Pleiotrophin deletion alters glucose homeostasis, energy metabolism and brown fat thermogenic function.

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Aims/hypothesis: Pleiotrophin, a developmentally regulated and highly conserved cytokine, exerts different functions including regulation of cell growth, migration and survival. Here we hypothesize that this cytokine can play a regulatory role for glucose and lipid homeostasis.

Methods: To test our hypothesis, we used gene targeted Ptn deficient female mice and their corresponding wild-type counterparts, and performed a longitudinal study characterizing the metabolic profile of our animal model at different ages from the young adulthood (3 months) to older ages (15 months).

Results: Our results reveal for the first time that pleiotrophin is a key player to preserve insulin sensitivity, driving the dynamics of adipose tissue lipid turnover and plasticity and regulating energy metabolism and thermogenesis.

Conclusions/interpretation This study opens therapeutic avenues for the treatment of metabolic disorders by targeting pleiotrophin in the white-brown adipose tissue crosstalk.
What is already known about this subject?

Pleiotrophin signaling may be involved in the inhibition of white preadipocytes differentiation *in vitro*.

What is the key question?

Is pleiotrophin a modulator of lipid and glucose homeostasis regulating fat accumulation, body composition and brown fat thermogenic function?

What are the new findings?

Pleiotrophin deficiency ameliorates insulin sensitivity in later life that is associated with an altered expansibility of periovaric adipose fat as well as with an enhanced cell differentiation and thermogenesis of brown adipose tissue.

The impaired lipid and glucose homeostasis in *Ptn* deficient mice can be attributed to a defective PPAR-γ activation.

How might this impact on clinical practice in the foreseeable future?

This study opens therapeutic avenues for the treatment of metabolic disorders by targeting pleiotrophin in the white-brown adipose tissue crosstalk.
INTRODUCTION

Pleiotrophin (PTN) is a highly conserved cytokine that belongs to a family of heparin-binding growth factors [1, 2]. PTN is found in cells in early differentiation stages, especially during embryonic development [3, 4]. PTN contributes to the epithelial-mesenchymal interactions in organs undergoing branching morphogenesis [5-7] and participates in bone formation [8].

In adult rodents there is a residual Ptn expression restricted to uterine cells, and discrete populations of the nervous system [9]. An almost identical and limited expression profile was found in human adult tissues [10]. On the contrary, PTN levels are upregulated in the uterus and placenta during pregnancy [9], in early stages of differentiation of cell types involved in repair [11, 12], and in inflammatory processes [13].

Besides its role in tumor growth, PTN functions include regulation of cell growth, migration and survival [14]. Importantly, PTN signaling might have an inhibitory role in preadipocytes differentiation in vitro [15], that is attributable to the cross-talk between the PTN/PI3K/Akt/GSK-3β/β-catenin and the Wnt/β-catenin signaling pathways to repress adipogenesis [16]. In fact, after induction of white adipocyte differentiation endogenous PTN decreases [17], and the in vitro administration of rPTN inhibits white adipocyte differentiation, including a decreased expression of white adipocyte markers, like Pparγ2 [16, 17].

Our hypothesis points out a novel role of pleiotrophin as a modulator of lipid and glucose homeostasis that might be involved in fat accumulation and body composition, due to adipose tissue-specific actions. To address this question, we monitored and phenotyped a whole-body constitutive pleiotrophin knockout-out mouse model (Ptn−/−) at different stages of adult life.
METHODS

Animals

PTN genetically deficient (Ptn−/−) mice on a C57BL/6J background were generated as described [18, 19]. Female Ptn−/− and wild-type (Ptn+/+) mice were housed at 22–24°C with 12-h/12-h light/dark cycles, from 08:00 to 20:00 h, and free access to water and chow diet. All the animals were maintained in accordance with European Union Laboratory Animal Care Rules (86/609/ECC directive) and the protocols approved by the Animal Research Committee of CEU-San Pablo University. Fasted mice of 3, 6, 12 and 15-months of age from each genotype were exposed to CO2 and killed by decapitation. Plasma and organs were collected and preserved at -80°C.

**Determination of body fat.**

Total body fat was extracted of the dried mummies of 6-months old mice in a soxhlet using chloroform-methanol (3:1).

**Plasma analysis, estimation of insulin resistance and glucose tolerance tests (GTT)**

Glucose (GOD-PAP, Roche Diagnostics), triacylglycerides (LPL/GPO-Trinder, Roche Diagnostics), and nonesterified fatty acids (NEFA) (ACS-ACOD; Wako Chemicals GmbH, Germany) were determined by enzymatic colorimetric tests. Plasma insulin (Mercodia), and free T4 (DRG) were determined using immunoassay kits. Plasma leptin was measured with a Bioplex pro-Mouse diabetes-Immunoassay kit (Bio-Rad). Insulin sensitivity was estimated as previously described [20]. GTT were performed in 6-h-fasted mice after intraperitoneal glucose administration (2g/kg) and the areas under the curve for glucose were calculated.
**q-PCR in white adipose tissue.**

RNA was isolated with Rneasy Mini Kits (Qiagen). First-strand cDNA were synthesized using the iScript cDNA-Synthesis kit (Bio-Rad), and were used for real-time qPCR analysis in a CFX96 Real-Time System (Bio-Rad). See Table1 for primer sequences.

**Histological studies**

Periovaric adipose tissue (AT) was stained with hematoxylin-eosin and analyzed in an optical microscope.

**Lipolysis analysis in isolated adipocytes.**

Adipocytes isolation from periovaric AT of 15-months old mice was performed as described [21]. To test catecholamine-stimulated lipolysis, isolated adipocytes were incubated with 1 U/ml of adenosine deaminase (Sigma-Aldrich, Spain) in the absence or presence of isoproterenol (Sigma-Aldrich). Lipolysis was quantified as liberation of glycerol into the media, determined by the GPO-Trinder method (Sigma-Aldrich). Basal lipolysis, half-maximal effective agonist concentration (EC50) and maximum lipolytic effect (Emax) values were estimated from the concentration-effect curves of glycerol. To measure the effect of insulin on lipolysis, isolated adipocytes were incubated with 0, 1, 10 or 100 nM insulin (Sigma, Madrid) prior to addition of isoproterenol (100 nM), and incubated for 90 min. Half-maximal effective agonist concentration (IC50) and maximum effect (Imax) values were estimated.

**Indirect calorimetry.**

6 month-old Ptn+/+ and Ptn−/− mice were individually housed in chambers maintained at 24±2°C with free access to chow and water, and adapted to the new environment for 72h. After adaptation mice were housed three days at 24°C, and seven days at 30°C. Oxygen consumption (VO₂) and CO₂ production (VCO₂) of each individual mouse were measured
every 30 min during 24h in labMaster metabolic cages. The respiratory exchange ratio (RER) was calculated as \( \frac{VCO_2}{VO_2} \). Data of total energy expenditure (EE) were used for calculation of cold-induced thermogenesis as a fraction of total daily EE as described [22].

**Analysis in brown adipose tissue (BAT).**

Total T3 and T4 concentrations were determined in BAT from 6-months old mice by radioimmunoassays, as described [23]. The Dio2 activity was assayed in BAT homogenates as previously described [24]. Mouse Dio2 mRNA was quantified by qRT-PCR using specific Taqman probes for mouse Dio2. Results were normalized to cyclophilin. Ucp-1 was determined by western blot, using a specific anti-UCP1 antibody (Chemicon) followed by corresponding secondary HRP-antibody (Sigma-Aldrich), and visualization by ECL (Amersham Biosciences); values were normalized with β-actin.

**Cell culture and differentiation of brown adipocytes**

Immortalized mouse brown adipocytes (mBA) were generated as described [25], grown in DMEM supplemented with 10% fetal serum, 1% penicillin/streptomycin and 2mM HEPES, and differentiated until they exhibited numerous multilocular cytoplasmatic lipid droplets [26]. Undifferentiated (day 0) preadipocytes were treated with 0.1 mg/ml pleiotrophin (PTN, Sigma) until day 6 of differentiation, when typically, non-treated preadipocytes achieve a fully differentiated phenotype. Markers of mBA differentiation (Cidea, Pgc1a and Prdm16) and Ptn mRNA levels were measured by qPCR (Table 1).

**Statistical analysis**

Results are expressed as mean ± SEM. When data were not normally distributed, log-transformed values were used for statistical analysis. Statistical comparisons between two groups were made using the Student’s t-test; comparisons between three or more
groups were made by ANOVA with 1 or 2 ways, followed by Student-Newman Keuls as 
post hoc test, using GraphPad Prism (v.5).

RESULTS

*Ptnt/* mice show reduced body weight and adiposity*

Survival rate was similar in both genotypes (Fig.1a). We first investigated whether *Ptnt* deletion affects body weight and fat distribution. Body weight of *Ptnt/* was significantly lower during the whole-time course of the study (Fig.1b). Furthermore, we found that the weights (Fig.1c-d) of periovaric and retroperitoneal AT were significantly higher in young *Ptnt/* mice, whereas in older mice, the weight of both fat depots was significantly lower in *Ptnt/* mice, pointing to a switch in the fat distribution by deletion of *Ptnt*, which evolved from a higher degree of adiposity in young animals to a slim-like condition in older mice. Accordingly, the analysis of total body composition in a subset of 6-months-old mice corroborated these data. *Ptnt/* mice showed a significant reduction in total body fat and a significant increase in water content as compared to controls (Fig.1e).

The increased adiposity observed in young *Ptnt/* mice parallels their higher leptin levels as compared to their age-matched controls (Fig.1f). Moreover, *Ptnt/+* mice exhibited an age-related hiperleptinemia, while circulating leptin in *Ptnt/* animals did not increase with age, remaining lower than in their wild-type counterparts in older ages. These significantly lower leptin levels in older *Ptnt/* mice can be explained by their reduced adiposity.

*Altered circulating lipid profile, impaired glucose tolerance and insulin resistance are age-related phenomena in *Ptnt/* mice.*
Energy imbalance and altered adiposity are related to deteriorated lipid and glucose homeostasis. Accordingly, Ptn−/− mice showed lower plasma triacylglycerides from 6-15 months of age than Ptn+/+ controls (Fig.2a). Similar results were found in plasma NEFA although, in this case, we only found significantly lower circulating NEFA in 15 months Ptn−/− mice (Fig.2b). Thus, Ptn−/− mice exhibited an altered lipid profile, as their plasma triacylglycerides and NEFA did not increase with the age as it physiologically happens in Ptn+/+ mice.

Next, we explored the impact of Ptn deficiency in glucose homeostasis. Fasting plasma glucose was similar between groups (Fig.2c). However, again we found a switch in plasma insulin of the Ptn−/− mice, departing from lower insulin levels in the youngest mice to hyperinsulinemia in 15 month-old Ptn−/− animals (Fig.2d).

Then, we performed glucose tolerance tests and estimated insulin sensitivity. We did not find any differences in the areas under the curve of glucose (AUC-G) at 3 and 6 months of age (Fig.2e). However, at 9, 12 and 15 months of age, AUC-G were significantly higher in Ptn−/− than in Ptn+/+ mice, indicating a deterioration of glucose tolerance in these animals with age. Furthermore, although the HOMA-IR (Fig.2f) at 3 months of age revealed higher insulin sensitivity in Ptn−/− mice, 15 month-old Ptn−/− mice showed a significantly higher HOMA-IR (Fig.2f) and a lower QUICKI (Fig.2g) indexes than the Ptn+/+ mice, suggesting impaired insulin sensitivity in later life.

**Deletion of Ptn is associated with differential expression of genes involved in lipid and glucose metabolism in visceral adipose tissue.**

For gene expression analysis, we focused our attention in the periovaric AT. 3month-old Ptn−/− mice showed a downregulation in the mRNA of the Ppar-γ isoforms 1 and 2 (Fig.3a-b) and their cofactor, Pgc1a (Fig.3c), as compared to wild-type controls. Although the
expression of these genes decreased with age (12 versus 3 months) in both genotypes, the age-related decrease in their expression was less pronounced in $Ptn^{-/-}$ mice. These results suggest that the phenotype observed in our animal model can be attributable to a defective $Ppar-\gamma$ activation, thus inducing the previously described impairments in lipid and glucose homeostasis.

To reinforce this notion, we analyzed two pivotal genes regulated by PPAR, responsible for controlling lipid and glucose metabolism in white adipocytes, $Lpl$ and $Glut-4$. In 3-month old mice, the expression of $Lpl$ and $Glut-4$ was significantly lower in $Ptn^{-/-}$ mice than in their age-matched wild-type controls (Fig.3d-e). Although, in both genotypes there was an age-related reduction in the expression of these genes, this reduction was lower in $Ptn^{-/-}$ mice. These results demonstrate that both $Ptn$ deletion and aging can cause, in a similar extent, a downregulation of these genes and, as expected, their expression parallels the changes in $Ppar-\gamma_1$ and $Ppar-\gamma_2$ mRNA. Indeed, there was a significant positive correlation between the expression of $Lpl$ and $Glut-4$ with the expression of $Ppar-\gamma_1$ and $Ppar-\gamma_2$ ($P<0.001$ for all Pearson coefficients).

As all these changes could be related to an altered cellular energy balance, we then investigated the mRNA levels of $Cpt-1$ and $Ucp-2$ in the periovaric AT, and found that $Cpt-1$ expression was downregulated, whereas $Ucp-2$ was upregulated (Fig.3f-g, respectively) by $Ptn$ deletion in 3 month-old mice. Furthermore, although in $Ptn^{+/+}$ animals aging was accompanied by a decrease in $Cpt-1$ expression, and a significant increase in $Ucp-2$, no change was observed in $Ptn^{-/-}$ mice (Fig.3f-g). Then, we analyzed the mRNA of genes related with lipid mobilization, as $\beta_3$-adrenoceptor. We found that the expression of $\beta_3$-adrenoceptor in periovaric AT of young $Ptn^{-/-}$ animals was lower than in $Ptn^{+/+}$ mice (Fig.3h). Interestingly, in $Ptn^{-/-}$ mice aging is accompanied by a significant increase in $\beta_3$-adrenoceptor mRNA, whereas in controls is associated with a decreased expression of this receptor.
All these changes can be related to an increased inflammation affecting visceral adipocytes, which can reflect a low grade of inflammation under a defective Ptn condition. Indeed, the mRNA expression of Tnf-α was increased in the adipocytes of 3-month-old Ptn−/− mice (Fig.3i). Although, in Ptn+/− animals aging was accompanied by an increase in expression of Tnf-α, no change was observed in Ptn−/− mice.

**Genetic inactivation of Ptn affects adipocyte size and β-adrenergic-stimulated lipolysis.**

We found a significant decrease in the adipocyte size in periovaric AT of 15 month-old Ptn−/− mice as compared to Ptn+/+ controls (Fig.4a-b). To further characterize the alteration of AT by Ptn deletion, catecholamine-stimulated lipolysis was analyzed in periovaric-isolated adipocytes. Basal lipolysis was significantly lower (Fig.4c) and the lipolytic response to isoproterenol was significantly higher in isolated adipocytes from Ptn−/−. Finally, the maximum inhibitory actions of insulin on catecholamine-induced lipolysis was significantly lower in isolated adipocytes from Ptn−/− than in the ones from control Ptn+/+ mice (Fig.4d).

**Metabolic activity under standard and thermoneutral conditions, and brown adipose tissue activity in Ptn−/− mice**

The effect of Ptn deletion on whole-body metabolic activity at 24°C was monitored in metabolic cages in 6 month-old female mice for 3 days, and subsequently for 7 days under thermoneutrality (30°C). Ptn+/+ mice were more active than Ptn−/− mice (Fig.5a) during the light and dark phase. No differences were observed in total energy expenditure (EE) between genotypes at none of the housing conditions (Fig.5b). Then, we calculated the contribution of cold-induced thermogenesis to the EE, finding that at 24°C, the
fractions of EE corresponding to cold-induced thermogenesis were higher in the $\text{Ptn}^{-/-}$ mice than in controls, both in the light ($50.8 \pm 3.5\%$ in $\text{Ptn}^{-/-}$ vs $39.8 \pm 2.1\%$ in $\text{Ptn}^{+/+}$ mice, $P<0.05$), and in the dark phase ($35.9 \pm 1.9\%$ in $\text{Ptn}^{-/-}$ vs $27.1 \pm 2.3\%$ in $\text{Ptn}^{+/+}$ mice, $P<0.05$), with a mean daily contribution of thermogenesis to EE of $42.6\%$ and $33.6\%$ for $\text{Ptn}^{-/-}$ and $\text{Ptn}^{+/+}$ mice, respectively. These differences disappeared when the animals were housed at thermoneutrality.

Analysis of food intake revealed that $\text{Ptn}^{+/+}$ mice tend to eat more at 24ºC, but no statistically differences were observed neither at 24ºC nor 30ºC, compared to $\text{Ptn}^{-/-}$ mice (Fig.5c). As shown in Fig.5d-e, we found that, at 24ºC, the respiratory exchange ratio (RER), was lower in $\text{Ptn}^{-/-}$ than in $\text{Ptn}^{+/+}$ mice. The analysis of the maximal and minimal RER shows that $\text{Ptn}^{+/+}$ mice consumed carbohydrates as the main source of fuel during the dark period and a combination of glucose and fatty acids during the light period. However, $\text{Ptn}^{-/-}$ mice consumed already fatty acids during the dark period and this is exacerbated in the light period, indicating that the use of fatty acids for energy production is clearly increased by deletion of $\text{Ptn}$. Nevertheless, in thermoneutral conditions no differences were found in RER between genotypes, neither during the dark nor light period (Fig.5d-e).

After 7 days housed under thermoneutrality, no significant differences were found in the weight of neither the retroperitoneal ($0.137 \pm 0.013g$ for $\text{Ptn}^{+/+}$ vs $0.189 \pm 0.018g$ for $\text{Ptn}^{-/-}$ mice) nor the periovaric AT ($0.279 \pm 0.028g$ in $\text{Ptn}^{+/+}$ vs $0.429 \pm 0.134g$ in $\text{Ptn}^{-/-}$ mice). Moreover, thermoneutrality also revert the altered lipid profile in $\text{Ptn}^{-/-}$ 6 old-month mice, as no differences were found in circulating triacylglycerides ($113.9 \pm 17.4$ mg/dL in $\text{Ptn}^{+/+}$ vs $95.2 \pm 16.2$ mg/dL in $\text{Ptn}^{-/-}$ mice) and the levels of circulating NEFA were even higher in $\text{Ptn}^{-/-}$ ($1.70 \pm 0.05 \, \mu\text{M}$) than in the $\text{Ptn}^{+/+}$ mice ($1.48 \pm 0.16 \, \mu\text{M}$, $P<0.05$).

To deepen into the mechanisms associated with the altered thermogenic function in the $\text{Ptn}^{-/-}$ mice, thyroid hormones and brown adipose tissue (BAT) activity were analyzed in
6 month-old female mice. Body temperature (Fig.6a) was significantly higher and plasma free-T4 (Fig.6b) lower the in \( Ptn^- \) than in \( Ptn^{+/+} \) mice. The analysis of thyroid hormones in BAT reveals that, although T4 levels were similar between groups (Fig.6c), T3 concentration (Fig.6d) was significantly higher in \( Ptn^- \) mice. This result was supported by the higher activity and expression of deiodinase (\( Dio2 \)), enzyme responsible for the tissular T3 synthesis, in BAT of \( Ptn^- \) mice (Fig.6e-f). Finally, the expression of the mitochondrial uncoupling protein-1 (UCP-1), responsible of facultative thermogenesis, was increased in BAT of \( Ptn^- \) mice (Fig.6g).

**Effect of PTN in brown adipocyte differentiation**

To study the effect of PTN in brown adipocyte differentiation, the mRNA of the cytokine was analyzed during the brown cells (mBA) differentiation. At day 3, we found a ten-fold reduction in the expression of \( Ptn \) compared to the undifferentiated cells that was maintained until day 6, when cells were fully differentiated (Fig.7a). Next, we treated mBA with recombinant PTN along differentiation, finding that this supplementation had no effect on the expression of endogenous \( Ptn \) (Fig.7b).

However, PTN supplementation significantly decrease the expression of the brown adipocyte markers, \( Cidea, Prdm16 \) and \( Pgc1a \) (Fig.7c-e), compared to the untreated cells, reflecting the inhibitory role of PTN in the differentiation programing of brown adipocytes.
DISCUSSION

The major leading causes accounting for the high prevalence of metabolic disorders are the imbalanced energy metabolism, deranged hormone biology and impaired adipocyte turnover. These conditions possess a multifactorial pathophysiology, making it difficult to approach the diseases due to the absence of a defined etiology. Therefore, characterization of new targets for the modulation of energy metabolism is of special interest for the treatment and prevention of metabolic disorders. Here, we aim to clarify if pleiotrophin can be a key player in the regulation of energy homeostasis and insulin sensitivity due to adipose tissue-specific actions.

In the present study, we found that Ptn deletion modulates adiposity and fat distribution through AT lipolytic activity and gene expression. Our Ptn−/− deficient mice model showed a clear alteration in body weight and metabolic activity. In fact, the age-related increase in body weight was significantly lower in Ptn−/− mice. Despite Ptn deletion in 3month-old mice was associated with a slight increase in the visceral AT depots, the age-related increase in adiposity was significantly reduced in Ptn−/− mice, suggesting a deterioration of AT expandability, which was further supported by the analysis of total fat mass in these animals.

Apart from the clear reduction in the overall total body fat mass by Ptn deletion, we found alterations in adipocyte cell size and turnover, which are important factors underlying the development of obesity and metabolic disorders. In fact, at 15 months of age, adipocytes from periovaric AT were smaller in Ptn−/− than in Ptn+/+ mice. The genotypic differences in the adipocyte size can be related to their lipolytic activity. In fact, catecholamine-induced lipolysis was significantly higher in periovaric adipocytes of Ptn−/− mice than in controls. Interestingly, in 12 month-old mice, the β3-adrenoceptor expression parallels the catecholamine-activated lipolytic activity of the tissue, being significantly higher in periovaric AT of Ptn−/− mice than in the control animals. Thus, the increased flux into the
circulation of fatty acids and glycerol released from AT in response to the increase adrenergic stimulation of lipolysis, may be responsible for the increased de novo synthesis of glucose and impaired glucose tolerance that we have found in Ptn\(^{-/}\) mice.

Importantly, the inhibitory response to insulin in the catecholamine-stimulated lipolysis in periovaric adipocytes from Ptn\(^{-/}\) mice was significantly lower than in control adipocytes. These results suggest that Ptn\(^{-/}\) mice develop insulin resistance and/or an amelioration of insulin sensitivity in later life, that is concomitant with the higher lipolytic activity and the inability of accumulate fat in white AT.

Additionally, we found that periovaric AT of Ptn\(^{-/}\) mice exhibits a low-grade inflammation even at 3-months of age, as suggested by the increased Tnf-\(\alpha\) expression. Tnf-\(\alpha\) is a well-known inducer of insulin resistance [27], and locally produced Tnf-\(\alpha\) may act within AT as a potent autocrine and paracrine regulator of diverse metabolic processes [28]. In fact, the deletion of Ptn was associated in young mice with a decreased gene expression in AT of Ppar\(\gamma_1\) and Ppar\(\gamma_2\). Although, it has previously found that, in vitro rPTN decreased expression of Ppar\(\gamma_2\) in white primary adipocyte precursors [17], in the same study, the injection of a PTN-neutralizing antibody to control animals did not increase Ppar\(\gamma_2\) in adipose tissue, but rather a slightly reduced expression of this transcription factor [17].

PPAR-\(\gamma\) is essential for the long-term survival and homeostatic function of the adipocyte [29], regulating different target genes involved in both lipid and glucose homeostasis. In particular, PPAR-\(\gamma_2\) prevents peripheral lipotoxicity promoting AT expansion, increasing the lipid-buffering capacity of peripheral organs [30]. The deletion of Ppar-\(\gamma_2\) in obese mouse models leads to reduced AT expandability that is associated with severe insulin resistance [31]. Moreover, Smad3-ko mice that have a reduced expression of Ppar-\(\gamma_2\), also show an impaired adipogenesis and altered lipid accumulation as a result of a decrease in the number and the size of adipocytes [32]. Thus, the reduction of Ppar, and its coactivator Pgc1, associated to Ptn deletion in periovaric AT may be in the molecular
origin of the phenotype of this mouse model with reduced visceral adiposity (Fig. 8).

Remarkably, the expression of Lpla and Glut-4, both regulated by PPARγ, was also downregulated in young Ptn<sup>-/-</sup> periovaric AT. We also found that in the periovaric AT of young Ptn<sup>-/-</sup>, Cpt-1 mRNA was downregulated, whereas Ucp-2 mRNA levels were upregulated to a similar degree of the expression than in the old 12-months Ptn<sup>+/+</sup> mice. Moreover, Tnf-α has been proposed to play a role in the upregulation of Ucp-2, in broadly distributed tissues including BAT, white AT and skeletal muscle [33]. Thus, it is tempting to suggest that deletion of Ptn is associated with increased inflammation in periovaric AT, accelerating the age-related alterations in lipid and glucose metabolism, including insulin resistance and a decreased capacity to store lipids (Fig. 9).

We next explored if Ptn-deficiency is associated with the development of whole-body impaired glucose tolerance and insulin resistance. Plasma glucose was similar between groups. Accordingly, one might speculate that Ptn deletion does not have any impact on glucose homeostasis. However, there is evidence that show that is possible to develop impaired glucose tolerance and insulin resistance under normoglycemia as a consequence of an impaired lipid metabolism [34, 35]. In fact, although glucose tolerance and insulin responsiveness were significantly higher in young 3 month-old Ptn<sup>−/−</sup> mice, both parameters were clearly impaired with aging, suggesting that Ptn deletion could favor a prediabetic state being these animals more prone to develop diabetes mellitus with elderly.

Finally, we further investigated the energy homeostasis in our mouse model. Ptn deletion induces impairments in energy metabolism favoring lipid oxidation. Ptn<sup>−/−</sup> mice already consumed a combination of glucose and fatty acids during the dark period and this was exacerbated in the light period, suggesting that energy production from fatty acids is increased in these animals [36]. The main oxidative tissue in which fatty acids are used as energy substrates by β-oxidation is the skeletal muscle. However, Ptn<sup>−/−</sup> mice exhibited reduced physical activity, suggesting that fatty acids are redirected for oxidation in other
tissues. Precisely, the fraction of the energy expenditure (EE) that accounts for the cold-induced thermogenesis was significantly higher in Ptn−/− than in Ptn+/+ mice. These results are in good agreement with previous studies showing that at 20–24°C, mice require over one-third of EE to maintain core body temperature [22]. Hence, as in the Ptn−/− mice cold-induced thermogenesis represents more than 40% of EE, this can contribute to the higher body temperature found in these animals.

Accordingly, we analyzed whether this could be related to alterations in BAT, as its main function is the production of heat by facultative thermogenesis. Brown fat thermogenesis depends on fatty acid utilization provided by endogenous lipolysis, the mitochondrial machinery involved in fatty translocation and oxidation, and the uncoupling of ATP synthesis by UCP-1. On this regard, we found a higher UCP-1 protein levels in BAT of Ptn−/− than in wild-type mice. Expression and activation of Ucp-1 is regulated by fatty acids, cold exposure or noradrenaline, and T3 is able to increase the stimulation exerted by noradrenaline [37]. Subsequently, the conversion of T4 to T3 by deiodinase is required for the thermogenic function of BAT [38]. The analysis of BAT revealed an increased deiodinase activity and expression, and higher concentrations of T3 in this tissue. Interestingly, Ptn−/− mice had lower levels of plasma free T4. Thus, it is tempting to propose that Ptn deletion is associated in BAT with an increased conversion of T4 to T3 by dio2 deiodinase, accounting for an increase in UCP-1 and the concomitant elevation in body temperature. This is maintained by an increased oxidation of fatty acids as energetic substrates, available from the increased lipolytic activity of AT despite the enough glucose disposal. This preferential oxidation of fatty acids may contribute to the diminished plasma concentration of fatty acids and triacylglycerides, and to the reduced adiposity observed in periovaric and retroperitoneal AT in Ptn−/− mice.

Deeping into the mechanism of the altered thermogenesis in the Ptn−/− mice, we found that differentiation of brown preadipocytes (mBA) blunted Ptn expression, and that treatment of mBA with rPTN diminished the expression of brown markers like Cidea,
Prdm16 and Pgc1a. Thus, although it is known that Ptn expression is suppressed during differentiation of white adipocytes [16], our results reveal for the first time, that the differentiation of brown adipose tissue is coordinated by changes in Ptn expression, and suggests an inhibitory role of this cytokine in brown fat differentiation and thermogenesis.

On the whole, the lipodystrophic phenotype of Ptn−/− mice seems to be related to an enhanced thermogenesis in BAT that is maintained by an active lipid mobilization from white fat. In favor of this hypothesis, when animals were housed at thermoneutrality, a condition that blunted thermogenesis, the differences found at 24ºC in adiposity and circulating triacylglycerides disappeared, and the NEFA were even higher in the Ptn−/− than in the control mice. These results together with the increased in RER at 30ºC point to a clear inhibition of the unrestrained lipolysis at thermoneutrality in the Ptn−/− mice

**Final remarks**

This is the first study demonstrating that pleiotrophin expression is essential to preserve the dynamics of adipose lipid turnover and plasticity, especially in visceral and brown fat pads, rendering Ptn−/− mice more prone to develop insulin resistance and/or an amelioration of insulin sensitivity in later life. These conditions are concomitant with an altered expansibility of periovaric AT and with an impaired thermogenesis of brown adipose cells. In this scenario, Ptn deletion and aging induce a similar expression profile of genes related to lipid uptake and utilization. Hence, we propose that the observed phenotype in our animal model can be attributable to a defective PPAR-γ activation, thus inducing the impairments in lipid and glucose homeostasis that we found upon Ptn deficiency (Fig.8).

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**DUALITY OF INTEREST.**

No potential conflicts of interest relevant to this article were reported.

**AUTHORS CONTRIBUTION**

J Sevillano, MG Sánchez-Alonso, M. Viana and I Valladolid-Acebes, performed experiments, analyzed data, and contributed to the edition of the manuscript; D Horrillo, G Medina-Gómez, and MJ Obregón, performed experiments and analyzed data; B Zapatería, M Limones, J Pita, E Gramage, M Vicente-Rodríguez, M Calderón y M Alcalá performed experiments; Herradón, G: contributed to edition of the manuscript; Ramos, MP: designed the study, performed data analysis, coordinated data interpretation, and contributed to edition of the manuscript. Ramos, MP is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

J.S and MG.S are Co-first authors

All authors reviewed and accepted the manuscript.
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**Table 1. Primer sets used for quantitative-PCR analysis.**

The SYBR green RT-PCR method (Bio-Rad, Hercules, CA) was used for the gene studies using the following primer sets (forward and reverse). The relative expression of each gene was normalized against HPRT and RPL13, used as reference standards.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5′-3′)</th>
<th>Reverse Primer (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rpl13</td>
<td>5′GGTGCCCTACAGTTAGATACCAC3′</td>
<td>5′TTTGGTTTCCGCTCTTTGGGTGTC3′</td>
</tr>
<tr>
<td>Hprt</td>
<td>5′TGCTCGAGATGTCATGAAGG3′</td>
<td>5′TATGTCCCCCGTGGACTGAT3′</td>
</tr>
<tr>
<td>Lpl</td>
<td>5′TGGAGAAGCCATCCGTGTG3′</td>
<td>5′TCATGCGAGCCTTCACCCAG3′</td>
</tr>
<tr>
<td>Ucp-2</td>
<td>5′ACAGGCTCTCTGACTCCTG3′</td>
<td>5′GGCTGGGAGACGAACACT3′</td>
</tr>
<tr>
<td>Pparγ2</td>
<td>5′GATGCAGCCTATGAGCAGCTT3′</td>
<td>5′AGAGGTCCAGACTGATTCC3′</td>
</tr>
<tr>
<td>Tnf-α</td>
<td>5′AGGCACTCCCCCAAAGATG3′</td>
<td>5′TGAGGTGCTGGGCCATAGAA3</td>
</tr>
<tr>
<td>Ctp1α</td>
<td>5′CCTGGTCCACAGGAAGCAT3′</td>
<td>5′CAATGCGAGCCACGACTT3′</td>
</tr>
<tr>
<td>Pgc1-α</td>
<td>5′GAAAGGGCCAACAGAGAGA3′</td>
<td>5′GTAAATCACACGGCGCTT3′</td>
</tr>
<tr>
<td>Cidea</td>
<td>5′-GCCTCGAGGAACCTATCAGC-3′</td>
<td>5′-AGAAGGCTCTCTGTGTCACC-3′</td>
</tr>
<tr>
<td>Prdm16</td>
<td>5′-CCTAAGGTGTCGCCCAGCA-3′</td>
<td>5′-CACCTCCCGCTTTCTACCC-3′</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. *Ptn*<sup>−/−</sup> mice show decreased body weight and reduced adiposity.

(a) Survival curve, (b) body weight, (c) periovaric adipose tissue weight, (d) retroperitoneal adipose tissue weight, (e) body fat and water content analysis, (f) circulating plasma leptin in *Ptn*<sup>+/+</sup> (grey line) and *Ptn*<sup>−/−</sup> (black line) female mice. Data is presented as mean ± SEM of n= 8 mice/group. Different capital letters show differences regarding the effect of ageing in *Ptn*<sup>+/+</sup> female mice (white circles); different lowercase letters regarding the effect of ageing in *Ptn*<sup>−/−</sup> female mice (black circles). Differences between *Ptn*<sup>−/−</sup> versus *Ptn*<sup>+/+</sup> mice are shown by: *: P<0.05; **: P<0.01.

Figure 2. Altered lipid profiles, impaired glucose tolerance and insulin resistance are age-related phenomena in *Ptn*<sup>−/−</sup> mice.

Plasma biochemistry and insulin sensitivity indexes in *Ptn*<sup>+/+</sup> female mice (white circles) and *Ptn*<sup>−/−</sup> (black circles) female mice at 3, 6, 12 and 15 months of age. (a) Triacylglycerides, (b) NEFA, (c) glucose, (d) insulin, (e) AUC-glucose, (f) HOMA-IR and (g) QUICKI. AUC-glucose represents the area under the curve for glucose during the intraperitoneal glucose tolerance test in 6-h-fasted female mice. Data is presented as mean ± SEM of n= 8 mice/group. Different capital letters show differences regarding the effect of ageing in *Ptn*<sup>+/+</sup>; different lowercase letters regarding the effect of ageing in *Ptn*<sup>−/−</sup> mice. Differences between *Ptn*<sup>−/−</sup> versus *Ptn*<sup>+/+</sup> mice are shown by: *: P<0.05; **: P<0.01; ***: P<0.001.

Figure 3. Deletion of *Ptn* is associated with differential expression of genes involved in lipid and glucose metabolism, in periovaric adipose tissue.

Real-time PCR analyses of (a) peroxisome proliferator activated receptor gamma 1 (*Pparγ1*), (b) peroxisome proliferator activated receptor gamma 2 (*Pparγ2*), (c) PPARγ coactivator-1α (*Pgc1-α*), (d) lipoprotein lipase (*Lpl*), (e) glucose transporter-4 (*Glut-4*), (f) carnitine palmitoyltransferase-1 (*Cpt-1*), (g) uncoupling protein-2 (*Ucp-2*), (h) β3 adrenergic receptor (*Adrb-β3*) and (i) tumor necrosis factor-α (*Tnf-α*) in periovaric adipose tissue of 3 and 12 months-old *Ptn*<sup>+/+</sup> (white bars) and *Ptn*<sup>−/−</sup> (black bars) female mice. Data is presented as mean ± SEM of n= 5 mice/group.
Differences between Ptn\textsuperscript{-} versus Ptn\textsuperscript{+/-} mice are shown by: \#: $P<0.05$; \#: $P<0.01$. Differences regarding the effect of ageing (12 versus 3 months) are shown by: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

**Figure 4. Genetic inactivation of Ptn affects adipocyte size and $\beta$-adrenergic agonists-stimulated lipolytic activities.**

Hematoxylin and eosin staining of (a) formalin-fixed periovaric adipose tissue cryosections (10X for top left and right panels; 20X for bottom left and right panels). Scale bars: 200 $\mu$m in the top left and right panels; 100 $\mu$m in the bottom left and right panels. (b) Quantification of periovaric adipocyte cell surface area.

(c) Concentration-response curves of isoproterenol-stimulated lipolysis in isolated adipocytes from 15-months old Ptn\textsuperscript{+/-} (white circles) and Ptn\textsuperscript{-} (black circles) female mice. Data represent lipolytic activities normalized in each experiment to the lipolytic activity in the absence of isoproterenol (basal). (d) Concentration-dependent inhibition of isoproterenol-induced lipolysis by insulin in isolated adipocytes from 15-months old Ptn\textsuperscript{+/-} (white circles) and Ptn\textsuperscript{-} (black circles) female mice. Data represent lipolytic activities normalized in each experiment to the lipolytic value in the absence of insulin (control). Data is presented as mean ± SEM of n= 7 mice/group. Differences between Ptn\textsuperscript{-} versus Ptn\textsuperscript{+/-} mice are shown by: *: $P<0.05$; ***: $P<0.001$.

**Figure 5. Altered metabolic activity under standard and thermoneutral conditions by Ptn deletion**

(a) Activity and (b) energy expenditure during light and dark cycle in 6-month old Ptn\textsuperscript{+/-} (grey line) and Ptn\textsuperscript{-} (black line) female mice housed 3 days at 24°C and 7 days at 30°C. (c) Food intake during the dark and light periods. RER during the (d) dark and (e) light cycles. Data are presented as mean ± SEM of n= 4 mice/group. Differences between Ptn\textsuperscript{-} versus Ptn\textsuperscript{+/-} mice are shown by: *: $P<0.05$; **: $P<0.01$.

**Figure 6. Genetic inactivation of Ptn is associated with impaired thyroid hormones and brown adipose tissue activity in 6-month old mice**
(a) Body temperature. (b) Plasma concentration of free T₄. Concentration of (c) T₄ and (d) T₃ in brown adipose tissue. Deiodinase (Dio2) (e) activity and (f) expression in brown adipose tissue. (g) Ucp-1 protein in brown adipose tissue from Ptn⁻/⁻ (white bars) and Ptn⁰/⁰ (black bars) female mice. Data is presented as mean ± SEM of n= 7 mice/group. Differences between Ptn⁻/⁻ versus Ptn⁰/⁰ mice are shown by: *: P<0.05; **: P<0.01.

**Figure 7. Effect of PTN in brown adipocyte differentiation**

(a) Ptn expression during mice brown preadipocyte differentiation. Real-time qPCR analyses of (b) Ptn expression (c), Cidea (d) Prdm16, (e) and Pgc1a in undifferentiated (day 0) preadipocytes and differentiated adipocytes (day 6) in the presence or absence of 0.1 mg/ml of pleiotrophin. Data is presented as mean ± SEM of n= 6 experiments. Differences between undifferentiated (day 0) versus differentiated adipocytes are shown by: *: P<0.05; **: P<0.01, ***: P<0.001. Differences regarding the effect of pleiotrophin on differentiation are shown by: #: P<0.05; #: #: P<0.01 #: #: #: P<0.001.

**Figure 8. Synoptic figure highlighting the molecular mechanisms by which PTN deficiency alters the dynamics of adipose lipid turnover and impairs energy metabolism**
**Periovaric adipose tissue**

**Glycerol released (% basal)**

**Basal lipolysis**

<table>
<thead>
<tr>
<th></th>
<th>$Ptn^{+/-}$</th>
<th>$Ptn^{-/-}$</th>
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<tbody>
<tr>
<td>(nm glycerol/ mg lipids)</td>
<td>30.7 ± 5.0</td>
<td>16.6 ± 2.0 *</td>
</tr>
</tbody>
</table>

**$E_{MAX}$ %**

<table>
<thead>
<tr>
<th></th>
<th>$Ptn^{+/-}$</th>
<th>$Ptn^{-/-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>189.1 ± 16.8</td>
<td>273.2 ± 43.3 *</td>
</tr>
</tbody>
</table>

**$EC_{50}$ nM**

<table>
<thead>
<tr>
<th></th>
<th>$Ptn^{+/-}$</th>
<th>$Ptn^{-/-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>81.1 ± 28.3</td>
<td>407.8 ± 145.8 *</td>
</tr>
</tbody>
</table>
Aging \rightarrow PTN^{-/-} \rightarrow Dio2 \rightarrow T3 \rightarrow Ucp-1

Visceral Adipocytes

PPAR-\gamma_{1/2} \rightarrow Expansibility \rightarrow TNF-\alpha \rightarrow Inflammation

PGC1\alpha

Glut-4 \rightarrow FA

Glucose Uptake \rightarrow IGT \leftarrow Insulin Resistance

Brown adipocytes

Altered energy metabolism

Dio2 \rightarrow Ucp-1

FA

T3

Activation

Inhibition

Interaction

Inhibited Interaction