

## Research Article

# Diet-Induced Swine Model with Obesity/Leptin Resistance for the Study of Metabolic Syndrome and Type 2 Diabetes

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The objective of the present study was to determine the suitability of a swine breed with leptin resistance and predisposition to obesity (the Iberian pig) as model for studies on metabolic syndrome and type 2 diabetes. Thus, six Iberian sows had *ad libitum* access to food enriched with saturated fat (SFAD group; food consumption was estimated to be 4.5 kg/animal/day) whilst four females acted as controls and were fed with 2 kg/animal/day of a commercial maintenance diet. After three months of differential feeding, SFAD animals developed central obesity, dyslipidemia, insulin resistance and impaired glucose tolerance, and elevated blood pressure; the five parameters associated with the metabolic syndrome. Thus, the current study characterizes the Iberian pig as a robust, amenable, and reliable translational model for studies on nutrition-associated diseases.

## 1. Introduction

Obesity is currently declared a global pandemic by the World Health Organization (WHO; <http://www.who.int/mediacentre/factsheets/fs311/en/index.html/>), with at least 2.6 millions of people dying each year as a result of being overweight or obese. The incidence of overweight and obesity is dramatically rising and, according to WHO predictions, approximately 2.3 billion of people will be overweight and more than 700 million will be obese by the year 2015 (<http://www.who.int/features/factfiles/obesity/en/index.html>). Furthermore, obesity predisposes to the development of metabolic abnormalities, clustered in the term *metabolic syndrome*. The metabolic syndrome is characterized by the presence of at least three of five symptoms: central obesity, insulin resistance, impaired glucose tolerance, dyslipidemia (increased triglyceridemia and low plasma high-density lipoproteins (HDL) cholesterol), and/or hypertension [1–5]. Moreover, metabolic syndrome is the

main risk factor for developing type 2 diabetes [6, 7]. From these considerations, there is an urgent necessity for increasing knowledge about obesity and its effects. However, mechanistical experimentation is not affordable in human beings and animal models are needed.

Most of the experimental studies on obesity and metabolic disorders are carried out on laboratory rodents despite the marked differences in metabolism and adipose tissue biology between rodents and humans [8]. However, different species of large animals offer numerous profitable characteristics [9]. The pig is emerging rapidly as a biomedical model for energy metabolism and obesity in humans because it shares several similarities with humans: omnivorous habits, propensity to sedentary behaviour and fattening, and similar metabolic and cardiovascular features [10–13].

The objective of the present study was to determine the propensity of a swine breed with predisposition to obesity (the Iberian pig) for developing features of metabolic

syndrome and type 2 diabetes. The Iberian pig is worldwide known for the production of a unique highly priced dry-cured product, the Iberian ham, with a unique taste due to its abundance in intramuscular fat. In fact, the Iberian pig has a high potential for fat accumulation under its skin and among the muscular fibres [14], due to a polymorphism of the leptin receptor gene (*LEPR*) with effects on food intake, body weight, and fat deposition [15, 16]. As a consequence, Iberian *LEPR* allele increases insatiability and obesity. Such state in human medicine is called *leptin resistance*, the failure, in obese individuals with elevated leptin levels, for suppressing feeding and mediating weight loss [17, 18] due to *LEPR* polymorphisms associated with food preferences and obesity [19]. Thus, having in mind these considerations, our hypothesis was that eating excess and obesity in Iberian pigs would develop a condition similar to the human metabolic syndrome. The final purpose of our study was to characterize a suitable pig model for studies on leptin resistance, obesity, metabolic syndrome, and type 2 diabetes.

## 2. Material and Methods

**2.1. Animals and Handling.** Ten adult Iberian sows (2-3 years old) were used. All the animals had been genotyped for polymorphism on *LEPR* gene with protocols previously described [15] and found to be homozygous for the allele LEPRc.1987T, previously associated with increased appetite, fattening, and bodyweight [15, 16]. The experimental procedure was performed in collective pens at the facilities of the INIA Animal Laboratory Unit (Madrid, Spain). The INIA Animal Unit meets the requirements of the European Union for Scientific Procedure Establishments. The experiment was carried out under Project License from the INIA Scientific Ethic Committee. Animal manipulations were performed according to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 about the protection of animals used in experimentation.

Animals were fed, prior to the experimental procedure, with a standard grain-based diet fulfilling their daily maintenance requirements (2 kg/animal/day); mean values of the diet were 89.8% of dry matter, 15.1% of crude protein, and 2.8% of polyunsaturated fat. At the beginning of the experimental procedure, the animals were divided in two different pens corresponding to different diets. Four of the sows acted as controls (control group or group C) and continued being fed with the same diet and amount. The remaining six animals had *ad libitum* access to the same diet but enriched with saturated fat (3.7%; *saturated fat ad libitum* group or group SFAD); during the experimental period (100 days), food consumption was estimated to be 4.5 kg/animal/day.

**2.2. Evaluation of Body Weight, Size, and Fatness.** Body weight, thoracic and abdominal circumferences (obtained with a measuring tape) and back-fat depth (ultrasonically determined at P2 point, at the level of the head of the last right rib) were measured at days 0, 45, and 90 after

starting the differential feeding. Thoracic circumference has been shown to be predictive for the amount of carcass fat whilst abdominal circumference is predictive of visceral and subcutaneous fat, obtained by quantitative dissection, in pigs and minipigs [20–22].

**2.3. Evaluation of Metabolic Status.** Blood samples were drawn concurrently with body measures, after a fasting period of around 18 hours, by jugular venopuncture with 5 mL sterile heparin blood vacuum tubes (Vacutainer Systems Europe). Immediately after recovery, the blood was centrifuged at 1500 g for 15 min and the plasma was separated and stored at  $-20^{\circ}\text{C}$  until assayed.

Parameters related to metabolism of lipids (triglycerides, total cholesterol, high-density lipoproteins cholesterol (HDL-c) and low-density lipoproteins cholesterol (LDL-c)) were measured with a clinical chemistry analyzer (Screen Point, Hospitex Diagnostics, Sesto Fiorentino, Italy). Plasma HDL-c ratio and LDL-c ratio were calculated by dividing total cholesterol by HDL-c and LDL-c concentrations, respectively; plasma LDL-c/HDL-c ratio was obtained by dividing LDL-c levels by HDL-c concentrations.

Parameters related to metabolism of glucose (glucose and insulin) were measured with a clinical chemistry analyzer for glucose (Screen Point, Hospitex Diagnostics, Sesto Fiorentino, Italia) and with a Porcine Insulin ELISA kit (Mercodia AB, Uppsala, Sweden), respectively. The assay sensitivity was 0.26 UI/L; the intraassay variation coefficient was 3.5%.

Possible changes in  $\beta$ -cell function and insulin resistance (IR) during the experimental protocol were assessed by the homeostasis model assessment (HOMA), using the equations  $\text{HOMA-IR} = (\text{FINS} \times \text{FPG})/22.5$  to assess insulin resistance [23] and  $\text{HOMA-}\beta = (20 \times \text{FINS})/(\text{FPG} - 3.5)$  to assess beta cell function [24]; FINS is fasting plasma insulin concentration in U/L and FPG is fasting plasma glucose concentration in mmol/L. Furthermore, an oral glucose tolerance test (OGTT) was performed at day 100 after the beginning of experimental procedure, by using the protocol described by Liu et al. [25]. In brief, animals were given 2 g/kg live weight of D-glucose, after a fasting period of around 18 hours, by gavage through a gastric tube inserted into the stomach. Blood samples were obtained for determining plasma glucose and insulin at 0, 15, 30, 60, 90, and 120 minutes after glucose administration, centrifuged, and stored at  $-20^{\circ}\text{C}$  until assayed.

**2.4. Measurement of Cardiovascular Features.** Blood pressures were monitored at the beginning and end of the experimental period (days 0 and 100) by using a tail cuff sphygmomanometer.

**2.5. Statistical Analyses.** Effects of diet on body, metabolic and cardiovascular features were assessed by analysis of variance for repeated measures (split-plot ANOVA). Results were expressed as the mean  $\pm$  SEM, and statistical significance was accepted from  $P < 0.05$ .

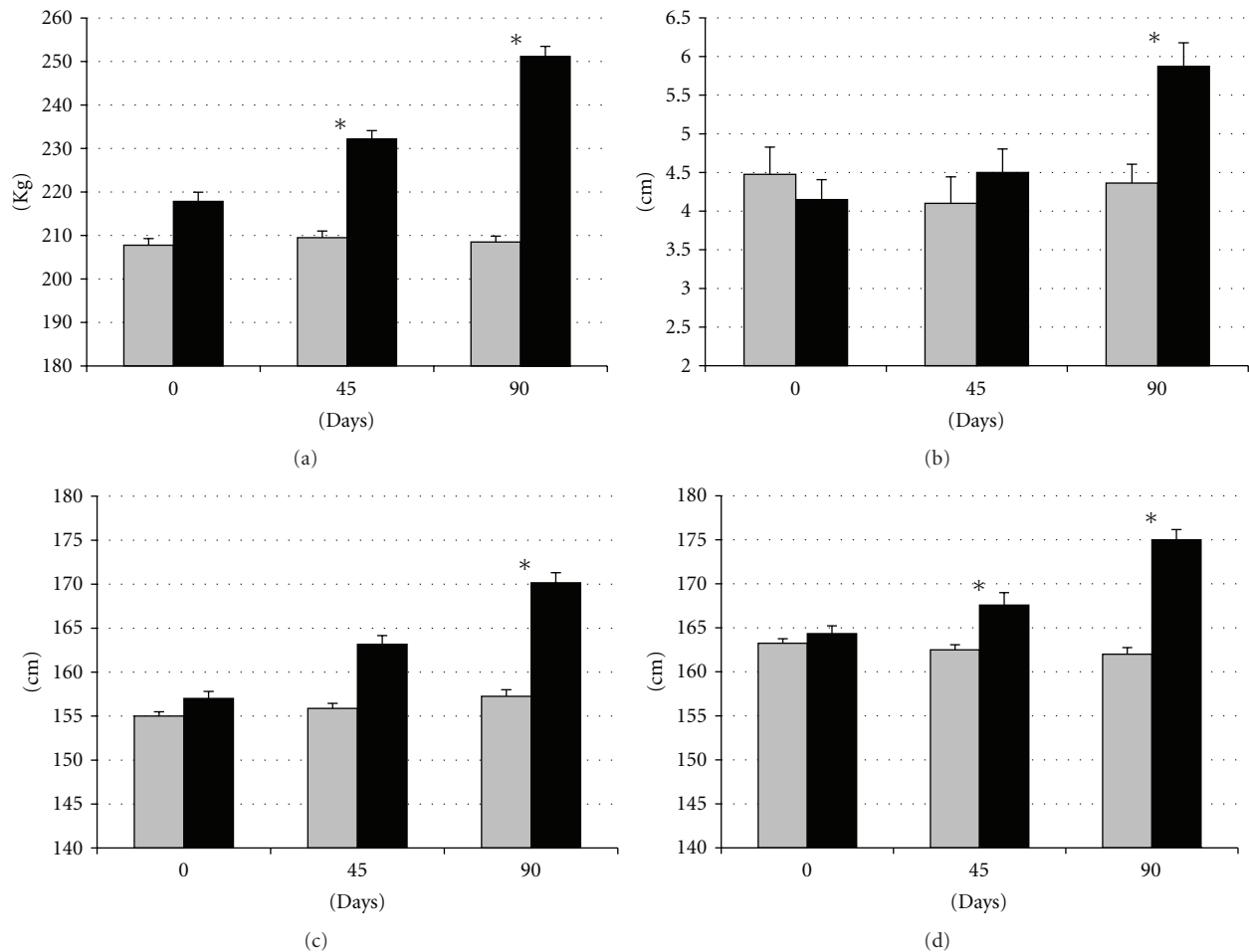


FIGURE 1: Changes in mean live weight (a), back-fat depth (b), and abdominal and thoracic circumferences ((c) and (d), resp.) over time after differential feeding in control sows (grey bars) and sows with *ad libitum* access to a diet enriched with saturated fat (black bars). Asterisks indicate significant differences.

### 3. Results

**3.1. Effects of Diet on Body Weight and Fatness: Central Obesity.** Live body weight, back-fat depth, and measurement of thoracic and abdominal circumferences were similar between groups at starting the differential feeding period (Figure 1). Values remained unchanged in control animals throughout the experimental period (with increases around 750 g for weight, 3 cm for the circumferences, and 1 mm for back-fat depth) but increased with time in the group SFAD (around 35 kg for weight, 13 and 6 cm for thoracic and abdominal circumferences, resp., and 18 mm for back-fat depth;  $P < 0.05$  for body weight and circumferences and  $P < 0.005$  for back-fat depth). Differences in body weight and thoracic circumferences between groups reached statistical significance from day 45 onwards ( $P < 0.05$ ), whilst abdominal circumference and back-fat content were different only at day 90 ( $P < 0.05$  and  $P < 0.001$ , resp.). Thus, animals in the SFAD group showed obesity and, specifically, *central obesity* after three months of differential feeding, a first symptom of *metabolic syndrome*.

**3.2. Effects of Diet on Metabolic Features: Dyslipidemia, Insulin Resistance, and Glucose Intolerance.** Assessment of plasma indexes of lipid metabolism showed no significant differences between groups at the beginning of the experimental protocol (Figure 2). After 45 days, differences were not significant. However, after 90 days, SFAD group had significantly ( $P < 0.05$ ) higher plasma levels of triglycerides and cholesterol and higher ratios of HDL-c (total cholesterol divided by HDL-c) and LDL-c/HDL-c (LDL-c levels divided by HDL-c concentrations), since the increase in total cholesterol was accompanied by the increase in LDL-C but not in HDL-c. Such enlarged triglyceridemia with low HDL-c ratios evidenced *dyslipidemia*, which is a second symptom of *metabolic syndrome*.

There were no significant differences between groups in glucose and insulin levels at the beginning of the study (Figure 3). Analysis of plasma glucose showed higher, but not significant, levels in SFAD group after 45 and 90 days of differential feeding. Conversely, plasma insulin concentrations showed different profiles between groups. Insulin level did not significantly differ in group C throughout

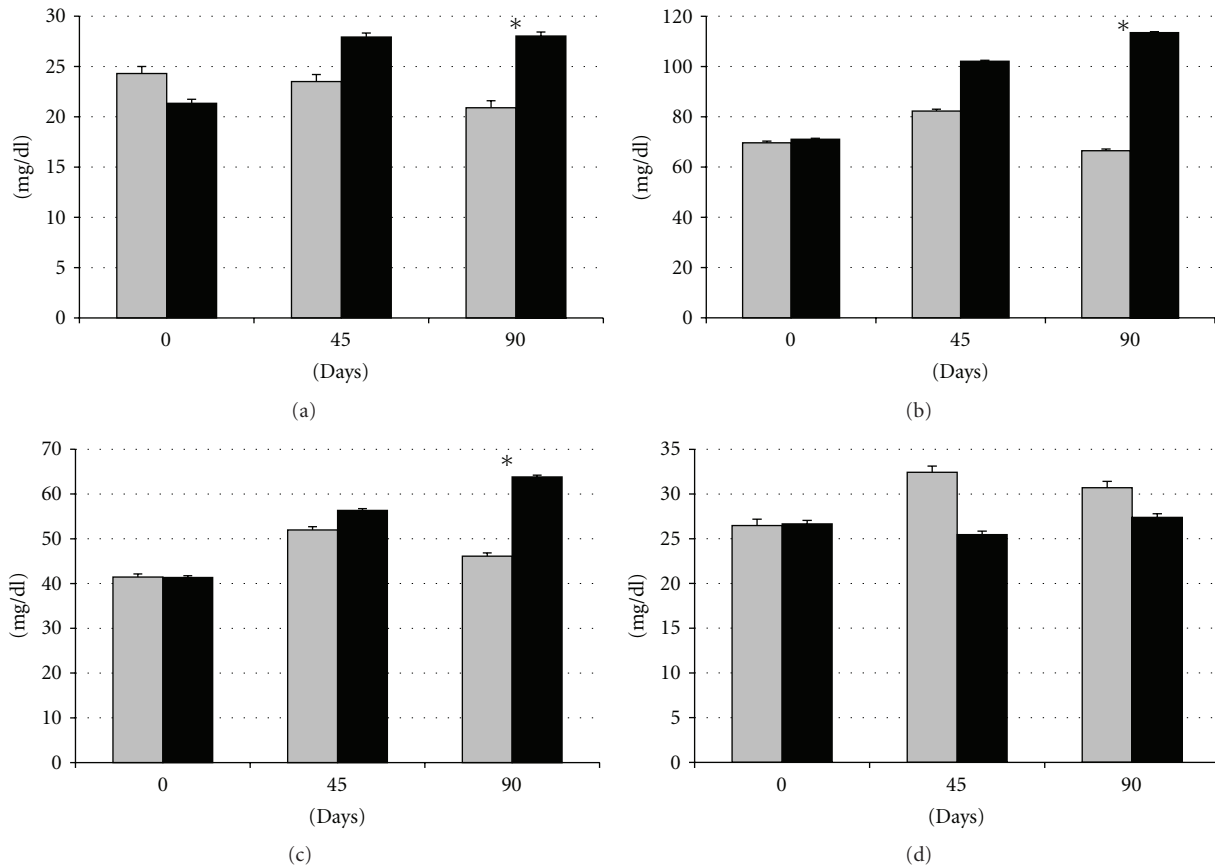


FIGURE 2: Changes in plasma concentration of triglycerides (a), cholesterol (b), LDL-c (c), and HDL-c (d) over time after differential feeding in control sows (grey bars) and sows with *ad libitum* access to a diet enriched with saturated fat (black bars). Asterisks indicate significant differences.

the experimental period, but plasma insulin concentration increased at day 45 in the group SFAD ( $P < 0.05$ ) and decreased again at day 90 ( $P < 0.01$ ).

Both HOMA-IR and HOMA- $\beta$  indexes remained nearly unchanged throughout the period of study in the group C. Conversely, both indexes increased significantly after 45 days in the group SFAD ( $P < 0.01$  for both) and returned to the control values at day 90 ( $P < 0.05$  and  $P < 0.01$ , resp.), when these indexes reached numerically higher but not statistically different values to control females. Thus, both HOMA-IR and HOMA- $\beta$  were higher in SFAD at day 45 ( $P < 0.05$  and  $P < 0.01$ , resp.), evidencing *insulin resistance*, the third symptom of *metabolic syndrome*, and *impaired  $\beta$ -cell function*, respectively.

The oral glucose tolerance test (OGTT; Figure 4) showed that, in control animals, the plasma glucose levels started to increase after 15 minutes and reached a peak around one hour later ( $P < 0.01$ ) for rapidly declining to starting values afterwards. Assessment of insulin levels in these pigs evidenced a well-characterized acute insulin response. In contrast, the OGTT showed a deficient glucose elimination (i.e., *glucose intolerance*, fourth symptom of the *metabolic syndrome*) in the group SFAD, since glucose levels did not decrease for the period of study. Moreover, SFAD sows had

a deficient insulin secretion, confirming the diet-induced  $\beta$ -cell dysfunction suggested by prior changes in HOMA- $\beta$  and evidencing the prodrome of *type 2 diabetes*.

**3.3. Effects of Diet on Cardiovascular Features: Hypertension.** Assessment of blood pressure showed similar values in both groups at the beginning of study (Figure 5). At the end of the treatment, all the parameters remained stable in control females but increased in obese females; these differences were statistically significant for diastolic and mean blood pressure ( $P < 0.01$  and  $P < 0.005$ , resp.) evidencing a *hypertensive state*, the fifth symptom of a *metabolic syndrome*.

## 4. Discussion

The results found in the present study indicate that adult females of the Iberian pig, a swine breed with *leptin resistance*, can develop the prodrome of *metabolic syndrome* and *type 2 diabetes* when allowed to freely eat a diet enriched with saturated fat. This feature was found as early as in three months. Thus, current experiment reinforces previous evidences about the susceptibility of this breed to changes in nutritional inputs [26, 27] and characterizes the Iberian pig

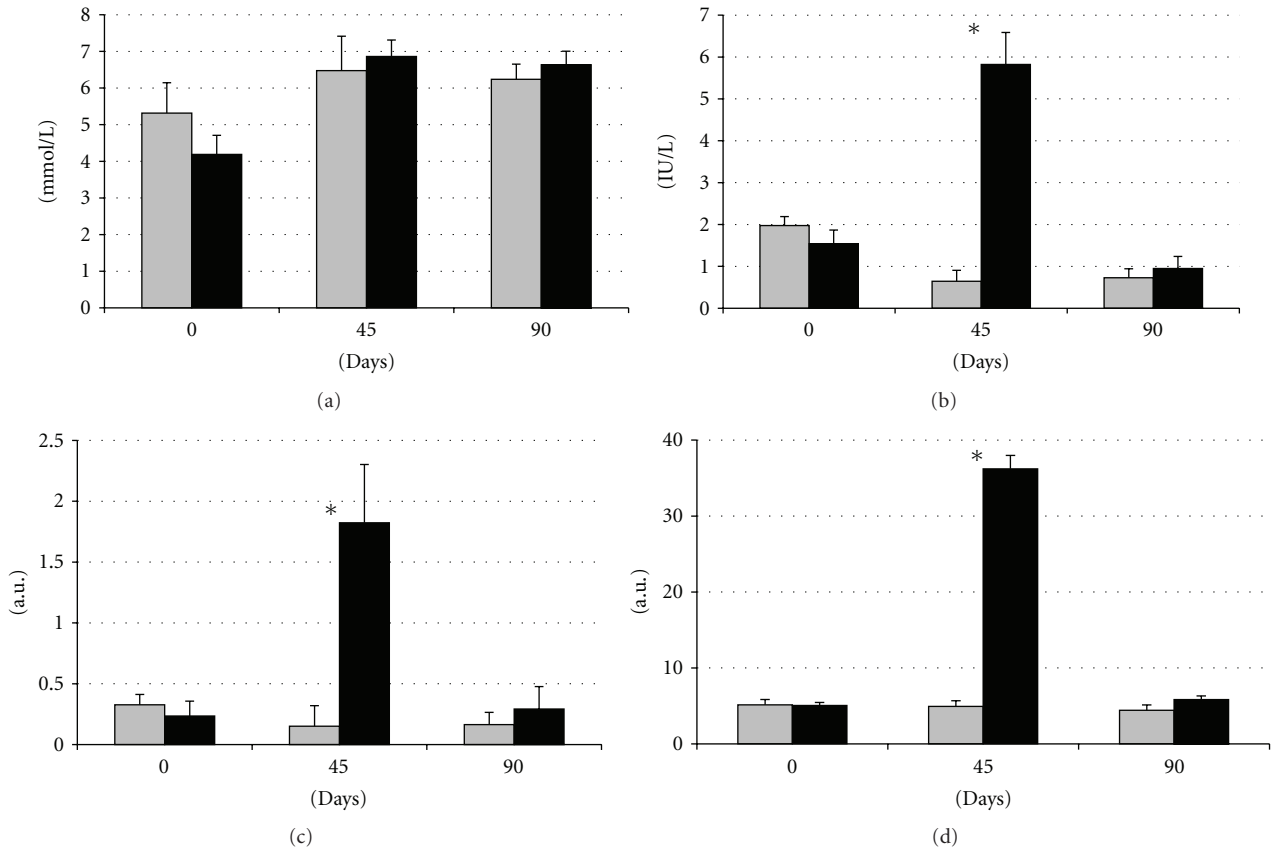


FIGURE 3: Changes in plasma concentration of glucose (a), insulin (b), and indexes of HOMA-IR and HOMA-β ((c) and (d), resp.) over time after differential feeding in control sows (grey bars) and sows with *ad libitum* access to a diet enriched with saturated fat (black bars). Asterisks indicate significant differences.

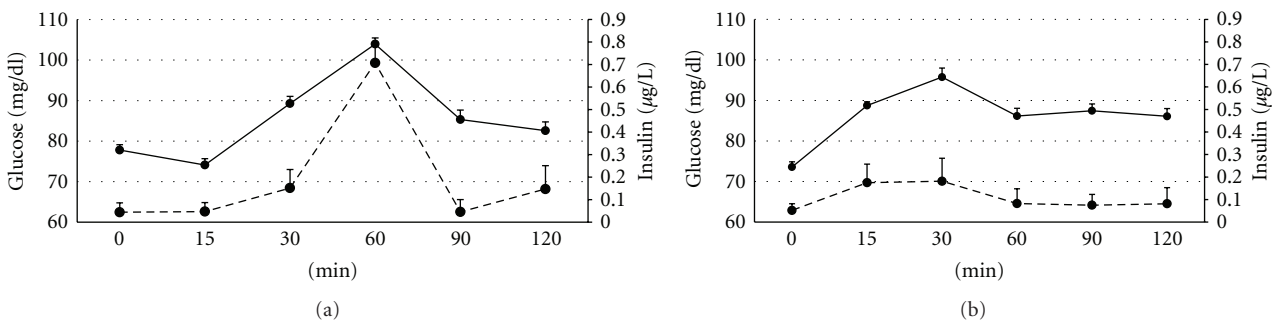


FIGURE 4: Changes in plasma concentration of glucose (continuous line) and insulin (discontinuous line) over time after oral administration of 2 g/kg live weight of D-glucose in control sows (a) and sows with *ad libitum* access to a diet enriched with saturated fat (b).

as a robust, amenable, and reliable translational model for studies on nutrition-associated diseases.

In these three months, pigs developed alterations in the five parameters associated with the metabolic syndrome. Obesity was associated with different degrees of dyslipidemia, insulin resistance and impaired glucose tolerance, and elevated blood pressure. Such finding reinforces the strength of our model. There are no other animal models consistently showing four or more risk factors of the syndrome [13]. Establishment of the five parameters of

the metabolic syndrome has been previously found in the Ossabaw pig [21]. However, it is known that Ossabaw pigs are derived from the Iberian pigs introduced by Spanish and Portuguese colonizers, since there were no pigs in the New World before their arrival in the 15th century. Iberian pigs, conversely to Ossabaw pigs, are numerous (the estimated census of breeding sows, only in Spain, is around 238.000) and, hence, easy to get and reasonably priced. Finally, the robustness of the model is also reinforced by the fact that homeostatic changes were found when feeding the sows with

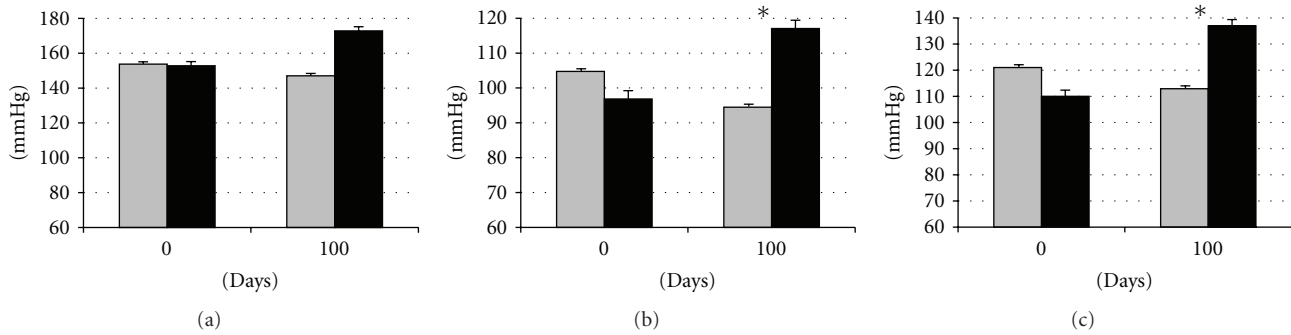


FIGURE 5: Changes in systolic (a), diastolic (b), and mean (c) blood pressures over time after differential feeding in control sows (grey bars) and sows with *ad libitum* access to a diet enriched with saturated fat (black bars). Asterisks indicate significant differences.

diets having a considerably lower level of fat content and energy than provided in previous studies with other swine breeds [21, 28, 29].

The effects of the diet on body weight, size, and fat content were remarkable and were observed very early after starting the differentiated feeding, but the pattern of fattening seemed to be affected by time. Differences in body measurements were firstly found in body weight and thoracic circumference and thereafter in subcutaneous fat and abdominal circumference. Thus, we can assume that fat deposition when there is a positive energy balance is firstly intramuscular in the whole carcass. At later stages, fat deposition is also subcutaneous and, which is more important, visceral. Visceral deposition of fat gives way to *central obesity*, the former and causal factor for metabolic syndrome.

The current study also indicates that effects on carbohydrate metabolism were found earlier than the effects on lipid metabolism. There were no significant differences in plasma glucose concentrations throughout the study as it has been described in the first stages of the human disease [30]. However, high intake of saturated fat impaired glucose regulation and induced some degree of *insulin resistance* and *altered  $\beta$ -cell function* (main parameters of metabolic syndrome and type 2 diabetes) at 45 days after starting the differentiated feeding. This finding is in agreement with previous studies in rats also fed with saturated fat, which developed insulin resistance in a very short term [31–33], but also with observational data in human beings [34]. Animals of the current experiment were capable of counterbalancing thereafter the prodrome of metabolic syndrome and type 2 diabetes as evidenced by the plasma insulin concentrations and HOMA-IR and HOMA- $\beta$  found at day 90. However, the equilibrium was unstable and the challenge of glucose during the OGTT evidenced the weakness of this adaptation; animals confronted with such acute high supply of glucose showed *glucose intolerance*, other parameter of the *metabolic syndrome*, and alterations in  $\beta$ -cell function, which are the main cause of *type 2 diabetes* [35].

Establishment of insulin resistance has been linked to elevated levels of triglycerides in blood and tissues [32, 36–38], and *hypertriglyceridemia* is another parameter of

metabolic syndrome. However, in the current study, hypertriglyceridemia was found in a later stage than insulin resistance; simultaneously with the increase in visceral fat, which confirms earlier hypothesis indicating that hypertriglyceridemia is related primarily to the amount of such kind of fat [39]. Obese sows of this study showed a well-established dyslipidemic profile with significantly higher plasma triglycerides but also total cholesterol and LDL-c/HDL-c ratio, suggesting higher levels of LDL-c than HDL-c. These features resemble hyperlipidemia type 2 (increased total cholesterol and LDL-c) and the so-called *lipid triad* in human medicine (elevated triglycerides, elevated LDL-c, and low HDL-c), [40, 41]. The rise of cholesterol caused by elevations of LDL-c may occur both in type 1 and type 2 diabetic patients; however, concurrently low HDL-c levels are indicative of obese individuals with type 2 diabetes [42].

Alterations of carbohydrate and lipoprotein metabolism were also early identified as risk factors for *hypertension*, the fifth symptom in the metabolic syndrome [43, 44]. In our study, higher blood pressures were also found in the obese sows, indicating that they have a hyperdynamic circulatory state, similar to previously described in Ossabaw pigs [45]. In our study, changes were mainly found in diastolic pressure, coincidentally with studies in human beings [46, 47]. Hyperdynamic circulatory states, characterized by increased blood pressure, total blood volume, cardiac output, and tissue metabolic demand, have been hypothesized to be the main cause of cardiac and hemodynamic alterations in obese individuals [48–50].

In conclusion, this study indicates that Iberian pigs freely eating saturated fat diets are prone to central obesity, abnormalities in insulin-glucose regulation, dyslipidemia, and elevated blood pressure, the symptoms of metabolic syndrome. Thus, the current data characterize the Iberian pig as a robust, amenable, and reliable translational model for studies on nutrition-associated diseases.

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