



**HAL**  
open science

## The Yucatan minipig model: A new preclinical model of malnutrition in obese patients with acute or chronic diseases

Annaëlle Couvert, Laurence Lacaze, Steve Touboulic, Sandrine Gautier, Sylvie Guerin, Gwenaelle Randuineau, Véronique Romé, Charles-Henri Malbert, David Val-Laillet, Frédéric Derbré, et al.

### ► To cite this version:

Annaëlle Couvert, Laurence Lacaze, Steve Touboulic, Sandrine Gautier, Sylvie Guerin, et al.. The Yucatan minipig model: A new preclinical model of malnutrition in obese patients with acute or chronic diseases. *Clinical Nutrition*, 2024, *Clinical Nutrition (edinburgh, Scotland)*, 43 (2), pp.357-365. 10.1016/j.clnu.2023.12.003 . hal-04415280

**HAL Id: hal-04415280**

**<https://hal.science/hal-04415280v1>**

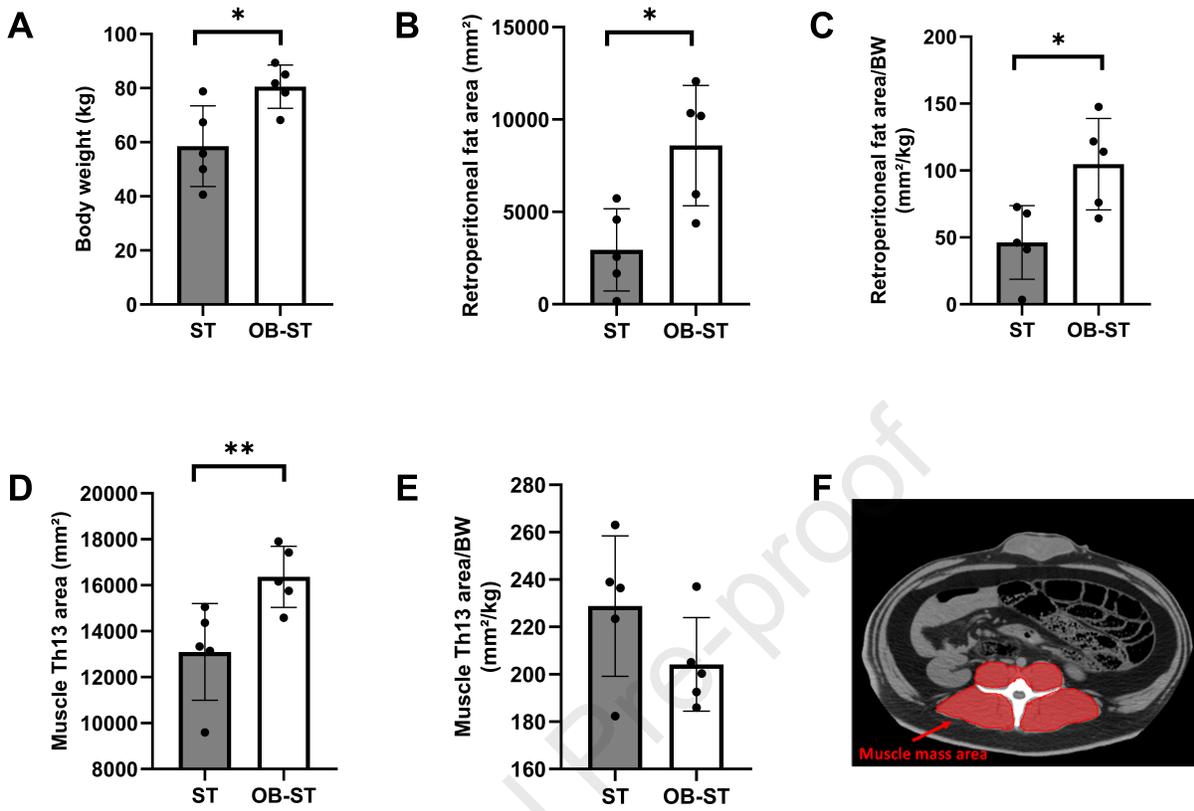
Submitted on 14 Mar 2024

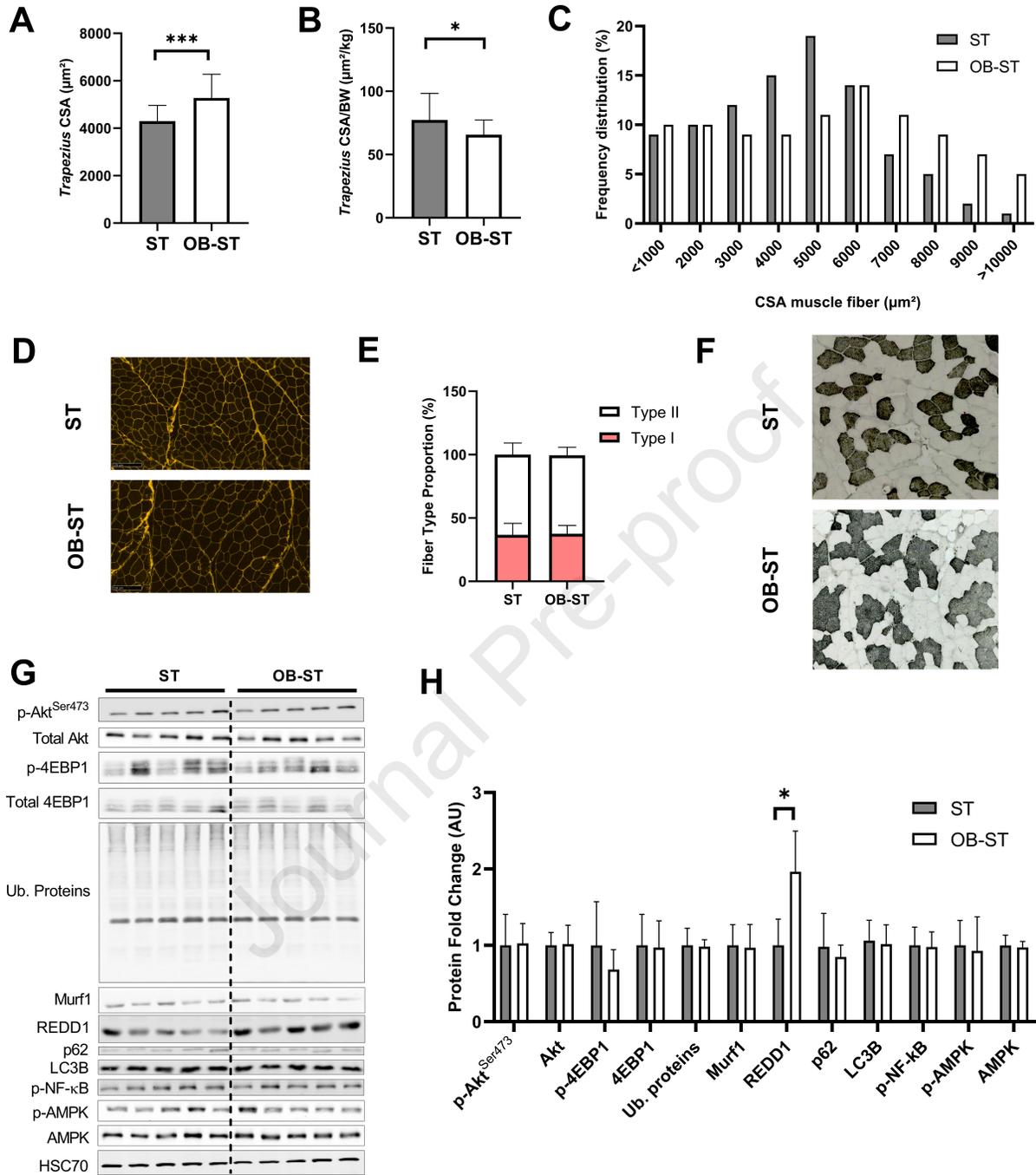
**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

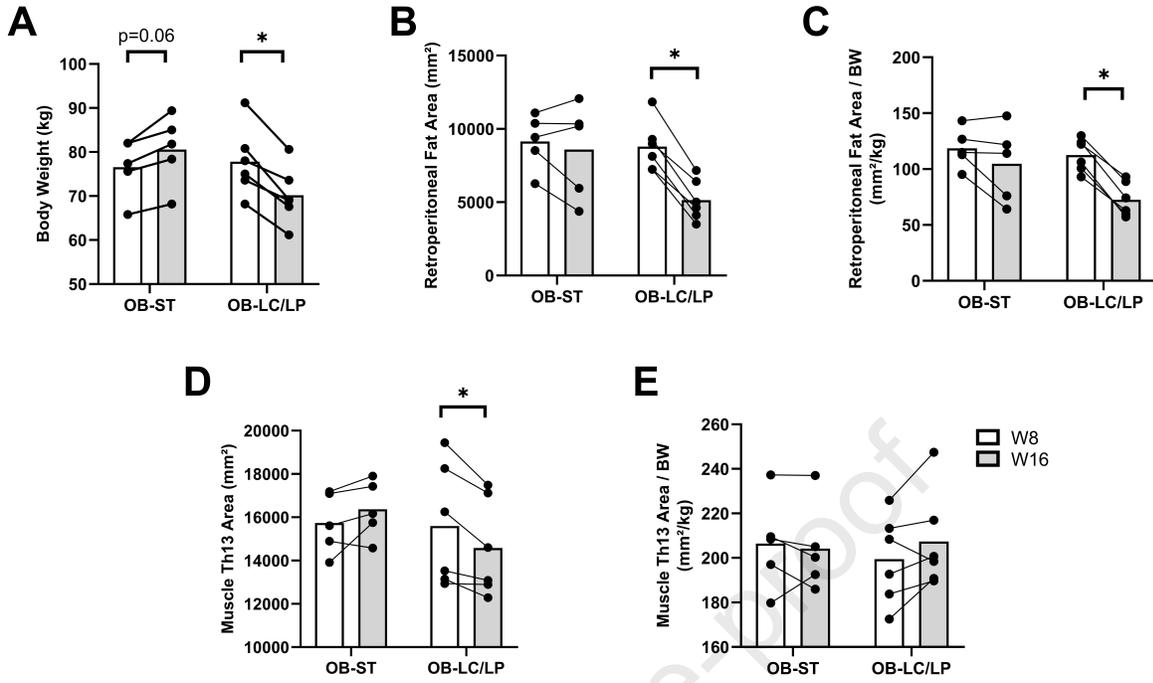
L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

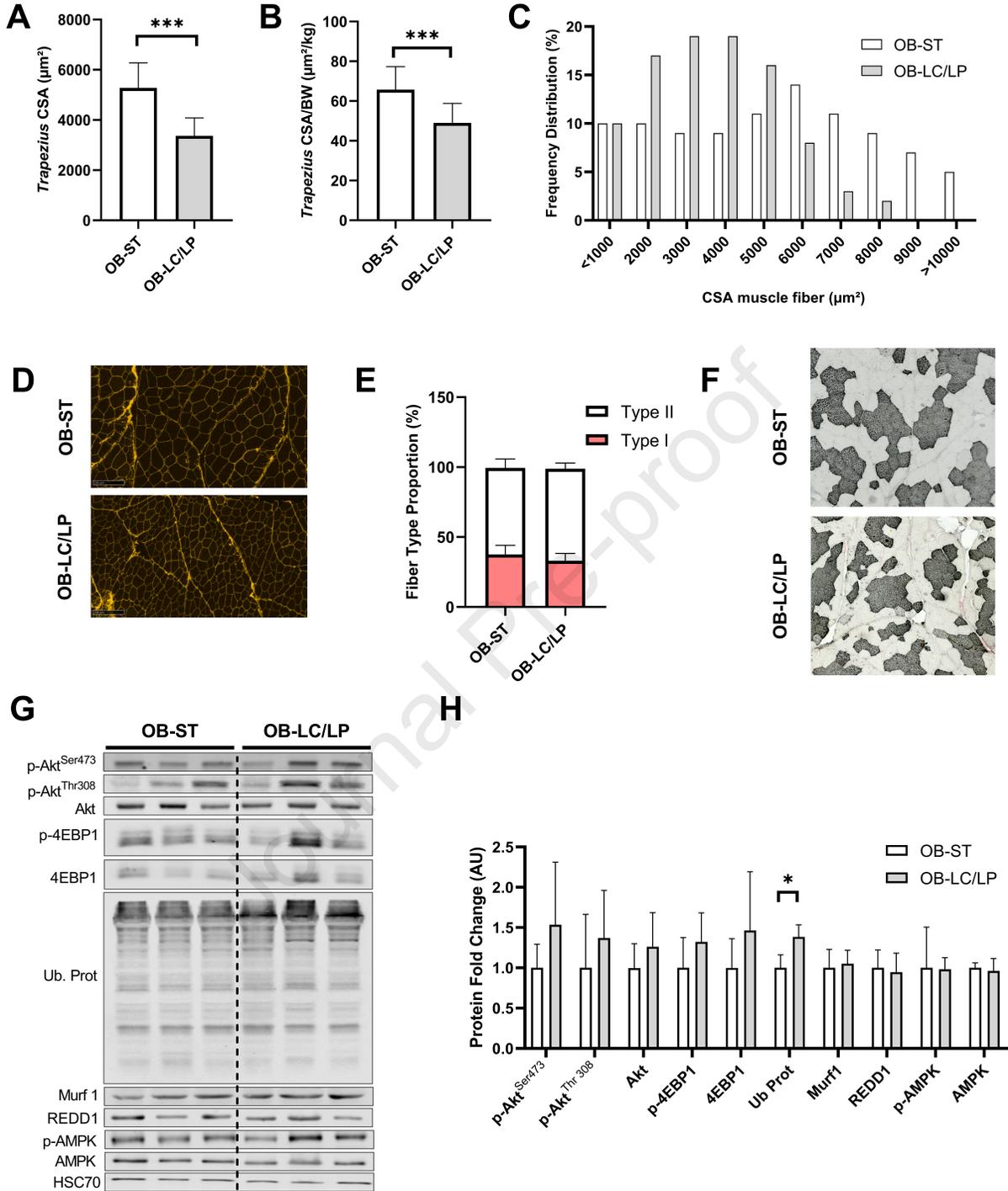


Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License









**The Yucatan minipig model: a new preclinical model of malnutrition in obese patients with acute or chronic diseases**

Annaëlle Couvert,<sup>a,b,e,\$</sup> Laurence Lacaze,<sup>b,c,\$</sup> Steve Touboulic,<sup>c</sup> Sandrine Gautier,<sup>a</sup> Sylvie Guérin,<sup>c</sup> Gwénaëlle Randuineau,<sup>c</sup> Véronique Romé,<sup>c</sup> Charles-Henri Malbert,<sup>d</sup> David Val-Laillet,<sup>c,#</sup> Frédéric Derbré,<sup>a,#</sup> Ronan Thibault,<sup>b,c,#</sup>

<sup>a</sup> Laboratory “Movement Sport and Health Sciences” EA 7470, University of Rennes, ENS Rennes, 35170 Bruz, France

<sup>b</sup> Service Endocrinologie-Diabétologie-Nutrition, Centre labellisé de nutrition parentérale au domicile, CHU Rennes, Rennes, France

<sup>c</sup> INRAE, INSERM, Univ Rennes, NuMeCan, Nutrition Metabolisms Cancer, Rennes, France

<sup>d</sup> INRAE, PRISM Ani-Scans, St Gilles, Rennes, France

<sup>e</sup> Laboratory of the Metabolic Adaptations to Exercise under Physiological and Pathological Conditions (AME2P), Clermont Auvergne University, Clermont-Ferrand, France

*\$: These authors are both first-authors and contributed equally to this work*

*#: These authors contributed equally to this work*

Corresponding authors:

Prof. Ronan THIBAUT, PhD, MD

Centre labellisé de nutrition parentérale au domicile - CHU Rennes

2, rue Henri Le Guilloux

35000 Rennes, France

Tél. +33 2 99 28 96 46

Fax +33 2 99 28 24 34

E-mail [ronan.thibault@chu-rennes.fr](mailto:ronan.thibault@chu-rennes.fr)

Dr Frédéric DERBRÉ, PhD

Laboratory “Movement, Sport and Health Sciences” EA 7470

University of Rennes, ENS Rennes,

35170 Bruz, France

Email: [frederic.derbre@univ-rennes2.fr](mailto:frederic.derbre@univ-rennes2.fr)

**ABSTRACT**

**Background & aims.** Malnutrition can develop in patients with obesity suffering from acute or chronic illness or after obesity surgery, promoting sarcopenic obesity. A better understanding of this pathophysiology and the development of new therapeutics for chronic diseases, that are often complicated with malnutrition and obesity, justify the development of new animal experimental models close to the human physiology. This study aims to characterize the effects of obesity and underfeeding on Yucatan obese minipigs, assessing its validity as a preclinical model for obesity-related malnutrition.

**Methods.** Sixteen 30-month-old Yucatan minipigs were divided into two groups for 8 weeks: a standard diet group (ST, n=5) and an obesogenic diet group (OB, n=11). After 8 weeks, the OB group was further divided into two sub-groups: a standard diet group (OB-ST, n=5) and a low-calorie/low-protein diet group (OB-LC/LP, n=6) for 8 weeks. Body composition by CT-Scan and blood parameters were monitored, and trapezius muscle biopsies were collected to analyse signaling pathways involved in protein turnover and energy metabolism.

**Results.** At W8, OB-ST animals exhibited significantly higher body weight (+37.7%,  $p=0.03$ ), muscle mass (+24.9%,  $p=0.02$ ), and visceral fat (+192.0%,  $p=0.03$ ) compared to ST. Trapezius cross sectional area (CSA) normalized to body weight was lower in OB-ST animals (-15.02%,  $p=0.017$ ). At W16, no significant changes were observed in protein turnover markers, although REDD1 increased in OB-ST (96.4%,  $p=0.02$ ). After 8 weeks of low-caloric/ low protein diet, OB-LC/LP showed decreased body weight (-9.8%,  $p=0.03$ ), muscle mass (-6.5%,  $p=0.03$ ), and visceral fat (-41.5%,  $p=0.03$ ) compared to OB-ST animals. Trapezius fiber CSA significantly decreased in OB-LC/LP (-36.1%,  $p<0.0001$ ) and normalized to body weight (-25.4%,  $p<0.0001$ ), combined to higher ubiquitinated protein content (+38.3%,  $p=0.02$ ).

**Conclusion.** Our data support that the Yucatan minipig model mimics nutritional and skeletal muscle phenotypes observed in obese patients, with or without protein-energy malnutrition. It also reproduces muscle atrophy observed in chronic diseases or post-obesity surgery, making it a promising preclinical model for obesity-related malnutrition.

**KEYWORDS:** obesity; undernutrition; muscle atrophy; sarcopenia; obesity surgery.

#### **NON-STANDARD ABBREVIATIONS**

4EBP1: eukaryotic translation initiation factor 4E binding protein 1; Akt: protein kinase B, ALT: alanine transaminase; AMPK; adenosine monophosphate-activated protein kinase, AST: aspartate transaminase; CSA: cross sectional area; CT-scan: computed tomography; EASO: European Association for the Study of Obesity; ESPEN: European Society of Clinical Nutrition and Metabolism ; LC3B: light chain 3 Beta; mTORC1: mammalian target of rapamycin 1; MuRF1: muscle ring finger-1; NF- $\kappa$ B: nuclear factor-kappa B; REDD1: Regulated in Development DNA damages responses 1; WGA: wheat germ agglutinin.

## INTRODUCTION

According to the European Society of Clinical Nutrition and Metabolism (ESPEN) terminology [1], ‘malnutrition’ or ‘undernutrition’ can be defined as “a state resulting from lack of intake or uptake of nutrition that leads to altered body composition (decreased fat-free mass) and body cell mass leading to diminished physical and mental function and impaired clinical outcome from disease” [1]. Patients suffering from malnutrition face numerous clinical consequences, including increased hospital length of stay, morbidity, mortality, and decreased quality of life [2,3]. Among the various forms of malnutrition, protein-energy malnutrition (referred to as ‘malnutrition’ here) can occur in certain clinical situations including obesity [4]. However, diagnosing malnutrition in the obese population remains challenging due to the excess of fat mass. A recent study found that 23% of elderly individuals with a BMI over 25 kg/m<sup>2</sup> were at risk of malnutrition, and 2% were malnourished [5]. Besides aging, other factors such as repeating dieting, acute or chronic diseases or obesity surgery could also promote malnutrition in obese patients [6,7]. Nonetheless, inadequate energy and protein intake can induce muscle atrophy and lead to the development of secondary sarcopenic obesity (SO) at any age [8]. SO is defined by the co-existence of an excess of fat mass and low muscle mass/function [8]. The risk of developing SO could be increased by repeated dieting and related weight cycles, which have been identified as risk factors for low muscle mass [9]. Decreased skeletal muscle mass could also be observed after obesity surgery, particularly during the first months post-surgery, when the primary objective is fat mass loss [10,11]. This loss of muscle mass is of significant concern for obese patients, as SO is well-known to induce adverse clinical consequences and increase the risk of mortality [12,13].

Muscle atrophy is promoted by an exacerbation of proteolysis mediated by different pathways as calpains/caspases, ubiquitin-proteasome or autophagy-lysosome, and/or an alteration in

protein synthesis, primarily regulated by the PI3K/Akt/mTORC1 signaling pathway [14]. However, the mechanisms involved in muscle atrophy related to malnutrition are poorly understood, especially in people with obesity, highlighting the necessity to develop preclinical models. Among these models, the Yucatan minipig has emerged as an interesting alternative to rodent models for biomedical research [15,16] due to its greater phylogenetic proximity to humans [17]. Recently, our laboratory has highlighted that the Yucatan minipig is a valid model to mimic changes in body composition, as well as histological and molecular patterns in skeletal muscle, occurring in human marasmus malnutrition [18]. However, the effects of underfeeding on body composition and skeletal muscle mass in the context of obesity and the underlying mechanisms involved remain unknown.

The context of the obesity pandemics and increased incidence of chronic diseases motivates the development of new animal experimental models close to the human physiology mimicking the clinical situations of chronic diseases complicated with obesity and malnutrition. The Yucatan minipig preclinical model of malnutrition combined with obesity would be a solid basis to make research possible that cannot be done in humans, such as in pathophysiology (to study the intra-organ molecular regulation during the genesis of malnutrition), drug pharmacokinetics (e.g.: chemotherapy, to prevent the situations of toxicity observed in obese malnourished oncology patients), or preclinical studies for new therapeutics for chronic diseases taking into account the frequent association of these diseases with obesity and malnutrition.

Therefore, the aims of this study were to characterize the effects of obesity itself, and then underfeeding, on body composition and skeletal muscle phenotype in Yucatan obese minipigs, and so, to assess its validity as a model for obesity-related malnutrition in humans. We first examined the consequences of obesity on body composition and skeletal muscle phenotype. We subsequently investigated in obese Yucatan minipigs the effects of a low-

calorie/low-protein (LC/LP) diet on weight, body composition, blood parameters, as well as histological and molecular patterns in skeletal muscle.

## **MATERIALS AND METHODS**

**Animal ethics.** Animal experiments were approved by The Regional Ethics Committee in Animal Experiment of Brittany (APAFIS # 16413-2018080711214660 v2) and validated by the French Ministry of Research in accordance with the current ethical standards of the European Community (Directive 2010/63/EU as amended by Regulation (EU) 2019/1010). All experiments were performed at the INRAE UE3P experimental facilities for pig physiology and phenotyping, under Agreement No. C35-275-32 and individual Authorization No. 35-88 (DVL).

**Animals and diets.** Sixteen 30-month-old Yucatan minipigs were randomly assigned to two experimental groups for eight weeks: a standard diet group (ST, n=5) and an obesogenic diet group (OB, n=11). The obesogenic diet consisted of a high-fat/high sucrose (HF/HS) diet. OB animals received 1 kg of the HF/HS feed per day during the first week, 1.5 kg during weeks 2 and 3, and 2 kg later on. When OB animals reached a body weight of 80 kg, their daily individual feed ration was decreased to 1 kg to prevent further excessive weight gain. The animals that did not reach this body weight continued to received 2 kg of HF/HS feed per day until the beginning of the experimental diets. After these 8 weeks of HF/HS diet, the ST group maintained the same standard diet for an additional period of 8 weeks (ST, n=5). The OB group animals were weighed at 8 weeks (W8). They were then divided into two diet sub-groups according to body weight in order to ensure a homogeneous weight distribution between the two groups at the start of the diets: a standard diet group (OB-ST, n=5) to maintain obesity, or a low-calorie/low-protein diet (OB-LC/LP, n=6) for 8 weeks to induce malnutrition as previously published [18]. The ST diet was calculated based on the metabolic

weight (MW) of the animals ( $MW=LW^{0.75}$ , where LW is the live weight in kg), energy requirements and the energy composition of the food. The usual energy intake for a normal-weight adult Yucatan minipig is 0.3017 MJ/kg MW (15% of digestible proteins). LC/LP diet consisted of half of the daily intake in terms of energy, *i.e.*, 0.1508 MJ/kg, with 43% less of proteins (8.63% of digestible proteins). Therefore, it can be inferred that the protein intake in the LC/LP group was 28% of the one of the ST group. The food intake for each animal was calculated and adjusted based on individual weight every two weeks. The composition of the different diets is detailed in **Table 1**. All animals were housed in individual pens (120 cm x 85 cm) under standard conditions of temperature (22-23°C), light/dark cycle (12h/12h), and provided with free access to tap water. At W8, animals subjected to the obesogenic diet were weighed to be evenly divided into two groups. A CT-Scan was also performed on these minipigs. All animals were weighed at the end of the study, *i.e.* at week 16 (W16). At W16, fasted minipigs were euthanized under electronarcosis followed by immediate exsanguination in the morning. Skeletal muscles (*i.e. trapezius*) were collected immediately after euthanasia, weighed and either frozen in liquid nitrogen or fixed in 4% paraformaldehyde (PFA). Blood samples were collected in heparin lithium tubes, and then centrifuged (1500 g for 10 min) to collect plasma.

**Computed tomography (CT)-scan for body composition measurement.** CT-scans were performed under general anesthesia as previously described [16], at W8 for the OB group animals, and at W16 for all the animals. Cross-sectional scans were acquired at the level of the 13<sup>th</sup> thoracic vertebra (Th13) to determine the areas of adipose tissue and muscle (*longissimus dorsi, psoas major and large muscle*). Muscle and fat areas were measured using ImageJ software (National Institutes of Health, Rockville, MD, USA). Muscle density range was considered between -29 and +150 Hounsfield units (HU), and fat density range, between -190 and -30 HU, as classically did in humans [19].

**Plasma analyses.** Alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, creatinine, albumin, glucose, triglycerides (TG) and cholesterol were measured using a Konelab automated system and specific detections kits for each parameter purchased from Thermo Fisher Scientific (Illkirch, France).

**Histological analyses.** *Trapezius* muscle samples were fixed in 4% PFA, embedded in paraffin, and then cut into serial 7- $\mu$ m-thick slices using a LEICA microtome (Leica CM3050S). Subsequently, these sections were stained using the Wheat Germ Agglutinin (WGA) method for reticulin stain. Complementary slices were also stained with monoclonal antibodies targeting type 2 and type I myosin isoforms as previously described [20]. Image acquisitions were performed at x20 magnification using a microscope (Nikon Eclipse 80i) and analyzed using Image J software [21]. Eight muscle fields per animal were analyzed, and all muscle fibers in each group were pooled for statistical analysis (7600-13300 fibers per group).

**Cytosolic protein extraction.** Skeletal muscles were ground in liquid nitrogen, and the obtained powder was used for extraction. Cytosolic protein extraction was performed from *trapezius* using a cold lysis buffer containing 10.0 mM Tris·HCl, pH 7.4, 0.5 M sucrose, 50.0 mM NaCl, 5.0 mM EDTA, 30.0 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1% NPosP-40, 0.25% sodium deoxycholate, 50.0 mM NaF, 100.0M sodium orthovanadate, and protease inhibitor cocktail (51/ml, P8340; Sigma, St. Louis, MO). Each sample was then incubated on ice for 30 min, followed by three sets of 5 s of sonication. The homogenates were then transferred to microcentrifugation tubes and centrifuged at 12,000g for 12 min at 4°C. The protein concentration of the supernatant was determined using a Lowry assay with bovine serum albumin as a standard.

**Western blotting.** Samples were diluted in SDS-PAGE sample buffer (50 mM Tris·HCl, pH 6.8, 2% SDS, 10% glycerol, 5%-mercaptoethanol, and 0.1% bromophenol blue) and heated for 5 min at 95°C before analysis. Samples containing 50  $\mu$ g of proteins were resolved on 8% to 14% SDS-PAGE. The proteins were transferred at 120 mA/gel for 90 min onto a 0.2- $\mu$ m

nitrocellulose membrane. Membranes were blocked with 5% BSA or non-fat dry milk in Tris-buffered saline/0.05% Tween-20 (TBST) for 1h at room temperature. Membranes were incubated overnight at 4°C with appropriate primary antibodies (**Suppl Table 1**). Afterward, membranes were washed with TBST and incubated for 1h at room temperature with infrared dye-conjugated secondary antibodies (LI-COR, Lincoln, NE). After being washed, blots were captured using the Odyssey Imaging System (LI-COR). All blots were scanned, and densitometry analysis of the bands was conducted using GS-800 imaging densitometer and Quantity One software (Bio-Rad Laboratories, Inc.). All blots were normalized based on HSC70 expression as a loading control.

**Statistical analyses.** All data are presented as the mean  $\pm$  standard deviation (SD). The analysis was separated into two parts. The first part compared the OB-ST group to the ST group to determine the related consequences of the induced obesity on body composition and skeletal muscle phenotypes. Mann-Whitney tests were performed to compare the OB-ST group to ST group for body weight, CT-scan data, biological and histological parameters, at W16. The second part compared the OB-ST and OB-LC/LP groups to determine the effects of the LC/LP diet on obese animals, and whether they could be considered as malnourished. Wilcoxon signed-rank tests were performed to compare the effects of OB-ST and OB-LC/LP diets on body weight and CT-scan data between W8 and W16. Mann-Whitney tests were performed to compare biological and histological parameters at W16. The significance level was set at 0.05 for all statistical analyses. Statistical trends were considered for  $0.05 < P$ -value  $< 0.10$ . Data were analyzed using GraphPad Prism version 8.00 for Windows (GraphPad Software, La Jolla, California).

## RESULTS

**Effects of the obesogenic diet on body weight and body composition.** Body weight and body composition at W16 are shown in **Figure 1**. We observed a significantly higher body weight in the OB-ST group compared to the ST group (+37.7%,  $p=0.032$ , **Figure 1A**). The retroperitoneal fat area was significantly higher in the OB-ST group than in the ST group, both in absolute value (+192.0%,  $p=0.032$ , **Figure 1B**) and relative (normalized to weight: +126.5%,  $p=0.032$ , **Figure 1C**, or to total surface area: +135.5%,  $p=0.032$ , data not shown) values. This indicates that the obese minipigs reproduce the excess in fat mass observed in human obesity. Absolute values of muscle area were also significantly higher in the OB-ST group than in the ST group (+24.9%,  $p=0.0079$ , **Figure 1D and 1F**). However, relative (normalized to body weight) values of muscle mass area did not significantly differ between the two groups ( $228.8 \pm 29.7$  vs.  $204.2 \pm 19.8$  mm<sup>2</sup>/kg in the ST and OB-ST group respectively, **Figure 1E and 1F**).

**Effects of obesity on muscle fiber size.** Muscle fiber cross-sectional areas (CSA) were determined by histological analyses in the *trapezius* muscle (**Figure 2**). At W16, muscle fiber CSA were significantly higher in the OB-ST group than in the ST group (+22.8%,  $p<0.0001$ , **Figure 2A**). When expressed relative to body weight, muscle fiber CSA were significantly lower in the OB-ST group than in the ST group (-15.02%,  $p=0.017$ , **Figure 2B, 2C and 2D**). We did not observe any difference in the proportion of types II and I muscle fibers between groups (**Figure 2E and 2F**).

**Effects of obesity on the expression of the signaling proteins involved in muscle mass regulation (atrophy) and energy metabolism.** At W16, there were no significant differences between OB-ST and ST groups regarding the expression of Akt and 4EBP1, well known markers of protein synthesis pathway (**Figures 2G-H**). However, the expression of REDD1

was significantly higher in the OB-ST group than in the ST group (+96.4%,  $p=0.016$ ) (**Figures 2G-H**). The markers characterizing the activity of the ubiquitin-proteasome (Murf1, ubiquitinated proteins), autophagy (p62, LC3B), and apoptosis (p-NF- $\kappa$ B) did not significantly vary between the two groups (**Figures 2G-H**). Total AMPK and p-AMPK did not significantly differ between the two groups (**Figures 2G-H**).

**Effects of obesity on biological plasma parameters.** At W16, glucose levels tended to be higher in the OB-ST than in the ST group (**Table 2**). Transaminases levels were significantly lower in the OB-ST group than in the ST group. The other parameters did not differ between the two groups (**Table 2**).

**Effects of the LC/LP diet on body weight and body composition of obese minipigs.** Body weight and body composition at W8 and W16 are shown in **Figure 3**. We observed a tendency of an increase in body weight between W8 and W16 in the OB-ST group (+5.2%,  $p=0.063$ ), whereas it significantly decreased in the OB-LC/LP group (-9.8%,  $p=0.031$ , **Figure 3A**). The absolute values of retroperitoneal fat and muscle areas significantly decreased in the OB-LC/LP group between W8 and W16 (-41.5%,  $p=0.031$ , **Figure 3B**, and -6.5%,  $p=0.031$ , **Figure 3D**, respectively). No differences were observed concerning the evolution of the retroperitoneal fat and muscle areas in the OB-ST group between W8 and W16. A significant decrease in relative (normalized to body weight) retroperitoneal fat area was observed in the OB-LC/LP group (-35.5%,  $p=0.031$ , **Figure 3C**), whereas the mean relative muscle area did not vary across the time (**Figure 3E**).

**Effects of the LC/LP diet on muscle fiber size in obese minipigs.** Muscle fiber CSA were determined by histological analyses in the *trapezius* muscle at W16 (**Figure 4**). Muscle fiber

CSA were significantly lower in the OB-LC/LP group than in the OB-ST group, both in absolute (-36.1%,  $p < 0.0001$ , **Figure 4A, 4C and 4D**) and relative (to body weight) (-25.4%,  $p < 0.0001$ , **Figure 4B**) values. The proportion of types II muscle fibers and I did not differ between groups (**Figure 4E and 4F**).

**Effects of the LC/LP diet on the expression of the signaling proteins involved in muscle mass regulation (atrophy) and energy metabolism in obese minipigs.** At W16, the expression of ubiquitinated proteins were significantly higher in the OB-LC/LP group than in the OB-ST group (+38.3%,  $p = 0.015$ , **Figures 4G-H**). However, no significant changes were observed between the groups regarding other markers of protein synthesis (p-Akt<sup>Thr308</sup>, p-Akt<sup>Ser473</sup>, Akt, p-4EBP1, 4EBP1) or proteolysis (i.e. Murf1) (**Figures 4G-H**), as well as AMPK phosphorylation (**Figures 4G-H**).

**Effects of the LC/LP diet on biological plasma parameters in obese minipigs.** At W16, alanine aminotransferase levels were significantly lower in the OB-LC/LP group than in the OB-ST group (**Table 3**). Creatinine tended to be higher in the OB-LC/LP group. The other parameters did not differ between the two groups (**Table 3**).

## DISCUSSION

Obesity induces changes in body composition, notably an increase in fat mass, that is often accompanied by an increase in absolute muscle mass and strength due to the mechanical stress exerted by the individuals' body weight [22–24]. However, this muscle adaptation appears to be modulated by age, as it is observed in young adults but not in older individuals [25,26]. Expressed in relative values, muscle mass and strength can be significantly reduced in obese individuals, contributing to the onset of SO, even in the absence of skeletal muscle atrophy [8,23,24]. Therefore, the last recommendations from the European Association for the

Study of Obesity (EASO) and ESPEN suggest introducing the concept of “relative or adequate (regarding the excess in fat mass) muscle mass” to define reduced muscle mass as a criterion of SO diagnosis [27]. Consistent with observations in humans, we found that Yucatan minipigs fed with an obesogenic diet, and thus becoming obese, exhibit higher body weight, retroperitoneal fat, and muscle (i.e. *longissimus dorsi*, *psaos major* and *large muscles*) areas in absolute values than animals fed with a standard diet. The increase in *trapezius* fiber CSA in absolute values in OB-ST minipigs confirms the increase in muscle mass in obese animals. Nonetheless, muscle fiber CSA normalized to body weight is significantly reduced in the OB-ST compared to the ST minipigs. Overall, these results demonstrate that an 8-week obesogenic diet promotes an increase in muscle mass in minipigs, but this increase appears insufficient compared to the increase in fat mass and body weight, indicating an inadequate relative muscle mass, as reported in obese patients and patients with SO [27].

We then investigated the impact of the obesogenic diet on muscle protein turnover. Skeletal muscle proteolysis is mainly mediated by the ubiquitin-proteasome and autophagy-lysosome systems [14]. Here, we found that 8 weeks of an obesogenic diet do not affect proteolysis markers (i.e. ubiquitinated proteins, MurF1, LC3B, p62) in the *trapezius* muscle, which is finally consistent with the increase in muscle mass occurring in these animals. However, the inadequate muscle mass we observed can be explained by a reduction in muscle anabolic response, as previously observed in obese humans and rodents [28,29]. Indeed, protein synthesis is primarily modulated at the translation level through the Akt/mTORC1 pathway [14]. Although the phosphorylation levels of Akt and 4EBP1 remain unchanged between the ST and the OB-ST animals under basal fasted conditions, we observe, in the OB-ST group, a higher expression of REDD1, known to be a negative regulator of the Akt/mTOR pathway [30]. This result is in accordance with previous studies, indicating that high-fat-fed mice exhibit higher REDD1 expression in skeletal muscle than control mice [31,32]. The increase

in REDD1 may be due to the hyperactivity of mTORC1, which is observed at the basal state in patients with obesity [28]. Unfortunately, we were unable to detect the mTORC1 protein content by Western blot despite different tested antibodies, to confirm these findings in minipigs. Furthermore, in the post-prandial state, overweight or obese patients present a poor responsiveness of muscle protein synthesis compared to normal-weight subjects [28,29]. Unfortunately, due to the high costs of minipig experiments, we have not yet explored these post-prandial responses in our experiments. Despite these limitations, our data support the notion that Yucatan minipigs subjected to an 8-week obesogenic diet, exhibited changes in body composition and muscle mass similar to those observed in the human obese population, making our experimental model highly relevant to explore the pathophysiological mechanisms and new therapies of SO.

The second objective of the present study was to characterize the histological and molecular skeletal muscle patterns in malnourished obese Yucatan minipigs, and so, to assess whether this model could be a good preclinical model of protein-energy malnutrition (i.e., marasmus) combined with obesity, as observed in obese patients with chronic disease or after obesity surgery. While a low BMI is often used as a criterion of malnutrition [33], it cannot be applied to obese patients. Therefore, weight loss compared to a previous weight is the main phenotypic criterion of malnutrition in this population. According to the Global Leadership Initiative on Malnutrition (GLIM) consensus, one of the five criteria to diagnose severe malnutrition is a non-volitional body weight loss of more than 10% in the past six months [34]. Moreover, it is well established that malnutrition is associated with muscle mass loss [35]. In the present study, we reproduced in obese animals the experimental model of marasmus-type protein-energy malnutrition, previously validated by our research team in lean Yucatan minipigs [18]. Obese Yucatan minipigs subjected to the similar low-energy and low-protein diet exhibited a decrease of 9.8% in body weight and of 6.5% in muscle area after

only eight weeks. We also reported a significant decrease in muscle fiber CSA of the *trapezius* muscle in the OB-LC/LP group compared to the OB-ST group, both in absolute and relative values, indicating a state of muscle atrophy, therefore confirming malnutrition in accordance with body composition data. These results are in line with skeletal muscle mass loss reported in patients after obesity surgery [11], and support the validity of our experimental Yucatan minipig model to induce similar body composition and muscle mass losses as those observed in malnourished obese patients.

Currently, few scientific data are available concerning the cellular mechanisms involved in muscle atrophy related to protein-energy malnutrition [18,36], and none in obese populations to our knowledge. Here, we observed that malnourished obese minipigs exhibited a higher expression of ubiquitinated proteins, suggesting that muscle atrophy would be partly due to protein degradation by the ubiquitin-proteasome system. These results are in line with studies performed on lean aged rodents, showing an increase in the activity of the ubiquitin-proteasome system in malnourished animals [36,37]. Furthermore, it is well established that the protein fraction synthesis rate (FSR) is reduced in the skeletal muscle of malnourished animals [37]. However, in our study, we did not observe any changes in the levels of phosphorylation of Akt and 4EBP1. As mentioned above, we did not explore the post-prandial response of Akt/mTORC1, which would have allowed us to determine whether the anabolic sensitivity is affected or not in the skeletal muscle of malnourished obese minipigs. This should be the objectives of future studies.

In conclusion, our findings indicate that the high fat/high sucrose diet in Yucatan minipigs was able to induce obesity and changes in body composition and skeletal muscle mass, similar to those observed in obese humans. Moreover, the low-calorie/low-protein diet applied for 8 weeks to obese Yucatan minipigs also led to fat mass and muscle mass losses, validating this experimental model to mimic protein-energy marasmus malnutrition in the context of obesity.

This model opens promising perspectives to develop new therapeutic strategies, and to characterize the pathophysiological mechanisms of muscle atrophy associated with malnutrition in the context of obesity, as observed in patients suffering from chronic diseases or after obesity surgery.

#### **ACKNOWLEDGEMENTS**

We thank all the staff from the Rennes pig experimental facilities (UE3P), and especially Julien George, Josselin Delamarre, Francis Le Gouevéc, Alain Chauvin and Serge Dubois, for taking care of the animals and helping during the experimental period. We also thank all the staff from the NuMeCan research department who was involved in the biological sampling and treatment, and especially Armelle Cahu, Laurence Le Normand, Isabelle Nogret and Régis Janvier.

#### **FUNDING STATEMENT**

Laurence Lacaze received two research grants from the Société Francophone de Nutrition Clinique et Métabolisme (SFNCM) and the Association de Chirurgie Hépatobiliaire-Pancréatique et Transplantation Hépatique (ACHBT). Frédéric Derbré received a research grant from the Association Française d'Etude et de Recherche sur l'Obésité (AFERO). Part of the project was also funded by INRAE.

#### **CONFLICT OF INTEREST**

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

#### **CREDIT AUTHOR STATEMENT**

**Annaëlle Couvert:** Investigation, Formal analysis. Writing- Original Draft. **Laurence Lacaze:** Conceptualization, Methodology, Investigation, Visualization, Formal analysis, Funding acquisition. **Steve Touboulis:** Investigation, Formal analysis. **Sandrine Gautier :** Investigation, Formal analysis **Sylvie Guérin:** Investigation, Formal analysis. **Gwénaëlle Randuineau:** Formal analysis. **Véronique Romé:** Formal analysis. **Charles-Henri Malbert:** Methodology, Investigation. **David Val-Laillet:** Methodology, Investigation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Frédéric Derbré:** Methodology, Investigation, Writing- Original Draft, Visualization, Formal analysis, Supervision, Funding acquisition. **Ronan Thibault:** Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

## REFERENCES

- [1] Cederholm T, Barazzoni R, Austin P, Ballmer P, Biolo G, Bischoff SC, et al. ESPEN guidelines on definitions and terminology of clinical nutrition. *Clin Nutr* 2017;36:49–64. <https://doi.org/10.1016/j.clnu.2016.09.004>.
- [2] Norman K, Pichard C, Lochs H, Pirlich M. Prognostic impact of disease-related malnutrition. *Clin Nutr* 2008;27:5–15. <https://doi.org/10.1016/j.clnu.2007.10.007>.
- [3] Reber E, Strahm R, Bally L, Schuetz P, Stanga Z. Efficacy and Efficiency of Nutritional Support Teams. *J Clin Med* 2019;8:1281. <https://doi.org/10.3390/jcm8091281>.
- [4] Barazzoni R, Gortan Cappellari G. Double burden of malnutrition in persons with obesity. *Rev Endocr Metab Disord* 2020;21:307–13. <https://doi.org/10.1007/s11154-020-09578-1>.
- [5] Sulmont-Rossé C, Van Wymelbeke-Delannoy V, Maître I. Prevalence of Undernutrition and Risk of Undernutrition in Overweight and Obese Older People. *Front Nutr* 2022;9:892675. <https://doi.org/10.3389/fnut.2022.892675>.
- [6] Faintuch J, Matsuda M, Cruz MELF, Silva MM, Teivelis MP, Garrido AB, et al. Severe protein-calorie malnutrition after bariatric procedures. *Obes Surg* 2004;14:175–81. <https://doi.org/10.1381/096089204322857528>.
- [7] Lupoli R, Lembo E, Saldalamacchia G, Avola CK, Angrisani L, Capaldo B. Bariatric surgery and long-term nutritional issues. *World J Diabetes* 2017;8:464–74. <https://doi.org/10.4239/wjd.v8.i11.464>.
- [8] Donini LM, Busetto L, Bischoff SC, Cederholm T, Ballesteros-Pomar MD, Batsis JA, et al. Definition and Diagnostic Criteria for Sarcopenic Obesity: ESPEN and EASO Consensus Statement. *Obes Facts* 2022;15:321–35. <https://doi.org/10.1159/000521241>.
- [9] Rossi AP, Rubele S, Calugi S, Caliari C, Pedelini F, Soave F, et al. Weight Cycling as a Risk Factor for Low Muscle Mass and Strength in a Population of Males and Females with Obesity. *Obesity (Silver Spring)* 2019;27:1068–75. <https://doi.org/10.1002/oby.22493>.

- [10] Chaston TB, Dixon JB, O'Brien PE. Changes in fat-free mass during significant weight loss: a systematic review. *Int J Obes (Lond)* 2007;31:743–50. <https://doi.org/10.1038/sj.ijo.0803483>.
- [11] Nuijten MAH, Eijssvogels TMH, Monpellier VM, Janssen IMC, Hazebroek EJ, Hopman MTE. The magnitude and progress of lean body mass, fat-free mass, and skeletal muscle mass loss following bariatric surgery: A systematic review and meta-analysis. *Obes Rev* 2022;23:e13370. <https://doi.org/10.1111/obr.13370>.
- [12] Atkins JL, Wannamathee SG. Sarcopenic obesity in ageing: cardiovascular outcomes and mortality. *Br J Nutr* 2020;124:1102–13. <https://doi.org/10.1017/S0007114520002172>.
- [13] Zhang X, Xie X, Dou Q, Liu C, Zhang W, Yang Y, et al. Association of sarcopenic obesity with the risk of all-cause mortality among adults over a broad range of different settings: a updated meta-analysis. *BMC Geriatr* 2019;19:183. <https://doi.org/10.1186/s12877-019-1195-y>.
- [14] Schiaffino S, Dyar KA, Ciciliot S, Blaauw B, Sandri M. Mechanisms regulating skeletal muscle growth and atrophy. *FEBS J* 2013;280:4294–314. <https://doi.org/10.1111/febs.12253>.
- [15] Miller ER, Ullrey DE. The pig as a model for human nutrition. *Annu Rev Nutr* 1987;7:361–82. <https://doi.org/10.1146/annurev.nu.07.070187.002045>.
- [16] Val-Laillet D, Blat S, Louveau I, Malbert CH. A computed tomography scan application to evaluate adiposity in a minipig model of human obesity. *Br J Nutr* 2010;104:1719–28. <https://doi.org/10.1017/S0007114510002667>.
- [17] Wernersson R, Schierup MH, Jørgensen FG, Gorodkin J, Panitz F, Staerfeldt H-H, et al. Pigs in sequence space: a 0.66X coverage pig genome survey based on shotgun sequencing. *BMC Genomics* 2005;6:70. <https://doi.org/10.1186/1471-2164-6-70>.
- [18] Lacaze L, Rochdi S, Couvert A, Touboulis S, Guérin S, Randuineau G, et al. The Yucatan minipig model: A new preclinical model of malnutrition induced by a low-calorie/low-protein diet. *Clin Nutr* 2022;41:2077–86. <https://doi.org/10.1016/j.clnu.2022.08.002>.
- [19] Mitsiopoulos N, Baumgartner RN, Heymsfield SB, Lyons W, Gallagher D, Ross R. Cadaver validation of skeletal muscle measurement by magnetic resonance imaging and computerized tomography. *J Appl Physiol* (1985) 1998;85:115–22. <https://doi.org/10.1152/jappl.1998.85.1.115>.
- [20] Behan WMH, Cossar DW, Madden HA, McKay IC. Validation of a simple, rapid, and economical technique for distinguishing type 1 and 2 fibres in fixed and frozen skeletal muscle. *J Clin Pathol* 2002;55:375–80. <https://doi.org/10.1136/jcp.55.5.375>.
- [21] Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012;9:671–5. <https://doi.org/10.1038/nmeth.2089>.
- [22] Garcia-Vicencio S, Coudeyre E, Kluka V, Cardenoux C, Jegu A-G, Fourot A-V, et al. The bigger, the stronger? Insights from muscle architecture and nervous characteristics in obese adolescent girls. *Int J Obes (Lond)* 2016;40:245–51. <https://doi.org/10.1038/ijo.2015.158>.
- [23] Lafortuna CL, Tresoldi D, Rizzo G. Influence of body adiposity on structural characteristics of skeletal muscle in men and women. *Clin Physiol Funct Imaging* 2014;34:47–55. <https://doi.org/10.1111/cpf.12062>.
- [24] Tomlinson DJ, Erskine RM, Morse CI, Winwood K, Onambélé-Pearson G. The impact of obesity on skeletal muscle strength and structure through adolescence to old age. *Biogerontology* 2016;17:467–83. <https://doi.org/10.1007/s10522-015-9626-4>.

- [25] Maffiuletti NA, Ratel S, Sartorio A, Martin V. The Impact of Obesity on In Vivo Human Skeletal Muscle Function. *Curr Obes Rep* 2013;2:251–60. <https://doi.org/10.1007/s13679-013-0066-7>.
- [26] Tomlinson DJ, Erskine RM, Winwood K, Morse CI, Onambélé GL. The impact of obesity on skeletal muscle architecture in untrained young vs. old women. *J Anat* 2014;225:675–84. <https://doi.org/10.1111/joa.12248>.
- [27] Donini LM, Busetto L, Bischoff SC, Cederholm T, Ballesteros-Pomar MD, Batsis JA, et al. Definition and Diagnostic Criteria for Sarcopenic Obesity: ESPEN and EASO Consensus Statement. *OFA* 2022:1–15. <https://doi.org/10.1159/000521241>.
- [28] Beals JW, Sukiennik RA, Nallabelli J, Emmons RS, van Vliet S, Young JR, et al. Anabolic sensitivity of postprandial muscle protein synthesis to the ingestion of a protein-dense food is reduced in overweight and obese young adults. *Am J Clin Nutr* 2016;104:1014–22. <https://doi.org/10.3945/ajcn.116.130385>.
- [29] Paulussen KJM, McKenna CF, Beals JW, Wilund KR, Salvador AF, Burd NA. Anabolic Resistance of Muscle Protein Turnover Comes in Various Shapes and Sizes. *Front Nutr* 2021;8:615849. <https://doi.org/10.3389/fnut.2021.615849>.
- [30] Britto FA, Dumas K, Giorgetti-Peraldi S, Ollendorff V, Favier FB. Is REDD1 a metabolic double agent? Lessons from physiology and pathology. *Am J Physiol Cell Physiol* 2020;319:C807–24. <https://doi.org/10.1152/ajpcell.00340.2020>.
- [31] Dungan CM, Li J, Williamson DL. Caloric Restriction Normalizes Obesity-Induced Alterations on Regulators of Skeletal Muscle Growth Signaling. *Lipids* 2016;51:905–12. <https://doi.org/10.1007/s11745-016-4168-3>.
- [32] Williamson DL, Li Z, Tudor RM, Feinstein E, Kimball SR, Dungan CM. Altered nutrient response of mTORC1 as a result of changes in REDD1 expression: effect of obesity vs. REDD1 deficiency. *J Appl Physiol (1985)* 2014;117:246–56. <https://doi.org/10.1152/jappphysiol.01350.2013>.
- [33] Cederholm T, Bosaeus I, Barazzoni R, Bauer J, Van Gossum A, Klek S, et al. Diagnostic criteria for malnutrition - An ESPEN Consensus Statement. *Clin Nutr* 2015;34:335–40. <https://doi.org/10.1016/j.clnu.2015.03.001>.
- [34] Cederholm T, Jensen GL, Correia MITD, Gonzalez MC, Fukushima R, Higashiguchi T, et al. GLIM criteria for the diagnosis of malnutrition – A consensus report from the global clinical nutrition community. *Clinical Nutrition* 2019;38:1–9. <https://doi.org/10.1016/j.clnu.2018.08.002>.
- [35] Sieber CC. Malnutrition and sarcopenia. *Aging Clin Exp Res* 2019;31:793–8. <https://doi.org/10.1007/s40520-019-01170-1>.
- [36] Barbé C, Salles J, Chambon C, Giraudet C, Sanchez P, Patrac V, et al. Characterization of the Skeletal Muscle Proteome in Undernourished Old Rats. *Int J Mol Sci* 2022;23:4762. <https://doi.org/10.3390/ijms23094762>.
- [37] Salles J, Chanet A, Berry A, Giraudet C, Patrac V, Domingues-Faria C, et al. Fast digestive, leucine-rich, soluble milk proteins improve muscle protein anabolism, and mitochondrial function in undernourished old rats. *Mol Nutr Food Res* 2017;61. <https://doi.org/10.1002/mnfr.201700287>.

## TABLES

**Table 1 - Composition and nutritional values of the Yucatan minipig diets: the high-fat/high-sucrose diet (HF/HS), the standard diet (ST) or the low-calorie/low-protein (LC/LP) diet. ME, metabolizable energy; NE: net energy; DNM: digestible nitrogenous matter.**

<b>Nutrients / compounds</b>	<b>HF/HS diet</b>	<b>ST diet</b>	<b>LC/LP diet</b>
Soft wheat (%)	6.25	10	30.77
Barley (%)	12	33	10
Soybean meal 48 (%)	12	6	-
Soybean oil (%)	10	-	-
Dry beet pulp (%)	-	-	5
Molasses (%)	-	1	1
Soft wheat bran (%)	14	25	25
Unshelled sunflower meal (%)	8	10	-
Soybean hulls (%)	8	12	12
Sugar (%)	20	-	-
Cornstarch (%)	6.50	-	8.14
Cellulose (%)	-	-	5
Dicalcium phosphate (%)	0.60	0.60	0.60
Calcium carbonate (%)	1.30	1.30	1.10
Sodium chlorure(%)	0.60	0.60	0.89
Mineral Vitamin complement (%)	0.75	0.50	0.50
<b>Total (%)</b>	<b>100</b>	<b>100</b>	<b>100</b>
<b>ME (MJ/kg)</b>	<b>14.53</b>	<b>10.31</b>	<b>10.37</b>
<b>ME (kcal/kg)</b>	<b>3473</b>	<b>2463</b>	<b>2479</b>
<b>DNM/NE (%)</b>	<b>20.31</b>	<b>15</b>	<b>8.63</b>

**Table 2. Biological plasma analyses of the Yucatan minipigs subjected to the standard diet (ST) or the obesogenic diet (OB-ST) for 16 weeks.** Values are expressed as mean  $\pm$  SD. Data were compared between groups using the Mann-Whitney test. Significant differences are indicated in bold/italic. ALT, alanine aminotransferase; AST, aspartate aminotransferase; TG, triglycerides.

	<b>ST group (n=5)</b>	<b>OB-ST group (n=5)</b>	<b>P value</b>
Glucose (g/l)	0.66 $\pm$ 0.15	0.81 $\pm$ 0.05	0.079
Albumin (g/l)	46.39 $\pm$ 3.19	45.24 $\pm$ 3.16	>0.999
ALT (IU/l)	55.20 $\pm$ 13.02	37.30 $\pm$ 6.49	<b>0.032</b>
AST (IU/l)	79.33 $\pm$ 19.67	38.82 $\pm$ 10.49	<b>0.008</b>
Creatinin (g/l)	15.55 $\pm$ 0.89	15.31 $\pm$ 1.50	0.695
Urea (mg/l)	371.28 $\pm$ 88.15	313.44 $\pm$ 65.00	0.548
TG (mg/l)	386.48 $\pm$ 119.98	445.56 $\pm$ 271.18	>0.999
Total cholesterol (mg/l)	831.36 $\pm$ 162.75	761.35 $\pm$ 136.01	0.548

**Table 3. Biological plasma analyses of the obese Yucatan minipigs subjected to the standard diet (OB-ST) or the low-calorie/low-protein diet (OB-LC/LP) for 8 weeks.** Values are expressed as mean  $\pm$  SD. Significance was checked using Mann-Whitney test. Significant differences are indicated in bold/italic. ALT, alanine aminotransferase; AST, aspartate aminotransferase; TG, triglycerides.

	<b>OB-ST group (n= 5)</b>	<b>OB-LC/LP group (n=6)</b>	<b>P value</b>
Glucose (g/l)	0.81 $\pm$ 0.05	0.87 $\pm$ 0.06	0.18
Albumin (g/l)	45.24 $\pm$ 3.16	44.95 $\pm$ 2.82	0.66
ALT (IU/i)	37.29 $\pm$ 6.49	27.19 $\pm$ 2.66	<b>0.02</b>
AST (IU/l)	38.83 $\pm$ 10.49	33.21 $\pm$ 13.44	0.43
Creatinin (g/l)	15.31 $\pm$ 1.50	18.18 $\pm$ 2.08	0.052
Urea (mg/l)	313.44 $\pm$ 64.99	253.09 $\pm$ 75.19	0.33
TG (mg/l)	447.56 $\pm$ 271.18	348.22 $\pm$ 134.22	0.66
Cholesterol (mg/l)	761.35 $\pm$ 136.01	770.89 $\pm$ 54.31	>0.99

**FIGURE LEGENDS**

**Figure 1. Body weight and body composition assessed by computed tomography (CT)-scan at the 13<sup>th</sup> thoracic vertebra (Th13) level in Yucatan minipigs subjected to the standard diet (ST, n=5) or the obesogenic diet (OB-ST, n=5) for 16 weeks.** A: Evolution of Body weight. B-C: Evolution of retroperitoneal fat areas (absolute and normalized to body weight). D-E: Evolution of muscle T13 areas (absolute and normalized to body weight). F: Representative image of a CT-scan slice at the Th13 level measuring muscle mass area. Values are expressed as mean  $\pm$  SD. Significance was checked using Mann-Whitney test. Significant differences between ST and OB-ST groups: \* $p < 0.05$ ; \*\*  $P < 0.01$ . BW, body weight.

**Figure 2. Histological and Western Blot analyses performed in *trapezius* muscle from Yucatan minipigs subjected to the standard diet (ST, n=5) or the obesogenic diet (OB-ST, n=5) for 16 weeks.** A-B: Muscle fiber cross-sectional area (CSA) histological analyses. C: Frequency distribution of muscle fibers according to their surface. D: Representative images of immunostaining (WGA). E: Proportion of type I and type II muscle fibers determined by histological analyses (MHCI and MHCII staining). F: Representative immunostaining images for type I (dark) and type 2 (blank) fibers. G: Representative images of Western Blot analyses. H: Content of total or phosphorylated proteins involved in muscle protein turnover and energy metabolism. Data are presented as fold change in the OB-ST compared to the ST group (mean  $\pm$  SD, n=5/group). Significance was checked using Mann-Whitney test. Significant differences between ST and OB-ST groups:  $P < 0.05$ ; \*\*\* $P < 0.001$ .

**Figure 3. Body weight and body composition assessed by computed tomography (CT)-scan at the 13<sup>th</sup> thoracic vertebra (Th13) level in obese minipigs subjected to the standard diet (OB-ST, n=5) or to low-calorie/low-protein diet (OB-LC/LP, n=6) for 8 weeks.** A: Evolution of Body weight. B-C: Evolution of retroperitoneal fat areas (absolute and normalized to body weight). D-E: Evolution of muscle T13 areas (absolute and normalized to body weight). F: Representative image of a CT-scan slice at the Th13 level measuring muscle mass area. Values are shown as paired individual mean values (n = 5-6/group). Significance was checked using Wilcoxon test. Significant differences between OB-ST and OB-LC/LP groups: \* $p < 0.05$ . BW, body weight.

**Figure 4. Histological and Western Blot analyses performed in *trapezius* muscle from obese Yucatan minipigs subjected to the standard diet (OB-ST, n=5) or the low-calorie/low-protein diet (OB-LC/LP, n=6) for 8 weeks.** A-B: Muscle fiber cross-sectional area (CSA) histological analyses. C: Frequency distribution of muscle fibers according to their surface. D: Representative images of immunostaining (WGA). E: Proportion of type I and type II muscle fibers determined by histological analyses (MHCI and MHCII staining). F: Representative immunostaining images for type I (dark) and type 2 (blank) fibers. G: Representative images of Western Blot analyses. H: Content of total or phosphorylated proteins involved in muscle protein turnover and energy metabolism. Data are presented as fold change in the OB-LC/LP group compared to the OB-ST group (mean  $\pm$  SD, n=5-6). Data were compared using the Wilcoxon test. \* $p < 0.05$ . AU, arbitrary units.

Journal Pre-proof