Moustaid-Moussa, 2001; Yamauchi et al., 2001; Steppan et al., 2001; Milan et al., 2002;).Adiponectin is an adipocyte-derived hormone,and one of the most abundant circulating proteins (Stumvoll et al., 2002). In contrast to other adipocyte-released hormones,adiponectin seems to protect from insulin resistance and type 2 diabetes (Stumvoll et al., 2002). Leptin, another adipocyte derived hormone, plays a key role in obesity by regulatingfood intake and energy expenditure and, consequently, adipose-tissue mass and body weight (Paracchini et al., 2005).

Literature has reported that approximately 600 genes are closely related to the obesity phenotypes (Rankinen et al., 2006). It is known that obesity is accompanied by complex changes in gene expression across various functional categories, making them suitable candidates for a large-scale gene expression analysis (Rankinen et al., 2006; Kim & Park, 2010). Actually, gene expression profile has long been of interest because alterations in transcriptome, in response to specific biological stimuli, provide valuable insights for interpreting functional elements of the genome and understanding disease pathogenesis. Gene expression profiling of blood cells or tissue samples has been used to assess disease-specific signatures and to distinguish from that of healthy individuals (Cheok et al., 2003; Holleman et al., 2004; Martinez et al., 2006; Liu et al., 2006;Tan et

al., 2008; Greg et al., 2008; Lockstone et al., 2008; Sortiriou & Pusztai, 2009;). Differentially expressed genes, involved in a wide variety of biological processes (immune response, lipid metabolism, energy production, cell adhesion and glucose metabolism), have been detected in abdominal subcutaneous adipose tissue of eutrophic and obese men (Shea et al., 2009).

Tissue and cell specific transcriptomic changes during stress condition (disease or in response to therapies) are of particular interest for prevention and establishment of treatment strategies (Liu et al., 2013). Therefore, in the present study, we analyzed the gene expression profiling of peripheral blood leukocytes from morbid obese and eutrophic women using DNA-microarrays. Additionally, we evaluated the expression of leptin (*LEP*) and adiponectin *(ADIPOQ)* genes in adipocytes of both populations by qRT-PCR.

2. Material and Methods

Subjects

The Ethics Committee for Human Research from the Botucatu Medical School – UNESP approved this study protocol (Document nº 3361-2009). Signed informed consent was obtained from all the study subjects.

Two groups were included in the study: 10 morbid obese women (BMI > 40 Kg/m^2), with age range from 30 to 50 years; and 10 eutrophic women (BMI ≤ 24.9 Kg/m^2), matched by age (control group). Women included in the obese group were registered for bariatric surgery at the Center of Gastroenterology and Bariatric Surgery, - Piracicaba, SP, Brazil. The eutrophic women were registered for abdominoplasty at Misericórdia Hospital, Botucatu – SP, Brazil. From both groups, blood and adipose tissue samples were sampling at the moment of surgery. The exclusion criteria for the obese group were: alcohol consumption (> 40g alcohol/ day), presence of genetic syndromes associated to obesity, Cushing syndrome, hypothyroidism, kidney or liver diseases, neoplasias, HIV infection and use of corticoids or estrogen replacement. For control group, women should not be in use of any medicine, should not have diabetes, hypercholesterolemia, hypertension or obesity, nor family history of one of these diseases, and should not have exercised at least 24h before blood sampling.

Blood Sampling

A peripheral blood sample (10 mL) was collected through venipuncture in PAXgene Blood RNA Tubes (Qiagen/PreAnalitiX, Switzerland), for RNA stabilization. The tubes were maintained at room temperature for 12 h and, then, kept in freezer -20° C, until the procedures.

Adipose tissue sampling and adipocyte isolation

At surgery, a sample of subcutaneous adipose tissue was collected and processed as previously described (20). Briefly, 4g of the AT were taken and sliced until reach homogeneous consistence. Small fragments were transferred to a Falcon's tube contained 10 mL of previously prepared digestion medium (200 mL of DMEN, 0.6 g of HEPES, 4 g of BSA, 0.035 g of $NaHCO_3$) and incubated for 45 min. at 37º C, in an orbital shaker. After digestion, cells were transferred to another tube through nylon filter. DMEN medium (25 mL) was added to wash the cells and centrifugation (30 s at

400 g) was performed. The floating adipocytes were transferred to separate tubes (Junior et al., 2005 (20B)).

RNA extraction

From blood samples, RNA was isolated using DNAse treatment and the PAXgene Blood RNA kit, according to manufacturer's protocol (Qiagen/PreAnalitiX, Switzerland). Total RNA was measured by spectrophotometry (NanoVue – GE, Sweden); purity was assessed by absorbance at 260/280 and between 1.9 and 2.1; integrity was evaluated using the Agilent 2100 Bioanalyzer, under standard conditions. From the isolated adipocytes, total RNA was extracted by using a TRIzol solution, according to the manufacturer's specifications. During the extraction, RNA was treated with DNAse (RNAse-free DNAse Set, Qiagen) andthen stored at -80°C. RNA was reverse-transcribed (cDNA) using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems – ABI, USA), according to the manufacturer's protocol.

Microarray

An amount of 140 ng of total RNA was hybridizated onto whole human genome 4x44K oligo microarrays (G4845A, Agilent Technologies) using one color (Cy3) Quick Amp labeling kit (Agilent Technologies), according to manufacturer instructions. Briefly, dilutions of stock solution One-color Spike Mix were performed and 2 µL of fourth dilution was added to 25 ng of total RNA. After 2 µL of Spike Mix, 1.8 µL of T7 Promoter Primer Mix were also added. The reaction was incubated at 65 ° C for 10 min and, then, kept on ice for 5 min. Afterwards, 4.7 µL cDNA Master Mix (5X First Strand buffer, 0.1 M DTT, 10mM dBTP mix and Affinity Script RNase Block mix) were added in each tube and first incubated at 40 °C, for 2 hours, then to 70 °C for 15 min, and on ice for 15 min. At the end, Transcription Master Mix (nuclease free-water, 5X transcription buffer, 0.1 M DTT, NTP mix, T7 RNA polymerase blend and cyanine 3-CTP) was added to each tube and incubated at 40 °C, for 2 hours. The labeled cRNA was purified with the RNeasy kit (Qiagen), according to manufacturer's instructions. cRNA was quantified in a spectrophotometer (NanoVue - GE Healthcare). Sample fragmentations were performed using the fragmentation mix (cyanine 3-labeled reagent, 10X blocking agent, nuclease free water and 25X fragmentation buffer), that was added to each microcentrifuge tube containing the cRNA and then, incubated at 60 ºC for 30 min and immediately placed on ice for 1 minute. Hybridization buffer was added to stop

the fragmentation reaction. Hybridizations were performed for 17 hours at 65 ° C using an automated system (SureHyb, GE healthcare). Subsequently, the slides were washed with the wash buffer solutions 1 and 2. The Agilent's Stabilization and Drying solutions were also used to protect the cyanine probes against ozone degradation. The hybridization signals were captured using the GenePix 4000B scanner (Molecular Devices). Data quantification and quality control were performed using the Feature Extraction (FE) software version 10.7 (Agilent Technologies).

Background adjustment was performed by using normexp method and offset=16. Data were log2-transformed and then normalized using a quartile function. Differentially expressed genes were identified using T-test, for comparing control to the obese group. The eBayes of RBioconductor was used to perform these statistical analyses. All clusters of co-regulated genes were submitted to functional analysis using the Biological Networks Gene Ontology tool (BINGO). Score values lower than 0.05 were considered.

Analysis of ADIPOQ and LEP mRNA by quantitative real-time PCR (qRT-PCR)

After reverse transcription, cDNA samples were stored at -20°C until PCR procedures. TaqMan/FAM-MGB probes and primers (Applied Biosystems, USA) for adiponectin (*ADIPOQ*) (Hs00605917_m1) and leptin (*LEP*) (Hs00174877_m1) were used for amplification. Reactions were performed at 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. β-actin gene was used as an endogenous control. Taqman Universal PCR Master Mix was purchased from Applied Biosystems, and quantitative real-time PCR was performed in duplicate using the 7500 FAST PCR system (Applied Biosystems, USA). Relative gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method (20C).

Statistical analysis

To generate standard curves, four serial dilutions of adipocytes derived mRNA were used. To the first dilution (i.e., the highest concentration) of the standard RNA was given the relative value of 100. The samples amount of mRNA was expressed relative to the standard curve. Results were analyzed using a general linear model with gamma distribution. ANOVA was used in case of normalization.

3. Results

From 40,000 genes analyzed in each array, 2,984 were differentially expressed in the obese group compared to control. From these genes, 1,404 were downregulated, and distributed into 618 different biological processes; other 1,580 were upregulated and belonged to 857 biological processes, according to the BINGO tool. Just to cite a few, these biological processes include: immune/defense response, regulation of metabolic processes, regulation of cytokine production, cellular response to stress and inflammatory response. Table 1 summarizes the biological processes related to the genes differentially expressed (obese X control).

In Table 2 are presented some genes and biological processes that we selected to discuss, because of their probably implication in obesity: stress response and food intake and satiety (*HTR3C*, *GHRLOS*, *LEPROTL1* and *NPY*), immune/defense response (*MARCO*, *IL-4R*, *IL-2RA*, *IL-5RA* and *TNFAIP8L2*) and DNA repair mechanisms (*ERCC2*, *ERCC4* and *ERCC6*).

Data obtained by the real-time qPCR demonstrated that *ADIPOQ* was downregulated in adypocytes of morbid obese women. On the other hand, no significant difference was detected for the *LEP* expression (Figure 1).

Table 1. Gene Ontology Biological Process classification of differentially expressed genes, as indicated by the microarray data

Biological Process	Number of genes
Downregulated genes	
Regulation of Metabolic Process	343
Immune System Process	153
Apoptosis	78
Defense Response	101
Response to Stimulus	302
Regulation of Cell Communication	151
Coagulation	40
Inflammatory Response	43
Leukocyte Activation	37
Wound Healing	46
Cell Cycle	77
Regulation of Cytokine Production	34
Regulation of Insulin Secretion	16
Cellular Response to Stress	71
Cell Division	27
Upregulated genes	
Response to Stress	268
Immune System Process	186
Response to Stimulus	372
Apoptosis	83
Regulation of Cytokine Production	48
Leukocyte Activation	41
Coagulation	46
Inflammatory Response	76
Cell Differentiation	179
Toll-like receptor 4 Signaling Pathway	12
Regulation of Cell-Cell Adhesion	8
Lipid Oxidation	8
Fatty Acid Oxidation	8
Nucleotide Excision Repair	10

Table 2. Differentially expressed genes in leukocytes of morbid obese women, as indicated by the microarray data

Gene ID Number	Gene	Symbol	*p*-value#	Biological Process (GO)*
Upregulated				
NM_006770	Macrophage receptor with collagenous structure	MARCO	0.013578	Defense response, immune response, regulation of response to stimulus
NR_004431	Ghrelin opposite strand (non-protein coding)	*GHRLOS*	0.003486	Regulation of metabolic processes, food intake
NM_000400	Excision repair cross-complementing rodent repair deficiency, complementation group 2	*ERCC2*	0.034245	Transcription induction, cell cycle checkpoints
NM_005236	Excision repair cross-complementing rodent repair deficiency, complementation group 4	*ERCC4*	0.039915	Response to stress, metabolic processes
NM_000124	Excision repair cross-complementing rodent repair deficiency, complementation group 6	*ERCC6*	0.035088	Cell response to stress, response to reactive oxygen species
NM_000417	Interleukin2 receptor, alpha	*IL-2RA*	0.002635	Immune system, regulation of biological processes, defense response
NM_000418	Interleukin4 receptor	*IL-4R*	0.00955	Immune response, regulation of cellular response to stress
NM_175725	Interleukin5 receptor, alpha (IL5RA)	*IL-5RA*	0.047961	Immune response, cytokines production
NM_004843	Interleukin 27 receptor, alpha	*IL-27RA*	0.005654	Regulation of biological processes, immune response

NM_052945	Tumor necrosisfactor receptor superfamily, member13C	*TNFSRF13C*	0.006153	Immune system, cytokines production, lymphocytes activation
NM_024575	Tumor necrosis factor, alpha-induced protein 8-like 2	*TNFAIP8L2*	0.012759	Lymphocytes activation, immune response
Downregulated				
NM_015344	Leptin receptor overlapping transcript-like 1	*LEPROTL1*	0.007216	Food intake, immune system
NM_000905	Neuropeptide Y	*NPY*	0.017908	Stress response, food intake, cardiovascular function, cell proliferation
NM_003853	Interleukin18 receptor accessory protein	*IL-18RAP*	0.00058	Immune system, regulation to stimulus
NM_003855	Interleukin 18 receptor 1	*IL-18R1*	0.000629	Defense response, immune system
NR_023392	Zinc finger protein 252	*ZNF252*	0.049975	Regulation of biological processes, metabolic processes
NM_207333	Zinc finger protein 320	*ZNF320*	0.049479	RNA metabolic processes, cellular metabolic processes, regulation of gene expression
NM_032164	Zinc finger protein 394	*ZNF394*	0.037125	Regulation of biological processes, cellular metabolic processes

# $p<0.05$; *GO – Gene Ontology

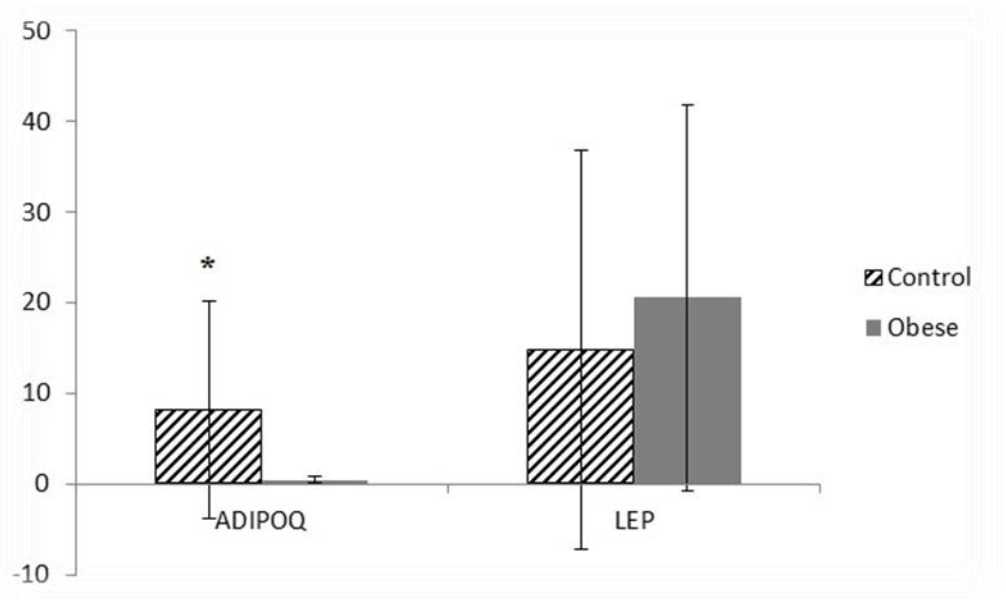

Figure 1.*ADIPOQ* and *LEP* mean expression in obese and eutrophic (control) women. * $p<0.05$

4. Discussion

Genetic mechanisms related to obesity or to hyperplasia and hypertrophy of the adipose tissue are not yet completely understood (Gómez-Ambrosi et al., 2003). Therefore, because of the epidemic proportions reached by obesity worldwide, special attention has been directed to identify deregulated genes that can be causes of this metabolic dysfunction (Nadler et al., 2000; Mokdad et al., 2001; Soukas et al., 2001; Jagoe et al., 2002). Herein, we used DNA-microarrays to evaluate gene expression profiling from leukocytes of morbid obese and eutrophic women, and we detected 2,984 differentially expressed genes between these two groups. The downregulated genes (1,404) were involved in 618 processes and the upregulated ones (1,580) were involved in 857 different biological processes. Literature has demonstrated the suitability of peripheral blood mononuclear cells (PBMCs) for obesity-related studies is in part related to their active metabolism (Kussman et al., 2006). Furthermore, gene expression patterns have shown that 86% of the expressed genes in blood cells are also expressed in the adipose tissue(Brattbakk et al., 2013).

Data reported in literature have demonstrated that the differentially expressed genes in obese subjects are mostly related to inflammation and immune responses (Lee etal., 2005), to lipid and glucose metabolism, to cell adhesion and energy production (Shea et al., 2009). Herein, in despite of the large number of differently expressed genes between the two groups of volunteers, we decided to focus our discussion onthose genes involved in food intake, satiety, immune/defense responseand DNA repair mechanism, once we had previous found higher level of oxidative DNA lesions in obese women (data still not published). Among the upregulated genes in blood cells of obese women, we detected the interleukin receptors*IL-2RA*, *IL-4R,IL-5RA, IL-27RA,* and cytokine genes like *TNFSRF13C* and *TNFAIP8L2*. Therefore, these findings are in accordance with the chronic low-grade inflammationstatus of obesity caused by infiltration of activated immune cells and overproduction of cytokines (Samaras et al., 2012). In fact, the two mostly significant comorbities associated to obesity, cardiovascular disease and type II diabetes have been linked to the presence of systemic inflammation (Illán-Gomez et al., 2012). Numerous evidences have shown that the IL-4 receptor (IL-4R), for instance, plays central role in regulating the production of IgE, influencing the development of allergic inflammatory diseases (Hershey et al., 1997; Nelms et al., 1999; Tanaka et al., 2002). Association between *IL-4R* polymorphisms andincreased BMI (Ha et al., 2008), and between *IL-5RA*, *TRNT1*, *CRBN* and *LRRN1* polymorphisms and obesity and blood

pressure have been also described (Kraja et al., 2012). The pro-inflammatory cytokine TNF-α (tumor necrosis factor-α) was the first identified adipocytokine associated to obesity and related to insulin resistance (Hotamisligil et al., 1993). Isolated and differentiated adipocytes are the principal source of elevated TNF-α level in obesity. However, more recently it has been recognized that macrophages from the stromal vascular fraction are the primary source of adipose derived TNF-α (Weisberg et al., 2003). Tumor necrosis factor-α induced protein 8 like-2 (TNFAIP8L2, TIPE2) is the second member of tumor necrosis factor-α induced protein 8 (TNFAIP8) family, which was recently defined as a novel protein involved in negative regulation of both adaptative and innate immune systems, thus manifesting a negative regulatory effect in the maintenance of immune homeostasis (Sun et al., 2008; Li et al., 2009; Zhang et al., 2010). According to some investigators, TIPE2 is highly expressed in inflammatory, but not in normal tissues (Sun et al., 2008). Nevertheless, in murine, this protein is also expressed in lymphoid tissues, including T cells and small group of non-lymphoid tissues, such as endocrine tissues, skeletal muscle and monocyte/macrophage derived cell lines (Zhang et al., 2010). Therefore, the role ofTIPE2in obesity and obesity-related diseases still need to be better investigated.

Still regarding to interleukin receptors, we also detected some downregulatedgenes (*IL-18RAP*, *IL-18R1*, *IL-18R2*, and others) in morbid obese women. It was previously reported that IL-18 deficiency is associated with obesity through hyperphagia and insulin resistance in knockout mice, due to the accumulation of fat tissue caused by increase of food intake. As IL-18 activates intracellular phosphorylation of STAT3, which is involved in modulating intracellular effects of leptin, it was tempting to speculate that the absence of IL-18 would induce leptin resistance and hyperphagia. In contrast, intracerebral (but not intravenous) administration of rIL-18 (recombinant IL-18) was able to revert hyperphagia, showing that the effect of IL-18 on food intake takes place centrally, and thus it has a role in the central regulation of food intake (Pittenger et al., 1999). Therefore, the *IL-18Rs* modulation could reduce the interaction between receptor/ligand, favoring obesity. Other underexpressed genes that caught our attention were *ZFPs* which are related to the zinc finger proteins (ZFP). *ZFP* genes act as key transcriptional regulators involved in adipogenesis. In fact, numerous zinc finger proteins have been implicated in regulating adipocyte commitment (Chen et al., 2005; Birsoy et al., 2009). Moreover, it was described that the ZFP36 inactivation in mice leads to a complex inflammatory

syndrome due to increased TNF-α production (Carballo et al., 1998). Therefore, the lower *ZFP* expression observed in our group of morbid obese women confirms the relevance of this gene in obesity, favoring adipogenesis and the inflammatory status.

Among the modulated genes directly related to appetite and food intake control we found *GHRLOS*(overexpressed),*LEPROTL1* (downregulated), and*NPY* (downregulated).*GHRLOS*is an antisense gene that seems to be involved in the regulation of the *GHRL* expression (acting in *cis*) and in the expression of a large number of genes that are outside its locus and potentially unrelated to the ghrelin (acting in*trans*). Actually, *GHRLOS* is a non-coding RNA geneon the opposite strand of *GHRL,* but its genomic structure, expression pattern and potential function remains to be investigated (Tack et al., 2006). Since ghrelin acts regulating appetite, food intake, gut motility and energy balance (Bednarek et al., 2000; Heijboer at al., 2006; Neary et al., 2006; Tack et al., 2006), we might suppose *GHRLOS* may play important regulatory and functional roles in the ghrelin axis in obese individuals. However, it is important to notice that we evaluated *GHRLOS* expression in blood cells and, usually, ghrelin expression occurs in stomach, where it is produced and released. Therefore, *GHRLOS* overexpression in blood cells might be explained by its role in regulating other genes and not only those related to ghrelin pathway.

Differently from *GHRLOS,LEPROTL1* (leptin receptor overlapping transcript-like 1) and *NPY*(neuropeptide Y) were downregulatedin the obese women. Initially, it is important to remind that leptin is an adipocyte-derived hormone that suppresses food intake and increases energy expenditure, and its receptor, which has several isoforms, is a single transmembrane protein belonging to the cytokine receptor family (Tartaglia et al., 1995). Leptin activity is observed from the activation of specific receptors presented in different organs. There are two types of receptors for leptin, the long-chain receptors, with higher level of expression in the hypothalamus, and the short-chain receptors with their expression found in many organs, including peripheral blood leukocytes, stomach, lung, skeletal muscle and others. (Romero & Zanesco, 2006).*LEPROTL1* is probably involved in the leptin direct effects on tissues outside the central nervous system, which results in the leptin activity on extra-brain tissues (Shimabukuro et al., 1997; Muller et al., 1997). Literature has reported that mice with mutations in leptin or leptin receptor genes have obese phenotypes (Tilg & Moschen, 2006). Furthermore, data have demonstrated that the overexpression of these genes in transgenic mice decreased body fat by reducing food intake and increasing catabolic activity (Considini, 2005).

Regarding to the neuropeptide Y (NPY), it is a potent hypothalamic orexigenic peptide. Evidence has indicated that the control of *NPY* expression in the arcuate nucleus (ARC) differs from its regulation in the dorsomedial hypothalamus (DMH). While ARC NPY is under the control of circulating leptin, the controls of DMH NPY are leptin-independent (Bi et al., 2003). A previous study showed that *NPY* expression is increased in the ARC in response to acute food deprivation, when circulating leptin levels are significantly decreased, whereas DMH *NPY* expression is significantly increased in rats only after chronic food restriction (Bi et al., 2003). Therefore, our data corroborated literature, once we found *NPY* downregulation in morbid obese women.

Not directly acting on appetite and food intake control, *5-HTR3C* is involved in the transport and re-uptake of serotonin. Serotonin (5-HT) is known to be an important neurotransmitter found in various areas of the central nervous system, being associated to several physiological mechanisms (Veenstra-VanderWeele et al.,2000). This neurotransmitter mediates cellular effects through several proteins that are involved in neurotransmission, synthesis, metabolism and membrane re-uptake (Veenstra-VanderWeele et al.,2000; Cravchik et al., 2000). 5-HT can regulate appetite and food intake, probably by promoting satiety. While 5-HT interacts with multiple subtypes of specific membrane receptors, there is evidence that its effects on appetite and food intake are, in part, mediated, by activation of receptors in the hypothalamus (Kennett & Curzon, 1991; Kennett et al., 1994). It is known that depletion of brain serotonin promotes hyperphagia and obesity, whereas administration of drugs increasing serotonergic transmission, serotonin receptor agonists and serotonin itself all reduce food intake, decrease body weight and enhance energy expenditure (Lam & Heisler, 2007).Since the levels o serotonin mRNA in blood sample did not differ in both groups, we could speculate that the 5-HTR3C overexpression would be related to a compensatory mechanism in attempt to reduce hyperphagia, however this mechanism needs further investigation since few data are found in literature.

As commented before, in a previous study we observed high amount of oxidative DNA lesions in blood cells of obese women, and we suggested the low-grade systemic inflammation and the consequent generation of ROS, as responsible for this genotoxicity. In accordance with these previous finding we observed an upregulation of genes related to DNA repair (*ERCC2, ERCC4* and *ERCC6*). *ERCC* (excision repair cross-complementing) genes are involved in the nucleotide excision repair (NER) pathway, which is a particularly important mechanism forrepairing bulky lesions such

as pyrimidine dimmers, photo-products, large chemical adducts and cross-links (Wood, 1997). There are strong evidences showing that this repair pathway might act as backup for repairing oxidative DNA damage (Reardon et al., 1997; Tilg & Moschen, 2006; Lopes, 2007; Fernández-Sánchez et al., 2011). Therefore, a plausible explanation for *ERCC* overexpression in obese women is because the increased amount of DNA damage caused by the inflammatory status

Regarding to the two genes analyzed in adipocytes (*ADIPOQ* and *LEP*), only *ADIPOQ* was modulated (downregulated) in morbid obese women. It is important to say that this gene was not modulated in the blood cells. Differently, *LEP* was not modulated in both cell types. Adiponectin is a protein which physiological role has not yet been fully elucidated, but it is believed that it has ability to reduce glucose, triglycerides and free fatty acids and also plays a major role in pathogenesis of metabolic syndrome (Shehzad et al., 2011). It is secreted by the adipose tissue and can be found in large amounts in plasma, being inversely related to the body fat in adults (Ukkola, 2002). Therefore, our data demonstrated that the lower level of adiponectin in obese individuals is closely related to the *ADIPOQ* downregulation. Differently, we did not detected changes in the expression of*LEP* both inadipocytes and blood cells. Leptin is also secreted by adipocytes, but it acts in the hypothalamus regulating food intake and energy expenditure, thereby limiting the adiposity (Halaas et al., 1995). Therefore, based on literature (van Heek et al., 1997; Jeanrenaud & Rohner-Jeanrenaud, 2001), it would be reasonable to think about restricted entry of leptin into the brain and/or failure in the response of leptin receptive neurons of the obese subjects. In fact, the transport of leptin into the brain and neuronal sensitivity to the leptin signal depends on the availability of leptin receptors at the blood-brain barrier and leptin receptive neurons, respectively. Both alterations in receptor gene, as well as the endocytosis and trafficking of ligand-activated cell surface receptors can regulate receptor availability and response to ligand (Mitchell et al., 2003; Stratigopoulos et al., 2009). Therefore, a weak interaction between receptor/ligand or changes in the affinity to leptin could also be a plausible explanation for our findings.

In summary, our data showed how the gene expression profile is in blood leukocytes of morbid obese women. Genes related to food intake, immune/defense,DNA repair and too many other different mechanisms were modulated, confirming the multifactorial and multigenic characteristics of obesity. *ADIPOQ*, but

not *LEP*, was downregulated in adipocytes. Gene networks will be build in order to check relationships among them.

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5. Considerações Finais

Desde o início deste século vem crescendo o número de estudos que buscam identificar os fatores genéticos e ambientais que determinam, ou favorecem, o desenvolvimento da obesidade. Esta, já vem sendo considerada um sério problema de saúde pública em vários países do mundo, especialmente devido às comorbidades associadas. Foi nesse cenário, portanto, que propusemos o presente estudo, com o objetivo de contribuir para o estabelecimento do perfil genético e genômico de portadores de obesidade mórbida e para avaliar se o estado inflamatório inerente à obesidade favorece a indução de danos no DNA, os quais podem ser agentes iniciadores das comorbidades.

Nossos resultados mostraram que mulheres obesas apresentavam maiores níveis de danos primários no DNA, incluindo quebras de cadeia e oxidação das bases púricas e pirimídicas. Tal achado foi associado ao estado inflamatório crônico de baixa intensidade que ocorre pelo excesso de tecido adiposo e à consequente produção de espécies reativas de oxigênio (EROs), que podem induz danos em estruturas celulares, proteínas, lipídeos e macromoléculas, como o DNA (Tilg & Moschen, 2006; Fernández-Sánchez et al., 2011). Recentemente, Karbownik-Levinska et al. (2012) realizaram estudo no qual avaliaram os níveis de peroxidação lipídica (PL) e de 8-oxodG (biomarcador de dano oxidativo) em indivíduos obesos, e observaram que tanto a PL quanto a 8-oxodG estão positivamente relacionadas ao índice de massa corporal (IMC), à pressão arterial elevada, circunferência de cintura aumentada e a elevados níveis séricos de PCR (proteína C reativa – marcador de inflamação). Estudos clínicos e experimentais também indicam o estresse oxidativo como importante mecanismo relativo à obesidade, à síndrome metabólica, ao desenvolvimento da diabetes e suas complicações (Furukawa et al., 2004). Portanto, a fim de certificarmos se os danos oxidativos no DNA estariam realmente associados ao processo inflamatório e não à comorbidades da obesidade (como por exemplo, a síndrome metabólica), dividimos o grupo de obesas em dois: mulheres com síndrome metabólica e sem síndrome metabólica. A síndrome metabólica é uma condição multifatorial que leva ao aumento do risco de aterosclerose e diabetes, e é caracterizada pela combinação de três ou mais das seguintes desordens: obesidade abdominal, hipertensão, hiperglicemia e dislipidemia (Isomaa et al., 2001; Grattagliano et al., 2007). Do grupo com obesidade mórbida, 33% das mulheres apresentavam síndrome metabólica, e quando comparamos seus níveis de danos aos das mulheres obesas sem a síndrome, não observamos diferença estatisticamente significativa, levando-nos a acreditar que o fato das obesas

terem apresentado maiores níveis de danos que as mulheres eutróficas não estaria relacionado a comorbidades da obesidade, mas confirmaria o estado inflamatório como o principal evento genotóxico.

Antigamente, acreditava-se que o tecido adiposo possuia apenas a função de estocagem de gordura. Sabe-se, hoje, que se trata de um órgão endócrino capaz de secretar inúmeras adipocinas, citocinas e quimiocinas, incluindo a leptina, adiponectina, resistina, fator de necrose tumoral-α (TNF-α), interleucina (IL)-1β, IL-6 e a proteína quimiotática de monócitos-1 (MCP-1), todos com papel central na regulação energética, vascular e da homeostase do sistema imune, atuando tanto localmente como à distância, e influenciando vários processos metabólicos e imunológicos (Berg & Scherer, 2005; Tilg & Moschen, 2006). De fato, nossos dados obtidos por meio de *microarrays* confirmaram que inúmeros genes pertencentes à família das interleucinas e do TNF-α estavam diferencialmente expressos em células do sangue periférico das mulheres com obesidade mórbida, assim como genes responsáveis pelo sistema de reparo do DNA.

A comparação entre o perfil de expressão gênica de mulheres obesas e eutróficas mostrou que cerca de 2.900 genes estavam diferencialmente expressos, sendo que desses, 1.580 estavam superexpressos e 1.404 subexpressos em obesas. De maneira geral, os genes superexpressos participavam de 857 processos biológicos, principalmente aqueles relacionados à resposta de defesa, sistema imune, consumo de alimento, regulação do apetite e sistemas de reparo do DNA, enquanto os subexpressos a 618 processos.Os genes da família *ERCC* (superexpressos em obesas), codificam enzimas que atuam no reparo por excisão de nucleotídeos (NER), o qual, segundo alguns autores, é reforço para o reparo dos danos oxidativos induzidos pela inflamação (Lopes, 2007; Fernández-Sánchez et al., 2011). Portanto, o aumento da expressão dos genes *ERCCs* deve ter ocorrido em resposta ao aumento de danos observados no teste do cometa em mulheres obesas.No caso do aumento da expressão de genes de citocinas uma possível explicação seria o excesso de gordura no tecido adiposo, que é capaz de ativar células do sistema imune e aumentar a produção de citocinas pró-inflamatórias. O tecido adiposo de indivíduos obesos contém grande quantidade de macrófagos, que são fonte adicional de mediadores solúveis nesse tecido, uma vez que são os adipócitos que contribuem com cerca de um terço da produção de interleucinas circulantes (Weisberg et al., 2006; Xu et al., 2003; Fantuzzi, 2005). Portanto, estando os macrófagos ativos no tecido adiposo, juntamente com os adipócitos e outros tipos

celulares, perpetua-se um ciclo de recrutamento de macrófagos e produção de citocinas pró-inflamatórias (Tilg & Moschen, 2006).

Além da análise do perfil de expressão gênica em células do sangue periférico, também avaliamos a expressão dos genes *ADIPOQ* e *LEP* em adipócitos. Como a amostra de tecido adiposo dos participantes do estudo não eram suficientes para extrair a quantidade necessária de RNA para a realização de *microarrays*, optamos por selecionar dois genes intimamente relacionados à obesidade e avaliá-los por real-time qPCR. Como isso, além de contribuir para o estabelecimento do perfil de expressão gênica em adipócitos, seria possível, também, comparar os padrões de expressões em dois tecidos distintos (tecido adiposo e sangue). No caso do gene *ADIPOQ*, observamos, em primeiro lugar, que este estava menos expresso em mulheres obesas que em eutróficas, o que já era de se supor, uma vez que a adiponectina tem relação inversa com a obesidade e teria função "protetora" contra essa desordem, pois possui a capacidade de reduzir glicose, triglicérides e ácidos graxos, além de ter papel importante no controle da síndrome metabólica (Ukkola & Santaniemi, 2002; Shehzad et al., 2012). Quanto ao gene *LEP*, seu nível de expressão não foi diferente entre os dois grupos, seguindo o mesmo padrão de expressão tanto nos adipócitos quanto em células do sangue periférico, levando-nos a pensar que sua entrada no cérebro não estaria acontecendo de maneira adequada, ou que houvesse falha na ligação entre a leptina e os neurônios, onde ficam localizados seus receptores. De fato, o transporte da leptina para o cérebro e a sensibilidade neuronal à leptina depende da biodisponibilidade de seus receptores, assim como de sua qualidade, e tanto as alterações no gene receptor quanto o seu transporte propriamente dito podem levar a uma fraca interação entre o receptor e a leptina ou a alterações na afinidade desta com o receptor (van Heek et al., 1997; Mitchell et al., 2003; Stratigopoulos, 2009).

Além das possíveis alterações no padrão de expressão, avaliarmos se determinados polimorfismos gênicos (genes da grelina e leptina e de seus respectivos receptores, do receptor da serotonina e da interleucina-6) podem favorecer o desenvolvimento da obesidade. Dos seis SNPs investigados, quatro se localizavam na região promotora dos genes e dois em regiões codificadoras. Hoje, sabe-se que SNPs em regiões promotoras estão associados a mudanças no transcriptoma, incluindo a quantidade de proteína produzida. No caso de nosso estudo, os SNPs em regiões codificadoras estavam relacionados aos genes que codificam receptores e, portanto, com potencial para interferir na interação entre o receptor e proteína (Nakayama et al., 1999;

Stratigopoulos et al., 2009). Contudo, nossos resultados demonstraram que nenhuma das variantes gênicas estudadas estava relacionada com o peso e o IMC, isto é, as frequências alélicas e genotípicas eram semelhantes entre as mulheres obesas e eutróficas.

6. Conclusões

Nossos resultados mostraram que:

- as variantes dos genes *GHRL* (-501A>C, RS 26802), *GHSR* (477G>A, rs 572169), *LEP* (-2548G>A, rs 7799039), *LEPR* (668A>G, rs 1137101), *5-HT2C* (-759C/T, rs 3813929) e *IL-6* (-572G>C, rs 1800796) não estão diretamente relacionadas à obesidade mórbida e, portanto, não devem ser consideradas como bons biomarcadores para essa disfunção do organismo;

- as mulheres obesas apresentam maiores níveis de danos primários no DNA (quebras de cadeia simples e dupla, sítios álcali-lábeis e bases oxidadas) de leucócitos do sangue periférico do que as eutróficas;

- o aumento de danos no DNA das mulheres obesas não está relacionado à presença da síndrome metabólica;

- os genótipos *GHRL*-CC, *LEP*-GG e *IL-6*-GC + CC estão associados a maior nível de danos no DNA, tanto em mulheres obesas como eutróficas;

- cerca de 2.900 genes estavam diferencialmente expressos nas mulheres obesas em relação às eutróficas, sendo que desses, 1.580 estavam superexpressos e 1.404 subexpressos. De maneira geral, os genes superexpressos participam de 857 processos biológicos, enquanto os subexpressos de 618, processos esses principalmente relacionados à resposta de defesa, sistema imune, consumo de alimento, regulação do apetite e sistemas de reparo do DNA;

Finalizando, o grande volume de dados gerados possibilitará que sejam ainda construídas redes para avaliar as complexas interações gênicas e suas relações com a obesidade.

7. Referências

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8. Anexos

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

(TERMINOLOGIA OBRIGATÓRIO EM ATENDIMENTO A RESOLUÇÃO 196/96 –CNS-MS)

Título da Pesquisa: "Alterações genéticas relacionadas à obesidade: danos no DNA, perfil de expressão e polimorfismos gênicos"

Eu _____, RG_____, Estado Civil _____, Idade___ anos, Residente na _____, nº _____, Complemento _____, Bairro _____, Cidade _____, Telefone (__) _____,

Declaro ter sido esclarecido sobre os seguintes pontos:

1. O trabalho tem por finalidade avaliar os resultados da cirurgia bariátrica de mulheres desde a fila de espera para a cirurgia até a manutenção do peso corporal, levando em conta: 1) a herança genética; 2) o consumo de alimentos; 3) os gastos de caloria em repouso e em atividade física; 4) o colesterol e as gorduras do sangue; 5) as defesas contra as substâncias agressoras do ambiente; 6) os hormônios ligados à obesidade; 7) a saúde dos ossos e; 8) as reservas de ferro no sangue.
2. Ao participar desse trabalho estarei contribuindo para esclarecer os resultados da cirurgia sobre o estado nutricional, doenças associadas e qualidade de vida de indivíduos após a cirurgia bariátrica.
3. Para a realização dessa pesquisa, autorizo a consulta de todos os meus dados registrados na Clínica Bariátrica e no Hospital das Clínicas de Botucatu, autorizo ainda a retirada de uma amostra da gordura da parede abdominal durante a cirurgia, bem como me disponibilizo a responder questionários sobre meus hábitos de vida e de consumo de alimentos, os quais terão duração de cerca de 40 minutos, e ainda me comprometo a realizar:
   - testes do gasto de caloria em repouso e em atividade física, por meio de um equipamento que mede o gasto calórico diário. Este exame tem a duração de cerca de 1 hora;

- avaliação da composição corporal, em gordura e outros componentes, por meio da verificação do peso, altura, dobra de gordura e circunferências. Esta avaliação tem a duração média de 20 minutos;
- medida da pressão arterial;
- coleta de sangue.

4. A minha participação como voluntário deverá ter a duração de dois anos com entrevista para avaliação nutricional (aplicação dos questionários sobre hábitos de vida e de consumo de alimentos e realização da avaliação da composição corporal), coleta de sangue para exames e o teste de gasto calórico, os quais serão realizados antes da cirurgia, 1 (um), 3 (três), 6 (seis), 12 (doze) e 24 (vinte e quatro) meses após a cirurgia, em datas previamente agendadas pela clínica ou pelo hospital, de forma a coincidir com o meu acompanhamento de rotina.
5. A coleta dos dados não será desconfortável, sendo que terei a liberdade de responder ou não qualquer pergunta e me recusar à realização dos exames.
6. Não haverá a necessidade de ser indenizado por parte da equipe responsável por esse trabalho ou da Instituição (Instituto de Biociências de Botucatu - UNESP).
7. O sangue que será coletado para este estudo será congelado para a realização de futuras pesquisas, cujos projetos serão apresentados a um Comitê de Ética em Pesquisa quando forem ocorrer. Serei avisado(a) do desenvolvimento das mesmas devendo, se desejar, assinar um novo Termo de Consentimento Livre e Esclarecido.
8. Meu nome será mantido em sigilo, assegurando assim a minha privacidade e se desejar, deverei ser informado sobre os resultados dessa pesquisa.
9. Poderei me recusar a participar ou mesmo retirar meu consentimento a qualquer momento da realização dessa pesquisa, sem nenhum prejuízo ou penalização, isto é, sem interrupção do meu tratamento.
10. Qualquer dúvida ou solicitação de esclarecimentos, poderei entrar em contato com a equipe científica pelo telefone Maria Rita Marques de Oliveira, (14) 3811-6232 Ramal 219, ou Clínica Bariátrica, (19) 3421-9100.
11. Para notificação de qualquer situação, relacionada com a ética, que não puder ser resolvida pelos pesquisadores deverei entrar em contato com o Comitê de Ética em Pesquisa da Faculdade de Medicina de Botucatu, pelo telefone (0XX14) 3811-6143.

Diante dos esclarecimentos prestados, concordo em participar, como voluntária(o), do estudo "Alterações genéticas relacionadas à obesidade: damos no DNA e perfil de expressão e polimorfismos gênicos".

Piracicaba/Botucatu, _____/_____/_____

_____

Assinatura do Voluntário

_____

Assinatura do Pesquisador

unesp
Universidade Estadual Paulista
Faculdade de Medicina de Botucatu

Distrito Rubião Junior, s/nº - Botucatu – S.P.
CEP: 18.618-970
Fone/Fax: (0xx14) 3811-6143
e-mail secretaria: capellup@fmb.unesp.br
e-mail coordenadoria: tsarden@fmb.unesp.br

Comitê de Ética em Pesquisa
Ética

Registrado no Ministério da Saúde
em 30 de abril de 1997

Botucatu, 05 de outubro de 2.009

OF. 398/2009-CEP

Ilustríssima Senhora
Profª. Drª. Daisy Maria Fávero Salvadori
Departamento de Patologia da
Faculdade de Medicina de Botucatu.

Prezada Profª Daisy,

De ordem do Senhor Coordenador deste CEP, informo que o Projeto de Pesquisa, **(Protocolo CEP 3361-2009) "Alterações genéticas relacionadas a obesidade: danos no DNA e perfil de expressão gênica e polimorfismos gênicos"**, que será conduzido por Danielle Cristina Almeida, orientada por Vossa Senhoria, com a participação de Maria Rita Marques de Oliveira, Glenda Nicioli da Silva, Rozangela Verlengia, Celso Vieira de Souza Leite e Irineu Rasera Junior, recebeu do relator **parecer favorável**, aprovado em reunião de 05/10/09.

Situação do Projeto: APROVADO. Ao final da execução deste Projeto, apresentar ao CEP **"Relatório Final de Atividades"**.

Atenciosamente,

Alberto Santos Capelluppi
Secretário do CEP