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A novel role of the c-Jun NH_2-Terminal Kinase (JNK) in obesity-associated insulin resistance

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Memòria presentada per Melisa Morcillo Sánchez per optar al títol de doctora per la Universitat de Barcelona

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UNIVERSITAT DE BARCELONA

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"Los grandes logros de cualquier persona generalmente dependen de muchas manos, corazones y mentes"

WALTER ELIAS DISNEY

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“Agradezco haberte tenido como padre”

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INTRODUCTION

The Obesity epidemic

Obesity is one of the most important public health problems that has alarmingly increased worldwide in the last few decades. Changes in eating and physical activity habits have led to an imbalance between food intake and energy expenditure leading to obesity epidemic.

Obesity is currently defined as excess body weight for height (see below), although this simple definition does not represent the complex phenotype showed by obese patients. Excessive adiposity or body fatness not only affects body size, also disrupts relevant metabolic and immune system functions crucial to maintain full-body homeostasis.

This complex disease increases the risk of chronic metabolic alterations leading to a large number of disorders such as type 2 diabetes (T2D), hypertension, hyperlipidemia, nonalcoholic fatty liver disease, cardiovascular diseases, and certain cancers (Kopelman et *al.* 2000). Moreover, severe and prolonged obesity can result in early death.

Globally, regarding the World Health Organization (WHO) data, 39% of adults in the world were overweight in 2016. The share of people who are overweight tends to be higher in richer countries and lower at those with minor incomes (Fig. 1). This observation might be explained by the differences in physical activity, diet, lifestyle, and socioeconomic background, beside to ethnic and genetics.

Obesity is most commonly measured using the body mass index (BMI) scale that is weight in kilograms divided by the square of height in meters. BMI values are used to define whether an individual is considered to be underweight, healthy, overweight or obese. The WHO defines these categories using the cut-off points and, in this regard, an

individual with a BMI between 25.0 and 30.0 is considered to be "overweight"; and "obese" when this index is higher than 30.0.

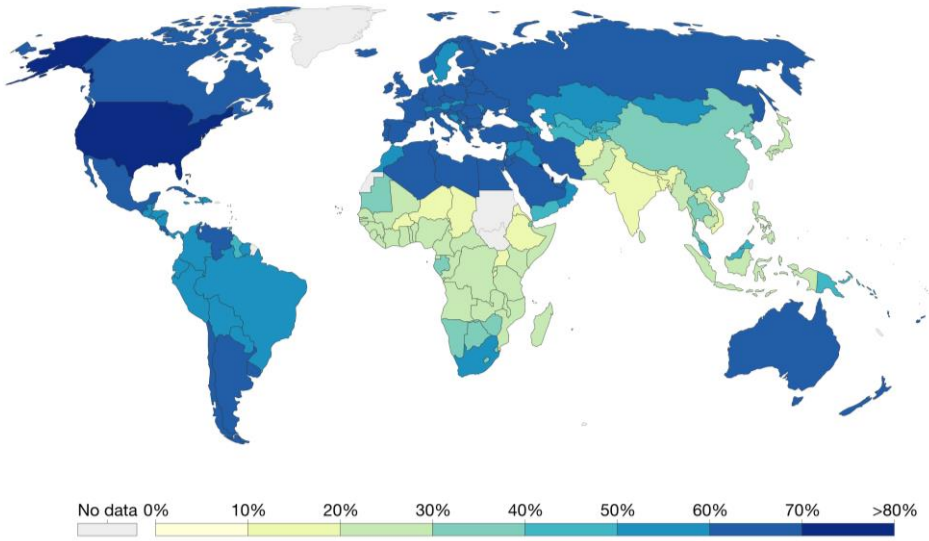


Figure 1. Distribution of overweigh/obesity across the world in 2016 (Source: WHO). Share of men and women older than 18 that were overweight (BMI equal or higher than 25.0) in 2016.

Obesity is strongly associated to insulin resistance and hyperinsulinemia. Insulin resistance is the result from the body's ineffective response to insulin and constitutes an early trait in T2D, a disease that comprises the majority of people with diabetes around the world. Therefore, the increase in T2D seems to be a consequence of the increase in obesity. Until recently, this type of diabetes was observed only in adults, but now, it is also increasingly occurring in children (WHO, 2018). Currently, there are some therapies that address different problems and stages of T2D. However, nowadays, it is important to focus our attention on the development of new anti-diabetic drugs and therapies that are more effective and have fewer side effects. (Röder *et al.* 2016).

Obesity-associated metaflammation and insulin resistance

The molecular link of obesity to its associated pathologies still remain largely unexplained, despite the identification of some gene variants and signal transduction pathways that collaborate in this multifactorial interaction. These genes and pathways could represent possible targets for therapeutic intervention in obese patients when, unfortunately, the therapy of dieting and exercise do not ameliorate the obesity-related pathologies. It is well established that obesity is associated with a chronic low-grade inflammation, known as metaflammation, thought to contribute to several metabolic disorders, including cardiovascular disease, liver disease, insulin resistance, T2D, cancer, and neurodegeneration. In this regard, the increased levels of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and others, have a major role in promoting the cell failure to respond normally to insulin leading to insulin resistance (Gregor & Hotamisligil 2011).

Metaflammation is a chronic low-grade inflammatory response initiated by obesity-associated excess of nutrients in adipocytes that, eventually, causes activation of specialized immune cells and leads to an unresolved inflammatory response that eventually, have systemic consequences. In general, metaflammation has inhibitory effects on insulin action through the activation of pro-inflammatory kinases c-Jun NH2-terminal kinase (JNK), inhibitor of nuclear factor κB kinase (IKK) and double-stranded RNA-dependent protein kinase (PKR) in metabolic tissues. Several studies have been demonstrated a crosstalk between immune and metabolic pathways (Hotamisligil 2017). It has been proposed the interaction between three evolutionary conserved pathways (Fig. 2):

- TNF-α and its receptor
- Insulin and insulin receptor (IR)
- TLR signaling pathway

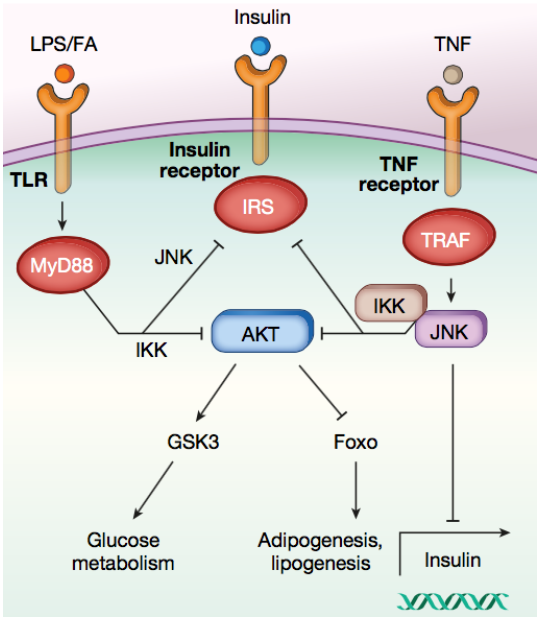


Figure 2. Immune and metabolic pathways crosstalk. The immune-response associated TLR and TNF-α receptor cascades regulate metabolism through their interaction with the IR pathway. Taken from Hotamisligil, 2017.

The activation of TNF-α and TLR signaling pathways disrupts metabolic homeostasis by blocking the IR signaling pathway, and, consequently, the inhibition of these inflammatory pathways ameliorates metabolic defects (Fig. 2). This relationship between immune and metabolic pathways determines that pro-inflammatory cytokines could act as metabolic hormones to provide adaptations to nutrient fluctuations. Therapeutic interventions to inhibit inflammatory pathways in obesity have shown beneficial effects on insulin sensitivity in mouse models

(Eldor *et al.* 2006, Todoric *et al.* 2006, Oh *et al.* 2010) and human trials (Gonzalez-Gay *et al.* 2006, Larsen *et al.* 2007, Stanley *et al.* 2011).

In this regard, pioneer studies showed that TNF-α expression was elevated in adipose tissue of obese mice and humans (Hotamisligil *et al.* 1995). Also, TNF-α-null mice have a significantly improved glucose tolerance and insulin sensitivity in response to a high fat diet (HFD) (Ventre *et al.* 1997). Moreover, it is well established that increased TNF-α expression induces insulin resistance (Hotamisgil 1999, Nieto-Vazquez *et al*. 2008). At mechanistic level, this pro-inflammatory cytokine binds to its receptor and activates IKK and JNK that inhibit IR signaling by disrupting the insulin-induced interaction between the IR and the IR substrate (IRS), thereby, promoting insulin resistance (Aguirre *et al.* 2000, Chen *et al.* 2002). Therefore, TNF-α is considered a potential link between obesity, insulin resistance and T2D (Nieto-Vazquez *et al.* 2008, Swaroop *et al.* 2012). In consequence, the inhibition of TNF-α production would be one of the strategies to prevent the development of obesity-induced insulin resistance and T2D pathogenesis (Akash *et al.* 2012). In this regard, several investigations have used anti-TNF-α treatments to prevent the pathogenesis of obesity-induced insulin resistance (Borst *et al.* 2002, Barbuio *et al.* 2007, Lo *et al.* 2007). Although some controversial studies have demonstrated that using TNF-α blockade has no effect on insulin resistance (Rosenvinge *et al.* 2007). For this reason, future studies are needed to improve clinical outcomes.

In addition to the induction of IKK and JNK pathways, TNF-α is closely linked to the enzyme phosphatase and tensin homologue (PTEN) regulation (Solomon *et al.* 2010). In human leukemic cells, TNF-α increases PTEN protein expression (Lee *et al.* 2007). Increased PTEN level is implicated as a negative regulator of insulin signaling pathway

(Song *et al.* 2012) and negatively affects insulin sensitivity (Gupta *et al.* 2012). Although PTEN mutations increase the risk of obesity and cancer, decrease the risk of T2D (Pal *et al.* 2012). In summary, it has been demonstrated a crosstalk between TNF-α and IR signaling but, unfortunately, the involved molecular mechanisms seem to be multiple and some of them still remain unresolved.

In addition, other conditions have been associated with obesity, such as induction of endoplasmic reticulum (ER) stress caused by adipocyte hypertrophy, the production of reactive oxygen species (ROS) caused by mitochondrial dysfunction and oxidative stress, and the increased plasma level of free fatty acids (FFA). These cellular and metabolic alterations also contribute to either local or systemic insulin resistance (Houstis et *al.* 2006, Solinas et *al*. 2006, Hotamisligil et *al.* 2010). Although, how insulin resistance progresses to T2D and its complications is still unknown.

JNK and insulin resistance

JNK is member of the mitogen-activated protein kinase (MAPK) family, and an important stress responsive transducer involved in obesity-induced insulin resistance. The MAPK kinase kinases (MAP3Ks) (TAK, ASK1, MLK, MEKK1, MEKK4) are the first kinases to respond to different signals that activate JNK, and then the downstream MAPK kinase (MAP2Ks), MKK4 and MKK7, are activated and phosphorylate JNK on Tyr185 and Thr183 and concomitantly activate it (Karin *et al.* 2005).

Several JNK isoforms have been reported that are encoded by 3 different genes: *jnk1*, *jnk2* and *jnk3*. *jnk1* and *jnk2*, which are widely expressed, promote the development of diet-induced obesity, metabolic inflammation and insulin resistance. It has been demonstrated that

exacerbated JNK1/2 activity participates in promoting insulin resistance in insulin target tissues (Hirosumi et *al*. 2002, Solinas & Karin et *al.* 2010, Solinas *et al*. 2016). On the contrary, *jnk3*, which expression is restricted to certain tissues such as brain, protects against excessive adiposity and insulin resistance. In this regard, JNK3 isoform is activated in hypothalamic neurons by HFD-feeding and this activation is needed for the correct control of food intake. HFD-fed mice lacking JNK3 show increased food intake and adiposity and develop insulin resistance compared with their HFD-fed control littermates (Vernia *et al.* 2016).

The stress-kinase effector JNK is not only activated by inflammatory cytokines, also other stimuli, such as ultraviolet (UV)-radiation, oxidative stress and ROS production, genotoxic damage, ER stress, FFA and microbial byproducts, can activate the JNK pathway. Some of these stimuli are also relevant in obesity, for example, ER stress, ROS and FFA. In relation to ER stress, it originates in the ER, a large cellular organelle responsible for ensuring proper protein maturation and folding, and any disruption in the homeostasis within ER could cause ER stress. Obesity shows many alterations that can promote ER stress such as lipid accumulation and disruptions in intracellular nutrient and energy fluxes. Obese animals present increased levels of several biochemical indicators of ER stress in liver and adipose tissue compared with their lean littermates (Özcan *et al.* 2004). Moreover, in β-cells, ER is important in ensuring proper insulin folding (Oh *et al.* 2018). These cells are very sensitive to ER stress inducing insulin signaling pathway blockade and reducing insulin transcription (Özcan *et al.* 2004). In addition, excess of FFA and inflammatory cytokines, in particular IL-1β, can contribute to β-cell failure (Guilherme *et al.* 2019).

Oxidative stress and ROS production also activate JNK (Haigis *et al.* 2011). Cancer cells frequently have increased burden of oxidative stress

and increased sensitivity to the damage promoted by ROS (Trachootham *et al.* 2009). Shi *et al.* (2014) demonstrated that JNK is a crucial player downstream of ROS leading to the synthetic lethality upon p53 activation. This investigation concluded that JNK activated by ROS converts p53 into an efficient inhibitor of oncogenes and elevated ROS production in malignant tumors might allow enhanced and sustained JNK-dependent p53 activation. In the context of obesity, inflammation, FFA and hyperglycemia induce ROS generation and, consequently, activation of different pathways, including the JNK pathway, that further block the IR pathway (Evans *et al.* 2002).

However, it should be noted that the metabolic effects of the JNK pathway activation depend on the specific context, magnitude and duration of this activation. JNK activation promotes survival, stress tolerance and proliferation, although also promotes various signals that leads to programmed cell death. Transient JNK activitation might result in proliferation while prolonged could lead to cell death (Solinas *et al.* 2017). In obesity, moderate and sustained JNK activation is observed in different insulin-target tissues such as adipose tissue and liver (Tournier *et al.* 2001, Hirosumi *et a*l. 2002, Díaz-Delfín *et al.*, 2007, Lopez-Bergami *et al.* 2008).

The use of genetically modified mice has enabled to establish the role of the JNK activity in physiology. In this regard, chronic JNK activation leads to different metabolic effects depending on the tissue within which is activated, as summarized in table 1.

Tissues	Effects of chronic JNK activation
Adipose tissue	Increased pro-inflammatory cytokines expression and adipocyte insulin resistance
Muscle	Myocytes insulin resistance and cardiomyocytes insulin resistance and death

Liver	Hepatosteatosis, hepatic insulin resistance, non-alcoholic fatty liver disease (NAFLD) and hepatocyte death
Macrophages	Increased pro-inflammatory cytokines expression and consequent recruitment of more macrophages
Pancreas	Reduced insulin secretion from pancreatic β-cells, locally insulin resistance and β-cell death
Brain	Central insulin resistance and subsequent deregulated energy homeostasis, inducing food intake and weight gain, regulation of glucose production and reduced insulin secretion by β-cells

Table 1. Different effects in different tissues due to chronic JNK activation. Table adapted from Yung and Giacca (2020).

The metabolic effects derived from chronic JNK activation are not the same in all tissues. In liver, JNK overactivation causes local and systemic insulin resistance (Nakatani *et al.* 2004) while, in pancreatic β-cells, there is a local insulin resistance although systemic insulin sensitivity is maintained (Lanuza-Masdeu *et al.* 2013). It has been demonstrated that constitutive JNK activation in β-cells of mice reduces the amount of insulin secreted in response to hyperglycemia due to a disruption of insulin signaling within these cells. Consequently, these mice display glucose intolerance (Lanuza-Masdeu *et al.* 2013).

Regarding inflammation, in liver, this response has been considered a hallmark of liver disease and may represent a cause of hepatocellular carcinoma (HCC) development (Sun & Karin, 2013). Inflammation is associated with JNK activation and JNK-deficient mice confirmed the important role of the JNK pathway in the development of HCC (Sakurai *et al.* 2006, Hui *et al.* 2008). Myeloid cells may represent a site of pro-inflammatory signaling where JNK activation can promote liver pathology such as hepatitis and HCC (Shirabe *et al.* 2012, Sun & Karin, 2013, Han *et al.* 2016). The inhibition of JNK in these cells may represent a therapeutic strategy for the treatment of inflammation-related liver disease.

JNK1-deficient mice show a protection against diet-induced insulin resistance, although these mice are also partially protected against adiposity (Hirosumi *et al.* 2002). Unexpectedly, hepatocyte-specific ablation of *jnk1* produces glucose intolerance, hepatic insulin resistance, inflammation and hepatic steatosis in HFD-fed mice (Sabio *et al.* 2009). However, previous studies of hepatic JNK1 contrast with these results. These studies employed intravenous delivery of adenoviruses that express dominant-negative JNK (Nakatani et al. 2004) or *Jnk* shRNA (Yang *et al.* 2007). On the contrary, adipose-specific JNK1-deficient mice show a protection against HFD-induced hepatic insulin resistance and steatosis despite the development of obesity (Sabio *et al.* 2008). The molecular mechanisms to explain the different phenotypes showed by these mouse models are unclear.

Nevertheless, hepatocyte-specific *jnk2*-deficient mice are less obese and showed slightly improved glucose tolerance and hepatic insulin resistance in response to a HFD when compared with hepatocyte-specific *jnk1*-deficient and wild type (WT) mice in the same condition (Vernia *et al* 2014). This observation suggests that JNK2 is the predominant isoform that induces insulin resistance in liver while hepatic JNK1 activation might not drive obesity-induced insulin resistance in this organ. Nevertheless, mice lacking *jnk1* and *jnk2* in hepatocytes developed larger tumors in a model of chemically induced HCC (Das *et al.* 2011). Severe forms of liver disorders have been observed in obese subjects, such as non-alcoholic steatohepatitis (NASH) by hepatocyte apoptosis (Kitade *et al.* 2017).

Due to these differences, nowadays, there have been proposed 4 distinct cellular mechanisms to explain how JNK activation leads to obesity-induced insulin resistance (Solinas & Becattini 2017):

- JNK activity in Macrophages (Increased inflammation)
- JNK activity in Pituitary Thyrotropic Cells (Reduction of Thyroid-Stimulating Hormone (TSH) production)
- JNK activity in the Hepatocyte (Fatty liver promotion)
- **JNK activity in Insulin-Target Cells (Disrupted Insulin signaling pathway)**

We focused our attention on the last mechanism which shows how JNK phosphorylation/activation disrupts IR signaling pathway and promotes insulin resistance in insulin-target cells. This molecular mechanism is a key determinant for the development of insulin resistance.

Upon hormone activation, the IR signaling pathway phosphorylates/activates JNK, and this activation leads to an interference on the interaction between the IR and, next step in the pathway, IRS as a result of a negative feedback mechanism (Fig. 3).

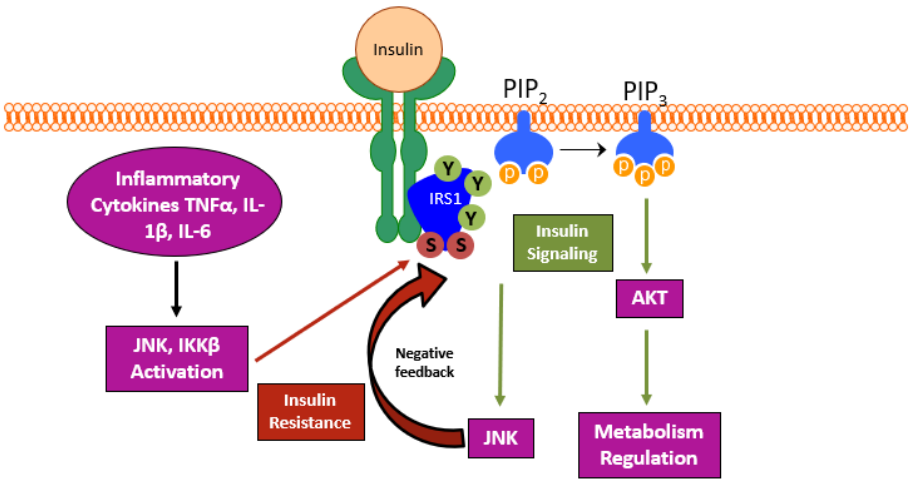


Figure 3. Inflammatory kinases suppress IR signaling resulting in insulin resistance. IRS phosphorylation on Ser-307 is an inhibitory signal to supress insulin signaling by inflammatory kinases, such as JNK and IKKβ.

Concretely, JNK functions as a physiological negative feedback mechanism to suppress IR signaling by direct IRS-1 phosphorylation on Ser-307 (Fig. 3). This modification disrupts the insulin-induced IR-IRS interaction and the concomitant IR-dependent Tyr phosphorylation of IRS. As a consequence, there is a decrease in the recruitment and activation of phosphatidylinositol 3-kinase (PI3K)-AKT (also known as protein kinase B, PKB) pathway and, consequently, its downstream effectors that promote glucose transporter (GLUT)4-mediated glucose internalization, protein synthesis, adipogenesis, glycogen synthesis and growth and cellular differentiation (Fig. 4). However, chronic inflammation constitutively activates JNK, which through IRS phosphorylation on Ser-307, interferes with the IR-IRS interaction leading to sustained insulin resistance (Fig. 3).

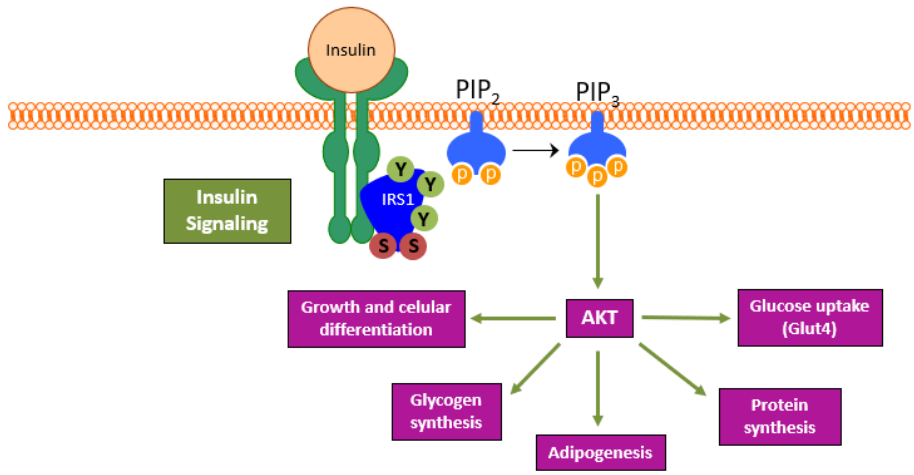


Figure 4. IR signaling pathway and downstream effectors and cellular response. Activation and regulation of different cellular and metabolic events in response to insulin.

Different investigations have demonstrated the concept of “selective insulin resistance” that would explain how insulin resistance is selective for the PI3K/AKT branch of IR signaling. In the insulin resistance condition, it has been observed reduced activation of the PI3K/AKT

pathway, while the insulin-induced activation of extracellular regulated protein kinase (ERK) pathway remains unaffected (Biddinger *et al.* 2008, Tan *et al.* 2015).

AKT is a key protein kinase required for insulin regulation of pathways that control systemic glucose homeostasis, including glucose transport, lipid and protein synthesis, respectively in adipocytes and skeletal muscle, and inhibition of gluconeogenesis and cell-autonomous activation of lipogenesis in hepatocytes (Czech *et al.* 2017). In obesity-associated insulin-resistance, AKT phosphorylation/ activation is greatly diminished in insulin-target tissues due to the mechanism described above and the decrease in the number of IRs.

White adipose tissue (WAT) and obesity

It has been suggested that one of the key factors that links excessive caloric intake and the positive energy balance with metabolic disturbance is the inability to appropriately expand the adipose tissue, which occurs in parallel to the decrease of its adipogenic capacity and the spill of lipids outside of this organ (Danforth *et al.* 2000, Kim *et al.* 2007). Adipose tissue has an important impact on energy homeostasis due to its capacity to store triglycerides but also to its endocrine function.

Disturbances in WAT are considered to be a potential link between adipokine secretion, obesity, chronic inflammation and insulin resistance. Obesity-induced adipose tissue inflammation is associated with the infiltration of macrophages and increased expression of pro-inflammatory cytokines, such as TNF-α, IL-6 and monocyte chemoattractant protein 1 (MCP1). Several studies have demonstrated that adipose tissue isolated from obese humans and mice had upregulation of pro-inflammatory cytokine expression (Hotamisligil *et al.* 1995, Enging 2017). This upregulation leads to reduced triglyceride

storage, increased lipolysis, and subsequent elevated FFA in plasma. Nevertheless, the main effector of the increased lipolysis is insulin resistance (Morigny *et al.* 2016). FFA can cause ER stress in adipocytes that also activates the JNK and IKK pathways (Guo *et al.* 2007, Hotamisligil 2010, Jiao *et al.* 2011).

In this tissue, TNF-α inhibits lipogenesis and adiponectin (an adipokine that promotes insulin sensitivity) expression by the inhibition of peroxisome proliferator-activated receptor-γ (PPAR)γ-mediated mechanisms (Ruan *et al.* 2003, Sung *et al.* 2010). PPARγ activators, such as thiazolidinediones (TZDs), have been used in numerous studies to establish that the transcriptional activity of PPAR-γ is required for the maintenance of insulin sensitivity and lipid metabolism (Spiegelman 1998). In fact, JNK inhibition by TZDs is required for their insulin sensitizing action (Díaz-Delfín *et al.* 2007)

Different investigations have observed ER stress in human adipose tissue of obese subjects, indicating that ER stress may play a crucial role in adipose tissue disorders, such as inflammation and apoptosis (Boden et *al.* 2008, Gregor et *al.* 2009). ER stress, insulin resistance and increased FFA levels in WAT associated to obesity may trigger the expression of anti/pro-inflammatory cytokines, such as TNF-α, IL-6, IL-1β, MCP1, IL-10 and decrease the expression of insulin-sensitizing effectors like IRS-1, IRS-2 and GLUT4. In WAT, ER stress promotes FFA efflux from adipocytes, and consequently, increases the level of circulating FFA. Therefore, ER stress is suggested to be the cellular basis of dyslipidemia, lipotoxicity and insulin resistance (Ozcan et *al.* 2004, Sharma et *al.* 2008).

Skeletal muscle and obesity

During food intake, insulin, secreted by β-cells in pancreas, enhances

glucose uptake into skeletal muscle cells through the insulin-sensitivite GLUT4, which translocates from intracelular vesicles to the plasma membrane in response to the hormone. In fact, due to the size of this tissue relative to adipose or liver, skeletal muscle is the major contributor to glucose uptake in response to insulin.

In addition to insulin, exercise plays also an important role in GLUT4 translocation and glucose uptake in this tissue. Contraction of muscle induces GLUT4 translocation in the absense of insulin (Messina et *al.* 2015). It has been demostrated that primary signals that allow GLUT4 translocation by insulin and exercise in skeletal muscle are distinct. Remarkably, under conditions of insulin resistance (obesity and/or T2D), exercise upregulates muscle GLUT4 expression in an insulin-independent manner.

In skeletal muscle, HFD and/or overfeeding deregulate GLUT4 response to insulin and consequently reduce glucose uptake contributing to hyperglycemia. In addition, obesity-associated hyperinsulinemia increases muscle glycolisis and, hence, lactate formation, which serves as a substrate for gluconeogenesis in the liver, a metabolic pathway that remains activated in insulin resistance and is an important contributor to hyperglycemia.

Liver and obesity

Liver has been considered an important tissue to maintain systemic glucose homeostasis by coordinating the production of glucose through glycogenolysis and gluconeogenesis pathways in times of fasting.

Under normal feeding conditions (Fig. 5a), IR signaling pathway in liver activates fatty acid synthesis from glucose and amino acids, conversion

to triglycerides and packaging into very-low-density lipoproteins (VLDLs) for export and uptake into peripheral tissues (Zhang et *al.* 2006, Biddinger et *al.* 2008).

The hyperinsulinemia associated to overfeeding conditions (Fig. 5b), could amplify the usual stimulation of this lipogenic pathway, leading to lipid overproduction and boosting the obese state. HFD activates Forkhead box protein O1 (FOXO1), a transcriptional activator of gluconeogenic genes, which is not supressed by AKT when IR signaling is downregulated.

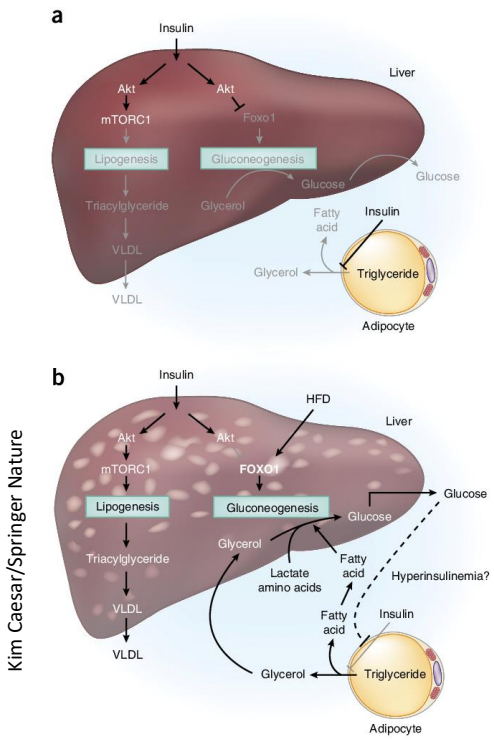


Figure 5. HFD effect on liver and adipose metabolism. Under normal feeding conditions **(a)** insulin supresses gluconeogenesis by AKT activation and inhibits adipocyte lipolysis, thereby, limiting the availability of glycerol, a gluconeogenic substrate. Under HFD conditions **(b)** hyperglycemia and chronic hyperinsulinemia might disrupt insulin suppression of adipocyte lipolysis promoting hepatic gluconeogenesis and glucose output.

HFD-feeding contributes to the development of hepatosteatosis, hepatic insulin resistance, and NAFLD. Obesity-associated hepatosteatosis is the first alteration before hepatic insulin resistance and NAFLD (Anstee *et al.* 2013, Kusminski *et al.* 2016). Prolonged HFD-feeding promotes the hallmarks of NAFLD, such as inflammation, fibrosis or hepatocellular ballooning (Hebbard *et al.* 2011). These alterations in liver can progress to NASH, cirrhosis and HCC.

Liver is the first insulin-target tissue that shows local insulin resistance in response to HFD. In this regard, hepatic insulin resistance ocurred after three days of HFD feeding, while in skeletal muscle and adipose develops later (Wang *et al.* 2001).

Insulin, leptin and obesity

Insulin is a principal anabolic hormone secreted by pancreatic β-cells for proper storage of nutrients and, together with leptin, is one of the major endocrine axes deregulated in obesity. An excessive accumulation of intra-islet fat can cause islet inflammation impairing glucose-stimulated insulin secretion and contributing to β-cell death (Cerf 2013). This hormone mediates the hypothalamic regulation of energy homeostasis and glucose metabolism (Woods *et al.* 1979, Flier 2004) and is responsible for glycemia maintenance by inhibition of hepatic glucose output and enhancement of glucose uptake into skeletal muscle and adipose tissue.

Leptin is encoded by the *obese* (*ob)* gene and is a well-established adipokine influencing appetite control and energy expenditure. It acts in the hypothalamus (anorexigenic action) and other regions in the brain, as well as, in peripheral tissues such as the adipose tissue (increases energy expenditure), where leptin receptor (LEPR) is expressed

(Frederich et *al.* 1995, Halaas et *al.* 1995, Vaisse et *al.* 1996). Obesity is associated with an increased leptinemia due to the development of leptin resistance. In addition, obesity decreases adiponectin expression and secretion. Low adiponectin levels promote IRS1 inhibition and causes insulin resistance through the activation of the mTOR signaling pathway (Hu *et al.* 1996, Menzaghi *et al.* 2002).

Insulin and leptin are major signals to the hypothalamus to regulate energy homeostasis and body adiposity. Both hormones target the same hypothalamic area in order to supress eating behaviour (Brüning *et al.* 2000). It has been suggested IR and LEPR share a number of signaling mediators, such as JAK2/STAT-3 (Janus kinase 2/signal transduction and activator of transcription 3) and PI3K (Y Benomar et *al*. 2005) leading to a cross regulation of both pathways. These findings would contribute to the understanding of the complex relationship between obesity-associated leptin- and insulin-resistance. Insulin was previously shown to potentiate leptin-induced signal transducer and activator of transcription (STAT)3 phosphorylation (Carvalheira *et al.* 2001).

Due to the abundant expression of their respective receptors in the hypothalamus, leptin and insulin exert parallel effects on reductions in food intake and the activation of adiposity signaling. Leptin and insulin were shown to be secreted in a body fat content dependent-manner (Bagdade *et al.* 1967, Schwartz *et al.* 1996). Prolonged excessive nutrition increases insulin and leptin secretion that promotes mTOR signaling pathway activation and, consequently, enhances IRS-1 serine phosphorylation to induce hypothalamic insulin resistance (Fig. 6).

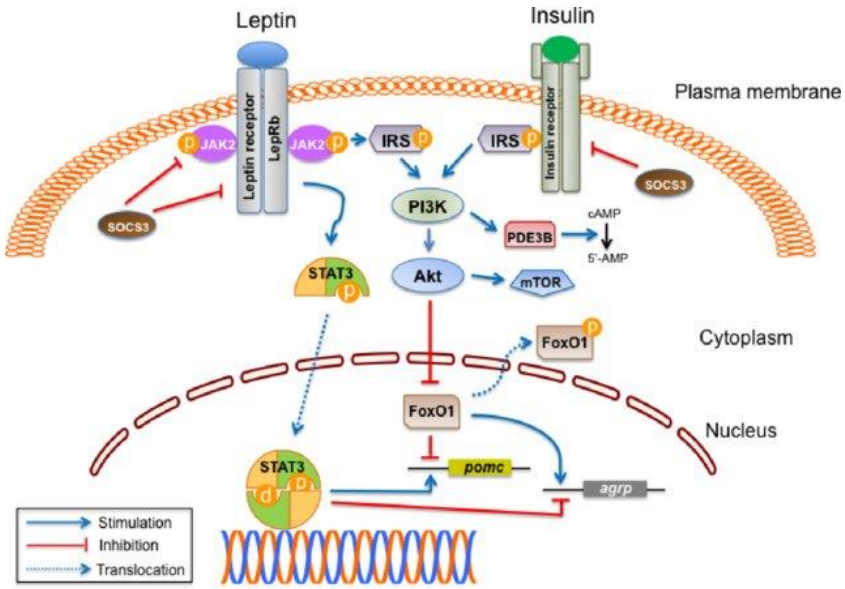


Figure 6. Interactions of LEPR and IR signaling in hypothalamus (Hyeong-Kyu Park, 2014). Leptin activates STAT3 when it binds to the long isoform of LEPR, LEPRb, in the arcuate nucleus, ventromedial hypothalamus, and other hypothalamic neurons.

It has been observed that there are differences among people who maintain their weight and those that gain weight more easily. Obesity could be evaluated by hormone analysis and hormone response profiles, particularly to insulin. In addition to other factors already commented, insulin resistance might be due to environmental obesogens and toxins (Karel Erion *et al.* 2017).

Consumption of a diet high in simple carbohydrates has been shown to consistently increase adiposity in rodents (Isken *et al.* 2009, Scribner *et al.* 2008). Insulin inhibits lipolysis and promotes fat/lipid storage. Administration of insulin in rodents leads to expansion of total fat mass (Beaton *et al.* 1956). Obesity leads to a release of FFA from adipose depots and, consequently, high plasma FFA levels are presented in obese subjects. The inhibition of the protein PI3K can prevent lipid accumulation (Ning *et al.* 2011). Moreover, daily administration of PI3K

inhibitor reduced adiposity and improved glycemia in the absence of any detectable toxicity (Ortega-Molina *et al.* 2015).

The obesity-associated tandem: hyperinsulinemia and insulin resistance

Obesity is strongly associated with a decreased response to insulin (insulin resistance) and an increased insulin level in blood (hyperinsulinemia). Several studies have tried to explain temporal and mechanistic connections between obesity, hyperinsulinemia and insulin resistance. In obesity there is an enormous expansion of the adipose tissue due to adipocyte hyperplasia and hypertrophy. Consequently, diverse responses are primary induced in this tissue, such as metaflammation, that are proposed to promote insulin resistance systemically and, consequently, insulin action is impaired not only in this tissue, but also in other insulin target tissues such as liver, skeletal muscle and pancreas (Houstis *et al.* 2006, Hotamisligil *et al.* 2010, Gregor & Hotamisligil 2011). For instance, insulin resistance leads to an increased circulating FFA that impedes insulin-mediated glucose uptake in skeletal muscle and increases glucose generation in liver (Samuel & Shulman 2012).

Although much attention has been addressed to the early steps of IR signaling cascade, obesity-induced insulin resistance seems to be caused by disruptions downstream of these steps. Most of the studies showed that insulin resistance induced by inflammation is associated with insulin signaling events downstream IR. New findings also connect insulin resistance to extensive metabolic cross-talk between the liver, adipose tissue, pancreas and skeletal muscle (Czech 2017) (Fig. 7). In conclusion, the development of insulin resistance seems to be complex and may occur at many different levels.

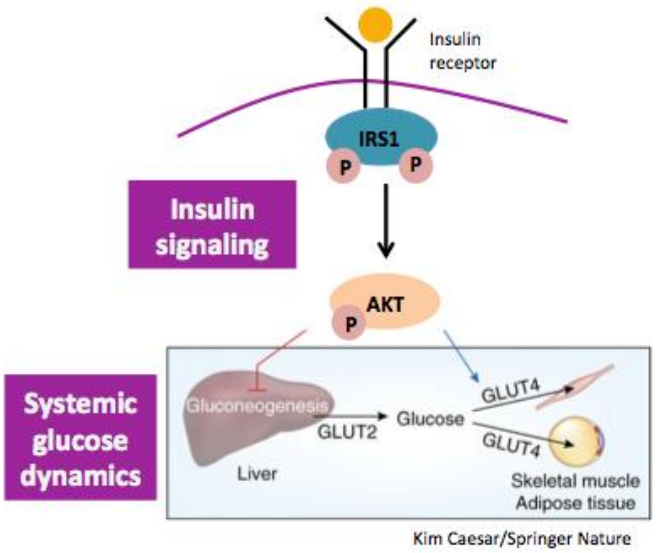


Figure 7. IR signaling pathway and systemic glucose dynamics. Cross-talk between different insulin-target tissues in response to insulin.

Hyperinsulinemia helps to maintain glucose and lipid homeostasis during states of increased insulin resistance such as illness or stress. Obesity-associated insulin resistance is proposed to be early compensated by a β-cell hyperplasia, which allows increased insulin secretion, but chronic insulin demand leads to a failure of the pancreatic insulin-producing cells (β-cells), and the consequence increase in blood glucose level or hyperglycemia, the hallmark of diabetes. Hyperglycemia and hyperinsulinemia have been investigated as promoters of diverse pathological conditions, nevertheless, some studies separate the causal role of hyperinsulinemia from hyperglycemia. In this regard, Zhang *et al.* (2019) showed that endogenous increase in insulin, but not glucose, contributes to pancreatic cancer development. It has been proposed that insulin-lowering interventions, such as exercise, diet, and metformin could represent effective strategies to prevent cancer or limit its progression.

Introduction

Currently, the most widely accepted view in the insulin resistance and hyperinsulinemia relationship is that in response to insulin resistance, in order to maintain normoglycemia, the organism increases the pancreatic secretion of insulin thereby producing hyperinsulinemia (Donath *et al.* 2013) (Model 1, Fig. 8). However, compelling evidence also supports for the connection between insulin resistance and hyperinsulinemia in the opposite way, that is, the chronic exposure to hyperglycemia would initially trigger hyperinsulinemia and, in turn, insulin resistance would be promoted (Model 2, Fig. 9). Unfortunately, the molecular mechanisms of how insulin resistance and hyperinsulinemia develop are still unclear and, nowadays, these two different viewpoints are in debate.

Proposed models

Model 1: Obesity associated metaflammation would be reversible for the promotion of primary insulin resistance, causing hyperglycemia and hyperlipidemia and, consequently, hyperinsulinemia would develop due to compensatory insulin secretion by pancreatic islet β-cells (Fig. 8). This explanation has been widely accepted and supported by the negative crosstalk between pro-inflammatory signals, such as the TNF-α, and the IR pathway described in a previous section.

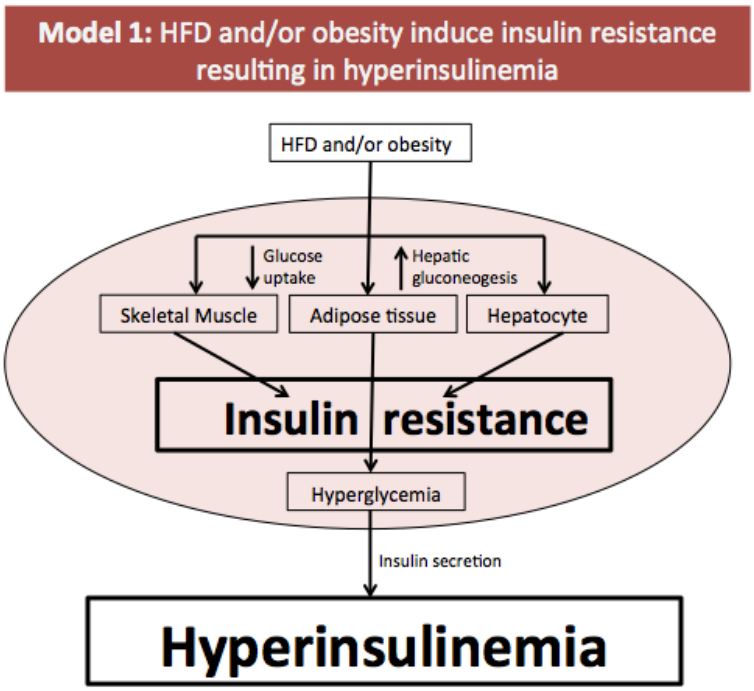


Figure 8. Obesity-induced insulin resistance as a primary cause of hyperinsulinemia. This model explains how HFD and/or obesity leads to pheripheral tissues disruptions that cause primary insulin resistance and this, triggers hyperinsulinemia.

Model 2: Frequent food ingest, specifically that with simple carbohydrates, associated to the obesity development leads to recurrent hyperglycemic episodes. Even in later stages, obesity deregulates hepatocyte effectors of gluconeogenesis, which causes increased glucose output in liver by GLUT2 and deregulate the GLUT4 response to insulin in skeletal muscle and adipose tissue, that causes a decreased glucose uptake by these tissues. Altogether, this also leads to hyperglycemia. In any of these scenarios, increased glucose in blood stimulates pancreatic islet β-cells to secrete insulin and, consequently, hyperinsulinemia develops. Thereafter, chronic exposure to high insulin levels would promote insulin resistance that will foster hyperglycemia and hyperlipidemia (Fig. 9).

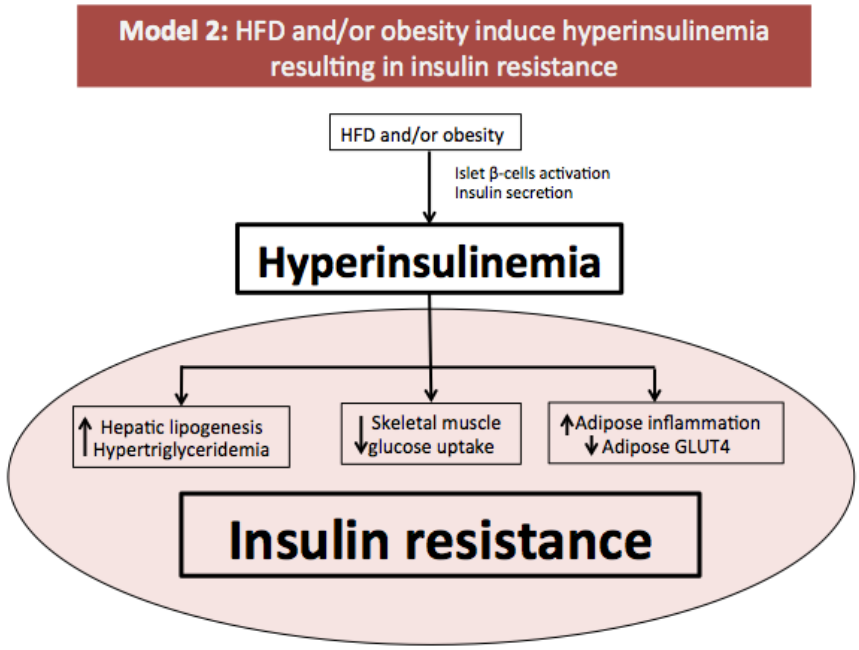


Figure 9. Obesity-induced hyperinsulinemia as a primary cause of insulin resistance. This model explains how HFD and/or obesity leads to an increased insulin level in blood (hyperinsulinemia) and this, causes systemic insulin resistance.

Different investigations suggested that an elevated insulin circulating

levels, may itself cause a disrupted IR signaling (Catalano *et al*. 2014) and an exacerbated insulin resistance (Shanik *et al.* 2008). Marban *et al.* (1996) investigated transgenic mice expressing multiple copies of the insulin gene. These mice were lean and normoglycemic although, remarkably, showed insulin resistance. On the contrary, *Ins1*$^{+/-}$*:Ins2*$^{-/-}$ transgenic mice, which show a decreased expression of insulin in pancreatic β-cells and no insulin secretion in response to hyperglycemia, were protected from diet-induced obesity and chronic hyperinsulinemia. This model showed energy expenditure genes activation in WAT and, in consequence, reduced inflammation, fatty acid spill over and hepatic steatosis (Mehran *et al.* 2012).

Some studies have demonstrated that reduced plasma insulin improved insulin sensitivity in obese mice. Flach et *al*. (2016) demonstrated that whole body Map4k4-depleted mice (M4K4 iKO), were characterized by reduced glucose-responsive plasma insulin and C-peptide levels, an impaired first phase of insulin secretion in response to glucose in islets, and protection against insulin resistance. However, this protection was abrogated by exogenous insulin administration. In summary, Map4k4 drives obesity-induced hyperinsulinemia and insulin resistance in part by promoting insulin secretion from β-cells.

Another study from Yang et *al.* (2014) demonstrated that chronic exposure to hyperinsulinemia induces T2D in chow-fed mice, ectopic fat accumulation in liver, and oxidative stress in liver and pancreas. Finally, non-obese patients of insulinoma develop insulin resistance that is reversed after tumor rejection (Furnica *et al.* 2017).

In summary, according to this model, primary hyperinsulinemia is what initially causes insulin resistance in target tissues, at least under conditions of nutrient excess. Genetic evidences have proved that

pathological hyperinsulinemia leads to metabolic complications induced by obesity (Mehran *et al.* 2012). The mechanism involved may include downregulation of IR signaling to AKT, though others indirect pathways are probably also important.

Transgenic mouse model

In this study I used a transgenic mouse model (C57BL/6J-Tg(GFPloxP-MKK7D)Ccf) that allows the activation of JNK in a Cre recombinase expression-dependent manner. These mice harbor the GFPloxP-MKK7D transgene (Fig. 10), in which MKK7D sequence encodes the MAP2K of JNK, MKK7, with two mutations (S271 and T275) that mimic the active form. In this transgene, expression of MKK7D is prevented by the presence of a stop-cassette coding for the green fluorescent protein (GFP) flanked by two *loxP* sites (Control mice). After Cre-dependent recombination specifically in pancreatic β-cells (MKK7D mice), MKK7D expression and consequent JNK activation leads to glucose intolerance as a result of impaired second phase of insulin secretion in response to hyperglycemia (Lanuza-Masdeu *et al.* 2013).

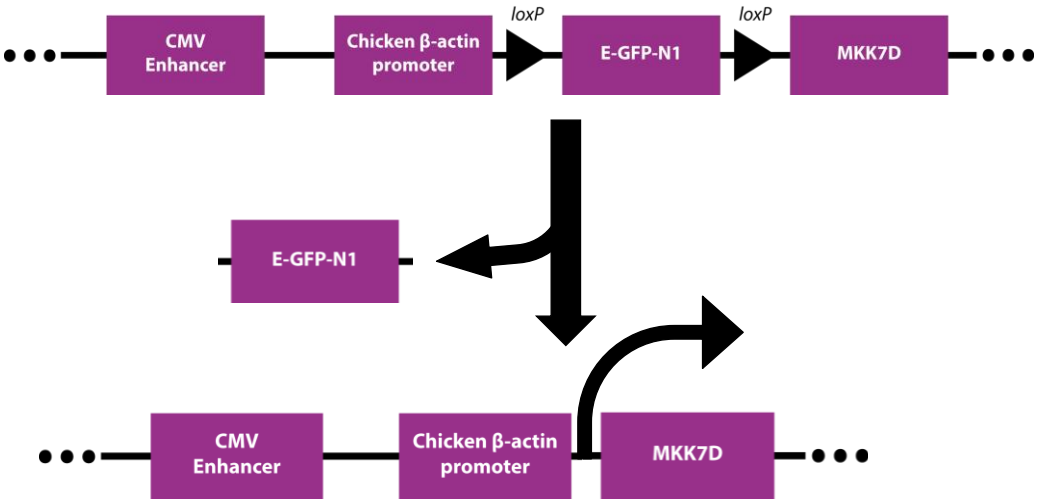


Figure 10. Diagram of the GFPloxP-MKK7D transgene. In this study, transgenic mice

constitutively express GFP under the control of the CMV enhancer/chicken β-actin promoter. After Cre recombinase expression in pancreatic β-cells, the GFP cassette is floxed, leading to the constitutive expression of MKK7D in this cell type (MKK7D mice). E-GFP-N1 (enhanced green fluorescent protein) encodes a red-shifted variant of wild-type GFP.

According to previous results, MKK7D overexpression in pancreatic β-cells leads to JNK activation and disrupts glucose homeostasis. This activation does not cause major morphostructural changes in Langerhans islets, although impairs IR signaling in β-cells and, thereby, the capacity to secrete insulin in response to hyperglycemia. Isolated pancreatic islets from MKK7D mice showed diminished insulin secretion in response to glucose compared to those from Control mice. Moreover, this impairment was overcome *in vitro* when islets were incubated with the JNK inhibitor peptide, JNKi-1, and *in vivo* by the PPARγ ligand and insulin-sensitizing TZD, rosiglitazone (Lanuza-Masdeu *et al.* 2013). In relation to the later, it has been demonstrated the ability of rosiglitazone to inhibit obesity-induced JNK activation, and inhibition that mediates the insulin-sensitizing action of TZDs (Díaz-Delfin *et al.* 2007).

In summary, overactivated JNK in pancreatic β-cells promotes insulin resistance in this cell type. Although in standard conditions, MKK7D mice are glucose intolerant due to the impairment to secrete insulin in response to hyperglycemia by pancreatic β-cells, in these conditions, they show similar insulin sensitivity than their Control littermates (Lanuza-Masdeu *et al.* 2013).

OBJECTIVES

Objectives

This study aimed to investigate the effect of impaired insulin paracrine action on obesity-disrupted glucose homeostasis. For this purpose, we took advantage of a transgenic mouse model (MKK7D mice), which do not secrete insulin in response to hyperglycemia due to the JNK-mediated inhibition of insulin receptor signalling in pancreatic β-cells (Lanuza-Masdeu *et al.* 2013). As the study progressed, diet-induced obese MKK7D mice demonstrated to be a suitable experimental model to test whether hyperinsulinemia might be the primary cause of obesity–induced insulin resistance. This hypothesis contrasts with the general idea that hyperinsulinemia arises from a compensatory pancreatic response to obesity-induced insulin resistance and its assessment would give support for the improvement of insulin resistance by controlling hyperinsulinemia. To achieve these goals the following objectives were proposed:

OBJECTIVE 1: **Effect on diet-induced obesity of JNK activation in pancreatic β-cells:** Comparative study of the development of obesity, glucose intolerance and insulin resistance in response to a HFD in MKK7D mice and Control littermates.

OBJECTIVE 2: Effect of chronic insulin administration on diet-induced obesity and insulin resistance in MKK7D mice: Study of the influence of a chronic insulin administration on the preservation of insulin sensitivity in obese MKK7D mice.

MATERIALS AND METHODS

Animals and *in vivo* studies

Transgenic mice C57BL/6J-Tg(Gfp^{loxP}-MKK7D)Ccf harbor the GFP^{loxP}-MKK7D transgene which allows the Cre recombinase-dependent overexpression of a constitutively activated form of MKK7 (Lanuza-Masdeu et *al*, 2013). Two independent lines were selected for this study and all the experiments were performed in both transgenic mouse lines. The RIP-Cre (C57BL/6-Tg(Ins2-Cre)25Mgn) mice strain was obtained from The Jackson Laboratory (Bar Harbor, Maine USA) (Ref: 003573). The mice analyzed in this study came from crosses of heterozygous Cre recombinase male mice with homozygous GFP^{loxP}-MKK7D female mice. At 4-weeks of age mice were divided in 4 (Objective 1) or 8 (Objective 2) groups (2n= 8 mice/group), according to the genotype, diet (SD or HFD) (Objective 1 and 2) and chronic insulin treatment (Objective 2); subjected to SD (BioServ #F4031) or HFD (BioServ #F3282, 60% fat calories); and, when relevant, a daily insulin administration (30 Units/Kg body weight long-lasting insulin glargine intraperitoneal (IP) injection for 12 weeks.

Mice were housed on a 12:12-h light-dark cycle and controlled temperature with access to food and water *ad libitum*.

All animal procedures were approved by the Animal Care Research Committee of the University of Barcelona.

Glucose tolerance test (GTT) and insulin tolerance test (ITT)

GTT and ITT were performed in 6 h-fasted mice by IP injection of glucose (2 mg/g body weight) or insulin (0.5 UI/Kg body weight), respectively. Blood was collected from the tail at 0 (before glucose or insulin injection), 30, 60 and 90 min, and glycemia was determined with an automatic glucometer (Elite; Bayer).

Tissue and blood isolation from mice

Blood was obtained by cardiac puncture and mice were euthanized by cervical dislocation, 15 minutes before euthanize half of the animals of each group were injected IP with 0.5 UI/Kg of insulin. Tissues were collected, frozen in liquid nitrogen, and stored at -80ºC.

Tissue protein extraction

Whole tissue protein extracts were performed using a lysis buffer (20 mM HEPES·Na pH7.5, 2.5 mM MgCl2, 10 mM EGTA, 40 mM β-glycerophosphate, 1% NP-40, 2 mM sodium orthovanadate, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for disgregation and dilution purposes. A small fragment of frozen tissue was disgregated using a *Polytron homogenizer*, in 0.5 ml of lysis buffer in 2 ml Eppendorf tubes at 4ºC. This temperature was carefully kept in the whole procedure to avoid sample degradation. Homogenates were centrifuged at 12.000 rpm at 4ºC, during 15 minutes.

Bradford protein quantification

Protein concentration was determined by Bio-Rad Protein Assay Dye Reagent following manufacturer's recommendations.

Blood insulin quantification

Blood insulin levels were determined by Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem, #90080) following manufacturer's recommendations.

Immunoblot analysis

25 µg protein extracts were denatured at 95ºC for 5 min in loading buffer (50 mM Tris·Cl pH=6.8, 2% SDS, 10% glycerol, 100 mM mercaptoethanol and 0.1% bromophenol blue) and resolved in 8% SDS-

PAGE. Proteins in gels were transferred onto PVDF membranes (iBlot 2 PVDF Regular Stacks, Thermo Fisher Scientific) and blocked with TBS (20 mM Tris·Cl, 150 mM NaCl, pH= 7,6) containing 0.1% Tween 20 (TBS-T) and 5% non-fat milk or 5% bovine serum albumin, BSA, when anti-phospho specific primary antibodies were used, for 1 h. Membranes were probed with the primary and secondary antibodies shown in Table 1 and 2. ECL Chemiluminiscense (Thermo Scientific) detection system was used to visualize immunoreactivity following the manufacturer's recommendations. Bands were quantified by densitometry analysis using ImageJ 1.44 (National Institutes of Health, USA).

Antigen	Company / Reference
p-AKT (EP2109Y)	Abcam / ab81283
AKT	Santa Cruz / sc-1619
Ob-R (B-3)	Santa Cruz / sc-8391
p-JNK	MerckMillipore / #07-175
γ-Tubulin	Sigma #T5326
Actin	Chemicon / #1501
ERK2 (D-2)	Santa Cruz / sc-1647

Table 2. Primary antibodies used for immunoblot analysis.

Antigen	Company / Reference
Rabbit IgG	Jackson / 115-095-003
Mouse IgG	Jackson / 115-035-174

Table 3. Secondary antibodies used for immunoblot analysis.

RNA extraction and purification

Tissues were homogenized in TRIZOL® reagent using a *Polytron*

homogenizer and RNA was extracted following manufacturer's recommendations. Briefly, after centrifugation of the tissue homogenate, the supernatant was transferred to a new tube, mixed with chloroform and centrifuged again. The aqueous phase was transferred and mixed with isopropanol to precipitate the RNA. After centrifugation, the pellet was washed in 75% ethanol, dissolved in Rnase-free water and incubated for 10 min at 60ºC.

Gene expression analysis; Quantitative real-time PCR (qRT-PCR)

Total RNA isolated from tissues homogenates was reverse transcribed with M-MLV reverse transcriptase (Invitrogen) and quantified by quantitative qRT-PCR using SYBR Green (Applied Biosystems). Gene expression levels were determined by using a standard curve. Each gene was normalized to the housekeeping gene L14 and/or β-actin and both genes were analysed in duplicate. The following pairs of primers were used by qRT-PCR (Table 3):

Gene	Forward primer	Reverse primer
TNFα	CAAAGGGATGAGAAGTTCCC	TGGTGGTTTGCTACGACGT
IL6	CCAGAGATACAAAGAAATGATGG	ACTCCAGAAGACCAGAGGAAAT
IL1β	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCG
IL10	AGGCGCTGTCATCGATTTCT	ATGGCCTTGTAGACACCTTGG
LEP	CAGGATCAATGACATTTCACACA	GCTGGTGAGGACCTGTTGAT
LepRb	TTGGAAGCCCCTGACGAAAA	GAGGGAATTGACAGCCAGAA
β-actin	CTAGGCACCAGGGTGTGAT	CCATGTTCAATGGGGTACTT
L14	TCCCAGGCTGTTAACGCGGT	GCGCTGGCTGAATGCTCTG

Table 4. Genes analyzed by qRT-PCR.

RNA relative expression was quantificated by standard curve method. The calibration curve result for each gene of interest is normalized to that of a housekeeping gene (L14 and β-actin) in the same sample, and then the normalized numbers are compared between samples to get a fold change expression.

Procartaplex Multiplex Immunoassay

Blood IL1, IL6, IL10, TNFα, Leptin levels were quantified by Procartaplex Multiplex Immunoassay following manufacturer's recommendations.

Statistical analysis

All data are presented as mean ± SEM and analysed by GraphPad Prism version 8.0.0 for Windows (San Diego, California USA).

Two-tailed unpaired Student's *t*-test was used for single variable comparison between two groups, significance level represented by # ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$).

Two-way or three-way ANOVA (Tukey's), as appropriate, were used to examine interactions between multiple variables comparing with UT, SD Control mice as a reference and as indicated, significance level represented by * (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

RESULTS

OBJECTIVE 1: Effect of JNK activation in pancreatic β-cells on diet-induced obesity

Induction of obesity by diet in MKK7D mice

In standard chow conditions MKK7D mice show insulin resistance specifically in pancreatic insulin-producing cells but preserve systemic insulin sensitivity (Lanuza-Masdeu *et al.* 2013). To further analyse this phenotype, mice were fed with HFD to challenge them to develop obesity. Before starting with the diet (SD or HFD) at 4-weeks of age, MKK7D mice, and Control littermates, were subjected to a GTT. Compared to Control mice, MKK7D mice were glucose intolerant, as previously described (Lanuza-Masdeu *et al.* 2013) (Fig. 11).

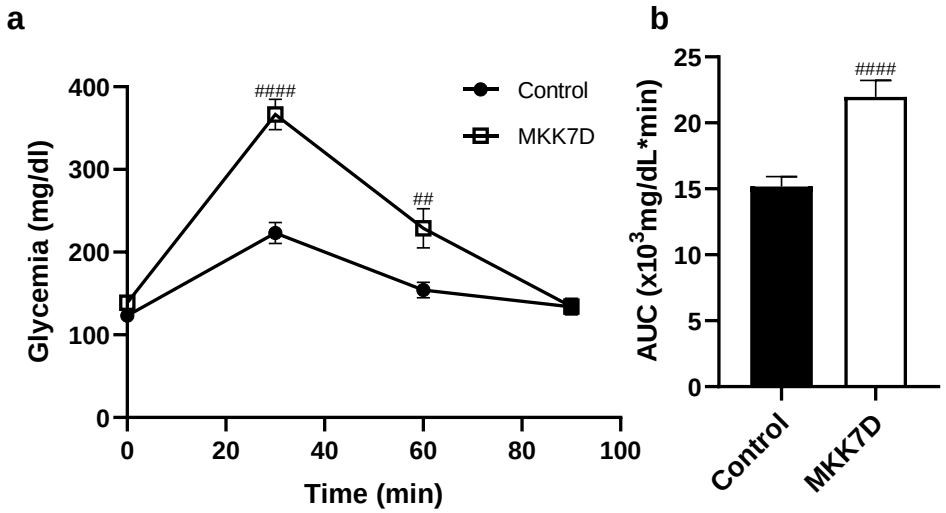


Figure 11. GTT analysis at 4-weeks of age of Control and MKK7D mice. (a) GTT curves, and **(b)** representation of the 90-min area under the curve (AUC) of the GTT analysis shown in **(a)** (n= 16 mice/group).

Afterwars, mice from each genotype, Control and MKK7D, were divided in two groups and, respectively, subjected to a SD or HFD from 4 to 14 weeks of age. Weight gain was followed weekly, and results are shown in figure 12. As can be observed, and according to previous

data (Lanuza-Masdeu *et al.* 2013), MKK7D mice in SD showed a slight less weight gain that Control littermates. However, no differences were observed between groups of Control and MKK7D mice fed with HFD as all animals developed obesity to the same extent.

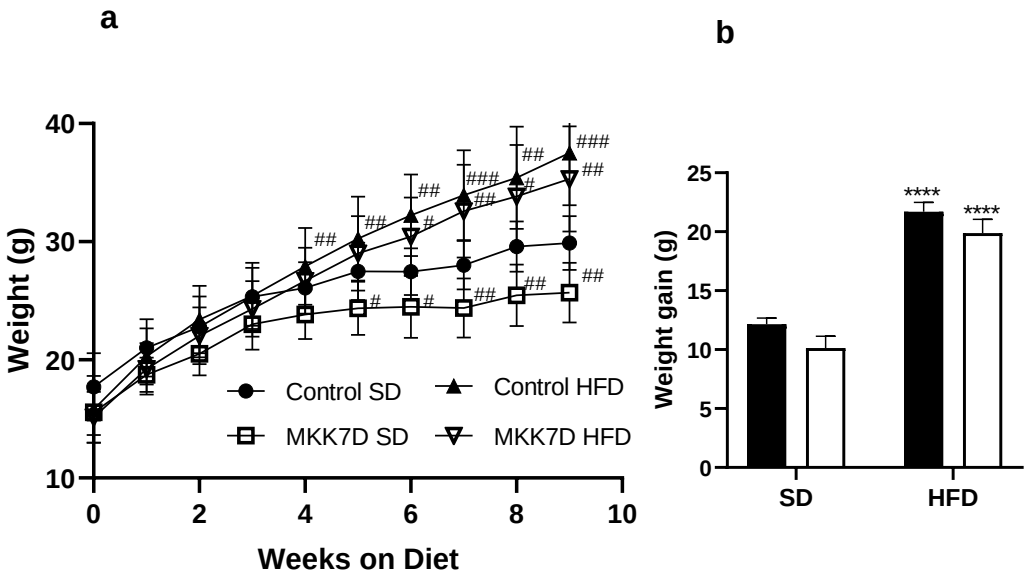


Figure 12. HFD promoted obesity similarly in Control and MKK7D mice. (a) Growth curves along the weeks on SD or HFD, and **(b)** weight gain representation of the indicated Control (black bars) and MKK7D (empty bars) animal groups (n= 8 mice/group).

Analysis of obesity-associated glucose intolerance and insulin resistance in MKK7D mice

On week 9 on SD or HFD, mice were subjected to a GTT and to an ITT to analyse glucose tolerance and insulin sensitivity, respectively. As in week 4 of age (Fig. 11), MKK7D mice on SD showed a glucose intolerant phenotype, in comparison to Control animals in this same condition, which was worsened by HFD (Fig. 13). As expected, Control mice developed glucose intolerance in response to HFD-induced obesity (Fig. 13).

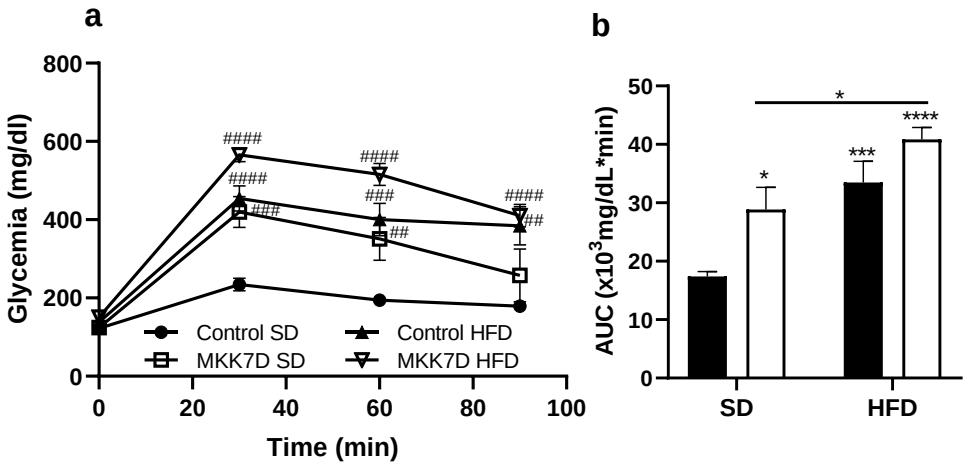


Figure 13. HFD-induced obesity produced glucose intolerance in Control and MKK7D mice. (a) GTT curves and **(b)** AUC analysis from the GTT shown in **(a)** of the Control (black bars) and MKK7D (empty bars) animal groups (n= 8 mice/group).

Regarding the ITT, Control and MKK7D mice on SD showed a similar response, as previously described in Lanuza-Masdeu *et al.* 2013. As expected, HFD-induced obesity promoted the development of an insulin resistance phenotype in Control animals. Unexpectedly, MKK7D mice on HFD preserved sensitivity to insulin despite of developing adiposity to the same extent as Control animals (Fig. 14).

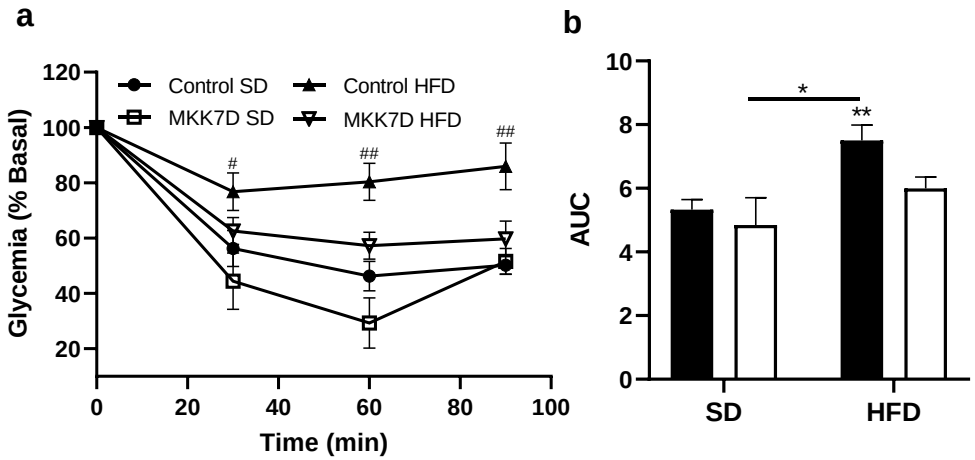


Figure 14. Obese MKK7D mice preserved insulin sensitivity. (a) ITT curves, and **(b)** AUC representation from ITT shown in **(a)** of the Control (black bars) and MKK7D (empty bars) experimental groups (n= 8 mice/group).

The blood insulin level (insulinemia) is a sensitive marker of early diagnosis of T2D and positively correlates with systemic insulin resistance being a good proxy for this trait. As shown in figure 15, obese MKK7D mice preserved normoinsulinemia while Control animals developed hyperinsulinemia in response to obesity. These results are consistent with the ITT data, and, altogether, indicate that MKK7D mice are protected from the development of obesity-associated insulin resistance and hyperinsulinemia.

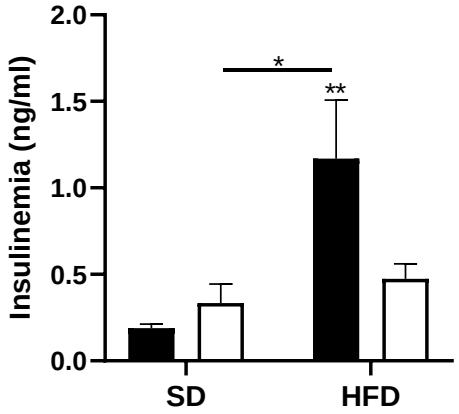


Figure 15. Obese MKK7D mice did not develop hyperinsulinemia. Plasma insulin level analysis of Control (black bars) and MKK7D (empty bars) mouse groups (n= 3 mice/group).

Tissue-specific analysis of the IR signaling pathway activation

Next we analysed at molecular level the response to insulin of relevant insulin-target tissues, namely adipose tissue, liver and skeletal muscle. For this purpose, half of the animals of each group was injected with insulin (0,5 UI/Kg) 15 min before to euthanize them, and insulin-induced activation of AKT, a very good proxy of the IR pathway

activation, was determined by immunoblot analysis of AKT phosphorylation on Ser473 ($pAKT^{Ser473}$) in protein extracts of these tissues.

Obesity originates from the overexpansion of the adipose tissue that is mediated by adipocyte hyperplasia and hypertrophy. These cellular changes promote metabolic, immune and endocrine disturbances, which eventually lead to systemic consequences on glucose, as well as in other metabolites, homeostasis. Therefore, we initially compared insulin-induced IR pathway activation in WAT, concretly, in epididymal WAT (e-WAT) of lean and obese Control and MKK7D mice, respectively. Results showed that AKT phosphorylation in response to insulin was induced to the similar extent in lean Control and MKK7D mice and, remarkably, also in obese MKK7D mice. On the contrary, in Control mice on HFD AKT phosphorylation was significantly diminished (Fig. 16). These results indicate that despite of developing a similar level of adiposity than Control, MKK7D mice preserve insulin sensitivity while obese Control mice developed insulin resistance in this tissue.

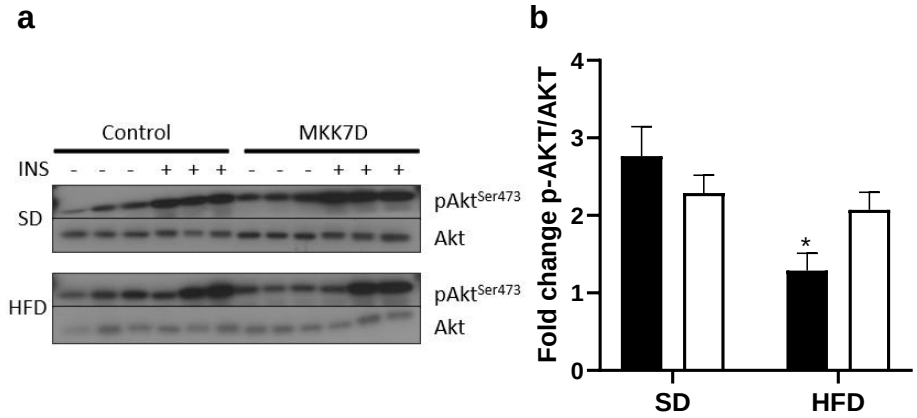


Figure 16. IR pathway activation by insulin in e-WAT. (a) Representative immunoblot analysis of $pAKT^{Ser473}$ and AKT in e-WAT protein extracts from non- or insulin (INS)-stimulated Control and MKK7D mice on SD or HFD, as indicated. **(b)**

Graph represents the fold change in the pAKT^{Ser473} versus AKT ratio between insulin (INS)-stimulated and non-stimulated Control (black bars) and MKK7D (empty bars) animals, determined by densitometry analysis of the x-ray films obtained from immunoblots (n=6 mice/group).

A similar comparative analysis of insulin-induced AKT phosphorylation was conducted in protein extracts from liver and skeletal muscle, and results are shown in figures 17 and 18, respectively. Analogously to adipose tissue, obese MKK7D preserved IR pathway activation in response to insulin in these tissues showing an opposite phenotype to Control mice on HFD.

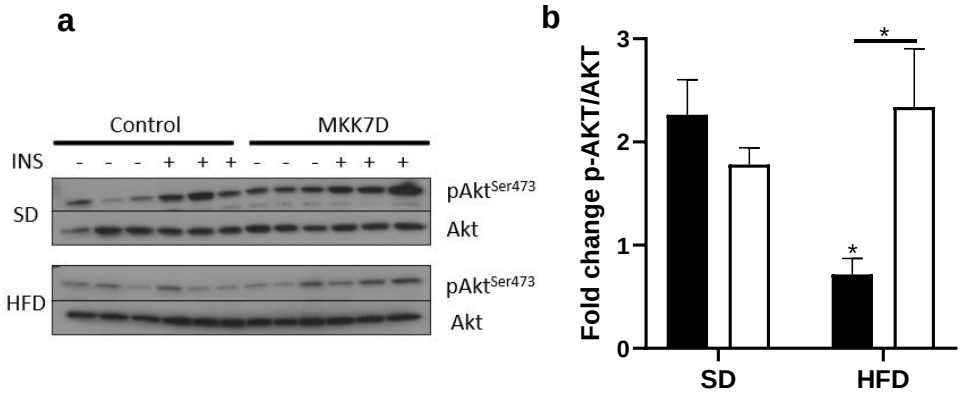


Figure 17. IR pathway activation by insulin in liver. (a) Representative immunoblot analysis of pAKT^{Ser473} and AKT in liver protein extracts from non- or insulin (INS)-stimulated Control and MKK7D mice on SD or HFD, as indicated. **(b)** Graph represents the fold change in the pAKT^{Ser473} versus AKT ratio between (INS)-stimulated and non-stimulated Control (black bars) and MKK7D (empty bars) animals, determined by densitometry analysis of the x-ray films obtained from immunoblots (n=6 mice/group).

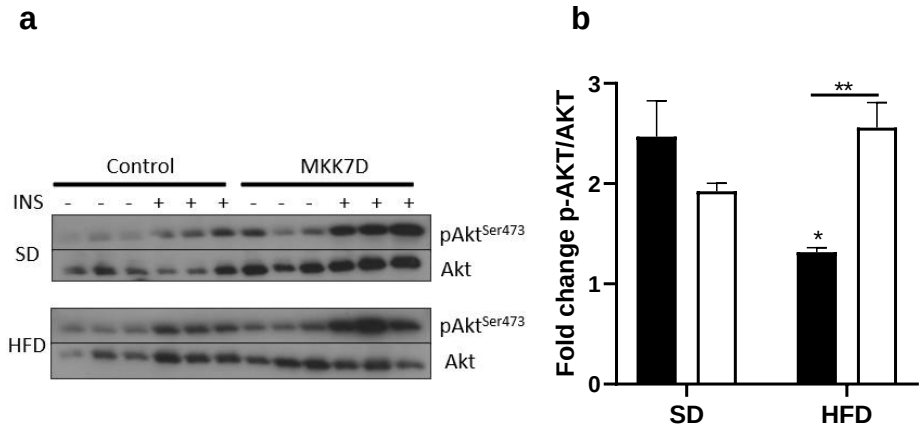


Figure 18. IR pathway activation by insulin in skeletal muscle. (a) Representative immunoblot analysis of pAKT^{Ser473} and AKT in skeletal muscle protein extracts from non- or insulin (INS)-stimulated Control and MKK7D mice on SD or HFD, as indicated. **(b)** Graph represents the fold change in the pAKT^{Ser473} versus AKT ratio between (INS)-stimulated and non-stimulated Control (black bars) and MKK7D (empty bars) animals, determined by densitometry analysis of the x-ray films obtained from immunoblots (n=6 mice/group).

Overall, these results demonstrate that regardless the development of adiposity, MKK7D mice preserve IR pathway response to insulin in all of insulin-target tissues analysed so far (e-WAT, skeletal muscle and liver), a feature that is in complete concordance with the preservation of systemic insulin sensitivity in these animals.

Results

OBJECTIVE 2: Effect of chronic insulin administration on diet-induced obesity and insulin resistance in MKK7D mice

Effect of chronic insulin administration on diet-induced obesity in MKK7D mice

We took advantage of the obese MKK7D mouse phenotype to assess the cause-and-consequence in the obesity-associated insulin resistance-hyperinsulimenia tandem by chronically exposing the animals to exogenous insulin. For this purpose, MKK7D and Control mice were subjected to a similar protocol to induce obesity as described in the development of the objective 1, except that the long lasting insulin glargine (30 Units/Kg body weight) was administered intraperitonealy (IP) once daily to half of the animals in each of the four experimental groups (one group on SD or HFD for each genotype). As before and previously to start with the specific diet, at 4 weeks of age Control and MKK7D mice were subjected to a GTT to assess the glucose intolerant phenotype of MKK7D mice (Fig. 19), and then, placed on SD or HFD, respectively, for 12 weeks. When relevant, glargine administration started on week 5 of age and animal weight was followed weekly. Chronic insulin treatment did not influence animal growth neither in SD nor in HFD, moreover, Control and MKK7D mice developed adiposity in response to HFD to the same extent regardless insulin administration and gender (Fig. 20 and Fig. 34 and 40 in the Annex section).

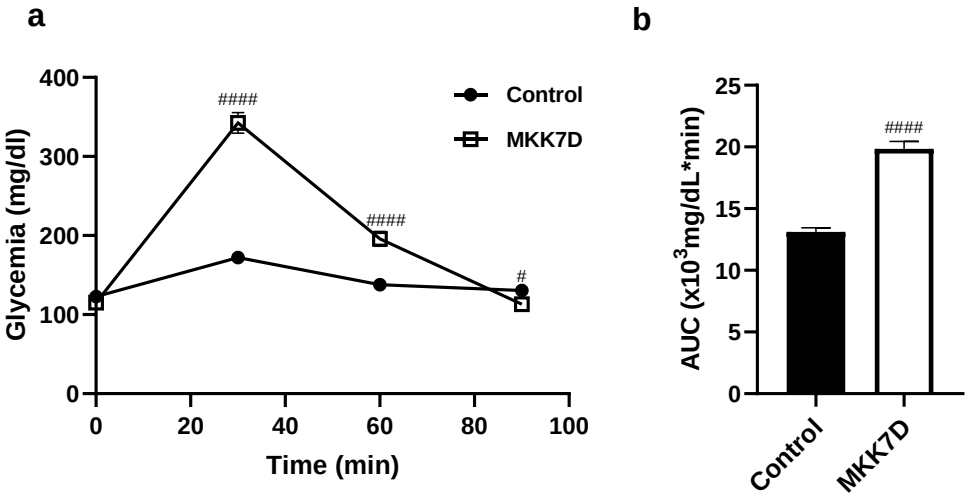
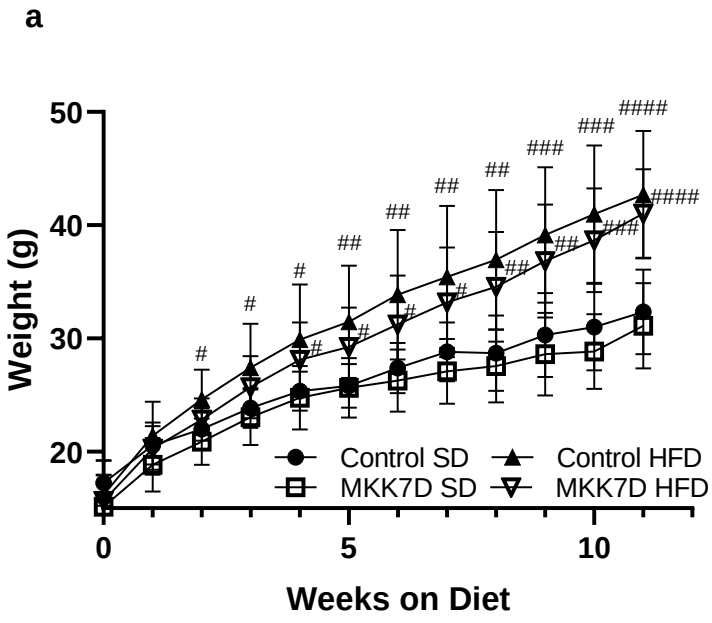


Figure 19. GTT analysis at 4-weeks old of Control and MKK7D mice. (a) GTT curves and **(b)** AUC representation of GTT shown in **(a)** of Control and MKK7D mice, as indicated (n= 32 mice/group).



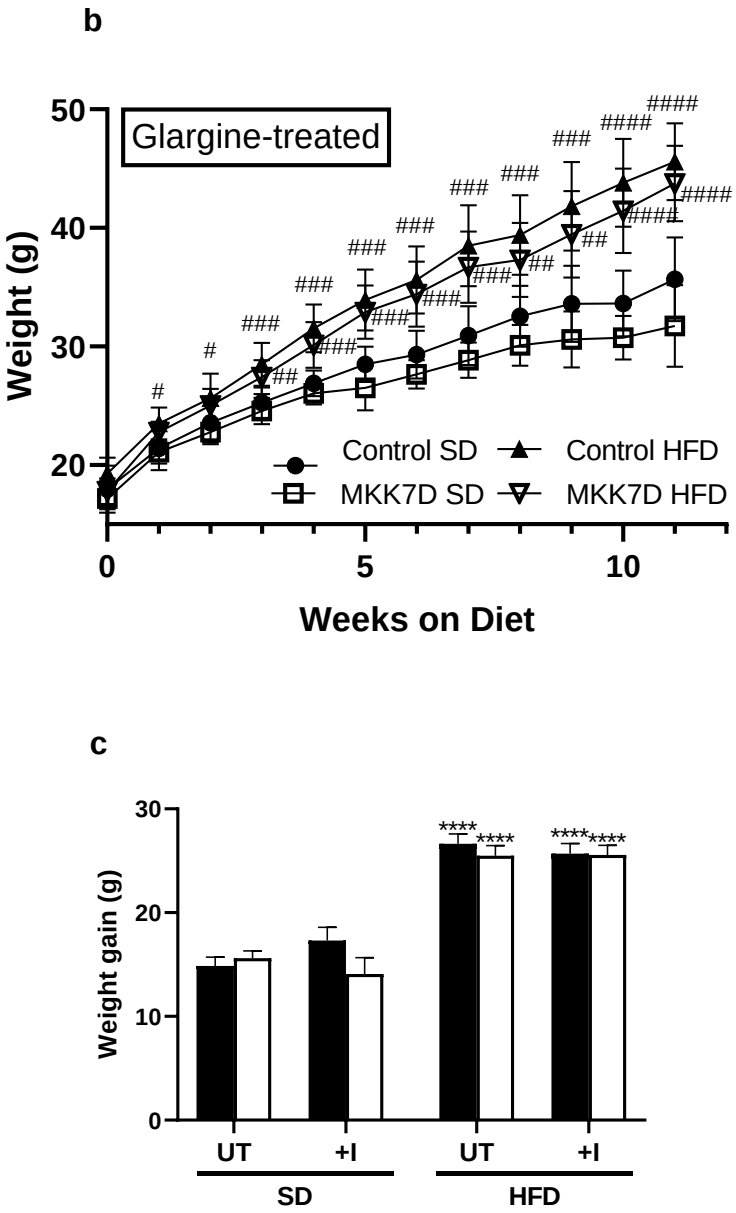


Figure 20. HFD promoted obesity in Control and MKK7D mice independently of genotype and chronic insulin treatment. Growth curves of Control and MKK7D mice along the weeks on SD or HFD of non- **(a)** and daily glargine-treated **(b)**, as indicated, and weight gain **(c)** of the indicated Control (black bars) and MKK7D (empty bars) experimental groups (UT, non-treated and +I) (n= 8 mice/group).

Effect of chronic insulin administration on the preservation of insulin sensitivity in obese MKK7D mice

At 15 weeks of age, all mice were subjected to a GTT and ITT to analyse glucose tolerance and insulin sensitivity, respectively**.** Regarding GTT, and as observed in the previous experiment (see Fig. 13), MKK7D mice were already glucose intolerant in SD, a phenotype that was aggravated by HFD, and Control mice became glucose intolerant only in those animals on HFD. In any case, chronic insulin administration did not modify this parameter neither in any diet condition nor for any of the two tested genotypes (Fig. 21, and Fig. 35 in the Annex section).

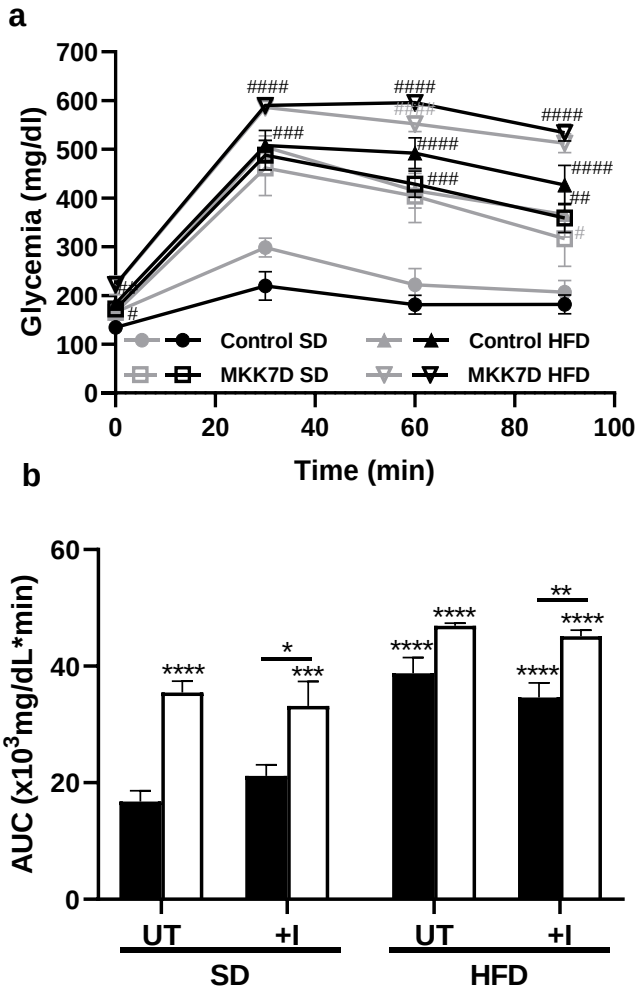
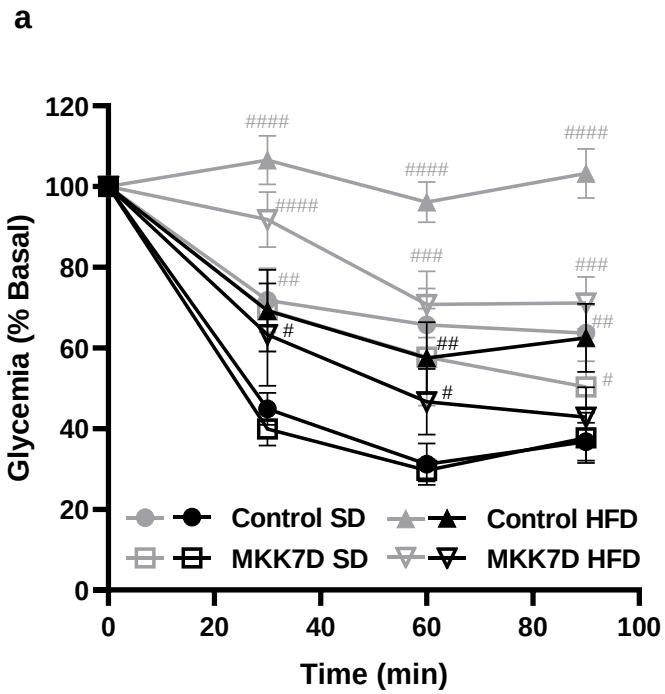


Figure 21. No effect of chronic insulin treatment on glucose tolerance in any analysed condition. (a) GTT curves of the indicated mice and diets (non- and glargine-treated mice are shown in black and grey lines, respectively), and **(b)** AUC representation of GTT shown in **(a)** of the indicated Control (black bars) and MKK7D (empty bars) mice and conditions (UT, non-treated; +I, glargine-treated) (n= 8 mice/group).

Recapitulating the results of the previous experiment (see Fig. 14), despite obese Control and MKK7D mice obtained similar weight gain after 12 weeks on HFD (Fig. 20), obese MKK7D mice were again protected from diet-induced insulin resistance, as shown by ITT (Fig. 22, Fig. 36 in the Annex section). Of note, chronic insulin administration reversed this protective phenotype, thus this treatment induced the development of insulin resistance in obese MKK7D mice. In fact, this treatment even induced insulin resistance in Control and, to some extent, also in MKK7D mice on SD, and further aggravated this condition in obese Control mice (Fig. 22, Fig. 36 in the Annex section).



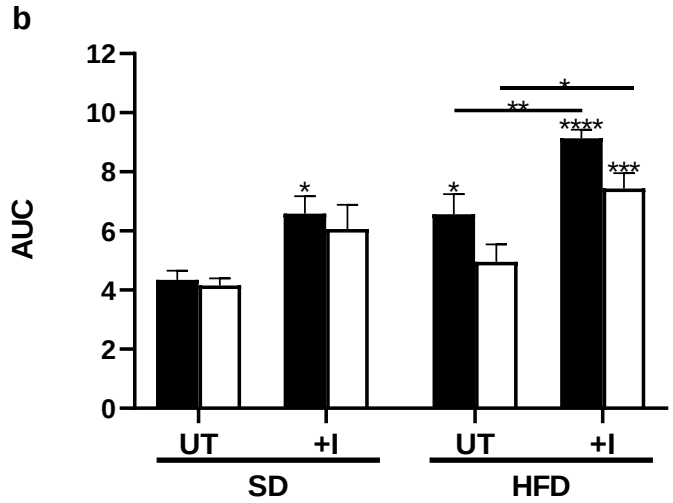


Figure 22. Chronic insulin treatment promotes insulin resistance. ITT curves of the indicated mice and diet (non- and glargine-treated mice are shown in black and grey lines, respectively) (**a**) and AUC representation from ITT shown in (**a**) (**b**) of the indicated Control (black bars) and MKK7D (empty bars) mice and conditions (UT, non-treated; +I, glargine-treated) (n= 8 mice/group).

As stated above, insulinemia is positively linked to insulin resistance. Moreover, both parameters also show a positive correlation with leptinemia and leptin resistance, respectively. In this regard, and similarly as in the previous experiment (see Fig. 15), MKK7D mice did not develop obesity-associated hyperinsulinemia (Fig. 23a, Fig. 37 in the Annex section). A similar profile was observed in relation to leptin, while obese Control mice showed the expected hyperleptinemia, remarkably, obese MKK7D mice maintained a normal level of leptin in blood; however, chronic insulin treatment reversed this protective phenotype again abrogating the difference between Control and MKK7D mice (Fig. 23b).

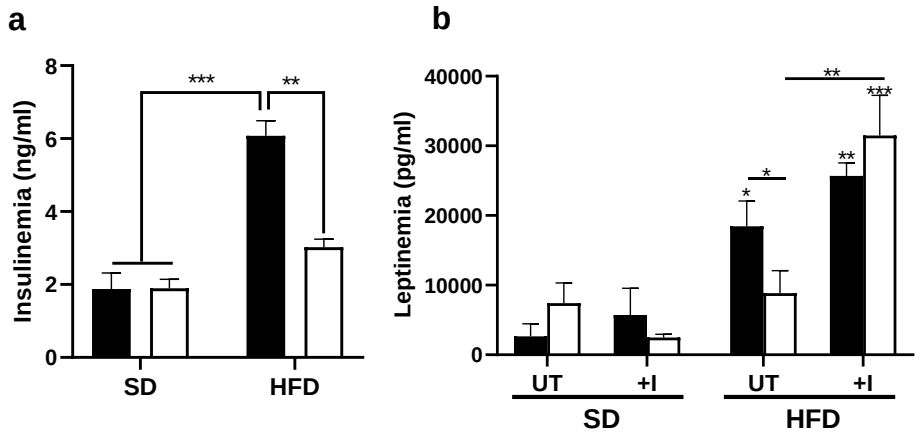
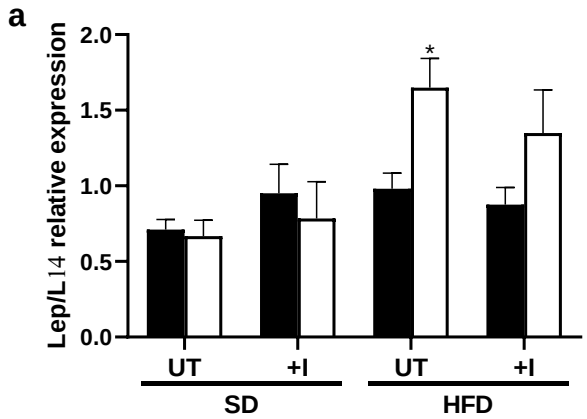


Figure 23. Chronic insulin administration reversed the protection from the development of hyperleptinemia of obese MKK7D mice. (a) Plasma insulin (n=3 mice/group) and **(b)** leptin (n=4 mice/group) level analysis of the indicated Control (black bars) and MKK7D (empty bars) mice and conditions (UT, non-treated; +I, glargine-treated).

Next we analysed leptin and its receptor (LepRb, long form) gene expression in e-WAT by qRT-PCR. Despite the results on leptinemia, leptin expression was only significantly increased in response to obesity in MKK7D mice (Fig. 24a). Interestingly, LepRb expression was higher in MKK7D mice than in Control animals in all experimental settings but, remarkably, with the exception of chronic insulin treatment combined with HFD (Fig 24b).



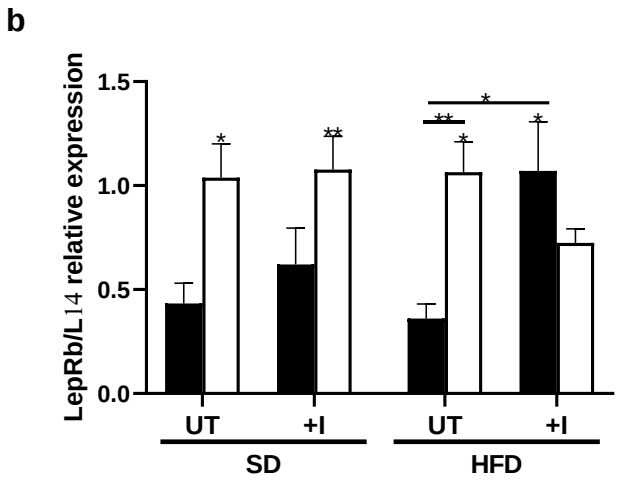


Figure 24. Leptin and LepRb expression in adipose tissue. **(a)** Leptin (Lep) and **(b)** LepRb expression analysis in e-WAT of Control (black bars) and MKK7D (empty bars) mice at the indicated conditions (UT, non-treated; +I, glargine-treated) (n= 6 mice/group).

Effects of the chronic insulin administration on the IR signaling pathway activation in specific tissues

Using the same approach as described in objective 1, IR pathway activation in response to insulin was determined in e-WAT, liver and skeletal muscle. In adipose tissue, chronic insulin administration already promoted insulin unresponsiveness in e-WAT of lean Control mice, while lean MKK7D mice preserved IR signaling pathway activation in response to hormone (Fig. 26, Fig. 38 in the Annex section). As in the initial experiment, in HFD IR pathway failed to respond to insulin in Control mice while this response was preserved in MKK7D animals (see Fig. 16), although chronic insulin treatment abrogated this protective effect in this tissue of these mice (Fig. 26, Fig. 38 in the Annex section).

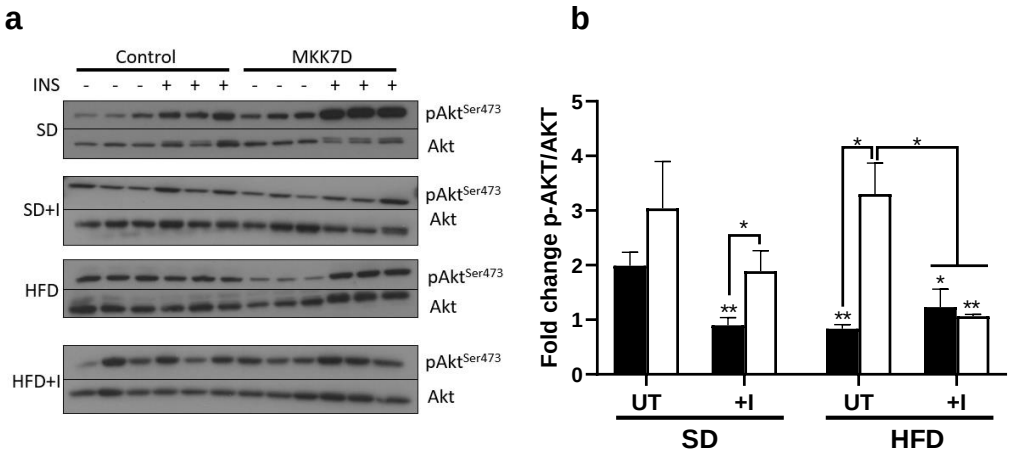


Figure 26. Effects of chronic insulin treatment in IR pathway activation in e-WAT. **(a)** Representative immunoblot analysis of pAKT^{Ser473} and AKT in e-WAT protein extracts from non- or insulin (INS)-treated Control and MKK7D mice on SD or HFD and without or with (+I) chronic glargine administration, as indicated. **(b)** Graph represents the ratio of pAKT^{Ser473} versus AKT determined by densitometry analysis of the x-ray films obtained from immunoblots of Control (black bars) and MKK7D (empty bars) mice (UT, non-treated; +I, glargine-treated) (n= 6 mice/group).

Regarding IR pathway activation in response to insulin in liver, chronic insulin administration strongly downregulated IR pathway activation in lean animals, though MKK7D mice response was significantly better. A similar observation was found in mice in the HFD condition, however, similarly to the e-WAT, this was abrogated by chronic insulin administration (Fig. 27).

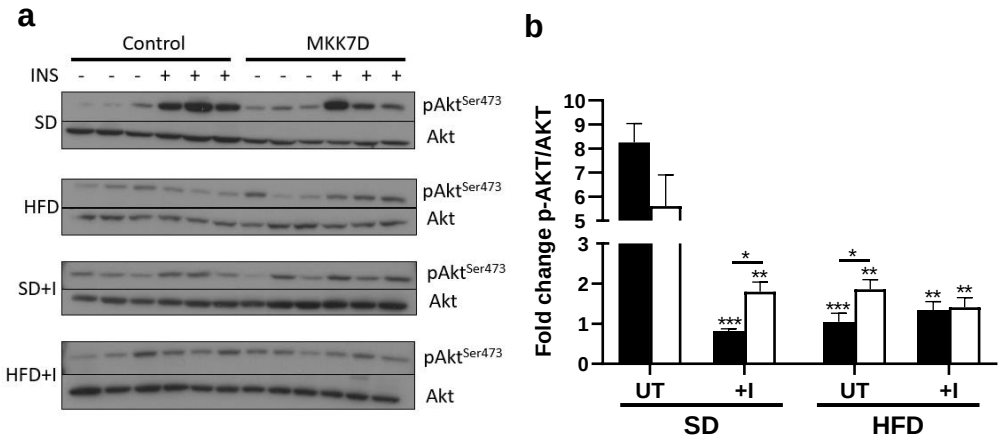


Figure 27. Effects of chronic insulin treatment in IR pathway activation in liver. (a) Representative immunoblot analysis of pAKT^{Ser473} and AKT in liver protein extracts from non- or insulin (INS)-treated Control and MKK7D mice on SD or HFD and without or with (+I) chronic glargine administration as indicated. **(b)** Graph represents the ratio of pAKT^{Ser473} versus AKT determined by densitometry analysis of the x-ray films obtained from immunoblots of Control (black bars) and MKK7D (empty bars) mice (UT, non-treated; +I, glargine-treated) (n= 6 mice/group).

In relation to skeletal muscle, the major contributor to glucose uptake in response to insulin, consistently with the previous experiment (see Fig. 18), obese Control mice showed reduced IR pathway activation compared with obese MKK7D mice in which activation level was similar as in the lean condition. However, chronic insulin treatment completely abrogated insulin-dependent activation of the IR pathway in obese MKK7D mice (Fig. 28). Of note, in this tissue, chronic insulin administration did not inhibit insulin response in lean animals (Fig. 28). Due to the importance of skeletal muscle in insulin-induced glucose uptake, this observation is in accordance with the results obtained in the GTT and ITT analysis of these animals.

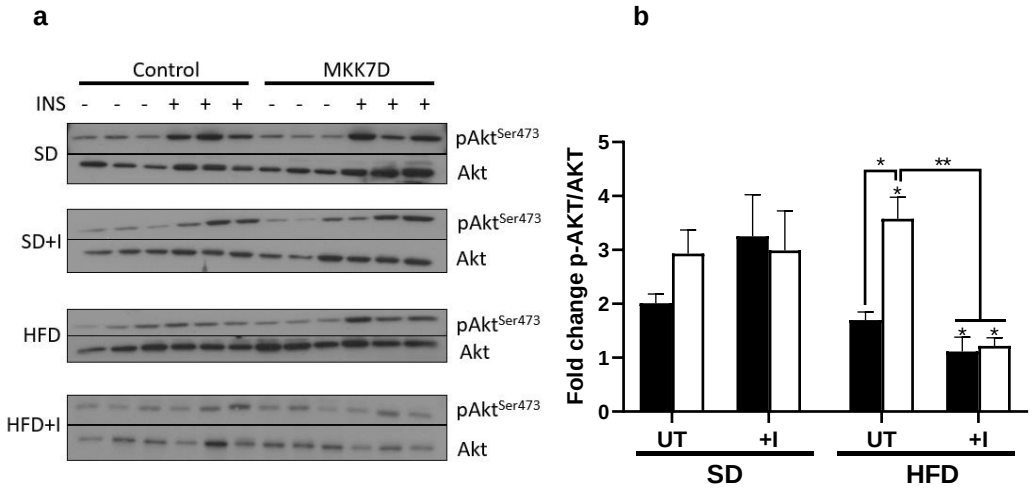
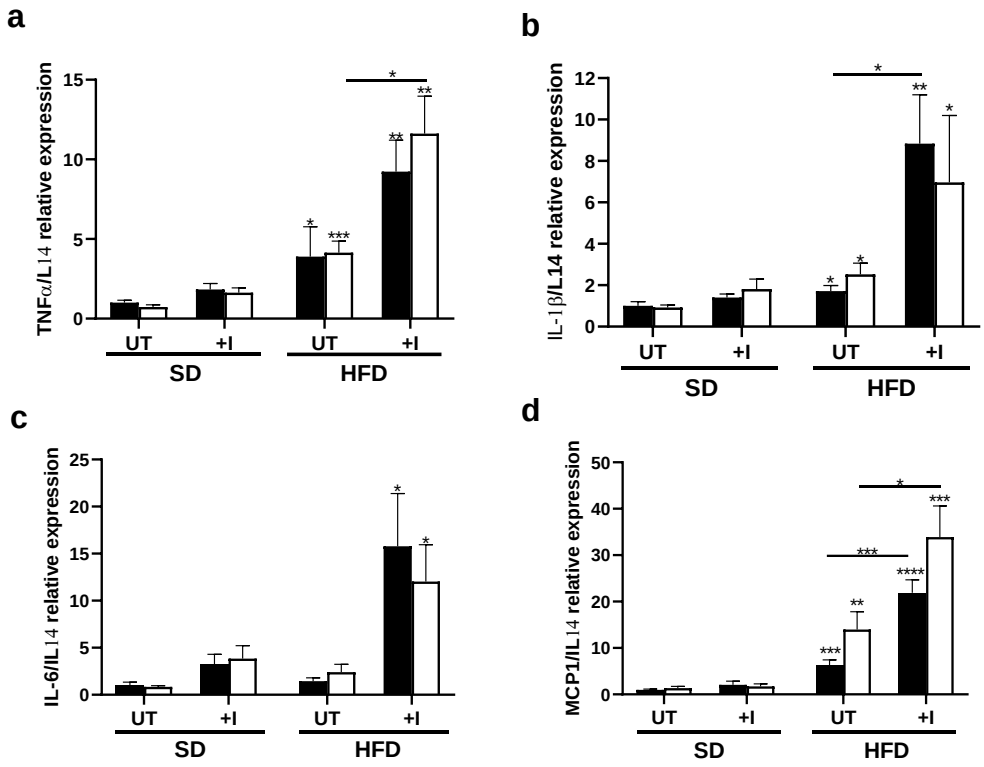


Figure 28. Effects of chronic insulin treatment in IR pathway activation in skeletal muscle. (a) Representative immunoblot analysis of pAKT^{Ser473} and AKT in skeletal muscle protein extracts from non- or insulin (INS)-treated Control and MKK7D mice on SD or HFD and without or with (+I) chronic glargine administration as indicated. **(b)** Graph represents the ratio of pAKT^{Ser473} versus AKT determined by densitometry analysis of the x-ray films obtained from immunoblots of Control (black bars) and MKK7D (empty bars) mice (UT, non-treated; +I, glargine-treated) (n= 6 mice/group).

Effects of chronic insulin administration on the obesity-associated inflammatory response in the adipose tissue

Obesity is associated with a low-grade chronic inflammatory response in the adipose tissue, which correlates with an increased expression of pro-inflammatory cytokines, some of which are responsible for insulin unresponsiveness (Hotamisligil *et al.* 1995). Due to the differences in insulin sensitivity observed in obese MKK7D *versus* obese Control mice, expression of cytokines associated to the inflammatory response, namely TNFα, IL-1β, IL-6, MCP1 and IL-10, was analysed by qRT-PCR in e-WAT of lean and obese Control and MKK7D mice, respectively. The expression of the pro-inflammatory markers, TNFα, IL-1β and MCP1, was increased, as expected, in obese and insulin-

resistant Control mice but, unpredictably, also and to a similar extent in obese and insulin sensitive MKK7D mice (Fig. 29a, b and c). No induction of IL-6 expression by obesity was observed (Fig. 29d). Notably, chronic-insulin administration strongly enhanced to a similar extent the expression of all pro-inflammatory markers, including also IL-6, in e-WAT of both, Control and MKK7D mice on HFD, while no significant effect was observed in lean animals (Fig. 29a, b and c). Remarkably, a difference was observed in the expression of the anti-inflammatory cytokine IL-10, which in contrast to obese Control mice, its expression was not increased by obesity in MKK7D mice, however, chronic insulin treatment eliminated such difference (Fig. 29e). Therefore, the expression of this cytokine was the only that correlated with insulin resistance.



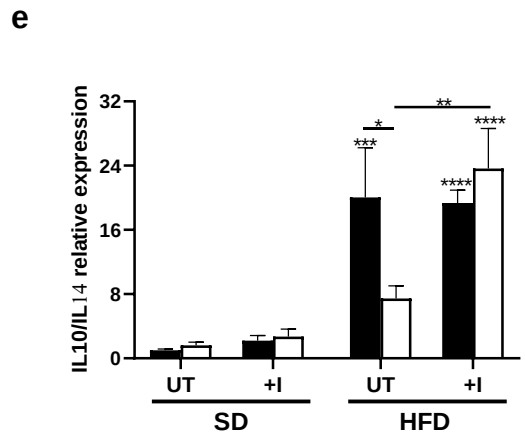
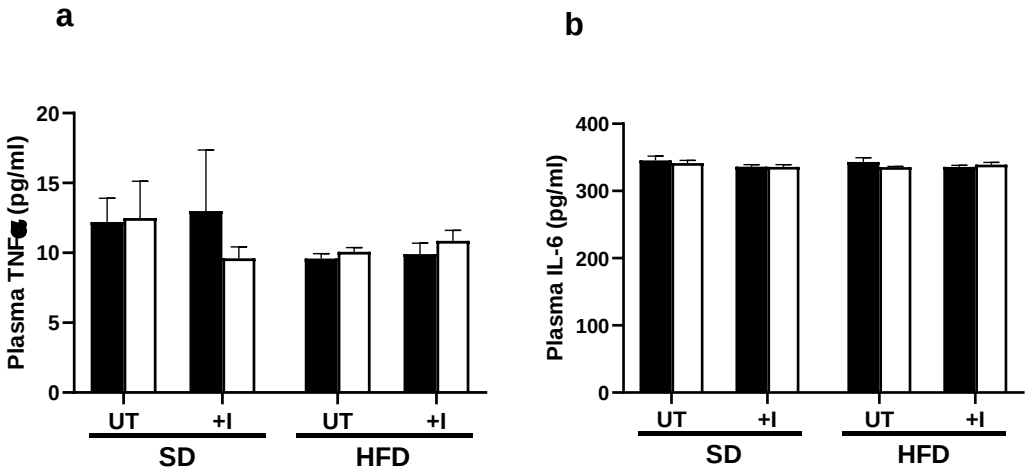


Figure 29. Obesity and chronic insulin treatment promoted increased expression of inflammatory-response associated cytokines in e-WAT. Relative expression of TNFα (**a**), IL-1β (**b**), IL-6 (**c**), MCP1 (**d**) and IL-10 (**e**) determined by qRT-PCR análisis in e-WAT of the indicated Control (black bars) and MKK7D (empty bars) mice and conditions (n=6 mice/group).

Next, the level in plasma of TNFα, IL-6 and IL-10 was analysed and, in contrast to their expression pattern in e-WAT, there were no differences between animals in any condition (Fig. 30).



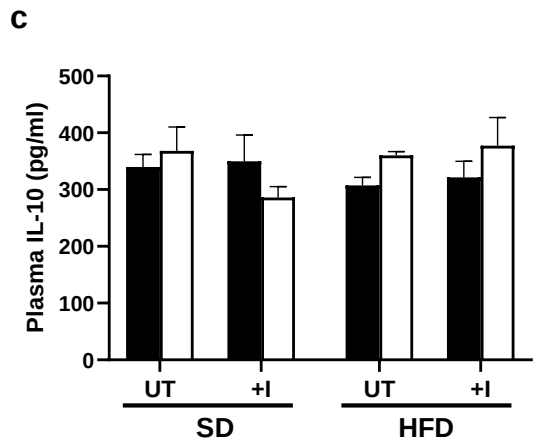


Figure 30. Similar blood circulating levels of pro/anti-inflammatory cytokines in Control and MKK7D mice. Concentration of TNFα, IL-6 and IL-10 was analysed by Procartaplex Multiplex Immunoassay in plasma from Control (black bars) and MKK7D (empty bars) mice subjected to the indicated diet (SD/HFD) and treatment (UT, non- or glargine-treated) (n= 4 mice/group).

We also analysed the activation of JNK, another pro-inflammatory marker responsible for inhibitory IRS phosphorylation and, consequent IR pathway downregulation. According to the literature and previous result from the group, JNK phosphorylation was increased in obese Control mice, but, unpredictably, no JNK activation was observed in adipose tissue of obese MKK7D mice, In addition, chronic insulin treatment increased JNK phosphorylation in obese Control a MKK7D mice to a similar extent (Fig. 31). These unexpected results showed that JNK activation correlated with insulin resistance but not with the obesity-associated activation of pro-inflammatory gene expression.

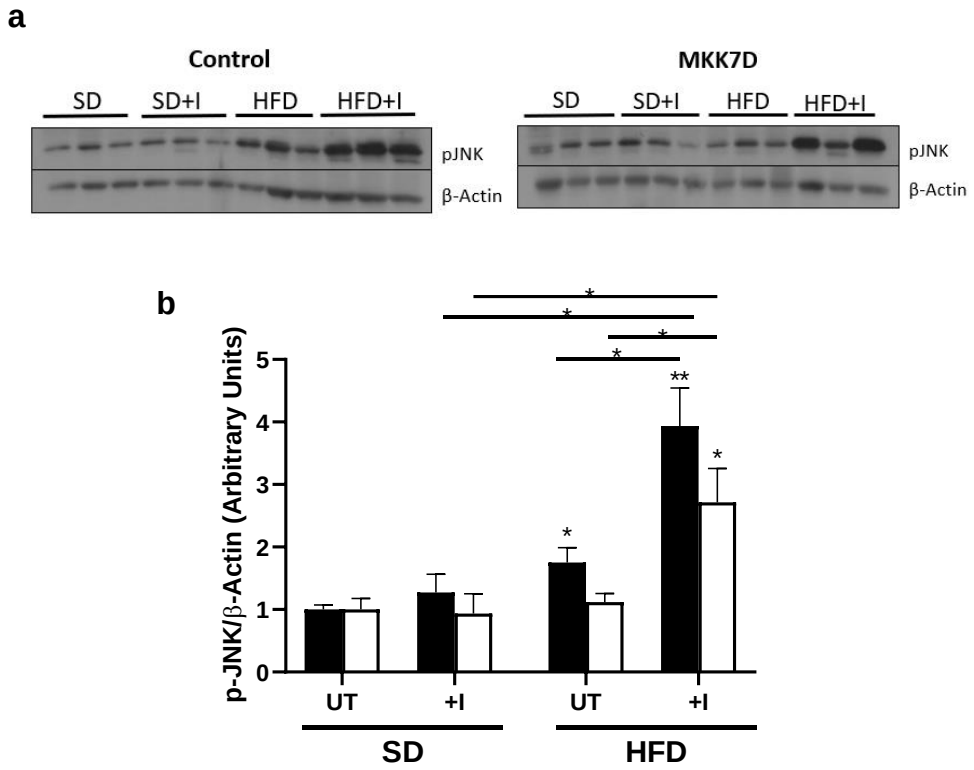


Figure 31. JNK phosphorylation/activation correlated with insulin resistance. (a) Immunoblot analysis of JNK phosphorylated on Thr183/Tyr185 (p-JNK) and β-actin, as loading control, in e-WAT protein extracts from Control and MKK7D mice subjected to the indicated diet and treatment (UT, non-treated; +I, glargine-treated), and **(b)** graphs represent the ratio of pJNK versus β-actin determined by densitometry analysis of the x-ray films obtained from immunoblots of Control (black bars) and MKK7D (empty bars) mice (n= 6 mice/group).

DISCUSSION

Impaired IR signaling in pancreatic β-cells by JNK activation and the development of diet-induced obesity.

In MKK7D mice, the MKK7D-mediated activation of JNK in pancreatic β-cells interferes with IR signaling in these cells and consequently, blocks the second phase of insulin release in response to glucose. According to our data, this defect does not prevent, in neither male nor female mice, the development of adiposity in response to a HFD though, interestingly, avoids the associated hyperinsulinemia and insulin resistance.

These observations are in agreement with those described by Flach *et al.* (2016) using a tamoxifen-inducible Map4K4-deficient mouse in which they reported an impairment in the first phase of insulin release in response to glucose, though this phenotype was only evident in HFD fed Map4K4-deficient mice. These mice were not protected from diet-induced obesity despite they did not develop neither hyperinsulinemia nor insulin resistance (Danai *et al.* 2015, Flach *et al.* 2016). Even though these mice display a similar phenotype to MKK7D mice, it should be taken in consideration that their phenotype may be the result of the diverse Map4K4 functions in different tissues in addition to pancreatic β-cells, as genetic deficiency was induced by tamoxifen in cells expressing CreERT2 recombinase under the control of ubiquitin C promoter.

On the other hand, our results are in contrast to those reported by Mehran *et al*. (2012), whose study of a transgenic mouse with a complete ablation of *ins2* (which drives insulin expression in the brain) and partial ablation of *ins1* (responsible for insulin expression in pancreatic β-cells) concluded that the prevention of HFD-induced hyperinsulinemia protects from obesity. This discordance may be due to

functional differences generated by the different genetic interventions performed on pancreatic β-cells, and further studies are required to elucidate the role of insulin in the development of adiposity. Nevertheless, the usefulness of MKK7D mice dissociating obesity from hyperinsulinemia and insulin resistance is that mice develop adiposity in response to diet, contrary to the mice from Mehran *et al.* (2012) study whose mice did not develop neither hyperinsulinemia nor obesity. Therefore, MKK7D mice are one is the few examples in which diet-induced obesity is developed at the same time as normoinsulinemia and insulin sensitivity is preserved.

Impaired IR signaling in pancreatic β-cells by JNK activation and the development of obesity-associated insulin resistance, hyperinsulinemia and hyperleptinemia.

Insulin, leptin and estrogens are the major endocrine axes deregulated in obesity. Indeed, disturbances in these hormones and their signal transduction pathways, together with the adipose chronic inflammation seem to underlie the nexus between obesity and the increased risk to develop some types of cancer such as breast, prostate and colorectal cancer (Nimptsch & Pischon 2016). Insulin and leptin play essential physiological roles in the regulation of fed and starved states and their circulating levels are increased in obesity due to the development of insulin and leptin resistance, respectively, by mechanisms that remain largely unsolved (Flier 2019).

Insulin resistance is one of the most important pathological conditions associated to obesity and a common trait with other disorders from the metabolic syndrome. In addition, insulin resistance is an early trait and a principal hallmark of T2D. Despite the strong association of T2D with obesity, how the latter drives to T2D is still unresolved. Several

investigations have focused their attention on the inflammatory kinase JNK that seems to play a central role in the pathogenesis of obesity (Hirosumi *et al.* 2002, Lee *et al.* 2003, Solinas *et al.* 2017). This protein is activated by numerous stress signals and, the metabolic consequences, as well as other JNK-mediated actions, depend on the specific context, magnitude and duration of this activation. Whole-body JNK1-deficient mice are protected from obesity-induced insulin resistance and inflammation (Hirosumi *et al.* 2002), although upon genetic dissection not all tissues with JNK1 ablation show this protective effect (Sabio *et al.* 2009). Nevertheless, the inhibition of JNK might represent a potential therapeutic target to restore insulin sensitivity and maintain β-cell function. Regarding the molecular mechanisms involved in JNK activation, numerous studies focus their investigations on different signals such as elevated inflammatory cytokines, FFA, ROS production, and blood glucose. In summary, this conserved and complex signaling pathway can be regulated by many factors and in a cell type specific manner mediates different outcomes.

Most of the studies oriented to determine the role of JNK in obesity-associated insulin resistance are based on the use of genetically deficient mouse models which show whether JNK is required for a specific function but cannot assesses about its sufficiency. Only few studies have addressed this latter aspect in metabolic relevant cell types. In this regard, adenovirus-mediated overexpression of JNK in liver decreases local and systemic insulin sensitivity and induces hyperinsulinemia and hepatic gluconeogenesis and glucose output (Nakatani *et al.* 2004). Similarly, expression of a constitutively activated form of JNK in skeletal muscle impairs IR signaling and normal glucose clearance in this tissue, though the reduced number of cells that achieved JNK activation precluded the study of the consequences of this activation to whole body glucose homeostasis (Henstridge *et al.*

2012). Finally, our initial characterization of MKK7D mice demonstrated that MKK7D-mediated activation of JNK in pancreatic β-cells induces local insulin resistance by JNK-dependent interference with the IR pathway which produces a glucose intolerant phenotype, but systemic insulin sensitivity is preserved (Lanuza-Masdeu *et al.* 2013). In this current study, we demonstrate that obese MKK7D mice maintain insulin sensitivity despite the development of obesity to the same extent than Control littermates, which in this condition are insulin resistant. Of note, obese MKK7D mice did not show JNK activation in adipose tissue in agreement with the preservation of IR pathway responsiveness to insulin in this tissue. In summary, though regulation of JNK activity represents a therapeutic strategy to ameliorate insulin resistance, isoform and/or cell type-specific drug targeting would be required due to the opposite consequences regarding systemic insulin resistance of JNK activation in specific cell types.

Blood insulin and leptin analysis confirmed that obese MKK7D mice maintained normoinsulinemia and normoleptinemia, in contrast to obese Control mice that presented increased circulating levels of both hormones. In summary, the impaired capacity of MKK7D mice to secrete insulin in response to hyperglycemia, results in a protection from obesity-associated hyperinsulinemia but also hyperleptinemia. This observation is relevant since the leptin circulating level correlates with the amount of body fat (Crujeiras *et al.* 2015), therefore, to our knowledge, obese MKK7D mice are a unique example in which adiposity and leptinemia are segregated. Normoleptinemia in obese MKK7D mice may be an indicator of the maintenance of leptin sensitivity, which may be at least partially explained by the increased expression of LepR in adipose tissue of these mice, a feature known to promote leptin paracrine action paracrine increasing energy expenditure and browning of the white adipose tissue (Wang *et al.* 2005).

The role of hyperinsulinemia in the development of obesity-associated insulin resistance.

To discern the causal and the effect in the obesity-associated insulin resistance and hyperinsulinemia is currently on intensive debate (Czech 2017). The initial idea that hyperinsulinemia is a compensatory mechanism to overcome insulin resistance is being challenged by compelling evidence pointing to the interaction in the reverse direction. For instance, patients of insulinoma or those treated with increasing dosis of insulin show insulin resistance (Shanik *et al.* 2008)

Taking in account the maintenance of normoinsulinemia in obese MKK7D mice, we wonder whether this feature might provide a protection from obesity-associated insulin resistance, giving further support to the causal role of hyperinsulinemia for insulin resistance. To confirm this hypothesis, hyperinsulinemia was induced by chronic insulin treatment, taking advantage of the long-lasting insulin glargine, along with HFD feeding. Chronic exposure to insulin abrogated the protection of MKK7D mice to obesity-associated insulin resistance. Moreover, chronic insulin administration abrogated IR pathway responsiveness to insulin in all three insulin target tissues tested, adipose tissue, liver and skeletal muscle. Moreover, Yang et al. 2014 demonstrated that chronic exposure to glargine caused insulin resistance, hyperinsulinemia and relative insulin deficiency in lean mice. This observation is in close agreement with our results showing that lean Control mice already develop insulin resistance in response to chronic in insulin administration and, with the exception of skeletal muscle, IR pathway unresponsiveness to insulin.

Altogether, these observations strongly support for hyperinsulinemia, probably promoted by frequent food consume, as the initial trait leading

to obesity-associated insulin resistance. If so, it would be worthwhile to explore the decrease in blood insulin level as a strategy to ameliorate insulin resistance. Hyperinsulinemia is not generally recognized as a primary therapeutic target although strong evidence implicates it as an important precursor to metabolic diseases associated with obesity (Pories *et al.* 2012). Indeed, it has been demonstrated that hyperinsulinemia blockade via genetic manipulation could provide lifelong protection against obesity (Templeman *et al.* 2015). Also, genetic prevention of hyperinsulinemia diminished weight gain and adiposity in leptin-deficient mice (D'Souza *et al.* 2016). In the other direction, weight loss is associated with improvement in hyperinsulinemia while weight gain is associated with worsening of hyperinsulinemia and reduced glucose effectiveness (Escalante-Herrera *et al.* 2003, Weiss *et al.* 2015). Finally, if hyperinsulinemia is causative of insulin resistance, it should be taken in consideration regarding the treatment of T2D with insulin or drugs that increase insulin secretion.

Notably, chronic insulin treatment of obese MKK7D mice significantly augmented leptin circulating level and diminished LEPR expression in adipose tissue, strongly suggesting that this treatment was inducing leptin resistance, in addition to insulin resistance, and evidencing the intense crosstalk between these two hormones and their signaling pathways. This observation indicates that hyperinsulinemia is needed to develop hyperleptinemia and that the high leptin level associated to obesity could not be due to adipose tissue overexpansion, but to leptin resistance development. On the light of these results, it would be of interest to test whether chronic leptin treatment of obese MKK7D mice would also negatively influence insulinemia and insulin sensitivity and, if so, it might be meaningful to explore decreasing obesity associated hyperinsulinemia as a strategy to alleviate insulin resistance.

The chronic low-grade inflammation in adipose tissue and the induction of obesity-associated insulin resistance.

It is established that obesity is associated with a chronic low-grade inflammatory response, known as metaflammation, in adipose tissue as a consequence of an induccion of pro-inflammatory gene expression. Some pro-inflammatory cytokines, such as TNF-α or IL-1β, are good activators of the JNK, which in turn will interfere with the IR pathway by serine phosphorylation of IRS-1/2 leading to insulin resistance. This unresolved inflammatory response seems to be triggered by processes that are induced in response to adipocyte hypertrophy such as ER stress (Gregor & Hotamisligil, 2011). In our study, both Control and MKK7D mice developed metaflammation in parallel to obesity supporting for the link between adipocyte disturbances and an exacerbated inflammatory response. However, obese MKK7D mice, despite presenting the same increase in adiposity and in expression of pro-inflammatory cytokines than Control mice, retain insulin sensitivity. Moreover, obese MKK7D mice do not show exacerbated JNK activity in adipose tissue, which is in harmony with the maintenance of insulin sensitivity but argues against a direct link between the obesity-associated inflammatory response and the activation of JNK. Notably, chronic insulin treatment induced both insulin resistance and JNK activation in adipose tissue of obese MKK7D mice, but not in lean animals. These results, together with others in the literature, suggest that the obesity-associated chronic low-grade inflammatory response and hyperinsulinemia are required to induce insulin resistance while alone by themselves are not sufficient. The cooperation of insulin with the inflammatory response is evidenced by the increased expression of pro-inflammatory markers, specially IL-6, upon chronic insulin treatment of obese MKK7D and Control mice.

Surprisingly, we found that the expression of the anti-inflammatory cytokine IL-10 in e-WAT presented a positive correlation with insulin resistance. This cytokine is secreted locally from immune cells due to inflammatory signals and characterized for its ability to inhibit macrophage activation (Moore *et al.* 2001). It has been suggested that IL-10 could be involved in T2D-related inflammation due to its hyperresponsiveness against chronic inflammation (Barry *et al.* 2016). Some studies have demonstrated that ablation of IL-10 improves insulin sensitivity, protects against diet-induced obesity and identify the IL-10 axis as a regulator of thermogenesis (Rajbhandari *et al.* 2018). IL10 expression leads to the activation/phosphorylation of signal transducer and activator of transcription 3 (STAT3) (Moore *et al.* 2001). This protein is essential for the anti-inflammatory activity of IL-10 (Lang *et al.* 2002). Obesity-associated hyperglycemia was shown to impair the ability of IL-10 to activate STAT3. Regarding our results, IL-10 could be a link between the obesity associated inflammatory response and insulin resistance. Further investigations are needed to mechanistically understand these observations.

In contrast to the increased gene expression of cytokines associated to the inflammatory response in e-WAT of obese Control and MKK7D mice, no differences were found in IL-1β, TNFα, IL-6 and IL-10 level in blood, not even in obese mice subjected to chronic insulin treatment that furthers their gene expression. Of note, in most investigations, circulating TNF-α concentrations in obese rodents and humans are not detectable. However, genetic ablation of TNF-α or its neutralization improves metabolic profile in obese rodents (Hotamisgil *et al.* 1993, Uysal *et al.* 1997). This suggests that TNF-α mainly mediates autocrine or paracrine actions, as a consequence, the effects of TNF-α activation might be spatially restricted (Xu *et al.* 2002). In addition, different studies in obese humans support a local role of TNF-α in the regulation of insulin

sensitivity in adipose tissue (Löfgren P *et al.* 2000, Kern P *et al.* 2001). Therefore, more investigations are required to identify the signaling molecules released by the overexpanded adipose tissue to induce systemic insulin resistance.

CONCLUSIONS

1. Independently of the gender, blockade of the IR signaling in pancreatic β-cells by JNK activation does not impair the development of diet-induced obesity.

2. Blockade of the IR signaling in pancreatic β-cells by JNK activation prevents the development of obesity-associated insulin resistance, hyperinsulinemia and hyperleptinemia.

3. Hyperinsulinemia has a casual role in the development of obesity-associated insulin resistance.

4. A chronic low-grade of inflammation in adipose tissue may be required but is not sufficient to induce obesity-associated insulin resistance.

5. JNK activation and IL-10 expression in adipose tissue correlates neither with the development of obesity nor with the obesity-associated chronic low-grade inflammatory response but with hyperinsulinemia and insulin resistance.

REFERENCES

References

Aguirre V, Uchida T, Yenush L, Davis R, White MF (2000) The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). J Biol Chem 275;9047-9054.

Akash MSH, Shen Q, Rehman K, Chen S (2012) Interleukin-1 receptor antagonist: a new therapy for type 2 diabetes mellitus. J Pharm Sci 101;1647-1658.

Anni MY Zhang, Jamie Magrill, Twan JJ de Winter, Xiaoke Hu, Søs Skovsø, Davis F Schaeffer, Janel L Kopp and James D Johnson (2019) Endogenous Hyperinsulinemia Contributes to Pancreatic Cancer Development. Cell Metabolism 30;403-404.

Anstee QM, Targher G, Day CP (2013) Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis. Nat Rev Gastroenterol Hepatol 10;330-344.

Bagdade JD, Bierman EL & Porte DJr (1967) The significance of basal insulin levels in the evaluation of the insulin response to glucose in diabetic and nondiabetic subjects. J Clin Invest. 46;1549–1557.

Barbuio R, Milanski M, Bertolo MB, Saad MJ, Velloso LA (2007) Infliximab reverses steatosis and improves insulin signal transduction in liver of rats fed a high-fat diet. J Endocrinol 194;539-550.

Barnes PJ, Karin M (1997) Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 336;1066-1071.

References

Barry JC, Shakibakho S, Durrer C, Simtchouk S, Jawanda KK, Cheung ST, Mui AL, Little JP (2016) Hyporesponsiveness to the anti-inflammatory action of interleukin-10 in type 2 diabetes. Sci Rep 17;6;21244.

Beaton GH, Curry DM (1956) A comparison of the effects of growth hormone and of insulin administration. Endocrinology 58;797-801.

Bellucci PN, González Bagnes MF, Di Girolamo G, González CD (2017) Potential Effects of Nonsteroidal Anti-Inflammatory Drugs in the Prevention and Treatment of Type 2 Diabetes Mellitus. J. Pharm. Pract. 30;549–556.

Biddinger SB, Hernandez-Ono A, Rask-Madsen C, Haas JT, Alemán JO, Suzuki R, Scapa EF, Agarwal C, Carey MC, Stephanopoulos G, *et al.* (2008) Hepatic Insulin Resistance is Sufficient to Produce Dyslipidemia and Susceptibility to Atherosclerosis. Cell Metab 7;125–134.

Boden G, Duan X, Homko G, *et al.* (2008) Increase in endoplasmic reticulum stress-related proteins and genes in adipose tissue of obese, insulin-resistant individuals. Diabetes 57;2438-2444.

Borst MD, Prakash J, Melenhorst W, van den Heuvel M, Kok RJ, Navis G, van Goor, H (2007) Glomerular and tubular induction of the transcription factor c-Jun in human renal disease. J. Pathol. 213;219–228.

Borst SE, Bagby GJ (2002) Neutralization of tumor necrosis factor reverses age-induced impairment of insulin responsiveness in skeletal muscle of Sprague-Dawley rats. Metabolism 51;1061-1064.

References

Brittanie J. Atkinson et *al.* (2013) Moderate GLUT4 Overexpression Improves Insulin Sensitivity and Fasting Triglyceridemia in High-Fat Diet-Fed Transgenic Mice. Diabetes 62;2249-2258.

Brüning JC et al. (2000) Role of brain insulin receptor in control of body weight and reproduction. Science 289;2122-2125.

Calo E, Quintero-Estades JA, Danielian PS, Nedelcu S, Berman SD, Lees JA (2010) Rb regulates fate choice and lineage commitment *in vivo*. Nature 466;1110–1114.

Carvalheira JB et *al.* (2001) Insulin modulates leptin-induced STAT3 activation in rat hypothalamus. FEBS Lett. 500;119–124.

Catalano KJ, Maddux BA, Szary J, Youngreen JF, Goldfine ID, et al. (2014) Insulin Resistance Induced by Hyperinsulinemia Coincides with a Persistent Alteration at the Insulin Receptor Tyrosine Kinase Domain. PloS ONE 31;273-281.

Cerf ME (2013) Beta Cell Dysfunction and Insulin Resistance. Front Endocrinol 4;37.

Chen G, Goeddel DV (2002) TNF-R1 signaling: a beautiful pathway. Science 296;1634-1635.

Cobrinik D. (2005) Pocket proteins and cell cycle control. Oncogene 24;2796–2809.

Crujeiras AB, Carreira MC, Cabia B, Andrade S, Amil M, Casanueva FF (2015) Leptin resistance in obesity: An epigenetic landscape. Life Sci 1;140;57-63.

References

Cui J, Zhang M, Zhang Y, Xu Z (2007) JNK pathway: Diseases and therapeutic potential. Acta Pharmacol. Sin. 28;601–608.

Dali-Youcef N, Mataki C, Coste A, et *al.* (2007) Adipose tissue-specific inactivation of the retinoblastoma protein protects against diabesity because of increased energy expenditure. Proc Natl Acad Sci USA 104;10703–10708.

Danforth E Jr (2000) Failure of adipocyte differentiation causes type II diabetes mellitus? Nat Genet 26;13.

Das M, Garlick DS, Greiner DL, Davis RJ (2011) The role of JNK in the development of hepatocellular carcinoma. Genes & Development 25;634-645.

DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP (1981) The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. Diabetes 30;1000–1007.

Després JP, and Lemieux I. (2006) Abdominal obesity and metabolic syndrome. Nature 444;881-887.

Díaz-Delfin J, Morales M, Caelles C (2007) Hypoglycemic action of thiazolidinediones/peroxisome proliferator-activated receptor gamma by inhibition of the c-Jun NH2-terminal kinase pathway. Diabetes 56;1865-1871.

Donath et *al*. (2013) Inflammation in obesity and diabetes: Islet dysfunction and therapeutic opportunity. Cell Metabol 17;860-872.

D'Souza AM, Johnson JD, Clee SM, Kieffer TJ (2016) Suppressing hyperinsulinemia prevents obesity but causes rapid onset of diabetes in leptin-deficient Lepob/ob mice. Mol Metab. 5;1103–1112.

Eldor R, Yeffet A, Baum K, Doviner V, Amar D, *et al.* (2006) Conditional and specific NF-κB blockade protects pancreatic βcells from diabetogenic agents. Proc Natl Acad Sci USA 103;5072–5077

Engin A (2017) The Pathogenesis of Obesity-Associated Adipose Tissue Inflammation. In Obesity and Lipotoxicity. Advances in Experimental Medicine and Biology; Springer International Publishing: Cham, Switzerland, pp. 221–245.

Escalante-Pulido M, Escalante-Herrera A, Milke-Najar ME, Alpizar-Salazar M (2003) Effects of weight loss on insulin secretion and in vivo insulin sensitivity in obese diabetic and non-diabetic subjects. Diabetes Nutr Metab. 16;277–283.

Esser N, Paquot N, Scheen AJ (2015) Anti-inflammatory agents to treat or prevent type 2 diabetes, metabolic syndrome and cardiovascular disease. Expert Opin. Investig. Drugs 24;283–307.

Evans JL, Goldfine ID, Maddux A, Grodsky GM (2002) Oxidative stress and stress-activated signaling pathways: A unifying hypothesis of type 2 diabetes. Endoc Rev 23;599–622.

Ezzat et *al.* (2013) The Cancer-Associated FGFR4-G388R Polymorphism Enhances Pancreatic Insulin Secretion and Modifies the Risk of Diabetes. Cell Metabol. 17;929-940.

Falsig J, Pörzgen P, Lotharius J, Leist, M (2004) Specific Modulation of Astrocyte Inflammation by Inhibition of Mixed Lineage Kinases with CEP-1347. J. Immunol. 173;2762–2770.

References

Flier J. S. (2004) Obesity wars: molecular progress confronts an expanding epidemic. Cell 116;337-350.

Frederick A. Dick and Nick Dyson (2003) pRB contains an E2F1-Specific Binding Domain that Allows E2F1-Induced Apoptosis to Be Regulated Separately from Other E2F Activities. Molecular Cell 12;639-649.

Frederich RC, Lollmann B, Hamann A, Napolitano-Rosen A, Kahn BB, Lowell BB & Flier JS (1995) Expression of ob mRNA and its encoded protein in rodents. Impact of nutrition and obesity. Journal of Clinical Investigation 96;1658–1663.

Frolov MV, Dyson NJ (2004) Molecular mechanism of E2F-dependent activation and pRb-mediated repression. J Cell Sci 117;2173-2181.

Furnica RM, Istasse L, Maiter D (2017) A severe but reversible reduction in insulin sensitivity is observed in patients with insulinoma. Ann Endocrinol (Paris) 79;30-36.

Gao YJ, Ji RR (2008) Activation of JNK pathway in persistent pain. Neurosci. Lett. 437;180–183.

Gregor & Hotamisligil (2011) Inflammatory mechanisms in obesity. Annu Rev Immunol 29;415-455.

Gregor MF, Yang L, Fabbrini E, *et al.* (2009) Endoplasmic reticulum stress is reduced in tissues of obese subjects after weight loss. Diabetes 58;693-700.

Grynberg, K, Ma FY, Nikolic-Paterson DJ (2017) The JNK Signaling Pathway in Renal Fibrosis. Front. Physiol. 8;829.

Gonzalez-Gay MA, De Matias JM, Gonzalez-Juanatey C, Garcia-Porrua C, Sanchez-Andrade A, *et al.* (2006) Anti-tumor necrosis factor-α blockade improves insulin resistance in patients with rheumatoid arthritis. Clin Exp Rheumatol 24;83–86.

Guilherme A, Henriques F, Bedard AH, Czech MP (2019) Molecular pathways linking adipose innervation to insulin action in obesity and diabetes mellitus. Nat Rev Endocrinol 15;207–225.

Guo W, Wong S, Xie W, Lei T, Luo Z (2007) Palmitate modulates intracellular signaling, induces endoplasmic reticulum stress, and causes apoptosis in mouse 3T3-L1 and rat primary preadipocytes. Am J Physiol Endocrinol Metab 293;576–586.

Gupta A, Dey CS (2012) PTEN, a widely known negative regulator of insulin/PI3K signaling, positively regulates neuronal insulin resistance. Mol Biol Cell 23;3882-3898.

Haigis KM, Sweet-Cordero (2011) New insights into oncogenic stress. Nat Genet 43;177–178.

Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK & Friedman JM (1995) Weight-reducing effects of the plasma protein encoded by the obese gene. Science 269;543–546.

Han MS, Barret T, Brehm MA and Davis RJ (2016) Inflammation Mediated by JNK in Myeloid Cells Promotes the Development of Hepatitis and Hepatocellular Carcinoma. Cell Rep 15;19-26.

References

Hansen JB, Jørgensen C, Petersen RK, et *al.* (2004) Retinoblastoma protein functions as a molecular switch determining white versus brown adipocyte differentiation. Proc Natl Acad Sci USA 101;4112–4117.

Hansen PA, Han DH, Marshall BA, et *al.* (1998) A high fat diet impairs stimulation of glucose transport in muscle. Functional evaluation of potential mechanisms. J Biol Chem 273;26157–26163.

Hebbard L, George J (2011) Animal models of nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol 8;35-44.

Hein TW, Xu W, Xu X, Kuo L (2016) Acute and Chronic Hyperglycemia Elicit JIP1/JNK-Mediated Endothelial Vasodilator Dysfunction of Retinal Arterioles. Investig. Ophthalmol. Vis. Sci. 57;4333–4340.

Henstridge DC, Bruce CR, Pang CP, Lancaster GI, Allen TL, Estevez E, Gardner T, Weir JM, Meikle PJ, Lam KSL, Xu A, Fujii N, Goodyear LJ, Febbraio MA (2012) Skeletal muscle-specific overproduction of constitutively activated c-Jun N-terminal kinase (JNK) induces insulin resistance in mice. Diabetologia 55;2769-2778.

Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS (2002) A central role for JNK in obesity and insulin resistance. Nature 420;333–336.

Hoerger et *al.* (2008) Is glycemic control improving in US adults? Diabetes Care 31;81-86.

Hotamisligil GS, Shargill NS, Spiegelman BM (1993) Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259;87-91.

Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM (1995) Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J Clin Invest 95;2409-2415.

Hotamisligil GS (1999) Mechanisms of TNF-alpha-induced insulin resistance. Exp Clin Endocrinol Diabetes 107;119-125.

Hotamisligil GS (2010) Endoplasmic reticulum stress and the inflammatory basis of metabolic diseases. Cell 140;900-17.

Houstis et *al*. (2006) Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature 440;944-948.

Hsu (2009) Consequences of delaying progression to optimal therapy in patients with type 2 diabetes not achieving glycemic goals. South Med J 102;67-76.

Hu E, Liang P and Spiegelman BM (1996) AdipoQ is a novel adipose-specific gene dysregulated in obesity. J Biol Chem 271;10697-10703.

Hui L, Zatloukal K, Scheuch H, Stepniak E, Wagner EF (2008) Proliferation of human HCC cells and chemically induced mouse liver cancers requires JNK1-dependent p21 downregulation. J Clin Invest 118;3943–3953.

Ikemoto S, Thompson KS, Itakura H, Lane MD, Ezaki O (1995) Expression of an insulin-responsive glucose transporter (GLUT4) minigene in transgenic mice: effect of exercise and role in glucose homeostasis. Proc Natl Acad Sci USA 92;865–869.

References

Isken F, Weickert MO, Tschop MH, Nogueiras R, Mohlig M, Abdelrahman A, *et al.* (2009) Metabolic effects of diets differing in glycaemic index depend on age and endogenous glucose-dependent insulinotrophic polypeptide in mice. Diabetologia 52;2159-2168.

Ivy JL, Kuo CH (1998) Regulation of GLUT4 protein and glycogen synthase during muscle glycogen synthesis after exercise. Acta Physiol Scand 162;295–304.

Jeffrey S Flier (2019) Starvation in the Midst of Plenty: Reflections on the History and Biology of Insulin and Leptin. Endrocr Rev 1;40;1-16.

Jiao P, Ma J, Feng B, Zhang H, Alan-Diehl J, Eugene-Chin Y, Yan W, Xu H (2011) FFA-Induced Adipocyte Inflammation and Insulin Resistance: Involvement of ER Stress and IKKβ Pathways. Obesity 19;483–491.

Kahn BB (1994) Dietary regulation of glucose transporter gene expression: tissue specific effects in adipose cells and muscle. J Nutr 124(Suppl.):1289S–1295S.

Karin M, Gallagher E (2005) From JNK to Pay Dirt: Jun Kinases, their Biochemistry, Physiology and Clinical Importance. IUBMB Life 57;283–295.

Kern P, Ranganathan G (2001) Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. Am J Physiol Endocrin Metabol 280;745-751.

Kim JY, van de Wall E, Laplante M, et *al.* (2007) Obesity-associated improvements in metabolic profile through expansion of adipose tissue. J Clin Invest 117;2621–2637.

Kitade H, Chen G, Ni Y, Ota T (2017) Nonalcoholic Fatty Liver Disease and Insulin Resistance: New Insights and Potential New Treatments. Nutrients 9;387.

Kusminski CM, Bickel PE, Scherer PE (2016) Targeting adipose tissue in the treatment of obesity-associated diabetes. Nat Rev Drug Discov 15;639-660.

Lang R, Patel D, Morris JJ, Rutschman RL, Murray PJ (2002) Shaping gene expresión in activated and resting primary macrophages by IL-10. J Immunol.; 169;2253-2263.

Lanuza-Masdeu et *al.* (2013) *In vivo* JNK activation in pancreatic β-cells leads to glucose intolerance due to insulin resistance in pancreas. Diabetes 62;2308-2317.

Larsen CM, Faulenbach M, Vaag A, Volund A, Ehses JA, *et al.* (2007) Interleukin-1-receptorantagonist in type 2 diabetes mellitus. N Engl J Med 356;1517–1526.

Lee YR, Yu HN, Noh EM, Youn HJ, Song EK, Han MK, Park CS, Kim BS, Park YS, Park BK, Lee SH, Kim JS (2007) TNF-alpha upregulates PTEN via NF-kappaB signaling pathways in human leukemic cells. Exp Mol Med 39;121-127.

Lim AKH, Tesch GH (2012) Inflammation in Diabetic Nephropathy. Mediators Inflamm. 2012;1–12.

Lipinski MM, Jacks J. (1999) The retinoblastoma gene family in differentiation and development. Oncogen 18;873–883.

References

Löfgren P, van Harmelen V, Reynisdottir S, Näslund E, Rydén M, Rössner S, Arner P (2000) Secretion of tumor necrosis factor-α shows a strong relationship to insulin-stimulated glucose transport in human adipose tissue. Diabetes 49;688-692.

Lo J, Bernstein LE, Canavan B, Torriani M, Jackson MB, Ahima RS, Grinspoon SK (2007) Effects on TNF-alpha neutralization on adipocytokines and skeletal muscle adiposity in the metabolic síndrome. Am J Physiol Endocrinol Metab 293;102-109.

Lopez-Bergami P, Ronai Z (2008) Requirements for PKC-augmented JNK activation by MKK4/7. Int J Biochem Cell Biol 40;1055–1064.

Lu Z, Marcelin G, Bauzon F, Wang H, Fu H, Dun SL, *et al.* (2013) pRb is an obesity suppressor in hypothalamus and high-fat diet inhibits pRb in this location. EMBO J 32;844-57.

Marban SL, Roth J, editors (1996) Transgenic hyperinsulinemia: a mouse model of insulin resistance and glucose intolerance without obesity. Boston: Birkhauser 201-224p.

Mehran et *al.* (2012) Hyperinsulinemia drives diet-induced obesity independently of brain insulin production. Cell Metabol 16;723-37.

Menzaghi C, Ercolino T, Di Paola R, *et al.* (2002) A haplotype at the adiponectin locus is associated with obesity and other features of the insulin resistance syndrome. Diabetes 51;2306-2312.

Mercader J, Ribot J, Murano I, et *al.* (2009) Haploinsufficiency of the retinoblastoma protein gene reduces diet-induced obesity, insulin resistance, and hepatosteatosis in mice. Am J Physiol Endocrinol Metab 297;184–193.

Messina et *al.* (2015) Exercise Causes Muscle GLUT4 Translocation in an Insulin-Independent Manner, *Biol Med* (Aligarh), 1;2.

Michael P Czech (2017) Insulin action and resistance in obesity and type 2 diabetes. Nat Med 11;23;804-814.

Moore KW, de Waal Malefyt R, Coffman RL & O'Garra A (2001) Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol. 19; 683-765.

Moreno-Navarrete JM, Petrov P, Serrano M, Ortega F, García-Ruiz E, Oliver P, et *al.* (2013) Decreased RB1 mRNA, protein, and activity reflect obesity-induced altered adipogenic capacity in human adipose tissue. Diabetes 62;1923–1931.

Morigny P, Houssier M, Mouisel E, Langin D (2016) Adipocyte lipolysis and insulin resistance. Biochimie 125;259–266.

Nakatani Y, Kaneto H, Kawamori D, Hatazaki M, Miyatsuka T, Matsuoka TA, Kajimoto Y, Matsuhisa M, Yamasaki Y, Hori M (2004) Modulation of the JNK pathway in liver affects insulin resistance status. J Biol Chem 279;45803–45809.

Nathan et *al.* (2009) Medical management of hyperglycemia in type 2 diabetes: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes. Diabetes Care 32;193-203.

Nieto-Vazquez I, Fernandez-Veledo S, Kramer DK, Vila-Bedmar R, Garcia-Guerra L, Lorenzo M (2008) Insulin resistance associated to obesity: the link TNF-alpha. Arch Physiol Biochem 114;183-194.

References

Ning J, Hong T, Yang X, Mei S, Liu Z, Liu HY, *et al.* (2011) Insulin and insulin signaling play a critical role in fat induction of insulin resistance in mouse. Am J Physiol Endocrinol Metab. 301;391-401.

Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, *et al.* (2010) GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell 142;687–698

Oh YS, Bae GD, Baek DJ, Park E-Y, Jun H-S (2018) Fatty Acid-Induced Lipotoxicity in Pancreatic Beta-Cells During Development of Type 2 Diabetes. Front Endocrinol 9;384.

Ortega-Molina A et al. (2015) Pharmacological Inhibition of PI3K Reduces Adiposity and Metabolic Syndrome in Obese Mice and Rhesus Monkeys. Cell Metabolism 21;558-570.

Özcan U, Cao Q, Yilmaz E, Lee A-H, Iwakoshi NN, Özdelen E, Tuncman G, Görgün C, Glimcher LH, Hotamisligil GS (2004) Endoplasmic Reticulum Stress Links Obesity, Insulin Action, and Type 2 Diabetes. Science 306;457–461.

Pal A, Barber TM, Van de Bunt M, Rudge SA, Zhang Q, Lachlan KL, Cooper NS, Linden H, Levy JC, Wakelam MJ, Walker L, Karpe F, Gloyn AL (2012) PTEN mutations as a cause of constitutive insulin sensitivity and obesity. N Engl J Med 367;1002-1011.

Pickard A, Cichon AC, Barry A, et al. (2012) Inactivation of Rb in stromal fibroblasts promotes epithelial cell invasion. EMBO J 31;3092-3103.

Pischon T, Nimptsch K (2016) Obesity and Risk of Cancer: An Introductory Overview. Recent Results Cancer Res 208;1-15.

Pories WJ, Dohm GL. Diabetes: have we got it all wrong? Hyperinsulinism as the culprit: surgery provides the evidence (2012) Diabetes Care 35;2438–2442

Puigserver P, Nadal-Ginard B, Palou A (1994) Expression and interaction of C/EBPalpha adipogenic transcription factor and retinoblastoma protein in adipocytes during differentiation. Int J Obes Relat Metab Disord 18;113.

Prashant Rajbhandari et *al.* (2018) IL-10 Signaling Remodels Adipose Chromatin Architecture to Limit Thermogenesis and Energy Expenditure. *Cell.* Author manuscript;172;218-233.

Rachel J. Roth Flach et *al.* (2016) Protein Kinase Mitogen-activated Protein Kinase Kinase Kinase Kinase 4 (MAP4K4) Promotes Obesity-induced Hyperinsulinemia. The journal of biological chemistry 291;16221-16230.

Razani B, Chakravarthy MV, Semenkovich CF (2008) Insulin Resistance and Atherosclerosis. Endocrinol. Metab. Clin. N. Am. 37;603–621.

Rosenvinge A, Krogh-Madsen R, Baslund B, Pedersen BK (2007) Insulin resistance in patients with rheumatoid arthritis: effect of anti-TNFalpha therapy. Scand J Rheumatol 36;91-96.

Roth Flach RJ, Danai LV, DiStefano MT, Kelly M, Menendez LG, Jurczyk A, Sharma RB, Jung DY, Kim JH, Kim JK, Bortell R, Alonso LC, Czech MP (2016) Protein Kinase Mitogen-activated Protein Kinase Kinase Kinase Kinase 4 (MAP4K4) Promotes Obesity-induced Hyperinsulinemia. J Biol Chem. 29;291;16221-16230.

References

Ruan H, Pownall HJ and Lodish HF (2003) Troglitazone antagonizes tumor necrosis factor-alpha-induced reprogramming of adipocyte gene expression by inhibiting the transcriptional regulatory functions of NF-kappaB. J Biol Chem 278;28181-28192.

Sabio G, Das M, Mora A, Zhang Z, Jun JY, Ko HJ, Barrett T, Kim JK, Davis RJ (2008) A stress signaling pathway in adipose tissue regulates hepatic insulin resistance. Science 322;1539–1543.

Sabio G, Cavanagh-Kyros J, Ko HJ, Jung DY, Gray S, Jun JY, Barret T, Mora A, Kim JK, Davis RJ (2009) Prevention of Steatosis by Hepatic JNK1. Cell Metab 10;491-498.

Sakurai T, Maeda S, Chang L, Karin M (2006) Loss of hepatic NF-kappa B activity enhances chemical hepatocarcinogenesis through sustained c-Jun N-terminal kinase 1 activation. Proc Natl Acad Sci U S A 103;10544–10551.

Samar I. Itani, Neil B. Ruderman, Frank Schmieder, and Guenther Boden (2002) Lipid-Induced Insulin Resistance in Human Muscle Is Associated With Changes in Diacylglycerol, Protein Kinase C, and IκB-α. Diabetes 51;2005-2011.

Samuel VT and Shulman GI (2012) Mechanism for insulin resistance: common threads and missing links. Cell 148;852-871.

Scheen (2001) Obesity and diabetes. In the management of obesity and related disorders. Kopelman PG, ed. London, M. Dunitz, p 11-44.

Schwartz MW, Peskind E, Raskind M, Boyko EJ & Porte DJr (1996) Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans. Nat Med. 2;589–93.

Scimè A, Grenier G, Huh MS, et *al.* (2005) Rb and p107 regulate preadipocyte differentiation into white versus brown fat through repression of PGC-1alpha. Cell Metab 2;283–295.

Scribner KB, Pawlak DB, Aubin CM, Majzoub JA, Ludwig DS (2008) Long-term effects of dietary glycemic index on adiposity, energy metabolism, and physical activity in mice. Am J Physiol Endocrinol Metab. 295;1126-1131.

Shanik MH, Xu Y, Skrha J, Dankner R, Zick Y, et al. (2008) Insulin resistance and hyperinsulinemia: is hyperinsulinemia the cart or the horse? Diabetes Care 31 Suppl 2;262-268.

Sharma NK, Das SK, Mondal AK, et *al.* (2008) Endoplasmic reticulum stress markers are associated with obesity in nondiabetic subjects. J Clin Endocrinol Metab 93;4532-4541.

Sheng J, Li H, Dai Q, Lu C, Xu M, Zhang J, Feng J (2019) DUSP1 recuses diabetic nephropathy via repressing JNK-Mff-mitochondrial fission pathways. J. Cell. Physiol. 234;3043–3057.

Shirabe K, Mano Y, Muto J, Matono R, Motomura T, Toshima T, Takeishi K, Uchiyama H, Yoshizumi T, Taketomi A, *et al.* (2012) Role of tumor-associated macrophages in the progression of hepatocellular carcinoma. Surg Today 42;1–7.

Shi Y, Nikulenkov F, Zawacka-Pankau J, Li H, Gabdoulline R, Xu J, Eriksson S, Hedstroöm E, Issaeva N, Kel A, Arnér ESJ and Selivanova G (2014) ROS-dependent activation of JNK converts p53 into an efficient inhibitor of oncogenes leading to robust apoptosis. Cell Death and Differentiation 21;612-623.

References

Solinas et *al.* (2006) Saturated free fatty acids inhibit induction of insulin gene transcription by JNK mediated phosphorylation of insulin-receptor substrates Proc Natl Acad Sci USA 103;16454-16459.

Solinas & Karin (2010) JNK1 and IKKbeta: molecular links between obesity and metabolic dysfunction. FASEB J 24;2596-2611.

Solinas G, Becattini B (2017) JNK at the crossroad of obesity, insulin resistance, and cell stress response. Molecular metabolism 6;174-184.

Solomon SS, Odunusi O, Carrigan D, Majumdar G, Kakoola D, Lenchik NI, Gerling IC (2010) TNF-alpha inhibits insulin signaling in liver and adipose tissue: a model of metabolic syndrome. Horm Metab Res 42;115-121.

Song MS, Salmena L, Pandolfi PP (2012) The functions and regulation of the PTEN tumour supressor. Nat Rev Mol Cell Biol 13;283-296.

Spiegelman BM (1998) PPAR-gamma: adipogenic regulator and thiazolidinedione receptor. Diabetes 47;507-514.

Stanley TL, Zanni MV, Johnsen S, Rasheed S, Makimura H, *et al.* (2011) TNF-α antagonism with Etanercept decreases glucose and increases the proportion of high molecular weight adiponectin in obese subjects with features of the metabolic syndrome. J Clin Endocrinol Metab 96;146–150.

Straczkowski M, Kowalska I, Nikolajuk A, Krukowska A, Gorska M (2005) Plasma interleukin-10 concentration is positively related to insulin sensitivity in young healthy individuals. *Diabetes Care* 28;2036-2037.

Ström K, Hansson O, Lucas S, et *al.* (2008) Attainment of brown adipocyte features in white adipocytes of hormone-sensitive lipase null mice. PLoS One 3;1793.

Sun B, Karin M (2013) Inflammation and liver tumorigenesis. Front Med 7;242–254.

Sung HY, Hong CG, Suh YS, *et al.* (2010) Role of (-)-epigallocatechin-3-gallate in cell viability, lipogenesis, and retinol-binding protein 4 expression in adipocytes. Naunyn Schmiedebergs Arch Pharmacol 382;303-310.

Swaroop JJ, Rajarajeswari D, Naidu JN (2012) Association of TNF-alpha with insulin resistance in type 2 diabetes mellitus. Indian J Med Res 135;127-130.

Tan SX, Fisher-Wellman KH, Fazakerley DJ, Ng Y, Pant H, Li J, Meoli CC, Coster ACF, Stöckli J, James DE (2015) Selective Insulin Resistance in Adipocytes. J Biol Chem 290;11337–11348.

Templeman NM, Clee SM, Johnson JD (2015) Suppression of hyperinsulinaemia in growing female mice provides long-term protection against obesity. Diabetologia 58;2392–2402.

Thallas-Bonke V, Thorpe SR, Coughlan MT, Fukami K., Yap FYT, Sourris KC, Penfold SA, Bach LA, Cooper ME, Forbes JM (2008) Inhibition of NADPH Oxidase Prevents Advanced Glycation End Product–Mediated Damage in Diabetic Nephropathy Through a Protein Kinase C-α–Dependent Pathway. Diabetes 57;460–469.

Thomas DM, Yang HS, Alexander K, Hinds PW (2003) Role of the retinoblastoma protein in differentiation and senescence. Cancer Biol Ther 2;124–130.

References

Todoric J, Loffler M, Huber J, Bilban M, Reimers M, *et al.* (2006) Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n-3 polyunsaturated fatty acids. Diabetologia 49;2109–2119.

Tournier C, Dong C, Turner TK, Jones SN, Flavell RA, Davis RJ (2001) MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. Genes Dev 15;1419–1426.

Trachootham D, Alexandre J, Huang P (2009) Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov 8;579–591.

Tricò D, Natali A, Arslanian S, Mari A, Ferrannini E (2018) Identification, pathophysiology, and clinical implications of primary insulin hypersecretion in nondiabetic adults and adolescents. JCI Insight. 3;124912.

Tuncman G, Hirosumi J, Solinas G, Chang L, Karin M, Hotamisligil G.S. (2006) Functional *in vivo* interactions between JNK1 and JNK2 isoforms in obesity and insulin resistance. Proceedings of the National Academy of Sciences of the United States of America 103;10741–10746.

Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS (1997) Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature 9;389;610-614.

Vaisse C, Halaas JL, Horvath CM, Darnell JE Jr, Stoffel M & Friedman JM (1996) Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. Nature Genetics 14;95–97.

van Exel E, Gussekloo J, de Craen AJ, FrÖlich M, Bootsma-Van Der Wiel A, Westendorp RG, the Leiden 85 Plus Study (2002) Low production capacity of interleukin-10 associates with the metabolic syndrome and type 2 diabetes: the Leiden 85-Plus Study. *Diabetes* 51;1088-1092.

Vázquez-Jiménez JG et al. (2017) Role of free fatty acids on insulin resistance. Gac Med Mex 153;773-783.

Ventre J, Doebber T, Wu M, MacNaul K, Stevens K, Pasparakis M, Kollias G, Moller DE (1997) Targeted disruption of the tumor necrosis factor-alpha gene: metabolic consequences in obese and nonobese mice. Diabetes 46;1526-1531.

Vernia S, Cavanagh-Kyros J, Garcia-Haro, Sabio G, Barret T, Jung DY, Kim JK, Xu J, Shulha HP, Garber M, *et al.* (2014) The PPARα-FGF21 hormone axis contributes to metabolic regulation by the hepatic JNK signaling pathway. Cell Metab 20;512-525.

Vernia S, Morel C, Madara JC, Cavanagh-Kyros J, Barrett T, Chase K, *et al.* (2016) Excitatory transmission onto AgRP neurons is regulated by c-Jun NH2-terminal kinase 3 in response to metabolic stress. Elife 5;10031.

Viatour P, Sage J (2011) Newly identified aspects of tumor suppression by RB. Dis Model Mech 4;581–585.

Wang J, Obici, S, Morgan K, Barzilai N, Feng Z, Rossetti L (2001) Overfeeding Rapidly Induces Leptin and Insulin Resistance. Diabetes 50;2786–2791.

References

Wang M-Y, Orci L, Ravazzola M, and Unger RH (2005) Fat storage in adipocytes requires inactivation of leptin's paracrine activity: Implications for treatment of human obesity. PNAS 102;18011-18016.

Weiss R, Magge SN, Santoro N, Giannini C, Boston R, Holder T, Shaw M, Duran E, Hershkop KJ, Caprio S (2015) Glucose effectiveness in obese children: relation to degree of obesity and dysglycemia. Diabetes Care 38;689–695.

Woods SC, Lotter EC, McKay LD & Porte DJr (1979) Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. Nature 282;503-505.

Xu H, Uysal KT, Becherer JD, Arner P, Hotamisligil GS (2002) Altered tumor necrosis factor-alpha (TNF-alpha) processing in adipocytes and increased expression of transmembrane TNF-alpha in obesity. Diabetes 51;1876-1883.

Xuefeng Yang *et al.* (2014) Exposure to excess insulin (glargine) induces type 2 diabetes mellitus in mice fed on a chow diet. Journal of Endocrinology 221;469-480.

Yang R, Wilcox DM, Haasch DL, Jung PM, Nguyen PT, Voorbach MJ, Doktor S, Brodjian S, Bush EN, Lin E, *et al.* (2007) Liver-specific knockdown of JNK1 up-regulates proliferator-activated receptor gamma coactivator 1 beta and increases plasma triglyceride despite reduced glucose and insulin levels in diet-induced obese mice. J Biol Chem 282;22765–22774.

Yung JHM and Giacca A (2020) Role of c-Jun N-terminal Kinase (JNK) in Obesity and Type 2 Diabetes. Cells 9;706.

Zhang W *et al.* (2006) FoxO1 regulates multiple metabolic pathways in the liver: effects on gluconeogenic, glycolytic, and lipogenic gene expression. *J. Biol. Chem.* 281;10105-10117.

Zhou J, Deo BK, Hosoya K, Terasaki T, Obrosova IG, Brosius FC, Kumagai AK (2005) Increased JNK Phosphorylation and Oxidative Stress in Response to Increased Glucose Flux through Increased GLUT1 Expression in Rat Retinal Endothelial Cells. Investig. Ophthalmol. Vis. Sci. 46;3403–3410.

ANNEX

Some experiments described in results were also performed in an independent MKK7D transgenic mice line and results are shown in this section. In summary, recorded data recapitulated those described in the Results section giving further support to the conclusions of this study.

MKK7D and Control mice were subjected to a similar protocol to induce obesity as described for the development of objective 2. Previously to start with the specific diet, at 4 weeks of age Control and MKK7D mice were subjected to a GTT to assess the glucose intolerant phenotype of MKK7D mice (Fig. 33), and then, placed on SD or HFD for 12 weeks. HFD induced obesity regardless genotype and chronic insulin treatment (not shown).

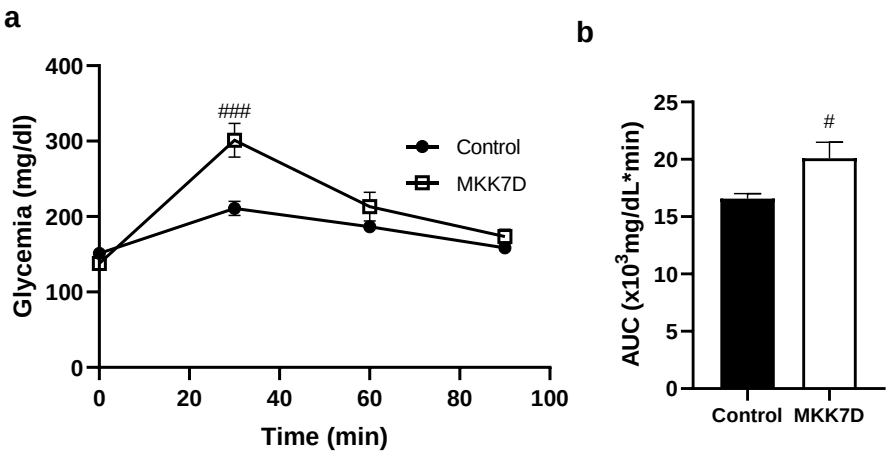


Figure 33. GTT analysis at 4-weeks of age of Control and MKK7D mice. (a) GTT curves, and **(b)** representation of the 90-min area under the curve (AUC) of the GTT analysis shown in **(a)** (n= 16 mice/group).

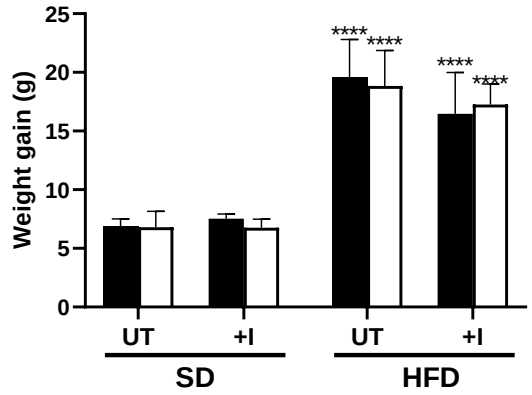


Figure 34. HFD promoted obesity in Control and MKK7D mice independently of genotype and chronic insulin treatment. Weight gain of the indicated Control (black bars) and MKK7D (empty bars) experimental groups (SD standard diet, HFD high fat diet, UT, non-treated and +INS, glargine-treated) (n= 8).

On week 12 on the specific diet, all mice were subjected to different test to assess glucose tolerance and sensitivity to insulin. Results of AUC of GTT, AUC of ITT and insulinemia analyzes are shown in figures 35, 36 and 37, respectively.

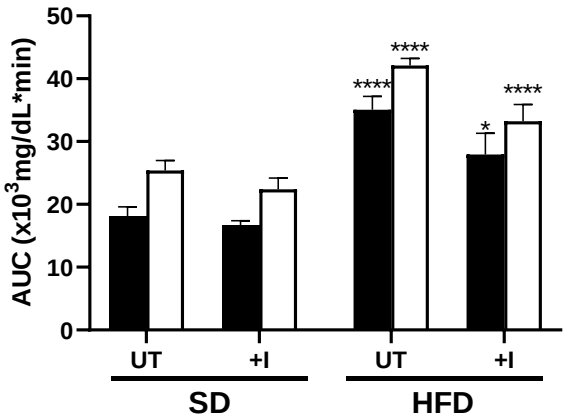


Figure 35. No effect of chronic insulin treatment on glucose tolerance in any analyzed condition. AUC representation of GTT (data not shown) of the indicated

Control (black bars) and MKK7D (empty bars) mice and conditions (UT, non-treated; +I, glargine-treated) (n= 8 mice/group).

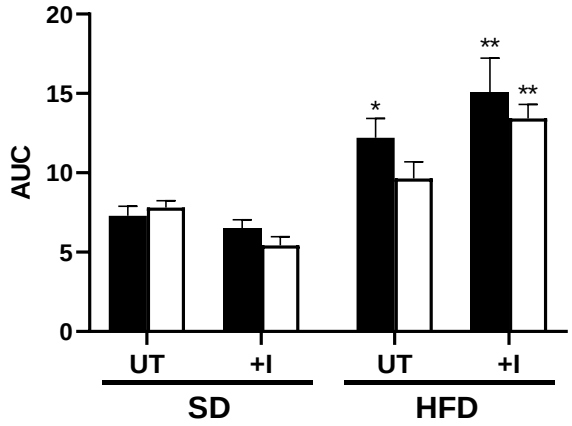


Figure 36. Chronic insulin treatment promotes insulin resistance in obese MKK7D mice. AUC representation from ITT (data not shown) of the indicated Control (black bars) and MKK7D (empty bars) mice and conditions (UT, non-treated; +I, glargine-treated) (n= 8 mice/group).

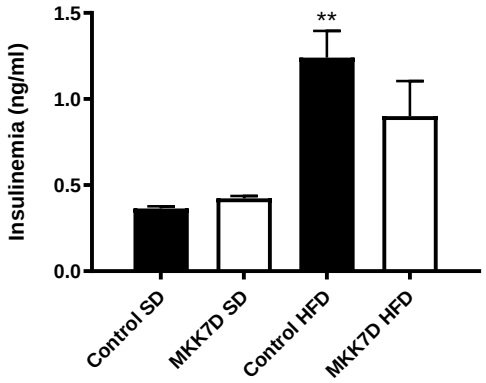


Figure 37. Obese MKK7D mice did not develop hyperinsulinemia. Plasma insulin level analysis of Control (black bars) and MKK7D (empty bars) mouse groups (n= 3 mice/group).

Annex

Activation of the IR signaling pathway was also analyzed in eWAT using AKT phosphorylation as a biomarker, and results are shown in figure 38.

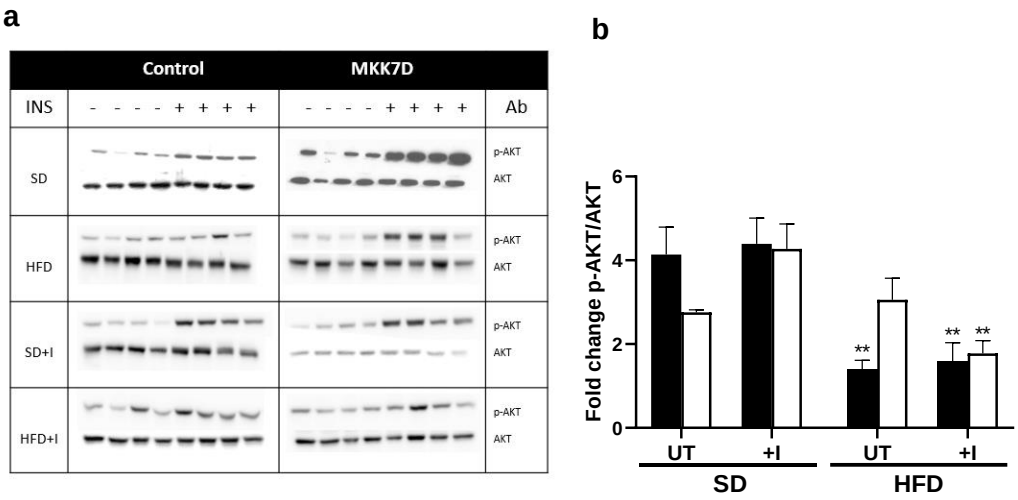


Figure 38. MKK7D mice are protected from obesity-induced insulin resistance in eWAT and chronic insulin treatment abrogates this protective phenotype. (a) Representative immunoblot analysis of pAKT^{Ser473} and AKT in e-WAT protein extracts from non- or insulin (INS)-treated Control and MKK7D mice on SD or HFD and without or with (+I) chronic glargine administration, as indicated. **(b)** Graph represents the ratio of pAKT ^{Ser473} versus AKT determined by densitometry analysis of the x-ray films obtained from immunoblots in e-WAT and shown in **(a)** (UT, non-treated; +I, glargine-treated) (n= 6 mice/group).

In this experiment Control and MKK7D female mice were also included and results are shown below. In summary, obese female mice demonstrated to be protected from the development of obesity-induced glucose intolerance and insulin-resistance and, this unresponsiveness precluded further analysis.

MKK7D and Control female mice were subjected to a similar protocol to induce obesity as described for the development of objective 2.

Previously to start with the specific diet, at 4 weeks of age Control and MKK7D female mice were subjected to a GTT to assess the glucose intolerant phenotype previously described also in female mice (Lanuza-Masdeu *et al.* 2013) (Fig. 39), and then, placed on SD or HFD for 12 weeks. Equally to male mice, HFD induced obesity regardless genotype and chronic insulin treatment (Fig 40).

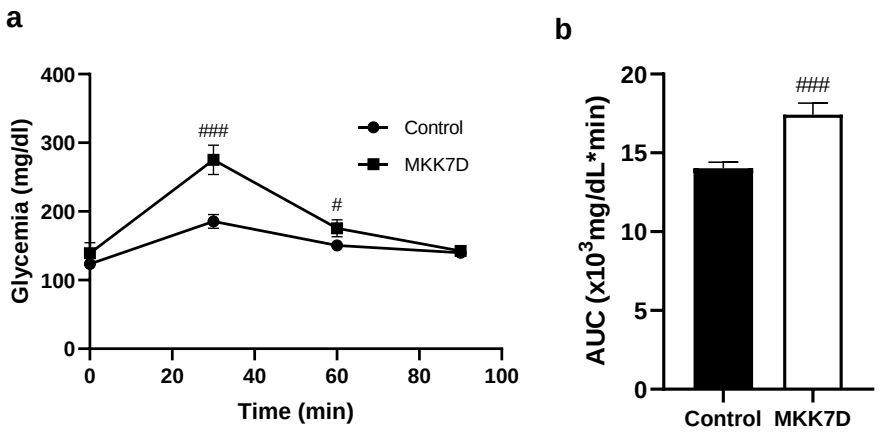


Figure 39. GTT analysis at 4-weeks of age of Control and MKK7D female mice. (a) GTT curves, and **(b)** representation of the 90-min area under the curve (AUC) of the GTT analysis shown in **(a)** (n= 16 mice/group).

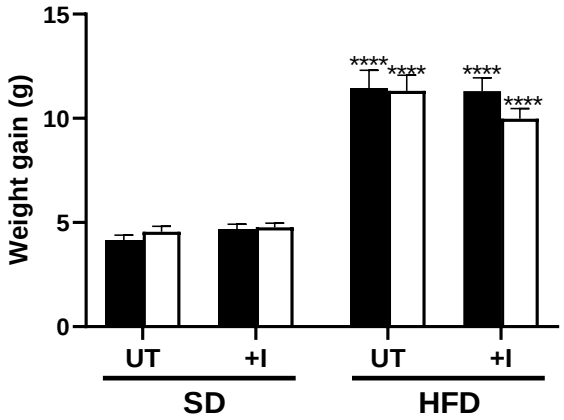


Figure 40. HFD promoted obesity in Control and MKK7D female mice independently of genotype and chronic insulin treatment. Weight gain of the indicated Control (black bars) and MKK7D (empty bars) experimental groups (UT, non-treated and +INS, glargine-treated) (n= 8 mice/group).

On week 12 on the specific diet, all mice were subjected to different test to assess glucose tolerance (GTT) and sensitivity to insulin (ITT) and as shown respectively in figures 41 and 42, HFD did not induced glucose intolerance in Control female mice, and the effect on insulin sensitivity was also minor regardless the genotype and insulin treatment. These results ruled out further analysis in female mice.

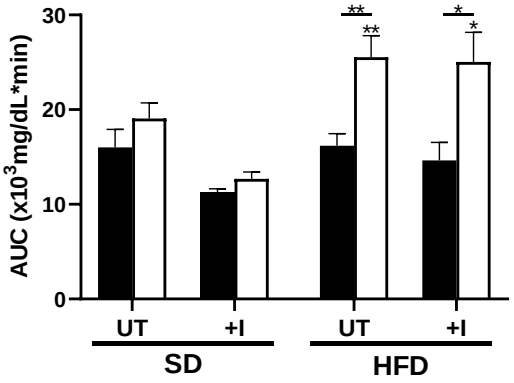


Figure 41. Obese Control female mice did not develop glucose intolerance. AUC analysis from GTT of the indicated Control (black bars) and MKK7D (empty bars) animal groups and conditions (UT, non-treated; +I, glargine-treated) (n= 8 mice/group)

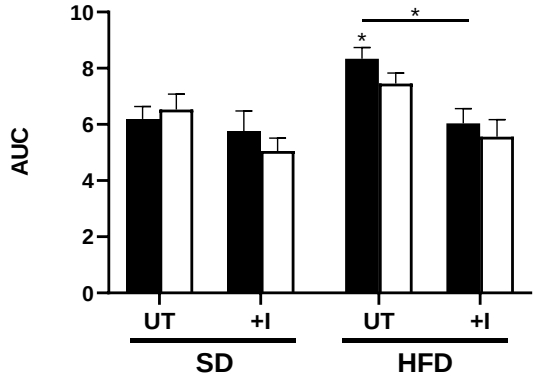


Figure 42. In Control and MKK7D female mice, obesity did not consistently induce insulin resistance. AUC analysis from ITT of the indicated Control (black bars) and MKK7D (empty bars) animal groups and conditions (UT, non-treated; +I, glargine-treated) (n= 8 mice/group).

LIST OF ABREVIATIONS

List of abreviations

BMI	Body Mass Index
ER	Endoplasmic Reticulum
ERK	Extracellular Regulated Protein Kinase
FFA	Free Fatty Acids
GLUT	Glucose Transporter
GFP	Green Fluorescent Protein
GTT	Glucose Tolerance Test
HCC	Hepatocellular Carcinoma
HFD	High Fat Diet
IL	Interleukin
ITT	Insulin Tolerance Test
IR	Insulin Receptor
IRS	Insulin Receptor Substrate
JAK2	Janus Kinase 2
LEPR	Leptin Receptor
MAPK	Mitogen-Activated Protein Kinase
MAP4K4	M4K4
MCP1	Monocyte Chemoattractant protein 1
NAFLD	Non-Alcoholic Fatty Liver Disease
OB	Obese
PI3K	Phosphatidylinositol 3-Kinase
PKB	Protein Kinase B

List of abreviations

PKR	Double-stranded RNA-dependent Protein Kinase
PPAR	Peroxisome Proliferator-Activated Receptor
PTEN	Phosphatase and Tensin Homolog
qRT-PCR	Quantitative Real-Time PCR
ROS	Reactive Oxigen Species
SD	Standard Diet
STAT3	Signal Transduction and Activator of Transcription 3
T2D	Type 2 Diabetes
TLR	Toll Like Receptor
TNFα	Tumor Necrosis Factor-α
TZDs	Thiazolidinediones
TSH	Thyroid-Stimulating Hormone
VLDLs	Very-Low-Density Lipoproteins
WAT	White Adipose Tissue
WHO	World Health Organization
WT	Wild Type