UNIVERSIDADE FEDERAL DO PARANÁ

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MODULATION OF *CHREBP* (*MLXIPL*) GENE EXPRESSION INDUCED BY WHEY PROTEIN CONSUMPTION IN MICE TREATED WITH HIGH REFINED CARBOHYDRATE-CONTAINING DIET

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Dissertação apresentada ao Programa de Pós-Graduação em Genética, Setor das Ciências Biológicas, Universidade Federal do Paraná, como requisito à obtenção do título de Mestre em Genética.

Orientadora: Profª. Dra. Lupe Furtado Alle
Co-orientador: Profª. Dra. Ana Claudia Bonnato
Co-orientador: Prof. Dr. Michel Fleith Otuki

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em GENÉTICA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **BRENO CESAR PERONI** intitulada: **Modulation in the expression of the chrebp (mlxipl) gene induced by whey protein consumption in mice treated with high refined carbohydrate-containing diet.**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 28 de Março de 2019.

LUPE FURTADO ALLE
Presidente da Banca Examinadora (UFPR)

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RESUMO

Objetivo: Os efeitos de uma dieta rica em carboidratos refinados (dieta HC), assim como a suplementação de *whey protein* isolado em uma dieta rica em carboidratos refinados (dieta HCW) e de uma dieta basal normocalórica e normoglicídica (dieta BD), na expressão de *Chrebpα* e *Chrebpβ* no tecido adiposo e fígado são pouco entendidos. O objetivo deste estudo foi avaliar se uma dieta rica em açúcar refinado com ou sem a adição de *whey protein* isolado, ou uma dieta basal são capazes de influenciar a saciedade, induzir mudanças na expressão de *Chrebp*, alterar a infiltração de células imunes no tecido adiposo e modular o desenvolvimento de esteatose hepática em camundongos.

Desenho e métodos: Camundongos machos de linhagem suíça (*Mus musculus*) foram divididos em três grupos com 10 animais em cada, e foram alimentados com três tipos diferentes de dietas: 1) dieta HC (grupo HC), 2) dieta HC mais whey protein isolado (grupo HCW) e 3) dieta basal (grupo BD), em um total de 12 semanas de experimento. Ao fim das 12 semanas, foram mensurados o consumo alimentar e de energia, a expressão de *Chrebpα* e *Chrebpβ* no fígado e no tecido adiposo, a atividade enzimática da mieloperoxidase (MPO) no tecido adiposo, e também a análise histológica do fígado.

Resultados: Observou-se que as dietas podem influenciar significativamente a expressão gênica em ambos os tecidos, e que, diferente do grupo HC, o grupo HCW foi protegido da esteatose hepática. *Chrebpβ* no fígado teve sua expressão influenciada pela atividade da MPO, provavelmente devido a mecanismos de inflamação sistêmica de baixo grau, gerados pelo aumento da adiposidade visceral. A expressão de *Chrebpβ* mostrou-se muito alta em relação a *Chrebpα* no fígado do grupo HC, um padrão que ocorre apenas em um ambiente rico em glicose, sinalizando uma potencial resistência à insulina nos tecidos periféricos.

Conclusões: As dietas foram capazes de influenciar a expressão de *Chrebp α* e *β*, tanto no tecido adiposo quanto no fígado. A dieta HC parece induzir um aumento na infiltração de células imunes no tecido adiposo, e o grupo de HCW foi protegido da esteatose hepática.

Palavras-chave: Fator de transcrição. Lipogênese de novo. Whey protein isolado.

ABSTRACT

Objective: The effects of a high refined carbohydrate-containing diet (HC diet), as well as the supplementation of whey protein isolate in a high refined carbohydrate-containing diet (HCW diet) and a basal normocaloric and normoglicid diet (BD diet), in the expression of *Chrebpα* and *Chrebpβ* in adipose tissue and liver are poorly understood. The objective of this study was to evaluate whether a high refined carbohydrate-containing diet with or without whey protein isolate supplementation, or a basal diet are able to influence satiety, induce changes in *Chrebp* expression, alter the infiltration of immune cells in adipose tissue and modulate the development of hepatic steatosis in mice.

Design and methods: Male *Mus musculus* Swiss lineage mice were divided into three groups with 10 animals each, and fed three different types of diets: 1) HC diet (HC group), 2) HC diet plus whey protein isolated (HCW group) and 3) basal diet (BD group), in a total of 12 weeks of experiment. At the end of the experiment, food and energy consumption, *Chrebpα* and *Chrebpβ* expression in liver and adipose tissue, myeloperoxidase enzymatic activity (MPO) in adipose tissue, as well as liver histological analysis were measured.

Results: It was observed that diets can strongly influence the gene expression in both tissues, and that the HCW group was protected from hepatic steatosis, but not HC group. *Chrebpβ* in the liver had its expression influenced by MPO activity, probably due to mechanisms of low-grade systemic inflammation generated by increased visceral adiposity. *Chrebpβ* expression was very high in relation to *Chrebpα* in the liver of the HC group, a pattern that occurs only in a glucose-rich environment, signaling a potential insulin resistance in the peripheral tissues.

Conclusions: Diets were able to influence the expression of *Chrebp α* and *β*, both in adipose tissue and in the liver. The HC diet appears to induce an increase in the infiltration of immune cells into adipose tissue, and the HCW group was protected from hepatic steatosis.

Key-words: Transcription factor. *De novo* lipogenesis. Whey protein isolate.

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LIST OF ACRONYMS

ChREBP - Carbohydrate Responsive Element Biding Protein Transcription Factor

ChREBP α - Carbohydrate Responsive Element Biding Protein Transcription Factor Alpha Isoform

ChREBP β - Carbohydrate Responsive Element Biding Protein Transcription Factor Beta Isoform

Chrebp - Carbohydrate Responsive Element Biding Protein Gene Expression

Chrebpα - Carbohydrate Responsive Element Biding Protein Alpha Gene Expression

Chrebpβ - Carbohydrate Responsive Element Biding Protein Beta Gene Expression

DNL - *De Novo* Lipogenesis

WAT - White Adipose Tissue

WPI - Whey Protein Isolate

WPH - Whey Portein Hydrolyzed

SUMMARY

1 INTRODUCTION

Obesity is a central risk factor for the development of various metabolic disorders, and may lead to comorbidities such as insulin resistance, systemic arterial hypertension (SAH) and dyslipidemias, a group of symptoms characteristic of the metabolic syndrome (MS) (MONDA et al., 2017).

It is known that many of the pathological characteristics of an individual are influenced by their genetic makeup, which can result in metabolic changes and generate differentiated nutritional requirements. To aid in this complex problem, several methods of molecular analysis have emerged to elucidate the interactions between genes and nutrients, how nutrition influences the regulation of metabolic pathways, and how this regulation affects the development of diseases (TUCKER et al., 2015).

One of the energy metabolism pathways is *de novo* lipogenesis (DNL), which generates lipids primarily from glucose. In obese individuals, DNL is deficient in white adipose tissue (WAT) because adipocytes are resistant to glucose uptake, mainly due to reduced glucose transporter 4 (*Glut4*) translocation from the cytoplasm to the cell membrane (TANG et al., 2016). In addition, liver DNL is increased in obese individuals, potentially generating fat accumulation and lipotoxicity. Interestingly, according to Cao et al. (2008), an increase in WAT DNL is capable of suppressing hepatic DNL.

DNL is mainly coordinated by the transcription factor ChREBP (carbohydrate responsive element binding protein), which binds in its DNA response element and activates the expression of genes that favor lipogenesis (NELSON et al., 2014).

The goal of this work was to investigate the modulation of *Chrebp α* and *β* expression in liver and adipose tissue, aiming to shed light upon a possible alternative pathway that leads to the increase of WAT DNL and insulin resistance in mice treated with high-refined carbohydrate-containing diet (HC), through the modulation of *Chrebp* expression, influenced by whey protein isolate (WPI) supplementation.

2 LITERATURE REVIEW

2.1 OBESITY AND ASSOCIATED COMORBITIES

The World Health Organization defines overweight and obesity as abnormal or excessive accumulation of fat that can harm health. The most commonly used measure for obesity, is the body mass index (BMI), which is defined as the weight in kilograms divided by the square of the person's height in meters (kg/m^2) (WHO, 2016). Although BMI is a benchmark and does not provide a direct measure of body fat content, many studies have shown that it correlates well with fat content in the vast majority of people. Adults with BMI between 25 and 30 are considered overweight, and those with BMI greater than or equal to 30 are considered obese (XIA e GRANT, 2013).

The world prevalence of obesity is high, with 39% of adults over 18 years considered overweight and 13% considered clinically obese (WHO, 2016). This is a major problem that affects both developed and developing countries.

In the Brazilian population, the weighted prevalence of abdominal obesity is 39.9%, and it reaches the highest percentage (72.6%) in the urban population of the state of São Paulo (VIGIDAL *et al*, 2013). According to a survey released by the Ministry of Health in April 2017, one in five people in Brazil is overweight and the prevalence of obesity increased from 11.8% in 2006 to 18.9% in 2016 (MINISTÉRIO DA SAÚDE, 2017).

According to Nelson et al. (2014) in the US population, 30% of adults are obese, and another 35% are overweight, according to the Body Mass Index (BMI). As reported by the Centers for Disease Control and Prevention (CDC), there has been a steady increase in adult obesity rates in the United States over the past two decades, from 19.4% in 1997 to 24.5% in 2004, 26,6% in 2007, 33.8% in 2008 and 35.7% in 2010 and child obesity exceeds 17% in the United States (XIA e GRANT, 2013).

Obesity can lead to a significant increase in the chance of developing comorbidities such as type 2 diabetes mellitus, cerebrovascular accident, heart attack, and some types of cancer (NELSON et al., 2014). In addition, weight gain and body fat accumulation, characteristic of obesity, coupled with individual predisposition to visceral fat (ectopic fat in the liver, pancreas and heart) accumulation may lead to

the development of metabolic syndrome (HAN e LEAN, 2016). The metabolic syndrome is characterized by a group of interrelated cardiovascular disease and diabetes risk factors, such as hyperglycemia (insulin resistance), systemic arterial hypertension, high levels of serum triacylglycerols, reduction in levels of HDL-cholesterol, and visceral obesity. Among these, hyperglycemia and visceral obesity are considered as key components for the development of the metabolic syndrome (VIDIGAL et al., 2013).

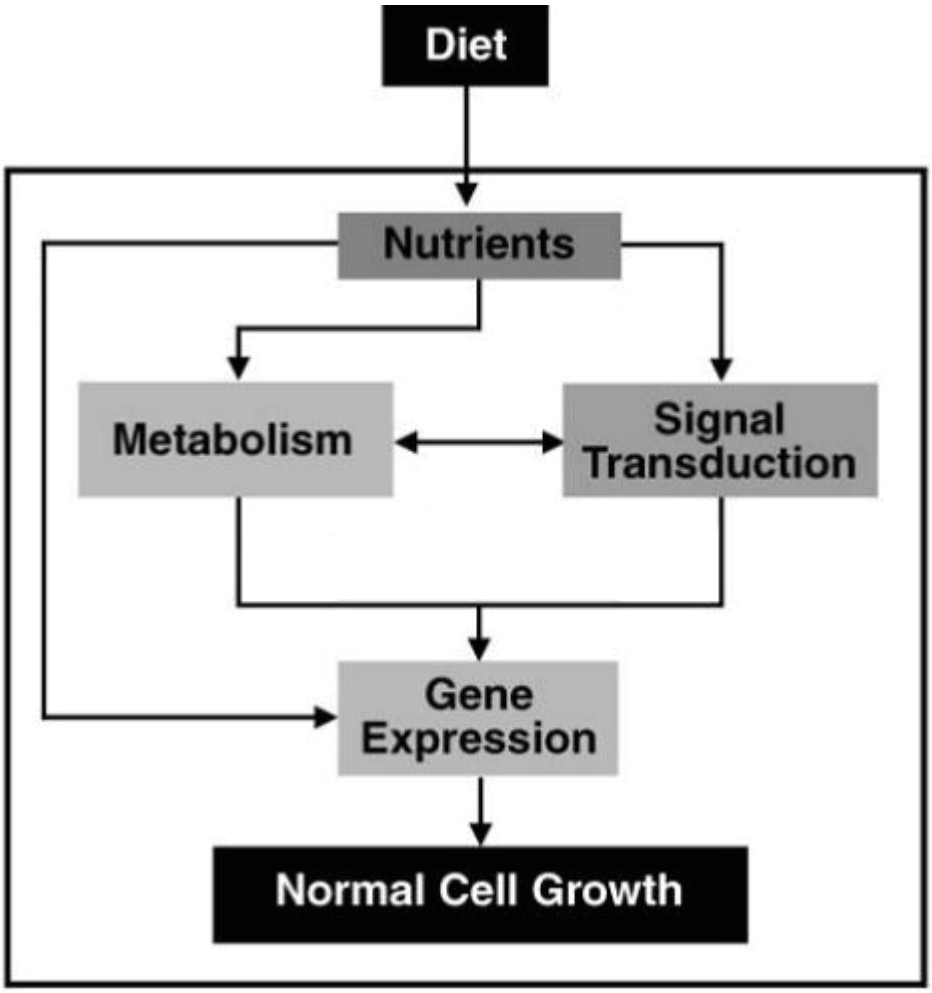
2.2 NUTRIGENOMICS

In view of metabolic changes, such as hyperglycemia (insulin resistance), systemic arterial hypertension, high levels of serum triacylglycerols, reduction in levels of HDL-cholesterol, and visceral obesity, some branches of science are concerned with developing efficient ways to avoid and/or reverse this situation, formulating strategies that lead, for example, to the improvement of tissue sensitivity to insulin, to the reestablishment of the normolipemic pattern (increase of HDL-c and reduction of triacylglycerols), and reduction of visceral obesity.

Among these branches is nutrigenomics, which is characterized by investigating an area of nutrition using molecular tools to understand the responses to certain diets or nutrients (PAVLIDIS et al., 2015). Nutrigenomics evaluates the impact of dietary components on the genome, epigenome, transcriptome, proteome, and metabolome (MEAD, 2007). It is an emerging field that attempts to understand the role of nutrition in gene expression, bringing together the science of bioinformatics, nutrition, molecular biology, genomics, epidemiology and molecular medicine (NEEHA e KINTH, 2013).

Nutrigenomics seeks to engender a molecular understanding of how nutrients (chemical molecules) affect health by altering gene expression. The fundamental concepts in this field of study are that the progression from a healthy to a chronic disease phenotype must occur through changes in gene expression, or through differences in protein and enzyme activities, and that nutrients directly or indirectly regulate these modifications. At the cellular level, nutrients may act as ligands for transcription factors, be metabolized and alter the concentrations of substrates and/or intermediates, and positively or negatively affect signaling pathways (FIGURE 01) (KAPUT and RODRIGUEZ, 2004).

Figure 1 - Schematic representation of the fate and activities of nutrients in the cell. Nutrients can act directly as ligands for transcription factors; can be metabolized by primary or secondary metabolic pathways, thus altering the concentrations of substrates or intermediates can be involved in gene regulation or signaling; or change the signal transduction and signaling pathways.



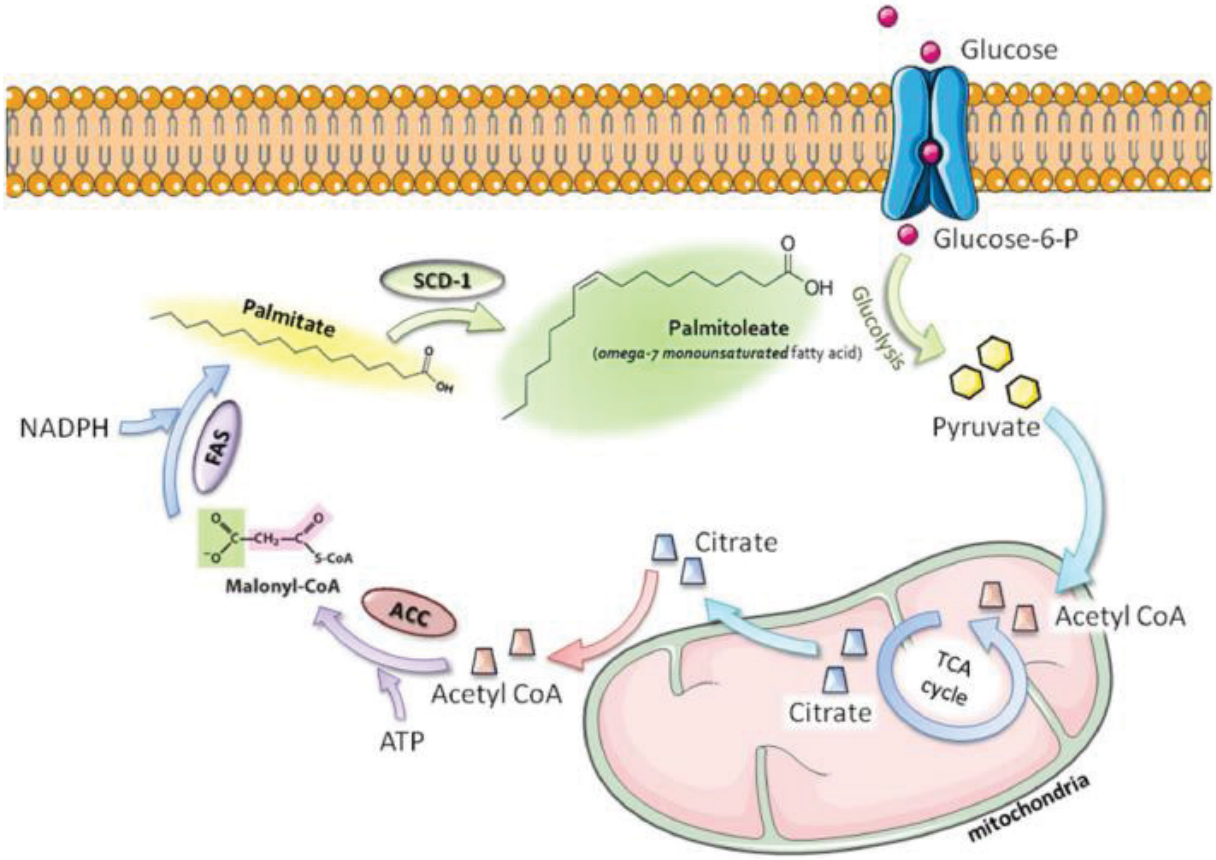
Source: adapted from Kaput and Rodriguez (2004).

2.3 *DE NOVO* LIPOGENESIS IN METABOLIC BALANCE

It has been demonstrated by Farese et al. (2012); Rosen and Spiegelman (2006); Virtue and Vidal-Puig (2010) that lipid metabolism in white adipose tissue (WAT) and liver contributes to metabolic homeostasis. One of the forms of regulation performed by these tissues is called *de novo* lipogenesis (DNL), which is characterized by the synthesis of fatty acids from non-lipid molecules. The WAT DNL includes uptake of glucose from the bloodstream and conversion of citrate (glucose derivative) into acetyl-CoA (by ATP-citrate lyase (ACL)), which is subsequently converted to malonyl-CoA (by acetyl-CoA carboxylase (ACC)), which in turn can be

converted to palmitate (by fatty acid synthase (FASN)), which may be modified by elongases and desaturases to produce other lipids (FIGURE 2) (TANG et al., 2016).

Figure 2. Schematic representation of white adipose tissue DNL and palmitoleic acid biosynthesis. Glucose-6-P, glucose-6-phosphate; TCA Cycle, tricarboxylic acid cycle; ACC, citrate lyase; FAS, fatty acid synthase, NADPH, nicotinamide adenine dinucleotide phosphate in reduced form; SCD-1, stearoyl-CoA desaturase.



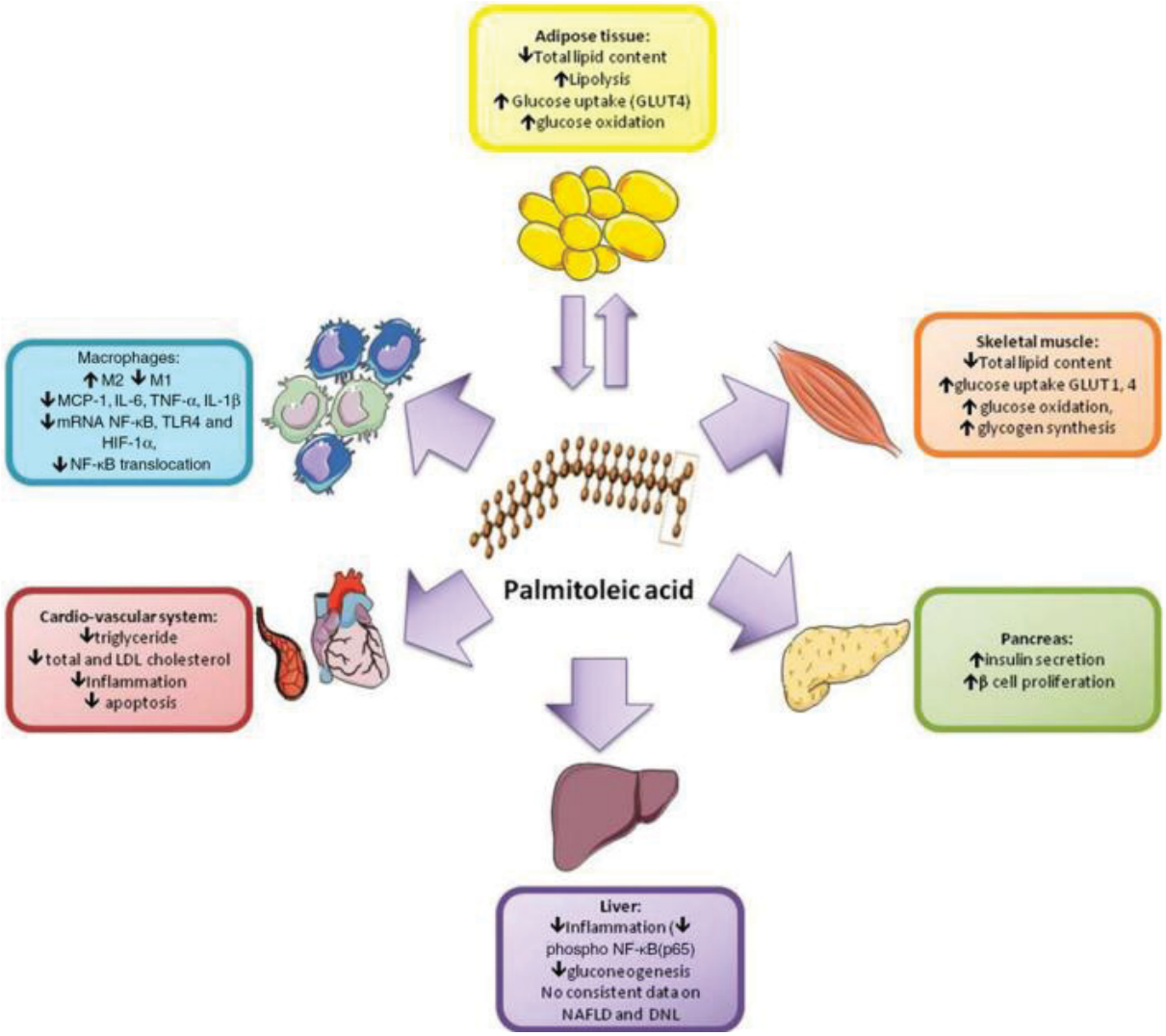
Source: Adapted from De Souza et al. (2018)

In rodent studies it was observed that in obese animals there was a reduction in WAT DNL and when DNL was restored in this tissue, there was a reversion in obesity induced insulin resistance (CAO et al., 2008 e HUO et al., 2012). In contrast, in obese rats and humans, DNL is increased in the liver, where it can cause lipotoxicity, insulin resistance, non-alcoholic fatty liver disease (NAFLD), and atherogenic dyslipidemia (POSTIC e GIRARD, 2008). Interestingly, there is evidence that increased WAT DNL can suppress hepatic DNL and, as a consequence, reduce liver fat accumulation (CAO et al., 2008).

Increased WAT DNL is associated with increased PPARγ (*Peroxisome Proliferator Activated Receptor γ*) activity, probably due to the formation of binding lipids, and is associated with improved insulin sensitivity and thermogenesis (WITTE

et al., 2015). Moreover, according to Souza et al. (2017), in mice fed with a high-fat diet, palmitoleic acid supplementation (a lipocin produced from WAT DNL) stimulated the uptake of glucose in liver through activation of AMPK (*AMP-activated protein kinase*) and FGF-21 (*Fibroblast growth factor 21*), dependent on PPARα (*Peroxisome Proliferator Activated Receptor α*), which are important molecules in the regulation of insulin sensitivity and energy metabolism. In contrast, the reduction of DNL in WAT impairs the synthesis of lipids, such as palmitoleic acid, which generate beneficial metabolic effects such as anti-inflammatory and improved insulin and glucose sensitivity (DE SOUZA *et al*, 2018) (FIGURE 3). In addition, when the obesity-induced condition of insulin resistance is established, WAT secretes molecules that antagonize the effects of insulin, such as retinol-4 binding protein (*RBP4*), tumor necrosis factor α (*TNF-α*), interleukin-6 (*IL-6*) and interleukin-1β (*IL-1β*), which promote systemic inflammation. Moreover, it reduces the secretion of molecules related to the improvement of insulin sensitivity, such as adiponectin (SMITH e KAHN, 2016).

Figure 3 - Schematic representation of Immune and metabolic effects of palmitoleic acid in different tissues. GLUT, glucose transporter; HIF, hypoxia inducible factor; IL, interleukin; LDL, low density lipoprotein; MCP, monocyte chemoattractant protein; NAFLD, nonalcoholic fatty liver disease; NF*κ*B, nuclear factor kappa b; TLR, toll-like receptor; TNF, tumor necrosis factor.



Source: De Souza et al. (2018)

2.4 *DE NOVO* LIPOGENESIS REGULATION

An important transcription factor that regulates DNL and carbohydrate metabolism, expressed primarily in the liver, adipose tissue and kidneys, is ChREBP (*Carbohydrate Responsive Element Binding Protein*) (NELSON et al., 2014). This transcription factor has leucine zipper and basic helix-loop-helix binding motifs. The proposed mechanism of functioning consists of its dephosphorylation in the cytosol, causing its entry into the nucleus, where it is dephosphorylated again (both reactions catalyzed by phosphoprotein-phosphatase 2A (*PP2A*)), to form heterodimers with the

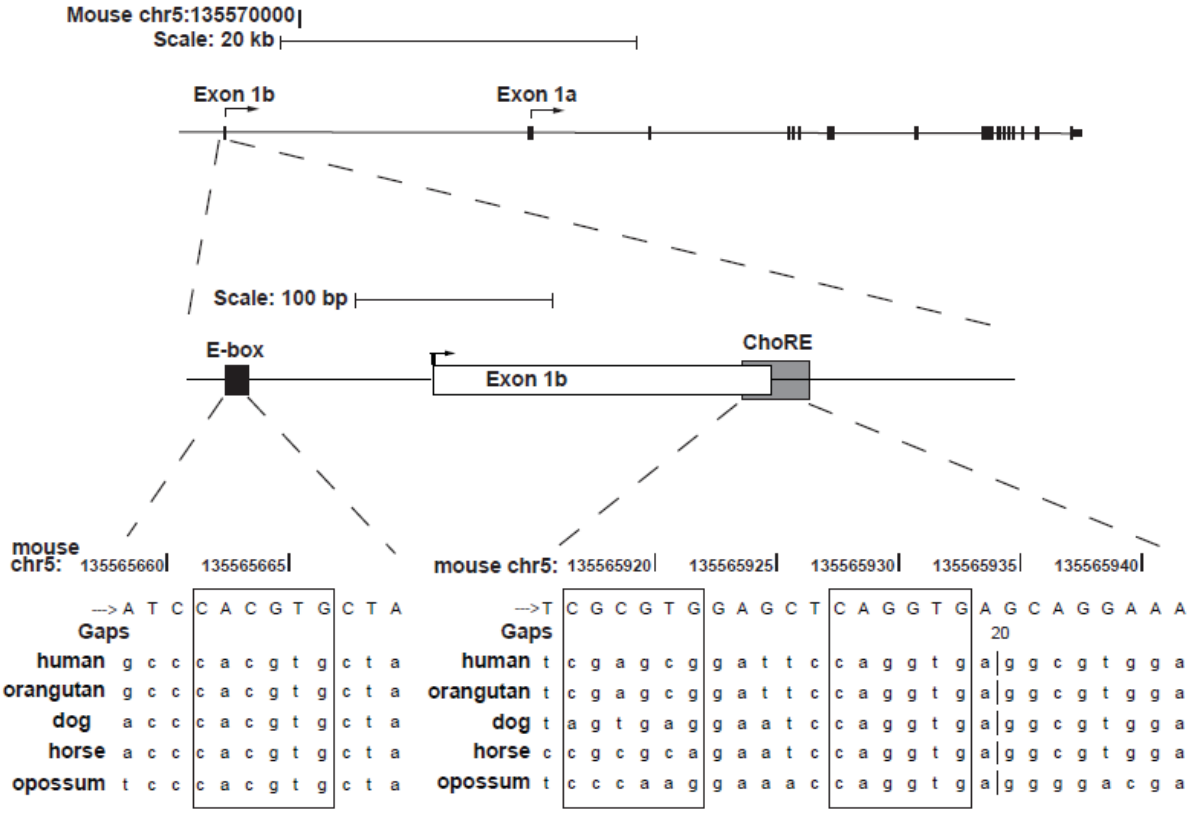
protein MLX (*Max-like protein X*), and then bind to the carbohydrate response element (*ChoRE*), which is composed of two E-boxes separated by five base pairs. The ChREBP/MLX heterodimer controls lipid and carbohydrate metabolism by regulating the expression of genes responsible for the coding of glycolytic, gluconeogenic and lipogenic enzymes, such as pyruvate kinase, the catalytic subunit of glucose-6-phosphatase, fatty acid synthase and acetyl-CoA carboxylase, and also by the translocation of glucose transporters 2 and 4 (*Glut2* and *Glut4*) (IIZUKA, 2013).

The gene encoding the ChREBP transcription factor was first described by Meng et al. (1998) and was best defined by De Luis et al. (2000), while investigating a chromosomal deletion characteristic of the Willians-Beuren syndrome, describing a gene located in the 7q11.23 region, designated by the authors of WBSCR14 (*Willians-Beuren Syndrome Chromosome Region 14*), with 33 kb, 17 exons and homology with a region of mice chromosome 5 (85% similarity with humans), with higher equivalences in the amino and carboxy terminal regions, where the main functional domains are located. Currently, the correct gene name is *MLXIPL* (*MLX-Interaciting Protein-Like*), due to the dependence of ChREBP on dimerizing with the MLX (Max-Like Factor X) protein in the cell nucleus to connect to *ChoRE* (FILHOULAUD et al., 2013). But, in the literature, it is most commonly named *ChREBP*.

There are two isoforms of ChREBP, which are called ChREBPα (found in the nucleus and in the cytosol) and ChREBPβ (found only in the nucleus) (IIZUKA, 2013). The ChREBPβ isoform was discovered by Herman et al. (2012) while investigating the mechanism by which the transcription factor modulates its own transcription. The authors found a *ChoRE* sequence at 17 kb upstream of the only known transcription start site of *Chrebp* (exon 1a) in mice (defined by two E-boxes (CACGTG) separated by five nucleotides), and an E-box of 255 base pairs upstream of this newly discovered *ChoRE*, suggesting an alternative promoter region and first exon (exon 1b) (FIGURE 3). The existence of mRNA's transcribed from this new promoter revealed the existence of a more compact isoform generated by alternative splicing (exon 1b to exon 2), removing exon 1a but including all the other 15 exons. However, the translational initiation site (ATG) of the alternative isoform is located in exon 4 (instead of the exon 1a removed in the splicing process), generating a

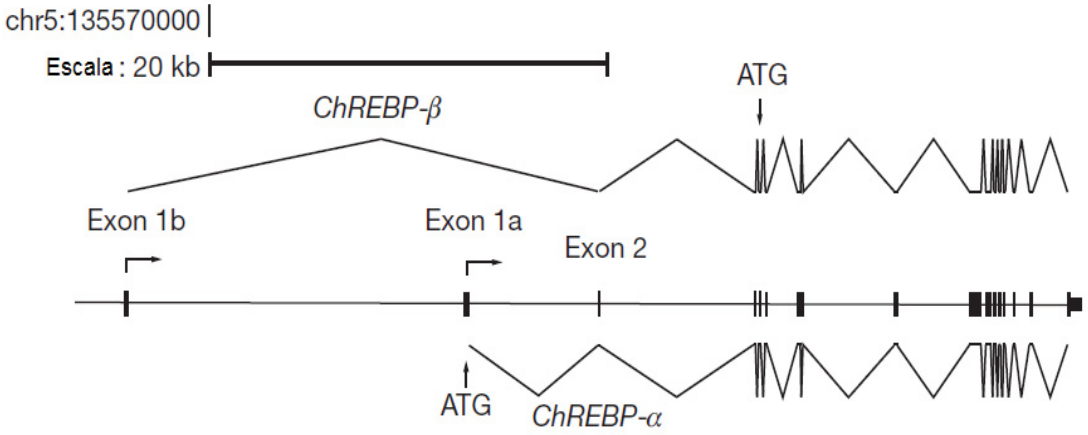
polypeptide of 687 amino acids (ChREBPβ), instead of 854 amino acids (ChREBPα) (FIGURE 4).

Figure 4 - E-box and ChoRE conserved in the MLXIPL gene.



Source: Herman, Mark A. et al. (2012).

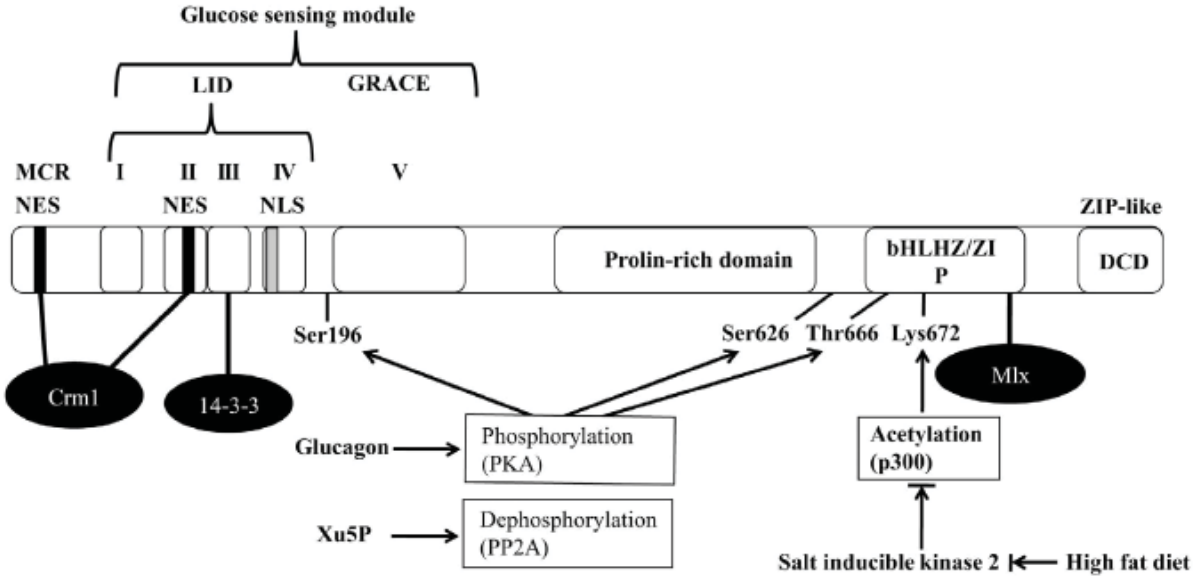
FIGURE 5 - ChREBPα and ChREBPβ genes structure model with indication of splice sites and translation start sites (ATG).



Source: Herman, Mark A. et al. (2012).

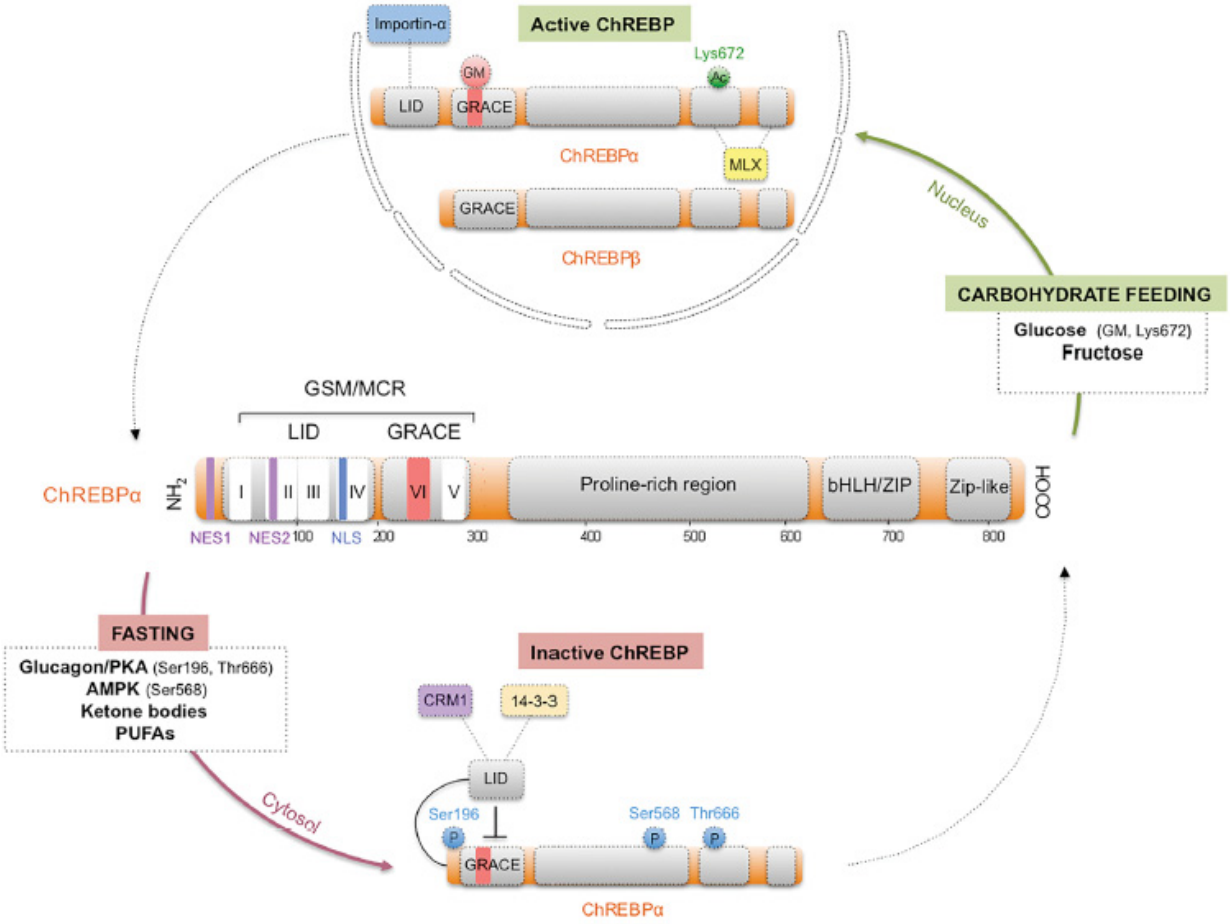
ChREBPα has a glucose detection module (GSM), which contains a low glucose inhibitory domain (LID) and a conserved glucose response element (GRACE). GSM has a conserved Mondo region (MCR), which is composed of five distinct subdomains, MCR I to V, among which MCR I to IV correspond to the LID region. ChREBPα also contains a nuclear localization signal (NLS) in MCR IV, and two nuclear export signals (NES1 and NES2) connected by the nuclear export factor (Crm1). MCR III is constitutively bound to a 14-3-3 protein, which is required for glucose response, and also contributes to the cytoplasmic localization of the transcription factor. ChREBP-β, on the other hand, has only the GRACE region (FIGURE 5). Therefore, the two isoforms perceive glucose concentrations through distinct mechanisms. ChREBPα isoform is regulated by insulin, hepatic X receptor and thyroid hormones (not by glucose), whereas ChREBPβ isoform is regulated directly by glucose concentration, and the two isoforms are regulated by different promoters (IIZUKA (2013). In addition, the expression of *Chrebpβ* requires the transactivation of *Chrebpα*, however, ChREBPβ isoform predominantly responds to changes in glucose flux mediated by Glut-4, and in adipose tissue is the most potent isoform in the stimulation of DNL. As a consequence, ChREBPβ is a potential target for the treatment and prevention of insulin resistance and obesity-associated comorbidities (HERMAN et al., 2012)**.** ChREBP protein structure and regulation upon nutritional and hormonal signals is shown in figure 7.

Figure 6 - Schematic representation of the ChREBPα protein. Serines 196 and 626 and threonine 666 are phosphorylation sites for the cAMP-dependent protein kinase (PKA), which inhibits the activity of ChREBPα. The same amino acids are dephosphorylation sites for the protein phosphatase 2A (PP2A), induced by xylulose-5-phosphate (intermediate molecule of the pentoses phosphate cycle), which allows the entry of the protein into the nucleus and binding to the response element a carbohydrates (chore). Lysine 672 is an acetylation site for the p300 coactivating histone acetyltransferase, which promotes the binding of ChREBPα to DNA. Acetylation by p300 is down regulated by salt-inducible kinase 2 (SIK2), which is activated under malnutrition conditions. SIK2 is inhibited by a lipid-rich diet. NES: nuclear export signal; NLS: nuclear localization signal; bHLHZ: leucine zipper and basic helix-loop-helix type binding motif. The ChREBPβ protein has only the GRACE region of the glucose detection module (GSM).



Source: Iizuka (2013).

Figure 7 - Schematic representation of ChREBP protein structure and its regulation upon nutritional and hormonal signals. Lys, lysine; Ser, serine; PKA, protein kinase A, AMPK, AMP dependent kinase; PUFAs, polyunsaturated fatty acids; Ac, acetylation; P, phosphorylation.



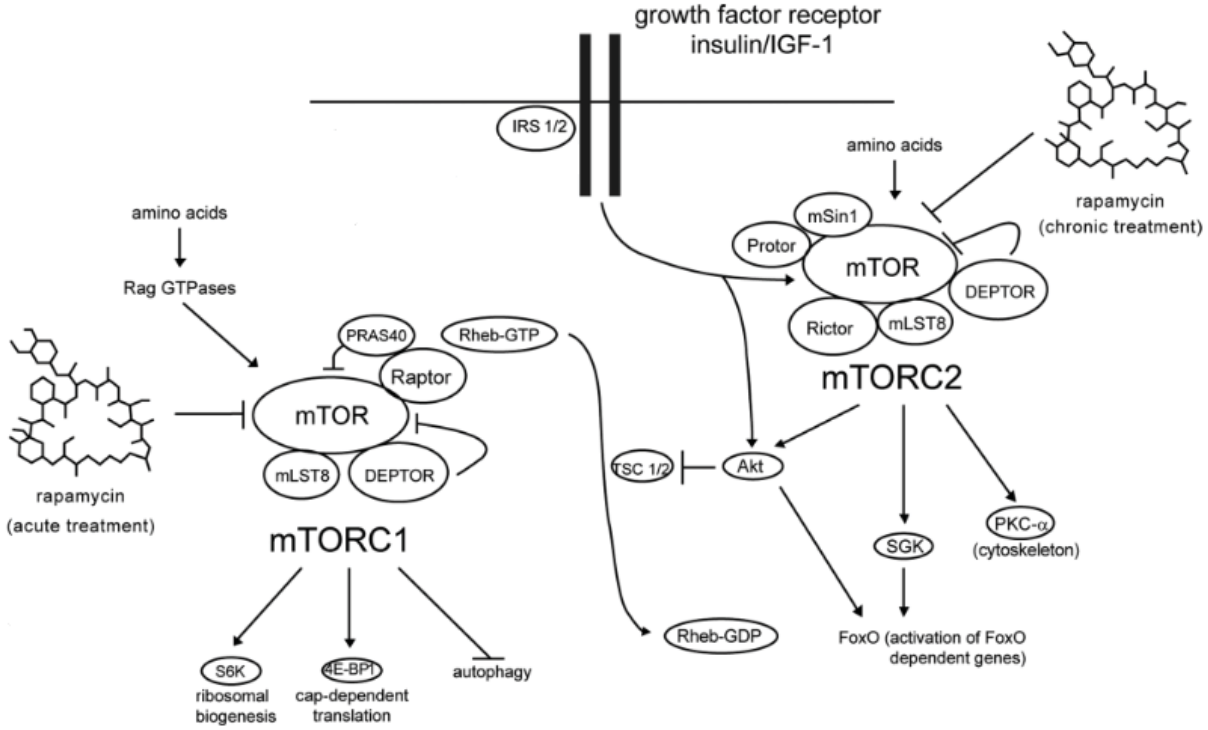
Source: Abdul-Wahed et al. (2017).

Classical insulin signaling for Glut-4 translocation into the adipocyte is initiated by phosphorylation of a tyrosine on the insulin receptor substrate (IRS-1), which activates a cascade of phosphorylation reactions beginning with the activation of phosphoinositide-3-kinase (PI3K) to convert phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) on the membrane. When bound to PIP3, protein kinase B (PKB, also called Akt, in this case the Akt2 isoform) is phosphorylated and activated by other protein kinases: mTORC2 and PDK1 (mTORC2 phosphorylates Akt at the hydrophobic sites (S473 in Akt1 and S474 in Akt2) while PDK1 phosphorylates akt in the kinase domains (T308 in Akt1 and T309 in Akt2). Akt2, in turn, phosphorylates its 160 kDa substrate (AS160) and by inhibiting it facilitates the movement of Glut4 from internal vesicles to the plasma membrane,

increasing the uptake of glucose by the cell (KLIP et al., 2014; NELSON et al., 2014 e TANG et al., 2016).

Mammalian Target of Rapamycin (mTOR) is a protein kinase with important intracellular signaling functions both in health and disease processes. mTOR is a large multi-domain protein and can bind to several proteins to form two distinct complexes called mTOR complexes 1 and 2 (mTORC1/2), which differ in protein components, substrate specificity, and regulation (WANG e PROUD, 2015). Both isoforms are stimulated by amino acids, hormones and growth factors (TATO et al., 2011). mTORC1 contains a protein called Raptor, which allows it to phosphorylate substrates such as the ribosomal proteins S6 kinases (S6Ks), and have its effect blocked by rapamycin. mTORC2 contains Rictor instead of Raptor, and phosphorylates a distinct set of substrates. This includes regulatory sites for a family of protein kinases such as Akt (FIGURE 6). Rapamycin does not directly inhibit mTORC2 function, but may impair it in long-term treatments (WANG e PROUD, 2015).

Figure 8 - Schematic representation of mTOR complexes 1 and 2. IRS 1/2, insulin receptor substrate 1/2; PRAS40, proline-rich Akt substrate of 40 kDa; mLST8, Target of rapamycin complex subunit LST8; S6K, Ribosomal protein S6 kinase; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; TSC 1/2, tuberous sclerosis complex 1/2; DEPTOR, DEP domain-containing mTOR-interacting protein; Raptor, regulatory-associated protein of mTOR; AKT, protein kinase B; Protor, rictor-binding component of mTOR complex-2; mSin1, mammalian SAPK interacting protein 1; FoxO, Forkhead box O; SGK, serine/threonine-protein kinase SGK; PKC-α, protein kinase c alpha; RICTOR, rapamycin-insensitive companion of mammalian target of rapamycin.



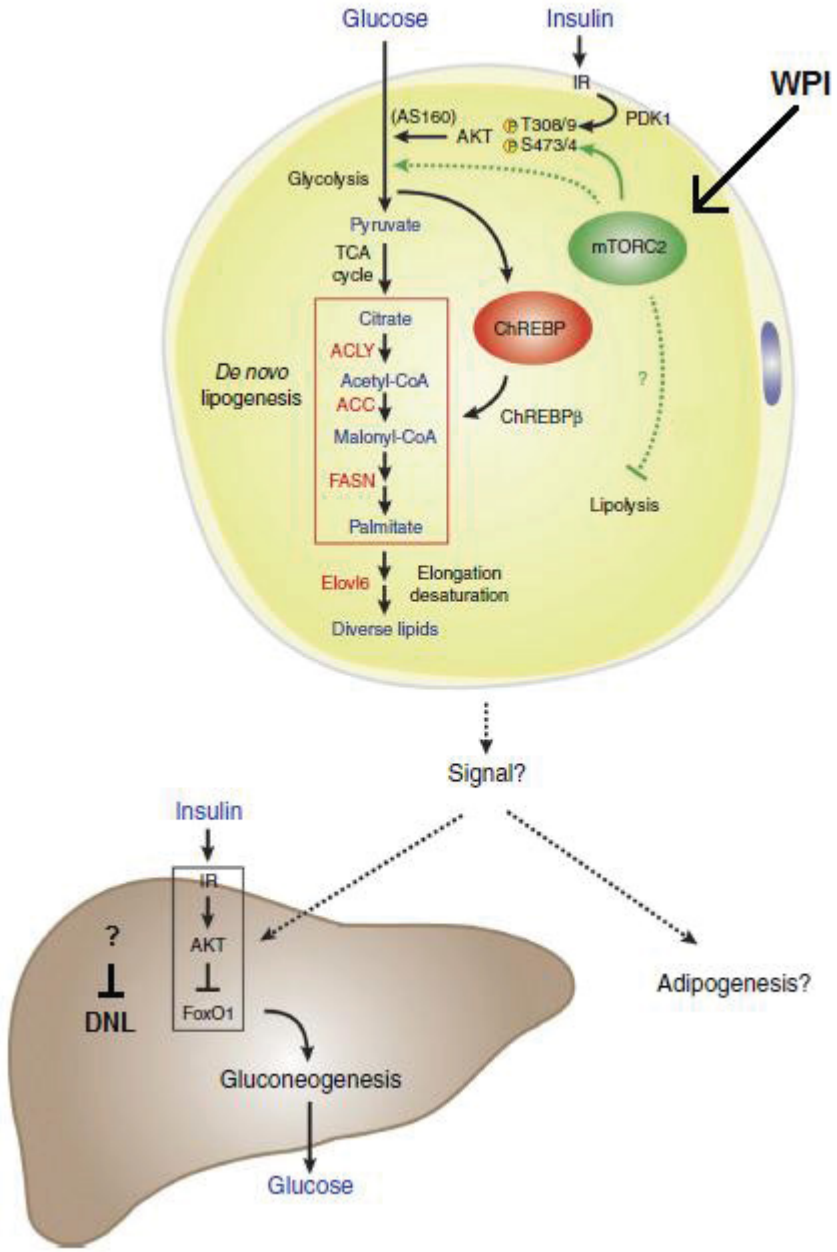
Source: Adapted from Lamming et al. (2012)

To better understanding mTORC2 role in DNL, Tang et al. (2016) generated Rictor protein absent white preadipocytes to analyze the acute consequences of mTORC2 inactivity on cell differentiation and function. Although they differentiated normally, when in a glucose-rich culture condition, Rictor-deficient cells (Rictor-iKO) failed to increase the expression of *Chrebpβ*, and also to increase the expression of lipogenic enzymes mRNA's (ACLY, ACC and FASN), in congruence with the reduction of DNL. In addition, Rictor-iKO cells showed higher insulin resistance, and lower ability to convert glucose to free fatty acids (FFA) and triacylglycerols (TAG). One of the proposed mechanisms is that, at least in part, mTORC2 promotes the expression of *Chrebpβ* and DNL by controlling the entry of glucose into the cell. Interestingly, phosphorylation of AS160 is normal in the Rictor-iKO adipocytes, apparently not interfering with the classical mechanism of translocation of Glut-4 to the cell membrane, suggesting there must be other mechanisms by which mTORC2

regulates DNL. Despite uncertainty about regulatory mechanisms, it is possible to infer that mTORC2 in the WAT, acts as an extrahepatic nutrient sensor that relays nutritional body status to the liver to control insulin sensitivity and glucose homeostasis, possibly through signaling performed by bioactive lipids resulting from DNL. Interestingly, it has been demonstrated by Tato et al. (2011) that *in vitro* cell stimulation with a mixture of essential amino acids, non-essential amino acids and L-glutamine, triggers the phosphorylation of Akt serine 473 via mTORC2/PI3K class I, revealing for the first time the stimulation of mTORC2 by amino acids. This result is in accordance with the experiment of Morato, Priscila e Neder et al. (2013), in which the consumption of whey protein hydrolysate (WPH) for 9 days in rats resulted in a greater translocation of Glut-4 to the cell membrane of muscle cells concomitantly with the significant increase in the phosphorylation of the Akt serine 473, with no change in serum concentration of insulin. In addition, the authors found insulin independent Glut-4 translocation, in agreement with Tang et al. (2016). The results also coincide with the findings of Morato et al. (2013), in which the dipeptide L-leucyl-isoleucine (apparently the most insulinogenic fraction of WPH) increased the phosphorylation of the Akt 473 serine, and L-isoleucine increased Glut-4 translocation to the rat sarcoplasmic membrane in an insulin-independent manner , reducing serum glucose.

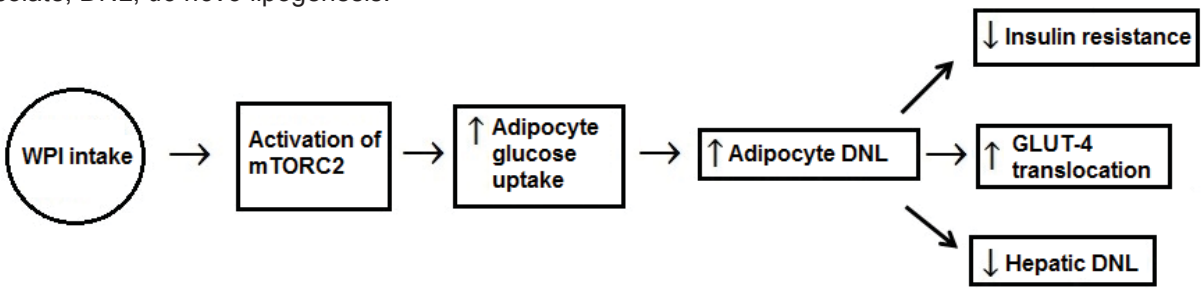
Considering these literature findings, we infer that the use of whey protein can modulate the activity of mTORC2, which controls the expression of *Chrebpβ* in the WAT, partly via regulation of Glut-4, increasing glucose uptake and positively modulating DNL in this tissue, thereby reducing glycemic levels and hepatic overload. This hypothesis is in agreement with Hamad et al. (2011), who observed oral administration of WPH, WPI and some peptides from whey protein resulted in reduced production of triglycerides in the liver and improved glycemia in rats with fatty liver disease induced by a high carbohydrate diet. Therefore, oral administration of WPI may help in the treatment of metabolic syndrome (FIGURE 7 and FIGURE 8).

Figure 9 - Schematic representation of the possible mechanism of regulation of *de novo* lipogenesis via WPI/mTORC2/ChREBPβ and its consequent metabolic effects. AS160, AKT subunit of 160 kDa; IR, insulin receptor; PDK1, phosphoinositide-dependent kinase 1; TCA cycle, tricarboxylic acid cycle; ACLY, ATP Citrate Lyase; ACC, acetyl-CoA carboxylase; FASN, fatty acid synthase; Elovl6, elongation of very long chain fatty acids protein 6; T308/9, threonine 308/9; S473/4, serine 473/4; P, phosphorilation.



Source: Adapted from Tang *et al* (2016).

Figure 10 - Simplified schematic representation of the possible mechanism of regulation of *de novo* lipogenesis via WPI/mTORC2/ChREBPβ and its metabolic effects. WPI, whey protein isolate; DNL, *de novo* lipogenesis.



Source: Present study.

3 JUSTIFICATION

Due to global increase in obesity and metabolic syndrome prevalence, research is needed to assist in the development of effective treatments that help reestablish homeostasis in affected individuals, in order to protect them from the deleterious metabolic effects generated by these pathologies. Nutrigenomics is a fundamental tool for this process, since it seeks to understand the molecular basis of nutrients and their genetic and epigenetic targets interactions and their role in biological processes modulation, aiming to generate efficient treatment and also the maintenance of health status. Based on the literature, it was possible to infer that oral intake of whey protein could regulate *Chrebpβ* expression in the WAT of insulin resistant animals, through the activation of mTORC2 (target of mammalian rapacin complex 2), thus increasing the use of glucose by adipocytes, culminating in the reduction of glycemia and hepatic *de novo* lipogenesis.

4 OBJECTIVES

4.1 GENERAL OBJECTIVE

To investigate the effect of oral whey protein isolate intake on the modulation of *Chrebp* expression in WAT and liver tissues and if the supplementation modulates satiety, infiltration of immune cells into adipose tissue and generate protection against hepatic steatosis in mice submitted to a high refined carbohydrate-containing diet capable of inducing increased visceral adiposity, inflammation, insulin resistance and hypertriglyceridemia.

5 MATERIALS AND METHODS

5.1 ANIMALS AND DIETS

The experiment was approved by the Ethics Committee on the Use of Animals, Biological Sciences Sector, Universidade Federal do Paraná (no. 23075.006828/2018-31). Male *Mus musculus* Swiss lineage mice, 3–4 weeks old, were maintained at 23ºC, with a 12 h light–12 h dark cycle (06.00–18.00 hours). The animals had *ad libitum* access to food and water throughout the trial (MCALLAN et al., 2013). The high refined carbohydrate-containing diet was prepared daily in the pharmacology laboratory, department of pharmacology, Universidade Federal do Paraná, and the basal diet was purchased from the vivarium of Universidade Federal do Paraná. The experimental diets consisted in a basal diet, a high refined carbohydrate-containing diet and a high refined carbohydrate-containing diet with whey protein isolate (FIGURE 11). The basal diet (BD; normocaloric and normoglycidic) (Presence® InVivo) totalized (for 100g) 314 kcal, of which 46.5g of carbohydrates, 23g of proteins, 4g of fats and 5g of dietary fiber. The high refined carbohydrate-containing diet (OLIVEIRA; MENEZES-GARCIA et al., 2013) (HC: 45% energy as condensed milk (Nestle®) consisted of 10% energy as sugar (Caravelas®); 45% energy as basal diet (Presence®), totaling (for 100g) 325.3 kcal, of which 55.68g of carbohydrates, 13.5g of proteins, 5.4g of fats and 2.25g of dietary fiber). The high refined carbohydrate-containing diet with whey protein isolate (HCW; 45% energy as condensed milk; 10% energy as sugar; 45% energy as basal diet + 15.74g of WPI (BlackSkull) totalized (for 100g) 337.17 kcal, of which 48.99g of carbohydrates, 23.49g of proteins, 5.25g of fats and 1.94g of dietary fiber). The diets HC, HCW and BD were given to groups of animals named HC, HCW and BD (10 animals per cage), respectively, during the 12 weeks of study.

Figure 11 - Nutritional table of the diets containing the amounts of energy, carbohydrates, proteins, fats and fibers. (A), basal diet; (B), high refined carbohydrate-containing diet; (C), high refined carbohydrate-containing diet with whey protein isolate.

(A) BASAL DIET (BD)

NUTRITIONAL INFORMATION
Portion of 100g

Energy	314 Kcal
Carbohydrates	46.5 g
Proteins	23 g
Total fat	4 g
Fiber	5 g

(B) HIGH REFINED CARBOHYDRATE-CONTAINING DIET (HC)

NUTRITIONAL INFORMATION
Portion of 100g

Energy	325.3 Kcal
Carbohydrates	55.68 g
Proteins	13.5 g
Total fat	5.4 g
Fiber	2.25 g

Composition: 45% condensed milk; 10% sugar; 45% basal diet.

(C) HIGH REFINED CARBOHYDRATE-CONTAINING DIET WITH WPI (HCW)

NUTRITIONAL INFORMATION
Portion of 100g

Energy	337,17 Kcal
Carbohydrates	48.99 g
Proteins	23.49 g
Total fat	5.25 g
Fiber	1.94 g

Composition: 45% condensed milk; 10% sugar; 45% basal diet + 15,74g of WPI.

Source: Present study.

5.1.1 EXPERIMENTAL PROTOCOL

The application of diets was performed on group-housed mice (ten per home cage) following a 1-week acclimatization period, during which the animals were fed a basal diet.

For 12 weeks, three groups of mice were fed the HC, the HCW or the BD diet (n = 10). Body weights were measured weekly. The mean energy intake was calculated based on the amount of food consumed by each group daily during the study period. At week 12, blood was collected through the tail vein to measure the glycemia of the animals using the Accu Chek Performa Kit (Roche®).

After blood collection, the animals were euthanized by cervical dislocation without the use of anesthesia to avoid the influence of the drugs over gene expression (AL-MOUSAWI et al., 2010). Samples of the perigonadal adipose tissue (WAT) and liver were collected and fixed in paraffin to make histological slides, and

parts of both organs were stored in RNA*later* (Thermo Fisher Scientific®) at -80 ºC according to the manufacturer instructions, for further extraction of mRNA's and retrotranscription.

5.1.2 HISTOLOGICAL EVALUATION

Collected liver and adipose tissue samples were fixed in ALFAC solution (80% ethanol, 40% formalin and glacial acetic acid). The tissues were then dehydrated, embedded in paraffin, and sectioned into 5 µm slices. Slices were hydrated in xylene and a descending sequence of ethanol, then stained with hematoxylin and eosin. Fat accumulation in liver sections was observed according to Brunt et al. (1999). To evaluate hepatic steatosis, slices were photographed at a magnification of 40× with an Olympus microscope DX51 endowed with an Olympus DP72 camera.

5.1.3 MYELOPEROXIDASE ENZYMATIC ACTIVITY ASSAY

The biopsies of adipose tissue with an average weight of 40 milligrams were added in 0.75 ml of 80 mM PBS pH 5.4 containing 0.5% of HTBA and homogenized (45 s at 0 °C) in a motor-driven homogenizer. The homogenate was decanted into microtubes and added to 0.75 ml of buffer previously described. The samples (1.5 ml) were placed in microfuge tubes and centrifuged at 11.200 ×g at 4 °C for 20 min. Triplicates of 30 µl of the supernatant were placed on a 96-well plate, to which 200 µl of a mixture containing 100 µl of 80 mM PBS pH 5.4, 85 µl of 0.22 M PBS pH 5.4 and 15 µl of 0.017% hydrogen peroxide into each well was subsequently added. The addition of 20 µl of 18.4 mM TMB in dimethylformamide promoted reaction initialization. The plate was then incubated at 37 °C for 3 min and the reaction stopped by addition of 30 µl of 1.46 M sodium acetate, pH 3.0, as described by Mendes et al. (2012). The enzymatic activity was determined colorimetrically using a plate reader (EL808; BioTech Instruments, INC) set to measure absorbance at 630 nm and expressed as OD/Biopsy.

5.1.4 RNA EXTRACTION AND COMPLEMENTARY DNA SYNTHESIS

Total RNA was isolated from hepatic and perigonadal adipose tissues with mirVana™ PARIS™ Kit, according to the manufacturer instructions. To remove any

potential genomic DNA contamination, a DNase treatment (DNase I, RNase-free**,** Thermo Scientific™) was performed after RNA isolation. Complementary DNA was synthesized from 1µg of total RNA (diluted in a solution of 10µL of DNase I, RNase-free**,** Thermo Scientific™) using the High Capacity cDNA Reverse Transcription Kit (20 µL reaction) (Applied Biosystems™), according to the manufacturer instructions. A parallel reaction without the inclusion of the MultiScribe™ Reverse Transcriptase enzyme was also performed as a negative control.

5.1.5 REAL-TIME PCR

The amplification of complementary DNA was performed in ViiA 7 Real-Time PCR System (Applied Biosystems™) using 0.6 µL (10 µM) forward and reverse primers (Eurofins Genomics™), 4.4 µL complementary DNA, 5.0 µL EvaGreen® qPCR System ROX Free Master kit (Biotium™), and 0.2 µL of ROX reference dye (Thermo Scientific™), according to the manufacturer instructions. Real-time PCR conditions were as follows: hold stage (50^{o}C for 2 min, followed by 95^{o}C for 10 min) followed by forty cycles (PCR stage) at 95^{o}C for 15 s; 60^{o}C for 60 s. The primer sequences are given in Table 1. Data obtained as Ct values were normalized to the expression of *GAPDH* according to ΔΔCt = ΔCt target gene - ΔCt housekeeping gene. For both the adipose tissue and the Liver, *GAPDH* have been shown to be the appropriate housekeeping gene. The relative gene expression was calculated using $2^{-\Delta\Delta Ct}$, and it is shown in comparison with the control group and between treated groups.

Table 1. Sequences of primers used for real-time PCR. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *ChREBPα*, carbohydrate responsive element-binding protein alfa; *ChREBPβ,* carbohydrate responsive element-binding protein beta.

Genes	Forward primer (5`—3`)	Reverse primer (5`—3`)
GAPDH	5`-tgtgtccgtcgtggatctga-3`	5`-cctgcttcaccaccttcttga-3`
ChREBPα	5`-cgacactcacccacctcttc-3`	5`-ttgttcagccggatcttgtc-3`
ChREBPβ	5`-tctgcagatcgcgtggag-3`	5`-cttgtcccggcatagcaac-3`

Source: Present study.

6 STATISTICAL ANALYSIS

Data are shown as means and standard deviation (SD). Statistical analysis was performed by two-tailed, unpaired, Student's t-test for the variables with normal distribution and Mann-Whitney U test for non-parametric variables. Multiple regression analysis was used to find independent variables exerting influence over gene expression. The normality of the variables was examined by the Kolmogorov-Smirnov test with Lilliefors correction. Statistical significance adopted was 0.05 (5%).

7 RESULTS AND DISCUSSION

7.1 GENE EXPRESSION, HISTOLOGICAL ANALYSIS AND MYELOPEROXIDASE ENZYMATIC ACTIVITY IN ADIPOSE AND LIVER TISSUES

As expected, the dietary pattern of each group was able to significantly influence gene expression in both tissues: adipose *Chrebpα* ($p = 0.0066$; intercept, $p = 0.0034$) *Chrebpβ* ($p = 0.0017$; intercept, $p = 0.026$) and liver *Chrebpα* ($p = 0.023$; intercept, $p = 0.012$), which occurred independently of body weight or of MPO enzymatic activity, with the exception of *Chrebpβ* expression in the liver, which had its expression influenced by MPO activity ($p = 0.037$; intercept, $p = 0.0074$),

In adipose tissue, gene expression of both isoforms was significantly higher in the control group compared to the two treated groups (Table 4). This shows that a diet low in refined sugar actually favors DNL in this tissue, according to our initial hypothesis. However, we found no significant differences in gene expression between the treated groups (HC and HCW), and therefore we can state that whey protein isolate supplementation does not appear to significantly alter the expression of these genes in adipose tissue in relation to HC group, which had the same diet rich in refined sugar, but without the addition of WPI. Although there was no statistical difference, there was a greater expression of *Chrebpα* and *Chrebpβ* in the adipose tissue of the HC group compared to the HCW group, a finding that corresponds to the inverse of our initial hypothesis. Thus, we confirm that both diets are able to directly influence gene expression, but the mechanisms by which this expression has been altered remain an enigma.

A limiting factor of our work was that the lineage of mice used was heterogenetic, which may have generated a different responsiveness among individuals due to variations in their genotypic constellation. In addition, we can infer that molecular signaling of excess sugar, which probably generated some degree of insulin resistance (HENRIQUES et al, 2013), was apparently "stronger" than the signaling of amino acids in the activation of mTORC2 and the consequent increase in the alternative glucose uptake pathway in adipocytes, which would lead to an increased gene expression, and consequently increased DNL.

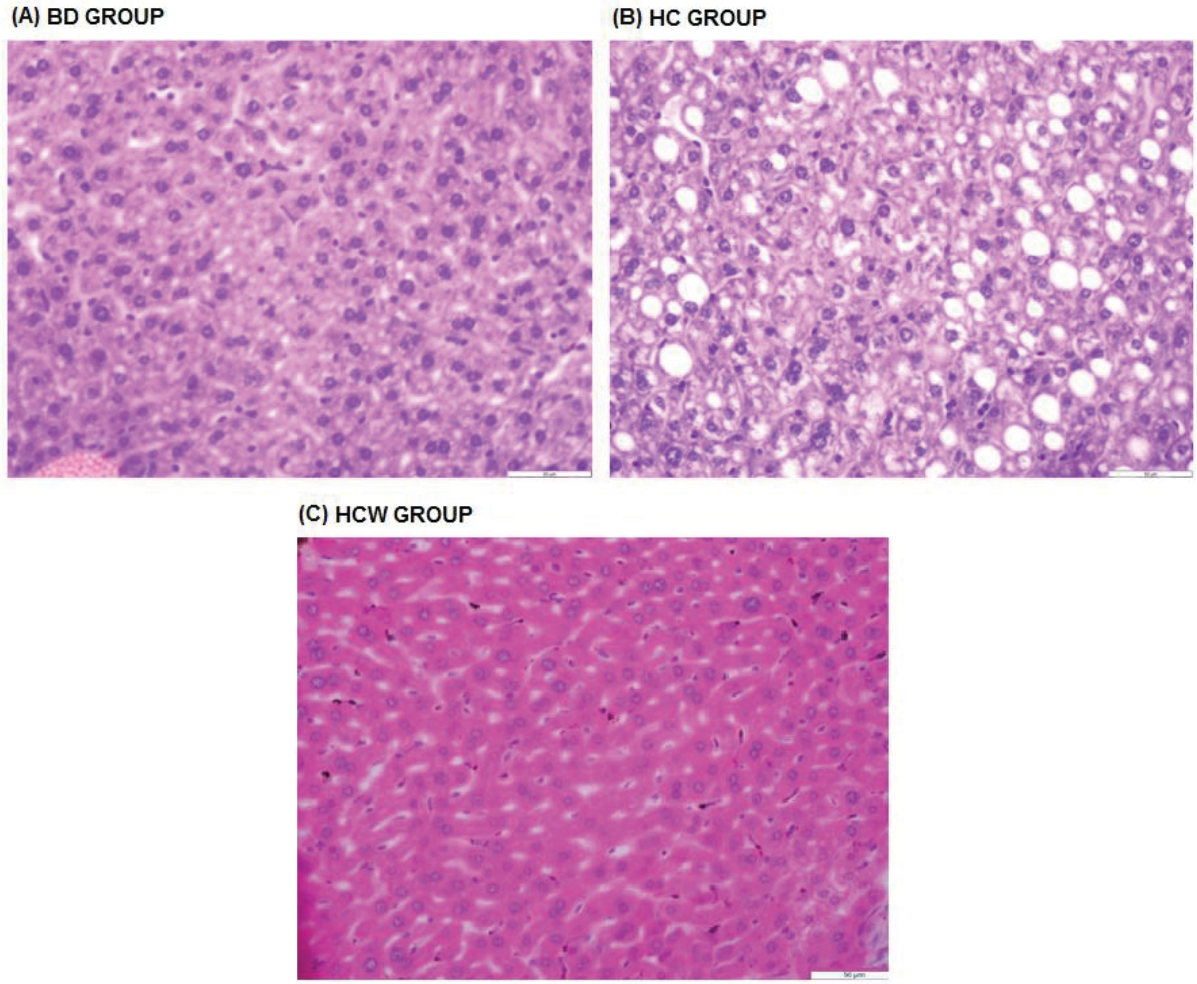
Table 2. Mean gene expression in adipose and liver tissues in each group.

Group	Gene name	Tissue	Mean gene expression	(SD)	Ratio (α:β) - Liver	Ratio (α:β) - Adipose tissue
BD	ChREBPα	Liver	1.8770 **	1.9517	1.8770:1.6860	
		Adipose	0.8453 **	0.5510		0.8453:1.3874
	ChREBPβ	Liver	1.6860	2.0427		
		Adipose	1.3874 **	0.9586		
HC	ChREBPα	Liver	0.2313	0.3670	0.2313:1.3501	
		Adipose	0.1816	0.3948		0.1816:0.0338
	ChREBPβ	Liver	1.3501	1.0086		
		Adipose	0.0338	0.0486		
HCW	ChREBPα	Liver	0.6751	0.2770	0,6751:0.5718	
		Adipose	0.0265	0.0282		0.0265:0.0076
	ChREBPβ	Liver	0.5718	0.2767		
		Adipose	0.0076	0.0071		

Table 4: Mean comparisons were made considering the same genes in each tissue between different groups. SD = Standard Deviation.
Mean values with ** were significantly different (P<0.05).
Source: Present study.

An interesting finding was that at first, whey protein supplementation in the HCW group had a protective effect against hepatic steatosis, corroborating our initial hypothesis. It can be seen in figure 9 that the HCW group did not develop hepatic steatosis, while the HC group developed it. From this, we could hypothesize that supplementation had a protective effect, however, it is difficult to determine whether supplementation was determinant in this process, whether it was the caloric restriction (almost 2000 Kcal) in relation to the HC group, or (and more likely) whether supplementation plus caloric restriction generated overlapping effects on liver protection. It is known that caloric restriction is able to protect obese mice from hepatic steatosis (KIM et al. 2016). Therefore, we can state that caloric restriction plus WPI supplementation can protect the liver of mice from hepatic steatosis while consuming a diet rich in refined sugar.

Figure 12. Liver histological slides taken from the BD (A), HC (B) and HCW (C) groups. Hepatic steatosis can be noticed by the white spaces in the B image, which represent fat accumulation.



Source: Present study.

Gene expression in the liver had very interesting results. First, we found a significant difference in *Chrebpα* gene expression between BD and HC groups, in which the mean expression in the HC group was lower, which initially seemed confusing. As observed in figure 9, we found the presence of hepatic steatosis in the animals of the HC group, but not in the BD group. Thus, in the first view they appear contradictory results, because we expected a higher expression of *Chrebpα* in the livers of the HC group that would culminate in increased DNL, which would result in hepatic steatosis.

However, gene expression of *Chrebpβ* has been shown to be very high in relation to *Chrebpα* in the livers of HC group (1.3501:0.2313 ratio) (Table 4). It is important to note that this pattern occurs exclusively in the HC group, a fact that according to Zhang et al. (2015) occurs only in a glucose-rich environment where

there is a rapid increase in *Chrebpβ* gene expression and only a small change in gene expression of *Chrebpα*, at least in rodent models. For instance, in INS-1–derived 832/13 cells, the ChREBPβ-to-ChREBPα ratio was 1:8 in low glucose and rapidly increased to 1:0,2 after 18 h in a high glucose environment (15 to 20 mmol/L). Therefore, we believe that in the HC group there was a greater abundance of glucose in the liver, in part due to the reduced glucose uptake in the adipose tissue of the animals (inferred by the low gene expression of the *Chrebpβ* in adipose tissue, which is directly influenced by the concentration of glucose). Interestingly, there was no significant difference in the mean serum glucose from the groups at the end of the experiment (data not shown), but most likely there were insulin variations (unmeasured data), corroborating the findings of Oliveira et al. (2013).

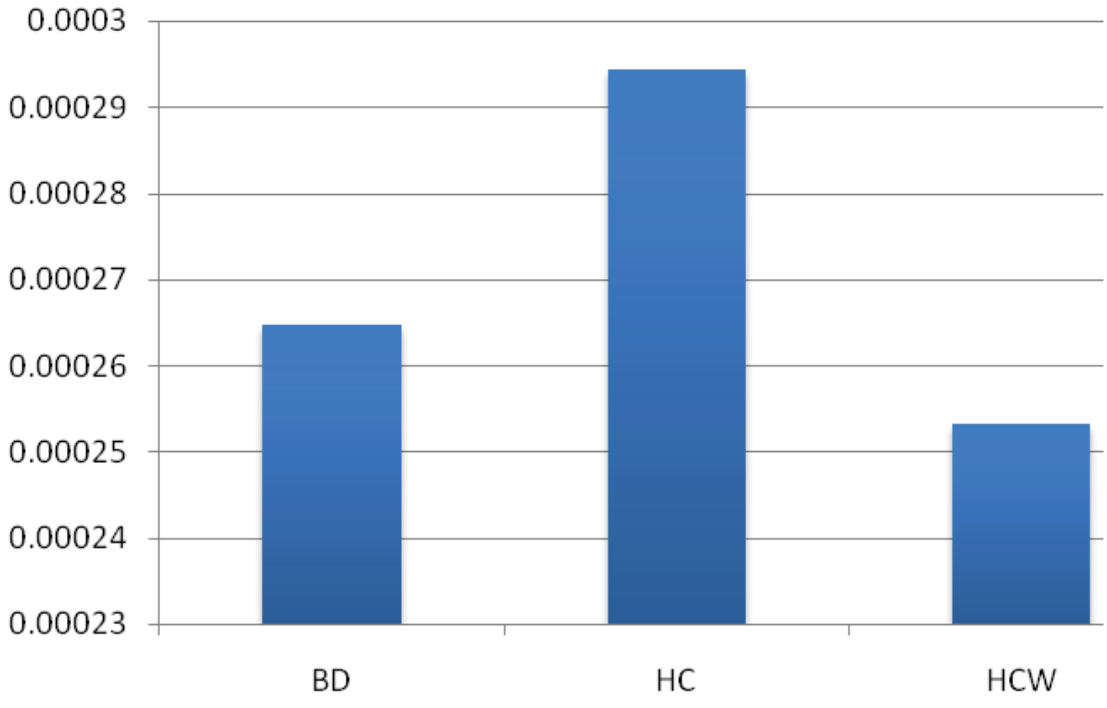
We also noticed that in the HC group there is a greater discrepancy between the mean values of the gene expression of the isoforms *α* and *β* in the liver, which can be justified by the fact that *Chrebpβ*, initially induced through *Chrebpα*, can in turn inhibit *Chrebpα* expression through a negative feedback loop (ABDUL-WAHED et al, 2017), and as there was an overexpression of *Chrebpβ* in the HC group it was expected to find greater inhibition of *Chrebpα* Interestingly, although the HCW group also consumed a diet rich in refined sugar, there was no such disproportion in gene expression. At least two possibilities may be considered to explain this positive modulation (lower hepatic DNL) in the HCW group. The first is that WPI supplementation led to a better utilization of glucose in the cytoplasm of hepatocytes, through anti-inflammatory mechanisms and improvement of insulin sensitivity (GO et al. (2018) and McAllan et al. (2013)). The second is that the caloric restriction *per se* generated a reduction in glucose uptake in hepatocytes due to the lower availability by the food consumption itself, generating a less lipogenic environment. However, it is most likely that these two conditions worked together for the modulation of gene expression. Despite the interesting findings, there are some complications, such as the fact that the regulation of *Chrebpβ* does not only depend on the α isoform, but also on other factors. For example, the expression of the β isoform demonstrates a clear circadian rhythm, unlike the α isoform (MONTAGNER et al. 2016).

Another factor that attracted attention was an apparent influence of MPO activity on adipose tissue on *Chrebβ* expression in the liver ($p = 0.037$; intercept, $p = 0.0074$). We know that as adipose tissue increases in size, there is a chronic and systemic increase of low-grade inflammation in these individuals, due to increased

infiltration of immune cells in this tissue and increased production of pro-inflammatory cytokines. This chronic inflammation seems to play a fundamental role in comorbidities associated with obesity, such as insulin resistance (DAM et al, 2016). Like Oliveira et al. (2013), we observed in the HC group an increased visceral adiposity, which occurred independently of weight gain (data not shown). It is known that the MPO enzyme is produced by neutrophils, and is a marker of the infiltration of these cells into adipose tissue. Zhang et al. (2014) found that mice that consumed a hyperlipidic diet had a greater infiltration of neutrophils in the perigonous adipose tissue, a fact marked by an increase in the enzymatic activity of MPO. In addition, body weight gain and defects in insulin signaling were observed.

As we can see in figure 10, there was no significant difference between the enzymatic activity of MPO between the groups, despite a trend of higher enzymatic activity in the HC group. However, this variable influenced the gene expression in adipose tissue only in the HC group, and we believe that the possible higher low-grade inflammation generated in these individuals may have influenced insulin sensitivity, contributing to the unusual pattern of *Chrebβ* expression in the liver of these animals.

Figure 13. **Myeloperoxidase enzymatic activity (MPO) in adipose tissue.**



Mean values with ** were significantly different (P<0.05).
Souce: present study.

7.2 ENERGY INTAKE AND BODY WEIGHT

The food intake in grams and Kcal was measured to evaluate if any of the diets would have power to generate greater satiety (Table 2). According to Westerterp-Plantenga et al. (2012), a high-protein diet can increase satiety. Moreover, according to Zapata et al. (2018), a diet rich in whey protein and its fractions is able to increase the satiety of obese rats in part by enhancing the expression of the mRNAs of peptide YY (PYY) in the colon and plasma of the animals, generating an anorectic effect. Interestingly, although the diets of the BD and HCW groups had practically the same amount of protein per serving, in the first six weeks of the experiment the HCW group consumed on average 205 Kcal less than the BD group and averaged 807 Kcal less at the end of the experiment. Therefore, it is believed that even though the HCW diet was more palatable, the anorectic influence of whey protein generated a lower energy consumption in this group. This effect can be confirmed by the fact that the lack of whey protein in the HC group generated an average increase in consumption of 1652 Kcal in the first six weeks and 1550 Kcal at the end of the study, in relation to BD and HCW groups. However, it is important to note that this diet had a smaller dose of protein per serving than that of the BD and HCW groups.

As shown in table 02, the food and energy consumption during the study period had interesting fluctuations. The consumption of food and energy was higher in the HC group during the first 4 weeks of study, probably due to the high palatability of the diet in relation to the others (DENIS et al, 2015), but this difference was ablated in the subsequent weeks of the study, probably due to the phenomenon of sensory specific satiety (SSS), which describes the decline in pleasantness associated with a food as it is eaten relative to a food that has not been eaten before (WILKINSON and BRUNSTROM, 2016). In other words, they probably "got sick" of the diet.

In the 4th to 8th week, the BD group had the greatest food consumption, at least significantly higher than the HCW group. In the last four weeks, the BD group had the highest food intake and energy consumption. Interestingly, in the first 6 weeks, the HC group consumed a total of 1550 Kcal more than the BD group and

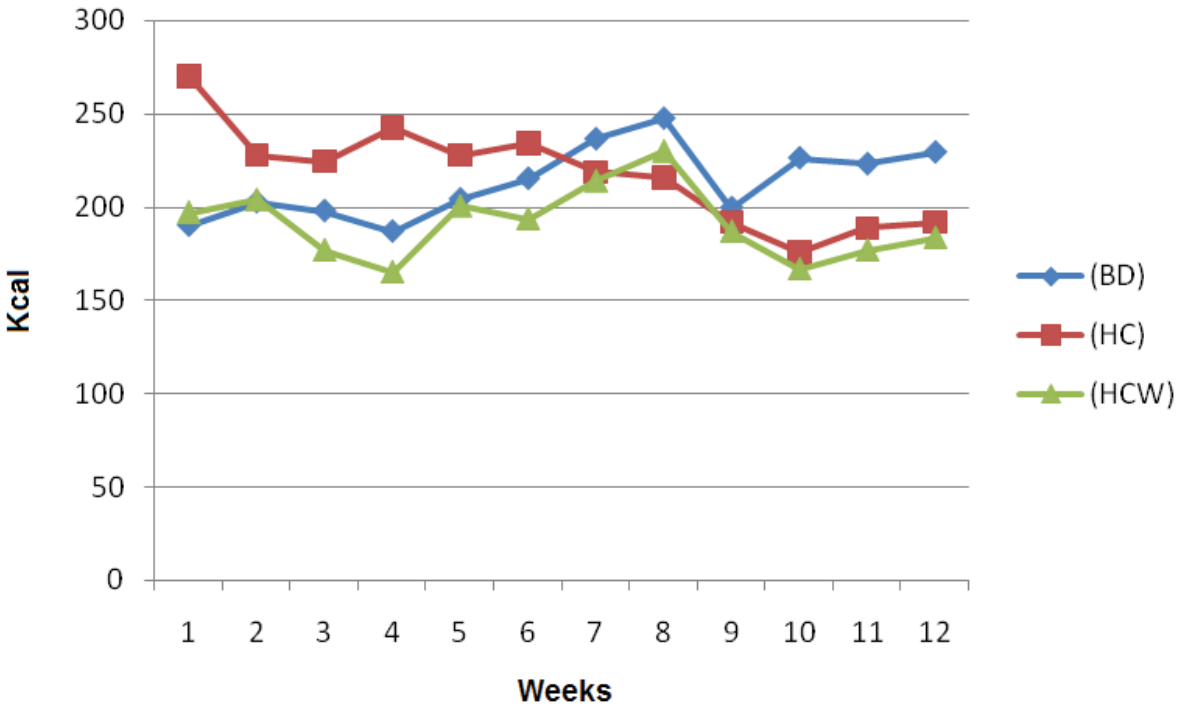
1755,5 Kcal more than the HCW group. This difference in energy consumption, which can be seen in the graph of figure 14, was enough to generate a statistical difference in the weight of the animals in that period (Table 03). This fact is important to reinforce that the determinant factor for body weight gain is the excessive energy consumption, not the type of diet practiced.

Table 3. Average daily food intake (in grams and calories) every four weeks. BD, basal diet; HC, high refined carbohydrate-containing diet; HCW, high refined carbohydrate-containing diet with WPI. The BD, HC and HCW groups are being compared to each other in the three periods.

	Week 1 - 4		Week 4 - 8		Week 8 - 12	
Groups	Mean	(SD)	Mean	(SD)	Mean	(SD)
BD	61.71 (g)	11.92	73.24 (g)*	12.83	70.56 (g)**	10.33
	193.78 (Kcal)	37.43	229.97 (Kcal)	40.28	221.56 (Kcal)**	32.42
HC	73.57 (g)**	9.5	71.34 (g)	13.58	58.82 (g)	8.62
	239.33 (Kcal)**	30.9	232.07 (Kcal)	43.43	191.35 (Kcal)	28.05
HCW	56.55 (g)	7.94	63.84 (g)	15.4	54.95 (g)	10.44
	190.68 (kcal)	26.76	215.25 (Kcal)	51.93	185.26 (Kcal)	35.2

Mean values with ** were significantly different (P<0.05). The mean values with * were significantly different (P<0.05) only in relation to the HCW group.
Source: Present study.

Figure 14. Graphical representation of the average weekly energy consumption of the BD, HC and HCW groups.



Source: Present study.

In the total of 12 weeks, the HC group consumed 17,860.59 Kcal, the BD group consumed 16,714.22 Kcal and the HCW group consumed 15,907.68 Kcal. Thus, the HC group consumed a total of 1147.4 Kcal more than the BD group and 1952.9 Kcal more than the HCW group. Despite this difference, there was no statistical difference between the mean weight of the groups at the end of the experiment.

Table 3 shows the evolution of the weight of the animals during the study period represented by weeks 1, 6 and 12. As expected, the higher energy consumption of the HC group, at least during weeks 4 to 8, generated a significant weight gain in this group during this period.

Table 4. Mean weight of mice (in grams) in the first week, sixth week and twelfth week. Comparisons were made between groups in the same time period.

	Week 01		Week 06		Week 12	
Groups	Mean	(SD)	Mean	(SD)	Mean	(SD)
BD	28	2.95	34	3.39	40	4.49
HC	28.35	1.56	37.55 **	3.11	41.56	5.34
HCW	27.45	2.96	34.65	2.76	38.78	3.59

Mean values with ** were significantly different (P<0.05).
Source: Present study.

8 CONCLUSIONS

Gene expression was significantly modulated by diet in adipose tissue and liver. In addition, the WPI intake was able to modulate the satiety of the animals, and potentiate the effects of high-protein diet, probably due to the regulation of the centers of hunger and satiety via peptide YY modulation.

The high refined carbohydrate-containing diet with WPI (HCW group) was able to protect the livers against hepatic steatosis, probably due to the overlap of the WPI supplementation plus the caloric restriction performed by this group in relation to the HC group, which in turn developed hepatic steatosis.

There was a probable greater infiltration of immunological cells into the adipose tissue of the HC group, and although the difference in means was not statistically significant, the increased MPO enzymatic activity in that group influenced *Chrebβ* hepatic expression, probably due to low-grade inflammation generated in these individuals.

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