

TATIANA FICHE SALLES TEIXEIRA

**INTER-RELATION OF FECAL MICROBIOTA, INTESTINAL  
PERMEABILITY, ENDOTOXEMIA AND INTESTINAL INFLAMMATION  
MARKERS ON OBESITY AND THE DEGREE OF INSULIN RESISTANCE**

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência da Nutrição, para obtenção do título de *Doctor Scientiae*.

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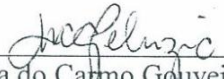
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*Só sei que nada sei, e o fato de saber isso, me coloca em vantagem sobre  
aqueles que acham que sabem alguma coisa (Sócrates)*

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## LISTA DE ABREVIATURAS

**ALT:** alanine aminotransferase  
**ANOVA:** analysis of variance  
**AOAH:** acyloxyacyl hydrolase  
**AP:** alkaline phosphatase  
**AST:** aspartate aminotransferase  
**AT:** adipose tissue  
**BA:** bile acids  
**BMI:** body mass index  
**CRP:** C-reactive protein  
**DXA:** dual-energy X-ray absorptiometry  
**eCB:** endocannabinoid system  
**EU/ml:** endotoxin units per milliliter  
**FIAF:** fasting-induced adipose factor  
**FXR:** farnesoid X receptor  
**HDL:** high-density-lipoprotein  
**HF:** high fat  
**HOMA:** homeostasis assessment model  
**IEC:** intestinal epithelial cells  
**IGT:** impaired glucose tolerance  
**IP:** intestinal permeability  
**IR:** insulin resistance  
**IRO:** insulin-resistant obese  
**IRS:** insulin receptor substrate  
**ISO:** insulin-sensitive obese  
**L/M:** lactulose/mannitol ratio  
**LBP:** LPS binding protein  
**LDL:** low density lipoprotein  
**LPS:** lipopolysaccharides  
**LTA:** lipoteichoic acids  
**MCP-1:** Monocyte chemotatic protein-1  
**MetS:** metabolic syndrome  
**MHNW:** metabolically healthy normal weight  
**MHO:** metabolically healthy obese

**MONW:** metabolically obese normal weight  
**MyD88:** myeloid differentiation factor-88  
**NEFA:** non-esterified fatty acids  
**NFκB:** nuclear factor kappa beta  
**OHR:** overweight or obese at higher risk  
**SAT:** subcutaneous adipose tissue  
**SHP:** small heterodimer partner  
**T2DM:** type 2 diabetes mellitus  
**TG:** triglycerides  
**TJ:** tight junctions  
**TLR:** toll-like receptors  
**TNF:** tumor necrosis factor alpha  
**VAI:** visceral adiposity index  
**VAT:** visceral adipose tissue  
**VLDL:** very low density lipoprotein

## RESUMO

TEIXEIRA, Tatiana Fiche Salles, D.Sc., Universidade Federal de Viçosa, dezembro de 2013. **Inter-relation of fecal microbiota, intestinal permeability, endotoxemia and intestinal inflammation markers on obesity and the degree of insulin resistance.** Orientadora: Maria do Carmo Gouveia Peluzio. Coorientadores: Leandro Licursi de Oliveira e Ângela Aparecida Barra.

O excesso de peso é considerado um sinal de problema de saúde atual ou futuro. Múltiplos fatores contribuem para o desenvolvimento e manutenção da obesidade e complicações associadas. Evidências recentes sugerem que existe uma complexa relação entre LPS, dieta, microbiota, permeabilidade intestinal, resistência à insulina (RI) e obesidade. No intuito de contribuir para o melhor entendimento desta complexa relação a presente tese apresenta 6 artigos. Os 3 primeiros são artigos de revisão que abordam os seguintes temas: 1) A complexidade da relação entre adiposidade (distribuição e hipertrofia do tecido adiposo) e alterações metabólicas, incluindo RI. O uso de termos como “obesos metabolicamente saudáveis” e “magros metabolicamente obesos” para definir diferentes fenótipos nas diferentes faixas de índice de massa corporal (IMC). 2) O envolvimento de endotoxinas, mais especificamente os lipopolissacarídeos (LPS) provenientes da microbiota gastrointestinal, como gatilho da ativação inflamatória e RI, e a complexidade de fatores que interagem neste contexto. 3) Os fatores que influenciam a alteração da permeabilidade intestinal, assim como aspectos metodológicos de avaliação da mesma. Em seguida são apresentados 3 artigos originais, cada qual acompanhado do resumo dos objetivos, métodos e resultados. Em geral, não foi observada associação da obesidade com permeabilidade intestinal aumentada e níveis elevados de LPS plasmático, como sugerido por modelos animais. No entanto, alguns resultados indicam a necessidade de que futuros estudos utilizem metodologias diferentes do teste de lactulose/manitol para avaliação da permeabilidade intestinal na obesidade. Indivíduos sobrepeso apresentaram a maior concentração de LPS plasmático, sem, no entanto, apresentar o maior grau de RI. Por outro lado, indivíduos com maiores concentrações de LPS plasmáticos apresentaram maior percentual de gordura androide e da enzima hepática AST em comparação com indivíduos com menores concentrações de LPS plasmático. O delineamento do nosso estudo não permite afirmar que os níveis de LPS plasmático não estejam envolvidos no desenvolvimento da RI. No entanto, é possível que durante a transição do estado de

sobrepeso para a obesidade os níveis de LPS plasmático influenciem o acúmulo de adiposidade central e o metabolismo hepático, o que em longo prazo pode contribuir para o desenvolvimento da RI. Além disso, demonstramos que a obesidade está associada a alterações da microbiota intestinal, confirmando achados de estudos anteriores. Estabelecer o impacto do LPS transpondo a barreira intestinal, e não aquele diretamente infundido na circulação, na RI em humanos não é uma tarefa fácil. Estudos de seguimento epidemiológicos, incluindo um maior número de indivíduos e comparando os possíveis fenótipos metabólicos em indivíduos com mesmo IMC, são necessários para esclarecer como as concentrações plasmáticas de LPS influenciam o metabolismo, e se alterações da microbiota fecal e da permeabilidade intestinal contribuiriam para o aumento de LPS plasmático em alguma fase.

## ABSTRACT

TEIXEIRA, Tatiana Fiche Salles, D.Sc., Universidade Federal de Viçosa, December, 2013. **Inter-relation of fecal microbiota, intestinal permeability, endotoxemia and intestinal inflammation markers on obesity and the degree of insulin resistance.** Adviser: Maria do Carmo Gouveia Peluzio. Co-advisers: Leandro Licursi de Oliveira and Ângela Aparecida Barra.

Excess body weight has been considered a signal of current or future health problems. Multiple factors contribute for the development and maintenance of obesity and complications associated. Recent evidences suggest a complex relationship between LPS, diet, microbiota, intestinal permeability, insulin resistance (IR) and obesity. To contribute for a better understanding of this complex relationship this thesis presents 6 articles. The first 3 are review articles that address the following themes: 1) The complexity of the relation between adiposity (distribution and hypertrophy of adipose tissue) and metabolic alterations, including IR. The use of terms such as “metabolically healthy obesity” and “metabolically obese normal weight” to define different phenotypes within categories of body mass index (BMI). 2) The involvement of endotoxins, more specifically lipopolysaccharides (LPS) from gastrointestinal microbiota, as a trigger of inflammatory activation and IR, as well as the complexity of factors that interacts in this context. 3) The factors that influence alteration of intestinal permeability, as well as methodological aspects of its evaluation. Next, 3 original articles are presented, each one presenting the summary of aims, methods and results. In general, association between obesity with higher intestinal permeability and higher plasma LPS concentration, as suggested by animal models, was not observed. Nevertheless, some of our findings indicate that future studies should use methodologies different from lactulose/mannitol test to evaluate intestinal permeability in obesity. Overweight subjects presented the highest plasma LPS concentration even so they did not show the highest degree of IR. On the other hand, subjects presenting the highest LPS concentration also showed the highest android fat percentage and the hepatic enzymes AST in comparison to subjects of lower plasma LPS. Our study design does not allow rulling out that plasma LPS levels are not involved in IR development. However, it is possible that during the transition of overweight to obese state plasma LPS concentration influences the accumulation of central fat and hepatic metabolism,

which in the long term could lead to development of IR. Additionally, we demonstrated that obesity is associated with alteration of microbiota composition, confirming findings from previous studies. Establishing the impact of LPS transposing gut barrier, not directly infused into the circulation, on IR in humans is not an easy task. Follow-up studies, including a higher number of subjects and comparing the possible metabolic phenotypes within subjects of the same BMI, are needed to clarify how plasma LPS concentration influences metabolism and if alteration of fecal microbiota and intestinal permeability could contribute to increase plasma LPS during a specific period.

## 1. GENERAL INTRODUCTION

The interaction between biological, social and psychological factors contributes to the establishment and maintenance of obesity, which becomes a chronic and progressive condition associated with health complications. However, the expansion of adipose tissue does not necessarily lead to diseases in humans. The tolerable threshold level of adiposity differs between subjects and is possibly influenced by environmental and genetic factors.<sup>1</sup> Therefore, there is a current trend to use terms such as benign/metabolically healthy or malign/unhealthy obese condition in accordance with the absence or presence of metabolic alterations, respectively.<sup>2-3</sup>

The main metabolic alteration associated with the malign/unhealthy condition of obesity is insulin resistance (IR),<sup>3</sup> which in turn associates with other dysfunctions such as glucose intolerance, dyslipidemia and endothelial dysfunction. Hence, the risks for the development of cardiovascular diseases, diabetes and hepatic steatosis are higher in the presence of both obesity and IR.<sup>4</sup>

The development of insulin resistance has been classically attributed to the production and secretion of inflammatory mediators, due to adipose tissue hypertrophy (induced by excessive caloric intake), associated with infiltration of specialized immune cells (such as macrophages) in this tissue. The progression of this condition increases the activation of inflammatory pathways and secretion of cytokines, such as TNF, that reduces the ability to store triglycerides (from diet or endogenous origin) into adipose tissue and stimulates lipolysis. In consequence, there is an increased delivery of free fatty acids and triglycerides into the circulation, which can be deposited in other organs such as the liver, skeletal muscle and heart. The ectopic deposition of fat impairs cellular processes such as oxidative mitochondrial phosphorylation and glucose transport induced by insulin, triggering IR.<sup>5</sup> Therefore, the restoration of metabolic functions seems to depend on the resolution of the chronic inflammatory state, which is suggested as a central biological aspect of the morbidities associated with obesity.

The identification of toll-like receptors (TLRs) in adipocytes, epithelial and immune cells<sup>6</sup> and their role in the activation of inflammation brought about new perspectives regarding the triggers of IR. The activation of these receptors has been considered a molecular mechanism correlated to the interaction between the diet (more specifically the lipids), inflammation, activation of innate immune system and sensitivity to the

action of insulin.<sup>7</sup> Additionally, these receptors are specialized in the recognition of pathogen-associated molecular patterns.<sup>1</sup> The endotoxins, among which lipopolysaccharides (LPS) derived from microorganisms stands out, are true ligands to TLRs able to induce inflammatory responses. Higher concentration of plasma endotoxins seems to increase the risk of chronic diseases related to subclinical inflammatory state.<sup>8-9</sup>

In fact, the subcutaneous infusion of LPS causes similar consequences to the high fat intake by animals: deregulation of inflammatory tonus, increased fasting glucose and insulin, increased body weight, liver and adipose tissue.<sup>10</sup> The definition of how the concentration of LPS increase in the circulation is as complex as the molecular mechanisms activated by LPS signaling. Two main mechanisms have been suggested: incorporation of LPS into chylomicrons<sup>11</sup> and passage through the paracellular space due to the increase in intestinal permeability.<sup>12-13</sup> Changes in the composition of gastrointestinal microbiota have been evidenced in obesity and associated to the increase of LPS absorption and intestinal permeability in animals.<sup>13-14</sup>

Evidences that demonstrate that obese subjects show increased intestinal permeability and that this favors the occurrence of endotoxemia are still scarce. The studies that detect higher level of circulating LPS in subjects with diabetes, obesity and/or cardiovascular diseases did not assess intestinal permeability.<sup>9,15-18</sup> It has been demonstrated in humans, animals and cell culture that exposure to higher fat content increases the concentration of LPS in the circulation.<sup>11,18,19,20</sup>

Therefore, it is not clear if both mechanisms – higher intake of fat and higher intestinal permeability – are related to increment of LPS concentration in the circulation in obesity. Few gaps in this area still need further investigation. The endotoxins (LPS) have been increasingly related to diabetes and cardiovascular diseases, suggesting the involvement of intestinal microbiota in metabolic disturbances. The hypothesis of higher intestinal permeability, one of the possible routes that allow increase of LPS into the circulation, has been tested in animal models and confirmed in different clinical situations, but not in obese individuals. The evidences that support the link between obesity, higher intestinal permeability, endotoxemia and type of intestinal microbiota in humans have been provided by studies that do not include assessment of all these



aspects in the same group of obese subjects. Therefore, more studies in this area are still needed.

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## **2. AIMS OF THE STUDY**

### **2.1. General aim**

Investigate the association between intestinal permeability, intestinal inflammation markers, endotoxemia and fecal microbiota with obesity and the degree of insulin resistance.

### **2.2. Specific aims**

- Correlate intestinal permeability and the concentration of plasma LPS, as well as their association with the degree of insulin resistance;
- Correlate the concentration of fecal markers of intestinal inflammation with intestinal permeability and endotoxins;
- Investigate the inter-relation between body adiposity, plasma LPS and the degree of insulin resistance;
- Compare the abundance of specific bacteria from fecal microbiota between lean and obese subjects;
- Correlate the abundance of specific bacteria with a marker of insulin resistance and endotoxemia.

### **3. ARTICLES**

#### **3.1 . Article 1 (review): Metabolically obese normal weight and metabolically healthy obese: what are the main characteristics of these phenotypes?**

Tatiana F S Teixeira, Raquel D M Alves, Ana Paula B Moreira, Maria do Carmo G Peluzio

#### **Abstract**

The aim of this review is to discuss the influence of fat depots on insulin resistance and the main characteristics of metabolically obese normal weight and metabolically healthy obese phenotypes. Medline/Pubmed and Science Direct were searched for papers related to the terms metabolically healthy obesity, metabolically obese normal weight, adipose tissue and insulin resistance. Normal weight and obesity might be heterogeneous in regard to its effects. Fat distribution and lower insulin sensitivity are the main factors defining phenotypes within the same body mass index. There are still some controversies to be solved regarding these terms. Future studies exploring these phenotypes will help to better understand the role of adiposity and/or insulin resistance in the development of metabolic alterations.

**Key words:** insulin resistance, adiposity, obesity

## 1. Introduction

The role of total adiposity in metabolic disorders is not precisely defined. Adiposity increases due to positive energy balance, sedentary lifestyle, genetic predisposition, psychosocial factors,<sup>1-3</sup> and possibly the gut microbiota profile.<sup>4-5</sup> A progressive increase in the prevalence and/or severity of morbidities and in the risk of mortality occurs as adiposity increases and obesity is established.<sup>2-3</sup> Hyperglycemia, dyslipidaemia, and hypertension are often associated with abdominal obesity and insulin resistance (IR) and their concomitant occurrence identify subjects at great risk (i.e., metabolic syndrome, MetS) of developing chronic diseases.<sup>6-7</sup>

It has been more than 20 years since IR was suggested to be the central metabolic disability that in long-term entails type 2 diabetes mellitus (T2DM), hypertension, and cardiovascular diseases.<sup>7-9</sup> IR occurs when higher insulin levels are necessary to maintain normal or only slightly impaired glycemia, while  $\beta$ -cell dysfunction with decrease in insulin levels leads to severe glucose intolerance and T2DM.<sup>8-10</sup> Although there is a strong association between obesity and IR, an obese subject can abstain from T2DM if a compensatory pancreatic  $\beta$ -cell response is nearly perfect. On the other hand, even normal weight subjects may develop IR, T2DM, and other metabolic disorders.<sup>8</sup>

A link between generalized or central obesity and metabolic disorders such as IR is currently assumed.<sup>11-14</sup> The degree of IR can rise with fat mass.<sup>11</sup> However, as stated by Virtue and Vidal-Puig<sup>11</sup> 'at the individual level, the association between the degree of obesity and development of IR may not be so clear cut'. Besides, the role of different fat depots on the development of metabolic complications is still open to controversy.<sup>15</sup>

Surprisingly, a body mass index (BMI) over 30 kg/m<sup>2</sup> *per se*, does not necessarily lead to metabolic disorders.<sup>16</sup> Indeed, some obese subjects, classified by means of their BMI, may have better metabolic profile than predicted.<sup>17</sup> Obesity may represent an adaptation to re-establish a new homeostatic state under a high availability of food/calories<sup>18</sup> in a way that expansion of adipose tissue might help to maintain a normal blood glucose and lipid profile.

In this context, two main terms have been used to identify different phenotypes in relation to the body size and the metabolism: metabolically obese normal weight (MONW) and metabolically healthy obese (MHO). They indicate that obese subjects will not necessarily present metabolic disorders while normal weight will not be necessarily 'healthy'. Thus, the aim of this review is to discuss how fat depots may influence the metabolic profile and about

the anthropometric, body composition, and biochemical characteristics of MONW and MHO subjects as well as the controversies regarding these terms.

## **2. Fat depots and metabolic disorders**

Adipose tissue is a clustering of cells (adipocytes and stromal cells) specialized in fat storage and capable of secreting adipokines and impacting on whole metabolism and immune cells.<sup>2,15</sup> Brown and white adipose tissues differ in their functionality: the first dissipate energy as heat (thermogenesis), while the latter is more associated with the endocrine and storage functions. The white adipose tissue can be found deeply and superficially beneath the skin (subcutaneous adipose tissue - SAT) and within the peritoneal cavity (visceral adipose tissue - VAT).<sup>11,19-22</sup> Conversely, abdominal fat is not synonymous of VAT. Therefore, waist circumference is a measurement of abdominal fat but does not discriminate between VAT and SAT.<sup>21,23-25</sup> Lam *et al.*<sup>22</sup> emphasizes the importance of carefully interpreting studies that uses the collective term 'visceral fat'. Different anatomical localization within peritoneal cavity (e.g. perirenal, omental, mesenteric) may imply different impact on metabolism.<sup>22,25</sup>

The distribution of fat, particularly the VAT, may be influenced by aging, gender (usually in men is higher), menopause, smoking, sedentary lifestyle, and nutritional factors (high-energy and high-fat diet, fructose).<sup>13,21,25</sup> The development of metabolic diseases may be a consequence of body weight and fat gain, but it is also related to fat depot location (visceral vs. subcutaneous, central vs. peripheral), hypertrophy or hyperplasia of adipocytes, liver fat and IR, as well as to the adipokines profile.<sup>2,3,15</sup> Therefore, the use of body mass index by itself for obesity diagnostics could lead to misclassification of risk if the percentage and localization of body fat is not considered.

### *2.1. Fat depot location*

VAT is often considered 'hazardous'<sup>13,21,23,26</sup> even representing only 7-15% of total body fat.<sup>27</sup> Liposuction of abdominal SAT did not significantly alter metabolic profile in the short-term<sup>28</sup> or even after a long-term longitudinal assessment.<sup>29</sup> The reduction of VAT might be more appropriate for metabolic improvement.

Positive association between VAT and IR are often reported.<sup>30</sup> Increased non-esterified fatty acids (NEFA) flux is the main mechanism to explain the association between visceral fat depot expansion and metabolic disabilities, including IR.<sup>31</sup> Visceral adipocytes in obese

subjects release large amounts of NEFA and glycerol. The excess of substrates availability affects different sites. In the liver, these substrates are converted into triglycerides (lipogenesis) and glucose (gluconeogenesis). The increase in intramyocellular lipids in skeletal muscle cells impairs insulin sensitivity and decreases the glucose uptake and glucose partitioning to glycogen. There is also impairment of insulin secretion in pancreatic islets leading to glucose intolerance. In parallel, insulin sensitivity in adipocytes decreases increasing lipolysis and NEFA supply. This partially explains the complex relationship between obesity, NEFA, IR, and dyslipidemia.<sup>7,31,32</sup>

In fact, Nielsen *et al.*<sup>33</sup> verified that obese had higher plasma NEFA than lean subjects and also a greater splanchnic NEFA uptake.<sup>33</sup> As visceral fat increases, its lipolysis accounts for an increasing proportion of hepatic NEFA delivery. However, the relative contribution of visceral fat mass in NEFA pool varies among subjects differing in their body composition and fat distribution.<sup>33</sup> The proportion of portal NEFA derived from VAT was greatly lower than the relative amount derived from lipolysis of SAT. Fatty acids released by SAT depots get into the venous circulation and reach splanchnic tissues by the arterial circulation. The excessive fatty acid released from VAT could be an important factor in developing hepatic IR, but it is unlikely to be the major factor in the pathogenesis of IR in skeletal muscle.<sup>34</sup> Thus, both fat depots are important suppliers of NEFA to the liver and SAT may play a key role as an initiating factor in the process of fat overflow to other ectopic sites.

Higher level of the mRNA expression of pro-inflammatory genes such as chemotactic factors is a clear distinction between VAT and deep and superficial SAT.<sup>20</sup> Tumor necrosis factor- $\alpha$ , macrophage inflammatory protein, and interleukin-8 were also highly expressed within VAT from T2DM subjects.<sup>35</sup> Additionally, fasting glucose was positively correlated with mRNA expression of these molecules in VAT, while fasting insulin was positively associated with expression of serum amyloid-A and IL-1 $\alpha$ .<sup>35</sup> The 'bad' fame of VAT is also related to higher propensity to express inflammatory mediators related to the recruitment and activation of immune cells.

Alvehus *et al.*<sup>20</sup> made an important consideration regarding gene expression and the pure mass effect. Gene expression is often expressed in relation to total RNA and does not consider tissue weight and/or cell size for the results adjustments. In their study, the volume of VAT was significantly smaller than SAT depots, which indicates that the impact of SAT on inflammation and metabolism may be underestimated. Whether considering tissue weight

and/or cell size may alter the interpretation of expression of genes of interest still needs elucidation.<sup>20</sup>

In an epidemiological study, an increment in fat depots, including subcutaneous, increased the risk of calcification in vascular beds.<sup>14</sup> The higher expression of nuclear factor kappa beta (NFkB) and leptin in SAT and the positive association between fasting insulin and the expression of a molecule regulating adipogenesis (cAMP response element-binding protein) in SAT indicates the possibility that this tissue contributes to the systemic inflammation and IR.<sup>35</sup> The differences found in gene expression of different regions of SAT (upper abdomen, lower abdomen, flank, and hip) may have pathophysiological implications when adiposity increases. Genes involved in the complement and coagulation cascades, immune responses, insulin signaling, urea cycle, and amino acids metabolism were highly expressed in the lower abdomen compared to the flank or hip.<sup>36</sup> It seems that both, VAT and SAT in the abdominal area are unfavorable to the metabolism. However, McLaughlin *et al.*<sup>27</sup> observed that SAT might exert a protective role. Insulin sensitive subjects showed significantly larger SAT depots and regression analysis indicated that increased SAT was associated with a decrement in the risk of being insulin resistant.<sup>27</sup>

Impairment in  $\beta$ -cell function might not be due to obesity per se. Elevated plasma NEFA concentration can be a metabolic derangement contributing to defects in compensatory  $\beta$ -cell response, as proposed by the lipotoxicity hypothesis. However, it is also possible that increased NEFA is a consequence of the reduced anti-lipolytic effect of insulin in cases where impaired insulin secretion is observed.<sup>37</sup> Lower VAT, lower fat intermediates in ectopic sites, greater capacity of organs such as muscle and liver for fat utilization rather than storage, and higher capacity for storing fat in SAT may help to preserve insulin sensitivity in some obese subjects.<sup>6,38,39</sup>

## 2.2. Hypertrophy and hyperplasia

The adipocyte size is an important histological characteristic to be considered in metabolic disabilities.<sup>30</sup> Hypertrophied intra-abdominal adipocytes are characterized by a hyperlipolytic state, which is resistant to the anti-lipolytic effect of insulin and provides large amounts of NEFA.<sup>31</sup>

Cell size from SAT and VAT depots correlated with waist-to-hip ratio and it was larger in subjects with metabolic syndrome (MetS) and hypertension. VAT adipocytes size correlated



positively with fasting glucose, insulin, homeostasis model assessment (HOMA), and the hepatic enzyme  $\gamma$ -glutamyl transferase.<sup>40</sup> Of note, subcutaneous adipocytes were larger than visceral.<sup>40</sup> However, adipocytes hypertrophy in omental depots can be more hazardous than in subcutaneous depots.<sup>30</sup> In fact, higher omental-adipocyte diameter was found in obese women with IR,<sup>41,42</sup> and it was correlated with the degree of IR and hepatic steatosis. Curiously, subcutaneous adipocytes size was also associated with the degree of liver fatness, but had no association with metabolic parameters.<sup>41</sup> Therefore, VAT hypertrophy seems to be more linked to IR.

The hyperplasia of visceral adipocytes is possibly dependent on the overflow of chemical energy from the inefficient storage of fat by the subcutaneous depots. Probably, an enhanced adipogenic capacity of subcutaneous depots protects against metabolic syndrome since it may contribute to a lower rate of omental adipocytes hypertrophy.<sup>15,41,42</sup>

### *2.3. Liver fat and insulin resistance*

Tarantino *et al.*<sup>43</sup> observed positive correlation between HOMA and severity of hepatic steatosis in young individuals. In addition, IR was not associated with BMI and adiposity. They questioned if high fat content in liver could be the breaking point between “benign” and “progressive malign” obesity.<sup>43</sup>

Non-alcoholic fatty liver disease (NAFLD) is considered to be one of the consequences of adipose tissue IR. NAFLD can progress toward more severe stages such as steatohepatitis, fibrosis, and cirrhosis. Nevertheless, in some subjects it is maintained as ‘simple steatosis’. Therefore, the terms ‘metabolically malign’ and ‘metabolically benign’ are also being used to describe the phenotypes of liver disease.<sup>44</sup>

Insulin signaling is required for storing energy as fat in healthy humans. However, in the presence of IR, triglycerides (TG) synthesis is decreased in adipose tissue and increased in liver,<sup>45</sup> impairing glucose, and lipid metabolism. Hepatic TG synthesis is recognized as an adaptive process under abundance of lipogenic precursors that allows fat to be stored in its least toxic form. An effective hepatic TG synthesis, lipid desaturation, and inhibition of lipid-induced inflammatory signaling are mechanisms that explain why fatty liver is not always accompanied by metabolic alterations, characterizing a metabolically benign state. When these compensatory mechanisms are overwhelmed, fatty acids induce damage to cells resulting in impairment of metabolism. A metabolically malignant condition of the liver is a

consequence of fat accumulation and is characterized by dyslipidemia and increased hepatic glucose production with hepatic IR.<sup>44</sup> Subjects with fatty liver showed a high-risk metabolic profile compared to subjects without fatty liver. This profile was characterized by higher BMI, waist circumference, SAT and VAT, fasting glucose, HOMA, TG, blood pressure, higher prevalence of T2DM, IR and MetS, as well as lower high-density lipoprotein (HDL). Fatty liver remained associated with dyslipidemia and dysglycemia even after adjusting analysis for VAT.<sup>46</sup>

Ectopic fat in the liver may be more important than visceral fat in the determination of metabolic disabilities in obesity.<sup>38</sup> Magkos *et al.*<sup>47</sup> found that a marked increased BMI, total body fat, and VAT was not associated with increased IR or alterations in very low density lipoprotein (VLDL) and VLDL-apo-B-100 metabolism in obese subjects without increased intra-hepatic TG content. The fat content of liver was associated with metabolic dysregulation, supporting the conclusion that increasing whole-body adiposity does not cause additional metabolic disabilities in the absence of increased intra-hepatic TG. Subjects classified as class III obese had nearly twice the volume of VAT than those classified as class I obese, despite having the same amount of intra-hepatic TG.<sup>47</sup>

#### *2.4. Adipokines profile and inflammation*

A chronic inflammatory status is often associated with obesity and IR.<sup>48</sup> Adipose tissue plays a central and primary role in inflammation level, which influences insulin sensitivity.<sup>49</sup> The infiltration of immune cells is an orchestrating event to induce inflammation and is higher in VAT than SAT.<sup>40</sup> The mechanisms for the accumulation of immune cells within the adipose tissue are not fully understood. Changes in the degree of adiposity might modulate the number and phenotype of immune cells. Adipocytes and stromal cells express signaling mediators that attract inflammatory cells (such as neutrophils, macrophages, mast cells, lymphocytes).<sup>49</sup> These cells secrete various cytokines (IL-1 $\beta$ , IL-6, IL-8, TNF, and MCP-1) that alter the pattern of expression and secretion of adipokines and cytokines in adipose tissue. This may constitute both a cause and a consequence of adipose tissue inflammation. These mediators in turn, entail adipose tissue dysfunction and impairment of insulin sensitivity, both locally and systemically.<sup>15,50</sup>

Insulin resistant obese (IRO) subjects showed higher infiltration of macrophages in omental adipose tissue, but not in SAT, than insulin sensitive subjects. The numbers of macrophages infiltrating omental adipose tissue and circulating adiponectin were the two single best

correlate with insulin sensitivity that explained 98% of the variation in glucose infusion rate.<sup>30</sup> It is suggested that increased VAT mass in obesity without an adequate support of vascularization might lead to hypoxia, macrophage infiltration, and inflammation.<sup>30</sup>

Recently, gut microbiota has also been suggested to be involved in systemic inflammation and metabolic disorders.<sup>22,51,52</sup> The main hypothesis is that gut inflammation, which can be induced by genetic, high fat diet and microbial dysbiosis, leads to increased intestinal permeability and delivery of bacteria and/or bacterial molecules, such as lipopolysaccharides (LPS) to the circulation.<sup>22,52,53</sup> As mesenteric fat is contiguous with the gut it would be directly affected by these inflammatory triggering molecules. This would activate mesenteric adipocytes hypertrophy, and increase pro-inflammatory gene expression and cytokine production. Consequently, macrophage infiltration and its activation would be increased in this fat depot. Furthermore, expanding mesenteric fat mass would provide increased fatty acid flux to the liver, which in the long term could result in an inflamed, steatotic, and insulin resistant liver.<sup>22</sup>

Three human studies partially support this hypothesis. Positive correlations between intestinal permeability markers and waist/abdominal circumferences,<sup>54,55</sup> visceral and liver fat,<sup>54</sup> insulin and HOMA indices were reported.<sup>55</sup> Microbiota composition differed between lean and obese women, while LPS levels were similar.<sup>56</sup> Even so, there are reports of higher LPS in obese and diabetic subjects.<sup>57-59</sup> In animal model, high saturated fat diet (HFD) increased adipocytes size in all fat depots and also macrophage infiltration in mesenteric and epididymal fat. Mesenteric fat from HFD mice showed higher mRNA levels of TNF- $\alpha$  and IL-6 and was considered 'as a metabolically distinct visceral fat depot with the most prominent pro-inflammatory nature'. In parallel, changes in microbiota and intestinal permeability were also reported.<sup>51</sup>

In general, an unfavorable or pathogenic phenotypic profile is characterized by adipocytes hypertrophy, visceral and ectopic fat deposition, and pro-inflammatory mediators' profile. Considering the association of visceral fat, NEFA flux, and dyslipidemia (hypertriglyceridemia), 'Visceral adipose index' has been proposed by Amato *et al.*<sup>24</sup> as a possible marker of adipose tissue dysfunction. Its equation encompasses waist circumference, BMI, plasma TG and HDL and may help assess cardiometabolic risk.<sup>24</sup>

In summary, three theories may explain how obesity is associated with IR: 1) *The Adipokine Hypothesis*: adipose tissue, especially VAT, from obese secretes more/less adipokines that

modulate insulin sensitivity; 2) *The Inflammation Hypothesis*: VAT from obese secretes chemokines that promote macrophage infiltration and activation. The activation of immune cells, by LPS for example, results in secretion of inflammatory molecules that interfere with insulin signaling; and 3) *The Adipose Tissue Expandability Hypothesis*: when an individual's capacity to increase fat mass is reached, lipid is deposited in ectopic sites and through a lipotoxic mechanism causes IR. These theories are not necessarily unrelated, conversely, one probably complements the other.<sup>11,21,25,31</sup>

### **3. Clinical and anthropometric characteristics of different metabolic phenotypes**

Among European, Canadian, and North-American subjects, the prevalence of normal weight with metabolic alterations varies from 2.6 to 8.1%, while overweight/obese without MetS represented 2.1 to 37% of the overall sample.<sup>17,60-63</sup> According to Wildman's study, as a percentage of each BMI group, 51.3% of overweight and 31.7% of obese subjects were classified as MHO, while 23.5% of normal-weight subjects were MONW.<sup>62</sup> The high prevalence of MetS in normal-weight and slightly overweight subjects (BMI 18.5-26.9 kg/m<sup>2</sup>) indicates that metabolic disabilities may also need to be screened in persons with a BMI at the upper end of the normal-weight and lower end of the overweight spectrum.<sup>64</sup> The purpose of this section is to present the different criteria used to define MHO and MONW phenotypes (Table 1) and to present physical and biochemical characteristics found in different studies (Tables 2 and 3).

#### *3.1. Metabolically obese normal weight (MONW)*

In 1980's, Ruderman *et al.*<sup>65</sup> discussed about individuals who are not obese by standard weight tables, but who have metabolic disabilities that are characteristically associated with adult-onset obesity. Hyperinsulinism and hypertrophied adipocytes were pointed as major characteristics of MONW.<sup>65</sup>

IR, hyperinsulinemia, and dyslipidemia may go undetected for years because young age and normal body weight mask the need for early detection and treatment in MONW subjects.<sup>66</sup> In general, MONW subjects are younger and more responsive to therapy (diet and exercise) than obese patients with already established disease. Thus, the early identification of MONW subjects may help to prevent the development of T2DM and other diseases.<sup>10,67</sup> A scoring method has been proposed by Ruderman *et al.*<sup>10</sup> Points are allotted for characteristics associated with IR and a score of seven or greater identifies a MONW individual.<sup>10</sup>

Screening adiposity in subjects with a normal BMI could also help to identify those at higher risk for metabolic disabilities.<sup>68</sup> MONW women showed higher levels of inflammatory markers such as C-reactive protein (CRP), TNF, IL-6, IFN- $\gamma$ , IL-1 $\beta$ , which were correlated with higher adiposity.<sup>69</sup> Upper body fat percentage tertile was accompanied by higher age, BMI, waist and hip circumferences, LDL, TG, and HOMA, and lower lean mass, HDL, and insulin sensitivity. Lean subjects with MetS were more prevalent in upper tertiles of body fat than in lower tertiles.<sup>68</sup> MONW subjects showed larger total and central body fat<sup>70</sup>, subcutaneous and visceral abdominal adiposity.<sup>66-67</sup> Adiposity was positively correlated with HOMA,<sup>70</sup> while visceral fat areas were also positively correlated with serum levels of TG, glucose infusion rate, and fasting insulin in MONW subjects.<sup>67</sup> Visceral adiposity, even in lean women, might be the key for an accentuated unfavorable metabolic profile, characterized by higher glucose, insulin, and total cholesterol levels than non-MONW women.<sup>69</sup>

Physical activity, energy expenditure<sup>66</sup> and resting metabolic rate<sup>71</sup> were lower in MONW subjects compared to control group. Sedentary lifestyle may lead to adiposity increment and higher cholesterol among MONW women since hormones such as leptin, adiponectin, and ghrelin did not differ between these group of women.<sup>70</sup>

Young women with a BMI lower than 26 kg/m<sup>2</sup> could be at a higher risk for impaired insulin sensitivity and for associated comorbidities if body fat percentage is higher than 30%.<sup>66,71</sup> Most of the studies involving MONW have different criteria and usually a small sample size. However, Conus *et al.*<sup>72</sup> highlighted the consistency of some observations: (i) the prevalence of MONW can reach values as high as 45% of a group, depending on the criteria, age, BMI, and ethnicity; (ii) the main characteristics that distinguishes MONW from control subjects are altered insulin sensitivity, atherogenic lipid profile, higher blood pressure, and abdominal/visceral adiposity, as well as, lower physical activity; and (iii) MONW subjects are at higher risks for T2DM and cardiovascular diseases.<sup>72</sup>

### *3.2. Metabolically healthy obese (MHO)*

Some obese individuals are quite healthy from a metabolic standpoint despite an outward risky appearance. MHO group did not show increased all-cause, cardiovascular and cancer mortality, when compared with normal weight insulin sensitive subjects.<sup>17</sup> Thus, it is important to cluster obese subjects into subgroups.

There is no standardized method to identify MHO individuals for research protocols or in clinical practice. Usually, most of the studies use the BMI for the definition of obesity ( $\geq 30$  kg/m<sup>2</sup>). The use of body fat percentage ( $\geq 25\%$  for men and  $\geq 30\%$  for women) would increase the prevalence of obesity in comparison to BMI as shown by Ortega *et al.*<sup>73</sup> Stratification of subjects into quartiles based on clamp, Matsuda and HOMA indices are used to define MHO or insulin sensitive obese (ISO), and insulin resistant obese (IRO).<sup>74</sup>

The use of different methods to identify MHO subjects resulted in differences in the mean values for peripheral fat mass and HDL. Still, it was possible to cluster biochemical characteristics for MHO subjects:<sup>39</sup> lower plasma TG, apolipoprotein B, ferritin as well as lower TG/HDL ratio, fasting insulin, and HOMA values in comparison to 'at risk' subjects.<sup>39,75</sup> Other studies also reported lower glucose,<sup>76</sup> total-cholesterol, and LDL as well as significantly higher values of HDL.<sup>60,63,75</sup> A better renal function is also reported for MHO compared to IRO subjects, who showed higher serum creatinine levels and lower glomerular filtration rate.<sup>76</sup> In one study, diet composition and physical activity did not differ between obese phenotypes.<sup>77</sup>

When the group of comparison is composed of metabolically healthy normal weight (MHNW) subjects, MHO showed higher waist circumference,<sup>74,76</sup> fat mass, blood pressure, carotid intima-media thickness,<sup>74</sup> insulin, non-HDL cholesterol, CRP levels, and lower HDL.<sup>32,74</sup> This could indicate that the concept of MHO is not appropriate. However, Sesti *et al.*<sup>76</sup> reported that MHO subjects - although exhibited, by selection, significantly higher BMI, and waist circumference - showed no differences in blood pressure, total cholesterol, TG, fasting plasma glucose, fasting insulin, insulin like growth factor-1, and insulin sensitivity compared to MHNW after adjusting for age, gender, and BMI. In this type of analysis, obesity *per se* is not the biggest issue for metabolic complications. Corroborating this hypothesis, Calori *et al.*<sup>17</sup> verified that insulin sensitive groups (non-obese vs. obese) presented similar metabolic profile. The insulin-sensitive groups were younger, had lower heart rates, higher plasma HDL, lower fibrinogen and TG, as well as a lower prevalence of T2DM and MetS compared to insulin resistant groups.<sup>17</sup>

Subjects at risk of T2DM but with different prediabetes categories (normal glucose tolerance, isolated impaired fasting glucose, isolated impaired glucose tolerance and both) showed differences in the visceral and liver fat accumulation, despite having similar BMI, waist circumference, and total body fat.<sup>78</sup> VAT correlated positively with hepatic enzymes

alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which were lower in MHO women compared to women classified as 'at risk'.<sup>79</sup> Non-obese and obese subjects with IR also showed higher levels of hepatic enzymes compared to non-obese insulin sensitive subjects.<sup>17</sup> Higher levels of these enzymes seem to reflect fat accumulation in the liver, which could entail hepatic IR.<sup>79</sup>

Hormonal differences after a oral glucose tolerance test may explain propensity to impaired glucose homeostasis of 'at risk' obese phenotype. 'At risk' obese subjects showed higher plasma glucose-dependent insulintropic polypeptide (GIP), lower post-glucose load glucagon-like peptide-1 (GLP-1), higher glucagon levels in baseline and after glucose load, indicating inappropriate glucagon suppression.<sup>80</sup>

As discussed earlier, inflammatory status may influence metabolic alterations. Philips & Perry<sup>81</sup> found lower concentrations of the protein C3, an acute-phase response protein with a central role in the innate immune system, in MHO and metabolically healthy non-obese subjects. An important consideration is that other inflammatory markers such as TNF- $\alpha$ , CRP, IL-6, PAI-1 and white blood cells count were lower in MHO, but depending on the metabolic health definition.

#### **4. Benefits of weight loss**

Weight loss should lead to metabolic benefits, especially on insulin sensitivity, independently of the type of obesity. Preliminary data showed that a 6-month energy-restricted diet reduced similarly and significantly the body weight (6-7%, including 7-10% loss of fat mass) in MHO and 'at risk' obese postmenopausal women. However, only 'at-risk' group improved the insulin sensitivity (26%), while MHO group showed a reduction of 13%.<sup>82</sup> The authors concluded that an energy-restricted diet associated with small reductions in body fat may improve whole body insulin sensitivity, except for a subset of individuals.<sup>82</sup>

Reduction of 5% body weight, waist circumference, VAT, and liver fat depot was also achieved after a low fat diet followed by IRO and MHO subjects. Nevertheless, reduction of total and liver fat and improvement of insulin sensitivity were significant only in IRO subjects. Although a significant increase in insulin sensitivity was observed in the IRO group, it barely exceeded 50% of the insulin sensitivity in the MHO group at follow-up. Improvement of insulin sensitivity through dietary intervention seems to be less effective in MHO individuals and is clearly positive for IRO subjects. However, this intervention alone might not be adequate to protect from T2DM and cardiovascular disease, when IR is

considered a key pathophysiological feature of these diseases. An early pharmacological treatment of IRO subjects in association with a lifestyle intervention may be considered as an appropriate therapeutic approach.<sup>83</sup>

The lack of homogeneity in treatment responses between obese individuals indicates that a phenotypic characterization may be needed to tailor the treatment according to the individual's characteristics/demand. The 'fit-fat' or metabolically healthy but obese individuals are under interest because they constitute a model that may provide insight into the pathogenesis of IR. It is unclear why these obese subjects are at lower risk of metabolic complications. Lower visceral adiposity and ectopic accumulation of fat, despite a high body fat content, lower pro-inflammatory systemic activation may be involved in this protection.<sup>84</sup>

### **5. Controversies**

Metabolic risk status is heterogeneous according to the BMI range. IR was observed in 7.7% and 55.7% of normal weight and obese subjects, respectively. Regardless of BMI, those with MetS or IR, were at a significant 4- to 11-fold increased multivariable relative risk of incident T2DM in comparison to normal weight subjects without MetS or IR. Overweight or obese without MetS and overweight insulin-sensitive subjects were not at increased risk for T2DM. However, ISO subjects were at about 3-fold increased risk relative to normal-weight subjects without IR. A quick look to this finding would indicate that even in the absence of IR, obesity by itself might be diabetogenic. Nevertheless, in the absence of metabolic disabilities, obesity did not increase the risk for cardiovascular disease and was a relatively weak risk factor for incident T2DM.<sup>61</sup>

According to Durward *et al.*,<sup>85</sup> the prevalence of the different phenotypes for lean and obese subjects varies according to the definition used for its characterization. They found that the prevalence of healthy obesity varied from 8.5 to 44.2% of total obese (n=1160), while unhealthy were 55.8 to 91.5% depending on the criteria. Regarding all of obese participants, only 3.4% (n=40) in contrast to 48.9% (n=567) were identified respectively as healthy and unhealthy by the definitions adopted. Concerning the total lean subjects (n=1737), the variations were between 46.7 to 95.6% for healthy and 4.4 to 53.3% for unhealthy.<sup>85</sup> Corroborating with this approach, Hinnouho *et al.*<sup>86</sup> as well as Soriguer *et al.*<sup>87</sup> also reported that the identification of metabolically healthy obesity ranged from 9-41% and 3-16.9%, respectively, depending on the definition considered. Thus, it is clear that establishment of



cut-off points or standardized criteria are still a need to strengthen the discussion of limits for benign and malign obesity classification, if this really exists.

The dynamism of fat storage is more complicated than simply 'eat less, spend more' formula. The use of drugs such as antibiotic shows that changes in the gut microbiome may also modulate adiposity, hepatic lipid, cholesterol, and TG metabolism.<sup>88</sup> Depending on the changes induced in the microbiota, an increase<sup>88</sup> or a decrease in body weight may be observed.<sup>89</sup> This portrays the complexity of the relation between adiposity, IR, and metabolic complications.

Insulin sensitivity is the main differentiating factor between benign vs. malign obesity, 'metabolically healthy' vs. 'at risk' or insulin resistant.<sup>17,90</sup> Nevertheless, Czech *et al.*<sup>45</sup> emphasize the huge challenges for understanding insulin signaling mechanisms and their dysfunctions. An enormous number of relevant studies associated with insulin metabolism are available (more than 100,000), making it time-consuming the task of 'separating fact from fiction'. Still, confirmatory studies remain necessary to solve controversies about insulin action.

The role of adipose tissue in IR development is not clear cut since even among class III obesity (BMI > 40 kg/m<sup>2</sup>) a relatively high percentage (58.3%) of MHO patients is reported.<sup>63</sup> Virtue and Vidal-Puig<sup>11</sup> raise interesting points that illustrate the complex relationship between IR and adipose tissue. At the same time that subjects with lipodystrophy, which is the inherent failure of adipose tissue development and/or function, may develop metabolic complications (IR, T2DM, dyslipidaemia), the differentiation and expansion of adipose tissue induced by drugs (e.g., thiazolidinedione) results in the improvement of insulin sensitivity. This suggests that increasing adipose tissue will not necessarily induce IR. Corroborating with this view, there are animal models that become more insulin resistant despite having less adipose tissue (PLO mice) or that remains insulin-sensitive with no ectopic fat deposition in liver despite having 50% greater body weight (AdTG-ob/ob mice).<sup>11</sup> In addition, Boyko *et al.*<sup>91</sup> presented controversies regarding the view that visceral obesity increases the risk of metabolic disturbances. Nondiabetic, second-generation Japanese-American men were followed for changes in visceral adiposity over 5 years. A higher IR and reduced insulin secretion (impaired  $\beta$ -cell function) were present earlier than visceral fat accumulation in some subjects that developed T2DM.<sup>92</sup> It is possible that an autocrine or paracrine action of cortisol generated by adipose stromal cells from

omental fat, but not subcutaneous, promotes abdominal obesity, since glucocorticoid receptors are expressed by adipocytes and stromal cells, and are also potent stimulators of adipocytes differentiation.<sup>26</sup>

Fat distribution has been suggested to be an important determinant of metabolic abnormalities. However, a prospective cohort study, compared mortality risk between different phenotypes with emphasis in abdominal obesity. Metabolically healthy abdominal obese had a significant higher risk than non-abdominal obese individuals, but not different from metabolically unhealthy abdominal obese.<sup>93</sup> Contrary, Mängee *et al.*<sup>94</sup> reported that total fat percentage did not differ between MHO and at risk subjects, while nuchal SAT thickness and VAT mass were significantly lower in MHO subjects.

Studies comparing all the phenotypes are still rare. The results from Sucurro *et al.*<sup>37</sup> accomplishing the normal weight and obese BMI range and the different metabolic phenotypes are depicted in Figure 1. The comparisons (MHNW *vs.* MHO; MONW *vs.* IRO and MHO *vs.* IRO) tend to show that being obese does worsen metabolic profile.<sup>37</sup> Another study, reported that MHO and IRO phenotypes were associated with higher mortality risk compared with MHNW. Obesity was associated with an increased risk for all cause mortality, regardless of whether the obese patients presented IR or a clustering of metabolic risk factors<sup>95</sup> or if they were classified as healthy or unhealthy.<sup>86</sup> These findings advocate to the importance of obesity reduction in all obese individuals

The comparison MHNW *vs.* MONW in Figure 1 shows that others factors rather than weight, total fat mass and waist circumference may be associated with a worse profile. Of note, both genders were included in this study, and for most parameters, the 'higher' levels does not necessarily mean beyond normal limits. Considering for example MetS criteria threshold<sup>96</sup>, only IRO group presented mean TG and waist circumference above threshold (> 150 mg/dl and > 102 cm, respectively), while the other groups (MHNW, MHO, MONW) showed values below the threshold.<sup>37</sup>

Hormonal (higher adiponectin)<sup>81,97</sup>, physical (better fitness), and behavioral (moderate alcohol intake and spending leisure-time in physical activity) factors may also be involved in a better metabolic phenotype.<sup>97</sup> It is noteworthy that the hazard ratios calculated by a model with no adjustments for fitness resulted in higher risk for all-cause mortality in MHO. However, using a model accounting for fitness showed no longer a higher risk compared

with normal-fat subjects. The authors suggested that fitness should be included in future research as it is a relevant confounder.<sup>73</sup>

Given that prevalence of MHO-like subjects is higher in younger-than 40<sup>63</sup> and obese subjects with MetS are older than MHO, during aging, transition from obese and apparently healthy to obese with a clustering of risk factors may occur.<sup>61-62</sup> Thus, duration of obesity might change the healthy phenotype. In a short follow-up period (3 y), MHO subjects showed a higher incidence of cardiometabolic risk factors and thicker intima-media of the common carotid than normal weight group. Weight gain was significantly associated with the development of these factors, independently of the BMI.<sup>98</sup> Other prospective cohort also describes that overweight/obese subjects were at higher risk of developing metabolic syndrome in comparison to normal weight.<sup>99</sup> The risk of becoming diabetic was higher in unhealthy obese subjects, while in MHO the risk was lower but still significant. Insulin resistance estimated by means of HOMA-IR at baseline contributed to the explanation of type 2 diabetes risk. The development of obesity in non-obese subjects was also significantly associated with the incidence of diabetes in the follow-up. In addition, depending on the criteria adopted for classification of phenotypes, 30.1-46.9% of MHO subjects at baseline became metabolically non-healthy by the 6-year follow-up.<sup>87</sup> As suggested by Pataky *et al.*<sup>90</sup>, the prevention of the aggravation of obesity is important to any subgroup of obese subjects. MHO individuals may still be at risk for other obesity related complications such as sleep apnea, cancer, and musculoskeletal problems.<sup>60</sup>

Interestingly, MONW Korean-elderly subjects had the highest risk of death from all causes during 10 years follow-up than overweight subjects without metabolic syndrome and MHO. In addition, MONW subjects had higher systolic blood pressure, serum glucose and triglycerides levels and prevalence of diabetes and hypertension than the MHO phenotype.<sup>101</sup> This may point to the importance of ethnicity and genetic factors.

Finally, in the majority of studies, the definition of phenotypes is based on insulin resistance markers and the 'worse' profile is stated based on statistical differences in biochemical parameters, irrespective if these values are within normal values or not. However, Figure 2 shows that although insulin sensitivity differs within phenotypes, the proportion of studies that in fact includes 'healthy' subjects, defined by means of reference values for biochemical parameters (glucose and lipid profile), is high even in studies assessing at risk/IRO subjects, being highest among those studies including MHO subjects. As expected, is more difficult to

find studies including subjects defined as at risk/IRO showing all biochemical values within desirable range. Even so, in the majority of studies (78.6%), IRO subjects did not present metabolic abnormalities (i.e., mean values above reference values), at least at the time of evaluation. Surprisingly, 40% of the studies including MONW subjects reported at least one biochemical alteration in this subgroup. Therefore, more studies in this field, especially follow-up studies, are needed and should investigate other blood markers that may distinguish better these phenotypes biochemically. Mangee *et al.*<sup>94</sup> results suggest uric acid as the best predictor of MetS among juveniles and adults classified as metabolically unhealthy and also as a considerable discriminator between obesity phenotypes.

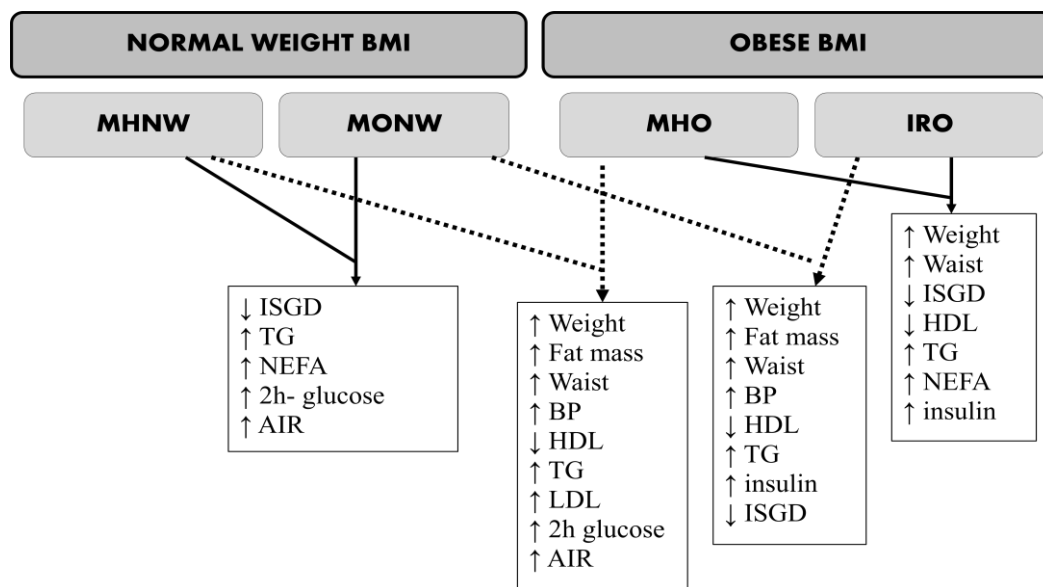
## **6. Conclusion**

In conclusion, excess weight has been considered a signal of current or future health problems. A subgroup of obese has emerged as a category that possibly escapes common metabolic disorders, at least for a certain period. Obesity and normal weight might be heterogeneous in regard to its effects and is less deleterious in the absence of IR. Metabolic abnormalities associated with MetS seem to depend on the absence or presence of IR, especially hepatic, and inflammatory signaling activation. A consensus regarding the criteria used to define metabolic health is needed.

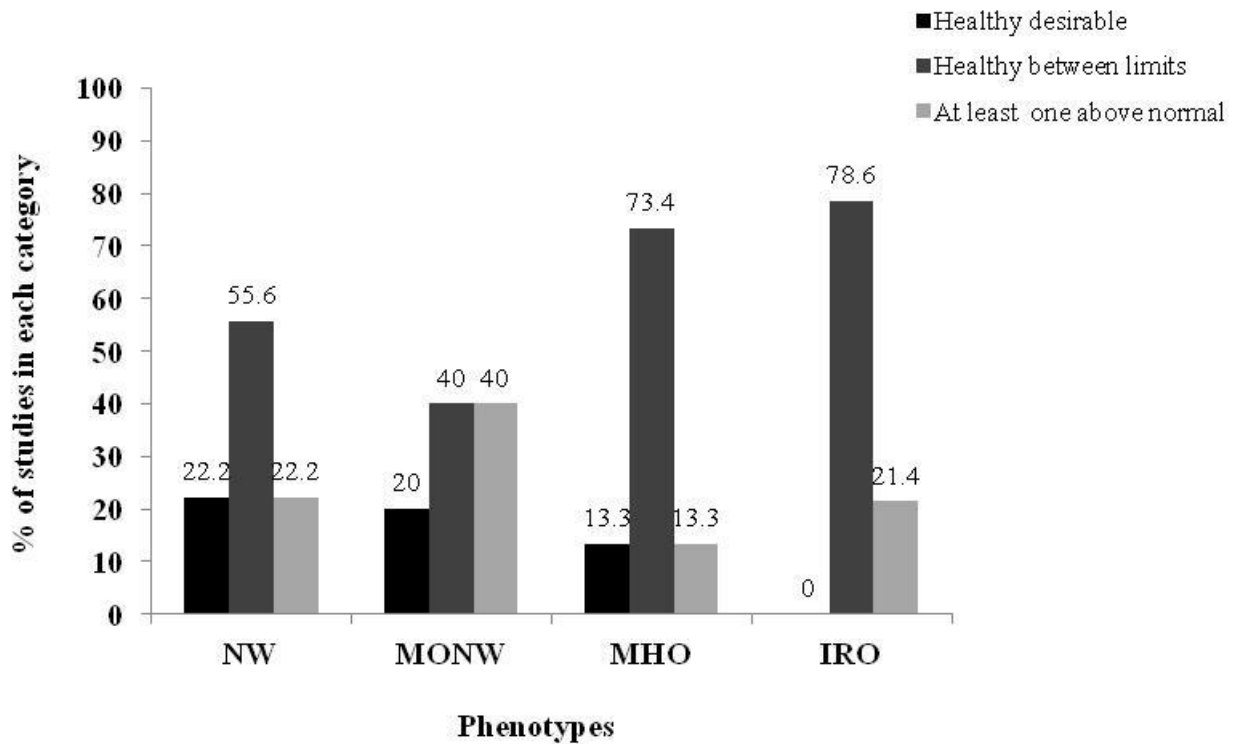
The relationship between adiposity and metabolic disabilities, including IR, or even mortality is more complex than it appears. The concept of ‘metabolic set point’ proposed by Virtue and Vidal-Puig<sup>11</sup> highlights the importance of individuality. The idea is that each individual has its own level of body weight and adipose tissue expansion beyond which metabolic homeostasis and capacity to buffer lipids will be compromised. This impairment may be even greater as visceral fat accumulation increases, as also demonstrated for normal weight subjects. Visceral adiposity seems to be a strong characteristic associated with higher risk, independently of body mass index. For some individuals, extra pounds may not be as detrimental as in others, especially if this excess is deposited in subcutaneous depots. However, the contribution of subcutaneous fat to metabolic disorders should not be underestimated.

Whether inflammatory signaling is triggered by excessive caloric intake and subsequent adipose tissue expansion, or by bacterial components delivered to liver and adipose tissue remains to be better explored, as well as the differences in LPS concentration and bacterial groups between the discussed phenotypes. There are not enough evidences to prove that

MHO subjects are permanently protected from the development of co-morbidities in long-term. The real meaning of the term ‘metabolically healthy obesity’ is still controversial and more studies in this field are of great interest. Although the term MHO makes sense, being obese may bring other problems related to joints, sleep apnea and respiratory problems, depression and several cancers, independently of phenotype. Finally, the ‘lean appearance’ is not necessarily synonymous of health. What MONW and obese at risk have in common? Of note, the influence of ethnicity, genetic polymorphisms and gender should be further explored in future studies including all body size phenotypes.



**Figure 1 – Comparison of different metabolic phenotypes described by Sucurro and co-workers:<sup>37</sup>** dotted lines connect the comparison between groups of similar insulin-stimulated glucose disposal but different BMI range (MHNW vs. MHO and MONW vs. IRO) and the resultant box describes the characteristics of obese in comparison to normal weight subjects. Full lines connect the comparison between same BMI range but different insulin-stimulated glucose disposal (MHNW vs. MONW and MHO vs. IRO) and the resultant box describes the characteristics of the ‘unhealthy’ group in comparison to ‘healthy’ phenotypes. AIR: acute insulin response during an intravenous glucose-tolerance test; BP: blood pressure; NEFA: free fatty acids; ISGD: insulin-stimulated glucose disposal; TG: triglycerides.



**Figure 2 – Categorization of glucose and lipid profile parameters means according to reference values from the 17 studies represented in table 3.** Biochemical parameters from the different phenotypes (NW, MONW, MHO, IRO) were classified as desirable, between limits and above normal according to the following reference values: glucose (desirable 3.8-5.6 mmol/l); total cholesterol (desirable < 5.18 mmol/L, between limits 5.18-6.19 mmol/l, above normal >6.2 mmol/l); HDL (desirable >1.55 mmol/l, between limits 1.04-1.55 mmol/l, above normal <1.04 mmol/l); LDL (desirable < 2.6 mmol/l, between limits 2.6-3.35 mmol/l, above normal > 4.11 mmol/l); triglycerides (desirable < 1.7 mmol/l, between limits 1.7-2.25, above normal > 2.26 mmol/l). For each phenotype, the number of studies describing mean values of biochemical parameters within the following categories are represented in percentage (%): healthy desirable (when glucose and lipid profile parameters were within desirable values), healthy desirable and between limits (when glucose and lipid profile parameters were within desirable and/or between limits values), at least one above normal (when glucose and/or one or more of the lipid parameters were above normal).

**Table 1 – Criteria for definition of different body size phenotypes in different studies: metabolically healthy normal weight (MHNW), metabolically obese normal weight (MONW), metabolically healthy obese (MHO) and insulin resistant obese (IRO)**

<b>Ref</b>	<b>Method</b>	<b>Criteria<sup>a</sup></b>
(68)	Body fat percentage (by bioelectrical impedance)	<i>MONW</i> : >23.1% for men (n=1017) and >33.3% for women (n=1045)
(69)	Body fat percentage (by DXA)	<i>MONW</i> : >30% for women (n=20)
(70)	HOMA	<i>MONW</i> : HOMA >1.69 (n=12) <i>Non-MONW</i> : HOMA <1.69 (n=84)
(17)	HOMA	<i>MHNW</i> : HOMA <2.5 (n=708) Nonobese-IR: HOMA ≥2.5 (n=923) <i>ISO</i> : HOMA <2.5 (n=43) <i>IRO</i> : HOMA ≥2.5 (n=337)
(32)	HOMA	<i>MHO</i> : absence of T2D, of IR (HOMA>3.6 for males and 3.13 for females), MetS and history of treatment with lipid-lowering drugs (n=314) <i>MHNW</i> : the same criteria as considered for MHO, but also normal weight (n=1173) <i>IRO</i> : HOMA >3.6 for males and 3.13 for females (n=843)
(43)	HOMA	<i>ISO</i> : HOMA <1.95 (n=21) <i>IRO</i> : HOMA ≥1.95 (n=21)
(66)	Euglycemic-hyperinsulinemic	<i>MONW</i> : <8 ml.min <sup>-1</sup> .kg <sup>-1</sup> of FFM (n=13)

	clamp (glucose disposal <sup>b</sup> )	<i>MHNW</i> : >8 ml.min <sup>-1</sup> .kg <sup>-1</sup> of FFM (n=58)
(74)	Euglycemic-hyperinsulinemic clamp (glucose disposal <sup>b</sup> )	<i>MHO</i> : >13.2 mg/min x kg <sub>FFM</sub> (n=20) <i>IRO</i> : <9.9 mg/min x kg <sub>FFM</sub> (n=40)
(82)	Euglycemic-hyperinsulinemic clamp (glucose disposal <sup>b</sup> ) (glucose disposal <sup>b</sup> )	<i>MHO</i> : ≥73.9 μmol min <sup>-1</sup> [kg FFM] <sup>-1</sup> (n=30) <i>Low insulin sensitivity</i> : ≤49.9 μmol min <sup>-1</sup> [kg FFM] <sup>-1</sup> (n=30)
(37)	Euglycemic-hyperinsulinemic clamp (glucose disposal <sup>b</sup> )	<i>MHO</i> : >12.3 mg/min x kg <sub>FFM</sub> (n=22) <i>IRO</i> : <8.7 mg/min x kg <sub>FFM</sub> (n=43) <i>MONW</i> : <10.2 mg/min x kg <sub>FFM</sub> (n=27) <i>MHNW</i> : >12.3 mg/min x kg <sub>FFM</sub> (n=55)
(97)	Euglycemic-hyperinsulinemic clamp (glucose disposal <sup>b</sup> )	<i>MHO</i> : ≥11.6 mg/min x kg <sub>FFM</sub> (n=18) <i>At risk</i> : <10.6 mg/min x kg <sub>FFM</sub> (n=18)
(30)	Euglycemic-hyperinsulinemic clamp (glucose disposal <sup>b</sup> )	<i>MHO</i> : >70 μmol x kg <sup>-1</sup> x min <sup>-1</sup> (n= 30) <i>IRO</i> : <60 μmol x kg <sup>-1</sup> x min <sup>-1</sup> (n= 30)
(38)	Oral glucose tolerance test to calculate ISI <sup>c</sup>	<i>ISO</i> : upper quartile of ISI (n=31) <i>IRO</i> : in the lower 3 quartiles of ISI (n=96)
(76)	Oral glucose tolerance test to calculate ISI <sup>c</sup>	<i>MHO</i> : ≥76.8 mg x L <sup>2</sup> x mmol <sup>-1</sup> x mU <sup>-1</sup> x min <sup>-1</sup> (n=106) <i>IRO</i> : ≤61.3 mg x L <sup>2</sup> x mmol <sup>-1</sup> x mU <sup>-1</sup> x min <sup>-1</sup> (n=212)
(39)	Comparison of 5 methods	<i>Euglycemic-hyperinsulinemic clamp</i> : <i>MHO</i> (upper quartile of glucose disposal rate; n=28); ‘ <i>at risk</i> ’ (lower quartile of glucose disposal rate; n=28)



*Matsuda index: MHO* (upper quartile; n=26); '*at risk*' (lower three quartiles, n=78)

*HOMA: MHO* (lower quartile; n=28); '*at risk*' (upper quartile; n=28)

*Wildman's criteria: MHO* having 0–1 cardiometabolic disabilities (SBP/DBP  $\geq$ 130/85 mmHg, TG  $\geq$ 1.7 mmol/l, glucose  $\geq$ 5.6 mmol/l, HOMA  $>$ 5.13, hsCRP  $>$ 0.1 mg/l, HDL-C  $<$ 1.3 mmol/l) (n=26); '*at risk*' ( $\geq$ 2 disabilities; n=84)

*Kareli's criteria: MHO* (meeting 4 out of 5 metabolic factors: HOMA  $\leq$ 2.7, TG  $\leq$ 1.7 mmol/l, HDL  $\geq$ 1.3 mmol/l, LDL  $\leq$ 2.6 mmol/l, hsCRP  $\leq$ 3.0 mg/l) (n=26); '*at risk*' (meeting less than 3; n=85)

(85) Comparison of 3 methods

*HOMA: MHO* (HOMA  $<$  2.5) (n=228); *MUO* (n=932)

*ATP-III: MHO*  $\leq$  2 MetS criteria (fasting glucose  $\geq$  5.6 mmol/L or T2D medication; SBP $\geq$ 130 or DBP  $\geq$  85 mmHg or antihypertensive medication; TG  $\geq$ 1.7 mmol/L or cholesterol-lowering medications; HDL  $<$ 1.04 mmol/L (males) and  $<$ 1.3 mmol/L (females); waist  $>$ 102 cm (males),  $>$ 88cm (females) (n=513); *MUO* (n=647)

*Combined: MHO*  $\leq$  1 criteria (HOMA  $\geq$ 1.95 or T2D medication; TG  $\geq$ 1.7 mmol/L or cholesterol-lowering medications; HDL  $<$ 1.04 mmol/L (males) and  $<$ 1.3 mmol/L (females); LDL  $\geq$ 2.6 mmol/L; total cholesterol  $\geq$ 5.2 mmol/L (or cholesterol-lowering medication) (n=99); *MUO* (n=1061)

(73) Biochemical parameters, BMI or BF%

*MHO: BF*  $\geq$ 25% (men) and  $\geq$ 30% (women) or BMI  $\geq$ 30 kg/m<sup>2</sup>+ meet  $\leq$ 1 of the metabolic disabilities (SBP/DBP  $\geq$  130/85 mmHg; TG  $\geq$ 1.7 mmol/L, HDL  $<$ 1.03 mmol/L (males) and  $<$ 1.3 mmol/L (females); fasting glucose  $\geq$  5.55 mmol/L; history of physician diagnosis of hypertension or T2D) (n=5959 for BF criteria) (n=1738 for BMI criteria)

(62) Biochemical parameters (n=5440)

*Cardiometabolic disabilities(CA):* BP ( $\geq$ 130/85 mmHg), fasting TG  $\geq$ 1.69 mmol/l, HDL  $<$ 1.03 mmol/l (men) and  $<$ 1.29 mmol/l (women), fasting glucose  $\geq$ 5.55 mmol/l, HOMA  $>$ 5.13, hsCRP  $>$ 0.1 mg/l

*MHNW: BMI*  $<$ 25 kg/m<sup>2</sup> and  $<$ 2 CA (n=26.4%)

*MONW: BMI*  $<$ 25 kg/m<sup>2</sup> and  $\geq$ 2 CA (n=8.1%)

*MHO: BMI*  $\geq$  30 kg/m<sup>2</sup> and  $<$ 2 CA (n=9.7%)

*MUO: BMI*  $\geq$  30 kg/m<sup>2</sup> and  $\geq$ 2 CA (n=20.9%)

(41)	Biochemical parameters	<p><i>MHO</i>: no history of cardiovascular, respiratory or metabolic diseases, not taking medications, normal thyroid status, glucose <math>\leq 5.6</math> mmol/L, blood pressure <math>\leq 135/85</math>, TG/HDL ratio <math>\leq 1.65</math> (men) e <math>\leq 1.32</math> (women) (n=15)</p> <p><i>MUO</i>: failure to meet at least one of the criteria above (n=14)</p>
(63)	Biochemical parameters	<i>MHO</i> : BMI $\geq 30$ kg/m <sup>2</sup> , HDL $\geq 40$ mg/dL, absence of T2D and absence of hypertension (n=36)
(42)	Biochemical parameters	<p><i>MHO</i>: without MetS (n=37)</p> <p><i>MetS</i>: three or more components: waist <math>\geq 85</math> cm, TG <math>\geq 1.7</math> mM; HDL <math>&lt; 1.29</math> mM; SBP <math>\geq 130</math> mmHg or DBP <math>\geq 85</math> mmHg; fasting glucose <math>\geq 5.6</math> mM (n=28)</p>
(60)	Biochemical parameters	<i>MHO</i> : when 4 out of 5 biochemical parameters are met below cut-off points proposed for lipid profile (TG $\leq 1.7$ mmol/l; total cholesterol $\leq 5.2$ mmol/l; HDL $\geq 1.3$ mmol/l and LDL $\leq 2.6$ mmol/l and HOMA $\leq 1.95$ ) (n=19)
(100)	Biochemical parameters	<i>MHO</i> : when 4 out of 5 biochemical parameters are met below cut-off points proposed for lipid profile (TG $\leq 1.7$ mmol/l; HDL $\geq 1.3$ mmol/l and LDL $\leq 2.6$ mmol/l) and HOMA $\leq 2.7$ , hs(?) -CRP levels ( $\leq 3$ mg/l) (n=32)

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MONW, metabolically obese normal weight; DXA, dual energy X-ray absorptiometry; HOMA, homeostasis model assessment; MHNW, metabolically healthy normal weight; ISO, insulin-sensitive obese; IRO, insulin resistant obese; MHO, metabolically healthy obese; hsCRP, high-sensitive C-reactive protein; MetS, metabolic syndrome; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides; MUO, metabolically unhealthy obese; T2D, type 2 diabetes.

<sup>a</sup>Normal weight group defined considering BMI  $> 18.5$  and  $< 24.9$  kg/m<sup>2</sup>, obese BMI  $\geq 30$  kg/m<sup>2</sup>, nonobese BMI  $> 18.5$  and  $< 30$  kg/m<sup>2</sup>.

<sup>b</sup>Glucose disposal (M) or glucose infusion rate (GIR): mean rate of glucose infusion during the last 45-60 min of the clamp examination (steady-state). Expressed as milligrams per minute per kilogram fat free mass ( $M_{FFM}$ ) or  $\mu\text{mol} \times \text{min}^{-1} \times [\text{kg FFM}]^{-1}$ .

<sup>c</sup>ISI: Insulin sensitivity index, which is based on 75g oral glucose tolerance test;

**Table 2 – Physical characteristics of different body size phenotypes: metabolically healthy normal weight (MHNW), metabolically obese normal weight (MONW), metabolically healthy obese (MHO) and insulin resistant obese (IRO)**

Ref	Sample	BMI	Fat mass (%)	Lean mass (kg)	Waist (cm)	Visceral fat (cm <sup>2</sup> )	SAT (cm <sup>2</sup> )
(17)	708 NW (392F/316M)	23.8 ± 2.8 <sup>a</sup>	-	-	82 ± 9 <sup>a</sup>	-	-
	923 MONW (512F/411M)	25.8 ± 2.3 <sup>b</sup>	-	-	89 ± 10 <sup>b</sup>	-	-
	43 MHO (31F/12M)	32.5 ± 4.3 <sup>c</sup>	-	-	94.4 ± 4 <sup>c</sup>	-	-
	337 IRO (191F/146M)	33.3 ± 3.4 <sup>d</sup>	-	-	104 ± 11 <sup>d</sup>	-	-
(37)	55 NW (44F/11M)	22.6 ± 1.9 <sup>a</sup>	27.5 ± 8.5 <sup>a</sup>	44.9 ± 7.9 <sup>a</sup>	76 ± 9 <sup>a</sup>	-	-
	27 MONW (18F/9M)	23.4 ± 1.6 <sup>a</sup>	29.6 ± 9.2 <sup>a</sup>	44.7 ± 10 <sup>a</sup>	79 ± 9 <sup>a</sup>	-	-
	22 MHO (19F/3M)	34.5 ± 4.7 <sup>b</sup>	42.1 ± 20.3 <sup>b</sup>	51.3 ± 12.2 <sup>b</sup>	98 ± 9 <sup>b</sup>	-	-
	43 IRO (28F/15M)	36.4 ± 6.4 <sup>b</sup>	45.7 ± 19.2 <sup>b</sup>	54.7 ± 15.5 <sup>b</sup>	106 ± 12 <sup>c</sup>	-	-
(66)	58 NW (F)	21.5 ± 2.0	27.4 ± 5.5 <sup>a</sup>	40.3 ± 4.0	-	35 ± 14 <sup>a</sup>	160 ± 78 <sup>a</sup>
	13 MONW (F)	22.5 ± 2.0	31.8 ± 5.9 <sup>b</sup>	38.9 ± 5.1	-	44 ± 16 <sup>b</sup>	213 ± 61 <sup>b</sup>
(69)	20 NW (F)	19.2 ± 1.5 <sup>a</sup>	23.3 ± 2.2 <sup>a</sup>	-	65.1 ± 3.9 <sup>a</sup>	-	-
	20 MONW (F)	22.6 ± 1.9 <sup>a,b</sup>	34.9 ± 5.0 <sup>b</sup>	-	72.3 ± 4.9 <sup>a,b</sup>	-	-
	20 OHR (F)	27.9 ± 4.6 <sup>b</sup>	42.9 ± 7.3 <sup>b</sup>	-	85.8 ± 10.2 <sup>b</sup>	-	-
(70)	84 NW (F)	21.8 ± 2.5	25.04 ± 5.8 <sup>a</sup>	41.6 ± 4.1 <sup>a</sup>	-	-	-
	12 MONW (F)	21.9 ± 3.4	32.2 ± 8.2 <sup>b</sup>	37.6 ± 3.2 <sup>b</sup>	-	-	-
(30)	30 MHO (20F/10M)	45.1 ± 1.3	50.5 ± 7.0	-	132 ± 5.2 <sup>a</sup>	138 ± 27 <sup>a</sup>	935 ± 124
	30 IRO (20F/10M)	45.2 ± 1.2	51.2 ± 5.8	-	138 ± 8.1 <sup>b</sup>	316 ± 91 <sup>b</sup>	890 ± 110
(32)	594 NW (M)	22.5 (22.4-22.7) <sup>b</sup>	-	-	84.5 (83.8-85.2) <sup>b</sup>	-	-
	120 MHO (M)	32.8 (32.3-33.3) <sup>a</sup>	-	-	110.2 (108.3-112.1) <sup>a</sup>	-	-
	579 NW (F)	22.2 (22.1-22.4) <sup>a</sup>	-	-	78.6 (78-79.3) <sup>a</sup>	-	-
	194 MHO (F)	34.4 (33.6-35.1) <sup>b</sup>	-	-	103.6 (101.9-105.3) <sup>b</sup>	-	-

(38)	54 NW (45F/9M)	-	26.9 ± 1.0 <sup>a</sup>	-	79.2 ± 1.0 <sup>a</sup>	-	-
	31 MHO (19F/12M)	-	36.6 ± 1.3 <sup>b</sup>	-	104.6 ± 1.7 <sup>b</sup>	-	-
	96 IRO (59F/37M)	-	36.9 ± 0.8 <sup>b</sup>	-	107.4 ± 1.0 <sup>b</sup>	-	-
(39)	28 MHO (F)	34.1 ± 3.0	-	42.4 ± 4.5 <sup>a</sup>	104.7 ± 9.1	190.2 ± 44.4 <sup>a</sup>	529.5 ± 97.4
	28 OHR (F)	34.6 ± 2.8	-	47.4 ± 6.6 <sup>b</sup>	107.5 ± 7.6	229.8 ± 54.3 <sup>b</sup>	501.4 ± 89.0
(42)	37 MHO (F)	27.2 ± 1.6	-	-	93.1 ± 5.6	-	-
	28 MetSO (F)	28.1 ± 2.3	-	-	95.4 ± 7.8	-	-
(60)	19 MHO (F)	33.5 ± 5.2	46.2 ± 9.7	44.7 ± 6.6	91.5 ± 5.9	-	-
	135 OHR (F)	34.4 ± 5.5	45.7 ± 11.4	45.4 ± 6.0	98.5 ± 9.7	-	-
(63)	36 MHO (34F/2M)	43.6 ± 8.6	50.0 ± 5.5	-	103.2 ± 12.2 <sup>a</sup>	-	-
	88 OHR (78F/10M)	43.4 ± 8.9	50.5 ± 4.0	-	116.7 ± 13.9 <sup>b</sup>	-	-
(74)	73 NW (F)	23.8 ± 2.8 <sup>a</sup>	26.3 ± 7.8 <sup>a</sup>	42.6 ± 6 <sup>a</sup>	76.8 ± 8 <sup>a</sup>	-	-
	20 MHO (F)	37.7 ± 9.9 <sup>b</sup>	51 ± 19 <sup>b</sup>	44 ± 15 <sup>a</sup>	100 ± 13 <sup>b</sup>	-	-
	40 IRO (F)	39 ± 7.4	43.5 ± 13.8 <sup>b</sup>	56 ± 10 <sup>b</sup>	108 ± 14 <sup>b</sup>	-	-
(75)	22 MHO (F)	32.3 ± 4.1	47.7 ± 4.8	40.4 ± 3.8 <sup>a</sup>	96.3 ± 8.6	-	-
	22 OHR (F)	34.8 ± 3.9	45.5 ± 4.4	47.4 ± 7.6 <sup>b</sup>	102.1 ± 9.2	-	-
(76)	122 NW (70F/52M)	23.9 ± 1.6 <sup>a</sup>	-	49 ± 9 <sup>a</sup>	86 ± 9 <sup>a</sup>	-	-
	106 MHO (62F/44M)	34.2 ± 5.6 <sup>b</sup>	-	55 ± 10 <sup>b</sup>	105 ± 10 <sup>b</sup>	-	-
	212 IRO (124F/88M)	35.2 ± 5.1 <sup>b</sup>	-	55 ± 12 <sup>b</sup>	111 ± 11 <sup>c</sup>	-	-
(79)	26 MHO (F)	33.6 ± 2.7	-	42.1 ± 4.1 <sup>a</sup>	103.6 ± 7.0	175.8 ± 43.9 <sup>a</sup>	-
	78 OHR (F)	34.2 ± 2.8	-	44.8 ± 6.2 <sup>b</sup>	107.2 ± 9.5	209.2 ± 47.8 <sup>b</sup>	-
(83)	26 MHO (12F/14M)	-	-	-	106.1 ± 1.9	-	-
	77 IRO (34F/43M)	-	-	-	108.1 ± 1.1	-	-

SAT, subcutaneous adipose tissue; NW, normal weight; F, female; M: male; MONW: metabolically obese normal weight; MHO, metabolically healthy obese; IRO, insulin resistant obese; OHR, overweight/obese higher risk; MetSO: metabolic syndrome obese. Different letters <sup>(a,b)</sup> within the same reference indicates that the values differs (statistically significant).

**Table 3 – Biochemical characterization of different body size phenotypes: metabolically healthy normal weight (MHNW), metabolically obese normal weight (MONW), metabolically healthy obese (MHO) and insulin resistant obese (IRO)**

Ref	Sample	Glucose (mmol/l)	Insulin (pmol/l)	HOMA	TC (mmol/l)	HDL (mmol/l)	LDL (mmol/l)	TG (mmol/l)
(17)	708 NW (392F/316M)	4.8 ± 0.5 <sup>a</sup>	50 ± 13 <sup>a</sup>	1.8 ± 0.5 <sup>a</sup>	5.9 ± 1.1 <sup>a</sup>	1.5 ± 0.4 <sup>a</sup>	3.9 ± 1.0 <sup>a</sup>	1.15 ± 0.6 <sup>a</sup>
	923 MONW (512F/411M)	5.4 ± 1.1 <sup>b</sup>	112 ± 70 <sup>b</sup>	4.6 ± 3.7 <sup>b</sup>	6.2 ± 1.1 <sup>b</sup>	1.3 ± 0.4 <sup>b</sup>	4.2 ± 1.0 <sup>b</sup>	1.56 ± 1.0 <sup>b</sup>
	43 MHO (31F/12M)	4.8 ± 0.3 <sup>a</sup>	56 ± 13 <sup>a</sup>	2 ± 0.4 <sup>a</sup>	6.2 ± 1.2 <sup>a</sup>	1.5 ± 0.3 <sup>a</sup>	4.1 ± 1.0 <sup>a</sup>	1.26 ± 0.6 <sup>a</sup>
	337 IRO (191F/146M)	6.0 ± 1.7 <sup>c</sup>	154 ± 70 <sup>c</sup>	7.2 ± 5.6 <sup>c</sup>	6.1 ± 1.1 <sup>a,b</sup>	1.2 ± 0.3 <sup>c</sup>	4.1 ± 1.1 <sup>b</sup>	1.7 ± 0.9 <sup>c</sup>
(37)	55 NW (44F/11M)	4.8 ± 0.6	55.5 ± 55.5 <sup>a</sup>	-	4.8 ± 0.9	1.6 ± 0.4 <sup>a</sup>	2.8 ± 0.7 <sup>a</sup>	0.86 ± 0.4 <sup>a</sup>
	27 MONW (18F/9M)	4.9 ± 0.5	55.5 ± 20.8 <sup>a</sup>	-	4.9 ± 0.9	1.5 ± 0.4 <sup>a</sup>	3.1 ± 0.9 <sup>a</sup>	1.0 ± 0.6 <sup>b</sup>
	22 MHO (19F/3M)	4.8 ± 0.5	76.4 ± 34.7 <sup>a</sup>	-	5.1 ± 1.0	1.4 ± 0.3 <sup>b</sup>	3.2 ± 0.8 <sup>b</sup>	1.1 ± 0.4 <sup>c</sup>
	43 IRO (28F/15M)	5.1 ± 0.5	118 ± 48.6 <sup>b</sup>	-	5.2 ± 0.9	1.2 ± 0.4 <sup>b</sup>	3.2 ± 0.8 <sup>a,b</sup>	1.8 ± 0.8 <sup>d</sup>
(66)	58 NW (F)	4.4 ± 0.3	49 ± 15 <sup>a</sup>	-	4.5 ± 0.7 <sup>a</sup>	1.5 ± 0.3	2.7 ± 0.8	2.4 ± 1.0
	13 MONW (F)	4.4 ± 0.4	60 ± 20 <sup>b</sup>	-	5.3 ± 0.9 <sup>b</sup>	1.7 ± 0.5	3.1 ± 0.9	2.4 ± 0.7
(69)	20 NW (F)	5.2 ± 0.18	45.8 ± 9.7	1.4 ± 0.1 <sup>a</sup>	4.6 ± 0.45 <sup>a</sup>	1.79 ± 0.17	2.77 ± 0.9	0.75 ± 0.12 <sup>a</sup>
	20 MONW (F)	5.1 ± 0.16	44.4 ± 12.5	1.5 ± 0.2 <sup>a,b</sup>	4.87 ± 0.67 <sup>a,b</sup>	1.76 ± 0.32	2.69 ± 0.63	0.97 ± 0.16 <sup>a,b</sup>
	20 OHR (F)	5.4 ± 0.11	63.2 ± 7.6	2.2 ± 0.6 <sup>b</sup>	5.65 ± 0.63 <sup>b</sup>	1.82 ± 0.51	3.0 ± 0.91	1.26 ± 0.19 <sup>b</sup>
(70)	84 NW (F)	4.65 ± 0.3 <sup>b</sup>	30.6 ± 12.1 <sup>b</sup>	0.91 ± 0.4 <sup>b</sup>	4.4 ± 0.9 <sup>b</sup>	1.68 ± 0.4	2.3 ± 0.7	0.82 ± 0.3
	12 MONW (F)	4.8 ± 0.3 <sup>a</sup>	70.3 ± 13.7 <sup>a</sup>	2.19 ± 0.5 <sup>a</sup>	5.1 ± 1.4 <sup>a</sup>	1.69 ± 0.4	3.0 ± 1.6	0.85 ± 0.3
(30)	30 MHO (20F/10M)	5.2 ± 0.2 <sup>a</sup>	29.8 ± 14 <sup>a</sup>	-	4.9 ± 0.9	1.4 ± 0.2 <sup>a</sup>	2.9 ± 0.9	1.2 ± 0.4 <sup>a</sup>
	30 IRO (20F/10M)	5.7 ± 0.4 <sup>b</sup>	104.7 ± 30 <sup>b</sup>	-	5.2 ± 1.0	1.0 ± 0.3 <sup>b</sup>	3.1 ± 0.9	1.9 ± 1.2 <sup>b</sup>
(32)	594 NW (M)	5.1 (5.1-5.2)	46.6 (44.5-48.1) <sup>a</sup>	1.5 (1.4-1.6) <sup>a</sup>	4.9 (4.8-5.0)	1.3 (1.3-1.4) <sup>a</sup>	4.9 (4.8-5.0)	1.2(1.1-1.3)
	120 MHO (M)	5.2 (5.1-5.3)	68.1 (63.1-73.2) <sup>b</sup>	2.2 (2.1-2.4) <sup>b</sup>	5.0 (4.8-5.2)	1.2 (1.2-1.3) <sup>b</sup>	5.0 (4.8-5.2)	1.3 (1.2-1.4)
	579 NW (F)	4.94 (4.9-4.98)	41.6 (39.5-43.7) <sup>a</sup>	1.3 (1.2-1.4) <sup>a</sup>	4.96 (4.87-5.06)	1.66(1.62-1.7) <sup>a</sup>	2.83(2.74-2.91) <sup>a</sup>	1.03(0.95-1.12)
	194 MHO (F)	4.97 (4.9-5.05)	63.8 (61.8-66.6) <sup>b</sup>	2.0 (1.9-2.1) <sup>b</sup>	4.99 (4.8-5.16)	1.44(1.39-1.49) <sup>b</sup>	3.03(2.89-3.18) <sup>b</sup>	1.13(1.03-1.24)
(38)	54 NW (45F/9M)	5.1 ± 0.08 <sup>a</sup>	37.0 ± 2.01 <sup>a</sup>	1.43 ± 0.1 <sup>a</sup>	5.1 ± 0.13	1.57 ± 0.05 <sup>a</sup>	3.12 ± 0.1	1.1 ± 0.05 <sup>a</sup>
	31 MHO (19F/12M)	5.06 ± 0.07 <sup>a</sup>	39.03 ± 2.01 <sup>a</sup>	1.45 ± 0.06 <sup>a</sup>	5.03 ± 0.08	1.37 ± 0.05 <sup>b</sup>	3.02 ± 0.1	1.6 ± 0.33 <sup>a,b</sup>
	96 IRO (59F/37M)	5.4 ± 0.004 <sup>b</sup>	90.9 ± 4.03 <sup>b</sup>	3.63 ± 0.15 <sup>b</sup>	4.98 ± 0.08	1.26 ± 0.02 <sup>b</sup>	3.27 ± 0.1	1.49 ± 0.11 <sup>a,b</sup>
(39)	28 MHO (F)	5.3 ± 0.4	87.5 ± 26.4 <sup>a</sup>	3.0 ± 1.0 <sup>a</sup>	5.1 ± 0.8	1.5 ± 0.3	3.0 ± 0.7	1.3 ± 0.5 <sup>a</sup>

	28 OHR (F)	5.5 ± 0.5	156.9 ± 68.7 <sup>b</sup>	5.6 ± 2.6 <sup>b</sup>	5.4 ± 0.9	1.3 ± 0.3	3.1 ± 0.8	2.2 ± 1.2 <sup>b</sup>
(42)	37 MHO (F)	5.1 ± 0.6 <sup>a</sup>	70.1 ± 22.2 <sup>a</sup>	2.3 ± 0.7 <sup>a</sup>	5.05 ± 1.0	1.57 ± 0.3 <sup>a</sup>	3.26 ± 0.9	1.16 ± 0.4 <sup>a</sup>
	28 MetSO (F)	5.5 ± 0.7 <sup>b</sup>	97.2 ± 52.8 <sup>b</sup>	3.2 ± 1.2 <sup>b</sup>	5.11 ± 0.6	1.10 ± 0.14 <sup>b</sup>	3.30 ± 0.6	2.39 ± 0.6 <sup>b</sup>
(60)	19 MHO (F)	-	-	2.3 ± 1.2 <sup>a</sup>	4.3 ± 0.5 <sup>a</sup>	2.6 ± 0.4 <sup>a</sup>	1.5 ± 0.2 <sup>a</sup>	1.1 ± 0.4 <sup>a</sup>
	135 OHR (F)	-	-	3.16 ± 1.8 <sup>b</sup>	5.4 ± 0.9 <sup>b</sup>	3.4 ± 0.8 <sup>b</sup>	1.3 ± 0.3 <sup>b</sup>	1.8 ± 0.7 <sup>b</sup>
(63)	36 MHO (34F/2M)	4.4 ± 0.8 <sup>a</sup>	-	-	4.5 ± 0.6 <sup>a</sup>	1.6 ± 0.2 <sup>a</sup>	2.5 ± 0.5 <sup>a</sup>	1.02 ± 0.4 <sup>a</sup>
	88 OHR (78F/10M)	5.1 ± 1.6 <sup>b</sup>	-	-	4.8 ± 0.7 <sup>b</sup>	1.3 ± 0.3 <sup>b</sup>	2.9 ± 0.6 <sup>b</sup>	1.34 ± 0.5 <sup>b</sup>
(74)	73 NW (F)	4.7 ± 0.5 <sup>a</sup>	48 ± 27.7 <sup>a</sup>	-	4.8 ± 0.9 <sup>a</sup>	1.6 ± 0.4 <sup>a</sup>	-	0.87 ± 0.4 <sup>a</sup>
	20 MHO (F)	4.7 ± 0.5 <sup>a</sup>	76.4 ± 20.8 <sup>b</sup>	-	4.7 ± 1.2 <sup>a,b</sup>	1.3 ± 0.2 <sup>b</sup>	-	1.1 ± 0.5 <sup>a</sup>
	40 IRO (F)	5.1 ± 0.5 <sup>b</sup>	138.9 ± 125 <sup>c</sup>	-	5.3 ± 1.0 <sup>b</sup>	1.3 ± 0.3 <sup>b</sup>	-	1.7 ± 1.1 <sup>b</sup>
(75)	22 MHO (F)	4.9 ± 0.5	84.0 ± 31.2 <sup>a</sup>	2.7 ± 1.2 <sup>a</sup>	5.6 ± 0.8	1.7 ± 0.4 <sup>a</sup>	3.4 ± 0.6	1.3 ± 0.5 <sup>a</sup>
	22 OHR (F)	5.1 ± 0.5	142.3 ± 58.3 <sup>b</sup>	4.7 ± 2.0 <sup>b</sup>	5.5 ± 0.9	1.3 ± 0.2 <sup>b</sup>	3.1 ± 0.9	2.2 ± 0.9 <sup>b</sup>
(76)	122 NW (70F/52M)	4.9 ± 0.5 <sup>a</sup>	48.6 ± 27.8 <sup>a</sup>	-	5.3 ± 1.1	1.5 ± 0.4 <sup>a</sup>	-	1.2 ± 0.6 <sup>a</sup>
	106 MHO (62F/44M)	4.9 ± 0.6 <sup>a</sup>	69.5 ± 27.8 <sup>a</sup>	-	5.3 ± 0.9	1.3 ± 0.3 <sup>b</sup>	-	1.5 ± 0.9 <sup>a</sup>
	212 IRO (124F/88M)	5.4 ± 0.7 <sup>b</sup>	125 ± 69.5 <sup>b</sup>	-	5.4 ± 1.0	1.2 ± 0.3 <sup>c</sup>	-	1.7 ± 0.9 <sup>b</sup>
(79)	26 MHO (F)	-	-	2.4 ± 0.7 <sup>a</sup>	-	1.4 ± 0.3	-	1.3 ± 0.5 <sup>a</sup>
	78 OHR (F)	-	-	4.2 ± 1.8 <sup>b</sup>	-	1.4 ± 0.3	-	1.7 ± 0.9 <sup>b</sup>
(83)	26 MHO (12F/14M)	5.07 ± 0.1	38.3 ± 1.9	1.16 ± 0.06	4.95 ± 0.18	1.37 ± 0.08	3.0 ± 0.13	1.71 ± 0.4
	77 IRO (34F/43M)	5.42 ± 0.1	91.4 ± 3.7	2.98 ± 0.13	5.02 ± 0.1	1.27 ± 0.03	3.29 ± 0.08	1.56 ± 0.12

HOMA, homeostasis assessment model; TC, total cholesterol; TG, triglycerides; NW, normal weight; F, female; M: male; MONW: metabolically obese normal weight; MHO, metabolically healthy obese; IRO, insulin resistant obese; OHR, overweight/obese higher risk; MetSO: metabolic syndrome obese. Different letters <sup>(a,b)</sup> within the same reference indicates that the values differs (statistically significant).

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### **3.2. Article 2 (review): Network between endotoxins, high fat diet, microbiota and bile acids on obesity**

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#### **Abstract**

Insulin resistance may favor metabolic abnormalities. The level of insulin sensitivity and  $\beta$ -cell response determine metabolic phenotypes. Distribution and hypertrophy of adipose tissue is often associated with insulin resistance. However, the involvement of inflammation has opened the discussion about the role of endotoxins, more specifically lipopolysaccharides (LPS), as triggers of inflammatory activation and insulin resistance. The consumption of high fat diet, in particular, can influence microbiota composition, LPS absorption and provide fatty acids that may activate the same receptors activated by LPS. In addition, it can increase bile secretion and influence bile acids profile. Bile acids and bile acid receptors seem to participate in glucose and lipid metabolism, influence insulin sensitivity and intestinal microbiota composition. Therefore, there is a complex relationship between endotoxins, diet, microbiota, bile acids, insulin resistance and obesity. The aim of this review is to provide a broad perspective of this network and to show the variety of factors that may influence outcomes and that should be taken into account in future studies in this field. We start discussing about endotoxins terminology and general aspects. Signaling pathways activated by insulin and LPS are summarized. Then, evidences of endotoxins effects on adipose tissue and intestines are presented. Because endotoxins and fatty acids share signaling pathways, the role of high fat diet in endotoxemia and inflammation is also accomplished. Additionally, the inter-relationship between microbiota, intestinal permeability, endotoxins and high fat diet is discussed. Furthermore, we propose that bile acids are a missing point to be better explored in this scenario.

**Key words:** insulin resistance, fatty acids, lipopolysaccharides, microbiota, intestinal permeability, bile acids

## 1. Introduction

According to Reaven, the clustering of high blood pressure, dyslipidemia and high fasting glucose levels does not evolve accidentally<sup>1</sup>, but may be a consequence of insulin resistance (IR).<sup>2-3</sup> Although it is accepted that the degree of IR may rise with one's fat mass, at the individual level, the causality between obesity and IR is not always a rule.<sup>4</sup> Terms such as benign vs. malign obesity, metabolically healthy obese vs. at risk and metabolically obese normal weight aroused as an attempt to highlight that for a same obese or lean body size different metabolic phenotypes can be expected. Higher and lower insulin sensitivity is the main differentiating factor for this categorization, in accordance with the concept that metabolic abnormalities will not necessarily occur due to obesity *per se*, but might be largely related to the presence of IR.<sup>5-6</sup>

Obesity is characterized by excessive growth of adipose tissue (AT).<sup>7</sup> There is a complex relationship between IR and AT. The balance between storage and utilization of energy sources is disturbed by the lack or excess of AT. In some cases, induction of AT differentiation and expansion by drugs (e.g., thiazolidinedione) improves insulin sensitivity. This indicates that increasing AT will not necessarily induce IR.<sup>4</sup> The occurrence of abnormalities associated with metabolic syndrome (IR, dyslipidemia, hypertension, fatty liver) will depend not only on the size, but also on the functionality of the AT.<sup>7</sup>

Each individual may present a threshold level of adiposity beyond which dysfunctionality is established.<sup>4,7</sup> Fat distribution and adipocytes size also influence the functionality of AT and occurrence of IR.<sup>7-8</sup> It is hypothesized that inefficiency of subcutaneous depots to store fat contributes to visceral depots expansion.<sup>9-10</sup> This would increase the supply of non-esterified fatty acids (NEFA) to ectopic sites, leading to IR<sup>2,11-12</sup> and abnormalities.<sup>4,13</sup> In addition, hypoxia caused by lack of adequate vasculature under AT expansion, activates recruitment and infiltration of immune cells, increasing production of pro-inflammatory molecules.<sup>14</sup> Inflammation within adipose tissue is believed to promote local dysfunctionality and systemic effects. This has led to the view that obesity is characterized by a state of chronic, low-grade, systemic inflammation, that would impair several cellular metabolic functions<sup>15</sup> including insulin signaling.<sup>14-18</sup>

In recent years, it has been suggested that the induction of inflammation in obesity might be triggered by molecules derived from the gut. Lipopolysaccharides (LPS) from gram-negative bacteria cell wall are considered potent inducers of innate immune cells activation

and inflammation. This has raised the possibility of LPS involvement in IR development, since higher levels are reported in diabetic subjects<sup>19</sup> and LPS also seems to regulate adipogenesis.<sup>20</sup> The hypothesis that higher levels of LPS may be one of the causes of IR is gaining strength, in parallel, with gut microbiota alteration, gut inflammation and visceral adipocyte inflammation.<sup>21</sup>

Within this context, it is important to remember that several factors should be taken into account to predict possible consequences of LPS. First, the level and distribution of adiposity, microbiota composition, the level and type of LPS in gastrointestinal lumen vary between individuals. Secondly, the gut act as a barrier for luminal LPS. Third, there are physiological mechanisms to detoxify or reduce LPS toxicity.<sup>22</sup> In addition, the diet can influence microbiota composition,<sup>23</sup> and LPS absorption.<sup>24-25</sup> Specific types of fatty acids may also activate the same receptors activated by LPS.<sup>26-27</sup> Besides providing fatty acids and increasing LPS absorption, the consumption of high fat (HF) diet also increases bile secretion<sup>28</sup> and influences bile acids (BA) profile.<sup>29</sup> BA and BA receptors seem to participate in glucose and lipid metabolism, influence insulin sensitivity<sup>30</sup> and microbiota composition.<sup>31</sup> This illustrates the complex relationship between LPS, diet, microbiota, BA, IR and obesity.

Thus, in the present review we start discussing about LPS and endotoxins terminology and general aspects. Subsequently, signaling pathways activated by insulin and LPS are summarized. Then, evidences of endotoxins effects on adipose tissue and intestines are presented. Because endotoxins and fatty acids share signaling pathways, the role of high fat diet on endotoxemia and inflammation is also accomplished. Additionally, the inter-relationship between microbiota, intestinal permeability, endotoxins and high fat diet is discussed. Furthermore, we propose that BA are a missing point to be better explored in the context of obesity, insulin resistance, microbiota, high fat diet and endotoxins.

## **2. Endotoxins: terminology and general aspects**

The term “endotoxin” is occasionally used to refer to any ‘toxin’ associated with microbial cells (flagellin, DNA, peptidoglycan, lipoteichoic acid) and to its biological activity. Although LPS is often interchangeably referred as an endotoxin, it is more associated with the chemical structure and composition of the cell wall molecule of gram-negative bacteria, which varies among species.<sup>32</sup> Even so, in the present review, we will also use LPS and endotoxin as synonymous.

The main components of LPS structure are: polysaccharide chain (O-antigen, the immunogenic site), oligosaccharides nucleus (core R) and lipid A.<sup>33-34</sup> The bioactivity of LPS molecule is determined by the lipid A moiety, whose fatty acids are saturated varying between 10 to 22 carbon atoms.<sup>34</sup> The toxicity of lipid A is also influenced by unsaturations of the fatty acid molecule, since lipid A containing unsaturated fatty acids is nontoxic or acts as antagonist.<sup>22,26-27</sup>

Many authors assume that all LPS types are toxic, which is not truth. The LPS from smooth types of gram-negative bacteria (as compared with rough-type)<sup>35</sup> and from *Rhodobacter capsulatus*, a non-enteric bacteria<sup>36</sup> for example, may actually reduce or inhibit the production of inflammatory cytokines. It is clear from infusion models in humans and animals that LPS from *E.coli*, one of the most commonly used, induce a strong, acute inflammatory response. This does not mean that bacterial parts from gram-positive bacteria will not induce this type of response and that the effects will be reproduced if LPS is translocating from gut instead of entering directly to circulation.

In general, the term LPS has often a negative connotation. Whereas recognition of LPS by host cells is implicated in beneficial consequences such as the mobilization of defense mechanisms.<sup>37</sup> The problems may arise when this response is exaggerated, such as in sepsis, or low grade, but chronic, as might be the case of obesity and type 2 diabetes mellitus (T2DM). This is why down-regulating responses and physiological mechanisms to remove LPS from circulation and tissues are important to the host.

Tolerance to endotoxins is a state of transitory hyporesponsiveness to LPS challenge after an initial exposure. It is a down-regulating mechanism that might be induced to protect the host against cellular damage, caused by hyperactivation of immune cells, especially in cases of persistent bacterial infection.<sup>38-39</sup> Neutralizing mechanisms, usually involving leukocytes, intestinal and liver enzymes also inhibit inflammatory activation. Human leukocytes express the enzymes acyloxyacyl hydrolases (AOAH) that are able to remove fatty acyl chains from lipid A moiety, inactivating LPS.<sup>40-41</sup> Alkaline phosphatase (AP) is another enzyme, expressed by hepatic and intestinal cells, also able to inactivate or reduce LPS biological activity by promoting its phosphorylation.<sup>42</sup>

The binding of LPS with lipoproteins such as chylomicrons and HDL is also another alternative to neutralize the endotoxic activity of LPS,<sup>35,43</sup> favoring its removal from circulation through the liver<sup>44</sup>. As lipoproteins help to control the effects of circulating LPS,

the levels of lipoproteins should be better explored *in vivo* to understand the host responses. Findings from *in vitro* human whole blood model suggest that there is a LPS-chemotype dependence over the kinetics of the interaction between LPS and lipoproteins, which may interfere in their toxicity. The polysaccharide chain length of LPS is presumably responsible for the velocity of the association with lipoprotein: the shorter the polysaccharide chain, the more hydrophobic the LPS molecule and the higher the apparent affinity of LPS for the lipoprotein phospholipid layer.<sup>45</sup>

In intestinal epithelial cells, the internalization of LPS molecules and subsequent intracellular destination is also dependent on LPS characteristics, which in turn determines both the consequences and the fate of the LPS. Large aggregates of LPS are internalized along with CD14 and deacylated via the lysosomal pathway (associated with reduction of potency), whereas monomeric LPS is transported to the golgi apparatus where initiates cell activation.<sup>46-47</sup>

Thus, biological responses may differ according to the size and composition of LPS, whether it is presented as component of intact bacteria or as isolated part,<sup>48</sup> as well as to the level and activity of hepatic and intestinal detox enzymes and the level of lipoproteins. This set of factors has not been usually considered and/or explored in the design of studies, especially *in vivo*.

### **3. Insulin signaling and resistance to its action**

A diverse serie of pathways are activated by insulin binding to its receptor. These pathways act in concerted fashion to coordinate the pleiotropic physiological effects of insulin over glucose, lipid and protein metabolism.<sup>17,49</sup> In the liver, insulin stimulates utilization and storage of glucose as lipid and glycogen, while repressing glucose synthesis and release. In adipocytes, insulin inhibits lipolysis and stimulates storage of glucose as lipid.<sup>49</sup>

The insulin receptor is a protein complex belonging to a subfamily of receptor tyrosine kinases. Intracellular substrates for the receptor-complex include the family of insulin-receptor substrate proteins (IRS 1/2/3/4), whose phosphorylated tyrosine residues act as a docking site for adaptor molecules, which in turn regulates the receptor activity. The serine phosphorylation is also possible, but attenuates the downstream signaling, being considered a negative feedback that leads to IR.<sup>49</sup> Several kinases are involved in phosphorylation of residues during the transmission of insulin signal, including phosphoinositide 3-kinase, protein kinase B, protein kinase C, and mitogen-activated protein kinase.<sup>49</sup>

The downstream signaling of the insulin receptor can be impaired by inflammatory signals, disturbing insulin action. Activation of the nuclear factor kappa beta (NF- $\kappa$ B) and activator protein-1 increase proinflammatory cytokines. Extracellular mediators (proinflammatory cytokines and non-esterified fatty acids (NEFA)) or intracellular stresses (endothelium reticulum stress or increased reactive oxygen species production by mitochondria) provide signals that converge to activation of multiple serine/threonine kinases. The activation of serine/threonine kinases, such as c-Jun N-terminal kinase, inhibitor of nuclear factor - $\kappa$ B kinase and protein kinase C, leads to direct inhibition of insulin signaling via serine phosphorylation of IRS-1 and may cause IR.<sup>17</sup>

One of the expected consequences of IR in the long term is glucose intolerance and hyperglycemia, which will not necessarily occur in all IR individuals. It will depend on the simultaneous occurrence of pancreatic islet  $\beta$ -cell dysfunction.<sup>12</sup> When a decrease in insulin sensitivity is compensated by a matched increase in insulin release, glucose tolerance is preserved. Potential cellular mechanisms of  $\beta$ -cell adaptation to IR are outlined by Kahn and co-workers.<sup>12</sup> A poor  $\beta$ -cell adaptation can result in decrement of insulin levels impairing its action in different sites. In the hypothalamus, this impairment could favor food intake and weight gain. Hepatic glucose production could be favored, uptake of glucose by muscle cells could be reduced, while in AT release of NEFA could increase. In ectopic sites, NEFA in excess would lead to IR and suppression of  $\beta$ -cell's adaptative response to IR.<sup>12</sup>

As reported by Ferrannini and co-workers,<sup>50</sup> IR is not as prevalent as previously thought in obese, and is less frequent than insulin hypersecretion, which might be a compensatory adaptation to the larger body surface.<sup>50</sup> Considering the same degree of IR, a different  $\beta$ -cell adaptation may occur depending on the degree and distribution of adiposity.<sup>51</sup> It is possible to encounter subjects with 1) IR and hyperinsulinemia, 2) IR without hyperinsulinemia and 3) hyperinsulinemia without IR.<sup>51</sup> These different situations may result in different metabolic abnormalities profile (Box 1).<sup>50,52</sup>

We believe that future studies exploring these different phenotypes and how LPS concentrations interact with them are of great importance to better define the involvement of LPS and adiposity in IR and other metabolic abnormalities.

#### **4. Lipopolysaccharides signaling pathways and insulin sensitivity**

Toll-like receptors (TLRs) are pattern-recognition receptors critical for inflammatory responses, since they recognize conserved pathogen-associated molecular patterns, such as LPS.<sup>17,53</sup>

LPS usually acts as agonist for TLR4, evoking inflammatory responses and cytokines secretion.<sup>17,22,54</sup> Activation of TLR4 by LPS is aided by auxiliary proteins including LPS binding protein (LBP), CD14 (soluble and membrane bound) and myeloid differentiation factor-2 (MyD-2). Activation of TLR4 results in activation of phosphoinositide 3-kinase and phosphorylation of protein kinase B. The phosphorylation cascade downstream protein kinase B includes p65, responsible for the transactivation of NF- $\kappa$ B. There is also another route of activation. Myeloid differentiation factor-88 (MyD88) is an immediate downstream adaptor molecule recruited by activated TLR4 that phosphorylates interleukin-1 receptor-associated kinases and tumor necrosis factor receptor-associated factor-6 (TRAF-6). The recruitment of the last to the receptor complex activates inhibitor of NF- $\kappa$ B kinases. This ends up with the activation and translocation of NF- $\kappa$ B into the nucleus and activation of mitogen-activated protein kinases. In the nucleus, the transcriptional factor NF- $\kappa$ B will induce the expression of target genes, including cyclooxygenase-2 and cytokines.<sup>17,22, 54-57</sup>

The response to LPS depends on the cell type. Some cells respond faster and are more sensitive to lower concentration than others. To illustrate, 30 min incubation of human aortic endothelial cells with LPS did not activate these cells, while an overnight incubation increased 4-fold IL-8 production. In contrast, human monocytes were more responsive and secreted significant amount of TNF already after 30 s of incubation.<sup>24</sup>

The acute administration of LPS in healthy subjects causes increase in plasma insulin and homeostasis model assessment indices (HOMA-IR) at 24h.<sup>58</sup> Higher insulin secretion could be an adaptive response to lower inflammatory activation, since insulin (at least exogenous) exerts anti-inflammatory properties. The insulin treatment during infusion of endotoxins in rats increased anti-inflammatory (IL-2, IL-4, IL-10) and decreased the proinflammatory cytokines (TNF, IL-1, IL-6).<sup>59</sup> Thus, an interaction between LPS and insulin levels and/or insulin sensitivity is currently assumed.

It is consistently reported in humans' studies of experimental endotoxemia (intravenous administration of LPS doses, from 0.6-3 ng/kg body weight) a mild, transient clinical and

biochemical response that involves: increases in temperature and heart rate, increased plasma levels of TNF, IL-6, IL-1 $\beta$ , C-reactive protein (CRP),<sup>58,60-63</sup> and also IL-10<sup>63</sup>. The cytokines released upon LPS challenge, particularly TNF, increases IRS-1 serine phosphorylation, leading to decreased insulin signaling.<sup>64</sup> IL-6 may exert insulin-sensitizing effect and was shown to enhance insulin-stimulated glucose disposal *in vivo*, increase fatty acid oxidation and glucose transport.<sup>65</sup> However, there are also contradictory results with possible deleterious effects of IL-6 in insulin action and glucose homeostasis. IL-1 $\beta$  is implicated in  $\beta$ -cell dysfunction and apoptosis, while IL-10 is a classical anti-inflammatory cytokine.<sup>64</sup> If LPS influences the secretion of these cytokines, then it is reasonable to accept the idea of their involvement in IR and T2DM.

Hormonal interactions during human endotoxemia might also help to explain the development of IR. In healthy humans infused with LPS, plasma adiponectin did not change significantly, while a modest increase in plasma leptin was observed. After LPS administration, whole blood and adipose samples resistin mRNA, and plasma resistin<sup>58</sup> and cortisol<sup>60</sup> increased sharply. The coordinated attenuation of adiponectin, increase in resistin and leptin during activation of innate immunity may converge to the insulin-resistant state, at least during acute LPS exposure. In healthy subjects, LBP was positively associated with leptin and insulin, while negatively associated with adiponectin.<sup>66</sup>

In table 1, studies describing the basal levels of endotoxins in different conditions are presented. It can be observed that in some cases where LPS levels are increased, higher insulin levels are also present.

In summary, the excessive activation of TLRs may lead to systemic inflammation and IR. Activation of NF- $\kappa$ B is a molecular target shared by proposed mechanisms of IR and LPS signaling pathways. Of note, others bacterial products (peptideoglican, flagelin) are the main agonists for the different TLRs, and endogenous molecules such as minimally oxidized LDL, heat shock proteins, fibrinogen and NEFA can also be recognized by these receptors.<sup>17,22</sup> This possibility turns difficult the task of defining the real impact of LPS in insulin signaling *in vivo* without the infusion of LPS. However, the current view is that cytokines released after LPS insult may lead to IR in several tissues.

## **5. Effects of LPS on adipose tissue and intestines**

The activation of TLRs is involved in the control of pathogens elimination, commensal homeostasis, and linkage to the adaptative immunity. There is considerable variation



between TLRs and perhaps also variations in TLRs effects between cell types and organs origin.<sup>67</sup> Here we briefly discuss the effects of LPS on adipose tissue and intestines.

### *5.1. Adipose tissue*

At first, white AT was seen as both a source and site of inflammation. The hypertrophy of adipocytes would trigger infiltration of immune cells, whose activation could lead to chronic inflammation and IR in AT.<sup>14,68</sup> The consequent delivery of NEFA from AT to other sites such as liver, muscle, heart and pancreas has been a mechanism strongly suggested in the literature to cause IR in these sites, contributing to dyslipidemia, fatty liver, glucose intolerance, and  $\beta$ -cell dysfunction.<sup>69</sup> However, the expression of TLR4 in 3T3-L1 adipocytes, isolated mouse adipocytes, and AT<sup>54</sup> raises the possibility that LPS triggers inflammation in AT and may directly cause IR in this site. Preadipocytes and adipocytes from visceral depots (i.e. mesenteric and omental) have been shown to express inflammatory cytokines after LPS exposure. These cytokines can attract immune cells, alter lipid metabolism and insulin signaling.<sup>21</sup>

The higher basal endotoxins levels in T2DM subjects<sup>70</sup> and obese pregnant women<sup>19</sup> (table 1) in comparison to their controls may be a possible explanation for the concomitant higher expression of molecules associated with LPS signaling cascades in subcutaneous AT samples<sup>70</sup> or in stromal vascular fraction cells isolated from AT.<sup>19</sup> In one of the studies, it was also reported paralleled to higher endotoxins, higher circulating levels of insulin (and HOMA-IR), leptin, CRP and IL-6 in obese women in comparison to lean.<sup>19</sup> The increased secretion of IL-6, IL-8 and TNF after exposure of human isolated adipocytes or stromal cells to LPS,<sup>19,70</sup> supports the view that AT, whether the adipocytes or other cells within AT, is responsive to LPS insult.

In fact, human studies using acute LPS infusion showed the modulation of gene expression in AT samples.<sup>61-62,71</sup> The degree of clinical, biochemical and gene expression changes seems to be dose dependent.<sup>61</sup> Increased expression of inflammatory ( $\uparrow$ mRNA of IL-6, TNF, MCP-1 and others) and insulin signaling markers ( $\uparrow$ mRNA of IRS-1 and SOCS-1 and -3) were observed in subcutaneous AT from gluteal site.<sup>61-62</sup> Concomitantly, there was a marked, rapid and transient induction of plasma TNF, IL-6, MCP-1, NEFA and cortisol in the earlier phase post-LPS infusion (0-8 h). At 24 h post-LPS, period of maximum high sensitive CRP, significant change in HOMA-IR occurred. Insulin sensitivity was inversely correlated with NEFA, while HOMA-IR was positively correlated with CRP and resistin.<sup>62</sup>

These results could advocate for a cause-effect relationship between acute endotoxemia and transient systemic IR, but not necessarily pancreatic  $\beta$ -cell dysfunction in humans. In addition, inflammatory modulation of adipose insulin signaling induced after LPS seems to precede the systemic IR.

The gene expression and protein production in both human omental and subcutaneous AT samples was also altered by open heart surgery with cardiopulmonary bypass.<sup>72</sup> A systemic IL-6 increase was observed together with a slightly different, but inflammatory, gene expression in both fat depots. Immunohistochemistry biopsies showed marked staining of NF- $\kappa$ B-p65 at protein level in adipocytes nucleus, endothelium and macrophages. These findings could indirectly be related to the occurrence of IR during surgery. Although plasma endotoxins were not evaluated pre and post-surgery,<sup>72</sup> major surgical procedures as cardiopulmonary bypass can cause intestinal hypoxia, which in turn may favor LPS translocation. Thus, it was unclear if AT induced-inflammation was “clean” or if involved LPS signaling. In another study, antibiotic therapy given previously to subjects undergoing the same type of surgery reduced gram-negative bacteria in rectum and also endotoxin and cytokines levels in comparison to the group that did not receive antibiotic treatment.<sup>73</sup>

Therefore, it seems reasonable to hypothesize that systemic LPS in plasma may represent an external stimulus to activate cellular signals leading to adipocytokines production toward inflammation and IR. However, there are still some open questions that further studies should try to address as follows.

The studies that evaluate basal endotoxin levels and gene expression in AT do not prove that higher endotoxins are the cause of local inflammation and systemic IR, as infusion models do. These studies do not control for example for food intake. As it will be discussed later, saturated fatty acids may also induce these inflammatory changes and also increase LPS absorption. In addition, penetration of LPS directly to the circulation (infusion models) may elicit different responses than the translocation of LPS from the intestines.

Cell culture experiments from Dasu and co-workers<sup>74</sup> showed that palmitate and stearate significantly amplified TLR2 and TLR4 expression via NF- $\kappa$ B activation and cytokine production in high glucose condition, while oleate had no effect.<sup>74</sup> High glucose combined with palmitate promoted production of superoxide via NADPH oxidase, which by themselves can induce inflammation. Inhibition of TLR-expression and NADPH oxidase attenuated the mentioned effect of high glucose and palmitate.<sup>74</sup> In their point of view, high

levels of glucose and NEFA in the circulation could result in different degree of TLR activation and proinflammatory factors production in monocytes. This could build systemic inflammation with impact on insulin signaling.<sup>74</sup> Nevertheless, it is worth mentioning that the reagents used were allowed to have less than 100 EU/mL of LPS. They argued that based on previous report, this low concentration does not interfere with TLR2/4 measurement.<sup>74</sup> The implication of these results to the *in vivo* setting should be considered in future studies.

Another open question is related to the issue of visceral adipose tissue accumulation as the fat depot highlighted to be involved in triggering IR and as the main site of inflammation and NEFA supply to liver. The evidences from different studies in humans investigating the effect of LPS on AT were based on subcutaneous adipose tissue samples mainly from gluteal site.<sup>19,61,70</sup>

Adipose tissue size can change by means of hyperplasia and hypertrophy. Adipogenesis is the process of adipocytes formation from precursor cells (hyperplasia). Lipogenesis is the synthesis of esterified fatty acids to form triglycerides (TG) to store fat (hypertrophy), being induced by insulin. The inability to increase cell number through adipogenesis reduces the ability to store lipids and this contributes to the development of metabolic diseases.<sup>7</sup> There are evidences that LPS may influence adipose tissue size. One study showed that chronic infusion of low dose of LPS stimulated adipose tissue expansion accompanied by IR,<sup>75</sup> while others showed that LPS inhibit adipogenesis.<sup>20,76</sup> It has been hypothesized that translocation of gut-derived molecules to adipose tissue localized in close proximity to the gut, such as mesenteric fat (a type of visceral fat), would trigger macrophage infiltration and inflammation, which in turn would stimulate expansion of this visceral depot. Expanding mesenteric fat mass would provide increased fatty acid flux to the liver, which in the long term could result in an inflamed, steatotic, and insulin resistant liver.<sup>77</sup> On the contrary, during sepsis, LPS levels increase the magnitude and duration of the systemic inflammatory response, which is usually associated with IR, hyperglycemia, but with a high rate of catabolism in muscle and fat cells.<sup>59</sup> Thus, it remains poorly understood the role of LPS in adipogenesis and lipogenesis, and how exactly this may affect metabolic control.

Finally, it should be further investigated if the infiltration of immune cells in the AT could be the result of hypoxia induced by adipocytes hypertrophy, delivery of LPS molecules or a direct effect of saturated fatty acids. In mice, it was shown that neutrophils transiently

infiltrated intra-abdominal AT early in the course of diet-induced obesity, preceding by weeks the well-described infiltration of macrophages. Unfortunately, circulating levels of LPS was not assessed.<sup>78</sup> There are evidences that neutrophils can induce glucose intolerance through the expression of neutrophil elastase, which was higher in AT from high fat fed mice. Both genetic and pharmacologic induced loss of function of neutrophil elastase improved glucose tolerance and insulin sensitivity. Incubation of mouse and human hepatocytes with neutrophil elastase caused IRS-1 degradation, lower insulin signaling, higher glucose production and cellular IR. The proinflammatory effects of neutrophil elastase seem to be dependent on TLR4.<sup>79</sup>

### *5.2. Intestines*

TLR4 dependent signals in intestinal cells are important to the host. LPS stimulation may prevent allergen induced Th2-type inflammation by upregulating Th1 responses via TLR4 in regulatory T cells. A “healthy” gut condition seems to depend on constant exposure of the intestinal surface to commensal derived TLRs ligands, a basal state of activation of downstream signaling pathways, rapid restitution and limited inflammatory responses.<sup>67</sup>

Mechanisms of hyporesponsiveness are essential to avoid aggressive reactions in the intestine, since exaggerated inflammatory responses in the absence of pathogenic bacteria would be deleterious. Molecular immune mechanisms that contribute to tolerance via TLRs in intestinal epithelial cells are cited by Cario<sup>67</sup>: 1) decreased surface receptor expression, 2) high expression levels of downstream signaling suppressor Tollip, 3) ligand induced activation of peroxisome proliferator activated receptor  $\gamma$  which uncouples NF- $\kappa$ B dependent targets genes, and 4) external regulators that suppress TLR mediated signaling pathways.<sup>67</sup>

Intestinal epithelial cells (IEC) are the frontline of the mucosal immune system expressing at least two TLRs (2 and 4). LPS-induced stimulation of different IEC lines involves selected activation of mitogen activated protein kinases pathways, culminating in NF- $\kappa$ B activation under addition of the serum protein sCD14. Constitutive expression of CD14 was not detected in three IEC lines. This may make IEC hyporesponsive and tolerant to the luminal content of the gastrointestinal tract. However, any release or expression of specific serum mediator proteins such as sCD14 may turn quiescent IEC into responsive cells.<sup>80</sup> IEC can release the acute phase proteins LBP and serum amyloid A (SAA) under stimulation of cytokines (IL-6, IL-1 $\beta$  and TNF) secreted by nearby cells.<sup>81</sup> In murine small intestinal crypt

epithelial cell line (m- IC<sub>cl2</sub>), CD14 mRNA was detected, and the exposure to LPS enhanced their LPS-binding capacity. TLR4 mRNA was detected within Golgi complex, not in the surface as found for peritoneal macrophages. The intracellular localization of TLR4 in intestinal epithelial cells might represent a regulatory barrier to prevent excessive stimulation, while in macrophages membrane localization might ensure highest LPS sensitivity. Another mechanism of protection against ongoing phagocyte infiltration and tissue damage upon LPS challenge in intestinal cell is the up-regulation of a serine protease inhibitor SLPI, which inhibits LPS transfer to CD14, internalization and prostaglandin synthesis.<sup>46</sup>

Internalization, cell traffic and intact function of Golgi apparatus are requirements for LPS-mediated stimulation through TLR4 in IC<sub>cl2</sub> cells.<sup>47</sup> In addition, a role for plasma membrane microdomains or lipid rafts was also implicated in LPS recognition. Incubation of cells with agents that impede their formation reduced LPS-mediated NFκB activation in a dose dependent manner. LPS-mediated cellular activation requires ligand internalization that occurs via a lipid raft-dependent formation of clathrin-coated pits and intracellular transport to Golgi compartment. The sub-cellular localization of the LPS recognition complex is influenced by the endothelium reticulum heat shock protein gp96.<sup>47</sup>

The lipid rafts represent versatile devices for compartmentalizing cellular membrane processes composed of sphingolipids, phospholipids, cholesterol and proteins. Their activation changes the conformation of a freely structure toward a larger platform where proteins meet into fluid microdomains to perform functions in signaling, processing and transport. The saturation/unsaturation of the hydrocarbon chains determines how this structure is packed and influence the freely movement of lipid rafts in cell membranes, which in turn may affect signaling. Cholesterol serves as spacer between hydrocarbon chains and as a glue to keep raft assembly, being essential for this structure to work properly. The removal of cholesterol turns the rafts nonfunctional.<sup>82</sup> It seems that even in the presence of LPS, the availability of cholesterol and fatty acids of different saturation/unsaturations degree might influence the response to LPS.

The interaction between IECs and microorganisms is the first step in the sequence of events leading to a host immune response intended to eradicate potential pathogens. Since the components of bacterial cell walls of both gram-negative (LPS) and gram-positive (lipoteichoic acids, LTA) can interact with IECs, the composition of gut microbiota seems to

be of great importance.<sup>83</sup> *Lactobacillus johnsonii* strain La1 and *Lactobacillus acidophilus* strain La10, as well as their purified LTA did not stimulate cytokine production in IEC (HT29) in the presence of sCD14, in contrast to LPS.<sup>83</sup> However, in peripheral mononuclear cells LTA did induce IL-8 release. In intestinal cells, a marked decrease in the LPS-induced IL-8 and TNF by LTA was observed. Similarly to LPS, deacylation of LTA weakened their inhibitory effect toward IL-8 secretion induced by LPS. Therefore it is suggested that the lipid moiety of LTA from these gram-positive bacteria tempered the LPS-mediated activation of these cells.<sup>83</sup>

Taken together, the different tissues present particularities in regard to LPS response. AT is more responsive, while IECs seems to have mechanisms to control activation. The equilibrium in gut microbiota composition is essential for a healthy gut mucosa and might influence LPS signaling and/or absorption. It is possible that the availability of cholesterol, saturated and unsaturated fatty acids affects the lipid rafts assembly, and consequent cellular signaling.

## **6. Endotoxins and fatty acids signaling pathways**

NEFA are often involved in the mechanistic explanations of IR (ectopic deposition, lipotoxicity) and there is also suggestion of their ability to promote TLR4 signaling.<sup>54</sup> The fact that monocytes/macrophages activation and the propensity for endotoxemia can be modulated by types of fatty acids<sup>26,84</sup> highlights the difficulty in defining the real impact of LPS on insulin signaling and obesity *in vivo*.

Fatty acids, more specifically saturated, and endotoxins are closely related. As discussed earlier, the endotoxic activity of LPS seems to depend on the acylated form of the hydroxy saturated fatty acids (mainly lauric, myristic, palmitic) in lipid A. This dependence is suggested by the fact that the deacylation of these fatty acids by the hepatic enzyme AOAII leads to loss of endotoxic activity.<sup>22</sup>

An increased expression of mRNA of IL-6 and TNF was stimulated in adipocytes exposed to LPS or saturated fatty acids mixture (palmitate and oleate). Similarly, a lipid infusion administrated to mice caused stimulation of TNF, IL-6 and MCP-1 mRNA in their AT. After lipid infusion, inhibition of insulin-stimulated IRS-1 phosphorylation in skeletal muscle was observed, which was attenuated in TLR4<sup>-/-</sup> mice. Despite an increased adiposity in TLR4<sup>-/-</sup> mice under high fat diet, they were more insulin sensitive than wild-type mice.<sup>54</sup> This may indicate that adiposity does not lead necessarily to IR as long as inflammatory

signaling is inhibited. TLR4, besides being an obligatory receptor for LPS, is also a sensor for endogenous lipids that may contribute to the inflammatory pathogenesis of lipid-induced IR. Although TLR4 deficiency substantially limits impairment of insulin signaling and IR in muscle caused by lipid infusion, it is not possible to conclude that TLR4 is the exclusive mechanism.<sup>54</sup>

It is worth mentioning that lipid infusion model will not necessarily provide the same effects of mice fed high fat diet. Yet, lipid infusion model reinforces the role of fatty acids on inflammatory pathways activation independently of LPS. Nevertheless, high fat diet is associated with increased LPS, which will be discussed in the next section.

Similarly to LPS, fatty acids activate TLR signaling.<sup>26-27</sup> The cyclooxygenase-2 is one of the target genes products derived from NF- $\kappa$ B activation under LPS exposure, at least in macrophage cell line. It also seems to be induced by lauric acid through NF- $\kappa$ B activation, involving TLR4. In contrast, DHA inhibited NF- $\kappa$ B activation and also the LPS-induced expression of cyclooxygenase-2, inducible nitric oxide synthase and IL-1 $\alpha$ .<sup>26-27,54</sup> Lauric acid activated signaling pathways similar to the ones activated by LPS, while DHA inhibited the phosphorylation of protein kinase B induced by LPS or lauric acid.<sup>55</sup>

Saturated fatty acids also amplify the proinflammatory cytokine response to low, physiologically relevant concentration of LPS. To illustrate this, exposure of monocytes to LPS promoted 21-fold and 10-fold increase in IL-6 and IL-8 mRNA, respectively. In contrast, when palmitic acid was incubated the increase in these two cytokines was respectively 7-fold and 2-fold. The exposure of cells with both promoted an 80-fold increase in IL-6 and a 53-fold increase in IL-8 mRNA expression. Interestingly, IL-6 protein secretion did not increase due to LPS incubation, while exposure to palmitic acid followed by LPS increased IL-6 by nearly 4-fold. Protein secretion in response to 48 hours of LPS alone was not different from controls. These effects were mediated through a mechanism separated from, but paralleled to the TLR4 signaling. This included the uptake and metabolic processing of saturated fatty acids into ceramide, which in turn led to protein kinase C-mediated activation of the mitogen activated protein kinases. The conversion of saturated fatty acids into ceramide indicates that inflammation can also occur independently of TLR2 or TLR4.<sup>85</sup>

As discussed earlier, more than IR itself,  $\beta$ -cell failure is a crucial physiological event that leads to T2DM. Chronically elevated glucose levels, which increases generation of reactive oxygen species and mitochondrial dysfunction, endoplasmic reticulum stress and c-Jun N-terminal kinase signaling have been suggested to influence  $\beta$ -cell function and survival. Saturated NEFA also seem to impair  $\beta$ -cell function through ceramide synthesis, c-Jun N-terminal kinase activation, oxidative and endoplasmic reticulum stress.<sup>86</sup>

Whole human islets also express functional TLR4 and TLR2, whereas human  $\beta$ -cells express only functional TLR2. The addition of free fatty acids to cultured human and mouse islet cells and to the insulin-producing cell line (MIN6B1) stimulated cytokines and chemokines. In comparison to palmitate, oleate induced the strongest response of IL-1 $\beta$  and IL-6 mRNA through IL-1R signaling. These effects were further enhanced by glucose solution. Islets from TLR2 and TLR4 knockout mice were partially protected from the induction of proinflammatory factors by fatty acids. Of note, the fatty acids preparations were found to have endotoxins in the range of 6-58 pg/mL. However, dose response curves of LPS with human or mouse islets showed that at least 1000-fold higher LPS concentration was required to induce IL-1 $\beta$  mRNA expression.<sup>87</sup>

Igoillo-Esteve and co-workers<sup>86</sup> found that palmitate (but not oleate) or high glucose led to upregulation of NF- $\kappa$ B in human islets and induction of mRNA of inflammatory molecules. Protein secretion also increased for IL-6 and CXCL1. IL-1- $\beta$  and IFN- $\gamma$  induced a greater expression of the mRNA of cytokines and chemokines than palmitate. Interference of IL-1 $\beta$  signaling abolished palmitate-induced cytokine and chemokine expression, while the use of a synthetic endoplasmic reticulum stressor induced cytokine expression and NF- $\kappa$ B activation to a similar extent as palmitate. Thus, NF- $\kappa$ B activation and endoplasmic reticulum stress were induced in human pancreatic beta and non-beta cells by palmitate.<sup>86</sup> However, Erridge and Samani<sup>88</sup> highlight that previous studies were based on fatty acids complexed with bovine serum albumin. Although they confirmed that the complex stimulated TLR signaling, saturated fatty acids alone did not elicit a similar response.<sup>88</sup>

Somehow, the hypothesis of LPS as a cause of IR is still gaining strength, especially with the advances in the knowledge about the role of gut microbiota on metabolism and body composition.<sup>89</sup> On the other hand, protective effect of omega-3 fatty acids and detrimental action of saturated fatty acids demonstrated in cell culture models are in accordance with other *in vivo* studies.<sup>90-96</sup> Lombardo & Chicco<sup>94</sup> and Kennedy and co-workers,<sup>97</sup>



respectively, reviewed the mechanisms through which omega-3 and saturated fatty acids protect or induce IR in different body sites, not in the light of the possible role of LPS in the context. Because fatty acids may exert a role in inflammatory signaling, it is important for future studies analyzing correlation of endotoxins levels with other markers to control for plasma fatty acids and/or lipid (including fatty acid profile) intake.

### **7. Diet composition and the influence on endotoxins absorption**

Previous reviews have discussed about the role of dietary pattern on endotoxin translocation, with particular focus on HF<sup>98-101</sup> and high fructose intake.<sup>101-103</sup>

High fat diets are given to induce obesity and metabolic abnormalities in animal models and seem to be associated with increases in plasma LPS concentration. Subjects divided according to plasma endotoxin levels showed similar anthropometric and biochemical parameters, despite higher energy and fat intake by the group presenting the highest LPS concentration.<sup>104</sup> Although a follow-up study is needed, this may indicate that LPS, at least in the concentration found, does not necessarily represent a problem or a causative link to metabolic abnormalities.

In mice, both HF and high carbohydrate diets increased plasma LPS, but more efficiently in the first.<sup>104</sup> In fact, table 2 summarizes different human studies that confirm that HF intake in a meal promotes peaks in plasma LPS. As can be observed, the fat content and meals composition influence the occurrence and time of peak in LPS concentration. It is possible that the faster the peak of LPS and return to basal levels, the lower the inflammatory activation. Even though the net amount of fat was similar between three studies,<sup>24-25,105</sup> in one of them, inflammatory markers changes were not observed<sup>24</sup>. Interestingly, the inclusion of orange juice in a HF meal blunted the increase in LPS and inflammatory markers.<sup>105</sup>

One of the studies, showed that the chylomicrons fraction, at the time of LPS peak, contained higher LPS concentration than the remaining plasma fraction.<sup>106</sup> This may have implication for LPS signaling. A marked increase in the uptake of LPS by the liver occurs when it is bound to chylomicrons, decreasing the production of nitric oxide by hepatocytes.<sup>107</sup> Another study showed that chylomicrons, in comparison to others lipoproteins, has the highest LPS-neutralizing capacity, reducing cytokine secretion.<sup>108</sup> The kinetics of chylomicrons-LPS complex may be related to the TG levels in the postprandial period. In morbidly obese subjects, the increase in endotoxin levels (serum and chylomicrons fraction) was induced by fat overload. The subjects with higher postprandial

hypertriglyceridemia showed a significant increase in LPS after fat overload. Postprandial LPS increase was related to postprandial hypertriglyceridemia, but not to the degree of IR.<sup>109</sup>

Of note, the induction of oxidative stress happened before the LPS peak and the induction of TLR2/4 mRNA expression in mononuclear cells was faster and prior to LPS peak when a high glucose solution was associated with a HF meal. Additionally, the high glucose solution seems to anticipate the peak of LPS in comparison to HF meal alone.<sup>105</sup> Thus, an overload of glucose may also interfere with LPS absorption and/or clearance and may directly activate TLRs and oxidative stress.

There are many features of lipids that are shown to interfere with LPS absorption and effects. Emulsified lipids resulted in the highest accumulation of LPS and TG in comparison to the free oil in rats and/or in cell culture.<sup>106</sup> The size of fatty acid chain also interferes. Butyric acid did not induce chylomicrons formation or increase in plasma LPS, while oleic acid did. The chemical inhibition of chylomicrons formation blocked absorption of LPS, indicating the importance of chylomicrons in LPS translocation from the gut and transport to the mesenteric lymph nodes, where increased TNF mRNA levels were observed.<sup>110</sup> The fatty acid profile of a HF diet or meal influences the extent of induced inflammation. Fat sources (milk fat, palm, rapeseed and sunflower oils) differing in their fatty acid profile were given to mice. Inflammation onset was not correlated with body weight gain. Endotoxemia was not associated with fat content in the diet (22% vs. 3% of total caloric content), but rather with lipid quality. Despite apparently higher endotoxemia, rapeseed fed group showed lower inflammation than palm-fed group. The group fed palm oil had higher LBP than the other groups, and also higher IL-1 $\beta$ , TLR4, and CD14 expression in AT compared to chow diet group.<sup>111</sup> The LBP/sCD14 ratio may be one possible explanation to either efficiently triggering (high LBP in palm oil) or preventing (high sCD14 in rapeseed group) inflammation.<sup>111</sup> Palm oil from vegetable source triggered greater inflammation than the so condemned animal fat source. Because rapeseed and sunflower oils and milk fat resulted in similar plasma levels of proinflammatory cytokine, despite their different fatty acid and TG structure,<sup>111</sup> more studies are necessary to elucidate the differences and similarities between different fat sources. This finding brings into question the view that higher LPS will lead to higher inflammation, especially for an *in vivo* normal condition where we find inter and intra variations in meals composition. How much the responses in experiments of LPS infusion can be translated to a physiological day-to-day life should be further addressed.

Harte and colleagues<sup>112</sup> showed that the effects of a HF meal may also differ according to the current metabolic status of subjects (prediabetic, nonobese, obese, T2DM). At fasting, endotoxins levels were significantly lower in nonobese compared with impaired glucose tolerance (IGT) and T2DM subjects, but similar to obese subjects. This indicates that obesity *per se* is not associated with higher endotoxins. Intake of HF meal (75g fat) increased endotoxins levels in all groups at 3 and 4 h in comparison to baseline, except for nonobese, whose increase was observed only after 1 h. The magnitude of increase in endotoxins levels in comparison to nonobese was significantly higher in the T2DM subjects at 3 and 4 h (78.2 and 125.4% respectively). In contrast, in IGT and obese groups the increase was higher at 3h (34.5% and 41.8%, respectively) than at 4 h (19 % and 22.2 %). Despite different levels of endotoxins in fasted state and also in the postprandial period, TNF levels were similar between groups and comparing 4h and baseline for each group.<sup>112</sup>

Taken together, these evidences may indicate that luminal interactions might interfere with LPS absorption (especially, chylomicrons formation). Factors that contribute to fasten LPS clearance (probably related to the type of fatty acids consumed) and/or influence expression of proteins (CD14, LBP) may modulate inflammatory activation. The types of nutrients consumed and the combination of different food types in a meal offers new challenges for the endotoxemia research field as we have already highlighted. The net amount of fat consumed, the fatty acid profile and its physico-chemical properties<sup>100</sup> should be considered in light of the metabolic status, digestive and absorptive capacity of subjects, and protein secretion response differences. The paradigm “higher LPS, higher inflammation” should be put into question considering all the influent factors interacting.

### **8. Microbiota, intestinal permeability, endotoxins and high fat diet inter-relationship**

It is currently accepted that microbiota may contribute to different disorders inside and outside the gut.<sup>113</sup> In particular, Bäckhed and colleagues<sup>114-115</sup> suggested that the presence of microbiota regulates adipogenesis and metabolic traits. The reduction of 5´adenosine monophosphate-activated protein kinase phosphorylation, which decreases fat oxidation in the liver and muscle, and the inhibition of fasting-induced adipose factor (FIAF) expression, which is an inhibitor of lipoprotein lipase, would favor fat storage and higher adiposity in conventionalized mice.<sup>115</sup> Higher levels of microbial metabolites, such as short-chain fatty-acids (SCFA),<sup>116-118</sup> could favor energy harvest<sup>119</sup> and the transactivation of lipogenic and glucose metabolism factors (carbohydrate-responsive element binding protein and sterol

regulatory element binding protein) in the liver, regulating metabolic traits.<sup>114</sup> Important to mention that Fleissner and co-workers<sup>120</sup> showed that the absence of microbiota did not protect against obesity. The fatty acid profile of diet (more than the net macronutrient amount) was determinant in the extension of the protection against weight gain in germ-free.<sup>120</sup> In addition, there are evidences that the different germ-free species respond differently to the absent microbiota. While in C57Bl/6J mice the absence of microbiota reduced adiposity (attributed to increased intestinal FIAF), in the F344 rat model, adiposity was preserved (despite increased intestinal FIAF).<sup>121</sup>

Many reviews<sup>89,122-134</sup> discuss about potential mechanisms of microbiota influence's on metabolism. The involvement of LPS is only another part of the iceberg behind microbiota influences on host metabolism. Gut dysbiosis has been associated with nutritional (especially HF) and genetic (*ob/ob*) obesity. The dysbiosis would lead to increased intestinal permeability (IP) and consequent endotoxemia, triggering low-grade inflammation and IR in the liver, muscles and AT.<sup>128</sup> The cross-talk between gut microbiota and endocannabinoid system (eCB) in the intestines was proposed to regulate adipogenesis and endotoxemia. Modulation of gut microbiota with prebiotic promoted normalization of eCB responsiveness in both the gut and AT, associated with decreases in IP, endotoxemia and fat mass.<sup>20</sup>

Higher IP is regarded as a potential contributor to increased mucosal immune activity, and therefore to the development and/or progression of diseases. Luminal content, particularly microorganisms and their components (such as LPS), plays important roles in mucosal immune regulation. The activation of mucosal immune cells could lead to the release of pro-inflammatory cytokines (such as TNF and IFN- $\gamma$ ). If this is not counterbalanced by immunoregulatory responses, exacerbation of local inflammation and barrier loss may occur. Further leakage of luminal contents and immune deregulation would happen in consequence.<sup>135</sup>

The interaction between presence of microbiota and diet profile influences intestinal inflammation. Ding and co-workers<sup>136</sup> showed that a HF diet promoted significantly higher weight gain and adiposity than low fat diet in the presence of microbiota, while in the absence, these parameters did not differ between the diets.<sup>136</sup> High fat diet induced higher ileal TNF mRNA levels and NF- $\kappa$ B activation only in the presence of microbiota and was correlated with plasma insulin and glucose. The induced intestinal inflammation preceded diet-induced weight gain and adiposity.<sup>136</sup> Therefore, intestinal and metabolic homeostasis

may be disturbed by the interaction between microbiota and HF diet. However, the observation of biochemical parameters from germ-free and conventionalized mice with different diets (e.g., germ-free low fat vs. high fat and conventionalized mice low vs. high fat diet),<sup>115</sup> may lead to the conclusion that HF diet exerts a similar deleterious impact on metabolism of both mice (higher glucose, insulin, leptin, TG and cholesterol in comparison to low fat diet), independently of the presence of microbiota.

The view of metabolic endotoxemia as a causal factor for obesity and IR was provided by Cani and co-workers,<sup>75</sup> who also added HF diet in the inter-relationship between innate immune system, gut microbiota and obesity. High fat diet increased fecal and plasma LPS, that was denominated metabolic endotoxemia.<sup>75</sup> Because mice lacking CD14 receptor were protected against all the metabolic alterations observed for HF diet or LPS infusion, it was concluded that metabolic endotoxemia dysregulates the inflammatory tone and triggers body weight gain and diabetes.<sup>75</sup>

How much LPS really contributes directly to adiposity gain is questionable when HF diet is associated. To illustrate, mice under HF diet exhibited higher food intake and gained more weight and adiposity than chow diet group. Association of HF diet with cellulose or oligofructosaccharide reduced food intake and resulted in lower weight and fat depots in comparison to HF diet alone.<sup>137</sup> Mice supplemented with each fiber exhibited similar fat depots weight. However, oligofructosaccharide group showed lower inflammatory profile, coincident with lower endotoxin levels.<sup>137</sup> Chow and HF+ oligofructosaccharide groups showed similar endotoxin levels, while the last exhibited higher adiposity. Therefore, endotoxemia might not lead to obesity, but the HF diet does. In addition, adiposity itself might not promote inflammation, because mice receiving fibers showed similar amount and distribution of fat and different inflammatory and metabolic profile. Lower endotoxins were associated with lower cytokines and better insulin and glucose levels. High fat diet increased fecal LPS levels and reduced *Bifidobacterium* levels, while oligofructosaccharide improved *Bifidobacterium* levels and reduced LPS.<sup>137</sup> There is one report that *Bifidobacterium* were higher in the HF fed weaning C57BL/6 mice than control diet.<sup>138</sup> This led to the conclusion that “gut microbiota contribute towards the pathophysiological regulation of endotoxemia and set the tone of inflammation for occurrence of diabetes and/or obesity”.<sup>137</sup> However, reduction of *Lactobacillus* and *Bifidobacterium* by means of antibiotics (ampicillin and neomycin) improved endotoxins and IP in mice under HF diet. Similar changes in gut microbiota of control group did not exert any effect on endotoxin or IP.<sup>139</sup> The authors

suggested that gut bacteria were involved in the control of IP and furthermore in the occurrence of metabolic endotoxemia.<sup>139</sup> Noteworthy mentioning that one of the antibiotics used in this study, namely neomycin, has been reported to reduce excretion of secondary bile acids,<sup>140</sup> which could possibly affect LPS absorption. Similarly, in another study, antibiotics (norfloxacin and ampicillin) improved glucose tolerance without changes in insulin or adiponectin levels, body weight and body fat mass in obese mice, even though lactobacilli and bifidobacteria were targeted. Plasma LPS, cecal *E. coli* content and TNF in the intestinal mucosa were all reduced in the treated group. This has also led to the conclusion that improvement of glycemic control, despite similar adiposity, was a consequence of gut microbiota modulation.<sup>141</sup>

Later, with the use of HF diet and prebiotic, changes in gut microbiota (with emphasis on the increase in bifidobacteria) reduced IP and LPS, improved systemic and hepatic inflammation, modulated gut peptides (GLP-2) and adiposity. The conclusion was that gut microbiota was involved in HF-diet induced metabolic endotoxemia, adipose tissue inflammation and metabolic disorders through IP modulation.<sup>142</sup> Therefore, both decrease (by means of antibiotic) and increase (by means of prebiotic) of bifidobacteria were associated with decreases in LPS. Metabolic improvements can be due to a pleiotropic effect of the antibiotics, instead of gut microbiota modulation, or other bacterial groups, such as *E. coli*, might be more strongly associated with LPS reduction.

Important to mention that HF feeding to C57Bl/6 mice might result in different metabolic phenotypes as reported by Serino and co-workers:<sup>143</sup> obese diabetic, lean-diabetic resistant (HF-LDR) and lean-diabetic (HF-LD). They compared many features of HF-LDR and HF-LD. Different microbial signatures were found for each group. Diabetic mice showed higher plasminogen activator inhibition-1, IP and LPS concentration, but similar IL-6 and TNF concentration. In addition, the diabetic animals had higher subcutaneous and visceral fat mass, adipocytes size, stromal vascular cells number (including macrophages and lymphocytes), leptin, resistin and increased phosphorylation of NF- $\kappa$ B in visceral adipose tissue than HF-LDR.<sup>143</sup> As different time points were not evaluated, it is difficult to conclude that higher LPS is the main causative factor for the occurrence of diabetes since adiposity differed. Even so, as discussed earlier, it may be possible that LPS participates in the regulation of adipose tissue expansion.

Another study was conducted dividing rats into diet-induced obesity-prone (DIO-P) and obesity-resistant (DIO-R) according to the highest and lowest weight gain after HF diet.<sup>84</sup> DIO-P showed higher caloric intake and adiposity index than DIO-R and low-fat diet controls (LF), the last two showing similar adiposity. The ileal mucosa from DIO-P rats had higher myeloperoxidase activity, TLR4 activation and IP, while duodenal mucosa showed lower AP activity. This may explain also the higher plasma LPS concentration. There was an increase in Bacteroidales and Clostridiales with HF feeding, while Enterobacteriales were more abundant only in DIO-P animals.<sup>84</sup> The induced changes in microbiota of HF fed rats did not cause obesity in all rats, since DIO-R rats maintained similar body weight, food intake, and adiposity as those under LF diet, despite differences in gut microbiota.<sup>84</sup> This study also raises the possibility that LPS may be a differential factor that influenced adiposity gain in obesity-prone rats.

The endogenous intestinal AP is somehow involved in controlling LPS levels since the knockout mice suffered from endotoxemia. These animals also had overexpression of proinflammatory cytokines (TNF and IL-1 $\beta$ ), increased IP, glucose intolerance, hyperinsulinemia and also more adipose tissue than wild-type, including more intra-abdominal fat.<sup>144</sup> Oral supplementation of AP to knockout and wild-type mice prevented endotoxemia, increase in IP and glucose intolerance induced by HF diet. Supplemented animals had lower levels of total liver lipids and TG and higher HDL levels. The adiposity index decreased in the group supplemented in comparison to HF alone, despite similar food intake.<sup>144</sup> When the supplementation started after HF feeding had induced metabolic alterations, AP supplementation improved glucose intolerance, post glucose hyperinsulinemia, and serum TNF, IL-1 $\beta$ , despite no changes in body weight. This improvement was concomitant with reduction of endotoxin content in caecum.<sup>144</sup> It is possible that intestinal AP detoxify LPS within the intestinal lumen, preventing its effects.

Under no influence of HF diet, Brun and co-workers<sup>145</sup> also showed a relationship between IP and endotoxins. Although no microbiota assessment was undertaken, inflammatory status was proportional to the endotoxin levels. The alteration of IP could be a marker of genetic obesity *ob/ob* and *db/db*, since under chow diet they showed higher IP than wild-type mice. The *ob/ob* mice express a truncated inactive form of leptin, whereas *db/db* mice express a signaling-incompetent long isoform of leptin receptors. These molecular differences can be associated with the extent of IP alteration: *db/db* presented higher IP (and also LPS) than

*ob/ob*.<sup>145</sup> It is possible that leptin signals are involved in regulation of IP and that HF diet and/or microbiota may influence the hormonal secretion.

Previous reviews<sup>99-101,135</sup> and original studies suggest the possible routes of penetration of LPS into the circulation: through chylomicrons<sup>110</sup> or paracellular infiltration due to increased intestinal permeability.<sup>145,146</sup> In obese humans, despite gut microbiota differences were reported in comparison to lean, no IP alteration was detected.<sup>147</sup> On the other hand, our group found higher urine lactulose excretion (possibly indicating higher IP)<sup>148</sup> and difference in fecal microbiota composition, but similar LPS levels.<sup>149</sup> Insulin and HOMA-IR were inversely correlated with fecal *Bifidobacterium* and *Clostridium coccooides* levels.<sup>149</sup> In fact, various studies have reported differences in microbiota composition between lean and obese/diabetic subjects or animals,<sup>116,117,150-153</sup> suggesting that differential microbial signatures may predispose to metabolic risk factors. However, as reviewed by Lyra and co-workers<sup>154</sup> there is no consistencies in these microbial changes between studies.

There is a complex relationship between gut microbiota, LPS, high fat diet, obesity and IP. It is not clear whether increasing or decreasing bacterial groups considered beneficial such as *Lactobacillus* and *Bifidobacterium* will lead to reduction of LPS levels and beneficial metabolic effects. The HF diet directly impact in modulation of IP and LPS translocation. The fact that HF feeding may induce different metabolic phenotypes should be more explored in terms of genetic differences, adipose tissue morphology and other hormonal traits in humans.

### **9. Bile acids: the missing point**

We showed how complex, and sometimes contradictory, is the interpretation of the evidences presented so far. Under the Nutrition Science view, more than anything, the HF diet is a metabolic-mess inducer. As it is directly associated with biliary system, we sought to find the associations of this system with microbiota, IP and LPS.

Bile acids (BA) are amphipathic molecules synthesized in hepatocytes and actively secreted by the liver into bile and discharged into intestinal lumen upon ingestion of a meal. Besides the traditional role in facilitating lipid absorption, BA are also known to activate multiple nuclear receptors, G protein coupled receptor TGR5 and cell signaling pathways (including c-Jun N-terminal kinase 1/2, protein kinase B, ERK1/2) in the liver and gastrointestinal tract.<sup>155</sup> Particularly, the farnesoid X receptor (FXR) is considered a BA sensor expressed primarily in entero-hepatic tissues and immune cells such as macrophages.<sup>156</sup>



As discussed earlier, gut microbiota differs between obese/diabetic and lean. These differences may somehow impact on BA metabolism or the contrary may be also true. From the side of microbes, the ability of pathogens and commensals to tolerate bile is likely important for their survival and colonization.<sup>157</sup> Gram-negative bacteria, the LPS providers, are inherently more resistant to bile than gram-positive. The loss of the O-antigen creates a “rough” colony phenotype, which is less resistant. Thus, LPS *per se* and its structural composition play a major role in bacterial resistance to bile and survival.<sup>157</sup> Bacterial species that express bile salts hydrolases, enzymes that hydrolyze/ deconjugate bile salts, may have additional advantage to surveillance. These enzymes may represent a detoxification mechanism increasing bile tolerance and survival in gastrointestinal tract.<sup>157</sup> Microbes are also able to modify BA profile, producing secondary and tertiary forms, through a broad range of reactions, such as deconjugation, dehydroxylation, oxidation and sulfation.<sup>30,132,157</sup> The changes in BA composition may affect host’s physiology. From the host side, bile, BA and FXR expression contribute to suppression of significant bacterial colonization of the small intestine.<sup>30-31</sup> Obstruction of bile flow and lower biliary secretion are known to allow intestinal bacterial overgrowth. In contrast, administration of conjugated BA stimulated bile secretion, reduced bacterial counts and plasma endotoxins in cirrhotic animals.<sup>158</sup> Thus, the equilibrium in the interaction between microbiota and BA is important to the host.

The dysbiosis has been suggested to alter the IP and consequently increase LPS levels and inflammation. Suzuki and Hara<sup>28</sup> showed that fat intake increased BA secretion and IP in both genetically lean and obese mice, suggesting a role of biliary system in IP modulation. Further, Caco-2 cells exposed to bile juice also showed increased IP.<sup>28</sup> In this study, it was not possible to distinguish if any specific BA or a specific factor in the bile exerted modulation of IP and unfortunately there was no LPS and microbiota assessment.

Bile composition seems to be an important factor for intestinal homeostasis. This composition was changed through intravenous administration of LPS to rats, markedly increasing TNF concentration in bile. The external drainage of bile flux after LPS injection protected gastrointestinal mucosa, while infusion of TNF into duodenal lumen caused intestinal damage similar to the intravenous administration of LPS without external drainage.<sup>159</sup> On the other hand, LPS or TNF administered to animals decreased mRNA levels of BA transporters and reduced taurocholate transport in liver cells. The impairment of BA transport attributable to endotoxin and cytokine effects at the sinusoidal and

canalicular membrane domains may account for sepsis-associated cholestasis<sup>160</sup> and also NAFLD.<sup>161</sup>

Bile composition in regard to BA profile also seems to be an influent factor to host responses. There is documented evidence of dependence between type of BA and AP secretion in rat bile. Tauroursodeoxycholate caused a 3-fold, taurocholate a 14-fold, and taurochenodeoxycholate a 75-fold increase in enzyme secretion,<sup>162</sup> while bile duct ligation caused a threefold elevation of hepatic and intestinal AP.<sup>163</sup> As AP is capable of inactivating LPS, the composition of BA in bile may influence the effects induced by LPS. Another study showed that despite a general increase in BA levels induced by HF feeding, ursodeoxycholic acid was decreased and inversely correlated with IP. This diet also increased FXR expression, as well as TNF- $\alpha$  and IP, along the intestine.<sup>164</sup>

Not only microbiota and HF diet affect BA profile. Fat, starch and cellulose were shown to differently influence BA concentration. Higher fat consumption increased deoxycholic and total BA. In contrast, higher cellulose decreased deoxycholic acid,  $\beta$ -muricholic acid and total BA. Starch did not change de composition, but was able to bind BA, affecting the level of free BA. The level of free BA was lower in feces of animals fed high starch-diet.<sup>29</sup> Flavonoids may also interfere in BA metabolism, and subsequently influence endotoxemia. Flavonoids can bind to BA and sterols in the intestine, reducing their re-absorption. This in turn, influences lipid metabolism in liver. In rats, reduction of serum and tissue TG and cholesterol were observed after flavonoids administration, despite stimulated cholesterogenesis. The cholesterol synthesized endogenously might be eliminated as fecal sterols and BA, as higher levels of BA in the liver and feces were noted.<sup>165</sup> The study from Ghanim and colleagues<sup>25,105</sup> provides interesting results to discuss in light of the raised important role of BA in the obesity-gut microbiota-LPS scenario. They used two food components that are known to interfere in BA metabolism (fruits and fiber). Fruits and orange juice are rich sources of flavonoids, which may have blunted postprandial increase in LPS even in a HF meal.<sup>105,125</sup>

Similarly to what have been discussed about the form of lipids in inducing endotoxemia, emulsification of dietary lipids and the formation of micelles, lipid digestion and absorption of fatty acids can be impaired depending on the ratio between conjugated and unconjugated BA. Unconjugated BAs are less efficient to provide the above mentioned properties. In addition, their binding to transport sites for enterohepatic recirculation occurs with lower

affinity. Increased loss of bile salts may arise, and metabolic pathways may be activated to increase cholesterol synthesis, which may in turn lower serum cholesterol levels.<sup>157</sup>

The profile of BA in bile may also influence immune response as shown by an *in vitro* study. Bile acids differentially inhibited TNF production by monocytes: deoxycholic acid > chenodeoxycholic acid > ursodeoxycholic acid (ineffective in the concentrations tested).<sup>166-167</sup> The ability of BA to influence cytokines release by immune cells indicates a role for BA in modulating inflammation. FXR deficient mice show deregulated immune response. In macrophages, FXR expression exerts anti-inflammatory and immuno-regulatory activities. However, in the presence of IFN- $\gamma$  there is a STAT1-dependent repression of FXR mRNA and protein expression. This indicates that FXR is negatively regulated during inflammation.<sup>156</sup>

Another illustration of the possible role of BA on inflammation modulation is that FXR influences expression of the small heterodimer partner (SHP), an atypical orphan member of the nuclear receptor superfamily.<sup>30</sup> A recent report suggested a role for SHP as an intrinsic endogenous regulator of homeostasis of the innate immune system. SHP was shown to inhibit TLR-dependent inflammatory response by regulating adaptor MyD88-dependent and MyD88-independent pathways. Deficiency or knockout of SHP increases the expression of inflammatory cytokines (TNF, IL-1 $\beta$ , IL-6) and cyclooxygenase-2, while overexpression resulted in significantly less LPS-induced effects. SHP negatively regulates NF- $\kappa$ B signaling by physically interacting with p65, inhibiting its nuclear translocation. In addition, SHP regulates the activities of a variety of transcription factors involved, for example, in lipid and glucose homeostasis.<sup>168</sup> The effects mediated by the activation of TLR2 and TLR4 by bacterial components such as LPS are possibly modulated by FXR ligands.

Surprisingly, we could find association of BA with some of the mechanisms presented so far, indicating that they may be an important player in the complex network involving obesity, microbiota, LPS and metabolic abnormalities. FXR is already viewed as a promising target for development of compounds that can be used for those with metabolic syndrome.<sup>169-171</sup> Transcriptional responses are induced by ligand dependent FXR activation in a coordinated way to regulate bile acid, cholesterol, TG, glucose metabolism, energy expenditure and also to protect the intestinal mucosa from bacterial overgrowth and inflammatory insults (box 2).<sup>30,155,172</sup> Bile acids are not exclusively ligand for FXR, which explains the broad range of effects that they may induce.<sup>173-174</sup> In addition BA may also

interact with eCB, since eCB system is markedly up-regulated in the liver of patients with primary biliary cirrhosis.<sup>175</sup> This may also explain the broad impact that BA exert on physiological processes, since eCB system is involved in regulation of nociception, food intake, intestinal motility, lipogenesis and inflammation.<sup>176</sup>

Bile acid sequestrants are pharmacologic molecules that bind to BA in the intestine resulting in the interruption of BA homeostasis, and are considered possible candidates for lipid and glucose control.<sup>177</sup> If they affect LPS concentration is an interesting area for future research. The metabolic pathways regulated by FXR, in general, become altered within the course of obesity development. The higher frequency of disturbances in the biliary system (e.x. gallstone disease) in obese and diabetic subjects<sup>178,179</sup> highlights the possibility that BAs are a missing point for obesity and diabetes studies.

### **10. Final considerations**

In the last few years, microbiota was included in the IR scenario. Somehow, the HF diet/meal would affect gut microbiota composition and the dysbiotic state would increase the LPS amount and translocation (through increased IP and chylomicrons) contributing to obesity, chronic inflammatory status, insulin resistance and T2DM.

As BA function as metabolic regulatory molecules during the feed/fast cycle, and especially HF diet increases bile flux, it is reasonable to hypothesize that studying bile acid kinetics and regulated molecular targets during endotoxemia will add exciting evidences of the role of LPS (or BA) on metabolic abnormalities. FXR is an interesting molecular target linking gut, microbiota, HF diet, endotoxins, BA and metabolic abnormalities. Numerous genes in the liver, intestine and AT are induced by BA via a functional FXR element in their promoters. The knowledge about the interaction between bacteria and bile may help to develop drugs or probiotics that more efficiently changes metabolic syndrome traits.

Of note, in livestock, suppression of growth, particularly lean tissue accretion, is observed due to intestinal-derived endotoxin and inflammation. Suppression of appetite, activation of the immune system and partitioning of energy and nutrients away from growth toward supporting immune system requirements are some of the mechanisms that might explain lower production performance of agricultural animals under intestinal transport of endotoxin and the subsequent inflammation.<sup>180</sup> Why in humans LPS would lead to weight gain and adiposity? Why some obese do not develop IR and other metabolic abnormalities, while others do? Are their microbiota and LPS levels different? Or their fat distribution is

detrimental? Could their dietary pattern, BA profile and/or genetic background be more protective? If the terminology benign and malign obesity is really applicable, the differences in LPS, BA levels and profile, IP and microbiota between them should be further investigated.

For now, there are more questions than answers. Above all, the intervention in diet composition is obligatory as a treatment option in obesity and metabolic abnormalities. The diet also directly influences bile composition. Hence, both diet and gut microbiota may interact and alter bile acid pool composition. In turn, this could have an impact on physiological regulations in different organs that express FXR receptors such as immune cells, liver, gastrointestinal tract cells and adipose tissue. Once more, the exploration of the different metabolic phenotypes (insulin resistant, insulin resistant+hyperinsulinemic and hyperinsulinemic subjects) is of importance. The differences in LPS levels in basal and postprandial states should be explored between them, controlling for the level and distribution of adiposity in future studies. We suggest that BAs metabolism and composition should be included in the big picture microbiota-LPS as a driving force of metabolic abnormalities.

**Box 1 – Possible metabolic abnormalities profile depending on insulin sensitivity and secretion**

<b>Insulin states</b>	<b>Pure insulin resistant</b>	<b>Pure Hyperinsulinemia</b>	<b>Insulin-resistant and hyperinsulinemic</b>
<b>Definition<sup>a</sup></b>	M in the bottom quartile and FPI in the lower three quartiles	FPI in the top quartile but M in higher three quartiles	M in the bottom quartile and FPI in the top quartile
<b>Characteristics</b>	Central fat distribution Excessive lipolysis (↑ NEFA) ↑ serum TG ↑ EGP	Larger fat mass percent (peripheral distribution) Suppressed lipolysis (normal NEFA) Suppressed EGP and insulin clearance ↑ SBP and serum TG ↓ serum HDL	Fasting NEFA and rates of glucose production ‘normal’, even though ↑ EGP and lipolysis

M: insulin-mediated glucose disposal rate; FPI: fasting plasma insulin; EGP: endogenous glucose production; SBP: systolic blood pressure; TG: triglycerides

<sup>a</sup>Quartiles defined on the distribution values of lean subjects

Adapted from Ferrannini and co-workers<sup>50,51</sup>

**Table 1 – Fasting levels of endotoxins in human individuals**

Reference	Sample	BMI (kg/m <sup>2</sup> )	Endotoxin (EU/mL)*	Observations†
Creely <i>et al.</i> <sup>70</sup>	25 NDC	29.5±4.3	3.1 (1.7) <sup>a</sup>	Similar levels of insulin, leptin, IL-6. ↑Glucose, TNF-α and sCD14
	25 T2DM	31.8 ±4.5	5.5 (1.6) <sup>b</sup>	
Basu <i>et al.</i> <sup>19‡</sup>	55 lean	22.0±2.0 <sup>a</sup>	0.5±0.2 <sup>a</sup>	Similar TNF-α and sCD14 ↑ insulin, leptin and IL 6
	65 obese	38.4±6.0 <sup>b</sup>	1.0±0.5 <sup>b</sup>	
Harte <i>et al.</i> <sup>181</sup>	23 controls	26.4±4.5 <sup>a</sup>	3.9 (3.2-5.2) <sup>a</sup>	↑ insulin
	63 NAFLD	34.0±6.0 <sup>b</sup>	10.6 (7.8-14.8) <sup>b</sup>	↑ Glucose and sCD14
	92 NASH	35±6.0 <sup>c</sup>	10.9 (7.8-13.9) <sup>b</sup>	NASH ↓TNF-α in NAFLD
Lassenius <i>et al.</i> <sup>182</sup>	219 lean	22.2±1.7 <sup>a</sup>	60(44-80)	↑ insulin, glucose, ↓ HDL
	126 overweight	28.2±2.8 <sup>b</sup>	62(49-82)	
Pussinen <i>et al.</i> <sup>183</sup>	6,170 NDC	26.7 (4.1) <sup>a</sup>	61.06 (36.11) <sup>a</sup>	↑ glucose, TG, ↓ HDL
	462 incident diabetes	31.6(5.2) <sup>b</sup>	77.03 (42.03) <sup>b</sup>	
Harte <i>et al.</i> <sup>112</sup>	9 lean	24.9 ± 3.2 <sup>a</sup>	3.3 ± 0.15 <sup>a</sup>	Similar leptin, TG, HDL and TNF-α ↑ glucose in T2D
	15 obese	33.3 ± 2.5 <sup>b</sup>	5.1 ± 0.94 <sup>a</sup>	
	12 IGT	32.0 ± 4.5 <sup>b</sup>	5.7 ± 0.1 <sup>b</sup>	
	18 T2DM	30.3 ± 4.5 <sup>c</sup>	5.3 ± 0.54 <sup>b</sup>	

NDC: non-diabetic control; T2DM: type 2 diabetes mellitus; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; IGT: impaired glucose tolerance; TG: triglycerides

<sup>a,b</sup>Different letters represent statistical significance

\*Endotoxin levels expressed as mean±standard deviation or in parentheses as geometrical mean or interquartile range.

†Higher (↑) and lower (↓) in ‘diseased’ conditions in comparison to controls.

‡Pregnant lean and obese women classified according to pregravid BMI

**Table 2 –Human studies testing the effects of meals containing different fat contents and sources on the increase of endotoxins, triacylglycerols and inflammatory markers in the circulation.**

Ref	Sample	Fat (g)	Meal	Duration	LPS peak	LPS return to basal	TG	Inflammatory markers
24	12 H M BMI 23 kg/m <sup>2</sup>	50	Tea, toast and butter	240 min	30 min	50 min	↑ at 120 min, peak at 240 min	Changes NO
106	12 H M BMI 24.9 kg/m <sup>2</sup>	33	Enteral emulsion, margarine, butter, olive oil, bread, jam, banana (882 kcal)	240 min	60 min	120 min	↑ at 120 min, peak at 240 min	↑ IL-6 (120 min) ↑sCD14 (at 60 min, peak at 240 min)
25	5 H M BMI 23.1 kg/m <sup>2</sup>	51	Egg muffin, sausage muffin, hash browns (910 kcal)	180 min	180 min	NO	↑ at 60 min, peak at 180 min	↑ LBP (120 min) ↑ROS (120 min, peak at 180 min) ↑TBARS (60 min, peak at 180 min) ↑ NAPH-oxidase (60 min) ↑ NFkB (120 min) No change in TNF-α or CRP
25	6 H M BMI 23.1 kg/m <sup>2</sup>	15	Oatmeal, milk, orange juice, raisins, peanut butter, English muffin	180 min	Changes NO	NA	↑ at 120 min, peak at 180 min	Changes NO
105	10 H M+W BMI 20-25 kg/m <sup>2</sup>	51	Egg muffin, sausage muffin, hash browns (900 kcal) + water	300 min	300 min	NO	NA	↑ ROS by MNC (60 min onwards) ↑ NAPH-oxidase (60 min onwards) ↑TLR2/4 mRNA in



105	10 H M+W BMI 20- 25 kg/m <sup>2</sup>	51	Egg muffin, sausage muffin, hash browns (900 kcal) + 75g glucose solution (300 kcal)	300 min	180 min	Started to decrease at 300 min	NA	MNC (peak at 180 min) ↑ROS by MNC (60 min onwards) ↑ NAPH-oxidase (60 min onwards) ↑TLR2/4 mRNA in MNC (peak at 60 min)
105	10 H M+W BMI 20- 25 kg/m <sup>2</sup>	51	Egg muffin, sausage muffin, hash browns (900 kcal) + orange juice (300 kcal)	300 min	Changes NO	NA	NA	↑ROS by MNC (60 min onwards) No changes in NAPH-oxidase or TLR2/4 mRNA

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LPS, lipopolysaccharides; TG: triglycerides; H, healthy; M, men; W, women; NO, not observed; LBP, LPS binding protein ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; NF-κB, Nuclear factor kappa beta; CRP, c-reactive protein; NA, not applicable; MNC: mononuclear cells; TLR 2/4, toll-like receptors 2 and 4;

**Box 2- Evidences of FXR and bile acids role in lipoprotein metabolism, glucose, insulin sensitivity and energy expenditure**

LDL- cholesterol metabolism	<p>CYP7A1 is an enzyme that converts cholesterol into BA. Its induction ↑ LDL-receptor expression and activity, ↓ plasma LDL.</p> <p>Deficiency of CYP7A1 is associated with a resistant hypercholesterolemia phenotype. FXR receptor modulates CYP7A1 activity. CDCA induces LDL-receptor and FXR activation, ↓ plasma LDL.</p> <p>FXR controls intestinal absorption of cholesterol. FXR <i>-/-</i> is associated with ↑ cholesterol absorption.</p>
HDL- cholesterol	<p>FXR <i>-/-</i> mice show ↑ HDL levels due to a reduced, selective uptake of cholesteryl esters by the liver. FXR ↑ the expression of the phospholipid transfer protein and ↓ the expression of hepatic lipase, suggesting a role of FXR in HDL remodeling.</p> <p>BA sequestrants ↑ HDL concentration while CDCA administration results in opposite effect.</p>
Triglycerides	<p>Bile acids sequestrants ↑ plasma TG and VLDL.</p> <p>CA ↓ hepatic TG accumulation and VLDL secretion in mouse model of hypertriglyceridemia.</p> <p>FXR activation by BAs or synthetic agonists ↓ the expression of the transcription factor SREBP-1c and its lipogenic targets genes in mouse primary hepatocytes. FXR also controls genes governing TG clearance. FXR activation ↑ apoC-II expression (activator of LPL activity) and decreases apoC-III and ANGPTL3 (both LPL inhibitors).</p>
Glucose	<p>FXR activation ↑ phosphoenolpyruvate carboxykinase (PEPCK) expression, a rate controlling enzyme of gluconeogenesis. CA-enriched diet ↓ PEPCK in wild-type mice but not in FXR<i>-/-</i> and SHP<i>-/-</i>. FXR may ↓ gluconeogenic enzyme expression via induction of SHP.</p> <p>BA sequestrants ↓ glucose levels and improved glycemic control, possibly through induction of GLP-1 secretion.</p>
Insulin sensitivity	<p>Physiological concentration of insulin directly ↓ BA synthesis.</p> <p>FXR deficiency leads to impaired glucose tolerance and insulin resistance in mice, which could be associated with ectopic lipid deposition in insulin target genes.</p>
Energy expenditure	<p>SHP, a direct FXR target gene, appears to be a negative regulator of thermogenesis in brown adipose tissue by inhibiting PGC-1 expression. SHP <i>-/-</i> mice show ↑ energy expenditure and resistance to diet-induced obesity. FXR expression ↑ during adipocytes differentiation <i>in vitro</i>.</p>

CDCA: chenodeoxycholic acid; CA: cholic acid. Adapted from Cariou & Staels<sup>169</sup>; Staels *et al.*<sup>177</sup>

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### **3.3. Article 3 (review in Press) Intestinal permeability measurements: general aspects and possible pitfalls**

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#### **Abstract**

*Introduction:* Disturbances of the gut barrier function have been related to a variety of diseases, including intestinal and extra-intestinal diseases. The intestinal permeability tests are considered useful tools for evaluating disease severity and to follow-up patients after a therapeutic intervention and indirectly assess barrier function.

*Objective:* The aims of this review were to highlight the possible factors underlying higher intestinal permeability and the clinical conditions that have been associated with this in different age range; and also provide some insight into methodological aspects.

*Results and discussion:* Abnormal regulation of tight junction function is the main cause of altered intestinal barrier. The impaired barrier function results in higher permeation rates of administered probes through the intestinal mucosa. Lactulose and mannitol are one of the most commonly used probes. The innocuousness and easiness of intestinal permeability tests can be explored to expand the knowledge about the clinical situations in which intestinal barrier dysfunction can be an important feature. Many factors may influence the results of the test. Researchers and healthcare professionals should try to circumvent the possible pitfalls of the intestinal permeability tests to produce consistent evidences. The use of others markers of intestinal physiology may also contribute to understand the role of barrier function in different diseases.

**Key words:** intestinal permeability; gut barrier; lactulose; mannitol

## 1. Introduction

The gastrointestinal tract has the complex task of absorbing nutrients while excluding the uptake of dietary antigens, luminal microbes and their products. The intestinal mucosa exhibit a selectively permeable barrier property, which supports this task. The histological organization of the gastrointestinal tract mucosa and the interaction between cellular (polarized epithelial cell membrane, tight junctions (TJ), lymphocytes) and extracellular components (mucin, unstirred layer of fluid)<sup>1-4</sup> are essential for the gut barrier function. Homeostasis of gut barrier function is critical for the ability of gastrointestinal tract to articulate aggressive reactions against enteric microbes while developing oral tolerance for food antigens and commensal bacteria.<sup>5</sup>

Disturbances of the gut barrier function have been related to a variety of clinical conditions in different age range (Tables 1 and 2).<sup>2,6</sup> The investigation of gut barrier dysfunction and other intestinal abnormalities (such as polyps, tumors) can be done through methods such as collection of a biopsy sample using surgical and/or endoscopic procedures. However, these procedures are invasive, often inconvenient to the patient and usually imply high healthcare costs.<sup>7</sup> This has led to the development of alternative methods to assess gut barrier function while preventing patients from undergoing such kind of invasive methods.

Intestinal permeability (IP) tests represent one alternative method. The concept of intestinal epithelial barrier function is tightly related to the concept of permeability, which is the property of the membrane to allow non-mediated solute diffusion.<sup>8-9</sup> When the barrier is intact, the permeability of substances is highly selective and controlled. Disturbances in gut barrier function can affect the control of permeating substances.<sup>9-10</sup> Based on these principles the oral administration of specific probes has been commonly used to indirectly assess gut barrier dysfunction and measure IP. These probes are subsequently quantified in blood or more frequently in urine.<sup>11</sup> In a simplistic way, injuries in the intestinal mucosa can impair its barrier function. The impaired barrier function results in higher permeation rate of probes and intact proteins through the intestinal mucosa.<sup>12-13</sup>

Intestinal permeability tests are not widely used in clinical practice. Their use has been usually restricted for scientific purposes. However, evaluation of IP can be a useful tool in screening for small intestinal disease, in assessing the response in the follow-up period after a therapeutic intervention and in predicting the prognosis, especially in celiac disease.<sup>14-15</sup> The majority of probes used have been shown to be non-toxic to patients and relatively easy to quantify. These characteristics can be explored by medical professionals to expand the knowledge about the clinical situations in which intestinal barrier dysfunction can be an important feature.

In this context, the aims of this review were to highlight the possible factors underlying higher IP and the clinical conditions that have been associated with this in different age range; and also provide some insight into methodological aspects to be considered in future studies.

## **2. Methods**

Medline/Pubmed, Scielo and Lilacs were used to search for articles accomplishing the following terms (alone or associated): intestinal or gut permeability, intestinal or gut barrier, lactulose, mannitol, tight junctions. Review and original articles were selected and read critically.

## **3. Factors underlying increased intestinal permeability**

The intestinal epithelium is a single layer of columnar epithelial cells that separates the intestinal lumen from the underlying lamina propria. It is believed that there are two routes for substances permeation through the intestinal epithelial cells: transcellular (across the cells, both by active and passive processes), and paracellular (between adjacent cells, by a passive process).<sup>16-17</sup> The epithelial cells are tightly bound together by intercellular junctional complexes. They are formed by TJ, gap junctions, adherens junctions and desmosomes. The space between cells is called paracellular space. The permeability of molecules through this space is under control of the junctional complexes, which are crucial for the integrity of the epithelial barrier.<sup>17</sup>

Tight junctions are complex structures comprising over 50 types of proteins (claudin, occludin, zonulin, junctional adhesion molecules). They form a continuous, circumferential seal around cells through the interaction with the perijunctional actomyosin ring of epithelial cells.<sup>17</sup> It has been observed that TJ have a central role in processes that regulate epithelial proliferation and differentiation.<sup>18</sup>

Regulation of the assembly, disassembly and maintenance of TJ structure is influenced by various physiological and pathological stimuli. The knowledge of how TJ are modified in response to signals that alter their functional properties is of great importance in the context of diseases associated with altered IP.<sup>16,19-21</sup> Experimental studies using animal and cell culture models or human studies have shown that deregulated TJ are the main cause of altered intestinal barrier. This alteration can be induced by endogenous and exogenous factors (Table 3).

Recently, it has been demonstrated that increased IP can occur due to discontinuities in the epithelial cell layer in the gut. These discontinuities are called gaps and have been identified in the mouse and humans. They are formed when epithelial cells leave the epithelium. These gaps have the diameter of an epithelial cell and are devoid of cellular contents, but filled with an unknown substance that maintains local barrier function. The rate at which cells leave may have implications for the permeability of the epithelium as a unit. The processes that control the rate of cell egress have not been well defined. This mechanism of increased permeability may be important in human diseases.<sup>22-23</sup>

As summarized by Teshima and Meddings<sup>22</sup> “simply measuring an increase in permeability provides no information to the physician about the mechanisms underlying the abnormality. However, an understanding of these mechanisms may prove valuable in designing interventions”. Thus the main causes of increased IP that should guide the development of efficacious intervention are: genetic alterations of TJ proteins, abnormal microbiota, abnormal regulation of TJ function (increased zonulin release), mucosal inflammation and abnormal epithelial dynamics.<sup>22</sup>

#### **4. General aspects of intestinal permeability tests**

Intestinal permeability tests are based on probes of different molecular weight, which determines the route of permeation (Table 4). Smaller molecules usually permeate through membrane pores. They are expected to be present in urine in higher proportion (10 to 30% of an orally ingested dose).<sup>24</sup> Less than 1% of higher molecular weight molecules are expected to be recovered in urine after an oral dose.<sup>25</sup> These molecules need to cross the barrier through the paracellular route, which is more tightly regulated by protein complexes.

The choice of probes depends on the intention of what part of intestine is meant to be assessed. Usually, recovery of sucrose in the urine reflects gastroduodenal permeability<sup>26</sup>, since sucrose is rapidly hydrolyzed by sucrase-isomaltase upon entering the duodenum and reflects absorption only in the most proximal portion of the gut.<sup>27</sup> Lactulose and mannitol, which are one of the most commonly used probes, are destroyed in the caecum and provide information regarding the small intestinal epithelium.<sup>16</sup> Sucralose is an artificial sweetener with similar molecular weight of lactulose and is resistant to bacterial fermentation.<sup>28</sup> It spends most of a 24 hour exposure period in the large intestine.<sup>16</sup> Therefore, sucralose has been suggested as better suitable sugar for whole gut permeability assessment.<sup>29</sup>

An inconvenience of IP tests is the prolonged period of urine collection, usually 5 to 6 hours. The introduction of sucralose into permeability measurements might extend the test period up to 24 hours, making it less convenient in clinical practice. McOmber and co-workers recommend re-examining the usual 5 to 6 hours collection times to compare healthy individuals to those with abnormal permeability, because this period of time might not include the point of maximal urinary recovery. They studied the recovery of sucrose, lactulose, mannitol and sucralose over a 24 hours period in healthy adults and children.<sup>30</sup> It was suggested that by using different collection periods greater differences may be seen between groups with less inter-individual variation: 4 to 6 hours for sucrose, 13 to 15 hours for lactulose, mannitol and sucralose. If sucralose/lactulose ratio is to be measured, collection time might be extended to 16 to 18 hours.<sup>30</sup> However, Akram and co-workers<sup>31</sup> have compared different urine times collection and their results suggest that the use of Lactulose/Mannitol (L/M) ratio to assess IP could be simplified by shortening the time of urine collection.<sup>31</sup> The reduction of the time can also be achieved by measuring the probes in blood 60-90min post-ingestion of solution.<sup>32-33</sup> More studies are needed to confirm that prolonged time collection is not needed.

The calculation of the ratio between sugar probes used (such as L/M) is considered a good marker of small intestinal permeation.<sup>9</sup> It is meant to circumvent confounding factors as inter-individual variation of gastric emptying, intestinal transit and transport, blood distribution and renal clearance.<sup>34</sup>



In general, the integrity of intestinal barrier function is dependent on healthy epithelial cells and on the proper functioning of the paracellular route.<sup>9</sup> Theoretically, an increase in the sugar probes ratio – for example L/M ratio - would indicate altered IP. This alteration may reflect a decrease in smaller probes (e.g. mannitol) absorption and/or an increase in the absorption of higher weight probes (e.g. lactulose). Decreased small weight probes absorption can be the result of a diminished absorptive area. Increased permeation of higher weight probes may be due to a facilitated diffusion of this marker into the crypt region as a consequence of decreased villous height or TJ loosening.<sup>35</sup>

The results of IP tests are usually expressed as percentage of excretion of probes (Table 5). Other units can be also found (mg/mL, mmol/L, mg).<sup>11,31-32,36-37</sup>

### **5. Possible pitfalls in intestinal permeability tests**

Many factors may influence the results of the test, as shown in Table 3. Thus, possible pitfalls for the IP tests may be circumvented by researchers or healthcare professionals when considering some details.

Previous orientation of individuals to avoid - few days before the test - the use of non-steroidal inflammatory drug,<sup>38-39</sup> acute alcohol ingestion,<sup>32,40-41</sup> psychological and physical stressful situations<sup>42-44</sup> should be given as part of the protocol. Considering that some genetic background may exert negative influence on barrier function, family history of inflammatory bowel diseases should be considered before inclusion of patients in a study. Regarding the personal medical history some clinical factors influencing IP such as food allergy, human immunodeficiency virus, diabetes, starvation, iron deficiency, diarrhea, viral gastroenteritis, smoking<sup>45-48</sup> should be an exclusion criteria, except if this is the topic under investigation. Additionally, search for evidence of endoparasite infection in the stools should be ideally performed before inclusion of individuals in the study.<sup>49</sup>

Usually, all tests are performed under overnight fast (8 to 10 hours). Few authors mention the instruction of individuals to follow a diet free of the sugars used as probes in the test at least 24 hours before it.<sup>13,32,50</sup> Lactulose, mannitol and sucralose are commonly used in IP tests and can be present in some common foods (Table 6). An important issue mentioned in some protocols to circumvent the possible influence of the intake of the same sugars that will be used in the IP test is the collection of a urine sample before the administration of the sugar probes. The amount of sugar quantified in

this sample should be subtracted from the results in the urine collected after the ingestion of the probes.<sup>13, 28, 33, 50</sup> Avoidance of some foods should be also advised when they contain other sugars that can imply in methodological difficulties to properly quantify the probes. Farhadi and co-workers recommend subjects to avoid consumption of dairy products on the previous day of the test since lactose peak tend to overlap that of lactulose.<sup>51</sup> During the IP test, in some studies it is mentioned that subjects are encouraged to drink water and/or to have a snack after 1 to 2 hours of probes administration.<sup>11-13,37</sup> It is not clear if this can affect the results. However, an important detail of this practice is to standardize the type of food and the volume of liquid offered to all individuals. Mattioli and co-workers<sup>52</sup> found that the L/M ratio was significantly lower in subjects that excreted more than 500 mL of urine. The greater urine volume was associated with a higher mannitol recovery. Thus, they emphasized that urine volume may influence urinary excretion of sugar probes and intake of liquids should be carefully monitored before and during the test.<sup>52</sup>

It is noteworthy that Camilleri and co-workers question the concept that lactulose and mannitol in urine collected between 0 to 6 hours reflect small intestine permeability. They have investigated the administration of these probes (radiolabelled) in a liquid formulation or in a delayed-release methacrylate-coated capsule. It was showed that after 2h of liquid formulation intake around 50% of the probes was in the colon, suggesting that sugars may not be absorbed exclusively in the small intestine. Thus, they suggest that the interpretation of the 0 to 6 hours differential two sugar urine excretion as an exclusive marker of small IP should be done cautiously.<sup>24</sup>

Osmolarity of test solutions should be mentioned in every study, since stress induced by high osmolarity can stimulate intestinal motility<sup>53</sup> and change the rate of sugars permeation.<sup>8</sup> The amount of sugar administered and the volume of solutions vary between studies (see Tables 1 and 2). In addition, the volume of solution administered is fixed for all subjects. Exception is observed in some studies with children, that use body weight to calculate the volume of solution to be administered individually.<sup>50,54</sup> This might have been proposed based on pharmacokinetics studies. At least for children, drugs dosages are based on body weight or body surface area since body size, proportion, organ development and function affect the pharmacokinetic behavior of many drugs.<sup>55</sup> It should be further discussed the possibility of using weight to calculate the volume of solution to be administered also to adult subjects. The body weight or

body mass index (BMI) of subjects included in the majority of studies is not mentioned. Could this make any difference for the interpretation of IP results?

A higher BMI is associated with higher filtration fraction. This means that there is a higher glomerular filtration rate (GFR) relative to effective renal plasma flow, suggesting an altered afferent/efferent balance and higher glomerular pressure.<sup>56</sup> In obese subjects, the values for GFR exceeded by 61% the values for GFR of the control group and by 32% the value of renal plasma flow, suggestive of glomerular hyperfiltration. The obesity-related glomerular hyperfiltration ameliorates after weight loss.<sup>57</sup> It is a possible pitfall when subjects with excess of weight are included in studies: could a higher amount of excreted sugar be a consequence of higher intestinal absorption (due to higher IP) or of a higher glomerular hyperfiltration? This has not been investigated in humans. Whenever overweight and obese subjects are submitted to IP test it should be investigated if they present normal renal function (impaired renal function should be adopted as exclusion criteria).

Choosing the best method to assess renal function should consider population characteristics such as age and BMI. Serum creatinine levels, anthropometric and clinical characteristics of patients are often used to estimate GFR. Body weight is an imperfect reflection of creatinine generation because increased body weight is associated more commonly with an increase in body fat or body water, edematous disorders, rather than an increase in muscle mass.<sup>58-59</sup> Creatinine clearance is not recommended when obese subjects are involved, but would be advised to exclude individuals that present creatinine level higher than 250 mmol/l.<sup>14</sup> A decline in renal function (creatinine clearance) occurs with advancing aging. Interestingly, L/M ratio did not change with aging due to a parallel progressive decline in the ability to excrete both lactulose and mannitol with increasing age.<sup>60</sup>

The use of the ratio L/M may not detect differences in IP between groups if one considers the possibility that an individual may be absorbing and excreting proportionally higher quantities of both mannitol and lactulose. Although this is only a hypothesis, obese women showed higher lactulose excretion, a tendency to higher mannitol excretion, while L/M ratio was not significantly different from lean women.<sup>61</sup> It is critical to assess the L/M ratio, as well as lactulose and mannitol recoveries separately, when interpreting test results.<sup>62</sup> Ferraris & Vinnakota<sup>63</sup> showed in animal

model that genetic obesity is associated with increased intestinal growth, which augments absorption of all types of nutrients. Obese men with chronic hyperglycemia showed evidence of increased small intestinal enterocyte mass (higher plasma citrulline) and increased enterocyte loss (higher plasma intestinal fatty acid binding proteins, I-FABP), but IP was not assessed.<sup>64</sup> Circulating levels of insulin which is a hormone usually increased in obese subjects<sup>65</sup>, may also influence IP. The addition of insulin in a cell culture showed that the insulin-induced decline in transcellular resistance is receptor-mediated and that receptors are localized in the basolateral membrane. Increased mannitol flux was an observed effect paralleled to this altered paracellular permeability.<sup>66</sup>

Barrier dysfunction may not be expressed all the time in particular conditions. It can range from mild to severe dysfunction (manifesting continuously) or intermittent dysfunction (manifesting only when the intestine is challenged). This susceptibility to barrier dysfunction can be detected using a ‘challenge’ test, as established by Hilsden and co-workers using aspirin.<sup>67</sup> Accordingly, subjects are given 1300 mg of aspirin (four 325 mg tablets) the night before the test and again on the morning of ingestion of the probe mixture. The use of the aspirin challenge showed that patients with non-alcoholic steatohepatitis do not have abnormal IP all the time, but they could easily develop gut leakiness when they are exposed to intestinal barrier stressors such as aspirin.<sup>68</sup>

Of note is the discussion presented recently by Vojdani<sup>69</sup> in his review entitled “For assessment of intestinal permeability, size matters”. Mannitol and lactulose are considered small molecules. Their use for IP assessment will not necessarily indicate structural damage in the TJ barrier, which would in turn allow penetration of large molecules. The use of probes of higher size (polysugars of 12 000- to -15 000 Da) may be more suitable to extrapolate if IP is higher enough to allow macromolecules such as bacterial toxins (such as lipopolysaccharides) and food antigens to permeate. Small inert markers may not mimic large molecules because of the size selectivity of TJ.<sup>69</sup>

## **6. Additional markers to indicate alteration in barrier function**

There are other markers that could be associated to IP tests to improve the interpretation of dysfunctions of gut barrier. D-lactate is produced from carbohydrate fermentation by abnormal microbiota or when the number of bacteria elevates rapidly (bacterial

overgrowth and short bowel syndrome).<sup>70-72</sup> Plasma D-lactate had the lowest false-negative rate among C-reactive protein level and leukocyte counts to diagnose appendicitis, and acute inflammatory disorder.<sup>73</sup>

Circulating citrulline is an amino acid produced from glutamine by differentiated small intestinal enterocytes. Citrulline is a non-protein amino acid that seems to exert an important role in preserving gut barrier function and reducing bacterial translocation.<sup>74</sup> The circulating levels are dependent only on *de novo* synthesis from intestinal metabolic activity. It reflects the functional enterocyte mass and can be used as a biological tool to quantitatively investigate epithelial integrity and follow intestinal adaptation (i.e., post-surgical) at the enterocyte level. Loss of small bowel epithelial cell mass results in declined circulating levels of citrulline, such as for short bowel syndrome, chronic villous atrophy and chemotherapy.<sup>75</sup> Another situation in which the citrulline availability is decreased was shown to be during the course of induced endotoxemia in rats.<sup>76</sup> There some studies using animal models that show an association between endotoxemia and increased IP.<sup>77-79</sup> As citrulline is metabolized into arginine by kidney cells, the interpretation of its levels in patients with compromised renal function should not be reliable.<sup>80</sup>

The quantification of claudin-3 in the urine showed that its rapid appearance in this fluid correlated with immunohistochemically visualized loss of claudin-3, which is a major sealing TJ protein. Measurement of urinary claudin-3 can be used as noninvasive marker for intestinal TJ loss.<sup>81</sup>

The assessment of urinary concentration of endogenous cytosolic enterocyte proteins such as I-FABP and liver FABP (L-FABP) are potentially useful in reflecting enterocyte damage. Pelters and co-workers investigated the distribution of these proteins in segments of human intestine.<sup>82</sup> They showed similar pattern of tissue distribution along the duodenal to colonal axis, being the jejunum the segment with highest content. In each intestinal segment it is observed a more than 40-fold higher content of L-FABP than I-FABP. Elevated plasma levels of both proteins were found in patients with intestinal diseases.<sup>82</sup> Since FABP are small, water-soluble cytosolic proteins, the loss of enterocyte membrane integrity will lead to release of these proteins into the circulation.<sup>71, 83</sup> FABP are expressed in cells on the upper part of the villi. Thus, destruction of these cells can lead to increased release of these proteins to the

circulation. Results from a pilot study with celiac patients showed that circulating levels of FABP are significantly elevated in untreated patients with biopsy proven celiac disease compared with healthy controls.<sup>84</sup>

Local inflammation is associated with increased IP. An increased migration of granulocytes into the intestinal mucosa, usually due to conditions of inflammation, might result in the degranulation of their secondary granules, resulting in an increase in their proteins in feces.<sup>85</sup> Neutrophil derived proteins such as calprotectin, lactoferrin<sup>85-88</sup> and elastase<sup>89</sup> can be present in stool and also in plasma as a marker of inflammation.<sup>90</sup>

Finally, zonulin is a protein that exhibits the ability to reversibly modulate intercellular TJ similar to the toxin from *Vibrio cholera* known as zonula occluden toxin.<sup>91-92</sup> Proteomic analyses characterized zonulin as pre-haptoglobin-2 (pre-HP2), a multifunctional protein that contains growth factor-like repeats. In its single-chain form, zonulin has the molecular conformation required to induce TJ disassembly by indirect transactivation via proteinase-activated receptor-2.<sup>92</sup> Higher levels of zonulin are associated with disorders such as celiac disease and type 1 diabetes, and positive correlation between zonulin and IP has been demonstrated.<sup>92-93</sup>

## **7. Conclusion**

There are many clinical situations in which increased IP seems to be present. If this alteration is contributing to worsen the clinical condition of affected subjects is still a question without answer for different diseases. This field of research should be better explored. However, the possible pitfalls should be taken into account. It is important to consider the different factors that may influence IP tests result and there are open questions regarding renal function and body size that should be further tested. This could help to produce more consistent evidences. The use of larger probes may be more appropriate to affirm that macromolecules such as food antigens and bacterial derived-compounds are crossing the barrier. Besides the use of IP tests, the association with the mentioned markers would be also interesting to investigate the role of barrier function in different diseases.

**Table 1. Intestinal permeability markers for healthy and diseased infants, children and adolescents**

Ref	Sample	Volume, sugar and osmolarity	% Excretion (mean ± SD or median (range))		
		Urine collection (hours) and method			
34	6 term (fed human milk) 21 preterm infants (4 fed human milk and 17 fed formula milk)	300mg Lac and 60 mg MA dissolved in liquid diet or water 5h and GC vs HPLC	L/M: Term human milk: 0.18 ± 0.19 Pre term human milk: 0.20 ± 0.16 Pre term formula: 0.32 ± 0.31		
94	12 CMPSE (6m-2y) 28 AD (6m-15y) 39 H	10% MA and 65% Lac 0.1g/kg BW for each sugar; 1,001 mosm/L 5h and GC	<b><u>Control</u></b> Lac: 0.37 ± 0.18 MA: 15.6 ± 5.98 L/M: 2.45 ± 1.01	<b><u>CMPSE</u></b> Lac: 0.39 ± 0.14 MA: 15.07 ± 5.67 L/M: 2.88 ± 1.5	<b><u>AD</u></b> Lac: 0.52 ± 0.51 <sup>†</sup> MA: 15.5 ± 8.9 L/M: 3.6 ± 3.31 <sup>†</sup>
95	77 underweight (44M and 33F, mean 13.1m) 17 H (11M and 6 F; mean 13.2m)	400mg Lac and 100 mg MA/3ml Dose 3 ml/kg BW 5h and enzymatic	<b><u>Control</u></b> Lac: 0.44 (0.34-0.71) MA: 5 (3.87-8.71) L/M: 0.09 (0.05-0.12)	<b><u>Underweight</u></b> Lac: 0.55 (0.35-0.88) MA: 3.89 (2.14-5.69) <sup>†</sup> L/M: 0.15 (0.09-0.26) <sup>†</sup>	
50	28 H (12M and 16F; mean 9y) 28 GSE (10M and 18F; mean 10y)	0.55 mL/kg 18.2g LAC/100 mL and 18.2g MA/100 mL 1500 mosmol/L 5h and GC	<b><u>Control</u></b> Lac: 0.28 ± 0.04% MA: 15.61 ± 5.8% L/M: 0.022 ± 0.007 (all <0.035)	<b><u>GSE</u></b> Lac: 0.73 ± 0.5% <sup>†</sup> MA: 8.72 ± 3.5% <sup>†</sup> L/M: 0.084 ± 0.054 <sup>†</sup> (all > 0.035)	
96	49 infected (helminthiasis) (mean 7.2y) 95 H (mean 7.2y)	2 mL/kg 5g/100mL Lac and 2g/100mL MA	<b><u>Control</u></b> L/M: 0.031 ± 0.023	<b><u>Infected</u></b> L/M: 0.042 ± 0.018 <sup>†</sup>	

		5h and enzymatic		
37	30 H (13M and 17F; mean 7.4 y) 10 ileocolitis Crohn's (mean 14.7y) 10 Celiac (mean 5.8y) with severe or active phase	50-100 mL 5 or 10g Lac and 2 or 5g MA (younger than 12y had the lower dose) 6h and HPLC	<b><u>Control</u></b> Lac: 0.33 ± 0.13% MA: 14.1 ± 6.6 % L/M: 0.024 ± 0.006	<b><u>Crohn's</u></b> Lac: 2.25 ± 2.1% <sup>†</sup> MA: 11.91 ± 7.95% L/M: 0.2 ± 0.08
54	15 H (no diarrhea episode in last 2 wk) 15 Diarrhea (3 or more liquid stools in the last 24h) Both groups age < 5y of both genders	2 mL/kg 200 mg/mL Lac and 50 mg/mL MA 5h and HPLC	<b><u>Control</u></b> Lac 0.1183 ± 0.0855 % L/M ratio: 0.0394 ± 0.0235	<b><u>Diarrhea</u></b> Lac: 0.3029 ± 0.2846% <sup>†</sup> . L/M ratio: 0.1404 ± 0.1206 <sup>†</sup>
97	52 H (13M and 39F; 8.2y) 93 FAB/IBS (28M and 65F; 8.5y) Participants 7-10y	125 mL 5g/dL Lac; 1g/dL MA; 10g/dL S; 1g/dL SU + 240 mL water 3h and HPLC	<b><u>Control:</u></b> Lac: 0.09 ± 0.06 MA: 7.6 ± 4.7 S:0.02 ± 0.03 SU:0.42 ± 0.32 L/M: 0.07 ± 0.03 S/L: 0.36 ± 0.26 SU/L 0.81 ± 0.43	<b><u>FAB/IBS</u></b> L: 0.10 ± 0.08 MA: 7.6 ± 5.5 S: 0.02 ± 0.03 SU: 0.44 ± 0.42 L/M: 0.06 ± 0.03 S/L: 0.59 ± 0.50 <sup>†</sup> SU/L: 1.01 ± 0.67 <sup>‡</sup>

M: men; F: female; H: healthy (control); AD: atopic dermatitis; BW: body weight; CMPSE: cow's milk-sensitive enteropathy, FAB/IBS: functional abdominal pain and irritable bowel syndrome; GC: gas chromatography; HPLC: high-performance liquid chromatography; Lac: Lactulose; LGSE: gluten sensitive enteropathy; L/M: lactulose/mannitol ratio; MA: mannitol; S: sucrose; SU: sucralose; S/L: sucrose/lactulose ratio; SU/L: sucralose/lactulose ratio. <sup>†</sup> p<0.05 compared to the control, <sup>‡</sup> p =0.05 compared to the control.



**Table 2. Intestinal permeability markers for healthy and diseased adults**

Ref	Sample	Volume, sugar and osmolarity Urine or blood* collection (hours) and method	% Excretion (mean ± SD or median (range))		
33	10 H (7M and 3F)	300mL; 10g Lac and 5g MA	<b><u>Control</u></b>	<b><u>Normal biopsy:</u></b>	<b><u>Abnormal biopsy:</u></b>
	28 investigation for GSE	696 mmol/kg	Lac: 0.15 ± 0.09	Lac: 0.27 ± 0.13	Lac: 0.65 ± 0.26
	(16F and 12M)	5h and HPLC	MA: 11.8 ± 6.2 L/M: 0.02 ± 0.014	MA: 12.6 ± 4.6 L/M: 0.021 ± 0.013	MA: 9.0 ± 3.4 L/M: 0.146 ± 0.10 <sup>†</sup>
98	41 H (10M and 31 F; mean 29y)	200mL; 5g Lac and 2g MA 5h and HPAEC-PAD	<b><u>Control</u></b>	<b><u>FH</u></b>	<b><u>FA</u></b>
	20 FH (4M and 16F; mean 29y)		L/M: 1.85 ± 0.81	L/M: 5.34 ± 4.26 <sup>†</sup>	L/M: 6.17 ± 6.07 <sup>†</sup>
	21 FA (6M and 15F; mean 29y)				
99	30 mild pancreatitis	50 mL; 10g Lac and 5g MA	<b><u>Control</u></b>	<b><u>Pancreatitis</u></b>	<b><u>Pancreatitis</u></b>
	15 severe pancreatitis	5h and enzymatic	L/M:0.016 ± 0.014	Mild	Severe
	26 H			L/M: 0.029 ± 0.027 <sup>†</sup>	L/M: 0.20 ± 0.18 <sup>†</sup>
35	12H (6M and 6F)	65 mL; 10g Lac and 0.5g MA	<b><u>Control</u></b>	<b><u>Depleted</u></b>	<b><u>Non-depleted</u></b>
	26 for PN (13 depleted and 10 non-depleted)	5g X 6h and GLC	Lac: 0.5 ± 0.1 MA: 19..2 ± 2.6 X: 29.9 ± 1.8	Lac: 2 ± 0.5 <sup>†</sup> MA: 12.9 ± 3.5 <sup>†</sup> X: 20.6 ± 3.4 <sup>†</sup>	Lac: 0.9 ± 0.3 <sup>†</sup> MA: 11.5 ± 1.6 <sup>†</sup> X: 18.1 ± 4.2 <sup>†</sup>
100	15 F (27-60y) Before and after pelvic	100 mL; 18.2g Lac and 18.2g MA 1500 mosml/l; 0.55 ml/kg BW	<b><u>Before</u></b>	<b><u>After</u></b>	
			Lac:0.4 ± 0.3	Lac:0.7 ± 0.6 <sup>†</sup>	

	external radiation	5h and GC	MA:14.5 ± 4.8 L/M: 0.03 ± 0.019	MA:11.8 ± 4.4 L/M: 0.064 ± 0.062 <sup>†</sup>	
101	46 type I diabetic (28 M and 18F; mean 15.8y)	150 mL; 5g Lac and 2g MA 375 mOsm/L	<b><u>Control</u></b> Lac:0.26 (0.07-1.14) MA: 18.8 (5.0-47.5) L/M: 0.014 (0.004-0.027)	<b><u>Diabetic</u></b> Lac: 0.55 (0.03-5.52) <sup>†</sup> MA: 17.3 (0.85-86.9) L/M: 0.038 (0.005-0.176) <sup>†</sup>	
102	23 H (11 M and 12 F; mean 27.9y)	5h and HPAEC-PAD			
	36 type I diabetic 56 relatives of diabetic 43 H	150 mL; 5g Lac and 2g MA 5h and HPAEC-PAD	<b><u>Control</u></b> Lac:0.48 ± 0.12 MA: 23.2 ± 3.36 L/M: 0.017 ± 0.0018	<b><u>Diabetic</u></b> Lac: 0.79 ± 0.11 <sup>†</sup> MA: 21.2 ± 2.22 L/M: 0.037 ± 0.003 <sup>†</sup>	<b><u>Relatives</u></b> Lac: 0.63 ± 0.14 <sup>†</sup> MA: 24.7 ± 3.2 L/M: 0.025 ± 0.01 <sup>†</sup>
103	22 H (11M and 11F; 62y) 22 CHF (18M and 4F; 67y)	100 mL water; 5g SU; 10g Lac; 5g MA and 20g S 5h and HPLC	<b><u>Control</u></b> L/M:0.017 ± 0.001 SU: 0.20 ± 0.06 X: 37.4 ± 1.4	<b><u>CHF</u></b> L/M: 0.023 ± 0.001 <sup>†</sup> SU: 0.62 ± 0.17 <sup>†</sup> X: 26.7 ± 3.0 <sup>†</sup>	
104	57 H (mean 40y) 40 FM (8M and 32 F; 48y) 17 CRPS (4M and 13 F; 43y)	100 mL; 20g S; 10g Lac and 5g MA 5h and HPLC	<b><u>Control</u></b> S: 0.19 ± 0.075 L/M: 0.0155 ± 0.006	<b><u>FM</u></b> S: 0.22 ± 0.2 <sup>†</sup> L/M: 0.025 ± 0.012 <sup>†</sup>	<b><u>CRPS</u></b> S: 0.29 ± 0.27 <sup>†</sup> L/M: 0.026 ± 0.020 <sup>†</sup>
105	20 H (control I) 10 nonalcoholic (control II) 10 alcoholic NLD 10 alcoholic LD 10 nonalcoholic LD	150 mL; 7.5g Lac; 2g MA and 40g S 5h and GC	<b><u>Control I</u></b> Lac:0.17 (0.03-0.49) MA: 16 (3-72) S: 0.03 (0.005-0.09)	<b><u>Alcoholic NLD</u></b> Lac: 0.17 (0.05-0.55) MA: 12 (7-27) S: 0.11 (0.02-0.4)	<b><u>Non-alcoholic LD</u></b> Lac: 0.17 (0.05-0.8) MA: 13 (2-34) S:0.05 (0.01-0.15)
			<b><u>Control II</u></b> Lac: 0.08 (0.02-0.02) MA:4 (0.6-14) S: 0.02 (0.006-0.05) <sup>†</sup>	<b><u>Alcoholic LD</u></b> Lac:3.8 (0.03-10) <sup>†</sup> MA: 5 (2-9.5) S: 1 (0.04-2.1) <sup>†</sup>	

68	12 H (4M and 8F) 6 steatosis (3M and 3F) 10 NASH (6M and 4F)	1g SU; 7.5g Lac; 40g S and 2g MA 5h and CG	<b><u>Control</u></b> Lac: 0.07 ± 0.05 MA: 10.7 ± 9.1 L/M: 0.007 ± 0.003 SU: 2.49 ± 1.34	<b><u>Steatosis</u></b> Lac: 0.23 ± 0.15 MA: 15.0 ± 4.9 L/M: 0.015 ± 0.008 SU: 3.07 ± 0.87	<b><u>NASH</u></b> Lac: 0.14 ± 0.12 MA: 18.5 ± 12.1 L/M: 0.020 ± 0.035 SU: 2.79 ± 1.55
106	134 H (40 M and 94 F) 43 chronic hepatitis 40 cirrhosis	150 mL 5g Lac and 2g MA 5h and HPAEC	<b><u>Control</u></b> L/M: 0.016 ± 0.014	<b><u>CLD</u></b> Hepatitis: L/M: 0.037 ± 0.04 <sup>†</sup> Cirrhotics: L/M 0.056 ± 0.08 <sup>†</sup>	
107	11 H (7M and 4 F) 32 cirrhosis + SAI (26 M and 8F)	100 mL 10g Lac and 5g MA 6h and HPLC	<b><u>Control</u></b> Lac:0.001 ± 0.0001 MA: 0.0838 ± 0.007 L/M: 0.0209 ± 0.0009	<b><u>Cirrhosis</u></b> Lac:0.007 ± 0.0004 <sup>†</sup> MA: 0.074 ± 0.004 L/M: 0.1003 ± 0.003 <sup>†</sup>	
108	54 diarrhea-IBS 22 H	100 mL 5g Lac and 2g MA; 24h	<b><u>Control</u></b> All had L/M < 0.07	<b><u>IBS</u></b> 39% had L/M ≥ 0.07	
32	6 (3M, 3F) H 6 (2M, 4F) Celiac	50 mL 10g Lac and 2.5g MA 1070 mOsm 30, 60, 90, 120* and HPLC	<b><u>Control</u></b> Lac (1h): 0.125 (0.11-0.15) MA (1h): 0.156 (0.15- 0.19) L/M: 0.039 (0.028-0.043)	<b><u>Celiac</u></b> Lac (1h): 0.56 (0.29-0.94) <sup>†</sup> MA (1h): 0.06 (0.018-0.9) <sup>†</sup> L/M: 0.42 (0.15-8.3) <sup>†</sup>	
109	30 H (13M,17F, mean 37y) 18 Dermatitis herpetiformis (9M, 9 F, mean 38y) 30 Celiac (12M, 18F, mean 36y)	450 mL 5g Lac and 2g MA 5h and HPLC	<b><u>Control</u></b> L/M: 0.017 ± 0.0007	<b><u>Celiac</u></b> L/M:0.073 ± 0.017 <sup>†</sup>	<b><u>Dermatitis</u></b> L/M: 0.082 ± 0.013 <sup>†</sup>
110	11H 22 Celiac (11M and 11F;	120mL 6g Lac and 3g MA	<b><u>Control</u></b> Lac: 2.75 ± 1.71	<b><u>Celiac AGA+</u></b> Lac: 10.27 ± 3.37 <sup>†</sup>	<b><u>Celiac AGA -</u></b> Lac: 3.79 ± 1.46 <sup>†</sup>

	mean 41y) (1y after a gluten free diet)	6h and HPLC	MA: 22.56 ± 3.32 L/M: 0.12 ± 0.07	MA: 10.18 ± 3.82 <sup>†</sup> L/M: 1.02 ± 0.46 <sup>†</sup>	MA: 11.12 ± 5.64 <sup>†</sup> L/M: 0.39 ± 0.11 <sup>†</sup>
21	15 H (8M,7F; mean 36y)	120mL	<b><u>Control</u></b>	<b><u>Celiac</u></b>	<b><u>Crohn</u></b>
	22 Celiac > 1y GD (11M and 11F; mean 41y)	6g Lac and 3g MA 6h and HPLC	Lac: 0.07 (0.05-0.28) MA: 21 (18.3-28) L/M: 0.003 (0.002-0.013)	Lac: 0.15 (0.04-0.85) <sup>†</sup> MA: 10.9 (3.3-19.5) <sup>†</sup> L/M: 0.013 (0.005-0.07) <sup>†</sup>	Lac: 0.42 (0.15-0.99) <sup>†</sup> MA: 21 (13.5-29.5) L/M: 0.021 (0.07-0.046) <sup>†</sup>
	31 Crohn (18M and 20F; mean 37y)				
111	64 H (31 M and 33F; mean 40y)	50 mL 10g Lac and 5g MA	<b><u>Controls</u></b> Lac: 0.313 (0.047-1.240)	<b><u>Crohn</u></b> Lac: 0.418 (0.03-1.5) <sup>†</sup>	<b><u>First degree relatives</u></b> Lac: 0.27 (0.012-3.56) <sup>‡</sup>
	23 Crohn's disease (13 M and 10F; 43y) and 28 H first degree relatives of Crohn's patients (14M and 14F; 62y)	1300 mOsm/L 6h and enzymatic	MA: 26.83 (16.9-50)	MA: 8.27 (4.1-36) <sup>†</sup>	MA: 9.54 (3.2-28) <sup>‡</sup>
112	22H	100mL	<b><u>Control</u></b>	<b><u>Crohn:</u></b>	
	125 Crohn (66M and 59 F; median 36y)	5g Lac; 2g MA and 5g X 6h and enzymatic	Lac: 0.293 (0.0089-0.665) MA: 14.2 (4.95-30.8) L/M: 0.0164 (0.0018-0.0548) X: 1.89 (0.8-4.73)	Lac: 0.326 (0.0204-2.76) <sup>†</sup> MA: 12.5 (1.43-43.75) L/M: 0.027 (0.0029-0.279) <sup>†</sup> X: 1.45 (0.32-4.5) <sup>†</sup>	
61	20 H F	120mL	<b><u>Control</u></b>	<b><u>Obese</u></b>	
	20 OB F	6.25g Lac and 3g MA 5h, GC	Lac: 0.247 ± 0.087 MA: 17.32 ± 7.31 L/M: 0.0144 ± 0.006	Lac: 0.418 ± 0.267 <sup>†</sup> MA: 21.86 ± 7.77 L/M: 0.018 ± 0.008	

M: men; F: female; H: healthy (control); Lac: Lactulose; MA: mannitol; L/M: lactulose/mannitol ratio; S: sucrose; SU: sucralose; X: xylose; S/M: sucrose/mannitol ratio; BW: body weight; GC: gas chromatography; HPLC: high-performance liquid chromatography; HPAEC-PAD: High-performance

anion exchange chromatography coupled with pulsed amperometric detection; CCGC: capillary column gas chromatography; PGC: packed column gas chromatography; AGA: anti-gliadin antibody; CRPS: complex regional pain syndrome; CHF: Chronic heart failure; FA: food-allergy IgE-mediated; FH: food hypersensitivity non-IgE mediated; FM: fibromyalgia; GSE: gluten sensitive enteropathy; IBS: Irritable Bowel Syndrome; LD: with liver disease; NASH: nonalcoholic steatohepatitis; NLD: with no liver disease; OB: obese; PN: parenteral nutrition; SAI: spontaneous ascitic fluid infection. †p<0.05 disease vs healthy; ‡p<0.025 controls vs relatives.

**Table 3. Factors that influence tight junctions assembly**

<b>Endogenous or exogenous factors</b>	<b>Evidences from human, animal or cell culture models</b>
<b>Genetic susceptibility</b>	10-25% of first-degree relatives of inflammatory bowel disease patients have increased IP in the absence of clinical symptoms. <sup>45-47</sup> Divergent study can be found. <sup>111</sup>
<b>Gender</b>	Oestrogen receptors are expressed in intestinal epithelial cells. Oestradiol regulates epithelium formation, occludin and junctional adhesion molecule expression. <sup>113</sup> Female rats are more resistant to intestinal injury induced by hypoxia and/or acidosis. The administration of estradiol or blockade of the testosterone receptor in male rats mitigates the gender differences found for histomorphological changes. <sup>114</sup> It was found differences in the recovery of sugar probes with aging just in females. <sup>30</sup>
<b>Cytokines (TNF and interferon-<math>\gamma</math>)</b>	Inflammatory cytokines disrupt TJ structure through inductions of changes on lipid composition and fatty acyl substitutions of phospholipids in membrane microdomains of TJ. <sup>115</sup> They also modulate myosin II regulatory light chain (MLC) phosphorylation through MLC kinase upregulation <sup>116</sup> , which is involved in barrier function. TNF caused occludin depletion in Caco-2 intestinal epithelial monolayers through a progressive decrease in occludin mRNA level. <sup>117</sup>
<b>Recruitment of immune cells</b>	Th2 cell responses contribute to gastrointestinal inflammation and dysfunction. Intestinal mastocytosis predispose to increased IP and food allergy. <sup>118</sup>
<b>Microbial-host interaction</b>	Small intestinal bacterial overgrowth has been detected in diseases related to altered IP. <sup>119</sup> Probiotic bacteria can reduce IP <sup>120</sup> : they increase TJ resistance and reduce cellular permeability <sup>121-122</sup> through influence on cytoskeleton organization <sup>123</sup> and cytokine production. <sup>124</sup>
<b>Alcohol consumption</b>	Acetaldehyde accumulation and induction of nitric oxide production contributes to increased tyrosine phosphorylation of TJ and adherens junction proteins and damaged microtubules cytoskeleton, which in turn increase IP. <sup>40</sup>
<b>Non-steroidal anti-inflammatory drugs</b>	Exert detergent properties on phospholipids membrane causing direct damage on epithelial surface; uncoupling of mitochondrial oxidative phosphorylation reduce ATP availability, which is necessary for actin-miosin ATP-dependent complexes of

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intercellular junctions.<sup>38</sup>

<b>Enteric pathogens</b>	<i>Clostridium difficile</i> , enteropathogenic <i>Escherichia coli</i> ; <i>Bacteroides fragilis</i> , <i>Clostridium perfringens</i> , <i>Vibrio cholera</i> may activate inflammatory cascade in epithelial cells; directly modify TJ proteins and perijunctional actomyosin ring; induce fluid and electrolyte secretion. <sup>49, 125</sup>
<b>Nutrients</b>	<p><i>Retinoic acid</i>: Metabolic depletion of retinoic acid in cells, alters expression of genes related to TJ modulation.<sup>126</sup></p> <p><i>Zinc</i>: Supplementation reduces lactulose excretion.<sup>127-128</sup> Activation of the zinc finger transcription factor (Hepatocyte nuclear factor-4<math>\alpha</math>) is essential for enterocyte differentiation and regulation of TJ proteins.<sup>129</sup></p> <p><i>Polyunsaturated fatty acids</i> (particularly <math>\omega</math>-3): Stimulate intestinal cells differentiation and maturation, improves TJ formation through their proteins redistribution and reduction of TNF-<math>\alpha</math> effect.<sup>130-131</sup></p> <p><i>Vitamin D</i>: Critical for preserving junctional complexes integrity and renew epithelial ability.<sup>132</sup></p> <p><i>Magnesium</i>: its deficiency has been shown to reduce cecal content of bifidobacteria and to lower expression of TJ proteins (occludin and zonulin).<sup>133</sup></p> <p>Modify and redistribute TJ transmembrane protein occludin and the plaque protein zonula occludens-1<sup>134</sup> and alter epithelial cell turn-over.<sup>135</sup></p>
<b>Stress</b>	
<b>High fat diet</b>	It reduces TJ protein expression in the small intestine. <sup>136</sup> It may alter the bile acid metabolism, which in turn would increase IP. <sup>137</sup>
<b>Polyamines</b>	Spermine may loosen the TJ of the epithelium increasing the intestinal absorption of drugs via a paracellular route. <sup>138</sup>

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TNF: Tumor necrose factor; IP: intestinal permeability; TJ: tight junctions.

**Table 4. Frequently used probes for assessment of intestinal permeability**

<b>Lower molecular weight (Molecular weight &lt; 200 Da)</b>	<b>Higher molecular weight (Molecular weight &gt; 300 Da)</b>
D-mannitol	Lactulose
L-rhamnose	Lactose
L-arabinose	Sucrose
	Cellobiose
	Sucralose
	PEGs (polyethylene glycols)
	Raffinose
	<sup>51</sup> CrEDTA (51Cr-labelled ethylenediaminetetraacetic acid)
	<sup>99</sup> Tc-DTPA (99m Tc diethylenetriamine pentaacetate)
	Iohexol
	Other contrast media (iodixanol, etc.)

Source: Travis and Menzies<sup>48</sup>, Frias *et al*<sup>139</sup> and Andersen *et al*<sup>140</sup>

**Table 5. Calculation of percentage of sugar probes excretion (e.g.: lactulose and mannitol)**

<b>% Lactulose excretion</b>	<b>% Mannitol excretion</b>	<b>Lactulose/Mannitol ratio</b>
$Lactulose\ excreted\ (mg) =$ $mg/L\ lactulose \times L\ urine$	$Mannitol\ excreted\ (mg) =$ $mg/L\ mannitol \times L\ urine$	$L/M = \% \text{ of lactulose excretion} /$ $\% \text{ of mannitol excretion}$
$\% \text{ of lactulose excretion} =$ $(mg\ lactulose\ excreted /$ $mg\ lactulose\ consumed) \times 100$	$\% \text{ of mannitol excretion} =$ $(mg\ mannitol\ excreted /$ $mg\ mannitol\ consumed) \times 100$	



**Table 6. Possible dietary sources of the main sugar probes (lactulose, mannitol and sucralose)**

Lactulose (4-O-b-D-galactopyranosyl-D-fructose)	Mannitol	Sucralose
Prebiotic food additive (infant formulas and healthy foods). <sup>141</sup> Lactulose is not present as such in nature but it is produced from lactose during heat treatment, and may be naturally present in considerable amounts in heat-processed dairy (UHT milk, yogurt, soymilk). <sup>142</sup>	The most abundant polyol in nature. Some funghi, and brown seaweeds. Celery; Reduced-calorie sweetener. <sup>143</sup> Parsley, carrot, coconut, cauliflower, cabbage, pineapple, lettuce, watermelon, pumpkin, squash, cassava, manioc, pea, asparagus, olive, coffee. <sup>144</sup> Berries <sup>145</sup> , chewing gum.	Sweetener and diet/light products. <sup>146</sup>

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### **3.4. Article 4 (Original): Intestinal permeability, lipopolysaccharides and degree of insulin resistance in men: are they correlated?**

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#### **Abstract**

Animal models show association between higher intestinal permeability, higher plasma lipopolysaccharides (LPS) concentration, and insulin resistance. These associations are still not clear in humans. The aim of this study was to evaluate intestinal permeability and plasma LPS concentration as well as their association with the degree of insulin resistance in lean and obese men. Twenty-four lean and twenty-eight obese men participated in the study. Lactulose/mannitol test, fecal elastase and calprotectin were used to evaluate intestinal barrier. Homeostasis assessment model (HOMA) was used as a marker of insulin resistance. Plasma LPS concentration, insulin, glucose and creatinine were analyzed. Plasma LPS, as well as lactulose/mannitol ratio were not significantly different between lean and obese men ( $p>0.05$ ). Fecal elastase was higher in lean compared to obese men ( $p<0.05$ ). Subjects above lactulose/mannitol median showed higher BMI, waist, total body fat percentage and HOMA ( $p<0.05$ ), but similar plasma LPS concentration ( $p>0.05$ ) than those below the median. The group above plasma LPS median even though showed higher BMI, waist, HOMA, it was not significant. The frequency of obese subjects above the median of lactulose/mannitol ratio and plasma lipopolysaccharides was similar to the frequency of lean subjects ( $p>0.05$ ). There was a significant correlation between plasma lipopolysaccharides versus HOMA only in obese ( $p<0.05$ ). Our findings do not clearly confirm the association between higher intestinal permeability, plasma LPS and the degree of insulin resistance in obese men. But they suggest that this area still offers great opportunity of research.

**Key words:** intestinal permeability, obesity, lipopolysaccharides, insulin resistance



## 1. Introduction

Homeostasis of gut barrier is critical for health. The invasiveness of biopsy has led to the development of alternative methods to assess gut barrier. As disturbances in gut barrier can affect the control of permeating substances, oral administration of specific probes has been commonly used to measure intestinal permeability (IP), which indirectly assesses gut barrier dysfunction.<sup>1</sup> Lactulose (L) and mannitol (M) are probes frequently used. The ratio of the excreted probes in urine after an oral dose (L/M) is considered a marker of IP.<sup>1-4</sup> Markers of intestinal inflammation such as fecal elastase and calprotectin help to complement the evaluation of gut barrier dysfunction.<sup>5-6</sup>

An increased L/M ratio, i.e. increased IP, could be a consequence of mucosal inflammation, villous atrophy and intestinal tight junctions loosening. Multiple factors such as intestinal microbial dysbiosis, consumption of high fat and high fructose diets, and nutritional deficiencies could contribute to dysfunctions of IP.<sup>2</sup> A complex association between diet, gut microbiota, IP and metabolic endotoxemia (high levels of plasma lipopolysaccharides, LPS) has been proposed as a mechanistic explanation for the chronic inflammatory activation and insulin resistance often associated with obesity.<sup>7</sup>

Studies using animal models demonstrate that obesity is a condition associated with increased IP, either genetic (*ob/ob* or *db/db*)<sup>8-10</sup> or high fat diet-induced obesity.<sup>9,11</sup> This in turn could justify higher plasma LPS concentrations.<sup>8-10</sup> In particular, there is increasing interest to investigate IP in obese subjects due to insufficient number of studies within this topic. The few studies that evaluated IP in overweight/obese subjects do not clearly confirm the findings from animal models.<sup>12-13</sup>

Therefore, we aimed to evaluate gut barrier and plasma LPS concentration as well as their association with the degree of insulin resistance in lean and obese men.

## 2. Methods

### 2.1. Study design and Subjects

Men were recruited through written announcements and social networks. The inclusion criteria were: lean (body mass index, BMI >18.5 and < 25 kg/m<sup>2</sup>) or obese (BMI ≥30 and < 35 kg/m<sup>2</sup>) men, older than 18 and under 50 years of age, absence of chronic disease other than obesity, not smoking, not taking any medication, not under a weight loss diet and weight stable for the last 3 months (less than 3 kg change). This was a cross-sectional study, including the participation of 24 lean and 28 obese men.

Subjects interested in participate were instructed to fill a 3-day food record in the week preceding the scheduled evaluation. In addition, they received a standardized dinner (one instant noodles pack and 200 mL of grape juice) to consume in the night before the scheduled evaluation. After fasting for 10 h, they attended the laboratory for data collection under standardized environment and protocols.

All subjects provided informed consent and all procedures involving human subjects were approved by the Ethical Committee in Human Studies from Universidade Federal de Viçosa (protocol n° 196/2012/CEP/07-12-E4).

## *2.2. Anthropometry and body composition*

Body weight was measured under fasting conditions with subjects wearing underwear (200 kg capacity, TANITA, model TBF-300 A, Tanita Corporation of America Inc, Illinois, USA). Height was measured with a fixed stadiometer (Seca®, Germany) to the nearest millimeter. Waist circumference was measured with a flexible tape in the lowest circle between the lowest rib and umbilicus. Total body fat was determined by tetra polar bioimpedance system (BodySystems®, Washington, USA).

## *2.3. Biochemical parameters*

Blood was collected in the antecubital vein using EDTA and serum tubes. After 20 min at 2-8°C, the blood was centrifuged at 2,200 x g for 15 min at 4°C (Heraeus Megafuge 11R centrifuge, Thermo Scientific) to separate plasma and serum, which were stored at -80°C. Fasting glucose and plasma creatinine were analyzed through enzymatic colorimetric method in auto analyzer (COBAS MIRA Plus; Roche Diagnostic Systems) following the instructions of commercial kits manufacturers (Bioclin/Quibasa, Brazil). Serum fasting insulin was determined by electrochemiluminescence immunoassay (Elecsys-Modular Analytics E170, Roche Diagnostic Systems®). Homeostasis model assessment (HOMA) indices were used as a marker of the degree of insulin resistance and were calculated as follows: fasting glucose (mmol/L) x fasting insulin (mU/L)/22.5.<sup>14</sup> Plasma creatinine was used to estimate creatinine clearance (CrC) through the formula proposed by Saracino et al<sup>15</sup> as follows: [(140-age (years)) x weight (kg)/72 x plasma creatinine (mg/dL)] x [1.25 – 0.012 x BMI].

Plasma LPS concentrations were analyzed through the chromogenic Limulus Amebocyte Lysate assay (HIT302, Hycult Biotech, The Netherlands). Plasma samples were heated at 75°C for 5 min to inactivate inhibitors and were not diluted. The absorbance of pure samples and standards (*E. coli* O111:B4) was measured at 405 nm (Multiskan Go, Thermo Scientific, USA) before

adding the reagents. The following steps were in accordance to manufacturer's instructions. Final absorbance was subtracted from initial absorbance. A standard curve was constructed by plotting the log<sub>10</sub> concentration of standards (standard concentrations: 0, 0.04, 0.1, 0.26, 0.64, 1.6, 4 and 10 EU/mL) and their absorbance. The concentration of LPS was estimated by the equation generated. The concentration of LPS was expressed as endotoxin units *per* milliliter (EU/mL).

#### 2.4. Intestinal permeability

Subjects were also instructed not to consume alcohol, anti-inflammatory drugs and a list of foods containing mannitol, and lactulose, during the three days prior to the assessments.

Subjects received 200 mL of an isosmolar solution (238.1 mOsm/kg) containing 7.6 g of lactulose (obtained from 11.5 mL of Colonac® syrup) and 2.04 g of mannitol (99% P.A, Synth). After 2 h of solution administration, subjects were allowed to eat. All subjects received 600 mL of water (3 x 200 mL) in predetermined timepoints. The urine eliminated in the following 6 hours was collected. The final volume of urine was measured. Thimerosal (12 mg) was added to a 50 mL aliquot of urine to prevent bacterial growth and subsequently stored at -20°C.

The sugar probes were quantified in urine using high-performance liquid chromatography (Shimadzu® system, model SPD-10A VP) with refractive index detector - RID 6A. Urine samples were centrifuged (10,000 rpm, 10 min, 4 ° C) and two milliliters were filtered through a micropore membrane (0.22 µl, Millipore, Brazil). Mobile phase was composed of 5mM sulfuric acid in water, flow rate of 0.8 ml/min, 45 kgf of pressure into the column BIORAD (30 cm x 7.9 mm), which was heated to 80°C. Under these conditions, 20 µl filtered urine was injected. Standard curves were used to determine the concentration of sugar probes in urine samples. The net amount of sugar probes excreted was calculated multiplying the determined concentration of each sugar probe in the urine by the total volume of urine collected over 6 hours. Then, the dose of sugar probes administered was used to calculate the percentage of lactulose (%L) and mannitol (%M) doses that were excreted in the urine. These results were used to calculate the Lactulose/Mannitol ratio (L/M).

#### 2.5. Fecal inflammatory markers

Subjects were instructed to bring fecal samples (on the day or maximum 1 week after the attendance day) as fresh as possible otherwise they should keep collected feces under refrigeration for maximum 12h. Fresh feces were homogenized and aliquots were stored in microtubes at -80°C for posterior analyses.

About 100 mg of feces were resuspended with 1 mL of PBS buffer (pH 7.09) and homogenized for 30s. Then, samples were centrifuged 10,000 x g for 20 min at 4°C (Refrigerated microcentrifuge, HERMLE Z 216 MK; Hermle Labortechnik) and the supernatant transferred to a new tube. This supernatant was used to perform the procedures described in human elastase ELISA kit (HK319-02, Hycult Biotech, The Netherlands).

One milliliter of a buffer prepared from 0.1M Tris, 0.15M NaCl, 1M urea, 10 mM CaCl<sub>2</sub>, 0.1M citric acid monohydrate, 5g/L of bovine serum albumin and 0.25 mM thimerosal was added to 100 mg of feces. After 20 min under agitation, samples were centrifuged (10,000 x g, for 20 min at 4°C). The supernatant obtained was used to quantify calprotectin (Human calprotectin ELISA kit, HK325-02, Hycult Biotech, The Netherlands).

After specific sample preparation steps, all the steps were performed according to manufacturer's instructions. Standards and samples absorbance were measured at 450 nm (Multiskan Go, Thermo Scientific, USA). Elastase (0.8, 1.6, 3.1, 6.3, 12.5 and 25 ng/mL) and calprotectin (0, 1.6, 3.1, 6.3, 25 ng/mL) standards were used to construct a standard curve. The concentrations of these markers in fecal samples were estimated by the equation generated. Results were expressed as micrograms/gram of feces.

## *2.6. Macronutrient intake*

Food records were reviewed with the subjects by a dietitian to check for errors or omissions. Daily energy, carbohydrate, protein, fat, and fiber intake were estimated through the analysis of three days (two-week days and one weekend-day) food records using the software DietPro® (A.S. Sistemas, Viçosa, Brazil) by the same dietitian.

## *2.7. Statistical analysis*

Statistical analysis were performed using the software Intercooled Stata 9.1 for Windows® (StataCorp LP, USA). Shapiro-wilk test was used to test for normality. Whenever possible, variables were transformed to pass normality test. Student-t and Mann-whitney tests were used according to data distribution to compare variables from lean versus obese subjects. In addition, these tests were used to compare subjects allotted to the groups equal/bellow *vs.* above the medians from the variables L/M ratio (0.0296) and LPS (0.675 EU/mL), which were obtained considering all subjects. Spearman test was used to evaluate correlation between variables. Chi-square test ( $\chi^2$ ) was used to compare the frequency of lean and obese subjects allotted to the groups equal/bellow and above each median. Data are represented as median and inter quartile range. A 5% level of significance was adopted.

### 3. Results

#### 3.1. Anthropometrics, body composition, biochemical profile and food intake

Lean and obese subjects presented similar age ( $26.6 \pm 7.1$  vs.  $27.9 \pm 8.9$ ,  $p > 0.05$ ). As expected, anthropometric and body composition variables were higher in obese subjects ( $p < 0.01$ ). Insulin and glucose were also higher for obese subjects ( $p < 0.01$ ). Although plasma creatinine did not differ ( $p > 0.05$ ), estimated creatinine clearance was higher in obese group ( $p = 0.001$ ). Plasma LPS was not significantly different between lean and obese men ( $p = 0.17$ ) (Table 1).

Lean and obese reported the consumption of similar daily carbohydrate ( $361.5 \pm 121.8$  g vs.  $362.6 \pm 94.9$  g,  $p = 0.97$ ), protein ( $101.4 \pm 24.8$  g vs.  $110.1 \pm 36.9$  g,  $p = 0.39$ ), fat ( $84.9 \pm 25.9$  g vs.  $97 \pm 38.7$  g), fiber ( $29.2 \pm 11.5$  g vs.  $27.6 \pm 10.3$  g,  $p = 0.68$ ) and energy ( $2685 \pm 819.7$  kcal vs.  $2764.2 \pm 779.7$  kcal,  $p = 0.68$ ) intake.

#### 3.2. Intestinal permeability and fecal markers

Lactulose and mannitol urinary excretions ( $p = 0.24$  and  $0.27$ , respectively), as well as L/M ratio ( $p = 0.61$ ) did not differ between lean and obese subjects. Fecal elastase was approximately 112% higher in lean group compared to obese ( $p = 0.001$ ), while fecal calprotectin levels did not differ ( $p = 0.73$ ) (Table 2).

#### 3.3. Subdivision of subjects according to median of L/M ratio and LPS

The use of L/M ratio median to subdivide subjects showed that those above the median also had higher BMI ( $p = 0.03$ ), total fat percentage ( $p = 0.04$ ), HOMA ( $p = 0.04$ ) and estimated creatinine clearance ( $p = 0.01$ ). Although by design L/M ratio was significantly different between the groups (Table 2), plasma LPS concentrations were similar ( $p > 0.05$ ) (Table 1).

Although subjects above LPS median showed higher weight, BMI, waist, body fat percentage, insulin and HOMA compared to those equal/below the median, statistical significance was not observed (Table 1). L/M ratio and estimated creatinine clearance were similar between equal/below and above LPS median (Table 2).

When subjects were divided by the median of L/M ratio and LPS, the frequency of obese subjects above the median value did not differ from to the frequency of lean subjects ( $p > 0.05$ ). In both situations, 58.3% of lean subjects were at equal/below the median group, but they did not cluster the same individuals. Regarding obese subjects, the majority of individuals were above the median for L/M ratio (60.7%) and plasma LPS (57.2%) criteria (Table 3).

Food intake also did not differ when subdividing subjects according to the medians considered (data not shown).

### 3.3. Correlation analyses

When data obtained from all subjects were analyzed, correlation between plasma LPS, fecal elastase and calprotectin was not observed. These variables also did not correlate with lactulose, mannitol and L/M ratio (data not shown). However, when correlation analyses were carried out in lean and obese subjects separately, plasma LPS concentration was significantly correlated with HOMA in obese ( $r=0.37$ ,  $p=0.04$ ). However, LPS and L/M ratio did not correlate in this group ( $p>0.05$ ).

Table 4 shows other variables that significantly correlated with HOMA. Weight, BMI, waist, total fat percentage were positively correlated with HOMA only when all subjects were considered ( $p<0.0001$ ). Separate analysis showed that in obese group these correlations were not observed, while in lean group, total body fat tended to be positively and significantly correlated with HOMA ( $p=0.08$ ). Glucose levels were positively correlated with HOMA considering all subjects and obese ( $p<0.01$ ) and also tended to be correlated in lean subjects ( $p=0.06$ ). Fecal elastase and calprotectin were inversely correlated with HOMA only when all subjects were considered ( $p<0.05$ ) (Table 4).

## 4. Discussion

In the present study, in which only men participated, L/M ratio and plasma LPS levels did not differ between lean and obese men and were not themselves correlated and neither with HOMA when data obtained from all subjects were considered.

Higher plasma LPS concentrations have been more commonly reported in type 2 diabetes mellitus<sup>16-18</sup> than in obese subjects.<sup>19</sup> In addition, there is no evidence to assure that this could be a consequence of higher IP in humans.<sup>16-18</sup> In fact, previous reports in humans couldn't confirm that obesity is associated with increased IP by means of L/M ratio test<sup>12-13</sup> and neither with higher LPS.<sup>18,20</sup> These findings could advocate against the proposed causality between increased IP, higher plasma LPS concentration and degree of insulin resistance. Other factors than LPS and IP may be more strongly associated with insulin resistance.

Waist circumference and total body fat percentage were more strongly correlated with HOMA than LPS, considering all subjects. Waist circumference indirectly indicates abdominal adiposity, which is traditionally considered an important contributor for the development of insulin

resistance and metabolic disturbances. Fat localization influences the susceptibility to insulin resistance.<sup>21</sup> Curiously, BMI, waist and total body fat percentage were not correlated with HOMA in obese group. At the individual level, the association between the degree of obesity and development of insulin resistance and metabolic disorders may not be a rule.<sup>22</sup> It is noteworthy to mention that 25% of our obese subjects were not above HOMA median (>1.87), while 20.8% of lean subjects did (data not shown). Terms such as “metabolically obese normal weight”, “metabolically healthy obese” and “at risk” are being used in the literature to define different phenotypes within the same BMI range. These terms are based on insulin sensitivity and assume that metabolic abnormalities will not necessarily occur due to obesity *per se*, but might be largely related to the presence of insulin resistance.<sup>23</sup> There are evidences that increasing whole-body adiposity may not cause additional metabolic disabilities in the absence of increased intra-hepatic triglycerides,<sup>24</sup> which is a condition observed in subjects of higher HOMA, independently of visceral fat.<sup>25</sup>

Considering the existence of these phenotypes, higher plasma LPS levels could be a differential determinant factor for “at risk” condition among obese subjects, since there was a positive correlation between LPS and HOMA in obese group. The fact that the majority of obese subjects were above L/M ratio and plasma LPS median suggest at least for some obese individuals these factors could be somehow associated with a higher degree of insulin resistance. While 43% of obese subjects showed LPS levels below LPS median, 42% of lean subjects showed LPS levels above the median. “Metabolically obese normal weight” is also a terminology emphasizing the occurrence of metabolic abnormalities within lean subjects that show higher inflammatory markers, adiposity and insulin resistance.<sup>26-27</sup> Plasma LPS concentration was 178% higher in the group above LPS median compared to the other subjects. Because this group is composed of 61.5% of obese and 38.5% of lean subjects, even though BMI, waist, and total body fat, as well as HOMA were greater in the group above LPS median, significance was not observed. Therefore, future studies exploring IP, LPS, and insulin resistance among “healthy” and “unhealthy” lean and obese subjects will better clarify the association between these factors.

Animal models strongly suggest that higher intestinal permeability and plasma LPS are important features of obesity.<sup>8-10</sup> In animal models, weight gain and insulin resistance was shown to occur after chronic subcutaneous infusion of LPS in mice,<sup>28</sup> but could be also a consequence of high fat diet.<sup>28-29</sup> High fat intake has been shown to increase plasma LPS in mice<sup>28</sup> and also in humans.<sup>18,30</sup> It has been shown in the literature that high fat diet induced higher ileal expression of inflammatory markers (TNF, NF- $\kappa$ B) in mice,<sup>31</sup> which could be a contributing factor for

higher IP.<sup>32</sup> Stenman and co-workers<sup>33</sup> showed that genetically obese, hyperphagic ob/ob mice became obese by eating normal chow and did not demonstrate signs of altered barrier function. These authors and other researchers have demonstrated that luminal bile acid could be involved in barrier dysfunction often associated with the consumption of a fat-rich diet.<sup>34-35</sup> This indicates that increased IP appears to be exclusive to a fatty diet and not necessarily attributable to obesity.<sup>33</sup> The consumption of a high fat diet combined with soluble fiber has been shown to reduce endotoxins levels,<sup>29</sup> IP<sup>36</sup> or both in obese mice.<sup>10</sup> Together with IP improvement, other benefits such as reduction of body and adipose tissue weight gain, improvement of insulin sensitivity and glucose metabolism, down regulation of inflammation and immune response, adipogenesis and oxidative stress markers have been also observed with fiber supplementation.<sup>10,36</sup> In our study, lean and obese subjects reported similar macronutrient intake, including fat and fiber intake, which may be a consequence of food records limitations related to self-reporting. Or this could explain the similar IP and LPS found in these groups.

Therefore, evidences from animal models strongly suggest that evaluation and modulation of IP could be an interesting strategy in obesity. Regarding the assessment of IP, we question whether L/M ratio is a good marker to analyze IP in obese subjects based on our previous<sup>13</sup> and present findings, as well as fecal elastase and calprotectin. Obesity is often associated with intestinal dysbiosis, such as small intestine bacterial overgrowth.<sup>37</sup> This could lead to pitfalls in the use of sugar probes to evaluate IP, such as fermentation of these sugars by the microbes.<sup>2</sup> Another pitfall that could be associated with L/M ratio is the possibility of altered renal function, often associated with obesity.<sup>38</sup> Although plasma creatinine did not differ between lean and obese, estimated creatinine clearance indicated that obese subjects and also those above L/M ratio median presented a higher renal flux. We don't know how much this could influence the reliability of results, since the assessment of renal function and also BMI is not usually observed in studies evaluating intestinal permeability through sugar probes.<sup>12,39-41</sup> We found that a higher IP (subjects above L/M ratio) was not accompanied by higher plasma LPS concentration. Vojdani<sup>42</sup> highlights that “intestinal permeability to very small molecules (182-342 Da), as it is the case of lactulose and mannitol, may not be necessarily related to structural damage in the tight junction barrier that permits increased penetration of large molecules, such as LPS”.<sup>42</sup> Fecal elastase and calprotectin are expected to be in higher levels in the presence of intestinal mucosa inflammation.<sup>43</sup> We found lower fecal elastase levels in obese compared to lean, as well as an inverse association between fecal elastase and HOMA. Again, our obese subjects did not present higher fat intake, L/M ratio and LPS levels. This may be consistent with absence of intestinal inflammation within our subjects. But these results may also indicate that pancreatic



function is overwhelmed, since low fecal levels of elastase were associated with pancreas atrophy and exocrine deficiency, commonly observed in diabetic patients.<sup>44</sup>

Although our findings do not clearly suggest higher IP in obese subjects, there are reports of positive correlation between IP parameters with metabolic syndrome risk factors, including HOMA,<sup>13</sup> visceral and liver fat in humans.<sup>41</sup> Some authors have proposed that mucosal inflammation and increased IP could be involved in visceral fat accumulation and metabolic dysfunction, as previously demonstrated in animal model.<sup>11</sup> Therefore, the confirmation of alteration of IP in human obesity still needs further investigation. Considering the pitfalls of L/M ratio in the context of obesity, it is possible that the use of other markers for assessment of IP could advocate in favor of higher IP in obese subjects. Serum zonulin, another potential IP marker, was found to be higher in obese subjects compared to non-obese and in subjects with glucose intolerance compared to normal glucose tolerant subjects. Circulating zonulin concentration was positively correlated with BMI, waist to hip ratio, fasting insulin, triglycerides, uric acid, and IL-6 and negatively associated with HDL-cholesterol and insulin sensitivity.<sup>45</sup> Unfortunately, this study did not assess endotoxin concentration.

The dilemma “who comes first, the chicken or the egg” should be remembered by researchers to help delineate future study designs that allow understanding the role of adiposity within this scenario. If one considers two individuals of similar level and distribution of adiposity, differing in the degree of insulin resistance, could the IP and plasma LPS be a differential factor? During the course of obesity development, plasma LPS concentration is increasingly higher? Another important question for future studies is related to the assessment of fasting LPS. Could the differences between lean and obese individuals be in the post-prandial period? This aspect may also emphasize the importance of meals composition.

In conclusion, our findings do not clearly confirm the association between higher IP, LPS, and degree of insulin resistance in obese men. Nevertheless, they suggest that this area offers great opportunity of research. Future studies should explore these variables within the different metabolic phenotypes among lean and obese subjects. In addition, the evaluation of IP should be assessed with other markers besides lactulose and mannitol urinary excretions.

**Table 1 – Anthropometric, body composition and biochemical profile in lean and obese and in subjects subdivided according to the median of lactulose/mannitol ratio and lipopolysaccharides**

	BMI		L/M ratio median		LPS median	
	Lean (n=24)	Obese (n=28)	≤0.0296 (n=25)	> 0.0296 (n=27)	≤ 0.675 (n=26)	> 0.675 (n=26)
<b>Weight<sup>§</sup> (kg)</b>	68.2 (65.3 - 74.6)*	101.3 (97.8-109.1)*	77.8 (68 - 97.6)	98.1 (71.8 - 104.8)	81.3 (67.4-98.1)	97.3 (73.2-107.8)
<b>Height (m)<sup>‡</sup></b>	1.74 (1.69-1.79)	1.78 (1.73 - 1.81)	1.77 (1.71-1.81)	1.76 (1.72-1.79)	1.76 (1.71-1.80)	1.77 (1.72-1.81)
<b>BMI (kg/m<sup>2</sup>)<sup>§</sup></b>	22.8 (21.9-23.6)*	31.9 (31.4 - 33.3)*	24.4 (21.9-31.8)*	31.5 (23.2-33.3)*	24.6 (22.5-31.8)	31.3 (23.2-33.2)
<b>Waist (cm)<sup>§</sup></b>	80.1 (77.2 - 83.5)*	108.7 (104.7-111.6)*	88.7 (80.5-105)	106.2 (80.7-110.4)	88.1 (79.8-105)	106.3 (80.7-110.4)
<b>Fat %<sup>§</sup></b>	15.7 (13.9-19.6)*	28.5 (26.5-30)*	20.5 (14.4-26.6)*	26.9 (17.2-30)*	22.1 (14.2-26.4)	27.3 (17.8-29.5)
<b>Glucose (mmol/L)<sup>†</sup></b>	4.85 (4.55 - 5.19) <sup>a</sup>	5.16 (4.83-5.63) <sup>b</sup>	5.05 (4.66-5.38)	4.94 (4.66-5.61)	4.91 (4.66-5.22)	5.16 (4.66-5.61)
<b>Insulin<sup>†</sup> (pmol/L)</b>	35.4 (25.0 - 50.0)*	77.1 (55.5 - 104.2)*	44.4 (31.9-77.7)	62.5 (36.1-95.8)	45.8 (34.7-66.6)	61.8 (34.7-90.3)
<b>HOMA<sup>†</sup></b>	1.12 (0.72 - 1.41)*	2.49 (1.87 - 3.85)*	1.28 (1.0-2.7)*	1.98 (1.08-3.04)*	1.43 (1.03-2.42)	1.99 (1.25-2.79)
<b>LPS (EU/mL)<sup>†</sup></b>	0.59 (0.40-1.06)	0.75 (0.51-1.29)	0.69 (0.42-1.22)	0.64 (0.43-1.13)	0.42 (0.35-0.54)*	1.17 (0.89-1.91)*
<b>Creatinine (mmol/L)<sup>§</sup></b>	81.2 (75.1-92.7)	80.3 (74.1 - 91.8)	79.5 (73.2-86.5)	84.7 (75.9-94.5)	83.8 (75.9-90.9)	77.7 (72.4-93.6)
<b>Creatine clearance (mL/min)<sup>†</sup></b>	99.3 (87.9-112.6)*	134.8 (104.9-145.9)*	101.8 (87.9-129.9)*	127.3 (104.8-141.2)*	109.2 (91.5-133.8)	105.2 (89.9-141.1)

BMI, body mass index; HOMA, homeostasis assessment model; LPS, lipopolysaccharides; L/M, lactulose/mannitol ratio.

Data are represented as median and interquartile range (IQR).

\*Statistical significance (p<0.05) within each criteria (BMI and specific medians).

†Different symbols within each variable indicates the statistical test used to compare groups, according to data distribution. §Mann-whitney test. ‡Student t-test. †Student t-test with transformed variables.

**Table 2 – Intestinal permeability markers, fecal elastase and calprotectin in lean and obese and in subjects subdivided according to the median of lactulose/mannitol ratio, and lipopolysaccharides**

	BMI		L/M ratio median		LPS median	
	Lean (n=24)	Obese (n=28)	≤0.0296 (n=25)	> 0.0296 (n=27)	≤ 0.675 (n=26)	> 0.675 (n=26)
<b>Lactulose</b> <sup>§</sup> (%)	0.33 (0.24-0.47)	0.45 (0.23-0.59)	0.41 (0.26-0.53)	0.35 (0.23-0.54)	0.38 (0.24-0.54)	0.36 (0.23-0.52)
<b>Mannitol</b> <sup>†</sup> (%)	11.9 (8.55-16.1)	15.3 (7.1-20.1)	15.8 (8.7-20.5)	11.6 (6.9-16.1)	13.4 (8.5-18.1)	13.7 (7.2-20)
<b>L/M ratio</b> <sup>**</sup>	0.029 (0.026-0.031)	0.03 (0.024-0.036)	0.025 (0.024-0.027) <sup>*</sup>	0.032 (0.03-0.036) <sup>*</sup>	0.029 (0.027-0.033)	0.029 (0.024-0.033)
<b>Fecal elastase</b> <sup>***</sup>	0.017 (0.011-0.038) <sup>*</sup>	0.008 (0.004-0.01) <sup>*</sup>	0.012 (0.007-0.02)	0.009 (0.005-0.016)	0.009 (0.005-0.016)	0.015 (0.008-0.02)
<b>Fecal calprotectin</b> <sup>***</sup>	0.12 (0.10-0.18)	0.13 (0.11-0.16)	0.14 (0.11-0.18)	0.13 (0.10-0.15)	0.12 (0.11-0.14)	0.13 (0.10-0.17)

BMI, body mass index; L/M, lactulose/mannitol ratio; LPS, lipopolysaccharides.

Data are represented as median and interquartile range (IQR). Statistical difference<sup>(\*)</sup> (p<0.05)

<sup>\*</sup>Urine samples from lean (n=22) and obese (n=28)

<sup>\*\*</sup>Fecal samples from lean (n=22) and obese (n=24); equal/below (n=21) and above (n=25) L/M ratio median;

equal/below (n=25) and above (n=21) and LPS medians. Results from fecal elastase and calprotectin are expressed as micrograms/g (µg/g) of feces

<sup>§,†</sup>Different symbols within each variable indicates the statistical test used to compare groups, according to data distribution. <sup>§</sup>Mann-whitney test. <sup>‡</sup>Student t-test. <sup>†</sup>Student t-test with transformed variables.)

**Table 3 – Frequency of lean and obese subjects equal/below and above lactulose/mannitol ratio, and lipopolysaccharides**

	L/M ratio median		LPS median	
	≤0.0296	> 0.0296	≤ 0.675	> 0.675
<b>Lean</b>	14 (58.3%)	10 (41.7%)	14 (58.3%)	10 (41.7%)
<b>Obese</b>	11 (39.3%)	17 (60.7%)	12 (42.8%)	16 (57.2%)
<b>Total</b>	25(48.1%)	27 (51.9%)	26 (50%)	26(50%)
<b>p-value</b> <sup>§</sup>	0.17		0.26	

L/M, lactulose/mannitol ratio; LPS, lipopolysaccharides

<sup>§</sup>Chi-square test was used to compare the prevalence of lean and obese subjects into groups equal/below and above each medians. Data are represented as net number and percentage of total lean (n=24) or obese (n=28) in parentheses.

**Table 4 – Correlation analyses between homeostasis assessment model (HOMA) and other variables in overall, lean and obese groups separately**

	Overall (n=52)		Lean subjects (n=24)		Obese subjects (n=28)	
	r	p	r	p	r	p
<b>LPS</b>	0.25	0.07	-0.07	0.72	0.37	0.04
<b>Weight</b>	0.55	0.0000	0.29	0.16	0.04	0.81
<b>BMI</b>	0.55	0.0000	0.16	0.43	0.10	0.59
<b>Waist</b>	0.57	0.0000	0.29	0.15	0.05	0.77
<b>Fat %</b>	0.57	0.0000	0.35	0.08	0.05	0.77
<b>Insulin</b>	0.98	0.0000	0.94	0.0000	0.98	0.0000
<b>Glicose</b>	0.52	0.0001	0.38	0.06	0.49	0.007
<b>Fecal elastase*</b>	-0.41	0.004	-0.18	0.41	-0.05	0.81
<b>Fecal calprotectin*</b>	-0.3	0.04	-0.42	0.05	-0.31	0.12

r, Spearman correlation coefficient; LPS, lipopolysaccharides; BMI, body mass index.

\*Correlation analysis with 46 observations (all subjects), 22 observations (lean) and 24 observations (obese)

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### **3.5. Article 5 (original): Body mass index is better than plasma lipopolysaccharides in clustering subjects with higher degree of insulin resistance**

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#### **Abstract**

Insulin resistance associates with metabolic abnormalities. Infusion of lipopolysaccharides (LPS) and obesity, particularly central fat, may contribute to its development. Evidences of the association between these two factors are still lacking. The aim of this study was to investigate the relationship between body mass index (BMI), android fat, homeostasis assessment model (HOMA) and plasma LPS. BMI, body composition and biochemical profile, including plasma LPS were assessed. Ninety-seven men were subdivided according to BMI categories and tertiles of plasma LPS. Obese subjects showed higher waist, total, gynoid and android fat, insulin and HOMA than overweight and lean subjects ( $p < 0.05$ ). Glucose, total cholesterol, triglycerides, AST, ALT, CRP were higher in obese compared to lean subjects ( $p < 0.05$ ). Plasma LPS of obese was similar to lean ( $p > 0.05$ ) and both lower than overweight subjects ( $p < 0.05$ ). Subjects of the upper tertile of plasma LPS presented higher android fat and AST compared to low and middle tertiles ( $p < 0.05$ ). BMI and HOMA, as well as the other variables were similar between tertiles of plasma LPS ( $p > 0.05$ ). BMI seems to better cluster subjects with higher degree of insulin resistance than tertiles of plasma LPS. Obese subjects did not show higher plasma LPS concentration, despite presenting the highest HOMA, while subjects of higher LPS did not show highest HOMA. The higher android fat and AST in subjects of higher plasma LPS concentration may indicate that the relationship between android fat, HOMA index and plasma LPS concentration needs further investigation in humans.

**Key words:** obesity, insulin resistance, android fat, lipopolysaccharides, body mass index

## 1.0. Introduction

It is strongly suggested that severity of morbidities and risk of mortality progressively increase with the adiposity increase.<sup>1</sup> It is also assumed that the degree of insulin resistance (IR), which in turn may increase the risk of dyslipidemia, hypertension and hyperglycemia,<sup>2-3</sup> rises with body fat mass. But this is not necessarily a rule for all individuals.<sup>4</sup> Not only obesity, but also normal weight, might be heterogeneous in regard to its effects, according to the absence or presence of IR.<sup>5</sup>

The role of adipose tissue in IR development is not clear cut since there are animal models and also side effects of drugs used to improve insulin sensitivity that shows that increasing adipose tissue will not necessarily induce IR.<sup>4</sup> Even so, many features of adipose tissue, such as fat depot location (visceral *vs.* subcutaneous, central *vs.* peripheral), are thought to influence the functionality of adipose tissue and its impact over metabolism.<sup>1</sup> Central accumulation of fat, also denominated android fat, particularly visceral rather than subcutaneous, is considered hazardous for the development of IR and type 2 diabetes (T2DM). The ‘portal theory’, whose central components are elevated flux of non-esterified fatty acids and intra-hepatic fat accumulation, links visceral fat and IR with disturbances of metabolism.<sup>6</sup> Considering the mentioned link, Amato and co-workers<sup>7</sup> proposed the “Visceral adipose index” (VAI), that encompasses waist circumference, body mass index (BMI), plasma triglycerides and HDL, as a possible marker of adipose tissue dysfunction and cardiometabolic risk.

For years, the combination of genetic factors, sedentary lifestyle and excessive caloric intake (especially high fat) were considered the main causal factors for adiposity increase.<sup>8</sup> Recently, discoveries about the role of microbiota on the regulation of fat storage<sup>9</sup> opened new perspectives.

Lipopolysaccharides (LPS) are constituents of gram-negative bacteria cell wall that may influence the host through the activation of toll-like receptors 4 (TLR4) culminating in the release of inflammatory molecules.<sup>10</sup> Chronic infusion of low dose of LPS stimulated adipose tissue expansion accompanied by IR in mice,<sup>11</sup> while others showed that LPS inhibited adipogenesis in cell culture.<sup>12</sup> Infusion of LPS in healthy subjects was also shown to transiently increase plasma insulin and homeostasis model assessment (HOMA) index,<sup>13-14</sup> an indirect marker of IR.<sup>15</sup> In addition, LPS also altered gene expression in adipose tissue, transiently increased plasma non-esterified fatty acids, C-reactive protein (CRP) and other inflammatory cytokines.<sup>14</sup> The downstream signaling of the insulin receptor can be impaired by inflammatory

signals, disturbing insulin action,<sup>16</sup> and could be a mechanism through which LPS would induce IR.

These findings advocate in favor of increased systemic plasma LPS as an external stimulus activating cellular signals leading toward inflammation and IR. Although infusion models clearly show a causative relationship between higher plasma LPS and IR, there are contradictory reports to assure that higher plasma LPS concentrations affect obese subjects<sup>17-19</sup> under fasted state and also that this could be accompanied by a higher degree of insulin resistance.

Considering the possible role of android fat and plasma LPS in the development of IR, the aim of this study was to investigate the relationship between BMI, android fat, HOMA index and plasma LPS levels in adult men.

## **2.0. Methods**

### *2.1. Subjects*

Recruitment occurred through written announcements and social network in the local community of Viçosa city (Minas Gerais, Brazil). One hundred and seventy six men interested and were screened. Ninety seven men fulfilled the following inclusion criteria: BMI >18.5 and < 35 kg/m<sup>2</sup>, older than 18 and under 50 years old, absence of acute or chronic disease episodes other than obesity, not smoking, not taking any medication, not under weight loss diet and weight stable for the last 3 months (less than 3kg change). All subjects provided informed consent. The study was approved by the Ethical Committee in Human Studies from Universidade Federal de Viçosa (protocol n° 196/2012/CEP/07-12-E4).

### *2.2. Anthropometric and body composition*

Subjects were weighted in the fasted state wearing underwear (200 kg capacity, TANITA, model TBF-300 A, Tanita Corporation of America Inc, Illinois, USA). Height was measured with a fixed stadiometer (Seca®, Germany) to the nearest millimeter. BMI was calculated dividing weight (kg) by the square of height (m). Waist and hip circumferences were measured with a flexible tape. Waist was measured in the lowest circle between the lowest rib and umbilicus. Total body fat was evaluated through bioimpedance (200 kg capacity, TANITA, model TBF-300 A, Tanita Corporation of America Inc, Illinois, USA). Body composition (total, ginoid and android fat) was also assessed by the Dual-energy X-ray Absortiomerty (DXA, Lunar Prodigy Advance DXA System, 13.31 version, GE Lunar). The VAI was calculated according to the

equation proposed for men by Amato and co-workers<sup>7</sup>, as follows  $VAI = \text{Waist (cm)} / [39.68 + (1.88 \times \text{BMI (kg/m}^2\text{)})] \times (\text{Triglycerides (mmol/L)/1.03}) \times (1.31/\text{HDL (mmol/L)})$ .

### 2.3. Biochemical parameters

Subjects fasted for 10h overnight. EDTA and serum tubes were used to collect blood in the antecubital vein. Tubes were kept under 2-8°C for 20 min and then centrifuged at 2,200 x g for 15 min at 4°C (Heraeus Megafuge 11R centrifuge, Thermo Scientific). Plasma and serum were collected and stored at -80°C for posterior analyses. Auto analyzer (COBAS MIRA Plus; Roche Diagnostic Systems) and commercial kits (Bioclin/Quibasa, Brazil) based on enzymatic colorimetric method were used to quantify fasting glucose, triglycerides, total cholesterol, HDL, CRP, aspartate aminotransferase (ASL) and alanine aminotransferase (ALT). Friedwald formula<sup>20</sup> was used to determine LDL concentrations. Serum fasting insulin was determined by electrochemiluminescence immunoassay (Elecsys-Modular Analytics E170, Roche Diagnostic Systems®). HOMA indices were calculated as follows:  $\text{fasting glucose (mmol/L)} \times \text{fasting insulin (mU/L)} / 22.5$ .<sup>21</sup>

Limulus Amebocyte Lysate (LAL) commercial kit (Hycult Biotech, The Netherlands) was used to quantify plasma LPS concentration. Plasma samples were heated (75°C) for 5min. Fifty microliters of undiluted plasma and prepared standards (*E. coli* O111:B4) were pipetted into the pyrogen-free microplate. Absorbance was read at 405 nm (Multiskan Go, Thermo Scientific, USA). Reagents were added according to the manufacturer's instructions. Absorbance was read again. Standard curve and its equation ( $R^2 > 0.97$ ) were generated by plotting the concentration of standards (log10) (standard concentrations: 0, 0.04, 0.1, 0.26, 0.64, 1.6, 4 and 10 EU/mL) and their absorbance. Plasma LPS concentrations (endotoxins units per milliliter, EU/mL) were estimated using the delta of absorbance ( $\Delta = \text{final absorbance} - \text{initial absorbance}$ ).

### 2.4. Statistical analyses

Statistical analysis were performed using the software Intercooled Stata 9.1 for Windows® (StataCorp LP, USA). Shapiro-wilk test was used to test for normality. Variables were transformed to pass normality test whenever possible. Subjects were subdivided into lean, overweight and obese in accordance to their BMI. In addition, subjects were subdivided into tertiles of plasma LPS concentrations. Analysis of variance (ANOVA) or Kruskal-Wallis tests were used to compare parametric and non-parametric variables, respectively, between BMI categories and tertiles of plasma LPS. The *post hoc* Bonferroni test was used for multiple comparisons after ANOVA, while Mann-Whitney test was used for multiple comparisons after

Kruskal-Wallis. Spearman correlation test was used to test association between plasma LPS and other variables. Multiple linear regression was used to assess the association of independent continuous variables (anthropometric and biochemical) with HOMA index (dependent variable). Data are represented as median and interquartile range. A 5% level of significance was adopted.

### **3.0. Results**

#### *3.1. Comparison between lean, overweight and obese men*

From the 97 participants of the study, 26 were lean (BMI >18.5 & < 25 kg/m<sup>2</sup>), 43 overweight (BMI ≥25 & <30 kg/m<sup>2</sup>) and 28 obese (BMI >30 kg/m<sup>2</sup>). Age and height were similar between groups. Weight, BMI, waist, waist/hip ratio, total body fat percentage, gynoid and android fat percentages were increasingly higher from lean to obese (p<0.05). Fasting insulin and HOMA were also increasingly higher from lean to obese (p<0.05). Glucose was higher in obese in comparison only to lean men (p=0.017). Total cholesterol was higher in overweight compared to lean (p=0.016), while LDL and HDL levels, as well as total cholesterol/HDL and LDL/HDL ratios did not differ between groups. Triglycerides were similar between overweight and obese, and both higher than lean (p<0.01 and p<0.001, respectively). The levels of hepatic enzymes AST and ALT and CRP were also similar between overweight and obese, and both higher than lean (p<0.05). Plasma LPS levels were similar between lean vs. obese, while overweight showed higher levels than lean and obese (p<0.05). VAI was significantly higher in overweight and obese compared to lean (p<0.01 and p<0.001, respectively) (Table 1).

#### *3.2. Comparison between lower, middle and upper tertiles of plasma LPS*

Plasma LPS concentration below 0.52 EU/mL defined the lower tertile (n=32). Intermediary levels (≥0.52 and < 1.15 EU/mL) were considered middle tertile (n=32), while ≥1.15 EU/mL defined the upper tertile (n=33). There was a trend for higher total body fat in the upper tertile of plasma LPS (p=0.07). Android fat and AST were significantly higher in subjects from the upper tertile compared to middle and lower tertiles (p<0.05), while total cholesterol was higher compared only to lower tertile (p<0.05). CRP tended to be higher along plasma LPS tertiles (p=0.08) (Table 2). Of note, median of plasma LPS concentration was 533% higher in the upper tertile of LPS compared to the lower tertile, while HOMA was only 48% higher (but not statistically significant).

The frequency of lean, overweight and obese in the tertiles of plasma LPS is shown in Figure 1. The frequency of obese subjects in the upper tertiles (32.1%) seems to be similar to frequencies in the lower (32.1%) and middle tertiles (35.8%) of plasma LPS. Surprisingly, 46.5% of

overweight subjects were at the upper tertiles of plasma LPS (Figure 1). From the 33 subjects in the upper tertile of plasma LPS, 60.6% were overweight, 27.3% were obese and 12.1% were lean.

We also considered HOMA > 2.7 as a cut-off for identification of IR<sup>15</sup> for Brazilian population. From the 28 obese subjects, 53.6% didn't have IR, while one lean subject (3.9%) and eight (18.6%) overweight subjects presented IR. Considering plasma LPS tertiles, 50% of the total insulin resistant subjects (n=22) were in the upper tertile, in contrast to 29.3% of the total insulin sensitive subjects (n=75) (Figure 2). However, from the 33 subjects in the upper tertiles of LPS, the majority were insulin sensitive (66.7%), in contrast to 33.3% that presented IR.

### *3.3. Correlation analyses and multiple regression*

When considering all subjects, plasma LPS concentration showed a weak positive correlation with HOMA (r=0.21, p=0.03), total body fat (r=0.24, p=0.02), android fat (r=0.33, p=0.001), insulin (r=0.21, p=0.03), total cholesterol (r=0.21, p=0.03), triglycerides (r=0.21, p=0.03), CRP (r=0.2, p=0.04), AST (r=0.23, p=0.02) and ALT (r=0.23, p=0.02).

Simple linear regression indicated the association of HOMA with plasma LPS ( $\beta=0.18$  (95% CI 0.027-0.33), p=0.021), total fat percentage measured through bioimpedance ( $\beta=0.05$  (95% CI 0.03-0.07), p<0.001), ALT ( $\beta=0.61$  (95% CI 0.38-0.83), p<0.001), and CRP ( $\beta=0.17$  (95% CI 0.045-0.31), p=0.009). The coefficient of determination ( $R^2$ ) were higher for total fat percentage ( $R^2=0.27$ ) and ALT ( $R^2=0.23$ ) than for plasma LPS ( $R^2=0.05$ ) and CRP ( $R^2=0.06$ ). In addition, in a multiple linear regression model including all these independent variables, the influence of plasma LPS ( $\beta=0.04$  (CI -0.09-0.17), p=0.54) and CRP ( $\beta=0.01$  (CI -0.14 – 0.12), p=0.86) on the variation of the response variable (i.e., HOMA) lost its significance, while significance remained for total fat ( $\beta=0.04$  (CI 0.02-0.06), p=0.000) and AST ( $\beta=0.44$  (CI 0.22-0.67), p=0.000). This model explained 36% of the variation in HOMA values.

## **4.0. Discussion**

There are huge challenges for understanding insulin signaling mechanisms and their dysfunctions in obesity and T2DM.<sup>22</sup> Ferrarini and Balkau<sup>23</sup> highlighted that depending on the isolate or combined occurrence of IR and hyperinsulinemia, phenotypic characteristics (physical and biochemical) may differ.<sup>23-24</sup> In the present study, BMI and LPS were used to subdivide adult men into categories and tertiles, respectively. It seems that distinct metabolic risk profile is also revealed from the clustering of subjects using each criterion. The fact that the majority of obese subjects (53.6%) were insulin sensitive reinforces the view that increasing adipose tissue

will not necessarily be associated with IR and that different phenotypes in relation to the body size and the metabolism exists.<sup>25</sup>

If IR is supposedly a consequence of LPS insult, then, it would be expected that subjects with higher plasma LPS concentration would have higher HOMA index, which was not the case in the present study. According to our findings, the assumption “higher plasma LPS, higher IR” is not easily defensible. The main findings that advocates against this assumption were the fact that 1) obese subjects showed highest HOMA, but similar plasma LPS compared to lean; 2) at the upper tertile of LPS the majority of variables, including HOMA, did not differ; and 3) almost 30% of all insulin sensitive subjects had elevated concentration of LPS, while 66.7% of subjects in the upper tertile of plasma LPS were insulin sensitive. Therefore, our findings suggest that higher plasma LPS concentration is not a feature of obesity *per se* and may not explain the highest HOMA observed in obese group. Other authors also did not find differences in fasting plasma LPS concentrations between lean and obese.<sup>17-18</sup>

LPS insult may contribute to inflammatory activation, impairing insulin signaling.<sup>16</sup> CRP is an inflammatory marker, which was positively correlated with plasma LPS. Higher CRP concentration was a common feature observed in the comparison obese *vs.* lean, and tended to be higher comparing upper *vs.* lower tertile of LPS. However, plasma LPS was higher only comparing upper *vs.* lower tertiles of LPS. This may suggest that LPS may stimulate the increase in the concentrations of plasma CRP. Of note, plasma LPS and CRP showed a lower influence in the variations of HOMA in the simple regression, while their influence lost its significance in the multiple model. Albeit, the cross-sectional nature of our study, as well as regression analyses, does not allow establishing causality associations between LPS and IR or assuring that LPS does not play a role at all.

The higher plasma LPS concentration observed in overweight subjects is intriguing. Follow-up studies may help to determine if there is a chronological sequence of events in the course transition from overweight to obese states related to biological responses to LPS that may contribute to specific metabolic risks. Obese subjects with established T2DM<sup>18,26-27</sup> and also overweight subjects with type 1 diabetes<sup>28</sup> had higher plasma LPS than non-diabetic subjects. In a follow-up study, prevalent and incident diabetes were associated with endotoxemia.<sup>26</sup>

Total adiposity and the levels of the hepatic enzyme AST were the two independent variables that better explained the variations of HOMA in the simple and multiple linear regression model. More than total adiposity, distribution of adipose tissue is considered an important characteristic

in the determination of risk of metabolic abnormalities, including IR, particularly visceral fat accumulation.<sup>29</sup> Based on the view that dysfunctionality of visceral adipose tissue is closely associated with IR and consequent metabolic disturbances, VAI was proposed as a simple marker to evaluate visceral fat dysfunction, since it considers physical and biochemical measurements.<sup>7</sup> This index was higher in overweight and obese, whose HOMA was higher, in comparison to lean, while it did not differ between LPS tertiles. This may indirectly indicate the association between degree of IR and visceral adipose tissue dysfunctionality. An interesting finding was the fact that subjects of higher plasma LPS (upper tertile) also showed significantly higher android fat percentage than lower tertiles. In addition, LPS and android fat were positively correlated. Again, flow-up studies in the future should explore if higher plasma LPS may contribute to visceral fat accumulation or if the central accumulation precedes the increase in plasma LPS. Lam and co-workers<sup>30</sup> proposed a hypothetical model suggesting a chronological sequence of events based on the proximity between the gut and mesenteric fat that may support these findings. LPS could translocate from intestinal lumen and directly affect mesenteric fat physiology. This would activate mesenteric adipocytes hypertrophy, increase pro-inflammatory gene expression and cytokine production, attracting immune cells. In addition, expansion of mesenteric fat mass would increase fatty acid flux to the liver, which in the long term could result in an inflamed, steatotic, and insulin resistant liver.<sup>30</sup> The higher total cholesterol and AST found for subjects of higher plasma LPS may indirectly suggest that disturbances of liver metabolism could be a first sign of LPS insult, before the appearance of systemic IR.

Although infusion models clearly show a causative relationship between higher plasma LPS and IR, some considerations are to be made since LPS, from a huge diversity of gastrointestinal bacteria, may enter the circulation after overcoming gut barrier. Transposing the intestinal barrier may occur due to increased intestinal permeability<sup>31</sup> and by incorporation of LPS inside chylomicrons<sup>32</sup> as proposed by animal models. Biological responses to LPS may differ according to its size and composition. These characteristics will determine intracellular destination upon internalization by intestinal cells, whether it will be deacylated or processed by Golgi complex with consequent reduction or increase of its biological activity.<sup>33</sup> The passage of LPS through paracellular space between intestinal cells may deviate this cellular barrier. However, association of obesity with altered intestinal permeability and concomitant increase in LPS was demonstrated only in mice.<sup>31</sup> Additionally, there are contradictory reports to assure that intestinal permeability<sup>34-35</sup> and higher plasma LPS concentrations affect obese subjects.<sup>17-19</sup> High fat intake stimulates chylomicrons formation and increases plasma LPS.<sup>36</sup> On the other hand, lipid infusion, without concomitant increase in LPS, is also able to induce IR, indicating the direct



action of fatty acids.<sup>10</sup> There are evidences that depending on the type of fatty acids TLR4 can be activated or inhibited.<sup>37</sup> In addition, fatty acid profile of a high fat diet or meal may influence the extent of induced inflammation, independently of higher endotoxemia.<sup>38</sup> In addition, circulating levels of lipoproteins may also influence the response to LPS. The liver is able to clear LPS from circulation, which seems to be more efficiently done when LPS is bound to chylomicrons, eliminating it into bile. This possibly reduces the systemic detrimental effects.<sup>39</sup> The capacity of LPS clearance may affect both liver and systemic level of inflammation. Therefore, establishing the impact of LPS transposing gut barrier, not directly infused into the circulation, on IR in humans is not an easy task.

In summary, BMI seems to better cluster subjects with higher degree of IR with a worse biochemical profile than tertiles of plasma LPS did. Obese subjects did not show higher plasma LPS concentration, despite highest HOMA, while subjects of higher plasma LPS concentration did not show highest HOMA. The higher android fat and AST in subjects of higher plasma LPS concentration may indicate the participation of this bacterial molecule somewhere in the portal theory. Therefore, the relationship between android fat, HOMA index and plasma LPS concentration needs further investigation in humans.

**Table 1 – Anthropometric, body composition and biochemical data between lean, overweight and obese men**

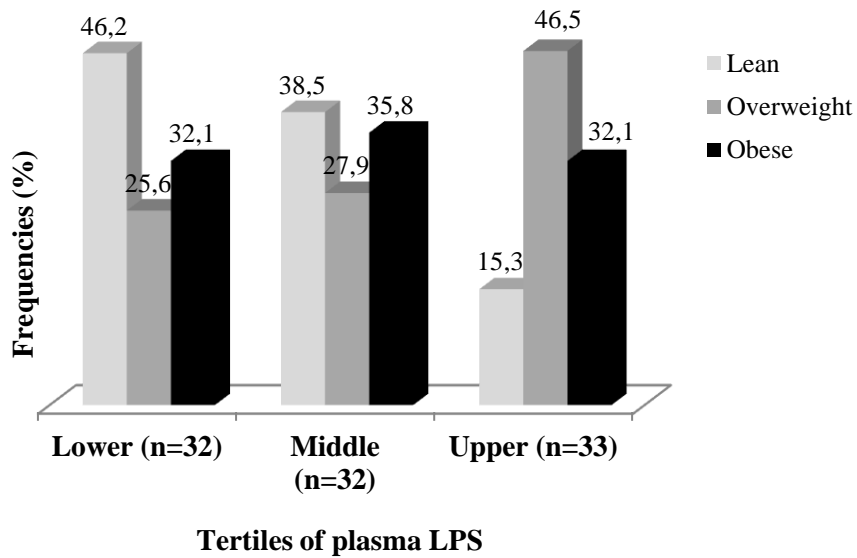
Variables	Lean (n=26)	Overweight (n=43)	Obese (n=28)
Age (y) <sup>§§</sup>	25 (21-31)	25 (22-29)	24.5 (22-31.5)
Weight (kg) <sup>§</sup>	69.7 (65.7-75) <sup>a</sup>	89.5 (81.7-95.6) <sup>b</sup>	101.3 (97.8-109.1) <sup>c</sup>
Height (m) <sup>§§</sup>	1.73 (1.7-1.79)	1.76 (1.72-1.84)	1.78 (1.73-1.81)
BMI (kg/m <sup>2</sup> ) <sup>†</sup>	22.9 (21.9-23.9) <sup>a</sup>	28.1 (27.4-28.5) <sup>b</sup>	31.9 (31.4-33.3) <sup>c</sup>
Waist (cm) <sup>§</sup>	80.6 (77.7-86.3) <sup>a</sup>	97 (93.8-100.8) <sup>b</sup>	108.7 (104.7-111.6) <sup>c</sup>
Waist/hip <sup>§</sup>	0.84 (0.82-0.89) <sup>a</sup>	0.91 (0.89-0.93) <sup>b</sup>	0.96 (0.93-0.99) <sup>c</sup>
Fat - DXA(%) <sup>†</sup>	17.2 (15.8-21.9) <sup>a</sup>	31.3 (27.5-34.8) <sup>b</sup>	37.4 (34.7-41.1) <sup>c</sup>
Ginoid fat (%) <sup>§</sup>	25.2 (21.9-27.3) <sup>a</sup>	36.6 (31.6-39.7) <sup>b</sup>	41.7 (39.3-45.6) <sup>c</sup>
Android fat (%) <sup>†</sup>	14.7 (12.1-16.4) <sup>a</sup>	31.4 (26.4-35) <sup>b</sup>	40.3 (36.4-46.9) <sup>c</sup>
Insulin (pmol/L) <sup>§§</sup>	35.4 (25.0-44.4) <sup>a</sup>	45.8 (31.9-69.4) <sup>b</sup>	77.1 (55.5-104.2) <sup>c</sup>
HOMA <sup>§§</sup>	1.12 (0.75-1.28) <sup>a</sup>	1.51 (1.07-2.21) <sup>b</sup>	2.49 (1.87-3.85) <sup>c</sup>
Glucose (mmol/L) <sup>§§</sup>	4.85 (4.61-5.16) <sup>a</sup>	4.94 (4.72-5.33) <sup>a,b</sup>	5.16 (4.83-5.63) <sup>b</sup>
TC(mmol/L) <sup>§</sup>	4.27 (3.76-4.71) <sup>a</sup>	4.82 (4.09-5.52) <sup>b</sup>	4.91 (4.22-5.53) <sup>a,b</sup>
LDL (mmol/L) <sup>†</sup>	2.72 (2.47-3.26)	3.15 (2.45-3.92)	3.04 (2.37-3.77)
HDL (mmol/L) <sup>§§</sup>	0.98 (0.83-1.17)	1.09 (0.88-1.19)	1.01 (0.84-1.11)
TC/HDL <sup>§</sup>	4.33 (3.59-5.18)	4.48 (3.63-5.84)	4.82 (4.32-5.79)
LDL/HDL <sup>§</sup>	2.89 (2.3-3.45)	2.88 (2.04-3.62)	3.2 (2.48-3.39)
TG (mmol/L) <sup>§§</sup>	0.79 (0.71-0.97) <sup>a</sup>	1.16 (0.87-1.76) <sup>b</sup>	1.53 (1.14-2.29) <sup>b</sup>
AST (U/I) <sup>†</sup>	28.5 (25-32) <sup>a</sup>	35 (26-48) <sup>b</sup>	36 (25.5-42.5) <sup>b</sup>
ALT (U/I) <sup>§§</sup>	14.5 (10-21) <sup>a</sup>	22 (15-29) <sup>b</sup>	25 (18-29) <sup>b</sup>
CRP (mg/L) <sup>§§</sup>	0.36 (0.15-0.9) <sup>a</sup>	1.01 (0.5-1.98) <sup>b</sup>	1.53 (0.86-2.13) <sup>b</sup>
LPS (EU/mL) <sup>§§</sup>	0.56 (0.4-1.04) <sup>a</sup>	1.06 (0.48-2.37) <sup>b</sup>	0.75 (0.5-1.29) <sup>a</sup>
VAI <sup>§§</sup>	1.03 (0.78-1.54) <sup>a</sup>	1.63 (1.0-2.76) <sup>b</sup>	2.02 (1.49-3.82) <sup>b</sup>

Data are presented as median (interquartile range). <sup>§</sup>One way ANOVA, post hoc Bonferroni; <sup>§§</sup>One way ANOVA (variable transformed), post hoc Bonferroni; <sup>†</sup>Kruskal-Wallis, followed by Mann-Whitney BMI, body mass index; DXA, Dual-energy X-ray Absortimetry; HOMA, homeostasis model assessment; TC, total cholesterol; LDL, Low-density lipoprotein; TG, triglycerides; HDL, high density lipoprotein; CRP, C-reactive protein; LPS, lipopolysaccharides; VAI, visceral adiposity index  
<sup>a,b,c</sup>Different letters in the same line represent statistical significance (p<0.05)

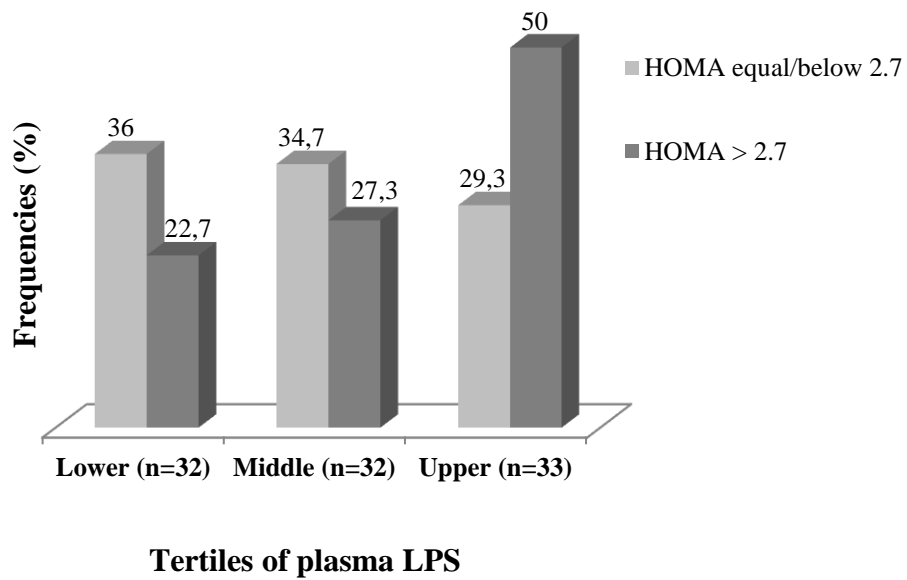
**Table 2 - Anthropometric, body composition and biochemical data between lower, middle and upper tertiles of plasma lipopolysaccharides**

Variables	LPS <0.526 (n=32)	LPS ≥0.526 and <1.15 (n=32)	LPS ≥ 1.15 (n=33)
Age (y) <sup>§§</sup>	24 (21.5-31)	26 (22-29)	25(22-31)
Weight (kg) <sup>§</sup>	83.6 (71.6-96.5)	90.2 (77.1-99.7)	89.6 (80.5-100)
Height (m) <sup>§§</sup>	1.74 (1.71-1.81) <sup>a</sup>	1.79 (1.76-1.86) <sup>b</sup>	1.75 (1.72-1.81) <sup>a,b</sup>
BMI (kg/m <sup>2</sup> ) <sup>†</sup>	27.3 (23.8-30.5)	27.9 (23.7-1.7)	28.4 (27.3-30.5)
Waist (cm) <sup>§</sup>	93.8 (86.4-101.5)	97.5 (86.7-106)	99 (93.9-105.4)
Waist/hip <sup>§</sup>	0.92 (0.87-0.94)	0.92 (0.86-0.95)	0.92 (0.89-0.95)
Fat-DXA (%) <sup>†</sup>	28.7 (22.1-34.1)	30.1 (20.9-35.2)	34.6 (29.6-37.1)
Ginoid fat (%) <sup>§</sup>	32.7 (27.3-39.7)	35.5 (27.7-39.2)	37.6 (33-41.9)
Android fat (%) <sup>†</sup>	27.1 (17.7-34.5) <sup>a</sup>	29.5 (16.2-36.1) <sup>a</sup>	36 (30.3-41.6) <sup>b</sup>
Insulin (pmol/L) <sup>§§</sup>	36.8 (28.5-66.6)	51.4 (40.3-77.7)	54.2 (36.1-88.9)
HOMA <sup>§§</sup>	1.12 (0.92-2.15)	1.62 (1.27-2.41)	1.66 (1.12-3.19)
Glucose (mmol/L) <sup>§§</sup>	4.88 (4.69-5.24)	5.05 (4.66-5.5)	5.0 (4.72-5.55)
TC(mmol/L) <sup>§</sup>	4.57 (3.79-5.15) <sup>a</sup>	4.72 (4.1-5.2) <sup>a,b</sup>	4.77 (4.14-6.2) <sup>b</sup>
LDL (mmol/L) <sup>†</sup>	2.8 (2.45-3.2)	3.06 (2.49-3.38)	2.96 (2.42-4.14)
HDL (mmol/L) <sup>§§</sup>	0.98 (0.84-1.09)	1.01 (0.84-1.17)	1.06 (0.91-1.19)
TC/HDL <sup>§</sup>	4.52 (3.57-5.51)	4.67 (3.93-5.19)	4.83 (3.56-5.84)
LDL/HDL <sup>§</sup>	3.07 (2.2-3.55)	2.95 (2.4-3.41)	2.89 (2.12-4.36)
TG (mmol/L) <sup>§§</sup>	0.96 (0.74-1.54)	1.11 (0.82-1.43)	1.47 (0.92-1.83)
AST (U/I) <sup>†</sup>	30 (25.5-36) <sup>a</sup>	28.5 (24.5-38.5) <sup>a</sup>	41 (29-54) <sup>b</sup>
ALT (U/I) <sup>§§</sup>	20 (13-24.5)	19.5 (13.5-25.5)	26 (15-31)
CRP (mg/L) <sup>§§</sup>	0.62 (0.32-1.28)	1.03 (0.68-1.86)	1.01 (0.41-2.14)
LPS (EU/mL) <sup>§§</sup>	0.36 (0.28-0.44) <sup>a</sup>	0.78 (0.64-0.96) <sup>b</sup>	2.28 (1.32-3.77) <sup>c</sup>
VAI <sup>§§</sup>	1.43 (0.99-2.66)	1.52 (0.99-2.04)	1.78 (1.1-2.52)

Data are presented as median (interquartile range).<sup>§</sup>One way ANOVA, post hoc Bonferroni; <sup>§§</sup>One way ANOVA (variable transformed), post hoc Bonferroni; <sup>†</sup>Kruskal-Wallis, followed by Mann-Whitney  
 BMI, body mass index; DXA, Dual-energy X-ray Absortimetry; HOMA, homeostasis model assessment; TC, total cholesterol; LDL, Low-density lipoprotein; HDL, high density lipoprotein; TG, triglycerides; CRP, C-reactive protein; LPS, lipopolysaccharides; VAI, visceral adiposity index  
<sup>a,b,c</sup>Different letters in the same line represent statistical significance (p<0.05)



**Figure 1 – Frequencies (%) of total lean, overweight and obese men in the tertiles of plasma LPS**



**Figure 2 - Frequencies (%) of men without (HOMA≤2.7) and with (HOMA>2.7) insulin resistance in the tertiles of plasma LPS**

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### 3.6. Article 6 (original published) Faecal levels of *Bifidobacterium* and *Clostridium* *coccoides* but not plasma lipopolysaccharide are inversely related to insulin and HOMA index in women

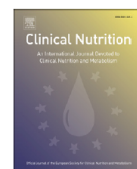
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Original article

## Faecal levels of *Bifidobacterium* and *Clostridium* *coccoides* but not plasma lipopolysaccharide are inversely related to insulin and HOMA index in women



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#### SUMMARY

**Background & aims:** The abundance of specific microbes might be associated with plasma lipopolysaccharide and insulin levels. The aims were to quantify the abundance of specific microbes and plasma LPS in females and assess their association with anthropometric, body composition and biochemical measurements.

**Methods:** Seventeen lean (BMI 19–24.99 kg/m<sup>2</sup>) and fifteen obese females (BMI > 30 kg/m<sup>2</sup>) participated. Anthropometry, body composition, food intake and biochemical analyses were assessed. Bacterial groups in faeces were analysed by qPCR method.

**Results:** *Lactobacillus plantarum* prevalence was higher ( $p = 0.005$ ) and its counts tended to be higher in lean vs. obese group ( $p = 0.06$ ). *Bifidobacterium* genus, *Bifidobacterium longum*, *Clostridium coccoides* and *Clostridium leptum* counts were higher in lean women ( $p < 0.05$ ); prevalence and counts of *Akkermansia muciniphila* tended to be higher in lean group ( $p = 0.09$ ,  $p = 0.06$ , respectively). Plasma LPS levels were similar between the study groups ( $p > 0.05$ ). No association was found between LPS and bacterial levels or insulin. Bifidobacteria and *C. coccoides* counts were inversely associated with insulin and HOMA index. **Conclusions:** Abundance of specific microbes is distinct between obese and lean women, but is not associated with LPS level. Bifidobacterial and *C. coccoides* levels are associated with insulin sensitivity. These bacterial groups may be capable of modulating insulin action.

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#### 1. Introduction

Waist circumference (abdominal obesity), blood pressure, glucose, triglyceride, cholesterol and insulin levels are frequently assessed parameters in clinical practice to estimate the person's risk of developing diabetes and cardiovascular diseases.<sup>1</sup> Insulin and glucose levels can be used to measure the degree of insulin resistance through the homeostasis model assessment (HOMA)

index.<sup>2</sup> Insulin resistance often affects overweight and obese people, and increases the chance of presence of other metabolic alterations such as dyslipidemia and hypertension.<sup>3</sup>

Insulin resistance can be triggered by a chronic inflammatory state. Systemic inflammation is thought to result from adipose tissue enlargement due to continuous metabolic overload (positive energy balance). This leads to recruitment and infiltration of immune cells, which secrete large amounts of inflammatory cytokines.<sup>4</sup> However, it has been suggested that the gut microbiota is a source of the inflammatory factor lipopolysaccharide (LPS).<sup>5</sup> LPS activates the immune cells to secrete inflammatory cytokines and trigger the onset of insulin resistance, obesity and diabetes in mice.<sup>6</sup> In fact, subjects with prevalent and incident diabetes showed higher body mass index (BMI), waist and endotoxin activity than the non-diabetic individuals.<sup>7</sup>

The increase in circulatory levels of LPS can be induced by a high fat diet,<sup>6,8</sup> which in turn can shift the gut microbiota composition, e.g., reducing *Bifidobacterium* levels. A negative correlation

**Abbreviations:** BMI, body mass index; HDL, High Density Lipoprotein; HOMA, homeostasis model assessment; LDL, low density lipoprotein; LPS, lipopolysaccharide; qPCR, Quantitative real-time polymerase chain reaction; TC, total cholesterol; TC/HDL, total cholesterol/high density lipoprotein ratio.

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between endotoxins and *Bifidobacterium* levels has been reported in mice fed with a high fat diet.<sup>8</sup> In addition, the high fat diet and consequently the changes in gut microbiota composition were found to have an impact on modulation of gut permeability.<sup>9,10</sup> Gut permeability might be the interface between gut microbiota, endotoxins and high fat diet in the core of metabolic dysfunctions.<sup>11,12</sup>

The quantity of specific bacterial groups in gut microbiota composition may influence weight management and/or predispose to obesity. This can be demonstrated by differences in the levels of specific bacterial groups e.g. *Bifidobacterium*, *Akkermansia*, *Lactobacillus* and *Clostridium* in subjects with higher vs. lower weight. However, insulin and LPS levels nor the association between the quantity of bacterial groups in the gut microbiota with these biochemical markers were not assessed.<sup>13–15</sup> In addition, characterisation of gut microbiota in lean and obese individuals at species level is scant. Numerous *Bifidobacterium* and *Lactobacillus* species are considered probiotics,<sup>16</sup> and among them some may have a higher potential to positively affect body weight and metabolic profile.

We hypothesised that obese subjects have higher levels of LPS, which could be associated with differences in gut microbiota composition and potentially with insulin levels. Therefore, we analysed the levels of faecal *Bifidobacterium*, *Akkermansia*, *Lactobacillus* and *Clostridium* species and plasma LPS in lean and obese female adults. Association between study bacteria with anthropometric, body composition and biochemical measurements such as insulin and LPS were also assessed.

## 2. Materials and methods

### 2.1. Subjects

Participants were recruited through written advertisements in Viçosa city in Minas Gerais state, Brazil. The inclusion criteria comprised being lean (BMI 19–24.99 kg/m<sup>2</sup>) or obese (BMI > 30 kg/m<sup>2</sup>), nor any diagnosed disease, no pregnancy or lactation, no use of regular medications and no intake of antibiotics in the last three months. Sixty-five women intended to participate in the study. Out of these, 20 lean and 20 obese women fulfilling the criteria were enrolled in the study. Seventeen lean and fifteen obese females of similar age (mean age of the lean and obese group 28.05 ± 6.9 y vs. 30.7 ± 5.7 y,  $p = 0.23$ ) provided a faecal sample and were included in the study. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethical Committee of the Federal University of Viçosa (protocol number 001/2010). A written informed consent was obtained from each participant.

### 2.2. Body composition and blood pressure

In the morning time, fasted participants wearing light clothes, without shoes, were weighed using a digital scale (150 kg, Toledo<sup>®</sup>, São Paulo, Brazil). Height was measured with a fixed stadiometer (Seca<sup>®</sup>, Germany) to the nearest millimeter. Body composition was analysed using a tetra polar bioimpedance system (BodySystems<sup>®</sup>, Washington, USA). Waist circumference was measured in the lowest circle between the lowest rib and umbilicus with a flexible tape. Blood pressure was assessed in the right arm of the volunteer in the sitting position, at rest, with the mercury sphygmomanometer.

### 2.3. Biochemical analysis

After 10 h fasting, the blood samples were collected from an antecubital vein using EDTA tubes; the blood was centrifuged at 2136 × g for 15 min (4 °C) to isolate the plasma. Then the plasma

was divided into aliquots and sent to be immediately processed at the Laboratory of Clinical Analysis of the Health Division at the Federal University of Viçosa or stored at –80 °C. The following assessments were made according to the laboratory standards: haemogram (Coulter T-890/Beckman Coulter<sup>®</sup>, USA), total cholesterol and lipoproteins (enzymatic colourimetric method), fasting plasma glucose (enzymatic colourimetric method of glucose-oxidase), (all kits used were from Bioclin/Quibasa, Brazil), and insulin by electrochemiluminescence using the Modular Analytics E170 e Elecsys 2010 (Roche Diagnostics<sup>®</sup>). Homeostasis model assessment (HOMA) indices were calculated as follows: fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5<sup>2</sup>.

### 2.4. Plasma LPS

The plasma LPS levels were determined using Limulus Amebocyte Lysate kit (QCL-1000, Lonza, Maryland, USA). Briefly, 50 µL of plasma was diluted with 450 µL of bacterial endotoxin-free water (1:10 v/v) and incubated at 70 °C for 15 min. A final plasma dilution of 1:70 was obtained after the addition of 3050 µL of bacterial endotoxin-free water. Endotoxin standard solutions were prepared at the concentrations ranging from 0.1 to 1.0 EU/mL to construct a standard curve. Diluted plasma samples and endotoxin standards solutions were dispensed (50 µL) into pre-equilibrated microplate wells (at 37 °C). The addition of all reagents and incubation times were followed according to manufacturer's instructions. The absorbance from each microplate well was measured at 405 nm (Multiskan Go, Thermo Scientific, USA). A linear prediction equation was generated after plotting the absorbance of standard solutions against its known concentration. The endotoxin concentration ( $x$ ) in the samples was determined using the equation ( $y = 0.3159x + 0.1047$ ,  $r^2 = 0.996$ ) and subsequently multiplied by 70 to correct plasma dilution.

### 2.5. Faecal samples

The subjects were asked to provide a faecal sample preferably within a few hours of collection; otherwise the material was kept at 4 °C for up to 12 h until it was stored at –20 °C prior to lyophilisation. One hundred milligrams of lyophilised faeces were weighed, diluted 1:10 (w/v) in phosphate buffer PBS (pH 7.4), homogenised by thorough agitation and centrifuged (453 × g, 2 min) to collect the supernatant. A 200-µl aliquot of the supernatant was used to extract the DNA using QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

### 2.6. Quantitative real-time polymerase chain reaction (qPCR) analysis of gut microbiota

The qPCR was used to characterise the faecal microbiota using group- and species-specific primers. These oligonucleotides were purchased from the Thermo Electron Corporation (Thermo Biosciences, Ulm, Germany). PCR detection and amplification of the 16S rRNA gene were performed with an ABI PRISM 7300-real-time PCR system (Applied Biosystems, Foster City, California). Each reaction mixture of 25 µL was composed of Power SYBR Green PCR Master Mix (Applied Biosystems), 1 µL of each of the specific primers at a concentration of 0.2 mol/L, and 1 µL of template DNA. The fluorescent products were detected in the last step of each cycle. A melting curve analysis was made after amplification to distinguish the targeted from the non-targeted PCR product. The bacterial concentration in each sample was calculated by comparing the  $C_t$  values obtained from standard curves. A standard curve was made from serial dilutions of DNA isolated from each pure culture of the different reference strains. A linear relationship



was observed between cell numbers and  $C_f$  values ( $r^2 = 0.99–0.97$ ). The following reference strains were used to construct the corresponding standard curves: *Bifidobacterium longum* (DSM 20219) (this strain was also used as the standard strain for quantification of the *Bifidobacterium* genus), *Bifidobacterium catenulatum* (JCM 7130), *Bifidobacterium bifidum* (DSM 20456), *Bifidobacterium adolescentis* (DSM 20083), *Bifidobacterium breve* (DSM 20213), *Akkermansia muciniphila* (ATCC BAA- 835), *Clostridium coccoides* (DSM 935<sup>1</sup>), *Clostridium leptum* (DSM 753<sup>1</sup>). Additionally, we used the following strains, with specific annealing temperatures set in our laboratory: *Lactobacillus acidophilus* DSM 20079 (63 °C), *Lactobacillus paracasei* DSM 20020 (60 °C), *Lactobacillus plantarum* DSM 20174 (58 °C), *Lactobacillus rhamnosus* DSM 20021 (61 °C) and *Lactobacillus casei* DSM 20011 (58 °C). The primer sequences of the reference strains are shown in supplemental Table 1. The amplification conditions for *Lactobacillus* strains were those set in our laboratory and were as follows: 1 cycle 95 °C/10 min, 40 cycles 95 °C/15 s, specific annealing temperatures/40 s, 72 °C/30 s and a dissociation step of 1 cycle 95 °C/15 s, 60 °C/1 min.

## 2.7. Food intake assessment

Participants were instructed to complete three one-day food records on non-consecutive days including one weekend day. Food records were analysed by the software DietWin Professional<sup>®</sup> (Brazil), and the mean value for the three days was used for subsequent statistical comparison of the main macronutrients and total fiber intake between the groups.

## 2.8. Statistics

Anthropometry, body composition and biochemical variables were compared between lean and obese women using Mann–

**Table 1**  
Anthropometric, body composition and biochemical characteristics of lean and obese adult women.

Variables	Lean <sup>a</sup> (n = 17)		Obese (n = 15)		p <sup>b</sup>
	Median	IQR	Median	IQR	
Weight (kg)	53.7	51.9–57.4	88.2	79.4–92.6	<0.001
Height (cm)	158.5	156.4–160.6	159	155.6–160.7	0.98
BMI (kg/m <sup>2</sup> )	21.2	20.6–21.9	34.5	32.8–36.7	<0.001
Waist circumference (cm)	68.0	66.5–72.0	93.5	89.5–97.0	<0.001
Body fat (%)	22.7	18.0–23.8	37.5	36.7–38.9	<0.001
Systolic BP (mmHg)	100	100–110	120	110–120	0.008
Diastolic BP (mmHg)	60	60–70	80	70–80	0.003
Fasting glucose (mmol/L)	4.66	4.55–4.85	4.99	4.82–4.99	0.005
Fasting insulin (pmol/L)	53.4	40.9–62.5	79.1	72.2–145.1	<0.001
HOMA	1.6	1.25–1.86	2.6	2.3–4.4	<0.001
TC (mmol/L)	4.58	4.05–5.06	4.27	3.80–4.81	0.37
HDL (mmol/L)	1.44	1.18–1.62	1.08	0.96–1.22	0.001
LDL (mmol/L)	2.60	2.24–3.11	2.59	2.23–3.19	0.92
TGL (mmol/L)	0.82	0.59–1.12	0.98	0.81–1.24	0.23
TC/HDL	3.0	2.8–3.5	3.6	3.4–4.5	0.02
LDL/HDL	1.8	1.5–2.2	2.3	1.8–3.2	0.03
Leukocytes ( $\times 10^9/L$ )	5.9	4.45–7.0	6.4	5.3–7.1	0.25
Eosinophils ( $\times 10^9/L$ )	0.13	0.06–0.25	0.13	0.06–0.19	0.59
Lymphocytes ( $\times 10^9/L$ )	1.84	1.5–2.4	2.06	1.8–2.2	0.23
Monocytes ( $\times 10^9/L$ )	0.11	0.05–0.15	0.13	0.091–0.16	0.52
Platelets ( $\times 10^9/L$ )	195	173–242	215	184–269	0.41
LPS (EU/mL)	15.1	11.0–22.0	14.9	8.9–28.9	0.81 <sup>b</sup>

BP, blood pressure; HOMA, Homeostasis Model Assessment; TC, Total Cholesterol; TGL, Triglycerides; TC/HDL, Total Cholesterol/High Density Lipoprotein ratio, LPS, Lipopolysaccharide.

<sup>a</sup> Biochemical data from lean group n = 16.

<sup>b</sup> Mann–Whitney test.

Whitney *U* test and are shown as median with interquartile range (IQR). The bacterial prevalence (%) and counts (log cells/g) were analysed. For the comparison of bacterial species prevalence between the study subject groups, chi-square test or Fisher's exact test, as appropriate, was used. Logarithmic numbers of bacteria are represented as medians with IQR. Comparison between the groups was analysed using Mann–Whitney *U* test. Bacterial species counts, which showed significant differences between lean and obese subjects were further assessed for correlation with other analytical variables (Spearman's correlation test was applied). Associations between bacterial log numbers (above the detection limit) and response variables that could be influenced by dietary factors (weight, BMI, fat percentage, waist circumference, HDL, TGL, glucose, insulin and HOMA) were further studied using multiple regression analysis (GLM procedure) controlling for carbohydrate, fat and energy intake. In the case of *L. plantarum* the association between response variables and the actual level of the variable was based solely on observations with values above the detection limit, also using multiple regression analysis controlling for carbohydrate, fat and energy intake. Since BMI can also interfere with anthropometric and biochemical variables, a second model was run controlling for BMI and energy intake (GLM procedure or REG procedure for *L. plantarum*) for those variables that reached significance in the first model. Statistical analyses were performed using SAS (for windows version 9.2, SAS Institute Inc., USA). Differences were considered significant at  $p < 0.05$ .

## 3. Results

### 3.1. Subject characteristics and food intake

Women from both groups were of similar height but all anthropometric and body composition measurements were higher in the obese group ( $p < 0.05$ ), as expected by the study design. Blood pressure and fasting glucose, although within normal limits for both groups, were lower in the lean compared to obese group ( $p < 0.05$ ). Fasting insulin and HOMA indices were higher in the obese group ( $p < 0.001$ ). Regarding lipid profile, the only lipoprotein fraction, which differed between the study groups was HDL, and it was lower in the obese group ( $p = 0.001$ ), while the ratio of total cholesterol or LDL to HDL was higher in the obese group ( $p < 0.05$ ). The immune cell counts were similar in both groups. Plasma LPS concentration did not differ between lean and obese women (Table 1). The obese group had a higher net intake of energy ( $9224.77 \pm 2152$  kJ/d vs.  $7361.6 \pm 1289.5$  kJ/d;  $p = 0.01$ ), carbohydrate ( $300 \pm 89$  g/d vs.  $230 \pm 45.6$  g/d;  $p = 0.01$ ) and fat ( $79.8 \pm 18.7$  g/d vs.  $63.9 \pm 16.0$  g/d;  $p = 0.03$ ) in comparison to the lean group. A tendency for a higher protein intake ( $73.3 \pm 22.3$  g/d vs.  $60.7 \pm 12.1$  g/d,  $p = 0.06$ ) was noted in the obese subjects. Regarding the intake of fiber there was no difference observed between obese and lean groups ( $19.5 \pm 11.8$  g/d vs.  $19.8 \pm 8.9$  g/d;  $p = 0.42$ ).

### 3.2. Microbiota analysis

*L. casei* was the only bacterial species not detected in the faecal samples from either group. The only bacterial species that differed regarding the prevalence was *L. plantarum*, whose presence was more common in the lean group ( $p = 0.005$ ), (Table 2). A tendency for a less frequent presence of *A. muciniphila* in obese subjects was detected ( $p = 0.09$ ).

*Bifidobacterium* genus, *B. longum*, *C. coccoides* and *C. leptum* counts were lower ( $p < 0.05$ ) in obese subjects, while a tendency for lower counts in obese subjects was also observed for *L. plantarum* and *A. muciniphila* ( $p = 0.06$ ).

**Table 2**  
Detection of bacterial groups and counts in faecal samples from lean and obese adult women.<sup>a</sup>

Bacteria	Lean (n 17)			Obese (n 15)			p <sup>b</sup>
	Prevalence (%)	Log cells/g (median)	IQR	Prevalence (%)	Log cells/g (median)	IQR	
<i>A. muciniphila</i>	100	8.9	5.7–9.4	80	6.6	4.4–8.8	0.06
<i>Bifidobacterium</i> genus	100	10.4	9.7–10.6	100	9.3	8.9–9.6	0.0005
<i>B. adolescentis</i>	71	6.3	0–6.7	47	0	0–6.0	0.10
<i>B. bifidum</i>	82	7.06	6.16–7.8	60	6.4	0–7.3	0.11
<i>B. breve</i>	18	0	0–0	27	0	0–4.9	0.54
<i>B. catenulatum</i>	94	7.6	5.8–9.3	80	6.2	3.7–8.2	0.21
<i>B. longum</i>	100	9.8	8.8–10.1	100	8.2	7.8–9.0	0.009
<i>C. coccoides</i>	100	10.6	10.4–10.8	100	10.0	9.7–10.7	0.036
<i>C. leptum</i>	100	9.9	9.7–10.0	100	9.2	8.9–9.6	0.0014
<i>L. acidophilus</i>	6	0	0–0	13	0	0–0	0.47
<i>L. paracasei</i>	41	0	0–6.3	27	0	0–6.4	0.38
<i>L. plantarum</i>	76*	5.6	5.2–5.7	27*	0	0–5.7	0.06
<i>L. rhamnosus</i>	65	6.33	0–6.8	47	0	0–6.7	0.32
<i>L. casei</i>	0			0			1.0

\*p value = 0.005 for comparison of percentages of positive samples for *L. plantarum* using chi-square test. The percentage of positives of other microbial groups did not differ between lean and obese.

<sup>a</sup> Faecal microbiota analysed by quantitative real-time polymerase chain reaction.

<sup>b</sup> p value for comparison of bacterial log numbers between lean and obese using Mann–Whitney U test.

### 3.3. Correlation between microbiota and metabolic risk factors

Bacterial species whose counts differed statistically or tended to differ between lean and obese individuals (Table 2) were included in the correlation analyses. When all subjects were evaluated together, weight correlated with *A. muciniphila* ( $r = -0.42$ ,  $p = 0.01$ ), *Bifidobacterium* genus ( $r = -0.61$ ,  $p = 0.0002$ ), *B. longum* ( $r = -0.53$ ,  $p = 0.001$ ), *C. leptum* ( $r = -0.61$ ,  $p = 0.0002$ ) and *L. plantarum* ( $r = -0.47$ ,  $p = 0.006$ ). The level of these bacterial groups and *C. coccoides* showed negative correlations with BMI and waist circumference ( $p < 0.05$ ). Body fat percentage correlated inversely with *Bifidobacterium* genus, *B. longum*, *C. leptum*, *C. coccoides* and *L. plantarum* levels ( $p < 0.05$ ). Regarding the immune cells, only the number of lymphocytes correlated negatively with *A. muciniphila* ( $r = -0.45$ ,  $p = 0.01$ ) and *B. longum* ( $r = -0.37$ ,  $p = 0.03$ ) counts. HDL levels showed positive correlation with *A. muciniphila*, *Bifidobacterium* genus, *B. longum* and *C. leptum* ( $p < 0.05$ ), while triglycerides correlated negatively with *C. leptum* ( $r = -0.38$ ,  $p = 0.03$ ) and tended to with *C. coccoides* ( $r = -0.35$ ,  $p = 0.05$ ) levels. Glucose levels tended to inversely correlate with *A. muciniphila* ( $r = -0.33$ ,  $p = 0.06$ ) and *B. longum* ( $r = -0.32$ ,  $p = 0.07$ ) levels. Counts of *Bifidobacterium* genus, *B. longum*, *C. coccoides* and *C. leptum* correlated inversely with insulin levels and HOMA index ( $p < 0.05$ ). However, there was no correlation of plasma LPS levels with the counts of bacterial groups studied (Table 3) neither with weight, BMI, waist, fat or energy intake, triglycerides, glucose and insulin levels (data not shown).

The food intake could bias the correlation between microbial counts and the predictor variables analysed. Since the obese group showed a higher intake of carbohydrate, fat and energy intake these variables were included in the regression analysis model. In the present model, the associations observed for *Bifidobacterium* genus, *B. longum*, *C. coccoides* and *C. leptum* were still significant ( $p < 0.05$ ). HDL and triglycerides no longer showed significant association with *Bifidobacterium* genus, *B. longum* and *C. leptum*. For *Akkermansia* all previously observed associations were no more seen by the regression analysis. The second regression analysis model included BMI and energy intake. It was found that *Bifidobacterium* genus and *C. coccoides* still inversely correlated with insulin and HOMA index while all the other associations were no longer significant (Table 4).

We have previously evaluated intestinal permeability of these volunteers.<sup>17</sup> Then, we also assessed the correlation between these

bacterial counts with intestinal permeability parameters. It was found a tendency for significant inverse correlation only between *Bifidobacterium* genus versus mannitol ( $r = -0.33$ ,  $p = 0.05$ ) and lactulose excretions ( $-0.33$ ,  $p = 0.06$ ).

## 4. Discussion

The present study demonstrates differences in the targeted microbial groups and species in the faeces of lean and obese adult women. Our study is also the first to characterise common *Lactobacillus* species in obese and lean women. The main species detected were *L. paracasei*, *L. plantarum* and *L. rhamnosus*, while *L. casei* was absent in either group. The lean group presented higher concentrations of *Bifidobacterium* genus, *B. longum*, *C. coccoides*, *C. leptum*, and also tended to have higher concentrations of *A. muciniphila* and *L. plantarum*. These bacterial groups might thus represent interesting targets in weight management.

To ascertain whether these bacterial groups could have physiological implications in metabolism, we adjusted the association analyses with dietary intake and BMI. It was uncovered that the majority of significant associations were no longer observed. Associations at the species level might be less significant to the host metabolism than at the bacterial groups studied. A very high variability of abundance (12- to 2200-fold) for the most common species across the individuals studied by Qin and co-workers<sup>18</sup> illustrates the difficulty in defining the importance of the abundance of specific species for the host metabolism. On the other hand, even after adjustments, the groups *Bifidobacterium* genus and *C. coccoides* were shown to inversely correlate with insulin and HOMA index. Thus, these bacteria at higher numbers might be associated with a better metabolic profile. In animal models, an association between gut microbiota, especially bifidobacteria and insulin levels was also found.<sup>6,8</sup>

Lower levels of the *Bifidobacterium* genus in individuals of higher weight have also been reported previously.<sup>13,14,19</sup> Bifidobacteria represent 3–6% of the adult faecal microbiota and their presence has been generally associated with beneficial health effects.<sup>20</sup> Decrease in *Bifidobacterium* group might be induced by a high fat diet, at least in mice. This type of diet induces weight gain and adiposity, and is also accompanied by a higher inflammatory tone in the adipose tissue and metabolic alterations such as glucose intolerance and insulin resistance. It has been reported that the promotion of expansion of *Bifidobacterium* population through



**Table 3**  
Associations between microbial counts and anthropometric and biochemical variables.<sup>a</sup>

	<i>A. muciphinila</i>	<i>Bifidobacterium</i> genus	<i>B. longum</i>	<i>C. coccoides</i>	<i>C. leptum</i>	<i>L. plantarum</i>
Weight	-0.42 (0.01)	-0.61 (0.0002)	-0.53 (0.001)	-0.33 (0.06)	-0.61 (0.0002)	-0.47 (0.006)
BMI	-0.49 (0.003)	-0.57 (0.0006)	-0.52 (0.002)	-0.35 (0.04)	-0.63 (<0.0001)	-0.40 (0.022)
Fat %	-0.27 (0.12)	-0.56 (0.0007)	-0.46 (0.007)	-0.40 (0.02)	-0.63 (<0.0001)	-0.36 (0.04)
Waist	-0.45 (0.009)	-0.58 (0.0004)	-0.52 (0.002)	-0.38 (0.03)	-0.59 (0.0003)	-0.40 (0.02)
Lymphocytes	-0.45 (0.01)	-0.31 (0.08)	-0.37 (0.03)	-0.22 (0.23)	-0.15 (0.39)	-0.13 (0.46)
HDL	0.54 (0.0017)	0.35 (0.04)	0.35 (0.04)	0.20 (0.27)	0.40 (0.02)	0.07 (0.69)
TGL	-0.03 (0.86)	-0.14 (0.42)	-0.03 (0.84)	-0.35 (0.05)	-0.38 (0.03)	0.38 (0.03)
Glucose	-0.33 (0.06)	-0.27 (0.12)	-0.32 (0.07)	-0.004 (0.97)	-0.21 (0.24)	-0.11 (0.55)
Insulin	-0.25 (0.16)	-0.69 (<0.0001)	-0.47 (0.006)	-0.42 (0.01)	-0.47 (0.007)	-0.20 (0.27)
HOMA	-0.28 (0.12)	-0.65 (<0.0001)	-0.46 (0.009)	-0.38 (0.03)	-0.47 (0.006)	-0.18 (0.30)
LPS	-0.19 (0.31)	-0.01 (0.92)	0.04 (0.84)	0.08 (0.67)	-0.05 (0.79)	0.05 (0.79)

TGL, Triglycerides; HOMA, Homeostasis Model Assessment; LPS, lipopolysaccharide. The *p* values for each association are shown in parenthesis.

<sup>a</sup> Data are given as correlation coefficient (*r*), which was calculated using of Spearman correlation test.

prebiotic use reduced the harmful effects of high fat diet on metabolism.<sup>8</sup> In the present study, obese women had higher net intake of fat (g/d) and lower levels of *Bifidobacterium* species in faeces. This is consistent with the view that gut microbiota composition can be associated with variations in the diet. Specifically, a diet with higher amount of fat may be unfavourable to bifidobacteria.

A high fat diet also seems to increase the intestinal absorption of LPS, commonly known as metabolic endotoxemia. High serum endotoxin activity was strongly associated with metabolic alterations such as dyslipidemia and insulin resistance in diabetic patients.<sup>21</sup> The proposed mechanisms are related to the ability of bacterial LPS to activate Toll-like receptors (TLRs), whose abnormal activation has been linked to insulin resistance through the induction of secretion of inflammatory mediators.<sup>22</sup> Thus, LPS may play a role in the low-grade inflammation and insulin resistance, which can be observed in obesity.<sup>23,24</sup> It is suggested that disturbances in gut permeability relate to a higher LPS levels in obese mice.<sup>9,25,26</sup> Gut permeability is showed to be influenced by a high fat diet<sup>10</sup> and also by modulation of gut microbiota (e.g. increase in *Bifidobacterium*).<sup>9</sup> Recently it has been reported that bifidobacteria seem to be able to improve gut barrier function and attenuate

bacterial/endotoxin translocation.<sup>9,27</sup> The administration of anti-biotic to obese mice modulated the gut microbiota, which was associated with reductions of the LPS levels and improvement of the insulin sensitivity.<sup>26,28</sup>

In this context, we expected to find higher LPS levels in obese subjects. Firstly, because we found a higher fat intake and lower levels of faecal bifidobacteria in obese subjects; secondly, we have previously showed that insulin and HOMA index were higher in women excreting higher quantities of mannitol and lactulose, indicative of higher intestinal permeability.<sup>17</sup> We also observed inverse association between *Bifidobacterium* genus and intestinal permeability parameters. Surprisingly, LPS levels between lean and obese study women were found to be similar. In addition, LPS levels did not correlate with bacterial groups, insulin, HOMA index or fat intake. The reason for this could be that: 1) our sample size is too small to detect statistical difference in LPS levels between the groups and 2) our obese women did not present serious metabolic alterations such as diabetes. Pussinen and co-workers<sup>7</sup> reported significantly higher LPS levels in individuals who presented at the same time diabetes and higher BMI in comparison to those without diabetes. Therefore, further studies are needed to clarify the association between the gut microbiota and LPS in humans. Since our

**Table 4**  
Association between bacterial counts and clinical variables in two multiple regression model.<sup>a,b</sup>

	<i>A. muciphinila</i>	<i>p</i>	<i>Bifidobacterium</i> genus	<i>p</i>	<i>B. longum</i>	<i>p</i>	<i>C. coccoides</i>	<i>p</i>	<i>C. leptum</i>	<i>p</i>	<i>L. plantarum</i> <sup>c</sup>	<i>p</i>
Weight	-1.9 (1.7)	0.28	-12.5 (4.1)	0.005	-6.7 (2.5)	0.01	-11.2 (5.9)	0.07	-18.0 (4.5)	0.0005	-20.8 (4.4)	0.0001
BMI	-0.8 (0.7)	0.24	-5.0 (1.6)	0.004	-2.6 (0.99)	0.01	-4.5 (2.3)	0.06	-7.2 (1.7)	0.004	-7.9 (1.7)	0.0001
Fat %	-0.89 (0.92)	0.34	-6.9 (2.0)	0.002	-3.5 (1.2)	0.01	-6.5 (3.0)	0.03	-9.6 (2.2)	0.0002	-10.0 (2.5)	0.0007
Waist	-1.85 (1.3)	0.17	-9.0 (3.0)	0.006	-4.8 (1.8)	0.01	-8.5 (4.3)	0.06	-12.7 (3.3)	0.0009	-14.6 (3.3)	0.0002
HDL	1.8 (1.4)	0.21	3.4 (3.8)	0.38	2.3 (2.2)	0.31	3.7 (5.2)	0.48	6.5 (9.8)	0.16	2.3 (5.1)	0.64
TGL	-0.74 (3.3)	0.82	-0.20 (8.4)	0.98	0.5 (4.9)	0.92	-11.3 (11.1)	0.32	-10.0 (4.5)	0.32	-7.4 (10.4)	0.48
Glucose	0.007 (0.55)	0.98	-1.7 (1.5)	0.26	-1.44 (0.89)	0.11	-0.39 (2.1)	0.85	-1.5 (1.8)	0.42	-1.4 (2.1)	0.51
Insulin	-0.12 (0.52)	0.80	-4.0 (1.2)	0.002	-1.9 (0.77)	0.02	-5.8 (1.5)	0.001	-4.5 (1.49)	0.005	-1.6 (1.8)	0.39
HOMA	-0.02 (0.11)	0.83	-0.93 (0.28)	0.003	-0.4 (0.17)	0.01	-1.3 (0.37)	0.001	-1.0 (0.35)	0.006	-0.35 (0.43)	0.42
			-0.67 (0.31)	0.04	-0.29 (0.18)	0.12	-0.72 (0.38)	0.06	-0.36 (0.43)	0.41	0.75 (0.49)	0.14

TGL, Triglycerides; HOMA, Homeostasis Model Assessment.

<sup>a</sup> Data are given as regression coefficient (*b*) and standard error (SE) in parenthesis. For each bacterial group *p* values for the association between bacterial count and clinical variables are presented.

<sup>b</sup> For each clinical variable, first line refers to results from the model controlling for energy, carbohydrate and lipid intake. The second line refers to results from the model controlling for BMI and energy intake.

<sup>c</sup> Multiple regression analysis based solely on observations above detection limit.

study subjects were young adults, it is possible that the differences seen in the specific microbial groups may in future lead to the increase in LPS levels. This could concomitantly occur with the development of metabolic alterations and associated disorders.

Our findings also suggest that higher concentrations of *C. coccoides* may potentially be beneficial since lean women had higher levels of this species. Moreover, this bacterium was also inversely correlated with insulin and HOMA index. *C. coccoides* is considered to be among the most predominant bacterial groups in adult individuals.<sup>29</sup> However, controversial findings regarding the abundance of these bacterial groups in obese subjects in comparison to controls have been reported in the literature. In one study, similar levels were observed between lean and morbidly obese individuals.<sup>30</sup> The results from other studies suggest that higher levels of this bacterial group may be unfavourable to weight management.<sup>13,15</sup> On the other hand, evidences from animal studies are inconclusive about the importance of *C. coccoides*. It was shown that a high fat diet significantly lowered the numbers of *C. coccoides*, while prebiotic use increased its levels in mice. A similar trend was observed for *Bifidobacterium*, however, no significant associations between *C. coccoides* and endotoxin or other parameters were observed in mice receiving high fat diet.<sup>8</sup> Thus, the implication of the abundance of this bacterial group to the host physiology still needs further investigation.

Taken together, our results indicate that faecal microbiota of obese women is distinct from lean, regarding the abundance of *Bifidobacterium* genus, *Bifidobacterium longum*, *C. coccoides*, *C. leptum*, *Akkermansia muciniphila* and *L. plantarum* bacteria. The physiological importance of these differences seems to be more related to *Bifidobacterium* genus and *C. coccoides*, which might play a role in insulin sensitivity. Further studies are needed to ascertain whether the associations observed represent greater involvement of microbiota on health and whether these bacterial groups may be capable of modulating insulin action.

### Conflict of interest

None declared.

### Acknowledgements

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### Appendix A. Supplementary data

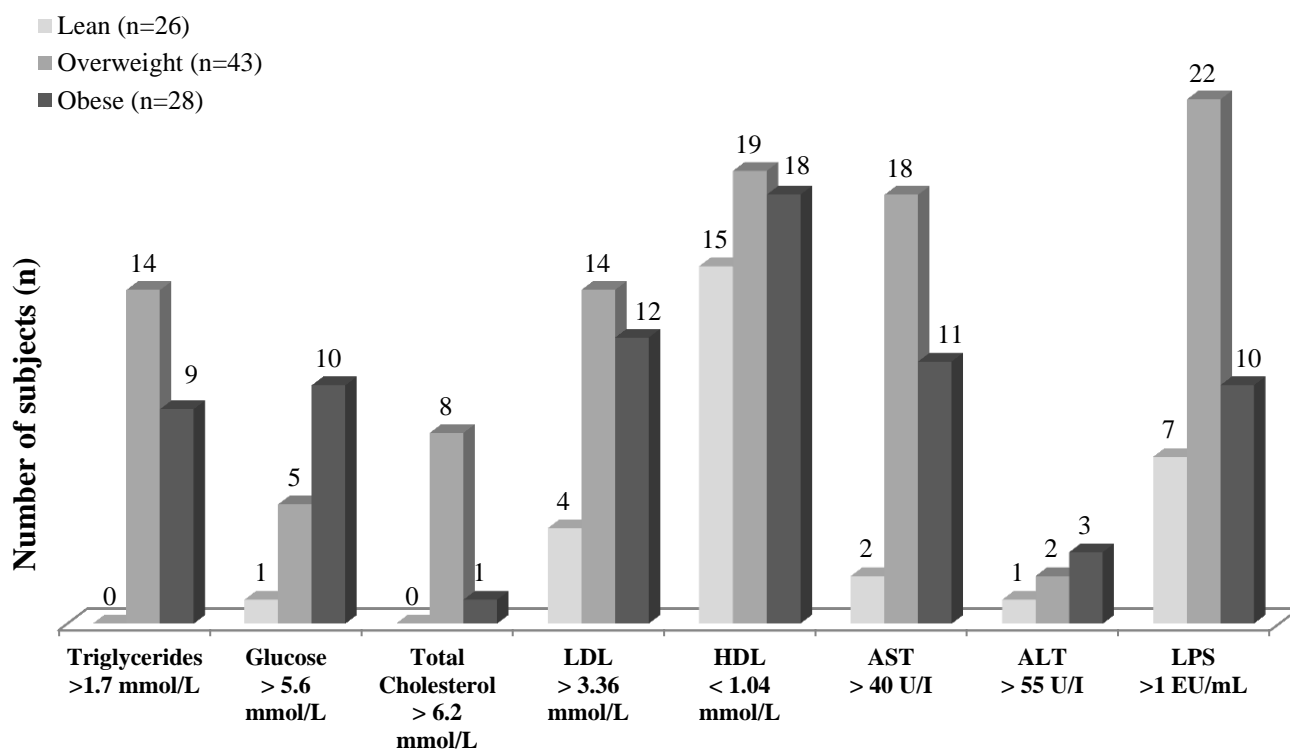
Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.clnu.2013.02.008>.

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#### 4. FINAL CONSIDERATIONS

Obese subjects, as a group, in fact demonstrate an unfavorable metabolic profile compared to lean subjects. This unfavorable profile is here referred as higher concentrations, not necessarily above reference values. This view needs to be better explored in future studies. The figure below show the number of subjects in each BMI category that present altered biochemical values according to reference values.



#### Reference values for biochemical parameters above normal

In this figure, it is possible to observe that a lower proportion of lean subjects showed biochemical alterations compared to those with excess of weight. Nevertheless, the majority of obese subjects did not present biochemical alterations. This is in accordance with the use of terms “metabolically healthy obesity” and “metabolically obese normal weight”. Because the number of subjects in our study is not expressive as the number of subjects usually included in epidemiological studies, it is possible that statistical analyzes using criteria that does not consider biochemical alteration may include individuals “healthy” and “with alterations” in the same group, diluting the strength of the associations that are demonstrated mainly in animal models.

We did not find increased intestinal permeability assessed through lactulose/mannitol test as well as plasma LPS concentrations in obese compared to lean subjects in both

men and women. It is possible that other methods to assess barrier function may show different results and confirm the findings from studies in animal models. These studies show that an altered intestinal microbiota may modulate intestinal permeability. We analyzed fecal microbiota only from women and we found that differences in the prevalence and abundance of bacterial groups between lean and obese women. In particular, the analysis showed that *Bifidobacterium* and *Clostridium coccooides* may influence the degree of insulin resistance. This indicates the importance of more studies analyzing microbiota and intestinal permeability through other method than lactulose and mannitol test.

An important aspect of the present study was that we didn't do only correlation analysis. Specifically, when we investigated the influence of fecal microbiota, most of the significant associations found became insignificant after controlling the analysis for the level of food intake. Similarly, the association between plasma LPS concentration with the degree of insulin resistance, also commonly shown in the literature, lost its significance after controlling the model by the level of hepatic enzymes and fat percentage. The cross talk between adipose tissue and the liver is traditionally considered an important aspect of the development of insulin resistance. How LPS interferes in this cross talk in physiological conditions, i.e., not in infusion models, requires further studies.

The fact that overweight subjects presented the highest concentrations of plasma LPS suggest that there is a need for follow-up studies. This type of study would help to understand if the transition to the obese state is associated with this higher concentration or if it is accompanied by the reduction of plasma LPS concentration. It is also possible that higher plasma LPS concentration remains only in those obese subjects that develop insulin resistance.



## ANNEX 1 – Ethical Committee Approval



MINISTÉRIO DA EDUCAÇÃO

UNIVERSIDADE FEDERAL DE VIÇOSA

COMITÊ DE ÉTICA EM PESQUISA COM SERES HUMANOS-CEP/UFV

*Campus Universitário – Divisão de Saúde - Viçosa, MG - 36570-000 - Telefone: (31) 3899-3783 – e-mail: cep@ufv.br*

Of. Ref. Nº 196/2012/CEP/07-12-E4

Viçosa, 18 de dezembro de 2012

Prezada Pesquisadora:

Cientificamos Vossa Senhoria de que o Comitê de Ética em Pesquisa com Seres Humanos, em sua 7ª Reunião de 2012, 2ª sessão, realizada em 17 de dezembro de 2012, analisou e **APROVOU** o projeto de pesquisa intitulado “*Efeitos do Consumo Agudo do Amendoim Rico em Ácido Graxo Oléico sobre o Apetite, Metabolismo Energético, Parâmetros Bioquímicos, Composição da Microbiota e Permeabilidade Intestinal em Homens Eutróficos e com Excesso de Peso*”.

Esclarecemos que, quando da conclusão do projeto, é necessário o envio à Secretaria do CEP/UFV do relatório final nos termos do item VII, 13, da Resolução CNS n. 196/96, com o fim de verificar os aspectos éticos da realização do trabalho.

Atenciosamente,

A handwritten signature in black ink, appearing to read 'Patrícia Aurélio Del Nero'.

*Professora Patrícia Aurélio Del Nero*

Comitê de Ética em Pesquisa com Seres Humanos – CEP/UFV

Presidente

À Pesquisadora

Neuza Maria Brunoro Costa

Departamento de Nutrição e Saúde - DNS

## ANNEX 2 – Statement of informed consent



Universidade Federal de Viçosa  
Centro de Ciências Biológicas e da Saúde  
Departamento de Nutrição e Saúde

Estou ciente de que:

1. Os procedimentos que serão adotados na pesquisa “Efeitos do consumo de amendoim na composição corporal, metabolismo energético, apetite, marcadores de inflamação e do estresse oxidativo e na microbiota e permeabilidade intestinal em obesos” consistem em: aplicação de questionários para obtenção de dados pessoais, ingestão alimentar e nível de atividade física; avaliações antropométricas (peso, altura, circunferência da cintura/quadril e composição corporal); de medida da pressão arterial; de exames de sangue (por punção digital e venosa) e de gasto energético; coleta de urina e fezes. O estudo completo terá duração de 4 semanas consecutivas, sendo que o voluntário seguirá durante este período uma dieta hipocalórica e receberá ou não uma porção de amendoim para ser consumida diariamente.
2. Como participante do estudo não serei submetido a nenhum tipo de intervenção que possa causar danos à minha saúde, visto que as condutas a serem adotadas objetivam a promoção da mesma e são respaldadas na literatura científica.
3. Estou ciente de que não terei nenhum tipo de vantagem econômica ou material por participar do estudo, além de poder abandonar a pesquisa em qualquer etapa do desenvolvimento, sem qualquer prejuízo.
4. Estou em conformidade que meus resultados obtidos estarão disponíveis para a agência financeira e para a equipe envolvida na pesquisa e poderão ser publicados com a finalidade de divulgação das informações científicas obtidas, sempre resguardando minha individualidade e identificação.

De posse de todas as informações necessárias, concordo em participar do projeto.

Data: \_\_\_/\_\_\_/\_\_\_

\_\_\_\_\_

*Voluntário*

Prof<sup>ª</sup> Rita de Cássia G. Alfenas  
Responsável pelo projeto

Prof<sup>ª</sup> Neuza Maria Brunoro Costa  
Responsável pelo projeto

Ana Paula Boroni Moreira  
Doutoranda

Raquel Duarte Moreira Alves  
Doutoranda