Université de Montréal

Impact of (pro)renin receptor deficiency in adipose tissue using a genetically engineered mouse model

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Cette mémoire intitulée:

Impact of (pro)renin receptor deficiency in adipose tissue using a genetically engineered mouse model

Présentée par :
Basma Ahmed

a été évaluée par un jury composé des personnes suivantes :

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Résumé

La stimulation du récepteur de la rénine/prorénine [(P) RR], un membre récemment découvert du système rénine-angiotensine (SRA), augmente l'activité du SRA et des voies de signalisation angiotensine II-indépendante. Pour étudier l'impact potentiel du (P)RR dans le développement de l`obésité, nous avons émis l'hypothèse que les souris déficientes en (P)RR uniquement dans le tissus adipeux (KO) auront une diminution du poids corporel en ciblant le métabolisme du tissu adipeux, l'activité locomoteur et/ou la prise alimentaire. Ainsi, des souris KO ont été générées en utilisant la technologie Cre/Lox. Le gain de poids et la prise alimentaire ont été évalués hebdomadairement dans les mâles et femelles KO et de type sauvage (WT) pendant 4 semaines alors qu'ils étaient maintenu sur une diète normal. De plus, un groupe de femelles a été placé pour 6 semaines sur une diète riche en gras et en glucides (HF/HC). La composition corporelle et l'activité ambulatoire ont été évaluées par l'EchoMRI et à l'aide de cages Physioscan, respectivement. Les tissus adipeux ont été prélevés et pesés. De plus, les gras péri-gonadaux ont été utilisés pour le microarray. Finalement, le niveaux d'expression d'ARNm du (P)RR ont été évalués.

Comme le gène du (P)RR est situé sur le chromosome X, les mâles étaient des KOs complets et les femelles étaient des KOs partielles. Les souris KO avaient un poids corporel significativement plus petit par rapport à WT, les différences étant plus prononcées chez les mâles. De plus, les femelles KOs étaient résistantes à l'obésité lorsqu'elles ont été placées sur la diète HF/HC et donc elles avaient significativement moins de masse grasse par rapport aux WTs. L'analyse histologique des gras péri-gonadaux des KOs nous ont dévoilés qu'il avait une réduction du nombre d'adipocytes mais de plus grande taille. Bien qu'il n'y ait eu aucun changement dans la consommation alimentaire, une augmentation de près de 3 fois de l'activité ambulatoire a été détectée chez les mâles. De plus, nous avons observé que leurs tibias étaient de longueur réduite ce qui suggère fortement l'affection de leur développement. Les gras péri-gonadaux des souris KO avaient une expression réduite de l`ABLIM2 (Actin binding LIM protein family, member 2) qui est associé avec le diabète de type II chez l'humain. Ainsi, les données recueillies suggèrent fortement que le (P)RR est impliquée dans la régulation du poids corporelle.

Mots-clés: Le récepteur de la rénine/prorénine, tissue adipeux, obésité, le système rénine-angiotensine, Souris KO

Abstract

Stimulation of the (pro)renin receptor [(P)RR], a recently discovered member of the renin-angiotensin system (RAS), increases the activity of the RAS and stimulates angiotensin II-independent signaling pathways. To investigate the possible impact of the (P)RR on obesity development, we hypothesized that mice deficient in the (P)RR specifically in their adipose tissue (KO) would have a decrease in body weight by targeting adipose tissue metabolism, locomotor activity and/or food intake. As such, KO mice were generated using the Cre/Lox technology. Weekly weight gain and food intake were assessed in both male and female KO and wild-type (WT) littermates for 4 weeks on a normal diet. A group of females were also placed for an additional 6 weeks on a high-fat/high-carbohydrate diet (HF/HC). Body composition and physical activity were evaluated using EchoMRI and Physioscan cages, respectively. Adipose tissues were collected and weighed at sacrifice. Moreover, perigonadal fat was used for Gene assay and histological analysis. (P)RR mRNA expression levels were evaluated using real-time PCR. Different circulating metabolites and proteinuria were measured by ELISA kits.

As the (P)RR gene is located on the X chromosome, males were complete KOs and females were partial KOs. KO body weights were significantly lower compared to WTs, the differences being more pronounced in males. Female KOs were resistant to obesity development when placed on a HF/HC diet and as such, had significantly smaller fat mass as well as lower circulating leptin levels compared to WTs. All KO perigonadal fat had a reduced number of adipocytes but of bigger size. Although there were no changes in food intake, an almost 3-fold increase in activity was detected in males. Moreover, they presented with shorter tibial length which strongly suggests that they may have developmental issues. Gonadal fat of KO mice showed a reduced expression of ABLIM2 gene (Actin binding LIM protein family, member 2) which is associated with type II diabetes in humans. Conversely, no obvious changes in glycemia were detected while tendencies for lower proteinuria could be observed.

The data collected thus strongly suggests that the (P)RR is implicated in body weight regulation.

Keywords: (Pro)renin receptor, Adipose tissue, Renin-Angiotensin system, Obesity, Knocked out mice

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1. ACE: Angiotensin-converting enzyme
2. ACEi: ACE inhibitor
3. AGT: Angiotensinogen
4. Ang I: Angiotensin I
5. Ang II: Angiotensin II
6. AP2-Cre: AP2-Cre recombinase mice
7. AT1R and AT2R: Ang II type 1 or type 2 receptors
8. ATP6AP2: ATPase, H+ transporting, lysosomal accessory protein 2
9. BAT: Brown adipose tissue
10. BMI: Body mass index
11. COX-2: Cyclooxygenase-2
12. DEXA: Dual energy X- ray absorptiometry
13. EC domain: Extracellular domain
14. EDTA: Ethylendiaminetetra acetic acid
15. ERK1/2: Extracellular signal regulated kinases 1 and 2
16. FAS: Fatty acid synthase
17. HDL: High density lipoprotein
18. HEK-293T cells: HEK cells expressing the large T-antigen of simian virus
19. HF/HC: High-fat/high-carbohydrate diet
20. HSP 27: Heat shock protein 27
21. IC domain: Intracellular domain
22. IGF2R: Mannose-6-phosphate/insulin-like growth factor II receptor
23. IPITT: Intra-peritoneal insulin tolerance test
24. JNK: c-jun N-terminal kinase
25. KO: Knock out
26. LDL: Low density lipoprotein
27. M 8-9: 8.9 kDa membrane-sector fragment
28. MAPK: Mitogen-Activated Protein Kinase

29. NAFLD: Non-alcoholic fatty liver disease
30. NEP: Neutral endopeptidase 24.11
31. OGTT: Oral Glucose Tolerance Test
32. PAI-1: Plasminogen activator inhibitor-1
33. PCP: Planar cell polarity
34. PCR: Polymerase chain reaction
35. PEP: Prolyl-endopeptidase
36. PGF: Perigonadal fat
37. PI3K: Phosphotidylnositol-3 kinase
38. PLZF: Promyelocytic Zinc Finger
39. PPARγ: Peroxisome proliferator-activated receptor γ
40. PRF: Perirenal fat
41. PRRB: (P)RR blocker
42. (P)RR: renin/prorenin receptor
43. (Pro)renin: Renin and prorenin
44. RAS: Renin-angiotensin system
45. RnBP: Renin binding protein
46. s(P)RR: Soluble form of the renin/prorenin receptor
47. SCF: Subcutaneous fat
48. (TGF)-ß1: Fibrogenic cytokine transforming growth factor
49. UCP-1: Uncoupling protein 1
50. V-ATPase: Vacuolar H+-ATPase
51. VLDL: Very low density lipoprotein
52. WAT: White adipose tissue
53. WHO: World health organization
54. WT: Wild-type

Dedication

To my mother, father, husband, big and small family for their continuous encouragement to complete that work.
Thank you!

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Basma Ahmed

Chapter 1 : Introduction

1.1 The renin-angiotensin system (RAS) cascade

The renin-angiotensin system (RAS) has an important role in controlling blood pressure, fluid homeostasis and salt balance [1, 2]. The reaction cascade typically starts in the juxtaglomerular (JG) cells of the kidney (Figure 1). There, the inactive prorenin (the renin proenzyme) is synthesized to be converted into active renin (an aspartyl protease) mainly in response to a decrease in blood pressure or increase in sympathetic activity. Mature renin is stored in granules in the JG cells and then released into the circulation while a smaller percentage remains in the kidneys to exert different intra-renal actions. Renin has been found to be synthesized in other sites such as brain, adrenal gland and visceral adipose tissue [3].

Renin, in turn, works on the N-terminal of a liver plasma protein of type globulin called angiotensinogen (AGT), its only known substrate, to release a decapeptide, angiotensin I (Ang I). Ang I has mild vasoconstrictor effects but not sufficient to affect blood pressure [3].

Further cleavage at the C-terminal of Ang I occurs via the angiotensin-converting enzyme (ACE), from the blood vessel endothelium in the lung, to form an 8 amino acids peptide, angiotensin II (Ang II), the most physiologically active component of the system [3].

Ang II acts through cell surface receptors, Ang II type 1 or type 2 receptors (AT1R and AT2R), which are classified as G protein-coupled receptors [4]. They have a wide distribution and are expressed by many cell types such as in the lung, liver, brain, kidneys and adrenals [2, 5].

Ang II has been shown to act mainly via AT1R to produce most of its known physiological effects. For example, it elevates the arterial blood pressure by producing a potent arteriolar vasoconstriction as well as by stimulating thirst.

In addition, it decreases renal sodium and water excretion as it stimulates the secretion of aldosterone from the cortex of adrenal glands [1, 2, 6]. Ang II also plays a role in the regulation of cell growth, inflammatory responses and oxidative stress mainly via AT1R. On the other hand, AT2R has been found to mainly oppose AT1R action in many aspects, for example, it causes vasodilatation, inhibits cell differentiation, cell growth and apoptosis [7, 8]. This receptor is highly expressed in foetal tissues and its expression levels decrease after birth [9].

Recently, it was shown that other metabolites of Angs could have different biological activities. For example, Ang III is derived from Ang II by removal of one amino acid from its N- terminal. This has been shown to occur in the brain as well as the kidneys by the action of aminopeptidase A where it has been demonstrated to have a role in the regulation of blood pressure by acting through AT1R. Further degradation of Ang III by aminopeptidase N reveals Ang IV [10, 2].

Ang (1-9) can be produced from Ang I by the action ACE 2. Ang (1-7) can then be produced from Ang (1-9) by the ACE or directly from Ang II through the activity of ACE 2 at its C-terminal [11, 12]. In contrast to ACE, ACE 2 does not convert Ang I into Ang II and is unaffected by ACE inhibitors (ACEi). Ang (1-7) acts via a specific receptor (Mas receptor) to produce vasodilatation, natriuresis in addition to its cardioprotective effects [10].

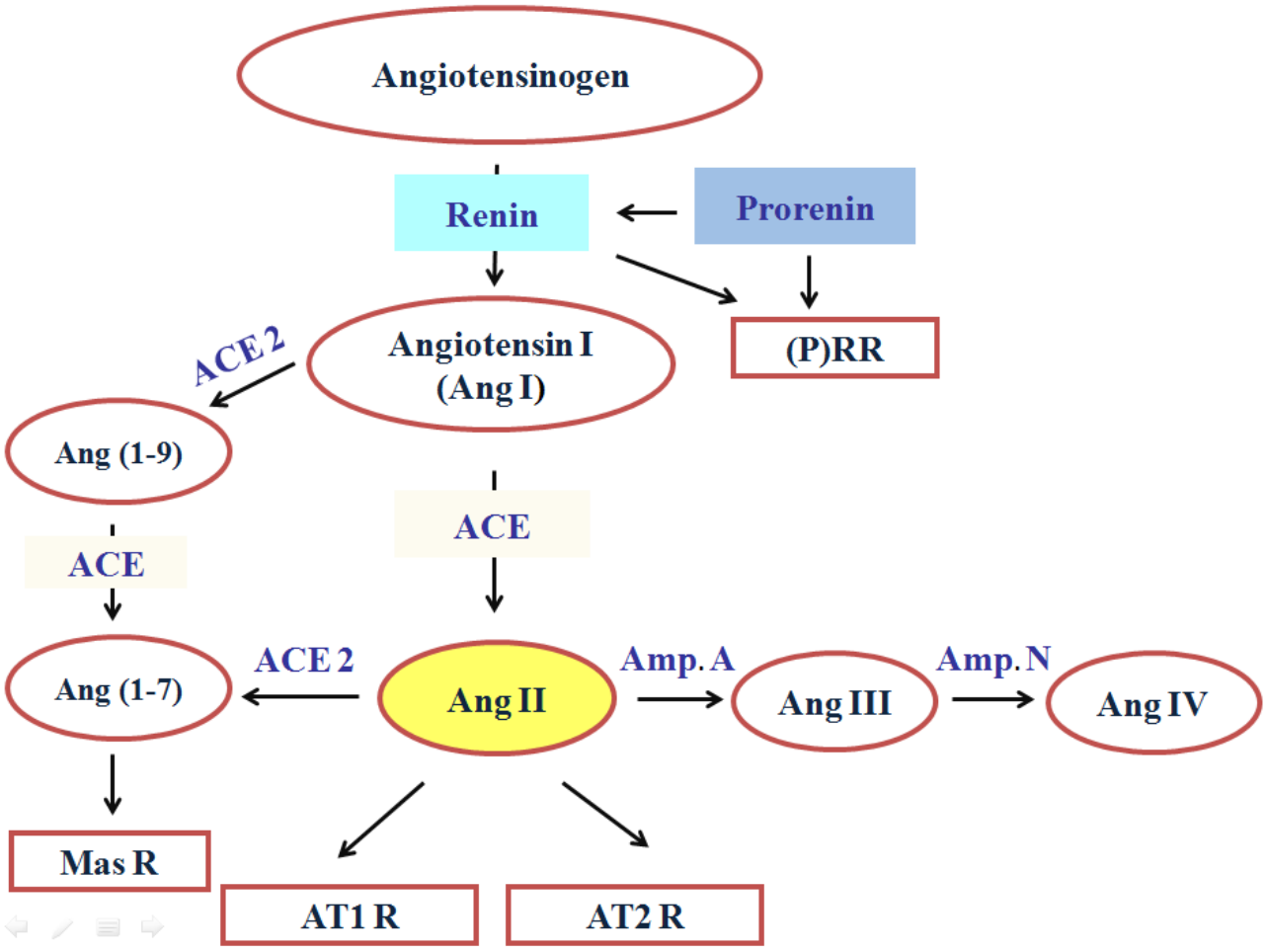

Figure 1: The renin-angiotensin system (RAS) cascade

Classical RAS cascade starts by the production of Ang I from Angiotensinogen which further be converted into Ang II that works via two receptors; AT1R and ATR2. Other Angiotensins can be also produced such as Ang III, Ang IV, Ang (1-9) and Ang (1-7) that works through the Mas receptor. The (pro)renin receptor ((P)RR) was added recently to the cascade. Ang I, Angiotensin I; Ang II, Angiotensin II; Ang III, Angiotensin III; Ang IV, Angiotensin IV; Ang (1-7), Ang (1-7); Ang (1-9), Ang (1-9); ACE, Angiotensin converting enzyme; ACE 2, Angiotensin converting enzyme 2; Mas R, Mas receptor; AT1R, Angiotensin receptor type 1; AT2R, Angiotensin receptor type 2; Amp. A, Aminopeptidase A; Amp. N, Aminopeptidase N.

1.2 Renin and prorenin

As mentioned before, prorenin is the inactive renin precursor. Structurally, renin has 2 lobes with a cleft in-between carrying its active site with two aspartyl residues. Prorenin has 43 amino acid residues at its N-terminal called the prosegment that overlies the renin active site cleft and thus hinders the access to AGT [13].

Prorenin can be activated by two mechanisms: 1) Irreversible proteolytic, by cleavage of the prosegment which occurs via both proconvertase 1 and cathepsin B, *in vivo,* mainly in the juxtaglomerular cells of the kidney [14, 15]; 2) Reversible nonproteolytic, by unfolding the prosegment from the active site cleft via low pH or partial activation by low temperatures which have never been demonstrated *in vivo* [16, 17].

Many tissues such as the eye, adrenal gland, adipose tissue, placenta and brain secrete inactive prorenin, but not active renin, into surrounding tissues and plasma [18-21]. Of note, the kidney is the only known tissue to secrete active renin and as such, only prorenin can be detected in the circulation after bilateral removal of the kidneys [22]. However, local RAS activity has been found in non-renal sites which suggests that there may be a way by which prorenin gets activated locally [23]. Moreover, under physiological conditions, plasma prorenin levels are about 10 folds higher than renin [24]. These are even higher, for undetermined reason, in certain physiological states such as in pregnancy and some pathological conditions as the diabetic nephropathy and retinopathy where plasma prorenin is used as a marker for microvascular complications [19, 24, 25].

On the other hand, other non-renal tissues such as the heart and vascular wall are unable to synthesize prorenin and rely on the circulation as their major source of prorenin which would then be responsible for the local activation of the RAS [26, 27].

In subsequent studies, tissue prorenin was found to be activated in these organs and could lead to local production of angiotensin peptides [21, 28] although there were originally no known mechanisms for its activation in non-renal tissues. In addition, Ang II-independent actions of renin per se were found in cultured human mesangial cells [29].

Taken together, this suggests that there is a mechanism by which prorenin in different tissues can be activated locally. Consequently, many have proposed a renin/prorenin (which we will collectively refer to as (pro)renin) binding protein (RnBP) or receptor to be implicated.

1.3 Renin binding proteins (RnBPs)

Several RnBPs have been demonstrated. One of these was found to be similar to the enzyme N-acyl-D-glucosamine 2 epimerase which can inhibit renin *in vitro*. However, its role in regulating renin activity *in vivo* was not determined [30]. Another RnBP, the mannose-6-phosphate/insulin-like growth factor II receptor (IGF2R) was proposed. However, that receptor is now considered to be mainly a clearance mechanism for (pro)renin since binding was found to produce rapid internalization of the (pro)renin/receptor complex, followed by rapid prorenin cleavage to active renin which is then slowly degraded [31]. Moreover, no extracellular or intracellular Ang production has been associated with this receptor [32].

Chapter 2 : The (pro)renin receptor

2.1 Discovery

Studies in the last 5 years have reported the presence of a new promising (pro)renin receptor [(P)RR] which has been shown to activate the RAS as well as Ang II-independent signaling pathways [29, 33, 34]. Using radio-labeled renin, Nguyen's group identified a receptor that could specifically bind to both renin and prorenin with very high affinity in cultured human mesangial cells [29]. In contrast to the IGF2R, no renin internalization or degradation followed the binding.

In addition, an increase in ^{3}H-thymidine incorporation without any effect on the cell count and a marked increase in plasminogen activator inhibitor-1 (PAI-1) antigen were observed subsequent to the binding. They suggested that renin produced mesangial cell activation which was characterized by a change in fibrinolytic capacity of the cells. Furthermore, they demonstrated that these effects were Ang II-independent as they were unaffected by either ACE inhibition or AT1R and AT2R blockade [33]. Subsequently, they cloned the receptor using an adult human kidney library and transfected the (P)RR cDNA into cells which lack the ability to bind renin [34].

2.2 Structure

The (P)RR is a 45-kDa membrane protein that specifically binds (pro)renin although prorenin has a higher affinity for the receptor compared to renin [35, 36]. Indeed, Nabi *et al*. showed that both rat prorenin and renin can bind to rat (P)RR that was expressed by baculovirus expression system with a dissociation constant (Kd) values of 8 nm and 20 nm respectively [36]. Structurally, (P)RR is a 350 amino-acids protein which has a small 20 amino acids intracellular domain (IC) and a single transmembrane domain (TM)[34] as well as an extracellular domain (EC) where (pro)renin binds [37].

As mentioned earlier, the prorenin has a peptide sequence near its N-terminal called the prosegment which covers the renin active site. It was found that the prorenin can bind to its receptor via a small amino acid sequence of the prosegment called the handle region [37-39]. However, renin, which lacks the prosegment, can bind to its receptor although the mechanism is still unclear. Nabi *et al.* demonstrated that a new segment present in both renin and prorenin, called the hinge region, could bind to the (P)RR but with less affinity than that of the handle region [38]. Later, it was discovered that the (P)RR is the full form of a smaller protein associated with the vacuolar H+-ATPase (V-ATPase) [40]. Consequently, the name of the gene coding for the (P)RR is the ATPase, H+ transporting, lysosomal accessory protein 2 *(ATP6AP2)*. The gene is located on the short arm of the X chromosome (Xp11.4) and encodes for a unique protein that gets further processed intracellularly [41].

2.3 Distribution

In humans, (P)RR mRNA has been detected with high levels in the heart, placenta and brain while lower levels are present in the kidney and liver in addition to barely detectable levels in the lung and skeletal muscles [34]. In the human brain, it has been shown that the (P)RR mRNA is highly expressed specifically in the frontal lobe as well as in the pituitary. Moreover, cells of the human anterior pituitary paraventricular and supraoptic nuclei were positively stained for the receptor by immunohistochemistry and were found to co-localize with arginine vasopressin and oxytocin [42]. The receptor has also been localized in the mesangium of renal glomeruli as well as in the sub-endothelium of renal and coronary arteries of normal human kidney and heart [34].

Interestingly, the (P)RR gene has been detected in isolated human adipocytes by Engeli *et al.* [43].

Furthermore, the same group detected the (P)RR mRNA in abdominal subcutaneous adipose tissue extracts of obese menopausal women [43]. However, since the tissue extract is rich in blood vessels which also highly express the (P)RR mRNA, Achard V *et al.* later did further studies on the stromal areas and isolated stromal cells of human visceral as well as subcutaneous adipose tissues. They were thus able to demonstrate specific synthesis of the functional (P)RR in human adipose tissue [44].

2.4 Signaling pathways of the (P)RR

2.4.1 Ang I production

When renin binds to the (P)RR, it displays a four-fold increase in its catalytic efficiency to convert AGT into Ang I while prorenin shows a non-proteolytic activation [34]. Thus, the (P)RR could have a very important role in the local production of Ang II in many tissues given that renin has been proposed to be the rate limiting step of the RAS (Figure 2) [34].

The mechanism implicated in the non-proteolytic activation of prorenin observed with the binding to the (P)RR could be due to conformational changes [17, 35, 45, 46]. Furthermore, renin activation has been proposed to be due to the proximity of the different components of the RAS, that is, Ang I produced by membrane-bound renin may be more easily converted to Ang II by membrane-bound ACE. However, a recent study investigating the presence of different forms of AGT proposes an alternate mechanism. The authors demonstrated that in both human and mice, AGT is present in two different forms, oxidized and reduced. The reduced form was found to be less easily transformed to Ang I as the cleavage site is buried within the structure of the molecule. Furthermore, the oxidized form was more susceptible to enzymatic cleavage by renin bound to the (P)RR which resulted in a 4-fold increase in Ang II production [47].

2.4.2 Ang II-independent signaling

2.4.2.1 Mitogen-Activated Protein Kinase (MAPK)

It has been reported that binding of (pro)renin to its receptor triggers intracellular signaling which is Ang II-independent since it occurs in the presence of renin and ACEi as well as AT1R blockers. Ang II-independent pathways include rapid phosphorylation of the serine and tyrosine residues of the (P)RR and phosphorylation as well as activation of mitogen-activated protein kinase (MAPK) pathways such as extracellular signal regulated kinases 1 and 2 (ERK1/2), P38 and c-jun N-terminal kinase (JNK), as well as heat shock protein 27 (HSP 27) (Figure 2) [48]. The prorenin activation of the latter pathway has been shown to lead to changes in actin filament dynamics which are known to keep the integrity of the cell architecture and growth. Of note, no changes in intracellular Ca^{+2} or cAMP have been observed with (P)RR stimulation [34]. Following ERK1/2 activation via the (P)RR, an increase in the fibrogenic cytokine transforming growth factor (TGF)-ß1 has been observed (Figure 2) [49]. This in turn activates profibrotic molecules such as, PAI-1, fibronectin protein and collagen I, independently of Ang II [49, 50]. Furthermore, these effects were found to be blocked by TGF-ß1 antibody administration [50]. It has recently been suggested that this occurs through an increased expression of Nox4 with a subsequent increase in superoxide anion production [51].

2.4.2.2 Promyelocytic Zinc Finger (PLZF)

Another (P)RR signal transduction pathway which has been reported *in vitro* involves direct interaction between the C-terminal domain of the (P)RR and the promyelocytic zinc finger (PLZF) transcription factor [52]. Following renin stimulation, the transcription of the p85α subunit of the phosphotidylnositol-3 kinase (PI3K-p85α) is activated which leads to translocation of the PLZF.

This in turn inhibits the transcription of the (P)RR, creating a short negative feedback loop (Figure 2). Thus, high levels of (pro)renin could inhibit (P)RR expression and prevent excessive receptor activation [53].

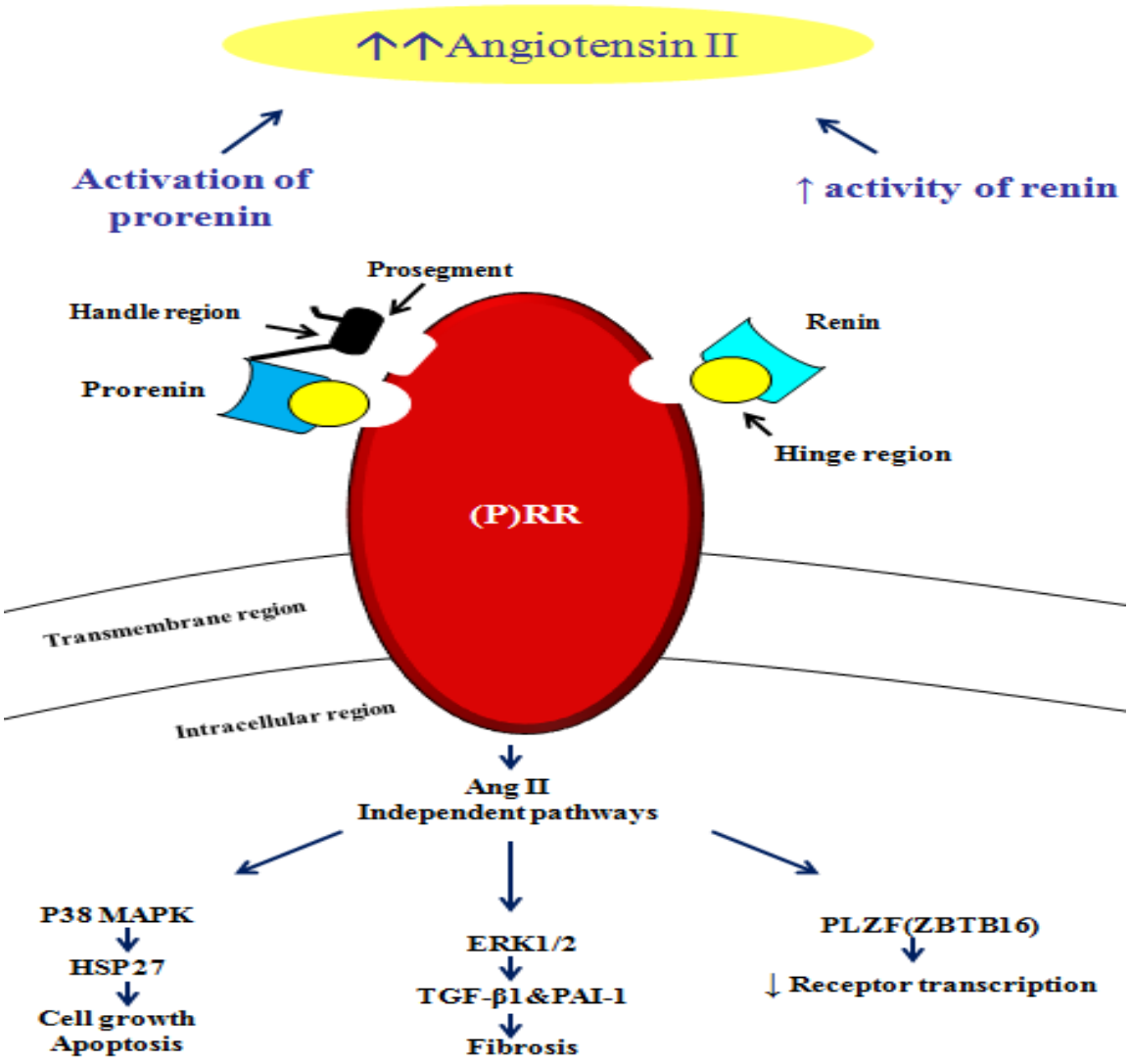

Figure 2: Mechanism of action of (P)rorenin receptor
Binding of the (pro)renin to the (P)RR leads to increased enzymatic activity of renin and renders prorenin non-proteolytically active which, taken together, increases the production of Ang II. At the same time, this binding leads to simultaneous activation of Ang II-independent intracellular signaling cascades. Binding sites for renin and prorenin to the receptor, hinge (yellow circles) and handle region (black rectangle), are also shown. ERK1/2, extracellular signal regulated kinases 1 and 2; HSP 27, heat shock protein 27; p38 MAPK, mitogen-activated protein kinase p38; PAI-1, plasminogen activator inhibitor-1; PLZF, promyelocytic zinc finger; TGF-ß1, transforming growth factor ß1. Modified from Ahmed B. and Lavoie J.L. [48]

2.4.2.3 Wnt/β-catenin signaling

The implication of the (P)RR in the Wnt/β-catenin signaling pathway (Figure 3) has recently been reported. Wnt proteins have been shown to be essential for normal embryogenesis and regulate many cellular aspects in adult tissues. For example, dysregulation of Wnt signaling has been associated with cancer development [48, 54].

In a study to understand the mechanism by which the Wnt receptors get activated, Cruciat *et al.* showed that the (P)RR was required for the activation of the Wnt-canonical pathway. Using a genome wide siRNA screen in HEK-293T cells (HEK cells expressing the large T-antigen of simian virus), they found that three siRNA directed towards the (P)RR resulted in inhibition of Wnt3 signaling at the level of, or upstream to the LRP 5/6 receptor [55]. Moreover, they demonstrated that the (P)RR could bind to the components of the Wnt receptor complex, Frizzled and LRP 5/6, via it's EC domain. They also found that the (P)RR acts as an adaptor between the LRP 5/6 receptor and the V-ATPase which induces LRP 5/6 phosphorylation and as such, activates the β-catenin pathway [55].

On the other hand, (P)RR was also found to be involved in the Wnt non-canonical pathway; the Wnt/PCP (planar cell polarity) [56, 57]. Indeed, two groups have confirmed that the (P)RR can regulate PCP in Drosophila as well as the convergent-extension movements in Xenopus gastrulae. Interestingly, these events are independent of renin as neither the Drosophila nor Xenopus expresses renin [56, 57].

2.5 Alternate molecular forms of the (P)RR

As mentioned previously, the receptor has 3 main domains: the TM, EC and IC domains. Previous studies investigating the receptor have mainly focused on the role of the full length (P)RR. Lately, it has become obvious that other forms of the receptor might also be playing important roles.

2.5.1 (P)RR and V-ATPases

Indeed, Ludwig *et al*. discovered by chance in 1998 a truncated form of the receptor composed of only the TM and the IC domains [58]. They described the C-terminal part of the (P)RR protein as a 8.9 kDa membrane-sector fragment (M 8-9) that associates with the V-ATPase present in chromaffin cell membranes (Figure 3). (P)RR and M 8-9 protein seem to be derived from the same (P)RR gene transcript. Accordingly, the (P)RR gene was named; ATP6AP2 [58]. V-ATPases are ATP-dependent proton pumps. Structurally, they are composed of a peripheral V1 and an integral V0 domain that are formed of 8 and 6 different subunits respectively. The V1 domain is responsible for ATP hydrolysis while the V0 domain translocates protons. Thus, the main roles of V-ATPases are the acidification of intracellular compartments, such as lysosomes and secretory vesicles, and proton pumping in different cell types such as in osteoclasts, macrophages and renal cells [59].

Later, another functional link between the (P)RR and the V-ATPase was demonstrated by Advani *et al*. as they first reported a high expression of the (P)RR on the apical membrane of rat collecting ducts intercalated A cells as well as in the convoluted and distal tubules of human kidneys [60]. They further determined the co-localization of (P)RR with the V-ATPase B 1/2 in this subtype of intercalated cells [60]. Moreover, the implication of the (P)RR/V-ATPase interaction in the (P)RR Ang II-independent pathways has been shown.

They demonstrated that the (P)RR ERK1/2 activation was attenuated when they pre-treated Madin Darby canine kidney cells, a collecting duct/ distal tubule lineage, with bafilomycin; a selective V-ATPase inhibitor [60].

Lately, additional data supporting the (P)RR/V-ATPase relation has been reported. For example, it was found that mice deficient in the (P)RR specifically in cardiomyocytes, developed severe heart failure and died by 3 weeks of age, although no cardiac anomalies could be detected at birth. Interestingly, cardiomyocytes from these knock-out mice showed depleted levels of the V0 domain without any effect on the V1 domain of the V-ATPase. This could be reproduced in cultured embryonic fibroblasts with specific ablation of the (P)RR gene [40]. As a result, (P)RR loss in cultured cells impaired vesicular acidification. In addition, cultured cardiomyocytes treated with bafilomycin showed similar phenotype to what was observed in isolated cardiomyocytes from knock-out animals [40]. Similarly, two groups reported that the (P)RR/V-ATPase is crucial for podocyte function and survival using mice with specific deletion of the (P)RR in podocytes [61, 62]. Knock-out mice were born without any detectable kidney abnormalities but developed a disruption of the glomerular filtration barrier that lead to a nephrotic syndrome which produced sever renal failure. As such, mice died as early as 2 to 4 weeks of age and their podocytes showed marked foot process effacement, alteration of their actin cytoskeleton, reduction of the slit diaphragm proteins and further numerous autophagic vacuoles. Interestingly, and in line with what was noticed with cardiac specific deletion of the (P)RR, (P)RR deletion in podocytes was found to be associated with a suppression of the V0 domain of the V-ATPase. This produced V-ATPase dysfunction that resulted in deacidification of intracellular vesicles. Moreover, previous abnormalities could also be reproduced by treating cultured podocytes with bafilomycin [61, 62].

Taken together, we can conclude that the (P)RR plays an important role in regulating V-ATPase activity and that the V-ATPase is implicated in (P)RR Ang II-independent signaling.

2.5.2 Soluble form of the (P)RR

Recently, a truncated soluble form of the (P)RR [s(P)RR] of 28 kDa that contains only the EC domain has been described. It is present in both plasma and urine and retains the ability to bind renin and prorenin (Figure 3) [63, 64]. Similarly to the full length (P)RR, the s(P)RR has been shown to activate prorenin and thus stimulate the production of Ang I [64-66] although there is no data concerning its impact on renin activity. Studies have suggested the possible implication of several enzymes such as furin or metalloproteases in the production of the s(P)RR [63, 65]. If we take into consideration the data showing that oxidized AGT is more readily cleaved by renin bound to the (P)RR, we can conclude that the s(P)RR may constitute a novel mechanism for the development of hypertension as well as other RAS-associated diseases.

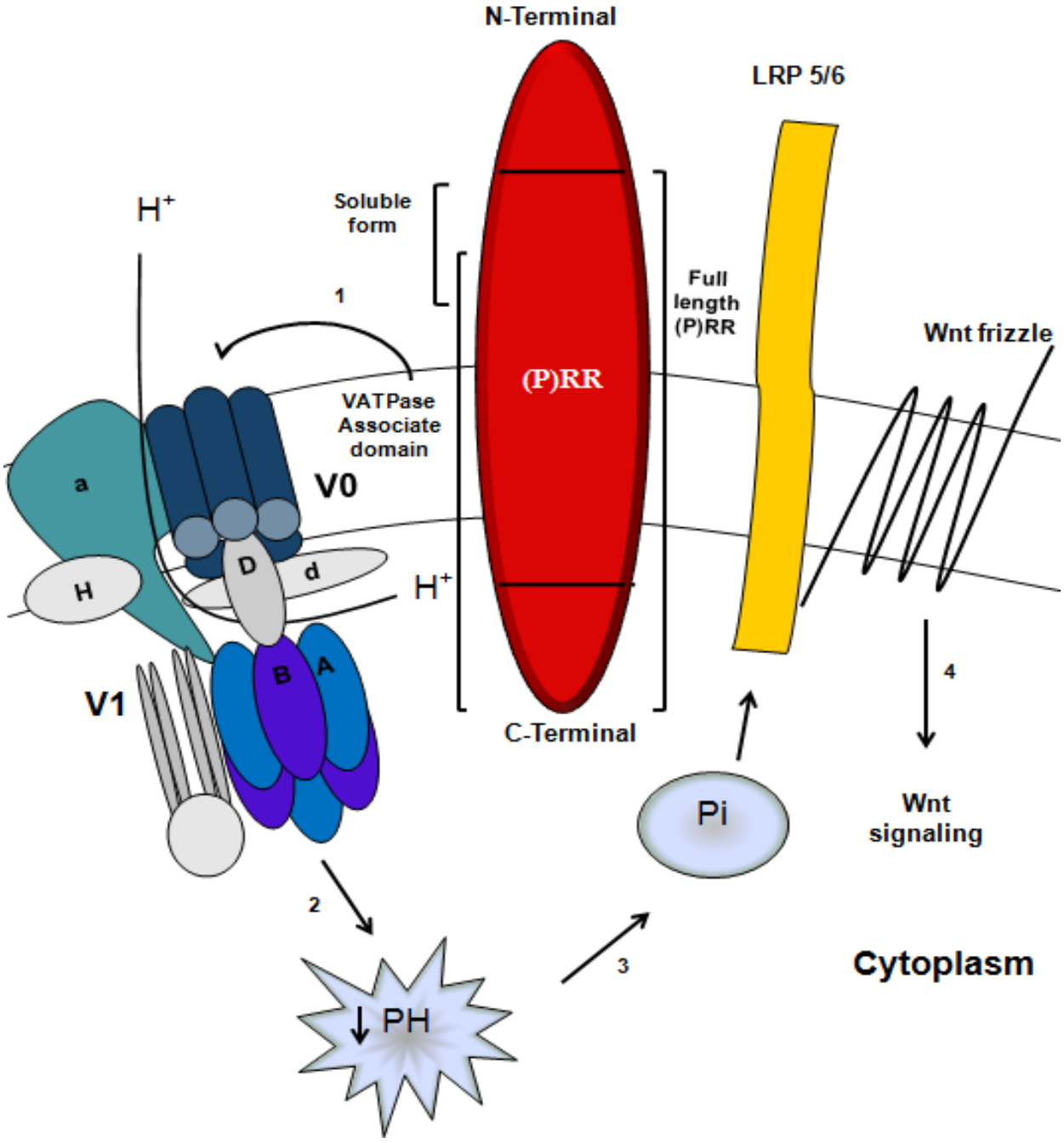

Figure 3: (P)RR and Wnt signaling

(P)RR acts as an adaptor between LRP 5/6 and V-ATPase (1) the later generates a proton gradient (2) that is essential for the phosphorylation and activation of LRP 5/6 (3) which in turn activates β-catenin signaling. LRP 5/6, low-density lipoprotein receptor-related protein 5/6; (P)RR, (Pro)renin receptor; Pi, phosphate ion. Modified from Ahmed B. and Lavoie J.L.[48].

2.6 Animal models of the (P)RR

2.6.1 Deletion of the (P)RR

Generating a knock-out model of the (P)RR is an extremely interesting tool to study the receptor's physiological roles. In fact, it has been found that ubiquitous deletion of the receptor is lethal, which reflects the importance and necessity of the (P)RR gene in embryonic development [41].

For instance, mutation of the (P)RR gene by gene trap in zebrafish embryos has been shown to be lethal before the end of embryogenesis and was associated with developmental abnormalities including neuronal cell death as well as body and eye hypopigmentation [41]. As mentioned earlier in the text, the (P)RR was found to be implicated in the Wnt signaling pathways which could explain these results. In contrast, tissue specific deletion of the receptor is not developmentally lethal although animals develop lethal defects after birth. For instance, knocking down the (P)RR in mice cardiomyocytes produced fatal heart failure at 3 weeks of age [40] while specific deletion of the (P)RR in podocytes resulted in severe renal failure and animal death by 2 to 4 weeks of age [61, 62].

2.6.2 Overexpression of the (P)RR

Transgenic animals which overexpress the (P)RR gene are an interesting alternative that has been employed to define the pathogenic effects and evaluate the *in vivo* relevance of the receptor. For instance, rats overexpressing the human (P)RR ubiquitously presented with slow progressive nephropathy produced mainly by Ang II-independent signaling. Indeed, these rats showed significant proteinuria with aging and glomerulosclerosis at 28 weeks of age as well as enhanced renal TGF-ß1 expression without any elevation in renal Ang II, blood glucose or blood pressure [67]. In contrast to imidapril, an ACEi, complete prevention of all mentioned symptoms could be achieved by chronic administration of a (P)RR blocker (PRRB), also known as the handle region peptide [67]. Moreover, renal enhancement of ERK, p38, and JNK immunostaining was observed.

The ERK and p38 activation were also completely inhibited by the PRRB while JNK activation was only attenuated but all three were unaffected by ACEi [67]. By using the same model, it was shown that transgenic rats had no changes in their blood pressure however their renal cortical cyclooxygenase-2 (COX-2) mRNA and protein levels were significantly increased. In addition, urine sodium excretion was found to be also increased which could be explained by activation of both RAS dependent and independent mechanisms as a result of increased PRR expression. Interestingly, COX-2 inhibition produced a significant decrease in renal cortical blood flow specifically in the transgenic animals [68]. Given that cortical COX-2 has been suggested to be implicated in diabetic hyperfiltration, these results suggest that the (P)RR may be involved in this effect.

On the other hand, rats overexpressing human (P)RR specifically in vascular smooth muscle developed progressive cardiovascular symptoms. Rats developed normally but exhibited spontaneous cardiovascular phenotypes at the age of 6 months such as marked increase in systolic blood pressure and an unexpected elevation in heart rate [69]. These symptoms were aggravated with age and earlier appearance of these symptoms could be observed in rats with higher transgene expression. In addition, this was accompanied by an increased plasma aldosterone levels as well as a significant increase in plasma aldosterone/renin ratio [69]. This demonstrates that aldosterone production was enhanced via the increased adrenal Ang II as a result of (P)RR overexpression without changing the renin levels.

Chapter 3 : Obesity

3.1 Epidemiology and definition

The dramatic increase in the prevalence of obesity among Canadians over the past 30 years has been deemed to constitute an "epidemic." In 2011, 18.3 %, approximately 4.5 million, of Canadian adults were reported to be obese [70]. In 2010, the IASO/IOTF (International Obesity Taskforce) analysis revealed that approximately 1.0 billion adults are overweight and a further 475 million are obese worldwide. Globally, 150 million school-aged children were found to be overweight and 50 million were obese [71]. Indeed, the World Health Organization (WHO) refers to the escalating global epidemic of obesity as "globesity,"

WHO defines obesity as an abnormal or excessive fat accumulation that presents a risk to health. WHO classifies obesity using the body mass index (BMI) which is calculated by dividing the body weight by the square of the height (Kg /m^2). According to WHO, people with BMI equal to or greater than 25 are considered overweight while obese individuals are characterized by a BMI equal to or greater than 30. Obesity is a multifactorial problem in regards to its etiology. Many theories have been proposed in trials to find a defined etiology such as gene-environmental and gene-behavioral interactions. However, the corner stone for occurrence of obesity remains the increased energy intake in the form of caloric consumption over the energy expenditure [72]. Lately, many factors were found to be implicated in increased caloric intake such as increased portion size, ready-made food and sweetened beverages as well as attractive advertising to promote them [72]. The continuous change in life style towards a more sedentary behavior leads to a decrease in energy expenditure [72].

Several studies have reported that an increase in BMI is positively correlated with higher mortality rates [73, 74] as a result of the increased the incidence of many health problems [75]. For instance, obesity is strongly associated with the development of type II diabetes in men and women of all ethnic groups [76-78] by causing insulin resistance. One of the proposed mechanisms for this effect is the increased production of the adipokines (elements that are produced by the adipose tissue) that enhance insulin resistance, such as resistin, and the decrease in those that stimulate the insulin sensitivity, such as adiponectin [79]. Another theory is that the increased secretion by adipose tissue of inflammatory chemokines enhances macrophage activation and infiltration into the adipose tissue. Activated macrophages in turn secrete different cytokines that affects the insulin sensitivity [80]. As obesity is associated with an increase in circulatory and visceral free fatty acids, excess fatty acids also promote the development of insulin resistance in the liver as well as in skeletal muscles [79]. Obesity is also associated with a disturbed lipid profile (dyslipidemia) in the form of decreased levels of circulatory high density lipoprotein (HDL) cholesterol while increased levels of circulatory triglycerides, cholesterol, low density lipoprotein (LDL) cholesterol, very low density lipoprotein (VLDL) cholesterol and apolipoprotein B [75]. Moreover, it has been reported that obesity is associated with elevated risk for cerebrovascular strokes [75]. For example, a study reported that a BMI above 30 Kg /m^2 was associated with increased the risk for total and ischemic but not for hemorrhagic strokes [81]. Also, obese individuals have been reported to be more prone to develop heart diseases [75]. For instance, the risk of developing heart failure was found to be increased by x-fold in obese subjects compared to those with a normal BMI [82]. Obesity is also a major risk factor for some respiratory diseases such as obstructive sleep apnea [75]. In addition, pregnant obese women are at higher risk of developing gestational diabetes and preeclampsia as well as more subject to caesarean sections [83]. Moreover, the incidence of other health problems

was found to be increased with obesity such as the osteoarthritis, psychological disorders, and even some types of cancers such as breast, colon, esophagus and kidney [72].

3.2 Obesity treatment

Many treatments for obesity have been proposed such as lifestyle modifications including dieting and exercise, psychological therapies as well as bariatric surgeries including Roux-en-Y bypass or gastric banding [84]. In addition, several anti- obesity drugs have been tested to control weight gain but have been stopped due to the increased risk of developing cardiovascular disorders, such as desoxyephedrine, phentermine and diethylpropion (amphetamine derivatives) as well as fenfluramine and dexfenfluramine (serotonin-releasing agents) [84]. Sibutramine (noradrenaline and serotonin-reuptake inhibitor) was believed to reduce weight gain by decreasing food intake and increasing energy expenditure, but it was reported to have serious side effects on the cardiovascular system. For example, long term use of Sibutramine in subjects with high cardiovascular risk was found to increasing the risk of nonfatal myocardial infarction and nonfatal stroke [85]. Conversely, Orlistat has been approved as an anti-obesity drug that decreases fat absorption by inhibiting the pancreatic lipases [86]. Apart from associated gastrointestinal symptoms such as bloating and diarrhea, Orlistat doesn't seem to have any serious side effects and as such, is a more promising treatment option.

3.3 White adipose tissue

White adipose tissue (WAT) (Figure 4) is formed of adipocytes and a stromal vascular compartment or fraction where the preadipocytes are located [87]. Almost all adipose tissue in the adult human body is WAT with a very wide distribution with the exception of certain tissues such as the eyelids, the penis and the scrotum. WAT can be divided according to the pattern of

distribution into subcutaneous and internal fat. Subcutaneous fat lies between the skin dermis and the outer layer of the muscles (fasciae). Internal fat includes visceral fat and non-visceral fat such as bone marrow. Visceral fat includes all intra-abdominal and pelvic fat such as omental, peri-renal, mesenteric fat as well as ectopic fat sites including heart, liver and muscles. The distribution and density of adipose deposits depends on the age, sex and ethnic group [88, 89]. For instance, the subcutaneous fat of women is noticed to be located mainly in the buttocks, thigh (gluteofemoral) and breast while around the abdomen (lumbosacral), neck and upper arm for men and this distribution becomes more obvious with age [89]. Of note, mature adipocytes count no more than half of the total cell content since WAT also contains other cell types such as fibroblasts, endothelial cells, preadipocytes, and macrophages [90].

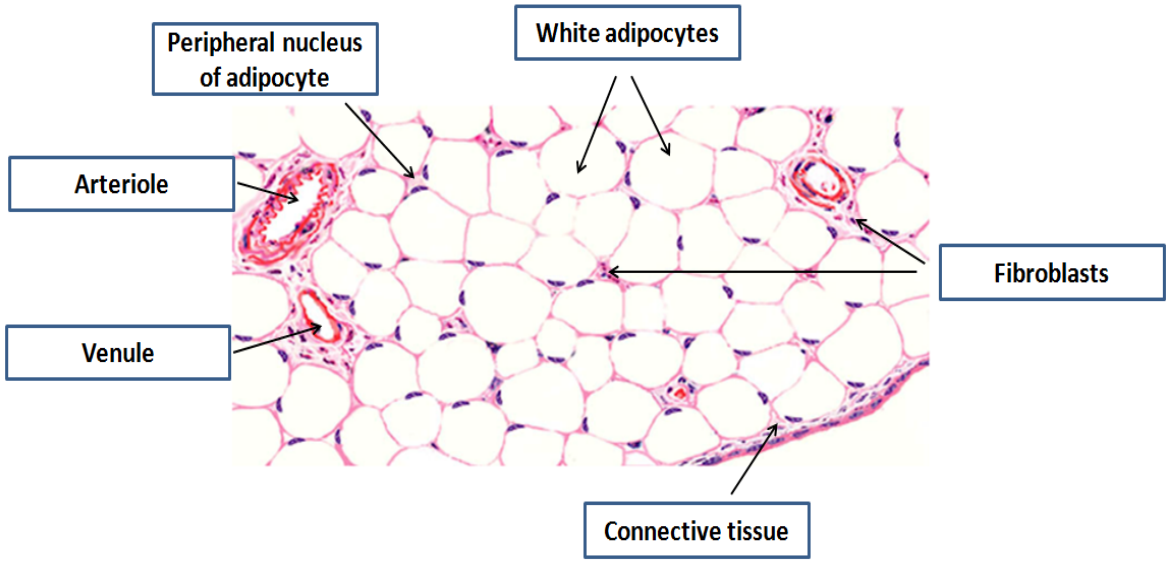

Figure 4: White adipose tissue

Hematoxylin and eosin stained section of mesenteric fat showing unilocular white adipocytes. Fat dissolved during the staining and cells appeared as empty spaces with small rim of cytoplasm and peripheral nuclei. Cells are separated by connective tissue that entangles fibroblasts and blood vessels. Modified from DiFiore's Atlas of histology [91].

Adipose tissue cells are derived initially from the mesenchymal stem cell (Figure 5). As can be seen, a series of differentiation cascades that end by the production of mature adipocytes are required and are under the control of several transcription factors [89]. The peroxisome proliferator-activated receptor γ (PPARγ) is one of the key transcription factors. It is a very important nuclear receptor that has been found to induce adipogenesis[92] and plays a role in determining WAT distribution [93, 94].

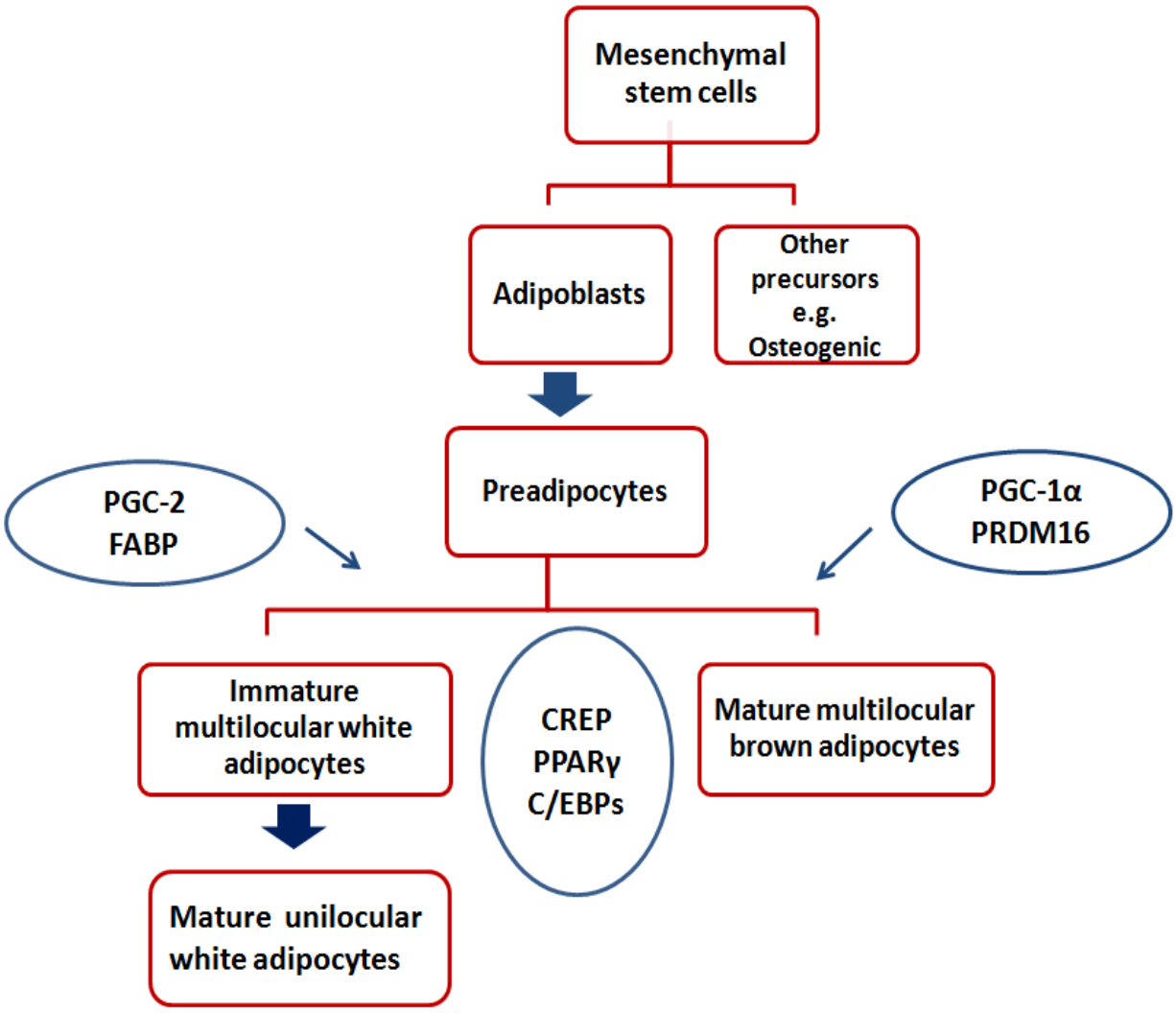

Figure 5: White and brown adipocytes' differentiation

The differentiation cascade of adipocytes from mesenchymal stem cells which is enhanced by several transcription factors is presented in this figure. CREB: (cAMP-response-element)-binding protein; PPARγ: peroxisome proliferator-activated receptor-gamma; C/EBPs: CCAAT/enhancer-binding proteins; PGC-2: peroxisome proliferator-activated receptor-gamma co-activator-2; FABP: fatty-acid binding protein; PGC-1α: peroxisome proliferator-activated receptor-gamma co-activator-1alpha; PRDM16: PR domain containing 16. Modified from obesity science to practice [89].

The main function of WAT seems to be energy storage that could easily be released during periods of negative energy balance and supply the different organs with fatty acids to be oxidized [95]. The WAT mass is determined by both the number and the size of adipocytes. It was proposed that during states of positive energy balance, adipocytes first become hypertrophic (increase in size) and then hyperplasia follows (increase in number). As such, adipocyte hyperplasia occurs only when adipocytes attain their maximal capacity of expansion as mentioned above. This capacity seems to be genetically determined [96].

However, WAT has other roles; for example, it acts as a heat insulator as well as it protects and supports internal organs [97]. It was also found that WAT plays an important role in several homoeostatic mechanisms such as the regulation of blood cell precursors, immunity and insulin sensitivity [97]. Moreover, it has been found in the last decade that adipocytes are able to release many factors, named adipokines, that play an important role not only in the regulation of energy homoeostasis but also on other physiological processes [98]. For instance, it has been found that WAT secretes a 16-kDa protein called leptin [99] which was found to be a product of the *Ob* gene. Mutation of this gene results in severe obesity as can be seen in the *Ob*/*Ob* mice [100, 101]. In addition, plasma leptin levels correlate directly with adipocyte size and body fat mass thus it increases markedly with the development of obesity in both human and rodents [99]. Indeed, leptin informs the brain about the energy status of the body. For example, in positive energy balance state, leptin crosses the blood brain barrier to stimulate its receptor in the brain which is highly expressed in the hypothalamic neurons [102, 103]. Thus it inhibits the orexigenic and enhances the anorexiogenic pathways of the hypothalamus to strongly suppress food intake and increase the energy expenditure. However, as leptin levels usually increase with the development of human and rodent obesity, this strongly suggests the development of leptin resistance in this condition [102, 103].

Leptin also has other metabolic functions. For example, it was found that leptin can suppress the expression of the insulin gene as well as insulin secretion in human pancreas [104].

Another adipokine called adiponectin is also secreted by white adipocytes. Conversely to what is found with leptin, adiponectin levels were found to be decreased with the development of obesity and to be increased by food deprivation [105]. Adiponectin acts through its receptors, AdipoR1 and AdipoR2, which are widely distributed in skeletal muscles and liver respectively. In skeletal muscles, adiponectin increases fatty acid oxidation and glucose uptake [105]. Decreased adiponectin levels in humans and total ablation of adiponectin in rodents were associated with the development of hepatic insulin resistance, inflammation as well as vascular injuries thus it also has an insulin sensitizing action [105].

3.4 Brown adipose tissue

Brown adipose tissue (BAT) in mammals plays a role in the regulation of both basal and inducible energy expenditure through thermogenesis. BAT cells have a lot of mitochondria that express a specific type of protein called the uncoupling protein 1 (UCP-1) at their inner membranes. UCP-1 allows the dispersion of the electro-chemical gradient that is generated through the respiratory chain in the form of heat rather than ATP, a process called uncoupling of the oxidative phosphorylation [106]. In rodents, BAT is found to be abundant in newborns especially in their inter-scapular areas [107]. Of note, brown adipocytes can also be found in other WAT sites especially in the inguinal, retroperitoneal and peri-ovarian region of reproductive fat. Interestingly, the number of brown adipocytes as well as the expression of UCP-1 was found to be increased in these sites after cold exposure [108]. In humans, it has been thought for a long time that BAT is found only in newborns but it has been shown lately that adults also have functional BAT [107].

Chapter 4 : The renin-angiotensin system and obesity

4.1 Human studies

A study reported that, plasma AGT concentrations are significantly and positively correlated with BMI as well as plasma leptin levels in normotensive non-obese young men suggesting a relation between adipose mass and circulating AGT levels [109]. Many reports showed the positive correlation of circulatory AGT and obesity [110-113]. It was also found that the expression of the AGT gene was increased in subcutaneous adipose tissue of obese men in correlation to the body weight [114]. In addition, the expression level in subcutaneous and omental fat was positively correlated with an increase in waist circumference [115]. Another group observed an increase in circulating RAS activity with a decrease in adipose tissue AGT gene expression in obese menopausal women compared to their lean counterparts [43]. However, although AGT expression has sometimes been found to be decreased, it is thought that the increase in adipose mass produces an overall increase in AGT adipose tissue content which would contribute to the increase in circulating AGT observed with obesity. Interestingly, in this study a significant decrease in RAS activity and further reduction in adipose tissue AGT expression were noticed after only 5 % reduction in body weight [43]. In line with these results, a study using Telmisartan, an AT1R antagonist, demonstrated the implication of the RAS on the distribution of body fat in patients with metabolic syndrome. Indeed, Telmisartan lead to significant and selective decrease in visceral but not subcutaneous fat which was associated with an improvement in insulin sensitivity [116]. Similar results were obtained by another group also using Telmisartan when administered to Japanese hypertensive patients which was associated with an increase in serum adiponectin levels [117].

It was also found that the use of Enalapril (an ACEi) for 16 weeks in moderately hypertensive patients was associated with a significant reduction of their body weights [118].

4.2 Animal studies

Many *in vivo* animal studies have found that the RAS might play a role in the pathogenesis and occurrence of obesity. For instance, renal ACE activity was found to be increased in association with obesity development in mice [119]. Moreover, it was noticed that mice with ubiquitous AGT deficiency were resistant to weight gain in response to both a normal and high fat diet which may be as a result of their significant increase in locomotor activity [120]. No changes in the dietary fat excretion were detected while an altered white adipose tissue development in the form of adipocyte hypotrophy and a decrease in their triglyceride content with an associated decrease in fatty acid synthase (FAS) activity were shown [120]. It was reported that AT1Ra/AT1Rb null mice showed slower rate of body weight gain after birth which was mainly as a result of a decrease in fat mass [121]. Another study showed that mice lacking AT2R were also resistant to diet induced weight gain and obesity as well as obesity related glucose intolerance [122]. They also showed increased levels of lipid oxidation. As a result, adipose tissue displayed adipocyte hyperplasia and hypotrophy. The authors reported that the small sized adipocytes could be explained by the associated decrease in the activity of the FAS in addition to decreased gene expression of PPARγ, the lipoprotein lipase (LPL) as well as the fatty acid transporters CD36 and fatty acid protein (aP2) which stimulate the fatty acid uptake and storage [122]. Conversely, the authors couldn't find the exact mechanism implicated in the adipocyte hyperplasia since Ang II has been reported to have a stimulatory effect on preadipocytes differentiation via AT2R [123].

However, they suggested that Ang II may have an inhibitory effect on preadipocytes proliferation *in vivo* via the AT2R as what was reported in smooth muscles and endothelial cells [122].

Moreover, it was observed that knocking down the AT1Ra in mice attenuated the high-fat diet induced obesity, weight gain and adiposity as a result of the increased energy expenditure and sympathetic activity without any changes in food intake. In addition, these mice were protected from some components of the metabolic syndrome as they had low blood pressure and showed an attenuation of the impaired glucose tolerance and insulin resistance as compared to their wild type littermates [124]. Thus it could be concluded that AT1R and AT2R may have synergistic effects in regards to adipose tissue development *in vivo.*

On the other hand, *in vitro* studies revealed conflicting data about the role of AT1R and AT2R in adipocytes. For instance, AT1R but not AT2R protein was detected throughout the differentiation process of 3T3-L1 preadipocytes[125] while a hypertrophic effect on 3T3-L1 and human adipocytes in primary culture via AT2R was also reported [126].

Of note, an inhibitory effect of Ang II through the AT1Ra on human preadipocytes conversion in primary culture was reported [127, 128] while other group showed Ang II stimulatory effect on Ob1771 mouse adipocytes differentiation through AT2R [123]. The discrepancy of the results might be explained by the variability in adipocyte sources and /or the experimental duration.

In another study, mice lacking the renin gene presented with lighter body weights compared to their wild-type littermates and these differences were accentuated by the administration of a high-fat diet. This was associated with an increase in metabolic rate as well as a decrease in intestinal dietary fat absorption [129]. In addition, no changes in food intake or in locomotor activity were observed and they were more insulin sensitive compared to their wild-type littermates [129].

Similarly, mice deficient in the ACE gene were leaner as they presented with a reduced body weight and showed less fat depots. This was associated with an increase in their total and resting energy expenditure with no changes in their food consumption and locomotor activity although their fecal fat excretion was not affected [130]. The controversy in regards to fat excretion data may be due to the different methods used. For instance, chloroform: methanol solution was used for ACE null mice versus petroleum benzene for AGT knocked out mice [130, 120] while fecal acid steatocrit was measured in mice lacking the renin gene [129].

As such, it was hypothesized that the administration of RAS blockers might show similar results to what had been observed with the deletion of the different RAS genes. Indeed, Sprague–Dawley rats receiving a normal diet that were treated with Perindopril, an ACEi, had a reduced weight gain as well as a decreased plasma leptin levels compared to the untreated group. This was not associated with any changes in their food intake and was considered to be mainly as a result of a reduction in their body fat mass as measured by Dual energy X- ray absorptiometry (DEXA) with no changes in their lean or bone mass [131]. Authors suggested that these effects may be due to a reduction in both plasma and tissue angiotensin which is known to promote adipocytes differentiation and adipogenesis although angiotensin levels were not assessed. Similar results were observed in Long Evans rats which received another ACEi, captopril, on either normal or high-fat diet but with noticeable changes in food intake [132]. Administration of the perindopril in the drinking water of Sprague–Dawley rats since birth was also associated with a decrease in body weight and food intake [133]. It has been suggested that the discrepancy in the food intake results might be explained by the difference between each ACEi to access and affect the brain nuclei at different doses [132]. Indeed, it has been shown that chronic treatment, over ten months, with captopril or irbesartan, an AT1R blocker, in NZO/BL6 F1, a genetic mouse model of the metabolic

syndrome, was beneficial in preventing obesity and hyperinsulinemia while reducing hypertension without changing food intake [134]. Authors proposed that blocking the RAS might increase insulin secretion and thus enhance catabolism as suggested by the increased plasma urea levels with normal kidney functions in the captopril treated group. Based on these studies, we can conclude that the RAS has a role in the pathogenesis of obesity in rodents.

Chapter 5 : Adipose tissue renin-angiotensin system

5.1 Local tissue RAS

It was demonstrated that the RAS is not only active systemically but that there are also complete RAS in many tissues (Figure 6). Indeed, critical components of the RAS have been described in several organs such as the brain, kidney, gonads, heart, pancreas and adipose tissue (Figure 6) [2]. This strongly suggests a potential autocrine and paracrine role of the RAS [135]. For example, brain Ang II has been found to act as a neurotransmitter [136]. Moreover, local RAS could be involved in different pathologies. For instance, brain and kidney RASs have been demonstrated to contribute to the development of hypertension [2].

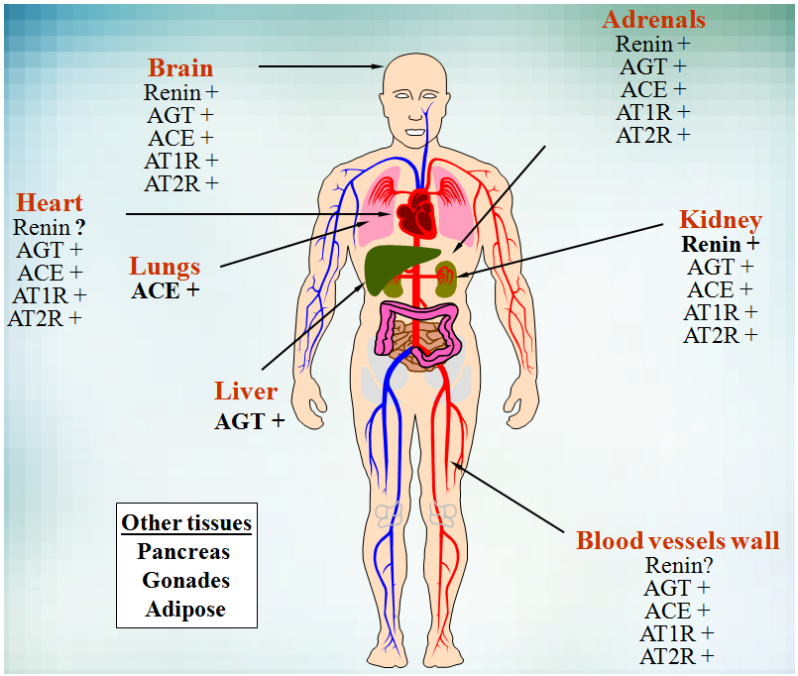

Figure 6: Expression of the RAS
Sites of expression of the different components of the RAS are shown. Classical sites of synthesis for the endocrine RAS are in bold. AGT, Angiotensinogen; ACE, Angiotensin-converting enzyme; AT1R, Angiotensin receptor type 1; AT2R, Angiotensin receptor type 2. Modified from Lavoie J.L. and Sigmund C.D. [2].

5.2 Adipose tissue RAS and obesity

Local RAS in adipose tissue has recently been a rich research area. In an early report, transgenic mice which overexpressed human AGT under the control of its own promoter showed high expression of the gene mRNA in their white as well as brown adipose tissue with stronger expression in male mice compared to females [137]. In another report, the AGT mRNA expression was found to be 4 times higher in visceral fat compared to subcutaneous fat of human subjects with a BMI of less than 30 kg/m^2. In obese subjects, AGT gene expression was significantly higher than leaner subjects in subcutaneous adipose without noticeable changes in visceral fat [138]. In line with these results, another group found that AGT gene expression and protein secretion were significantly higher in visceral as compared to subcutaneous isolated adipocytes from normal male rats. Interestingly, a 50% reduction in the AGT secretion from different adipose tissue depots occurred after castration and was reversed by the administration of testosterone [139]. Furthermore, several studies have shown that the high expression of AGT in adipose tissues and its constitutive excretion from mature adipocytes was present in rodents as well as in humans [140]. Indeed, adipose tissue was found to be responsible for up to 30% of the circulating levels of AGT in rodents [141]. Moreover, targeted AGT overexpression in mice adipose tissue was found to be associated with obesity development and hypertension [141]. It is important to know that adipose tissue also expresses other components of the RAS such as renin, ACE and ACE2. As such, this contributes to the synthesis of Ang II and other angiotensin peptides in the adipose tissue [123, 142]. Hence, adipose tissue not only contributes to Ang II production but it also allows for its autocrine and paracrine action since AT1R, AT2R and MasR are expressed in both adipocytes and periadipocytes [143]. In addition, it was observed that the production of adipose tissue AGT is concomitantly increased

with the development of both genetic and diet induced obesity not only in rodents [144, 145] but also in humans [114].

Ang II enhances the lipogenesis in adipocytes by increasing the activity[146] and the gene transcription of the adipocyte FAS through the AT2 receptors [126, 147]. It also stimulates the activity of other lipogenic enzymes such as glycerol-3-phosphate dehydrogenase [146]. Through the same receptor, Ang II was also found to work as a lipogenic hormone since it promotes the accumulation of triglycerides in murine adipocytes and in human adipose cells in primary culture which promotes adipocyte growth [126]. Another group found that Ang II had a dose dependent anti-lipolytic effect on human subcutaneous adipose tissue and skeletal muscle in both normal weight and obese subjects [148]. Later, the same authors found that this effect occurred mainly via AT1R [149]. Furthermore, an enhanced lipolytic activity with a subsequent increase in plasma free fatty acids and significant decrease in epididymal fat weight were noticed in fasted mice treated with valsartan, an AT1R antagonist [150]. Of note, Ang II was found to enhance the differentiation of preadipocytes into mature adipocytes by different mechanisms via both AT1R [142] and AT2R [123, 151]. Overall, Ang II seems to play an evident role in enhancing adipocyte lipid storage and expanding white adipose tissue by different mechanisms.

5.3 Adipose tissue RAS and insulin sensitivity

Generally, acute Ang II administration is associated with an improved insulin sensitivity and adipocyte glucose consumption in rodents as well as in type 2 diabetic subjects. Conversely, chronic Ang II infusion in rats was found to induce significant insulin resistance and impaired insulin signaling such as, reduced adipocyte and skeletal muscle glucose uptake due to increased oxidative stress [152].

Locally, overexpression of AGT in mice adipose tissue was found to be associated with increased insulin resistance in parallel to obesity development [153].

Chapter 6 : Hypothesis

As mentioned above, RAS activation is associated with obesity development. As a new member of the RAS, the (P)RR was found to increase renin enzymatic activity and thus stimulates the production of Ang I, and as a result, Ang II. In addition, the (P)RR was found to have specific Ang II-independent signaling pathways as mentioned earlier. Therefore, we expect that the (P)RR might also have a role in regulating body weight and obesity development although little is known about this role.

We have recently demonstrated in our laboratory that the (P)RR mRNA levels are increased specifically in white adipose tissue depots in a mouse model of diet-induced obesity and that systemic administration of a (P)RR blocker reduces weight gain and appetite [154]. Furthermore, this is accompanied by a normalization of many circulating metabolites such as free fatty acids, triglycerides and glucose although the exact mechanism is unknown. Hence, to determine the implication of adipose tissue in this novel role of the (P)RR, mice deficient for this receptor (AP2-KO) specifically in their white adipose tissue were studied. We hypothesize that knocking down the receptor in white adipose tissue will decrease weight gain and prevent the development of obesity and its related complications, such as type II diabetes.

Chapter 7 : Materials and Methods

7.1 Animals

Mice deficient for the (P)RR in their white adipose tissue were generated using the Cre/Lox technology. (P)RR floxed mice (a generous gift from Merck Frosst Canada) in which exon 2 of the ATP6AP2 gene was flanked between loxP sites, were bred with AP2-Cre recombinase mice (AP2-Cre mice; Jackson Laboratories) that express the Cre-recombinase enzyme under the control of a white adipocyte promoter (Figure 7).

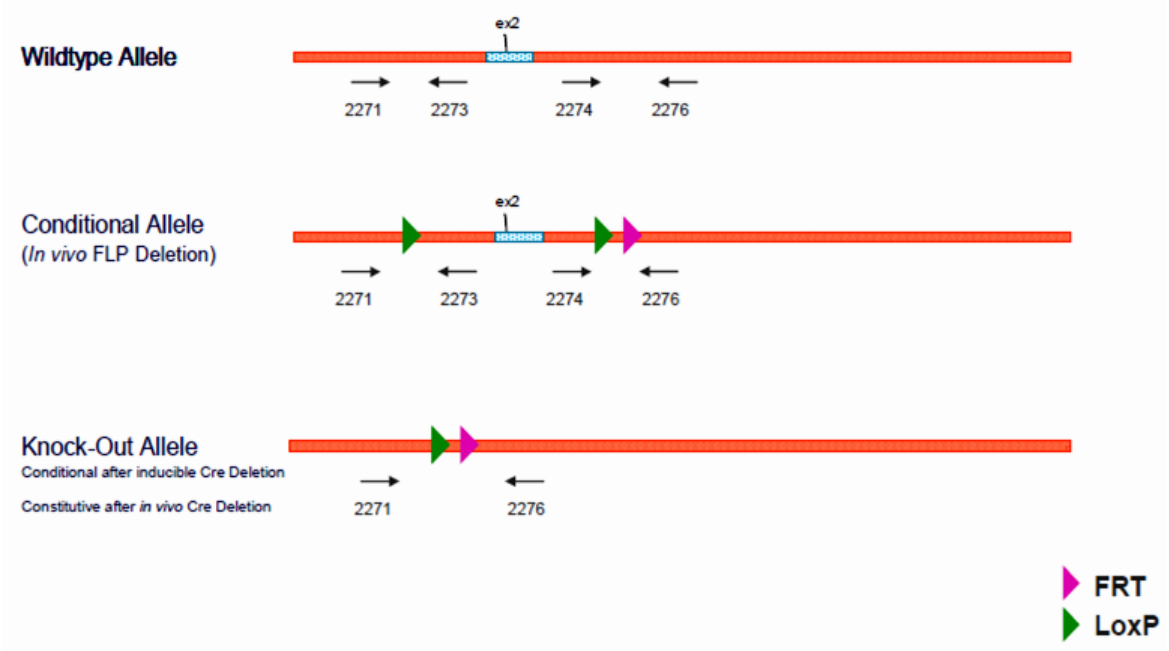

Figure 7: Genotype strategy for AP2-KO mice.
Cre- recombinase enzyme excises the floxed exon 2 of ATP6AP2 gene.

The produced Cre+/ PRR-L +/- mice specifically lack the (P)RR in adipose tissue (AP2-KO). Since the (P)RR gene is carried on the X- chromosome, we had homozygote complete knock-out males while heterozygote partial knocked out females. We used Cre-/ PRR-L -/- male and female littermates as control mice (wild-type). We also looked at Cre+ and PRR-L +/- mice and they were not different then the Cre-/ PRR-L -/- wild-types.

Animals were maintained on 12-hour light/dark cycle with free access to food and water. At 10 weeks of age, mice were separated one per cage to acclimate to this housing condition before the start of the experiments. At 12 weeks of age, we started monitoring mice body weight gain, food and water consumption on a weekly basis.

Mice were divided into 2 groups, AP2-KO and wild-type (WT) mice, both adult males and females, were maintained for 4 weeks on a normal diet; standard laboratory chow (2018; Teklab Premier Laboratory Diets, Madison, WI) *ad libitum*. One group of females was placed for an additional 6 weeks on a high-fat/high carbohydrate (HF/HC) diet (Bio serv; 545 kcal/100g; 60% fat) to induce obesity. Care of the mice used in these experiments complied with standards for the care and use of experimental animals set by the Canadian Council for the Protection of Animals, and all procedures were approved by the University Animal Care and Use Committee at the CHUM Research Center.

7.2 Genotyping

Tail snips were obtained and DNA was purified in order to genotype all mice using polymerase chain reaction (PCR). For DNA extraction, tails were digested by heating the samples for 60 minutes at 90°C in 100 µl of basic digestion buffer (pH 12.0) (25mM NaOH and 0.2mM EDTA disodium dissolved in sterile water) [155]. Samples were preserved at -20°C for future analysis.

PCR was done to detect the presence of both Ap2-Cre recombinase and the (P)RR loxP genes. A final volume of 14 µl was used for the PCR reaction of the Ap2-Cre transgene which contained 0.5 µl of DNA in addition to 20 µM of each of the 4 primers, 10x reaction buffer, 10 µM dNTP (deoxynucleotide phosphate) mix (CTP, GTP, ATP and TTP), 50 mM $MgCl_2$, 0,1 µl of 5 U/µl Taq polymerase and 7.2 µl of sterile water. The sequences for the primers used are indicated in table 1. The DNA was amplified over 35 cycles with a primer annealing temperature of 51°C. DNA was then loaded on 3% agarose gel and migrated for 30 minutes at 100 volts. Positive samples showed a band around 100 bp in addition to the internal control band which was around 324 bp (Figure 8).

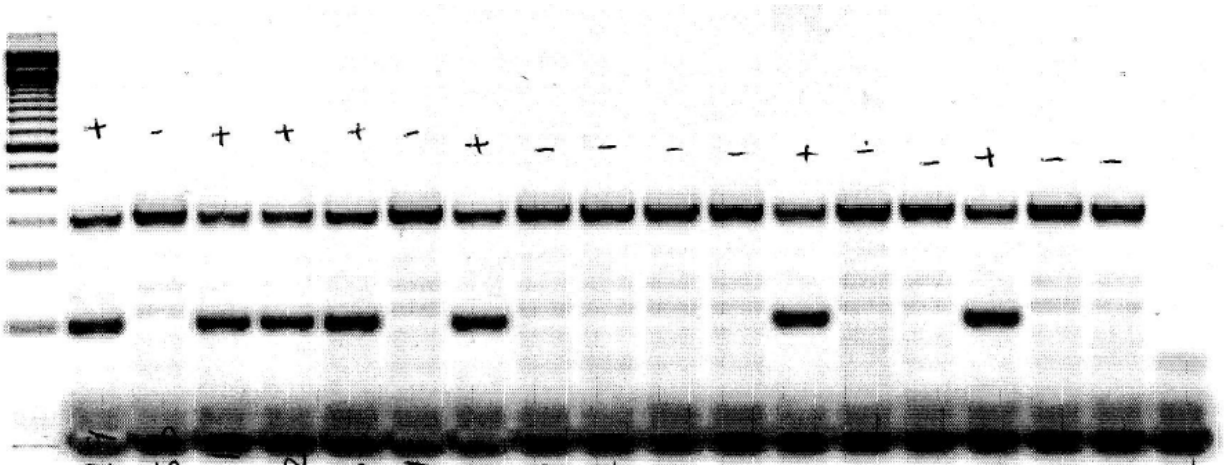

Figure 8: A gel image for the Ap2-Cre recombinase genotyping PCR

Table 1: Primers for Ap2-Cre recombinase and (P)RR loxP genes

Ap2-Cre recombinase	
oIRM0042	5' CTA GGC CAC AGA ATT GAA AGA TCT -3'
oIRM0043	5' GTA GGT GGA AAT TCT AGC ATC ATC C -3'
oIRM1084	5' GCG GTC TGG CAG TAA AAA CTA TC -3'
oIRM1085	5' GTG AAA CAG CAT TGC TGT CAC TT -3'
(P)RR loxP	
2271	5' AGCACTCTCTTCCAGGTATGTTGTG 3'
2273	5' CTGGATCCCGGAGCATGGGTAAAGG 3'
2274	5' CAGGTGTGCTGCTATTAATAGG 3'
2276	5' GCCCCTCTCTTACAGTTCTATCAGT 3'

For the (P)RR loxP amplification, 0.5 µl of DNA was added to a final volume of 25 µl mix as mentioned before but with the use of 100 µM of each of the primers. The sequences of the used primers are indicated in table 1.

The PCR was run over 30 cycles with an annealing temperature of 62°C. The DNA was also loaded on 3% agarose gel and migrated for 30 minutes at 100 volts. All mice showed a control band of 289 bp while mice with the lox-p sites on one X chromosome displayed a band of 326 bp.

A wild type band around 179 bp representing the other X chromosome will appear only in female mice as male mice that carry the lox-p sites had no "normal" (P)RR gene (Figure 9).

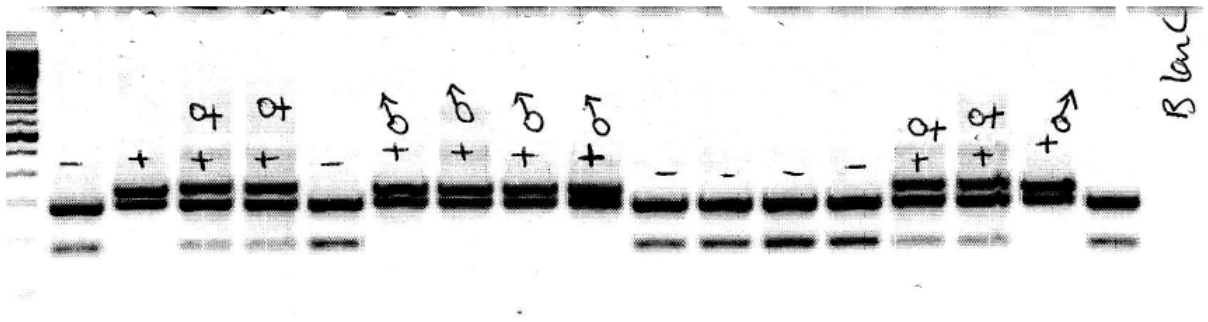

Figure 9: A gel image for the (P)RR loxP genotyping PCR.

7.3 Body composition analysis

Body composition was assessed via quantitative nuclear magnetic resonance spectroscopy (whole body composition analyzer; Echo medical system: EchoMRItm - 700) in live, conscious mice at 16 weeks of age, before placing them in the physioscan cages. At the beginning of each measurement session, a system test was performed and the equipment was calibrated by scanning a calibration holder containing a known amount of fat to test the validity of the measurements. The instrument measures the body composition in terms of fat tissue, lean tissue, free water and total water.

7.4 Locomotor activity

Mice maintained on a normal diet were placed in sophisticated rodent cages (Physioscan from Accuscan) at 16 weeks of age to assess their locomotor activity. Body weight of each mouse was measured before inserting the animals one per cage. Mice had free access to food and water. Data was collected over four nights and five days. Data for the first 3 days and 3 nights was omitted to allow mice to adapt to the new housing environment (Figure 10).

The system estimated the total horizontal distance traveled by each mouse every 2 minutes. A quantitative analysis of the total distance traveled was conducted with the data collected on the fourth day and fourth night. At the end of monitoring, mice were weighed and then returned back to their home cages and kept for one week for acclimatization.

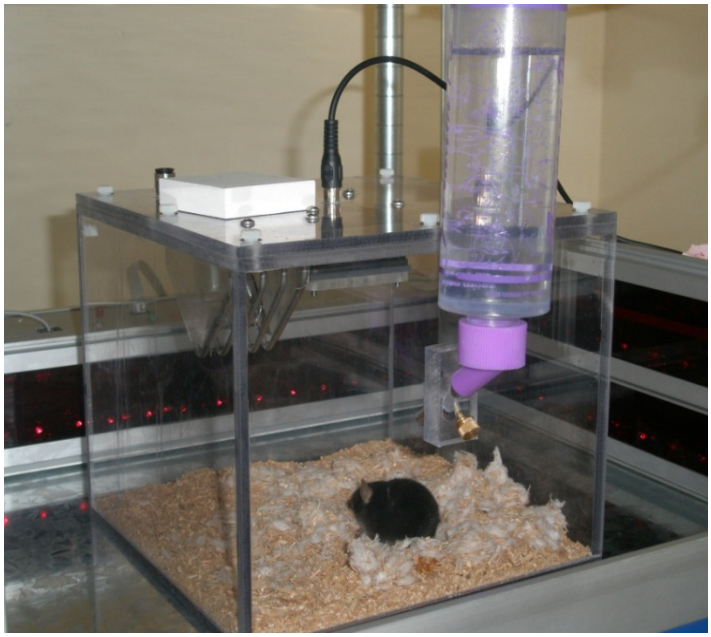

Figure 10: Physioscan cage

7.5 Tissue collection

At 22 weeks of age, animals were fasted overnight in clean cages with new bedding before tissue collection. Mice were sacrificed by CO_2 asphyxiation. Blood was collected by heart puncture. Mouse body length was measured from the nose to the hip-joint. The different fat depots were collected, weighed and snap-frozen in liquid nitrogen and kept at -80^{o}C for future analysis. This included the subcutaneous fat (SCF), perigonadal fat (PGF) and perirenal fat (PRF) as well as BAT. Tibias were also collected and their lengths were measured using a caliper.

In addition, many other tissues were excised such as aorta, diaphragm, pancreas, spleen, skeletal muscles, submandibular glands, gonads, heart, liver, brains, adrenals and kidneys. The weights of the later 5 tissues were monitored. Hearts were separated into their four chambers; each part was weighed and snap-frozen in liquid nitrogen. All other collected tissues were snap-frozen in liquid nitrogen and kept at -80^{o}C.

7.6 Histology and adipose tissue cellularity

At sacrifice, parts of the PGF were fixed for 24 hours in 10% formalin at room temperature. Embedding, sectioning and staining were manipulated via the histology platform of the Research institute in Immunology and Cancer at the University of Montreal as follows: Each sample was paraffin-embedded. Adipose tissues were then cut with a microtome producing 4 μm sections.

Images of the histological sections were obtained by light microscope fitted with a camera at 10x magnification lens. Image J software was used to detect the number and size of adipocytes in 3 measurement frames (696×520 μm) for each section of adipose tissue (3 sections/mouse). For cell count, following actions were used: Plugins, analyze, cell counter. For cell area: manual tracing of each cell was done and then the area was calculated by the following actions: Analyze, measure.

7.7 Real-time PCR for (P)RR expression

Frozen tissues were grinded up into a fine powder using a stainless steel mortar and pestle which were kept frozen by being placed in liquid nitrogen. Total RNA was extracted from the tissue powders using 1 ml of TRIzol (Invitrogen, Burlington, ON, Canada). After adding chloroform and centrifugation at 4°C , an upper aqueous phase containing the RNA was separated from the middle interphase (DNA) and the lower red phase (Trizol, chloroform, protein and lipid), then transferred into new tubes. RNA was then precipitated using isopropranolol. The pellet was further washed in

75% ethanol and left to dry under a chemical hood. RNA was re-suspended in RNase free water according to pellet size and incubated for 10 minutes at 60°C and further quantified at 260/280 nm using a spectrophotometer. A modified protocol was used for adipose tissues in which an additional step is added to remove the lipid layer before the chloroform step.

Extracted RNA was used to synthesize single-stranded cDNA. To remove genomic DNA, 1 μg RNA was incubated with a mix of 1U deoxyribonuclease I (DNase I; Invitrogen)/ug RNA , RNase free water and 10X DNase I reaction buffer for 15 minutes at room temperature. DNase reaction was inactivated by heating samples with 1 μl of 25 mM EDTA for 10 minutes at 65°C. A mix of random hexamer primers (200 ng/μl) and dNTP 10 mM was added and samples were heated for 5 minutes at 65°C. Single-stranded cDNA was synthesized using SuperScriptII Reverse Transcriptase (Invitrogen) with the following program in a thermocycler: 10 minutes at 25°C followed by 50 minutes at 42°C and finishing with a 15 minutes at 70°C step.

The real-time PCR final volume of 25 μl contained 0.3 μmol/l of the specific forward (F) and reverse (R) primers for (P)RR (F (5'TTTGGATGAACTTGGGAAGC-3'); R (5'-CACAAGGGATGTGTCGAATG-3')) or 18s (F (5' AAACGGCTACCACATCCAAG-3'); R (5'-CCTCCAATGGATCCTCGTTA-3')) as well as 5 μl of 1:5 diluted single-stranded cDNA template in Quantitec SYBR Green PCR mix (Roche, Laval, QC, Canada) (1X final)**.** Each sample was analyzed in duplicate. mRNA levels are expressed as values relative to 18s ribosomal RNA which was used as an internal control.

7.8 Plasma metabolites

Collected blood samples were placed in 1.5 ml tubes containing 15 µl of EDTA 0.5 M pH 8.0, and spun down at 6000 g for 10 minutes at 4°C to recuperate plasma which was then frozen at -80°C until further assayed.

Quantitative determination of glucose by enzymatic method using a commercially available kit (Wako) was done. The working solution was prepared by adding equal volumes of the reaction buffer and the color reagent. For the standard curve, distilled water was used to prepare serial dilutions of the standard II solution. For the plasma, 2 µl of each sample were incubated with 200 µl of the working solution for 5 minutes at 37^{o}C. Each sample was done in duplicate. A FLUOstar OPTIMA spectrophotometer was used to read the absorbance of the plates at a wavelength of 505 nm.

The concentration of glucose (mg/dl) was calculated as follows:

$$\text{Glucose Concentratio}(mg/dl) = \frac{Absorbance\ of\ sample}{Absorbance\ of\ standard}\text{X Concentration of the standard}$$

Serum lipoprotein analyses were performed by High-Performance Liquid Chromatography (HPLC) with gel permeation columns (LipoSEARCH; Skylight-Biotec, Inc., Akita, Japan), which measured cholesterol and triglyceride levels in each lipoprotein fraction and lipoprotein particle size distribution simultaneously. Serum lipoproteins, triglycerides and cholesterol were analyzed by an HPLC system at Skylight Biotech (Akita, Japan) according to the procedures described by Usui et al [156, 157]. Leptin and adiponectin were measured by ELISA kits at Morinaga Institute of Biological Science, Inc. (Kanagawa, Japan) and Otsuka Pharmaceuticals (Tokyo, Japan) respectively.

7.9 Proteinuria

At 16 weeks of age, urine samples were collected manually in 1.5 ml tubes by restraining the mice mildly. Samples were kept at -80^{o}C for future analysis. Before measurement, each sample was thawed at room temperature. Albumin and creatinine urinary concentrations were measured in duplicate using the Albuwell and Creatinine companion mouse ELISA kits (Exocell, Philadelphia, PA).

For albumin, serial dilutions of the standard were prepared using the diluent supplied with the kit as follows 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 µg/ml. A 1:20 dilution of the urine samples was prepared in the supplied diluent. Each sample was incubated for 30 minutes with the primary Rabbit Anti-murine Albumin Antibody. Plates were washed 10 times with water. Samples were further incubated for 30 minutes with 100 µl of Anti- rabbit HRP conjugate. Plates were washed again and samples were incubated for 10 minutes with 100 µl of the developer and then 100 µl of the color stopper were added. The absorbance for albumin was read at 490 nm.

For creatinine measurement, a fresh picrate working solution was prepared by adding 2 ml of 1 N NaOH to a 10 ml bottle of picrate reagent. Urine samples were diluted 1:20 in distilled water. Serial dilutions of the creatinine standard were prepared as follow: 10, 3 and 1 mg/dl. 20 µl of the diluted samples were incubated for 10 minutes with 100 µl of working solution. Absorbance was read at 500 nm then samples were incubated with 100 µl of acid reagent for 5 minutes and absorbance was read again at 500 nm. Proteinuria was evaluated as the albumin/creatinine ratio.

7.10 Gene Affymetrix transcriptome

For these experiments, total RNA was extracted from PGF of both males and females using TRIzol® reagent (Invitrogen, Carlsbad, CA) and purified with the RNeasy® MinElute cleanup kit (Qiagen, Valencia, CA) following the manufacturer's recommendations. The quality of the total RNA was evaluated on an Agilent 2100 Bioanalyzer system (Agilent, Palo Alto, CA). Microarray experiments were performed using the GeneChip® Mouse Gene 1.0 ST array (interrogates 28 853 genes), where each gene is represented by approximately 27 probes along the entire length of the transcript (Affymetrix, Santa Clara, CA). 100 ng of total RNA for each sample were processed using the Ambion® WT Expression Kit (Invitrogen). This kit uses a reverse transcription priming method that specifically primes non-ribosomal RNA, including both poly(A) and non-poly(A) mRNA, and generates at the end of the process sense-strand cDNA. 5.5 µg of the single-stranded cDNA were fragmented and labeled using the Affymetrix GeneChip® WT Terminal Labeling Kit and 2.0 µg of the resulting cDNA were hybridized onto the chip. The whole hybridization procedure was performed using the Affymetrix GeneChip® system according to the protocol recommended by Affymetrix. The hybridization was evaluated with Affymetrix GeneChip® Command Console Software (AGCC) and quality of the chips with Affymetrtix Expression Console. Partek Genomics Suite (Partek, St. Louis, Missouri) was used for data analysis. First, the data were normalized by Robust Multichip Average (RMA) algorithm, which uses background adjustment, quintile normalization and summarization. Then, the transcripts found to be significantly differentially expressed between WT and KO groups by more than 20% were included in the gene enrichment and pathway analyses, which were performed using web-based DAVID functional enrichment analysis, and Ingenuity Pathways Analysis (Ingenuity Systems, Inc., Redwood City, CA). Experiments were conducted at the CRCHUM genomic platform.

7.11 Statistical analysis

All values are expressed as mean ± standard error (SE). A repeated measures 2-way ANOVA was applied for weekly body weight, weight gain and food consumption. If any interactions were detected, a Tukey post-hoc test was used. A T-test was used for EchoMRI, physioscan and metabolic parameters as well as the (P)RR mRNA. A $p \leq 0.05$ was considered statistically significant.

Chapter 8 : Results

Knocking down of the receptor in white adipose tissues in both male and female mice was confirmed by comparing the levels of receptor expression in different white adipose tissue deposits and all other organs such as liver, kidneys, spleen, etc. A significant and specific decrease in the receptor expression was noticed in all white adipose tissue pads of KO males and females as compared to their WT littermates (Figure 11 and 12) and the level of expression was observed to be much lower in complete KO males as expected (Figure 11 and 12). No differences in the receptor expression were noticed in all other examined tissues (Figure 13 and 14).

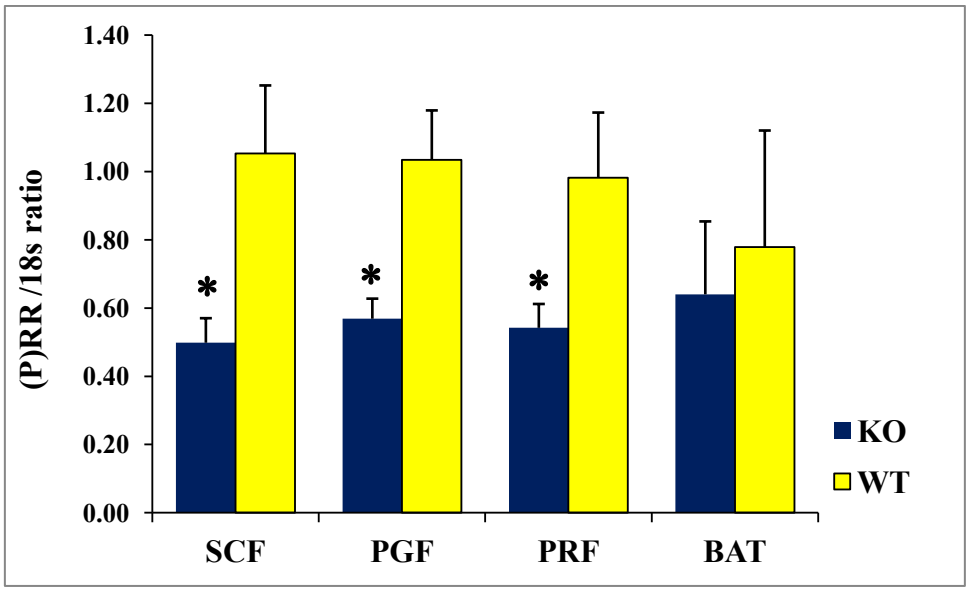

Figure 11: (P)RR expression in different adipose tissues of male mice.
Male KO showed significantly lower levels of the (P)RR mRNA in their white adipose tissue pads as compared to their WT littermates while no changes could be detected in the brown adipose tissue. * $p \leq 0.05$, statistically different from WTs. N= 4 KOs and 4 WTs. Brown adipose tissue, BAT; Subcutaneous fat, SCF; Perigonadal fat, PGF; Perirenal fat, PRF.

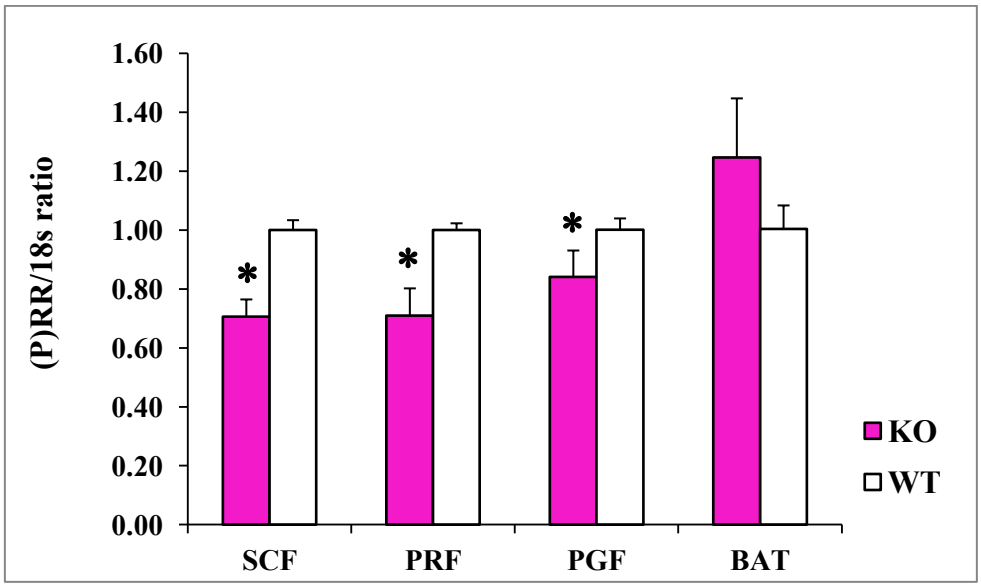

Figure 12: (P)RR expression in different adipose tissue pads of female mice.
Female KO showed significantly lower levels of (P)RR mRNA in their white adipose tissue pads as compared to their WT littermates while, no changes could be detected in the brown adipose tissue. * $p \leq 0.05$, statistically different from WTs. N= 3 KOs and 3 WTs. Brown adipose tissue, BAT; Subcutaneous fat, SCF; Perigonadal fat, PGF; Perirenal fat, PRF.

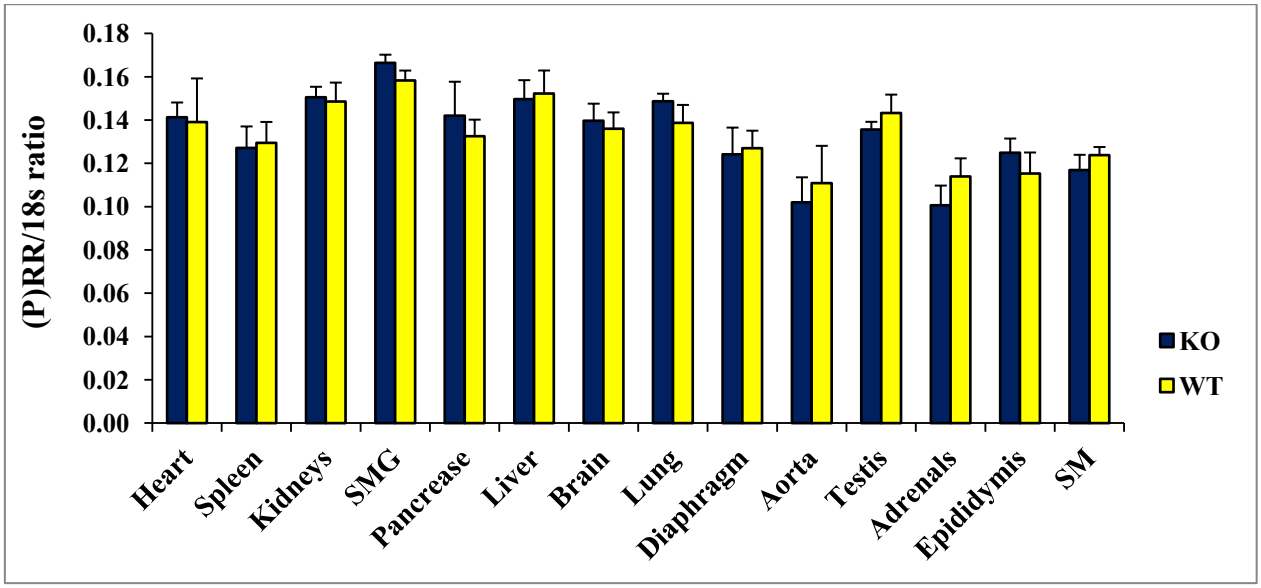

Figure 13: (P)RR expression in different tissues of male mice.
Male KO had no changes in the levels of (P)RR mRNA in the different organs assessed as compared to their WT littermates. N= 5 KOs and 5 WTs. Submandibular gland, SMG; Skeletal muscles; SM.

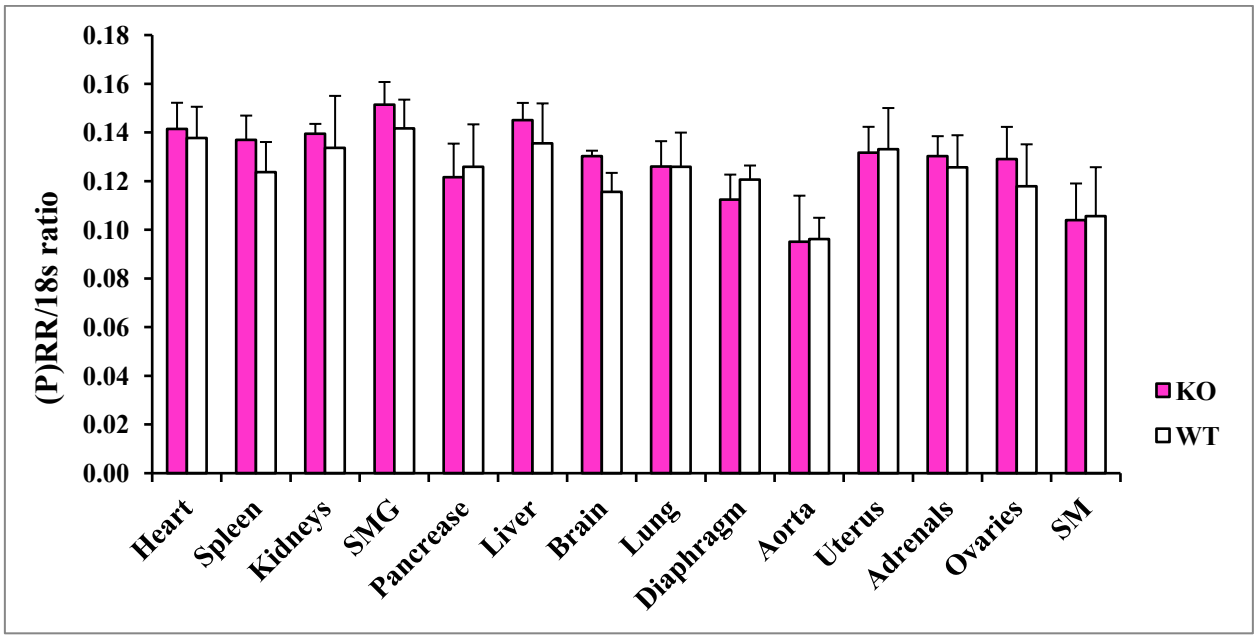

Figure 14: (P)RR expression in different organs of female mice.
Female KO had no changes in the levels of (P)RR expression in their different organs as compared to their WT littermates. N= 3 KOs and 3 WTs. Submandibular gland, SMG; Skeletal muscles; SM.

At 12 weeks of age, KO males presented with a significantly smaller body weights (28.3% less body weight) as compared to their WT littermates (22.2 ± 0.81 g versus 28.5 ± 0.98 g) (Figure 15). By following their weekly body weight and food intake for 4 weeks on normal diet, we determined that KO males maintained significantly smaller body weights as compared to their WTs (Figure 15 and 16) but without any significant differences in their food intake when calculated as daily consumption in grams or as daily caloric consumption (Figure 17A and B).

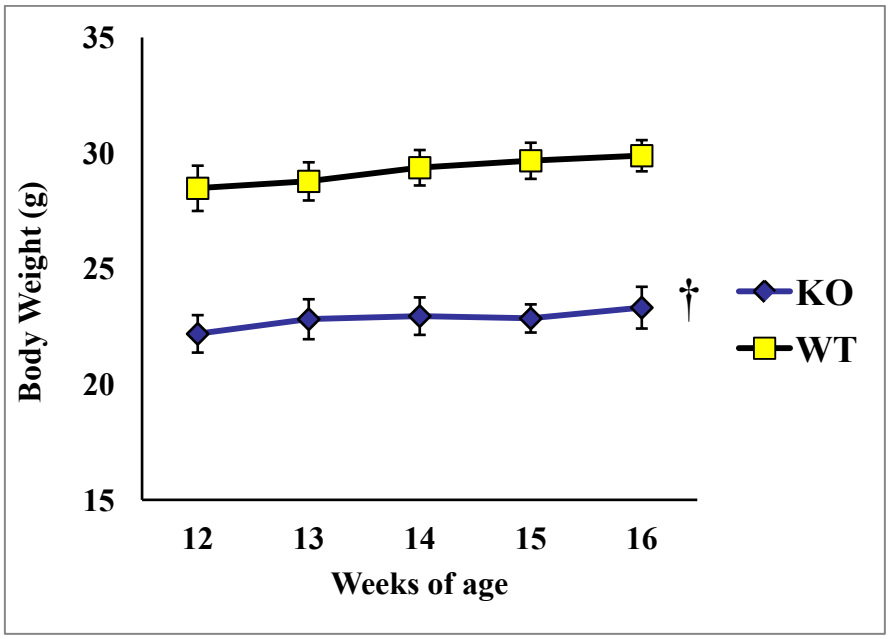

Figure 15: Body weight of male mice.
Male KO showed significantly lighter body weights over four weeks on normal diet as compared to their WT littermates. † $p \leq 0.001$, statistically different from WTs. N= 6 KOs and 10 WTs.

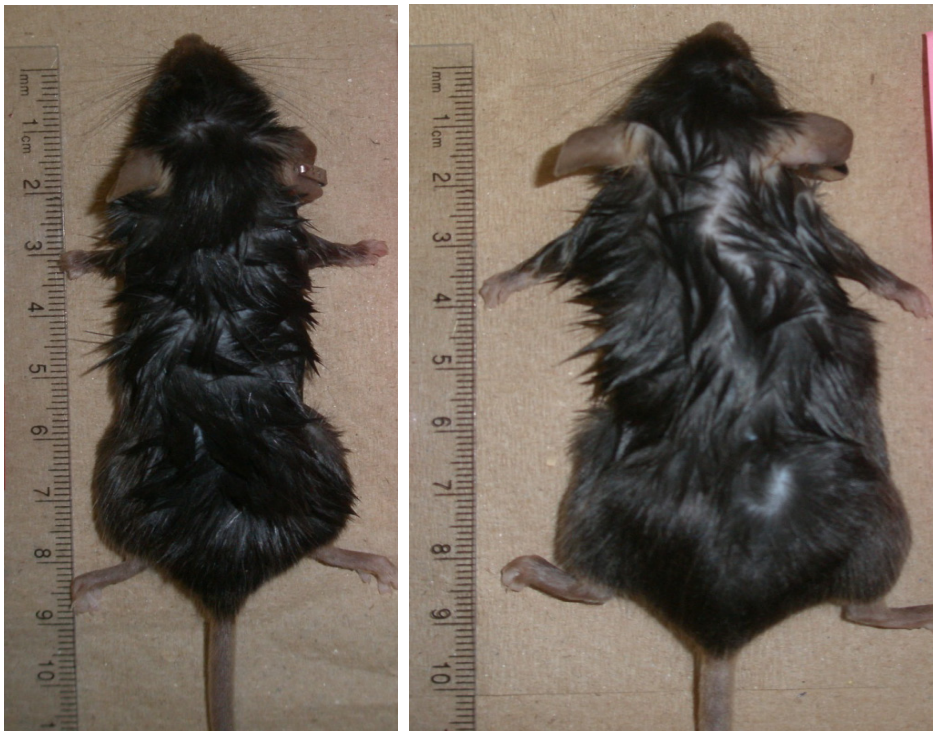

Figure 16: Male mice.
Pictures of complete KO male (on the left) and its WT littermate (on the right) at 16 weeks of age.

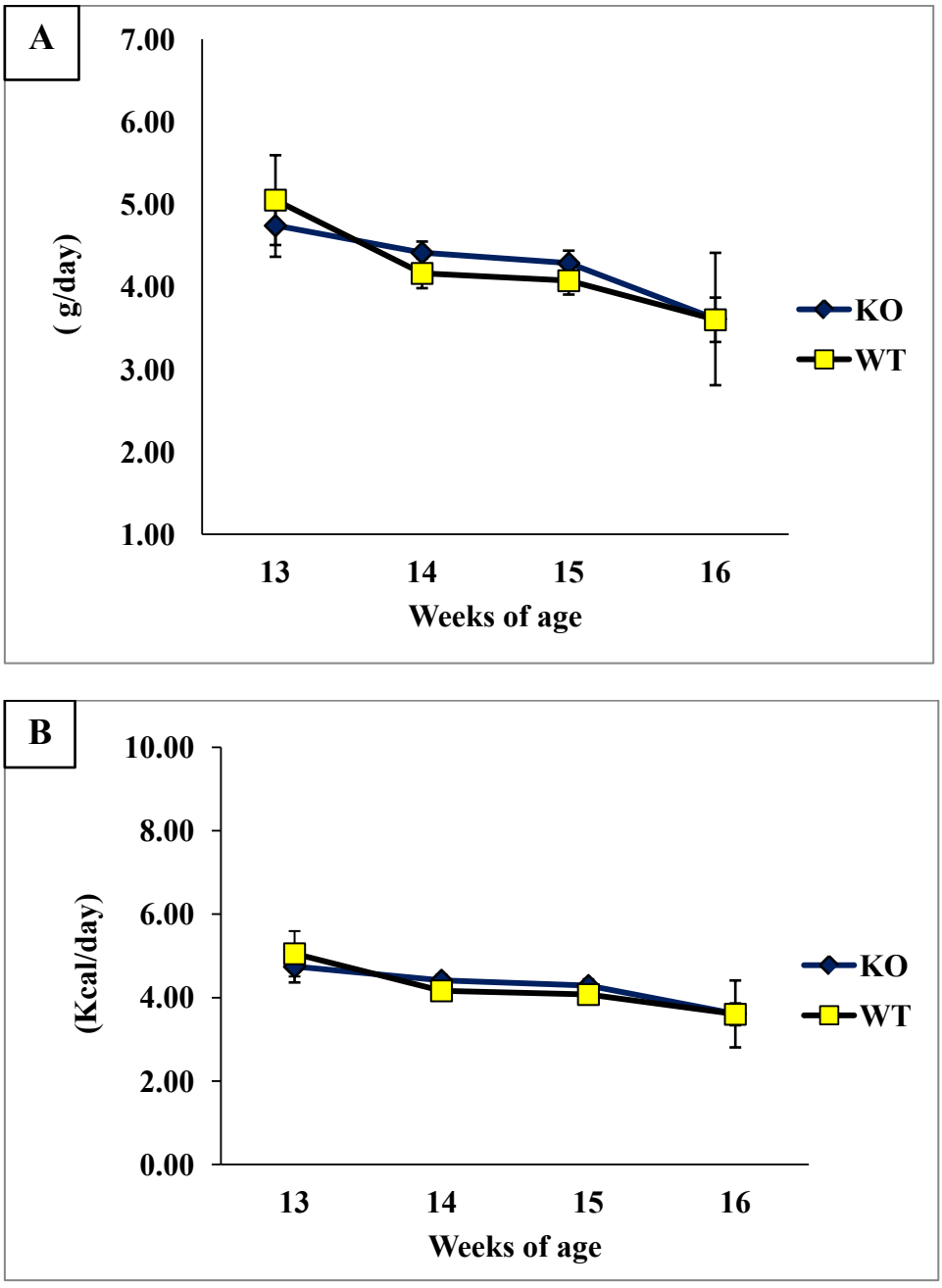

Figure 17: Food intake of male mice
Over four weeks on normal diet, KO males showed no differences in their daily food consumption as expressed in grams (A) and in kilocalories (B) as compared to their WT littermates. N= 6 KOs and 10 WTs.

By EchoMRI analysis of their body composition, KO males displayed a significant decrease in their fat mass in grams that was maintained when it was corrected for mice tibial length. However, they also had significant reduction in their lean body mass in grams (16% less lean mass) that was maintained when corrected for mice tibial length as compared to their WTs (Figure 18). Given that the male KO mice also had shorter tibial length (1.68 ± 0.03 versus 1.81 ± 0.02) this strongly suggests that these mice may have developmental issues potentially due to their extreme leanness.

The decrease in fat mass observed by EchoMRI was confirmed by the significant reduction in PRF, SCF, PGF and BAT weights per se (Figure 19A) and corrected by tibial length (Figure 19B) as measured at sacrifice. In fact, KO males showed significantly lighter brain weights as compared to their wild type littermates even when it was corrected for tibial length (Table 2) while only a tendency for smaller heart weights could be observed which disappeared when heart weights were corrected for tibial length. Moreover, KO males tended to have smaller left ventricles (LV) which disappeared when corrected for mice tibial length (Table 3). They also showed no differences in the size of any of the other heart chambers, even when corrected for tibial length, as compared to their WT littermates (Table 3).

Interestingly, KO males showed significant increase in their locomotor activity as detected by the physioscan cages. KO males showed a 2-folds rise in the total horizontal distance traveled in both dark and light cycles as compared to their WT littermates (Figure 20). No obvious differences could be detected between the complete KO males and their WT littermates when looking at fasting glycemia (223 ± 20.49 versus 243 ± 32.03 mmol/l).

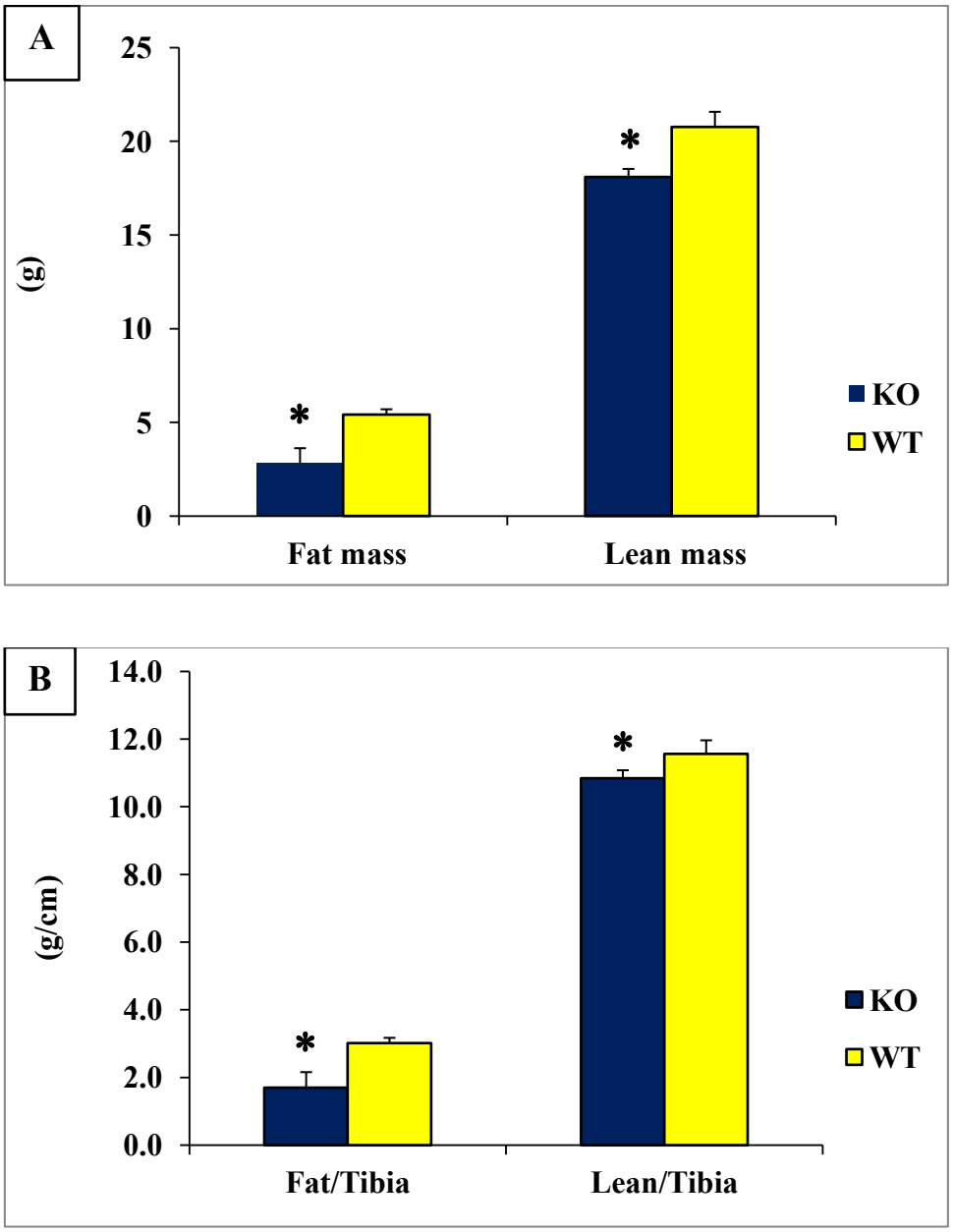

Figure 18: EchoMRI data analysis for male mice.
Body composition analysis measured by EchoMRI demonstrated that KO males presented with not only a significant reduction in their total fat mass weight, but also in their lean body mass as compared to their WT littermates (A). When corrected for the tibial length (B), these differences were maintained. Values are expressed as means ± SE. * $p \leq 0.05$, statistically different from WTs. N= 4 KOs and 4 WTs.

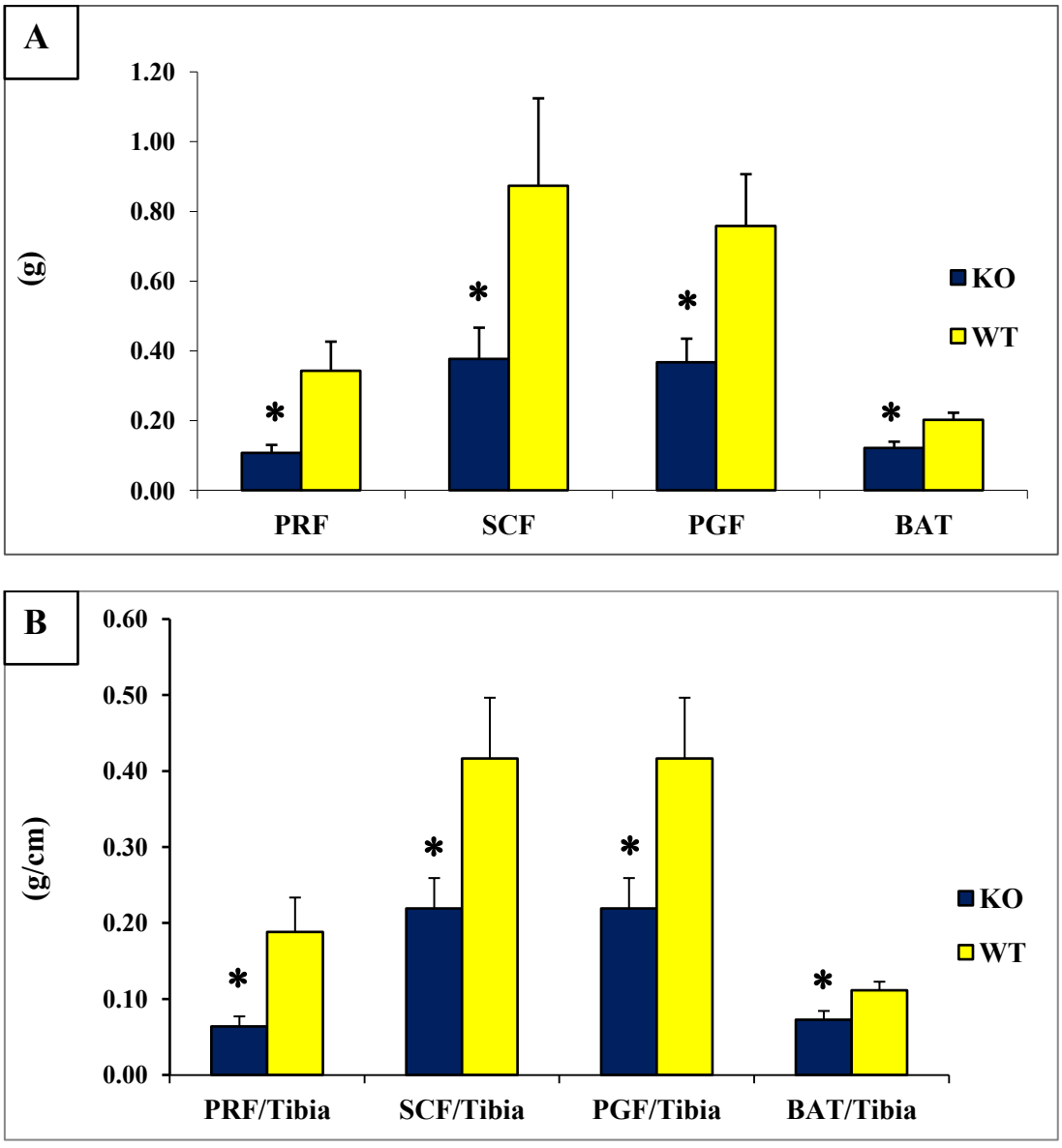

Figure 19: Weights of different fat pads.
Male KOs displayed significantly lighter white adipose tissue pads, weighed separately at sacrifice, in addition to lighter brown adipose tissue pads as compared to their WT littermates (A). These differences were maintained when values were corrected for tibial length **(**B**)**. Values are expressed as means ± SE. * $p \leq 0.05$, statistically different from WTs. N= 6 KOs and 6 WTs . Brown adipose tissue, BAT; Subcutaneous fat, SCF; Perigonadal fat, PGF; Perirenal fat, PRF.

Table 2: Weights and lengths of different tissues in male mice

	WT		KO	
	Weight (g)	Weight/tibia length ratio (g/cm)	Weight (g)	Weight/tibia length ratio (g/cm)
Liver	1.118 ± 0.103	0.618 ± 0.060	1.022 ± 0.082	0.610 ± 0.053
Heart	0.170 ± 0.023	0.091 ± 0.012	0.123 ± 0.007	0.073 ± 0.004
Brain	0.471 ± 0.013	0.260 ± 0.007	0.355 ± 0.015†	0.215 ± 0.009‡
kidneys	0.384 ± 0.029	0.156 ± 0.029	0.343 ± 0.020	0.187 ± 0.020
Adrenal	0.010 ± 0.004	0.006 ± 0.002	0.008 ± 0.001	0.005 ± 0.001

Values are expressed as means ± SE. † $p \leq 0.001$, ‡ $p \leq 0.005$ statistically different from WTs. N= 6 KOs and 6 WTs. Whole organs were weighed separately at sacrifice. Red color: KO males showed significantly lighter brain weights as compared to their wild type littermates even when it was corrected to tibial length.

Table 3: Weights of different heart chambers in male mice

	WT		KO	
	Weight (g)	Weight/tibia length ratio (g/cm)	Weight (g)	Weight/tibia length ratio (g/cm)
Left ventricle	0.087 ± 0.006	0.049 ± 0.004	0.081 ± 0.007	0.050 ± 0.005
Left atrium	0.012 ± 0.003	0.007 ± 0.002	0.003 ± 0.000	0.002 ± 0.000
Right ventricle	0.021 ± 0.001	0.012 ± 0.001	0.022 ± 0.005	0.013 ± 0.003
Right atria	0.007 ± 0.004	0.004 ± 0.002	0.007 ± 0.002	0.004 ± 0.002

Values are expressed as means ± SE. N= 3 KOs and 3 WTs. Heart chambers were separated and weighed at sacrifice.

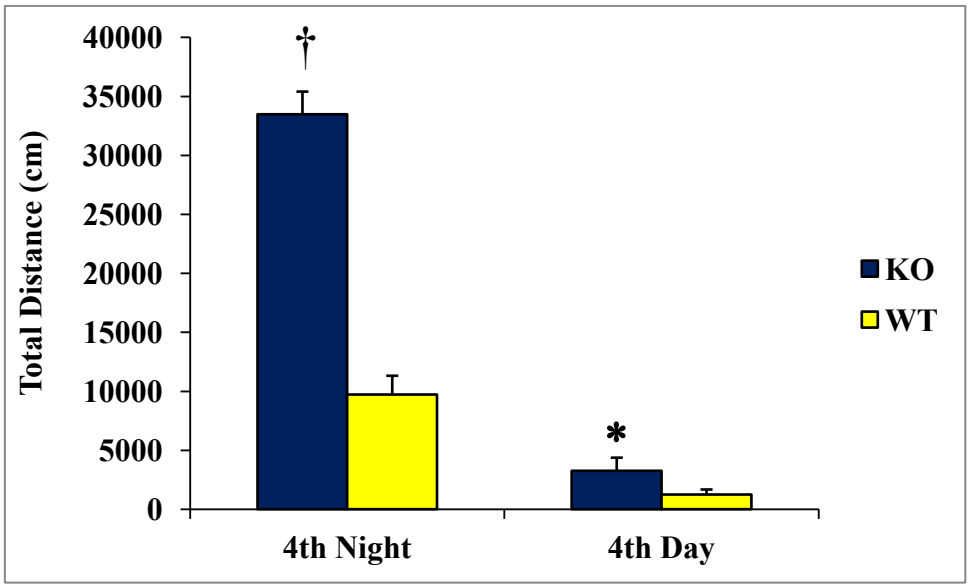

Figure 20: Locomotor activity of male mice.
KO males traveled significantly longer total distances in physioscan cages during both dark and light cycle as compared to their WT littermates. † p≤0.001, * p≤0.05 statistically different from WTs. N= 4 KOs and 6 WTs.

As expected, female partial KO mice presented with similar phenotypes then the males but with less pronounced differences in comparison to their WT littermates. As such, partial KO females had initially significant lighter body weights (10.9% less body weight) which was maintained throughout the four weeks of measurement while they were on a normal diet (14% less body weight) (Figure 21). Interestingly, when a group of these mice was transferred to a HF/HC diet, they showed significant resistance to weight gain and had significantly lighter body weights over the 6 weeks of administration (Figure 22A, B and Figure 23). Moreover, similarly to what was observed in male mice, no significant differences in food intake could be detected when calculated as daily consumption in grams or as daily caloric consumption on either the normal diet or the HF/HC diet (Figures 24 and 25).

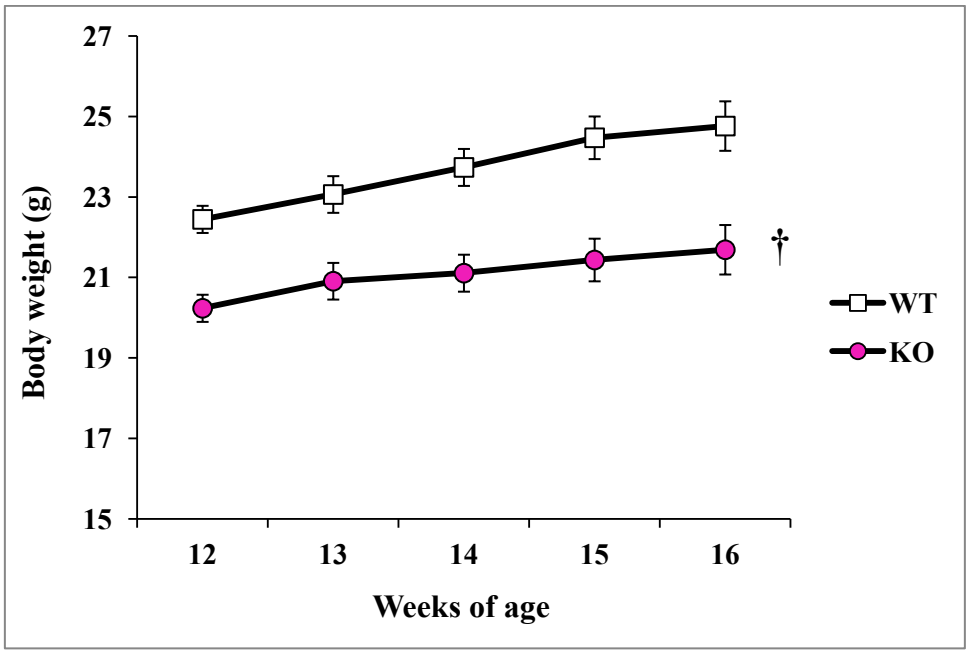

Figure 21: Body weight of female mice on normal diet.
Female KO mice showed significantly lighter body weights over four weeks on a normal diet as compared to their WT littermates. Values are expressed as means ± SE. † $p \leq 0.001$, statistically different from WTs. N= 11 KOs and 11 WTs.

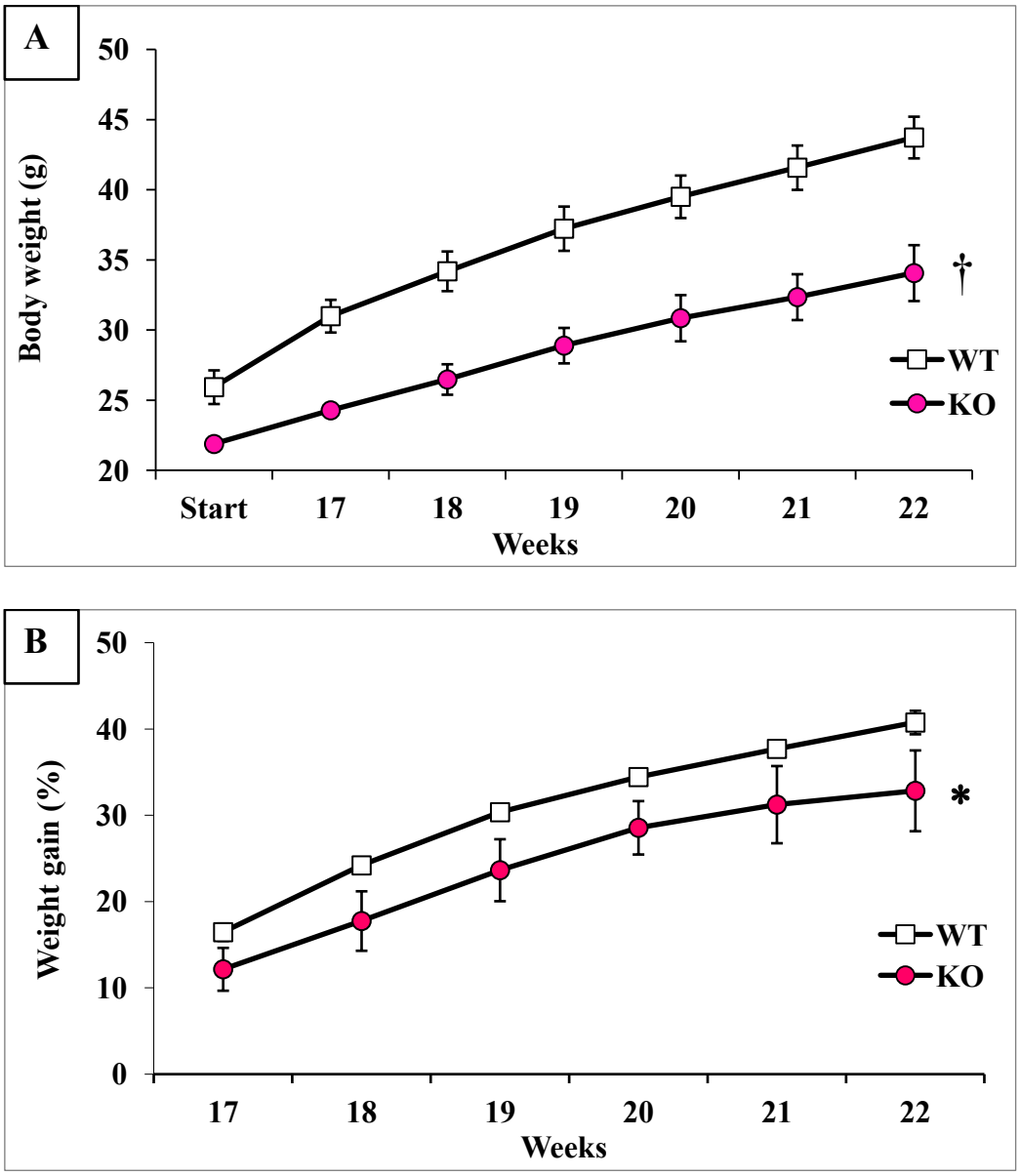

Figure 22: Body weight and weight gain of female mice on HF/HC diet.
Female KO mice showed significantly lighter body weights when kept for six weeks on HF/HC diet (A), and as such, they were significantly resistant to weight gain (B). Values are expressed as means ± SE. † $p \leq 0.001$, ∗ $p \leq 0.05$, statistically different from WTs. N= 7 KOs and 8 WTs.

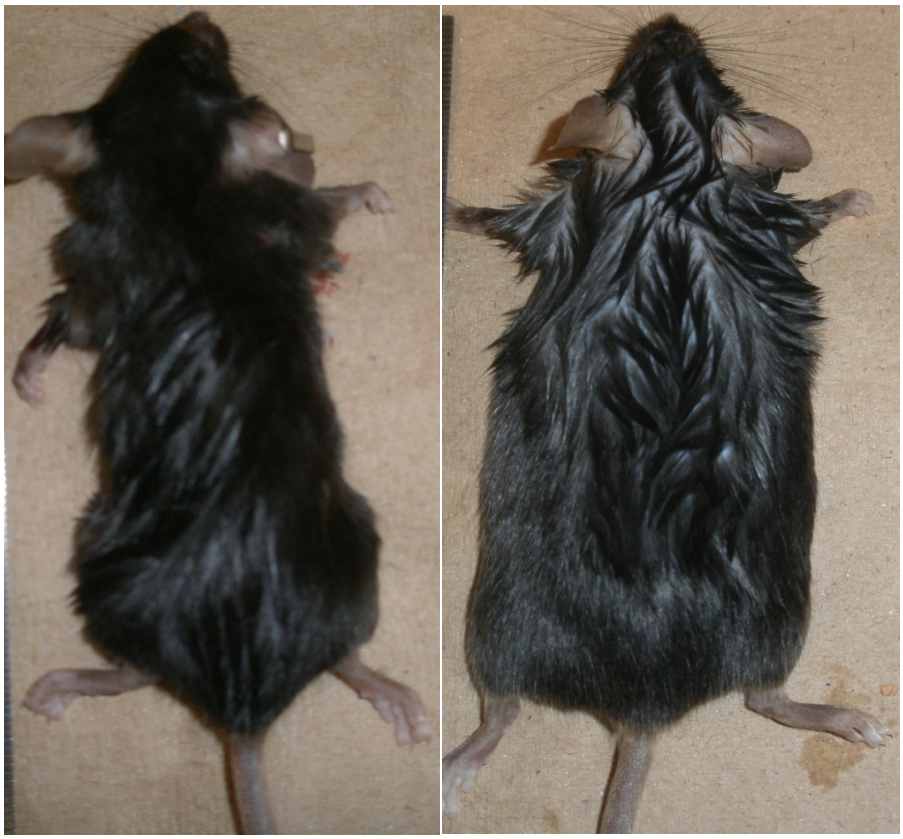

Figure 23: Female mice on HF/HC diet:
Pictures of partial KO female (on the left) and its WT littermate (on the right) at 22 weeks of age.

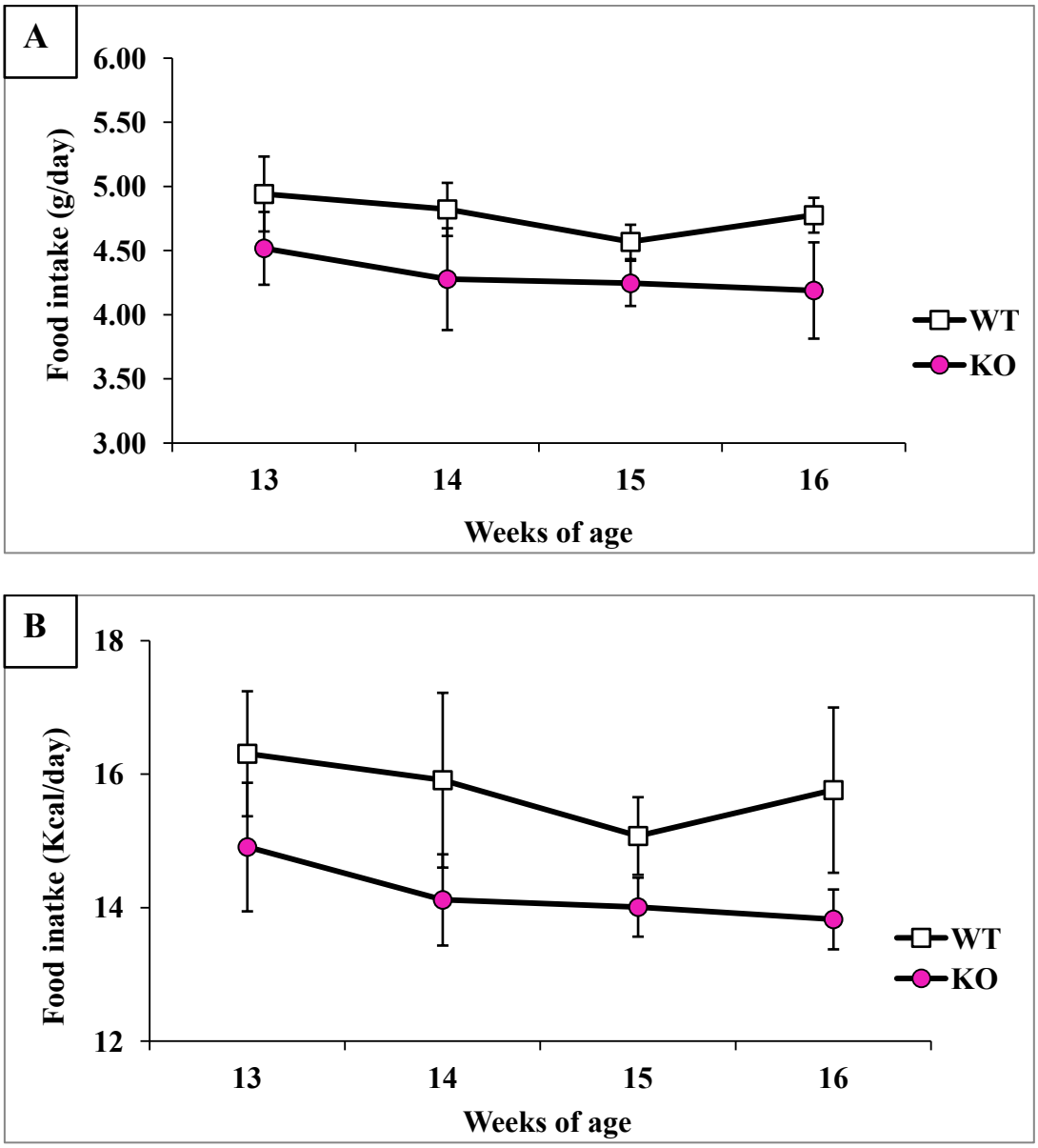

Figure 24: Food intake of female mice on normal diet.
Over four weeks on normal diet, female KO mice showed no differences in their daily food consumption as expressed in grams (A) and in kilocalories (B) as compared to their WT littermates. Values are expressed as means ± SE. N= 11 KOs and 11 WTs.

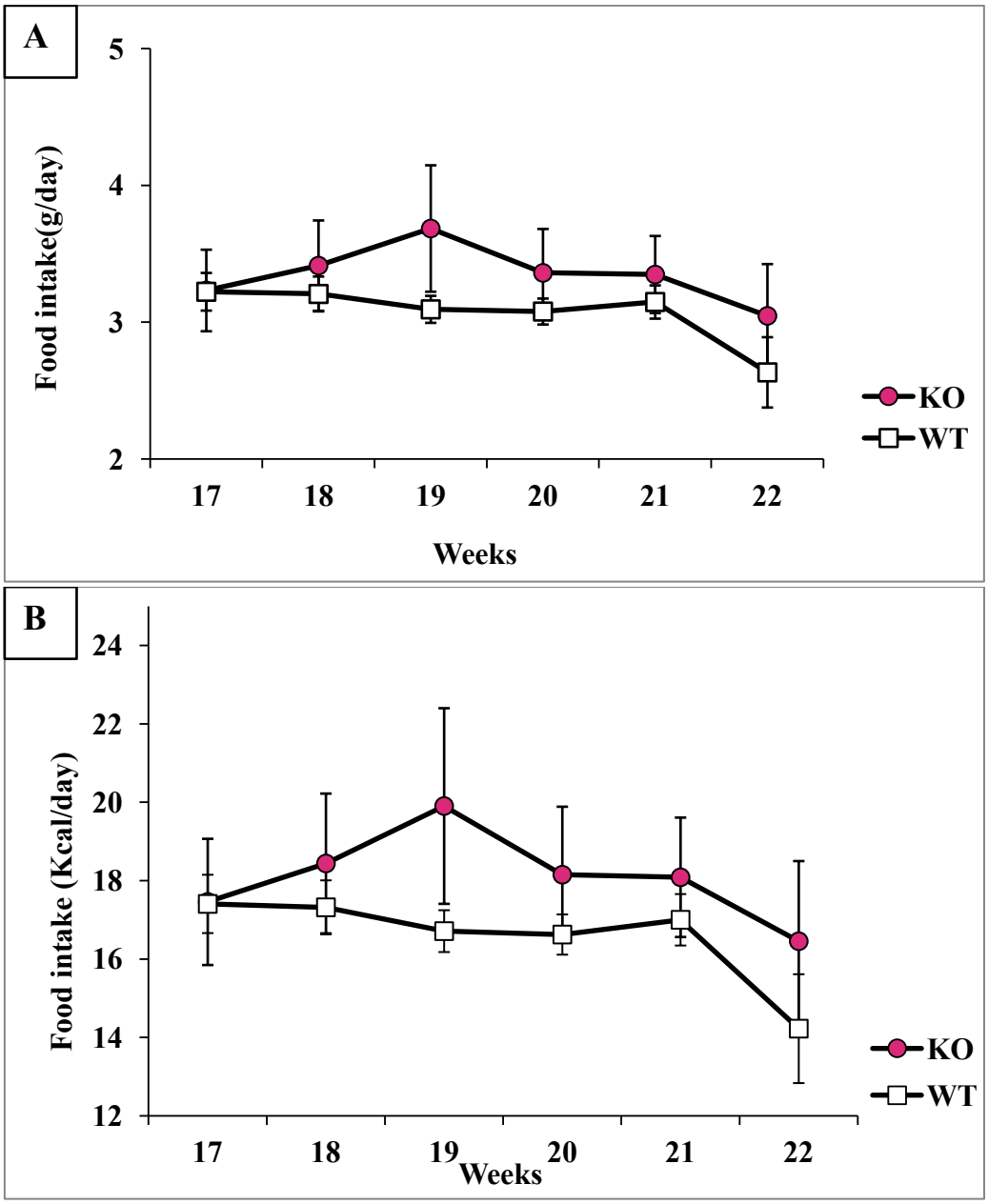

Figure 25: Food intake of female mice on HF/HC diet.
Over six weeks on a HF/HC diet, female KO showed no differences in their daily food consumption as expressed in grams (A) and in kilocalories (B) as compared to their WT littermates. Values are expressed as means ± SE. N= 7 KOs and 8 WTs.

Also, as determined in the male mice, EchoMRI analysis of the body composition for the partial KO females on the normal diet demonstrated that they had significantly less fat and lean body mass in grams (Figure 26A) that was maintained when corrected for mice tibial length (Figure 26B). Similarly to what was observed on the normal diet, the group of females that was transferred to the HF/HC diet showed significantly less fat mass in grams as compared to their WT littermates but without detectable differences in mice lean body mass (Figure 27A) even when corrected to mice tibial length (Figure 27B). The EchoMRI observations were associated with a significant reduction in subcutaneous and perigonadal fat in addition to a strong tendency for less perirenal fat on both normal and HF/HC diet (Figures 28A and 29A) even when corrected to mice tibial length (Figures 28B and 29B).

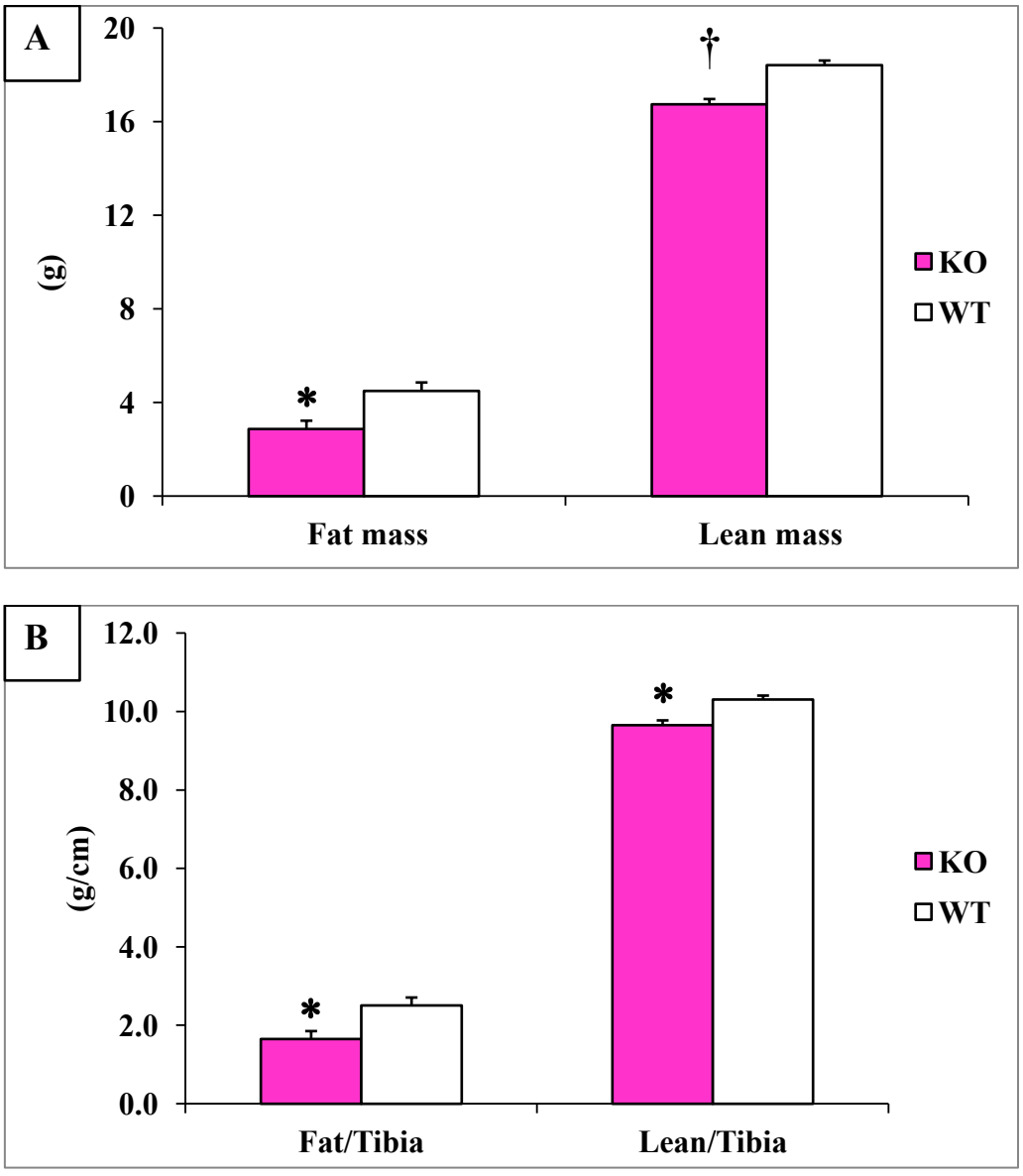

Figure 26: EchoMRI analysis for female mice on normal diet.
After 4 weeks on a normal diet, body composition evaluated by EchoMRI demonstrated that female KO showed not only significantly lower fat mass weight but also less lean body mass as compared to their WT littermates (A).These differences were also maintained when values were corrected for tibial length (B). Values are expressed as means ± SE. † $p \leq 0.001$, * $p \leq 0.05$, statistically different from WTs. N= 11 KOs and 11 WTs.

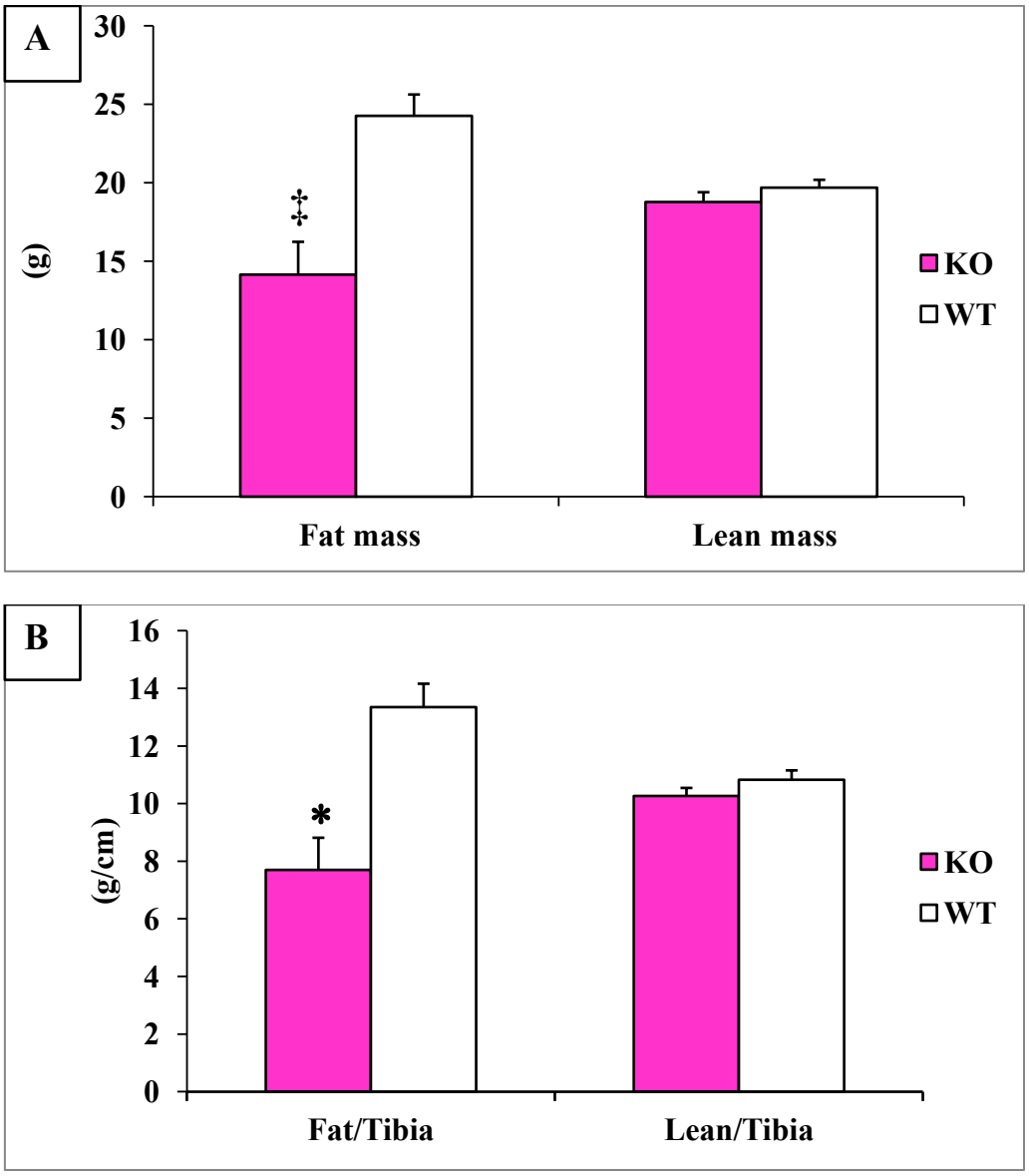

Figure 27: EchoMRI analysis for female mice on HF/HC diet.
When female KO were placed on a HF/HC diet, evaluation of the body composition by EchoMRI demonstrated that they had significantly less fat mass but without any significant differences in their lean body mass as compared to their WT littermates (A). These differences were maintained when values were corrected for tibial length. Values are expressed as means ± SE. ‡ $p \leq 0.005$, * $p \leq 0.05$ statistically different from WTs. N= 7 KOs and 7 WTs.

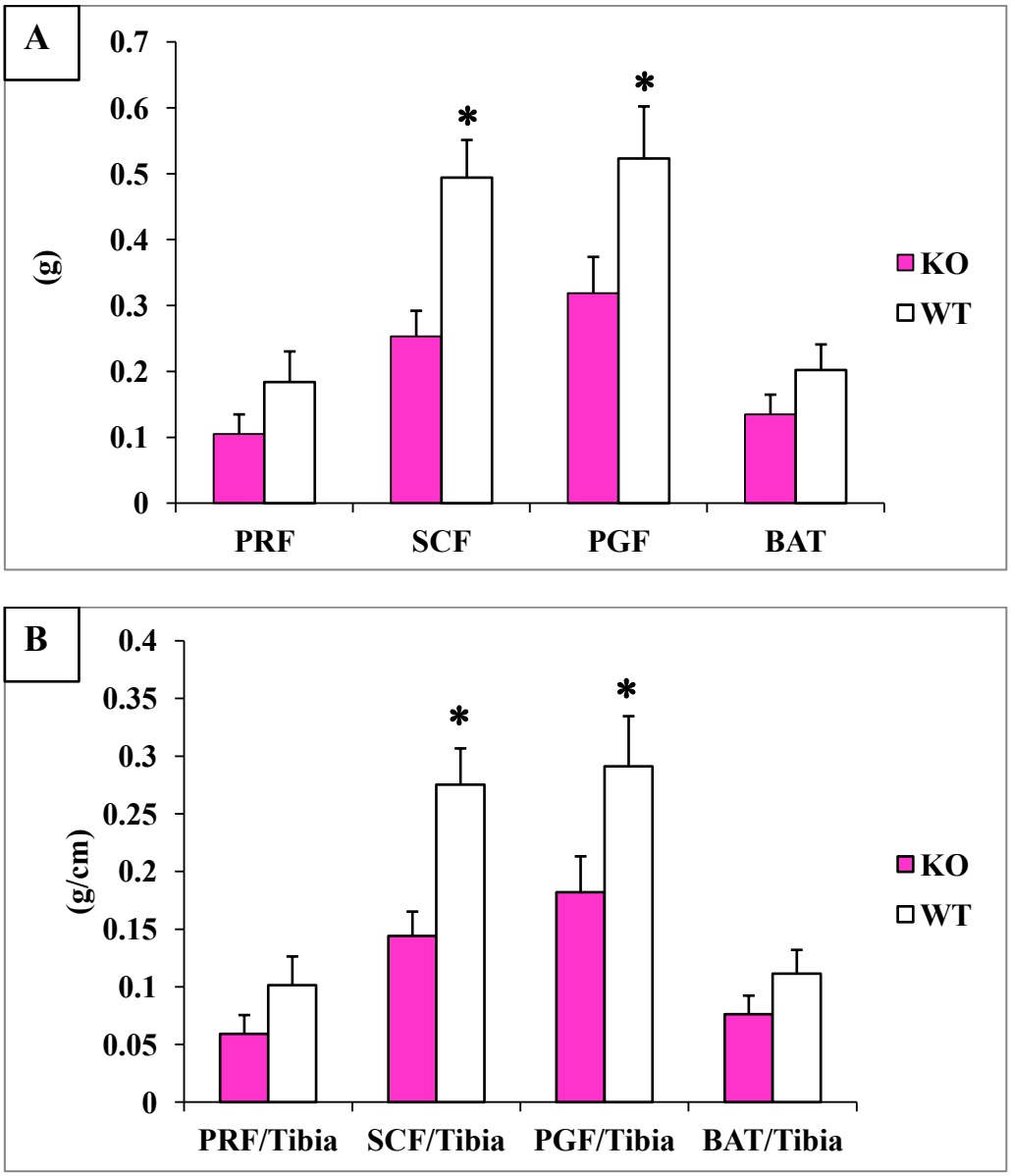

Figure 28: Weights of different fat pads for female mice on normal diet.
After 4 weeks on a normal diet, female KO showed significantly lighter subcutaneous (SCF) and perigonadal (PGF) fat , weighed separately at sacrifice, as compared to their WT littermates (white) while perirenal (PRF) and brown adipose tissue (BAT) pads only tended to be lighter (A). These differences were maintained when the weights of these tissues were corrected for tibial length (B). Values are expressed as means ± SE. * $p \leq 0.05$, statistically different from WTs. N= 11 KOs and 11 WTs.

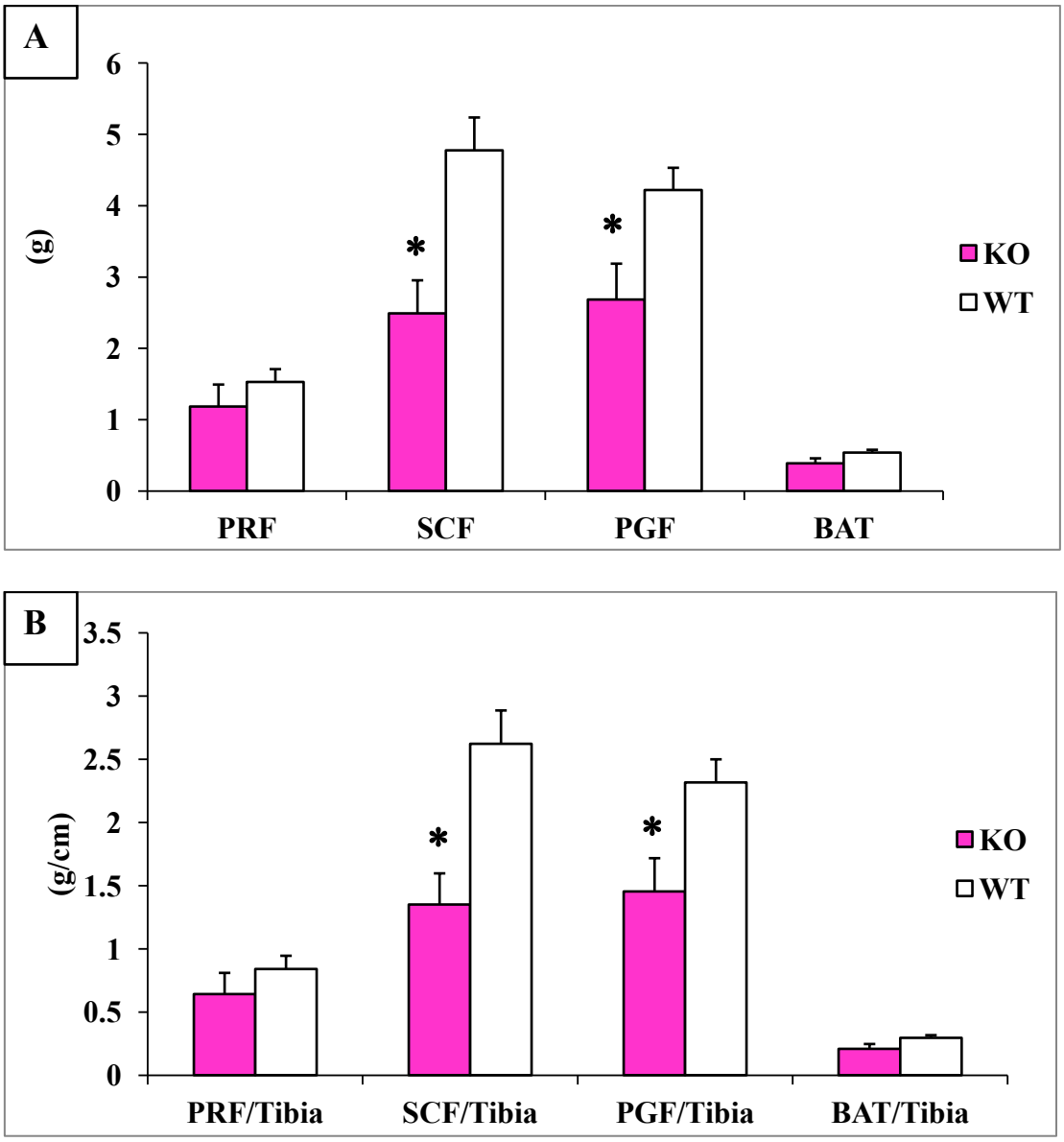

Figure 29: Weights of different fat pads for female mice on HF/HC diet.
After 4 weeks on a HF/HC diet, female KO showed significantly lighter subcutaneous (SCF) and perigonadal (PGF) fat, weighed separately at sacrifice, as compared to their WT littermates (white) while perirenal (PRF) and brown adipose tissue (BAT) pads only tended to be lighter (A). These differences were maintained when the weights of these tissues were corrected for tibial length (B). Values are expressed as means ± SE. * $p \leq 0.05$, statistically different from WTs. N= 6 KOs and 6 WTs.

In addition, partial KO females on the normal diet showed no diffrences in tibial lengths as compared to their WT littermates (1.793 ± 0.017 versus 1.759 ± 0.018 cm). They also tended to have a lighter brain and heart weights compared to the WT mice with no differences in liver and kidney weights (Table 4) but these tendencies disappeared when the weights were corrected for the tibial length (Table 4). Moreover, partial KO females tended to have smaller right ventricles (RV) even when corrected for tibial length (Table 5) and also tended to have smaller left ventricles (LV) which disappeared when corrected for mice tibial length (Table 5). They also showed no differences in the size of the left and right atria (LA and RA) even when corrected for tibial length as compared to their WT littermates (Table 5).

On the other hand, KO females that were placed on a HF/HC diet tended to have lighter liver weights as compared to their WT littermates that were maintained when corrected for tibial length without any differences in heart and kidney weights (Table 6).

As in males, no obvious differences in glycemia were detected between partial KO females and their WT littermates either on the normal diet (207 ± 29.09 versus 163 ± 30.17 mmol/l) or on the HF/HC diet (144±20.99 versus 130±19.60 mmol/l).

Interestingly, KO females on the normal diet tended to have less proteinuria as estimated by albumin/creatinine ratio compared to their WT littermates (0.10 ± 0.01 versus 0.23 ± 0.06 μg/mg).

Table 4: Weights and lengths of different tissues in female mice on normal diet

	WT		KO	
	Weight (g)	Weight/tibia length ratio (g/cm)	Weight (g)	Weight/tibia length ratio (g/cm)
Liver	1.118 ± 0.063	0.623 ± 0.033	1.002 ± 0.053	0.569 ± 0.028
Heart	0.130 ± 0.007	0.072 ± 0.003	0.111 ± 0.011	0.063 ± 0.006
Brain	0.476 ± 0.007	0.010 ±0.003	0.445 ± 0.017	0.253 ± 0.010
kidneys	0.147 ± 0.004	0.082 ±0.002	0.144 ± 0.005	0.082 ± 0.002
Adrenal	0.012 ± 0.003	0.007 ±0.002	0.010 ± 0.002	0.006 ± 0.001

Values are expressed as means ± SE. N= 11 KOs and 11WTs. Whole organs weighed at sacrifice

Table 5: Weights of different heart chambers in female mice on normal diet.

	WT		KO	
	Weight (g)	Weight/tibia length ratio (g/cm)	Weight (g)	Weight/tibia length ratio (g/cm)
Left ventricle	0.089 ± 0.004	0.050 ± 0.002	0.085 ± 0.004	0.049 ± 0.002
Left atrium	0.003 ± 0.0005	0.001 ± 0.000	0.003 ± 0.0005	0.001 ± 0.000
Right ventricle	0.024 ± 0.002	0.013 ± 0.001	0.017 ± 0.003	0.010 ± 0.002
Right atrium	0.004 ± 0.001	0.002 ± 0.001	0.006 ± 0.001	0.003 ± 0.001

Values are expressed as means ± SE. N= 8 KOs and 8 WTs. Heart chambers were separated and weighed at sacrifice.

Table 6: Weights and lengths of different tissues in female mice on HF/HC diet.

	WT		KO	
	Weight (g)	Weight/tibia length ratio (g/cm)	Weight (g)	Weight/tibia length ratio (g/cm)
Liver	1.211 ± 0.058	0.664 ± 0.031	1.031 ± 0.073	0.562 ± 0.041
Heart	0.149 ± 0.005	0.082 ± 0.004	0.147 ± 0.006	0.080 ± 0.003
kidneys	0.333 ± 0.010	0.182 ± 0.007	0.343 ± 0.013	0.187 ± 0.007

Values are expressed as means ± SE. N= 6 KOs and 6 WTs. Whole organs were weighed at sacrifice.

However, in contrast to the male KOs, no significant differences could be observed in the locomotor activity of female KOs as compared to their WTs (Figure 30).

In accordance with their decreased weight gain with the administration of a HF/HC diet, partial KO females strongly tended to have less plasma leptin levels (Figure 31) while no detectable differences of adiponectin levels (32.2 ± 4.97 versus 32.5 ± 3.59). In addition, no differences in fasting plasma total cholesterol levels (87.643 ± 18.706 versus 87.428 ± 12.563 mg/dl) or fasting plasma total triglyceride levels (41.695 ± 8.917 versus 38.395 ± 7.697 mg/dl) were detected between female KOs and WTs.

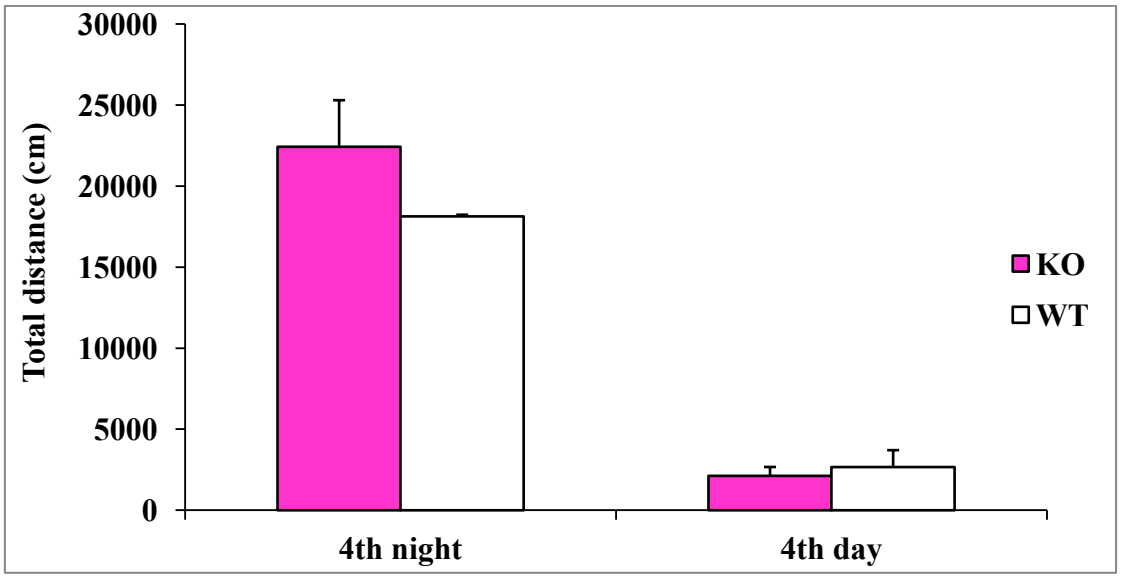

Figure 30: Locomotor activity of female mice.
Partial KO females on normal diet didn't show significant differences in their total distance traveled at physioscan cages during both dark as well as light cycle as compared to their WT littermates. N= 7 KOs and 7 WTs.

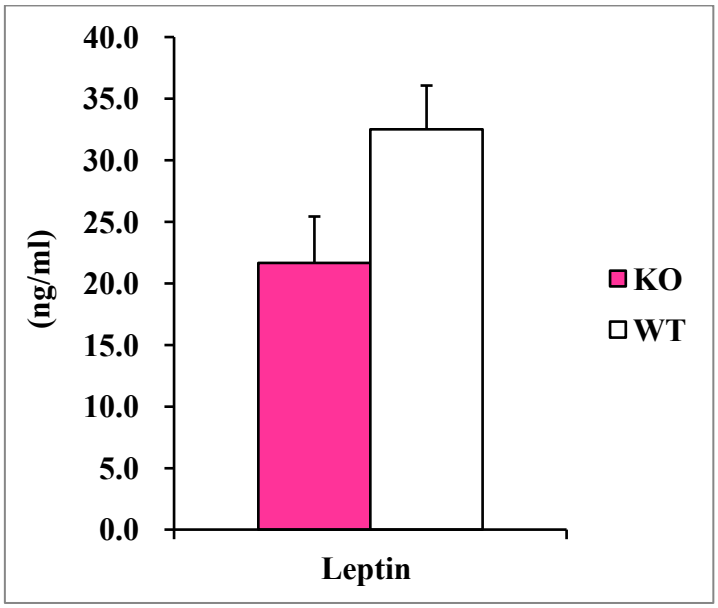

Figure 31: Plasma leptin level of female mice on HF/HC diet.
Partial KO females on HF/HC diet tended to have less plasma leptin levels compared to their WT littermates. Values are expressed as means ± SE. N= 6 KOs and 6 WTs.

The histological analysis of the perigonadal fat pads of female mice on a normal diet revealed a significant increase in the average size of adipocytes accompanied by a significant decrease in adipocyte number (Figure 32).

The Gene Affymetrix transcriptome of the perigonadal fat of male and female KOs mice demonstrated an associated change in the regulation of different genes as shown in Table 7. Of note, one of the genes which was found to be down regulated is the Mutant human ABLIM2 gene (SNP substitution mutation, allelic variations: A/G (rs1574131)) which has been found to be associated with non-insulin-dependent diabetes mellitus (Table 7).

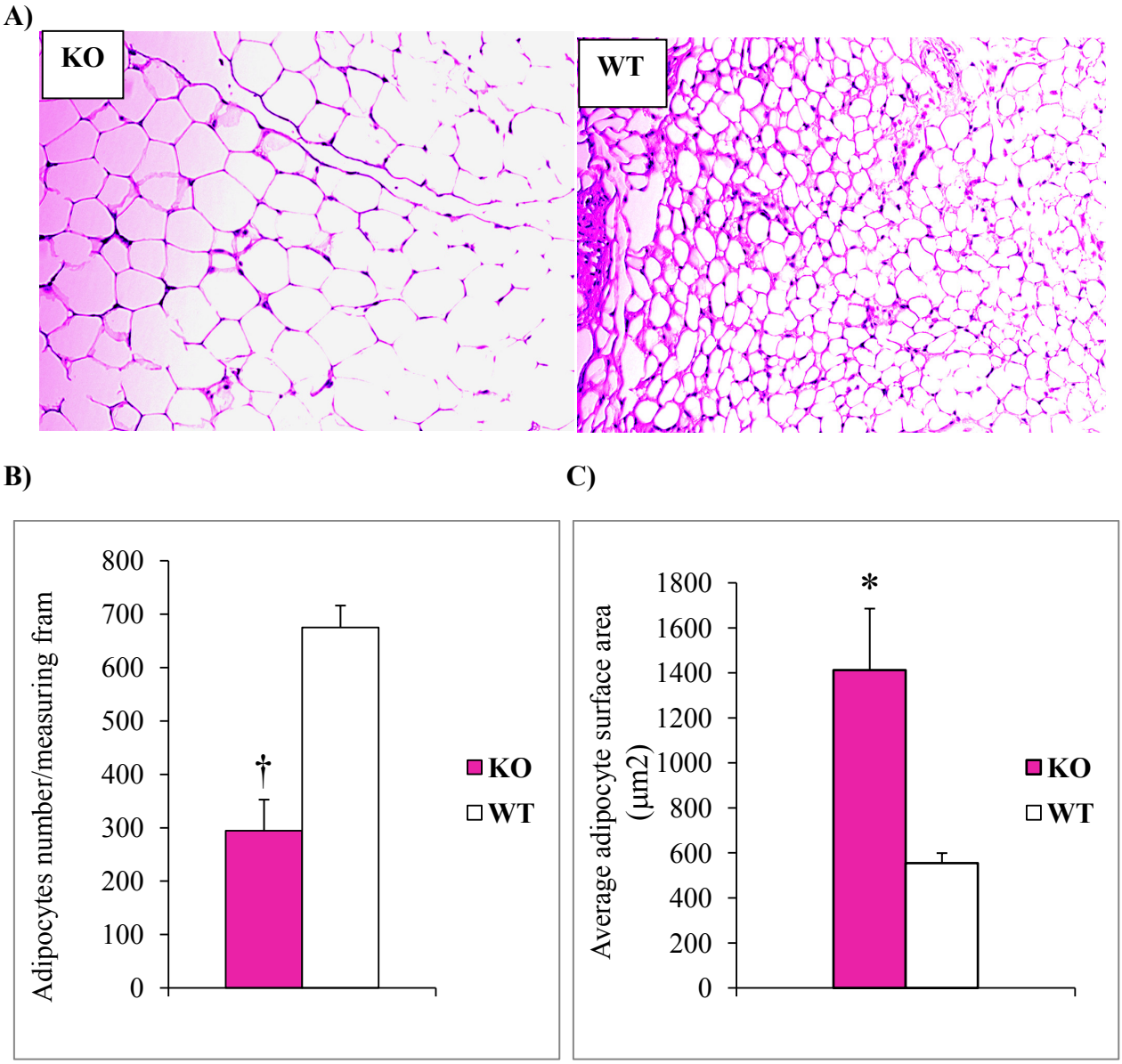

Figure 32: White adipose tissue histology.
(A) Stained sections (hematoxylin and eosin) of perigonadal fat of female KO (left) and WT (right) mice on normal diet. Scale bar: 100 µm. **(**B) female KO on a normal diet displayed a significantly less number of adipocytes in their perigonadal fat associated with signifcantly larger adipocytes (C) as compares to their WT littermates. Values are expressed as means ± SE. † $p \leq 0.001$, * $p \leq 0.05$ N= 4 KOs and 4 WTs.

Table 7: Microarray analysis: gene regulation.

Symbol	Synonym(s)	Gene Name	Fold Change(F)	Fold Change(M)	Location	Symbol
SPIB	MGC109046	Spi-B transcription factor (Spi-1/PU.1 related)	-1,409	-1,485	Nucleus	Transcription regulator
ABLIM2†	AI606905, C230091L11, DKFZp761F129, FLJ26534, FLJ39684, KIAA1808, LOC100128350, MGC141918	Actin binding LIM protein family, member 2	-1,345	-1,375	Cytoplasm	Other
SELL	A.11, AI528707, CD62L, L-SELECTIN, LAM1, LECAM-1, LEU8, LNHR, LSEL, Ly-22, Ly-m22, LYAM1, lymphocyte adhesion molecule 1, Mel14, PLNHR, TQ1	Selectin L	-1,325	-1,303	Plasma Membrane	Other
GPR56	BFPP, Cyt28, DKFZp781L1398, TM7LN4, TM7XN1	G protein-coupled receptor 56	-1,207	1,366	Plasma Membrane	G-protein coupled receptor
PIP5K1B	AI844522, MGC105713, MSS4, PI4P5K IA, Pip 5 Kinase alpha, PIP5K BETA, Pip5k1a, PIPK5A, Pipk5b, Pipki, Pipki alpha, STM7, TYPE I PIPKINASE BETA	Phosphatidylinositol-4-phosphate 5-kinase, type I, beta	-1,135	1,429	Cytoplasm	Kinase
UNC5B	6330415E02Rik, A630020F16, D10BWG0792E, p53RDL1, UNC5H2	unc-5 homolog B (C. elegans)	1,142	1,614	Plasma Membrane	Transmembrane receptor
CDR2L	D030068L24, D030068L24Rik, Gm21, HUMPPA, RGD1306881	Cerebellar degeneration-related protein 2-like	1,167	1,362	unknown	Other
KIF1A	ATSV, C2orf20, C630002N23Rik, DKFZp686I2094, FLJ30229, Gm1626, HUNC-104, Kin1a, Kinesin-1A, Kns1, MGC133285, MGC133286, UNC104	Kinesin family member 1A	1,186	1,508	Cytoplasm	Other
FGF21		Fibroblast growth factor 21	1,223	1,356	Extracellular Space	Growth factor
MDK	FLJ27379, Mek, MIDKINE, MK, NEGF2	Midkine (neurite growth-promoting factor 2)	1,233	1,548	Extracellular Space	Growth factor
THBD	AHUS6, AI385582, BDCA3, CD141, THRM, THROMBOMODULIN, TM	Thrombomodulin	1,267	1,202	Plasma Membrane	Transmembrane receptor

† $p \leq 0.001$

Table7: Microarray analysis: gene regulation (Continued).

Symbol	Synonym(s)	Entrez Gene Name	Fold Change(F)	Fold Change(M)	Location	Symbol
SEMA3B	AW208495, FLJ34863, LUCA-1, SEMA, SEMA5, SEMAA, semaV	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	1,299	1,419	Extracellular Space	Other
RRAGD	5730543C08Rik, AI467523, bA11D8.2.1, C030003H22RIK, D4Ertd174e, DKFZP761H171, FLJ44176, RAGD	Ras-related GTP binding D	1,331	1,679	Cytoplasm	Enzyme
ALDH1L2	D330038I09RIK, DKFZp686A16126, DKFZp686M064, DKFZp686P14145, FLJ36769, FLJ38508, MGC119536, MGC119537, MGC19039, mtFDH, RGD1309458	Aldehyde dehydrogenase 1 family, member L2	2,273	2,876	unknown	Enzyme

Chapter 9 : Discussion

To our knowledge, we are the first group to knock down the (P)RR in white adipose tissue. We validated our model by evaluating the (P)RR mRNA expression in many organs, comparing KO mice to their WT littermates. We found a unique and tissue specific decrease in the receptor expression level in white adipose tissue pads (subcutaneous, perirenal and perigonadal fat) in the KO mice while no differences could be detected in all other evaluated tissues. The percentage of the deletion of the receptor gene was around 50% in the white adipose tissue of the complete KO males and around 30% in those of the partial KO females. However, in these experiments, we examined whole adipose tissue fat pads which are a mix of adipocytes and other cells such as fibroblasts, endothelial cells, preadipocytes, and macrophages as well as many small blood vessels. In this type of experiment around 70-80% deletion has been often observed with other genes using the AP2 promoter [158]. However, since adipocytes compose approximately 50% of the cells in adipose tissue[90] and that the (P)RR is known to be quite ubiquitous[34] it is not that surprising that we observed lesser levels of gene expression deletion. As such, we anticipate that this deletion probably targeted adipocytes specifically although to verify this, adipocyte isolation will be required.

As expected, we noticed a more accentuated phenotype in male KO mice compared to females since the (P)RR gene is carried on the X chromosome [41]. Indeed, because of our breeding strategy, male mice were complete KOs whereas females were only partial KO. We have noticed that we seemed to have a reduced number of male KO mice per litter as determined after weaning than what would be expected by the Mendelian genetics. This suggests that there are some lethality occurred as a result of complete knocking down of the receptor in adipose tissue.

In fact, a similar but more sever shortening of mice life span was shown when (P)RR was knocked down uniquely in other cell types such as cardiomyocytes as well as podocytes [40, 61]. In addition, we were able to determine which males were KOs directly in the cage because of their much lower body weights. Furthermore, complete KO males presented with a 28% lighter body weights at 16 weeks of age when maintained on a normal diet which was not associated with any changes in daily caloric food intake. These weight changes were characterized by a significant reduction in fat mass. However, a significant reduction in their lean body mass was also detected. Given that they also showed significantly shorter tibias, this strongly suggests that these mice may have developmental issues as a result of the adipose tissue (P)RR deletion or because of the extreme leanness of these animals. However, given that female KO mice also exhibited a decrease in lean body mass with a less pronounced decrease in fat mass, this suggests that specific deletion of the (P)RR in adipose tissue may contribute to maturation potentially through the modulation of different adipokines.

As expected, female partial KO mice had similar but less pronounced phenotypes than that of the males regarding the body weight and composition changes as well as food intake on a normal diet and these phenotypes were accentuated when the females were transferred to HF/HC diet although no detectable changes could be measured for their locomotor activity. However, female KO mice had no significant changes in their tibial length.

Ang II was reported to have a lipogenic effect as it enhances adipocytes accumulation of triglycerides[126] and stimulates the activity of lipogenic enzymes[146] which contributes to expanding the adipose tissue mass. As mentioned earlier in the text, activation of the (P)RR leads to an increase in renin's catalytic efficiency and to a non-proteolytic activation of prorenin [34]. As such, it

may contribute to an increase in the local production of Ang II which could further expand the white adipose tissue mass.

Therefore, one of the underlying mechanisms for the phenotype observed in our KO mice could be through decreasing the renin and prorenin activity which would produce a decrease in Ang II-dependent effects on white adipose tissues and decrease its growth.

It was reported that complete knocking down of the AT1Ra in their adipocytes showed no changes in their body weight and fat mass but displayed a significant adipocytes hypertrophy as a result of a decrease in adipocyte differentiation. Authors suggest that the increase in expression of other RAS receptors (AT1Rb and AT2R) may have compensated for the decrease of AT1Ra which may have contributed to a less striking phenotype [158]. However, our partial KO female mice had significantly lower body weights in association with a decrease in fat mass on normal diet and were resistant to body weight gain on HF/HC diet. In addition, their perigonadal fat showed similar histological phenotype on normal diet in the form of marked increase in adipocytes size in association with decrease of the adipocytes number. This suggests that RAS mechanisms are involved but that additional Ang II-independent pathways may enhance this phenotype so that a marked decrease in fat mass is observed. It will be important however to establish if compensatory mechanisms involving an increase in the expression of other RAS components in adipose tissue may mask the extent of the impact of the (P)RR deletion as what was observed in mice lacking AT1Ra. It will be also interesting to examine the differentiation capacity of preadipocytes to identify the potential mechanisms implicated in this histological phenotype.

As such, the (P)RR may impact adipose tissue mass through its Ang II-independent pathways, for instance, by producing MAPK activation and thus releasing TGF-ß1and profibrotic molecules such as, PAI-1, fibronectin protein and collagen I. On the other hand, it can also phosphorylate and

activate p38 MAPK as well as the HSP27. Through these factors, the (P)RR is thought to play a role in regulating the cell integrity, growth, motility and apoptosis. Therefore, these pathways could be implicated in the decreased white adipose tissue mass observed in the KO mice.

In addition, the functional (P)RR/V-ATPase relation could be implicated in the histological changes noticed for KO mice. As mentioned earlier in the text, the principle roles of V-ATPases are the acidification of intracellular compartments and proton pumping in different cell types. As such, it was found that deletion of (P)RR specifically in cardiomyocytes as well as in podocytes resulted in lethal histological abnormalities in these cell types later in life in association with the disruption of the V0 domain of V-ATPase. Hence, given the similar phenotype that is observed in adipocytes following the deletion of the (P)RR, evaluating the different domains of V-ATPase in adipocytes will be an important continuation for this study. Furthermore, the potential developmental issues observed in the KO mice could be as a result of the interaction with the V-ATPase. In fact, it was found that deletion of the receptor ubiquitously in Zebra fish is lethal and was associated with developmental abnormalities, which means that the (P)RR gene is essential for embryonic development [41]. As mentioned earlier in the text, the (P)RR was found to be implicated in the Wnt/β-catenin signaling pathway via its interaction with the V-ATPase [55]. Since the Wnt proteins have been shown to be essential for normal embryogenesis [54], it may be suggested that the complete deletion of the (P)RR lead to dysregulation of Wnt signaling, resulting in developmental malformation in KO mice. However, other experiments will be needed to determine the exact mechanism implicated in the developmental issues, such as by investigating the proteins of Wnt/β-catenin signaling in the adipocytes.

An important factor that could also contribute to the decrease in fat mass observed in our male and female KO mice is the possible increase in energy expenditure.

We would expect energy expenditure to be increased in our KO mouse model due to a rise in lipid oxidation given the reported effect of increased RAS activity in adipose tissue. Furthermore, although we did not evaluate this parameter in the current study, the increased locomotor activity observed in the male mice strongly suggests that they also have an increase in their daily energy expenditure. Although it is hard to determine the exact mechanism for this effect, similar findings have also been found in mice lacking AGT in their adipose tissue [141]. This thus suggests that adipose tissue RAS may have remote effects on the central nervous system. In fact, sympathetic activity was previously found to be increased when evaluated in AT1Ra null mice although unfortunately, locomotor activity was not evaluated in this study. So, overstimulation of the sympathetic activity could be a potential mechanism. Assessment of sympathetic activity as well as thermogenesis via the measurement of urinary catecholamines, mice body temperature as well as the expression and protein level of the UCP-1 gene in collected brown adipose tissues are to be done in the future.

We have previously shown in our laboratory that using the (P)RR blocker systemically improved the metabolic profile in mice treated with a HF/HC diet. However, we were unable to see these improvements in the current study although female mice were not treated with the HF/HC diet for a prolonged period of time as what had been done previously. Moreover, it is possible that systemic administration of the (P)RR blocker may have affected other organs such as the pancreas, which may have also contributed to the improvement of the metabolic profile. We have however noticed a down regulation of the ABLIM2 gene which was found to be associated with the development of type II diabetes in humans [159]. Additional experiments will be needed to further

investigate the regulation of glycemia and insulinemia through the oral glucose tolerance tests (OGTT) or intra-peritoneal insulin tolerance test (IPITT).

Moreover, when female KO mice were placed on a HF/HC diet, they tended to show lighter liver weights. This may suggest that they accumulated less fat in their livers. If this is the case, it suggests that KO females probably will be less susceptible to develop non-alcoholic fatty liver disease (NAFLD). NAFLD is excess accumulation of triglycerides and fats in adipocytes so that it exceeds %5 of the liver weight and it is a part of the metabolic changes that accompanies the development of obesity and could lead to metabolic disorders such as hepatic insulin resistance[160, 161].

In conclusion, KO males and females showed lighter body weights on normal diet in association with decreased fat and lean body mass. The KOs gonadal fat showed reduced adipocyte numbers of bigger size and reduced expression of ABLIM2 gene that is associated with human diabetes. In addition, the KO females were resistant to obesity development when placed on a HF/HC diet and thus showed less fat mass. Male KOs seemed to have developmental abnormalities as they presented with shorter tibial length. Moreover, we observed marked increase in locomotor activity in male KOs which may have contributed to their decreased fat mass and total body weight.

Chapter 10 : Conclusion and perspectives

The current study shows the implication of the (P)RR in the development of obesity through a mouse model that lacks the receptor in white adipocytes. As the (P)RR gene is located on the X-chromosome, we had homozygote complete knock-out males while heterozygote partial knocked out females. Males and females' whole white adipose tissue pads showed 50% and 30% reduction in the receptor expression respectively. Thus, given that the adipose tissue is constituted of many different cell types, looking at the receptor expression in isolated adipocytes will be needed for a better validation of the model. KO males and females showed lighter body weights on normal diet with decreased fat and lean body mass although no changes in the food intake could be observed. We used the Cre/loxp recombination technology and thus produced the KO in the fetus; however, it will be interesting to use this technology to induce the knock down the receptor in adult mice to avoid possible compensating mechanisms that might occur during mice development. As such, this may allow a better evaluation of the phenotype related specifically to a deficiency in the (P)RR. It would be interesting to investigate if knocking down the receptor might have decreased the intestinal fat absorption similarly to what has been observed mice lacking the renin gene [129]. This might indeed contribute to the weight change observed in these animals.

On the other hand, the gonadal fat showed reduced adipocytes number of bigger size. Since these histological results are more pronounced than what have been reported in mouse KOs of other RAS members in white adipose tissue, we suspect that Ang II-independent pathways of the (P)RR are also implicated in these changes. Thus assessment of both adipocytes Ang II-dependent and -independent pathways would be needed to verify the underlying mechanisms of the reported phenotypes. As such, it would be of interesting to measure renin activity, Ang II levels as well as the total and phosphorylated ERK1/2 in adipocytes. In addition, female KOs were resistant to the

development of obesity when placed on a HF/HC diet however this treatment has not yet been assessed in male KOs. Hence, evaluating the effect of a HF/HC diet in male KOs will be required and it will be interesting to see if lean body mass is still decreased in these animals as we observed a normalization of the lean body mass weight with the administration of the HF/HC diet to the females.

Also, as mentioned earlier, marked increases in locomotor activity was observed in male KOs which could also be implicated in the smaller body weights of these mice. Moreover, sympathetic activity has been reported to be increased in AT1Ra null mice and so, investigating the sympathetic activity in the male KOs could be suggested as one of the possible implicated mechanism for the increased locomotor activity. As type II diabetes is a common complication of obesity, we assessed the fasting glycemia in these mice although no obvious change could be observed. However, we did measure a reduced expression of the ABLIM2 gene that is associated with human diabetes in gonadal fats of KOs. Thus a more sensitive evaluation of the glycemia would be needed either by the oral glucose tolerance tests (OGTT) or intra-peritoneal insulin tolerance test (IPITT) in order to evaluate this parameter more closely. Moreover, evaluating glucose homeostasis in mice which have been placed on a HF/HC diet may be required to better distinguish the differences produced by the (P)RR deficiency.

Although no detectable differences in lipid profile could be observed in female mice on a HF/HC diet, given their mild phenotype, we may not have sufficient accuracy to detect small changes. As such, it would be of interest to investigate this parameter in the males since they have a more pronounced phenotype. In fact, studies have reported an association between the metabolic syndrome and the increased albumin in urine [162, 163]. In addition, obesity is a great risk factor for

developing hypertension and type II diabetes and thus it is considered as an important risk factor for the chronic kidney diseases [164]. Thus, looking at the proteinuria in mice on HF/HC diet will be important to see if the KOs are more prone to develop proteinuria with obesity or not.

Based on the data that we obtained from partial knocking down of the (P)RR, we would expect that the receptor could potentially be a new regulator of the adipose tissue mass and body weight. We have recently demonstrated in our laboratory that systemic administration of a (P)RR blocker in obese mice resulted in a decrease in weight gain and normalization of glycemia and triglycerides as well as a reduction in insulinemia. In addition, it prevented the fall in cardiac functions observed with increased obesity [154]. Taken together, the (P)RR and its blocker seem to be a new therapeutic target in the prevention of obesity and its associated metabolic disorders.

Chapter 11 : Reference List

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