# Exploratory study of egg white hydrolysate mechanisms of action in insulin sensitive tissues of high fat diet-induced insulin resistant rats

by

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# Abstract

**Background:** Chronic diseases related to the metabolic syndrome, such as type 2 diabetes and hypertension lead to complications when not treated properly. Natural approaches able to tackle multiple aspects of the metabolic syndrome and cause minimal side effects are desired. Bioactive peptides are short amino acid sequences that exert benefits beyond their nutritional value, improving health. Egg white is a rich source of bioactive peptides that are promising candidates as functional foods. Egg white peptides and hydrolysates (EWH) have been shown to reduce blood pressure, inflammation and fat mass, all related to the metabolic syndrome. Previously, feeding EWH for 6 weeks improved glucose and insulin tolerance in insulin resistant (IR) rats. Here, I aimed to identify EWH mechanism(s) of action related to glucose homeostasis. In this work I hypothesized that EWH supplementation affects multiple signaling pathways in insulin sensitive tissues to improve glucose tolerance in high fat diet (HFD)-induced insulin resistant rats.

**Methods:** Sprague-Dawley rats were fed HFD for 6 weeks and then divided into 2 groups, HFD (n=7) and HFD+4% EWH (n=8) for another 6 weeks. 10 min prior to euthanization insulin was injected in half of the animals in each group. Insulin sensitive tissues (skeletal muscle, liver and white adipose tissue (WAT)) were tested, when applicable, for changes in insulin signalling, renin-angiotensin system (RAS) components, gluconeogenesis and lipolysis enzymes by western blot. In addition, WAT inflammatory markers and adipokines were measured by ELISA, PPARγ activation by DNA binding activity and adipocyte size by histological analysis.

**Results:** EWH enhanced Akt phosphorylation in muscle and WAT of EWH-treated animals. RAS components abundance presented minimal changes, but notably there was increased angiotensin type 2 receptor (AT2R) in liver and WAT after EWH supplementation. The abundance of liver gluconeogenesis enzymes did not change. PPARγ DNA binding activity was enhanced and lipolytic capacity reduced by EWH in WAT. Moreover, the treatment reduced adipocyte size in epididymal and retroperitoneal WAT, despite no changes in tissue inflammatory cytokines, resistin or adiponectin levels.

**Conclusion:** In this study WAT was more responsive to EWH effects than other insulin sensitive tissues. In summary, the main findings of this study were that EWH changed adipocyte morphology, reduced adipocyte lipolytic capacity, enhanced insulin sensitivity in peripheral tissues, had little effect on local inflammation and induced only AT2R of the RAS components examined. The results are in accordance with our hypothesis showing that EWH is affecting insulin sensitive tissue to improve glucose tolerance through a variety of mechanisms and acts independently of changes in local inflammatory markers or adipokines. In WAT, a model linking EWH to AT2R activation, with downstream effects on PPARγ and Akt activation is proposed for further investigation. This research contributes to evidence for EWH as a potential product in the functional food market.

# Preface

This thesis is an original work done by Stepheny Carneiro de Campos Zani. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Effect of bioactive peptides on metabolic syndrome", No. 1472. This research project was funded by Alberta Livestock and Meat Agency (ALMA) and the Natural Sciences and Engineering Research Council of Canada (NSERC) discovery grant. I was funded by Faculty of Graduate Studies and Research (FGSR) from University of Alberta, the Faculty of Medicine and Dentistry and Alberta Diabetes Institute (ADI) during the execution of this project.

Chapter 1 (sections 1.10 and 1.11; table 1.1 and table 1.2) of this thesis has been modified from the published paper: De Campos Zani, S. C., Wu, J., & Chan, C. B. (2018). Egg and soy-derived peptides and hydrolysates: A review of their physiological actions against diabetes and obesity. *Nutrients*, *10*(5). https://doi.org/10.3390/nu10050549. I was responsible for the manuscript composition and C.B.C and J.W. edited the manuscript. The remaining sections of this chapter are not published anywhere else.

Chapter 2 (section 2.3, table 2.1) and 3 (sections 3.1, 3.2 and 3.3) of this thesis contain data that together with data from Forough Jahandideh's PhD thesis (Jahandideh, 2017) has been submitted as: Jahandideh F, de Campos Zani SC, Son M, Proctor SD, Davidge ST, Chan CB, Wu J. Egg white hydrolysate enhances insulin sensitivity in high fat diet induced insulin resistant rats via AKT activation. *British journal of nutrition*. Oct. 2018. F.J. and I were responsible for the manuscript composition and for the *ex vivo* data collection and analysis presented in this manuscript. *In vivo* experimentation and data collection were done by F.J with assistance from Nicole Coursen. PPARγ abundance

measurement in the manuscript was done by M.S. C.B.C, W.J., S.T.D and S.D.P edited the manuscript.

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***"And so let us always meet each other with smile, for the smile is the beginning of love…"***

*Mother Teresa*

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# LIST OF ABBREVIATIONS

ACE: Angiotensin converting enzyme

ACE2: Angiotensin converting enzyme 2

ADD1/SREBP1c: Basic helix-loop-helix family

ADP: Adenosine diphosphate

AGEs: Advanced glycation end products

Akt: Protein kinase B

ALT: Alanine aminotransferase

ANOVA: Analysis of variance

AS160: Akt substrate of 160 kDa

AST: Aspartate aminotransferase

AT1R: Angiotensin II type 1 receptor

AT2R: Angiotensin II type 2 receptor

ATP: Adenosine triphosphate

ATGL: Adipocyte triglyceride lipase

C/EBP: CAAT/ enahncing binding protein

Ca2+: Calcium

cAMP: Cyclic adenosine monophosphate

cGMP: Cyclic guanosine monophosphate

CHOL: Total Cholesterol

COX-2: Cyclooxygenase-2

CVDs: Cardiovascular diseases

DG: Diglycerides

DPPH: 1,1-diphenyl-2-picrylhydrazyl

DPP-IV: Dipeptidyl peptidase-IV

ERK 1/2: Extracellular signal regulated kinase

EW: Egg white

EWH: Egg white hydrolysate

FAS: Fatty acids synthase

FFA: Free fatty acids

FGS: Focal glomerulosclerosis

G6PDH: Glucose 6-phosphate dehydrogenase

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GI: Gastrointestinal

GIP: Glucose-dependent insulinotropic polypeptide

GLP-1: Glucagon-like peptide 1

GLP1R: Glucagon-like peptide 1 receptor

GLUT: Gucose transporter

GLY-3-P: Glycerol-3-phosphate

HbA1C: Glycated hemoglobin A

HDL: High density lipoprotein

HFD: high fat diet

HOMA-IR: Homeostasis model assessment of insulin resistance

HOMA-β: Homeostasis model assessment of insulin secretion

HSL: Hormone sensitivy lipase

IDF: International Diabetes Federation

IL-6: Interleukin 6

IpGTT: Intraperitoneal glucose tolerance test

IR: Insulin receptor

IRS: Insulin receptor substrate

LPL: Lipoprotein lipase

MetS: Metabolic Syndrome

MG: Monoglyceride

MGL: Monoglyceride lipase

mTORC1: Mammalian target of rapamycin complex 1

NAFLD: Non alcoholic fatty liver disease

NEFA: non-esterified fatty acids

OGTT: Oral glucose tolerance test

PDE3B: Phosphodiesterase 3B

PEPCK: Phosphoenolpyruvate carboxykinase

PI3K: Phosphatidylinositol-3 kinase

PKA: Protein kinase A

PKG: Protein kinase G

PPAR-γ: Peroxisome proliferator associated receptor gamma

QUICKI: Quantitative index of insulin sensitivity

RAS: Renin-angiotensin system

ROS: Reactive oxygen species

RT: Room temperature

SCD: Stearoyl-CoA desaturase

SCFA: Short chain fatty acids

SHRs: Spontaneous hypertensive

SNS: Sympathetic nervous system

SREBP-1: Sterol element binding protein 1

T2D: Type 2 diabetes

TBA: Total bile acids

TG: Triglycerides

TNF-α: Tumor necrosis factor alpha

TXA2R: Thromboxane A2 receptor

TZD: Thiazolidinediones

VLDL: very- low density lipoprotein

WAT: White adipose tissue

α-SMA: Anti-a-smooth muscle actin

# CHAPTER 1: Literature Review

Section 1.10 and 1.11 in this chapter are modified from the published paper: De Campos Zani, S. C., Wu, J., & Chan, C. B. (2018). Egg and soy-derived peptides and hydrolysates: A review of their physiological actions against diabetes and obesity. *Nutrients*, *10*(5). https://doi.org/10.3390/nu10050549 (Appendix 1).

## 1.1 Metabolic syndrome definition and prevalence

Metabolic syndrome (MetS), also known as insulin resistance syndrome, syndrome X or the deadly quartet (McCracken, Monaghan, & Sreenivasan, 2018), is a cluster of abdominal obesity, insulin resistance, hypertension and dyslipidemia (high triglyceride (TG) and total cholesterol (CHOL) and low high-density lipoprotein (HDL)-cholesterol) (Alberti et al., 2009).

Due to different definitions and threshold criteria, in 2009 a consensus established the criteria for MetS diagnosis as the presence of 3 out of the 5 risk factors listed below (Alberti et al., 2009):

- Elevated waist circumference (population and country specific)
- High TG levels (> 1.7 mmol/L)
- Low HDL levels (> 1.0 mmol/L - males /> 1.3 mmol/L - females)
- High blood pressure (Systolic > 130 mmHg and/or diastolic > 85 mmHg)
- High fasting glucose (> 5.5 mmol/L)

To determine MetS prevalence is complicated due to different criteria and parameters involved. Keeping this in mind, it is estimated that globally MetS affects 25%

of the population (Nolan, Carrick-Ranson, Stinear, Reading, & Dalleck, 2017). Country-specific studies show that in the United States more than 30% of adults have the syndrome (Moore, Chaudhary, & Akinyemiju, 2017). In Canada, MetS prevalence is around 20% in adults (Rao, Dai, Lagace, & Krewski, 2014), although the numbers are slightly out of date. The global burden of MetS in young adults (18-30 years) is believed to be 7% (Nolan et al., 2017).

The high prevalence has been linked to the rising rates of type 2 diabetes (T2D) and hypertension among the population (Moore et al., 2017), which is a consequence, in part, of lifestyle and diet changes such as high carbohydrate and fat diets, sedentary lifestyle, smoking, as well as family history (McCracken et al., 2018). It is worth mentioning that MetS prevalence increases with aging (DeFronzo & Ferrannini, 1991).

## 1.2 Type 2 diabetes definition and prevalence

Diabetes affects 425 million people (20-79 years) in the world, with an expected increase to 629 million people affected by 2045 and T2D accounts for 90% of those cases. Interestingly, around half of the people suffering from diabetes is undiagnosed (International Diabetes Federation, 2017). Plasma glucose concentration is the gold standard method used to diagnose diabetes and the thresholds following IDF diabetes atlas (International Diabetes Federation, 2017) are:

**Impaired fasting glucose**

- Fasting plasma glucose: between 6.1-6.9 mmol/L and
- 2-hour post 75g oral glucose challenge: less than 7.8 mmol/L

**Prediabetes / Impaired glucose tolerance**

- Fasting plasma glucose: less than 7.0 mmol/L and
- 2-hour post 75g oral glucose challenge: between 7.8 and 11.0 mmol/L
- HbA1C: between 6.0-6.4%

**Diabetes:**

- Fasting plasma glucose: higher than 7.0 mmol/L or
- 2-hour post 75g oral glucose challenge: higher than 11.1 mmol/L or
- Random glucose higher than 11.1 mmol/L or
- HbA1C: higher than 6.5%

T2D is a complex metabolic disease that results from insufficient production of insulin by the pancreas (β-cell dysfunction or reduced mass) and/or the inefficient response to insulin by insulin-sensitive tissues (insulin resistance), leading to hyperglycemia (Del Prato, Bianchi, & Daniele, 2017).

Diabetes complications can cause death and people with diabetes have an increased risk of developing health problems; around 10.7% of all-cause mortality in people between 20-79 years is due to diabetes. In addition to being a life-threatening condition, diabetes causes a burden in the health care system accounting for a total of approximately USD 727 billion worldwide in 2017. In Canada the heath care expenditure with diabetes in 2017 was around USD 15 billion (International Diabetes Federation, 2017).

Insulin action is intrinsically related to both MetS and T2D. Thus, it is essential to understand the role of insulin in regulating glucose homeostasis in physiological and

pathophysiological conditions to better understand both diseases. Insulin physiology will be discussed in the following section.

## 1.3 Insulin physiology

### *1.3.1 Insulin production and secretion*

Insulin is responsible for regulating blood glucose and lipids; as such, the physiology and molecular mechanisms of insulin synthesis, secretion and signaling are complex. Insulin is a hormone produced by the endocrine pancreas, more specifically by the β-cells localized in the islets of Langerhans. Insulin helps to regulate plasma glucose concentrations (glycemia) and lipids (lipidemia) by acting in insulin sensitive tissues (skeletal muscle, adipose tissue and liver). Upon meal ingestion, insulin secretion is enhanced, thereby stimulating energy storage and preservation of endogenous energy sources (Boron & Boulpaep, 2005).

Proinsulin is the precursor of insulin, which is encoded by the insulin gene. After cleavages by endopeptidases, insulin is complexed with zinc and stored inside granules in β-cells, alongside C-peptide and other β-cells-derived substances, until exocytosis occurs (Z. Fu, Gilbert, & Liu, 2013). Insulin secretion is triggered by nutrient ingestion, mostly by an increase in glucose plasma concentration. Physiologically, glucose plasma concentration is maintained between 4-7 mmol/L.

Briefly, a rise in plasma glucose concentration and consequent increase in glucose metabolism in the β-cell enhance ATP/ADP ratio leading to closure of $K_{ATP}$ channels and β-cell membrane depolarization; which, in turn, increases intracellular $Ca^{2+}$ levels and triggers insulin exocytosis (Henquin, 2000). Besides the classical triggering pathway cited

above, there is a metabolic amplification pathway, which is a result of nutrient metabolism potentializing insulin secretion and it is independent of the rise in intracellular $Ca^{2+}$ (Kalwat & Cobb, 2017).

Upon stimulation, insulin is secreted in a biphasic manner. First, insulin is rapidly secreted reaching a peak (first phase), which is followed by a prolonged steady secretion of insulin (second phase) (Henquin, Dufrane, Kerr-Conte, & Nenquin, 2015). The first phase of insulin secretion is a consequence of previously docked granules, while the second phase is due to the recruitment of intracellular insulin granules from the reserve pool (Ohara-Imaizumi et al., 2004). After exocytosis, insulin reaches the portal vein and the systemic circulation acting in insulin sensitive tissues and facilitating glucose uptake.

Insulin action is counter-regulated by different hormones, such as glucagon, adrenaline, cortisol and growth hormone (Smith et al., 1993). Generally, those hormones act to inhibit insulin action of enhancing glucose uptake by peripheral tissues or supressing hepatic glucose production. Glucagon and adrenaline are released first, during the first period of hypoglycemia, while cortisol and growth hormone act during prolonged hypoglycemia (Smith et al., 1993). In this way insulin and counter-regulatory hormones crosstalk maintain the body's glucose balance.

### *1.3.2 Insulin signaling and glucose uptake in peripheral tissues*

Insulin exerts its effects in multiple tissues, such as skeletal muscle, cardiac muscle, adipose tissue and liver via the insulin receptor (IR). Skeletal muscle is the major tissue responsible for glucose clearance (Shulman et al., 1990), although liver and adipose tissue play an important role in glucose metabolism. Insulin signaling is complex and multiple

molecules are involved. The main steps of this signaling will be described in the following paragraphs.

The binding of insulin to its cell membrane receptor (IR), which is a heterotetramer comprised of 2 alpha- (IR-α) and 2 beta-subunits (IR-β), leads to IR autophosphorylation and activation of tyrosine kinase. The now phosphorylated site of IR-β phosphorylates insulin receptor substrate (IRS), which activates phosphatidylinositol-3 kinase (PI3K) (White, 1997). PI3K converts phosphatidylinositol (PIP)-2 into PIP3, and PIP3 phosphorylates protein kinase B (Akt), which activates Akt substrate of 160 kDa (AS160) located in the glucose transporter (GLUT)-4 vesicles. AS160 is essential to release GLUT4 from the intracellular vesicles and initiate its trafficking to the plasma membrane. During basal states GLUT4 is stored in the cytoplasm but after insulin stimulation translocation occurs and GLUT4 fuses with the plasma membrane allowing for the influx of glucose (X. Zhou, Shentu, & Xu, 2017).

There are different classes of GLUT, for this work class I is the most important. Class I GLUT includes GLUT1-4. GLUT4, which is highly responsive to insulin, is present in skeletal muscle, adipose tissue and brain (Navale & Paranjape, 2016). In adipocytes, apart from glucose uptake via GLUT4, insulin signaling suppresses lipolysis (the breakdown of lipids to generate energy) and promotes lipid storage. These processes will be discussed next.

### 1.4 Adipose tissue physiology

Adipose tissue is a heterogenous tissue composed of adipocytes and the stromal-vascular fraction (fibroblasts, macrophages and blood cells). Fat depots throughout the

body vary in cell composition and size and can be divided into two main types, white and brown adipose tissue (Hausman, 1985). White adipose tissue (WAT) is no longer thought to be only an energy reservoir; in fact, it is considered an endocrine organ which secretes substances sometimes called *adipokines* that affect physiology and energy balance, such as leptin, adiponectin, resistin, inflammatory cytokines, and angiotensinogen (Trayhurn & Beattie, 2001).

### *1.4.1 Adipogenesis*

Adipogenesis defines the formation and maturation of new adipose cells or adipocytes. Adipogenesis can be separated in two phases, the differentiation of a mesenchymal cell into preadipocyte and the further maturation of the former. Mature adipocytes, besides the changes in gene expression, are characterized by the presence of a large lipid droplet, occupying almost the entire cytoplasm (Ali, Hochfeld, Myburgh, & Pepper, 2013).

Peroxisome proliferator associated receptor gamma (PPARγ) is a transcription factor highly associated with adipogenesis, adipose tissue modulation and glucose metabolism. PPARγ is a nuclear receptor and lipid sensor, which modifies metabolism depending on fatty acid abundance and its metabolite concentration and composition (Evans, Barish, & Wang, 2004).

An extensive review of PPARγ and its array of actions is outside of the scope in this thesis. Nevertheless, three main transcription factors are found to highly influence adipocyte differentiation: PPARγ, CAAT/enhancer binding protein (C/EBP) and basic

helix–loop–helix family (ADD1/SREBP1c). In simplified words, PPARγ is activated both early in adipogenesis and later together with C/EBPα, leading to transcription of adipocyte phenotype specific genes and eventually to adipocyte differentiation and the formation of the lipid droplet. PPARγ is required for adipogenesis *in vitro* and *in vivo* (Rosen, Walkey, Puigserver, & Spiegelman, 2000) and is not only essential for adipogenesis, but also to maintain adipose tissue functionality (Evans et al., 2004).

### *1.4.2 Lipolysis and lipogenesis*

The amount of fat stored is determined by the balance of energy input (through our meals) and output (energy expenditure). After meal ingestion, the gastrointestinal (GI) tract works to break down carbohydrates, fats and proteins to small absorbable molecules, which later reach the bloodstream (Boron & Boulpaep, 2005). Excess energy is stored in the form of TG (mainly in WAT) or glycogen (mainly in liver and skeletal muscle), serving as future reservoirs.

The focus in this thesis is on lipid metabolism in adipose tissue. Lipogenesis and lipolysis are the two regulatory pathways in lipid metabolism and the balance between them determines the size of the lipid droplet in adipocytes. Both processes are reviewed in detail elsewhere (Lafontan & Langin, 2009; S. Wang et al., 2008).

Briefly, lipogenesis promotes the storage of TG by adipocytes under the control of insulin (Fig. 1.1). Insulin stimulates lipogenesis in both liver and WAT by increasing glucose uptake and activating lipogenic and glycolytic enzymes after its binding to the insulin receptor. In addition, insulin is believed to control lipogenesis via sterol element binding protein 1 (SREBP-1) activation, a transcriptional factor related to CHOL and fatty

acid metabolism, leading to lipid storage (Kersten, 2001). After a meal, dietary TG is hydrolyzed in the GI tract and absorbed in the form of non-esterified fatty acids (NEFA) and glycerol by the enterocytes. The enterocytes will re-esterify NEFA and glycerol into TG and phospholipids, forming the chylomicrons. Chylomicrons reach the bloodstream and subsequently the cell surface of metabolically important tissues (skeletal muscle, adipose tissue and liver), where they are hydrolyzed by lipoprotein lipase, regenerating NEFA and glycerol to be absorbed. NEFA is stored as TG by the adipocytes, while glycerol is exported to the liver to be used in the gluconeogenesis or glycolysis cycle (Boron & Boulpaep, 2005). In humans, very-low density lipoprotein (VLDL) and chylomicrons and are the main source of fatty acids delivered to adipocytes (Lafontan & Langin, 2009).

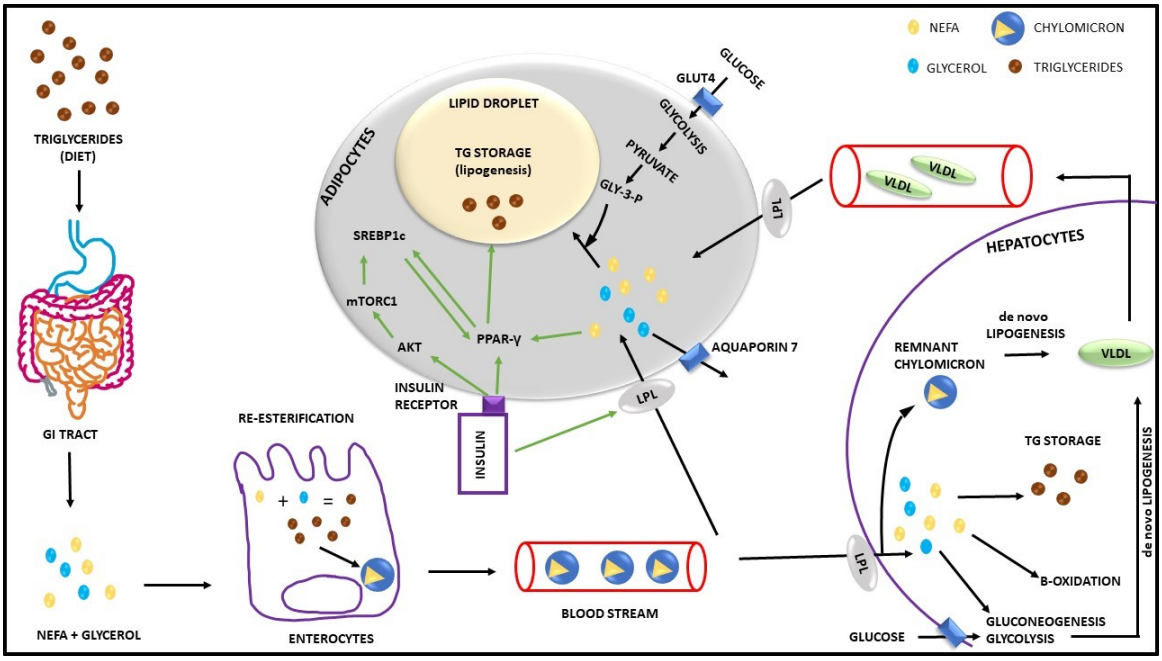


**Figure 1. 1 - Simplified lipogenesis pathway.** (See text for explanation). Abbreviations: GI, gastro-intestinal; NEFA, non-esterified fatty acids; TG, triglycerides; LPL, lipoprotein lipase; VLDL, very low density lipoprotein; Akt, protein kinase B; mTORC1, mammalian target of rapamycin complex 1 ; SREBP1c, Sterol regulatory element-binding transcription factor 1; GLY-3-P, glycerol-3-phosphate; GLUT4, glucose transporter 4; PPARγ, Peroxisome proliferator-activated receptor gamma.

The remnant chylomicrons go through *de novo* lipogenesis in the liver and are exported to the bloodstream in the form of VLDL (Boron & Boulpaep, 2005). *De novo* lipogenesis utilizes remnant lipoproteins and non-lipid products from glycolysis to produce TG and eventually VLDL, serving as molecules to transport lipids out of the liver back into the systemic circulation (Sanders & Griffin, 2016).

Lipolysis, on the other hand, is the opposite metabolic route allowing for the catabolism of lipids and the release of NEFA and glycerol from adipocytes thereby controlling plasma NEFA concentration (Fig. 1.2). It is highly activated during fasting to

supply the energy demand. Catecholamines are the major physiological regulators of lipolysis via PKA pathway; they are hormones that increase cAMP by binding to β-adrenergic receptors stimulating lipolysis, whereas hormones that decrease cAMP (notably insulin) inhibit the process. Insulin inhibits lipolysis by activating PI3K pathway and phosphodiesterase 3B (PDE3B), which in turn degrades cAMP preventing the phosphorylation of PKA and subsequent effects (Lafontan & Langin, 2009). Insulin was also shown to inhibit lipolysis via mTORC1-Egr1-adipose triglyceride lipase (ATGL) pathway in WAT of mice and in adipocyte cell line, suggesting an alternative route, probably independent of PKA inhibition to supress NEFA release into the bloodstream. Interestingly, this pathway may be activated by nutrients as well, such as amino acids (P. Chakrabarti et al., 2013). Another lipolysis stimulation pathway occurs via natriuretic peptides, leading to increase in cGMP activation of protein kinase G (PKG) and lipolytic enzyme activation (Morigny, Houssier, Mouisel, & Langin, 2016). However, this pathway is not the focus of this work.

Several lipases are involved in lipolysis but the main regulatory enzymes are adipocyte triglyceride lipase (ATGL), which mainly cleaves TG into diacylglycerol (DG) and hormone sensitive lipase (HSL), which also cleaves TG into DG but has a stronger affinity to convert DG into monoacylglycerol. (S. Wang et al., 2008).

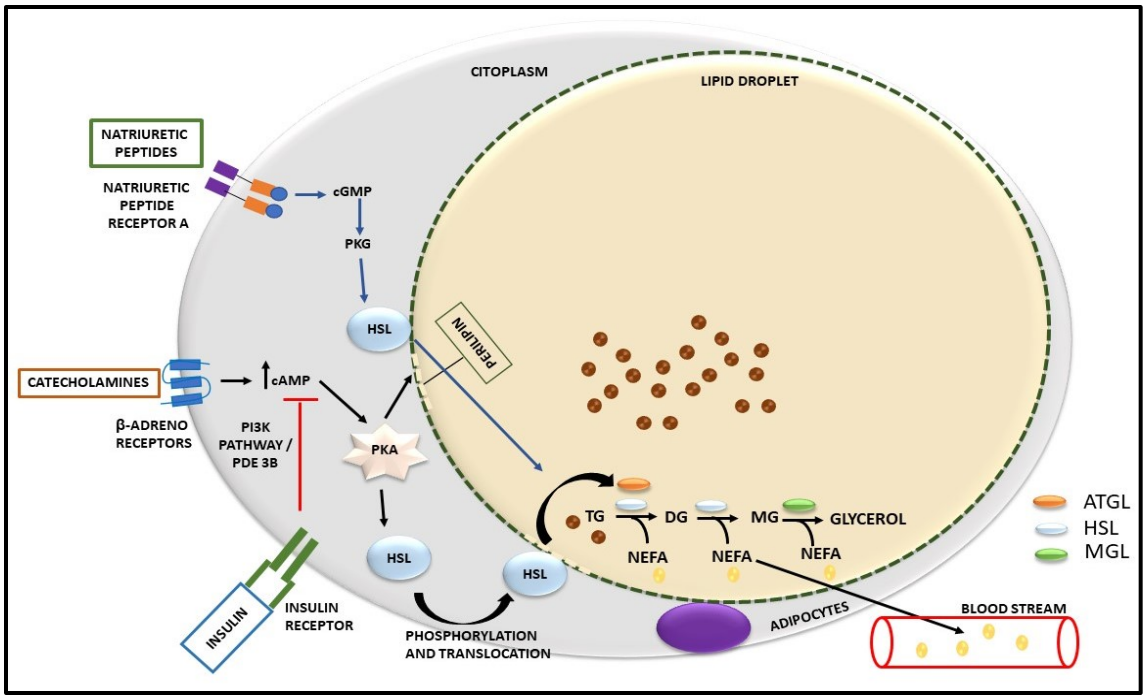


**Figure 1. 2- Simplified lipolysis pathway** (See text for explanation). Abbreviations: cAMP, Cyclic adenosine monophosphate; PKA, protein kinase A; HSL, hormone sensitive lipase; PI3K, Phosphoinositide 3-kinase; NEFA, non-esterified fatty acids; TG, triglycerides; DG, diglycerides; MG, monoglyceride; cGMP, Cyclic guanosine monophosphate; ATGL, adipose triglyceride lipase; MGL, monoglyceride lipase; PDE3B, phosphodiesterase 3B; PKG, protein kinase G.

In the classical PKA stimulated lipolysis pathway subsequent to lipolysis stimulation, the increase in cAMP leads to PKA phosphorylation. Activated p-PKA phosphorylates HSL, which is translocated to the lipid droplet surface (Langin, 2006). P-PKA also activates perilipin, which is a protein covering the lipid droplet's surface preventing the access to TG during basal state and exposing the lipid droplet during lipolytic activation. Once activated, perilipin causes a re-organization in the lipid droplet's surface, further exposing its content to cytoplasmic lipases (e.g. HSL) and facilitating the breakdown of TG into NEFA and glycerol; furthermore, perilipin is essential for HSL

translocation to the lipid droplet surface after p-PKA stimulation (Sztalryd et al., 2003). PKA-mediated phosphorylation of perilipin leads to release of an ATGL co-activator, also known as CG1-58 which forms a complex with ATGL, fully activating this enzyme, therefore, PKA is not the direct effector in ATGL activation (Morigny et al., 2016). Finally, NEFA and glycerol are exported from adipocytes to the bloodstream reaching the systemic circulation. In addition, NEFA can be re-esterified into TG within the adipocyte, a process that is stimulated by insulin and reduces NEFA in the circulation (Campbell, Carlson, Hill, & Nurjhan, 1992).

Lipolysis and lipogenesis imbalance leads to increased fat mass (obesity), lipid accumulation in non-adipose tissue and further development of metabolic diseases such as T2D (Lafontan & Langin, 2009). In fact, visceral adiposity is strongly related to metabolic diseases (Kang et al., 2017; Wanderley Rocha, Jorge, Braulio, Arbex, & Marcadenti, 2017); in addition, visceral fat is highly metabolically active and contributes to the circulating amount of NEFA, potentially affecting hepatic and peripheral insulin sensitivity. On the other hand, subcutaneous adipose tissue presents a better lipid storage capacity, preventing ectopic fat accumulation (Bjorndal, Burri, Staalesen, Skorve, & Berge, 2011; Goossens, 2017).

## 1.5 Insulin pathophysiology in MetS and T2D

### *1.5.1 Metabolic Syndrome*

The pathophysiology of MetS is not fully defined, and the syndrome is a consequence of genetic and environmental factors (Roberts, Hevener, & Barnard, 2013). While some believe that hyperinsulinemia and elevated NEFA are the underlying causes

of the syndrome (DeFronzo & Ferrannini, 1991; McCracken et al., 2018; Reaven, 1988), another theory points to obesity or overnutrition as the triggering factor of MetS (Grundy, 2015; Roberts et al., 2013). Regardless, insulin resistance is a key component of MetS. Insulin resistance is characterized by reduced glucose uptake by insulin sensitive tissues and impaired gluconeogenesis suppression by insulin (Roden, Petersen, & Shulman, 2017).

The hyperinsulinemia/NEFA theory suggests that excessive lipolysis leads to insulin resistance, which leads to the failure of insulin to inhibit lipolysis and creates a cycle that culminates in cardiovascular diseases (CVDs) and T2D (DeFronzo & Ferrannini, 1991; Groop, Bonadonna, Shank, Petrides, & DeFronzo, 1991; McCracken et al., 2018). In fact, MetS increases the risk for CVDs by 2-fold and for T2D by 5-fold (Alberti et al., 2009).

Physiologically, insulin stimulates cellular glucose uptake and regulates circulating amount of NEFA (Groop et al., 1991; Reaven, 1988). In normal conditions insulin has an antilipolytic effect in WAT and muscle. In the presence of insulin resistance, elevated circulating NEFA and glycerol are observed. This may lead to tissue NEFA uptake and lipid accumulation, exacerbating peripheral insulin resistance (Jocken et al., 2013). In addition, individuals with T2D have higher glycerol content and lower expression of HSL in muscle than obese but normal glucose tolerant subjects (Jocken et al., 2013). Furthermore, obese individuals have lower expression and activation rates of HSL than lean subjects, which contributes to dysfunctional adipose tissue (Large et al., 1999). Impaired insulin-stimulated glucose uptake due to insulin resistance alongside higher rates of lipid oxidation, which fail to stop hepatic glucose output and exacerbate peripheral insulin resistance, may be the primary causes of hyperglycemia in MetS (Reaven, 1988).

Obesity is a known risk factor for hypertension and excessive circulating NEFA/glycerol act in the liver as a substrate to produce atherogenic lipids (Grundy, 2016; Reaven, 1988), contributing to the development of hypertension and CVDs. In addition, hyperinsulinemia can result in hypertension via several mechanisms such as increased sodium/water reabsorption by the kidneys, activation of sympathetic nervous system (SNS), high intracellular calcium accumulation and stimulation of growth factors. The feedback between insulin and SNS maintains the insulin resistant state and high blood pressure (DeFronzo & Ferrannini, 1991).

The second theory relies on overnutrition eventually leading to abdominal fat accumulation and initiating the cycle previously mentioned. Abdominal fat is believed to be more responsive than lower body fat to catecholamines and its accumulation leads to higher lipolysis rates, thereby increasing the amount of NEFA reaching the liver and peripheral tissues and culminating in insulin resistance and hyperinsulinemia (Grundy, 2015; Kaplan, 1989; Roberts et al., 2013). However, improvement of metabolic parameters after bariatric surgery, in the presence of residual obesity (Clifton, 2011; Ikramuddin & Buchwald, 2011), suggests that obesity may be just a marker of MetS instead of the underlying cause (Grundy, 2015).

The crosstalk between all the conditions found in MetS is clear; however, the real trigger for the syndrome development is not well defined. More investigation is needed to define the primary cause of MetS and exclude manifestations that are simply markers of the syndrome.

### *1.5.2 Type 2 Diabetes*

In T2D, insulin resistance is usually detected before defects in β-cell function. In skeletal muscle insulin resistance reduces glucose uptake and glycogen synthesis (Shulman et al., 1990) contributing to elevated plasma glucose concentration. In fact, impaired insulin signaling (less Akt phosphorylation) reduces GLUT4 trafficking to the plasma membrane (Camps et al., 1992; Karlsson et al., 2005); in addition, elevated lipid content in muscle, found in diabetes, impairs insulin signaling and mitochondrial dysfunction, causing insulin resistance (Roden et al., 2017).

In WAT, GLUT4 translocation and fusion to the plasma membrane are reduced in T2D (Garvey, Huecksteadt, Matthaei, & Olefsky, 1988; Lizunov et al., 2013; Maianu, Keller, & Garvey, 2001). Furthermore, insulin resistance elevates lipolysis rates in adipose tissue contributing to elevated NEFA in the circulation (Lafontan & Langin, 2009) causing the cycle present in MetS as mentioned above.

In liver, endogenous glucose production is elevated in obese and T2D individuals (Basu, Chandramouli, Dicke, Landau, & Rizza, 2005; Boden, Chen, & Stein, 2001); however, the observed hyperglucagonemia (high plasma glucagon concentration) is not the only factor increasing glucose concentration (Basu et al., 2005); in fact, impaired insulin suppression of endogenous glucose production, increased fatty acids (Basu et al., 2005) and reduced glucose uptake (Boden et al., 2001) in those individuals highly contribute to hyperglycemia.

Individuals with T2D often present decreased insulin secretion or impaired β-cell function (Del Prato et al., 2017). The readily available granules docking and fusion to the plasma membrane are reduced in diabetic rats, decreasing the response of the first phase of

insulin secretion (Ohara-Imaizumi et al., 2004). Furthermore, β-cell mass is usually reduced by the time of T2D diagnosis (Yoneda et al., 2013). In addition, incretin effect is impaired in T2D, contributing to less insulin secretion via the amplification pathway (Knop et al., 2012; Toft-Nielsen et al., 2001). Incretin effect is the greater insulin secretion upon oral glucose/nutrient ingestion compared to intravenous injection (Elrick, Stimmler, Hlad, & Arai, 1964) due to the release of glucagon-like-peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) by the intestinal enteroendocrine cells (Siegel, Schulze, Schmidt, & Creutzfeldt, 1992).

In summary, hyperglycemia is a diagnostic characteristic of T2D caused by defects in insulin secretion and resistance to insulin actions in skeletal muscle, liver and adipose tissue. Several pathways involved in insulin signaling and glucose metabolism are affected by insulin resistance leading to further complications as discussed next.

## 1.6 The consequences of hyperglycemia and hyperlipidemia: diabetes complications

The regulation of plasma glucose levels is not only important to meet the body's energy demand, but also to control blood glucose concentration because high plasma glucose concentration (hyperglycemia) is toxic, termed *glucotoxicity*. Diverse cell types can regulate the amount of glucose entering them; however, those that have poor mechanisms of glucose uptake regulation suffer most from hyperglycemia, such as smooth muscle cells (Brownlee, 2005; Kaiser et al., 1993) leading to cell damage and death.

Hyperglycemia generates oxygen reactive species (ROS) and is the common link between different pathways leading to micro and macrovascular complications of diabetes,

such as protein kinase C activation and hexosamine pathway (changes in gene expression), polyol pathway (increases oxidative stress damage), and accumulation of advanced glycation end products (AGEs) (Brownlee, 2005; Huebschmann, Regensteiner, Vlassara, & Reusch, 2006). AGEs are formed due to non-enzymatic reduction of glucose, lipid and proteins irreversibly due to hyperglycemia, and lead to vascular remodeling, atherosclerosis, nephropathy, retinopathy and neuropathy (Huebschmann et al., 2006). In addition, glucotoxicity even in the presence of mildly elevated glucose concentration, if for prolonged periods, leads to impaired insulin secretion/β-cell function (Henquin et al., 2015). The macrovascular complications of diabetes are mainly related to cardiovascular diseases (atherosclerosis, coronary heart disease and stroke) and accounts for a great proportion of health care costs associated with diabetes (Fowler, 2008).

In the same manner, regulation of lipid plasma concentration is very important because lipotoxicity may occur. Lipotoxicity is characterized by the deleterious effect of lipid accumulation in non-adipose tissues, due to high NEFA and TG in circulation. The outcomes of this toxic environment are inflammation, mitochondrial dysfunction and insulin resistance (Engin, 2017). Lipid accumulation causes muscle insulin resistance (Roden et al., 2017) and β-cell dysfunction (Y. P. Zhou & Grill, 1994).

Glucose and lipid homeostasis are clearly important in the pathophysiology of MetS and T2D. The imbalance between lipid storage and oxidation is associated with obesity; therefore, adipose tissue is a possible target to prevent or reduce complications associated with metabolic diseases. Therefore, it is necessary to discuss its pathophysiology in greater detail.

## 1.7 Adipose tissue dysfunction

Although adipocytes are highly plastic, which means that they modify their morphology to adapt to the environment (e.g. expanding after taking up lipids when fat is plentiful) but at a certain point their buffer capacity plateaus and the tissue physiology is affected. In addition, impairment of adipocytes' hyperplastic capacity leads to hypertrophy and adipose tissue dysfunction (Goossens, 2017; Kim et al., 2015). WAT dysfunction can take different forms, from impaired hyperplasia or hypertrophy to dysregulation of adipokine secretion. Interestingly, pharmaceutical agents that target adipose tissue, the thiazolidinediones (TZD), reduce insulin resistance by normalizing some aspects of adipose tissue physiology and were considered a promising new therapy for diabetes until their side-effects (weight gain, edema, heart failure) (Rizos, Elisaf, Mikhailidis, & Liberopoulos, 2009) forced their recall from the market (FDA, 2000). Still, finding ways to improve adipose tissue function in diabetes remains an important goal. An overview of WAT dysfunction in insulin resistance is shown in Figure 1.3 with details following.

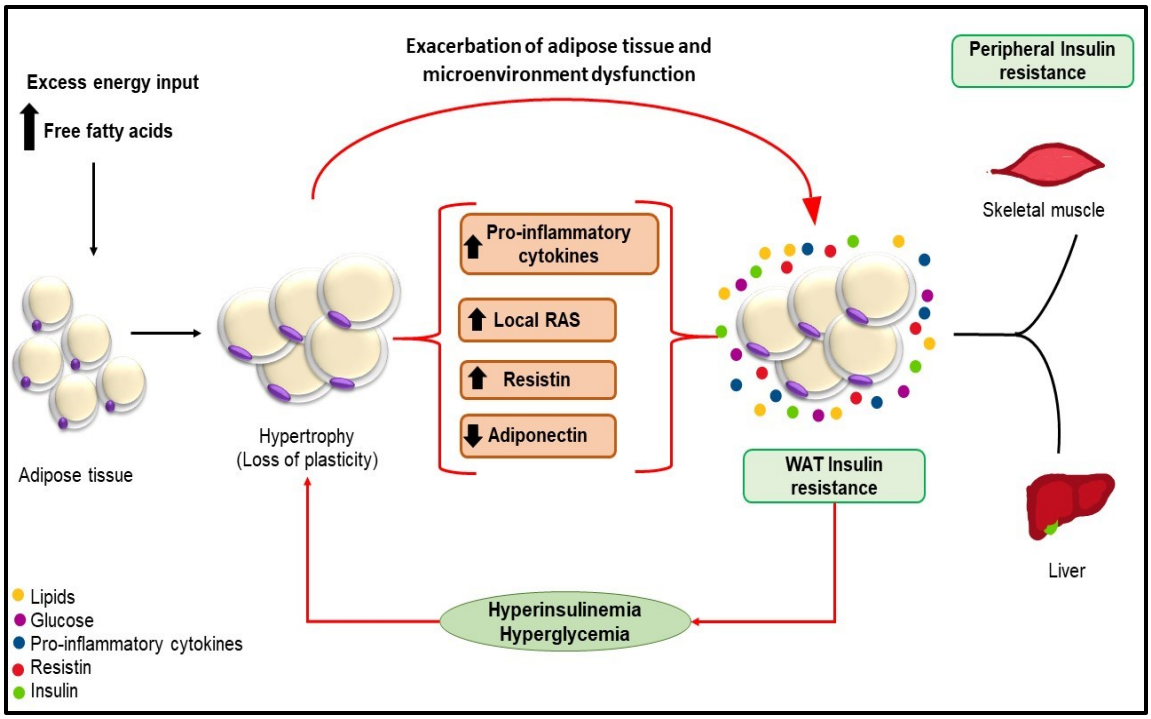


**Figure 1. 3- Central role of adipose tissue in insulin resistance.** After reaching its buffer capacity, adipose tissue becomes hypertrophied and dysfunctional leading to insulin resistance locally and peripherally. Abbreviations: RAS, renin-angiotensin system.

### *1.7.1 Dysfunctional adipocyte plasticity, lipolysis dysregulation and insulin resistance*

Excess energy intake causes fat accumulation and obesity is associated with altered adipocyte phenotype. In fact, larger adipocytes are found in obese children (Landgraf et al., 2015) and adults (Langin et al., 2005; Ryden et al., 2016). The number of adipocytes is believed to be determined in childhood and remains constant in adulthood (Spalding et al., 2008).

Decrease in NEFA delivery from adipose tissue to non-adipose tissues after lipolysis or impaired insulin suppression of lipolysis after a meal are thought to be the main reasons contributing to elevated NEFA in obesity (Lafontan & Langin, 2009). In addition,

alterations in lipase expression or function are found in T2D and obesity, suggesting WAT dysfunction (Jocken et al., 2007; Lafontan & Langin, 2009). In fact, lipolysis rate (over basal lipolysis) is reduced or blunted after adrenergic stimulation in obesity (Langin et al., 2005; Ryden et al., 2016). Interestingly, per cell number, both basal and adrenergic stimulated lipolysis rate are increased in obesity, which is due to the presence of larger adipocytes (Langin et al., 2005; Ryden et al., 2016). In addition, after weight loss upon bariatric bypass, the evaluation of lipolysis shows restored parameters and reduced cell size (Ryden et al., 2016).

However, WAT functionality is more important in preventing metabolic diseases development than changes in WAT mass. Despite higher volume of adipose tissue and elevated TG, CHOL, glucose and insulin plasma concentration in obese individuals, plasma NEFA concentration is similar compared to lean subjects during fasting state; however, in postprandial state, obese subjects have impaired lipid storage trafficking which contributes to ectopic fat accumulation (McQuaid et al., 2011).

Furthermore, adipocyte hypertrophy is more strongly correlated with insulin resistance than WAT mass. Changes in adipocyte morphology have a better correlation with insulin resistance than weight loss (Eriksson-Hogling et al., 2015). Although studies show the relationship between larger adipocytes and insulin resistance (Eriksson-Hogling et al., 2015; Varlamov et al., 2010) (Fig. 1.3), when examining small and large adipocytes from the same fat depot, basal NEFA/glycerol release is higher in large than in small adipocytes, but the cells have no differences in insulin signaling (Wueest, Rapold, Rytka, Schoenle, & Konrad, 2009). Interestingly, larger fat cells, from the same fat depot, present reduced intracellular glucose metabolism, most apparent under high glucose concentrations

(Czech, 1976). These results suggest that the adipose tissue dysfunction and insulin resistance crosstalk occurs at the intracellular level, rather than in the first steps of insulin signaling.

### *1.7.2 Dysfunctional adipokine release, inflammation and insulin resistance*

Obesity and WAT dysfunction are often associated with inflammation and insulin resistance (Fig. 1.3). In fact, WAT macrophage infiltration is found in obesity and is directly proportional to WAT mass and adipocyte size (Weisberg et al., 2003). The presence of macrophages, which are inflammatory cells, and higher levels of proinflammatory cytokines in WAT in obesity provide experimental support for the link between obesity, chronic inflammation and insulin resistance (Landgraf et al., 2015; Weisberg et al., 2003; H. Xu et al., 2003); however, Kim et al. (2015) showed that impaired insulin sensitivity in hypertrophied adipocytes occurs regardless of inflammation. The authors suggest that it happens, in part, due to defective actin remodeling, which affects GLUT4 trafficking to the membrane (Kim et al., 2015). This theory is in accordance with the classic study done by Czech (1976) showing that large adipocytes have a lower intracellular glucose metabolism.

Resistin and adiponectin are the main adipokines highly linked to obesity and insulin resistance. In fact, obese and MetS subjects have lower adiponectin plasma concentration than non-obese subjects (Arita et al., 1999; Bahia et al., 2006). On the other hand, obese but insulin sensitive subjects show lower adiponectin and resistin compared to lean insulin resistant subjects, suggesting that resistin may be related to insulin resistance rather than obesity. In addition, inflammatory markers are higher in lean insulin resistant

than in obese insulin sensitive subjects (Moscavitch et al., 2016). Nevertheless, the link between obesity, resistin and insulin resistance in humans is still controversial (X. Huang & Yang, 2016).

During childhood adiponectin concentration does not correlate with adipocyte size but it correlates with macrophage infiltration; while resistin is positively associated with obesity, adipocyte size and macrophage number (Landgraf et al., 2015). In animal models, elevation of plasma resistin is associated with obesity (Steppan et al., 2001) and is believed to lead to insulin resistance (X. Huang & Yang, 2016). Adiponectin stimulates adipocyte differentiation and maturation, and improves adipocyte insulin sensitivity *in vitro* (Y. Fu, Luo, Klein, & Garvey, 2005); adiponectin's anti-diabetic effects are believed to be due to stimulation of fatty acid oxidation in skeletal muscle, decreasing fatty acid influx in liver and eventually improving peripheral insulin sensitivity (Yamauchi et al., 2001).

In summary, several mechanisms account for adipose tissue dysfunction in obesity, including changes in morphology, intracellular signaling and extracellular environment. The evidence presented here indicates that a dysfunctional adipose tissue and a lipotoxic environment may be the underlying causes of insulin resistance in obesity. Nevertheless, further research is needed to understand the molecular basis of this crosstalk.

## 1.8 Renin angiotensin system (RAS)

### *1.8.1 RAS overview and hypertension*

While the above-mentioned mechanisms linking inflammation, WAT dysfunction and insulin resistance can explain many facets of disordered metabolism in MetS and T2D, the linkage between cardiovascular risk factors such as hypertension, obesity and T2D is

not fully explained. However, in recent years, the renin angiotensin system (RAS), which is mainly known for its action in the systemic regulation of blood pressure has been proposed as a linking mechanism (Putnam, Shoemaker, Yiannikouris, & Cassis, 2012). The molecules involved in this system are angiotensin I, angiotensin II, angiotensin II receptor type 1 (AT1R), angiotensin II receptor type 2 (AT2R), angiotensin converting enzyme (ACE) and angiotensin converting enzyme 2 (ACE2) (Chu & Leung, 2009; Shum et al., 2013).

RAS works in the whole body maintaining vascular tone and hydromineral balance (Sparks, Crowley, Gurley, Mirotsou, & Coffman, 2014). The systemic RAS can be summarized as follows: the liver (other tissues in minor proportions) produces the precursor angiotensinogen, which is cleaved by renin giving rise to Ang I. The cleavage of Ang I by ACE (which is produced mainly in the lungs) forms Ang II, which is the most active hormone in the system. Ang II exerts its effects in several tissues by high affinity binding to angiotensin II receptors (AT1R and AT2R) to regulate the blood pressure. A detailed review can be found in Sparks et al., 2014.

### *1.8.2 RAS, obesity and insulin resistance*

Besides the systemic RAS, studies show a local RAS in different tissues, for example, in WAT (Giacchetti et al., 2002; Sysoeva et al., 2017) and skeletal muscle (Agoudemos & Greene, 2005; Chai, Wang, Dong, Cao, & Liu, 2011). Modulation of RAS components affects these tissues' physiology and has a direct relationship with insulin resistance (Chu & Leung, 2009).

An important finding is that the use of blockers or stimulators of RAS components affects insulin sensitivity and glucose homeostasis, suggesting a relationship between hypertension and insulin resistance. For instance, after the onset of MetS (obesity, hyperglycemia and hypertension), AT1R chronic blockade improves glucose homeostasis and lipid metabolism, while reducing adipocyte size and fat mass. Those changes occurred without affecting pancreatic function (Rodriguez et al., 2018). On the other hand, insulin secretion was enhanced after ATIR blockade in another study (Chu, Lau, Carlsson, & Leung, 2006).

Despite the controversial effect of AT1R blockade on insulin secretion, AT1R blockade reduced adiposity and improved glucose tolerance and peripheral insulin sensitivity (Rodriguez et al., 2012; Rong et al., 2010; Shum et al., 2013). These effects were partially attributed to enhanced GLP1 receptor (GLP1R) expression in β cells and in higher plasma GLP-1 after AT1R blockade in one of the studies (Rodriguez et al., 2012), while others believe that improved WAT function is the reason for the changes observed (Rong et al., 2010; Shum et al., 2013). Interestingly, mice deficient in AT1R (at1r-KO) showed the same improvement in insulin sensitivity and reduction in lipid accumulation in liver and skeletal muscle after treatment with AT1R blockers, suggesting the presence of an AT1R-independent pathway to improve glucose homeostasis. The authors suggested PPARγ activation as one of the pathways (Rong et al., 2010).

RAS modulators may affect insulin signaling and glucose and lipid metabolism via AT2R as well. In mice, AT2R blockade had no effect on glucose, insulin, TG and CHOL plasma concentration, but impaired insulin signaling in WAT and liver without changes in skeletal muscle. This suggests that AT2R actions may be tissue-specific (Munoz et al.,

2017). In skeletal muscle, AT1R/AT2R balance is responsible for controlling blood flow and insulin action. The inhibition of AT2R had a more prominent effect than AT1R blockade in reducing glucose tolerance. Although both receptors are essential for an optimal vascular and metabolic action of insulin in muscle, higher AT2R abundance may provide better outcomes (Chai et al., 2011). In addition, chronic AT2R stimulation is believed to be essential for adipocyte differentiation *in vitro* and to restore adipose tissue morphology and function improving glucose tolerance (Ohshima et al., 2012; Quiroga et al., 2018; Shum et al., 2013).

Liver is also affected by RAS components modulation. Non-alcoholic fatty liver disease (NAFLD) is associated with T2D and an ACE inhibitor reduced HFD-induced NAFLD, improving insulin sensitivity and glucoregulation (Frantz, Penna-de-Carvalho, Batista, Aguila, & Mandarim-de-Lacerda, 2014). Another ACE inhibitor prevented the development of liver fibrosis and reduced hepatic steatosis improving lipid and glucose profile and leading to higher insulin sensitivity in obese rats (Toblli et al., 2008).

The expression of RAS components is higher in visceral than in subcutaneous WAT (Giacchetti et al., 2002), and is higher in obese and obese hypertensive subjects (Faloia et al., 2002; Van Harmelen et al., 2000) and in HFD-induced obese mice (Rahmouni, Mark, Haynes, & Sigmund, 2004); furthermore, WAT RAS overactivation induced local inflammation and peripheral insulin resistance in mice (Kalupahana et al., 2012). ACE inhibitors also improved insulin sensitivity by reducing adipocyte size, but not fat mass (Furuhashi et al., 2004).

Thus, an interplay between hypertension, obesity and insulin resistance exists, but no conclusive studies show the causal relationship between them. The crosstalk between

hypertension and insulin resistance may not be unidirectional and both share several factors and pathways, perhaps not being the cause and effect of each other (Hu & Stampfer, 2005). From what is known and based on the previous studies, adipose tissue seems to have a key role in the insulin sensitizing effects of RAS modulators.

## 1.9 Management of MetS and T2D

The first line of therapy for metabolic diseases is lifestyle changes, which include interventions on dietary patterns and physical activity to avoid progression of the symptoms (Diabetes Canada clinical practice guidelines, 2018). In fact, lifestyle interventions dramatically reduce the incidence of T2D in glucose intolerance individuals (Knowler et al., 2002). In addition, weight reduction in obese and overweight individuals has been positively correlated with lower risk of developing uncontrolled hypertension (Sabaka et al., 2017) and better renal function associated with improvement in other metabolic parameters such as fasting insulin and glucose levels (Straznicky et al., 2011).

With MetS development, most people will eventually rely on a lifetime polydrug therapy to treat the symptoms of T2D, hypertension and atherogenic dyslipidemia, and to avoid related complications. The down-side of polypharmacy includes reduced adherence to treatment, cost of treatment and drug interactions (Grundy, 2006). Another aspect to consider when managing a multi-factorial syndrome is the short and long-term risk. People with low short-term risk can rely on lifestyle changes during the first of years of treatment; however, if their long-term risk is high for one of the conditions (e.g., hypertension or diabetes) drug therapy may be included in the beginning (Eckel, Grundy, & Zimmet, 2005). In addition, drugs to alleviate one of the symptoms may worsen others, which is the case

for TZD. The management of MetS needs expert evaluation, therapy risk-benefit assessment and close follow-up to provide a better prognosis.

A novel approach to improve health and treat symptoms of disease is utilization of functional foods. Food-derived bioactive peptides have been shown potential in metabolic diseases management. The approach involving food-derived peptides, more specifically egg-derived peptides is discussed further in the next sections.

## 1.10 Food derived bioactive peptides / functional foods

Identifying natural products that can improve a disease state while exerting fewer side-effects is an exciting research trend. Many studies have explored the potential of biologically active peptides derived from food, which are referred to as functional food ingredients. In the absence of a common definition, The European Commission Concerted Action on Functional Food Science in Europe together with The International Life Sciences Institute Europe published a consensus document and defined functional foods as those that beyond their nutritional value can exert one or more physiological effects in the body in a manner that can improve health/well-being or reduce the risk of diseases ("Scientific concepts of functional foods in Europe-consensus document," 1999). These peptides are produced enzymatically or using fermentation under controlled conditions of pH and temperature.

### *1.10.1 Food-derived peptides and MetS conditions*

Several food-derived bioactive peptides have shown potential to manage MetS conditions. Soy-derived bioactive peptides for example, have properties against obesity and T2D in vitro and in vivo (Aoyama et al., 2000; Goto, Mori, & Nagaoka, 2013; Lammi, Zanoni, & Arnoldi, 2015; Lu et al., 2012; Roblet et al., 2014; Tsou, Kao, Lu, Kao, & Chiang, 2013). Milk, another food with several effects promoting health, generates peptides (such as IPP and VPP) that improve insulin signaling, glucose tolerance, and have adipogenic and anti-inflammatory properties in vitro (S. Chakrabarti, Jahandideh, Davidge, & Wu, 2018; S. Chakrabarti & Wu, 2015; Sawada et al., 2015). The work described in this thesis focusses on egg-white derived peptides and the potential of egg as a functional food.

### *1.10.2 Egg components and acceptability*

Eggs are composed of three main parts, the shell, the white and the yolk. The yolk, which has nutritional and organoleptic properties, is composed of water, proteins (lipovitellins, livetins, phosphovitins and low-density lipoproteins (LDL)), lipids and carbohydrates. While the egg white is mainly composed of water and proteins (mainly ovalbumin, ovotransferrin, ovomucoid, ovomucin, and lysozyme) (Chang, Lahti, Tanaka, & Nickerson, 2018).

One of the main concerns about consuming egg is the potential for allergenic reactions. Interestingly, studies have shown that after thermal or hydrolytic processes the allergenicity of egg parts is reduced, both in rodents and in humans (Ballmer-Weber et al., 2016; Watanabe et al., 2014). The main reason for the reduced allergenicity is the changes

in egg protein structures and reduced IgE-reactive epitopes (Chang et al., 2018; Watanabe et al., 2014). Nevertheless, cautious should be taken regarding this aspect because while reduction in allergic reactions is present, most of them are not allergy-free products (Lopez-Exposito et al., 2008). Egg has been widely used in the food industry, and due to its general acceptance regardless of religion or ethnicity, egg is a functional food with the potential to reach a wide range of the population.

### *1.10.3 Production, diversity and bioavailability of egg-derived peptides and hydrolysates*

Although several processes can be used to generate bioactive peptides, the most common method is enzymatic hydrolysis. Substrate specificity of enzymes generates peptides of different amino acid sequences and can be used to optimize the production of peptides with desired biological effects, as can be seen in tables 1 and 2. Nevertheless, the peptides are complex and the use of a purification step following hydrolysis is common (Roblet et al., 2014; Tsou et al., 2013; Zambrowicz et al., 2015). Alternatively, synthetic production of peptides (Lammi, Zanoni, Arnoldi, & Vistoli, 2016; Son, Chan, & Wu, 2018) can be used to obtain specific peptides and study their physiological action.

Several groups have used the whole hydrolysate instead of individual peptides when studying their effects (Garces-Rimon et al., 2016a; Gonzalez-Espinosa de los Monteros, Ramon-Gallegos, Torres-Torres, & Mora-Escobedo, 2011; Goto et al., 2013; Martinez-Villaluenga, Dia, Berhow, Bringe, & Gonzalez de Mejia, 2009; Ochiai, Kuroda, & Matsuo, 2014; Ochiai & Matsuo, 2014; Requena et al., 2017). In those cases, effects cannot be attributed to a specific peptide, because the enzymatic hydrolysis can generate myriad bioactive peptides, raising the possibility that the effect observed could be due a to

a combination of numerous peptides presented in the hydrolysate. Another variable of enzymatic hydrolysis process is the processing duration, which can impact both the peptide sequences and concentration in the hydrolysate (Garcés-Rimón, López-Expósito, López-Fandiño, & Miguel, 2016b).

It is worth mentioning that some of the enzymes used in hydrolysate production are not naturally produced by the human body, such as thermoase (Jahandideh et al., submitted), flavourzyme and neutrase (Garcés-Rimón et al., 2016b; Tsou et al., 2013), which means the peptides produced may not replicate those generated by the natural digestion process in human body. Even though some studies used enzymes that are naturally produced in humans, such as pepsin and pancreatin (Gonzalez-Espinosa de los Monteros et al., 2011; Lammi et al., 2016; Requena et al., 2017; Zambrowicz et al., 2015) there is also no guarantee that the desired peptides would be produced or stable after further GI digestion.

Due to the diversity of peptides obtained after enzymatic hydrolysis, multiple mechanisms of action of the peptides may influence outcomes. The length of the peptides can influence the absorption process in the gut (Miner-Williams, Stevens, & Moughan, 2014) and specific amino acids can have a greater influence in the interaction with enzymes, for example the regions of interaction between a soy peptide and the enzyme dipeptidyl peptidase-IV (DPP-IV) correlated with the presence of the amino-acids glutamine and arginine (Lammi et al., 2016).

There is little evidence that accounts for the mechanisms of action of the peptides and important questions remain unanswered. For instance, are the peptides absorbed intact, or can they initiate a cascade reaction by binding to receptors in the gut cells? Is the

integrity and stability of the peptides after GI digestion a requirement for them to exert their physiological effects?

### *1.10.4 Egg peptides digestion and absorption*

The concept that oligopeptides can be absorbed intact is still controversial and their bioavailability after oral administration is poorly understood (Ding, Wang, Yu, Zhang, & Liu, 2016). The main site of amino acid absorption is the small intestine; however, a significant amount of amino acids is found in the large intestine. Nevertheless, animal studies show a low degree of absorption in the large intestine (van der Wielen, Moughan, & Mensink, 2017).

Once ingested, peptides must overcome several barriers to reach their target organ or system. First, there is GI digestion by pepsin and trypsin, which hydrolyze the peptide into smaller amino acids sequences or free amino acids; second, the brush border peptidases act in the intestine, hydrolyzing the peptides one more time. If the peptide passes those barriers and is then absorbed, there are serum peptidases to overcome before its final action (Vermeirssen, Van Camp, & Verstraete, 2004).

Peptides and amino acids can be absorbed by different mechanisms when ingested orally: (1) paracellularly, (2) through passive diffusion, (3) by endocytosis or (4) by carrier mediated transport (Vermeirssen et al., 2004). Peptide movement by solute carrier transporters is the main mechanism responsible for amino acids absorption and Pept1 (SLC15A1) is the most studied peptide transporter in the distal colon. (van der Wielen et al., 2017).

The GI stability and absorption of peptides are influenced by several factors. For instance, small peptides are more stable than larger peptides, but both are rapidly degraded in humans and in simulated gastric fluid. Peptide stability is determined through peptide length, the amino acid sequence, and the presence of complex structures (e.g. cyclic structures, structural flexibility) (J. Wang, Yadav, Smart, Tajiri, & Basit, 2015).

To predict peptide bioavailability in humans, simulated GI digestion can be used in vitro (J. Wang et al., 2015). To study peptide transport in the human intestine, the transepithelial transport assay using Caco-2 cells is often used (Ding et al., 2016).

In 1984, Hara and colleagues compared the absorption of di- and tri- peptides with an equivalent mixture of single amino acids prepared from egg white hydrolysis using portal cannulation in rats. The egg white hydrolysate presented the same molecular weight range as the one used in the work presented here (~350 kDa). Small peptides presented a 70-80% higher absorption rate than the single amino acids in the mixture, when administered directly in the duodenum. This rate is suggested to be due to a lower affinity and competition of the amino acid for the same transporter (Hara, Funabiki, Iwata, & Yamazaki, 1984).

Ovalbumin- and ovotransferrin-derived peptides, TNGIIR and RVPSL respectively, presented high stability under simulated GI digestion (~94% remained intact). However, during transepithelial absorption by Caco-2 cells, a significant portion of the peptides was degraded (TNGIIR ~17%; RVSPL ~36%), likely by the brush border membrane peptidases. The use of DDP-IV inhibitor reduced RVSPL hydrolysis, showing the susceptibility of the peptide to other brush border peptidases (Ding et al., 2016; Ding, Wang, Zhang, & Liu, 2015). The authors showed evidence that the TNGIIR and RVSPL

mechanism of transport occurred paracellularly via tight junctions (Ding et al., 2016; Ding et al., 2015).

In another study, simulated GI digestion completely hydrolyzed RADHPFL, FRADHPFL and YAEERYPIL into their smaller respective peptides. When applied to the apical side of the cell culture chamber, some tested peptides (FRADHPFL, YAEER, YPI) were found intact on the basolateral side, except for these three: RADHPFL, RADHP and YAEERYPIL. This shows that small and large peptides can be absorbed intact by Caco-2 cells (Miguel et al., 2008). Interestingly, the antihypertensive activity of the large peptides in vitro was reduced after digestion (Miguel, Aleixandre, Ramos, & Lopez-Fandino, 2006). Since RADHP did not cross Caco-2 cells and it can exert antihypertensive effects in vivo (Miguel et al., 2006), the authors suggest that RADHP exerts its effects via receptors in the intestine epithelial cells. In contrast, YPI, which resisted the brush border peptidases hydrolysis, was absorbed intact and likely transported by the Pept1 transporter (Miguel et al., 2008).

Food and digestive matrix interfere with the peptides' bioavailability by affecting their permeation. A protein-rich food matrix both increased and slowed tri-peptide absorption, in contrast to the pure peptides (without the matrix)(Grootaert et al., 2017a). While YAEERYPIL and RADHPFL were absorbed intact by Caco-2 cells (Miguel et al., 2008),when tested in a simulated GI digestion, both were degraded, giving rise to their respective smaller peptides (Grootaert et al., 2017a). This suggests that they must resist the GI digestion to be absorbed intact.

Different peptides have different absorption mechanisms, and a single peptide can be absorbed through more than one mechanism. For instance, IRW, LKP and IQW are

partially resistant to the brush border peptidases and are absorbed intact by intestinal epithelial cells; moreover, the tri-peptides are absorbed both via PepT1 transporter and paracellularly (Bejjani & Wu, 2013; Q. Xu, Fan, Yu, Hong, & Wu, 2017). Presenting several variables accounting for their absorption and bioavailability, the study of each individual peptide is required for a complete understanding of their mechanism of action and future application in functional foods.

## 1.11 Egg-derived bioactive peptides and physiological pathways

Several studies have emphasized the potential of egg proteins in affecting different pathways *in vitro* and *in vivo* as was meticulously reviewed by (Liao, Fan, & Wu, 2018). Egg derived peptides and hydrolysate have shown antioxidant (Nimalaratne, Bandara, & Wu, 2015; Noh & Suh, 2015; Yoo, Bamdad, Gujral, Suh, & Sunwoo, 2017), antimicrobial (Carrillo, Lucio, Gaibor, Morales, & Vasquez, 2018), anti-inflammatory (W. Huang et al., 2010; Meram & Wu, 2017; Son et al., 2018), ACE-inhibitor/antihypertensive (Abeyrathne, Lee, & Ahn, 2013; Eckert et al., 2014; Jahandideh et al., 2016; Majumder et al., 2013; Z. Yu et al., 2017). The egg peptide and hydrolysate effects against obesity and type 2 diabetes are discussed next.

### *1.11.1 In vitro effects of egg-derived peptides and hydrolysates in obesity and type 2 diabetes*

A summary of *in vitro* studies and identified peptide sequences are provided in Table 1.1. Multiple metabolic pathways in several organs are involved in glucoregulation.

One possibility to help manage diabetes is inhibition of intestinal α-glucosidase, which is an effective method to delay carbohydrate absorption (Zhipeng Yu et al., 2011) and reduce blood glucose concentrations. Peptides obtained after pepsin hydrolysis of egg white (EW) exhibited α-glucosidase IC50 values ranging from 365 to 1694 μg/mL (Zambrowicz et al., 2015), while peptides obtained from alcalase hydrolysis of egg yolk yielded IC50 values ranging from 23 to 40 μmol/L (Zhipeng Yu et al., 2011).

Beside α-glucosidase inhibition, the peptides from EW exerted multiple activities, for instance, ACE-inhibitory capacity with IC50 ranging from 9 to 27 μg/mL and DPP-IV-inhibitory activity with IC50 from 223 to 1402 μg/mL. The only exception was the peptide YIEAVNKVSPRAGQPF, which did not present either α-glucosidase or DPP-IV inhibitory activity (Zambrowicz et al., 2015). The results suggest that egg peptides can potentially exert more than one physiological effect. Multiple activities exerted by the egg white hydrolysate (EWH) were found in other studies using cell lines as well (Garcés-Rimón et al., 2016b; Jahandideh, Chakrabarti, Davidge, & Wu, 2017).

EWH obtained with different enzymes (Table 1.2) exerted concomitantly anti-inflammatory, antioxidant, hypocholesterolemic, DPP-IV- and ACE-inhibitory activity (Garcés-Rimón et al., 2016b). The EWH derived from pepsin and peptidase-mediated hydrolysis had the highest potential against disorders associated with MetS such as hypertension, obesity and T2D, presenting IC50 against DPP-IV of <10 mg protein/mL and against ACE ranging from 47 to 151 μg/mL (Garcés-Rimón et al., 2016b).

In the 3T3-L1 adipocyte cell line, thermoase + pepsin-prepared EWH not only sensitized the cells to insulin action but also mimicked insulin signaling. The EWH stimulated adipocyte differentiation by enhancing PPARγ and C/EBP-α expression, which

led to enhanced adiponectin release and intracellular lipid accumulation. Moreover, these EWH enhanced the phosphorylation of proteins involved in the insulin signaling pathway, such as extracellular signal regulated kinase 1/2 (ERK 1/2), IRS-1, IRβ and Akt (Jahandideh et al., 2017). In adipocytes, the same EWH also presented anti-inflammatory properties by reducing cyclooxygenase-2 (COX-2) expression and C-Jun phosphorylation induced by tumor necrosis factor-α (TNF-α) (Jahandideh et al., 2017). Thus, the effect of thermoase + pepsin-prepared EWH in 3T3-F442A cells is exerted via insulin receptor and downstream proteins in the insulin signaling pathway. The adipogenic effect observed was partially mediated by PPARγ, because the hydrolysate upregulated PPARγ abundance *in vitro* (Jahandideh et al., 2017). Eleven peptides were identified from the previous hydrolysate and all of them enhanced PPARγ abundance; however, only two of them presented a significant increase (Jahandideh, Liu, & Wu, 2018). In macrophages, no effects were observed regarding TNF-α using peptidase or pepsin or flavourzyme EWH, but peptidase-prepared EWH reduced interleukin-6 (IL-6) after lipopolysaccharides stimulation (Garcés-Rimón et al., 2016b).

An improvement in insulin sensitivity was also observed in a muscle cell line exposed to EW peptides. IRW, a peptide from egg ovotransferrin improved insulin resistance induced by angiotensin-II in skeletal muscle cells (Son et al., 2018). The peptide reversed the impaired insulin signaling and glucose uptake by normalizing phosphorylation of the serine307 residue in IRS and increasing Akt phosphorylation (Ser473), which contributed to increased translocation of GLUT4 to the plasma membrane. It was shown that these effects were exerted partly by reducing AT1R expression and ROS production

(Son et al., 2018). In contrast, IQW and LPK egg white-derived peptides only exhibited antioxidant activity (Son et al., 2018).

Although anti-diabetic activity is exerted by specific peptides, others presented low or no activity as antidiabetic agents (Garcés-Rimón et al., 2016b; Son et al., 2018; Zhipeng Yu et al., 2011; Zambrowicz et al., 2015), a fact that was attributed to their different amino acid sequences once they all were tested under the same conditions (Garcés-Rimón et al., 2016b; Son et al., 2018). This fact indicates that the effects observed were due to the presence of specific peptides; however, there is a lack of experiments studying the relationship between the amino acid sequence in the peptides and their actions.

In summary, in vitro studies show that EWH or peptides derived from EW and egg yolk can exert multiple biological activities, including antidiabetic, by inhibiting enzymes such as DDP-IV and α-glucosidase or improving insulin sensitivity or signaling. However, the peptide amino acid sequence is important in determining the peptides' ability to act as antidiabetic agents. Therefore, there is a need for more in vitro experiments to specifically identify the interaction between the peptides, their amino acid sequence and the targets involved in the insulin signaling pathway.

### *1.11.2 In vivo effects of egg-derived peptides and hydrolysates in obesity and type 2 diabetes*

*In vivo* EWH presents multiple biological activities as demonstrated in Table 1.2. All these in vivo studies were done in rodents and the specific peptides in EWH were not reported. One study found reduced fasting insulin levels after treatment with pepsin-prepared EWH (Garces-Rimon et al., 2018), one observed a reduction in blood glucose

concentration with protease-prepared EWH (Ochiai et al., 2014) and both found reduced homeostasis model assessment of insulin resistance (HOMA-IR) (Garces-Rimon et al., 2018; Ochiai et al., 2014). Another three studies reported no changes in blood glucose levels with EWH obtained from protease and alcalase hydrolysis (Ochiai & Matsuo, 2014; Ochiai et al., 2017; Y. Wang et al., 2012). Serum leptin concentrations were not statistically different (Ochiai et al., 2017; Y. Wang et al., 2012), and reduction or no changes were observed in plasma adiponectin levels (Garces-Rimon et al., 2016a; Ochiai et al., 2014).

Alongside enlarged adipose tissue, ectopic fat accumulation can lead to insulin resistance and consequently T2D. Analysis of the lipid content in liver and muscle, and total body fat percentage in rats showed reduced values after protease- and pepsin-prepared EWH treatment (Garces-Rimon et al., 2016a; Ochiai et al., 2014; Ochiai & Matsuo, 2014). The steatotic state was improved (reduced size and number of fat vesicles), but no histological changes were seen in the adipose tissue with the pepsin-prepared EWH groups presenting similar adipocyte size as the obese control (Garces-Rimon et al., 2016a). Stearoyl-CoA desaturase (SCD) is an enzyme involved in fat synthesis and responsible for converting a saturated fatty acid to its respective unsaturated fatty acid (Ochiai et al., 2014). The SCD index is the ratio between those fatty acids and is related to obesity and insulin resistance. Dietary supplementation with protease-prepared EWH decreased SCD index in serum, muscle and liver in rodents (Ochiai et al., 2014; Ochiai & Matsuo, 2014; Ochiai et al., 2017).

Several hypotheses were tested in an attempt to elucidate the mechanisms responsible for reducing fat accumulation; for instance, SCD-1 is an enzyme essential in fat synthesis and because the abundance of lipogenic enzymes such as lipoprotein lipase

(LPL) and fatty acids synthase (FAS) were not altered by EWH, the decrease in non-adipose tissue lipid content was attributed to the reduced SCD index (Ochiai et al., 2014). Garcés-Rimón et al. postulate that the reduction in liver fat accumulation could be due to the ability of pepsin-prepared EWH to stimulate free fatty acids (FFA) oxidation in the hepatocytes but this hypothesis has not been tested (Garces-Rimon et al., 2016a). Another possibility is that the reduction in fat accumulation occurred due to increased fat excretion. Indeed, two studies reported increased excretion of CHOL and/or TG and total bile acids in feces after protease-prepared EWH treatment (Ochiai et al., 2014; Ochiai et al., 2017). In serum, reduction of CHOL or TG and/or FFA was observed (Garces-Rimon et al., 2016a; Ochiai & Matsuo, 2014; Ochiai et al., 2017). However, no improvement in serum lipid profile was seen in another two studies (Ochiai et al., 2014; Y. Wang et al., 2012). An interesting corollary finding was that protease-prepared EWH increased muscular mass while decreasing fat accumulation, although the mechanism by which the hydrolysate acts remains unclear (Ochiai & Matsuo, 2014).

A study of the gut microbiota revealed that pepsin-prepared EWH treatment improved dysbiosis in obese rats; furthermore, short chain fatty acid (SCFA) and lactate concentrations in feces were lower compared to the obese group (Requena et al., 2017). SCFAs are produced by gut microbiota through fermentation of dietary fiber, carbohydrates and peptides and are shown to improve glucose homeostasis and insulin sensitivity in rodents (Gao et al., 2009); in addition, increased fecal SCFA content is found in obese human subjects (Schwiertz et al., 2010). Mechanisms that could explain the lower fecal SCFA in the pepsin-prepared EWH-fed group include maintenance of intestinal microbiota homeostasis or prevention of absorptive dysfunction by EWH; nevertheless,

Requena et al. hypothesized that the change in microbiota occurred secondary to peptide absorption, with their actions on target tissues as antioxidant and anti-inflammatory agents leading to modulation of the gut microbiota (Requena et al., 2017) but there is as yet no evidence for this hypothesis.

Anti-inflammatory and antioxidant activity can contribute towards obesity and diabetes management (Requena et al., 2017). In two studies *in vivo*, treatment with pepsin- and alcalase-prepared EWH reduced TNF-α in plasma and kidney and malondialdehyde levels in plasma and urine indicating antioxidant properties (Garces-Rimon et al., 2016a; Y. Wang et al., 2012); these results are compatible with those observed *in vitro* previously mentioned (Table 1.1).

Only one study investigated the EWH effect on β-cell function and compared to their fatty control, isolated β-cells of the pepsin-prepared EWH treated animals responded secreting insulin after a higher concentration of glucose stimulation (8mM and 10mM of glucose, respectively). The authors suggest a lower insulin resistance in the pepsin-prepared EWH group (Garces-Rimon et al., 2018).

When not treated, diabetes can lead to several complications including nephropathy. Although NWT-03, an alcalase-prepared EWH, exerted in vitro DPP IV-inhibitory activity, *in vivo* it was not efficient in improving the diabetic state; however, the treatment reduced renal injury development and albuminuria in T2D rats (Y. Wang et al., 2012). When compared with vildagliptin a currently used DPP-IV inhibitor, both NWT-03 and vildagliptin exerted renal protection effects but only vildagliptin increased GLP-1 levels. Therefore, it is believed that NWT-03 and vildagliptin can act via similar mechanisms but independently of their DPP-IV inhibitory activity (Y. Wang et al., 2012).

It is worth noting that when administered in a single dose, protease-prepared EWH did not alter lipid profile, inhibit pancreatic lipase or slow food transit (Ochiai et al., 2017). Interestingly, both protease-prepared EWH and EW prevented fat accumulation and increased muscle mass, but EW increased fat excretion compared to EWH (Ochiai & Matsuo, 2014; Ochiai et al., 2017).

To summarize, EWH presented antidiabetic properties *in vivo*, reducing ectopic fat accumulation in liver and muscle, which can enhance insulin sensitivity, and increasing fat excretion, which reduces absorption of calories and could contribute to weight loss. It also protected against diabetes complications (nephropathy), but little or no change was observed regarding blood glucose, adiponectin or insulin levels and regarding inhibition of DPP-IV. The discrepancies in the results observed *in vivo* could be attributed to the difference in the physiological background of the animals used but is more likely due to variation in the mixture of peptides present in the hydrolysates. Furthermore, the studies suggest that bioactive peptides present in the EWH were responsible for at least part of the effects observed; nevertheless, no measurement of the peptides in plasma, identification of those peptides or any other specific assay was conducted. There is a gap in the literature to explain the mechanism of absorption and action of these peptides.

**Table 1. 1-** *In vitro* studies of egg-derived hydrolysates/peptides and their effects related to diabetes and obesity (adapted from de Campos Zani, Wu, & Chan, 2018).

| | Aims | Hydrolysis | Main Findings | Additional assays | Peptides |
|---|---|---|---|---|---|
| **Egg yolk specific peptides**<br>**Enzymatic activity** | | | | | |
| (Zambrowicz et al., 2015) | Investigate multiple biological properties of peptides | Pepsin (120 min) | Three out of four peptides inhibited ACE, α-glucosidase and DPP-IV activity. The peptides presented antioxidant and ion chelating activity. | **DPPH - radical scavenging**<br>All peptides tested presented radical scavenging properties (from 1.5 to 2.3 µMTroloxeq/mg) | YINQMPQKSRE<br>YINQMPQKSREA<br>VTGRFAGHPAAQ<br>YIEAVNKVSPRAGQPF |
| **Egg white specific peptides**<br>**Enzymatic activity** | | | | | |
| (Zhipeng Yu et al., 2011) | Investigate the inhibitory activity of hydrolysates against α-glucosidase and α-amylase and identify peptides | Alcalase (180 min) | Peptides from EW inhibit α-glucosidase but not α-amylase. | N/A | **Ovotransferrin**<br>RVPSLM TPSPR DLQGK AGLAPY<br>**Ovalbumin**<br>RVPSL DHPFLF HAGN WIGLF |
| **Egg white specific peptides**<br>**Cell culture** | | | | | |
| (Garcés-Rimón et al., 2016b)<br>264.7 RAW macrophages | Investigate multiple biological properties of related to the metabolic syndrome | Alcalase<br>Flavourzyme<br>Neutrase<br>Trypsin<br>Pepsin<br>Pancreatin<br>Peptidase Promod 144P<br>(0, 2, 4, 8, 12, 24, 36 and 48 hours) | Pepsin hydrolysate: ↓ACE.<br>Peptidase hydrolysate:<br>↓ ROS, CHOL and IL-6. | **Peptidase hydrolysate (24 h)**<br>**Hypocholesterolemic activity**<br>0.259 ± 0.01(mmol bound/mg protein)<br>**ORAC test**<br>1099.9 ± 0.6 (µmol Trolox/g protein) | **Peptidase hydrolysate (24 h)**<br>LPDEVSG DDNKVED GVDTKSD IESGSVEQA GGLVVT |
| | | | | **Pepsin hydrolysate (8 h)**<br>**Hypocholesterolemic activity**<br>0.154 ± 0.011 (mmol bound/mg protein)<br>**ORAC test**<br>574.2 ± 4.0 (µmol Trolox/g protein) | **Pepsin hydrolysate (8 h)**<br>FRADHPPL FSL SALAM YQIGL RADHPFL IVF YAEERYPIL YRGGLEPINF RDILNQ ESIINF |

| | | | | | |
|---|---|---|---|---|---|
| (Jahandideh et al., 2017)<br><br>3T3-F442A Pre-adipocyte | Investigate the effect of hydrolysate on differentiation, insulin signaling and inflammation markers in pre-adipocytes | Thermoase (90 min) + Pepsin (180 min) | ↑ intracellular lipid accumulation, adiponectin levels. ↑PPARγ and C/EBPα. ↑ p-ERK 1/2, p-IRβ and p-IRS-1. ↓ COX-2 and TNF-α - mediated C-Jun phosphorylation. ↑ p-AKT after insulin treatment. | PPARγ expression enhanced in dose-dependent manner with EWH at 2.5, 5 and 10 mg/mL | ERYPIL<br>VFKGL<br>WEKAFKDED<br>QAMPFRVTEQE |
| (Son et al., 2018)<br><br>Rat L6 myoblasts | Study the effect of specific ACE inhibitory peptides on insulin resistance induced by Ang-II and their mechanisms of action in muscular cells | N/A | IRW prevented the decrease in glucose uptake induced by Ang-II, normalized serine phosphorylation of IRS and GLUT4 expression and ↑ p-AKT. IRW ↓ AT1R, no effect on AT2R; ↓ ROS and NADPH activity. IQW and LPK peptides had anti-oxidant but no other actions. | N/A | **Ovotransferrin**<br>IRW<br>IQW<br>LPK |
| (Jahandideh et al., 2018)<br><br>3T3-F442A pre-adipocytes | Identify EWH bioactive peptides with adipogenic potential | Thermoase (90 min) + Pepsin (180 min) | All the identified peptides enhanced PPARγ; however, only QAMPFRVTEQE and VFKGL presented significant results - ↑PPARγ | N/A | WEKAFKDED ERYPIL<br>QAMPFRVTEQE<br>WEKAFKDEDTQA<br>ILQMCS ERYDDES<br>ASEKMKILEL KILEL<br>VFKGL IVFKGL<br>YRGGLEPINF |

Abbreviations: ACE, angiotensin converting enzyme; Ang-II, Angiotensin II; DPP IV, Dipeptidyl peptidase IV; EW, Egg white; IRS-1, Insulin receptor substrate 1; IRS, Insulin receptor; IRβ, Insulin receptor β; COX-2, cyclooxygenase 2; PPARγ, peroxisome proliferator associated receptor gamma; C/EBP-α, CAAT/ enhancer binding protein alpha; AKT, protein kinase B; ERK1/2, Extracellular signal regulated kinase 1 /2; TNF-α, Tumor necrosis factor alpha; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ROS, Reactive oxygen species; CHOL, Cholesterol; IL-6, Interleukin 6; GLUT4, Glucose transporter 4; AT1R, Angiotensin II type 1 receptor; AT2R, Angiotensin II type 2 receptor.

**Table 1. 2-** *In vivo* studies of egg-derived hydrolysates/peptides and their effects related to diabetes and obesity in rodents (adapted from de Campos Zani et al., 2018).

| **Egg white hydrolysate Studies in rodents** | **Aims** | **Hydrolysis** | **Treatment details** | **Food intake and body weight (BW)** | **Blood/ Feces / urine analysis** | **Tissue analysis** | **Main Findings** |
|---|---|---|---|---|---|---|---|
| (Y. Wang et al., 2012)<br><br>Zucker obese rats | Measure effect of hydrolysate NWT-03 on renovascular damage | Alcalase (6 h) | Aqueous **NWT-03** (1g/kg/day)<br>15 weeks | **Food intake** - not given<br>**BW** - no effect | No effect on blood glucose, insulin, HBA1C, cholesterol and FFA levels.<br>↑ **GLP-1** only by VIL<br><br>**URINE:** Reduced MDA levels and decreased albuminuria | **KIDNEY -** Reduced inflammatory interleukins (IL-1β, IL-13) and TNF-α. Improved FGS, reduced expression of α-SMA and increased TXA2R expression. | No changes in the diabetic profile of the rats; renovascular damage reduced by NWT-03 treatment |
| (Ochiai & Matsuo, 2014)<br><br>Wistar rats | Investigate the effect of EW and EWH on fat metabolism and TG content in non-adipose tissues | Protease (duration not specified) | **Casein** (297 g/kg)<br>**EWH** (394 g/kg)<br>**EW** (286 g/kg)<br>8 weeks | **Food intake**<br>EWH ↓, EW ↓↓<br><br>**BW**<br>EW ↓ | **EWH vs casein-** ↓ TG, ALP activity and FFA by EWH.<br>**EW vs EWH-** ↓ HDL-CHOL, FFA and ↑ total- CHOL by EWH.<br><br>**FECES**<br>**EWH vs casein -** ↑CHOL excretion by EWH.<br>**EW vs EWH-** ↑ TG, TBA and CHOL excretion by EW. | **EWH vs Casein -** Similar results in all parameters, except for ↓ fat mass. ↑ mass; ↓ SCD index, TG content and G6PDH activity (MUSCLE). ↓CHOL, TG and SCD index (LIVER).<br>**EW vs EWH -** Similar results in all parameters, except for ↑ mass and ↓ SCD (MUSCLE) ↓ SCD (LIVER) by EW. | EW and protease EWH reduced fat in adipose and non-adipose tissues Inhibited enzymes involved in lipogenesis and increased muscular mass and lipid excretion |
| (Ochiai et al., 2014)<br><br>Goto-Kakizaki rats | Feeding trial with EWH to study fat and glucose diabetic or normal rats | Protease (duration not specified) | **Casein** (200 g/kg) And **EWH** (267 g/kg)<br>6 weeks | **Food intake** Not different<br>**BW**<br>↓ by EWH | Glucose, HOMA-IR, SCD Index - ↓<br><br>**No difference** between any other parameters tested. | **MUSCLE -** ↓ TG and SCD. LPL, FAS and G6PDH similar.<br>**LIVER -** TG similar, ↓ SCD index.<br>Liver, adipose tissue and muscle similar weight. | Improved blood glucose levels and HOMA-IR, but not insulin secretion. Reduced TG in muscle and decreased lipid accumulation in tissues |
| Wistar rats | | | **Casein** (200 g/kg) And **EWH** (267 g/kg)<br>6 weeks | **Food intake and BW**<br>not different | **No difference** in any of the parameters tested. (glucose, insulin, HOMA-R, HOMA-P, TG, NEFA, TC, HDL-CHOL, non-HDL-CHOL, adiponectin and SCD index) | **MUSCLE -** ↓ SCD but LPL, FAS and G6PDH similar<br>**LIVER** - TG similar, ↓ SCD index.<br>Liver, adipose tissue and muscle similar weight | Reduced lipid content in muscle |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (Garces-Rimon et al., 2016a)<br><br>Zucker obese rats | Demonstrate EWH effects related to obesity, lipid metabolism, inflammation and oxidative stress | Pepsin (8 or 14 h) | Aqueous EWH (750 mg/kg/day) 12 weeks | No difference in food intake and BW regardless of the hydrolysate | ↓TNF-α, FFA and adiponectin, MDA. No changes in blood TG and CHOL. | **ADIPOSE TISSUE -** ↓ weight but no changes in histology.<br>**LIVER -** ↓ steatosis, ↑ GSH. Similar kidney and liver weight.<br>Longer duration of hydrolysis negated effects. | Reduced fat accumulation improved hepatic steatosis and dyslipidemia. Decreased inflammatory and oxidative stress markers in plasma |
| (Ochiai et al., 2017)<br><br>Wistar rats | Study the effect of EW and low allergenic EWH on fat accumulation | Protease (duration not specified) | **Equicaloric Diets**<br>**Casein** (297 g/kg)<br>**EWH** (394 g/kg)<br>**EW** (286 g/kg)<br>8 weeks | No difference in food intake and body weight between the three groups. | **EWH vs Casein**<br>↓, total CHOL, ALP. Similar glucose, TG, NEFA, HDL-CHOL, non-HDL-CHOL, HOMA-β and insulin.<br>**EW vs EWH**<br>Similar results in all parameters.<br><br>**FECES**<br>**EWH & EW vs Casein**<br>↑ TG, CHO and TBA | **EWH vs Casein-** Similar results in all parameters, except for ↓ weight, TG and NEFA, SCD index (LIVER). ↓ TG (MUSCLE)<br><br>**EWH vs EW-** Similar results in all parameters, except for ↑ G6PDH activity (muscle), SCD (adipose tissue) ↓ FAS (liver) in EWH. | Reduced fat accumulation in non-adipose tissues, reduced intestinal absorption of lipid by increasing lipid excretion. Similar results as EW, however EWH was less allergenic |
| (Requena et al., 2017)<br><br>Zucker obese rats | Observe the effect of EWH on the gut microbiota of rats | Pepsin (8 h) | Aqueous EWH (750 mg/kg/day) 12 weeks | **Food intake**<br>N/A<br>**BW**<br>no difference. | **FECES**<br>↓ lactate and SCFA. *Lactobacillus/ Enterococcus* and *C. leptum* similar to lean control. | **N/A** | Partially reverted dysbiosis present in Zucker obese rats |
| (Garces-Rimon et al., 2018)<br><br>Zucker obese rats | Observe the effect of EWH in glucose metabolism related complications | Pepsin (8 h)<br><br>Peptidase (24 h) | Aqueous EWH (750 mg/kg/day) 12 weeks | **Food intake**<br>N/A<br>**BW**<br>no difference. | **Pepsin EWH-** ↓ insulin, HOMA-IR, HOMA-β and ↑QUICKI compared to obese control. | **PANCREAS –** Enlarged islets by peptidase hydrolysate compared to obese control. | Pepsin EWH rescued insulin secretion after glucose stimulation and improved insulins sensitivity |

Abbreviations: EWH, Egg white hydrolysate; FFA, free fatty acids; MDA, Malondialdehyde; EW, Egg white; TG, Triglyceride; CHO, Cholesterol; ALP, Alkaline phosphatase; TBA, Total bile acids; SCD, Stearoyl CoA desaturase; NEFA, Non esterified fatty acids; ; FGS, Focal glomerulosclerosis; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; G6PDH, Glucose 6-phosphate dehydrogenase; LPL, Lipoprotein lipase; FAS, Fatty acid synthase; TNF-α, Tumor necrosis factor alpha; α-SMA, Anti-a-smooth muscle actin; VIL, Vildagliptin; HOMA-R, homeostasis model assessment of insulin resistance; HOMA-β, Homeostasis model assessment of insulin secretion; GSH, Reduced Glutathione; HBA1C, Glycated hemoglobin A1C; GLP-1, Glucagon like peptide-1; TXA2R, Thromboxane A2 receptor; SCFA, Short chain fatty acids; MetS, Metabolic syndrome; QUICKI, Quantitative index of insulin sensitivity.

## 1.12 Highlights of previous work by our group on EWH in an insulin resistance model and adipocytes

Work done by our group has been focusing on EWH effects in a model of HFD-induced insulin resistance and adipocyte cell culture. Feeding EWH improves glucose tolerance, insulin signaling and systemic inflammation, and enhances PPARγ abundance (Jahandideh et al., submitted). *In vitro*, EWH mimics insulin action, reduces production of inflammatory markers and has adipogenic potential (Jahandideh et al., 2017; Jahandideh et al., 2018) but these experiments were performed in a cell line that might not perfectly replicate a physiological system. Based on these results, in this work I focused on the exploration of EWH mechanisms of action to improve glucose tolerance in insulin resistant rats (Fig. 1.4). Because peripheral insulin resistance and adipose tissue physiology are important factors to consider when talking about MetS and T2D, insulin sensitive tissues were the primary focus.

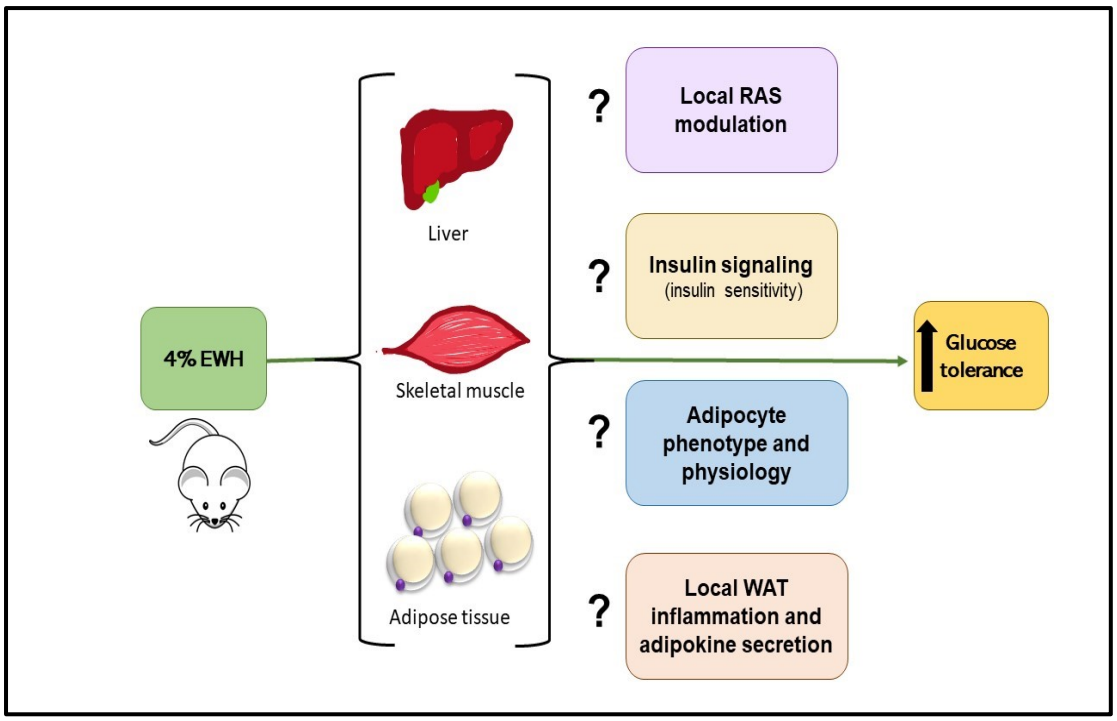


**Figure 1. 4- Thesis focus.** This work focusses on insulin sensitive tissues as the main pathway for EWH improve glucose tolerance. Different pathways were explored. Abbreviations: EWH, egg white hydrolysate; RAS, renin-angiotensin system; WAT, white adipose tissue.

# CHAPTER 2: Objectives, Hypothesis and Methods

Section 2.3 in this chapter is modified from the submitted paper: Jahandideh F, de Campos Zani SC, Son M, Proctor SD, Davidge ST, Chan CB, Wu J. Egg white hydrolysate enhances insulin sensitivity in high fat diet induced insulin resistant rats via AKT activation. *British Journal of Nutrition*. Submitted Oct. 2018. Revisions submitted Feb. 2019 (Appendix 2).

## 2.1 Thesis objectives

The overall objective of this thesis was to evaluate the effect of egg white hydrolysate (EWH) provided in the diet on metabolic syndrome related pathophysiology, focusing on insulin-regulated pathways and factors that contribute to insulin resistance (Fig. 1.4). The specific objectives were to investigate:

- RAS components (ACE, ACE2, AT1R, AT2R) in skeletal muscle, liver and WAT.
- Local inflammatory markers and plasma and WAT resistin and adiponectin.
- Insulin signaling in insulin sensitive tissues by quantifying Akt phosphorylation in skeletal muscle and liver, and insulin receptor β (IR-β) phosphorylation, GLUT4 and AS160 abundance in white adipose tissue (WAT).
- Gluconeogenesis enzymes (PEPCK and G6Pase) abundance in liver.
- Adipocyte morphology by microscopy and quantifying precursors of adipogenesis such as, phosphorylation of ERK 1/2 and activation of PPARγ by measuring its DNA binding activity.
- Lipolytic potential by measuring phosphorylation of PKA and HSL.

## 2.2 Hypothesis

I hypothesize that EWH improved glucose tolerance in HFD-induced insulin resistance rats by downregulating the abundance of RAS components AT1R and/or ACE and upregulating AT2R and/or ACE2 in insulin sensitive tissues, such as skeletal muscle, liver and/or WAT and by stimulating adipogenesis. I speculate that EWH will affect several pathways involved in insulin sensitivity such as local inflammation, insulin signaling and lipolysis.

## 2.3 Methods

### *2.3.1 Animals and diets*

The animal care protocol was approved by the University of Alberta Animal Care and Use Committee (Protocol # 1472) in accordance with the guidelines issued by the Canadian Council on Animal Care. The study also adhered to the Guide for the Care and Use of Laboratory Animals, United States National Institutes of Health. Male Sprague Dawley (SD) rats aged 8 weeks were purchased from Charles River Canada (St. Constant, QC, Canada) and housed 2 per cage with ad libitum access to normal chow and water for 1 week. After 1 week of acclimatization, all rats received a high fat diet (HFD) (20% w/w) for 6 weeks to induce glucose intolerance as shown before (Hashemi et al., 2015). Rats were then randomly assigned to one of the following groups: HFD, or HFD+4% EWH (n=7-8 each). The amount of the EWH used in this study could reasonably be expected to be achieved in the human population. These diets continued for another 6 weeks with *ad libitum* access to food and water. The diet composition is shown in Table 2.1. The EWH preparation has been described elsewhere

(Jahandideh et al., 2017). Casein was used to make all diet groups isonitrogenous. After the treatment period, half of the animals (n=4) in each group were injected with insulin (2 IU/kg body weight (BW) or saline intraperitoneally 10 min before euthanizing to study insulin signaling in muscle and fat. The diet composition was calculated previously (Jahandideh et al., submitted) and is used in this thesis as a complement to the results shown later. The diets are matched for carbohydrate, fat (including saturated and unsaturated fats) and fibre as well as total protein.

**Table 2. 1-** Composition of the experimental diets (g/kg). (adapted from Jahandideh et al., submitted)

| Ingredients (g/kg) | HFD | HFD+4%EWH |
|---|---|---|
| **Casein** | **280** | **245** |
| **EWH** | **0** | **40** |
| **Sucrose** | **200** | **200** |
| **Lard** | **195** | **195** |
| **Maltodextrin** | **115** | **115** |
| **Corn starch** | **85** | **85** |
| **Cellulose** | **58** | **58** |
| **Mineral mix** | **43** | **43** |
| **Soybean oil** | **30** | **30** |
| **Vitamin mix** | **19** | **19** |
| **L-Cystine** | **3.5** | **3.5** |

| | | |
|---|---|---|
| **Calcium phosphate** | **3.4** | **3.4** |
| **Choline bitartrate** | **3** | **3** |

### *2.3.2 Tissue collection*

At the end of the study, rats were euthanized using isofluorane anesthesia after a 16-h fast. A 3-5 mL blood sample was taken by cardiac puncture from all rats in both animal trials except for the insulin-injected rats and stored at -80 °C. Muscle, liver and fat pads were excised, washed in phosphate buffered saline, blotted, weighed, and immediately frozen at -80°C until further analysis. Small sections of fresh fat pads were fixed in 10% formalin (48 hours) then embedded in paraffin.

### *2.3.3 Tissue homogenization and protein extraction*

Hepatic and muscle proteins were extracted using an extraction buffer consisting of lysis buffer (Mitosciences Abcam, Toronto, ON, Canada), aprotinin 2 µg/mL (Calbiochem, Oakville, ON, Canada), sodium fluoride 42 mg/L, sodium orthovanadate 184 mg/L, and protease inhibitor cocktail 2mg/mL (Sigma-Aldrich, Oakville, ON, Canada). Proteins from fat pads were extracted using a commercially available kit AT-022 (Invent Biotechnologies Inc., Plymouth, MN, USA) as per manufacturer's instructions. The protein content was measured using total protein assay and sample aliquots were stored at -80 °C.

### *2.3.4 Inflammatory markers*

Inflammatory cytokines in plasma and adipose tissue were assessed using a commercial Rat Inflammation ELISA strip #EA-1201 (Signosis, Inc. Santa Clara, CA, USA) following manufacturer's instructions.

### *2.3.5 Resistin and adiponectin assay*

Resistin and adiponectin were assessed in plasma, retroperitoneal and epididymal adipose tissue lysate using an electro chemiluminescent commercial kit- Rat adiponectin and Rat resistin # K153BXC-1 (Mesoscale Discovery, Gaithersburg, MD, USA). Samples were analyzed in duplicates and in a single batch following the manufacturer's instructions. For tissue lysate 50µg/50µl of protein were added in each well.

### *2.3.6 Adipocyte size and distribution*

Adipose tissue paraffin blocks were cut into 5 µm sections and affixed in glass slides. One slide per sample was stained with hematoxylin-eosin, and 10 photomicroscopic images of each slide were taken in a grid formation using microscope 20x objective lens and Axion Vision 4.8 software. A scale bar of 100 µm was placed in the images. ImageJ software "freehand selections" tool was used to measure adipocyte area ($mm^2$) of 47-52 cells/sample.

### *2.3.7 Western blotting*

Protein extracts were separated by SDS-PAGE electrophoresis on 10-12% polyacrylamide gels, transferred to nitrocellulose membranes, and incubated with primary antibodies as indicated (Table 2.2). PPARγ, ACE, ACE2, AT1R, AT2R, PEPCK, G6Pase-α, GLUT-4, IR-β, ERK 1/2 and AKT bands were normalized to β-actin. HSL, PKA and AS160 bands were normalized to GAPDH. All the phosphorylated proteins (p-Akt, p-IR-β, p-ERK 1/2, p-HSL and p-PKA) bands were normalized to its correspondent total protein. Protein bands were detected by a Li-cor Odyssey BioImager and quantified by densitometry using corresponding software Image Studio Lite 5.2.

### *2.3.8 Nuclear protein extraction*

Nuclear protein was extracted from retroperitoneal and epididymal adipose tissue using a commercial nuclear extraction kit #40010 (Active Motif Inc, Carlsbad, CA, USA) following the manufacturer's instructions for frozen tissue. 100 mg of tissue was used for each extraction.

### *2.3.9 PPARγ binding activity*

PPARγ DNA binding activity was assessed using a TransAM$^{TM}$ PPARγ kit (Active Motif Inc, Carlsbad, CA, USA), which is an ELISA based assay, following the manufacturer's instructions using 10µg/10µL of nuclear protein extract from retroperitoneal and epidydimal adipose tissue per well.

### *2.3.10 Statistical analysis*

All data presented are expressed as means ± SEM of 3-6 rats from each treatment group as indicated in figure legends. Statistical analysis was performed using GraphPad Prism software, version 6.0. Data were checked for normal distribution by the Shapiro-Wilk test. For AKT, ERK 1/2 and IR-β two-way analysis of variance (ANOVA) was used. For all other data, one-way ANOVA, Kruskal-Wallis test, and student's t-test were used as indicated. Tukey's and Dunn's post-hoc tests were performed to assess differences between groups when a significant main effect was observed. A p-value of 0.05 was considered statistically significant. The number of animals in this study was determined by a power calculation based on oral glucose tolerance test (OGTT) as described elsewhere (Jahandideh et al., submitted).

**Table 2. 2 -** Antibodies information

| ANTIBODY | COMPANY / CATALOG NUMBER | CONCENTRATION / INCUBATION TIME |
|---|---|---|
| Anti-protein kinase B-( Akt 1/2/3) | Santa Cruz / #sc-81434 | 3:1000 / overnight / 4 °C |
| Anti-phospho protein kinase B-(Akt) | Cell Signaling / #9271 | 1:1000 / overnight / 4 °C |
| Anti-angiotensin converting enzyme 2 (ACE2) | Abcam / #ab87436 | 1:1000 / overnight / 4 °C |
| Anti-angiotensin converting enzyme 1 (ACE) | Abcam / #ab75762 | 1:1000 / overnight / 4 °C |
| Anti-angiotensin II type 1 receptor (AT1R) | Santa Cruz / #sc-515884 | 1:1000 / overnight / 4 °C |
| Anti-angiotensin II type 2 receptor (AT2R) | Abcam / #ab92445 | 1:1000 / overnight / 4 °C |
| Anti-glucose-6 phosphatase α (G6Pase-α) | Santa Cruz / #sc-25840 | 1:1000 / overnight / 4 °C |

| | | |
|---|---|---|
| Anti-phosphoenolpyruvate carboxykinase (PEPCK) | Cayman Chemical / #10004943 | 5:1000 / overnight / 4 °C |
| Anti-GLUT 4 | Cell Signaling / #2113S | 1:1000 / overnight / 4 °C |
| Anti-insulin receptor (IR)- β | Cell Signaling / #3025 | 1:1000 / overnight / 4 °C |
| Anti-phospho IR-β | Santa Cruz / #sc-8501 | 1:1000 / overnight / 4 °C |
| Anti-phospho-ERK 1/2 | Cell Signaling / #9101S | 1:1000 / overnight / 4 °C |
| Anti-ERK 1/2 | Cell Signaling / # 4695P | 1:1000 / overnight / 4 °C |
| Anti-hormone sensitive lipase (HSL) | Abcam / #ab45422 | 1:1000 / overnight / 4 °C |
| Anti-phospho hormone sensitive lipase (p-HSL) (Ser660) | Cell Signaling / #4126 | 1:1000 / overnight / 4 °C |
| Anti-protein kinase A (PKA) | Cell Signaling / #58425 | 1:1000 / overnight / 4 °C |
| Anti-phospho protein kinase A (p-PKA) | Cell Signaling / #5661S | 1:1000 / overnight / 4 °C |
| Anti-Akt substrate of 160KDa (AS160) | Thermo Fisher / #MA5-14840 | 2:1000 / overnight / 4 °C |
| Anti-beta- actin (β-actin) | Sigma-Aldrich / #A5441 | 1:10000 / 15 min / RT |
| Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) | Thermo Fisher / #MA 5-15738 | 2:10000/ overnight/ 4 °C |
| Anti-rabbit Green | Li-COR Biosciences / # 926-32213 | 1:10000 / 1 hour / RT |
| Anti-mouse Green | Li-COR Biosciences / # 926-32212 | 1:10000 / 1 hour / RT |
| Anti-rabbit Red | Li-COR Biosciences / # 925-68071 | 1:10000 / 1 hour / RT |
| Anti-mouse Red | Li-COR Biosciences / # 925-68072 | 1:10000 / 1 hour / RT |

Abbreviations: RT, room temperature.

# CHAPTER 3: Results

Sections 3.1, 3.2 and 3.3 contain figures adapted from the submitted paper: Jahandideh F, de Campos Zani SC, Son M, Proctor SD, Davidge ST, Chan CB, Wu J. Egg white hydrolysate enhances insulin sensitivity in high fat diet induced insulin resistant rats via AKT activation. *British Journal of Nutrition*. Submitted Oct. 2018. Revisions submitted Feb. 2019 (Appendix 2).

## 3.1 EWH effects on RAS system components (ACE, ACE2, AT1R and AT2R) of insulin sensitive tissues from HFD-induced obese rats and adipose tissue inflammation

Because the EWH preparation used in this thesis had been previously shown to reduce blood pressure in rats (Jahandideh et al., 2016), I investigated changes in local RAS components in several metabolically important tissues (liver, skeletal muscle and adipose tissue). The western blot analysis showed no changes in abundance of RAS components ACE, ACE2, AT1R (Figs. 3.1-3.4). Most importantly, increased AT2R protein abundance in liver ($p=0.0152$) and in retroperitoneal adipose tissue ($p=0.0279$) was observed (Fig. 3.4B and C, respectively). ACE had generally low abundance and its protein was only found in quantifiable amounts in liver (Fig. 3.1).

Local inflammation was analyzed in both epididymal and retroperitoneal adipose tissue by measuring several inflammatory markers. The results show no major changes in local inflammatory markers (Fig. 3.5), except a 10% increase in IL-6 in epididymal adipose tissue after EWH treatment (Fig. 3.5A). In addition, resistin and adiponectin were measured in adipose tissue and plasma (Fig. 3.6). 4% EWH treatment

did not change the concentration of these adipokines locally (epidydimal and retroperitoneal adipose tissue lysate) or systemically (plasma).

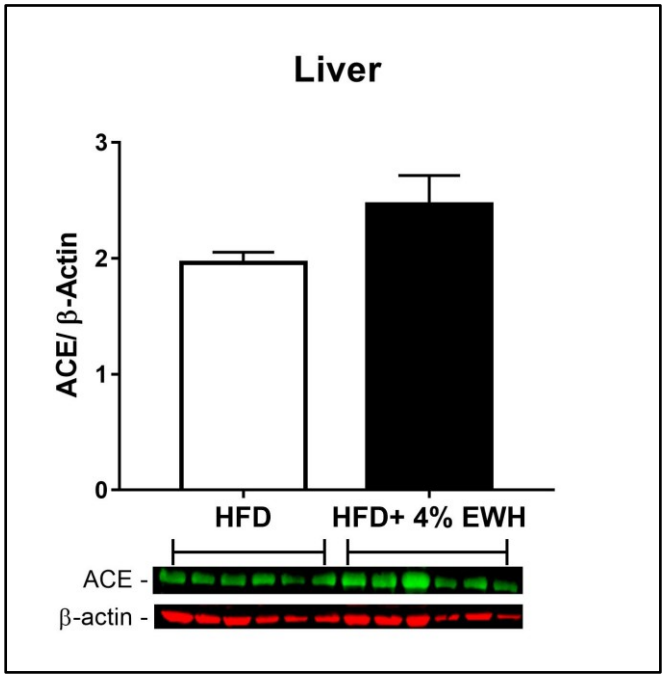


**Figure 3. 1- Angiotensin converting enzyme (ACE) abundance in Liver.** ACE abundance measured by western blot. Data expressed as Mean ± SEM of n=6 rats and analyzed by two-tailed t-test.

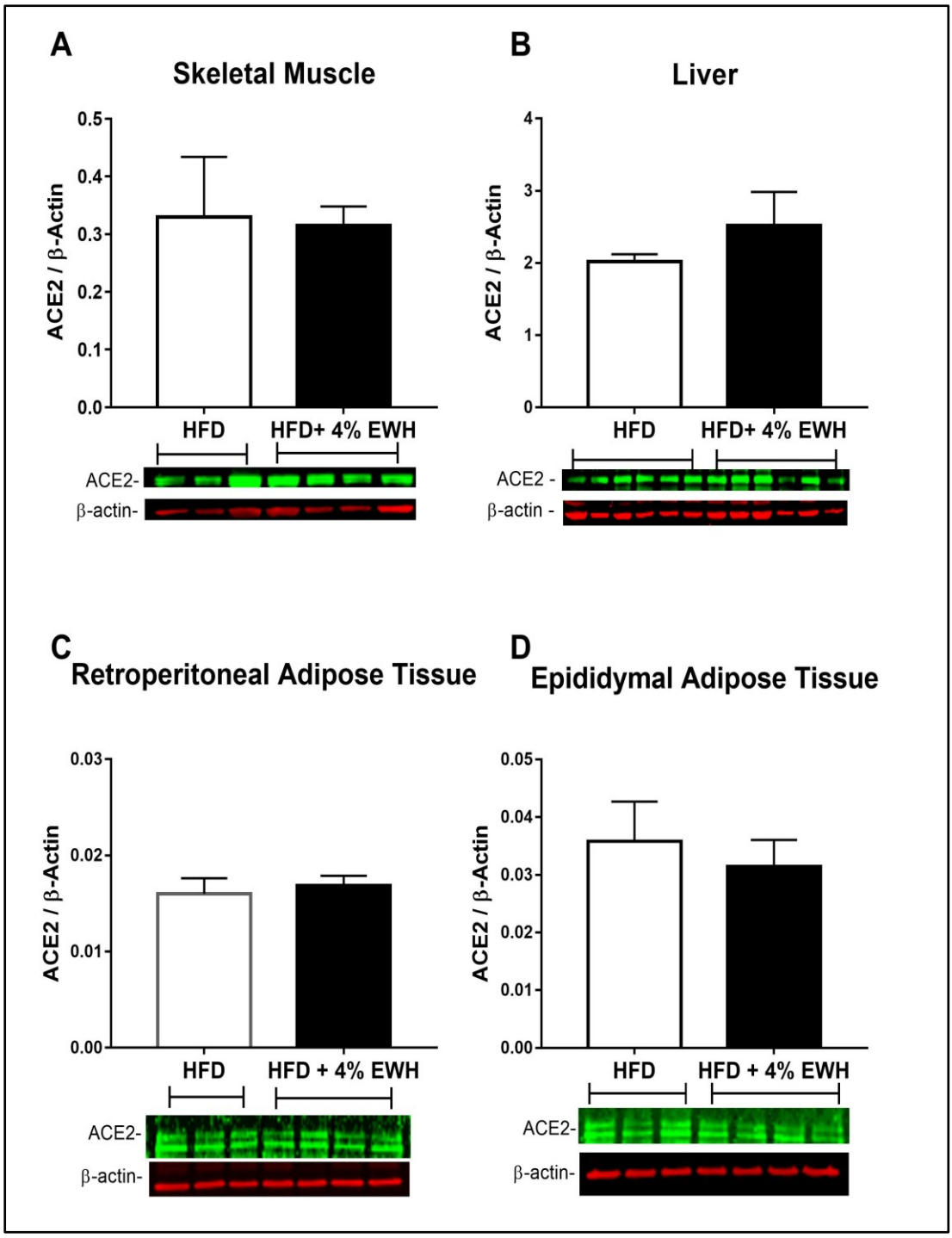


**Figure 3. 2- Angiotensin converting enzyme 2 (ACE2) tissue abundance.** ACE2 abundance measured by western blot. **(A)** Skeletal muscle **(B)** Liver **(C)** Retroperitoneal WAT **(D)** epididymal WAT. Data expressed as Mean ± SEM of n=3-6 rats and analyzed by two-tailed t-test. WAT, white adipose tissue.

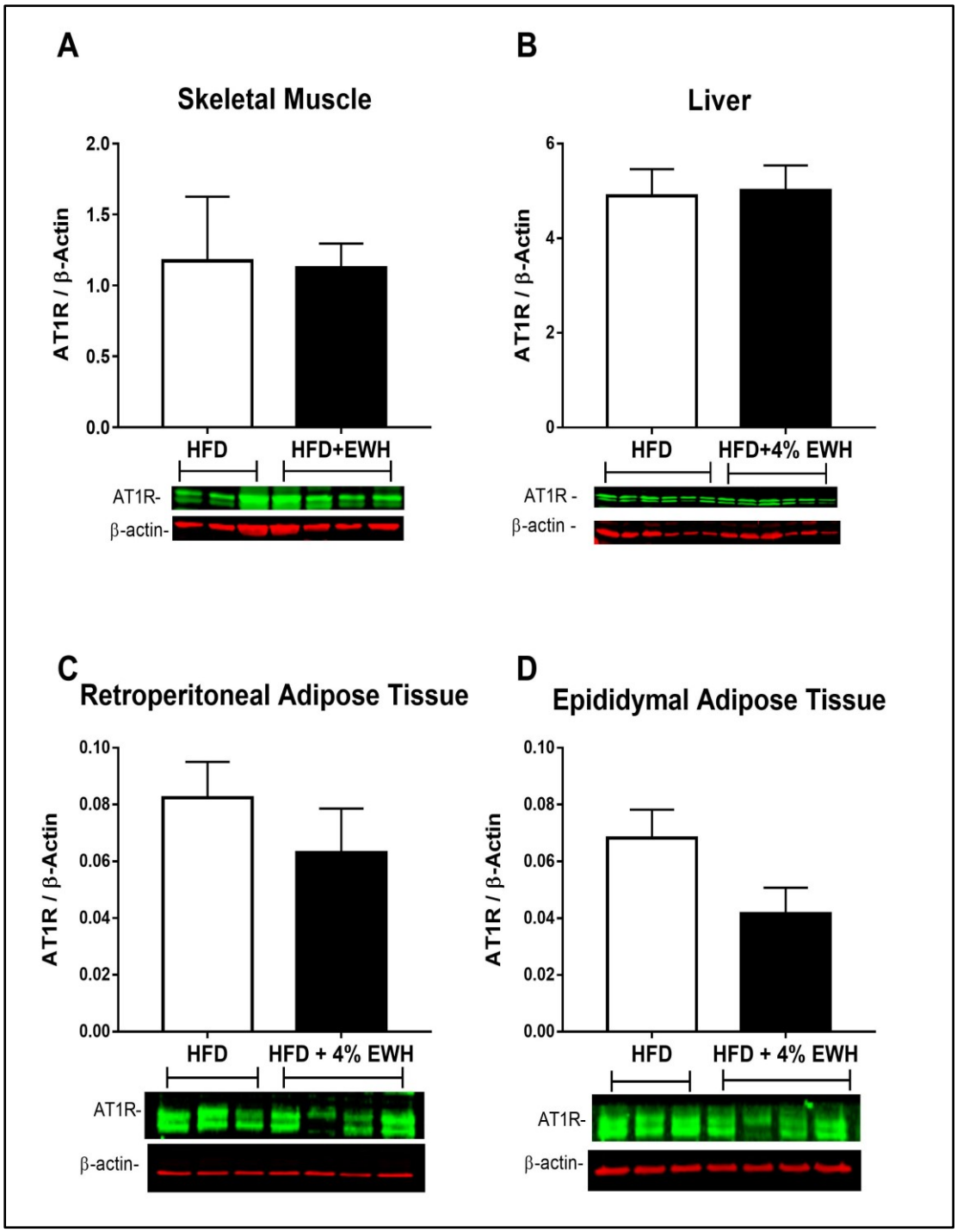


**Figure 3. 3- Angiotensin II type 1 receptor (AT1R) tissue abundance.** AT1R abundance measured by western blot. **(A)** Skeletal muscle **(B)** Liver **(C)** Retroperitoneal WAT **(D)** epididymal WAT. Data expressed as Mean ± SEM of n=3-6 rats and analyzed by two-tailed t-test.

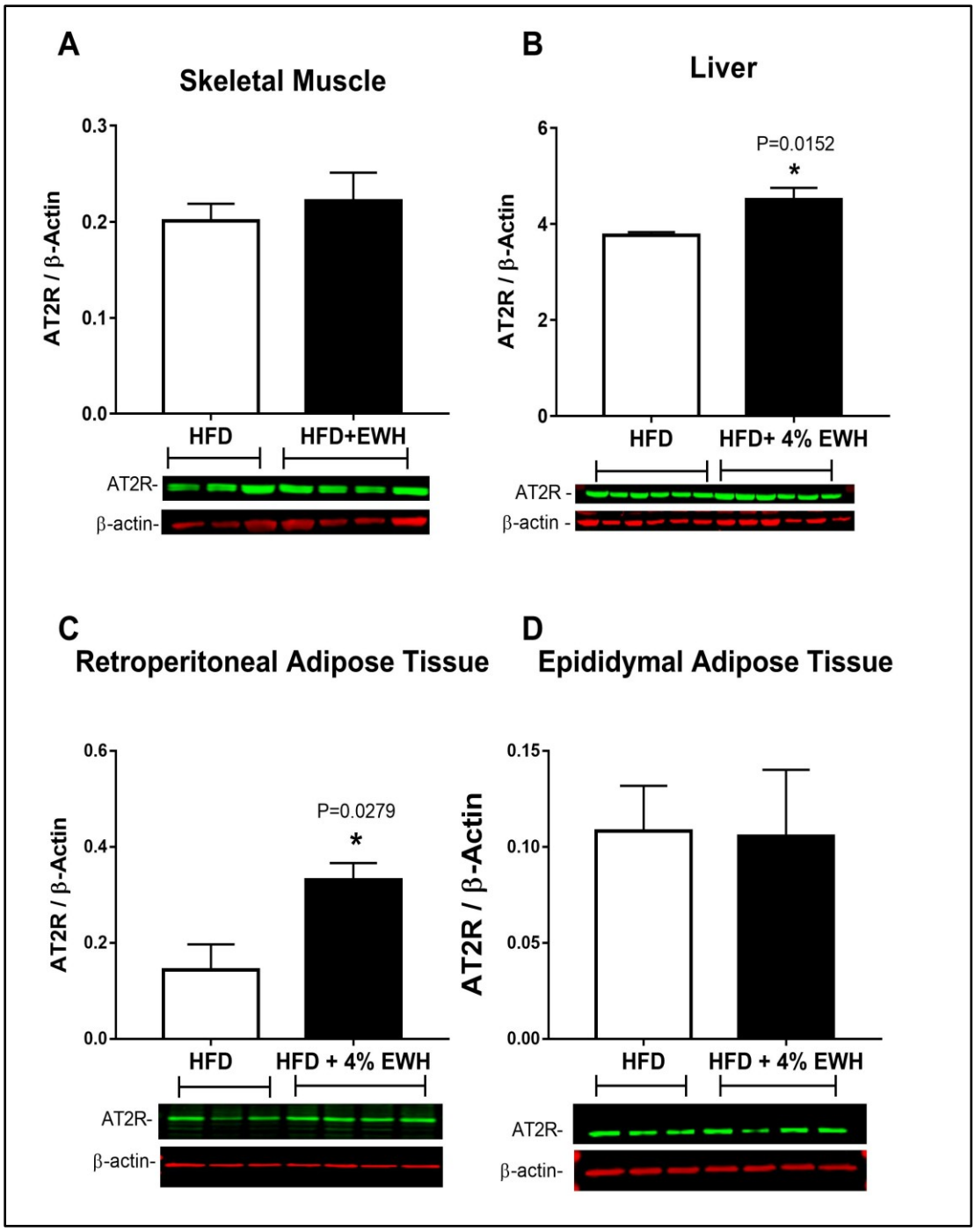


**Figure 3. 4- Angiotensin II type 2 receptor (AT2R) tissue abundance.** AT2R abundance measured by western blot. **(A)** Skeletal muscle **(B)** Liver **(C)** Retroperitoneal WAT **(D)** epididymal WAT. Data expressed as Mean ± SEM of n=3-6 rats and analyzed by two-tailed t-test. * shows significant difference at $p< 0.05$.

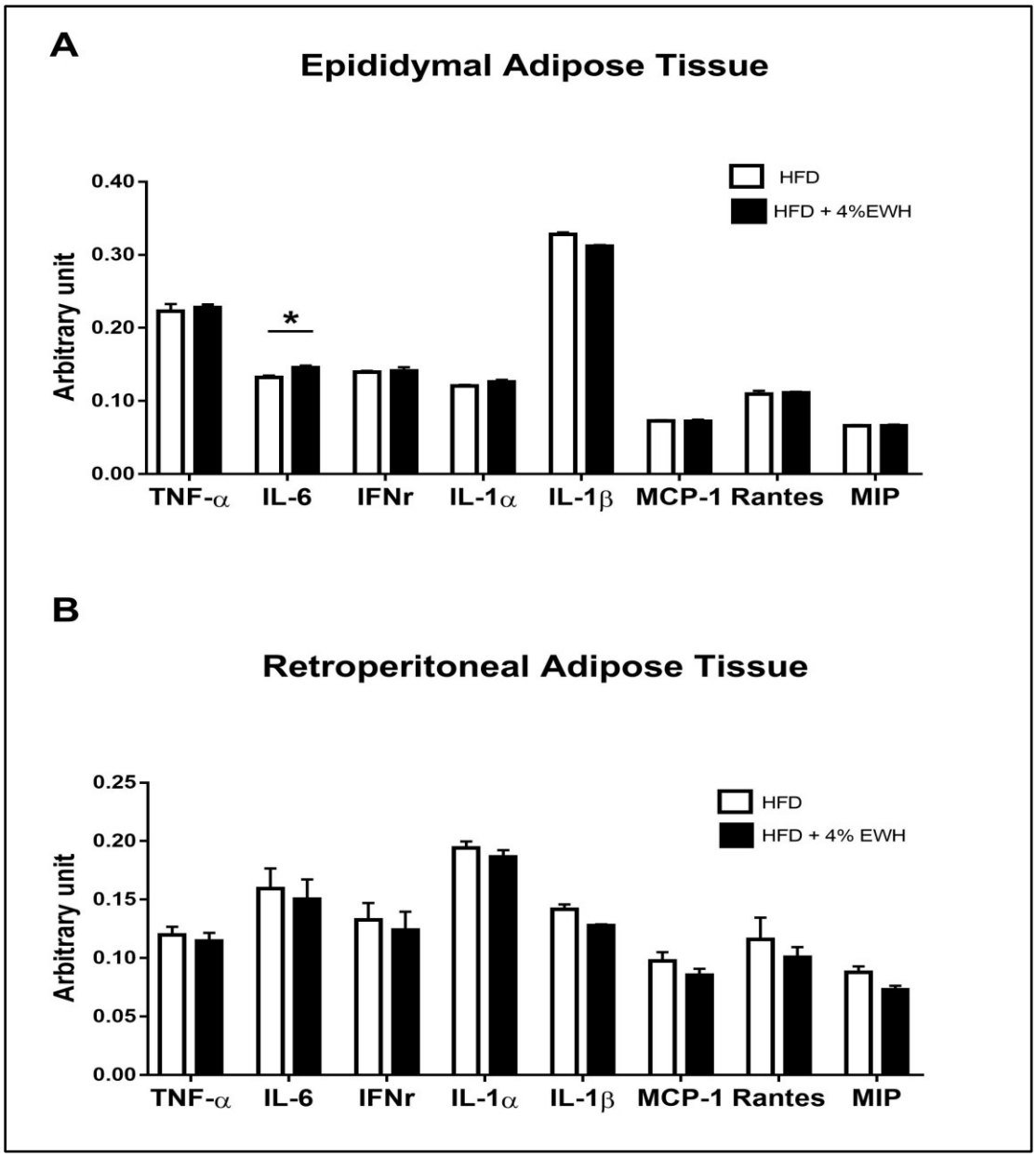


**Figure 3. 5- Epididymal and retroperitoneal adipose tissue inflammatory markers. (A)** Epididymal WAT, n=3 rats. **(B)** Retroperitoneal WAT, n=6 rats. Data expressed as Mean ± SEM and analyzed by two-tailed t-test. * shows significant difference at $p< 0.05$ analyzed by two-tailed t-test. TNF-α, Tumor necrosis factor; IL-6, Interleukin-6; IFNr, Interferon production regulator; IL-1a, Interleukin-1 alpha; IL-1b, Interleukin- 1 beta; MCP-1, Monocyte chemoattractant protein-1; Rantes, regulated on activation, normal T cell expressed and secreted; MIP, Macrophage inflammatory protein.

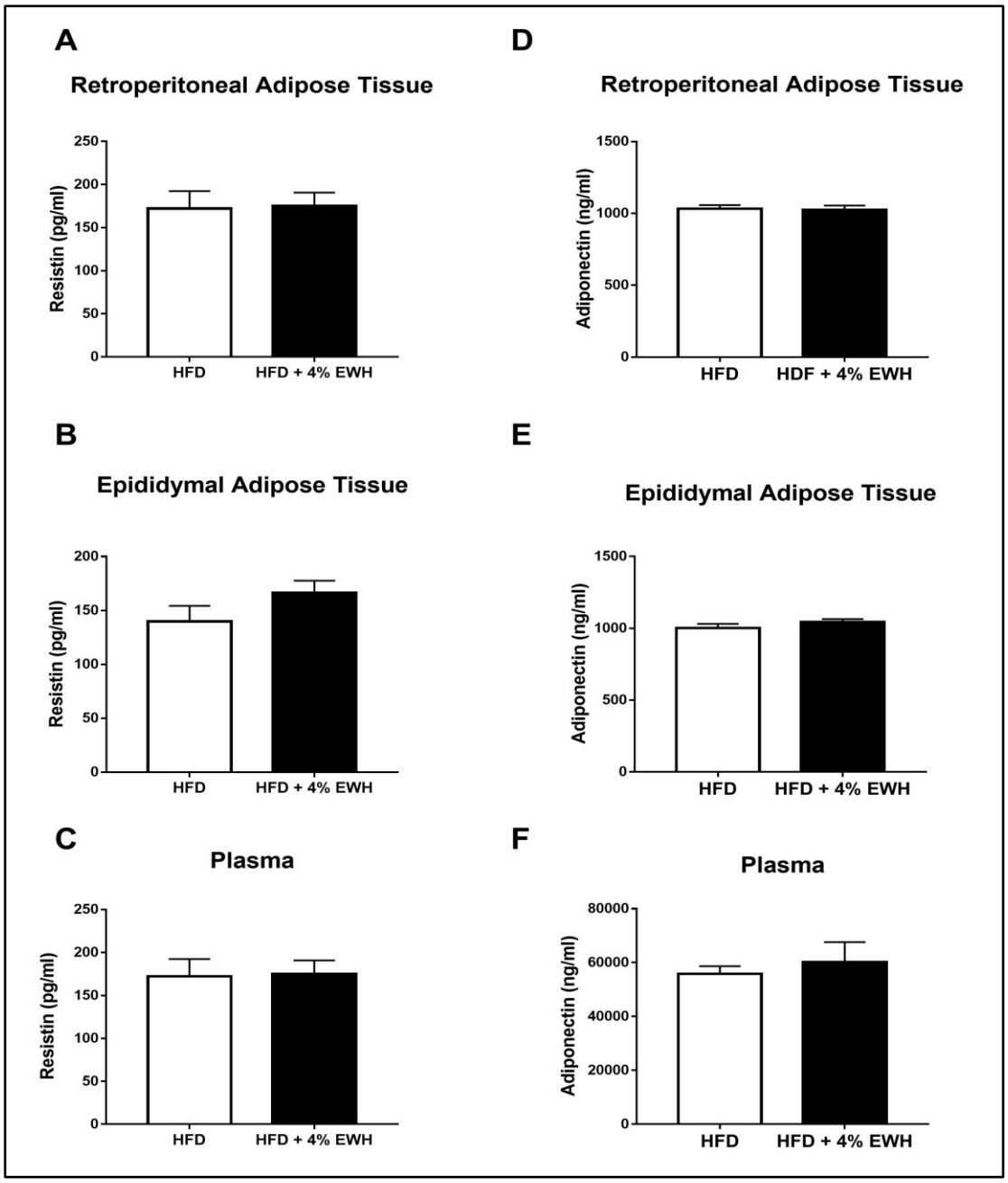


**Figure 3. 6- Adipokine concentration in adipose tissue and plasma.** Adipokines were measured by ELISA as follows: **(A)** retroperitoneal WAT resistin, **(B)** epidydimal WAT resistin, **(C)** plasma resistin, **(D)** retroperitoneal WAT adiponectin, **(E)** epidydimal WAT adiponectin, **(F)** plasma adiponectin. Data expressed as Mean ± SEM of n=6-7 rats and analyzed by two-tailed t-test.

### 3.2 EWH effects on insulin signaling pathway and gluconeogenesis of HFD-fed, insulin resistant rats

EWH effects on insulin signaling were measured by Akt and IRβ phosphorylation. In addition, abundance of the downstream proteins AS160 and GLUT4 was measured. The results indicate that 4% EWH treatment enhanced Akt (Ser473) phosphorylation in skeletal muscle, but not in liver (Fig. 3.7A and B, respectively). There was no difference in total Akt relative to housekeeping protein in both tissues (data not shown). Since a previous study in our lab showed enhanced Akt phosphorylation in adipose tissue (Jahandideh et al., submitted), I investigated a downstream protein in the Akt signaling, called AS160 and an upstream regulator of Akt phosphorylation, IRβ (Tyr1322) (Fig. 3.8). For AS160, the 2-way ANOVA analysis shows a significant interaction ($p=0.0252$) and treatment ($p=0.0096$) effect in epididymal WAT; furthermore, post-hoc analysis shows that 4% EWH treatment enhanced AS160 abundance in epididymal fat pad after insulin stimulation ($p=0.0415$) while no significant increase was observed in the HFD control (Fig. 3.8B). No changes were observed regarding IRβ (Tyr1322) phosphorylation (Fig. 3.8A and D) or total protein abundance (data not shown) in WAT.

AS160 activation catalyzes GLUT4 translocation to the plasma membrane. In this work GLUT4 couldn't be isolated only from the membrane, and the analysis of total (cytosolic + membrane) GLUT4 protein abundance showed no effect of 4% EWH in either fat pad (Fig. 3.8C and F).

To investigate if endogenous glucose production could have changed after 4% EWH treatment, I investigated the abundance of gluconeogenesis enzymes in liver. No changes were observed after 4% EWH regarding PEPCK or G6Pase abundance (Fig. 3.9A and B, respectively).

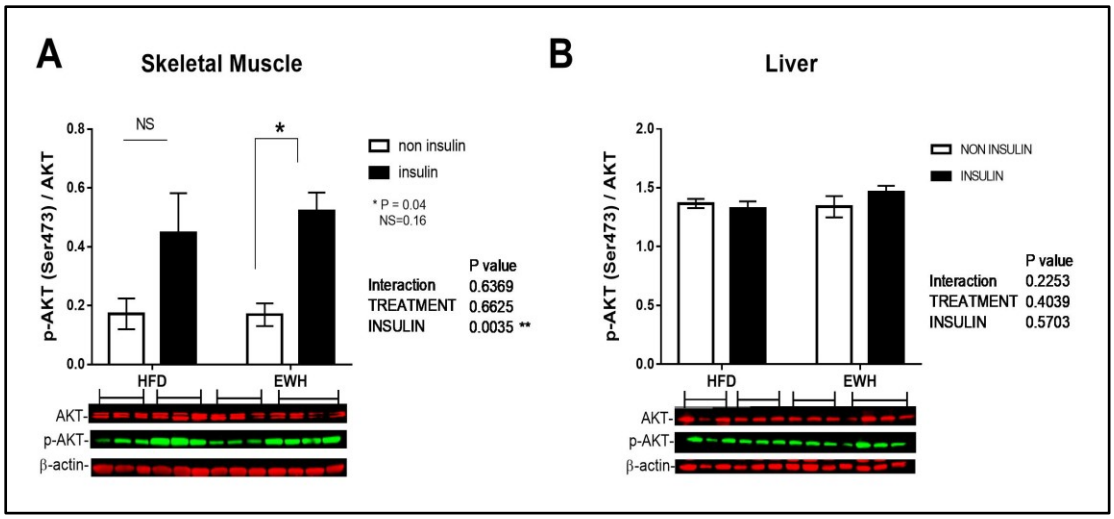


**Figure 3. 7- Tissue insulin sensitivity assessed by phosphorylation of Akt (Ser473**). Insulin sensitivity was measured in **(A)** skeletal muscle and **(B)** liver in HFD and HFD+4% EWH treated rats. p-Akt protein band was normalized to total Akt as a measure of insulin sensitivity in both groups. Data are expressed as Mean ± SEM for n= 3-4 rats and analyzed by 2-way ANOVA followed by Tukey's post-hoc comparison test. * $p<0.05$.

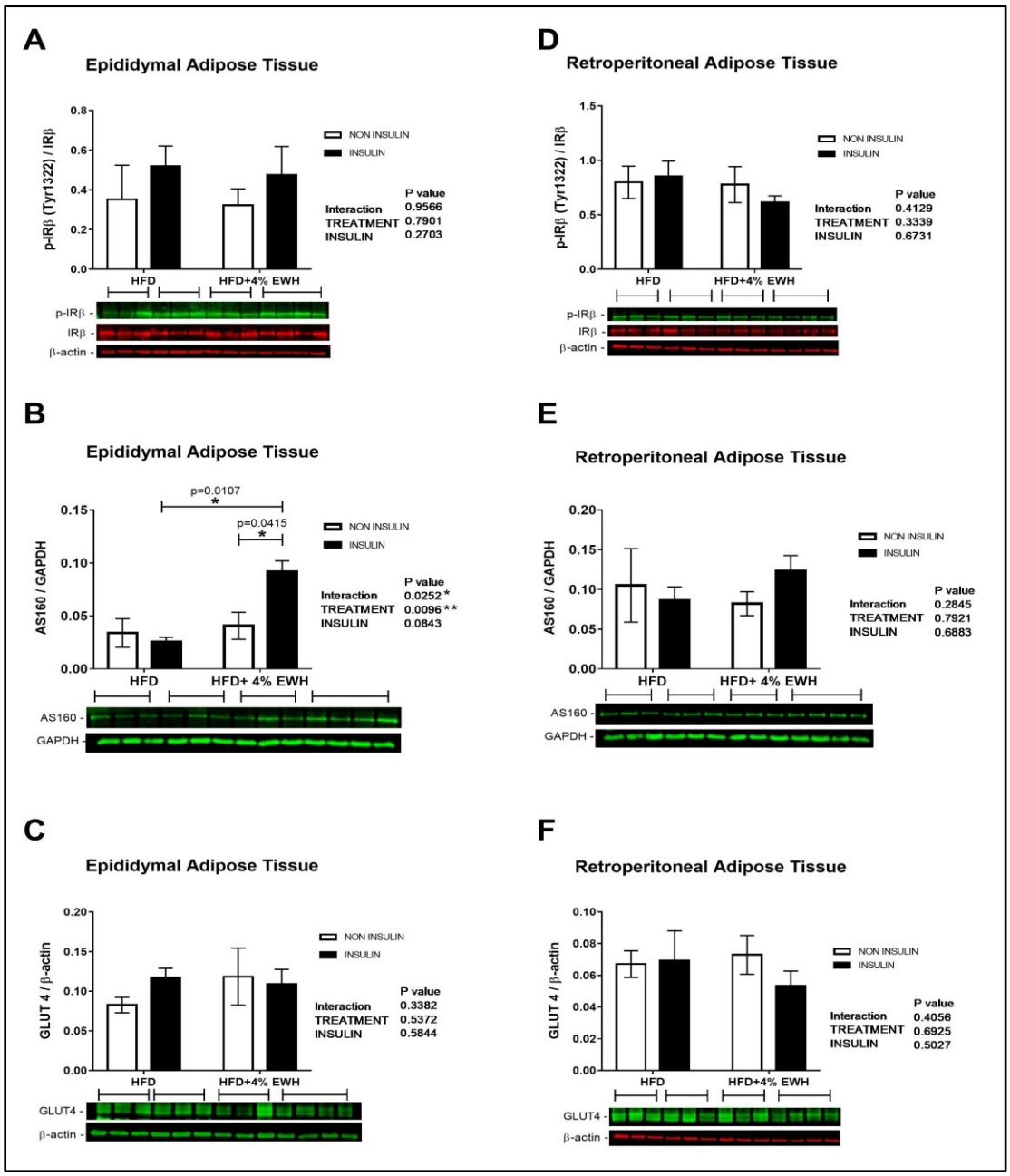


**Figure 3. 8- Epididymal and retroperitoneal WAT insulin signaling measured by IRβ (Tyr1322) phosphorylation, AS160 and GLUT 4 abundance. (A)** Epididymal IRβ, **(B)** epididymal AS160, **(C)** epididymal GLUT4, **(D)** retroperitoneal IRβ, **(E)** retroperitoneal AS160, **(F)** retroperitoneal GLUT4 in HFD and HFD+4% EWH treated rats. p-IRβ protein band was normalized to total IRβ in both groups. Data expressed as Mean ± SEM for n= 3-4 rats and analyzed by 2-way ANOVA followed by Tukey's post-hoc comparison test. * $p<0.05$, ** $P<0.01$.

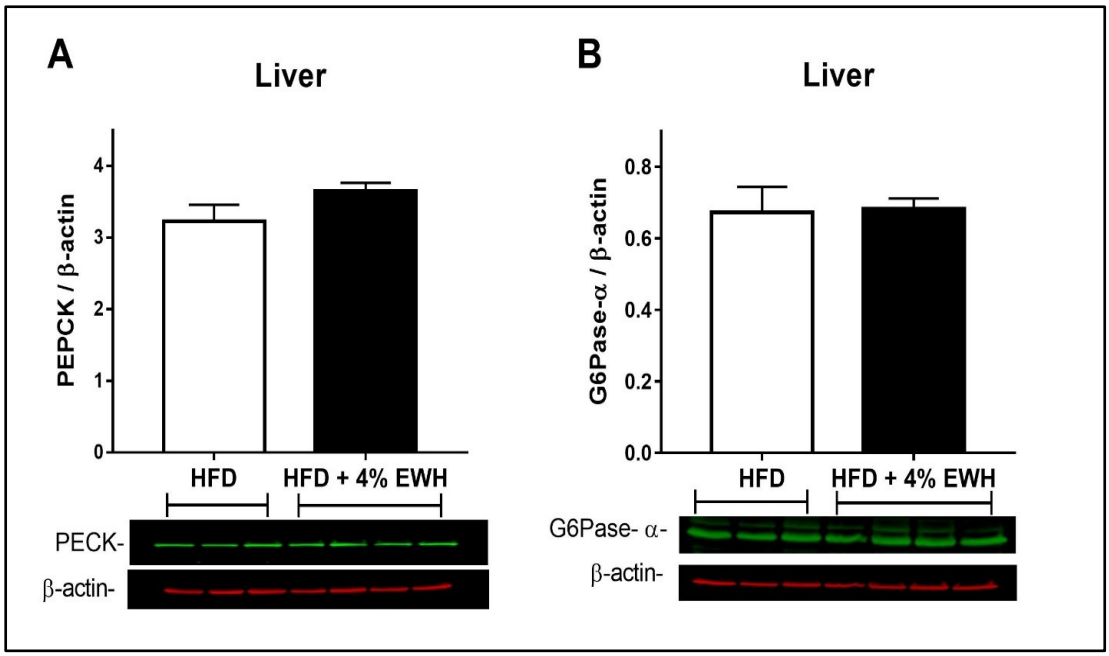


**Figure 3. 9- Liver PEPCK and G6Pase-α abundance.** PEPCK and G6Pase protein bands were normalized to β-actin as the loading control. **(A)** PEPCK and **(B)** G6Pase in HFD and HFD+4%EWH treated groups. Data expressed as Mean ± SEM for n= 3-4 rats and analyzed by two-tailed t-test. PEPCK, Phosphoenolpyruvate carboxykinase; G6Pase-α, Glucose 6 phosphatase-α.

### 3.3 EWH effects on adipocyte morphology in retroperitoneal and epididymal adipose tissue

Previous work in 3T3 cells and primary WAT indicated that total PPARγ abundance was increased by EWH treatment, suggesting that 4% EWH might influence WAT morphology (Jahandideh et al., 2017; Jahandideh et al., submitted). To address this question, adipocyte morphology and related signaling pathways, PPARγ DNA binding activity, ERK phosphorylation and adipocyte area were measured. In epididymal adipose tissue, ERK phosphorylation was enhanced after insulin stimulation in both HFD control and 4% EWH (Fig. 3.10A), but no changes were observed in retroperitoneal fat pad (Fig. 3.10B). There was no difference in total ERK 1/2 relative to housekeeping protein in both epididymal and retroperitoneal WAT (data not shown). PPARγ DNA binding activity was enhanced in retroperitoneal ($p= 0.0242$) (Fig. 3.11B), but not in epididymal adipose tissue (Fig. 3.11A).

Retroperitoneal adipocyte mean area was significantly reduced (from 0.025 $mm^2$ to 0.022 $mm^2$) after 4% EWH treatment ($p=0.0383$) (Fig. 3.12B). Despite no changes in PPARγ DNA binding activity or mean adipocyte area (from 0.021 $mm^2$ to 0.017 $mm^2$) in epididymal adipose tissue (Fig 3.11A and 3.12A), adipocyte distribution analyzed by area intervals revealed that 4% EWH treatment decreased the number of larger adipocytes and increased the number of smaller adipocytes in both fat pads (Fig. 3.13). In retroperitoneal adipose tissue the number of adipocytes within the range area of 0.012 to 0.013 $mm^2$ increased, while larger adipocytes (0.020 to 0.029 $mm^2$) decreased significantly (Fig. 3.13B). Whereas in epididymal adipose tissue the number of adipocytes within the area range of 0 to 0.009 $mm^2$ increased, while larger adipocytes (0.020 to 0.029 $mm^2$) decreased significantly (Fig. 3.13A).

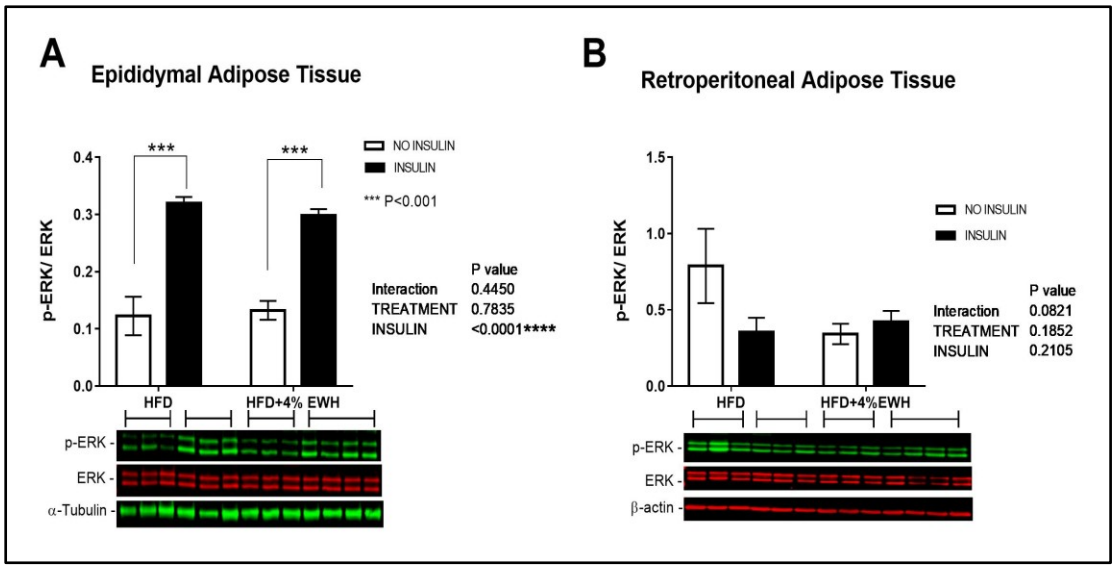


**Figure 3. 10- ERK 1/2 phosphorylation in retroperitoneal and epididymal WAT. (A)** Epidydimal and **(B)** retroperitoneal WAT. p-ERK protein band was normalized to total ERK in both groups. Data are expressed as Mean ± SEM for n= 3-4 rats and analyzed by 2-way ANOVA followed by Tukey's post-hoc comparison test. **shows significant difference at p< 0.01.

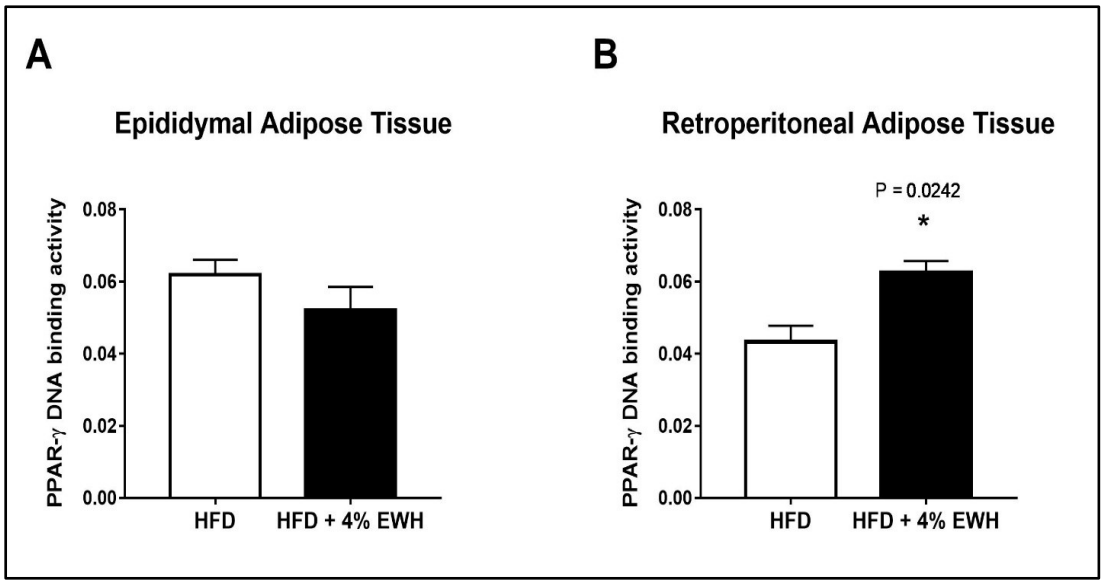


**Figure 3. 11- PPARγ DNA binding activity in retroperitoneal and epididymal adipose tissue. (A)** Epidydimal and **(B)** retroperitoneal WAT. Data expressed as Mean ± SEM for n= 3 rats and analyzed by two-tailed t-test. * shows significant difference at $p< 0.05$.

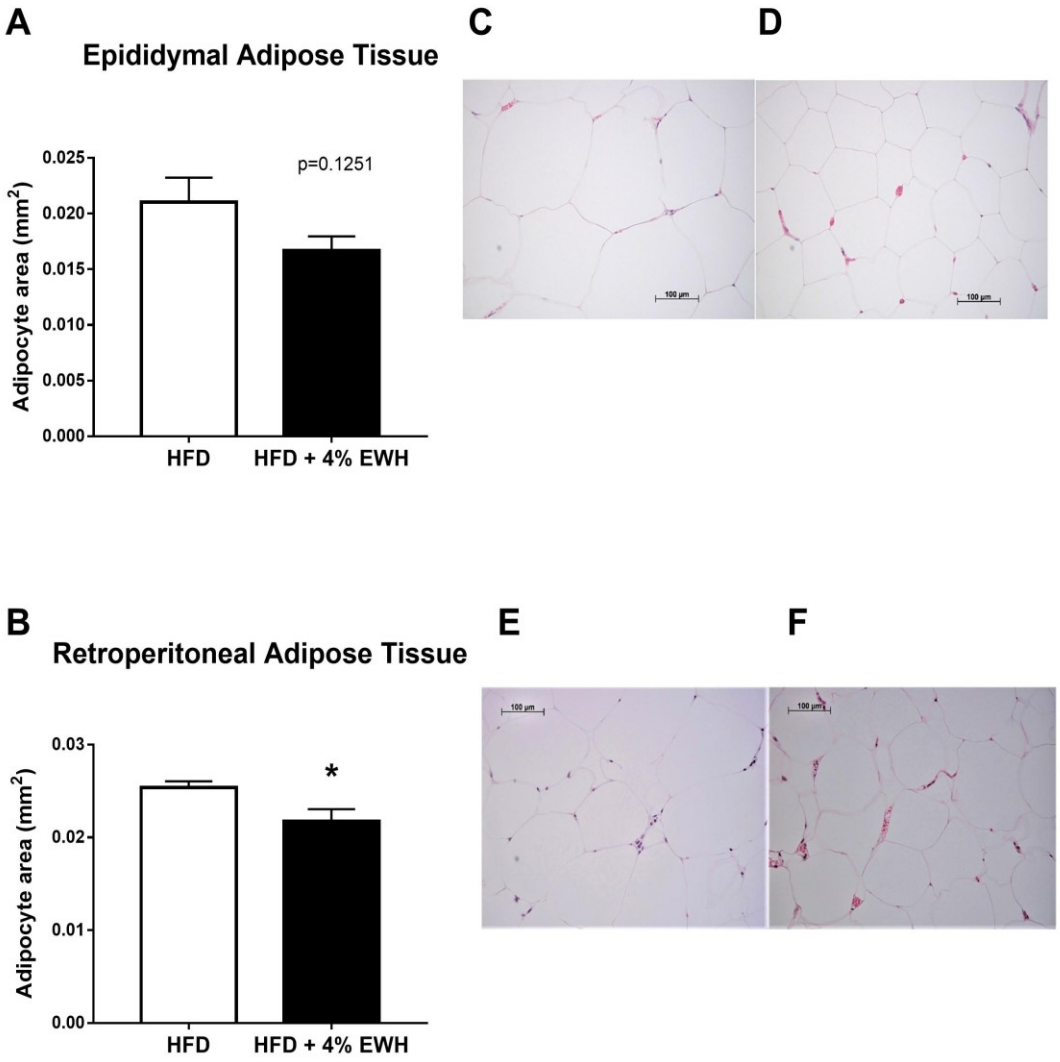


**Figure 3. 12- Mean adipocyte area in retroperitoneal and epididymal adipose tissue. (A)** Epidydimal and **(B)** retroperitoneal WAT. Photomicroscopic images of epidydimal adipose tissue using 20X objective lens. **(C)** epididymal HFD, **(D)** epididymal HFD+4% EWH, **(E)** retroperitoneal HFD, **(F)** retroperitoneal HFD+4% EWH. Scale bar indicates 100 µm. Data expressed as Mean ± SEM for n= 5 rats and analyzed by two-tailed t-test. * shows significant difference at $p< 0.05$.

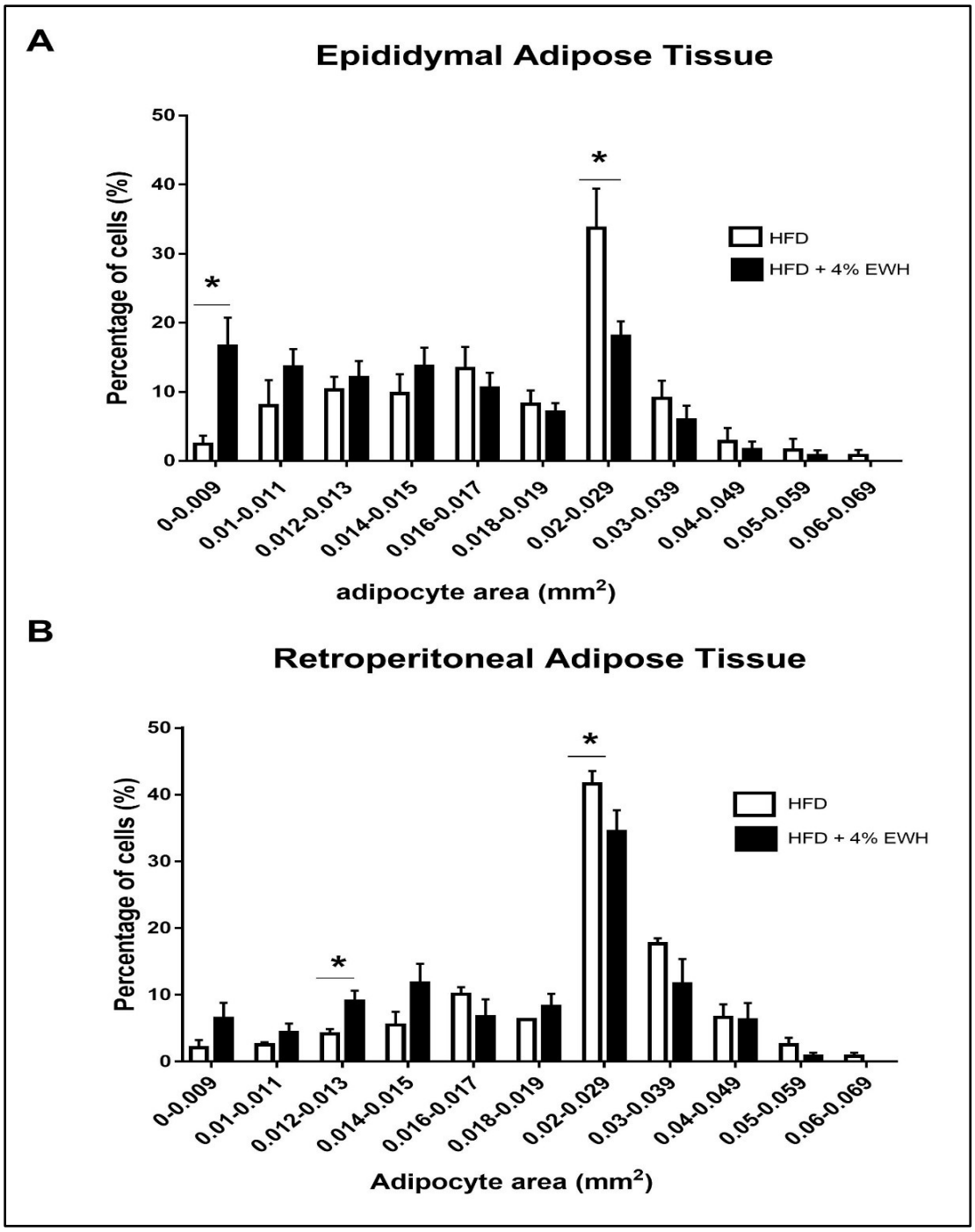


**Figure 3. 13- Adipocyte distribution in retroperitoneal and epididymal adipose tissue.** Mean adipocyte area and adipocyte area distribution were analyzed in **(A)** epididymal and **(B)** retroperitoneal WAT. Data expressed as Mean ± SEM for n= 5 rats and analyzed by two-tailed t-test. * shows significant difference at $p< 0.05$.

### 3.4 EWH effects on lipolysis in retroperitoneal and epididymal adipose tissue

To investigate EWH effects on the lipolytic pathway, PKA and HSL phosphorylation were measured. 4% EWH treatment had a significant effect ($p<0.05$) in enhancing PKA phosphorylation in both epididymal and retroperitoneal fat pads, independent of insulin exposure (Fig. 3.14A and C). Although no effects were seen regarding total PKA abundance in retroperitoneal fat pad (Fig. 3.14D), a reduction in total PKA abundance was observed after chronic treatment with 4% EWH in epididymal adipose tissue (Fig. 3.14B).

HSL is a protein downstream of PKA activation and 4% EWH treatment had a general effect of enhancing HSL phosphorylation (Ser660) in retroperitoneal adipose tissue (Fig. 3.15C), while no effect was observed in epididymal fat pad (Fig. 3.15A). Post-hoc analysis showed reduced p-HSL after insulin stimulation in the EWH-treated retroperitoneal adipose tissue (Fig. 3.15C); however, in HFD control the reduction was not significant, suggesting that the control group presented less insulin sensitivity in this fat pad than the treated group (Fig. 3.15C). Total HSL abundance was reduced in epididymal adipose tissue (Fig 3.15B). No changes were observed in retroperitoneal adipose tissue regarding total HSL abundance (Fig. 3.15D).

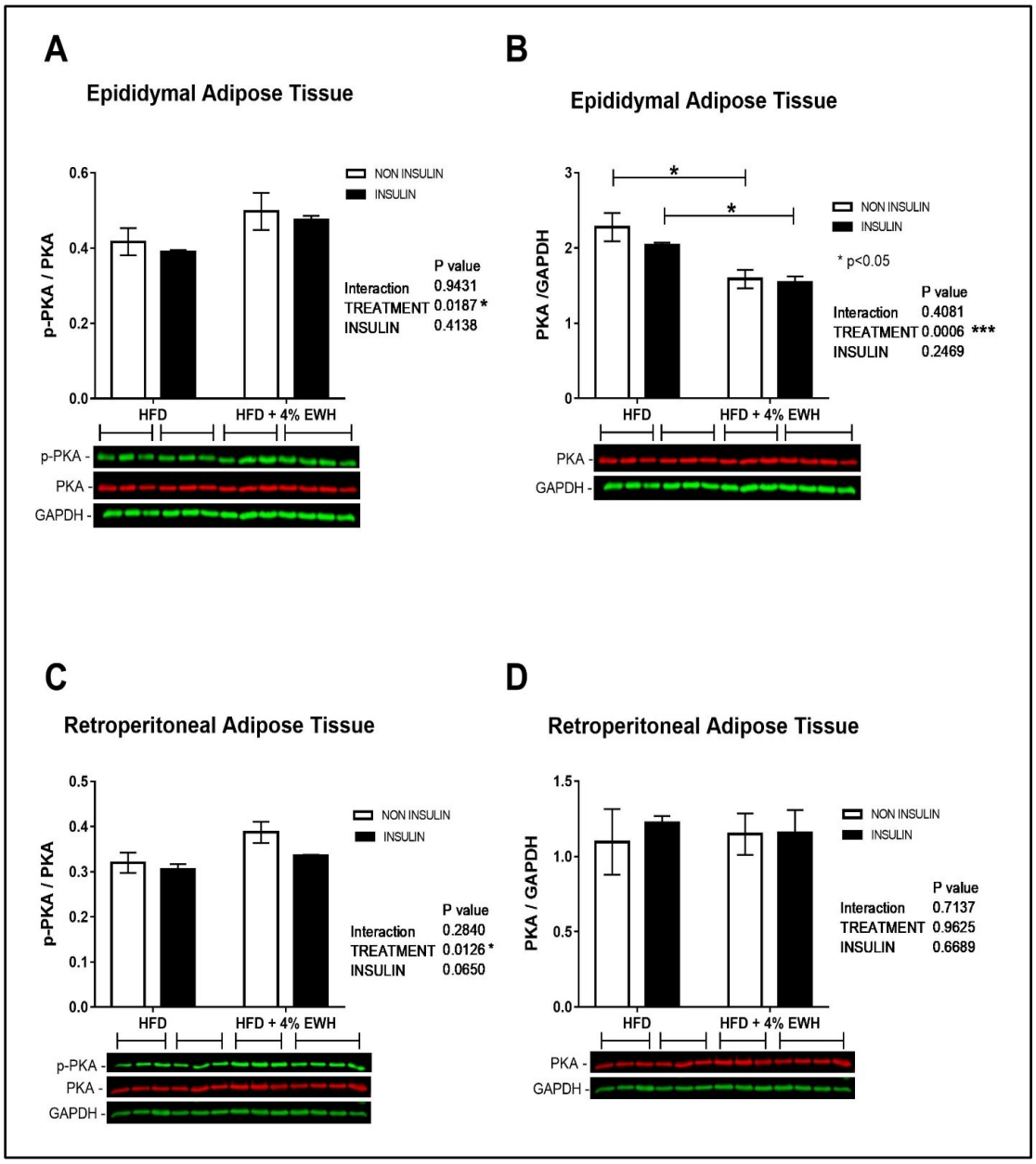


**Figure 3. 14- PKA and p-PKA abundance in retroperitoneal and epididymal WAT. (A)** Epidydimal p-PKA, **(B)** epidydimal PKA, **(C)** retroperitoneal p-PKA, **(D)** retroperitoneal PKA. p-PKA protein band was normalized to total PKA in both groups. Data expressed as Mean ± SEM for n= 3-4 rats and analyzed by 2-way ANOVA followed by Tukey's post-hoc comparison test. *shows significant difference at p< 0.05.

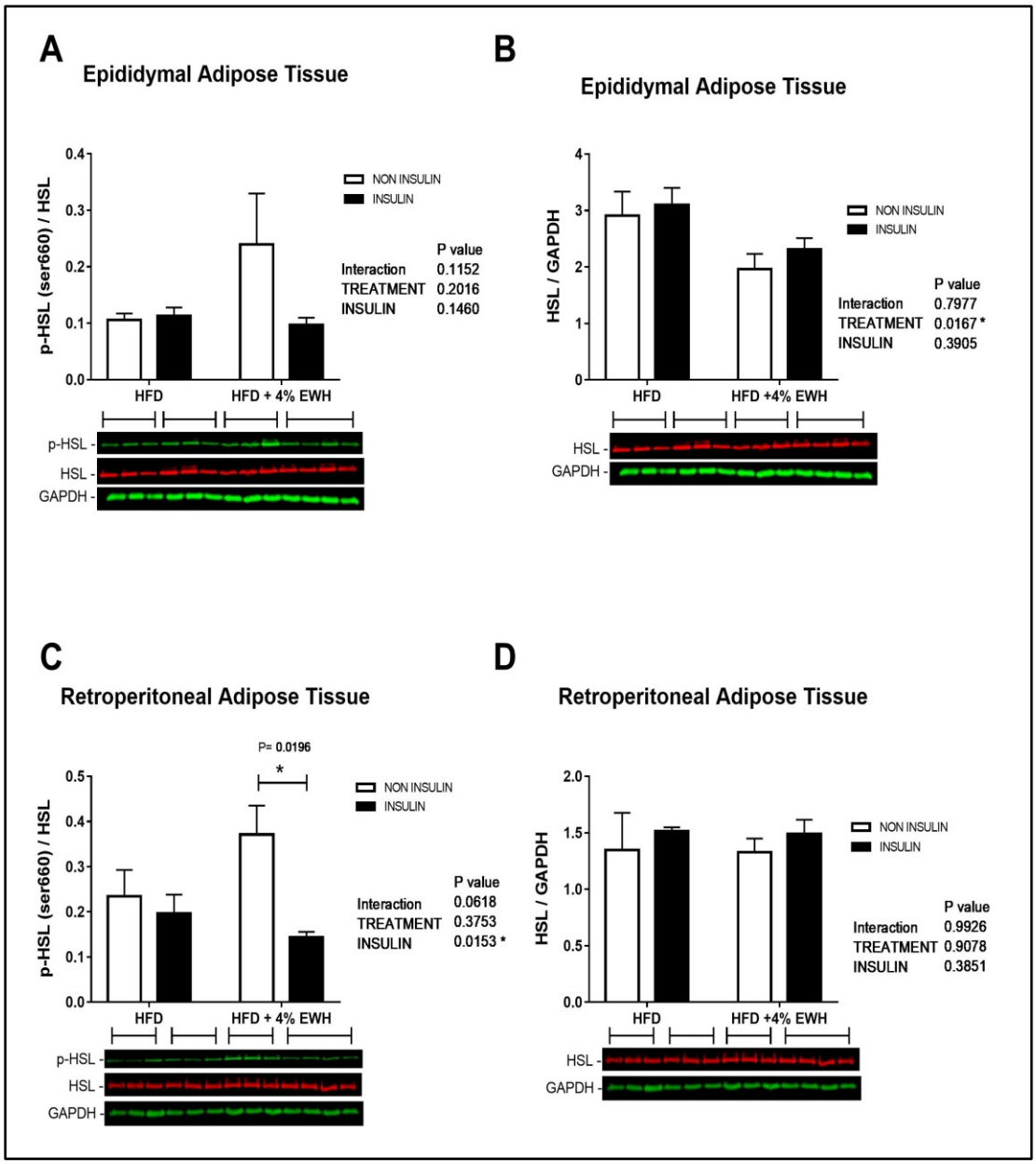


**Figure 3. 15- HSL and p-HSL (Ser660) abundance in retroperitoneal and epididymal WAT. (A)** Epidydimal p-HSL (Ser660), **(B)** epidydimal HSL, **(C)** retroperitoneal p-HSL (Ser660), **(D)** retroperitoneal HSL. p-HSL protein band was normalized to total HSL in both groups. Data expressed as Mean ± SEM for n= 3-4 rats and analyzed by 2-way ANOVA followed by Tukey's post-hoc comparison test. *shows significant difference at $p< 0.05$.

# CHAPTER 4: Discussion and Conclusion

The functional food market is growing worldwide, and food derived bioactive peptides have received attention due to their potential to treat diseases. Although most of the studies using egg as a source of bioactive peptides focus on ACE-inhibitor or anti-inflammatory and antioxidant properties (Grootaert et al., 2017b; Kovacs-Nolan, Phillips, & Mine, 2005), previous work by our group showed a beneficial effect of EWH in improving glucose tolerance (Jahandideh et al., submitted). However, little evidence in the literature investigates the mechanisms of EWH improving conditions associated with metabolic diseases (de Campos Zani et al., 2018). Furthermore, to my knowledge, we are the only group using the specific EWH preparation described previously (Jahandideh et al., 2017) and because different peptide amino acid sequences produce different outcomes as seen in Table 1.2, the effects and efficacy of each must be characterized in pre-clinical trials before progressing to human studies.

In this work I hypothesized that EWH supplementation affects insulin sensitive tissues through multiple mechanisms to improve glucose tolerance in HFD-induced insulin resistant rats. To test this hypothesis, various metabolic pathways in liver, skeletal muscle and adipose tissue were investigated. The exploratory nature of this work provides valuable information regarding the mechanism of action of EWH. Overall, 4% EWH chronic treatment (Fig 4.1) (1) changed adipocyte morphology; (2) reduced adipocyte lipolytic capacity; (3) enhanced insulin sensitivity in peripheral tissues; and (4) had little or no effect on local inflammation and RAS components modulation, with the exception of AT2R, which may be important as discussed later. The results support the hypothesis; furthermore, I show evidence that the improvement in glucose tolerance happens regardless of changes in local inflammatory markers or adipokines abundance. It is worth mentioning that there were no differences in food

intake (HFD: 129.7 ± 3.80 kcal/day ; HFD+4% EWH: 127.0 ± 5.20 kcal/day) or body weight (HFD initial: 358 ± 8.52 g / final: 828 ± 21.65 g; HFD+ 4% EWH initial: 352 ± 10.14 g / final: 813 ± 20.17 g) between the groups throughout the period of 12 weeks of treatment (Jahandideh et al., submitted), eliminating these as confounders of the interpretation.

It should be first confirmed that the HFD rats used in this study were insulin resistant. Importantly, the effect of insulin on Akt phosphorylation is non-significant in all 3 tissues tested for the HFD group (data from skeletal muscle and liver p-Akt can be confirmed from Forough Jahandideh's PhD thesis (Jahandideh, 2017)) and seen together with WAT p-Akt, in (Jahandideh et al., submitted - appendix 2). In addition, no effect of insulin is seen regarding IR-β phosphorylation in WAT. Furthermore, insulin did not suppress phosphorylation of HSL in HFD WAT. These observations are consistent with the characteristics of insulin resistance described in the literature (Reaven, 1988; Roden et al., 2017). Therefore, these rats can be considered a diet-induced insulin resistant model.

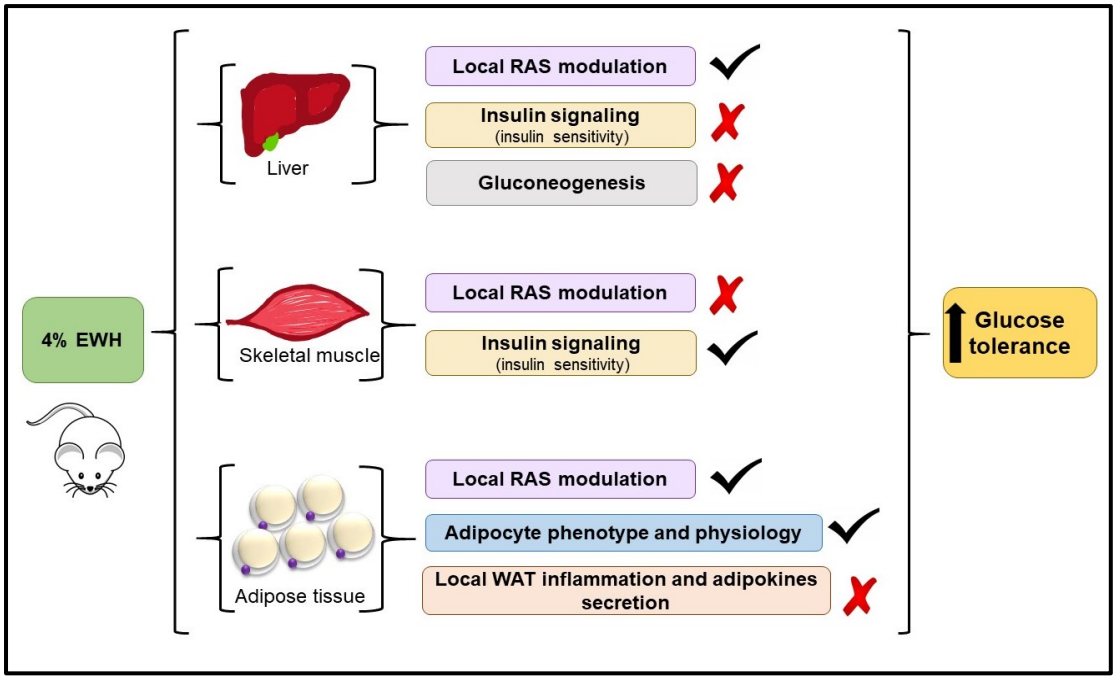


**Figure 4. 1- Summary of EWH effects on insulin sensitive tissues of HFD-induced insulin resistant rats** (See text for explanation). Red "X" means no effect of EWH, black "check marks" means effect of EWH on that pathway. Abbreviations: EWH, egg white hydrolysate; RAS, renin-angiotensin system.

## 4.1 EWH effects on adipose tissue

### *4.1.1 EWH changes adipose tissue morphology consistent with improved insulin sensitivity*

Adipogenesis is a process that maintains adipose tissue physiology and contributes to preventing ectopic fat accumulation and metabolic diseases such as T2D (as reviewed by Ghaben & Scherer, 2019). PPARγ is a lipid sensor involved in adipose tissue metabolism and acts by controlling and activating genes redirecting metabolism and proliferation; its expression is most abundant in adipose tissue, but it is also found in other tissues. Due to its role in lipid metabolism PPARγ has been considered a potential target to reduce lipotoxicity by activating gene transcription of proteins

involved in lipogenesis leading to improved insulin sensitivity; this, in part, clarifies the mechanism of crosstalk between lipids and glucose homeostasis (Evans et al., 2004). PPARγ is also considered the master regulator of adipogenesis and its knockout impairs adipogenesis *in vivo* and *in vitro* (Rosen et al., 2000). Previously our group demonstrated that PPARγ total abundance was enhanced in adipose tissue after EWH treatment (Jahandideh et al., submitted) and adipogenic peptides were identified in the egg hydrolysate used in this study using a cell culture assay (Jahandideh et al., 2018); here I show that EWH treatment increases PPARγ protein abundance and transcriptional activation, because PPARγ DNA binding activity was enhanced 44% in retroperitoneal WAT by EWH. Therefore, EWH may have PPARγ as one of its targets but more studies are necessary to conclude the dependence of EWH on PPARγ to exert the observed effects.

TZD are a class of drugs used in T2D treatment that act by enhancing insulin sensitivity. Interestingly, TZD are PPARγ agonists and pharmacotherapy with TZD improves insulin signaling by promoting adipogenesis (Kim et al., 2015), reducing inflammatory cytokines and macrophage infiltration in WAT (H. Xu et al., 2003). Furthermore, PPARγ agonists reduce resistin (Steppan et al., 2001) and increase adiponectin plasma concentration (Yamauchi et al., 2001) in mice. However, TZD was shown to improve insulin sensitivity by both adiponectin-dependent and -independent pathways, depending on the dose and target tissue (Kubota et al., 2006). Although in 3T3 adipocyte cell line EWH shows similar effects as TZD such as reduction of inflammatory cytokines, increase in adiponectin and enhanced Akt phosphorylation (Jahandideh et al., 2017), *in vivo* those effects are attenuated; however, it should be noted that *in vivo* EWH treatment was chronic while an acute treatment was performed *in vitro*. Therefore, it is possible that a transient effect happened during the initial

treatment *in vivo* and is not seen with the same intensity after chronic treatment. Nevertheless, PPARγ activation and improvement in insulin sensitivity are still outcomes of the EWH treatment in this study.

The mechanism by which PPARγ agonists improve insulin sensitivity has not been clarified yet, but TZD specifically target adipose tissue to enhance insulin sensitivity (Jiang et al., 2002). Likewise, Akt phosphorylation was enhanced by EWH in muscle and adipose tissue (Jahandideh et al., submitted). In liver, however, only prolonged treatment with TZD enhanced Akt phosphorylation in their study (Jiang et al., 2002), while in ours it was not enhanced after chronic treatment with EWH. This supports the idea of PPARγ agonists having adipose tissue, not the liver, as its main target. In rats, PPARγ agonists reduced both NEFA and TG within the first two days of treatment, leading to enhanced Akt phosphorylation in adipose tissue (Jiang et al., 2002). PPARγ agonists are shown to reduce plasma TG concentration by reducing hepatic TG production and enhancing TG clearance (Oakes, Thalen, Jacinto, & Ljung, 2001). Although I did not perform any experiments regarding lipid clearance or release, it is consistent with previous results showing that EWH reduced plasma TG concentration (Jahandideh et al., submitted). Despite no clearly defined mechanism to reduce NEFA and TG by PPARγ activation, it is accepted that its activation will lead to adipogenesis and promote better lipid storage by adipose tissue, preventing lipotoxicity and insulin resistance in peripheral tissues (Evans et al., 2004; Ghaben & Scherer, 2019).

Because of the essential role of PPARγ in adipose tissue metabolic health, I investigated if EWH was affecting upstream proteins in the adipogenesis cascade, such as ERK 1/2. *In vitro*, ERK activation promotes adipogenesis by activating PPARγ, CEBP-α, perilipin and fatty acid binding protein (aP2) (Prusty, Park, Davis, & Farmer,

2002). In addition, insulin promotes adipogenesis by activating ERK 1/2 during early phases of the process (Prusty et al., 2002). In the same manner our results show that after insulin stimulation ERK 1/2 phosphorylation was enhanced, but no differences were seen regarding EWH treatment *in vivo*, despite enhanced ERK 1/2 phosphorylation by EWH *in vitro* (Jahandideh et al., 2017). This suggests that although EWH did not affect ERK activation *in vivo* within the chronic timeframe examined, it may affect downstream of the adipogenesis cascade, possibly at PPARγ level.

Another mechanism proposed involves PPARγ activation by the RAS component AT2R leading to adipogenesis. Interestingly, despite containing ACE-inhibitory peptides, EWH in this study produced little effect on RAS components modulation locally but enhanced significantly AT2R abundance in adipose tissue by 130%. AT2R activation and its role in insulin sensitivity and adipocyte physiology is still unclear and controversial. Nevertheless, AT2R activation in rodent primary adipocyte cell culture stimulated PPARγ in the initial steps of adipogenesis, with increased AT2R mRNA shown during adipocyte differentiation; in addition, AT2R knockdown is correlated with decrease or absence of PPARγ in adipocytes (Shum et al., 2013).

*In vivo*, AT2R agonism restores adipocyte size and improves insulin sensitivity in rodent models of diet and genetically induced T2D (Ohshima et al., 2012; Shum et al., 2013) and increases PPARγ DNA binding activity in adipose tissue (Ohshima et al., 2012), results similar to ours. In addition, similarly to our results, adipose tissue TNF-α levels were not reduced in the study by Quiroga et al., 2018, but contrary to our results adiponectin was enhanced. The enhancement of AT2R abundance may have a role in improving adipocyte insulin sensitivity; however, I did not investigate AT2R activation by EWH in this study.

Considering that EWH may be stimulating PPARγ, directly or indirectly (via AT2R) I looked for evidence of adipogenesis as a downstream indicator of its activation. Adipogenesis leads to formation of new and smaller fat cells and maintains the metabolic health of adipose tissue, contributing to safer lipid storage and preventing local and peripheral insulin resistance and/or lipotoxicity (Ghaben & Scherer, 2019). EWH reduced adipocyte size in this study, switching adipocyte morphology towards a less dysfunctional phenotype. The reduction of adipocyte size in our study is consistent with the enhancement in PPARγ abundance and activation in adipose tissue. Adipose tissue expandability (hyperplasia) has been linked to improved glucose tolerance and insulin sensitivity also in the context of lipectomy, where PPARγ has shown to be essential to maintain adipose tissue function in mice by adipogenesis, generating smaller adipocytes, keeping the regulation of glucose homoeostasis and preventing ectopic lipid accumulation, probably due to enhanced lipid uptake by the new adipocytes (Booth et al., 2017). Therefore, EWH is shown here to act, partially, by stimulating adipogenesis via PPARγ activation directly and/or indirectly.

Accordingly to the general theory found in the literature, the outcome of PPARγ stimulation is adipogenesis leading to improvement in insulin sensitivity. As an indication of enhanced insulin sensitivity, enhanced Akt phosphorylation in adipose tissue was observed (Jahandideh et al., submitted) and in skeletal muscle, but not in liver. Akt phosphorylation is a key step in leading to GLUT4 translocation to the plasma membrane allowing glucose uptake by the cells (X. Zhou et al., 2017), and is used as a downstream measurement of insulin signaling activation. No other study measuring Akt phosphorylation after EWH consumption is available for comparison, but soy derived peptides have also shown to enhance Akt phosphorylation and GLUT4 translocation in rodents (Lu et al., 2012). Interestingly, AT2R stimulation improved

glucose tolerance in mice by reducing adipocyte size and enhancing Akt phosphorylation after insulin stimulation in adipose tissue compared to control insulin stimulated group (Quiroga et al., 2018). *In vitro*, EWH has been shown to improve insulin sensitivity by enhancing Akt phosphorylation in adipocyte cell culture (Jahandideh et al., 2017) and to restore GLUT4 translocation to the plasma membrane of angiotensin II-induced insulin resistance muscle cells *in vitro* (Son et al., 2018). No changes in total adipose tissue GLUT4 abundance was observed in this work; however, I show that EWH treatment had a significant effect on AS160 abundance after insulin stimulation in adipose tissue and AS160 is a requirement for GLUT4 translocation (X. Zhou et al., 2017). Unfortunately, the isolation of GLUT4 from the plasma membrane was not feasible during the project. Therefore, I am unable to state conclusively if EWH in fact enhanced GLUT4 translocation to the plasma membrane of adipocytes contributing to enhanced glucose uptake in WAT.

There is a lack of *in vivo* studies using egg derived peptides or hydrolysate to compare with the results obtained in our study. However, the results in this work suggest that, in fact, EWH may be contributing to enhanced insulin sensitivity systemically, and may be doing so by acting locally in adipose tissue via PPARγ activation to stimulate adipogenesis and further contributing to reduced lipotoxicity peripherally.

### *4.1.2 EWH reduced adipocyte lipolytic capacity*

Physiologically, insulin inhibits lipolysis and promotes lipogenesis (Fig. 1.1) and its failure to suppress lipolysis may lead to increase in circulating NEFA (Bjorndal et al., 2011; Goossens, 2017). Increased lipolysis rates have been one of the concerns in obesity. Elevated basal lipolysis rates are associated with lipotoxicity, inflammation

and insulin resistance as reviewed (Morigny et al., 2016). In this study, I measured phosphorylation of PKA and HSL as indicators of lipolysis potential, because this pathway is known to be inhibited by insulin. In fact, inhibition of PKA activity and HSL phosphorylation by insulin has shown to be partially dependent on Akt phosphorylation in adipocytes. Although the authors show a certain independency of Akt phosphorylation in PKA-mediated perilipin phosphorylation, the HSL phosphorylation remained p-Akt dependent (Choi et al., 2010). I observed different responses to EWH treatment by epidydimal versus retroperitoneal fat pads. In epidydimal adipose tissue EWH decreased the total amount of PKA and HSL but enhanced phosphorylation of PKA independent of insulin. No changes in PKA or HSL abundance were seen in retroperitoneal WAT although the enhanced phosphorylation of PKA was similarly detected. Also, in retroperitoneal adipose tissue EWH enhanced HSL phosphorylation in the fasting state, an effect blunted by insulin, indicating restored insulin sensitivity. In another study, TZD stimulation increased basal and stimulated lipolysis in subcutaneous and visceral WAT, which was accompanied by an increase in mRNA of ATGL and MGL in visceral WAT, but not HSL (while the three mRNA of all three lipases was increased in subcutaneous WAT) (Festuccia, Laplante, Berthiaume, Gelinas, & Deshaies, 2006). Other authors showed that only ATGL mRNA levels were increased in visceral WAT of normal chow TZD treated mice, but in HFD TZD treated mice neither ATGL or HSL mRNA levels were enhanced; however, after TZD treatment the total protein amount of both enzymes in the cytosolic WAT fraction appeared to be reduced, while the protein amount in the fat cake tended to be increased, yet no statistical significance was reached (Shen, Patel, Yu, Jue, & Kraemer, 2007). In our study, basal phosphorylation of lipolytic enzymes (PKA, HSL) appears to be stimulated by EWH. In addition, these changes occurred with minimal changes (slightly

reduced) in total HSL and reduced total PKA protein amount in epidydimal fat pad. This illustrates the complexity of the lipolysis process and the need to investigate multiple lipases to fully understand it. Nevertheless, I cannot exclude the possibility of EWH having stimulatory targets other than PPARγ or even producing a weaker stimulatory effect compared to TZD, which may contribute to the differences observed in terms of lipase abundance.

It should be noted that different fat pads have different proliferative potential (hyperplasia or hypertrophy), response to a stimulus (e.g obesity or adrenergic stimulation) and adipokine release; these differences are influenced by genetic and environmental factors. For instance, after glucocorticoid treatment, epidydimal and retroperitoneal WAT responded differently in terms of glyceroneogenesis and degree of insulin resistance (Ferreira et al., 2017) and under a calorie restricted diet, the three fat pads (subcutaneous, epididymal ad retroperitoneal) presented differences in terms of adipocyte size reduction, activation of lipolytic proteins, insulin signaling, adipokine secretion and WAT inflammatory markers (Narita et al., 2018). This highlights that even fat pads nominally characterized in the same group (subcutaneous or visceral) can have different degrees of sensitivity to a similar stimulus and emphasizes the importance of evaluating multiple fat pads in the context of metabolic diseases. It is worth mentioning that despite the discrepancies between human and rodent fat depots, the hormonal regulation of adipose tissue metabolism appears to be similar between humans and rodents. In addition, retroperitoneal and epidydimal fat pads are strong candidates to study metabolic diseases and associate with human visceral adiposity (Chusyd, Wang, Huffman, & Nagy, 2016).

In the fasting state it is expected to have higher lipolysis rates stimulated by the SNS and counter regulatory hormones via PKA-mediated phosphorylation of HSL.

However, in the fed state, in the presence of insulin, lower PKA and HSL phosphorylation is expected, consequently reducing lipolysis postprandially. Despite our results showing similar phosphorylation of HSL between the groups in the fasting state, in the fed state (mimicked here by the presence of insulin), our treatment reduced phosphorylation of HSL. Reduced lipolytic capacity has been suggested as a potential target to manage T2D (Morigny et al., 2016). This suggests that EWH may be sensitizing adipose tissue to be more responsive during fasting conditions and facilitate lipolysis, while reducing total lipolysis capacity to prevent exacerbation of NEFA release in the fed (insulin-stimulated) state, once again contributing to reduced lipotoxicity. Another possibility is that while adipocyte sensitization by EWH leads to enhanced PKA and HSL phosphorylation and eventually to higher NEFA release through lipolysis during fasting (which was not measured dynamically), the new adipocytes (smaller and insulin sensitive) may be taking up NEFA and storing them as TG upon PPARγ activation, reflected by the reduced plasma TG concentration in the treated group (Jahandideh et al., submitted). In fact, PPARγ agonists enhance adipose tissue capacity to taken up and store NEFA (Oakes et al., 2001) and AT2R agonist improved insulin effects of reducing NEFA and TG during clamp studies (Shum et al., 2013). I cannot exclude the possibility that EWH-treated rats had a higher rate of NEFA re-esterification as a contribution to decreased plasma NEFA concentration, as was shown to happen after PPARγ agonism using TZD (Tordjman et al., 2003). Lastly, I should consider the possibility of the observed results being attributed to a different adipose tissue composition in terms of adipocyte size and sensitization. For example, at this stage adipose tissue may be in a transition/compensation phase where NEFA release is equal to NEFA uptake. It is possible that in the future the net effect of NEFA

release and uptake may change, with more numerous sensitized adipocytes overcoming the number of insulin resistant cells. However, at this point these are just speculations.

In summary, in this study EWH showed a greater effect in adipose tissue than in other insulin sensitive tissues (liver and muscle). I showed evidence that EWH activates PPARγ in adipose tissue, which leads to adipogenesis stimulation and, possibly, to higher rates of lipid uptake and storage; together with adipocytes' reduced lipolytic capacity, this system is contributing to reduced lipotoxicity and enhanced insulin sensitivity locally (adipose tissue) and systemically (skeletal muscle), which are partially the reasons for the previously observed improved glucose tolerance (Jahandideh et al., submitted).

## 4.2 EWH effects on inflammation, RAS modulation and gluconeogenesis

### *4.2.1 Modulation of RAS components and inflammation are not the main mechanisms by which EWH improved glucose tolerance in HFD fed rats.*

RAS overexpression has a proven link with inflammation, obesity and insulin resistance, thus becoming a target in the management of metabolic diseases. Previous work by our group on spontaneous hypertensive (SHR) rats showed that EWH and one of its peptides (IRW) reduce blood pressure (Jahandideh et al., 2016; Majumder et al., 2013). Of interest, modulation of RAS components also locally influences insulin action (Chai et al., 2011; Chu & Leung, 2009; Rodriguez et al., 2018). In this work there was no decrease in abundance of most RAS components, but there was an increase in abundance of AT2R in adipose tissue and liver. AT2R activation in the context of adipose tissue has been previously described in this thesis. As for the increase in AT2R in liver, I do not have an explanation. However, combined with lack of increase in Akt

phosphorylation in liver compared to control, I can say that EWH may be not targeting the liver as a primary target for the insulin sensitization observed. Moreover, the results suggest that RAS was not the main mechanism by which insulin signaling was enhanced in skeletal muscle, due to no change observed in skeletal muscle RAS components abundance. In summary, despite systemic action related to regulation of blood pressure, EWH does not affect RAS locally as a main mechanism to enhance insulin sensitivity in our study; although AT2R modulation in adipose tissue may possibly have a role as discussed earlier.

Adipose tissue inflammation is characteristic of obesity (Landgraf et al., 2015; Weisberg et al., 2003; H. Xu et al., 2003) and EWH treatment reduced systemic inflammation in HFD fed rats (Jahandideh et al., submitted). Similarly to the egg-derived bioactive peptides effect of reducing inflammatory markers (Jahandideh et al., 2017; Majumder et al., 2015), soy (Kwak et al., 2016; Young, Ibuki, Nakamori, Fan, & Mine, 2012) and milk peptides (Aihara, Osaka, & Yoshida, 2014; S. Chakrabarti & Wu, 2015) have also shown anti-inflammatory properties *in vitro* and *in vivo*. Although reduced inflammation and resistin are related to improved insulin sensitivity (Moscavitch et al., 2016), here I show that glucose tolerance and systemic inflammation were improved (Jahandideh et al., submitted) despite no decrease in adipose tissue local inflammation or changes in production of adipokines. Different egg-derived hydrolysates tested in rodents have been shown to reduce systemic (Garces-Rimon et al., 2016a; Moscavitch et al., 2016) and kidney inflammatory markers (Y. Wang et al., 2012). Nevertheless, an increase in insulin sensitivity may occur without improvement of inflammatory status (Kim et al., 2015) and insulin resistance due to obesity may occur before any changes in adipose tissue inflammation, as verified in humans (Tam et al., 2010). These results suggest that our EWH preparation did not affect local

adipose tissue inflammatory markers or adipokine secretion to improve insulin sensitivity.

### *4.2.2 EWH affects glucose homeostasis but not gluconeogenesis enzymes abundance*

Glucose homeostasis is maintained by counter-regulatory hormones, which shifts metabolism from endogenous glucose production by the liver (e.g. glucagon) to cellular glucose uptake (insulin) (Smith et al., 1993), and chronic treatment with EWH is shown to improve glucose tolerance and insulin sensitivity (Jahandideh et al., submitted). In the context of improved insulin sensitivity by EWH, particularly in the second phase of the insulin tolerance test, which is thought to reflect endogenous glucose production, I would expect a reduction in gluconeogenesis. Although in this work, EWH treatment did not decrease the abundance of gluconeogenic enzymes, I cannot exclude the possibility that insulin suppressed the activity of PEPCK and G6Pase more robustly in the treated rats as suggested by the insulin tolerance test (Jahandideh et al., submitted). This supports the idea of insulin suppressing gluconeogenesis, as expected in the context of enhanced insulin sensitivity by EWH. Performance the pyruvate tolerance test would be able to clarify the role of gluconeogenesis after EWH treatment, but this was not tested in this study. In addition, other mechanisms cannot be excluded, for instance Ochiai and Azuma showed evidence, based on improvements in both OGTT and IpGTT, that a different EWH affected intestinal and non-intestinal factors involved in glucose homeostasis, besides lipid metabolism, accounting for improved glucose tolerance (Ochiai & Azuma, 2017). Therefore, different physiological mechanisms may be accounting for the observed EWH effects.

### *4.2.3 IR-β phosphorylation*

As an upstream mediator of Akt phosphorylation, I predicted enhanced IR-β phosphorylation would be detected in WAT of EWH-treated rats because of enhanced Akt phosphorylation (Jahandideh et al., submitted) and of the insulin mimetic effect of EWH in 3T3 adipocyte cell line (Jahandideh et al., 2017) shown previously. However, the increase in Akt phosphorylation occurred despite no effect in WAT IR-β phosphorylation by EWH treatment in this study. This suggests that EWH, *in vivo*, may be affecting the pathway downstream of the insulin receptor.

Interestingly, Quiroga et al. showed that improved insulin signaling in adipose tissue after AT2R agonist treatment happened without changes in IR-β phosphorylation compared to control group (Quiroga et al., 2018). Therefore, the AT2R increase observed in our study could be contributing to the enhanced insulin signaling shown by increased phosphorylation of Akt, but I did not perform any experiments to test this hypothesis.

Although mTOR pathway is more related to mitogenic activity, a role for its pathway in glucose and lipid metabolism has been shown recently and I cannot exclude the possibility of mTORC2 being a possible candidate to enhance Akt phosphorylation after amino acid ingestion (specially leucine and arginine) (Lee, Jung, & Guertin, 2017). Thus, it is worth investigating mTOR pathway as an upstream activator of Akt after EWH hydrolysate in future studies, especially considering that EWH is predominantly composed of protein.

## 4.3 Conclusion

Based on the results of this study a proposed mechanism of action of EWH to improve glucose tolerance is presented in Fig. 4.2. Despite WAT being more responsive to EWH effects than other insulin sensitive tissues, at least in the pathways investigated here, different fat pads showed different responses to EWH treatment, illustrating the importance of studying multiple sites in order to fully appreciate the full spectrum of EWH effects. Nevertheless, this study demonstrates that EWH may be acting by several mechanisms to improve glucose tolerance in HFD-induced insulin resistant rats, which includes changes in adipose tissue morphology (reduced adipocyte size) and physiology (sensitization of adipocytes to insulin), improvement in insulin signaling, and possibly increase of AT2R (Fig 4.2). It is also possible that AT2R is the adipocyte membrane receptor for EWH peptide(s), whose activation elicits the mechanism of action hypothesized here.

The EWH preparation use in this thesis was used in different contexts as well, table 4.1 summarizes the effects of EWH in different tissues and cell line obtained from the present thesis, the papers already mentioned (Jahandideh et al., 2017; Jahandideh et al., 2016; Jahandideh et al., submitted) and Forough Jahandideh's PhD thesis (Jahandideh, 2017). Those results highlight the potential of EWH applicability in the management of metabolic diseases and in the functional food market.

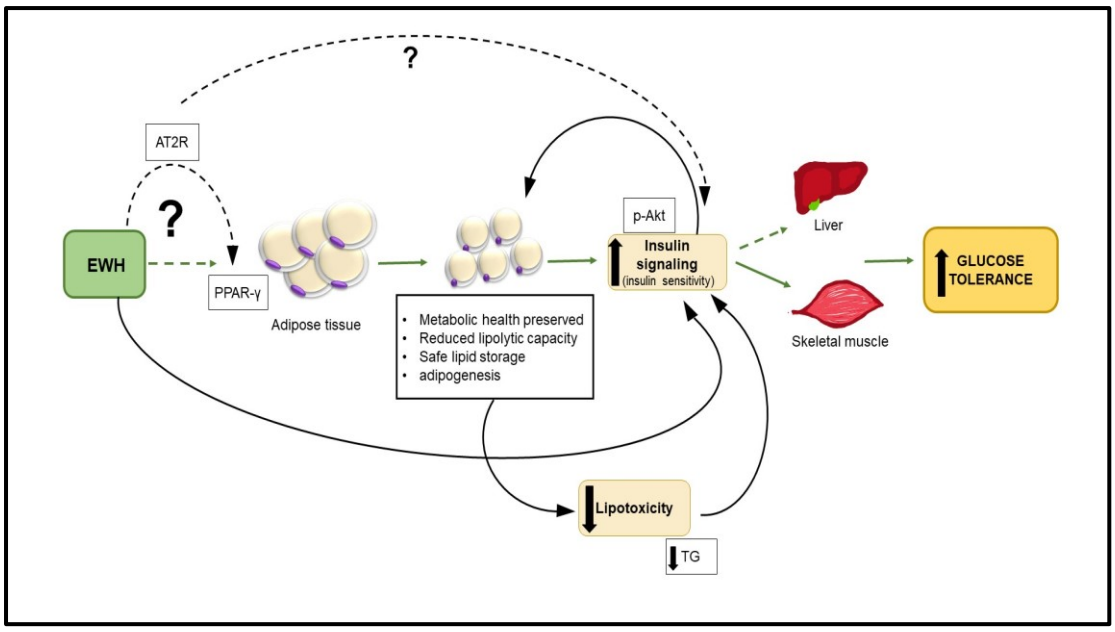


**Figure 4. 2- EWH proposed mechanisms of action to improve glucose tolerance.**

(See text for explanation). Abbreviations: AT2R, angiotensin II type 2 receptor; PPARγ, Peroxisome proliferator-activated receptor gamma; Akt, protein kinase B.

**Table 4. 1-** Summary of EWH effects in different tissues and pre-adipocyte cell line.

| | Liver | Skeletal Muscle | Retro WAT | Epi WAT | Vasculature | 3T3 -F442A cell-line |
|---|---|---|---|---|---|---|
| **Insulin Signaling** | | | | | | |
| p-Akt | X | ↑ | ↑ | ↑ | --- | ↑ |
| p-IRS-1 | --- | --- | --- | --- | --- | ↑ |
| p-IRβ | --- | --- | X | X | --- | ↑ |
| AS160 | --- | --- | X | ↑ | --- | --- |
| **Adipose tissue differentiation** | | | | | | |
| C-EBPα | --- | --- | --- | --- | --- | ↑ |
| p-ERK | --- | --- | X | X | --- | ↑ |
| PPARγ abundance | --- | --- | X | ↑ | --- | ↑ |
| PPARγ activity | --- | --- | ↑ | X | --- | --- |
| Number large adipocytes | --- | --- | ↓ | ↓ | --- | --- |
| Number small adipocytes | --- | --- | ↑ | ↑ | --- | --- |
| Adiponectin | --- | --- | X | X | --- | ↑ |
| Lipid accumulation | --- | --- | --- | --- | --- | ↑ |
| **Lipolysis** | | | | | | |
| PKA | --- | --- | X | ↓ | --- | --- |
| p-PKA (overall) | --- | --- | ↑ | ↑ | --- | --- |
| HSL | --- | --- | X | ↓ | --- | --- |
| p-HSL (after insulin) | --- | --- | ↓ | X | --- | --- |
| **RAS components** | | | | | | |
| AT2R | ↑ | X | ↑ | X | ↑ | X |

| | | | | | | |
|---|---|---|---|---|---|---|
| AT1R | X | X | X | X | ↓ | ↓ |
| ACE | X | X | X | X | ↓ | --- |

**Inflammation and vasculature**

| | | | | | | |
|---|---|---|---|---|---|---|
| COX-2 | --- | --- | --- | --- | --- | ↓ |
| p-c-Jun | --- | --- | --- | --- | --- | ↓ |
| BP | --- | --- | --- | --- | ↓ | --- |
| Nitrotyrosine | --- | --- | --- | --- | ↓ | --- |
| Relaxation | --- | --- | --- | --- | ↑ | --- |

(Adapted from the results obtained in this thesis and Jahandideh, 2017; Jahandideh et al., 2017; Jahandideh et al., 2016; Jahandideh et al., submitted) --- N/A, ↑ enhanced compared to control group, ↓ decreased compared to control group, X- no changes compared to control group. Abbreviations: Akt, protein kinase B; IRS-1, insulin receptor substrate 1; AS160, Akt substrate of 160 kDa; C-EBPα; ERK, extracellular signal-regulated kinase 1/2; PPARγ, Peroxisome proliferator-activated receptor gamma; PKA, protein kinase A; HSL, hormone sensitive lipase; AT2R, angiotensin II type 2 receptor; AT1R, angiotensin II type 1 receptor; ACE, angiotensin converting enzyme; COX-2, cyclooxygenase-2; BP, blood pressure; Retro, retroperitoneal; Epi, epididymal.

## 4.4 Limitations

As any other study, ours is not exempt of limitations. First, I recognize that the results presented here cannot be translated directly to humans; therefore, I do not know if the effects of 4% EWH treatment have enough magnitude to exert a physiological effect in the human body. Second, the EWH used in this study is a mixture of peptides, variable in length and amino acid sequence. Thus, I cannot attribute the changes observed to any of the peptides specifically; which makes difficult to speculate possible pathways of absorption, degradation in the GI tract or to correlate specific amino acids to the physiological changes showed. On the other hand, the peptide mixture may be more efficacious than any of the individual peptides. Third, no dynamic measurements of endogenous glucose production or lipid metabolism were done in our study. For this reason, I can only rely on the evidence under static conditions given by tissues lysates

and plasma analysis to support our hypothesis. In addition, all experiments were performed after chronic EWH administration, which may contribute to failure to observe some effects observed after acute treatment of adipocyte cell lines. Finally, I did not measure AT2R activation or its blockade to investigate the dependency of the effects observed on AT2R. Therefore, I cannot conclude that AT2R is the EWH receptor or upstream activator of PPARγ in the present study although the data are consistent with such a role.

## 4.5 Future directions

The exploratory study presented here shows evidence that EWH may be primarily targeting adipose tissue to cause the metabolic effects observed. Taking in consideration the results presented, it would be interesting to further investigate the working hypothesis proposed in Fig. 4.2, particularly the dependence on AT2R of EWH activation of PPARγ. Moreover, the investigation of EWH dependency on PPARγ activation would help to clarify the exact intracellular pathway essential for EWH action in adipose tissue.

Because EWH seems to have an effect in decreasing total amount of lipolytic enzymes (PKA and HSL), a study of dynamic changes in lipolysis rate in the absence and presence of insulin would help clarify the effect of EWH related to lipid profile and adipose tissue physiology. Importantly, it would be interesting to identify the peptides that are present in the mixture of the EWH and which ones are acting in adipose tissue; some studies in this regard are already in progress in our group which will allow for future clarification of the peptides' absorbance and stability in GI tract, action (e.g. receptor-mediated or not) and magnitude of effects. Importantly, the possibility of a synergistic or additive effect of the EWH peptides to available drugs in the market for

the management of metabolic diseases, would be valuable. These are but several of many possibilities to enhance the knowledge regarding EWH and derived peptides in the human physiology and clarify their mechanism of action, contributing to the advancement of science in this field and their introduction to the functional food market.

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**Appendix 1**

*nutrients*

*Review*

# Egg and Soy-Derived Peptides and Hydrolysates: A Review of Their Physiological Actions against Diabetes and Obesity

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**Abstract:** Type 2 diabetes and obesity are two chronic conditions associated with the metabolic syndrome and their prevalences are increasing worldwide. The investigation of food protein-derived bioactive peptides that can improve the pathophysiology of diabetes or obesity while causing minimal side effects is desired. Egg and soy proteins generate bioactive peptides with multiple biological effects, exerting nutritional and physiological benefits. This review focuses on the anti-diabetic and anti-obesity effects of egg- and soy-derived peptides and hydrolysates in vivo and in vitro relevant to these conditions. Studies using the intact protein were considered only when comparing the results with the hydrolysate or peptides. In vivo evidence suggests that bioactive peptides from egg and soy can potentially be used to manage elements of glucose homeostasis in metabolic syndrome; however, the mechanisms of action on glucose and insulin metabolism, and the interaction between peptides and their molecular targets remain unclear. Optimizing the production of egg- and soy-derived peptides and standardizing the physiological models to study their effects on diabetes and obesity could help to clarify the effects of these bioactive peptides in metabolic syndrome-related conditions.

**Keywords:** diabetes; obesity; egg; soy; peptides; hydrolysate; bioactive peptides

## 1. Introduction

Diabetes is a chronic disease marked by the presence of hyperglycemia that occurs when the pancreas cannot produce enough insulin, or the body cannot effectively use the insulin that is produced. Uncontrolled diabetes can lead to several serious complications such as cardiovascular disease, nephropathy, retinopathy, amputation and nerve damage [1,2]. Well-managed diabetes can reduce the risk of these complications and increase life expectancy [1]. The treatment of diabetes requires long-term self-management and adherence to therapy, but several commonly used drugs can cause side-effects, which could negatively impact adherence [3].

Identifying natural products that can improve the disease state while exerting fewer side-effects is a current research trend. Many studies have explored the potential of biologically active peptides derived from food sources, which could

be referred to as functional food ingredients. In the absence of a common definition, The European Commission Concerted Action on Functional Food Science in Europe together with The International Life Sciences Institute Europe published a consensus document and defined functional foods as those that beyond their nutritional value can exert one or more physiological effects in the body in a manner that can improve health/well-being or reduce the risk of diseases [4]. These peptides are produced enzymatically or using fermentation under controlled conditions of pH and temperature.

Two interesting sources of bioactive peptides are hen egg and soy. Egg is relatively cheap, found in almost every country and is nutrient-dense, which means it could be affordable and beneficial to a broad range of the world's population. Although the physiological effects of egg hydrolysates (EH) and peptides have been tested, the majority of publications investigated the angiotensin converting enzyme (ACE)-inhibitory, antioxidant or anti-inflammatory effects as reviewed by Liu et al. [5]. Soy, which is also protein-dense and broadly available, has been mostly studied regarding its antioxidant and anti-inflammatory actions as reviewed by Masilamani et al. [6]. Since an upregulated renin angiotensin system, inflammation and oxidative stress are observed in obesity and diabetes, which in turn are components of the metabolic syndrome (MetS) along with hypertension, it is of interest to study egg and soy in terms of their anti-diabetic and anti-obesity properties.

This review focuses on the effects of EH and soy hydrolysate (SH) or peptides in improving or preventing type 2 diabetes (T2D) and obesity in vivo or relevant in vitro endpoints such as insulin signaling pathways. The inclusion criteria were studies using egg, egg white (EW), egg yolk or soy in the form of hydrolysate or peptides. Studies where the results of intact protein were compared to the hydrolysate were also included. Only studies using oral administration of the hydrolysate or peptides in vivo were considered.

## 2. Diversity of Bioactive Peptides

Although several processes can be used to generate bioactive peptides, the most common method is enzymatic hydrolysis. Substrate specificity of enzymes generates peptides of different amino acid sequences and can be used to optimize

the production of peptides with desired biological effects, as can be seen in Tables 1–4. Nevertheless, the peptides are complex and the use of a purification step following hydrolysis is common [7–9]. Alternatively, synthetic production of peptides [10,11] can be used to obtain specific peptides and study their physiological action.

Several groups have used the whole hydrolysate instead of individual peptides when studying their effects [12–18]. In those cases, effects cannot be attributed to a specific peptide, because the enzymatic hydrolysis can generate a myriad bioactive peptides, raising the possibility that the effect observed could be due to a combination of numerous peptides presented in the hydrolysate. Another variable of enzymatic hydrolysis process is the processing duration, which can impact both the peptide sequences and concentration in the hydrolysate [19].

It is worth mentioning that some of the enzymes used in hydrolysate production are not naturally produced by the human body, such as thermoase [20], flavourzyme [8,19] and neutrase [19], which means the peptides produced may not replicate those generated by the natural digestion process in the human body. Even though some studies used enzymes that are naturally produced in humans, such as pepsin and pancreatin [7,11,15,17] there is also no guarantee that the desired peptides would be produced or stable after further gastrointestinal digestion.

Due to the diversity of peptides obtained after enzymatic hydrolysis, multiple mechanisms of action of the peptides may influence outcomes. The length of the peptides can influence the absorption process in the gut as reviewed by Miner-Williams et al. [21] and specific amino acids can have a greater influence in the interaction with enzymes, for example the regions of interaction between a soy peptide and the enzyme dipeptidyl peptidase-IV (DPP-IV) correlated with the presence of the amino acids glutamine and arginine [11].

There is little evidence that accounts for the mechanisms of action of the peptides and important questions remain unanswered. For instance, are the peptides absorbed intact? Or can they initiate a cascade reaction by binding to receptors in the gut cells? Is the integrity and stability of the peptides after gastrointestinal digestion a requirement for them to exert their physiological effects?

## 3. In Vitro Study of Egg Hydrolysate (EH)/Peptides

A summary of in vitro studies and identified peptide sequences are provided in Table 1. Multiple metabolic pathways in several organs are involved in glucoregulation. One possibility to help manage diabetes is inhibition of intestinal α-glucosidase, which is an effective method to delay carbohydrate absorption [22] and reduce blood glucose concentrations. Peptides obtained after pepsin hydrolysis of egg white (EW) exhibited α-glucosidase $IC_{50}$ values ranging from 365 to 1694 μg/mL [7], while peptides obtained from alcalase hydrolysis of egg yolk yielded $IC_{50}$ values ranging from 23 to 40 μmol/L [22]. Beside α-glucosidase inhibition, the peptides from EW exerted multiple activities, for instance, ACE-inhibitory capacity with $IC_{50}$ ranging from 9 to 27 μg/mL and DPP-IV- inhibitory activity with IC50 from 223 to 1402 μg/mL. The only exception was the peptide YIEAVNKVSPRAGQPF, which did not present either α-glucosidase or DPP-IV inhibitory activity [7]. The results suggest that egg peptides can potentially exert more than one physiological effect. Multiple activities exerted by the egg white hydrolysate (EWH) were found in other studies using cell lines as well [19,20]. EWH obtained with different enzymes (Table 2) exerted concomitantly anti-inflammatory, antioxidant, hypocholesterolemic, DPP-IV- and ACE-inhibitory activity [19]. The EWH derived from pepsin and peptidase-mediated hydrolysis had the highest potential against disorders associated with MetS such as hypertension, obesity and T2D, presenting $IC_{50}$ against DPP-IV of <10 mg protein/mL and against ACE ranging from 47 to 151 μg/mL [19].

In the 3T3-L1 adipocyte cell line, thermoase + pepsin-prepared EWH not only sensitized the cells to insulin action but also mimicked insulin signaling. The EWH stimulated adipocyte differentiation by enhancing peroxisome proliferator associated receptor gamma (PPAR-γ) and CAAT/enhancer binding protein alpha (C/EBPα) expression, which led to enhanced adiponectin release and intracellular lipid accumulation. Moreover, these EWH enhanced the phosphorylation of proteins involved in the insulin signaling pathway, such as extracellular signal regulated kinase 1/2 (ERK 1/2), insulin receptor substrate 1 (IRS-1) and insulin receptor and protein kinase B (AKT) [20]. In adipocytes, the same EWH also presented anti-inflammatory properties by reducing cyclooxygenase-2 (COX-2) expression and C-Jun phosphorylation induced by tumor necrosis factor-α (TNF-α) [20]. Thus, the effect of thermoase + pepsin-

prepared EWH in 3T3-L1 cells is exerted via insulin receptor and downstream proteins in the insulin signaling pathway. The adipogenic effect observed was partially mediated by PPAR-γ, because peptides identified in the hydrolysate upregulated PPAR-γ expression in vitro [20]. In macrophages, no effects were observed regarding TNF-α using peptidase or pepsin or flavourzyme EWH, but peptidase-prepared EWH reduced IL-6 after lipopolysaccharides stimulation [19].

An improvement in insulin sensitivity was also observed in a muscle cell line exposed to EW peptides. IRW, a peptide from egg ovotransferrin improved insulin resistance induced by angiotensin-II in skeletal muscle cells [10]. The peptide reversed the impaired insulin signaling and glucose uptake by normalizing phosphorylation of the serine residue in IRS and increasing AKT phosphorylation, which contributed to increased translocation of glucose transporter 4 (GLUT4) to the plasma membrane. It was shown that these effects were exerted partly by reducing angiotensin II type 1 receptor expression and reactive oxygen species (ROS) production [10]. In contrast, IQW and LPK egg white-derived peptides only exhibited antioxidant activity [10].

Although anti-diabetic activity is exerted by specific peptides, others presented low or no activity as antidiabetic agents [7,10,19,22], a fact that was attributed to their different amino acid sequences once they all were tested under the same conditions [10,19]. This fact indicates that the effects observed were due to the presence of specific peptides; however, there is a lack of experiments studying the relationship between the amino acid sequence in the peptides and their actions.

In summary, in vitro studies show that EWH or peptides derived from EW and egg yolk can exert multiple biological activities, including antidiabetic, by inhibiting enzymes such as DDP-IV and α-glucosidase or improving insulin sensitivity or signaling. However, the peptide amino acid sequence is important in determining the peptides' ability to act as antidiabetic agents. Therefore, there is a need for more in vitro experiments to specifically identify the interaction between the peptides, their amino acid sequence and the targets involved in the insulin signaling pathway.

**Table 1.** In vitro studies of egg-derived hydrolysates/peptides and their effects related to diabetes and obesity.

| | Aims | Hydrolysis | Main Findings | Additional assays | Peptides |
|---|---|---|---|---|---|
| **Egg yolk specific peptides**<br>**Enzymatic activity** | | | | | |
| Zambrowicz et al. 2015<br>[7] | Investigate multiple biological properties of peptides | Pepsin<br>(120 min) | Three out of four peptides inhibited ACE,<br>$\alpha$-glucosidase and DPP-IV activity. The peptides presented antioxidant and ion chelating activity. | **DPPH - radical scavenging**<br>All peptides tested presented radical scavenging properties<br>(from 1.5 to 2.3 µMTroloxeq/mg) | YINQMPQKSRE<br>YINQMPQKSREA<br>VTGRFAGHPAAQ<br>YIEAVNKVSPRAGQPF |
| **Egg white specific peptides**<br>**Enzymatic activity** | | | | | |
| Yu et al. 2011<br>[22] | Investigate the inhibitory activity of hydrolysates against $\alpha$-glucosidase and $\alpha$-amylase and identify peptides | Alcalase<br>(180 min) | Peptides from EW inhibit $\alpha$-glucosidase but not $\alpha$-amylase. | N/A | **Ovotransferrin**<br>RVPSLM TPSPR<br>DLQGK AGLAPY<br>**Ovalbumin**<br>RVPSL DHPFLF<br>HAGN WIGLF |
| **Egg white specific peptides**<br>**Cell culture** | | | | | |
| Garcés-Rimon et al. 2016<br>[19]<br>264.7 RAW macrophages | Investigate multiple biological properties of related to the metabolic syndrome | Alcalase<br>Flavourzyme<br>Neutrase<br>Trypsin<br>Pepsin<br>Pancreatin | Pepsin hydrolysate: ↓ACE.<br>Peptidase hydrolysate:<br>↓ ROS, CHOL and IL-6. | **Peptidase hydrolysate (24 h)**<br>**Hypocholesterolemic activity**<br>0.259 ± 0.01(mmol bound/mg protein)<br>**ORAC test** | **Peptidase hydrolysate**<br>**(24 h)**<br>LPDEVSG DDNKVED<br>GVDTKSD |

| | | | | | |
|---|---|---|---|---|---|
| | | Peptidase<br>Promod 144P<br>(0, 2, 4, 8, 12, 24, 36 and 48 hours) | | 1099.9 ± 0.6 (μmol Trolox/g protein) | IESGSVEQA GGLVVT |
| | | | | **Pepsin hydrolysate (8 h)**<br>**Hypocholesterolemic activity**<br>0.154 ± 0.011 (mmol bound/mg protein)<br>**ORAC test**<br>574.2 ± 4.0 (μmol Trolox/g protein) | **Pepsin hydrolysate (8 h)**<br>FRADHPPL FSL<br>SALAM YQIGL<br>RADHPFL IVF<br>YAEERYPIL<br>YRGGLEPINF<br>RDILNQ ESIINF |
| Jahandideh et al., 2017<br>[20]<br>3T3-F442A Preadipocyte cell culture | Investigate the effect of hydrolysate on differentiation, insulin signaling and inflammation markers in pre-adipocytes | Thermoase (90 min)<br>+<br>Pepsin (180 min | ↑ intracellular lipid accumulation, adiponectin levels. ↑PPAR-γ and C/EBPα. ↑ p-ERK 1/2, p-IRβ and p-IRS-1.<br>↓ COX-2 and TNF-α -mediated C-Jun phosphorylation.↑ p-AKT after insulin treatment. | PPAR-γ expression enhanced in dose-dependent manner with EWH at 2.5, 5 and 10 mg/mL | ERYPIL<br>VFKGL<br>WEKAFKDED<br>QAMPFRVTEQE |
| Son et al., 2017<br>[10]<br>Rat L6 myoblasts | Study the effect of specific ACE inhibitory peptides on insulin resistance induced by Ang-II and their mechanisms of action in muscular cells | N/A | IRW prevented the decrease in glucose uptake induced by Ang-II, normalized serine phosphorylation of IRS and GLUT4 expression and ↑ p-AKT. IRW ↓ AT1R, no effect on AT2R; ↓ ROS and NADPH activity. IQW and LPK peptides had anti-oxidant but no other actions. | N/A | **Ovotransferrin**<br>IRW<br>IQW<br>LPK |

Abbreviations: ACE, angiotensin converting enzyme; Ang-II, Angiotensin II; DPP IV, Dipeptidyl peptidase IV; EW, Egg white; IRS-1, Insulin receptor substrate 1; IRS, Insulin receptor; IRβ, Insulin receptor β; COX-2, cyclooxygenase 2; PPARγ, peroxisome proliferator associated receptor gamma; C/EBP-α, CAAT/enhancer binding protein alpha; AKT, protein kinase B; ERK1/2, Extracellular signal regulated kinase 1/2; TNF-α, Tumor necrosis factor alpha; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ROS, Reactive oxygen species; CHOL, Cholesterol; IL-6, Interleukin 6; GLUT4, Glucose transporter 4; AT1R, Angiotensin II type 1 receptor; AT2R, Angiotensin II type 2 receptor; ↑ enhanced/stimulated; ↓reduced/inhibited.

## 4. In Vivo Studies of Egg White Hydrolysate (EWH)/Peptides

In vivo EWH presents multiple biological activities as demonstrated in Table 2. All these in vivo studies were done in rodents and the specific peptides in EWH were not reported. Although no changes were found in circulating insulin, one study observed a reduction in blood glucose concentration and reduced homeostasis model assessment of insulin resistance (HOMA-IR) with protease-prepared EWH treatment [12]. Another three studies reported no changes in blood glucose levels with EWH obtained from protease and alcalase hydrolysis [13,23,24]. Serum leptin concentrations were not statistically different [23,24], and reduction or no changes were observed in plasma adiponectin levels [12,14].

Alongside enlarged adipose tissue, ectopic fat accumulation can lead to insulin resistance (IR) and consequently T2D. Analysis of the lipid content in liver and muscle, and total body fat percentage in rats showed reduced values after protease- and pepsin-prepared EWH treatment [12–14,23]. The steatotic state was improved (reduced size and number of fat vesicles), but no histological changes were seen in the adipose tissue with the pepsin-prepared EWH groups presenting similar adipocyte size as the obese control [14]. Stearoyl-CoA desaturase (SCD) is an enzyme involved in fat synthesis and responsible for converting a saturated fatty acid to its respective unsaturated fatty acid [12]. The SCD index is the ratio between those fatty acids and is related to obesity and insulin resistance. Dietary supplementation with protease-prepared EWH decreased SCD index in serum, muscle and liver in rodents [12,13,23]. Several hypotheses were tested in an attempt to elucidate the mechanisms responsible for reducing fat accumulation; for instance, SCD-1 is an enzyme essential in fat synthesis and because the abundance of lipogenic enzymes such as lipoprotein lipase (LPL) and fatty acids synthase (FAS) were not altered by EWH, the decrease in non-adipose tissue lipid content was attributed to the reduced SCD index [3]. Garcés-Rimon et al. postulate that the reduction in liver fat accumulation could be due to the ability of pepsin-prepared EWH to stimulate FFA oxidation in the hepatocytes but this hypothesis has not been tested [14]. Another possibility is that the reduction in fat accumulation occurred due to increased fat excretion. Indeed, two studies reported increased excretion of cholesterol (CHOL) and/or triglyceride (TG) and total bile acids in feces after protease-prepared

EWH treatment [12,23]. In serum, reduction of CHOL or TG and/or free fatty acids (FFA) was observed [13,14,23]. However, no improvement in serum lipid profile was seen in another two studies [12,24]. An interesting corollary finding was that protease-prepared EWH increased muscular mass while decreasing fat accumulation, although the mechanism by which the hydrolysate acts remains unclear [13].

A study of the gut microbiota revealed that pepsin-prepared EWH treatment improved dysbiosis in obese rats; furthermore, short chain fatty acid (SCFA) and lactate concentrations in feces were lower compared to the obese group [15]. SCFAs are produced by gut microbiota through fermentation of dietary fiber, carbohydrates and peptides and are shown to improve glucose homeostasis and insulin sensitivity in rodents [25]; in addition, increased fecal SCFA content is found in obese human subjects [26]. Mechanisms that could explain the lower fecal SCFA in the pepsin-prepared EWH-fed group include maintenance of intestinal microbiota homeostasis or prevention of absorptive dysfunction by EWH; nevertheless, Requena et al. hypothesized that the change in microbiota occurred secondary to peptide absorption, with their actions on target tissues as antioxidant and anti-inflammatory agents leading to modulation of the gut microbiota [15] but there is as yet no evidence for this hypothesis.

Anti-inflammatory and antioxidant activity can contribute towards obesity and diabetes management [15]. In two studies in vivo, treatment with pepsin- and alcalase-prepared EWH reduced TNF-α in plasma and kidney and reduced malondialdehyde levels in plasma and urine indicating antioxidant properties [14,24]; these results are compatible with those observed in vitro previously mentioned in Table 1.

When not treated, diabetes can lead to several complications including nephropathy. Although NWT-03, an alcalase-prepared EWH, exerted in vitro DPP IV-inhibitory activity, in vivo it was not efficient in improving the diabetic state; however, the treatment reduced renal injury development and albuminuria in T2D rats [24]. When compared with vildagliptin (VIL), a currently used DPP-IV inhibitor, both NWT-03 and VIL exerted renal protection effects but only VIL increased GLP-1 levels. Therefore, it is believed that NWT-03 and VIL can act via

similar mechanisms but independently of their DPP-IV inhibitory activity [24].

It is worth noting that when administered in a single dose, protease-prepared EWH did not alter lipid profile, inhibit pancreatic lipase or slow food transit [23]. Interestingly, when compared, protease-prepared EWH and EW, both prevented fat accumulation and increased muscle mass, but EW increased fat excretion compared to EWH [13,23].

To summarize, EWH presented antidiabetic properties in vivo, reducing ectopic fat accumulation in liver and muscle, which can enhance insulin sensitivity, and increasing fat excretion, which reduces absorption of calories and could contribute to weight loss. It also protected against diabetes complications (nephropathy), but little or no change was observed regarding blood glucose, adiponectin or insulin levels and regarding inhibition of DPP-IV. The discrepancies in the results observed in vivo could be attributed to the difference in the physiological background of the animals used but is more likely due to variation in the mixture of peptides present in the hydrolysates. Furthermore, the studies suggest that bioactive peptides present in the EWH were responsible for at least part of the effects observed; nevertheless, no measurement of the peptides in plasma, identification of those peptides or any other specific assay was conducted. There is a gap in the literature to explain the mechanism of absorption and action of these peptides.

**Table 2.** In vivo studies of egg-derived hydrolysates/peptides and their effects related to diabetes and obesity in rodents.

| | Aims | Hydrolysis | Treatment details | Food intake and body weight (BW) | Blood/ Feces / urine analysis | Tissue analysis | Main Findings |
|---|---|---|---|---|---|---|---|
| **Egg white hydrolysate** | | | | | | | |
| **Studies in rodents** | | | | | | | |
| Wang et al., 2012 [24]<br>Zucker obese rats | Measure effect of hydrolysate NWT-03 on renovascular damage | Alcalase<br>(6 h) | Aqueous **NWT-03**<br>(1g/kg/day)<br>15 weeks | **Food intake** - not given<br>**BW** - no effect | No effect on blood glucose, insulin, HBA1C, cholesterol and FFA levels.<br>↑ **GLP-1** only by VIL<br>**URINE:** Reduced MDA levels and decreased albuminuria | **KIDNEY -** Reduced inflammatory interleukins (IL-1β, IL-13) and TNF-α. Improved FGS, reduced expression of α-SMA and increased TXA2R expression. | No changes in the diabetic profile of the rats; renovascular damage reduced by NWT-03 treatment. |
| Ochiai and Matsuo 2014 [13]<br>Wistar rats | Investigate the effect of EW and EWH on fat metabolism and TG content in non-adipose tissues | Protease<br>(duration not specified) | **Casein** (297 g/kg)<br>**EWH** (394 g/kg)<br>**EW** (286 g/kg)<br>8 weeks | **Food intake**<br>EWH ↓, EW ↓↓<br>**BW**<br>EW ↓ | **EWH vs casein-** ↓ TG, ALP activity and FFA by EWH.<br>**EW vs EWH-** ↓ HDL-CHOL, FFA and ↑ total- CHOL by EWH.<br>**FECES**<br>**EWH vs casein -** ↑CHOL excretion by EWH.<br>**EW vs EWH-** ↑ TG, TBA and CHOL excretion by EW. | **EWH vs Casein -** Similar results in all parameters, except for ↓ fat mass. ↑ mass; ↓ SCD index, TG content and G6PDH activity (MUSCLE). ↓CHOL, TG and SCD index (LIVER).<br>**EW vs EWH -** Similar results in all parameters, except for ↑ mass and ↓ SCD (MUSCLE) ↓ SCD (LIVER) by EW. | EW and protease EWH reduced fat in adipose and non-adipose tissues Inhibited enzymes involved in lipogenesis and increased muscular mass and lipid excretion. |
| Ochiai et al., 2014 [12]<br>Goto-Kakizaki rats | Feeding trial with EWH to study fat and glucose diabetic or normal rats | Protease<br>(duration not specified) | **Casein** (200 g/kg) And<br>**EWH** (267 g/kg)<br>6 weeks | **Food intake** Not different<br>**BW**<br>↓ by EWH | Glucose, HOMA-IR, SCD Index - ↓<br>**No difference** between any other parameters tested. | **MUSCLE -** ↓ TG and SCD. LPL, FAS and G6PDH similar.<br>**LIVER -** TG similar, ↓ SCD index. Liver, adipose tissue and muscle similar weight. | improved blood glucose levels and HOMA-IR, but not insulin secretion. Reduced TG in muscle and decreased lipid accumulation in tissues. |
| Wistar rats | | | **Casein** (200 g/kg) And<br>**EWH** (267 g/kg)<br>6 weeks | **Food intake and BW**<br>not different | **No difference** in any of the parameters tested.<br>(glucose, insulin, HOMA-R, HOMA-P, TG, NEFA, TC, HDL-CHOL, non-HDL-CHOL, adiponectin and SCD index) | **MUSCLE -** ↓ SCD but LPL, FAS and G6PDH similar<br>**LIVER** - TG similar, ↓ SCD index. Liver, adipose tissue and muscle similar weight | reduced lipid content in muscle. |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Garcés-Rimon et al., 2016<br>[14]<br>Zucker obese rats | Demonstrate the effects of EWH related to obesity, lipid metabolism, inflammation and oxidative stress | Pepsin<br>(8 or 14 h) | Aqueous EWH<br>(750 mg/kg/day)<br>12 weeks | No difference in food intake and BW regardless of the hydrolysate | ↓TNF-α, FFA and adiponectin, MDA. No changes in blood TG and CHOL. | **ADIPOSE TISSUE -** ↓ weight but no changes in histology.<br>**LIVER -** ↓ steatosis, ↑ GSH. Similar kidney and liver weight.<br>Longer duration of hydrolysis negated effects. | reduced fat accumulation, improved hepatic steatosis and dyslipidemia. Decreased inflammatory and oxidative stress markers in plasma. |
| Ochiai et al., 2017<br>[23]<br>Wistar rats | Study the effect of EW and low allergenic EWH on fat accumulation | Protease<br>(duration not specified) | **Equicaloric Diets**<br>**Casein** (297 g/kg)<br>**EWH** (394 g/kg)<br>**EW** (286 g/kg)<br>8 weeks | No difference in food intake and body weight between the three groups. | **EWH vs Casein**<br>↓, total CHOL, ALP. Similar glucose, TG, NEFA, HDL-CHOL, non-HDL-CHOL, HOMA-β and insulin.<br>**EW vs EWH**<br>Similar results in all parameters.<br>**FECES**<br>**EWH & EW vs Casein**<br>↑ TG, CHO and TBA | **EWH vs Casein-** Similar results in all parameters, except for ↓ weight, TG and NEFA, SCD index (LIVER). ↓ TG (MUSCLE)<br>**EWH vs EW-** Similar results in all parameters, except for ↑ G6PDH activity (muscle), SCD (adipose tissue) ↓ FAS (liver) in EWH. | reduced fat accumulation non- adipose tissues, reduced intestinal absorption of lipid by increasing lipid excretion.<br>Similar results as EW, however EWH was less allergenic |
| Requena et al., 2017<br>[15]<br>Zucker obese rats | Observe the effect of EWH on the gut microbiota of rats | Pepsin<br>(8 h) | Aqueous EWH<br>(750 mg/kg/day)<br>12 weeks | **Food intake**<br>N/A<br>**BW** no difference. | **FECES**<br>↓ lactate and SCFA. *Lactobacillus/ Enterococcus* and *C. leptum* similar to lean control. | **N/A** | partially reverted dysbiosis present in Zucker obese rats. |

Abbreviations: EWH, Egg white hydrolysate; FFA, free fatty acids; MDA, Malondialdehyde; EW, Egg white; TG, Triglyceride; CHO, Cholesterol; ALP, Alkaline phosphatase; TBA, Total bile acids; SCD, Stearoyl CoA desaturase; NEFA, Non esterified fatty acids; FGS, Focal glomerulosclerosis; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; G6PDH, Glucose 6-phosphate dehydrogenase; LPL, Lipoprotein lipase; FAS, Fatty acid synthase; TNF-α, Tumor necrosis factor alpha; α-SMA, Anti-a-smooth muscle actin; VIL, Vildagliptin; HOMA-R, homeostasis model assessment of insulin resistance; HOMA-β, Homeostasis model assessment of insulin secretion; GSH, Reduced Glutathione; HBA1C, Glycated hemoglobin A1C; GLP-1, Glucagon like peptide-1; TXA2R, Thromboxane A2 receptor; SCFA, Short chain fatty acids; WK, week; ↑ enhanced/stimulated; ↓ reduced/inhibited.

## 5. In Vitro Studies of Soy Hydrolysates (SH)/Peptides

Soybean also contains bioactive peptides, and eight studies evaluating the in vitro effects of SH or peptides against diabetes and obesity are summarized in Table 3. Similarly to EH, during adipocyte differentiation SH obtained from pepsin hydrolysis increased lipid accumulation, expression of PPAR-γ and the expression and secretion of adiponectin in a dose dependent manner in 3T3-L1 pre-adipocytes; furthermore, this SH enhanced glucose uptake and expression of GLUT4, which could contribute to improve insulin sensitivity [16]. It is believed that this SH stimulated pre-adipocyte differentiation through PPAR-γ activation, although the SH did not present PPAR-γ ligand activity [16]. Interestingly, a study found that compared to pepsin + pancreatin-prepared SH from ungerminated soybeans, using germinated soybean hydrolysate reduced the number of adipocytes during the differentiation process and increased lipolysis in mature adipocytes, which could lead to less fat accumulation [17].

Higher lipolysis in 3T3-L1 mature cells was observed after treatment with flavourzyme-prepared SH even after gastrointestinal (GI)-simulated digestion as well [8]. Along similar lines, SH prepared with alcalase lowered lipid accumulation and downregulated LPL and FAS gene expression (enzymes involved in the lipid uptake and de novo fatty acid synthesis) in the absence of or following GI-simulated digestion [18]. A hydrolysate obtained only with naturally-occurring GI enzymes (pepsin + pancreatin) exerted similar effects, although to a lesser extent. It suggests that GI digestion in vivo may not markedly affect the bioavailability of that SH [18] although whether that is true for all hydrolysates remains to be determined. β-conglycin is a storage protein naturally found in soybean and it is interesting to note that the higher the β-conglycin concentration in the hydrolysate, the higher the inhibition of LPL and FAS [18].

IR in skeletal muscle and liver is a prominent state found in T2D. Pepsin + pancreatin- prepared SH and its fractions (peptides not identified) enhanced glucose uptake in L6-muscle cells; in addition, the fractions, but not the SH, were able to activate AMPK pathway in those cells [9]. Glucose uptake in C2C12 skeletal muscle cells was also enhanced by another soy peptide, named aglycin

[27]. In HepG2 cells soy peptides previously known to modulate cholesterol metabolism by activating AMPK and ERK1/2 pathway [28], affected glucose metabolism by enhancing AKT phosphorylation, which in turn inactivated glycogen synthase kinase 3 (GSK3) by phosphorylating its serine residue, which can lead to higher glucose storage [29]. Moreover, the peptides increased glucose uptake and enhanced the expression GLUT4 and GLUT1 in liver cells [28]. One of these peptides (IAVPTGVA) also presented DPP-IV inhibitory activity with an $IC_{50}$ value of 106 μM, and the regions of interaction between IAVPTGVA and DPP-IV were identified as the amino terminal Glu205 and Glu206 and carboxyl terminal Arg358 residues [11].

Inflammation is not the focus of this review, but it is linked with obesity and diabetes. Two studies reported changes in inflammatory markers by SH [18] or soy peptide [30] in co-cultured adipocytes and macrophages such as, reduced COX-2 and inducible nitric oxide synthase protein and lowered nitric oxide and prostaglandin E2 production [18]. The treatment with synthetized soy peptide FLV reduced the production and effect of inflammatory molecules and improved insulin sensitivity in adipocytes (higher IRS-1 and AKT phosphorylation) [30]. The authors showed evidence that peptide transport into 3T3-L1 cells occurred mainly via the peptide transporter PepT2 [30].

Taken together, the in vitro results show that SH obtained after hydrolysis by specific enzymes and some identified peptides can improve insulin sensitivity, inhibit DPP-IV, increase glucose uptake in muscle and liver, and reduce lipid accumulation and inflammation in adipose tissue (Table 3). Some of the studies suggest that the soy peptides can act through AMPK and AKT pathways to modulate glucose metabolism and via PPAR-γ to stimulate adipocyte differentiation. Although one peptide transporter in adipocytes and regions of interaction between soy peptide and DPP-IV was identified [30], there is still a lack of studies regarding the specific interactions between the peptides and enzymes involved in the glucoregulation process and the mechanism of absorption of those peptides.

**Table 3.** In vitro studies of soy-derived hydrolysates (SH)/peptides and their effects related to diabetes and obesity.

| | Aims | Hydrolysis | Outcomes | Main Findings | Peptides |
|---|---|---|---|---|---|
| **Soy specific peptides Enzymatic activity** | | | | | |
| Lammi et al., 2016 [11] | Verify that soy peptide inhibits DPP-IV in vitro and identify the regions of interactions | Pepsin and/or Pancreatin synthetized peptides | Only IAVPTGVA ↓ DPP-IV activity. Regions of interaction were n-terminus Glu205 and Glu206 and c-terminus Arg358; the peptide has a proline flanked by valine in the fourth n-terminal residue, predicts interaction with DPP-IV. | Soy peptide IAVPTGVA inhibits DPP-IV activity in vitro. YVVNPDNDEN and YVVNPDNNEN were inactive against DPP-IV. | IAVPTGVA YVVNPDNDEN YVVNPDNNEN |
| **Soy specific peptides Cell culture** | | | | | |
| Tsou et al., 2013 [8] 3T3-L1 adipocytes | Isolate and identify peptides from soy hydrolysate with lipolytic activity | Flavourzyme 1% (125 min) | Three peptides ↑ glycerol release. After *in vitro* GI simulated digestion, VHVV capacity was not affected; ILL and LLL had attenuated lipolytic activity. | Soy peptides stimulated lipolysis in 3T3-L1 adipocytes and were little or not affected by GI enzymes. | ILL LLL VHVV |
| Lammi et al., 2015 [29] Human HepG2 cells | Verify that soy peptides modulate glucose metabolism | Trypsin or pepsin - synthetized peptides | All three peptides ↑ p-AKT,↓ GSK3 activation, ↑ GLUT 4 and GLUT 1 mRNA, ↑ glucose uptake. IAVPTVGVA > IAVPGEVA > LPYP). IAVPGEVA and IAVPTVGVA ↑ GLUT1 mRNA more; LPYP ↑ GLUT4 mRNA more. | Soy peptides modulate glucose metabolism and enhance glucose uptake in liver cells by activation of AKT and AMPK pathways. | IAVPGEVA IAVPTGVA LPYP |
| Kuak et al., 2016 [30] RAW 264.7 macrophages and 3T3-L1 adipocytes | Demonstrate the mechanism of transport of soy peptide into adipocytes and evaluate TNF-α induced inflammation and insulin response | Synthetized peptide | FLV peptide ↓ TNF-α, MCP-1 and IL-6 in co-cultured cell line (macrophages + adipocytes). FLV ↓ TNF-α-induced p- JNK and p-IKK and ↓ degradation of IκBα. TNF-α induced insulin resistance in adipocytes was ameliorated by FLV (↑ p-IRS-1, p-AKT). PepT2 > PepT1 expressed in adipocytes, ↑ by LPS and TNF-α. | FLV is transported into adipocyte cells mainly through PepT2 action and FLV can reduce the inflammatory and insulin resistant states linked to obesity mainly by inhibiting TNF-α induced inflammatory pathways. | FLV |
| **Soy Hydrolysate Cell culture** | | | | | |
| Martinez-Villaluenga et al., 2009 | Study the effect of SH on lipid accumulation and inflammation | Alcalase (3 h) | **Alcalase SH** in 3T3-L1 cells: ↓ lipid accumulation, LPL and FAS mRNA. Further GI simulated digestion did not reduce the bioavailability of Alcalase SH; | SH reduced lipid accumulation and inflammatory marker expression, even after GI simulated digestion. | N/A |

| | | | | | |
|---|---|---|---|---|---|
| [18]<br><br>3T3-L1 adipocytes and RAW 264.7 macrophages | | **or**<br><br>Pepsin + Pancreatin (3 h each) | Compared to Pepsin + pancreatin SH, Alcalase SH ↓ LPL and FAS mRNA in a higher extension, before and after GI digestion.<br>**Alcalase SH** in RAW cells: ↓ LPL-induced nitrite formation, iNOS and COX-2 protein expression, PGE2 production.<br>**Pepsin + pancreatin SH** in 3T3-L1 cells: ↓ lipid accumulation, LPL mRNA, but not FAS mRNA. | Downregulation of LPL and FAS partially explain mechanism of action. Higher concentration of β-conglycin in the hydrolysate related to higher activity *in vitro.* | |
| González-Espinosa de los Monteros et al., 2011<br><br>[17]<br><br>3T3-L1 adipocytes | Investigate the effect of germinated vs ungerminated soybean hydrolysate on fat metabolism in adipocytes. Assess the interaction with soy phytochemicals. | Pepsin + Pancreatin (duration not specified) | Concentration > 1mg/mL ↓ cell viability during differentiation process (10 days incubation), but not during 24 h of exposure.<br>SH with and without phytochemicals ↓ lipogenesis, with higher germination time correlated to greater lipogenesis reduction.<br>Lipolysis were present in a dose-dependent manner only with SH without phytochemicals treatment. | Germination changed the amino acids composition in the SH and interfered with the responses. Overall, SH reduced the number of adipocytes during the differentiation process and increased lipolysis in mature adipocytes. | N/A |
| Goto et al., 2013<br><br>[16]<br><br>3T3-L1 pre-adipocytes | Observe effects of soybean peptic hydrolysate on adipocyte differentiation | Peptic hydrolysate (duration and enzymes not specified) | **During adipocyte differentiation** SH dose-dependently ↑ lipid accumulation, aP2 mRNA, adiponectin mRNA and secretion, PPAR-γ mRNA and protein expression, glucose uptake, GLUT4 mRNA. | SH stimulated adipocyte differentiation via PPAR-γ pathway and increased glucose uptake during differentiation process. | N/A |
| Roblet et al., 2014<br><br>[9]<br><br>L6-skeletal muscle cells | Verify the potential of EDUF to concentrate soy peptides and identify the mechanism of action of those peptides | Pepsin (45 min) + Pancreatin (120 min) | The initial hydrolysate, anionic and cationic peptides ↑ glucose uptake. Only the peptides ↑ p-AMPK. | Anionic and cationic soy stimulated glucose uptake and AMPK phosphorylation in L6-skeletal muscle cells i*n vitro*. | N/A |

Abbreviations: SH, Soy hydrolysate; Ap2, adipocyte fatty acid-binding protein; IRS-1, Insulin receptor substrate 1; COX-2, Cyclooxygenase 2; PPARγ, Peroxisome proliferator associated receptor gamma; AKT, protein kinase B; TNF-α, Tumor necrosis factor alpha; LPL, Lipoprotein lipase; FAS, Fatty acid synthase; GLUT4, Glucose transporter 4; GLUT1,glucose transporter 1; SH, Soy hydrolysate; GI- gastrointestinal; iNOS, Inducible nitric oxide synthase; PGE2, Prostaglandin E2; AMPK, Activated protein kinase; JNK, c-Jun N-terminal kinase; IKK, IκB kinase; PepT2, Peptide transporter 2; PepT1, Peptide transporter 1; IL-6, Interleukin 6; DPP-IV, Dipeptidyl peptidase IV; MCP-1, Monocyte chemoattractant protein-1; LPS, Lipopolysaccharide; GSK3, Glycogen synthase kinase 3; ↑ enhanced/stimulated; ↓ reduced/inhibited.

## 6. In Vivo Studies of Soy Hydrolysate (SH)/Peptides

The tests in vivo described in Table 4 show that SH can modulate glucose metabolism and reduce body weight. Two studies reported reduced blood glucose levels by SH or peptides [27,31]. Similarly to in vitro studies (Table 3), a 37-amino acid soy peptide named aglycin improved muscle glucose uptake by increasing the phosphorylation of insulin receptor, IRS-1 and AKT, and enhancing membrane GLUT4 levels, which contribute to improved insulin sensitivity in T2D mice [27]. In fact, treatment with aglycin led to similar results as those exerted by metformin in oral glucose tolerance (OGTT) and insulin tolerance tests [27]; furthermore, the release of insulin during OGTT was normal in the treated animals and, as expected, abnormal in T2D mouse controls, suggesting that the effect on glucose tolerance was primarily due to enhanced glucose uptake and insulin sensitivity [27]. It is noteworthy that intact aglycin-37 amino acids were found in blood samples from mice, indicating that it is stable after GI digestion and probably absorbed intact [27].

With regards to serum lipid profile and lipid excretion, protease-prepared SH reduced fat accumulation in genetically obese mice, enhanced lipid excretion and improved plasma CHOL levels in diet obese rats [31]. The reduction in fat accumulation could be due to the higher postprandial energy expenditure observed after intake of protease-prepared SH compared to casein [32]; furthermore, the major contributor to enhanced postprandial energy expenditure was increased exogenous carbohydrate oxidation. Although the effect on energy expenditure was not sustained after 24 h, total carbohydrate oxidation continued to be higher in the SH-treated group, perhaps due to higher plasma insulin levels and lower glucose concentrations during the postprandial period or due to lower lipid absorption and increased carbohydrate absorption [32]. No experiments were conducted to substantiate these hypotheses.

Tests in humans have only been done with respect to glucagon and insulin responses after intake of SH or intact soy protein. SH induced a slower response of insulin and glucagon compared to its intact protein and no effect in plasma glucose was observed. The concentration of soy protein or SH administered did not correlate with the increase in plasma levels of insulin but, interestingly, glucagon was sensitive to protein concentration

in a dose-dependent manner for both soy groups [33]. Another comparison, in rodents, showed that SH reduced body weight compared to whey isolate (WI) and whey isolate hydrolysate (WIH). In addition, soy intact protein and SH reduced liver and fat pad weight and maintained body protein percentage compared to WIH and WI, respectively [34].

The results in vivo herein, although scarce show that SH and soy peptides can potentially reduce tissue fat accumulation and increase fat excretion. Moreover, the soy peptide aglycin is resistant to GI digestion and can be absorbed intact by mice [27]. SH may also facilitate metabolic flexibility by shifting to carbohydrate utilization [32]. Nevertheless, only a few studies were done to test SH and peptides as antidiabetic agents in vivo and only one identified the peptide responsible for the effects.

**Table 4:** *In vivo* studies *of* soy-derived hydrolysates / peptides and their effects related to diabetes and obesity.

| | Aims | Hydrolysis | Treatment details | Food intake and body weight (BW) | Blood/ Feces / urine analysis | Tissue analysis | Main Findings | Peptides |
|---|---|---|---|---|---|---|---|---|
| **Soy Specific peptide Studies in rodents** | | | | | | | | |
| Lu et al., 2011 [27] BALB/c mice | Investigate effects of soy peptide aglycin as antidiabetic agent | Not specified | HFD + aglycin (50 mg/g) or Metformin (100 mg/kg/d) orally daily for 28 days | No difference in **BW or food intake** (compared with diabetic model control) | Intact peptide detected in plasma after oral administration. **Glucose after 28 days** ↓by Aglycin. **OGTT and ITT-** Aglycin similar effect as metformin. Insulin release not affected during OGTT. | **Skeletal Muscle** ↑ mRNA and total protein of IR and IRS-1. Total AKT and GLUT 4 mRNA not different. ↑ p-IR, p-IRS-1, p-AKT and GLUT4 on membrane. | Aglycin ameliorated glucose intolerance and insulin resistance in T2D mice mainly by increasing glucose utilization and insulin sensitivity after long-term treatment.***In vitro*** **-** glucose uptake ↑ in C2C12 skeletal muscle by aglycin in normal and insulin resistant cells. | Aglycin (37 aa) |
| **Soy hydrolysate Studies in rodents** | | | | | | | | |
| Aoyama et al., 2000 [31] Sprague-Dawley rats | Study the effect of soy isolate hydrolysate on weight reduction | Protease (duration not specified) | HFD for 12 weeks + SH (40.4%) or SPI or Casein (39.1%) for 4 weeks | Similar **BW**, **food intake** and body composition in all 3 groups | **SP XSH-** SPIH ↓Glucose, total CHOL and HDL. **SH X Casein-** SH ↓Glucose total CHOL and HDL. **SP X SH**- similar **SH X Casein-** SH ↑ protein and fat % and ↓ apparent fat digestibility | **Liver** SH ↓weight. **fat pad** similar weight | SH reduced fat accumulation and blood lipid profile levels by increasing fat excretion. SH reduced blood glucose in rats. | Mixture of peptides within five to six amino acids in length |
| Yellow KK mice | | | HFD for 31 days + SH (40.4%) or Casein (39.1%) for 4 weeks | No difference in **BW.** SH ↓ % fat and ↑ % protein (body composition). | N/A | **Liver** similar weight. **Fat pad** SPIH ↓ weight | SH reduced fat accumulation and increased total protein % in genetically obese KK mice. | |
| Aoyama et al., 2000 [34] | Study the effect of intact soy protein and hydrolysate as | Protease (duration not specified) | HFD for 4 weeks + SPI or SPIH or WI or WIH for | SH ↓ **BW** and carcass weight than WI and WIH. **SP and SH** ↓ fat %. | Glucose and TG similar between four groups. SP ↓ total-CHOL than WIH | SP and SH ↓ liver weigh than WIH and WI and ↓ fat pad than WI | No differences were observed between the SP and SH groups; however, compared to WI and WIH. SH ↓weight gain, liver | N/A |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Yellow KK mice | anti-obesity agents | | 2 weeks (energy restricted diet) | **Food intake** similar. | | | and fat pad weight while maintaining body protein. | |
| Ishihara et al., 2003 [32]<br><br>Yellow KK mice | Investigate the effect of soy isolate hydrolysate on energy expenditure | Protease<br><br>(duration not specified) | HFD for 28 days + high protein diet SH (404 g/kg) or Casein (391 g/kg) for 4 weeks | No difference in **BW or food intake** | SH ↑ lipid content | SH ↓ kidney weight. No difference in liver, muscle, fat pad, heart or spleen weights. | SH- ↑ postprandial energy expenditure, ↑ exogenous carbohydrate oxidation. No difference in postprandial exogenous lipid oxidation.<br><br>24-h energy expenditure similar; ↑ 24-h carbohydrate oxidation. SH excreted more TG in feces than casein group. | Mixture of peptides within five to six amino acids in length |
| **Soy hydrolysate Studies in Humans** | | | | | | | | |
| Claessens et al., 2008 [33]<br><br>Male, non-obese human (average 28 years, BMI 24 kg/m$^2$) | Compare glucagon and insulin response after ingestion of soy protein and SH | Not specified | Cross-over trial: consumed drinks containing 0·3, 0·4 or 0·6 g/kg BW of soy protein or SH | N/A | Intact soy protein > SH for insulin and glucagon response. Blood glucose not different. Enhanced glucagon response with increased protein load during intact and SH ingestion | N/A | Intact soy protein induced a more rapid insulin and glucagon response than the SH. Glucagon was more sensitive to protein load than insulin and responded in a dose dependent manner. No effects in blood glucose were observed. | N/A |

Abbreviations: IRS, Insulin receptor; IRS-1, Insulin receptor substrate 1; AKT, protein kinase B; GLUT4, Glucose transporter 4; SPIH, Soy hydrolysate; SP, intact soy protein ; HFD, High fat diet; CHOL-cholesterol; TG, Triglyceride; OGTT, Oral glucose tolerance test; ITT, Insulin tolerance test; T2D, Type 2 diabetes; BW, Body weight; BMI, Body mass index; WK, week

## 7. Conclusions

In conclusion, the research so far shows that both egg and soybean can be rich sources of bioactive peptides; furthermore, they can potentially exert multiple physiological activities, including anti-obesity and anti-diabetic effects, which is relevant in the management of MetS. Bioactive peptides can be produced by different methods such as, enzymatically, chemically or by molecular biology. However, there is a huge variability in the methods, consequently generating many different hydrolysates and peptides, as shown in Tables 1–4. The duration of the hydrolysis process and the enzymes used generate different amino acid sequences, which influence the type and intensity of activity exerted by the peptides. Although an in silico approach may help to investigate the predictability of peptide generation [11], the predictability, purity and cost-effectiveness of each method vary [35]; therefore, optimization of the production process and the identification of amino acid sequences that can potentially act as anti-diabetic agents are still in need.

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# Appendix 2

## Egg white hydrolysate enhances insulin sensitivity in high fat diet induced insulin resistant rats via AKT activation

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EWH effects on tissue insulin sensitivity

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Key words: egg white hydrolysate, insulin sensitivity, glucose metabolism, renin angiotensin system, adipose tissue.

**Abstract**

Agents that block the renin-angiotensin system (RAS) improve glucoregulation in the Metabolic Syndrome disorder. We evaluated the effects of egg white hydrolysate (EWH), previously shown to modulate the protein abundance of RAS component *in vivo*, on glucose homeostasis in diet-induced insulin resistant rats. Sprague Dawley rats were fed a high fat diet (HFD) for 6 weeks to induce insulin resistance. They were then randomly divided into 4 groups receiving HFD or HFD supplemented with different concentrations of EWH (1%, 2%, and 4%) for another 6 weeks in the first trial. In the second trial, insulin resistant rats were divided into two groups receiving only HFD or HFD+4% EWH for 6 weeks. Glucose homeostasis was assessed by oral glucose tolerance and insulin tolerance tests. Insulin signaling and protein abundance of RAS components, gluconeogenesis enzymes, and peroxisome proliferator-activated receptor gamma (PPARγ) were evaluated in muscle, fat, and liver. Adipocyte morphology and inflammatory markers were evaluated. In vivo administration of EWH increased insulin sensitivity, improved oral glucose tolerance ($P<0.0001$) and reduced systemic inflammation ($P<0.05$). EWH potentiated insulin-induced Akt phosphorylation in muscle ($P=0.0341$) and adipose tissue ($P=0.0276$) but minimal differences in the protein abundance of tissue RAS components between the EWH and control groups were observed. EWH treatment also reduced adipocyte size ($P=0.0383$) and increased PPARγ2 protein abundance ($P=0.0237$). EWH treatment yielded positive effects on the inflammatory profile, glucose tolerance, insulin sensitivity, and adipocyte differentiation in HFD-induced insulin resistance rats. The involvement of local RAS activity requires further investigation.

## Introduction

Unhealthy diet pattern, sedentary lifestyle, and genetic predisposition are the main determinants of obesity, cardiovascular diseases (CVDs), and metabolic syndrome (MetS). MetS is a cluster of abnormalities including hypertension, dyslipidemia, glucose intolerance, and abdominal obesity (1), increasing the risk for CVDs and type 2 diabetes (T2D) by two- and five-fold, respectively, compared with those without the syndrome (2). Lifestyle changes including dietary interventions and physical activity are the first line therapies for both CVDs and MetS (3). However, the long-term sustainability of these interventions is usually poor and patients progress to drug therapy. Given the serious side-effects associated with pharmacological drugs and their lack of effectiveness in some cases, scientific attention has been drawn towards application of naturally-derived compounds for the management of MetS (4, 5).

Bioactive peptides, encrypted within the primary sequences of food proteins but released by enzymatic digestion or food processing, can regulate blood pressure (6), reduce hyperlipidemia (7), and attenuate inflammation and oxidative stress (8). In addition, several whey protein-derived dipeptides increased glucose uptake in myotubes (9). Food proteins and peptides have also been reported to improve glucose tolerance and insulin sensitivity by stimulating Akt phosphorylation (10-14) and glycogen synthesis (9, 15) in rodents and patients (16).

Another mechanism to improve glucose homeostasis and insulin sensitivity is by affecting adipose tissue (12, 17-19). We recently reported the adipogenic, insulin mimetic and sensitizing effects of an egg white hydrolysate (EWH) in 3T3-F442A pre-adipocytes (20). *In vivo* studies show benefits of egg peptides on reducing fat mass and adipocyte size, hepatic steatosis and inflammation, contributing to improved insulin sensitivity (21-23). EWH also exerted anti-hypertensive effects in spontaneously hypertensive rats by modulating renin angiotensin system (RAS) (24). The abundance of the vascular angiotensin converting enzyme (ACE) and angiotensin type 1 (AT1R) receptor was

reduced, while vascular angiotensin type 2 receptor (AT2R) abundance increased. RAS is believed to be linked with insulin resistance in animals and humans (25-28).

Since EWH has shown potential benefits on several aspects of the MetS especially on adipose tissue, we aimed to study the effects of EWH on glucose tolerance and insulin sensitivity in a rat model of insulin resistance. We hypothesized that EWH supplementation affects insulin signaling and improves insulin sensitivity in peripheral tissues, such as adipose tissue and skeletal muscle. The primary experimental outcome of this study was oral glucose tolerance, while the effects of EWH on tissue insulin signaling, adipose tissue, and local RAS protein abundance were assessed as the secondary outcomes.

## Materials and Methods

### *Preparation of egg white hydrolysate*

Food grade egg white hydrolysate was prepared in the Food Processing Development Centre (Leduc, AB) according to the following conditions. Liquid egg white was diluted with water at a ratio of 1:1 (v/v) to obtain a solution with 5% protein solid. After adjusting the pH to 8.0 with 2MNaOH solution, and the temperature to 65°C, thermoase (0.1%, w/w) was added and protein digestion was carried out for 90 min. The enzyme was then inactivated by adjusting pH to 2.5 for pepsin digestion. The mixture was further hydrolyzed at 55°C by 1% pepsin for 180 min. The reaction was terminated by heating the solution at 95°C for 15 min and the hydrolysate was centrifuged (GEA Westfalia Separator Group, Oelde, Germany) at 8510 rpm and average flow rate of 450 L/h and then condensed to obtain approximately 10% solid. The hydrolysate was then spray dried at an inlet temperature of 300°C and outlet temperature of 90°C, the powder was collected and stored in food grade freezer (-20°C) for further experiments. EWH protein content was 77.7% with ≥85% of the peptides in the range of 1.36-6.51 KDa (Supplementary material S1).

### *Animals and diets*

The animal care protocol was approved by the University of Alberta Animal Care and Use Committee (Protocol # 1472) in accordance with the guidelines issued by the Canadian Council on Animal Care. The study also adhered to the Guide for the Care and Use of Laboratory Animals, United States National Institutes of Health. Eight weeks old

male Sprague Dawley (SD) rats (n=48) weighing 339.5 ± 11.7 g were purchased from Charles River Canada (St. Constant, QC, Canada) and housed 2 per cage (conventional cages) with ad libitum access to standard chow and water. Rats were acclimatized for one week at the University of Alberta animal facility, exposed to a 12:12 hour cycle of light:dark in a humidity and temperature-controlled (60% RH, and 23°C) environment. Two animal trials were conducted to fulfill the purpose of the study. The first trial was conducted to determine the effective dosage of the treatment while the second trial further verified the effectiveness of the treatment as compared to the control group. In both trials, after one-week of acclimatization, all rats received a high fat diet (HFD) (20% w/w) for 6 weeks to induce glucose intolerance as shown before (29). Rats were then randomly assigned to one of the following groups: HFD, HFD+1% EWH, HFD+2% EWH, and HFD+4% EWH (n=7-8 each) in the first trial and to HFD and HFD+4% EWH (n=7-8 each) in the second trial. The amount of the EWH used in this study could reasonably be expected to be achieved in the human population. These diets continued for another 6 weeks with ad libitum access to food and water. The diet composition is shown in Table 1. Casein was used to make all diet groups isonitrogenous. In the second study, half of the animals (n=4) in each group were injected with insulin (2 IU/kg body weight (BW)) or saline intraperitoneally 10 min before euthanizing to study insulin signaling in muscle and fat.

***Glucose and insulin tolerance tests***

After 5 weeks of experimental diets (11 weeks in total on HFD), an oral glucose tolerance test (OGTT) was performed on rats after an overnight fast (16 h). Fasting blood glucose was measured using a glucometer (Accu-Check Compact Plus, Roche Diagnostics, Laval, QC, Canada) and blood was collected from a tail vein for insulin determination. Then, rats were gavaged with 40% glucose solution (2g of glucose/kg BW) and blood glucose was measured at different time points. 50 µL of blood was taken at each time point during OGTT, centrifuged (1,000 x g, 20 min at 4°C) to obtain plasma, and stored at -20 ˚C for insulin measurement. For insulin tolerance test (ITT), rats were fasted for 4 hours after six weeks of EWH treatment (12 weeks in total on HFD). Fasting blood glucose was measured and then each rat received 1.5 IU/kg BW dose of insulin via an intraperitoneal injection. Blood glucose was determined at different time points post injection.

Homeostatic model assessment insulin resistance (HOMA-IR) was calculated based on the following equation: fasting glucose (mg/dL) × fasting insulin (μU/mL)/405.

***Body weight and food intake measurement***

BW was monitored weekly and food intake per 2 rats/cage was measured for 24 hours 3 times a week throughout the study.

***Tissue collection***

At the end of the study, rats were euthanized by exsanguination via excision of the heart under inhaled isoflurane anesthesia (isoflurane/oxygen; 1.0–2.5% mixture) after a 16-h fast. A 3-5 mL blood sample was taken from all rats in both animal trials except for the insulin-injected rats. Muscle, liver, kidneys, and fat pads were excised, washed in phosphate buffered saline, blotted, weighed, and immediately frozen at -80°C until further analysis. Small sections of fresh fat pads were fixed in 10% formalin (48 hours) for paraffin-embedded fat samples to determine adipocyte size and distribution.

***Tissue homogenization and protein extraction***

Hepatic and muscle proteins were extracted using an extraction buffer consisting of lysis buffer (Mitosciences Abcam, Toronto, ON, Canada), aprotinin (Calbiochem, Oakville, ON, Canada), sodium fluoride, sodium orthovanadate, and protease inhibitor cocktail (Sigma-Aldrich, Oakville, ON, Canada). Proteins from fat pads were extracted using a commercially available kit (Invent Biotechnologies Inc., Plymouth, MN, USA) as per manufacturer's instructions. The protein content was measured using total protein assay and sample aliquots were stored at -80 ˚C.

***Plasma analysis***

Plasma triglycerides (TG), total cholesterol, and non-esterified fatty acids (NEFA) (Wako Pure Chemical Industries Ltd.; Chuo-ku, Osaka, Japan) concentrations were measured using direct colorimetric enzymatic reactions as per the manufacturer's instructions. Samples from the OGTT were assayed for insulin using an ELISA kit (Alpco Diagnostics, Salem, NH, USA).

***Inflammatory markers***

Inflammatory cytokines in plasma and adipose tissue were assessed using a commercial Rat Inflammation ELISA strip (Signosis, Inc. Santa Clara, CA, USA) following manufacturer's instructions.

***Adipocyte size and distribution***

Adipose tissue paraffin blocks were cut into 5 μm sections and affixed in glass slides. One slide per sample was stained with hematoxylin-eosin, and 10 photomicroscopic images of each slide were taken in a grid formation using microscope 20X objective lens and Axion Vision 4.8 software. A scale bar of 100μm was placed in the images. ImageJ software "freehand selections" tool was used to measure adipocyte area ($mm^2$) of 47-52 cells/sample.

***Western blotting***

Protein extracts were separated by SDS-PAGE electrophoresis on 10-12% polyacrylamide gels, transferred to a polyvinylidene fluoride or nitrocellulose membrane, and incubated with antibodies against PPARγ and p-Akt (Cell Signaling Technology, Beverly, MA, USA), Akt, ACE, AT1R, AT2R, glucose 6 phosphatase-α (G6Pase-α), and β-actin (Santa Cruz, Dallas, Texas, USA), ACE2 (Abcam, Toronto, ON, Canada), and phosphoenolpyruvate carboxykinase (PEPCK) (Cayman chemical, Ann Arbor, Michigan, USA). PPARγ, ACE, ACE2, AT1R, AT2R, PEPCK and G6Pase bands were normalized to β-actin. Goat anti-rabbit and donkey anti-mouse conjugated secondary antibodies were purchased from Li-cor Biosciences (Lincoln, NB). Protein bands were detected by a Li-cor Odyssey BioImager and quantified by densitometry using corresponding software Odyssey v3.0 (Li-cor) or Image Studio Lite 5.2.

***Statistical analysis***

All data presented are expressed as means ± SEM of 3-10 rats from each treatment group as indicated in figure legends. Statistical analysis was performed using the GraphPad Prism software, version 6.0. Data was checked for normal distribution by the Shapiro-Wilk test. For analyses of OGTT, insulin ELISA, ITT, and insulin sensitivity in fat and muscle, we used a two-way analysis of variance (ANOVA). For all other data, one-way ANOVA, Kruskal-Wallis test, and student's t-test were used as indicated in figure legends. Bonferroni and Dunn's post-hoc tests were performed to assess differences between groups when appropriate. For the primary outcome, oral glucose tolerance, we conducted a post-

hoc power analysis using effect size of 0.25, alpha 0.05 and power 0.8 for an ANOVA with repeated measures, within factors using G*power (version 3.1, University of Kiel), which indicated a total sample size of 20 was required. A p value of 0.05 was considered statistically significant.

## Results

### Food intake, body and tissue weight

No significant differences were observed in the BW and food intake (Table 2) between the groups throughout the study, indicating that the palatability of the diets did not affect the results. Moreover, there were no significant differences in liver and kidney relative weights between groups. However, 4% EWH fed rats had lower fat mass compared to the HFD group as measured by the retroperitoneal and epididymal fat pad relative weights (Table 2). EWH had no adverse effects on rats in this study.

### OGTT and ITT

The glucose tolerance of the rats is shown in Fig. 1A. Rats receiving 4% EWH had significantly lower glucose response at t = 30, 60, 90, and 120 compared to the HFD group. Despite a better glucose tolerance in HFD+4% EWH group, no significant difference in insulin concentrations was observed during OGTT between HFD+4% EWH and HFD groups (Fig. 1B).

Rats in EWH treated groups tended to have lower blood glucose levels during the glucose disappearance phase (0–30 min) of the ITT, but not at a significant level. However, the difference became more evident between groups during the recovery phase (60–120 min), where HFD+4% EWH group exhibited significantly lower glucose levels at t = 90 and 120 min (Fig. 1C).

### Plasma metabolic profile

The metabolic profile of the rats is summarized in Table 2. There were no significant differences in fasting glucose and insulin concentrations between groups. HFD+4% EWH group showed lower fasting plasma TG compared to HFD group, while no differences were observed regarding NEFA and total cholesterol.

### Plasma and adipose tissue inflammatory markers

Plasma inflammatory markers in HFD and HFD+4% EWH groups showed comparable levels of TNF-α and Rantes. EWH feeding significantly reduced IL-1α, IL-β,

and MCP-1 compared to HFD group as shown in Fig. 2. In adipose tissue, except for a significant increase in epididymal fat IL-6 in HFD+4%EWH group compared to HFD, no other changes were observed in inflammatory cytokines (Supplementary material S2).

**Tissue insulin sensitivity measured by p-Akt**

HFD+4% EWH treatment improved glucose and insulin tolerance in rats as shown by OGTT and ITT. We further investigated tissue insulin sensitivity as one of the possible mechanisms of action of EWH since this treatment enhanced p-Akt in our previous cell study in adipocytes (20). Akt signaling plays a central role in insulin-stimulated glucose uptake in both muscle and adipose tissue (30). Muscle, liver, retroperitoneal, and epididymal fat pads were studied as the major tissues responsible for insulin-dependent glucose clearance. Fig. 3A-C shows enhanced p-Akt in muscle, retroperitoneal, and epididymal fat of the HFD+4% EWH group compared to HFD control. While insulin administration failed to induce phosphorylation of Akt in muscle and adipose tissue of the HFD group, an indication of insulin resistance state, 4% EWH supplementation enhanced insulin sensitivity significantly in muscle, epididymal, and retroperitoneal fat depots. However, liver p-Akt was unaffected by EWH (Fig. 3D).

**Adipocyte morphological changes in retroperitoneal and epididymal adipose tissue**

Because EWH enhanced insulin sensitivity in adipose tissue, we studied the effects of EWH treatment on markers of adipocyte differentiation, including adipocyte size and PPARγ abundance. Fig. 4A shows that EWH significantly reduced adipocyte size in retroperitoneal adipose tissue as measured by mean adipocyte area from 0.025 $mm^2$ to 0.022 $mm^2$, while the size reduction in epididymal fat (from 0.021 $mm^2$ to 0.017 $mm^2$) was not statistically significant (Fig. 4C). Nevertheless, both fat pads presented significant changes towards smaller adipocytes phenotype when the adipocyte distribution was analyzed by area intervals. The number of adipocytes within the range of 0.012 to 0.013 $mm^2$ was significantly increased in retroperitoneal fat pad whereas the number of larger adipocytes (0.02 to 0.029 $mm^2$) decreased significantly (Fig 4B). Similarly, epidydimal fat pad showed increased number of small adipocytes (from 0 to 0.009 $mm^2$), and decreased number of larger adipocytes (from 0.02 to 0.029 $mm^2$) as shown in Fig 4D ($p<0.05$). To further explore the effects of 4% EWH on adipocyte differentiation at the molecular level, we measured PPARγ2 protein abundance in adipose tissue. PPARγ is a transcription factor

mainly expressed in adipose tissue and involved in adipocyte differentiation by regulating several genes in lipid metabolism and enhancing insulin sensitivity (31). PPARγ2 abundance was significantly increased following 4% EWH treatment in epididymal fat compared to HFD group (Fig. 4A). Although PPARγ2 was enhanced in retroperitoneal fat, this increase was not significant (Fig 4B).

**Tissue protein abundance of RAS and gluconeogenesis components**

As one of the potential mechanisms for enhanced insulin sensitivity, we measured tissue protein abundance of different components of RAS namely ACE, ACE2, AT1R, and AT2R in HFD and HFD+4% EWH groups (Fig. 6 & Supplementary material S3-S5). Generally, no significant changes were observed in the protein abundance of tissue RAS components except for the AT2R which was increased significantly in the liver (Fig 6B) and retroperitoneal fat pad (Fig. 6C) in HFD+4% EWH group compared to HFD group. Hepatic glucose output contributes to blood glucose concentrations; however, no changes in liver PEPCK and G6Pase protein abundance were observed (Supplementary material S6).

**Discussion**

Our study revealed that supplementing a HFD with 4% EWH: (i) improved glucose tolerance with no changes in the postprandial insulin; (ii) enhanced overall insulin sensitivity as measured by ITT as well as enhanced muscle and fat insulin signalling in both fasted and insulin stimulated states; (iii) reduced systemic pro-inflammatory cytokines IL-1α, and IL-1β, and chemokine MCP-1; (iv) switched adipocyte phenotype towards smaller sizes in both retroperitoneal and epididymal adipose tissue along with increased PPARγ2 expression in epididymal adipose tissue, which is consistent with adipocyte differentiation.

We focused on the ability of EWH to alleviate insulin resistance, a key component of MetS (32). Prolonged HFD feeding is known to induce insulin resistance and glucose intolerance (29, 33, 34), adipocyte dysfunction and inflammation (35, 36), and dyslipidemia (37, 38) in rodents. Therefore, HFD induced insulin resistant SD rats were chosen as the animal model. Our data also confirmed the presence of insulin resistance in

HFD group as tissue Akt phosphorylation (especially in the muscle and adipose tissue) was not significantly increased after exogenous insulin injection in these rats.

Supplementing HFD with 4% EWH improved glucose tolerance in insulin resistant rats with no changes in plasma insulin concentrations, suggesting that 4% EWH treated rats are potentially more sensitive to insulin. The ITT results confirmed this hypothesis. Furthermore, 4% EWH tended to increase glucose disappearance in the first phase (0-60 min) and potentially suppressed hepatic glucose production in the second phase (60-120 min) of the ITT; however, analysis of gluconeogenesis enzymes such as PEPCK and G6Pase-α in liver revealed no differences after EWH treatment (Supplementary material S6). Although we did not observe any significant changes in the protein abundance of gluconeogenesis enzymes, EWH treatment may have affected the activity of these enzymes and/or other enzymes involved in the hepatic glucose production. Our data suggest that the improvement in glucose tolerance after EWH treatment was at least in part due to the increased insulin-stimulated glucose uptake. Food-derived bioactive peptides including egg-derived peptides have been recently reported to beneficially affect insulin signaling and glucose homeostasis (39).

We previously showed a significant increase in insulin sensitivity through enhanced p-Akt in EWH treated 3T3 adipocytes (20). To further explore whether 4% EWH treatment enhanced tissue insulin signaling, we assessed p-Akt in major insulin sensitive tissues namely liver, muscle, and adipose tissue. Interestingly, consistent with our previous data, insulin sensitivity was enhanced significantly in epididymal and retroperitoneal fat depots as well as muscle in EWH treated rats (Fig. 3). Therefore, we concluded that enhanced insulin signalling in adipose tissue and muscle contributed to the observed improvement in glucose tolerance after 4% EWH treatment. When assessing the rats' metabolic profile, no significant effect on plasma lipid components was observed except for reduced fasting plasma TG in the 4% EWH group. Our current data do not explain the change in plasma TG concentration, however, this may be due to reduced de novo lipid synthesis by the liver or enhanced tissue accumulation, especially in adipose tissue as opposed to remaining in the circulation (40). Smaller adipocytes having more capacity to store lipids contribute to reduced plasma TG and increased insulin sensitivity (41). Indeed, the reported enhanced insulin sensitivity in adipose tissue along with the significantly reduced adipocyte size and

higher PPARγ2 abundance after 4% EWH treatment in this study could indicate enhanced adipocyte differentiation leading to reduced plasma TG levels. WEKAFKDED, QAMPFRVTEQE, ERYPIL, and VFKGL are the bioactive peptides in EWH with PPARγ stimulatory activity in adipocytes (42). Pioglitazone, a PPARγ agonist, reduced adipocyte size and plasma TG and improve tissue insulin sensitivity in mice (43). In another study, pioglitazone improved insulin sensitivity and stimulated adipocyte differentiation in rats without affecting PEPCK expression level in liver (44), similar to our data. Furthermore, we observed reduced plasma pro-inflammatory markers (IL-1α and β and MCP-1) in the HFD+4% EWH fed rats indicating lower systemic inflammation compared to the HFD group. Obesity has been linked with a low-grade chronic inflammatory response characterized by altered adipokines production and increased markers of inflammation (45). Considering the critical role of inflammation in the occurrence of insulin resistance (46), the importance of the anti-inflammatory effect of the EWH in HFD treated rats is highlighted. Inflammation within white adipose tissue, induces insulin resistance locally and systematically due to the diffuse nature and close association of adipose tissue with other metabolically active tissues (47).

Although we did not target the specific association of adipose tissue to the less inflammatory phenotype of EWH treated rats, our data on enhanced PPARγ2 expression in the epididymal fat and tissue insulin sensitivity, consistent with the outcomes of our previous study in cells (20) points to a potential effect of the treatment on adipose tissue. When assessing the adipose tissue inflammatory markers, however, we did not observe any changes between the groups (Supplementary material S2).

Modulation of RAS components is another potential mechanism for the observed enhanced tissues insulin sensitivity following treatment with 4% EWH. RAS is mostly known for its systemic effects on blood pressure. In addition to systemic RAS, RAS components have also been reported to be expressed in tissues such as adipose tissue, liver, and pancreas known as local RAS (48). Local RAS activation has been linked to IR and T2D (28, 49). RAS blockade enhances glucose tolerance and whole-body insulin sensitivity in insulin resistant animal models or insulin resistant hypertensive humans (26, 50-54). AT1R and AT2R regulate insulin action in muscle (55) while RAS overexpression in skeletal muscle may impair insulin signaling and reduce glucose transporters expression

restricting glucose uptake (56). IRW, an egg derived tripeptide with antihypertensive effects (57), improves Ang II-induced insulin resistance in L6 cells, partially through reduced AT1R abundance and antioxidant activity (58). Despite the potential link between local RAS and tissue insulin sensitivity, this pathway appears not to be mainly involved in the observed effects of EWH on improved insulin sensitivity in the current study. This is because between all the studied RAS components including ACE, ACE2, AT1R and AT2R in four different tissues, only AT2R protein abundance in liver and fat was affected. Meanwhile, we cannot exclude the possibility that this change may have played a role in tissue insulin sensitivity in our study. The reported effects of AT2R on insulin sensitivity and adipocyte differentiation are controversial. Yvan-Charvet et al. showed the deleterious effects of AT2R-dependent Ang II signaling on adipose tissue mass and glucose intolerance in mice (59), whereas, Shum et al. documented the involvement of AT2R in early adipocyte differentiation and its role in restoring normal adipocyte morphology and improving insulin sensitivity in rats (60). In fact, increasing AT2R/AT1R activity ratio has been suggested as a pharmacological manipulation to improve muscle insulin sensitivity and glucose metabolism (55). Therefore, this aspect needs further investigations in future studies.

In conclusion, this study confirms the beneficial effects of chronic supplementation with 4% EWH on glucose tolerance and insulin sensitivity in HFD induced insulin resistant rats mainly through affecting insulin sensitivity in adipose tissue and skeletal muscle as well as enhancing adipocyte differentiation. Because the effective dosage of the EWH used in this study is feasible to be incorporated into a human diet, it is worthwhile to investigate the potential effects of this treatment in humans. It should be mentioned however, that one of the limitations of this study is the absence of a standard chow diet group, which would permit conclusions about whether EWH returned glucose tolerance to normal.

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**Conflict of interest:** None

**Authorship:** FJ, JW, and CBC designed the experiments, FJ conducted animal experiments, FJ and SCCZ performed tissue experiments, analyzed the data, and wrote the manuscript. MJ performed PPARγ analysis; STD, SP, CBC and JW edited the manuscript.

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**Table 1. Composition of the experimental diets (g/kg)**

| | Groups | | | |
|---|---|---|---|---|
| Ingredients (g/kg) | HFD | HFD+1%EWH | HFD+2%EWH | HFD+4%EWH |
| Casein | 280 | 271.3 | 262.5 | 245 |
| EWH | 0 | 10 | 20 | 40 |
| Sucrose | 200 | 200 | 200 | 200 |
| Lard | 195 | 195 | 195 | 195 |
| Maltodextrin | 115 | 115 | 115 | 115 |
| Corn starch | 85 | 85 | 85 | 85 |
| Cellulose | 58 | 58 | 58 | 58 |
| Mineral mix | 43 | 43 | 43 | 43 |
| Soybean oil | 30 | 30 | 30 | 30 |
| Vitamin mix | 19 | 19 | 19 | 19 |
| L-Cystine | 3.5 | 3.5 | 3.5 | 3.5 |
| Calcium phosphate | 3.4 | 3.4 | 3.4 | 3.4 |
| Choline bitartrate | 3 | 3 | 3 | 3 |

**Table 2. Food intake, body composition and metabolic profile of rats.**

| Diet groups | HFD | SEM | HFD+ 1% EWH | SEM | HFD + 2% EWH | SEM | HFD + 4% EWH | SEM |
|---|---|---|---|---|---|---|---|---|
| Initial BW (g) | 358.00 | 8.52 | 358.40 | 14.13 | 369.70 | 12.07 | 352.00 | 10.14 |
| Final BW (g) | 828.30 | 21.65 | 806.60 | 28.65 | 837.40 | 27.16 | 813.70 | 20.17 |
| Weight change (g) | 470.30 | 18.79 | 448.30 | 18.53 | 467.70 | 25.65 | 461.60 | 16.71 |
| Food intake (kcal/day) | 129.70 | 3.80 | 126.60 | 7.27 | 133.3 | 3.44 | 127.00 | 5.20 |
| **Relative tissue weight (g)/Kg BW** | | | | | | | | |
| Liver | 0.027 | 0.002 | 0.029 | 0.001 | 0.030 | 0.002 | 0.026 | 0.001 |
| Kidney | 0.005 | 0.000 | 0.005 | 0.000 | 0.005 | 0.000 | 0.005 | 0.000 |
| Epididymal Fat | 0.032 | 0.001 | 0.027 | 0.003 | 0.028 | 0.002 | 0.028* | 0.001 |
| Retroperitoneal Fat | 0.056 | 0.003 | 0.046 | 0.003 | 0.050 | 0.004 | 0.048* | 0.002 |
| **Metabolic profile (Fasting state)** | | | | | | | | |
| Glucose (mg/dL) | 92.54 | 3.06 | 102.6 | 3.46 | 101.4 | 2.49 | 98.59 | 1.85 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| insulin (uIU/ml) | 44.78 | 7.77 | 36.48 | 13.24 | 34.58 | 3.24 | 35.90 | 7.17 |
| HOMA-IR | 10.75 | 2.32 | 10.09 | 3.88 | 9.21 | 1.06 | 9.10 | 2.74 |
| TG (mg/dL) | 98.31 | 8.61 | 105.10 | 4.19 | 80.59 | 11.37 | 67.22* | 8.59 |
| NEFA (mEq/L) | 0.59 | 0.06 | 0.58 | 0.09 | 0.59 | 0.06 | 0.62 | 0.06 |
| Cholesterol (mg/dL) | 80.78 | 6.88 | 92.67 | 5.10 | 63.16 | 12.27 | 64.27 | 10.26 |

Body weight and food intake throughout 12-week study period (Mean ± SEM for n= 7-16 rats). Relative tissue weight after 12-week period (Mean ± SEM for n= 7-16 rats). Metabolic profile after 12-week period (Mean ± SEM for n =4-12 rats). HOMA-IR, homeostatic model assessment insulin resistance (fasting glucose (mg/dL) × fasting insulin (μU/mL) /405; TG, triglyceride; NEFA, non-esterified fatty acids; BW, Body weight; HFD, High fat diet; EWH, Egg white hydrolysate. ***** $p<0.05$ compared to HFD.

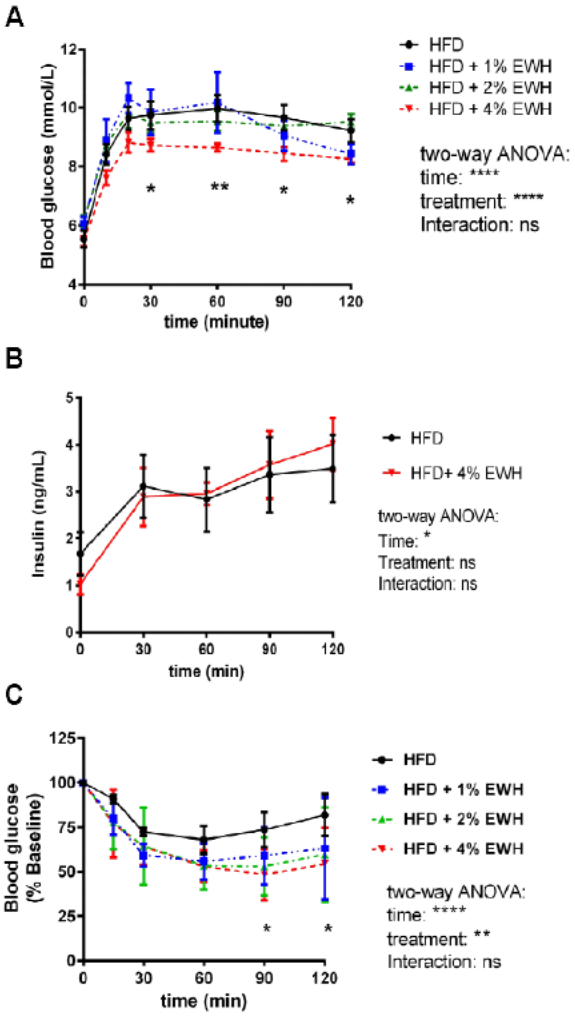



**Figure 1. *In vivo* characterization of glucose homeostasis. (A)** OGTT after 5 weeks of treatment with a HFD (20% w/w) supplemented with 1, 2, and 4% of EWH (Mean ± SEM for n= 4-11 rats). **(B)** Plasma insulin concentrations measured in blood samples collected during the OGTT (Mean ± SEM for n= 4 rats). **(C)** ITT after a 4 hour fast. Blood glucose levels are shown as % of basal glucose (Mean ± SEM for n= 5 rats). Analysis by two-way ANOVA followed by Bonferroni's post-hoc comparison test. * and ** show significant difference compared to HFD at $p<0.05$ and $p<0.01$, respectively.

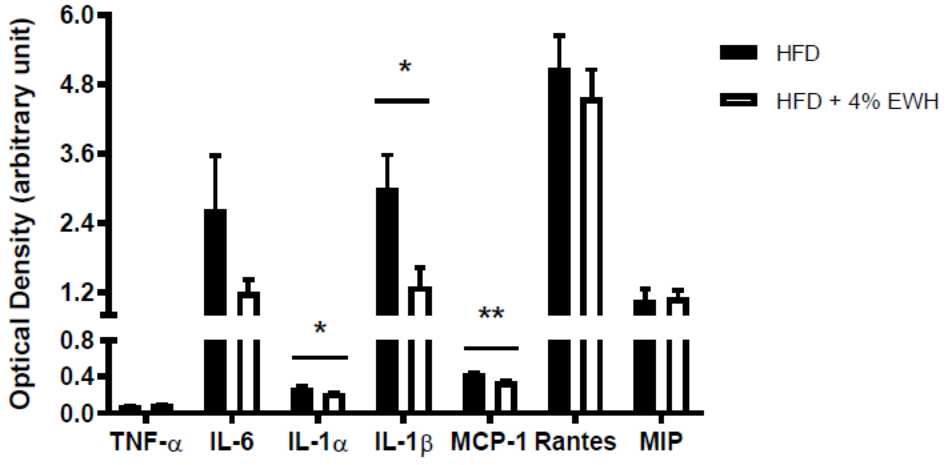



**Figure 2. The effect of EWH feeding on plasma inflammatory markers in insulin resistant (HFD-fed) rats.** Data are shown as Mean ± SEM for n = 4 analyzed by two-tailed t-test. * and ** show significant difference at $p<0.05$ and $p<0.01$, respectively. TNF-α, Tumor necrosis factor-α; IL-6, Interleukin-6; IL-1α, Interleukin-1α; IL-1β, Interleukin-1β; MCP-1, Monocyte chemotactic protein-1; Rantes, regulated on activation, normal T cell expressed and secreted.

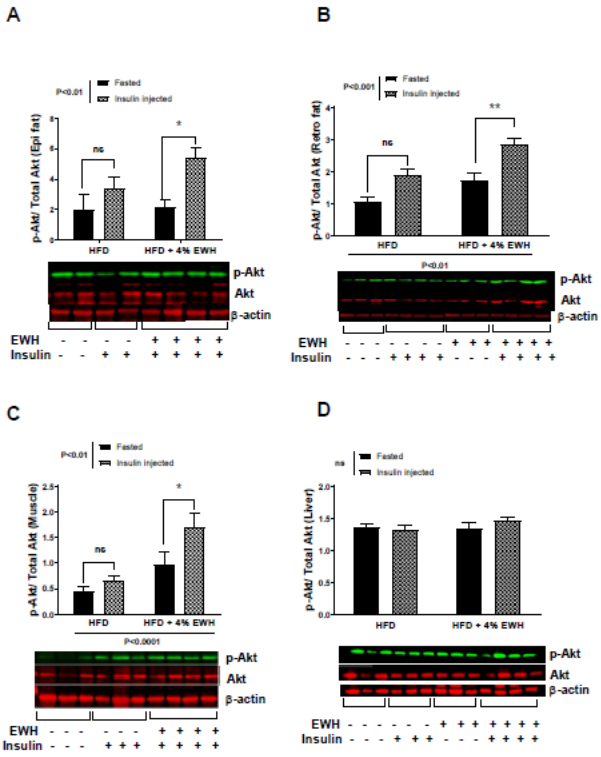


**Figure 3. Tissue insulin sensitivity assessed by phosphorylation of Akt.** Insulin sensitivity was measured in epididymal fat **(A)**, retroperitoneal fat **(B)**, muscle **(C)**, and liver **(D)** of rats euthanized in fasted and intraperitoneal insulin injected states in HFD and HFD+4% EWH treated rats. p-Akt protein band was normalized to total Akt as a measure of insulin sensitivity in both groups. Data are shown as the Mean ± SEM for n= 4-8 rats. Data were analyzed by two-way ANOVA followed by Bonferroni's post-hoc comparison test. * $p<0.05$.

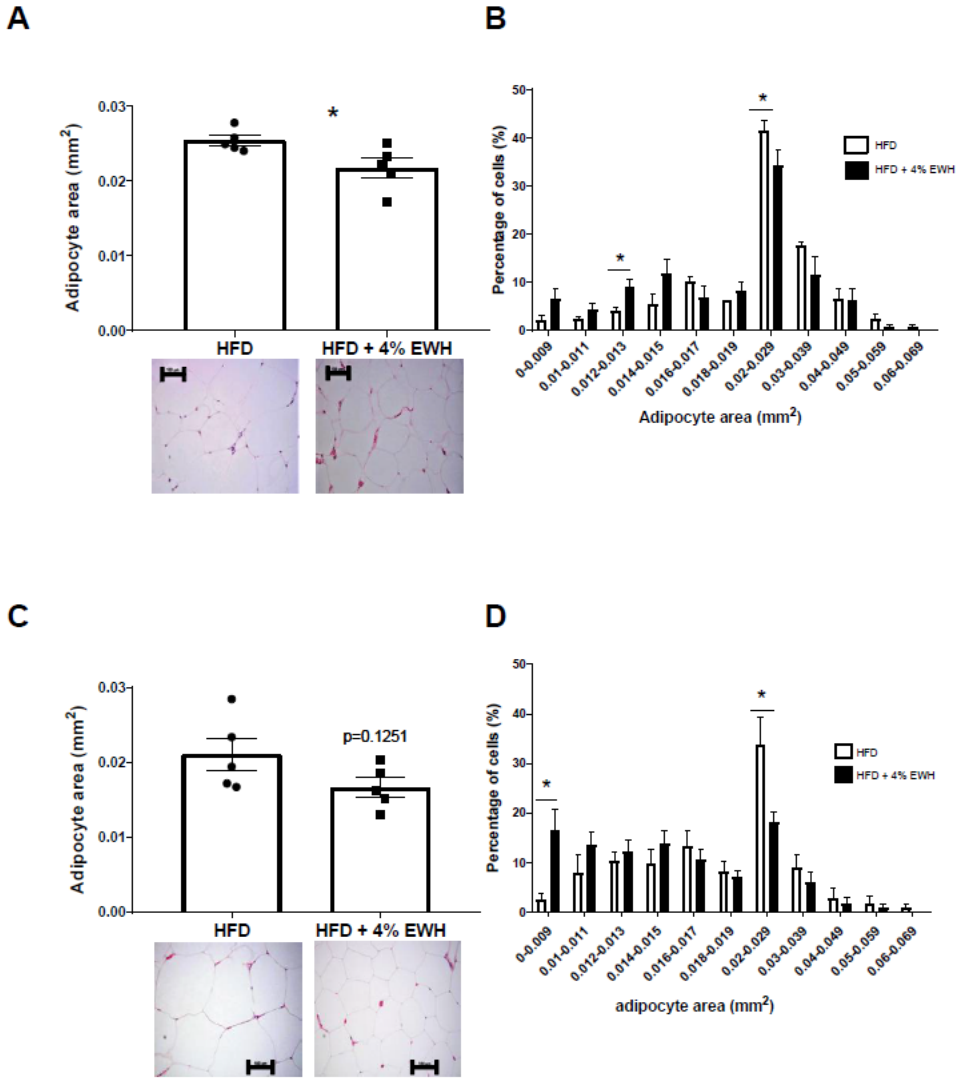



**Figure 4. Adipocyte distribution in retroperitoneal and epididymal adipose tissue.** Mean adipocyte area and adipocyte area distribution were analyzed in **(A, B)** retroperitoneal and **(C, D)** epididymal adipose tissue. Photomicroscopic images of

adipocytes using 20X objective lens for HFD and HFD+4%EWH groups are shown. Scale bar indicates 100 µm. Data are Means ± SEM for n= 5 rats. Data were analyzed by two-tailed t-test. * shows significant difference at $p< 0.05$.

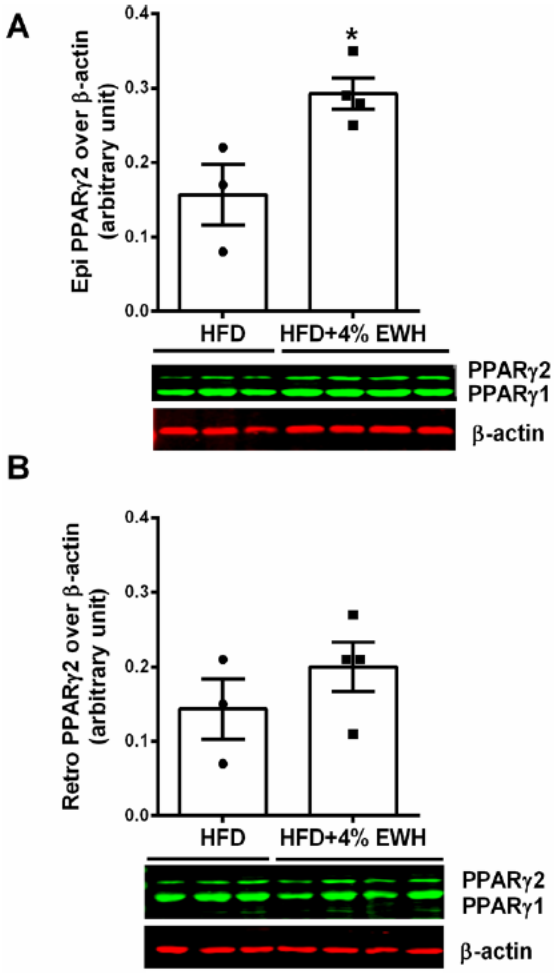



**Figure 5. Adipose tissue PPARγ abundance.** PPARγ2 protein band was normalized to β-actin as the loading control in **(A)** epididymal fat, and **(B)** retroperitoneal fat in HFD and HFD+4%EWH treated groups. Data are shown as the Mean ± SEM for n= 3-4 rats. Data were analyzed by two-tailed t-test. * shows significant difference at $p<0.05$. PPARγ, Peroxisome proliferator-activated receptor gamma.

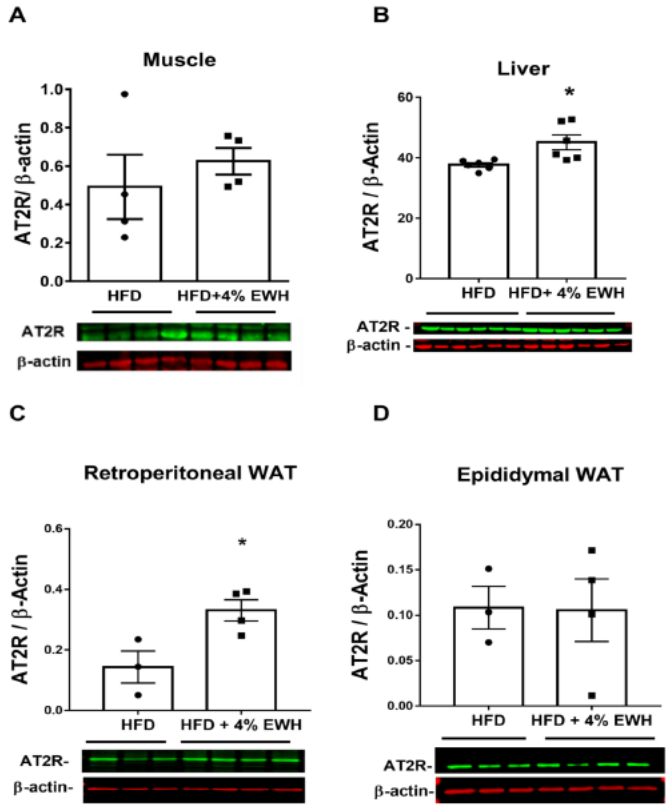
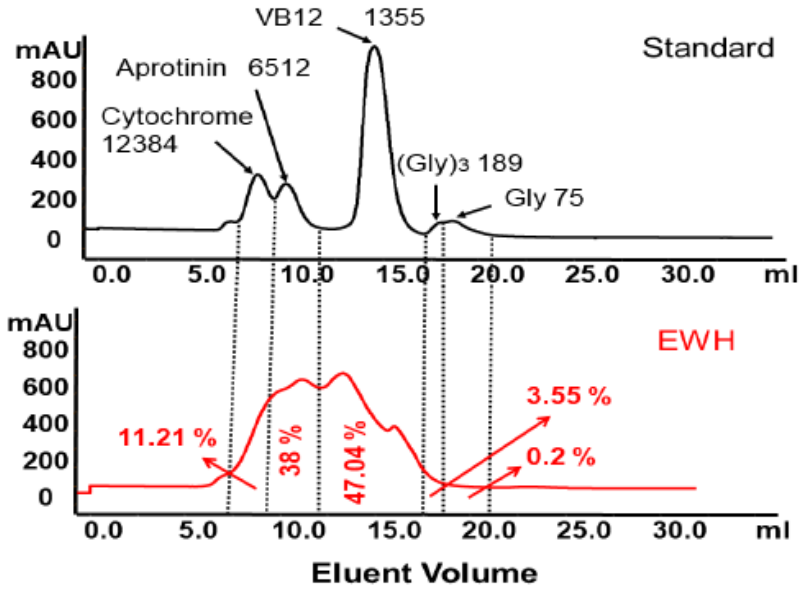
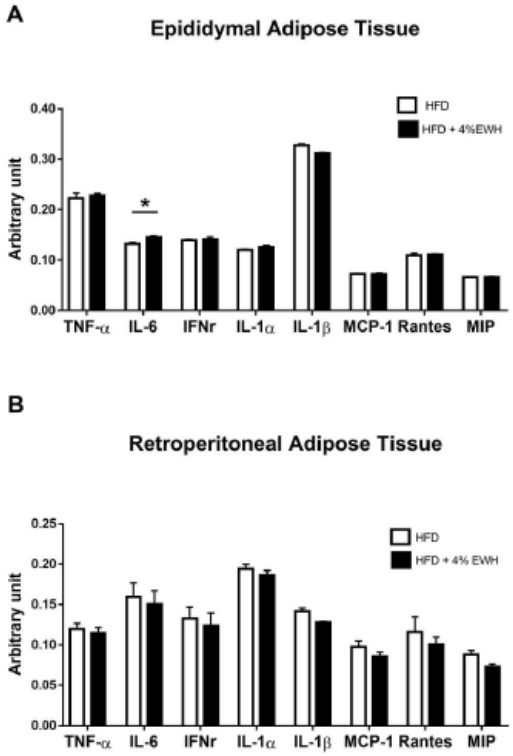



**Figure 6. AT2R protein abundance in skeletal muscle (A), liver (B) and adipose tissue (C and D).** The protein band of AT2R was normalized to β-actin as the loading control in HFD and HFD+4%EWH treated groups. Data is shown as the Mean ± SEM for n= 4-6 rats. Data has been analyzed by two-tailed t-test. * shows significant difference at $p<0.05$. AT2R, Angiotensin II type 2 receptor; WAT, white adipose tissue.

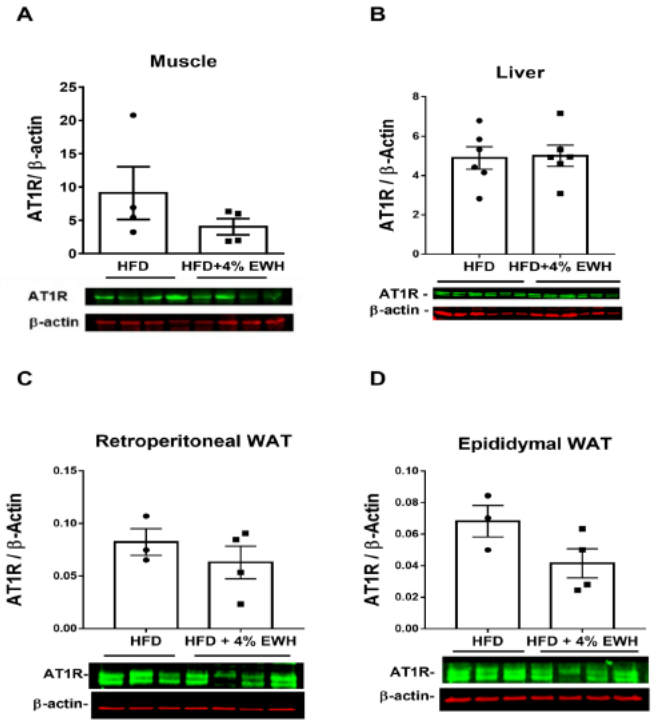
**Supplementary material**



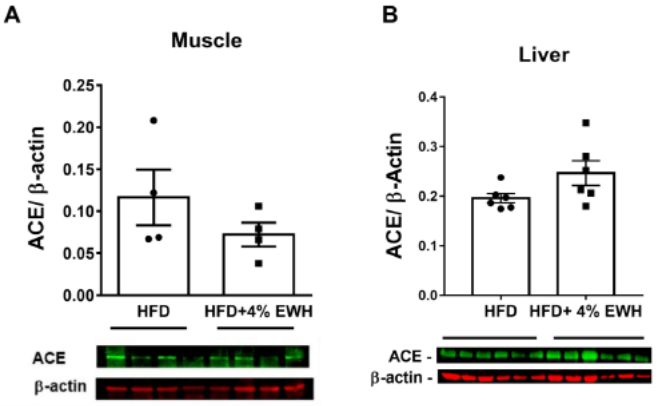

**S 1- Size profile of EWH peptides.** The majority of EWH-derived peptides (more than 85%) are in the range of 6.51 to 1.36 KDa. A small fraction of peptides (11.21%) with higher molecular weight between 12.38 to 6.51 KDa also present in the EWH. The molecular weight distribution of EWH peptides was determined using size-exclusion chromatography on an AKTA liquid chromatography system (GE Healthcare, Uppsala, Sweden) coupled with a Superdex Peptide 10/300GL column at room temperature. EWH was dissolved in 30% aqueous acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA) and filtered through 0.22 µm filters. 100 µL of the sample was injected into the column and separated using an isocratic elution at a flow rate of 0.6 mL/min with 30% ACN containing 0.1% TFA. The absorbance of the eluent was monitored at 215 nm. Molecular weight markers (cytochrome c, 12384 Da; aprotinin, 6512 Da; vitamin B12, 1355 Da; (glycine)3, 189 Da; and glycine, 75 Da) were run under identical conditions to obtain the standard curve.



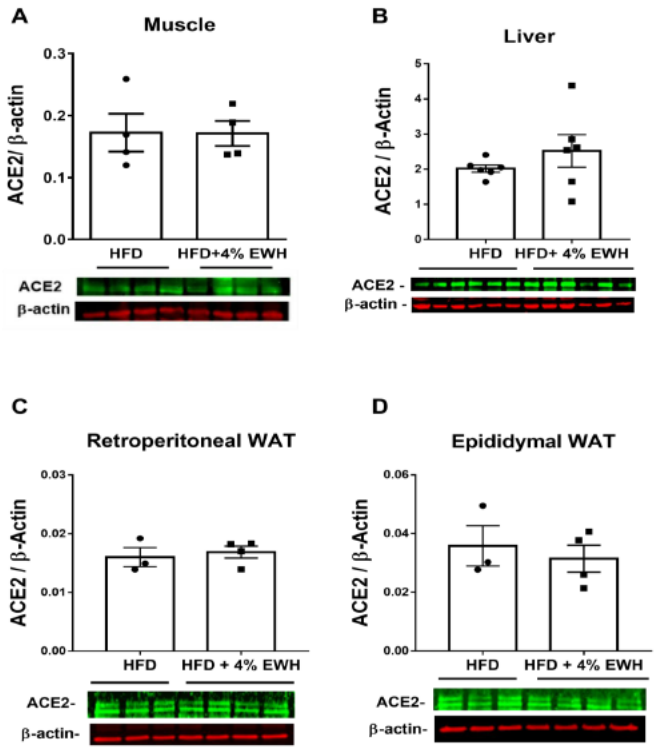

**S 2- Epididymal and retroperitoneal adipose tissue inflammatory markers in (A) Epididymal adipose tissue n=3 rats and (B) Retroperitoneal adipose n=6 rats.** Data are shown as the Mean ± SEM and were analyzed by two-tailed t-test. * shows significant difference at $p < 0.05$. TNF-α, Tumor necrosis factor; IL-6, Interleukin-6; IFNr, Interferon production regulator; IL-1α, Interleukin-1 alpha; IL-1β, Interleukin- 1 beta; MCP-1, Monocyte chemoattractant protein-1; Rantes, regulated on activation, normal T cell expressed and secreted; MIP, Macrophage inflammatory protein.



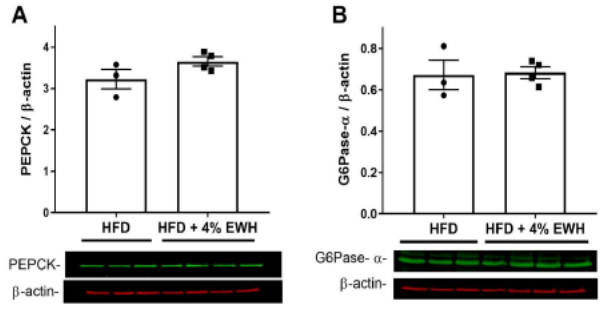

**S 3- AT1R protein abundance in skeletal muscle (A), liver (B), and adipose tissue (C and D).** The protein band of AT1R was normalized to β-actin as the loading control in HFD and HFD + 4% EWH treated groups. Data are shown as the Mean ± SEM for n= 4 rats and were analyzed by two-tailed t-test. AT1R, Angiotensin II type 1 receptor; WAT, white adipose tissue.




**S 4- ACE protein abundance in skeletal muscle (A) and liver (B).** The protein band of ACE was normalized to β-actin as the loading control in HFD and HFD+4%EWH treated groups. Data are shown as the Mean ± SEM for n= 4-6 rats and were analyzed by two-tailed t-test. ACE, angiotensin converting enzyme; WAT, white adipose tissue.




**S 5- ACE2 protein abundance in skeletal muscle (A), liver (B), and adipose tissue (C and D).** The protein band of ACE2 was normalized to β-actin as the loading control in HFD and HFD+4%EWH treated groups. Data are shown as the Mean ± SEM for n= 3-6 rats and were analyzed by two-tailed t-test. ACE2, angiotensin converting enzyme 2; WAT, white adipose tissue.




**S 6- Liver PEPCK and G6Pase-α abundance.** PEPCK and G6Pase protein bands were normalized to β-actin as the loading control. **(A)** PEPCK and **(B)** G6Pase in HFD and HFD+4%EWH treated groups. Data are shown as the Mean ± SEM for n= 3-4 rats and were analyzed by two-tailed t-test. PEPCK, Phosphoenolpyruvate carboxykinase; G6Pase-α, Glucose 6 phosphatase-α