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Impact of moderate dietary protein restriction on glucose homeostasis in a model of oestrogen deficiency

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Highlights:

- Low protein intakes drive fasting hyperglycaemia in ageing female rats.
- A low protein diet differentially affects the pancreas-liver-muscle axis based on oestrogen status.
- A low protein diet drives a down-regulation of GLUT4 protein expression in skeletal muscle of ageing SHAM rats.
- A low protein diet drives a hyperglucagonemia-associated hepatic stress in ageing OVX rats.

Abstract for Journal of Nutritional Biochemistry

The need to consume adequate dietary protein to preserve physical function during ageing is well recognized. However, the effect of protein intakes on glucose metabolism is still intensively debated. During age-related oestrogen withdrawal at the time of the menopause, it is known that glucose homeostasis may be impaired but the influence of dietary protein levels in this context is unknown. The aim of the present study is to elucidate the individual and interactive effects of oestrogen deficiency and suboptimal protein intake on glucose homeostasis in a preclinical model involving ovariectomy (OVX) and a 13-week period of a moderately reduced protein intake in 7-month-old ageing rats. To investigate mechanisms of action acting via the pancreas-liver-muscle axis, fasting circulating levels of insulin, glucagon, IGF-1, FGF21 and glycemia were measured. The hepatic lipid infiltration and the protein expression of GLUT4 in the gastrocnemius were analyzed. The gene expression of some hepatokines, myokines and lipid storage/oxidation related transcription factors were quantified in the liver and the gastrocnemius. We show that, regardless of the oestrogen status, moderate dietary protein restriction increases fasting glycaemia without modifying insulinemia, body weight gain and composition. This fasting hyperglycaemia is associated with oestrogen status-specific metabolic alterations in the muscle and liver. In oestrogen-replete (SHAM) rats, GLUT4 was down-regulated in skeletal muscle while in oestrogen-

deficient (OVX) rats, hepatic stress-associated hyperglucagonaemia and high serum FGF21 were observed. These findings highlight the importance of meeting dietary protein needs to avoid disturbances in glucose homeostasis in ageing female rats with or without oestrogen withdrawal.

Keywords:
Protein intake, oestrogen deficiency, hyperglycaemia, muscle, liver, glucagon, FGF21

1. Introduction:

Asymptomatic hyperglycaemia in association with progressive metabolic dysregulation represent important risk factors associated with age-related chronic diseases [1,2] and mortality [3]. Although the impact of diet on age-related health is largely recognized [4] including the role of fats and carbohydrates on dysregulation of glucose metabolism, the influence of dietary protein is still the subject of debate. Several clinical studies advocate that high protein consumption could be diabetogenic [5–7] while others argue that it does not affect fasting glycaemia adversely and even decreases glycated HbA1C levels significantly in patients with type 2 diabetes [8–10]. Higher protein intake could delay age-related muscle loss and have beneficial effects on glycemic control [11]. In studies considering risk of sarcopenia, a substantial proportion of ageing individuals have suboptimal protein intake [12–14] and this was shown to be associated with higher risk of functional decline, particularly in women [15]. During age-related menopause, it is still controversial whether or not the oestrogen status independently influences the regulation of glucose homeostasis [16]. While some studies did not observe any differences between pre- and postmenopausal women in fasting glycaemia and/or insulin sensitivity [17,18] others found a direct involvement of menopause in increased fasting glycaemia [3,19,20].

The maintenance of a normal glycaemic state relies on the synchronicity of complex regulatory mechanisms between organs mediated through a multiplicity of signals such as hormones and nutrients. The three main organs that interact for ensuring glucose homeostasis are: 1) the pancreas which orchestrates peripheral tissue glucose uptake and release through its secretion of insulin and glucagon respectively; 2) the liver, through glycogenesis and gluconeogenesis/glycogenolysis in response to insulin and glucagon respectively and 3) the skeletal muscle which is responsible for 50-60% of systemic glucose uptake mainly due to the expression of GLUT4, an insulin-responsive glucose transporter [21,22]. Moreover, fasting or postprandial hyperglycemia has been shown to be a steatogenic factor [23,24], a state of hepatic stress that could lead to the production of FGF21 [25,26]. FGF21, which is mainly produced by the liver, is a hepatokine that improves the whole-body insulin sensitivity [27] and inhibits the secretion of glucagon [28]. FGF21 circulating levels are increased in T2D obese patients [29] as well as in response to dietary protein restriction [30,31]. In preclinical models, low protein

intakes and impairment of oestrogen signalling have been shown to affect individually the skeletal muscle and/or the liver metabolic fitness (↑ hepatic fat accumulation, ↓ muscle oxidative metabolism) [30,32–36]. However, to date, how glucose homeostasis in response to suboptimal dietary protein is regulated by the liver and/or the skeletal muscle in the absence of oestrogen is unknown.

Thus, the aim of this study was to elucidate the individual and interactive effects of oestrogen deficiency and moderately reduced protein intake on glucose homeostasis in a rodent menopausal model and to understand the mechanisms of action via the pancreas-liver-muscle axis. Hence, fasting glycaemia, insulin, glucagon, IGF-1 and FGF21 circulating levels were assessed as well as the lipid infiltration in the liver and the protein expression of GLUT4 in the gastrocnemius. The gene expressions of some myokines, hepatokines and transcription factors involved in lipid storage or oxidation were evaluated at the liver and gastrocnemius levels. We found that a moderate protein restriction increases the fasting glycaemia in SHAM and OVX animals and this is associated with oestrogen status-specific metabolic alterations at the liver and muscle level.

2. Materials and Methods:

2.1. Animals and diets:

All animal procedures received approval from the Animal Ethics Committee of the University of Geneva, School of Medicine. Seven-month-old female Sprague-Dawley rats (n = 38; Charles Rivers, Iffa Credo, l'Arbresle, France) were housed individually at 25°C with 12: 12h light-dark cycle and had free access to demineralized water. After 2 weeks of acclimatization and pair feeding equilibration with the diet containing 14% casein, the rats underwent transabdominal ovariectomy (OVX) or a sham surgery (SHAM) under anesthesia with intraperitoneal ketamine hydrochloride (100 mg/kg body weight). Animals were strictly pair-fed isocaloric diets containing 14% (normal protein: NP) or 5% casein (low protein: LP) for 13 weeks. The low protein diet was made isocaloric by the addition of corn carbohydrates to ensure a similar energy intake for all animals (for diet composition see **supplementary table 1**). Rats were restricted to the average amount of food eaten by the SHAM-NP group the previous day. Four groups (SHAM-NP; SHAM-LP; OVX-NP; OVX-LP) containing 9 or 10 animals were investigated. Body weight was monitored weekly throughout the study. At the end of the dietary intervention, all rats were fasted for 6h before being sacrificed by an overdose of ketamine hydrochloride. Blood was withdrawn from the abdominal aorta for biochemical measurements. Liver and tibial muscles were weighed and immediately frozen in liquid nitrogen and stored at -80°C for later analysis.

2.2. Body composition:

Total fat mass and lean body mass were analyzed by using an EchoMRI-700 quantitative nuclear magnetic resonance analyzer (Echo Medical Systems, Houston, TX) at the end of treatment just before sacrifice.

2.3. Oil red O staining and image analysis of hepatic sections:

A section of frozen liver was embedded in NEG50TM compound (Thermo Fisher Scientific, UK) and sectioned at 5µm using a cryostat (MICROM HM 560). The liver section was then stained with Oil Red O and eosin using standard procedures. Images were acquired on Mirax 3DHistech microscope with 20x objective leading to calibration of 0.232 microns per pixels. To measure the fat content, subsequent image analysis was

performed with TissueStudio/Definiens software (Definiens AG, 80636 München Germany). Slides to be processed were assembled in workspaces and configurated for processing according to "IHC Dual Brown/Red Chromogene". The first step in image processing was selection of liver section areas where processing should be applied. This area was selected automatically by specifying parameters of tissue detection steps Tissue/Background Separation, Composer:Inialization, Composer:Training used for subsequent automatic analysis. The resulting areas were processed to isolate the red staining of Oil Red O. The biological considerations were based on the percentage of Marker objects with respect to the total number of objects expressed for each tissue area.

2.4. Quantitative Real-time PCR:

A section of frozen liver or gastrocnemius was homogenized in Tri Reagent® (Sigma Chemical Company, St Louis,Mo, USA) using a FastPrep system apparatus (QBiogene, Illkirch, France). Total RNA was extracted and then purified on mini-columns (RNeasy Plus Mini Kit, Basel, Switzerland) following the supplier's instructions. Total RNA (0.5µg) was reverse-transcribed using a mix of random hexamers – oligo d(T) primers and PrimerScript reverse transcriptase enzyme (Takara bio inc. Kit) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Green master mix (Applied Biosystems) on a SDS 7900 HT instrument (Applied Biosystem), and each sample was run in triplicate. Results were normalized to the mean of expression levels of *Actb* and *Gapdh* for the liver and *Ppia*, *Tubb4b* and *Gapdh* for the gastrocnemius and expressed as a percentage of the SHAM-NP group. Primer sequences are described in **supplementary table 2**.

2.5. Protein Immunoblotting:

Western blot analysis was performed using the NuPage System precast gels as described by the manufacturer (Novex, Life Technologies). Homogenization of gastrocnemius sample was prepared with TissueLyser (Qiagen) using ice-cold lysis buffer A (50 mmol/L Tris-HCL, pH 7.5, 1 mmol EDTA/L, 1 mmol EGTA/L, 10%glycerol, 1%triton-X, 50 mmol NaF/L, 5 mmolNa4P2O7/L, 1 mmoldithiothreitol/L, 10 mg trypsin inhibitor/mL, 2 mg aprotinin/mL, 1 mmol

benzamidine/L, and 1 mmol phenylmethylsulfonylfluoride/L). The lysate was centrifuged (12,000 x g, 20 min, 4°C) and after supernatant collection, the protein concentration was determined using the Pierce ®BCA Protein Assay Kit (Thermo Scientific). For all samples, equal amounts (5 μg) of protein were denatured by boiling for 10 minutes and loaded onto 4–12% Bis-Tris midi gels. Following electrophoresis, proteins were transferred to nitrocellulose membranes (iBlot gel Transfert Stacks, life technologies) and blocked for 1 hour with Odyssey Blocking Buffer at room temperature. The membranes were then incubated overnight at 4ºC with primary antibodies (GLUT4 (1:2000) ab654; Anti-AKT1 phospho (1:2000) ab66138; Anti-AKT1 (1:1000) ab91505 were purchased from Abcam; α-Tubulin (1:1000) #3873 were purchased from Cell Signaling Technology) which were diluted in 0.1% Tween Odyssey Blocking Buffer. After incubating with primary antibodies, the membranes were washed four times for five minutes, in PBS containing 0.1% Tween 20 (PBST). Then the membranes were incubated at room temperature for 1 hour in light restricted conditions with IRDye800CW-conjugated goat anti-rabbit or anti-mouse secondary antibodies (LI-COR Biosciences) diluted in Odyssey Blocking Buffer. The blots were then washed four times for five minutes, with PBST and rinsed with PBS. Proteins were visualized by scanning the membrane on an Odyssey Infrared Imaging System (LI-COR Biosciences) with 800-nm channels.

2.6. Biochemical assays /measures:

Plasma glucose, triglycerides (Roche Diagnostics, Basel, Switzerland) and free fatty acids (Wako, Neuss, Germany) were measured with commercial kits. Serum glucagon was evaluated using a glucagon radioimmunoassay kit (Linco Research, St Charles, MO). Serum insulin (Crystal Chem. USA), IGF-1 (IDS), FGF21 and adiponectin (Millipore) were evaluated by ELISA. The homeostasic model assessment of insulin resistance (HOMA-IR) was calculated as the product of the fasting glycaemia (mmol/L) and the fasting insulinemia (μU/mL), divided by the constant 22.5.

2.7. Statistical analysis:

Data were analyzed with Graphpad Prism (v8.4.0) and presented as mean ± SEM for all parameters measured except hepatic mRNA gene expression levels which are presented

as box plots. Data normality was tested with a D'Agostino and Pearson test. Normally distributed data were analyzed using a 2-way ANOVA to detect the OVX and protein diet effects and their interaction (O x D) after 13 weeks of treatment. Data that were determined to be non-normally distributed were transformed (square-root) and then re-analyzed with a 2-way ANOVA. Significance of main effects and their interaction were reported and Sidak's multiple comparison post-hoc tests were performed in order to assess differences between groups. Any data that were still determined to be non-normally distributed were analyzed using a non-parametric Kruskal-Wallis test (KW test). For body weight evolution a repeated measure ANOVA was applied. A *p*-value <0.05 was considered statistically significant. Pearson's correlation analyses were performed with variables normally distributed from untransformed and transformed (square-root) data. Although not removed from the statistical analysis, outlier values were identified according to the Grubb's test. For details of statistics for simple effects and interactions for hepatic gene expressions see **supplementary table 3** and for Pearson's correlation analyses see **supplementary table 4**.

3. Results:

3.1. Body weight gain, body composition and tissue weight

The reduced dietary protein diet resulted in a transient slower OVX-induced weight gain during the first 6 weeks following the surgery (**Figure 1A**) with complete catchup during the last 5 weeks of the study. Indeed, between week 0 and week 13, independently of the dietary protein intake, body weight gain was 3.8 and 3.9 times higher in the OVX group compared to the SHAM-NP group ($p < 0.001$) and the SHAM-LP group ($p < 0.001$), respectively (**Figure 1B**). This was associated with a rise of both fat mass (OVX effect; $p < 0.01$) and lean mass (OVX effect; $p < 0.001$) (**Figure 1C-D**) while the daily food intake between SHAM-NP and OVX-NP and between SHAM-LP and OVX-LP was not statistically different (Table 1). Moreover, regardless of the oestrogen status, the 5% casein isocaloric diet did not significantly change the final body weight and composition when compared to the NP groups (**Table 1** and **Figure 1C-D**). In NP and LP diet groups, OVX drastically decreased the uterus weight (g) and the uterus: body weight ratio (%), hence confirming the efficacy of the surgery (**Table 1**). In terms of skeletal muscle, OVX increased the gastrocnemius weight (g) (OVX effect; $p < 0.01$) whereas its relative weight (%) was not changed. Conversely,

OVX did not alter the soleus weight while its weight ratio was lowered (OVX effect; $p < 0.01$). At the liver level, although the weight (g) was not modified, in rats fed a 14% casein diet, OVX led to a reduction of the liver weight ratio (-15% vs. SHAM-NP group; $p < 0.0001$), whereas in the LP groups, no significant alteration was observed (**Table 1**).

3.2. Serum substrates and hormone concentrations

Independently of the oestrogen status, a 13-week period of a 5% casein isocaloric diet drastically increased the fasting glucose serum levels (+ 44% vs. SHAM-NP; $p < 0.01$; + 53% vs. OVX-NP; $p < 0.001$). Although serum TG levels tended to rise during dietary protein restriction, mostly in SHAM rats (+ 48% vs. SHAM-NP) this difference was not statistically significant ($p = 0.9561$ vs. SHAM-NP). Plasma FFA were not significantly altered (**Table 2**). In order to understand the LP diet-induced fasting hyperglycaemia, several hormones involved in the regulation of glucose homeostasis were analyzed. While fasting serum insulin concentration was not modified, circulating levels of glucagon and FGF21 were increased by the reduced protein intake in OVX animals (+ 78% vs. OVX-NP group; $p < 0.001$; + 153% vs. OVX-NP; group $p < 0.01$ respectively) (**Table 2**). For the serum glucagon, a significant interaction was observed ($p = 0.035$) indicating that the LP diet-induced hyperglucagonemia was related to the oestrogenous status of the animals. The concentrations of serum adiponectin and IGF-1 were not changed by the 5% casein isocaloric diet whereas OVX respectively increased them by 43% ($p < 0.001$) and 46% ($p < 0.001$) relative to the SHAM-NP group and by 52% ($p < 0.0001$) and 50% ($p < 0.001$) relative to the SHAM-LP group. Finally, the HOMA-IR, which is an index of insulin resistance, was increased by a low protein diet (Diet effect $p < 0.001$) mostly in OVX groups (+ 42% vs. SHAM-NP; $p = 0.069$; + 73% vs. OVX-NP; $p < 0.01$) (**Table 2**).

3.3. Hepatic lipid accumulation and related gene expression

Since the liver plays a crucial role in the maintenance of the fasting glycaemia and since alteration of glucose metabolism and hepatic lipid accumulation have been shown to be associated [23,24], we quantified the liver fat content following Oil Red O staining. A 5% casein isocaloric diet increased the % of Oil Red O staining relative to the total

surface analyzed and this statistically significant diet effect ($p < 0.05$) was mainly driven by the OVX-LP group (**Figure 2A**). Indeed, whereas 7 livers among 9 had more than 20% of the total surface stained with Oil-Red O in the OVX-LP group, there were only 3 livers out of 10 in OVX animals fed a NP diet. Thus, we hypothesized that fat accumulation in the liver may be the consequence of alterations in gene expression of key transcription (co-)factors involved in fat storage and/or oxidation. OVX increased the gene expression of *Pparg*, a lipogenic transcription factor (TF), by 45% and 68% (OVX effect; $p < 0.01$) when compared to the SHAM-NP and SHAM-LP groups, respectively (**Figure 2B**). Furthermore, in rats fed a 14% casein diet, OVX increased the gene expression of *Ppara* (+45% vs. SHAM-NP; $p < 0.05$) while, due to a significant outlier value in the SHAM-NP (Grubb's test), only a trend was observed for *Ppargc1a*. Conversely, in OVX groups, a LP diet led to lower mRNA levels of these two TF controlling FA oxidation (- 29% vs. OVX-NP; $p < 0.05$ for *Ppara* and - 40% vs. OVX-NP; $p < 0.05$ for *Ppargc1a*) (**Figure 2B**). These results suggest that a 13-week period of a 5% casein isocaloric diet dampened the oestrogen deficiency-related adaptation of the hepatic lipid metabolism in ageing pair-fed rats by notably involving a down-regulation of *Ppara* and *Ppargc1a* expression.

Fatty liver is associated with increased inflammation and altered production of hepatokines [37,38]. While the gene expression of *Tnfa* was not modified in our model, a LP diet led to lower *Hgf* and *Igf1* and higher *Inbha* mRNA levels in comparison with SHAM or OVX rats fed a 14% casein diet (Diet effect; $p < 0.01$; $p < 0.05$; KW $p < 0.01$; respectively) (**Figure 2C**). Besides, regardless of the dietary protein intakes, OVX drove a slight increase of *Igf1* gene expression and a ~2-fold rise of *Fst* mRNA levels when compared to SHAM groups (OVX effect; $p < 0.01$; respectively) (**Figure 2C**). Since a high *Inhba/Fst* mRNA ratio is associated with fatty liver [39], we evaluated how this ratio is modulated by OVX and/or dietary protein restriction. We did not observe any statistically significant differences due to the presence of two outlier values in the SHAM-LP and OVX-NP groups (Grubb's test) (**Figure 2D** and **supplementary Figure 1A**). Moreover, FGF21 was investigated here because it is a hepatokine produced in a situation of metabolic stress, hepatic fat accumulation [25,26] and dietary protein restriction [30,31]. Although the *Fgf21* gene expression was not significantly modulated (**Figure 2C**), a positive correlation with the FGF21 circulating levels was observed (r =

0.59 p = 0.0004), supporting that the liver was certainly the main FGF21-producing organ in our model (**Figure 2E**) as previously observed [30].

Finally, since a dietary protein restriction can affect the growth hormone receptor (GHR)/IGF-1/IGF-1 receptor (IGF-1R) signaling pathway which has been shown to be related to fatty liver [34,40], we analyzed the gene expression of *Ghr* and *Igf1r*. To decipher the involvement of other mechanisms, we investigated mRNA levels of *Gcgr*, which is implicated in the glucagon action, *Acvr2b*, which drove the Activin A action and *Sirt1* for which hepatic overexpression improves high fat diet (HFD)-related hepatosteatosis [41]. Gene expression of *Igf1r*, *Gcgr* and *Sirt1* was not modified by either OVX or the LP diet. Whereas *Ghr* mRNA levels were decreased by OVX mainly in the LP group (- 45% vs. SHAM-LP group; $p < 0.0001$; - 29% for OVX-NP group vs. SHAM-NP; $p < 0.001$), *Acvr2b* gene expression was increased by OVX (OVX effect; $p < 0.01$) and reduced by dietary protein restriction (Diet effect; $p < 0.01$) (**supplementary Figure 1**).

3.4. Expression of proteins and genes involved in protein synthesis and muscle energetic metabolism

Since AKT1 and GLUT4 are two essential proteins involved in protein synthesis and muscle glucose uptake, respectively [22,42], we analyzed whether our model could modify their expressions in the gastrocnemius. Regardless of the dietary protein intakes, a statistically significant OVX effect drove a reduction of phospho-AKT1 (pAKT1) / total AKT1 (tAKT1) ($p < 0.05$) (**Figure 3A-B**). The 13-week period of a 5% casein isocaloric diet dramatically decreased GLUT4 protein levels in SHAM rats (- 52% vs. SHAM-NP; $p < 0.05$) whilst no significant changes occurred in OVX groups (**Figure 3 A-C**). Since the muscle glucose uptake may be impaired by intramyocellular lipid accumulation [43], the gene expressions of *Pparγ* and *Fabp4*, two genes involved in the fatty acid uptake and storage, were evaluated. We observed no modifications of their mRNA levels in response to OVX and/or dietary protein restriction (**Table 3**). Finally we investigated the gene expression of several myokines implicated in the local and whole-body metabolic homeostasis [44]. *Igf1* and *Fgf21* mRNA levels did not change whereas *Il6* gene expression tended to increase in OVX groups (OVX effect; $p = 0.054$), mainly in rats fed a 14% casein diet (+ 72% vs. SHAM-NP group). The gene expression

of myonectin (*C1qtnf5*), a myokine which has a key function in the regulation of the lipid metabolism at the hepatic and systemic level [45,46], was significantly up regulated by OVX only in rats fed a LP diet (+ 87% vs. SHAM-LP group; $p < 0.001$) (**Table 3**).

4. Discussion:

A high fasting plasma glucose has been shown to be one of the ten leading risk factors for global disability-adjusted life-years from 1990 to 2015 in 195 countries and territories [47]. Based on this, aiming to reduce the extent of fasting hyperglycaemia is a critical clinical objective that unbalanced macronutrient composition diets can render arduous [48]. In this study we investigated the impact of dietary protein restriction on fasting blood glucose levels, related-hormone modulations and related liver and skeletal muscle metabolic responses according to oestrogen status. We found that, regardless of the oestrogen status, a 13-week period of a moderately LP diet (5% of daily energy intake) in seven-month-old female rats led to fasting hyperglycaemia. In SHAM animals, this circulating glucose disturbance was mainly associated with a down-regulation of the expression of GLUT4 protein in the gastrocnemius, while in OVX animals it was associated with higher glucagon and FGF21 serum concentrations and modifications of hepatic lipid metabolism.

In the present study, OVX led to higher body weight, fat and lean mass without modification of fasting glycaemia, HOMA-IR and hepatic lipid content. Consistent with the gain of weight observed in estrogen receptor-α knockout mice having unaltered daily energy intake but reduced energy expenditure (↓ voluntary physical activity, ↓ basal metabolic activity and ↓ heat production) [49,50], the oestrogen signaling disruption in the OVX groups drove body weight increase despite the pair-feeding-related limitation of food intake. Moreover, the prevention of OVX-related hyperphagia by pair-feeding has been shown to prevent hepatic lipid accumulation [51]. Accordingly, the pair-feeding that OVX animals sustained in our study, by limiting body weight gain, could prevent or delay insulin resistance and fatty liver establishment that some reports had noticed in response to OVX without food restriction [36,52,53]. At a molecular level, we showed that NP diet fed OVX rats presented an increased hepatic gene expression of both *Pparg* and *Ppara*, suggesting an effective adaptation of

the liver to oestrogen deficiency thanks to an adequate balance of lipogenesis and lipid oxidation to prevent lipid accumulation. While other studies found an OVX-related increase of *Pparg* expression in the liver in accordance with our results [54,55], discrepancies exist regarding the impact of oestrogen deprivation on the hepatic gene expression of *Ppara* (↓ [53]; ↔ [51,56]; ↑ [55] and the present study). Of note, the duration of fasting before sacrifice has a substantial impact on hepatic fat content [57] which may help to explain the differences between studies in terms of gene expression. In addition, we found that estrogen deficiency caused higher circulating levels of IGF-1, as reported in our previous study [58], and adiponectin. These two factors are known to directly sensitize the whole body to insulin [59–61] and their high serum concentrations may dampen OVX-related glucose and hepatic metabolic disturbances especially in a food restriction state. Moreover, although we did not observe any modification of fasting insulinemia and HOMA-IR in response to oestrogen depletion, to assess whole-body insulin resistance further insulin measurements under a glucose challenge would be needed as previously reported [52].

Although the impact of dietary protein levels on glucose homeostasis is still debated, the fact that protein intakes beyond the recommended dietary allowance (RDA) is beneficial to older people for the maintenance of muscle mass and health is now widely recognized [62]. In the present study we showed that moderate protein restriction drove fasting hyperglycaemia regardless of the oestrogen status. In preclinical studies, the impact of dietary protein restriction on glucose metabolism has been mainly evaluated in young growing rats for which protein needs are particularly high. In this specific context, protein malnutrition leads to an impairment of body growth due to changes of circulating hormone amounts and/or actions that aim at using nutrients very efficiently (↑ insulin sensitivity, glucose tolerance, ↓ hepatic gluconeogenesis) and preventing protein anabolism concomitantly (↓ serum IGF-1, ↑ FGF21) [34,63–65]. Here, we employed seven-month old SHAM and OVX rats, which required reasonably lower protein intakes than growing animals. We showed that a 5% casein isocaloric diet did not compromise the body weight evolution over time, the final body composition and muscle weight. In addition, this decrease in protein intake was not associated with lower serum IGF-1. In comparison with our previous studies, in which a 2.5% casein

isocaloric diet induced a long-term reduction of body weight and serum IGF-1 in ageing intact, SHAM or OVX female rats [66,67], the dietary protein restriction used here is considered as moderate amino-acid depletion.

Skeletal muscle and liver are two organs particularly involved in the regulation of glycaemia. A novel observation in this study was the differential response of these two organs to moderate dietary protein restriction in function of the oestrogen status. While the LP diet tested in this study had no impact on the weight of the liver and the gastrocnemius, important modifications of gene or protein expression were observed.

In oestrogen replete ageing animals, the expression of GLUT-4 in the gastrocnemius was reduced by the LP diet and this was not associated with changes in insulin, glucagon or FGF21 serum concentrations. Although the main mechanism responsible for muscle glucose uptake is the insulin-stimulated translocation of GLUT-4 from the cytoplasm to the plasma membrane, the total amount of GLUT-4 protein in the muscle is also crucial for controlling the glycaemia. This was well demonstrated previously by a princeps study using mice with muscle-specific deletion of GLUT-4 in which fasting glycaemia and insulin-stimulated muscle glucose transport were increased and blunted respectively when compared to control mice [22]. Another study has also shown that the insulin-independent upregulation of GLUT-4 protein in the skeletal muscle in response to exercise is related to posttranscriptional regulation and is associated with the reversal of some metabolic disturbances in obese mice [68]. These results support the major role of the total amount of GLUT-4 protein and not only its subcellular location for glycaemia control. Based on that, our results suggest a likely implication of the LP diet-induced downregulation of the GLUT-4 protein in the gastrocnemius for the altered fasting glycaemia in SHAM ageing rats. The observation of a negative association between the expression of GLUT4 and fasting glycaemia is also consistent with this hypothesis (**supplementary table 4**; r = -0.75 p = 0.005).

The liver is highly sensitive to protein deprivation, a stressful nutritional state leading to fatty liver and hepatic production of FGF21 [30,34,69]. In this study, the moderate dietary protein restriction-related hepatic lipid accumulation and FGF21 serum increase is mostly driven by the OVX-LP group. This is consistent with the 5% casein isocaloric diet-associated gene down expression of *Ppara* and *Ppargc1a*, two major TFs involved

in fatty acid oxidation. In agreement with our findings, chronic and medium reduction of PGC1α in the liver has been shown to lead to hepatic fat accumulation and hepatic insulin resistance [70]. Moreover, a LP diet could modify the protein expression pattern of insulin receptor substrate-2 (IRS-2) in mouse liver while IRS-2 signaling is important for insulin action and resistance in hepatocytes [71]. In the present study HOMA-IR is increased in the OVX rats fed a reduced protein intake suggesting that this type of diet favours insulin resistance in oestrogen depleted ageing pair-fed animals. Although we did not investigate the insulin resistance at the liver level directly, we observed in the OVX groups a negative correlation between fasting glycaemia and hepatic gene expression of *Ppargc1a* (**supplementary table 4**; r = -0.73; p = 0.002). This finding raises an interesting question on the role of this TF at the hepatic level and the need to better understand its implication in the disturbances of hepatic and systemic glucose metabolism in response to various diets particularly in an oestrogen withdrawal state. Indeed, the crosstalk between oestrogen signaling and PGC1α in the hepatic response to nutrient stressors is crucial and the disruption of both of them potentiates liver damage [72].

Consistent with the known role of glucagon in regulation of glycaemia via hepatic release of glucose, the results found here show a positive association between fasting glycaemia and the increase in the fasting circulating concentration of glucagon with moderate dietary protein restriction (**supplementary table 4**; r = 0.65; p = 0.003) in OVX rats. Oestrogen depletion has been shown to increase glucagon production both in mouse and human pancreatic α cells [73]. Although we did not find any modification of glucagon serum in response to OVX, the LP diet-associated hyperglucagonemia was observed only in oestrogen depleted rats highlighting the interaction between dietary protein restriction and oestrogen withdrawal on glucagon secretion in a fasted state. Moreover, glucagon stimulates hepatocyte fatty acid oxidation by specifically inducing the protein or gene expression of PPARα or *Ppargc1a*, respectively [74,75] and increases the hepatic amino acid catabolism [76]. In a state of oestrogen depletion, the dietary protein restriction induced- hyperglucagonemia was associated with lower hepatic mRNA levels of *Ppara* and *Ppargc1a* suggesting rather a reduced fatty acid oxidation and possibly a liver resistance to the glucagon lipolytic action

(**supplementary table 4)**. Importantly, whereas fasting hyperglucagonemia characterizes T2D and prediabetic patients, not all show alterations of fasting glucagon circulating levels. Hepatic steatosis has been proposed to discriminate patients with fasting hyperglucagonemia by inducing glucagon resistance and subsequent pancreatic α-cell over activity [77].

FGF21 is a hepatokine which transmits the metabolic status of the liver to other metabolically active tissues (adipose tissue, muscles, nervous system). While FGF21 has been shown to improve the sensitivity to insulin, its circulating levels are increased in humans and rats with obesity and diabetes due to a resistance state to FGF21 [78,79]. Consistent with these studies, even if serum FGF21 is increased in the OVX-LP group, the level of insulin resistance remains higher when compared with OVX rats fed a NP diet. These results support a resistance state to FGF21 in our model as well.

FGF21 is also a mediator of the glucagon actions [80,81]. FGF21 has been shown to inhibit glucagon secretion in isolated rat pancreatic islets [82] and to reduce plasma glucagon in mice [83]. In the OVX-LP group, although FGF21 is increased, serum glucagon levels remain high, possibly reflecting an impaired liver-α cells axis characterized by a pancreatic resistance to FGF21 in addition to a hepatic resistance to glucagon. Although further studies on the underlying mechanisms are necessary for understanding the LP diet-associated disturbances of glucose homeostasis in a state of oestrogen withdrawal, the current results suggested that the alteration of the crosstalk between the liver and the pancreas would be involved.

In conclusion, our findings show that a moderate dietary protein restriction in ageing females rats drives fasting hyperglycaemia mostly associated with an impairment of the skeletal muscle GLUT-4 expression in oestrogen-replete rats and an impairment of the liver-α cells axis in oestrogen depleted animals. Our study highlights the importance to consider the dietary protein intakes according to the protein requirements for a specific physiological state in order to avoid hepatic, muscular and systemic metabolic disorders potentially promoting the development of a pre-diabetic/diabetic state with ageing.

Author contributions:

P.A., E.O., M.N.H. and S.M.S. designed the initial experiment. C.F., L.G.K. and K.B. conceived the hypothesis. C.F. wrote the application for animal ethics approval, conducted experiments, performed data analyses and data interpretation and wrote the manuscript. L.G.K., S.M.S. and K.B. participated to experiment achievement. K.B., L.G.K., T.D. and P.A. participated to data interpretation and reviewed and edited the manuscript.

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Conflict of interest:

E.O., M.N.H., and L.G.K. are full-time employees of Société des Produits Nestlé SA.
The authors declare that they have no competing interests.

Figure legends:

Figure 1:

Design of the study (A). Evolution of body weight throughout the experimental period, including 2 weeks of diet equilibration and 13 weeks of isocaloric diets containing 14% (normal protein: NP) or 5% casein (low protein: LP) (B). Body weight gain between week 0 and week 13 in grams (C). Fat and lean masses in grams at the end of the experimentation (D-E). N = 9-10 per group. *P* value for differences between groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. SHAM group for corresponding protein intake; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. normal protein group for corresponding oestrogen status using a post-hoc Sidak's test (for the two-way ANOVA) or a post-hoc Dunn's test (for the Kruskal-Wallis test). When the interaction in the two-way ANOVA or the Kruskal-Wallis test is statistically significant ($p < 0.05$); groups were compared regardless the protein intake and the oestrogen status.

Figure 2:

Representative images of Oil Red O and eosin-staining of liver frozen sections after 13 weeks of experimentation. Quantification of the % of Oil Red O stained surface relative to the total surface analyzed. N = 8-10 per group (A). Hepatic expression of genes involved in lipid metabolism (B). Hepatic gene expression of soluble molecules with local and whole-body action. N = 7-8 per group (C). Hepatic *Inhba*/*Fst* mRNA ratio N = 7-8 per group (D). Scatterplot of serum FGF21 correlation with hepatic *Fgf21* gene expression using all groups (E). *P* value for differences between groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. SHAM group for corresponding protein intake; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. normal protein group for corresponding oestrogen status; ¤ $p < 0.05$ vs. OVX-NP using a post-hoc Sidak's test (for the two-way ANOVA) or a post-hoc Dunn's test (for the Kruskal-Wallis test). When the interaction in the two-way ANOVA or the Kruskal-Wallis test is statistically significant ($p < 0.05$); groups were compared regardless the protein intake and the oestrogen status.

Figure 3:

Western blots of pAKT1, AKT1, GLUT4 and α-Tubulin (A). Quantification of pAKT1/AKT1 ratio (B) and GLUT4/ α-Tubulin ratio (C). N = 5-6 per group. *P* value

for differences between groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. SHAM group for corresponding protein intake; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. normal protein group for corresponding oestrogen status using a post-hoc Sidak's test (for the two-way ANOVA) or a post-hoc Dunn's test (for the Kruskal-Wallis test). When the interaction in the two-way ANOVA or the Kruskal-Wallis test is statistically significant ($p < 0.05$); groups were compared regardless the protein intake and the oestrogen status.

Supplementary Figure 1:

Hepatic *Inhba*/*Fst* mRNA ratio without outliers. N = 6-8 per group (A). Hepatic gene expression of *Ghr*, *Igf1r*, *Acvr2b*, *Gcgr*, *Sirt1*. N = 8 per group (B). *P* value for differences between groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. SHAM group for corresponding protein intake; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. normal protein group for corresponding oestrogen status using a post-hoc Sidak's test (for the two-way ANOVA) or a post-hoc Dunn's test (for the Kruskal-Wallis test). When the interaction in the two-way ANOVA or the Kruskal-Wallis test is statistically significant ($p < 0.05$); groups were compared regardless the protein intake and the oestrogen status.

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Table 1: Final body weight, food intake and organ weight

					Statistical analysis (*p*)		
	SHAM-NP	SHAM-LP	OVX-NP	OVX-LP	OVX effect	Diet effect	Interaction
Final body weight (g)	334.1 ± 7.6	335.0 ± 6.3	375.6 ± 4.4****	372.4 ± 5.3***	< 0.0001	0.851	0.737
Food intake (g/day)	14.5 ± 0.2↓	14.8 ± 0.3	15.1 ± 0.1	15.2 ± 0.1	Kruskal-Wallis test : < 0.05		
Organ weight (g)							
Uterus	0.73 ± 0.07	0.77 ± 0.09	0.15 ± 0.01***	0.16 ± 0.01**	Kruskal-Wallis test : < 0.0001		
Liver	7.81 ± 0.28	7.74 ± 0.24	7.43 ± 0.22	7.97 ± 0.19	0.760	0.334	0.207
Gastrocnemius	1.58 ± 0.02	1.64 ± 0.03	1.75 ± 0.03**	1.70 ± 0.05	0.002	0.870	0.171
Soleus	0.138 ± 0.006	0.137 ± 0.003	0.138 ± 0.003	0.135 ± 0.006	0.888	0.623	0.834
Organ weight ratio (% of body weight)							
Uterus	0.22 ± 0.02	0.23 ± 0.03	0.04 ± 0.002***	0.04 ± 0.002**	Kruskal-Wallis test : < 0.0001		
Liver	2.34 ± 0.06	2.31 ± 0.07	1.98 ± 0.0005***	2.14 ± 0.0005(* p = 0.07)	< 0.0001	0.216	0.109
Gastrocnemius	0.47 ± 0.01	0.49 ± 0.01	0.47 ± 0.01	0.46 ± 0.01	0.064	0.808	0.233
Soleus	0.041 ± 0.002	0.041 ± 0.001	0.037 ± 0.001*	0.036 ± 0.001*	0.0010	0.605	0.983

The results are means ± SEM. They were obtained after 13 weeks of diet. N = 9-10 per group. *P* value for differences between groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. SHAM group for corresponding protein intake; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. normal protein group for corresponding oestrogen status; ↓ $p < 0.05$ vs. OVX-LP group using a post-hoc Sidak's test (for the two-way ANOVA) or a post-hoc Dunn's test (for the Kruskal-Wallis test). When the interaction in the two-way ANOVA or the Kruskal-Wallis test is statistically significant ($p < 0.05$); groups were compared regardless the protein intake and the oestrogen status.

Table 2: Biochemical measurements

					Statistical analysis (*p*)		
	SHAM-NP	SHAM-LP	OVX-NP	OVX-LP	OVX effect	Diet effect	Interaction
Glucose (mmol/L)	10.3 ± 0.6	14.8 ± 0.9$^{\#\#}$	10.6 ± 1.0	16.2 ± 0.6$^{\#\#\#}$	0.392	< 0.0001	0.469
FFA (mEq/L)	0.61 ± 0.03	0.49 ± 0.04	0.61 ± 0.06	0.58 ± 0.04	0.348	0.093	0.336
Triglycerides (mmol/L)	0.58 ± 0.05	0.86 ± 0.11$^{¤¤}$	0.43 ± 0.02	0.53 ± 0.04	Kruskal-Wallis test : 0.003		
Insulin (ng/mL)	0.34 ± 0.03	0.35 ± 0.03	0.32 ± 0.01	0.36 ± 0.04	Kruskal-Wallis test : 0.782		
Glucagon (pg/mL)	61.1 ± 6.9	70.2 ± 4.6$^{¤}$	44.5 ± 4.5	79.4 ± 7.2$^{\#\#\#}$	0.535	0.0007	0.035
FGF21 (pg/mL)	294.6 ± 45.0	465,1 ± 170.2	243.4 ± 30.5	616,0 ± 150,2$^{\#}$	Kruskal-Wallis test : 0.032		
Adiponectin (ng/mL)	15330.0 ± 1602.1	14785.0 ± 1658.3	21961.5 ± 716.8**	22533.3 ± 928.0***	< 0.0001	0.992	0.663
IGF-1 (ng/mL)	539,7 ± 59.0	570.2 ± 58.1	786.3 ± 36.9**	854.6 ± 52.1**	< 0.0001	0.345	0.716
HOMA-IR	4.72 ± 0.64	6.72 ± 0.76 ($\#\ p = 0.07$)	4.40 ± 0.52	7.61 ± 0.80$^{\#\#}$	0.694	0.0004	0.376

The results are means ± SEM. They were obtained after 13 weeks of diet. N = 8-10 per group. *P* value for differences between groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. SHAM group for corresponding protein intake; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. normal protein group for corresponding oestrogen status; ¤ $p < 0.05$; ¤¤ $p < 0.01$ vs. OVX-NP using a post-hoc Sidak's test (for the two-way ANOVA) or a post-hoc Dunn's test (for the Kruskal-Wallis test). When the interaction in the two-way ANOVA or the Kruskal-Wallis test is statistically significant ($p < 0.05$); groups were compared regardless the protein intake and the oestrogen status.

Table 3: Gene expressions in the gastrocnemius

					Statistical analysis (*p*)		
	SHAM-NP	SHAM-LP	OVX-NP	OVX-LP	OVX effect	Diet effect	Interaction
Ppary	100.0 ± 11.1	78.6 ± 9.9	90.9 ± 19.3	105.7 ± 18.7	0.564	0.832	0.252
Fabp4	100.0 ± 1.8	89.5 ± 4.4	104.7 ± 16.6	105.4 ± 16.4	0.398	0.689	0.641
Il6	100.0 ± 11.4	101.0 ± 21.8	171.6 ± 17.1	134.3 ± 41.5	0.054	0.487	0.463
Fgf21	100.0 ± 19.1	127.8 ± 15.2	124.2 ± 32.1	92.0 ± 14.1	0.706	0.960	0.203
Igf1	100.0 ± 10.4	95.4 ± 10.7	101.8 ± 8.6	117.2 ± 15.5	0.322	0.642	0.397
C1qtnf5	100.0 ± 10.1	73.0 ± 5.5	110.2 ± 9.0	136.3 ± 12.6***	0.001	0.961	0.012

The results are means ± SEM. They were obtained after 13 weeks of diet and were expressed in % of the SHAM-NP group. N = 6 per group. *P* value for differences between groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. SHAM group for corresponding protein intake; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. normal protein group for corresponding oestrogen status using a post-hoc Sidak's test. When the interaction in the two-way ANOVA or the Kruskal-Wallis test is statistically significant ($p < 0.05$), groups were compared regardless the protein intake and the oestrogen status

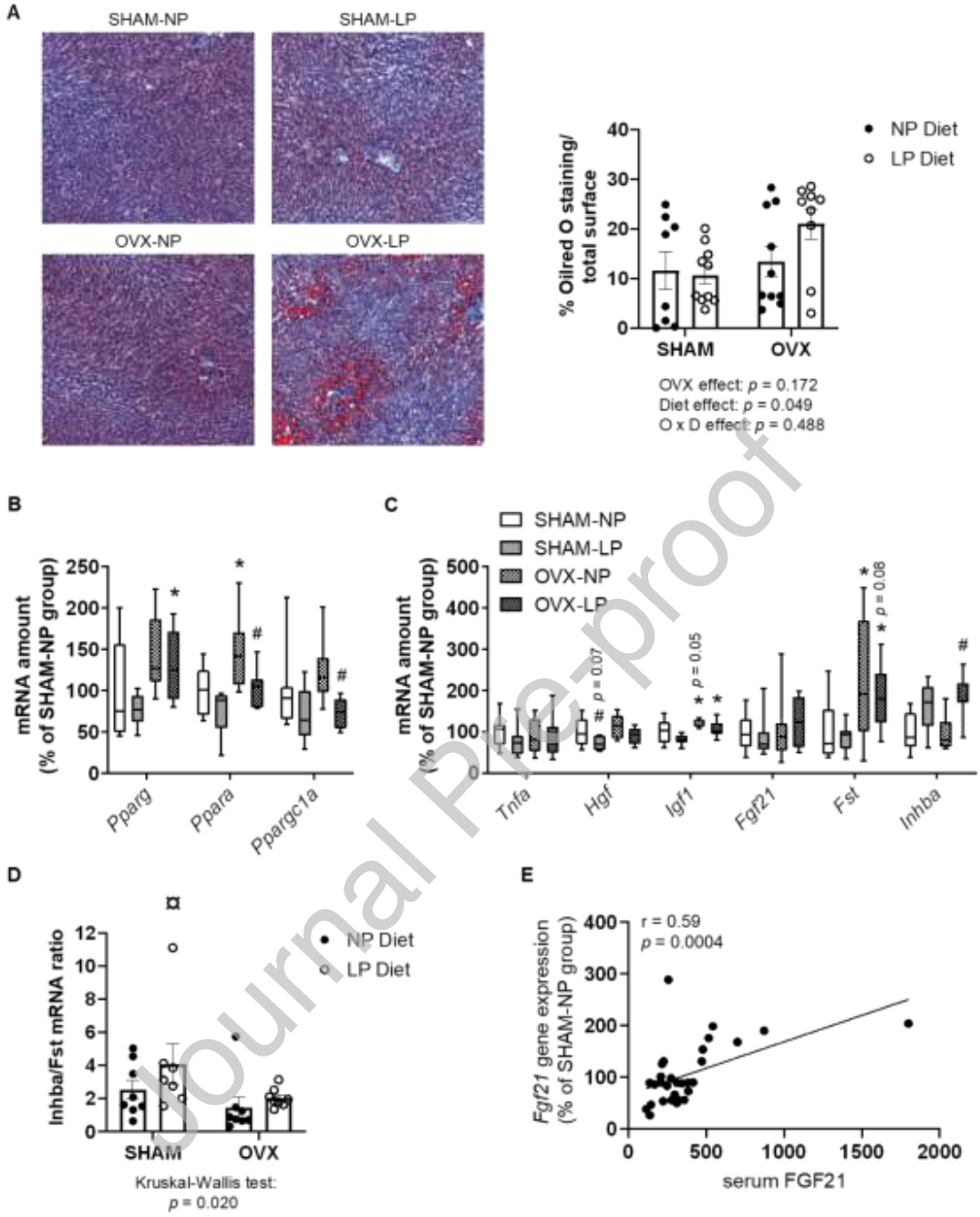

Figure 2: Fournier C et al.

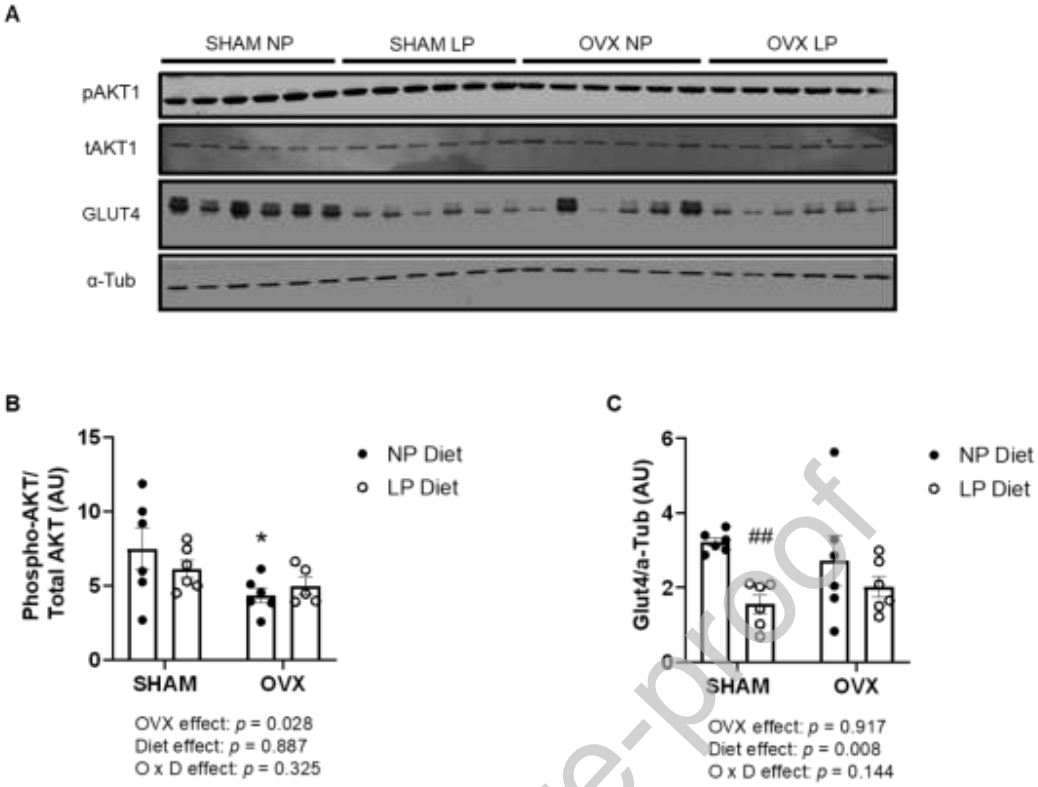

Figure 3: Fournier C et al.