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Novas perspectivas para o papel de amilóide sérica A (SAA) na obesidade e resistência à insulina

Edson Mendes de Oliveira

Tese para obtenção do grau de
DOUTOR

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Edson Mendes de Oliveira

Novas perspectivas para o papel de amilóide sérica A (SAA) na obesidade e resistência à insulina

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São Paulo, 02 de março de 2015.

Choose a job you love, and you will never have to work a day in your life.

Confucius

We are what we repeatedly do. Excellence, therefore, is not an act, but a habit.

Aristotle

Dedicated to the loving memory of my mother,
Ana Maria Mendes de Oliveira.

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RESUMO

OLIVEIRA, E.M. **Novas perspectivas para o papel de amilóide sérica A (SAA) na obesidade e resistência à insulina.** 2015. 119f. (Tese de Doutorado). Faculdade de Ciências Farmacêuticas. Universidade de São Paulo, São Paulo, 2015.

Endotoxemia crônica de baixo grau tem um importante papel na obesidade e resistência à insulina associada a uma ração hiperlipídica. Por outro lado, embora se saiba que a endotoxemia intensa e infecção reduzam o apetite e induzam a um intenso catabolismo, conduzindo a perda de peso durante a fase aguda da inflamação, os efeitos tardios da endotoxemia intensa nunca foram explorados. Aqui mostramos que, além dos efeitos correntes, a endotoxemia aguda provoca alterações bioquímicas prolongadas no tecido adiposo que intensificam os efeitos deletérios de uma ração hiperlipídica. Camundongos submetidos à endotoxemia aguda apresentaram aumento na expressão de TLR-4, CD14 e SAA3 no tecido adiposo, permanecendo alteradas após uma semana em recuperação. Quando associado a uma ração hiperlipídica, os camundongos previamente submetidos à endotoxemia aguda mostraram um ganho de peso mais pronunciado e uma maior resistência à insulina. Adotando a ração hiperlipídica como um estímulo obesogênico, foi avaliada a participação da proteína amilóide sérica A (SAA) no desenvolvimento da obesidade. Usando um oligonucleotídeo antisense anti-SAA, observamos que a depleção da SAA previne as alterações metabólicas, elevação de endotoxina, ganho de peso e resistência à insulina associadas a ração rica em gordura. O sono inadequado é outro fator importante a ser considerado na epidemia de obesidade. Descobrimos que a restrição do sono (SR) provoca alterações bioquímicas e morfológicas no tecido adiposo de camundongos. A concentração de resistina no soro e a expressão de mRNA no tecido adiposo de resistina, TNF-α e IL-6 foram aumentadas após SR. Quando associado a uma ração hiperlipídica, os camundongos submetidos previamente à SR ganharam mais massa com aumento da infiltração de macrófagos no tecido adiposo epididimal, e resistência à insulina. SAA também faz parte das alterações bioquímicas iniciais provocadas pelo SR. Observou-se que a expressão de SAA no fígado e tecido adiposo é regulada positivamente, com retorno ao basal quando o sono é restaurado. Além disso, 48

horas de restrição de sono total em voluntários humanos saudáveis também causou uma elevação nas concentrações séricas de SAA. Considerando que SAA induz proliferação, sugerimos que situações onde ocorra aumento na produção de SAA e a consecutiva proliferação celular, o tecido adiposo se tornaria predisposto a futura diferenciação e hipertrofia. Além disso, sugerimos que SAA altera a sinalização de LPS, possivelmente inibindo sua depuração. O mecanismo de associação entre a inflamação e a obesidade é complexo e inclui uma diversidade de fatores; a proteína inflamatória SAA pode ser um deles. Em conclusão, nossos dados descrevem a relação entre SAA, inflamação aguda, restrição do sono e obesidade.

Palavras-chave: adipócito, endotoxemia, fase aguda, inflamação, restrição de sono.

ABSTRACT

OLIVEIRA, E.M. **New insights into the role of serum amyloid A (SAA) on obesity and insulin resistance.** 2015. 119p. (Doctoral Dissertation). Faculdade de Ciências Farmacêuticas. Universidade de São Paulo, São Paulo, 2015.

Chronic low-grade endotoxemia is an important player in obesity and insulin resistance associated to a high-fat diet (HFD). On the other hand, although it is known that intense endotoxemia and infection reduce appetite and induce intense catabolism, leading to weight loss during the acute inflammatory phase, the late effects of an intense endotoxemia were previously unexplored. Here we report that, besides the concurrent effects, multiple and intense endotoxemia causes long lasting biochemical alterations in the adipose tissue that intensify the harmful effects of a HFD. Mice submitted to multiple and severe endotoxemia had increased the adipose tissue expression of TLR-4, CD14 and SAA3, remaining altered after one week in recovery. When associated to a HFD, mice previously submitted to acute endotoxemia showed a more severe weight gain and impaired insulin sensitivity. Adopting the HFD as an obesogenic stimulus, we evaluated the participation of the protein serum amyloid A (SAA) in obesity development. Using a SAA-targeted antisense oligonucleotide, we observed that the depletion of SAA prevented metabolic alterations, endotoxin elevation, weight gain and insulin resistance in a diet-induced obesity protocol. Inadequate sleep is another important factor to be considered in the obesity epidemic. We found that sleep restriction (SR) causes biochemical and morphological alterations in mice adipose tissue. The levels of serum resistin and the adipose tissue mRNA expression of resistin, TNF-α and IL-6 were increased after SR. When associated to a HFD, mice previously submitted to SR gained more weight with increased macrophage infiltration in the epididymal adipose tissue, and insulin resistance. SAA is also part of the initial biochemical alterations caused by SR. It was observed that the expression of SAA in liver and adipose tissue is upregulated, with return to baseline when sleep is restored. Furthermore, 48 hours of total sleep restriction in healthy human volunteers also caused a serum elevation in SAA concentrations. Considering that SAA induces cell proliferation, we suggest that situations with an increase in SAA production and the consecutive preadipocyte proliferation would prime the adipose tissue to further

adipocyte differentiation and hypertrophy. Furthermore, we suggest that SAA alter LPS signaling, possibly inhibiting its clearance. The mechanism associating inflammation and obesity is complex and encompass a diversity of factors; the inflammatory protein SAA may be one of them. In conclusion, our data describes the relationship between SAA, acute inflammation, sleep restriction and obesity.

Keywords: adipocyte, endotoxemia, acute inflammation, inflammation, sleep restriction

INTRODUCTION

This dissertation is organized as a literature review regarding the role of SAA in the adipose tissue and obesity, including the contributions made during my doctorate period, which are organized as three papers (**P1** – page 22, **P2** – page 27 and **P3** – page 36) and two manuscripts in preparation (draft **D1** – page 45 and draft **D2** – page 80), attached right after the introduction section.

Serum amyloid A (SAA)

Serum amyloid A protein (SAA) belongs to the family of apolipoproteins produced in countless vertebrates studied to date, especially many mammalian species including human, mouse, hamster, dog, rabbit, cow, sheep and horse. The high degree of conservation of SAA genes, which has been maintained through evolution of placental mammals and extending the other vertebrates including fish and marsupials, corroborate the evidence they have important biological functions[1].

In humans, the SAA family includes a number of genes that are closely related but differently regulated, showing four distinct genes localized on the short arm of chromosome 11p15.1[2; 3]. Inducible *SAA1* and *SAA2* genes specify the two acute phase SAA (SAA1 and SAA2) whose expression is induced in response to pro-inflammatory stimuli[4], encoding 104 amino acids, molded into a protein of 12.5 kDa and sharing 93% identity in its sequence of amino acids. A third gene, called *SAA3* which shows 71% identity with *SAA1* and *SAA2* is considered a pseudogene[5]. The constitutive SAA (SAA4) is another SAA family member being the product of the *SAA4* gene[6]. The SAA4 differs from SAA1 and SAA2 as regards the peptide chain, being eight amino acids longer, and showing an identity of approximately of 55% compared to both of them. In addition, SAA4 probably has a posttranslational modification, a glycosylation at a single point in the amino acid asparagine (N), number 76 in the polypeptide chain of the protein[7].

In mice, four functional and distinct isoforms of SAA have been identified, SAA 1-4[1]. Isoforms 1 and 2, whose expression and synthesis in the liver are induced in response to pro-inflammatory stimuli (inflammatory and/or infectious response)[4].The SAA3 is mainly expressed in extrahepatic tissue but also considered an acute SAA, being the most abundant isoform in adipose tissue[8; 9; 10] and the SAA4 that is

considered constitutively active as its serum levels do not change in the presence of acute inflammatory or infectious stimuli[7].

Although SAA have been sequenced in several species, there is a lack of studies concerning its structure. It is known that SAA has a α-helix structure in the amino terminal region, allowing its connection with the high-density lipoprotein (HDL)[11]. Besides the association between HDL and SAA be essential for the transport of SAA in the bloodstream, it is believed that this association is important for keeping the SAA inactive during transport[12]. Dissociated SAA acts as a powerful stimuli for inflammatory cells[13], featuring the HDL as a safe SAA carrier to specific sites[14].

Hepatocytes produce SAA from stimulus such as the inflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin 1 (IL-1) and interleukin 6 (IL-6)[11; 15]. The inflammatory response is responsible for activate a cascade of reactions, collectively known as the acute-phase response (APR), that contribute to the protection of the host from tissue damage, infection or trauma. In healthy subjects, the plasma concentration of SAA is about 10 µg/mL, which could increase 1000 times in 24 hours in response to tissue damage or infection[16]. A permanent but slight increase in serum SAA is observed in cases of chronic diseases such as diabetes[17], rheumatoid arthritis[18], cancer[19] and atherosclerosis[20] and is believed to contribute to their pathogenesis mechanisms. For instance, SAA directly accelerates the atherosclerosis development, leading an increased LDL retention in atherosclerotic lesions[20; 21].

The half-life of SAA in plasma is about 90 minutes[22], and its catabolism also occurs in the liver. However, the liver's ability to degrade SAA during the acute phase is reduced, which maintains the high protein concentration in the plasma[23]. SAA is also synthesized in extra-hepatic tissues, including monocytes, macrophages, endothelial cells and smooth muscle cells[11]. Adipocytes also express and secrete SAA, characterizing the adipose tissue as another important source of this protein in addition to the liver[24].

Free SAA is only found in inflamed tissues, suggesting a role in the local inflammatory response[25]. Although until now no receptor dedicated solely to SAA has been identified, several studies suggest different types of receptors for SAA: (i) FPR-2 (formyl-peptide receptor 2) a transmembrane G-protein coupled receptor that is responsive to pertussis toxin, and related to chemotactic activity, cytokine production

and angiogenesis[26; 27; 28]; (ii) CD36, also known as FAT (fatty acid translocase), a member of the class B scavenger receptor family of cell surfaces proteins, expressed by monocytes, macrophages, endothelial cells and adipocytes[29; 30]; (iii) SR-BI is another member of the class B scavenger receptor family that SAA can bind[31], both scavenger receptors are related to an inflammatory response when activated by SAA; (iv) RAGE, the receptor for advanced glycation endproducts found on mononuclear cells[32]; (v) Tanis (or selenoprotein S), a receptor expressed by the liver and regulated by glucose concentration[33]; (vi) Toll-like receptors family, specially the innate immune related receptors TLR-2[34] and TLR-4[35].

SAA has a high immunological activity and possesses many proinflammatory and cytokine-like properties. In neutrophils, SAA promoted the mRNA expression and release of cytokines related to the inflammatory response, such as TNF-α, IL-1β, IL-1ra and IL-8[13; 36; 37]. Also, SAA induced the production of nitric oxide (NO), CCL20, TNF-α, IL-1β, IL-6, IL-8 and the growth-promoting granulocyte-macrophage colony-stimulating factor (GM-CSF) in monocytic cells[35; 38; 39]. This induction of cytokines is dependent on nuclear factor (NF)-κB and involves the activation of ERK1/2, p38, JNK mitogen-activated protein kinases, and the phosphoinositide-3 kinase pathway[12; 39]. SAA is chemoattractant for polymorphonuclear leucocytes and monocytes in vitro and in vivo, and may be involved in the pathogenesis of several diseases[40]. For instance, neutrophils and monocytes from diabetic subjects responded more efficiently to SAA increasing both the production of cytokines and cell migration[41]. SAA is also able to increase proliferation in fibroblasts[42], endothelial cells[43] and preadipocytes (**P2**). In tumor progression, it is suggested a dual role for SAA, in some cases inducing proliferation, migration and invasion and in other inhibiting these processes [44; 45]. SAA was also identified as a molecule that functions in the placental microenvironment to regulate metalloprotease activity and trophoblast invasion, in a TLR-4-dependent manner, which are key processes in placentation and placental homeostasis[46]. Although SAA is a mediator of the innate immune system, it also participates in the regulation of adaptive immune responses. SAA, via IL-1β (activation of inflammasome), is able to induce a Th17 type of imunne response, being correlated to the pathogenesis of several diseases, including rheumatoid arthritis and asthma[47; 48].

SAA and obesity

Obesity is a disease characterized by excessive accumulation of subcutaneous and visceral fat. Its severity can be measured by complications such as dyslipidemia and cardiovascular disease, type 2 diabetes, certain cancers, respiratory problems, skin problems and disorders of the locomotor system[49; 50; 51]. Currently, obesity is a serious public health problem in developed countries and a growing problem in developing countries[52; 53].

The World Health Organization (WHO) tells that 2.8 million people die each year worldwide, due to diseases related to overweight or obesity. Between 1980 and 2008, the worldwide prevalence of obesity has nearly doubled. In 2008, more than 1.4 billion adults (20 and older) were overweight. Of these, over 200 million men and nearly 300 million women were obese, totaling a number of half a billion individuals, representing 10% of men and 14% of women in the world[54]. According to the Instituto Brasileiro de Geografia e Estatística (IBGE), is increasing the number of obese people in Brazil. Researches indicate that there are 101.8 million overweight adults (20 and older), representing 50.8% of the population and 35.1 million obese, representing 17.5% of the population[55; 56].

The development of obesity is characterized by an increase in the number of cells into the adipose tissue (hyperplasia) and intracellular accumulation of lipids (hypertrophy), the result of cellular proliferation and differentiation. These processes are regulated by endocrine, genetic, metabolic, neurological, pharmacological, environmental and nutritional factors[57; 58; 59]. The adipose tissue way to obesity, it is characterized by adipocyte hypertrophy and a reduction in local blood flow and insufficient angiogenesis, with consequent formation of hypoxic areas. Major inflammation-related adipokines has been shown to be modulated by hypoxia, including IL-6, MIF (macrophage migration inhibitory factor), VEGF, MMP-2[60; 61; 62] and also serum amyloid A (**P1**).

Over the past decades, the inflammatory response has been extensively associated with weight gain and related complications such as insulin resistance and cardiovascular risk[63; 64; 65; 66]. Furthermore, inflammation has been proposed as a link between obesity and its complications[64]. Among the studies correlating proinflammatory molecules with obesity, a meta-analysis comprising 11 cross-sectional studies and 10 prospective studies has established that SAA levels are

positively associated with BMI levels while weight loss is associated with decreased SAA levels[67]. Also, according to Yang et al, SAA derived from adipose tissue (both protein and gene expression) was associated with human BMI as well as its adipocyte size[64].

A causal relation between SAA and obesity is supported by its effect on the induction of pro-inflammatory cytokines and chemokines in adipocytes (**P2**), as in the case of immune cells[68; 69]. SAA also affects adipocyte biology enhancing preadipocyte proliferation mediated by the ERK1/2 signaling pathway (**P2**) and inhibiting adipocyte differentiation by the decreased expression of adipogenesis-related genes, such as *PPARγ2* (peroxisome proliferator-activated receptor γ 2), *C/EBPβ* (CCAAT/enhancer-binding protein β) and *GLUT4* (**P2**)[68; 69].

The inflammatory status associated with hyperplasia and hypertrophy of adipose tissue and the recruitment of macrophages into this tissue characterizes a state of mild chronic inflammation in obesity, leading to insulin resistance and type 2 diabetes[70]. In obese mice, the expression of TNF-α and SAA are strongly related to insulin resistance and metabolic syndrome[71]. It is important to emphasize that adipocytes stimulated with SAA, presented impaired insulin sensitivity (**P2**). SAA also induces an increased lipolysis (**P2**) through an ERK dependent pathway[69]. The increased lipolysis could contribute to the increased circulating levels of free fatty acids and further decreased glucose uptake by muscle and liver found in metabolic disorders[72]. In type 2 diabetes, neutrophils and monocytes are more activated and produces higher concentrations of IL-8, IL-1β, TNF-α and IL-1ra and reactive oxygen species[73]. This basal activation and the failure to appropriately respond to specific stimuli are related to the inflammatory status that accompanies diabetes and deficiencies in normal neutrophils responses, respectively[73]. Furthermore, diabetic patients have an increased serum levels of SAA, and that could be related to the higher responsivity of neutrophils from diabetic patients to SAA[41].

Besides the large amount of adipocytes, other cells contribute to the production of inflammatory mediators in adipose tissue[74]. Several research groups have demonstrated an effective participation of macrophages in inflammatory aspects of obesity[75]. The importance of macrophages in the development of metabolic complications in obesity is related to their ability to produce pro-inflammatory cytokines. In this way, the beginning of this process may be the migration of M1 macrophages into the adipose tissue. In this context, there are

several studies showing the importance of inflammatory mediators such as MCP-1 (monocyte chemotactic protein 1) and its receptor CCR2 and CCR4, that promote cell migration to the inflammatory site, when released by adipocytes[76; 77]. However, other important publications found mild influence of MCP-1 in macrophage infiltration or insulin resistance development[78; 79]. Others mechanisms that could contribute to the migration of macrophages to the adipose tissue may involve the chemoattractive properties of SAA (**P1**).

The identification of SAA as a key factor triggering obesity gain more soundness in recent published studies with SAA 1/2 knockout mice (acute-phase-related isoforms) showing that they gain less weight during theirs lifespan[80] and that SAA3 knockout mice (adipose tissue-induced isoform) gained less weight on a high-fat diet compared to controls, with reduced adipose tissue inflammation and macrophage content[81].

Despite the inflammation resulting from obesity is well characterized, it has been discussed if obesity could be a consequence of the inflammatory process. Although intestinal microbiota have important physiological roles increasing vascularization and blood flow to the intestine, promoting better absorption of nutrients, it also contributes to a moderate endotoxemia, especially with the adoption of a high-fat diet[82; 83]. Accordingly, continuous infusion of low doses of lipopolysaccharide (LPS), mimicking the metabolic endotoxemia caused by a high-fat diet, induces obesity, insulin resistance and diabetes, in addition to the expression and release of TNF-α and IL-6 by adipose tissue in an murine experimental model[84; 85; 86]

The importance of enteric and non-pathogenic bacterial flora in diet-induced obesity was also clearly evidenced by studies that have shown that germ-free animals have a lower percentage of body fat and do not develop obesity and insulin resistance when subjected to a high-fat diet[83]. Another recent study also shows that intestinal LPS is able to increase the migration of macrophages to adipose tissue, contributing to the tissue inflammation[87].

The identification of the role of endotoxemia and the TLR co-receptor CD14 in obesity occurred simultaneously[88]. After that, it was shown that TLR-4-deficient mice are protected from diet-induced insulin resistance, independently of germ-free conditions[89; 90] and that the activation of TLR-4 and its co-receptor CD14 is associated to insulin resistance in adipocytes and to the adipose tissue

development[88; 91; 92]. In 2012, Caricilli *et al.* showed that mice TLR2$^{-/-}$ have a change in the profile of the existing intestinal microbiota, followed by an increment in LPS absorption, subclinical inflammation, insulin resistance and later obesity. The molecular mechanisms involves the activation of TLR4 receptors associated with endoplasmic reticulum stress and activation of the JKN both in liver and in fat tissue, all associated with promotion of inflammation[93].

The ability of low-grade inflammation driven by metabolic endotoxemia to induce obesity led us to consider that intense endotoxemia, mimicking an acute inflammation, may also impact on adipose tissue. Although intense and multiple endotoxemia lead to weight loss, it also causes long-lasting adipose tissue expression of TLR-4, CD14 and SAA (**D1**). These modifications in the adipose tissue by themselves are not enough to impact on weight gain unless a high-fat diet is introduced. When associated to a HFD, mice previously submitted to acute endotoxemia showed a more severe weight gain and impaired insulin (**D1**). The association of acute inflammation with obesity observed by us could explain epidemiological data that show a relationship between children from low-income families with a higher prevalence of childhood diseases[94] and increased risk of obesity in adulthood[95]. In United States of America, from 2003 to 2007, the prevalence of obesity increased by 10% in children and 23% considering only poor children[95; 96]. This study of more than 40,000 children showed that individuals from low-income families have 2 times more chance to become obese than a middle-class child or high[95].Statistics also show that the possibility of becoming a morbid obesity is 1.7 times higher for poor children and adolescents[97]. Poverty associated with poor hygiene, no health education, no access to safe drinking water, inadequate nutrition and air pollution is considered the dominant factor for a higher prevalence of diseases in children[94; 98]. In 2001, Ford *et al.* published the hypothesis that the overweight in adulthood may be associated with an inflammatory condition present in childhood[99]. Although for bacterial infections a direct relation with obesity was not previously considered, viral diseases are considered in the etiology of human obesity. Several experimental models with different animals show a positive correlation between viral infection and obesity[100; 101; 102]. It was shown that a human adenovirus, adenovirus-36 (Ad-36) is able to induce obesity in experimental models using chickens, mice and nonhuman primates[103]. In adipocytes, Ad-36 increased the differentiation and accumulation of lipids and also decreases the release of leptin[103].

In humans, anti-Ad-36 antibodies are more prevalent in obese subjects (30%) than in non-obese (11%)[104].

The fact that SAA, a classical acute phase marker, has proliferative activity on preadipocytes, while inhibiting adipocyte differentiation (**P2**), led us to consider that SAA could participate in preadipocyte proliferation during endotoxemia, and under appropriate conditions differentiate to adipocyte with a consequent weight gain. In order to evaluate the participation of SAA as a triggering setting to insulin sensitivity and weight gain in a HFD-induced metabolic entodoxemia model, SAA-targeted antisense oligonucleotide was used. HFD-fed mice under ASO_{SAA} treatment did not show increase in endotoxemia neither other HFD-related outcomes, as weight gain, visceral and subcutaneous fat accumulation, macrophage infiltration into the adipose tissue or impaired insulin sensitivity (**D1**). This data points to a link between SAA and LPS in the establishment of obesity and insulin resistance. LPS stimulates SAA production and this can be part of the LPS signaling to obesity and insulin resistance. Furthermore, it is possible to predict that SAA compromises LPS clearance by the impairment of the SR-BI (**D1**)[31].

Inadequate sleep is another important factor to be considered in the obesity epidemic, which can be defined as decreased total sleep time or reduced sleep quality, considering the presence of sleep disorders[105]. Voluntary reduction of sleep time has become increasingly common in recent years, mainly due to the demands and opportunities of modern society[106].The sleep restriction may be total (when there is no period of sleep a) or partial (when it takes longer than usual to go to sleep, or when you need to wake up earlier)[107].

In humans, epidemiological studies have evidenced a clear relationship between sleep reduction and obesity[108; 109; 110]. The restriction of sleep is associated with two parallel endocrine changes that can alter food intake: decreased release of the anorectic hormone leptin[111; 112; 113] and increased release of the orexigenic hormone ghrelin[113; 114; 115] resulting in increased feelings of hunger and food intake. Changes in cortisol release profile and growth hormone (GH) are also present in sleep restriction, which promotes stress and the exaggerated stimulation of the sympathetic system, with consequent serum increase of the above hormones[106]. Increased cortisol and GH are related to the regulation of glucose uptake, contributing to insulin resistance and predicting the development of type 2 diabetes[107].

Despite already shown different perspectives by which sleep restriction contributes to the weight gain process, it is not yet fully elucidated all mechanisms involved[108]. In addition, sleep restriction appears to be able to promote an inflammatory response, with increased serum and adipose tissue inflammatory molecules as C-reactive protein (CRP) and proinflammatory cytokines such as IL-6 and TNF-α. Chronic elevation of serum concentration of proinflammatory cytokines and adipose tissue may favor a low-grade inflammation, very similar to that found in obesity[116]. Besides that, TLR-4 is activated in response to sleep loss[117; 118]. Probably the connection between sleep restriction and obesity occurs in persistent elevation of concentration of these cytokines[107].

It was demonstrated that obese patients with severe obstructive sleep apnea (OSA) have plasma SAA levels increased[119; 120]. Thus, in the proinflammatory context related to sleep restriction, defining a sleep restriction experimental model that lead to animal weight gain and insulin resistance could provide further conditions for the study of the participation of SAA in obesity. It was found that sleep restriction causes biochemical and morphological alterations in adipose tissue (**P3**). The levels of serum resistin and the adipose tissue mRNA expression of resitin, TNF-α and IL-6 are increased after sleep restriction. Although during the sleep restriction mice lose weight, they became more susceptible to the harmful effects of a diet-induced obesity protocol. When associated to a HFD, sleep restricted mice gain more weight with increased subcutaneous fat mass and macrophage infiltration in the epididymal adipose tissue. Furthermore, enhanced glucose tolerance and insulin resistance is also observed (**P3**).Using the same sleep restriction protocol, it was described that the expression of SAA in liver and adipose tissue is upregulated under sleep loss condition, with return to baseline when sleep is restored (**D2**).Furthermore, 48h of total sleep restriction in non-obese and non-OSA human volunteers also cause a elevation in SAA concentrations in serum (**D2**). This data points out that sleep restricted individuals are subjected to any outcome derived from the elevation of SAA (**D2**). Corroborating to this data, there are studies showing that sleep restriction leads to the upregulation of TLR-4 expression in leukocytes[117] and also to a low level endotoxemia[121].

Concluding, there is a clear relationship between SAA, endotoxemia and obesity development, possibly driven by a priming process in the adipose tissue via

activation of preadipocyte proliferation. The Figure 1 outlines the contributions included in this dissertation regarding the role of SAA in obesity.

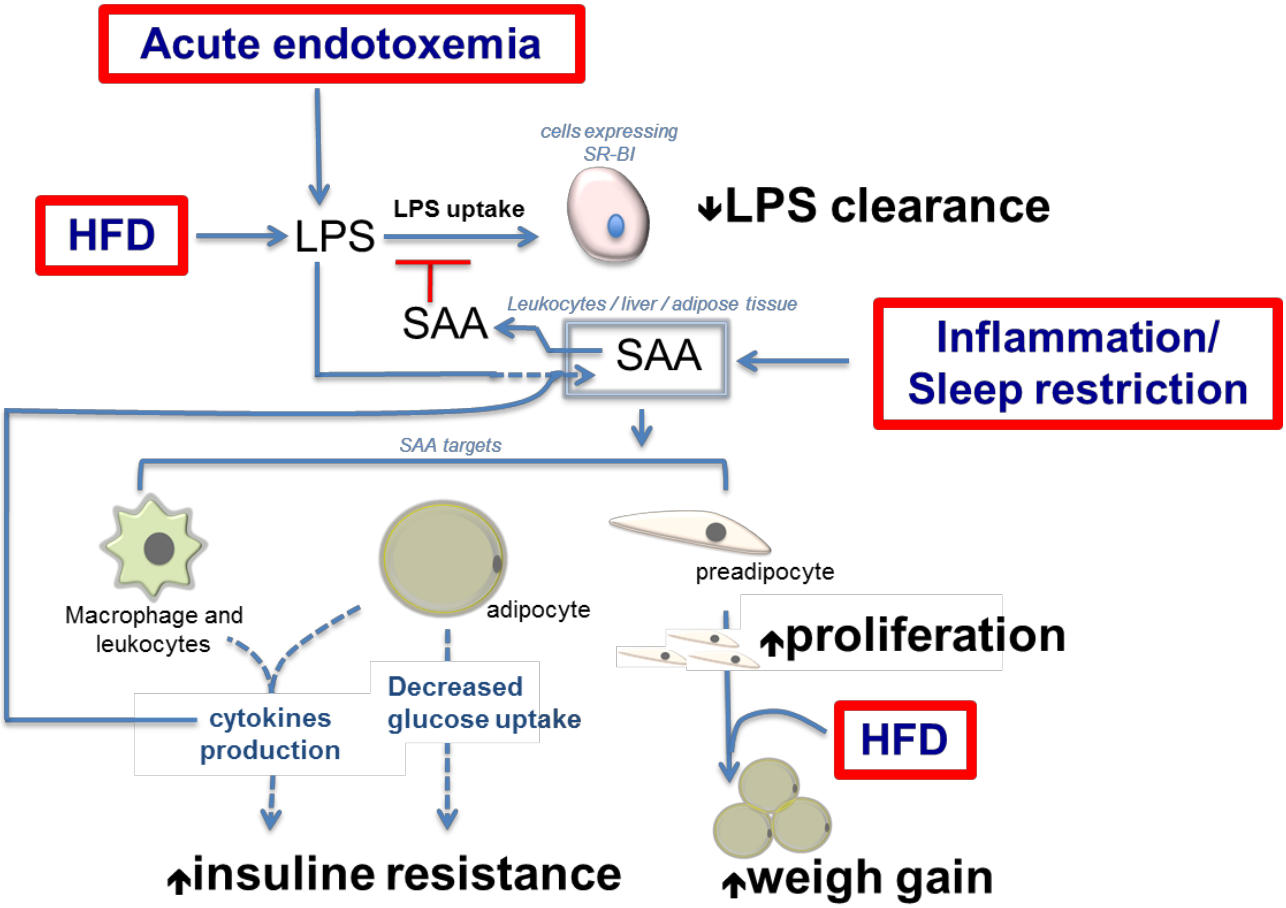

Figure 1. SAA participation in diet, inflammation and sleep restriction-induced insulin resistance and obesity.

Hypoxia Increases Serum Amyloid A3 (SAA3) in Differentiated 3T3-L1 Adipocytes

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Hypoxia Increases Serum Amyloid A3 (SAA3) in Differentiated 3T3-L1 Adipocytes

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Abstract—Hypoxia has been implicated as a possible cause of adipose tissue inflammation. Furthermore, the acute phase protein serum amyloid A (SAA) has been associated with the modulation of the adipogenic process, and it is well-known that obese individuals have increased levels of SAA. The effect of hypoxia in the expression and production of SAA was examined in murine 3T3-L1 adipocytes. Hypoxia leads to a substantial increase in SAA3 mRNA and protein level, apparently in a time-dependent manner (threefold in 48 h), in fully differentiated 3T3-L1, followed by reestablishment of gene expression to basal levels after 24 h of reoxygenation. Hypoxia-induced SAA may be one of the key molecules to the development of the inflammatory response in adipose tissue.

KEY WORDS: hypoxia; SAA; SAA3; adipocytes; obesity.

INTRODUCTION

The genesis of obesity is associated with the formation of hypoxic areas within the adipose tissue [1]. The physiological basis of adipose tissue hypoxia has several possible causes, including the reduction in local blood flow and insufficient angiogenesis, leading to reduction of capillary density. Also, the increase in adipocyte size contributes substantially to interstitial hypoxia, reported in both human and animal models [2].

Obesity is characterized as a state of low-grade inflammation with increased levels of inflammatory mediators in plasma and adipose tissue [2, 3], and some of them are driven in response to hypoxia [4]. Mature adipocytes are an important source of the protein serum amyloid A (SAA) [5], and the extensive immunomodulatory activities of this protein are well-known [6, 7]. Furthermore, SAA enhances cell proliferation, including 3T3-L1 cells, inhibits differentiation to adipocytes, and induces TNF-α, IL-6, IL-8, and monocyte chemotactic protein-1 (MCP-1) release, suggesting an action as a proinflammatory adipokine [8, 9]. In mice, the main isoform produced by adipocytes is SAA3, and it is strongly upregulated in the adipose tissue of obese animals [10]. Considering that SAA may contribute to the genesis of obesity and insulin resistance [8, 11], and that the effect of hypoxia on SAA profile has not yet been studied, the aim of this study was to evaluate if ambient hypoxia is able to modulate SAA3 in mice 3T3-L1 adipocytes and in fully differentiated 3T3-L1 adipocytes.

MATERIALS AND METHODS

Cell Culture and Hypoxia Treatment

Mice 3T3-L1 adipocytes were maintained and induced to differentiation as described previously [8]. The ambient hypoxia was generated filling a sealed acrylic chamber (self-designed) with low-oxygen air (1 % O_2, 5 % CO_2, and 94 % N_2), maintained at 37 °C. An oximeter (Dräger Pac® 5000) and H_2O were added inside the chamber in order to control the O_2 content and maintain an adequate humidity. The hypoxia assays, for 3T3-L1 and differentiated 3T3-L1, were performed in 6, 12, 24, and 48 h followed or not by reoxygenation for 24 h. The cell viability after hypoxia was performed by

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colorimetric thiazolyl blue tetrazolium bromide (MTT) assay [12].

Western Blot and ELISA

Cells were lysed, sonicated, and cleared as described previously [8]. Immunoblottings for hypoxia-inducible factor 1-alpha (HIF-1α) were performed using SDS-PAGE followed by the transference to a nitrocellulose membrane, and the protein was detected with anti-mouse HIF-1α (Abcam®, Cambridge, MA, USA). SAA3 was measured using the ELISA kit (Millipore Corporation, Billerica, MA, USA).

RNA Isolation and Quantitative Real-Time PCR

Total RNA from 3T3-L1 cells and the cDNA synthesis were performed as described previously [8]. Real-time PCR (*saa3* and *β-actin*) were performed on ABI Prism 7500 System using the TaqMan® gene expression assay (Applied Biosystems, USA) according to the manufacturer's instructions. The relative comparison method ($2^{-\Delta\Delta CT}$) was used to compare the expression levels of mRNA.

Statistical Analysis

The results were shown as the mean±standard error of the mean (SEM) of three independent experiments. Statistical analyses were performed with GraphPad Prism4 (GraphPad Software, Inc., San Diego, CA, USA). The *P* values of <0.05 were considered statistically significant.

RESULTS

Differentiation of 3T3-L1 Leads to SAA3 Expression and Production

Although both 3T3-L1 and differentiated 3T3-L1 (Fig. 1a, b) were able to express SAA3 mRNA (the magnitude in differentiated 3T3-L1 was notably higher in a ratio 1:30; Fig. 1c), the SAA3 protein was only detected in differentiated 3T3-L1, highlighting distinct profiles in different stages of maturation (Fig. 1d).

Hypoxia Leads to the Stabilization of HIF-1α and Does not Alter 3T3-L1 Cell Viability

To validate the experimental conditions, treatment with ambient hypoxia induced a significant accumulation of HIF-1α protein in 3T3-L1 and differentiated 3T3-L1 after 6 h. At longer times, HIF-1α was stabilized followed by a decrease in its concentration after 24 h (Fig. 1e). Reoxygenation, as expected, abolished the presence of HIF-1α. Furthermore, hypoxic treatment did not interfere in 3T3-L1 viability (Fig. 1f) but decreased in less than 20 %, the viability of differentiated 3T3-L1 in all performed times of hypoxia, including the reoxygenation (Fig. 1g), remaining constant over the time and not representing a substantial interference in the hypoxia assays.

Hypoxia Increases SAA3 Production in Differentiated 3T3-L1

SAA3 mRNA expression did not change under hypoxia in 3T3-L1 (Fig. 1h), and no protein was detected in intracellular compartment or supernatant. However, for differentiated 3T3-L1, a threefold increment in both SAA3 mRNA and protein under hypoxia condition in a time-dependent manner was observed. Surprisingly, after 24 h of reoxygenation, the expression of SAA3 returned to basal conditions, and the protein content decreased significantly (Fig. 1i, j).

DISCUSSION

In the current study, we used in vitro ambient hypoxia to verify whether the adipokine SAA is altered. The isoform SAA3 in 3T3-L1 and in differentiated 3T3-L1 under normoxia or hypoxia condition was evaluated. In the normoxia condition, differentiated 3T3-L1 cells were able to express mRNA and release SAA3 protein, in a 30-fold increment when compared to 3T3-L1. Under hypoxia, SAA3 was upregulated only in differentiated 3T3-L1, remaining undetected in 3T3-L1. However, during reoxygenation, the levels of SAA3 (both mRNA and protein) were restored at basal condition in differentiated 3T3-L1.

There are several transcription factors that are implicated in the molecular response to hypoxia, including nuclear factor kappa B (NFκB) and cAMP response element-binding protein. The activation of NFκB, which has been demonstrated also in adipocytes, modulates the expression of target genes such as TNF-α, and it is one of the proposed transcription factors described for SAA [4, 13]. The upregulation of SAA3 in adipocytes under hypoxia shown in this study might have important consequences in the

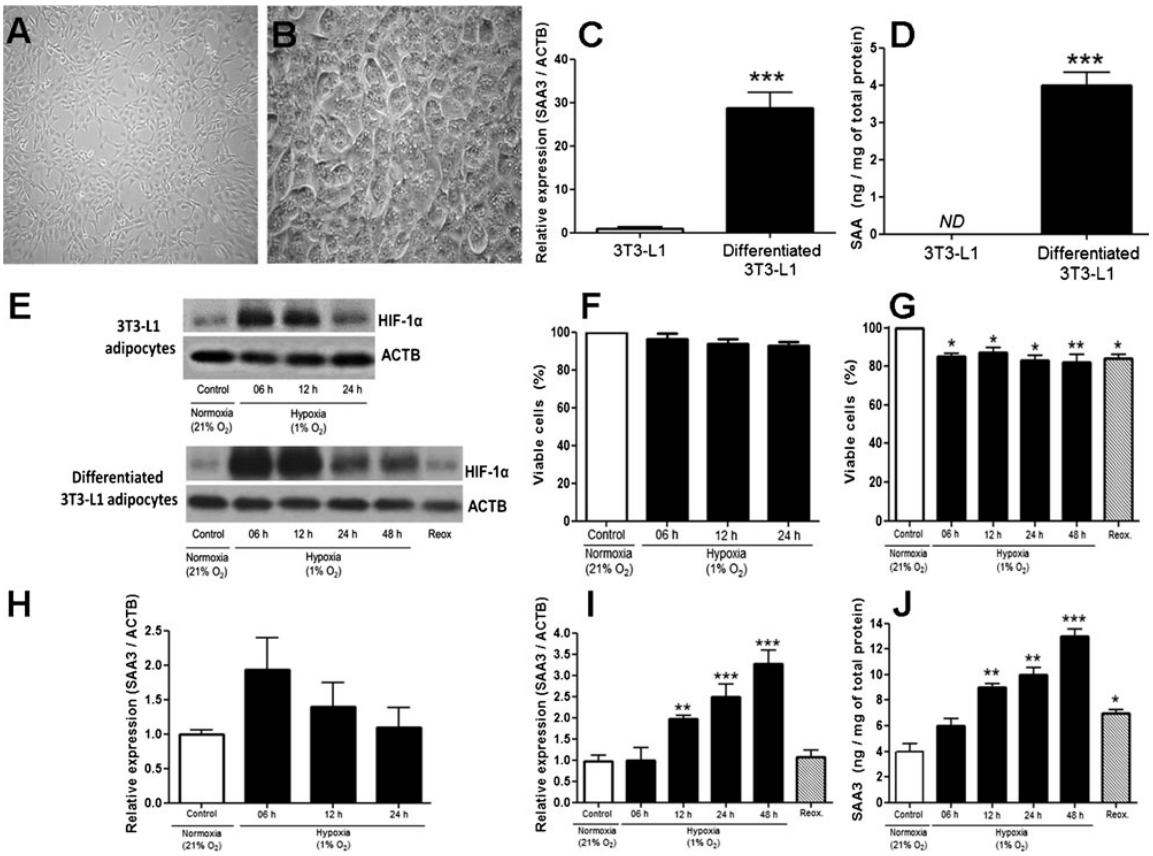

Fig. 1. Hypoxia enhances SAA3 production in differentiated 3T3-L1. **a** Representative light microscopy of 3T3-L1 and **b** differentiated 3T3-L1 under normoxia (×100 magnification). **c** Relative expression of SAA3 mRNA and **d** intracellular SAA3 protein content of 3T3-L1 and differentiated 3T3-L1. **e** Induction of HIF-1α under hypoxia or reoxygenation (24 h in 1 % O_2 followed by 24 h in normoxia) in 3T3-L1 and differentiated 3T3-L1. MTT viability assay for **f** 3T3-L1 and **g** differentiated 3T3-L1 after hypoxia or reoxygenation. **h** Relative expression of SAA3 mRNA in 3T3-L1 under hypoxia. **i** Relative expression of SAA3 mRNA and **j** intracellular SAA3 protein content of differentiated 3T3-L1 after hypoxia or reoxygenation. Data are the mean±SEM of three independent experiments. One-way ANOVA were performed in **c**, **d**, **f**, and **h**. Two-way ANOVA were performed in **g**, **i**, and **j** (*P<0.05; **P<0.01; ***P<0.001). *ND* not detected, *ACTB* β-actin, *Reox* reoxygenation.

comprehension of the pathophysiology of obesity, given its potent inflammatory action. We have previously demonstrated that SAA is a potent stimulus for the release of several cytokines from immune cells and adipocytes [6, 8, 14]. In addition, SAA-treated adipocytes increased the expression of SAA3, showing a positive feedback loop that maintains the inflammatory stimulus [8]. SAA has also been listed as a pro-angiogenic factor [15], which may be a physiologic response of the adipose tissue against hypoxia, independently of VEGF increase. The increase of SAA3 in adipose tissue under hypoxia also suggests that molecules other than MCP-1 must be involved in the recruitment and retention of macrophages, as demonstrated before [16–18], linking adipose tissue hypoxia with macrophage infiltration.

In this study, we highlighted the differences between undifferentiated and differentiated 3T3-L1 regarding SAA3 expression, suggesting that the maturation state of cells is related to the magnitude of production of this proinflammatory adipokine. Furthermore, a stimulation of SAA3 production under hypoxia in differentiated 3T3-L1 was demonstrated, a

regulated mechanism that might constitute an important element in the pathogenesis of obesity and its comorbidities.

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REFERENCES

1. Regazzetti, C., P. Peraldi, T. Gremeaux, R. Najem-Lendom, I. Ben-Sahra, M. Cormont, et al. 2009. Hypoxia decreases insulin signaling pathways in adipocytes. *Diabetes* 58: 95–103.
2. Ye, J. 2009. Emerging role of adipose tissue hypoxia in obesity and insulin resistance. *International Journal of Obesity* 33: 54–66.
3. Hotamisligil, G.S. 2006. Inflammation and metabolic disorders. *Nature* 444: 860–7.
4. Trayhurn, P., B. Wang, and I.S. Wood. 2008. Hypoxia in adipose tissue: a basis for the dysregulation of tissue function in obesity? *British Journal of Nutrition* 100: 227–235.
5. Poitou, C., N. Viguerie, R. Cancello, R. De Matteis, S. Cinti, V. Stich, et al. 2005. Serum amyloid A: production by human white adipocyte and regulation by obesity and nutrition. *Diabetologia* 48: 519–528.
6. Furlaneto, C.J., and A. Campa. 2000. A novel function of serum amyloid A: a potent stimulus for the release of tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8 by human blood neutrophil. *Biochemical and Biophysical Research Communications* 268: 405–408.
7. Sandri, S., D. Rodriguez, E. Gomes, H.P. Monteiro, M. Russo, and A. Campa. 2008. Is serum amyloid A an endogenous TLR4 agonist? *Journal of Leukocyte Biology* 83: 1174–1180.
8. Filippin-Monteiro, F.B., E.M. de Oliveira, S. Sandri, F.H. Knebel, R.C. Albuquerque, and A. Campa. 2012. Serum amyloid A is a growth factor for 3T3-L1 adipocytes, inhibits differentiation and promotes insulin resistance. *International Journal of Obesity* 36: 1032–9.
9. Faty, A., P. Ferre, and S. Commans. 2012. The acute phase protein serum amyloid A induces lipolysis and inflammation in human adipocytes through distinct pathways. *Plos One* 7(4): e34031. doi:10.1371/journal.pone.0034031.
10. Sommer, G., S. Weise, S. Kralisch, P.E. Scherer, U. Lossner, M. Bluher, et al. 2008. The adipokine SAA3 is induced by interleukin-1 beta in mouse adipocytes. *Journal of Cellular Biochemistry* 104: 2241–2247.
11. Ye, X.Y., Y.M. Xue, J.P. Sha, C.Z. Li, and Z.J. Zhen. 2009. Serum amyloid A attenuates cellular insulin sensitivity by increasing JNK activity in 3T3-L1 adipocytes. *Journal of Endocrinological Investigation* 32: 568–575.
12. van Meerloo J, Kaspers GJL, Cloos J. Cell sensitivity assays: the MTT assay. In: Cree IA, ed. Cancer cell culture: methods and protocols. 731, 2011:237–245. Totowa: Humana.
13. Reigstad, C.S., G.O. Lunden, J. Felin, and F. Backhed. 2009. Regulation of serum amyloid A3 (SAA3) in mouse colonic epithelium and adipose tissue by the intestinal microbiota. *Plos One* 4(6): e5842. doi:10.1371/journal.pone.0005842.
14. Sandri, S., E. Hatanaka, A.G. Franco, A.M.C. Pedrosa, H.P. Monteiro, and A. Campa. 2008. Serum amyloid A induces CCL20 secretion in mononuclear cells through MAPK (p38 and ERK1/2) signaling pathways. *Immunology Letters* 121: 22–26.
15. Connolly, M., A. Marrelli, M. Blades, J. McCormick, P. Maderna, C. Godson, et al. 2010. Acute serum amyloid A induces migration, angiogenesis, and inflammation in synovial cells in vitro and in a human rheumatoid arthritis/SCID mouse chimera model. *Journal of Immunology* 184: 6427–37.
16. Han, C.Y., S. Subramanian, C.K. Chan, M. Omer, T. Chiba, T.N. Wight, et al. 2007. Adipocyte-derived serum amyloid A3 and hyaluronan play a role monocyte recruitment and adhesion. *Diabetes* 56: 2260–2273.
17. Hatanaka, E., P.T. Monteagudo, M.S.M. Marrocos, and A. Campa. 2007. Interaction between serum amyloid A and leukocytes—a possible role in the progression of vascular complications in diabetes. *Immunology Letters* 108: 160–166.
18. Wang, B., I.S. Wood, and P. Trayhurn. 2007. Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. *Pflugers Archiv-European Journal of Physiology* 455: 479–492.

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ORIGINAL ARTICLE

Serum amyloid A is a growth factor for 3T3-L1 adipocytes, inhibits differentiation and promotes insulin resistance

FB Filippin-Monteiro, EM de Oliveira, S Sandri, FH Knebel, RC Albuquerque and A Campa

BACKGROUND/OBJECTIVES: Serum amyloid A (SAA) is an acute-phase protein that has been recently correlated with obesity and insulin resistance. Therefore, we first examined whether human recombinant SAA (rSAA) could affect the proliferation, differentiation and metabolism of 3T3-L1 preadipocytes.

DESIGN: Preadipocytes were treated with rSAA and analyzed for changes in viability and [^{3}H-methyl]-thymidine incorporation as well as cell cycle perturbations using flow cytometry analysis. The mRNA expression profiles of adipogenic factors during the differentiation protocol were also analyzed using real-time PCR. After differentiation, 2-deoxy-[1,2-^{3}H]-glucose uptake and glycerol release were evaluated.

RESULTS: rSAA treatment caused a 2.6-fold increase in cell proliferation, which was consistent with the results from flow cytometry showing that rSAA treatment augmented the percentage of cells in the S phase (60.9 ± 0.54%) compared with the control cells (39.8 ± 2.2%, ***$P < 0.001$). The rSAA-induced cell proliferation was mediated by the ERK1/2 signaling pathway, which was assessed by pretreatment with the inhibitor PD98059. However, the exposure of 3T3-L1 cells to rSAA during the differentiation process resulted in attenuated adipogenesis and decreased expression of adipogenesis-related factors. During the first 72 h of differentiation, rSAA inhibited the differentiation process by altering the mRNA expression kinetics of adipogenic transcription factors and proteins, such as PPARγ2 (peroxisome proliferator-activated receptor γ 2), C/EBPβ (CCAAT/enhancer-binding protein β) and GLUT4. rSAA prevented the intracellular accumulation of lipids and, in fully differentiated cells, increased lipolysis and prevented 2-deoxy-[1,2-^{3}H]-glucose uptake, which favors insulin resistance. Additionally, rSAA stimulated the secretion of proinflammatory cytokines interleukin 6 and tumor necrosis factor α, and upregulated SAA3 mRNA expression during adipogenesis.

CONCLUSIONS: We showed that rSAA enhanced proliferation and inhibited differentiation in 3T3-L1 preadipocytes and altered insulin sensitivity in differentiated cells. These results highlight the complex role of SAA in the adipogenic process and support a direct link between obesity and its co-morbidities such as type II diabetes.

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Keywords: Serum amyloid A; preadipocyte; proliferation; adipogenesis; insulin resistance; inflammation

INTRODUCTION

Obesity is a chronic metabolic disorder caused by an imbalance between energy intake and expenditure. This disorder occurs by hyperplastic growth caused by the mitotic activity in precursor cells (preadipocytes) and by hypertrophic growth due to intracellular lipid accumulation.[1] This scenario is closely associated with a state of chronic low-grade inflammation characterized by abnormal production of cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin 6, and by the activation of inflammatory signaling pathways in adipose tissue.[2,3] The expression and secretion of inflammatory molecules is increased in obesity, and this increase negatively affects insulin sensitivity in adipose tissue.[4–6]

In obese individuals, a chronic and systemic elevation of serum amyloid A (SAA) protein was recently associated with the size of adipocytes.[7] Until recently, it was thought that the expression and release of SAA occurred predominantly in the liver,[8] however, it is now known that human adipose tissue is a major SAA expression site during the non-acute-phase reaction condition.[9–11] In rodents, the main isoform found in adipocytes is SAA3,[4,12] and it is strongly upregulated in the adipose tissue of obese mice as compared with lean controls.[13]

In previous studies, we had demonstrated immunomodulatory activities of SAA in human leukocytes, such as the induction of expression and release of TNF-α, interleukin-1β, interleukin-8 and enhanced leukocyte migration and adhesion.[14–16] Recently, we showed that SAA is an inducer of nitric oxide production in macrophages and may be an endogenous agonist for the TLR4 complex.[17] Furthermore, we have demonstrated that SAA was triggered in chronic conditions, such as diabetes.[18] Fibroblasts and endothelial cells are also responsive to SAA. In Swiss 3T3 fibroblasts, SAA increased proliferation that was completely abolished by the addition of antioxidants.[19]

3T3-L1 preadipocyte, a murine cell line, is commonly used as a model of adipogenesis, lipogenesis and lipolysis. The differentiation of growth-arrested 3T3-L1 cells into adipocytes is induced using hormonal stimuli that initiate the mitotic clonal expansion of preadipocytes leading to phenotype modifications.[20,21] Expression of C/EBPβ, a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors, is rapidly induced in response to these stimuli (dexamethasone, 3-isobutyl-1-methylxanthine, insulin) and regulates peroxisome proliferator-activated receptor γ 2 (PPARγ2), a spliced variant of

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PPARγ in adipocytes, and C/EBPα, which have crucial roles in adipocyte maturation.[20,22]

Several roles of SAA in 3T3-L1 cell lines have already been investigated, including the expression of GLUT4, the serine phosphorylation rate of the IRS-1 (insulin receptor substrate 1) and glucose uptake in fully differentiated 3T3-L1 cells. It was demonstrated that SAA reduced insulin sensitivity, which was mediated by the JNK pathway.[23] Although these conclusions were relevant to understand the complications of obesity and metabolic syndrome, the low-grade inflammation state that occurs during the early and advanced stages of adipogenesis (proliferation and differentiation) was not elucidated. Therefore, to fully address the role of SAA in adipogenesis, we performed assays in three different stages of 3T3-cells. Firstly, we treated preadipocytes with rSAA and proliferation was assessed by [^{3}H-methyl]-thymidine incorporation and flow cytometry. Secondly, we verified the effect of rSAA on the differentiation of 3T3-L1 cells into adipocytes by lipid accumulation and the mRNA expression profiles of adipogenic factors. Finally, we observed the effect of rSAA on 2-deoxy-[1,2-^{3}H]-glucose uptake and glycerol release in differentiated 3T3-cells.

MATERIALS AND METHODS

Reagents

rSAA was purchased from Peprotech Inc. (Rocky Hill, NJ, USA). PD98059, SB203580, pertussis toxin and wortmannin were purchased from Calbiochem (La Jolla, CA, USA). Insulin, dexamethasone, 3-isobutyl-1-methylxanthine, Oil Red O, annexin V-fluorescein isothiocyanate (V-FITC), phenylmethylsulfonyl fluoride and propidium iodide (PI) were supplied by Sigma Chemical Co. (St Louis, MO, USA). [^{3}H-methyl]-thymidine and 2-deoxy-[1,2-^{3}H]-glucose were acquired from Amersham Biosciences (São Paulo, Brazil). Dulbecco's modified Eagle's medium (DMEM), calf serum (CS), penicillin, streptomycin and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents used were purchased from Merck (Darmstadt, Germany) unless otherwise indicated.

Cell culture and differentiation induction

Mouse 3T3-L1 preadipocytes were generously provided by Dr. Mari Cleide Sogayar (Instituto de Química, Universidade de São Paulo, Brazil). Cells were maintained in DMEM supplemented with 10% CS containing 100 IU ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin. Cells were cultured at 37 °C in a humidified atmosphere at 5% CO_2, and the medium was changed every 48 h. 3T3-L1 cells were induced to differentiate as described previously.[24] Starting on day 1 of differentiation, cells were treated with 5 μg ml^{-1} rSAA and during each medium change, rSAA was restored. At the times indicated, cells were stained with Oil Red O to detect cytoplasmic triglycerides, extracted and read spectrophotometrically.[25] In cell-free supernatants of the cultures, glycerol levels were determined using the Glycerol 3-phosphate Oxidase-Trinder Kit (Sigma). The cytokines interleukin 6 and TNF-α were measured using an enzyme-linked immunosorbent assay (ELISA) (DuoSet, R&D System, Minneapolis, MN, USA).

Cell proliferation

Mitogen-activated protein kinases (MAPK), especially ERK1/2 and p38MAPK, have been implicated in cell cycle control and differentiation. The phosphoinositide 3-kinase (PI3 K) pathway, an important signaling pathway in the mediation of cell survival, adipocyte differentiation and glucose transport was also evaluated. We used specific pharmacological inhibitors of ERK1/2 (PD98059), p38^{MAPK} (SB203580) and PI3 K (wortmannin) for 3T3-L1 cell pretreatment that was followed by rSAA stimulation in a cell proliferation assay. For that, cells were deprived of serum (1% CS) for 48 h, pretreated with specific inhibitors including PD98059 (10 μM), SB203580 (10 μM), pertussis toxin (100 ng ml^{-1}) or wortmannin (100 nM) and treated with rSAA (1, 5 and 10 μg ml^{-1}) and/or insulin (100 nM) at the times indicated. Then, 0.5 μCi of [^{3}H-methyl]-thymidine (specific activity 248 GBq mmol^{-1}) was added for 24 h. Cells were washed twice with cold phosphate-buffered saline (PBS), fixed with ice-cold 10% trichloroacetic acid, lysed with 0.5 M NaOH and transferred to filters (10 × 5 mm). The lysates were placed in vials containing 2 ml of scintillation fluid and were counted using the liquid scintillation counter (Beckman Instruments, Palo Alto, CA, USA).

Flow cytometry analysis

For the cell cycle and viability assays, cells were serum-starved in DMEM containing 1% CS for 48 h and treated with rSAA (1 and 5 μg ml^{-1}) for 24 h. Cells were harvested and washed twice with PBS. For cell cycle analysis, cells were gently resuspended in 300 μl of a solution containing 2 μg ml^{-1} PI, 0.1% sodium citrate and 0.1% Triton X-100. The proportion of cells in each phase of the cell cycle was determined using a flow cytometer set to 488 nm (excitation wavelength), and the data were analyzed using FlowJo software (Tree star, Inc., Ashland, OR, USA). For the viability assay using double staining (annexin V-FITC and PI), cells were resuspended in 100 μl of binding buffer (10 mM HEPES/NaOH, 140 mM NaCl and 2.5 mM $CaCl_2$). Next, 5 μl of annexin V-FITC was added. The cells were gently agitated and incubated for 20 min at room temperature in the dark. Subsequently, 40 μl of PI solution (2 μg ml^{-1}) and 400 μl of binding buffer were added, and the samples were analyzed using flow cytometry. The fluorescence of annexin V-FITC was measured in the FL1 channel (green fluorescence: 530/30 nm) and PI fluorescence was measured in the FL2 channel (orange-red fluorescence: 585/42 nm) using the FACSCanto flow cytometric equipment (Becton Dickinson, San Diego, CA, USA). The percentage of necrotic or apoptotic cells and the analysis of the cell cycle were calculated by Flowjo software (Tree star, Inc.).

RNA extraction and complementary DNA synthesis

Total RNA from 3T3-L1 cells was isolated at the indicated times using the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany). The complementary DNA was synthesized from 600 ng of RNA using the SuperScript First Strand kit (Invitrogen, Carlsbad, CA, USA).

Quantitative Real-Time PCR

The following primers were used: PPARγ2 (5′-CACAGAGATGCCATTCTGGC-3′ and 5′-GGCCTGTTGTAGAGCTGGGT-3′), perilipin (5′-CATGTCCCTATCCGAT GCC-3′ and 5′-TCGGTTTTGTCGTCCAGG-3′), C/EBPα (5′-GTGTGCACGTCTATG CTAAACCA-3′ and 5′-GCCGTTAGTGAAGAGTCTCAGTTTG-3′), C/EBPβ (5′-GT TTCGGGACTTGATGCAATC-3′ and 5′-AACAACCCCGCAGGAACAT-3′), FABP4 (5′-CCAATGAGCAAGTGGCAAGA-3′ and 5′-GATGCCAGGCTCCAGGATAG-3′), GLUT4 (5-GCTGTGCCATCTTGATGACGG-3′ and 5-TGAAGAAGCCAAGCAGGAG GAC-3′) and 18S (5′-GTAACCCGTTGAACCCCATT-3′ and 5′-CCATCCAATCGGT AGTAGCG-3′), which was used as a constitutive control. BLAST searches were conducted on all primer sequences to ensure gene specificity. Each amplification reaction was performed in triplicate and included the addition of the SyBr Green Master Mix (Applied Biosystems, Mount Holly, NJ, USA). Each data set also included a negative control (no complementary DNA). Reaction conditions were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 10 s (melting) and 60 °C for 1 min (annealing and elongation). Melting curve analyses from 76 to 84 °C were performed at the end of each run as a quality control step. The Ct (cycle threshold) for each run was set to 0.1, when amplification was observed in the log phase. Relative gene expression was determined using the ΔΔCt method, and the efficiency of each reaction was validated as previously described.[26] The expression levels of SAA3 mRNAs were quantified using the TaqMan PCR reagent kit's detection system according to the protocols provided by the manufacturer (Applied Biosystems). The levels of mRNA were normalized to the amount of β-actin RNA detected in each sample. PCR reactions were performed in the Gene AMP 7500 Sequence Detection System (Applied Biosystems).

2-deoxy-[1,2-^{3}H]-glucose uptake

Glucose uptake by differentiated 3T3-L1 cells was measured as previously described[27] with modifications. Briefly, 3T3-L1 cells were differentiated into adipocytes and incubated with or without rSAA (1, 5 and 10 μg ml^{-1}) in serum-free DMEM for 24 h. The adipocytes were washed twice with 37 °C

Krebs–Ringer phosphate buffer (pH 7.4) (128 mM NaCl, 4.7 mM KCl, 1.65 mM $CaCl_2$, 2.5 mM $MgSO_4$ and 5 mM Na_2HPO_4). Adipocytes were either untreated (basal, insulin-independent) or treated with insulin (100 nM) for 10 min in Krebs–Ringer phosphate buffer. Without changing the buffer, glucose uptake was initiated by adding 1.0 μCi per well of 2-deoxy-[1,2-^{3}H]-glucose (specific activity 740 GBq mmol^{-1}) for 10 min at 37 °C. The cells were gently washed three times with ice-cold PBS and lysed in 800 μl of solution containing 0.5 M NaOH and 0.1% sodium dodecyl sulfate. Samples were assayed for glucose uptake using a liquid scintillation counter. The level of glucose uptake induced by insulin (100 nM) was set at 100%.

Measurement of SAA3 protein levels in cell lysates

Cells were washed with PBS, harvested using sonication buffer (PBS pH 7.4 and 2 mM phenylmethylsulfonyl fluoride), centrifuged at 300 × *g* for 5 min and lysed by sonication (Branson Ultrasonics Corporation, Danbury, CT, USA) for 10 s at 40 W. The supernatants were cleared by centrifugation at 15 000 × *g* for 15 min. SAA3 was measured using the ELISA kit (Invitrogen).

Statistical analysis

Results were shown as the mean ± s.e. of 6–9 determinations from 2–3 experiments. Statistical analyses were performed with Graph Pad Prism4 (Graph Pad Software Inc., San Diego, CA, USA). When multiple samples were compared with one independent variable, one-way analysis of variance with Newman–Keuls *post hoc* test was performed. Data with two independent variables were tested by two-way analysis of variance, and tested with Bonferroni *post hoc* test, as indicated in figure legends. The level of significance was set at $P<0.05$.

RESULTS

rSAA prevented the necrosis induced by serum starvation

After 48 h of serum starvation, preadipocytes were treated with rSAA for 24 h before performing the flow cytometry analysis using simultaneous staining with annexin V–FITC and PI. Double-staining analysis demonstrated that rSAA reduced serum starvation-induced necrosis, which was assessed by PI-positive cells. rSAA at 1 and 5 μg ml^{-1} enhanced cell viability (81.9 ± 4.4% and 83.9 ± 2.5%, respectively) compared with unstimulated cells (55.5 ± 9%, ***$P<0.001$) (Figures 1a and b). However, a small fraction of rSAA-treated cells underwent apoptosis (annexin-positive cells), whereas the prevention of necrosis was markedly decreased.

rSAA stimulated preadipocyte proliferation

Subconfluent 3T3-L1 preadipocytes were stimulated with rSAA (1, 5 and 10 μg ml^{-1}) and cultured in serum-deprived medium (1% or 5% CS). The incorporation of radiolabeled thymidine into newly replicated DNA was used to assess the effects of rSAA on cell proliferation. As shown in Figure 1e, treatment of 3T3-L1

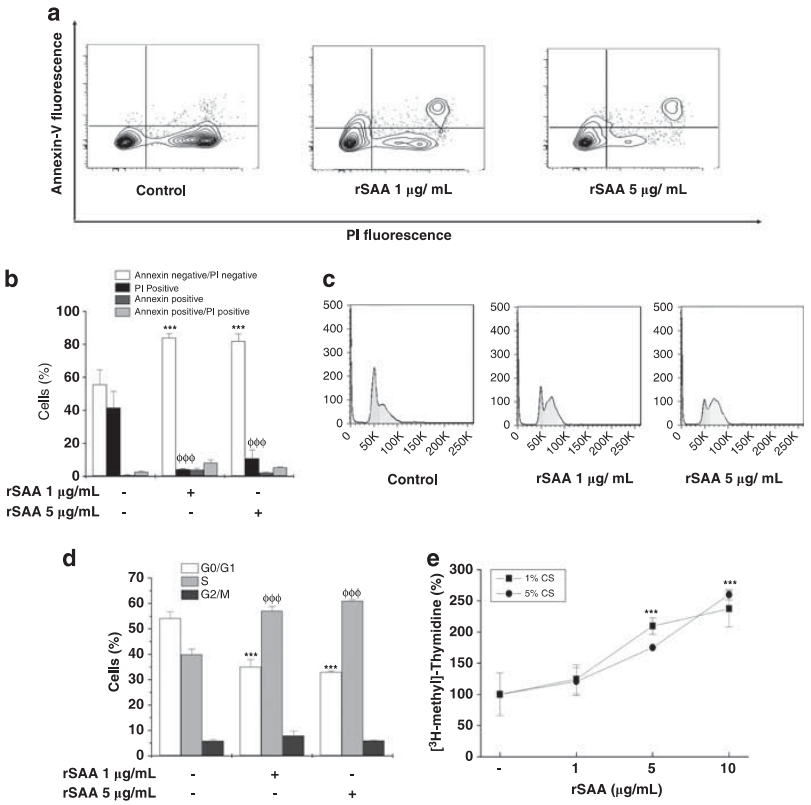

Figure 1. Enhanced cell viability and increased proliferation are induced by rSAA in 3T3-L1 preadipocytes. (**a**) Dot plots show the intensity of Annexin V fluorescence plotted on the Y-axis and PI fluorescence plotted on the X-axis. (**b**) The percentage of live cells (annexin-negative/PI-negative), necrotic cells (PI-positive), apoptotic cells (annexin-positive) and late apoptotic cells (annexin-positive/PI-positive) after analysis by flow cytometry. (**c**) Representative flow cytometry histograms showing cell cycle distributions. (**d**) The percentage of cells in each phase of the cell cycle stained with PI solution (2 μg ml^{-1}). (**e**) 3T3-L1 cells were treated with increasing concentrations of rSAA (1–10 μg ml^{-1}) in serum-deprived medium for 24 h. During treatment, the cells were labeled with [^{3}H-methyl]-thymidine. Data are the mean ± s.e. of three independent experiments, which were performed in triplicate and two-way analysis of variance were performed (***, ΦΦΦ$P<0.001$ vs control groups).

preadipocytes with rSAA promoted an increase in [^{3}H-methyl]-thymidine incorporation in a dose-dependent manner; that increase reached 260% of the control with 10 μg ml^{-1} rSAA at 24 h (***$P<0.001$). When we analyzed the cell cycle, rSAA also stimulated cell cycle progression from the G1 to S phase (Figures 1c and d), which was demonstrated by a higher percentage of cells in S phase (60.9 ± 0.54%, rSAA-treated cells) compared with the control cells (39.8 ± 2.2%) (***$P<0.001$).

rSAA induced proliferation through the ERK1/2 pathway

Because the mitogenic effects of rSAA in preadipocytes were pronounced, we explored the involvement of several signaling pathways that could be involved in the proliferative effects of rSAA. We hypothesized that rSAA-induced proliferation was mediated by the FPR2 receptor because the pertussis-sensitive G-proteins have been reported to be involved in SAA biological effects. To test this hypothesis, cells were pretreated with the pertussis toxin, and [^{3}H-methyl]-thymidine incorporation was determined in the absence and presence of rSAA. As shown in Figure 2a, no changes in proliferation were observed. However, PD98059, an ERK1/2 inhibitor, prevented the rSAA-induced increase in [^{3}H-methyl]-thymidine incorporation, which suggests that the effects of rSAA on preadipocyte proliferation were mediated through the ERK1/2 signaling pathway. A 24-h exposure to rSAA in the absence or presence of the PI3 K inhibitor wortmannin or the p38^{MAPK} inhibitor SB203580 revealed that the inhibitors affected proliferation in control cells and in rSAA-treated cells (Figure 2b). This result precludes the evaluation of the p38^{MAPK} and PI3 K signaling pathways in the rSAA-induced cell survival.

In 3T3-L1 preadipocytes, insulin activates two major signaling cascades, the PI3 K and MAPK pathways. The PI3K pathway was activated in cells using insulin, a well-known preadipocyte mitogen, and these cells were compared with cells stimulated with rSAA alone. As observed in Figure 2c, insulin increased the proliferation of preadipocytes, which was inhibited using wortmannin. Although the cells treated with both rSAA and insulin showed a higher rate of proliferation (174.7 ± 5.9%) compared with insulin-treated cells (141.1 ± 10.2%), the presence of wortmannin in both treatments significantly attenuated this effect (82.6 ± 10.1%).

rSAA inhibited the expression of adipogenic transcriptional regulators and adipocyte differentiation

To determine whether rSAA affects adipocyte differentiation, we maintained 3T3-L1 cells in DMEM containing a hormonal stimulus with or without rSAA during the entire process of differentiation. At the indicated times, we analyzed the effect of rSAA on the mRNA expression of transcription factors and adipocyte-specific genes during the first 72 h of the differentiation program by quantitative real-time PCR. Changes in the mRNA expression profiles of C/EBPβ, C/EBPα, PPARγ2, GLUT4, FABP4 and perilipin were observed in the cultures maintained with rSAA. At 48 h of differentiation, rSAA induced an increase in the mRNA expression of PPARγ2, perilipin and C/EBPα and β (Figure 3). However, at 72 h, rSAA significantly downregulated mRNA expression of PPARγ2, C/EBPα and C/EBPβ, which are the critical genes for the adipocyte phenotype. Interestingly, cultures maintained with rSAA showed a significant decrease in GLUT4 mRNA expression at 48 and 72 h after the hormonal stimulus compared with those cultures without rSAA. Oil red O elution after cell staining, which was conducted during the differentiation process (Figure 4a), revealed that the formation of lipid droplets, a marker for adipocyte differentiation, was significantly decreased in rSAA-treated cells (0.9395 ± 0.07) compared with control cells (1.056 ± 0.054). However, the glycerol measurements from the supernatants of cell cultures were not altered under either conditions (control and rSAA-treated cells), which suggests that the decreased lipid deposition in cells treated with rSAA was caused by the inhibition of differentiation and not

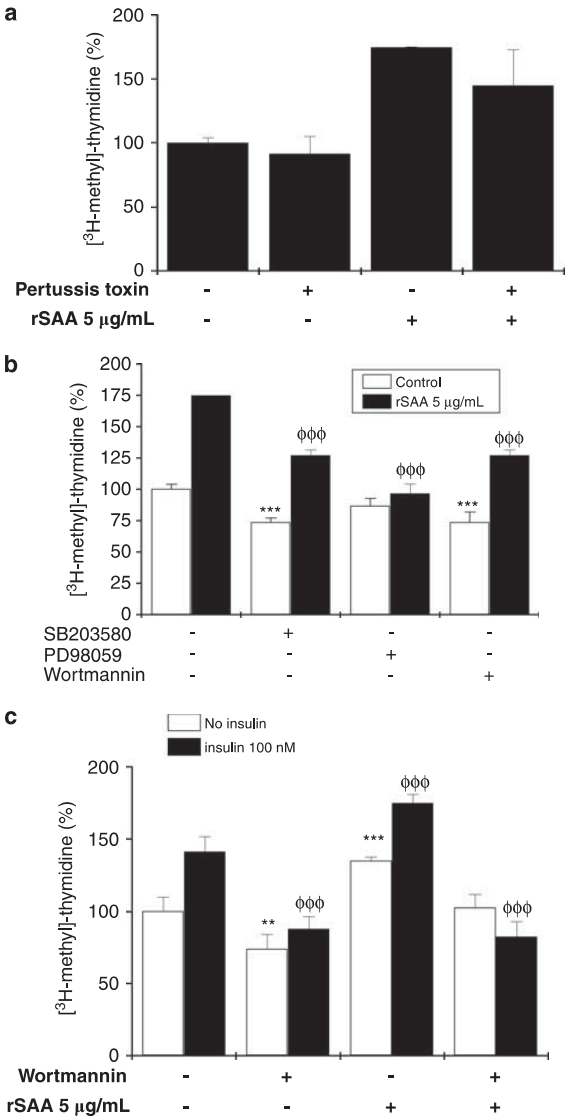

Figure 2. The involvement of proteins in different signaling pathways on the preadipocyte proliferation induced by rSAA. (**a**) Pretreatment of 3T3-L1 cells with pertussis toxin under the conditions described in the methods. (**b**) 3T3-L1 preadipocytes were pretreated with SB 203580, PD98059 and wortmannin and then stimulated with rSAA. (**c**) The influence of insulin (100 nM) and rSAA (5 μg ml^{-1}) on activation of the PI3 K pathway. Data are the mean ± s.e. of three independent experiments, which were each performed in triplicate and one-way analysis of variance was performed in A and two-way analysis of variance was performed in B and C (**$P<0.01$, ***, $^{\Phi\Phi\Phi}P<0.001$ vs control groups).

subsequent lipolysis (Figure 4b). These data were consistent with those shown in the microscopic analysis of lipid droplet deposition at day 7 of differentiation in control cells (Figure 4c) and rSAA-treated cells (Figure 4d).

rSAA increased lipolysis and decreased glucose transport

The differentiated 3T3-L1 cells were incubated with different concentrations of rSAA (1, 5 and 10 μg ml^{-1}) for 24 h. After the

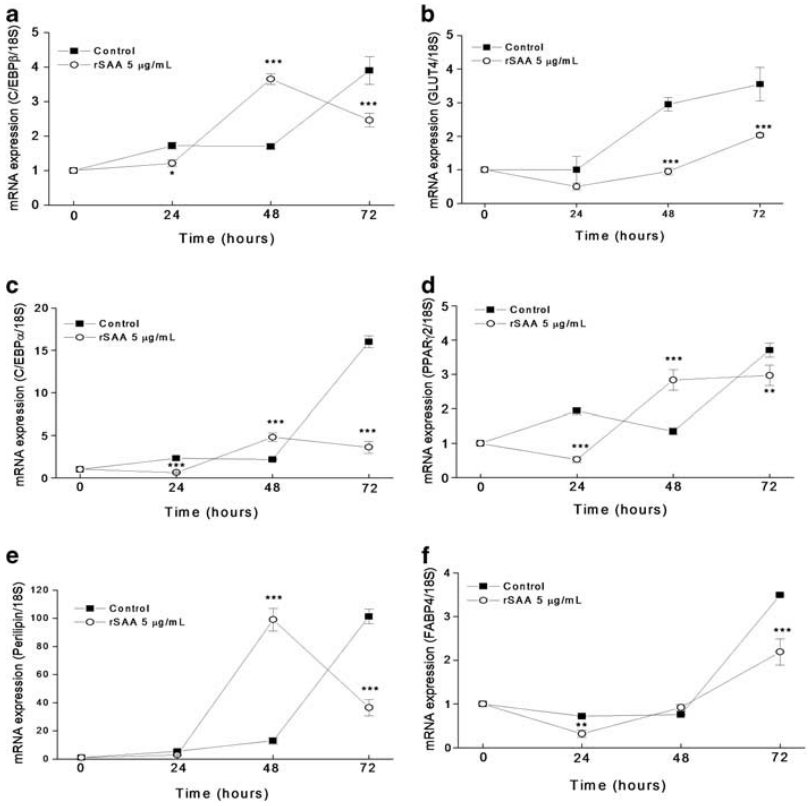

Figure 3. The effect of rSAA on 3T3-L1 preadipocyte differentiation. Quantitative real-time PCR was performed to assess the mRNA expression of (**a**) C/EBPβ, (**b**) GLUT4, (**c**) C/EBPα, (**d**) PPARγ2, (**e**) perilipin and (**f**) FABP4. Cells were treated with a hormonal stimulus in the presence (○) or absence (■) of rSAA. Data are the mean ± s.e. of three independent experiments and two-way analysis of variance was performed (**$P<0.01$, ***$P<0.001$ vs control groups).

24 h of rSAA stimulation at the indicated concentrations, glycerol release was measured. Figure 4e showed that rSAA increased glycerol release in the cell-free supernatant of cultures. Furthermore, glucose uptake was determined under basal conditions (without insulin) and stimulated conditions (100 nM insulin). The 24-h incubation with rSAA at 5 and 10 $\mu g\,ml^{-1}$ decreased glucose uptake in insulin-stimulated 3T3-L1 cells (Figure 4f).

rSAA induced secretion of cytokines and expression of SAA3

When the hormonal stimulus to induce differentiation was added, 3T3-L1 cells released cytokines, especially TNF-α. This release was determined by testing the supernatant of cultures using an ELISA (Figures 5a and b). The addition of rSAA induced a nine-fold and three-fold increase in interleukin 6 and TNF-α release, respectively, in 3T3-L1 cells at day 3 of differentiation. The SAA3 mRNA expression and protein synthesis were quantified during 3T3-L1 differentiation in the presence or absence of rSAA. SAA3 is usually expressed and produced during differentiation. The presence of rSAA caused a significant increase in SAA3 gene expression (almost 90-fold) (Figure 5c) and a modest increase in SAA3 protein synthesis (almost 2-fold) (Figure 5d).

DISCUSSION

In the current study, we reported that SAA had a strong influence on 3T3-L1 proliferation, differentiation and metabolism. SAA effectively increased the proliferation of 3T3-L1 preadipocytes in a dose-dependent manner. The cell cycle analysis showed an increased number of cells in the S phase, which is consistent with the results of the thymidine incorporation assay. Moreover, SAA increased cell viability that was verified by a marked inhibition of necrosis under the same conditions. A similar biological effect of SAA on cell proliferation was previously observed in Swiss 3T3 fibroblasts[19] and two glioma cell lines;[28] these results support a growth factor-like activity of SAA. SAA activity is dependent on its concentration and its intended cell. We reported here that SAA induced 3T3-L1 cell proliferation and inhibited cell death. This is an entirely unique contribution on the role of SAA in adiposity.

The signaling involved in SAA-induced proliferation is not well-known, and may involve multiple pathways. Although a signaling study was not our focus, we generated data showing the involvement of ERK1/2 on SAA-induced proliferation by using pharmacological inhibitors of the main signaling pathways involved in cell survival and proliferation.[29,30] However, we were unable to elucidate the roles of the PI3 K and $p38^{MAPK}$ pathways in our system. Although pretreatment with their specific inhibitors (wortmannin and SB203580, respectively) attenuated SAA-induced proliferation, the inhibitors also decreased the proliferation in control cells.

3T3-L1 cells were exposed to insulin (100 mM) and then subjected to a proliferation assay that included the SAA and wortmannin conditions described above. It is known that insulin-induced proliferation is mediated by the PI3K signaling pathway and uses the IGF-I receptor.[29,31,32] Our current study using wortmannin was noteworthy because the inhibitory effect of

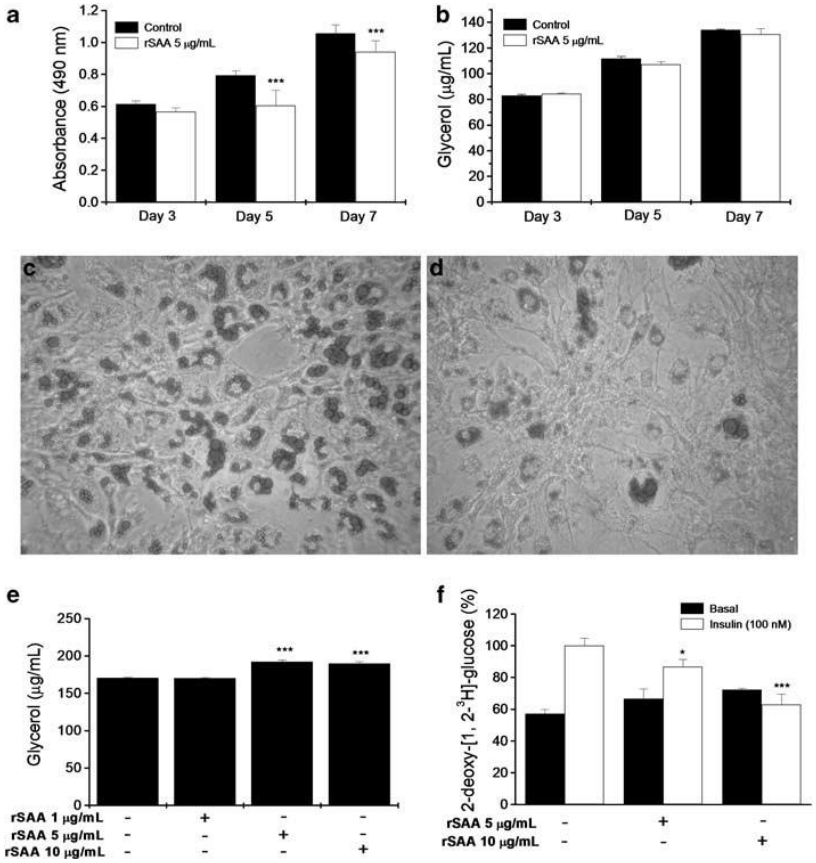

Figure 4. The effect of rSAA on adipocyte differentiation and metabolism. (**a**) Oil red O elution in 3T3-L1 cells treated with a hormonal stimulus in the presence (white bars) or absence (black bars) of rSAA at the indicated times. (**b**) Glycerol release into the culture medium under the same conditions that are described above. Oil red O staining at day 7 of differentiation (**c**) in the absence or (**d**) presence of rSAA (5 μg ml^{-1}). (**e**) Glycerol release after a 24-h treatment with rSAA in fully differentiated adipocytes. (**f**) 2-deoxy-[1,2-^{3}H]-glucose uptake under basal (black bars) and insulin-stimulated (white bars) conditions. Data are the mean ± s.e. of three independent experiments, and two-way analysis of variance was performed in **a**, **b** and **f** and one-way analysis of variance was performed in **e** (*$P < 0.05$, ***$P < 0.001$ vs control).

wortmannin on SAA-induced proliferation was equivalent to its inhibitory effect on insulin-treated cells. SAA and insulin had an additive effect on 3T3-L1 proliferation, which suggests that insulin and SAA exert mitogenic effects on 3T3-L1 cells that are mediated by both the ERK1/2 and PI3K signaling pathways.

FPR2 is a putative receptor for SAA present in adipocytes and is involved in the activation of MAPK.[33] However, our results imply that FPR2 is not involved in SAA-induced proliferation because the effects elicited by SAA on 3T3-L1 cells were not sensitive to pertussis toxin. Given the myriad effects triggered by SAA, it is likely that multiple receptors are involved in the biological effects described here. Adipocytes contain many of the SAA-sensitive receptors, for example, CD36,[34] selenoprotein S (SELS)[35], TLR4,[17] and FPR2.[33]

Although SAA appeared to be a potent mitogenic factor for adipocytes, SAA inhibited differentiation. The differentiation process is regulated by a network of transcription factors and adipocyte marker genes.[36,37] The exposure of 3T3-L1 cells to a hormonal stimulus caused differentiation. The induction of gene expression peaked at around 2 to 3 days and was followed by intracellular lipid accumulation, which was easily observed after 5 days of treatment. The addition of SAA caused a remarkable change in the expression profile of the adipogenic genes C/EBPβ, C/EBPα, PPARγ2, perilipin, FABP4, GLUT-4 and C/EBPα. These genes are the essential transcriptional regulators of adipogenesis and are targets of other adipogenic inhibitors.[21] Additionally, SAA prevented intracellular lipid deposition, although the glycerol release from 3T3-L1 cells during differentiation was not altered. The decrease in lipid accumulation may have been caused by the loss of adipogenic capacity or decreased glucose uptake rather than lipolysis.

Furthermore, production of the cytokines TNF-α and interleukin 6 during the differentiation of 3T3-L1 cells was demonstrated in the current study. The presence of SAA caused the pronounced release of these cytokines. We have previously demonstrated that SAA is a potent stimulus for the release of several cytokines from immune cells[14,16,38] and that this protein is a potent inflammatory stimulus. The current study presents the first evidence that SAA induces cytokines in cells other than immune cells.

In previous studies using fully differentiated adipocytes, secreted TNF-α stimulated lipolysis and inhibited lipogenesis, which elevated the high free fatty acid concentration in the culture medium.[6,39,40] Our data suggest that SAA by itself or SAA-induced TNF-α promoted lipolysis. The possible involvement of TNF-α in insulin resistance has been suggested in a number of studies. TNF-α increases plasma triglycerides and very low-density lipoprotein concentrations,[41,42] as well as lipolysis in mouse, rat and human adipocytes.[40,43–45]

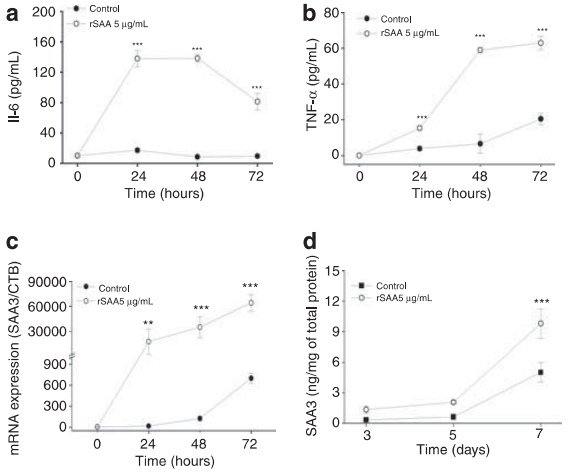

Figure 5. Cytokine release and SAA3 production during the course of differentiation. Using an ELISA assay, (**a**) interleukin 6 (IL-6) and (**b**) TNF-α release were assessed using the supernatant of cultured 3T3-L1 cells that underwent differentiation in the absence (●) or presence (○) of rSAA (5 µg ml^{-1}). (**c**) The effect of rSAA on the expression of SAA3 in cells treated with hormonal induction (■) or with rSAA (5 µg ml^{-1}) and hormonal induction (○). (**d**) SAA3 protein production assessed by ELISA assay in the absence (●) or presence (○) of rSAA (5 µg/ml). Data are the mean ± s.e. of three independent experiments and two-way analysis of variance was performed (**$P<0.01$, ***$P<0.001$ vs control).

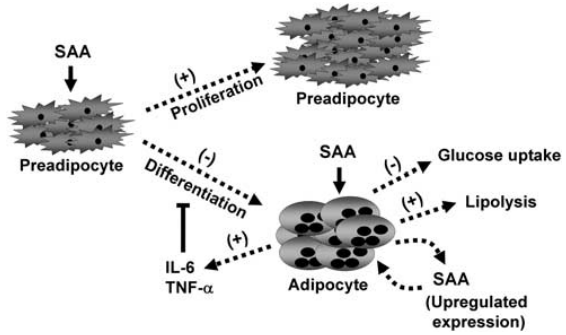

Figure 6. The influence of SAA on adipose cells. Adipocytes may be affected by SAA, which is produced by the liver during inflammatory processes and/or synthesized by adipose tissue. SAA alters the proliferation, differentiation and metabolism of adipocytes and contributes to the inflammatory state of adipose tissue.

Although the mechanism by which SAA promotes lipolysis is unknown, one reasonable possibility is the decrease in perilipin expression, a lipid droplet-associated protein that acts as a protective coating against lipases. Additionally, analysis of GLUT4 gene expression revealed that SAA caused a decrease in the mRNA levels observed at 48 and 72 h of differentiation. This finding was consistent with results from the glucose uptake assay, which was performed in fully differentiated adipocytes and showed that a decrease in glucose uptake occurred under insulin-stimulated conditions. These results show that SAA may contribute to insulin resistance in adipose tissue during the inflammatory state. In previous study using fully differentiated adipocytes, SAA attenuated cellular insulin sensitivity, upregulated the level of phosphor-JNK, and downregulated the level of phosphotyrosine-IRS-1.[23] Our data support these observations and also demonstrate the role of SAA in different stages of the adipogenic process, including the final step of differentiation with reduced glucose-uptake capacity.

Data from the current study support a role for SAA in the pathogenesis of obesity through the increase in preadipocyte proliferation and inhibition of the differentiation process. In differentiated adipocytes, SAA also impairs glucose metabolism, which may contribute to insulin resistance. Curiously, cells treated with SAA displayed an increase in the expression of SAA3 by almost two orders of magnitude. These results may represent a positive feedback loop that maintains the inflammatory condition (Figure 6).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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REFERENCES

1 Rodeheffer MS, Birsoy K, Friedman JM. Identification of white adipocyte progenitor cells *in vivo*. *Cell* 2008; **135**: 240–249.
2 Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose-tissue expression of tumor-necrosis-factor-alpha in human obesity and insulin-resistance. *J Clin Invest* 1995; **95**: 2409–2415.
3 Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H *et al.* Recent advances in the relationship between obesity, inflammation, and insulin resistance. *Eur Cytokine Netw* 2006; **17**: 4–12.
4 Scheja L, Heese B, Zitzer H, Michael MD, Siesky AM, Pospisil H *et al.* Acute-phase serum amyloid a as a marker of insulin resistance in mice. *Exp Diabetes Res* 2008; **2008**: 230837.
5 Virkamaki A, Ueki K, Kahn CR. Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. *J Clin Invest* 1999; **103**: 931–943.
6 Yang RZ, Lee MJ, Hu H, Pollin TI, Ryan AS, Nicklas BJ *et al.* Acute-phase serum amyloid A: an inflammatory adipokine and potential link between obesity and its metabolic complications. *Plos Medicine* 2006; **3**: 884–894.
7 Sjoholm K, Lundgren M, Olsson M, Eriksson JW. Association of serum amyloid A levels with adipocyte size and serum levels of adipokines: differences between men and women. *Cytokine* 2009; **48**: 260–266.
8 Urieli-Shoval S, Linke RP, Matzner Y. Expression and function of serum amyloid A, a major acute-phase protein, in normal and disease states, 2000; **7**: 64–69.
9 Jernas M, Palming J, Sjoholm K, Jennische E, Svensson PA, Gabrielsson BG *et al.* Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression. *Faseb Journal* 2006; **20**: 1540.
10 Poitou C, Viguerie N, Cancello R, De Matteis R, Cinti S, Stich V *et al.* Serum amyloid A: production by human white adipocyte and regulation by obesity and nutrition. *Diabetologia* 2005; **48**: 519–528.
11 Poitou C, Coussieu C, Rouault C, Coupaye M, Cancello R, Bedel JF *et al.* Serum amyloid A: a marker of adiposity-induced low-grade inflammation but not of metabolic status. *Obesity* 2006; **14**: 309–318.
12 Sommer G, Weise S, Kralisch S, Scherer PE, Lossner U, Bluher M *et al.* The adipokine SAA3 is induced by interleukin-1 beta in mouse adipocytes. *J Cell Biochem* 2008; **104**: 2241–2247.
13 Lin Y, Rajala MW, Berger JP, Moller DE, Barzilai N, Scherer PE. Hyperglycemia-induced production of acute phase reactants in adipose tissue. *J Biol Chem* 2001; **276**: 42077–42083.
14 Furlaneto CJ, Campa A. A novel function of serum amyloid A: a potent stimulus for the release of tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8 by human blood neutrophil. *Biochem Biophys Res Commun* 2000; **268**: 405–408.
15 Hatanaka E, Furlaneto CJ, Ribeiro FP, Souza GM, Campa A. Serum amyloid A-induced mRNA expression and release of tumor necrosis factor-alpha (TNF-alpha) in human neutrophils. *Immunol Lett* 2004; **91**: 33–37.
16 Ribeiro FP, Furlaneto CJ, Hatanaka E, Ribeiro WB, Souza GM, Cassatella MA *et al.* mRNA expression and release of interleukin-8 induced by serum amyloid A in neutrophils and monocytes. *Mediators Inflamm* 2003; **12**: 173–178.

17 Sandri S, Rodriguez D, Gomes E, Monteiro HP, Russo M, Campa A. Is serum amyloid A an endogenous TLR4 agonist? *J Leukoc Biol* 2008; **83**: 1174–1180.
18 Hatanaka E, Monteagudo PT, Marrocos MSM, Campa A. Interaction between serum amyloid A and leukocytes - A possible role in the progression of vascular complications in diabetes. *Immunol Lett* 2007; **108**: 160–166.
19 Hatanaka E, Dermargos A, Armelin HA, Curi R, Campa A. Serum amyloid A induces reactive oxygen species (ROS) production and proliferation of fibroblast. *Clin Exp Immunol* 2011; **163**: 362–367.
20 Farmer SR. Regulation of PPAR gamma activity during adipogenesis. *Int J Obes* 2005; **29**: S13–S16.
21 Hamm JK, Park BH, Farmer SR. A role for C/EBP beta in regulating peroxisome proliferator-activated receptor gamma activity during adipogenesis in 3T3-L1 preadipocytes. *J Biol Chem* 2001; **276**: 18464–18471.
22 Kawai M, Sousa KM, MacDougald OA, Rosen CJ. The many facets of PPAR gamma: novel insights for the skeleton. *Am J Physiol Endocrinol Metab* 2010; **299**: E3–E9.
23 Ye XY, Xue YM, Sha JP, Li CZ, Zhen ZJ. Serum amyloid A attenuates cellular insulin sensitivity by increasing JNK activity in 3T3-L1 adipocytes. *J Endocrinol Invest* 2009; **32**: 568–575.
24 Moon HS, Chung CS, Lee HG, Kim TG, Choi YJ, Cho CS. Inhibitory effect of (-)-epigallocatechin-3-gallate on lipid accumulation of 3T3-L1 cells. *Obesity* 2007; **15**: 2571–2582.
25 Zou C, Shen Z. One-step intracellular triglycerides extraction and quantitative measurement *in vitro*. *J Pharmacol Toxicol Methods* 2007; **56**: 63–66.
26 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* 2001; **25**: 402–408.
27 Hsu HF, Tsou TC, Chao HR, Shy CG, Kuo YT, Tsai FY *et al.* Effects of arecoline on adipogenesis, lipolysis, and glucose uptake of adipocytes-A possible role of betel-quid chewing in metabolic syndrome. *Toxicol Appl Pharmacol* 2010; **245**: 370–377.
28 Knebel FH, Albuquerque RC, Maria-Engler SS, Campa A. Dual effect of serum amyloid A protein on gliomas. *Inflamm Res* 2011; **60**: 195–195.
29 Cleveland-Donovan K, Maile LA, Tsiaras WG, Tchkonia T, Kirkland JL, Boney CM. IGF-I activation of the AKT pathway is impaired in visceral but not subcutaneous preadipocytes from obese subjects. *Endocrinology* 2010; **151**: 3752–3763.
30 Matthews LC, Taggart MJ, Westwood M. Modulation of caveolin-1 expression can affect signalling through the phosphatidylinositol 3-kinase/Akt pathway and cellular proliferation in response to insulin-like growth factor I. *Endocrinology* 2008; **149**: 5199–5208.
31 Benito M, Valverde AM, Lorenzo M. IGF-I: A mitogen also involved in differentiation processes in mammalian cells. *Int J Biochem Cell Biol* 1996; **28**: 499–510.
32 Gagnon A, Dods P, Roustan-Delatour N, Chen CS, Sorisky A. Phosphatidylinositol-3,4,5-trisphosphate is required for insulin-like growth factor 1-mediated survival of 3T3-L1 preadipocytes. *Endocrinology* 2001; **142**: 205–212.
33 Lee HY, Kim SD, Shim JW, Kim HJ, Yun J, Baek SH *et al.* A pertussis toxin sensitive G-protein-independent pathway is involved in serum amyloid A-induced formyl peptide receptor 2-mediated CCL2 production. *ExpMol Med* 2010; **42**: 302–309.
34 Baranova IN, Vishnyakova TG, Bocharov AV, Kurlander R, Chen ZG, Kimelman ML *et al.* Serum amyloid a binding to CLA-1 (CD36 and LIMPII analogous-1) mediates serum amyloid A protein-induced activation of ERK1/2 and p38 mitogen-activated protein kinases. *J Biol Chem* 2005; **280**: 8031–8040.
35 Du JL, Sun CK, Lü B, Men LL, Yao JJ, An LJ *et al.* Association of SelS mRNA expression in omental adipose tissue with Homa-IR and serum amyloid A in patients with type 2 diabetes mellitus. *Chin Med J* 2008; **121**: 1165–1168.
36 Rosen ED, MacDougald OA. Adipocyte differentiation from the inside out. *Nature Rev Mol Cell Biol* 2006; **7**: 885–896.
37 Gregoire FM, Smas CM, Sul HS. Understanding adipocyte differentiation. *Physiol Rev* 1998; **78**: 783–809.
38 Sandri S, Hatanaka E, Franco AG, Pedrosa AMC, Monteiro HP, Campa A. Serum amyloid A induces CCL20 secretion in mononuclear cells through MAPK (p38 and ERK1/2) signaling pathways. *Immunol Lett* 2008; **121**: 22–26.
39 Guilherme A, Tesz GJ, Guntur KVP, Czech MP. Tumor necrosis factor-alpha induces caspase-mediated cleavage of peroxisome proliferator-activated receptor gamma in adipocytes. *J Biol Chem* 2009; **284**: 17082–17091.
40 Souza SC, de Vargas LM, Yamamoto MT, Lien P, Franciosa MD, Moss LG *et al.* Overexpression of perilipin A and B blocks the ability of tumor necrosis factor alpha to increase lipolysis in 3T3-L1 adipocytes. *J Biol Chem* 1998; **273**: 24665–24669.
41 Lang CH, Dobrescu C, Bagby GJ. Tumor necrosis factor impairs insulin action on peripheral glucose disposal and hepatic glucose output. *Endocrinology* 1992; **130**: 43–52.
42 Peraldi P, Spiegelman B. TNF-alpha and insulin resistance: summary and future prospects. *Mol Cell Biochem* 1998; **182**: 169–175.
43 Langin D, Arner P. Importance of TNF alpha and neutral lipases in human adipose tissue lipolysis. *Trends Endocrinol Metabol* 2006; **17**: 314–320.
44 Spiegelman BM, Arner P, Caro JF, Atkinson RL, Hotamisligil GS. Elevation of adipose-tissue expression of TNF-alpha and TNF receptors in human obesity and insulin-resistance. *Diabetes* 1995; **44**: A45–A45.
45 Vassaux G, Negrel R, Ailhaud G, Gaillard D. Proliferation and differentiation of rat adipose precursor cells in chemically-defined medium - differential action of anti-adipogenic agents. *J Cell Physiol* 1994; **161**: 249–256.

Late effects of sleep restriction: Potentiating weight gain and insulin resistance arising from a high-fat diet in mice

Original Article
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

Late Effects of Sleep Restriction: Potentiating Weight Gain and Insulin Resistance Arising from a High-Fat Diet in Mice

Edson Mendes de Oliveira[1], Bruna Visniauskas[2], Silvana Sandri[1], Silene Migliorini[1], Monica Levy Andersen[2], Sergio Tufik[2], Ricardo Ambrósio Fock[1], Jair Ribeiro Chagas[2,3], and Ana Campa[1]

Objective: Epidemiological studies show the association of sleep restriction (SR) with obesity and insulin resistance. Experimental studies are limited to the concurrent or short-term effects of SR. Here, we examined the late effects of SR regarding weight gain and metabolic alterations induced by a high-fat diet (HFD).

Methods: C57BL/6 mice were subjected to a multiple platform method of SR for 15 days, 21 h daily, followed by 6 weeks of a 30% HFD.

Results: Just after SR, serum insulin and resistin concentrations were increased and glycerol content decreased. In addition, resistin, TNF-α, and IL-6 mRNA expression were notably increased in epididymal fat. At the end of the HFD period, mice previously submitted to SR gained more weight (32.3±1.0 vs. 29.4±0.7 g) with increased subcutaneous fat mass, had increments in the expression of the adipogenic genes *PPAR*γ, *C/EBP*α, and *C/EBP*β, and had macrophage infiltration in the epididymal adipose tissue. Furthermore, enhanced glucose tolerance and insulin resistance were also observed.

Conclusions: The consequences of SR may last for a long period, characterizing SR as a predisposing factor for weight gain and insulin resistance. Metabolic changes during SR seem to prime adipose tissue, aggravating the harmful effects of diet-induced obesity.

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Introduction

As a highly prevalent condition of a multifactorial nature, obesity is today a central public health issue. The way obesity is seen today is quite different from how it was seen before. New factors, never before proposed, such as microbiota composition, use of antibiotics, infections, and probably the dietary intake of inadequate amounts of microelements and vitamins have been linked with the increased incidence or the worsening of obesity and insulin resistance (1-5). In this complex scenario, sleep disorders have been proposed to be not only a consequence of obesity but also a cause contributing to weight gain (6). There is epidemiological evidence that the restriction of human sleep may contribute to increased weight, suggesting that short sleep duration is strongly and consistently associated with concurrent and future obesity (7-9). In addition, several studies have correlated inadequate sleep with clear changes in glucose metabolism and insulin sensitivity that may contribute to the development of diverse comorbidities, such as cardiovascular disease, insulin resistance, and type 2 diabetes (10,11). Significant weight gain may result in insulin resistance, a condition that, in turn, may promote further adiposity.

It is now generally accepted that chronic sleep restriction (SR) has negative effects on general health and, more specifically, on metabolism (12). Hypotheses regarding the mechanism that relates SR with insulin resistance and obesity in humans include the greater time awake, providing more opportunity for food intake, hormonal changes capable of affecting caloric intake, physical activity, basal metabolic rate, and proinflammatory cytokine production (13,14). Experimental models and several cross-sectional and cohort studies with humans show impaired insulin sensitivity in young and healthy individuals without pre-existing diabetes mellitus (11,15-18).

Our hypothesis is that the effects of SR can reverberate long after SR cessation. Animal experimental models usually describe the effects of SR just during and shortly after restriction. In humans, a long-term impact of SR was eventually assessed; however, the late effects of a past period of SR are difficult to recognize. The definition we adopted for late effects are side effects of SR that become apparent after SR has ended and the recovery sleep period completed. This study evaluates the late impact of a period of SR on weight gain, insulin resistance, and adipose tissue structure.

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A generally accepted animal model to study the consequences and related mechanisms of SR is the modified multiple-platform method (19) that suppresses mainly paradoxical sleep in rodents, but also suppresses around 30% of slow wave sleep and reduces the stress because of social isolation. Paradoxical sleep in rodents corresponds to the rapid eye movement period in humans. The preservation of sleep homeostasis in chronic SR models is still an open question (20,21). In the present study, mice were subjected to the multiple-platform method to create the SR period. After that, a high-fat diet (HFD) was introduced. Adipogenic, metabolic markers, and adipose tissue architecture were evaluated.

Methods

Animals

Male C57BL/6 mice (3 months of age) were obtained from CEDEME Universidade Federal de São Paulo (UNIFESP). The animals were housed in a room maintained at 20±2°C in 12:12 h light/dark cycle (lights on at 7:00 am and off at 7:00 pm) inside standard polypropylene cages. For each experimental group, 5-9 animals were used for the experimental protocol. All procedures used in the present study complied with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, 1996). The experimental protocol was approved by the Ethical Committee of UNIFESP (approval n°0474/09).

Sleep restriction

The method of SR was adapted from the multiple-platform method (19). Groups of 5-9 mice were placed in water tanks (41 × 34 × 16.5 cm), containing 13 platforms (3 cm in diameter) each, surrounded by water up to 1 cm beneath the surface. In this method, the animals were able to move inside the tank, jumping from one platform to the other, keeping diet and water *ad libitum*. All the control groups were kept in control home-cages allowing sleep *ad libitum* under standard rodent chow diet. For SR experiments, the animals were randomly assigned to two groups: the control group and the SR group, both under standard rodent chow diet. The SR group was sleep restricted for 15 days, 21 h daily. After each 21 h period of SR, the mice were allowed to sleep for 3 h (sleep opportunity beginning at 10:00 am). The euthanasia occurred immediately after the last SR period.

SR followed by HFD

For SR followed by 6 weeks of HFD (SR+HFD) experiments, the animals were randomly assigned to four different groups: the control, the SR, the HFD, and the SR+HFD groups. The control group was not submitted to SR or HFD. The SR mice were sleep restricted as described above followed by 7 weeks. The HFD mice were allowed to sleep *ad libitum* during the entire protocol and submitted to HFD for 6 weeks. The SR+HFD mice were sleep restricted as described above followed by 1 week of recovery period in standard home-cage plus 6 weeks on a 30% HFD. In our experimental model, we consider the recovery sleep period as 7 days, the time that we observed weight reestablishment. The diet was produced following the American Institute of Nutrition's recommendations for the adult rodent, and its composition is listed in Table 1. After this period, mice were submitted to euthanasia. All mice were euthanized by decapitation in the same day between 8:00 and 10:00 am. Body weight was measured weekly. Food and water intake were measured every 2 days. The entire experimental design is illustrated in Figure 3A.

TABLE 1 Experimental diet composition

Ingredient (g/kg)	Chow diet[a]	High-fat diet[a]
Sucrose	100	100
Casein	120	120
Corn oil	80	80
Lard	–	300
Cellulose	50	50
Mineral mix (Rhoster®)	35	35
Vitamin mix (Rhoster®)	10	10
DL-methionine	1.8	1.8
Choline bitartrate	2.5	2.5
Tert-butylhydroquinone	0.01	0.04
Cornstarch q.s.p.	1000	1000

[a]Chow diet and high-fat diet formulation according to AIN-93M.

Glucose and insulin tolerance tests and measurements of serum leptin, adiponectin, insulin, resistin, and free glycerol

Glucose and insulin tolerance tests (GTT and ITT) were performed by injecting glucose (2 g/kg body weight i.p.) or insulin (0.75 U/kg body weight i.p.), respectively, after a 4 h fasting period. Tail blood samples were collected at 0, 15, 30, 60, and 90 min for GTT and 0, 5, 10, 20, 30, 45, 60, and 120 min for ITT. Blood glucose levels were determined using a Contour TS Bayer glucometer. Fasting leptin, adiponectin, and insulin concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) from Millipore Corporation (Billerica, MA, USA). Resistin protein was measured using the ELISA kit (R & D Systems), and free glycerol content was determined by the colorimetric Free Glycerol Assay Kit (Abcam®, Cambridge, MA, USA), following manufacturer's instructions.

Light microscopy

Epididymal, retroperitoneal and subcutaneous white adipose tissue (WAT) depots were dissected and weighed to assess the adipose tissue mass in light microscopy and morphometry analysis. Epididymal and retroperitoneal fat depots from lean mice (chow diet) were not quantified because of their very small quantity. After dissection, epididymal fat pad was fixed by immersion in 4% formaldehyde in 10 mM phosphate buffer, pH 7.4, for 24 h, dehydrated, cleared, and then embedded in paraffin. Serial sections (5 μm thick) were obtained and then stained by hematoxylin and eosin to assess morphology. Tissue sections were observed with a Nikon Eclipse 80i microscope (Nikon®) using an ×10 objective, and digital images were captured with NIS-Element AR software (Nikon®). The macrophage infiltration was analyzed by morphology considering the frequency of occurrence of crown structures into the epididymal adipose tissue of each animal.

In vivo peripheral fat area quantification

The peripheral fat area was also quantified. Two X-ray images were taken at different energy levels: one low-energy X-ray

TABLE 2 PCR primers used in all quantitative PCR assays

Primer	Forward	Reverse
PPARγ	5′-TTC TGA CAG GAC TGT GTG ACA G-3′	5′-ATA AGG TGG AGA TGC AGG TTC-3′
C/EBPα	5′-GTG TGC ACG TCT ATG CTA AAC CA-3′	5′-GCC GTT AGT GAA GAG TCT CAG TTT G-3′
C/EBPβ	5′-GTT TCG GGA CTT GAT GCA ATC-3′	5′-AAC AAC CCC GCA GGA ACA T-3′
Leptin	5′-CCA AAA CCC TCA TCA AGA CC-3′	5′-CTT TCA TTT CCC CTC CTT TTC-3′
Perilipin	5′-CAT GTC CCT ATC CGA TGC C-3′	5′-TCG GTT TTG TCG TCC AGG-3′
Resistin	5′-CTT TCA TTT CCC CTC CTT TTC-3′	5′-CAG TCT ATC CTT GCA CAC TGG-3′
TNF-α	5′-GGT GCC TAT GTC TCA GCC TC-3′	5′-CAC TTG GTG GTT TGC TAC GA-3′
IL-6	5′-TGT GCA ATG GCA ATT CTG AT-3′	5′-ACC AGA GGA AAT TTT CAA TAG GC-3′
18S	5′-GTA ACC CGT TGA ACC CCA TT-3′	5′-CCA TCC AAT CGG TAG TAG CG-3′

(10 keV) to image soft tissue and one high-energy X-ray (15 keV) to image bone, using the Carestream® In-Vivo MS FX Pro multispectral imaging system. The image math tool was utilized to produce the low/high energy ratio images which allowed circumscribing and integrating different anatomical areas on the animals. The resulting ratiometric image was then displayed using a "Fire" intensity scale highlighting the subcutaneous fat area on the animals.

RNA extraction and cDNA synthesis

Total RNA from epididymal and subcutaneous adipose tissue was isolated after HFD or SR+HDF using Qiagen RNeasy® Mini kit (Qiagen, Hilden, Germany). cDNA was then synthesized from 1 μg of RNA using SuperScript® First-Strand Synthesis System for RT-PCR kit (Life Technologies®, Grand Island, NY, USA).

Quantitative real-time PCR

Real-time PCR was performed using the primers listed in Table 2. BLAST searches were conducted on all primer sequences to ensure gene specificity. Each amplification reaction was performed in quadruplicate and included the SyBr® Green Master Mix (Life Technologies®, Grand Island, NY, USA). Reaction conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 10 s, and 60°C for 1 min. Melting curve analyses were performed at the end of each run as a quality control step. The Ct (cycle threshold) for each run was set to 0.1, when amplification was observed in log phase. Relative gene expression was determined using the ΔΔCt method, and efficiency of each reaction was previously validated (22). PCR reactions were performed in Gene AMP® 7500 Sequence Detection System (Applied Biosystems, Grand Island, NJ, USA).

Statistical analysis

Results were presented as means ± SE and the number of independent experiments is indicated. Statistical analysis was performed with Graph Pad Prism4 (Graph Pad Software, San Diego, CA, USA). Comparisons between two groups were conducted with the unpaired Student's *t* test. Data with two independent variables were tested by two-way analysis of variance with Bonferroni *post hoc* test. The level of significance was set at $P < 0.05$.

Results

SR alters some metabolic and inflammatory parameters but no adipogenic markers

Mice were subjected to SR for 15 days, 21 h daily. Just after the SR period, serum concentrations of insulin, which was already described to be affected by SR, were measured. There was an increase of approximately 50% of serum insulin concentration (Figure 1A). Free glycerol, leptin, adiponectin, and resistin were measured to also assess the metabolic status of the animals. Free glycerol was reduced by approximately 20% (Figure 1B) and there were no alterations of leptin and adiponectin serum concentrations (Figure 1C, D). However, we observed almost twice the amount of resistin is SR mice (Figure 1E). To further assess the impact of SR on the adipose tissue, real-time PCR for adipokines, adipogenic markers, and proinflammatory cytokines was done. The relative amounts of PPARγ (Figure 2A), C/EBPα (Figure 2B), C/EBPβ (Figure 2C), perilipin (Figure 2D), and leptin (Figure 2E) mRNA were comparable between control and SR mice. In contrast, the relative amounts of resistin, TNF-α, and IL-6 transcript were markedly increased in epididymal fat of SR mice (Figure 2F-H).

SR leads to mass gain and macrophage infiltration in adipose tissue after 6 weeks of HFD

After the SR period, the animals spent 1 week in recovery followed by 6 weeks of a 30% HFD (Figure 3A). The total food intake in grams was similar among all groups of animals (chow diet or HFD) with an average 2.5 g per day. When we consider the caloric intake, the control and SR groups consumed approximately 10 kcal/day and the HFD and SR+HFD groups consumed 13.9 kcal/day (Figure 3B). The water intake was similar among all the groups (Figure 3C). As expected, animals lost weight during SR (Figure 3D). However, from the fourth week of the protocol there were no differences in weight between control and SR groups, while HFD and SR+HFD groups showed an increase in body weight evidenced from the seventh week. Furthermore, it was also possible to verify that the SR+HFD group gained more weight than the HFD group (Figure 3D and Table 3). From then on, as control and SR groups had similar weight gain, epididymal fat mass, and adipose tissue morphology, we focused on comparison between HFD and SR+HFD groups. The highest increment in total weight of the SR+HFD group was because of an increase in the subcutaneous depot weight

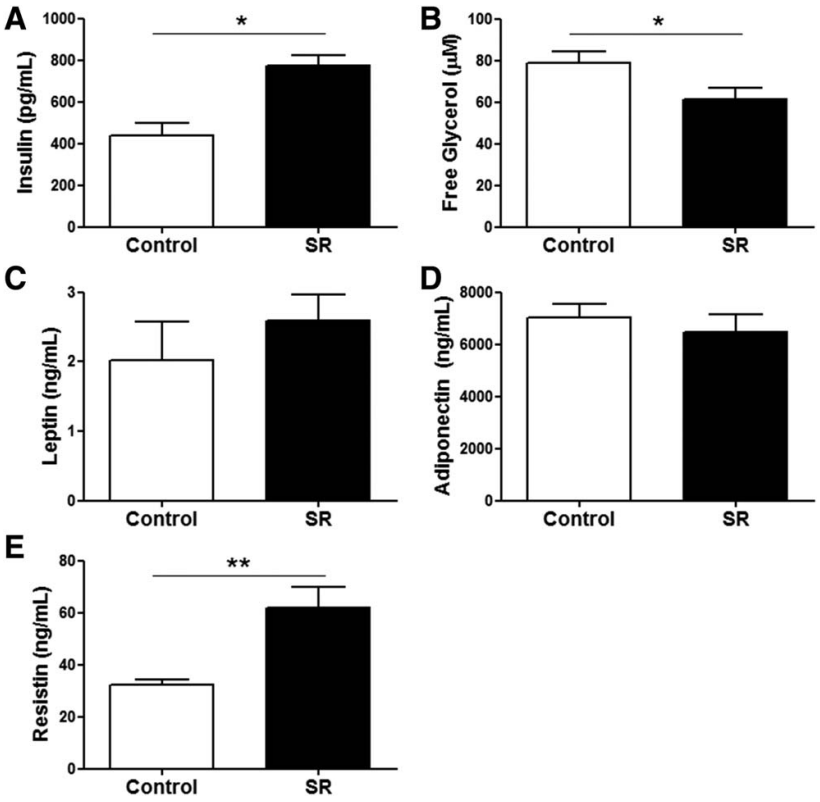

Figure 1 Insulin, glycerol, and resistin serum levels are altered just after sleep restriction (SR). C57BL/6 mice were submitted to SR for 15 days, 21 h daily, and metabolic markers were quantified after SR. (**A**) Insulin, (**B**) free glycerol, (**C**) leptin, (**D**) adiponectin, and (**E**) resistin were measured in serum and assessed by ELISA. Data are means ± SE from 5-9 mice per group (*$P < 0.05$, ** $P < 0.01$, *vs.* control).

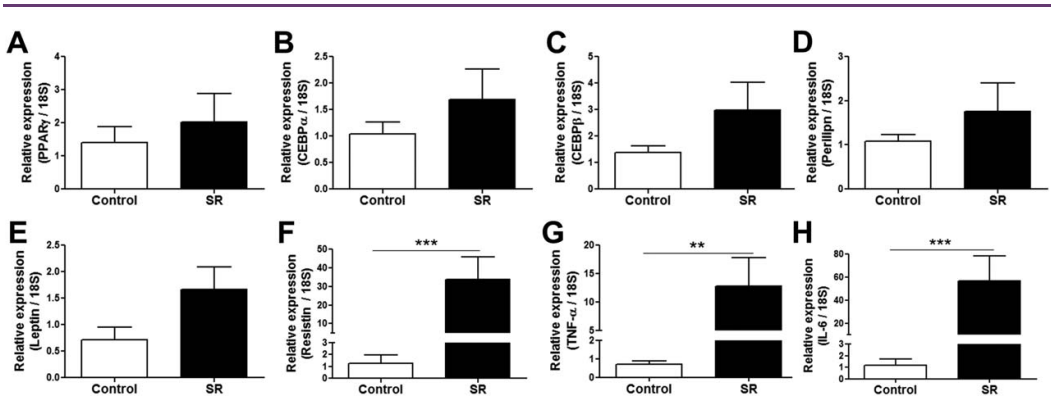

Figure 2 Resistin, TNF-α, and IL-6 are upregulated in adipose tissue after sleep restriction (SR). Quantitative real-time PCR was performed to assess mRNA expression of the adipogenic markers (**A**) PPARγ, (**B**) C/EBPα, (**C**) C/EBPβ, (**D**) perilipin, and (**E**) leptin, as well as the proinflammatory markers (**F**) resistin, (**G**) TNF-α, and (**H**) IL-6 in epididymal adipose tissue depot. Data are means ± SE from 5–9 mice per group (**$P < 0.01$, *** $P < 0.001$, *vs.* control).

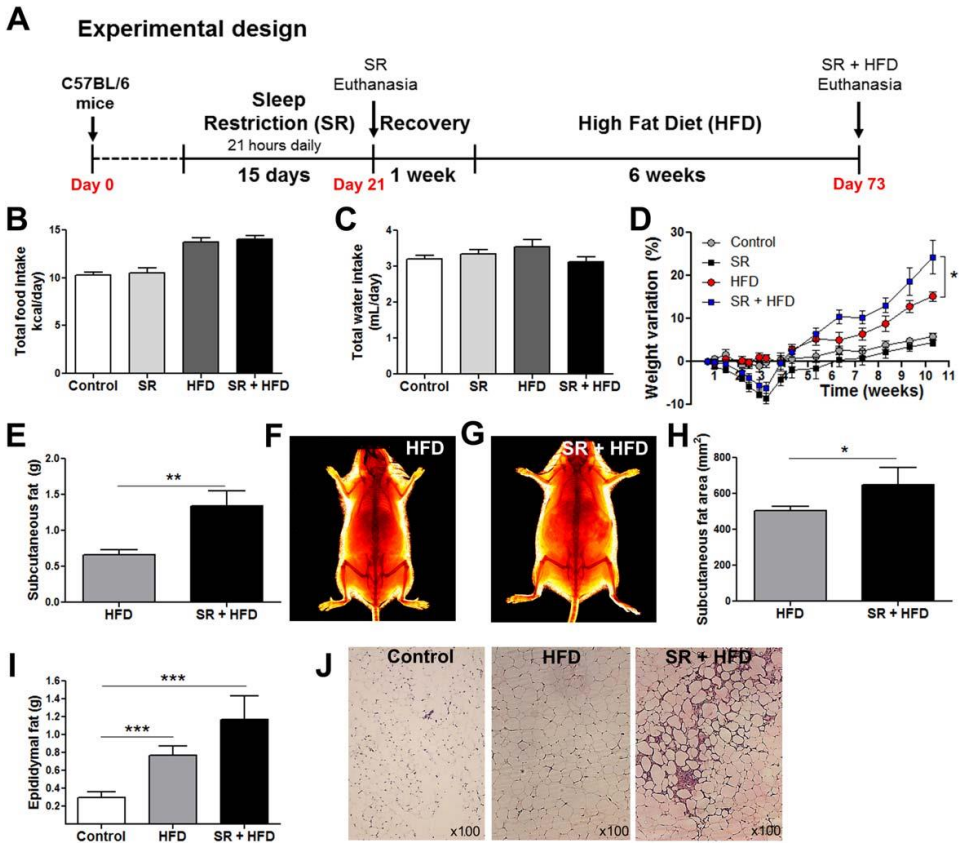

Figure 3 A previous period of sleep restriction (SR) potentiates weight gain and macrophage infiltration in adipose tissue induced by a high-fat diet (HFD). (**A**) C57BL/6 mice were subjected to a multiple-platform method of SR for 15 days, 21 h daily. After the SR period, the animals spent 1 week in recovery followed by 6 weeks of a 30% HFD. (**B**) Daily caloric intake. (**C**) Daily water intake. (**D**) Weight gain curve of control mice fed a chow diet (control group), Sr mice fed a chow diet (SR group), mice fed HFD (HFD group), and mice sleep restricted followed by HFD (SR+HFD group). (**E**) Subcutaneous fat pad weight. (**F**) Representative subcutaneous fat area in mice fed HFD and (**G**) representative subcutaneous fat area in SR+HFD mice. (**H**) Subcutaneous fat area quantification. (**I**) Epididymal fat pad weight. (**J**) Histological sections of epididymal fat pads from control, HFD, and SR+HFD mice. Data are means ± SE from 5–9 mice per group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, between groups, as indicated). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Figure 3E). These data were confirmed using X-ray images highlighting the subcutaneous fat area on the animals, showing that SR+HDF mice had a higher peripheral fat area when compared to HFD mice (Figure 3F-H). Although there was no statistical difference in epididymal fat mass between HFD and SR+HFD animals (Figure 3I), there were areas with a massive macrophage infiltration into the epididymal fat tissue of 33% of the animals submitted to SR+HFD (Figure 3J).

SR followed by HFD increases transcriptional regulators of adipogenesis

In both the HFD and SR+HFD groups, an increase was observed in the mRNA expression of some genes responsible for driving adipogenesis, such as PPARγ, C/EBPα and C/EBPβ, leptin, perilipin, and resistin in epididymal and subcutaneous adipose tissue when compared to control (chow diet) (Figure 4A-H). With the exception of perilipin, all the other adipogenic parameters were increased in at least one of the adipose tissue depots analyzed when animals in the HFD and SR+HFD groups were compared (Figure 4A-E). The same increment profile was observed for resistin but not for TNF-α and IL-6 (Figure 4F-H).

SR leads to insulin resistance after 6 weeks of HFD

The diet-induced obesity for 6 weeks was sufficient to alter the metabolic status of the animals. In contrast to control mice, HFD and

TABLE 3 Body weight of mice at specific time points of the experimental protocol

	Weight (g)			
	Control ($n = 5$)	SR ($n = 5$)	HFD ($n = 6$)	SR+HFD ($n = 9$)
Day 0	25.1 ± 0.1	25.6± 1.2	25.6 ± 0.3	26.0 ± 0.2
Day 21	25.2 ± 0.5	23.4 ± 0.8[a]	25.8 ± 0.2	24.4 ± 0.3[b]
Day 73	26.6 ± 0.5	26.8 ± 1.1	29.4 ± 0.7[c]	32.3 ± 1.0[d]

C57BL/6 mice were subjected to a multiple-platform method of SR for 15 days, 21 h daily. After the SR period, the animals spent 1 week in recovery followed by 6 weeks of a 30% HFD. Control group (mice fed chow diet), SR group (mice sleep restricted), HFD group (mice fed a HFD), and SR+HFD group (mice sleep restricted followed by HFD). Data are means ± SE from 5–9 mice per group.
[a]$P < 0.05$, SR day 0 *vs.* SR day 21.
[b]$P < 0.05$, SR+HFD day 0 *vs.* SR+HFD day 21.
[c]$P < 0.05$, control *vs.* HFD or SR *vs.* HFD.
[d]$P < 0.05$, control *vs.* SR+HFD, SR *vs.* SR+HFD, or HFD *vs.* SR+HFD).

SR+HFD mice showed decreased free glycerol and increased leptin levels (Figure 5A,B), though this period on HFD did not have significant changes in adiponectin, resistin, fasting glucose, and insulin concentrations (Figure 5C-F). No significant difference in free glycerol, leptin, adiponectin, resistin, glucose or insulin levels was observed when HFD and SR+HFD groups were compared (Figure 5A-F). However, the GTT and ITT were notably affected in the SR+HFD group, showing impaired insulin sensitivity (Figure 5G-H).

Discussion

In this study, we examined the late effects of SR regarding weight gain and metabolic status when associated with a subsequent adoption of HFD. Initially, SR was able to alter insulin and glycerol concentrations (Figure 1). The increase in insulin concentration and hepatic and peripheral insulin resistance was previously described in cross-sectional and cohort studies in human and in experimental models and therefore was expected (10,13,23). The reason for insulin increment may be directly related to SR, but it was also hypothesized by others to be a consequence of a stress condition, corticosterone increase, hyperphagia, or even enhanced energy expenditure (24-27). SR cannot be dissociated from a mild increase of nonspecific stress and, moreover, it also changes the HPA axis response to stress. So, it is quite reasonable to not expect that in nonexperimental situations SR and stress will be separated. The decreased free glycerol profile could also be explained by its utilization for glucose synthesis, associated with an enhanced hepatic gluconeogenesis derived from free fatty acid released during lipolysis (28).

Despite the fact that sleep disorders alter glucose and lipid metabolism, no effect was observed on the adipogenic markers in the epididymal fat pad shortly after SR. Our study, as well as studies from other authors (11,27,29), showed that mice tend to lose weight during SR (Table 3, Day 21), with enhanced lipolysis and thus diverting adipogenesis (11). Notably, adipose tissue produces several pro-inflammatory, procoagulant, and acute-phase molecules in direct proportion to adiposity. Among these molecules, TNF-α, IL-6, PAI-1, NO, and MCP-1 have been implicated in the development of adverse pathophysiological phenotypes associated with obesity and type 2 diabetes (30-33). Here, we confirmed previous studies showing that SR caused an increased expression of TNF-α and IL-6 in the adipose tissue (Figure 2). We also observed an important increase in resistin mRNA expression and protein content shortly after SR that was not previously reported (Figure 1 and 2) and that may resound on the subsequent modifications caused by the adoption of HFD. It is important to highlight that resistin was already described as being altered in obese patients with obstructive sleep apnea syndrome (OSAS) and is linked to subclinical inflammation and insulin resistance (34,35). Our findings point out that even in nonfat and non-OSAS mice, resistin levels are elevated after SR. Thus, the possible harmful effects of the increase of resistin such as cardiovascular diseases, obesity, and type 2 diabetes should be considered in sleep disorders (36,37).

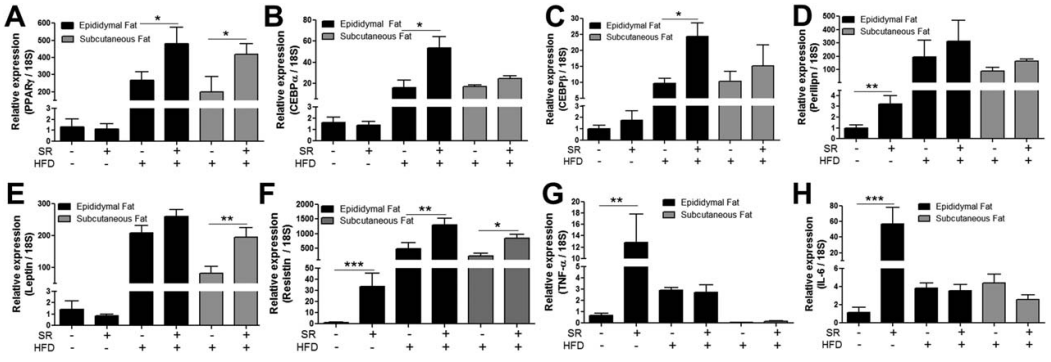

Figure 4 A previous period of sleep restriction (SR) increases adipogenic and inflammatory markers induced by a high-fat diet (HFD). Determination of the relative mRNA expression by real-time PCR of (**A**) PPARγ, (**B**) C/EBPα, (**C**) C/EBPβ, (**D**) perilipin, (**E**) leptin, (**F**) resistin, (**G**) TNF-α, and (**H**) IL-6 in epididymal adipose tissue depot. Data are means ± SE from 5–9 mice per group (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, between groups, as indicated).

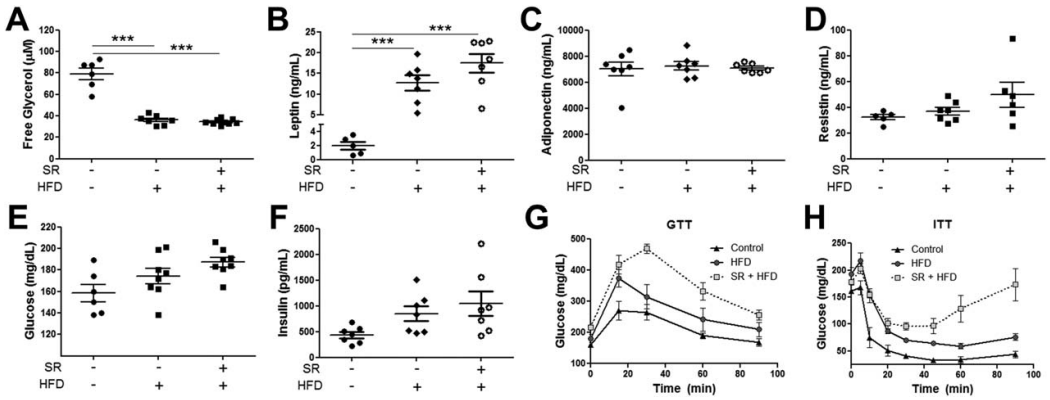

Figure 5 A previous period of sleep restriction (SR) increases glucose tolerance and insulin resistance induced by a high-fat diet (HFD). Determination of (**A**) free glycerol, (**B**) leptin, (**C**) adiponectin, (**D**) resistin, (**E**) glucose, (**F**) insulin, (**G**) glucose tolerance test (GTT), and (**H**) insulin tolerance test (ITT) in control, HFD, and SR+HFD groups. Data are means ± SE from 5–9 mice per group (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ between groups, as indicated).

Our main aim was to look for late effects of a period of SR related to complications arising from 6 weeks of HFD. HFD is a classic adipogenic inducer and we looked for any outcome exacerbation caused by a previous SR. Interestingly, we observed a strong synergism between SR and HFD. This is evident when we observe that SR animals gained weight faster (Figure 3D) and at the end of the experimental protocol there were a marked difference in weight (Table 3, Day 73) and in the subcutaneous adipose tissue size (an increment of approximately 12%) without change in food and water consumption (Figure 3). In this sense, as far as we know, our study is the first to show late weight gain associated with a previous SR, validating a prior suggestion that sleep disruption is able to induce prolonged physiological impairments, contributing to the development of obesity (38). Our data legitimate SR as a triggering factor to adipose tissue mass gain associated with a subsequent HFD period. The histological evaluation of the adipose tissue of these animals also showed a significant change in the cellular composition, clearly modifying the tissue architecture. Concomitantly with the adipose tissue hypertrophy, areas of a massive infiltration of macrophages in the epididymal depot were observed in 33% of the animals previously submitted to SR (Figure 3J). It is well established that adipose tissue macrophage accumulation is directly proportional to adiposity in mice and humans (30). The association between macrophage infiltration and adiposity provides a mechanism to explain obesity comorbidities, including the adipose tissue production of proinflammatory molecules (30,39). Although our experimental model of SR was a potent stimulus for TNF-α and IL-6 mRNA expression, it does not potentiate the increased expression of these cytokines caused only by the adoption of HFD (Figure 4).

After the adoption of HFD, a remarkable change in the expression profile of the adipogenic differentiation state-specific genes PPARγ, C/EBPα, C/EBPβ, leptin, and perilipin was also observed. Some of these genes are essential transcriptional regulators of adipogenesis and are targets for adipogenic inhibitors in obesity treatment (40). As expected, the large increment in the expression of these genes was already observed with the HFD induction when compared to chow diet. However, the increase in most of these adipogenic genes in SR animals was approximately twice that observed in animals only subjected to HFD in both the epididymal and subcutaneous adipose fat pads (Figure 4). It seems that the SR period primes the adipose tissue, predisposing it to hypertrophy. Although we do not yet know the biochemical triggers of this process, the large increase in TNF-α, IL-6, and resistin in adipose tissue during the SR draws a lot of attention. These molecules could be involved in the initial phase; however, they do not appear to be associated with the severity of the HFD-induced process, given that there was no difference in the levels of these molecules when the HFD and SR+HFD groups were compared.

In contrast to the subcutaneous adipose tissue, we did not observe an increase in weight of the epididymal fat pad at the experimental time here evaluated. However, the adipogenic markers were already elevated in mice submitted to SR followed by HFD, leading toward adipose tissue hypertrophy and metabolic consequences. In addition, the presence of areas of macrophage infiltration found in a third of SR+HFD samples points to a more severe outcome. The worsening of the metabolic condition of mice previously sleep deprived is clearly noticed from the GTT and ITT profiles (Figure 5). These findings raise important questions. How long should the period of SR be to adversely impact on the adipose tissue? How long do the marks of the SR period persist?

In conclusion, our data suggest that a history of SR potentiates future complications arising from HFD, such as obesity, insulin resistance, and type 2 diabetes. Although we are conscious of the limitations of SR experimental models and the difficulty of applying our findings to humans, it is unavoidable to consider that some of our findings may have a parallel for humans. This seems especially relevant considering the reduction of the average sleep period in the last decades as a consequence of the adoption of modern work and social habits and the current epidemic of obesity. O

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References

1. Cani PD, Bibiloni R, Knauf C, Neyrinck AM, Delzenne NM, Burcelin R. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* 2008;57: 1470-1481.
2. Trasande L, Blustein J, Liu M, Corwin E, Cox LM, Blaser MJ. Infant antibiotic exposures and early-life body mass. *Int J Obes* 2013;37: 16-23.
3. Gabbert C, Donohue M, Arnold J, Schwimmer JB. Adenovirus 36 and obesity in children and adolescents. *Pediatrics* 2010;126: 721-726.
4. Hegde V, Dhurandhar NV. Microbes and obesity-interrelationship between infection, adipose tissue and the immune system. *Clin Microbiol Infect* 2013;19: 314-320.
5. Suliburska J, Cofta S, Gajewska E, et al. The evaluation of selected serum mineral concentrations and their association with insulin resistance in obese adolescents. *Eur Rev Med Pharmacol Sci* 2013;17: 2396-2400.
6. Knutson KL. Does inadequate sleep play a role in vulnerability to obesity? *Am J Hum Biol* 2012;24: 361-371.
7. Hairston KG, Bryer-Ash M, Norris JM, Haffner S, Bowden DW, Wagenknecht LE. Sleep duration and five-year abdominal fat accumulation in a minority cohort: The IRAS family study. *Sleep* 2010;33: 289-295.
8. Watanabe M, Kikuchi H, Tanaka K, Takahashi M. Association of short sleep duration with weight gain and obesity at 1-year follow-up: A large-scale prospective study. *Sleep* 2010;33: 161-167.
9. Chaput JP, Despres JP, Bouchard C, Tremblay A. The association between sleep duration and weight gain in adults: A 6-year prospective study from the Quebec Family Study. *Sleep* 2008;31: 517-523.
10. Tasali E, Leproul R, Ehrmann DA, Van Cauter E. Slow-wave sleep and the risk of type 2 diabetes in humans. *Proc Natl Acad Sci US A* 2008;105: 1044-1049.
11. Barf RP, Meerlo P, Scheurink AJW. Chronic Sleep Disturbance Impairs Glucose Homeostasis in Rats. *Int J Endocrinol* 2010.
12. Copinschi G, Leproult R, Spiegel K. The Important Role of Sleep in Metabolism. *Front Horm Res* 2014;42: 13.
13. Zimberg IZ, Damaso A, Del Re M, et al. Short sleep duration and obesity: mechanisms and future perspectives. *Cell Biochem Funct* 2012;30: 524-529.
14. Padilha HG, Crispim CA, Zimberg IZ, et al. A link between sleep loss, glucose metabolism and adipokines. *Braz J Med Biol Res* 2011;44: 992-999.
15. Broussard JL, Ehrmann DA, Van Cauter E, Tasali E, Brady MJ. Impaired insulin signaling in human adipocytes after experimental sleep restriction a randomized, crossover study. *Ann Intern Med* 2012;157: 549-558.
16. Klingenberg L, Chaput J-P, Holmback U, et al. Acute sleep restriction reduces insulin sensitivity in adolescent boys. *Sleep* 2013;36: 1085-1090.
17. Wang Y, Carreras A, Lee S, et al. Chronic sleep fragmentation promotes obesity in young adult mice. *Obesity* 2014;22: 758-762.
18. Vetrivelan R, Fuller PM, Yokota S, Lu J, Saper CB. Metabolic effects of chronic sleep restriction in rats. *Sleep* 2012;35: 1511-1520.
19. Machado RB, Hipolide DC, Benedito-Silva AA, Tufik S. Sleep deprivation induced by the modified multiple platform technique: quantification of sleep loss and recovery. *Brain Res* 2004;1004: 45-51.
20. Leemburg S, Vyazovskiy VV, Olcese U, Bassetti CL, Tononi G, Cirelli C. Sleep homeostasis in the rat is preserved during chronic sleep restriction. *Proc Natl Acad Sci U S A* 2010;107: 15939-15944.
21. Clasadonte J, McIver SR, Schmitt LI, Halassa MM, Haydon PG. Chronic sleep restriction disrupts sleep homeostasis and behavioral sensitivity to alcohol by reducing the extracellular accumulation of adenosine. *J Neurosci* 2014;34: 1879-1891.
22. Filippin-Monteiro FB, de Oliveira EM, Sandri S, Knebel FH, Albuquerque RC, Campa A. Serum amyloid A is a growth factor for 3T3-L1 adipocytes, inhibits differentiation and promotes insulin resistance. *Int J Obes* 2012;36:1032-1039.
23. Knutson KL, Van Cauter E, Zee P, Liu K, Lauderdale DS. Cross-sectional associations between measures of sleep and markers of glucose metabolism among subjects with and without diabetes: the Coronary Artery Risk Development in Young Adults (CARDIA) sleep study. *Diabetes Care* 2011;34: 1171-1176.
24. Knutson KL, Cauter E. Associations between sleep loss and increased risk of obesity and diabetes. *Ann N Y Acad Sci* 2008;1129: 287-304.
25. Andersen ML, Martins PJF, D'Almeida V, Bignotto M, Tufik S. Endocrinological and catecholaminergic alterations during sleep deprivation and recovery in male rats. *J Sleep Res* 2005;14: 83-90.
26. Hipolide DC, Suchecki D, Pinto APD, Faria EC, Tufik S. Paradoxical sleep deprivation and sleep recovery: Effects on the hypothalamic-pituitary-adrenal axis activity, energy balance and body composition of rats. *J Neuroendocrinol* 2006;18: 231-238.
27. Martins PJF, Marques MS, Tufik S, D'Almeida V. Orexin activation precedes increased NPY expression, hyperphagia, and metabolic changes in response to sleep deprivation. *Am J Physiol Endocrinol Metab* 2010;298: E726-E734.
28. Chen XH, Iqbal N, Boden G. The effects of free fatty acids on gluconeogenesis and glycogenolysis in normal subjects. *J Clin Invest* 1999;103: 365-372.
29. Barf RP, Van Dijk G, Scheurink AJW, et al. Metabolic consequences of chronic sleep restriction in rats: Changes in body weight regulation and energy expenditure. *Physiol Behav* 2012;107: 322-328.
30. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003;112: 1796-1808.
31. Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc Natl Acad Sci USA* 2003;100: 7265-7270.
32. Samad F, Loskutoff DJ. Tissue distribution and regulation of plasminogen activator inhibitor-1 in obese mice. *Mol Med* 1996;2: 568-582.
33. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* 1997;389: 610-614.
34. Harsch IA, Koebnick C, Wallaschofski H, et al. Resistin levels in patients with obstructive sleep apnoea syndrome—the link to subclinical inflammation? *Med Sci Monit* 2004;10: CR510-CR515.
35. Yamamoto Y, Fujiuchi S, Hiramatsu M, et al. Resistin is closely related to systemic inflammation in obstructive sleep apnea. *Respiration* 2008;76: 377-385.
36. Reilly MP, Lehrke M, Wolfe ML, Rohatgi A, Lazar MA, Rader DJ. Resistin is an inflammatory marker of atherosclerosis in humans. *Circulation* 2005;111: 932-939.
37. Steppan CM, Bailey ST, Bhat S, et al. The hormone resistin links obesity to diabetes. *Nature* 2001;409: 307-312.
38. Husse J, Hintze SC, Eichele G, Lehnert H, Oster H. Circadian clock genes Per1 and Per2 regulate the response of metabolism-associated transcripts to sleep disruption. *Plos One* 2012;7.
39. Weisberg SP, Hunter D, Huber R, et al. CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest* 2006;116: 115-124.
40. Hamm JK, Park BH, Farmer SR. A role for C/EBP beta in regulating peroxisome proliferator-activated receptor gamma activity during adipogenesis in 3T3-L1 preadipocytes. *J Biol Chem* 2001;276: 18464-18471.

Acute endotoxemia exacerbates diet-induced obesity and insulin resistance: possible role for serum amyloid A

Acute endotoxemia exacerbates diet-induced obesity and insulin resistance: possible role for serum amyloid A

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Running title: SAA in endotoxemia-induced obesity.

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Abbreviation list: antisense oligonucleotide (ASO), cluster of differentiation 14 (CD14), glucose tolerance test (GTT), high-fat diet (HFD), insulin tolerance test (ITT), lipopolysaccharide (LPS), serum amyloid A (SAA), scavenger receptor BI (SR-BI), toll-like receptor 2 (TLR-2), toll-like receptor 4 (TLR-4).

ABSTRACT

From the canonical study from Cani, Burcelin and colleagues we have learned that low-grade endotoxemia, also named metabolic endotoxemia, causes weight gain and insulin resistance (Cani P.D. *et al*. Diabetes. 2007;56:1761). This low-grade inflammation is also achieved with a HFD. From there, several studies have consolidated the role of microbiota-derived endotoxemia and the importance for the receptor system CD14/TLR-4 in obesity and insulin resistance. Here we took hold of these observations and addressed whether serial intense endotoxemia mimicking a systemic acute inflammation can also be considered an aggravating factor to obesity development. Mice lost weight when submitted to multiple LPS i.p. administrations (10 mg/kg, every 3 days for 24 days) and, after the cessation of endotoxemia, they rapidly recovered and maintained the normal weight. However, acute endotoxemia caused a long-lasting adipose tissue expression of TLR-4, CD14 and SAA in mice, becoming more susceptible to the harmful effects of a high-fat diet (HFD). Animals previously submitted to acute endotoxemia showed a more severe weight gain (approximately in 15%), related to an increase of visceral and subcutaneous adipose tissue (33.3% and 60.1%, respectively), a higher increment in leptin and insulin serum levels (1.3 and 3.6 times higher, respectively), and impaired insulin sensitivity. In the second part of this study, we evaluated the participation of the inflammatory protein serum amyloid A (SAA) as a triggering setting to insulin sensitivity and weight gain in a HFD-induced metabolic entodoxemia model. HFD-fed mice under SAA-targeted antisense oligonucleotide (ASO_{SAA}) treatment did not show increase in endotoxemia neither other HFD-related outcomes, as weight gain, visceral and subcutaneous fat accumulation, macrophage infiltration into the adipose tissue or impaired insulin sensitivity. In conclusion, our data describes that acute endotoxemia primes the adipose tissue and we identified SAA as one of the molecules involved in this process. It is known that SAA is produced by adipose tissue and promote preadipocyte proliferation. We suggest that situations with an increase in SAA and the consecutive preadipocyte proliferation would prime the adipose tissue to further adipocyte differentiation and hypertrophy, when in appropriate conditions such as HFD. Furthermore, we suggest that SAA alter LPS signaling, promoting its greater intestinal absorption or inhibiting its clearance. The mechanism associating inflammation and obesity is undoubtedly complex and encompass a diversity of

factors, and the inflammatory protein SAA may be one of those. If these data are confirmed in humans, acute inflammation associated with a western diet should be recognized as one of the contributing factors of the outbreak of obesity and type 2 diabetes.

INTRODUCTION

The development of a chronic and low-grade inflammation is a hallmark of obesity and a key factor for the development of obesity comorbidities (1). Even more than a consequence of obesity, chronic inflammation is seen today as a key cause of the inflammatory status, weight gain, obesity and insulin resistance, specially triggered by the endotoxemia derived from intestinal microbiota (2; 3). Furthermore, high-fat feeding modulates gut microbiota and plasma concentration of endotoxin, correlating them to the occurrence of metabolic diseases (3).

The basis for the role of metabolic endotoxemia in obesity is robust and thus, it is unavoidable to question what is the impact of a transient and intense endotoxemia in adipose tissue and weight gain. High concentrations of LPS cause an inflammatory acute phase and it is well known that this is a catabolic process leading to weight loss and causing injury to various organs mainly mediated by inflammatory products (4). However, we wonder if intense endotoxemia primes the adipose tissue resulting in similar metabolic changes observed by Cani and colleagues. Would acute inflammation contribute to weight gain obesity comorbidities induced by a high-fat diet?

Another point addressed in this study was to extend the understanding of which are the factors that link inflammation, weight gain and comorbidities induced by a high-fat- diet.

The acute phase response is a complex reaction to infection or tissue injury characterized by fever, leukocytosis, changes in vascular permeability, altered metabolic responses and activation of nonspecific host defenses (5). These phenomena are primarily mediated by acute phase proteins mostly released by hepatocytes (5).

One acute phase protein of particular interest for human and also expressed in mice is serum amyloid A (SAA). This protein has been implicated not only in the regulation of inflammatory responses (6-8), but also in control of cell proliferation (9-

11) and metabolic profile (11-13). Although mainly secreted by the liver, SAA has also been reported to be produced by adipocytes, macrophages and several other cell types (14; 15). Furtheremore, SAA is an endogenous ligand to TLR-4, TLR-2 and SR-BI receptors, admittedly associated with the inflammatory response and also LPS receptors (6; 16; 17).

Although LPS is certainly one of the early factors in high-fat diet–induced metabolic diseases, in this study we identified the protein serum amyloid A (SAA) as an additional trigger setting insulin sensitivity and weight gain.

RESEARCH DESIGN AND METHODS

Animals. Male Swiss Webster mouse (21 days of age) were obtained from the Animal Facility of the Faculty of Pharmaceutical Sciences, University of São Paulo, Brazil, under approval by its Ethical Committee (CEEA n°297). The animals were housed inside standard polypropylene cages in a room maintained at 22±2°C in 12:12 h light/dark cycle (lights on at 7:00 am and off at 7:00 pm) and a relative humidity of 55±10%. Body weight was measured once a week during the entire protocol. Food and water intake were kept *ad libitum* and were measured every 2 days. At the end, mice were submitted to euthanasia by anesthetic overdose and ensured by cervical dislocation.

Acute endotoxemia. The method of multiple inductions of acute-phase comprises intraperitoneal administration of 8 consecutive injections (every 3 days) of LPS 10 mg/kg (Lipopolysaccharides from *Escherichia coli* 026:B6, Sigma-Aldrich®, St. Louis, MO, USA), in saline (NaCl 0,9%), starting at weaning (21 days of age) with end at 45 days of age of the animal. The time between two acute endotoxemia (3 days) was defined by the SAA profile. It was observed that after LPS injection, SAA concentration increases over a hundred times, with maximum values in 12 hours, approximately 1500 µg/mL, with return to basal in 72 hours (Supplementary Table 1). For acute endotoxemia experiments, mice (6 per group) were randomly assigned into 2 different groups: the Control group and the LPS group, with euthanasia occurring after the last acute-phase period. The experimental design is illustrated in Figure 1*A*.

Acute endotoxemia followed by High-Fat Diet (HFD). For acute-phase followed by 10 weeks on a high-fat diet (LPS+HFD) experiments, the animals (8 mice per group) were randomly assigned into 2 different groups: HFD group and LPS+HFD group. The HFD mice were submitted to a HFD for 10 weeks starting concurrently with the LPS+HFD group. The LPS+HFD mice were underwent to multiple inductions of acute-phase followed by 1 week of recovery period in standard chow-diet plus 10 weeks on a 30% HFD. In our experimental model we consider the recovery sleep period as 7 days, the time that we observed weight reestablishment. The diet was produced following the American Institute of Nutrition´s recommendations for the adult rodent and its composition is listed in Supplementary Table 2. Body weight was measured every 3 days during acute-phase period. The experimental design is illustrated in Figure 2*A*.

Diet-induced obesity protocol and SAA depletion. The animals were first randomly assigned into 2 different groups in order to receive chow or high-fat diet for 10 weeks. Each group was then split again to receive antisense oligonucleotide in vivo treatment for SAA mRNA translation inhibition (kindly provided by Isis Pharmaceuticals, Inc., Carlsbad, CA, USA), composing the following groups with 6 mice per group: Control (no ASO treatment), $ASO_{scramble}$ (ASO not specific to any murine transcript) and ASO_{SAA} (ASO specific for murine SAA 1/2 transcript). Both ASOs were intraperitoneal injected at the dose of 25 mg/kg of the animal, once a week. The antisense oligonucleotides sequences and chemistries are listed in Supplementary Table 3. The experimental design is illustrated in Figure 4*A*. SAA levels tend to increase when a high-fat diet is introduced. In our experimental model, the mRNA transcription inhibitor ASO_{SAA} was effective at decreasing the concentration of SAA even under HFD (Table 1).

Glucose and insulin tolerance tests and measurements of serum leptin, adiponectin, insulin, IGF-I, SAA1/2 and endotoxin. Glucose and insulin tolerance tests (GTT and ITT) were performed as described previously (18). Serum concentrations of the proteins below were determined using ELISA following the manufacturer's instructions: leptin, adiponectin and insulin (Millipore® Corporation, Billerica, MA, USA), SAA1/2 (Tridelta Development Ltd, Maynooth, Ireland) and IGF-I (R&D Systems®, Minneapolis, MN, USA). Endotoxin was measured with the Limulus

Amoebocyte Lysate (LAL) chromogenic end-point assay (Lonza®, Allendale, NJ, USA).

Histological Analysis. Paraffin-embedded sections (5 µm thick) from epididymal adipose tissue were stained by hematoxylin and eosin to assess morphology. Immunohistochemistry for F4/80 was performed using a rat anti-mouse F4/80 antibody (1:100 dilution, AbD Serotec®, Raleigh, NC, USA) subsequently incubated with the appropriate secondary biotinylated antibody (Vector Laboratories Inc., Burlingame, CA, USA) and visualized with Immpact AEC peroxidase (Vector Laboratories Inc., Burlingame, CA, USA). Immunofluorescence for F4/80, SAA and perilipin were performed using a rat anti-mouse F4/80 antibody and rabbit anti-mouse perilipin (both 1:100 dilution, Abcam®, Cambridge, UK), and a rabbit anti-mouse SAA (1:200 dilution, kindly produced and provided by Dr. de Beer laboratory, University of Kentucky, KY, USA), subsequently incubated with the appropriate secondary fluorescent antibody (Invitrogen®, Camarillo, CA, USA) and the slides mounted using Vectashield set mounting medium with 4,6-diamidino-2-phenylindol-2-HCl (DAPI; Vector Laboratories Inc., Burlingame, CA, USA). An isotype control was used to ensure antibody specificity in each staining. Tissue sections were observed with a Nikon Eclipse 80i microscope (Nikon®) and digital images were captured with NIS-Element AR software (Nikon®).

In vivo peripheral fat area quantification. Two X-ray images were taken at different energy levels; one low-energy X-ray (10 keV) to image soft tissue, and one high-energy X-ray (15 keV) to image bone, using the Carestream® In-Vivo MS FX Pro multispectral imaging system. A low/high energy ratio images were taken which allowed circumscribe and integrate different anatomical areas on the animals. The resulting ratiometric image was then displayed using a “Fire” intensity scale highlighting the subcutaneous fat area on the animals.

Quantitative Real-Time PCR. Total RNA from epididymal adipose tissue was isolated using Qiagen RNeasy® Lipid Tissue Mini kit (Qiagen, Hilden, Germany). cDNA was then synthesized from 1 µg of RNA using the High Capacity cDNA Reverse Transcription (Life Technologies®, Grand Island, NY, USA). Real-time PCR

were performed using SyBr® Green Master Mix (Life Technologies®, Grand Island, NY, USA). The primer sequences are detailed in Supplementary Table 4. Real-time PCR for SAA3 was performed using the TaqMan® assay (Applied Biosystems®, Grand Island, NJ, USA), catalog number Mm00441203_m1 – *Saa3 a*nd β-actin (ACTB), number 4552933E, as an endogenous housekeeping gene control. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.

Gene Set Enrichment Analysis of publicly available microarray data. We collected from GEO (http://www.ncbi.nlm.nih.gov/geo, GSE50647) the expression profiles of mouse visceral adipose tissue. In the study (19), authors exposed chow-fed apolipoprotein E (apoE) deficient mice to either 1) recurrent intravenous infection with *A. actinomycetemcomitans* or 2) a combination of recurrent intravenous infection with *A. actinomycetemcomitans* with a chronic intranasal infection with *C. pneumonia*. For the Gene Set Enrichment Analysis (GSEA) we ranked genes based on their mean log2 fold-change values between infected compared to uninfected mice. We then utilized custom gene sets, which contained genes related to: proliferation, adipogenesis, inflammation and the SAA. GSEA was performed using default parameters. Heat maps were used to display all genes from a statistically significant gene set.

Statistical analysis. Results were presented as mean ± SEM. Statistical analysis was performed with Graph Pad Prism4 (Graph Pad Software, Inc., San Diego, CA, USA). Comparisons between two groups were conducted with the unpaired Student´s *t* test. Data with two independent variables were tested by two-way analysis of variance with Bonferroni *post hoc* test. The level of significance was set at $p<0.05$.

RESULTS

Acute endotoxemia per se affects the adipose tissue but not lead to late weight change. In order to verify the effect of acute endotoxemia in adipose tissue, mice were subjected to 8 consecutives LPS challenges, every 3 days (Fig. 1*A*). During the acute phase, endotoxin and SAA levels raised over a hundredfold in serum (Fig. 1*B*

and *C*) and mice developed overt signs of endotoxemia (hunched posture, reduced movement and piloerection) with no animal death. It is know that acute endotoxemia change food intake behavior causing a reduced food intake leading to weight loss. During the acute endotoxemia period, LPS animals showed a reduced caloric intake, coming to be 40% lower (Fig. 1*D*), causing weight loss, approximately 12.5% of their total weight and 20% of epididymal adipose tissue mass (Fig. 1 *E* and *F*). In histological analysis from epididymal adipose tissue, it was also possible to verify a decrease in 30% of the adipocyte size (Fig. 1*G*). Besides weight loss, mice presented increased inflammatory markers, such as macrophage infiltration, SAA mRNA expression and protein production and TLR-4 and CD14 expression in adipose tissue (Fig. 1*H*, *I*, *J*, *K*, *L* and *M*). The increased serum levels of endotoxin and SAA after each LPS challenge remains the same, indicating that there was no tolerance in this period. After endotoxemia, the animals recovered their weight in the course of a week without showing any difference from Control group in the 6 consecutive weeks (Fig. 1*C* and *D*). One week without acute endotoxemia is also sufficient for the reestablishment of serum levels of endotoxin and SAA, however, the expression of SAA, TLR-4 and CD14 remain altered in the adipose tissue (Supplementary Figure 1).

A previous history of acute endotoxemia exacerbates HFD complications. After the LPS challenges and a 1-week recovery period, mice were submitted to a high-fat diet (HFD) for 10 weeks. Mice submitted to HFD were used as control (Fig. 2*A*). The shift of chow diet to HFD during the experimental protocol resulted in an increment of approximately twice in the caloric intake for both groups (Fig. 2*B*) and despite the fact that the caloric intake were similar between them, mice previously submitted to multiple acute endotoxemia (LPS+HFD) showed a different growth curve with increased total body weight (approximately in 15%) (Fig. 2*C*), due an increment in visceral and subcutaneous adipose tissue depot (Fig. 2*D*, *E* and *F*). The data were confirmed using X-rays images highlighting the subcutaneous fat area on the animals, showing that LPS+HDF mice have a higher peripheral fat area, an increment in 23% of adipose tissue (Fig. 2*G* and *H*). Besides that, the epididymal fat from LPS+HFD mice presented larger adipocytes than HFD group (Fig. 2*I*), even though both groups show hypertrophied adipose tissue, with a clear increase in

macrophage infiltration and SAA production when compared to lean control (chow diet) (Fig. 2*J*).

Under a high-fat diet, the endotoxin and SAA concentration reach levels about twice that observed in lean mice (Fig. 3*A* compared to 1*B* and Fig. 3*B* compared to 1*C*) but not differing between the groups HFD and LPS+HFD. In a similar manner, transcript levels of SAA3 in adipose tissue from HFD and LPS+HFD were comparable but increased when compared to a lean mice (Fig. 3*D* compared to 1*I*). The adipose tissue expression of SAA 1/2 was similar among all conditions (Fig. 3*C* and Fig. 1*H*). Nevertheless, mice previously submitted to multiple acute endotoxemia show an increment in TLR-4 and CD14 mRNA expression in the adipose tissue, with no change in TLR-2 transcript levels (Fig. 3*E*, *F* and *G*), and a worsened metabolic profile after the diet-induced obesity, with an increase in leptin and insulin levels (Fig. 3*H* and *I*), culminating with a glucose and insulin tolerance tests notably affected, showing impaired insulin sensitivity (Fig. 5*J* and *K)*. Fasting glucose, adiponectin and IGF-I concentrations were also measured in serum and no significant difference were observed (data not shown).

SAA depletion prevents weight gain and its comorbidities induced by HFD. From the data obtained, we addressed the investigation of the involvement of SAA protein in weight gain and insulin resistance. For that, mice fed a chow or HFD for 10 weeks and concurrently submitted to administration of SAA-targeted antisense oligonucleotide (Fig. 4*A*) were analysed regarding adipose tissue composition and architecture. Considering the type of diet used, no effect was observed in the amount and caloric intake when $ASO_{scramble}$ or ASO_{SAA} was administered (Fig. 4*B*). The growth curves of mice fed a chow diet did not show any difference in the animal development, comparing the groups Control, $ASO_{scramble}$ and ASO_{SAA}, demonstrating no toxic or side effect from the antisense oligonucleotide treatment (Fig. 4*C*). However, the ASO_{SAA} group was protected from the effects caused by a HFD The growth curve and the visceral and subcutaneous fat depot weight were comparable to a chow-diet fed animal (Fig. 4*D*, *E*, *F* and *G*). These data were confirmed using X-rays images highlighting the subcutaneous fat area on the animals, showing that HFD+ASO_{SAA} mice have a minor peripheral fat area when compared to HFD or HFD+$ASO_{scramble}$ mice (Fig. 4*H* and *I*).

The metabolic status of the animal treated with SAA-targeted antisense oligonucleotide was also assessed. After 10 weeks on a HFD, mice from ASO_{SAA} group did not show adipocyte hypertrophy or significant staining for F4/80 and SAA, demonstrating that SAA depletion prevents adipose tissue expansion and remodeling triggered by a diet rich in fat (Fig. 5*A* and *B*). Remarkably, endotoxin levels from mice treated with ASO_{SAA} under a HFD are comparable to a chow-diet fed animal (Fig. 5*C*). Moreover, it is clear the trend to maintain the metabolic balance, with no change in the levels of TLR-4 expression in adipose tissue (Fig. 5*D*), and the serum levels of leptin (Fig. 5*E*), adiponectin (Fig. 5*F*) and IGF-I (Fig. 5*G*). Also, treatment with ASO_{SAA} enables an improvement in glucose tolerance and insulin resistance notably affected when a HFD is implemented (Fig. 5*H*, *I*, *J* and *K*).

Recurrent infection upregulate proliferative and inflammatory genes in adipose tissue. We looked at the GEO database for studies similar to our experimental protocol and where it was performed transcriptome analysis in mice adipose tissue. From the study GSE50647 (Ref), where mice were infected with gram-negative bacteria (*A. actinomycetemcomitans* or coinfected with *A. actinomycetemcomitans* and *C. pneumonia)*, it was observed that a group of genes responsible for driving proliferation and inflammation were upregulated after infection, as well as SAA-related genes (SAA isoforms and receptors). On the other hand, the cluster of genes involved in adipogenesis were downregulated (Fig. 6).

DISCUSSION

Chronic low-grade endotoxemia is an important player in obesity and insulin resistance associated to a high-fat diet (2). On the other hand, although it is known that intense endotoxemia and infection reduce appetite and induce intense catabolism, leading to weight loss during the acute inflammatory phase (4; 5), the late effects of an intense endotoxemia were previously unexplored. Here we report that, besides the concurrent effects, multiple and intense endotoxemia also causes long lasting biochemical alterations in the adipose tissue that intensify the harmful effects of a high-fat diet. Mice submitted to multiple and severe endotoxemia had increased the adipose tissue expression of TLR4, CD14 and SAA3, that did not return to basal levels after one week in recovery, and became more prone to the

harmful effects of a HFD. This is the first description that acute inflammation is an aggravating factor for weight gain and insulin resistance derived from a fat-enriched diet. In the second part of this study, using the high-fat diet as an obesogenic stimulus, we evaluated the participation of the protein SAA in obesity development. The reasons underlying this study were the SAA's ability to induce proinflammatory cytokines production in several cell types, to induce preadipocyte proliferation and to inhibit adipocyte glucose uptake in vitro (7; 10; 11; 20; 21). The depletion of SAA in a diet-induced obesity protocol prevented metabolic alterations, endotoxin elevation, weight gain and insulin resistance.

The intense and transient endotoxemia we used, caused by an administration of 150 to 300 μg of LPS, led to an approximately 150 times increment in endotoxin levels in serum, reaching values near to 300 EU/mL being compatible with concentrations found in mice and humans during infectious processes and other pathological conditions (22). Endotoxemia induces the release of a large amount of inflammatory mediators, such as proinflammatory cytokines and highly reactive oxygen and nitrogen intermediates that were identified as contributors to the LPS-induced tissue damage (23; 24). The protein SAA, an acute phase reactant, increased near 1000 times and returned to basal levels after 72 hours in each LPS administration. During the endotoxemic phase the food intake dramatically dropped and a perceptible and expected depletion in fat depots with smaller adipocytes occurred (2). Interestingly, besides SAA production, others inflammatory markers were increased in adipose tissue after the last LPS injection, including macrophage infiltration and TLR-4 and CD14 (25). The rapid recovery of weight and maintenance of the growth curve after the suspension of endotoxemia tells us that the modifications derived from the acute phase are transient and apparently not essential to the adipose tissue homeostasis unless a high-fat diet is introduced. In this case, the devastating metabolic repercussions are clearly more pronounced.

The increase in TLR-4 and CD14 expression observed in adipose tissue after endotoxemia followed by HFD draws a lot of attention and may be the key to explain our data. It is known that LPS and nutritional fatty acids activates TLR-4 and the co-receptor CD14 triggering the secretion of proinflammatory cytokines (26-28). This is probably one of the elements in the inflammatory signaling cascade in adipose tissue linked to metabolic diseases. Thus, the simple fact that after the suspension of acute endotoxemia the levels of TLR-4 and CD14 mRNA remains elevated could justify the

higher responsiveness of adipose tissue to a HFD. Accordingly, it was imperative to investigate other TLR-4 ligands, especially those endogenous such as SAA.

SAA is the name of a highly conserved family of proteins. In human and mice, the inducible isoforms SAA1 and SAA2 are predominantly synthesized by the liver and carried by the high-density lipoproteins in the blood. During acute inflammation, the serum levels of SAA may increase up to 1000-fold the basal levels, whereas in chronic inflammatory conditions such as obesity, serum SAA is modest but persistently augmented. SAA is also expressed by several others cell types including macrophages, endothelial cells, smooth muscle cells and synoviocytes. Adipocytes are other known producers of SAA being positively correlated with the obesity grade and modulated under hypoxic conditions (15; 29). The role of SAA in adipose tissue began to be unraveled in the last years and may be associated to its action as a stimulus for the production of cytokines, reactive oxygen species and NO and also as a activator of the inflammasome pathway, being considered as a mediator of the innate and adaptive immune system (6-8; 30; 31). Furthermore, SAA is a potent chemotactic for a number of cell types and has proliferative activities (9-11; 32). A special feature of SAA that supports the rational of this study is its proliferative activity on preadipocytes, while inhibiting adipocyte differentiation (11; 33). This fact led us to consider that situations with an increase in SAA production would also promote preadipocyte proliferation. Thus, under appropriate conditions, the cells could be differentiated to adipocytes with a consequent hypertrophy of the adipose tissue. Also, it was recently reported that SAA3 knockout mice blunts weight gain and macrophage infiltration into the adipose tissue induced by an obesogenic diet (34). Moreover, knockout mice for both SAA1 and SAA2 gain less weight compared with their wild-type counterparts during the lifespan (35).

In addition, although until now no receptor dedicated solely to SAA has been identified, SAA can bind and activate several cell surface receptors, including the TLR-4 and TLR-2 (6; 29). This seems extremely relevant to our study given the identified roles of these receptors in obesity. It is known that the activation of TLR-4 and its co-receptor CD14 are associated to obesity and insulin resistance in a mice diet-induced obesity protocol (2; 36; 37). Besides that, TLR-2-deficient mice are protected from insulin resistance induced by a high-fat diet and show reduced tissue inflammation, in a process also dependent of the microbiota (38; 39). Although the expression of TLR-2 was not increased in our model of acute endotoxemia, it should

be considered that TLR-2 has the ability to interact with TLR-4 and the cooperation between them has been related to the responsiveness of cells to LPS.

In order to define the contribution of SAA in obesity we evaluated the effect of SAA depletion in a diet-induced obesity protocol using a SAA-targeted antisense oligonucleotide. Under a HFD the serum levels of SAA doubled and ASO_{SAA} treatment efficiently reduced it to the basal level. The treatment with ASO_{SAA} was characterized by a marked reduction of the deleterious alterations caused by a HFD underlying the importance of SAA in obesity. Furthermore, it is remarkable the fact that ASO_{SAA} prevented the elevation of endotoxin levels in serum, an increase that is expected when mice are submitted to a HFD, suggesting further interactions between LPS and SAA. Virtually all LPS molecules are rapidly complexed with circulating proteins and lipoproteins. When complexed with HDL (40; 41), LPS is cleared by the scavenger receptor BI (SR-BI) presented in hepatocytes (42) and in other cell types such as macrophages and monocytes as recently described (43). The clearance of LPS through SR-BI efficiently determines the magnitude of the inflammatory response. In this sense, it was shown that SAA was associated with impairment of SR-BI (13). This regulation may explain our data of lower endotoxemia when mice are submitted to a HFD under treatment with ASO_{SAA}. We suggest that the reduction in SAA serum levels could improve the SR-BI-mediated LPS clearance. The Figure 7 summarizes the main findings of our previous (11) and current study outlining the possible collaboration of LPS and SAA as factors defining obesity.

In order to evaluate the comprehensiveness of some of our conclusions, especially that related to our initial hypothesis that acute inflammation induces preadipocyte proliferation while triggering SAA production, we performed a Gene Set Enrichment Analysis in a publicly available microarray data (GSE50647) (19), where mice were infected with gram-negative bacteria. We identified an increment in SAA-related genes (SAA isoforms and receptors) and the upregulation of a cluster of genes responsible for driving proliferation and inflammation after infection while genes involved in adipogenesis were downregulated, thus supporting the assumption that cell proliferation occurs in adipose tissue during an infection process. Recently, preadipocyte proliferation was also observed under inflammatory conditions. Luch and colleagues demonstrated that low rates of LPS increased the proliferation of preadipocytes in vitro and in vivo through a CD14-dependent mechanism (44).

Considering that epidemiological data shows that low-income children have a higher prevalence of infectious diseases and are more susceptible to obesity (45; 46), is inevitable to consider that excess body weight in adulthood may be associated with an inflammatory state in children. Although obesity is to a large extend a lifestyle disease, the current scientific literature has shown previous unsuspected factors contributing to obesity development. For instance, viral infections have been linked to obesity, particularly by the human adenovirus-36 (47). In humans, anti-Ad-36 antibodies are more prevalent in obese subjects (30%) than in non-obese (11%) (48). Despite differences between viral and bacterial infections it is legitimate to assume that both types of infection share some major signaling pathways linking them to obesity, such as the upregulation of TLR-4 and SAA (49; 50).

In conclusion, our data describes that conditions leading to inflammation may resound for a long time, contributing to the obesity and type 2 diabetes epidemis when associated with a western diet. The mechanism is undoubtedly complex and encompasses a diversity of factors, and the inflammatory protein SAA may be one of those. If confirmed in humans, these results could lead to new medical recommendations for patients in post-infection recovery, as additional diet instructions.

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Duality of Interest. No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions. E.M.O. contributed to the study conception and design, performing experiments, acquisition and interpretation of data and the manuscript writing. T.P.A, J.C.S., S.S. and S.M. performed some experiments. H.T.I.N. design

experiments and interpreted data. R.A.F. designed experiments and A.C. contributed to the study conception and design, interpretation of data and the manuscript writing.

FIGURE LEGENDS

Figure 1. Acute endotoxemia per se affects the adipose tissue but not lead to late weight change. (A) Experimental design. Mice Swiss Webster were submitted to i.p. administration of 8 consecutive doses of 10 mg/kg LPS, every 3 days. **(B)** Endotoxin levels in serum. **(C)** SAA concentration in serum. **(D)** Daily caloric intake. **(E)** Weight gain curve of Control and LPS mice. The black arrows in *D* and *E* indicate the end of acute endotoxemia period. **(F)** Epididymal fat pad weight. **(G)** Adipocyte size after LPS challenges. Quantitative Real-Time PCR was performed to asses mRNA expression of **(H)** SAA1/2, **(I)** SAA3, **(J)** TLR-2, **(K)** TLR-4 and **(L)** CD14 in epidydimal adipose tissue. **(M)** Histological sections of epididymal fat pads after LPS challenges showing adipocyte morphology on hematoxylin and eosin staining, macrophage infiltration ($F4/80^+$) and SAA production. Data are means ± SE from 6 mice per group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, between groups, as indicated).

Figure 2. A previous history of acute endotoxemia potentiates weight gain induced by a high-fat diet (HFD). (A) Experimental design. Mice Swiss Webster were submitted to i.p. administration of 8 consecutive doses of 10 mg/kg LPS, every 3 days, followed by 10 weeks in HFD. **(B)** Daily caloric intake, considering the diet switch after acute endotoxemia period. **(C)** Weight gain curve of HFD and LPS+HFD groups. **(D)** Epididymal, **(E)** Retroperitoneal and **(F)** Subcutaneous fat pad weight after HFD period. **(G)** Representative subcutaneous fat area in HFD and LPS+HFD mice after HFD period. **(H)** Subcutaneous fat area quantification. **(I)** Adipocyte size after HFD period. **(J)** Histological sections of epididymal fat pads after HFD periods showing adipocyte morphology on hematoxylin and eosin staining, macrophage infiltration ($F4/80^+$) and SAA production. Data are means ± SE from 8 mice per group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, between groups, as indicated).

Figure 3. A previous history of acute endotoxemia potentiates glucose tolerance and insulin resistance induced by a high-fat diet (HFD). After a HFD

period, mice previously submitted to multiple acute endotoxemia were evaluated regarding its metabolic parameters. Determination of **(A)** endotoxin and **(B)** SAA in serum. Quantitative Real-Time PCR for mRNA expression of **(C)** SAA 1/2 **(D)** SAA3, **(E)** TLR-2, **(F)** TLR-4 and **(G)** CD14 in adipose tissue. At last, the measurement of **(H)** leptin**, (I)** insulin in serum and **(J)** Glucose tolerance test (GTT) and **(K)** Insulin tolerance test (ITT). Data are means ± SE from 8 mice per group (**p* < 0.05, ***p* < 0.01, between groups, as indicated).

Figure 4. SAA depletion prevents weight gain triggered by a diet-induced obesity protocol. (A) Experimental design. Mice Swiss Webster fed a HFD were submitted to i.p. administration of SAA-targeted antisense oligonucleotide (ASO_{SAA} 25 mg/kg/week) during 10 weeks. **(B)** Daily caloric intake. **(C)** Weight gain curve of chow diet fed animals treated with $ASO_{scramble}$ or ASO_{SAA}. **(D)** Weight gain curve of HFD fed animals animals treated with $ASO_{scramble}$ or ASO_{SAA}. **(E)** Epididymal, **(F)** Retroperitoneal and **(G)** Subcutaneous fat pad weight. **(H)** Subcutaneous fat area quantification and **(I)** representative subcutaneous fat area in mice under chow or high-fat diet submitted to $ASO_{scramble}$ or ASO_{SAA}. Data are means ± SE from 6 mice per group (Different letters represent statistical difference, $p < 0.05$).

Figure 5. SAA depletion prevents the harmful effects triggered by a diet-induced obesity protocol. (A) Histological sections of epididymal fat pads from mice under chow or high-fat diet and submitted to $ASO_{scramble}$ or ASO_{SAA}. Adipocyte morphology on hematoxylin and eosin staining, macrophage infiltration ($F4/80^{+}$) and SAA production are indicated by the white arrows. Determination of **(B)** adipocyte size in epididymal adipose tissue. **(C)** Endotoxin levels in serum. **(D)** TLR-4 mRNA expression in epididymal adipose tissue. Measurement of **(E)** leptin, **(F)** adiponectin and **(G)** IGF-I in serum. Glucose tolerance test (GTT) in **(H)** chow diet fed animals and **(I)** HFD fed animals. Insulin tolerance test (ITT) in **(J)** chow diet fed animals and **(K)** HFD fed animals. Data are means ± SE from 6 mice per group (Different letters represent statistical difference [$p < 0.05$] and *$p < 0.05$, between groups, as indicated).

Figure 6. Recurrent infection modulates proliferative, adipogenic, inflammatory and SAA-related genes in adipose tissue. Gene Set Enrichment Analysis (GSEA) revealed that proliferative, adipogenic, inflammatory and SAA-related gene sets in mouse adipose tissue were significantly associated (nominal *p*-value < 0.05) with infection with *A. actinomycetemcomitans* or co-infection with *A. actinomycetemcomitans* and *C. pneumonia* (see methods for details). Heat maps show the mean $\log_2$ fold-change of all genes of each gene set on each condition compared to uninfected mice.

Figure 7. LPS and SAA in obesity development.

Table 1. SAA levels under SAA-targeted antisense oligonucleotide treatment. SAA quantification in serum from Mice fed a chow diet or HFD and treated with $ASO_{scramble}$ and ASO_{SAA} .

Supplementary Figure 1. Alterations caused by acute endotoxemia are restored after a week without LPS treatment. Mice Swiss Webster were submitted to i.p. administration of 8 consecutive doses of 10 mg/kg LPS, every 3 days. After LPS period, mice were kept under standard conditions for one week for further metabolic analysis. Control group (empty bars). LPS-treated mice (black bars). LPS-treated mice followed 1 week in recovery (gray bars). **(A)** Endotoxin levels in serum. **(B)** SAA concentration in serum. Quantitative Real-Time PCR was performed to asses mRNA expression of **(C)**, SAA 1/2 **(D)** SAA3, **(E)** TLR-2, **(F)** TLR-4 and **(G)** CD14 in epididymal adipose tissue. Data are means ± SE from 4 mice per group (Different letters represent statistical difference, $p < 0.05$).

Figure 1

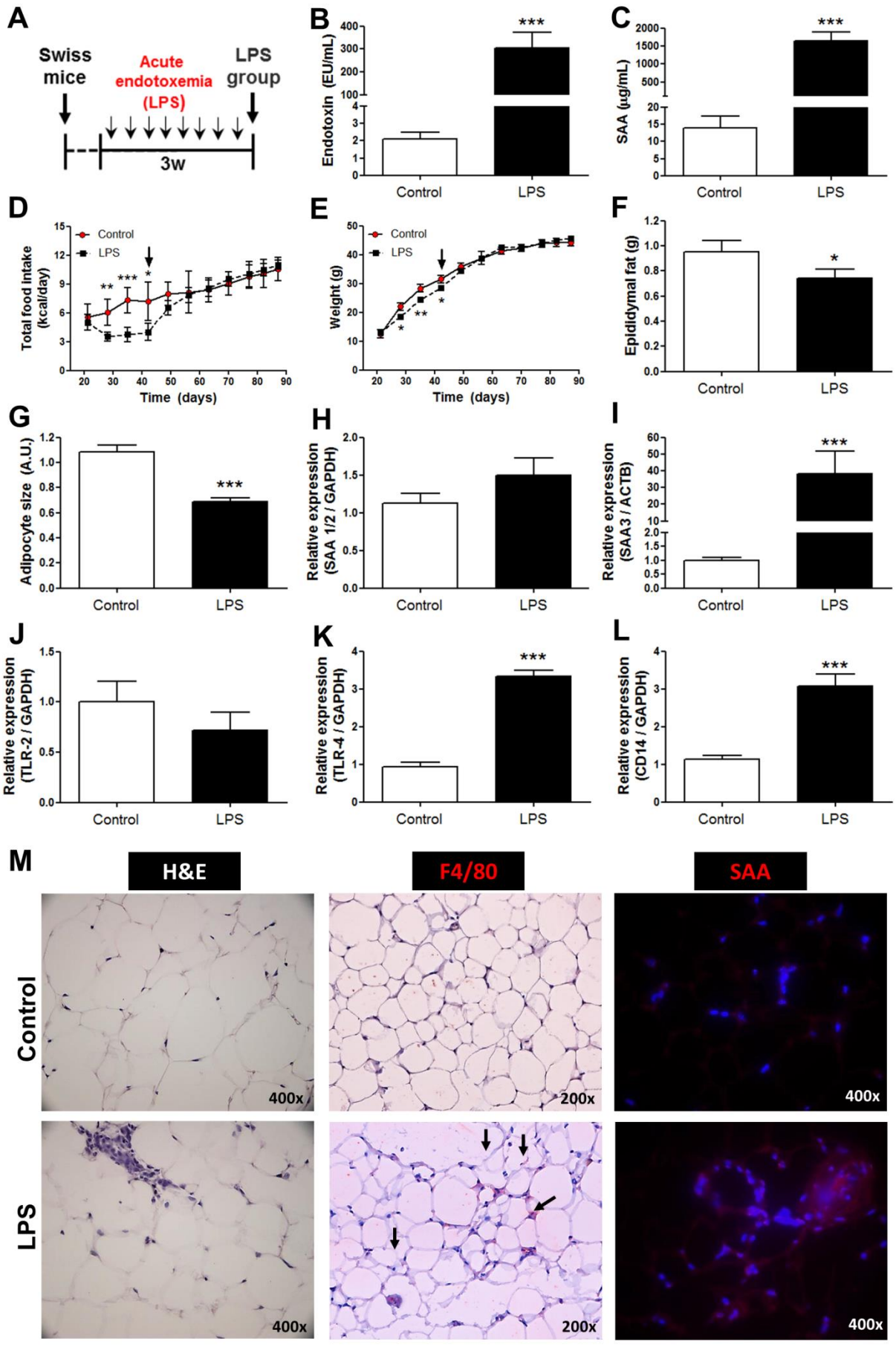

A
Swiss mice
Acute endotoxemia (LPS)
LPS group
3w
B
Endotoxin (EU/mL)
Control
LPS
C
SAA (μg/mL)
D
Total food intake (kcal/day)
Time (days)
E
Weight (g)
F
Epididymal fat (g)
G
Adipocyte size (A.U.)
H
Relative expression (SAA 1/2 / GAPDH)
I
Relative expression (SAA3 / ACTB)
J
Relative expression (TLR-2 / GAPDH)
K
Relative expression (TLR-4 / GAPDH)
L
Relative expression (CD14 / GAPDH)
M
H&E
F4/80
SAA
Control
LPS
400x
200x

Figure 2

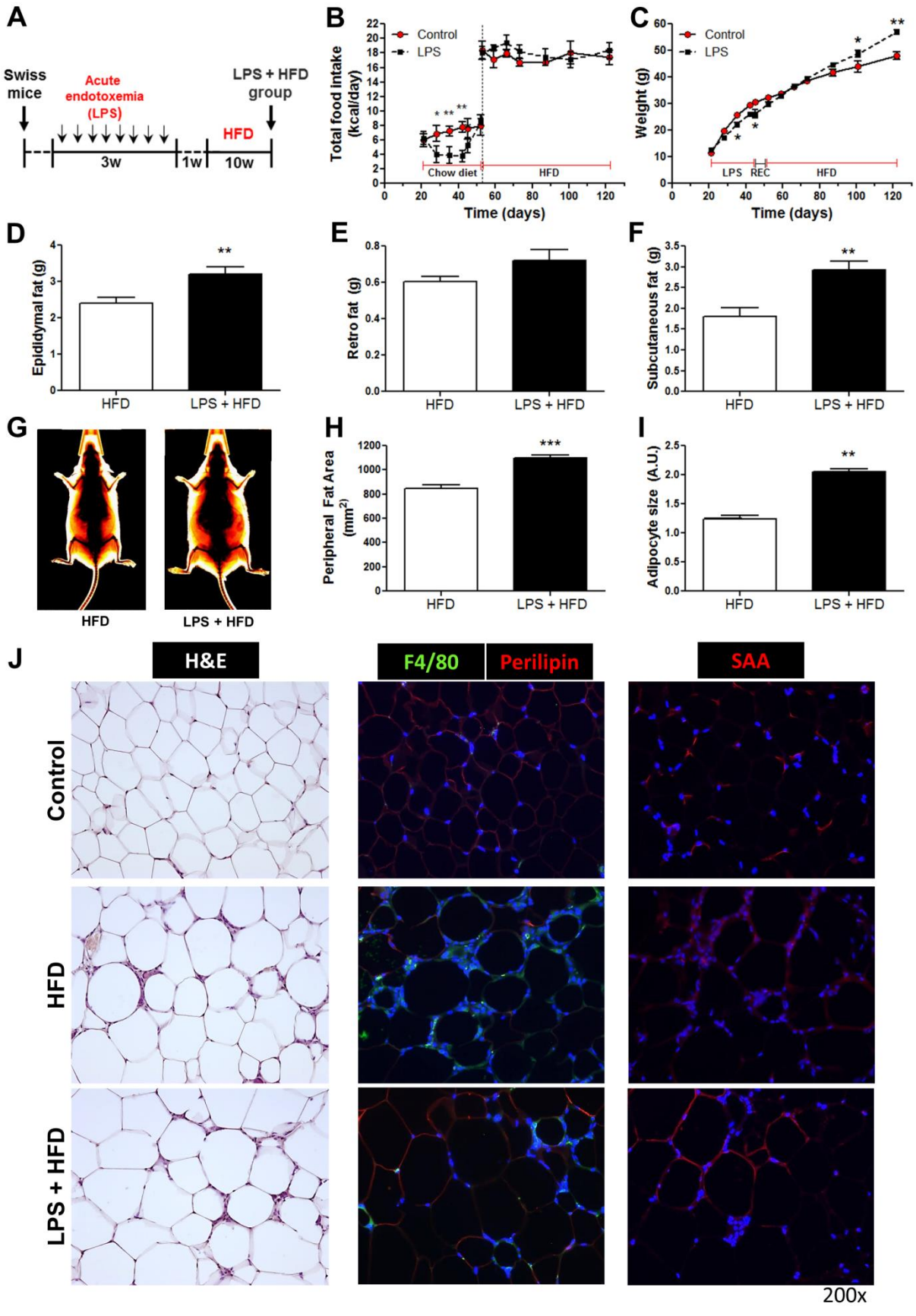

A
Swiss mice
Acute endotoxemia (LPS)
LPS + HFD group
HFD
3w
1w
10w
B
Control
LPS
Total food intake (kcal/day)
Chow diet
HFD
Time (days)
C
Control
LPS
Weight (g)
LPS
REC
HFD
Time (days)
D
Epididymal fat (g)
HFD
LPS + HFD
E
Retro fat (g)
HFD
LPS + HFD
F
Subcutaneous fat (g)
HFD
LPS + HFD
G
HFD
LPS + HFD
H
Peripheral Fat Area (mm2)
HFD
LPS + HFD
I
Adipocyte size (A.U.)
HFD
LPS + HFD
J
H&E
F4/80
Perilipin
SAA
Control
HFD
LPS + HFD
200x

Figure 3

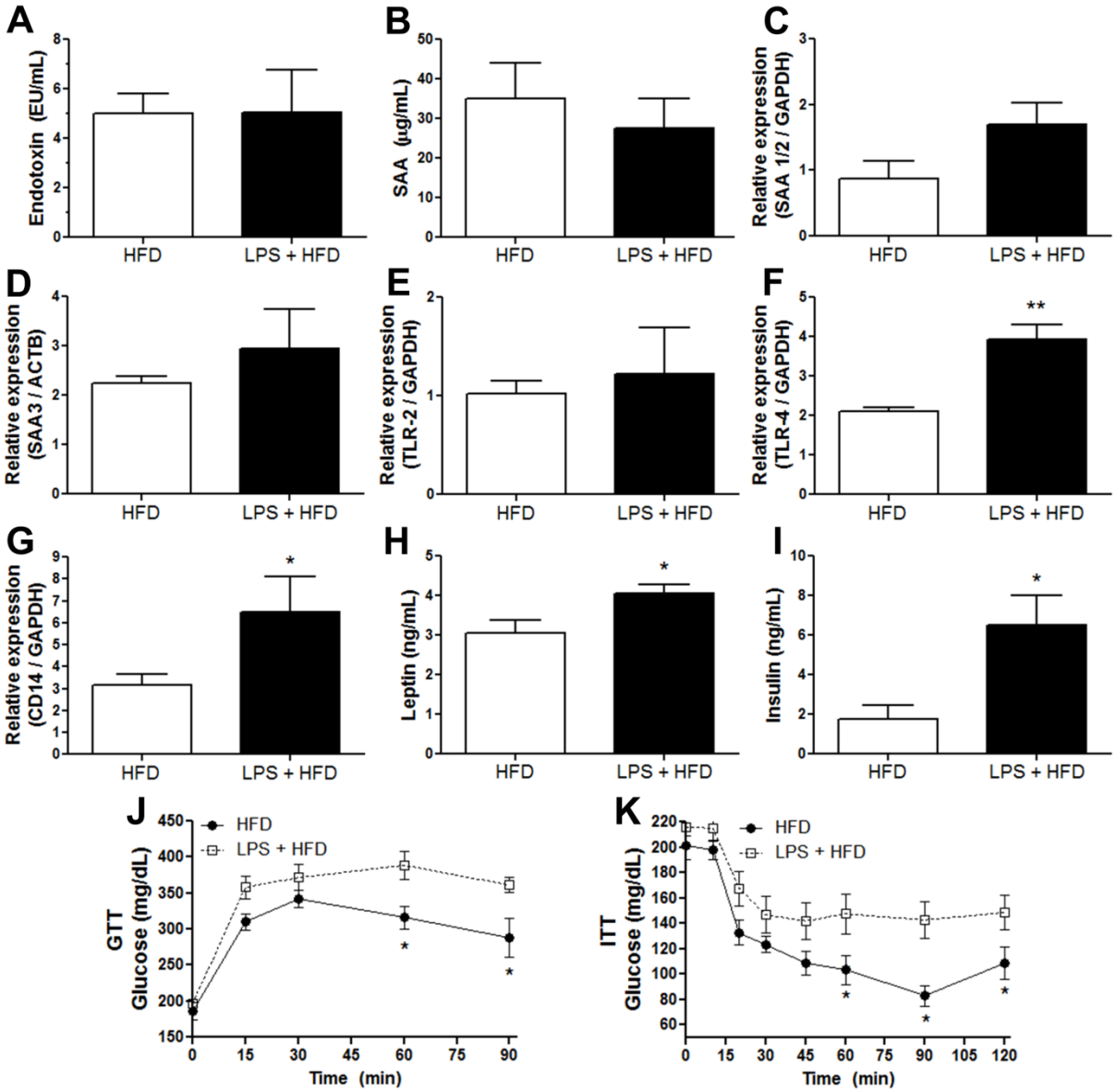

A
Endotoxin (EU/mL)
HFD
LPS + HFD
B
SAA (μg/mL)
C
Relative expression (SAA 1/2 / GAPDH)
D
Relative expression (SAA3 / ACTB)
E
Relative expression (TLR-2 / GAPDH)
F
Relative expression (TLR-4 / GAPDH)
**
G
Relative expression (CD14 / GAPDH)
*
H
Leptin (ng/mL)
I
Insulin (ng/mL)
J
GTT
Glucose (mg/dL)
Time (min)
K
ITT
Glucose (mg/dL)
Time (min)

Figure 4

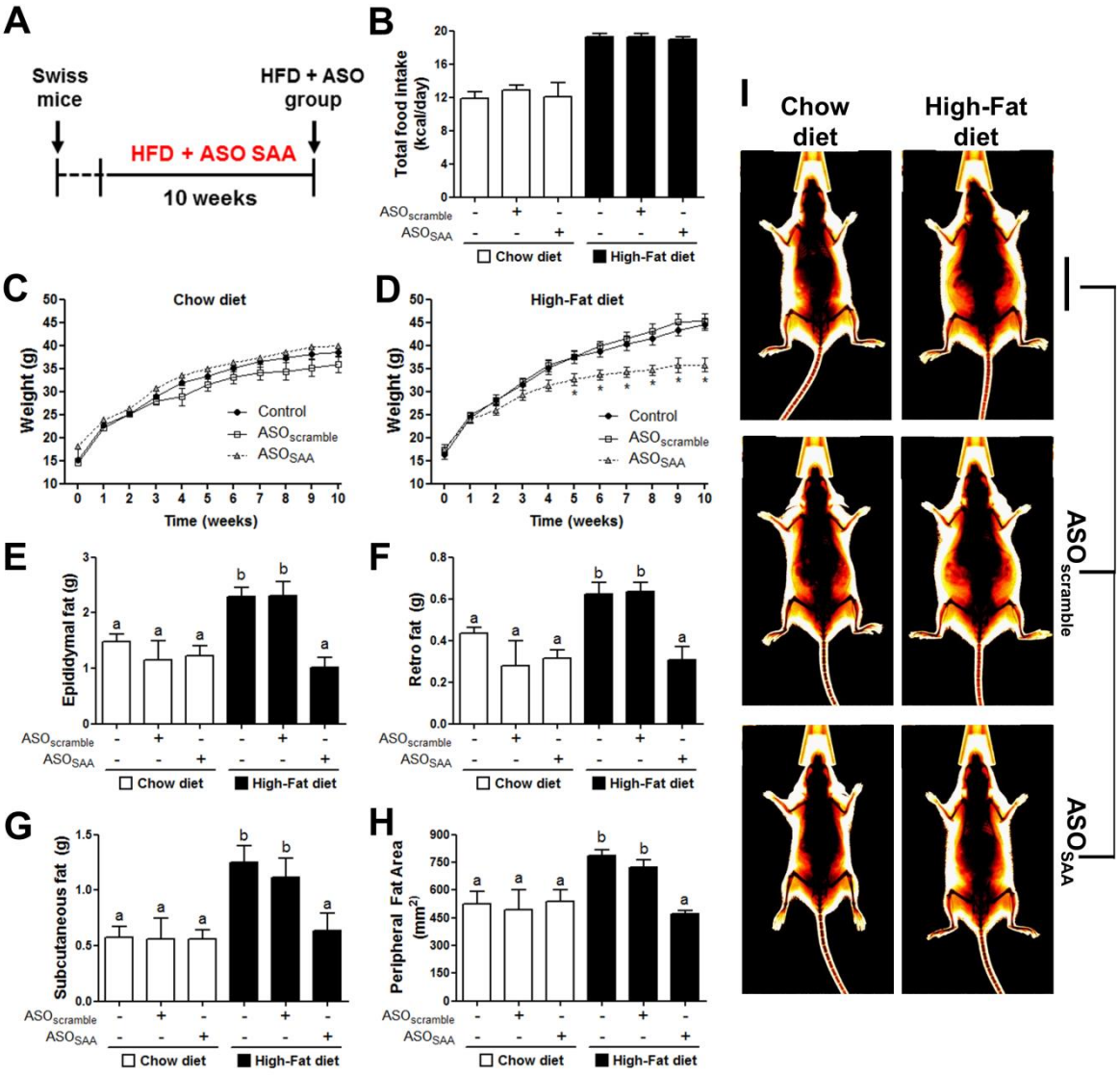

A
Swiss mice
HFD + ASO group
HFD + ASO SAA
10 weeks
B
Total food intake (kcal/day)
ASOscramble
ASOSAA
Chow diet
High-Fat diet
C
Chow diet
Weight (g)
Time (weeks)
Control
ASOscramble
ASOSAA
D
High-Fat diet
Weight (g)
Time (weeks)
Control
ASOscramble
ASOSAA
E
Epididymal fat (g)
ASOscramble
ASOSAA
Chow diet
High-Fat diet
F
Retro fat (g)
ASOscramble
ASOSAA
Chow diet
High-Fat diet
G
Subcutaneous fat (g)
ASOscramble
ASOSAA
Chow diet
High-Fat diet
H
Peripheral Fat Area (mm2)
ASOscramble
ASOSAA
Chow diet
High-Fat diet
I
Chow diet
High-Fat diet
ASOscramble
ASOSAA

Figure 5

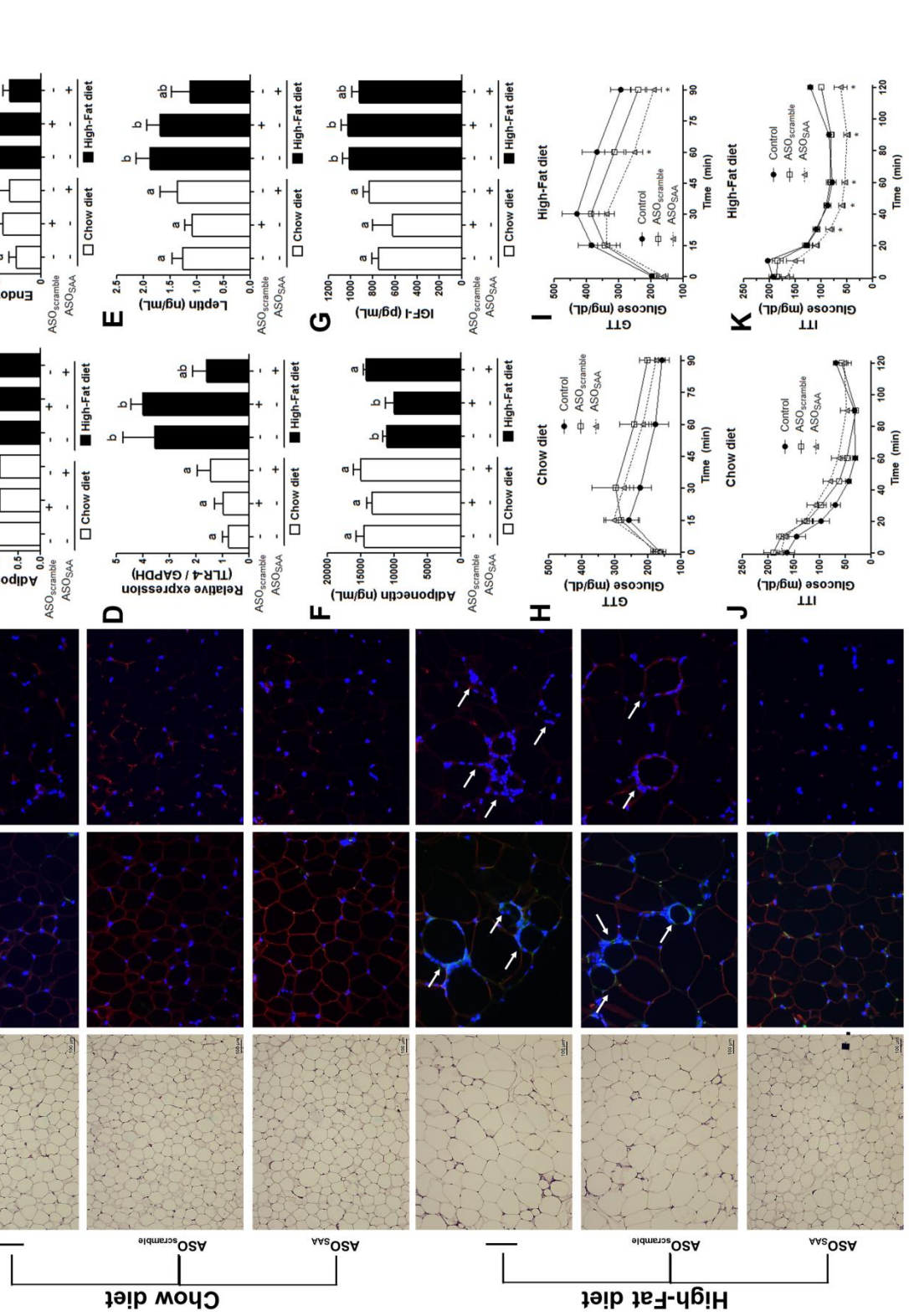

A
H&E
Perilipin
F4/80
SAA
Chow diet
High-Fat diet
ASOscramble
ASOSAA
B
Adipocyte size (A.U.)
C
Endotoxin (EU/mL)
D
Relative expression (TLR-4 / GAPDH)
E
Leptin (ng/mL)
F
Adiponectin (ng/mL)
G
IGF-1 (pg/mL)
H
GTT
Glucose (mg/dL)
Time (min)
Control
I
High-Fat diet
J
ITT
K

Figure 6

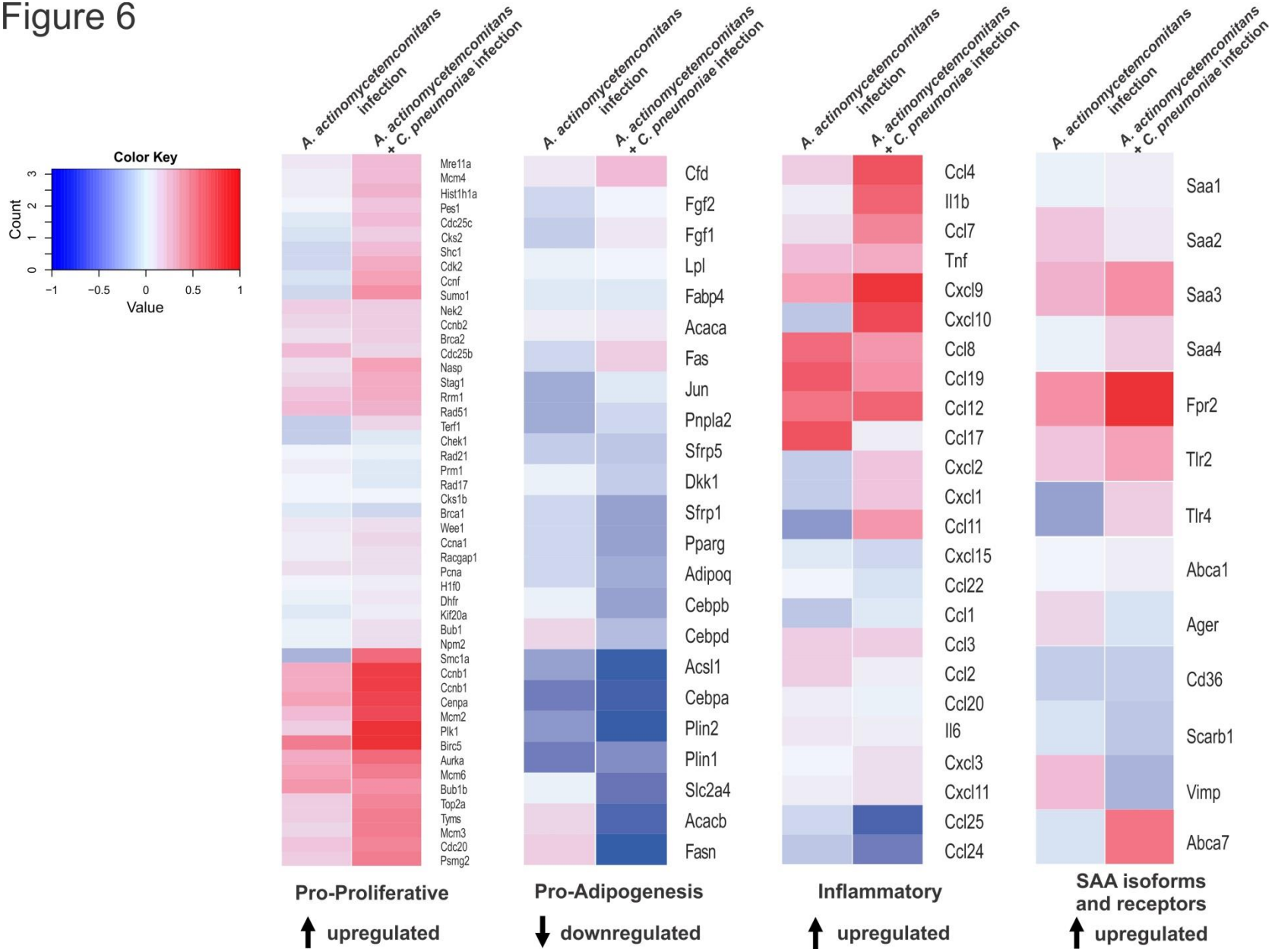

Color Key
Count
Value
A. actinomycetemcomitans infection
A. actinomycetemcomitans + C. pneumoniae infection
Mre11a
Mcm4
Hist1h1a
Pes1
Cdc25c
Cks2
Shc1
Cdk2
Ccnf
Sumo1
Nek2
Ccnb2
Brca2
Cdc25b
Nasp
Stag1
Rrm1
Rad51
Terf1
Chek1
Rad21
Prm1
Rad17
Cks1b
Brca1
Wee1
Ccna1
Racgap1
Pcna
H1f0
Dhfr
Kif20a
Bub1
Npm2
Smc1a
Ccnb1
Ccnb1
Cenpa
Mcm2
Plk1
Birc5
Aurka
Mcm6
Bub1b
Top2a
Tyms
Mcm3
Cdc20
Psmg2
Pro-Proliferative
upregulated
Cfd
Fgf2
Fgf1
Lpl
Fabp4
Acaca
Fas
Jun
Pnpla2
Sfrp5
Dkk1
Sfrp1
Pparg
Adipoq
Cebpb
Cebpd
Acsl1
Cebpa
Plin2
Plin1
Slc2a4
Acacb
Fasn
Pro-Adipogenesis
downregulated
Ccl4
Il1b
Ccl7
Tnf
Cxcl9
Cxcl10
Ccl8
Ccl19
Ccl12
Ccl17
Cxcl2
Cxcl1
Ccl11
Cxcl15
Ccl22
Ccl1
Ccl3
Ccl2
Ccl20
Il6
Cxcl3
Cxcl11
Ccl25
Ccl24
Inflammatory
upregulated
Saa1
Saa2
Saa3
Saa4
Fpr2
Tlr2
Tlr4
Abca1
Ager
Cd36
Scarb1
Vimp
Abca7
SAA isoforms and receptors
upregulated

Figure 7

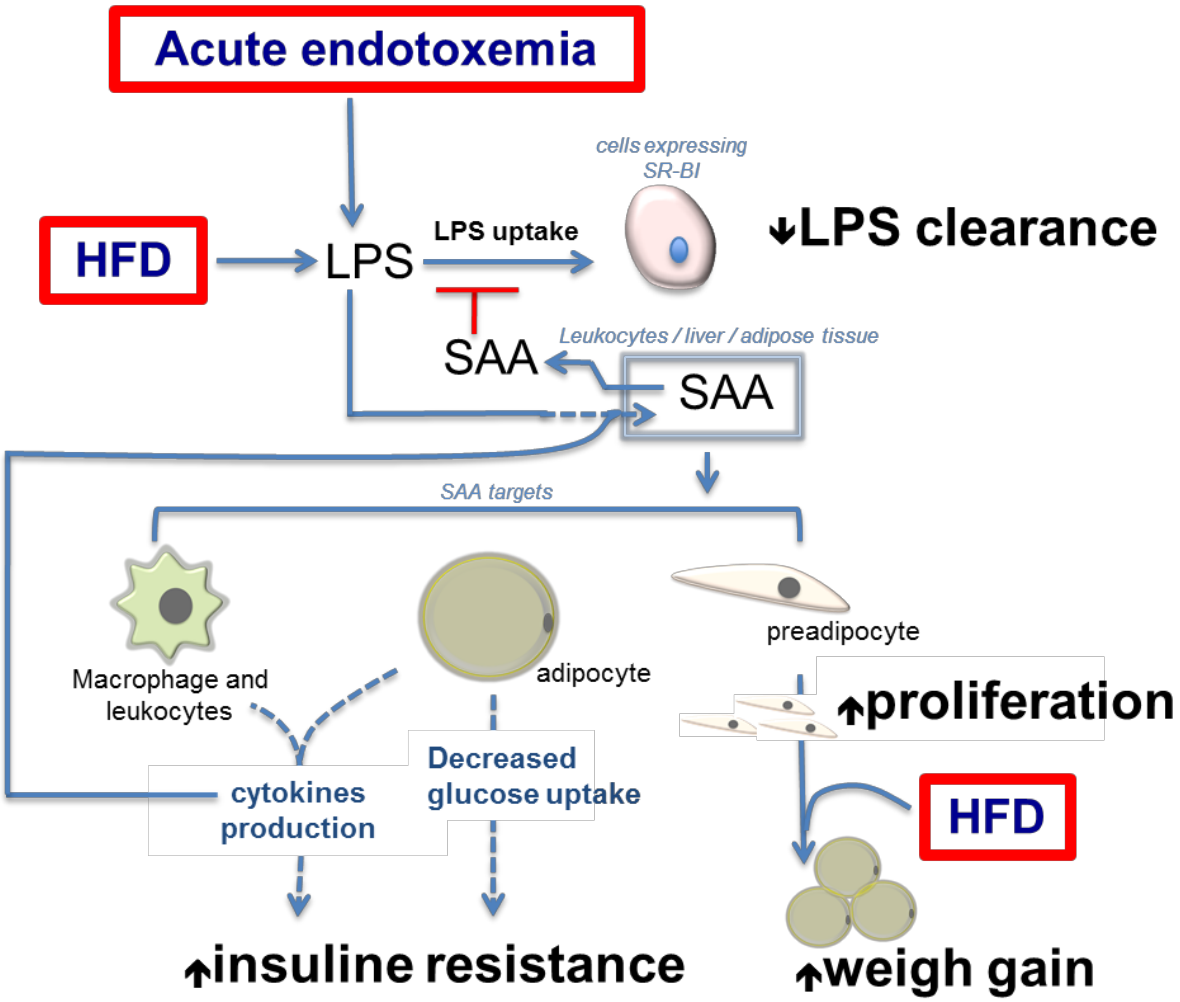

Acute endotoxemia
HFD
LPS
LPS uptake
cells expressing SR-BI
↓LPS clearance
SAA
Leukocytes / liver / adipose tissue
SAA
SAA targets
Macrophage and leukocytes
adipocyte
preadipocyte
↑proliferation
cytokines production
Decreased glucose uptake
HFD
↑insuline resistance
↑weigh gain

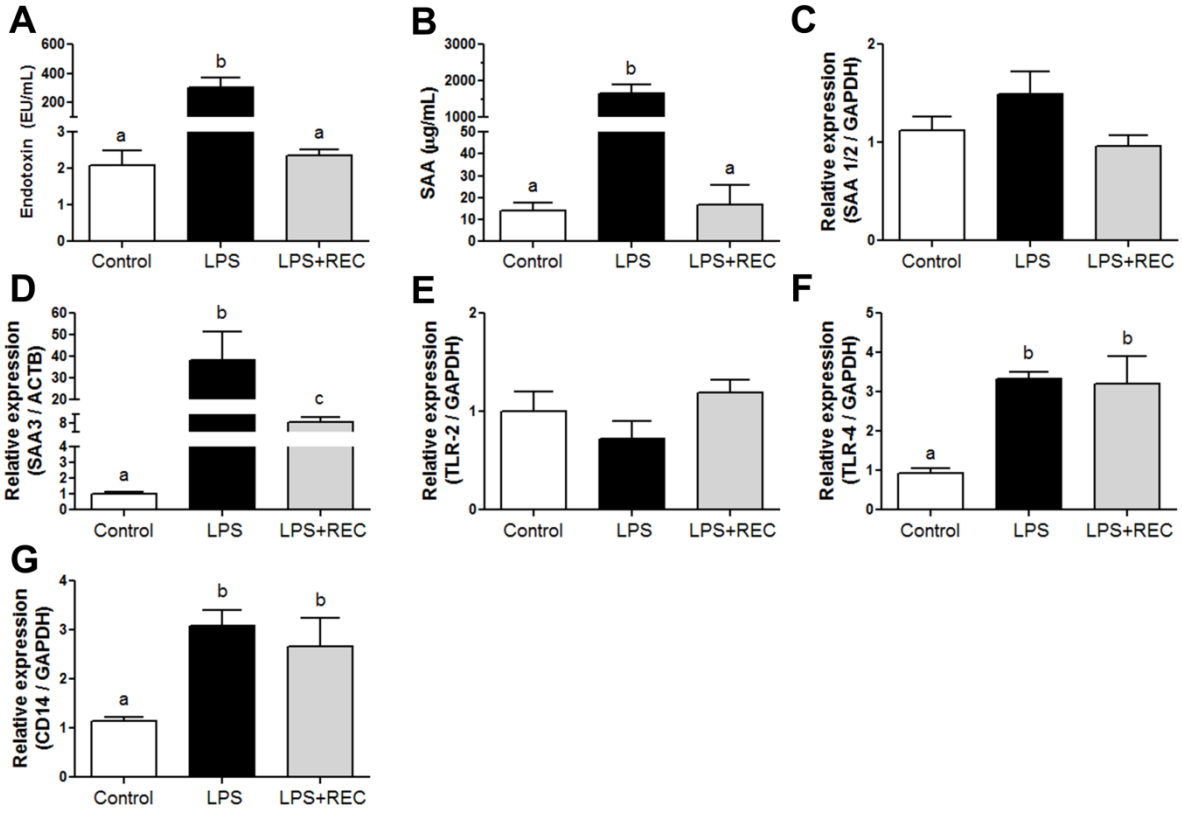
Table 1- SAA levels under SAA-targeted antisense oligonucleotide treatment

		SAA (µg/mL)
Chow diet	-	14.3 ± 4.9
	$ASO_{scramble}$	12.3 ± 3.3
	ASO_{SAA}	13.6 ± 4.1
HFD	-	27.0 ± 8.8**
	$ASO_{scramble}$	36.4 ± 16.5*
	ASO_{SAA}	10.5 ± 9.6

Data are means ± SD from 6 mice per group
(*p<0.05, **p<0.01, when compared to chow diet mice without ASO treatment)

Table 1. SAA levels under SAA-targeted antisense oligonucleotide treatment. SAA quantification in serum from Mice fed a chow diet or HFD and treated with $ASO_{scramble}$ and ASO_{SAA} .

Supplementary Figure 1



Supplementary Table 1- SAA profile during acute endotoxemia

	SAA (µg/mL)	
Time (hours)	Control	LPS (10 mg/kg)
0 h	16.8 ± 8.2	15.4 ± 6.8
6 h	21.7 ± 6.8	986.4 ± 75.8***
12 h	25.4 ± 13.5	1527.0 ± 193.6***
24 h	18.2 ± 10.7	888.4 ± 141.0**
48 h	7.1 ± 2.3	125.4 ± 40.1**
72 h	17.5 ± 8.7	29.6 ± 11.7

Data are means ± SD from 3 mice per group
(**p<0.01, ***p<0.001, between groups, as indicated)

Supplementary Table 1. SAA profile during acute endotoxemia. SAA was quantified in serum after 6, 12, 24, 48 and 72 hours of LPS-treatment (10 mg/kg).

Supplementary Table 2- Experimental diet composition

Ingredients (g/Kg)	Chow diet[1] 3.99 kcal/g	High-Fat diet[1] 5.55 kcal/g
Sucrose	100	133.56
Casein	120	186.98
Corn oil	80	53.42
Lard	--	300
Cellulose	50	66.78
Mineral Mix (Rhoster®)	35	46.74
Vitamin Mix (Rhoster®)	10	13.36
DL-Methionine	1.8	2.4
Choline Bitartrate	2.5	3.34
Tert-butylhydroquinone	0.01	0.04
Cornstarch q.s.p.	1000	1000

[1]According to AIN-93M

Supplementary Table 2. Experimental diet composition. Chow diet and high-fat diet formulation according to AIN-93M.

Supplementary Table 3- Antisense oligonucleotides sequences and chemistries

ASO	*Target*	*Species*	*Chemistry*	*Length*	*Sequence*
ASO_{SAA}	SAA 1/2	Mouse	10-3-10 (S)-cEt gapmer w / phosphorothioate backbone	16	5'-GTTTATTACCCTCTCC-3'
$ASO_{scramble}$	Control	Mouse	10-3-10 (S)-cEt gapmer w / phosphorothioate backbone	16	5'-GGCCAATACGCCGTCA-3'

All information was provided by the manufacturer Isis Pharmaceuticals, Inc., Carlsbad, CA, USA.

Supplementary Table 3. Antisense oligonucleotides (ASOs) sequences and chemistries. $ASO_{scramble}$ (ASO not specific to any murine transcript) and ASO_{SAA} (ASO specific for murine SAA 1/2 transcript) structural and chemical characteristics.

Supplementary Table 4 - PCR primers used in all quantitative PCR assays

Primer (gene / protein)	Forward	Reverse
Saa1/2 (SAA1 / SAA2)	5'-AGA CAA ATA CTT CCA TGC TCG G-3'	5'-CAT CAC TGA TTT TCT CAG CAG C-3'
Tlr2 (TLR-2)	5'-CAG CTG GAG AAC TCT GAC CC-3'	5'-CAA AGA GCC TGA AGT GGG AG-3'
Tlr4 (TLR-4)	5'-TCA TGG CAC TGT TCT TCT CCT-3'	5'-CAT CAG GGA CTT TGC TGA GTT-3'
Cd14 (CD14)	5'-GCG AGC TAG ACG AGG AAA GT-3'	5'-CAC GCT TTA GAA GGT ATT CCA G-3'
Gapdh (GAPDH)	5'-TGG CAA AGT GGA GAT TGT TGC C-3'	5'-AAG ATG GTG ATG GGC TTC CCG-3'

REFERENCES

1. Ouchi N, Parker JL, Lugus JJ, Walsh K: Adipokines in inflammation and metabolic disease. Nature Reviews Immunology 2011;11:85-97
2. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmee E, Cousin B, Sulpice T, Chamontin B, Ferrieres J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, Burcelin R: Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007;56:1761-1772
3. Cani PD, Bibiloni R, Knauf C, Neyrinck AM, Delzenne NM, Burcelin R: Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 2008;57:1470-1481
4. Gabay C, Kushner I: Mechanisms of disease: Acute-phase proteins and other systemic responses to inflammation. New England Journal of Medicine 1999;340:448-454
5. Kushner I: THE PHENOMENON OF THE ACUTE PHASE RESPONSE. Annals of the New York Academy of Sciences 1982;389:39-48
6. Sandri S, Rodriguez D, Gomes E, Monteiro HP, Russo M, Campa A: Is serum amyloid A an endogenous TLR4 agonist? Journal of Leukocyte Biology 2008;83:1174-1180
7. Furlaneto CJ, Campa A: A novel function of serum amyloid A: A potent stimulus for the release of tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8 by human blood neutrophil. Biochemical and Biophysical Research Communications 2000;268:405-408
8. Hatanaka E, Ribeiro FP, Campa A: The acute phase protein serum amyloid A primes neutrophils. Fems Immunology and Medical Microbiology 2003;38:81-84
9. Hatanaka E, Dermargos A, Armelin HA, Curi R, Campa A: Serum amyloid A induces reactive oxygen species (ROS) production and proliferation of fibroblast. Clinical and Experimental Immunology 2011;163
10. Knebel FH, Albuquerque RC, Massaro RR, Maria-Engler SS, Campa A: Dual Effect of Serum Amyloid A on the Invasiveness of Glioma Cells. Mediators of Inflammation 2013;
11. Filippin-Monteiro FB, de Oliveira EM, Sandri S, Knebel FH, Albuquerque RC, Campa A: Serum amyloid A is a growth factor for 3T3-L1 adipocytes, inhibits differentiation and promotes insulin resistance. International Journal of Obesity 2012;36:1032-1039
12. Liu LR, Lin SP, Chen CC, Chen YJ, Tai CC, Chang SC, Juang RH, Tseng YW, Liu BH, Mersmann HJ, Shen TL, Ding ST: Serum Amyloid A Induces Lipolysis by Downregulating Perilipin Through ERK1/2 and PKA Signaling Pathways. Obesity 2011;19:2301-2309
13. Tsun JGS, Shiu SWM, Wong Y, Yung S, Chan TM, Tan KCB: Impact of serum amyloid A on cellular cholesterol efflux to serum in type 2 diabetes mellitus. Atherosclerosis 2013;231:405-410
14. Upragarin N, Landman WJM, Gaastra W, Gruys E: Extrahepatic production of acute phase serum amyloid A. Histology and Histopathology 2005;20:1295-1307
15. de Oliveira EM, Sandri S, Knebel FH, Iglesias Contesini CG, Campa A, Filippin-Monteiro FB: Hypoxia Increases Serum Amyloid A3 (SAA3) in Differentiated 3T3-L1 Adipocytes. Inflammation 2013;36:1107-1110
16. Guo L, Zheng Z, Ai J, Huang B, Li X-A: Hepatic Scavenger Receptor BI Protects Against Polymicrobial-induced Sepsis through Promoting LPS Clearance in Mice. Journal of Biological Chemistry 2014;289:14666-14673
17. Cheng N, He R, Tian J, Ye PP, Ye RD: Cutting edge: TLR2 is a functional receptor for acute-phase serum amyloid A. Journal of Immunology 2008;181:22-26

18. de Oliveira EM, Visniauskas B, Sandri S, Migliorini S, Andersen ML, Tufik S, Fock RA, Chagas JR, Campa A: Late effects of sleep restriction: Potentiating weight gain and insulin resistance arising from a high-fat diet in mice. Obesity 2015;23:391-398
19. Hyvarinen K, Tuomainen AM, Laitinen S, Alfthan G, Salminen I, Leinonen M, Saikku P, Kovanen PT, Jauhiainen M, Pussinen PJ: The effect of proatherogenic pathogens on adipose tissue transcriptome and fatty acid distribution in apolipoprotein E-deficient mice. Bmc Genomics 2013;14
20. Sandri S, Hatanaka E, Franco AG, Pedrosa AMC, Monteiro HP, Campa A: Serum amyloid A induces CCL20 secretion in mononuclear cells through MAPK (p38 and ERK1/2) signaling pathways. Immunology Letters 2008;121:22-26
21. Franco AG, Sandri S, Campa A: High-density lipoprotein prevents SAA-induced production of TNF-alpha in THP-1 monocytic cells and peripheral blood mononuclear cells. Memorias Do Instituto Oswaldo Cruz 2011;106
22. Nymark M, Pussinen PJ, Tuomainen AM, Forsblom C, Groop P-H, Lehto M, Finndiane Study G: Serum Lipopolysaccharide Activity Is Associated With the Progression of Kidney Disease in Finnish Patients With Type 1 Diabetes. Diabetes Care 2009;32:1689-1693
23. Russell JA: Drug therapy: Management of sepsis. New England Journal of Medicine 2006;355:1699-1713
24. Cohen J: The immunopathogenesis of sepsis. Nature 2002;420:885-891
25. Ghosh C, Bishayi B: Characterization of Toll-Like Receptor-4 (TLR-4) in the Spleen and Thymus of Swiss Albino Mice and Its Modulation in Experimental Endotoxemia. Journal of Immunology Research 2015;2015:13
26. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS: TLR4 links innate immunity and fatty acid-induced insulin resistance. Journal of Clinical Investigation 2006;116:3015-3025
27. Schaffaeler A, Schoelmerich J: Innate immunity and adipose tissue biology. Trends in Immunology 2010;31:228-235
28. Lin Y, Lee H, Berg AH, Lisanti MP, Shapiro L, Scherer PE: The lipopolysaccharide-activated toll-like receptor (TLR)-4 induces synthesis of the closely related receptor TLR-2 in adipocytes. Journal of Biological Chemistry 2000;275:24255-24263
29. Eklund KK, Niemi K, Kovanen PT: Immune Functions of Serum Amyloid A. Critical Reviews in Immunology 2012;32:335-348
30. Niemi K, Teirila L, Lappalainen J, Rajamaki K, Baumann MH, Oorni K, Wolff H, Kovanen PT, Matikainen S, Eklund KK: Serum Amyloid A Activates the NLRP3 Inflammasome via P2X(7) Receptor and a Cathepsin B-Sensitive Pathway. Journal of Immunology 2011;186:6119-6128
31. Ather JL, Ckless K, Martin R, Foley KL, Suratt BT, Boyson JE, Fitzgerald KA, Flavell RA, Eisenbarth SC, Poynter ME: Serum Amyloid A Activates the NLRP3 Inflammasome and Promotes Th17 Allergic Asthma in Mice. Journal of Immunology 2011;187:64-73
32. Badolato R, Wang JM, Murphy WJ, Lloyd AR, Michiel DF, Bausserman LL, Kelvin DJ, Oppenheim JJ: SERUM AMYLOID-A IS A CHEMOATTRACTANT - INDUCTION OF MIGRATION, ADHESION, AND TISSUE INFILTRATION OF MONOCYTES AND POLYMORPHONUCLEAR LEUKOCYTES. Journal of Experimental Medicine 1994;180:203-209
33. Faty A, Ferre P, Commans S: The Acute Phase Protein Serum Amyloid A Induces Lipolysis and Inflammation in Human Adipocytes through Distinct Pathways. Plos One 2012;7
34. den Hartigh LJ, Wang SR, Goodspeed L, Ding YL, Averill M, Subramanian S, Wietecha T, O'Brien KD, Chait A: Deletion of Serum Amyloid A3 Improves High Fat High Sucrose

Diet-Induced Adipose Tissue Inflammation and Hyperlipidemia in Female Mice. Plos One 2014;9
35. De Beer MC, Wroblewski JM, Noffsinger VP, Rateri DL, Howatt DA, Balakrishnan A, Ji AL, Shridas P, Thompson JC, van der Westhuyzen DR, Tannock LR, Daugherty A, Webb NR, De Beer FC: Deficiency of Endogenous Acute Phase Serum Amyloid A Does Not Affect Atherosclerotic Lesions in Apolipoprotein E-Deficient Mice. Arteriosclerosis Thrombosis and Vascular Biology 2014;34:255-261
36. Song MJ, Kim KH, Yoon JM, Kim JB: Activation of Toll-like receptor 4 is associated with insulin resistance in adipocytes. Biochemical and Biophysical Research Communications 2006;346:739-745
37. Kim K-A, Gu W, Lee I-A, Joh E-H, Kim D-H: High Fat Diet-Induced Gut Microbiota Exacerbates Inflammation and Obesity in Mice via the TLR4 Signaling Pathway. Plos One 2012;7
38. Ehses JA, Meier DT, Wueest S, Rytka J, Boller S, Wielinga PY, Schraenen A, Lemaire K, Debray S, Van Lommel L, Pospisilik JA, Tschopp O, Schultze SM, Malipiero U, Esterbauer H, Ellingsgaard H, Rutti S, Schuit FC, Lutz TA, Boni-Schnetzler M, Konrad D, Donath MY: Toll-like receptor 2-deficient mice are protected from insulin resistance and beta cell dysfunction induced by a high-fat diet. Diabetologia 2010;53:1795-1806
39. Caricilli AM, Picardi PK, de Abreu LL, Ueno M, Prada PO, Ropelle ER, Hirabara SM, Castoldi A, Vieira P, Camara NOS, Curi R, Carvalheira JB, Saad MJA: Gut Microbiota Is a Key Modulator of Insulin Resistance in TLR 2 Knockout Mice. Plos Biology 2011;9:21
40. Ulevitch RJ, Johnston AR, Weinstein DB: NEW FUNCTION FOR HIGH-DENSITY LIPOPROTEINS - THEIR PARTICIPATION IN INTRA-VASCULAR REACTIONS OF BACTERIAL LIPOPOLYSACCHARIDES. Journal of Clinical Investigation 1979;64:1516-1524
41. Ulevitch RJ, Johnston AR, Weinstein DB: NEW FUNCTION FOR HIGH-DENSITY LIPOPROTEINS - ISOLATION AND CHARACTERIZATION OF A BACTERIAL LIPOPOLYSACCHARIDE HIGH-DENSITY LIPOPROTEIN COMPLEX FORMED IN RABBIT PLASMA. Journal of Clinical Investigation 1981;67:827-837
42. Guo L, Ai J, Zheng Z, Howatt DA, Daugherty A, Huang B, Li X-A: High Density Lipoprotein Protects against Polymicrobe-induced Sepsis in Mice. Journal of Biological Chemistry 2013;288:17947-17953
43. Li B, Yu M, Pan X, Ren C, Peng W, Li X, Jiang W, Zheng J, Zhou H: Artesunate Reduces Serum Lipopolysaccharide in Cecal Ligation/Puncture Mice via Enhanced LPS Internalization by Macrophages through Increased mRNA Expression of Scavenger Receptors. International Journal of Molecular Sciences 2014;15:1143-1161
44. Luche E, Cousin B, Garidou L, Serino M, Waget A, Barreau C, André M, Valet P, Courtney M, Casteilla L, Burcelin R: Metabolic endotoxemia directly increases the proliferation of adipocyte precursors at the onset of metabolic diseases through a CD14-dependent mechanism. . Molecular Metabolism 2013;2:281-291
45. Singh GK, Siahpush M, Kogan MD: Rising social inequalities in U.S. childhood obesity, 2003-2007. Annals of Epidemiology, 2010, p. 40-52
46. Singh AR, Singh SA: Diseases of Poverty and Lifestyle, Well-Being and Human Development. Mens Sana Monographs, 2008, p. 187-225
47. van Ginneken V, Sitnyakowsky L, Jeffery JE: Infectobesity: viral infections (especially with human adenovirus-36: Ad-36) may be a cause of obesity. Medical Hypotheses 2009;72:383-388
48. Atkinson RL, Dhurandhar NV, Allison DB, Bowen RL, Israel BA, Albu JB, Augustus AB: Human adenovirus-36 is associated with increased body weight and paradoxical reduction of serum lipids. International Journal of Obesity 2005;29:281-286

49. Miwata H, Yamada T, Okada M, Kudo T, Kimura H, Morishima T: SERUM AMYLOID A-PROTEIN IN ACUTE VIRAL-INFECTIONS. Archives of Disease in Childhood 1993;68:210-214
50. Machida K, Cheng KTH, Sung VMH, Levine AM, Foung S, Lai MMC: Hepatitis C virus induces toll-like receptor 4 expression, leading to enhanced production of beta interferon and interleukin-6. Journal of Virology 2006;80:866-874

Sleep restriction triggers the production of serum amyloid A (SAA) in humans and mice. Is SAA a link between sleep disorders and obesity?

Sleep restriction triggers the production of serum amyloid A (SAA) in human and mice. Is SAA a link between sleep disorders and obesity?

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Running title: SAA: a sign of inflammation in sleep restriction.

Keywords: sleep curtailment; sleep loss; obesity; type 2 diabetes; western diet

ABSTRACT

Recently we described that sleep restriction (SR) primes adipose tissue predisposing it to hypertrophy and becoming mice prone to obesity and insulin resistance when a high-fat diet was adopted. The biochemical triggers of this process may be related to inflammatory cytokines and resistin, given that we observed a huge increase in TNF-α, IL-6 and resistin in adipose tissue during the SR. Here, we moved ahead with this issue showing that the protein serum amyloid A (SAA) is also part of the initial biochemical alterations caused by SR. Based on a previously study from our lab that identified effects of SAA on adipocyte biology (Filippin-Monteiro *et al*. International Journal of Obesity. 2012;36:1032), we hypothesized that SAA has a role in the etiology of obesity and insulin resistance triggered by sleep restriction. Serum concentration and hepatic and adipose tissue expression of SAA, respectively SAA1/2 and SAA3, were increased in mice C57BL/6J when subjected to a multiple platform method of SR for 21 h daily, for 15 days. Given the wide interest to know how human are affected by shortening of sleep period, we also verified that a 72 h of paradoxal sleep deprivation in mice and a 48h of total sleep deprivation in healthy human volunteers causes an increase of SAA. Both humans and mice had an about 4 times increase in serum SAA with return to baseline after a recovery period. In this study we discuss that SAA, an inflammatory component considered to be important in the critical function of the inflammasome in obesity and insulin resistance is present in sleep restriction. The presence of SAA in the adipose tissue may be relevant in the weight gain caused by SR and may have a role in other comorbidities associated to poor sleep such as insulin resistance, CAD and cancer.

INTRODUCTION

Recently we describe that a history of sleep restriction potentiates future complications arising from a high-fat diet (1), corroborating epidemiological evidences that the restriction of human sleep may contribute to increased weight, and that short sleep duration is associated with concurrent and future obesity (2-4). This issue seemed especially relevant considering the reduction of the average sleep period in the last decades, the current epidemic of obesity and the increased risk of cardiovascular disease and diabetes (2; 5; 6).

Also recently, we identified the potential participation of the protein serum amyloid A in weight gain and insulin resistance induced by intense endotoxemia and high-fat diet (**D1**). SAA production is upregulated in liver and adipose tissue in the acute phase of an inflammatory process (7; 8). SAA has been considering to have a role in the activation of immune cells triggering inflammatory responses (8-10), to have growth factor-like activity, such as proliferative activity on different cell types, including preadipocytes (11-13), and to bind to receptors of the TLRs family and to SR-BI that are involved in the inflammatory process and metabolic control in obesity(14-16).

Here we found that sleep restriction lead to alteration in the production of SAA in mice and humans and we hypothesized that SAA is one of the molecules involved in the signaling linking sleep restriction with obesity and comorbidities.

MATERIALS AND METHODS

Animals – Male C57BL/6 mouse (3 months of age) were obtained from CEDEME Universidade Federal de São Paulo (UNIFESP). The animals were housed in a room maintained at 20±2°C in 12:12 h light/dark cycle (lights on at 7:00 am and off at 7:00 pm) inside standard polypropylene cages. For each experimental group, 5-9 animals were used for the experimental protocol. All procedures used in the present study complied with the 'Guide for the Care and Use of Laboratory Animals' (Institute of Laboratory Animal Resources, 1996). The experimental protocol was approved by the Ethical Committee of UNIFESP (approval n°0474/09).

Acute sleep restriction (SR) protocol. The method of SR was adapted from the multiple platform method, originally developed for rats (17-19). Groups of 5-9 mice were placed in water tanks (41 x 34 x 16.5 cm), containing 13 platforms (3 cm in diameter) each, surrounded by water up to 1 cm beneath the surface. In this method, the animals were able to move inside the tank, jumping from one platform to the other, keeping diet and water *ad libitum*. All the control groups were kept in control home-cages allowing sleep *ad libitum* under standard rodent chow diet. For SR experiments, the animals were randomly assigned into 2 groups: the control group and the SR group, both under standard rodent chow diet. The SR group was sleep restricted for 15 days, 21 h daily. After each 21 h period of SR, the mice were allowed to sleep for 3 h (sleep opportunity beginning at 10:00 am). The euthanasia occurred immediately after the last SR period.

Paradoxical sleep deprivation (PSP) protocol. For PSP experiments, the animals were randomly assigned into 3 groups: the control group, the PSP72 group and the RT24 group. PSP animals were sleep deprived for 72 consecutive hours. The RT24 mice were sleep deprived for 72 consecutive hours followed by 24 hours in sleep rebound period. The euthanasia occurred immediately after the last sleep deprivation period.

Human Sleep deprivation. The experimental protocol was performed as previously described (20). The study was conducted at the Sleep Laboratory of the Department of Psychobiology at the Universidade Federal de São Paulo (UNIFESP) with the

approval of the Ethics Committee of the University as well as the Radiation Protection Center (#1676/06). Thirty healthy male volunteers ranging from 19 to 29 years of age were randomly assigned to one of three experimental groups after giving written informed consent (10 non-sleep deprived, 10 total sleep deprivaved, and 10 REM sleep deprived). Total sleep deprivation was defined as 48 consecutive hours without sleep. REM sleep deprived group were inhibit to have rapid eye movement (REM) during 4 consecutive nights.

***Quantitative Real-Time PCR*.** Total RNA from epididymal adipose tissue liver was isolated using Qiagen RNeasy® Lipid Tissue Mini kit (Qiagen, Hilden, Germany). cDNA was then synthesized from 1 µg of RNA using the High Capacity cDNA Reverse Transcription (Life Technologies®, Grand Island, NY, USA). Real-time PCR were performed using SyBr® Green Master Mix (Life Technologies®, Grand Island, NY, USA) for SAA1/2 (F-5'-AGA CAA ATA CTT CCA TGC TCG G-3' and R-5'-CAT CAC TGA TTT TCT CAG CAG C-3'). Real-time PCR for SAA3 was performed using the TaqMan® assay (Applied Biosystems®, Grand Island, NJ, USA), catalog number Mm00441203_m1 – *Saa3 a*nd β-actin (ACTB), number 4552933E, as an endogenous housekeeping gene control. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.

SAA quantification. Serum concentrations of SAA was determined using ELISA following the manufacturer's instructions. Mouse SAA (Tridelta Development Ltd, Maynooth, Ireland) and human SAA (Invitrogen®, Camarillo, CA, USA).

Histological Analysis. Paraffin-embedded sections (5 µm thick) from epididymal adipose tissue were stained by hematoxylin and eosin to assess morphology. Immunohistochemistry for F4/80 was performed using a rat anti-mouse F4/80 antibody (1:100 dilution, AbD Serotec®, Raleigh, NC, USA) subsequently incubated with the appropriate secondary biotinylated antibody (Vector Laboratories Inc., Burlingame, CA, USA) and visualized with Immpact AEC peroxidase (Vector Laboratories Inc., Burlingame, CA, USA). Immunofluorescence for F4/80 and SAA were performed using a rat anti-mouse F4/80 antibody (1:100 dilution, Abcam®, Cambridge, UK), and a rabbit anti-mouse SAA (1:200 dilution, kindly produced and

provided by Dr. de Beer laboratory, University of Kentucky, KY, USA), subsequently incubated with the appropriate secondary fluorescent antibody (Invitrogen®, Camarillo, CA, USA) and the slides mounted using Vectashield set mounting medium with 4,6-diamidino-2-phenylindol-2-HCl (DAPI; Vector Laboratories Inc., Burlingame, CA, USA). An isotype control was used to ensure antibody specificity in each staining. Tissue sections were observed with a Nikon Eclipse 80i microscope (Nikon®) and digital images were captured with NIS-Element AR software (Nikon®).

Statistical analysis. Results were presented as mean ± SE and the number of independent experiments is indicated. Statistical analysis was performed with Graph Pad Prism4 (Graph Pad Software, Inc., San Diego, CA, USA). When multiple samples were compared with one independent variable, one-way analysis of variance with Newman-Keuls *post hoc* test was performed. Data with two independent variables were tested by two-way analysis of variance with Bonferroni *post hoc* test, as indicated in figure legends. The level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

In order to verify the effect of sleep restriction on SAA production, mice were subjected to SR for 21 h daily for 15 days. As already known during this period mice loses weight; approximately 10% in our protocol, and the serum concentration of SAA had an increase of 4-fold. This is an expressive increase considering that in obesity, diabetes and some chronic inflammatory conditions the rise in SAA serum levels reaches no more than 3-fold increase (21; 22). Serum levels of SAA mainly have a hepatic contribution from the upregulated mRNA expression of the SAA1/2 isoforms (23; 24). Besides liver, SAA expression is also upregulated in adipose tissue but in this case the SAA3 is the isoform affected (25; 26). The isoform SAA3 is the inducible isoform in mice adipose tissue (26). For instance, the same pattern was observed in mice under high-fat diet, acute endotoxemia and infection.

By morphological and immunostaining of the adipose tissue of SR mice, it was observed that SAA production is correlated to the presence of small adipocytes and macrophage infiltration. This finding are in accordance with the described

proliferative activity of SAA on preadipocytes (11) and the induction of chemoattractive cytokines by SAA (27).

The manner by which the expression of SAA1/2 and SAA3 are specifically regulated are not known but previous data supports the direct induction of adipose tissue SAA3 by the acute phase (hepatic) SAA1/2 (11). Sleep restriction and sleep reestablishment seemed to be a prompt stimulus for controlling the expression of SAA3 in adipose tissue given that an increase in SAA3 expression was observed in an experimental model of 72 consecutive hours without sleep with rapid return to the basal expression after 24 hours in recovery. Despite the fact that it was not possible to identify the specific SAA isoforms in serum, it is possible to assume that there is a strong correlation between the serum (SAA1/2) and adipose tissue (SAA3) isoforms, considering that it was observed the same profile in response to SR.

It was also possible to measure SAA from plasma derived from healthy human volunteers, kept during 48h in total sleep deprivation or 4 days in REM sleep deprivation. It was observed a 4-fold increase in SAA levels in 24 and 48h in total sleep deprived human. The increment was similar to that found in mice kept in 72h in total sleep restriction. Besides the differences in sleep habits and characteristics between mice and humans, the increased in serum levels of SAA in response to SR seems to be similar between the species.

It is unavoidable to analyze the data from this study with those recently published by us highlighting an acute and intense inflammation as an aggravating factor for diet-induced weight gain and insulin resistance (**D1**). As happened with acute inflammation (**D1**), the SR lead to weight loss but also lead to modification in adipose tissue expression of SAA3, that have been considered as an important factor to subsequently trigger obesity and insulin resistance. In these two cases mice became more prone to weigh gain (**D1** and **P3**). There are more similarities among conditions that lead to obesity. For instance, acute endotoxemia, low-grade endotoxemia and sleep restriction have TLR-4 upregulated in adipose tissue driving inflammation (28; 29). Furthermore, sleep deprivation also results in a low-grade endotoxemia (30; 31). The impairment of SR-BI by SAA (16; 32) may explain the increase of endotoxemia in sleep loss, similarly to ours previous hypothesis for the metabolic endotoxemia (**D1**).

In conclusion, our findings showed that SAA, a protein considered to be important in the critical function of the inflammasome, cytokine production,

proliferative and chemoattractive phenomena besides the development of obesity and insulin resistance, are upregulated in sleep restriction. More than a link between SR and obesity/insulin resistance, SAA could also explain the increased incidence of cardiovascular diseases and cancer in experimental models of sleep restriction (33; 34).

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

FIGURES AND LEGENDS

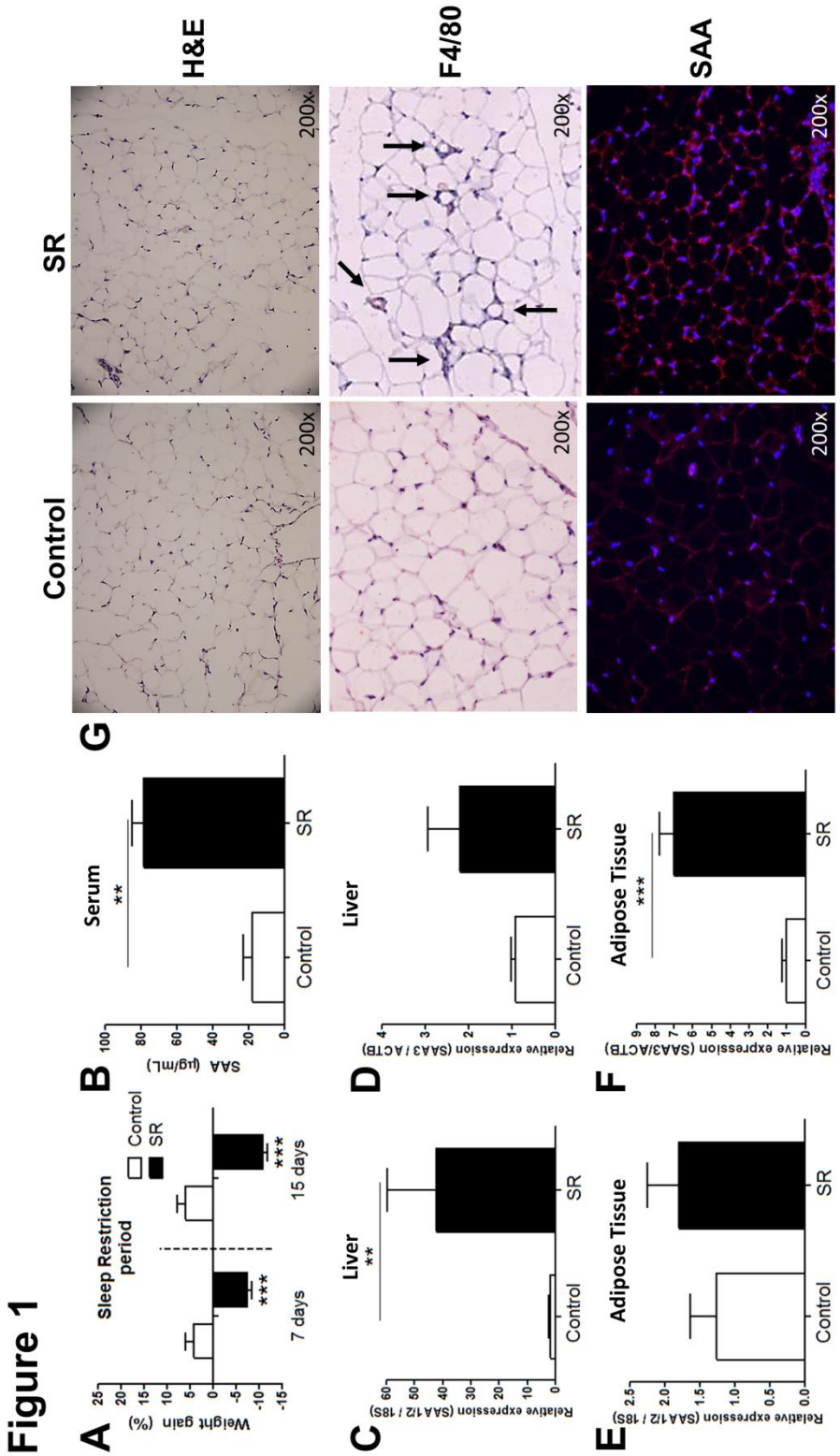

Figure 1. Chronic Sleep restriction (SR) causes weight mass lose and increases SAA (SAA1/2 in liver and SAA3 in adipose tissue). Mice C57BL/6 were submitted to SR for 21 h daily for 15 days. **(A)** Body weight of mice after 7 or 15 days of sleep restriction. **(B)** SAA concentration in serum, assessed by ELISA. Real-Time PCR were performed to asses mRNA expression of **(C)** SAA1/2 in liver. **(D)** SAA3 in liver. **(E)** SAA1/2 in adipose tissue and **(F)** SAA3 in adipose tissue. **(G)** Histological sections of epididymal fat pads after SR showing adipocyte morphology on hematoxylin and eosin staining, macrophage infiltration ($F4/80^{+}$) and SAA production Data are means ± SE from 6-12 mice per group and statistical analyses were performed by one-way ANOVA followed by Tukey's *post* test (** p<0.01, *** p<0.001, *vs.* control).

Figure 2

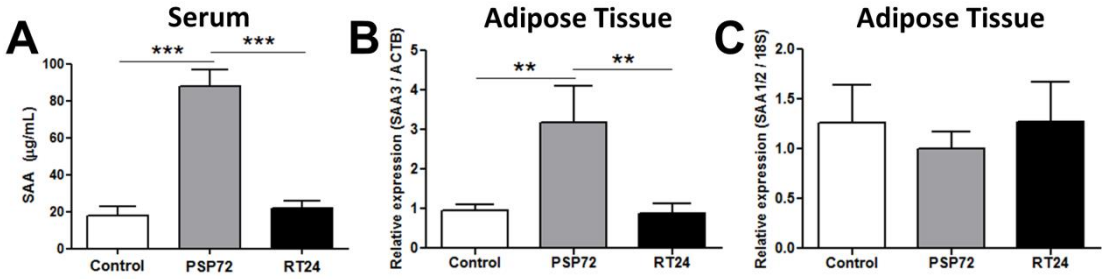

Figure 2. Acute sleep restriction is also able to increase SAA levels with return to basal after 24 hours of recovery. Mice C57BL/6 were submitted to paradoxical sleep deprivation (PSP) for 72 h uninterrupted followed by 24 h of recovery period. **(A)** SAA concentration in serum, assessed by ELISA. Real-Time PCR were performed to asses mRNA expression of **(B)** SAA3 and **(C)** SAA1/2 in adipose tissue. Data are means ± SE from 6-12 mice per group and statistical analyses were performed by two-way analysis of variance with Bonferroni *post hoc* test (** p<0.01, *** p<0.001, *vs.* control).

Figure 3

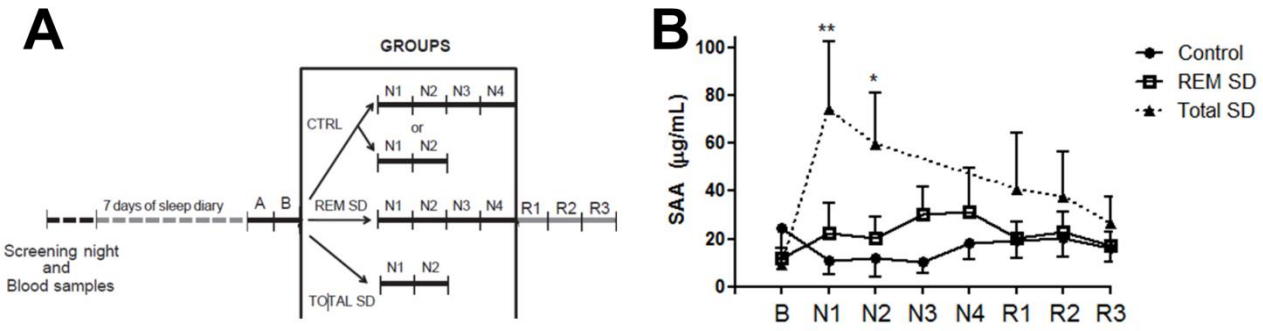

Figure 3. SAA is increased in human serum submitted to total sleep deprivation. (A) Schematic representation of the experimental protocol. Thirty healthy male volunteers ranging from 19 to 29 years of age were randomly assigned to one of the three experimental groups after giving written informed consent (10 non-sleep deprived, 10 total sleep deprived, and 10 REM sleep deprived). Exclusion criteria included the following: sleep disorders, obesity and obstructive sleep apnea (OSA). **(B)** Serum SAA concentration during the experimental protocol.

REFERENCES

1. de Oliveira EM, Visniauskas B, Sandri S, Migliorini S, Andersen ML, Tufik S, Fock RA, Chagas JR, Campa A: Late effects of sleep restriction: Potentiating weight gain and insulin resistance arising from a high-fat diet in mice. Obesity 2015;23:391-398
2. Knutson KL: Does inadequate sleep play a role in vulnerability to obesity? American Journal of Human Biology 2012;24:361-371
3. Knutson KL, Cauter E: Associations between sleep loss and increased risk of obesity and diabetes. Molecular and Biophysical Mechanisms of Arousal, Alertness, and Attention 2008;1129:287-304
4. Taheri S, Lin L, Austin D, Young T, Mignot E: Short sleep duration is associated with reduced leptin, elevated ghrelin, and increased body mass index. Plos Medicine 2004;1:210-217
5. Zimberg IZ, Damaso A, Del Re M, Carneiro AM, Souza HdS, de Lira FS, Tufik S, de Mello MT: Short sleep duration and obesity: mechanisms and future perspectives. Cell Biochemistry and Function 2012;30:524-529
6. Padilha HG, Crispim CA, Zimberg IZ, De-Souza DA, Waterhouse J, Tufik S, de-Mello MT: A link between sleep loss, glucose metabolism and adipokines. Brazilian Journal of Medical and Biological Research 2011;44:992-999
7. Jensen LE, Whitehead AS: Regulation of serum amyloid A protein expression during the acute-phase response. Biochemical Journal 1998;334:489-503
8. Eklund KK, Niemi K, Kovanen PT: Immune Functions of Serum Amyloid A. Critical Reviews in Immunology 2012;32:335-348
9. Hatanaka E, Ribeiro FP, Campa A: The acute phase protein serum amyloid A primes neutrophils. Fems Immunology and Medical Microbiology 2003;38:81-84
10. Furlaneto CJ, Campa A: A novel function of serum amyloid A: A potent stimulus for the release of tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8 by human blood neutrophil. Biochemical and Biophysical Research Communications 2000;268:405-408
11. Filippin-Monteiro FB, de Oliveira EM, Sandri S, Knebel FH, Albuquerque RC, Campa A: Serum amyloid A is a growth factor for 3T3-L1 adipocytes, inhibits differentiation and promotes insulin resistance. International Journal of Obesity 2012;36
12. Hatanaka E, Dermargos A, Armelin HA, Curi R, Campa A: Serum amyloid A induces reactive oxygen species (ROS) production and proliferation of fibroblast. Clinical and Experimental Immunology 2011;163
13. Cai XP, Ben Freedman S, Witting PK: Serum amyloid A stimulates cultured endothelial cells to migrate and proliferate: inhibition by the multikinase inhibitor BIBF1120. Clinical and Experimental Pharmacology and Physiology 2013;40:662-670
14. Sandri S, Rodriguez D, Gomes E, Monteiro HP, Russo M, Campa A: Is serum amyloid A an endogenous TLR4 agonist? Journal of Leukocyte Biology 2008;83:1174-1180
15. Vreugdenhil ACE, Rousseau CH, Hartung T, Greve JWM, van't Veer C, Buurman WA: Lipopolysaccharide (LPS)-binding protein mediates LPS detoxification by chylomicrons. Journal of Immunology 2003;170:1399-1405
16. Tsun JGS, Shiu SWM, Wong Y, Yung S, Chan TM, Tan KCB: Impact of serum amyloid A on cellular cholesterol efflux to serum in type 2 diabetes mellitus. Atherosclerosis 2013;231:405-410
17. Patti CL, Zanin KA, Sanday L, Kameda SR, Fernandes-Santos L, Fernandes HA, Andersen ML, Tufik S, Frussa R: Effects of Sleep Deprivation on Memory in Mice: Role of State-Dependent Learning. Sleep 2010;33:1669-1679

18. Zager A, Andersen ML, Lima MMS, Reksidler AB, Machado RB, Tufik S: Modulation of sickness behavior by sleep: The role of neurochemical and neuroinflammatory pathways in mice. European Neuropsychopharmacology 2009;19:589-602
19. Silva RH, Chehin AB, Kameda SR, Takatsu-Coleman AL, Abilio VC, Tufik S, Frussa R: Effects of pre- or post-training paradoxical sleep deprivation on two animal models of learning and memory in mice. Neurobiology of Learning and Memory 2004;82:90-98
20. Martins RCS, Andersen ML, Garbuio SA, Bittencourt LR, Guindalini C, Shih MC, Hoexter MQ, Bressan RA, Castiglioni MLV, Tufik S: Dopamine Transporter Regulation during Four Nights of REM Sleep Deprivation Followed by Recovery - An in vivo Molecular Imaging Study in Humans. Sleep 2010;33:243-251
21. Hatanaka E, Monteagudo PT, Marrocos MSM, Campa A: Interaction between serum amyloid A and leukocytes - A possible role in the progression of vascular complications in diabetes. Immunology Letters 2007;108:160-166
22. Hatanaka E, Monteagudo PT, Marrocos MSM, Campa A: Neutrophils and monocytes as potentially important sources of proinflammatory cytokines in diabetes. Clinical and Experimental Immunology 2006;146:443-447
23. Whitehead AS, Debeer MC, Steel DM, Rits M, Lelias JM, Lane WS, Debeer FC: IDENTIFICATION OF NOVEL MEMBERS OF THE SERUM AMYLOID-A PROTEIN SUPERFAMILY AS CONSTITUTIVE APOLIPOPROTEINS OF HIGH-DENSITY-LIPOPROTEIN. Journal of Biological Chemistry 1992;267:3862-3867
24. Uhlar CM, Whitehead AS: Serum amyloid A, the major vertebrate acute-phase reactant. European Journal of Biochemistry 1999;265:501-523
25. Sellar GC, Jordan SA, Bickmore WA, Fantes JA, Vanheyningen V, Whitehead AS: THE HUMAN SERUM AMYLOID A PROTEIN (SAA) SUPERFAMILY GENE-CLUSTER - MAPPING TO CHROMOSOME 11P15.1 BY PHYSICAL AND GENETIC-LINKAGE ANALYSIS. Genomics 1994;19:221-227
26. Sommer G, Weise S, Kralisch S, Scherer PE, Lossner U, Bluher M, Stumvoll M, Fasshauer M: The adipokine SAA3 is induced by interleukin-1 beta in mouse adipocytes. Journal of Cellular Biochemistry 2008;104:2241-2247
27. Sandri S, Hatanaka E, Franco AG, Pedrosa AMC, Monteiro HP, Campa A: Serum amyloid A induces CCL20 secretion in mononuclear cells through MAPK (p38 and ERK1/2) signaling pathways. Immunology Letters 2008;121:22-26
28. Wisor JP, Clegern WC, Schmidt MA: Toll-Like Receptor 4 Is a Regulator of Monocyte and Electroencephalographic Responses to Sleep Loss. Sleep 2011;34:1335-1345
29. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmee E, Cousin B, Sulpice T, Chamontin B, Ferrieres J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, Burcelin R: Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007;56:1761-1772
30. Everson CA: SUSTAINED SLEEP-DEPRIVATION IMPAIRS HOST-DEFENSE. American Journal of Physiology 1993;265:R1148-R1154
31. Kheirandish-Gozal L, Peris E, Wang Y, Kakazu MT, Khalyfa A, Carreras A, Gozal D: Lipopolysaccharide-Binding Protein Plasma Levels in Children: Effects of Obstructive Sleep Apnea and Obesity. Journal of Clinical Endocrinology & Metabolism 2014;99:656-663
32. Li B, Yu M, Pan X, Ren C, Peng W, Li X, Jiang W, Zheng J, Zhou H: Artesunate Reduces Serum Lipopolysaccharide in Cecal Ligation/Puncture Mice via Enhanced LPS Internalization by Macrophages through Increased mRNA Expression of Scavenger Receptors. International Journal of Molecular Sciences 2014;15:1143-1161
33. Thompson CL, Larkin EK, Patel S, Berger NA, Redline S, Li L: Short Duration of Sleep Increases Risk of Colorectal Adenoma. Cancer 2011;117:841-847

34. Thompson JC, Jayne C, Thompson J, Wilson PG, Yoder MH, Webb N, Tannock LR: A brief elevation of serum amyloid A is sufficient to increase atherosclerosis. Journal of Lipid Research 2015;56:286-293

REFERENCES

1 UHLAR, C. M.; WHITEHEAD, A. S. Serum amyloid A, the major vertebrate acute-phase reactant. **European Journal of Biochemistry,** v. 265, n. 2, p. 501-523, Oct 1999. ISSN 0014-2956. Disponível em: < <Go to ISI>://000083077100001 >.

2 SELLAR, G. C. et al. ORGANIZATION OF THE REGION ENCOMPASSING THE HUMAN SERUM AMYLOID-A (SAA) GENE FAMILY ON CHROMOSOME-11P15.1. **Genomics,** v. 23, n. 2, p. 492-495, Sep 1994. ISSN 0888-7543. Disponível em: < <Go to ISI>://A1994PK06000025 >.

3 _____. THE HUMAN SERUM AMYLOID A PROTEIN (SAA) SUPERFAMILY GENE-CLUSTER - MAPPING TO CHROMOSOME 11P15.1 BY PHYSICAL AND GENETIC-LINKAGE ANALYSIS. **Genomics,** v. 19, n. 2, p. 221-227, Jan 1994. ISSN 0888-7543. Disponível em: < <Go to ISI>://A1994NA60300003 >.

4 JENSEN, L. E.; WHITEHEAD, A. S. Regulation of serum amyloid A protein expression during the acute-phase response. **Biochemical Journal,** v. 334, p. 489-503, Sep 1998. ISSN 0264-6021. Disponível em: < <Go to ISI>://000076087600001 >.

5 SELLAR, G. C.; WHITEHEAD, A. S. THE PUTATIVE 5TH HUMAN SERUM AMYLOID A PROTEIN (SAA)-RELATED GENE SAA5 IS DEFINED BY SAA3. **Biochemical and Biophysical Research Communications,** v. 200, n. 1, p. 202-205, Apr 1994. ISSN 0006-291X. Disponível em: < <Go to ISI>://A1994NG38700029 >.

6 STEEL, D. M. et al. A CONSTITUTIVELY EXPRESSED SERUM AMYLOID-A PROTEIN GENE (SAA4) IS CLOSELY LINKED TO, AND SHARES STRUCTURAL SIMILARITIES WITH, AN ACUTE-PHASE SERUM AMYLOID-A PROTEIN GENE (SAA2). **Genomics,** v. 16, n. 2, p. 447-454, May 1993. ISSN 0888-7543. Disponível em: < <Go to ISI>://WOS:A1993LB92800021 >.

7 WHITEHEAD, A. S. et al. IDENTIFICATION OF NOVEL MEMBERS OF THE SERUM AMYLOID-A PROTEIN SUPERFAMILY AS CONSTITUTIVE APOLIPOPROTEINS OF HIGH-DENSITY-LIPOPROTEIN. **Journal of Biological Chemistry,** v. 267, n. 6, p. 3862-3867, Feb 1992. ISSN 0021-9258. Disponível em: < <Go to ISI>://A1992HE60700050 >.

8 MEEK, R. L.; BENDITT, E. P. AMYLOID-A GENE FAMILY EXPRESSION IN DIFFERENT MOUSE-TISSUES. **Journal of Experimental Medicine,** v. 164, n. 6, p. 2006-2017, Dec 1986. ISSN 0022-1007. Disponível em: < <Go to ISI>://A1986F117600014 >.

9 SCHEJA, L. et al. Acute-Phase Serum Amyloid A as a Marker of Insulin Resistance in Mice. **Experimental Diabetes Research**, 2008. ISSN 1687-5214. Disponível em: < <Go to ISI>://000267260300001 >.

10 SOMMER, G. et al. The adipokine SAA3 is induced by interleukin-1 beta in mouse adipocytes. **Journal of Cellular Biochemistry,** v. 104, n. 6, p. 2241-2247, Aug 2008. ISSN 0730-2312. Disponível em: < <Go to ISI>://000258387200026 >.

[11] URIELI-SHOVAL, S.; LINKE, R. P.; MATZNER, Y. Expression and function of serum amyloid A, a major acute-phase protein, in normal and disease states. **Current Opinion in Hematology,** v. 7, n. 1, p. 64-69, Jan 2000. ISSN 1065-6251. Disponível em: < <Go to ISI>://WOS:000165671100012 >.

[12] EKLUND, K. K.; NIEMI, K.; KOVANEN, P. T. Immune Functions of Serum Amyloid A. **Critical Reviews in Immunology,** v. 32, n. 4, p. 335-348, 2012 2012. ISSN 1040-8401. Disponível em: < <Go to ISI>://WOS:000313155200004 >.

[13] FURLANETO, C. J.; CAMPA, A. A novel function of serum amyloid A: A potent stimulus for the release of tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8 by human blood neutrophil. **Biochemical and Biophysical Research Communications,** v. 268, n. 2, p. 405-408, Feb 2000. ISSN 0006-291X. Disponível em: < <Go to ISI>://000085427400030 >.

[14] FRANCO, A. G.; SANDRI, S.; CAMPA, A. High-density lipoprotein prevents SAA-induced production of TNF-alpha in THP-1 monocytic cells and peripheral blood mononuclear cells. **Memorias Do Instituto Oswaldo Cruz,** v. 106, n. 8, Dec 2011. ISSN 0074-0276. Disponível em: < <Go to ISI>://WOS:000299068800014 >.

[15] STEEL, D. M. et al. BIOSYNTHESIS OF HUMAN ACUTE-PHASE SERUM AMYLOID A-PROTEIN (A-SAA) INVITRO - THE ROLES OF MESSENGER-RNA ACCUMULATION, POLY(A) TAIL SHORTENING AND TRANSLATIONAL EFFICIENCY. **Biochemical Journal,** v. 291, p. 701-707, May 1993. ISSN 0264-6021. Disponível em: < <Go to ISI>://A1993LA82400008 >.

[16] GABAY, C.; KUSHNER, I. Mechanisms of disease: Acute-phase proteins and other systemic responses to inflammation. **New England Journal of Medicine,** v. 340, n. 6, p. 448-454, Feb 1999. ISSN 0028-4793. Disponível em: < <Go to ISI>://WOS:000078571000007 >.

[17] KUMON, Y. et al. SERUM AMYLOID-A PROTEIN IN PATIENTS WITH NON-INSULIN-DEPENDENT DIABETES-MELLITUS. **Clinical Biochemistry,** v. 27, n. 6, p. 469-473, 1994. ISSN 0009-9120. Disponível em: < <Go to ISI>://WOS:A1994PZ36100007 >.

[18] HILLIQUIN, P. Biological markers in inflammatory rheumatic diseases. **Cellular and Molecular Biology,** v. 41, n. 8, p. 993-1006, 1995. ISSN 0145-5680. Disponível em: < <Go to ISI>://WOS:A1995TL81100002 >.

[19] BIRAN, H. et al. SERUM AMYLOID-A (SAA) VARIATIONS IN PATIENTS WITH CANCER - CORRELATION WITH DISEASE-ACTIVITY, STAGE, PRIMARY SITE, AND PROGNOSIS. **Journal of Clinical Pathology,** v. 39, n. 7, p. 794-797, 1986. ISSN 0021-9746. Disponível em: < <Go to ISI>://WOS:A1986D328100016 >.

[20] THOMPSON, J. C. et al. A brief elevation of serum amyloid A is sufficient to increase atherosclerosis. **Journal of Lipid Research,** v. 56, n. 2, p. 286-293, Feb 2015. ISSN 0022-2275. Disponível em: < <Go to ISI>://CCC:000348905000009 >.

[21] DONG, Z. et al. Serum Amyloid A Directly Accelerates the Progression of Atherosclerosis in Apolipoprotein E-Deficient Mice. **Molecular Medicine,** v. 17, n. 11-12, p. 1357-1364, Nov-Dec 2011. ISSN 1076-1551. Disponível em: < <Go to ISI>://WOS:000297958800026 >.

[22] TAPE, C.; KISILEVSKY, R. APOLIPOPROTEIN-A-I AND APOLIPOPROTEIN SAA HALF-LIVES DURING ACUTE-INFLAMMATION AND AMYLOIDOGENESIS. **Biochimica Et Biophysica Acta,** v. 1043, n. 3, p. 295-300, Apr 17 1990. ISSN 0006-3002. Disponível em: < <Go to ISI>://WOS:A1990DA04400010 >.

[23] GOLLAHER, C. J.; BAUSSERMAN, L. L. HEPATIC CATABOLISM OF SERUM AMYLOID-A DURING AN ACUTE PHASE RESPONSE AND CHRONIC INFLAMMATION. **Proceedings of the Society for Experimental Biology and Medicine,** v. 194, n. 3, p. 245-250, Jul 1990. ISSN 0037-9727. Disponível em: < <Go to ISI>://WOS:A1990DY44000012 >.

[24] POITOU, C. et al. Serum amyloid A: production by human white adipocyte and regulation by obesity and nutrition. **Diabetologia,** v. 48, n. 3, p. 519-528, Mar 2005. ISSN 0012-186X. Disponível em: < <Go to ISI>://000227741800015 >.

[25] O'HARA, R. et al. Acute-phase serum amyloid - A production by rheumatoid arthritis synovial tissue. **Arthritis Research,** v. 2, n. 2, p. 142-U4, 2000. ISSN 1465-9913. Disponível em: < <Go to ISI>://000167357300007 >.

[26] HE, R.; SANG, H. R.; YE, R. D. Serum amyloid A induces IL-8 secretion through a G protein-coupled receptor, FPRL1/LXA4R. **Blood,** v. 101, n. 4, p. 1572-1581, Feb 15 2003. ISSN 0006-4971. Disponível em: < <Go to ISI>://WOS:000180846700055 >.

[27] LEE, M.-S. et al. Serum amyloid A binding to formyl peptide receptor-like 1 induces synovial hyperplasia and angiogenesis. **Journal of Immunology,** v. 177, n. 8, p. 5585-5594, Oct 15 2006. ISSN 0022-1767. Disponível em: < <Go to ISI>://WOS:000241093100073 >.

[28] SU, S. B. et al. A seven-transmembrane, G protein-coupled receptor, FPRL1, mediates the chemotactic activity of serum amyloid A for human phagocytic cells. **Journal of Experimental Medicine,** v. 189, n. 2, p. 395-402, Jan 18 1999. ISSN 0022-1007. Disponível em: < <Go to ISI>://WOS:000078191700017 >.

[29] BARANOVA, I. N. et al. Serum amyloid a binding to CLA-1 (CD36 and LIMPII analogous-1) mediates serum amyloid A protein-induced activation of ERK1/2 and p38 mitogen-activated protein kinases. **Journal of Biological Chemistry,** v. 280, n. 9, p. 8031-8040, 2005. ISSN 0021-9258. Disponível em: < <Go to ISI>://WOS:000227395700073 >.

[30] VAN DER WESTHUYZEN, D. R. et al. Serum amyloid A promotes cholesterol efflux mediated by scavenger receptor B-I. **Journal of Biological Chemistry,** v. 280, n. 43, p. 35890-35895, Oct 28 2005. ISSN 0021-9258. Disponível em: < <Go to ISI>://WOS:000232726900015 >.

[31] TSUN, J. G. S. et al. Impact of serum amyloid A on cellular cholesterol efflux to serum in type 2 diabetes mellitus. **Atherosclerosis,** v. 231, n. 2, p. 405-410, Dec 2013. ISSN 0021-9150. Disponível em: < <Go to ISI>://WOS:000327537700038 >.

[32] CAI, H. et al. Serum amyloid A induces monocyte tissue factor. **Journal of Immunology,** v. 178, n. 3, p. 1852-1860, 2007. ISSN 0022-1767. Disponível em: < <Go to ISI>://WOS:000243820900071 >.

[33] WALDER, K. et al. Tanis: A link between type 2 diabetes and inflammation? **Diabetes,** v. 51, n. 6, p. 1859-1866, 2002. ISSN 0012-1797. Disponível em: < <Go to ISI>://WOS:000175936700025 >.

[34] HE, R. L. et al. Serum amyloid A induces G-CSF expression and neutrophilia via Toll-like receptor 2. **Blood,** v. 113, n. 2, p. 429-437, Jan 8 2009. ISSN 0006-4971. Disponível em: < <Go to ISI>://WOS:000262318600022 >.

[35] SANDRI, S. et al. Is serum amyloid A an endogenous TLR4 agonist? **Journal of Leukocyte Biology,** v. 83, n. 5, p. 1174-1180, May 2008. ISSN 0741-5400. Disponível em: < <Go to ISI>://000258019500012 >.

[36] RIBEIRO, F. P. et al. mRNA expression and release of interleukin-8 induced by serum amyloid A in neutrophils and monocytes. **Mediators of Inflammation,** v. 12, n. 3, p. 173-178, Jun 2003. ISSN 0962-9351. Disponível em: < <Go to ISI>://000184071300006 >.

[37] HATANAKA, E. et al. Serum amyloid A-induced mRNA expression and release of tumor necrosis factor-alpha (TNF-alpha) in human neutrophils. **Immunology Letters,** v. 91, n. 1, p. 33-37, Jan 2004. ISSN 0165-2478. Disponível em: < <Go to ISI>://000188972700006 >.

[38] SANDRI, S. et al. Serum amyloid A induces CCL20 secretion in mononuclear cells through MAPK (p38 and ERK1/2) signaling pathways. **Immunology Letters,** v. 121, n. 1, p. 22-26, Nov 2008. ISSN 0165-2478. Disponível em: < <Go to ISI>://000261280600004 >.

[39] SONG, C. J. et al. Serum amyloid A induction of cytokines in monocytes/macrophages and lymphocytes. **Atherosclerosis,** v. 207, n. 2, p. 374-383, Dec 2009. ISSN 0021-9150. Disponível em: < <Go to ISI>://WOS:000273566700014 >.

[40] BADOLATO, R. et al. SERUM AMYLOID-A IS A CHEMOATTRACTANT - INDUCTION OF MIGRATION, ADHESION, AND TISSUE INFILTRATION OF MONOCYTES AND POLYMORPHONUCLEAR LEUKOCYTES. **Journal of Experimental Medicine,** v. 180, n. 1, p. 203-209, Jul 1 1994. ISSN 0022-1007. Disponível em: < <Go to ISI>://WOS:A1994NR98900023 >.

[41] HATANAKA, E. et al. Interaction between serum amyloid A and leukocytes - A possible role in the progression of vascular complications in diabetes. **Immunology Letters,** v. 108, n. 2, p. 160-166, Feb 2007. ISSN 0165-2478. Disponível em: < <Go to ISI>://000244575100007 >.

[42] _____. Serum amyloid A induces reactive oxygen species (ROS) production and proliferation of fibroblast. **Clinical and Experimental Immunology,** v. 163, n. 3, Mar 2011. ISSN 0009-9104. Disponível em: < <Go to ISI>://WOS:000287091900011 >.

[43] CAI, X. P.; BEN FREEDMAN, S.; WITTING, P. K. Serum amyloid A stimulates cultured endothelial cells to migrate and proliferate: inhibition by the multikinase inhibitor BIBF1120. **Clinical and Experimental Pharmacology and Physiology,** v. 40, n. 9, p. 662-670, Sep 2013. ISSN 1440-1681. Disponível em: < <Go to ISI>://WOS:000323376800011 >.

[44] KNEBEL, F. H. et al. Dual Effect of Serum Amyloid A on the Invasiveness of Glioma Cells. **Mediators of Inflammation**, 2013 2013. ISSN 0962-9351. Disponível em: < <Go to ISI>://WOS:000315889300001 >.

[45] BELLÉ, L. P. **Ação da proteína amilóide sérica A em melanomas**. 2015. 164 (Doutorado). Departamento de Análises Clínicas e Toxicológicas, Universidade de São Paulo, São Paulo.

[46] SANDRI, S. et al. Serum Amyloid A in the Placenta and Its Role in Trophoblast Invasion. **Plos One,** v. 9, n. 3, Mar 2014. ISSN 1932-6203. Disponível em: < <Go to ISI>://WOS:000332839300049 >.

[47] ATHER, J. L. et al. Serum Amyloid A Activates the NLRP3 Inflammasome and Promotes Th17 Allergic Asthma in Mice. **Journal of Immunology,** v. 187, n. 1, p. 64-73, Jul 2011. ISSN 0022-1767. Disponível em: < <Go to ISI>://WOS:000291799300011 >.

[48] MIGITA, K. et al. Serum Amyloid A Induces NLRP-3-Mediated IL-1 beta Secretion in Neutrophils. **Plos One,** v. 9, n. 5, May 2014. ISSN 1932-6203. Disponível em: < <Go to ISI>://WOS:000339563400009 >.

[49] LAAKE, I. et al. A Prospective Study of Body Mass Index, Weight Change, and Risk of Cancer in the Proximal and Distal Colon. **Cancer Epidemiology Biomarkers & Prevention,** v. 19, n. 6, p. 1511-1522, Jun 2010. ISSN 1055-9965. Disponível em: < <Go to ISI>://WOS:000278507300016 >.

[50] LEITZMANN, M. F. et al. Prospective study of body mass index, physical activity and thyroid cancer. **International Journal of Cancer,** v. 126, n. 12, p. 2947-2956, Jun 15 2010. ISSN 0020-7136. Disponível em: < <Go to ISI>://WOS:000277551100020 >.

[51] SAMOLIS, S. et al. Relation between first trimester maternal serum leptin levels and body mass index in normotensive and pre-eclamptic pregnancies - Role of leptin as a marker of pre-eclampsia: A prospective case-control study. **Gynecological Endocrinology,** v. 26, n. 5, p. 338-343, May 2010. ISSN 0951-3590. Disponível em: < <Go to ISI>://WOS:000276624500006 >.

[52] BORNSTEIN, S. R. et al. Is the Worldwide Epidemic of Obesity a Communicable Feature of Globalization? **Experimental and Clinical Endocrinology & Diabetes,** v. 116, p. S30-S32, Sep 2008. ISSN 0947-7349. Disponível em: < <Go to ISI>://WOS:000260119400007 >.

[53] MELNYK, B. M.; SMALL, L.; MOORE, N. The worldwide epidemic of child and adolescent overweight and obesity: Calling all clinicians and researchers to intensify efforts in prevention and treatment. **Worldviews on Evidence-Based Nursing,** v. 5, n. 3, p. 109-112, 2008 2008. ISSN 1545-102X. Disponível em: < <Go to ISI>://WOS:000259228900001 >.

[54] WHO. **World Health Statistics 2012**: World Health Organization 2012.

[55] IBGE, I. B. D. G. E. E. **Síntese de indicadores sociais: uma análise das condições de vida da população brasileira**. Rio de Janeiro, Brazil: Estudos & Pesquisas: Informação Demográfica e Socioeconômica. 29 2012.

[56] MS. **Ministério da Saúde e Núcleo de Pesquisas Epidemiológicas em Nutrição e Saúde da USP. Vigitel.** 2013.

[57] KOPELMAN, P. G.; ALBON, L. Obesity, non-insulin-dependent diabetes mellitus and the metabolic syndrome. **British Medical Bulletin,** v. 53, n. 2, p. 322-340, 1997. ISSN 0007-1420. Disponível em: < <Go to ISI>://WOS:A1997XJ95600007 >.

[58] SPIEGELMAN, B. M.; FLIER, J. S. Obesity and the regulation of energy balance. **Cell,** v. 104, n. 4, p. 531-543, Feb 23 2001. ISSN 0092-8674. Disponível em: < <Go to ISI>://WOS:000167181500008 >.

[59] HASLAM, D. W.; JAMES, W. P. T. Obesity. **Lancet,** v. 366, n. 9492, p. 1197-1209, Oct 1 2005. ISSN 0140-6736. Disponível em: < <Go to ISI>://WOS:000232311300030 >.

[60] LOLMEDE, K. et al. Effects of hypoxia on the expression of proangiogenic factors in differentiated 3T3-F442A adipocytes. **International Journal of Obesity,** v. 27, n. 10, p. 1187-1195, Oct 2003. ISSN 0307-0565. Disponível em: < <Go to ISI>://000185625000005 >.

[61] HOSOGAI, N. et al. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. **Diabetes,** v. 56, n. 4, p. 901-911, Apr 2007. ISSN 0012-1797. Disponível em: < <Go to ISI>://000245697200001 >.

[62] WANG, B.; WOOD, I. S.; TRAYHURN, P. Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. **Pflugers Archiv-European Journal of Physiology,** v. 455, n. 3, p. 479-492, Dec 2007. ISSN 0031-6768. Disponível em: < <Go to ISI>://000250380800007 >.

[63] ROSS, R. Atherosclerosis - An inflammatory disease - Reply. **New England Journal of Medicine,** v. 340, n. 24, p. 1929-1929, Jun 1999. ISSN 0028-4793. Disponível em: < <Go to ISI>://WOS:000080863500029 >.

[64] YANG, R. Z. et al. Acute-phase serum amyloid A: An inflammatory adipokine and potential link between obesity and its metabolic complications. **Plos Medicine,** v. 3, n. 6, p. 884-894, Jun 2006. ISSN 1549-1277. Disponível em: < <Go to ISI>://000239479600030 >.

[65] SJOHOLM, K. et al. Association of serum amyloid A levels with adipocyte size and serum levels of adipokines: Differences between men and women. **Cytokine,** v. 48, n. 3, p. 260-266, Dec 2009. ISSN 1043-4666. Disponível em: < <Go to ISI>://000271916300014 >.

[66] BASTARD, J. P. et al. Recent advances in the relationship between obesity, inflammation, and insulin resistance. **European Cytokine Network,** v. 17, n. 1, p. 4-12, Mar 2006. ISSN 1148-5493. Disponível em: < <Go to ISI>://WOS:000237306600001 >.

[67] ZHAO, Y. L. et al. Association between serum amyloid A and obesity: a meta-analysis and systematic review. **Inflammation Research,** v. 59, n. 5, p. 323-334, May 2010. ISSN 1023-3830. Disponível em: < <Go to ISI>://000276660200001 >.

[68] FILIPPIN-MONTEIRO, F. B. et al. Serum amyloid A is a growth factor for 3T3-L1 adipocytes, inhibits differentiation and promotes insulin resistance. **International Journal of Obesity,** v. 36, n. 8, Aug 2012. ISSN 0307-0565. Disponível em: < <Go to ISI>://WOS:000307629500003 >.

[69] FATY, A.; FERRE, P.; COMMANS, S. The Acute Phase Protein Serum Amyloid A Induces Lipolysis and Inflammation in Human Adipocytes through Distinct Pathways. **Plos One,** v. 7, n. 4, Apr 19 2012. ISSN 1932-6203. Disponível em: < <Go to ISI>://WOS:000305336200005 >.

[70] HOTAMISLIGIL, G. S. Inflammation and metabolic disorders. **Nature,** v. 444, n. 7121, Dec 14 2006. ISSN 0028-0836. Disponível em: < <Go to ISI>://WOS:000242805400043 >.

[71] SHOELSON, S. E.; LEE, J.; GOLDFINE, A. B. Inflammation and insulin resistance. **Journal of Clinical Investigation,** v. 116, n. 7, p. 1793-1801, Jul 2006. ISSN 0021-9738. Disponível em: < <Go to ISI>://WOS:000238924900008 >.

[72] ARNER, P. CONTROL OF LIPOLYSIS AND ITS RELEVANCE TO DEVELOPMENT OF OBESITY IN MAN. **Diabetes-Metabolism Reviews,** v. 4, n. 5, p. 507-515, Aug 1988. ISSN 0742-4221. Disponível em: < <Go to ISI>://WOS:A1988P742500006 >.

[73] HATANAKA, E. et al. Neutrophils and monocytes as potentially important sources of proinflammatory cytokines in diabetes. **Clinical and Experimental Immunology,** v. 146, n. 3, p. 443-447, Dec 2006. ISSN 0009-9104. Disponível em: < <Go to ISI>://WOS:000241914000009 >.

[74] ROCHA, V. Z.; LIBBY, P. The multiple facets of the fat tissue. **Thyroid,** v. 18, n. 2, p. 175-183, Feb 2008. ISSN 1050-7256. Disponível em: < <Go to ISI>://000253722800011 >.

[75] WEISBERG, S. P. et al. Obesity is associated with macrophage accumulation in adipose tissue. **Journal of Clinical Investigation,** v. 112, n. 12, p. 1796-1808, Dec 2003. ISSN 0021-9738. Disponível em: < <Go to ISI>://000187348300007 >.

[76] KANDA, H. et al. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. **Journal of Clinical Investigation,** v. 116, n. 6, p. 1494-1505, Jun 2006. ISSN 0021-9738. Disponível em: < <Go to ISI>://000237979700012 >.

[77] WEISBERG, S. P. et al. CCR2 modulates inflammatory and metabolic effects of high-fat feeding. **Journal of Clinical Investigation,** v. 116, n. 1, p. 115-124, Jan 2006. ISSN 0021-9738. Disponível em: < <Go to ISI>://000234488700015 >.

[78] INOUYE, K. E. et al. Absence of CC chemokine ligand 2 does not limit obesity-associated infiltration of macrophages into adipose tissue. **Diabetes,** v. 56, n. 9, p. 2242-2250, Sep 2007. ISSN 0012-1797. Disponível em: < <Go to ISI>://000249304500008 >.

[79] KIRK, E. A. et al. Macrophage chemoattractant protein-1 deficiency fails to restrain macrophage infiltration into adipose tissue. **Diabetes,** v. 57, n. 5, p. 1254-1261, May 2008. ISSN 0012-1797. Disponível em: < <Go to ISI>://000255628700015 >.

[80] DE BEER, M. C. et al. Deficiency of Endogenous Acute Phase Serum Amyloid A Does Not Affect Atherosclerotic Lesions in Apolipoprotein E-Deficient Mice. **Arteriosclerosis Thrombosis and Vascular Biology,** v. 34, n. 2, p. 255-261, Feb 2014. ISSN 1079-5642. Disponível em: < <Go to ISI>://WOS:000335607300010 >.

[81] DEN HARTIGH, L. J. et al. Deletion of Serum Amyloid A3 Improves High Fat High Sucrose Diet-Induced Adipose Tissue Inflammation and Hyperlipidemia in Female Mice. **Plos One,** v. 9, n. 9, Sep 2014. ISSN 1932-6203. Disponível em: < <Go to ISI>://WOS:000342492700142 >.

[82] BACKHED, F. et al. The gut microbiota as an environmental factor that regulates fat storage. **Proceedings of the National Academy of Sciences of the United States of America,** v. 101, n. 44, p. 15718-15723, Nov 2004. ISSN 0027-8424. Disponível em: < <Go to ISI>://000224922300032 >.

[83] _____. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. **Proceedings of the National Academy of Sciences of the United States of America,** v. 104, n. 3, p. 979-984, Jan 2007. ISSN 0027-8424. Disponível em: < <Go to ISI>://000243761100054 >.

[84] CANI, P. D. et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. **Diabetes,** v. 57, n. 6, p. 1470-1481, Jun 2008. ISSN 0012-1797. Disponível em: < <Go to ISI>://WOS:000256611600008 >.

[85] _____. Metabolic endotoxemia initiates obesity and insulin resistance. **Diabetes,** v. 56, n. 7, p. 1761-1772, Jul 2007. ISSN 0012-1797. Disponível em: < <Go to ISI>://WOS:000247768000002 >.

[86] TURNBAUGH, P. J. et al. An obesity-associated gut microbiome with increased capacity for energy harvest. **Nature,** v. 444, n. 7122, p. 1027-1031, Dec 2006. ISSN 0028-0836. Disponível em: < <Go to ISI>://000242971100045 >.

[87] CAESAR, R. et al. Gut-derived lipopolysaccharide augments adipose macrophage accumulation but is not essential for impaired glucose or insulin tolerance in mice. **Gut,** v. 61, n. 12, p. 1701-1707, Dec 2012. ISSN 0017-5749. Disponível em: < <Go to ISI>://WOS:000310922000009 >.

[88] CANI, P. D. et al. Metabolic endotoxemia initiates obesity and insulin resistance. **Diabetes,** v. 56, n. 7, p. 1761-1772, Jul 2007. ISSN 0012-1797. Disponível em: < <Go to ISI>://000247768000002 >.

[89] TSUKUMO, D. M. L. et al. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. **Diabetes,** v. 56, n. 8, p. 1986-1998, Aug 2007. ISSN 0012-1797. Disponível em: < <Go to ISI>://WOS:000248473100005 >.

[90] SHI, H. et al. TLR4 links innate immunity and fatty acid-induced insulin resistance. **Journal of Clinical Investigation,** v. 116, n. 11, p. 3015-3025, Nov 2006. ISSN 0021-9738. Disponível em: < <Go to ISI>://000241810900025 >.

[91] SONG, M. J. et al. Activation of Toll-like receptor 4 is associated with insulin resistance in adipocytes. **Biochemical and Biophysical Research Communications,** v. 346, n. 3, p. 739-745, Aug 2006. ISSN 0006-291X. Disponível em: < <Go to ISI>://000238865900016 >.

[92] KIM, K.-A. et al. High Fat Diet-Induced Gut Microbiota Exacerbates Inflammation and Obesity in Mice via the TLR4 Signaling Pathway. **Plos One,** v. 7, n. 10, Oct 16 2012. ISSN 1932-6203. Disponível em: < <Go to ISI>://WOS:000310135800066 >.

[93] CARICILLI, A. M. et al. Gut Microbiota Is a Key Modulator of Insulin Resistance in TLR 2 Knockout Mice. **Plos Biology,** v. 9, n. 12, p. 21, Dec 2011. ISSN 1544-9173. Disponível em: < <Go to ISI>://WOS:000298673000003 >.

[94] STEVENS, P. **Diseases of Poverty and the 10/90 gap**: International Policy network 2012.

[95] SINGH, G. K.; SIAHPUSH, M.; KOGAN, M. D. **Rising social inequalities in U.S. childhood obesity, 2003-2007**: Annals of Epidemiology. 20**:** 40-52 p. 2010.

[96] DREWNOWSKI, A. et al. **Poverty and childhood overweight in California Assembly districts**: Health and Place. 15**:** 631-635 p. 2009.

[97] SKELTON, J. A. et al. **Prevalence and trends of severe obesity among U.S. children and**

adolescents: Academic Pediatrics. 9**:** 322-329 p. 2009.

98 SINGH, A. R.; SINGH, S. A. **Diseases of Poverty and Lifestyle, Well-Being and Human Development**: Mens Sana Monographs. 6**:** 187-225 p. 2008.

99 FORD, E. S. et al. C-reactive protein and body mass index in children: Findings from the Third National Health and Nutrition Examination Survey, 1988-1994. **Journal of Pediatrics,** v. 138, n. 4, p. 486-492, Apr 2001. ISSN 0022-3476. Disponível em: < <Go to ISI>://WOS:000168175400014 >.

100 CARTER, J. K.; OW, C. L.; SMITH, R. E. ROUS-ASSOCIATED VIRUS TYPE-7 INDUCES A SYNDROME IN CHICKENS CHARACTERIZED BY STUNTING AND OBESITY. **Infection and Immunity,** v. 39, n. 1, p. 410-422, 1983 1983. ISSN 0019-9567. Disponível em: < <Go to ISI>://WOS:A1983PV89900056 >.

101 BERNARD, A. et al. HYPERINSULINEMIA INDUCED BY CANINE-DISTEMPER VIRUS-INFECTION OF MICE AND ITS CORRELATION WITH THE APPEARANCE OF OBESITY. **Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology,** v. 91, n. 4, p. 691-696, 1988 1988. ISSN 0305-0491. Disponível em: < <Go to ISI>://WOS:A1988R440600015 >.

102 DHURANDHAR, N. V. et al. Increased adiposity in animals due to a human virus. **International Journal of Obesity,** v. 24, n. 8, p. 989-996, Aug 2000. ISSN 0307-0565. Disponível em: < <Go to ISI>://WOS:000088597000008 >.

103 VAN GINNEKEN, V.; SITNYAKOWSKY, L.; JEFFERY, J. E. Infectobesity: viral infections (especially with human adenovirus-36: Ad-36) may be a cause of obesity. **Medical Hypotheses,** v. 72, n. 4, p. 383-388, Apr 2009. ISSN 0306-9877. Disponível em: < <Go to ISI>://WOS:000264619800005 >.

104 ATKINSON, R. L. et al. Human adenovirus-36 is associated with increased body weight and paradoxical reduction of serum lipids. **International Journal of Obesity,** v. 29, n. 3, p. 281-286, Mar 2005. ISSN 0307-0565. Disponível em: < <Go to ISI>://WOS:000226981400005 >.

105 KNUTSON, K. L. Does inadequate sleep play a role in vulnerability to obesity? **American Journal of Human Biology,** v. 24, n. 3, p. 361-371, May-Jun 2012. ISSN 1042-0533. Disponível em: < <Go to ISI>://WOS:000302606700013 >.

106 PADILHA, H. G. et al. A link between sleep loss, glucose metabolism and adipokines. **Brazilian Journal of Medical and Biological Research,** v. 44, n. 10, p. 992-999, Oct 2011. ISSN 0100-879X. Disponível em: < <Go to ISI>://WOS:000295721600003 >.

107 ZIMBERG, I. Z. et al. Short sleep duration and obesity: mechanisms and future perspectives. **Cell Biochemistry and Function,** v. 30, n. 6, p. 524-529, Aug 2012. ISSN 0263-6484. Disponível em: < <Go to ISI>://WOS:000307787200013 >.

[108] SHARMA, S.; KAVURU, M. Sleep and Metabolism: An Overview. **International Journal of Endocrinology**, 2010. ISSN 1687-8337. Disponível em: < <Go to ISI>://WOS:000284934100001 >.

[109] TAHERI, S. et al. Short sleep duration is associated with reduced leptin, elevated ghrelin, and increased body mass index. **Plos Medicine,** v. 1, n. 3, p. 210-217, Dec 2004. ISSN 1549-1277. Disponível em: < <Go to ISI>://WOS:000227378900018 >.

[110] KNUTSON, K. L.; CAUTER, E. Associations between sleep loss and increased risk of obesity and diabetes. **Molecular and Biophysical Mechanisms of Arousal, Alertness, and Attention,** v. 1129, p. 287-304, 2008. ISSN 0077-8923. Disponível em: < <Go to ISI>://WOS:000256620900026 >.

[111] TAKAHASH.Y; KIPNIS, D. M.; DAUGHADA.WH. GROWTH HORMONE SECRETION DURING SLEEP. **Journal of Clinical Investigation,** v. 47, n. 9, p. 2079-&, 1968 1968. ISSN 0021-9738. Disponível em: < <Go to ISI>://WOS:A1968B779600011 >.

[112] NILSSON, P. M. et al. Incidence of diabetes in middle-aged men is related to sleep disturbances. **Diabetes Care,** v. 27, n. 10, p. 2464-2469, Oct 2004. ISSN 0149-5992. Disponível em: < <Go to ISI>://WOS:000224115700028 >.

[113] TUOMILEHTO, H. et al. Sleep duration is associated with an increased risk for the prevalence of type 2 diabetes in middle-aged women - The FIN-D2D survey. **Sleep Medicine,** v. 9, n. 3, p. 221-227, Mar 2008. ISSN 1389-9457. Disponível em: < <Go to ISI>://WOS:000255164300002 >.

[114] HONDA, Y. et al. GROWTH HORMONE SECRETION DURING NOCTURNAL SLEEP IN NORMAL SUBJECTS. **Journal of Clinical Endocrinology & Metabolism,** v. 29, n. 1, p. 20-+, 1969 1969. ISSN 0021-972X. Disponível em: < <Go to ISI>://WOS:A1969C510300004 >.

[115] VANCAUTER, E. et al. MODULATION OF GLUCOSE REGULATION AND INSULIN-SECRETION BY CIRCADIAN RHYTHMICITY AND SLEEP. **Journal of Clinical Investigation,** v. 88, n. 3, p. 934-942, Sep 1991. ISSN 0021-9738. Disponível em: < <Go to ISI>://WOS:A1991GD66900028 >.

[116] CALDER, P. C. et al. Dietary factors and low-grade inflammation in relation to overweight and obesity. **British Journal of Nutrition,** v. 106, p. S1-+, Dec 2011. ISSN 0007-1145. Disponível em: < <Go to ISI>://WOS:000298112400001 >.

[117] AHO, V. et al. Partial Sleep Restriction Activates Immune Response-Related Gene Expression Pathways: Experimental and Epidemiological Studies in Humans. **Plos One,** v. 8, n. 10, Oct 23 2013. ISSN 1932-6203. Disponível em: < <Go to ISI>://WOS:000326037000048 >.

[118] WISOR, J. P.; CLEGERN, W. C.; SCHMIDT, M. A. Toll-Like Receptor 4 Is a Regulator of Monocyte and Electroencephalographic Responses to Sleep Loss. **Sleep,** v. 34, n. 10, p. 1335-1345, Oct 1 2011. ISSN 0161-8105. Disponível em: < <Go to ISI>://WOS:000295624800010 >.

[119] SVATIKOVA, A. et al. Serum amyloid A in obstructive sleep apnea. **Circulation,** v. 108, n. 12, p. 1451-1454, Sep 23 2003. ISSN 0009-7322. Disponível em: < <Go to ISI>://WOS:000185467400008 >.

[120] POITOU, C. et al. Serum amyloid a and obstructive sleep apnea syndrome before and after surgically-induced weight loss in morbidly obese subjects. **Obesity Surgery,** v. 16, n. 11, p. 1475-1481, Nov 2006. ISSN 0960-8923. Disponível em: < <Go to ISI>://000241926700013 >.

[121] KHEIRANDISH-GOZAL, L. et al. Lipopolysaccharide-Binding Protein Plasma Levels in Children: Effects of Obstructive Sleep Apnea and Obesity. **Journal of Clinical Endocrinology & Metabolism,** v. 99, n. 2, p. 656-663, Feb 2014. ISSN 0021-972X. Disponível em: < <Go to ISI>://WOS:000333460300067 >.

ATTACHMENTS

UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas
Secretaria de Pós-Graduação

Informações para os Membros de Bancas Julgadoras de Mestrado/Doutorado

1. O candidato fará uma apresentação oral do seu trabalho, com duração máxima de trinta minutos.

2. Os membros da banca farão a argüição oral. Cada examinador disporá, no máximo, de trinta minutos para argüir o candidato, exclusivamente sobre o tema do trabalho apresentado, e o candidato disporá de trinta minutos para sua resposta.

2.1 Com a devida anuência das partes (examinador e candidato), é facultada a argüição na forma de diálogo em até sessenta minutos por examinador.

3. A sessão de defesa será aberta ao público.

4. Terminada a argüição por todos os membros da banca, a mesma se reunirá reservadamente e expressará na ata (relatório de defesa) a aprovação ou reprovação do candidato, baseando-se no trabalho escrito e na argüição.

4.1 Caso algum membro da banca reprove o candidato, a Comissão Julgadora deverá emitir um parecer a ser escrito em campo exclusivamente indicado na ata.

4.2 Será considerado aprovado o aluno que obtiver aprovação por unanimidade ou pela maioria da banca.

5. Dúvidas poderão ser esclarecidas junto à Secretaria de Pós-Graduação: pgfarma@usp.br, (11) 3091 3621.

São Paulo, 23 de maio de 2014.

Prof. Dr. Adalberto Pessoa Junior
Presidente da CPG/FCF/USP

Av. Prof. Lineu Prestes, 580, Bloco 13 A - Cidade Universitária - CEP 05508-900 - São Paulo - SP
Fone: (11) 3091 3621 - Fax (11) 3091 3141 – e-mail: pgfarma@usp.br

UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Comissão de Ética no Uso de Animais - CEUA

Ofício CEUA/FCF/06/2011

CERTIFICADO

A Comissão de Ética no Uso de Animais da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo Certifica que o Projeto "**"A inflamação aguda na gênese da obesidade: modelo experimental com foco na amilóide sérica A (SAA) como marcador inflamatório e de hipertrofia do tecido adiposo".**" (Protocolo CEUA/FCF/297), de responsabilidade **Profa. Dra. Ana Campa**, está de acordo com as normas do Conselho Nacional de Controle de Experimentação Animal - CONCEA e foi APROVADO em reunião de 7 de fevereiro de 2011.

São Paulo, 7 de fevereiro de 2011.

Prof. Dr. Marco Antonio Stephano
Coordenador da Comissão de Ética no Uso de Animais
CEUA/FCF/USP

Av. Prof. Lineu Prestes, 580 - Bloco 13 A - Cidade Universitária - CEP 05508-900 - São Paulo - SP
Fone: (11) 3091-3622 / Fax: (11) 3091-3677 - e-mail: ceuafcf@usp.br

UNIVERSIDADE FEDERAL DE SÃO PAULO

HOSPITAL SÃO PAULO

FORMULÁRIO PARA APRESENTAÇÃO DE DOCUMENTOS (EMENDAS/ATUALIZAÇÕES/COMUNICADOS/ETC)

CEP Nº **0474/09** **CONEP Nº**

Título do Protocolo:

Avaliação bioquimica e molecular de Receptores Ativados por Proteases (PARs) em modelos de privação de sono'

Nome Pesquisador Principal: Jair Ribeiro Chagas

TIPO DE DOCUMENTO:

Ementa

1. Quais fatores influenciaram na emissão deste documento?

A possibilidade de uma relação entre os fatores inflamatórios inicialmente avaliados, os receptores ativados por proteases (PARs) e a proteina amilóide sérica A (SAA) uma importante proteína marcadora de fase aguda da inflamação, cuja avaliação não foi proposta no projeto original.

2. O que se propõe no documento apresentado?

Solicitar autorização para o uso de mais 40 animais experimentais para avaliação paralela, nos mesmos animais, das concentrações plasmáticas e teciduais de SAA.

3. Qual a opinião do pesquisador principal em relação ao(s) documento(s) apresentados?

Recentemente tem-se demonstrado que a SAA está envolvida em vários quadros com características inflamatórias como diabetes, obesidade e disfunções imunes, muito semelhante às consequencias da privação de sono. Ao lado dos receptores PAR a SAA pode ser um importante mediador nesses processos na privação de sono. Parece-nos que as informações obtidas no experimento proposto serão relevantes para esclarecimento do papel da SAA e sua complementaridade aos PARs nas condições de privação de sono.

Declaro estar ciente e de acordo com as informações presentes neste formulário

Assinatura do Pesquisador Principal: **Jair Ribeiro Chagas** Data___/___/___

São Paulo, 20 de março de 2013

CEP Nº **0474/09**

CONEP Nº:

Ilmo(a) Sr(a)

Pesquisador(a): JAIR RIBEIRO CHAGAS

Disciplina/Departamento: Medicina e Biologia do Sono

Título do estudo: Avaliação bioquimica e molecular de receptores ativados por proteases (PARs) em modelos de privação de sono

Prezado(a) Pesquisador(a),

O Comitê de Ética em Pesquisa da Universidade Federal de São Paulo/Hospital São Paulo ANALISOU E APROVOU o(a) Emenda 1 (versão 15/mar/2013) Solicitação de autorização para o uso de mais 40 animais. do projeto de pesquisa acima referenciado.

Atenciosamente,

Prof. Dr. José Osmar Medina Pestana
Coordenador do Comitê de Ética em Pesquisa da
Universidade Federal de São Paulo/Hospital São Paulo

Edson Mendes de Oliveira
Curriculum Vitae

Personal Information

Full name Edson Mendes de Oliveira

Date of birth 10 February 1985

Name used in Bibliographic Citations OLIVEIRA, E. M.; de Oliveira, E M

Professional Address Clinical Chemistry and Toxicology Department, Faculty of Pharmaceutical Sciences, University of Sao Paulo (USP)
Avenida Professor Lineu Prestes, 580
Butantã - Sao Paulo
05508-900, SP - Brazil
Phone number: 11 30913741

e-Mail
contact e-mail : edsonmendes@usp.br
alternative e-mail : edson.fbq@gmail.com

Formal Education

2011 Doctorate in Pharmacy.
University of Sao Paulo, USP, Sao Paulo, Brazil
with *Sandwich Doctorate* in University of Kentucky, KY, United States of America (Advisor : Professor Maria C de Beer)
Title: New insights into the role of serum amyloid A (SAA) on obesity and insulin resistance.
Advisor: Professor Ana Campa
Scholarship from : Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP)
Keywords: SAA, acute inflammation, obesity, serum amyloid A,adipose tissue
Knowledge areas : Biochemistry, Adipose Tissue Fisiology, Cell biology.

2009 - 2011 Master's in Pharmacy.
University of Sao Paulo, USP, Sao Paulo, Brazil
Title: Serum amyloid A (SAA): pruduction of recombinant human protein SAA1 and SAA4 and its native expression. On adipose tissue cells submitted to hypoxia.
Year of degree: 2011
Advisor: Ana Campa
Scholarship from : Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)
Keywords: adipocytes, SAA, serum amyloid A,adipose tissu, hypoxia, recombinant protein, SAA1, SAA4
Knowledge areas: Biochemistry, Adipose Tissue Fisiology, Cell biology.

2004 - 2008 Bachelor's in Pharmacy and Biochemistry.
Universidade Estadual de Londrina, UEL, Londrina, Brazil

Professional Experience

1. Universidade de São Paulo - USP

Contract institutional

2011 - Current Contract: Student - Doctorate in Pharmacy.
Working hours (weekly): 40, Schemes of job: Full-time and exclusiveness
2009 - 2011 Contract: Student – Master´s in Pharmacy
Working hours (weekly): 40, Schemes of job: Full-time and exclusiveness

Activities

2011 - Current Research Projects, Clinical Chemistry and Toxicology Department, Faculty of Pharmaceutical Sciences

05/2010 - Current Councils, Comissions and Consulting, Clinical Chemistry and Toxicology Department, Faculty of Pharmaceutical Sciences, University of Sao Paulo (USP)
Specification:
Student representative of the Committee on Post-Graduate Program

06/2012 - 12/2012 Trainee Job, Clinical Chemistry and Toxicology Department, Faculty of Pharmaceutical Sciences, University of Sao Paulo (USP)
Trainee:
Internship in Teaching. Discipline Clinical Citologyy. Course in Pharmacy and Biochemistry

02/2010 - 06/2010 Trainee Job, Clinical Chemistry and Toxicology Department, Faculty of Pharmaceutical Sciences, University of Sao Paulo (USP)
Trainee:
Internship in Teaching. Discipline Clinical Biochemistry. Course in Pharmacy and Biochemistry

06/2009 - 12/2009 Trainee Job, Clinical Chemistry and Toxicology Department, Faculty of Pharmaceutical Sciences, University of Sao Paulo (USP)
Trainee:
Internship in Teaching. Discipline Clinical Citologyy. Course in Pharmacy and Biochemistry

Languages

English Understanding Fluent , Speaking Fluent, Writing Fluent, Reading Fluent

Español Understanding Functional , Speaking Basic, Writing Functional, Reading Functional

Portuguese Understanding Fluent , Speaking Fluent, Writing Fluent, Reading Fluent

Scientific Journal Referee

1. **2013 - Current** **Food and Chemical Toxicology**
2. **2013 - Current** **Brazilian Journal of Microbiology**
3. **2013 - Current** **Revista Brasileira de Pesquisa em Saúde**

4. **2014 - Current** **Neuropsychiatric Disease and Treatment**

5. **2014 - Current** **The International Journal of Chronic Obstructive Pulmonary Disease**

Awards

1. **2014 -** IASO Travelling Fellowship Award, International Association for the Study of Obesity.

2. **2014 -** Best Oral Presentation (Doctorate level). SUPFAB, Faculdade de Ciências Farmacêuticas - Universidade de São Paulo.

S, T & A Production

Bibliographic Production
Articles Published in Scientific Journals

1. DE OLIVEIRA, EDSON MENDES ; VISNIAUSKAS, BRUNA ; SANDRI, SILVANA ; MIGLIORINI, SILENE ; ANDERSEN, MONICA LEVY ; TUFIK, SERGIO ; FOCK, RICARDO AMBRÓSIO ; CHAGAS, JAIR RIBEIRO ; CAMPA, ANA . **Late effects of sleep restriction: Potentiating weight gain and insulin resistance arising from a high-fat diet in mice**. Obesity (Silver Spring, Md.), v. 23, p. 391-398, 2015.

2. TIAGO, MANOELA, **DE OLIVEIRA, EDSON MENDES**, BROHEM, CARLA ABDO, PENNACCHI, PAULA COMUNE, PAES, RAFAEL DUARTE, HAGA, RAQUEL BRANDÃO, CAMPA, ANA, BARROS, SILVIA BERLANGA DE MORAES, SMALLEY, KEIRAN S., MARIA-ENGLER, SILVYA STUCHI. **Fibroblasts Protect Melanoma Cells from the Cytotoxic Effects of Doxorubicin**. Tissue Engineering. Part A., v. 20, p.2412-2421, 2014.

3. GALDINO, A. G. S., **OLIVEIRA, E. M.**, FILIPPIN-MONTEIRO, F. B., ZAVAGLIA, C. A. C.. **Análise de ensaios in vitro do compósito de 50% HA-50% TiO2 fabricados pelo método da esponja polimérica**. Cerâmica (São Paulo. Impresso), v. 60, p. 586-593, 2014.

4. SANDRI, SILVANA, URBAN BORBELY, ALEXANDRE, FERNANDES, ISABELLA, **MENDES DE OLIVEIRA, EDSON**, KNEBEL, FRANCIELE HINTERHOLZ, RUANO, RODRIGO, ZUGAIB, MARCELO, FILIPPIN-MONTEIRO, FABIOLA, BEVILACQUA, ESTELA, CAMPA, ANA. **Serum Amyloid A in the Placenta and Its Role in Trophoblast Invasion**. Plos One. , v.9, p.e90881 - 2014.

5. GALVÃO BARROS, JANAINA ALINE ; FILIPPIN-MONTEIRO, FABIOLA BRANCO ; **DE OLIVEIRA, EDSON MENDES** ; CAMPA, ANA ; CATALANI, LUIZ HENRIQUE ; DE NOGUEIRA MORAES PITOMBO, RONALDO ; POLAKIEWICZ, BRONISLAW . **Cytotoxicity of PVPAC-treated bovine pericardium: A potential replacement for glutaraldehyde in biological heart valves**. Journal of Biomedical Materials Research. Part B, Applied Biomaterials, v. 102, p. 574-582, 2014.

6. OLIVEIRA, EDSON MENDES, SANDRI, SILVANA, KNEBEL, FRANCIELE HINTERHOLZ, CONTESINI, CAROLINE GARCIA IGLESIAS, CAMPA, ANA, FILIPPIN-MONTEIRO, FABÍOLA BRANCO. **Hypoxia Increases Serum Amyloid A3 (SAA3) in Differentiated 3T3-L1 Adipocytes.** Inflammation. , v.36, p.1107 - 1110, 2013.

7. MORENO, ANA C. R., CLARA, RENAN O., COIMBRA, JANINE B., JÚLIO, ARIANE R., ALBUQUERQUE, RENATA C., **OLIVEIRA, EDSON M.**, MARIA-ENGLER, SILVYA S., CAMPA, ANA. **The expanding roles of 1-methyl-tryptophan (1-MT): In addition to inhibiting kynurenine production, 1-MT activates the synthesis of melatonin in skin cells.** The FEBS Journal (Print). , v.280, p.4782 - 4792, 2013.

8. TOURINO, MELISSA CAVALHEIRO, **DE OLIVEIRA, EDSON MENDES**, BELLÉ, LUZIANE POTRICH, KNEBEL, FRANCIELE HINTERHOLZ, ALBUQUERQUE, RENATA CHAVES, DÖRR, FELIPE AUGUSTO, OKADA, SABRINA SAYORI, MIGLIORINI, SILENE, SOARES, IRENE SILVA, CAMPA, ANA. **Tryptamine and dimethyltryptamine inhibit indoleamine 2,3 dioxygenase and increase the tumor-reactive effect of peripheral blood mononuclear cells.** Cell Biochemistry and Function. , v.31, p.361 - 364, 2013.

9. Filippin-Monteiro, F B, **de Oliveira, E M**, Sandri, S, Knebel, F H, ALBUQUERQUE, R. C., Campa, A. **Serum amyloid A is a growth factor for 3T3-L1 adipocytes, inhibits differentiation and promotes insulin resistance.** International Journal of Obesity. , v.1, p.1 - 8, 2012.

Articles published in annals of international events (Abstracts)

1. **DE OLIVEIRA, EDSON MENDES**, VISNIAUSKAS, B., SANDRI, SILVANA, MIGLIORINI, SILENE, ANDERSEN, M., TUFIK, S., FOCK, R. A., CHAGAS, J. R., CAMPA, ANA A History Of Sleep Restriction Promotes Future Weight Gain And Insulin Resistance In: 12th International Congress on Obesity (ICO 2014), 2014, Kuala Lumpur. **Obesity Reviews**. Oxford: Wiley, 2014. v.15. p.126 – 126

2. **OLIVEIRA, E. M.**, VISNIAUSKAS, B., SANDRI, S., Migliorini, S., ANDERSEN, M., TUFIK, S., FOCK, R. A., CHAGAS, J. R., CAMPA, A. A history of sleep restriction promotes future weight gain and insulin resistance In: Keystone Symposia - Obesity: A multisystems perspective, 2014, Vancouver, BC - Canada. **Keystone Symposia - Obesity: A multisystems perspective**. , 2014.

3. **OLIVEIRA, E. M.**, WONG, P., ALBUQUERQUE, R. C., de BEER, M. C., FOCK, R. A., CAMPA, A. Acute inflammation induces obesity in mice: possible role for serum amyloid A (SAA) In: Keystone Symposia - Adipose Tissue Biology, 2013, Keystone, CO. U.S.A.. **Keystone Symposia - Adipose Tissue Biology**. , 2013.

4. **OLIVEIRA, E. M.**, WONG, P., SANDRI, S., Migliorini, S., ALBUQUERQUE, R. C., de BEER, M. C., FOCK, R. A., CAMPA, A. Acute inflammation induces obesity in mice: possible role for serum amyloid A (SAA) In: 11th World Congress on inflammation, 2013, Natal - RN - Brazil. **11th World Congress on inflammation**. , 2013.

5. ORSATI, R. C., COIMBRA, JANINE B., JULIO, A. R., ALBUQUERQUE, R. C., **OLIVEIRA, EDSON M.**, MARIA-ENGLER, S. S, CAMPA, A., MORENO, A. C. R. The expanding roles of 1-methyl-tryptophan (1-MT): In addition to inhibiting kynurenine production, 1-MT activates melatonin synthesis in skin cells In: Cancer and Metabolism, 2013, Amsterdam. **Cancer and Metabolism**. , 2013.

6. **OLIVEIRA, EDSON M.**, Tourino, M. C., Belle, L. P., KNEBEL, F. H., ALBUQUERQUE, R. C., DORR, F. A., OKADA, S. S., Migliorini, S., SOARES, I. S., CAMPA, A. Regulation of the kynurenine pathway by tryptamines: directing tryptophan metabolism and impacting tumor growth In: International Congress Natural Anticancer Drugs, 2012, Olomouc - Czech Republic. **Biomedical Papers**. , 2012.

7. TIAGO, M., **OLIVEIRA, E. M.**, CAMPA, A., MARIA-ENGLER, S. S The protective role of the melanoma microenvironment in the response to chemotherapy and target therapy In: Society for Melanoma Research Congress, 2012, Hollywood. **Pigment Cell and melanoma research**. , 2012. v.23. p.892 – 892

8. **OLIVEIRA, E. M**., FILIPPIN-MONTEIRO, F. B., SANDRI, S., KNEBEL, F. H., CONTESINI, C. G. I., KAMAMOTO, F., CAMPA, A.
Hypoxia increases the expression of SAA in differentiated 3T3-L1 and human adipocytes In: 10th World Congress on Inflammation, 2011, Paris.
Inflammation Research. Springer, 2011. v.60. p.S1 - S321

9. SANDRI, S., **OLIVEIRA, E. M**., KNEBEL, F. H., CONTESINI, C. G. I., BEVILACQUA, E., CAMPA, A.
Serum amyloid A in placental villous explants: effect of hypoxia In: 10th World Congress on Inflammation, 2011, Paris.
Inflammation Research. Springer, 2011. v.60. p.S1 - S321

10. MORENO, A. C. R., ALBUQUERQUE, R. C., ORSATI, R. C., **OLIVEIRA, E. M.**, MARIA-ENGLER, S. S, CAMPA, A.
1-Methyl-Tryptophan inhibited the expression of IL-8, HLA-ABC and HLA-DR in the human melanoma SK-MEL-147 independently of IDO activity In: XLVI Semana Universitária Paulista de Farmácia e Bioquímica (SUPFAB), 2011, São Paulo.
Brazilian Journal of Pharmaceutical Sciences (Impresso). São Paulo: Faculdade de Ciências Farmacêuticas, 2011. v.47.

11. FILIPPIN-MONTEIRO, F. B., OLIVEIRA, E. M., IACOVELLA, T., ALBUQUERQUE, R. C., CAMPA, A.
Poster Presentations In: 11th International Congress on Obesity, 2010, Estocolmo.
Obesity Reviews. , 2010. v.11. p.121 – 121

12. FILIPPIN-MONTEIRO, F. B., OLIVEIRA, E. M., IACOVELLA, T., ALBUQUERQUE, R. C., CAMPA, A.
3T3-L1 AdipocyteS Biology Is Affected By Serum Amyloid A (SAA): Effects on Cell Cycle, Proliferation and Death In: Keystone Symposia, 2010, Keystone CO.
Adipose Tissue Biology. , 2010. p.138 – 138

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Graduação:	Farmacêutico - Universidade Estadual de Londrina - Paraná - Brasil - 2007
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Curso:	Doutorado
Programa:	Farmácia (Análises Clínicas)
Área:	Análises Clínicas
Data de Matrícula:	02/03/2011
Início da Contagem de Prazo:	02/03/2011
Data Limite para o Depósito:	02/03/2015
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Proficiência em Línguas:	Inglês, Aprovado em 02/03/2011
Data de Aprovação no Exame de Qualificação:	Aprovado em 11/04/2013
Data do Depósito do Trabalho:	
Título do Trabalho:	
Data Máxima para Aprovação da Banca:	
Data de Aprovação da Banca:	
Data Máxima para Defesa:	
Data da Defesa:	
Resultado da Defesa:	
Histórico de Ocorrências:	Ingressou no Doutorado em 02/03/2011 Mudança de Norma em 18/08/2014 Mudança de Regulamento em 18/08/2014 Matrícula de Acompanhamento em 03/02/2015

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 6542 em vigor a partir de 20/04/2013)
Última ocorrência: Matrícula de Acompanhamento em 03/02/2015
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Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
QBQ5751-6/1	Bioquímica Avançada (Instituto de Química - Universidade de São Paulo)	15/03/2011	09/06/2011	180	12	100	B	N	Concluída
FBC5722-2/1	Controle Hormonal da Resposta Inflamatória	02/08/2011	22/08/2011	60	4	100	A	N	Concluída
BMA5887-2/1	Princípios de Tomografia Computadorizada e Ressonância Magnética Aplicados à Anatomia (Instituto de Ciências Biomédicas - Universidade de São Paulo)	09/08/2011	17/10/2011	30	0	0	-	N	Matrícula cancelada
FBC5705-4/1	Tópicos em Microscopia Quantitativa	03/10/2011	09/10/2011	30	2	100	A	N	Concluída
MCM5891-1/3	Estatística Instrumental (Faculdade de Medicina - Universidade de São Paulo)	04/10/2011	31/10/2011	60	4	87,5	A	N	Concluída
QBQ5747-6/3	Animais de Laboratório (Instituto de Química - Universidade de São Paulo)	21/11/2011	30/11/2011	15	1	100	A	N	Concluída
BMH5745-3/3	Compartimentalização e Metabolismo de Lípides (Instituto de Ciências Biomédicas - Universidade de São Paulo)	03/04/2012	15/05/2012	60	4	95	A	N	Concluída
MCM5797-4/3	Obesidade (Faculdade de Medicina - Universidade de São Paulo)	11/06/2012	01/07/2012	60	4	95	A	N	Concluída
FBC5734-2/1	Aplicações da Citometria de Fluxo em Modelos Experimentais	06/08/2012	12/08/2012	30	2	100	A	N	Concluída
BMF5869-2/1	Processo Inflamatório em Doenças Cardiovasculares e Metabólicas (Instituto de Ciências Biomédicas - Universidade de São Paulo)	04/09/2013	05/11/2013	90	6	90	A	N	Concluída
Credito Externo	Biologia do Sono (1)	10/02/2014	24/03/2014	-	3	81	T	-	-
FBC5792-3/1	Tópicos em Análises Clínicas III	11/03/2014	23/06/2014	15	1	90	A	N	Concluída
Credito Externo	Medicina do Sono (1)	02/04/2014	07/05/2014	-	3	90	T	-	-
FBC5766-4/1	Tópicos em Análises Clínicas IV	05/08/2014	17/11/2014	15	1	100	A	N	Concluída
BIB5748-1/2	Sequenciamento de Próxima Geração (Next Generation Sequencing) (Instituto de Biociências - Universidade de São Paulo)	13/10/2014	19/10/2014	30	0	0	-	N	Matrícula cancelada

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	20	47
Estágios:			
Total:	0	20	47

Créditos Atribuídos à Tese: 167

Observações:

1) Disciplina(s) cursada(s) na(o) Universidade Federal de São Paulo. Atribuição de créditos aprovada pela Comissão Coordenadora do Programa, em Sessão de 30/09/2014.

Conceito a partir de 02/01/1997:

A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.

Um(1) crédito equivale a 15 horas de atividade programada.

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Comissão julgadora da tese de doutorado:

NUSP	Nome	Vínculo	Função
55700	Ana Campa	FCF - USP	Presidente

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