Université de Montréal

Déterminer les mécanismes impliqués dans les effets du récepteur à la rénine et prorénine dans l'obésité et dans le diabète

par

Zulaykho Shamansurova (Akhmedova)

Département de Physiologie Moléculaire et Intégrative

Faculté de Médecine

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Présentée par :
Zulaykho Shamansurova (Akhmedova)
Département de Physiologie Moléculaire et Intégrative
Faculté de Médecine

a été évaluée par un jury composé des personnes suivantes :

Professeur Réjean Couture, *Président-rapporteur*
Dre Julie L. Lavoie, *Directrice de recherche*
Dr Ashok Srivastava, *Membre du jury*
Dre Louise Larose, *Examinateur externe*
Dr. John Chan, *Représentant de la doyenne*

University of Montreal

Determining mechanisms implicated in the effects of the renin and prorenin receptor in the development of obesity and diabetes

by
Zulaykho Shamansurova (Akhmedova)

Department of Molecular and Integrative Physiology
Faculty of Medicine

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Dr. John Chan, *Representative of the Dean*

Résumé

L'obésité est une épidémie mondiale qui augmente le risque de développer un diabète de type 2 ainsi que ses complications. Chez les individus obèses, le tissu adipeux sécrète de grandes quantités d'hormones et de cytokines qui affectent négativement le métabolisme du glucose et des lipides, ce qui provoque l'inflammation et la résistance à l'insuline. L'obésité augmente également l'activité du système rénine-angiotensine (RAS) localement au niveau de différents tissus et de façon systémique dans la circulation. L'angiotensinogène est convertie en angiotensine I par la rénine, ainsi que par la prorénine uniquement quand la prorénine est liée au récepteur de la rénine et prorénine [(P)RR] [1]. Ceci est la voie angiotensine-dépendante (Ang-D) du (P)RR. La liaison de la rénine et de la prorénine avec le (P)RR active également une voie angiotensine-indépendante (Ang-ND), ce qui produit une signalisation intracellulaire comportant la mitogen activated protein kinase (MAPK), la extracellular regulatory kinase 1/2 (ERK1/2), la promyelocytic leukemia zinc finger protein (PLZF) et le tumor necrosis factor alpha (TNF-a). Ceux-ci peuvent provoquer la croissance et la prolifération cellulaire, l'apoptose et la fibrose et pourraient donc être reliés aux dommages tissulaires et aux complications associées à l'obésité [1, 2]. Plusieurs effets bénéfiques d'un blocage pharmacologique du (P)RR ont été rapportés tels la prévention du développement d'une fibrose cardiaque et rénale ainsi que la prévention de la néphropathie et de la rétinopathie diabétique. Cependant, les effets du (P)RR dans le tissu adipeux ont été peu étudiés.

Par conséquent, notre objectif était d'étudier le rôle du (P)RR dans le développement de l'obésité et de la résistance à l'insuline par : 1) l'administration de HRP (un peptide bloquant l'effet du (P)RR) chez un modèle de souris obèse par l'administration d'une diète riche en gras (HFD), et 2) l'évaluation de souris ayant une délétion (KO) du gène (P)RR spécifiquement dans le tissu adipeux, qui a été généré dans notre laboratoire par la technologie Cre-LoxP.

L'expression du gène et de la protéine du (P)RR dans les tissus adipeux était augmentée chez les souris nourries avec une HFD indépendamment du traitement au HRP. Le traitement par le HRP a réduit le poids corporel et la masse adipeuse chez les souris nourries avec une HFD alors qu'une tendance pouvait être observée chez les souris sur diète normale (ND). De façon similaire, les souris (P)RR KO spécifiquement dans le tissu adipeux avaient une réduction du

poids corporel et de la masse adipeuse, même sur ND, ce qui suggère fortement l'implication du (P)RR dans le tissu adipeux dans le développement de l'obésité.

Le phénotype des souris KO incluait une augmentation de l'activité horizontale uniquement dans leur période active, ce qui pourrait contribuer à augmenter leur métabolisme énergétique et ainsi réduire leur poids corporel et leur masse adipeuse. De plus, les souris KO homozygotes mâles avaient un métabolisme de base plus élevé car nous avons observé une augmentation de la consommation d'oxygène et de la production de dioxyde de carbone pendant leur période active et de sommeil. Cette augmentation du métabolisme pourrait résulter, en partie, d'une augmentation de la thermogenèse comme en témoigne l'expression accrue du gène de brunissement, PRDM16, dans le tissu adipeux péri-rénale de souris mâles KO. Conformément à cela, des résultats récents provenant de notre laboratoire ont également démontré que le HRP pouvait induire du brunissement au niveau du tissu adipeux sous-cutanée [3].

Chez les souris traitées avec le HRP, bien que la glycémie eût été similaire aux souris recevant le placebo, l'insuline plasmatique et le rapport insuline/glucose était plus faible indépendamment de la diète. De façon similaire, les souris (P)RR KO avaient une insulinémie et un taux de peptide C plus faibles par rapport aux souris contrôles, sans aucune différence dans les courbes de la glycémie au cours d'un test de tolérance au glucose par voie orale. Les niveaux d'insuline dans l'état basal et stimulé étaient significativement plus faibles chez les souris KO, sans aucune modification du contenu pancréatique en insuline et du ratio insuline/peptide-C, ceci indique donc qu'il n'y a pas eu d'altération du niveau du métabolisme pancréatique de l'insuline. L'augmentation de l'adiponectine plasmatique chez les souris KO pourrait, entre autres, contribuer à une meilleure sensibilité à l'insuline observée. De plus, dans les groupes traités aux HRP, nous avons observé une amélioration du profil d'expression des gènes des transporteurs de glucose GLUT1 et GLUT4, du TNF-alpha, MCP-1, F4/80 et de la leptine dans le tissu adipeux ce qui pourrait contribuer à la meilleure sensibilité à l'insuline. Comme une meilleure sensibilité à l'insuline a été observée chez la souris suite au blocage pharmacologique et à la suppression génétique du (P)RR, ceci suggère que le (P)RR est impliqué dans la régulation de l'homéostasie du glucose.

De plus, un taux circulant réduit des triglycérides (TG) a été observé chez les souris traitées au HRP, alors que des niveaux inférieurs de TG ont été trouvés seulement dans les

muscles squelettiques chez les souris KO. Ces modifications du métabolisme des lipides et des taux circulants d'adiponectine résultent probablement d'un tissu adipeux plus sain tel que révélé par nos analyses histologiques démontrant une réduction de la taille des adipocytes chez les souris KO et traitées au HRP [3].

Nos résultats démontrent que le (P)RR, en particulier dans le tissu adipeux, est impliqué dans la régulation du poids corporel et de l'homéostasie du glucose probablement par la modulation de la morphologie et de la fonction des adipocytes. Le développement d'une nouvelle stratégie clinique axée sur le blocage du (P)RR pourrait aider à traiter l'obésité et ses pathologies associées telles la résistance à l'insuline et le diabète de type 2.

Mots-clés : Obésité, homéostasie des glucides, tissu adipeux, système rénine-angiotensine, récepteur à la rénine et prorénine, insuline, peptide C, adiponectin, brunissement du tissu adipeux blanc.

Abstract

Obesity is a worldwide epidemic and increases the risk of developing type 2 diabetes and its complications. In obesity, adipose tissue secretes large amounts of hormones and cytokines that negatively regulate glucose and lipid metabolism, causing inflammation and insulin resistance. Obesity also increases the activity of both local (tissue-specific) and circulating renin-angiotensin system (RAS). Angiotensinogen is converted to angiotensin I by renin, whereas prorenin may only do so upon binding to the (pro)renin receptor [(P)RR] [1]. This is thus the angiotensin-dependent (Ang-D) pathway of the (P)RR. The binding of renin and prorenin with the (P)RR also activates an angiotensin-independent pathway (Ang-ND), leading to intracellular signaling involving, for instance, the mitogen activated protein kinase (MAPK), the extracellular regulatory kinase ½ (Erk1/2), the promyelocytic leukemia zinc finger protein (PLZF) and tumor necrosis factor alpha (TNF-a) [1, 2]. These can produce cell growth and proliferation, apoptosis and fibrosis [1, 2], and as such may contribute to tissue damage and complications associated with obesity. The beneficial effects of pharmacological blockade of the (P)RR include prevention of the development of cardiac and renal fibrosis, as well as of diabetes-associated nephropathy and retinopathy. However, effects of the (P)RR in adipose tissue have been poorly investigated. Hence, our objective was to study the role of the (P)RR in the development of obesity and insulin resistance by: 1) administering HRP (a (P)RR blocker peptide) to mice fed a high-fat diet (HFD), and 2) in knock-out (KO) mice with adipose tissue-specific (P)RR gene deletion, which were generated in our laboratory by cre-loxp technology.

(P)RR gene and protein expression in adipose tissue were increased in mice fed a HFD independently of HRP treatment. HRP treatment also reduced mice body weight and fat masses in HFD-fed mice while they only tended to be lower in mice on normal diet (ND). Similarly, the adipose tissue specific (P)RR KO mice had reduced body weight and fat masses, even on ND, and as such confirmed the involvement of adipose tissue (P)RR in the development of obesity.

The KO phenotype included increased horizontal activity, only in the dark cycle (active period), which would increase energy expenditure and could contribute to their lower body weight and fat mass. Male hemizygous KO mice had higher basal metabolic rate as they had

increased oxygen consumption and carbon dioxide production during both their active and inactive period. This increased basal metabolism may result in part from an increase in thermogenesis as increased "beiging" gene expression, PRDM16, was observed in peri-renal fat of male KO mice. In line with this, recent results from our laboratory have also shown that HRP may induce "beiging" in subcutaneous fat [3].

In mice treated with the HRP, although glycemia was similar to placebo treated mice, plasma insulin and the insulin to glucose ratio were lower compared to untreated groups on both HFD or ND. Similarly, (P)RR KO mice had lower plasma insulin and C-peptide levels compared to controls, without any differences in the glycemia curves during an oral glucose tolerance test. Given that the basal and stimulated insulin levels were significantly lower in KO mice, without any changes in total pancreatic insulin content and with similar insulin to C-peptide ratio, this suggests that pancreatic insulin metabolism was not modified. The increased circulating adiponectin levels observed in KO mice may have contributed to the better insulin sensitivity present in the mice. In the HRP treated mice, we observed an improved gene expression profile of glucose transporters GLUT1 and GLUT4, TNF-alpha, MCP-1, F4/80 and leptin in adipose tissue, which may also contribute to the increased insulin sensitivity. Given that better insulin sensitivity was observed in mice with both (P)RR pharmacological blockade and genetic suppression, this suggests that the (P)RR is involved in the regulation of glucose homeostasis.

In addition, lower circulating triglycerides (TG) levels were found in mice treated with HRP, whereas lower TG levels were observed only in skeletal muscles in (P)RR KO mice. Put altogether, the lower lipid content and higher plasma adiponectin levels likely result from a healthier fat tissue as revealed by histological analysis which showed a reduction in adipocytes size in KO mice and was recently revealed in HRP treated HFD fed mice [3].

Our results demonstrate that the (P)RR, particularly in adipose tissue, is implicated in the regulation of body weight and glucose homeostasis via modulation of adipocytes morphology and function. The development of a new clinical strategy focused on blockade of the (P)RR specifically in adipose tissue could help to treat obesity and its associated pathologies such as insulin resistance and type 2 diabetes.

Keywords : Obesity, glucose homeostasis, adipose tissue, renin-angiotensin system, renin and prorenin receptor, insulin, C-peptide, adiponectin, beiging.

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Liste des abréviations

ACC	Acetyl-CoA carboxylase
ACE	Angiotensin converting Enzyme
ACEi	Angiotensin converting enzyme inhibitor
Adipo R1	Adiponectin receptor type 1
Adipo R2	Adiponectin receptor type 2
Agt	Angiotensinogen
Akt/PKB	Serine/threonine-specific protein kinase, or protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP activated protein kinase
AngI	Angiotensin I
AngII	Angiotensin II
Ang-D	Angiotensin-dependent
Ang-ND	Angiotensin-independent
ARB	Angiotensin II receptor blocker
ATP	Adenosine triphosphate
AT1R	Angiotensin II type 1 receptor
AT2R	Angiotensin II type 2 receptor
ATGL	Adipose transglycerol lipase
BAT	Brown adipose tissue
BW	Body weight
CAD	Coronary Artery Disease
CamK	Ca2+/Calmodulin-dependent protein kinase
C/EBP	(CCAAT)-enhancer binding protein
CVD	Cardiovascular disease
CREBP	cAMP responsible element binding protein
DGAT	Diglyceride acyltransferase
DM	Diabetes Mellitus
Erk1/2	Extracellular signal response kinase 1/2

FA	Fatty acids
FAS	Fatty acid synthase
FFA	Free Fatty Acids
FOXO1	Forkhead box protein O1
GFP	Green fluorescent protein
GLUT	Glucose transporter
GPCR40	G-protein coupled receptor 40 or FFA receptor 1
G6PD	Glucose-6 phosphate dehydrogenase
HFD	High-fat diet
HIF	Hypoxia induced factor
HMW	High molecular weight
HRP	Handle region protein
HSL	Hormone sensitive lipase
IL	Interleukin
IP-ITT	Intra-peritoneal insulin tolerance test
IRS	Insulin receptor substrate
KO	Knockout
MAPK	Mitogen activated protein kinase
MasR	Mas receptor
MCP	Monocyte chemoattractant protein
Mest/Peg	Mesoderm specific transcript protein/Paternally expressed gene
MSC	Mesenchymal stem cells
Myf5	Myocyte induced factor 5
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
ND	Normal Diet
NF-kB	Nuclear Factor kappa-light-chain enhancer of activated B cells
NOS	Nitric oxide synthase
NOX	NADPH Oxidase
OGTT	Oral glucose tolerance test

PAI-1	Plasminogen activator inhibitor type 1
PEPK	Phosphoenolpyruvate kinase
PGC1-a	Peroxisome-proliferator activated receptor gamma coactivator 1-alpha
PGF	Peri-gonadal fat
PI3K	Phosphoinositol-3 kinase
PLZF	Promyelocytic leukemia zinc finger
PKC	Protein kinase C
POMC	Pro-opiomelanocortin
PPAR	Peroxisome proliferator activated receptor
PRDM16	PR domain containing 16
PRA	Plasma renin activity
(P)RR	(Pro)Renin Receptor
RAS	Renin-angiotensin system
RBP	Retinol binding protein
ROS	Reactive oxygen species
SCF	Subcutaneous fat
SH2	Src homology 2 domain
SREBP	Sterol Regulatory Element Binding Protein
TG	Triglycerides
T2DM	Type 2 Diabetes Mellitus
TGF-beta	Transforming growth factor beta
TNF-a	Tumor necrosis factor alpha
TZD	Thiazolidinediones
UCP	Uncoupling protein
VAT	Visceral adipose tissue
WAT	White adipose tissue
WHO	World health organisation
Wnt	Wingless-related integration site
WT	Wild-type

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Introduction

Obesity and Type 2 Diabetes Mellitus (T2DM) are known to be worldwide pandemics. Statistics show that 33% of adults in the world have obesity or are overweight and 10% suffer from type 2 Diabetes Mellitus (T2DM) (http://www.who.int/mediacentre/factsheets/fs311/en/). However, during the past 30 years obesity levels have doubled and diabetes mellitus has tripled [4, 5]. The significant growth of yearly incidents caused by these conditions show a close relationship with life expectancy and cardiovascular diseases. For instance, recent studies have shown that there is an association between obesity and cancer, depression, mental illness and sleep apnea [6]. Moreover, both obesity and T2DM contribute to increased costs for medical care and social life [4, 7].

Obesity is characterised by an increase in body weight, but more specifically with an increase in fat mass. Adipose tissue is a multifunctional endocrine organ that participates in whole-body lipid and glucose metabolism as well as inflammation, through secretion of hormones and cytokines [8, 9 10]. In many epidemiological, clinical and experimental studies, obesity has been shown to increase the renin-angiotensin system (RAS) activity both systemically and locally in adipose tissue [9, 11-14]. Angiotensin II is also well known as a main player in the development of obesity and insulin resistance, however the molecular mechanisms of this association are not yet clear [9, 11-13,15]

Our knowledge of the RAS has also been updated throughout the past decades [15, 16]. It has been shown that its components can act locally in an autocrine or paracrine manner and also at a distance through secretion into the circulation [9, 17]. Moreover, fat tissue has been shown to be a major source of circulating angiotensinogen (Agt) which would explain the increase in circulating RAS activity observed with obesity as it would contribute to increase the production of angiotensin II [9, 11, 18, 19]. In addition, angiotensin II has been shown to be essential for the differentiation and function of adipocytes resulting in an increased production leading to fat mass growth [18, 20, 21]. It has also been shown to produce insulin resistance: through the disabling of GLUT4 translocation in skeletal muscle cells; also by the activation of the extracellular regulatory kinase ½ (Erk1/2) and the phosphorylation of IRS1,2 which prevents further phosphorylation in response to insulin; as well as by inhibition of Akt function by nitration [22-

[24]. Hence, given that hyperglycemia itself can also stimulate the expression of RAS genes in adipose tissue and liver, a vicious circle is started [25, 26].

Clinical studies using RAS inhibitors such as angiotensin-converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARBs) have also confirmed the role of the RAS in the development of the obesity and diabetes [27], as improved insulin sensitivity was reported, which can prevent diabetes and associated complications [28, 29]. It seems that the local RAS in adipose tissue can play three roles as a candidate in the development of obesity and insulin resistance: First, through the impact of adipocytes differentiation [9, 18, 30], second, through the modulation of adipokines secretion (increasing inflammatory cytokines such as TNF-alpha, IL6 and leptin, while decreasing adiponectin secretion), and third, through the secretion of angiotensinogen (Agt) into the circulation [9, 11, 31].

The classic angiotensin II formation cascade is initiated by converting angiotensinogen to angiotensin I by renin [15]. Then, angiotensin I is converted to angiotensin II by the angiotensin converting enzyme (ACE). The formed angiotensin II mainly acts through its type 1 receptor (AT1R) and produces vasoconstriction, cell proliferation and differentiation.

Recent studies have shown that renin, as well as prorenin, can bind to a specific receptor - the (Pro)Renin Receptor [(P)RR] which increases renin's catalytic activity by 4 folds and renders prorenin non-proteolytically active i.e. without cleaving the pro-segment [1, 32, 33]. The binding of renin/prorenin to the (P)RR has been shown to produce both angiotensin-II dependent and independent effects. Angiotensin-dependent effects (Ang-D), relate to the well-known angiotensin II formation cascade of the RAS which is stimulated. As mentioned above, this can impact adipose tissue, insulin resistance, oxidative stress and cell growth and proliferation [34, 35]. Angiotensin-independent effects (Ang-ND) occur through the activation of different signaling pathways, such as the mitogen activated protein kinase (MAPK), the extracellular regulatory kinase 1/2 (Erk1/2), the transforming growth Factor beta (TGF-β) and the promyelocytic leukemia zinc finger (PLZF) pathways which are linked to renin/prorenin binding to the (P)RR [32, 36-39]. There are also Ang-ND effects of the (P)RR which are not stimulated by renin/prorenin binding which have been shown to involve a (P)RR interaction with the V-ATPase [40]. Unexpectedly, Ang-ND effects also impact cell growth, proliferation, apoptosis, and fibrosis [32, 36-38]. Hence, it is possible that when RAS inhibitors are used in clinical practice the remaining tissue damage may result from the action of renin/prorenin on the (P)RR through Ang-ND,

particularly given the fact that angiotensin-converting enzymes inhibitors (ACEi) and angiotensin receptor blockers (ARBs) produce an increase in circulating renin [41].

Experimental studies have shown that administration of a (P)RR blocker peptide, or the handle-region peptide (HRP), a part of the pro-segment of the prorenin, can prevent diabetic retinopathy and nephropathy [42, 43], cardiosclerosis [37] and hypertension [36, 37]. In that context, the effects of the (P)RR in adipose tissue and its implication in the development of obesity are of interest but have been poorly studied.

To justify the goal and hypothesis of our studies, this introduction will present the data from the literature regarding obesity, type 2 diabetes mellitus (T2DM) and the renin-angiotensin system (RAS) in separate chapters. In the last chapter the role of the (P)RR and its relationship with different diseases will be discussed.

CHAPTER I. OBESITY

1.1.DEFINITION AND EPIDEMIOLOGY OF OBESITY

Obesity is a medical condition that is a complex disease defined as excessive body weight, specifically in fat mass, which may have negative effects on health and leads to the development of cardiovascular and metabolic complications (Mayo clinic, http://www.mayoclinic.org/diseasesconditions/obesity/basics/definition/con-20014834).

Obesity in adults can be determined by using the body mass index (BMI = weight in kg/height in meters squared), where a BMI of 18-25 is defined as normal, 25-30 as overweight and >30 as obese [44, 45]. The WHO 2014 statistics found that in developed countries, about 39% of adults aged 18 and over (38% men and 40% women) were overweight, and 13% of adults (11% men and 13% women) were obese [4] (http://www.who.int/mediacentre/factsheets/fs311/en/). In Canada, 25% of adults and 10% of children are overweight or obese (http://www.obesitynetwork.ca/obesity-in-canada).

Obesity increases the risk of developing other disorders such as hypertension, dyslipidemia, insulin resistance, type 2 diabetes mellitus (T2DM): this combination is named "metabolic syndrome" [22, 46, 47]. Obesity also increases the risk of developing cancer [48], depression, mental illnesses [49, 50], sleep apnea [6, 51-53], coronary heart diseases, atherosclerosis, polycystic ovarian syndrome and other disorders. Obesity increases mortality 4-fold and morbidity 10-fold [6].

Based on the health economic impact, WHO declared obesity as a worldwide epidemic in 2002 [4]. Obesity has a large economic impact worldwide due to high cost of treatment and also higher rate of disability and mortality [6, 54]. In Canada, the cost for prevention and treatment of obesity in 2010 was estimated to be $6 billion or 4% of the total Canadian Medicare budget (http://www.obesitynetwork.ca/obesity-in-canada).

1.2. RISK FACTORS FOR OBESITY AND ITS PATHOGENESIS

1.2.1. RISK FACTORS FOR OBESITY

There are a number of factors which have been shown to increase the risk of obesity[6, 47].

Nutrition. An excess calorie intake is a main risk factor for obesity**.** An imbalance between food intake and energy expenditure is important in the development of obesity [55, 56] . Ease of access to "fast-food" with an imbalanced amount of energy or ingredients, such as high-fat/high-carbohydrate, high salt, and additives (sodium benzoate, sweeteners, colorants, emulsions) used in food industry has been shown to increase the risk of obesity by 2-fold, especially among children and young people [57, 58].

Physical activity. Muscle contraction requires energy expenditure through the oxidation of fat and glucose and also increases heat production by the mitochondrial respiratory chain [59]. Physical inactivity increases the risk of developing obesity by ~30%, while physical activity protects against obesity [59].

Genetics. The family history of an individual also contributes to the risk of developing obesity [60, 61]. Maternal or paternal obesity increases the risk of developing obesity by causing cytoplasmic dysfunction in oocytes, chromatin damage, and by altering the embryo's gene transcription and translation which can have a powerful impact in more than one generation [60]. During the past decades, scientists have found ~58 loci involved in the development of obesity, where some of them were linked to single nucleotide polymorphisms (SNP) but most with polygenic nucleotide polymorphism (PNP) [62-64]

Aging increases the risk of being overweight and obese due to the hormonal and metabolic changes [44, 48, 65-68].

Central nervous system alterations may increase the risk of developing obesity as a result of emotional stress [69, 70]. Individuals affected by mental diseases and sleep disorders often develop obesity [49, 50, 53]. Hormones, which are released during emotional stress trigger central mechanisms of food intake and can control behavior, cause alterations in the neural adaptation of the brain reward circuitry, and by involving the arco-temporal nuclei in the brain [69, 71]. Stomach-brain axis disturbances induced by stress may alter neuronal activity in the hypothalamus, which regulates satiety, mood and eating behavior [72, 73]. Patients treated for anxiety and depression often report weight gain (~20 kg), probably due to side effects on dopamine and serotonin in several brain centers[49, 53, 69, 74, 75].

There are other factors, such as environmental and social factors which also can present a risk for obesity, such as a poor income, access to healthy food, hygiene, etc. [76]. In some cases, traditional and family habits may impact food choice and physical activity [4, 53].

1.2.2. Pathogenesis of obesity

The development of Obesity and related diseases. Expansion of adipose tissue is considered to be the first step in the development of obesity, which occurs by structural remodeling and functional abnormalities of adipose tissue [77-79]. The first triggering factor can be an excess of nutrients or energy surplus [80]. Enlargement of fat can occur through an increase in adipocyte number (proliferation/hyperplasia) or their volume (hypertrophy) measured directly by microscopy or through genetic markers of cell size, such as mesoderm-specific transcript (Mest), Paternally expressed gene 1 (Peg) [81-84]. The main risk factors in the development of obesity and related diseases are schematically shown in **Figure 1**. These factors can be subdivided as follows and will be described below: 1) life style, such as, nutrition and physical activity; 2) genetics, for instance, related to gene abnormalities, genetic diseases and aging; 3) other diseases such as endocrine or central nervous system disorders and 4) particular conditions, such as trauma and physical disability. Interestingly, the known mechanisms of the pathogenesis, such as adipocyte cell growth, differentiation, ischemia, inflammation,

dysfunction and remodelling have been linked with dysregulation of the renin-angiotensin system (**Figure 1**).

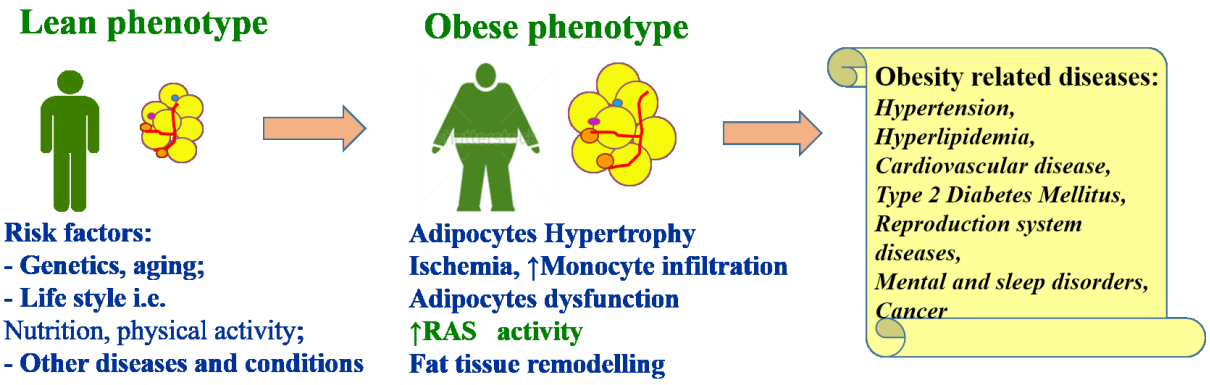


Figure 1. Development of obesity and related diseases

Leptin resistance. The adipocyte derived hormone leptin binds to its specific receptor and functions as a "body size sensor" in the brain, which regulates appetite and food intake [73, 85]. Unfortunately, in obesity, leptin resistance develops as a result of high circulating leptin levels which are produced by the increasing fat mass [68, 86-88]. Therefore, appetite and food intake are increased non-proportionally to body size, and nutrient overconsumption continues. Leptinemia is considered an important step in the development of obesity and is often accompanied by decreased circulating adiponectin level and decreased sensitivity to insulin which increases the risk of developing T2DM [69, 70, 89, 90].

Hypoxia in adipose tissue, via stimulation of the hypoxia induced factor -1 (HIF-1), can induce the expression of genes such as TNF-alpha, interleukins 1 and 6 (IL-1, IL-6), nuclear factor kappa-light-chain enhancer of activated B cells (NF-kB), monocyte attractant protein 1 (MCP-1) and peroxisomes proliferator-activated receptors (PPARs) [9, 78, 91-95]. These hypoxia induced pro-inflammatory factors in turn can change adipose tissue cell integrity through stimulation of the beta integrin, Erk1/2 and MAPK signaling pathways and promote development of obesity and insulin resistance [81].

Inflammation in adipose tissue is considered the next step in the development of obesity [96, 97], although the exact mechanism is not clear yet. Some authors have shown that low-grade inflammation in adipose tissue results from overfeeding and stimulated by nutrients, especially by lipids, through special cell surface receptors, such as G-protein coupled receptor 40 (GPCR40), that can trigger the gene transcription for growth factors, hormones and cytokines in adipose tissue [27, 68, 80, 96, 98-102]. Increasing production of inflammatory cytokines, especially tumor necrosis factor alpha (TNF-alpha), promotes insulin resistance by affecting insulin signaling pathways via inhibition of insulin receptor substrate 1 (IRS1) and decreasing adiponectin gene expression [55, 103, 104].

Increased local adipose tissue RAS activity is also considered as a main factor for the development of obesity and insulin resistance. Indeed, by promoting adipocyte proliferation and contributing to circulating RAS levels, the local adipose tissue RAS is a main contributor to the development of obesity [9, 11, 20, 30, 105]. Increased local adipose tissue production of Ang II stimulates vasoconstriction, hypoxia, adipogenesis, lipogenesis as well as lipolysis [9, 86, 97, 106]. It also modulates adipose tissue gene expression profile which have both local and systemic impacts on body weight and energy homeostasis [9, 86, 97, 106]. Increased Ang II formation also promotes reactive oxygen species (ROS) formation, and affects insulin signaling by decreasing of Akt/PKB phosphorylation and GLUT4 activity, which leads to insulin resistance [7, 23, 24, 46, 56, 96, 103, 107-109].

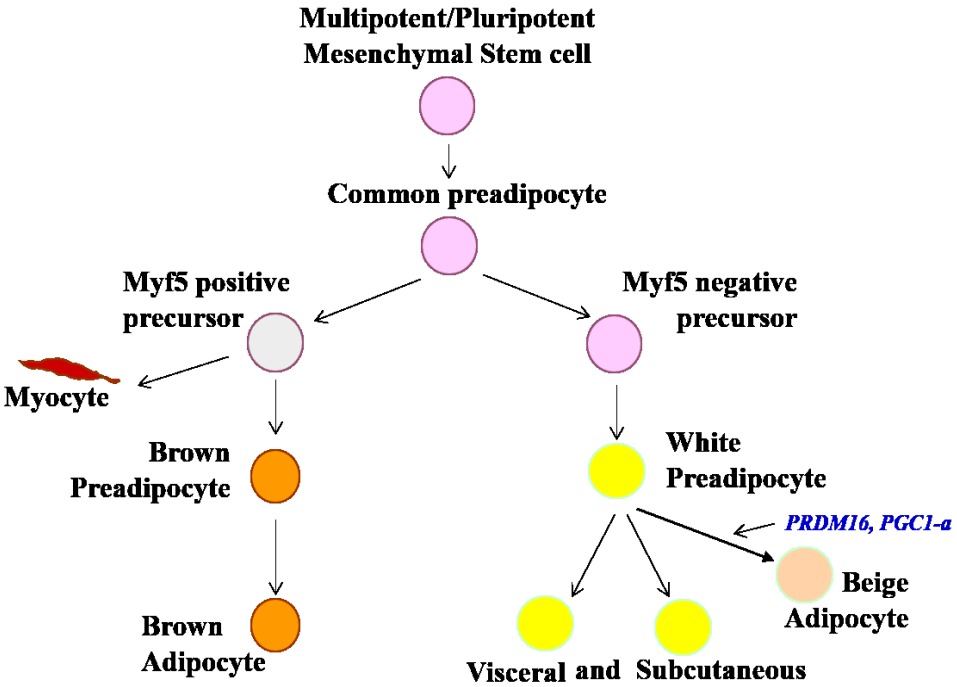
1.3 ADIPOGENESIS

Adipocytes in embryo develops (**Figure 2**) from pluripotent mesenchymal stem cells (MSC) into common preadipocytes, which is turn are converted into cells with and without myogenic factor 5 (Myf5). Myf5 negative precursor cells are converted to white preadipocytes and then to white adipocytes, both visceral and subcutaneous, whereas Myf5 positive precursor cells gives rise to brown preadipocytes and then to brown adipocytes and myocytes [77, 106, 110-112].

In adults, adipocytes develop from multipotent MSC and also from several precursor cells from the stromal-vascular fraction, from adipocyte progenitor stem cells, and also from bone marrow stromal cells [79, 113, 114], and continue as MSC shown in **Figure 2**.

The known transcriptional signals for adipocyte differentiation include the transforming growth factor beta (TGF-beta), the insulin-like growth factor 1 (IGF-1), the fibroblasts growth factor 1 (FGF-1), the bone morphogenetic protein (BMP) and activin [77]. Transcription factors such as a CCAAT (cytosine-cytosine-adenosine-adenosine-thymidine) enhancer binding protein (C/EBP) and peroxisome proliferation activating receptor gamma (PPARɣ) are the master regulator gene transcription factors, while the wingless-relates integration site (Wnt) signaling plays an important role in adipocyte differentiation [115].

The study of the gene transcription factors network regulation of adipogenesis showed that differentiation of progenitor cells occurred under the two waves of gene transcription factors



Figu **ıthetized in this figure. Cell colour identifies major changes. The text in light blue identifies factors involved in the alternative pathway of the white preadipocyte differentiation.**

activation, where the second waves of transcriptional factors can determine the cell type. By studying the role of gene transcriptional network many processes and diseases have been explained. For example, "beiging" of white adipose tissue [92] or the negative relationship between obesity and osteoporosis due the same precursor cells for adipocytes and osteoblasts [113].

1.4 ADIPOSE TISSUE STRUCTURE AND FUNCTION

Adipose tissue cell types. Adipocytes are the main cells present in adipose tissue and are classified generally as white, brown, and more recently an intermediate phenotype defined as beige adipocytes, which depends on the number of mitochondrion and the gene expression pattern present in those cells [103, 116]. Together with mature adipocytes there are (**Figure 3**) preadipocytes, T-cells, macrophage, stromal elements and blood vessels within the adipose tissue which constitute the stromal-vascular fraction [117].

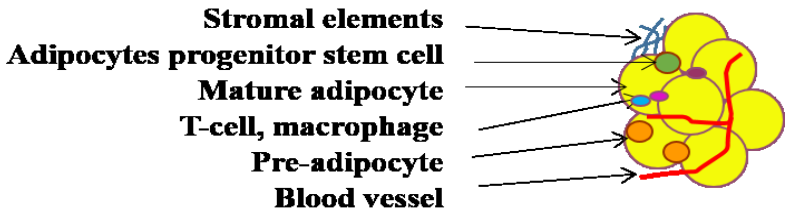


Figure 3. Adipose tissue structure

Adipose tissue enlargement by cells hyperplasia and/or hypertrophy. Fat enlargement may occur (**Figure 4**) through proliferation of adipocytes by PPARγ stimulated differentiation of

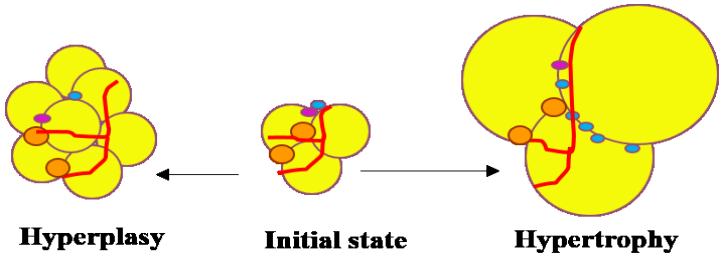


Figure 4. Adipose tissue enlargement

multipotent progenitor and precursor cells which increases cell number (hyperplasia) or by increasing the cell volume through increasing adipocyte TG storage in lipid droplet (hypertrophy) [82]. Angiotensin II, through inhibition of PPAR-gamma phosphorylation, arrests adipocyte proliferation and leads to an increase in cell volume [118].

Mature white adipocytes contain (**Figure 5**) a single large lipid droplet, which occupies ~ 90% of the volume, while the remaining 10% contains the nucleus and organelles. In addition to storing lipids, adipocytes have been shown to produce a number of hormones and cytokines [78, 92, 103, 119].

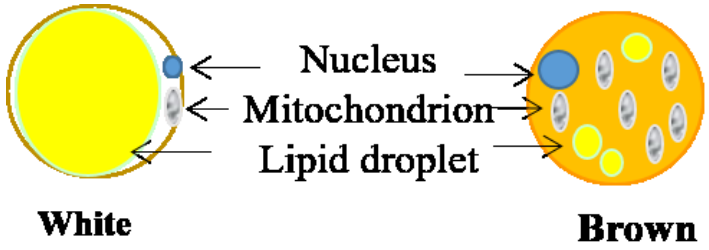


Figure 5. Structure of white and brown adipocytes

Brown adipocytes are the main cells responsible for the body's "heat" production and as such this tissue defends the body from obesity by increasing energy expenditure [92, 116]. Brown adipocytes have the same precursors as myocytes (**Figure 2**) and differ from white adipocytes (**Figure 5**) in size, shape, lipid droplets size and number, as well as by the mitochondrial content. Brown adipocytes contain larger numbers of small lipid droplets dispersed in the cytoplasm together with other organelles. They also contain a larger number of mitochondria The activation of fat oxidation in white adipose tissue (WAT) through increasing cyclic AMP or uncoupling protein 1 (UCP-1) gene/protein expression has been shown in mice to prevent obesity [92]. Indeed, altered cellular differentiation to produce a brown-like adipocyte rather than a white adipocyte, a phenomenon called beiging, has been demonstrated by several authors [92].

Beiging is a process where white adipocytes acquire a brown adipocyte specific gene expression pattern, such as PPAR gamma coactivator 1-alpha (PGC-1a) and UCP-1 gene transcription

factors, during differentiation (**Figure 2**) through the stimulation of the zinc finger transcription factor PRDM16. These white adipocytes become "beige" adipocytes [92, 112, 116, 120].

Other cell types present in adipose tissue include macrophages, monocytes, T-cells, endothelial and vascular smooth muscle cells, nervous filaments, and other connective tissue elements, which collectively are named the stromal-vascular fraction [9, 78, 121].

Adipose tissue location. By convention **(Figure 6),** fat tissue is named according to the location of each fat pad in the body, such as subcutaneous (SCF), peri-gonadal (PGF), peri-renal (PRF) and mesenteric white adipose tissue. The distribution of different fat depot in general in mammals is the same and may differ by localisation and amount. Despite the many similarities between adipocytes from different locations, there are still distinct differences between the adipocytes found in the different fat pads, that may relate to their origin from the different part of the mesenchymal layer during embryonic development [79, 113, 121].

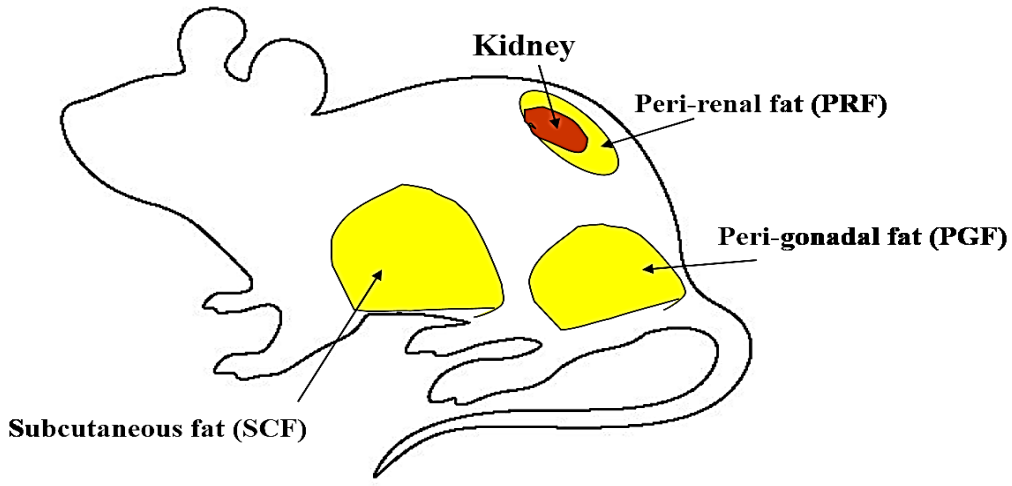


Figure 6. Conventional fat pad collected during the necropsy in the mouse

The other differences between the fat depots can be shown in the contents of precursor and stromal-vascular cells. The number of adipocyte precursor cells are highest in visceral adipose tissue (VAT), which may potentially explain why the VAT has been implicated in the development of obesity and insulin resistance [78, 121, 122].

Moreover, there are differences between the fat pads in adipocyte function and metabolic activity [78]. In mice, the VAT was shown to be more sensitive to beta adrenergic stimulation, and as a result, it has increased lipolysis compared to the subcutaneous fat (SCF), and contributes to the development of the metabolic syndrome [123, 124].

Occasionally, ectopic fat is found within the liver, muscles, pancreas, bone marrow, as well as in the peri-vascular and peri-cardiac area [82, 125, 126]. Some authors have suggested that ectopic fat should be considered pathological due to insulin resistance, increased lipolysis, higher levels of reactive oxygen species (ROS) formation and increased RAS activity with higher local and circulating AngII level and also its contribution to the development of local tissue remodelling and fibrosis [127-129].

1.5 ADIPOCYTE CELL SIZE

Fat tissue is unique because of its ability to easily change cell size by increasing or shrinking about 10-fold [81]. Adipocyte size changes has been shown to modify their properties and function [81], as shown in **Table 1**

Table 1. Relationship between Adipocyte cell size and function

Inflammatory cytokines release	Normal	Increased
Leptin secretion	Normal/High	Decreased
Adiponectin production and secretion	Normal	Increased
RAS gene expression, production	Normal	Increased
Glucose uptake	Normal	Increased
Fatty acids uptake	Normal	Increased
Lipolysis, Lipogenesis	Normal	Increased
TG storage	Normal	Increased
Insulin sensitivity	Normal	Decreased
MCP1, TLR4-expression	Normal	Increased

Monocytes infiltration	Normal	Increased

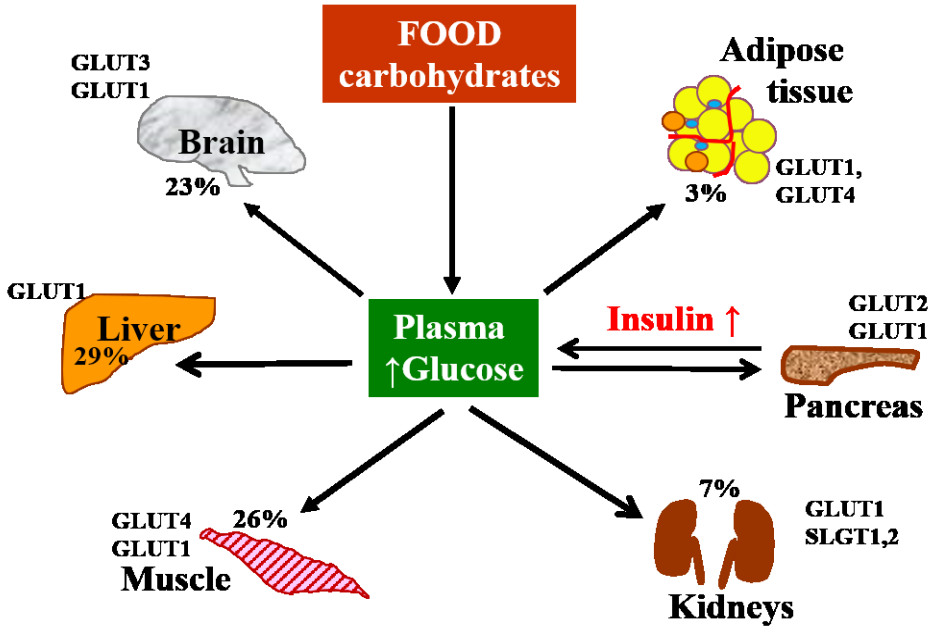
The response to insulin differs in adipocytes according to their size: small adipocytes are more insulin sensitive and although the lipid droplets are smaller their fatty acid uptake is higher than large adipocytes [91], whereas large adipocytes due to insulin resistance have slow glucose uptake and increased lipolysis and compensatory fat uptake. Some authors proposed that increased volume and excess fat storage in adipocytes viewed as an adaptive mechanism to prevent the toxic effects of hyperlipidemia [93, 121]. It has been reported that high levels of expression of an imprinted gene, the mesoderm-specific transcript/paternal expressed gene 1 (Mest/Peg1), in mice and humans is associated with an increased adipocyte size [84]. Surprisingly, the authors showed that the administration of thiazolidinediones (TZD) also caused fat enlargement, but through stimulation of hyperplasia. The mechanism implicated in this effect is thought to be the activation of the PPAR-ɣ pathway. Interestingly, following this treatment, Mest/Peg1 gene expression was found to be decreased, indicating that this gene is a good marker of adipocyte size independently of adipose tissue mass.

Peroxisome proliferation-activated receptors (PPARs) are nuclear receptors that function as transcription factors. Three forms of PPARs have been observed, alpha (a), beta (β/δ) and gamma (γ), the latter being highly expressed in adipose tissue [130]. PPARγ is implicated in adipocytes differentiation and development, and as such, it can prevent an increase in adipocyte cell size i.e. hypertrophy [110]. PPARγ and its agonists were shown to have beneficial effects in adipose tissue on adipokine and inflammatory cytokine secretion which produces a reduction in inflammation and a better sensitivity to insulin [131].

1.6 ADIPOSE TISSUE GLUCOSE AND LIPID METABOLISM

As was mentioned before, adipose tissue is a buffer for excess body glucose. Fat storing cells are found in protozoa and worms such as C. Elegans indicating their important role in evolution for whole body metabolism [79]. Ingested glucose enters the blood and increases plasma glucose.

It is then distributed to tissues as shown on **Figure 7** where about 29% of glucose is distributed to the liver and gastrointestinal system, 26% to skeletal muscles, 23% to the brain, 7% to the kidneys and about 3% to adipose tissue [132]. Adipose tissue acts as a buffer, mainly during the postprandial period (after a meal) to absorb the excess amount of fat and carbohydrates from the blood and protect cells from the harmful effects of simultaneous high levels of glucose and lipids, known as glucolipotoxicity [22, 89, 133-141]. A good example of a defect in adipose tissue function is lipodystrophy, which features insulin resistance and ectopic fat accumulation, despite



the extremely low fat mass and lower body weight [94, 142].

Figure 7. Distribution of ingested glucose in the body. % of glucose absorbed by each tissue following glucose ingestion is indicated. The type of glucose transporter present in each tissue is indicated. In response to the increased plasma glucose, the pancreas secretes insulin (red) to facilitate absorption of glucose by certain tissues.

Glucose transporters. Glucose enters adipocytes (**Figure 8**) via the facilitated transporter GLUT1 in basal (fasting) state and via GLUT4 after a meal [81, 143-145]. Most of the glucose that enters the adipocytes is stored as triglycerides (TG) in lipid droplets. In obesity, translocation of GLUT4 to the adipocyte cell surface is impaired and glucose uptake is inhibited [143, 146, 147]. This delays glucose elimination from the blood after a meal and affects whole-body glucose

homeostasis. Measures used for the treatment of obesity and type 2 diabetes mellitus (exercise, metformin, TZD) act via increasing the activity of GLUT4 [148-150].

Fatty acid transporters. Fatty acids can enter into adipocytes (**Figure 8**) by binding with the tissue-specific "fatty acid transport protein" – FATP (in adipose tissue, type 1 – FATP1) and may also enter adipocytes via the scavenger receptor (fatty acid translocase FAT/CD36), located on the cell surface [147, 151, 152].

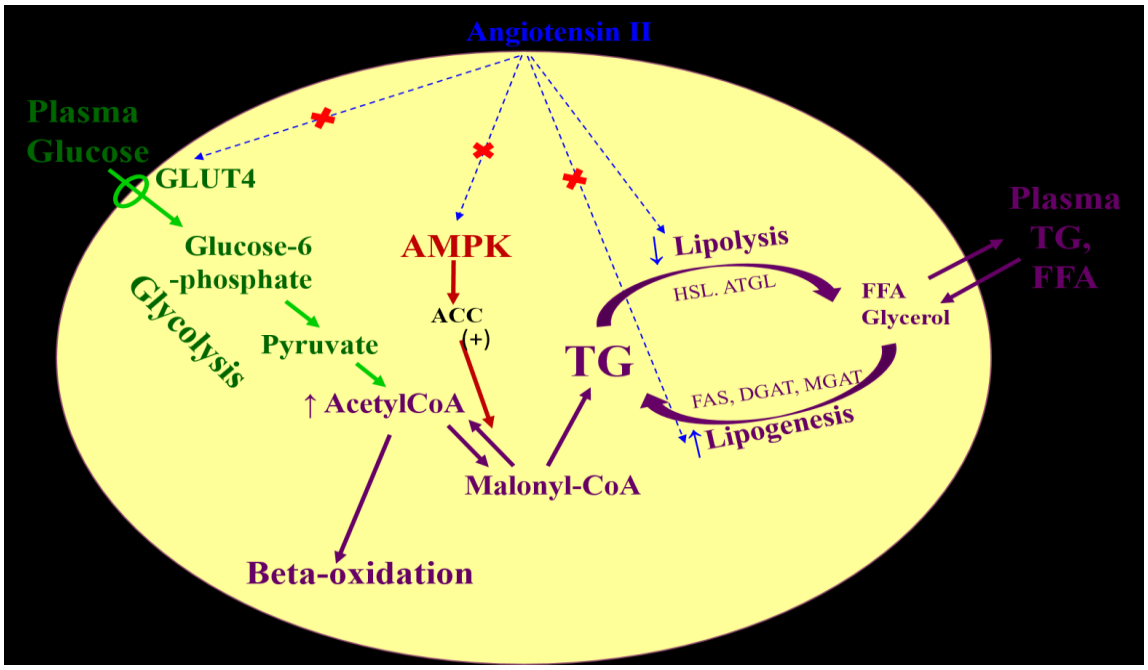


Figure 8. Adipose tissue lipid and glucose metabolism. This figures shows lipid (purple) and glucose (green) metabolism. In blue is shown the effect of angiotensin II on these 2 pathways. ACC, Acetyl-CoA co-carboxylase; TG, triglycerides; FFA, free fatty acids; ATGL, adipose triglyceride lipase; HSL, hormone sensitive lipase; FAS, fatty acid synthase; DGAT, di- and MGAT, mono-glycerol acyltransferase;

Fatty acid binding proteins (FABP) is another class of specific fatty acid transporters [152]. The FABP4/AP2 is a specific adipose tissue protein [152] which binds lipophilic substances, such as hormones and vitamins, which are transported from the outer side into the cell compartment [153]. FABP4 regulates lipid metabolism by modulating lipase activity and regulating adipocyte

differentiation as it attenuates the Janus kinase 2 (JAK2) and phosphatase and tensin homolog (PTEN) signaling stimulated by leptin [152-154]. In one study authors found that RAS inhibition by the ARB candesartan produced a ~ 20% decrease in the serum level of FABP4 and as such, ARBs were proposed as a method for suppression of cardiovascular events in hypertensive subjects [155]. These authors also found a relationship between increased FABP4 level and obesity, hypertension and cardiovascular events.

Adipocyte lipid uptake and synthesis is controlled by insulin, pyruvate dehydrogenase and acetyl Coenzyme-A (acetyl-CoA) in the case of lipid synthesis, and is also regulated by the sterol regulatory element binding protein 1 (SREBP1), a transcription factor for lipolytic enzyme gene expression[156, 157].

Lipid synthesis and lipolysis in adipocytes. As shown in **Figure 8**, when glucose, enters the adipocyte, it may be converted to acetyl-CoA and then be either oxidised or be involved in fat synthesis. The enzyme acetyl-CoA cocarboxylase (ACC) stimulates the synthesis of malonyl-CoA from acetyl-CoA for further fatty acid synthesis and adipocyte hypertrophy [82, 89, 158]. On the other hand, lipoprotein lipases remove fatty acids from chylomicrons, low density lipoprotein (LDL) and very low density lipoprotein (VLDL) in plasma and mobilize them into lipid droplets for TG synthesis and storing [150, 159, 160]. Adipocytes lipid droplet TG synthesis is sequentially carried out by mono- and di-glycerol-acyl-transferases (MGAT, DGAT). Fatty acid (FA) release is stimulated by lipases, such as the acyl-triglyceride lipase (ATGL) and the hormone sensitive lipase (HSL), which are responsible for about 95% of total TG hydrolysis in adipocytes (**Figure 8**) [161]. More specifically, FA and glycerol can be released from adipocytes by FAT or FABP, shown above, during fasting or exercising following the activation of the HSL by beta-adrenoreceptors [159, 162]. Lipogenic and lipolytic enzymes are key regulators in VAT as they are involved in increasing adipocyte cell size/volume (hypertrophy) by enlargement of the lipid droplet(s) [122, 138].

1.7 ADIPOKINES

Adipose tissue as an endocrine organ, as it secretes hormones (adipokines) and inflammatory cytokines [9, 103, 163]. There are more than 20 hormones and about 20 cytokines that have been shown to be produced by adipocytes [163]. Expression and secretion of most adipokines are altered by obesity [46, 164, 165]. Leptin, adiponectin and resistin are the most studied adipokines. Their level in the circulation are regulated by different parameters such as insulin sensitivity, inflammatory status, obesity and Type 2 Diabetes Mellitus (T2DM) [46, 103, 166-168].

Table 2. Leptin, Resistin and Adiponectin levels in Obesity, T2DM and RAS inhibition

Conditions	Leptin	Resistin	Adiponectin
Obesity	↑gene expression in adipose tissue [169] ↑plasma level [170]	↑gene expression in adipose tissue [171, 172] ↑plasma level [171]	↓gene expression in adipose tissue [173] ↓plasma level [174-176]
Type 2 Diabetes Mellitus	↑plasma level [176, 177]	↑protein expression [178]	↓plasma level [179] ↓protein expression [180, 181] [181]
Insulin resistance	↑plasma level [182]	↑plasma level [183]	↓plasma level [164]
RAS inhibition	↓plasma level [184]	↓plasma level [185]	↑plasma level [186]

Notes: ↑ (increased); ↓ (decreased)

Adiponectin effects promote better insulin sensitivity, whereas leptin and resistin lead to insulin resistance. Data from the literature about changes in leptin, resistin and adiponectin levels in obesity, insulin resistance and T2DM are presented in **Table 2.**

1.7.1 Leptin

Leptin is the first discovered adipose tissue derived hormone. It is secreted by mature adipocytes, mainly in white adipose tissue [86, 87, 103]. It was also found to be secreted by other

tissues, including bone marrow, mammary glands, pituitary gland, ovaries, skeletal muscle and gastric chief cells [79, 81, 103, 163]. Leptin synthesis is stimulated by dexamethasone, insulin, emotional stress, and decreased by exercise, fasting and sleep [187, 188].

Effects of leptin. Leptin targets the hypothalamus (**Figure 9**), where it binds to its specific receptor [87, 189]. Leptin acts as a brain sensor to protect the body from both excess and very small fat content [142]. Leptin decreases appetite by regulating the secretion of neuropeptide Y (NP-Y), melanocortin and Agouti-related peptide (AgRP) in the arcuate nucleus [190, 191]. It also negatively impacts locomotor activity during food restriction and fasting whereas it increases locomotor activity in a fed state [71, 182, 192, 193].

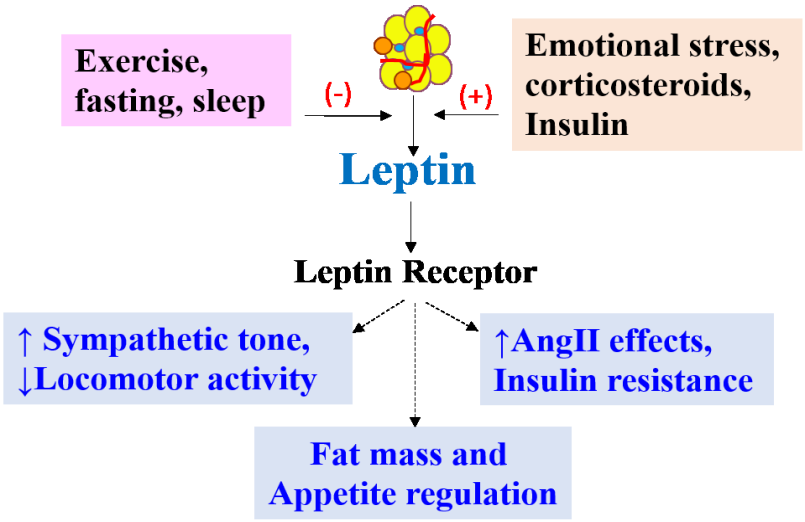


Figure 9. Leptin signaling and effects. Leptin signaling (black text) and effects (blue boxes) are shown on this figure. Elements which contribute to the stimulation (orange box) or inhibition (pink box) of leptin production are also presented.

Leptin and obesity. Leptin gene expression and its plasma level are increased with obesity in humans and rodents fed a HFD [55, 80, 163]. Obesity is often accompanied by hyperleptinemia which eventually leads to leptin resistance [87]. About 3% of all cases of obesity are due to genetic mutation of the gene for leptin (ob/ob) or its receptor (db/db) [87, 194, 195]. The role of leptin and its receptor in the development of obesity have been studied using ob/ob and db/db mouse models,

respectively [194]. Ob/ob mice are obese and insulin resistant. In this model, leptin replacement (infusion) decreases appetite, body weight and fat masses as well as improves insulin sensitivity [196]. The db/db mice have a more severe obese phenotype with the presence of fasting hyperglycemia, hyperlipidemia and hyperinsulinemia as well as diabetes [197]. Moreover, leptin has been shown to impair lipid and carbohydrate metabolism, leading to insulin resistance [87, 195, 198] although its effects on glucose homeostasis are complex and not yet fully understood.

1.7.2 Resistin

Resistin is another adipose tissue hormone for which plasma levels are increased with insulin resistance and inflammation [103, 167, 176]. In humans, it is also secreted by monocytes and the placenta, while in mice, resistin is exclusively secreted by adipocytes and the placenta [167, 172]. Resistin is synthesized in response to inflammation, hypoxia, and bacterial endotoxins, whereas thiazolidinediones (TZD) decreases its production. Interestingly, plasma leptin and resistin levels are decreased by RAS inhibition [185, 199]. For instance, administration of ACE inhibitors, such as perindopril and enalapril, in people with coronary artery disease improves insulin sensitivity [185].

Molecular mechanisms of action are not yet clear. In humans, resistin is proposed to bind with a specific cell surface receptor, the adenylate cyclase binding protein type 1 (CAP-1), which has not been confirmed in mouse studies [200]. Resistin binding to CAP-1 in monocytes leads to increasing cAMP level in the cytoplasm and activates PKA and NFk-β, which trigger inflammatory protein gene transcription [201]. By stimulating pro-inflammatory factor gene transcription and expression, resistin contributes to the development of insulin resistance.

Effects of resistin. A positive correlation has been found between circulating resistin level and conditions such as insulin resistance and coronary heart disease. This has been proposed to be through the action of resistin on NFkB, Toll-like receptor 4 (TLR4), eNOS, and p38MAPK pathways [167, 172]. Conversely, resistin was not changed in adipose tissue and plasma from

resistant to HFD mice, whereas in genetically obese KK-Ay mice and in isolated adipocytes of FVB mice strain, resistin level was lower in spite of obesity [171, 172], This disparity in correlation between resistin, body weight and insulin resistance may be because it is secreted by both adipocytes and circulating mononuclear cells [202].

1.7.3 Adiponectin

Adiponectin is mainly an adipose tissue-derived hormone, which is also synthesised in skeletal muscle, gastrointestinal cells, and the placenta [168, 173, 203, 204]. The adiponectin gene has been shown to be expressed differently in different fat pads [181]. Adiponectin gene expression is increased by PPAR-gamma, estradiol, insulin and starvation, while it is decreased by AngII, TNF-alpha, hypoxia, oxidative stress, forkhead box protein O1 (FOXO1), and C-reactive protein [173]. In addition, adiponectin is present in different molecular forms, such as a monomer, hexamer and a globular fraction as a result of posttranslational modifications [175, 205]. Amongst these different fractions, the high molecular weight (HMW) fraction of adiponectin has been shown to be the most physiologically active [175, 179, 206-208].

Effects of Adiponectin. Adiponectin enhances insulin sensitivity by decreasing the activity of phosphoenolpyruvate kinase (PEPK) and glucose-6 phosphate dehydrogenase (G6PD) which limit gluconeogenesis in the liver. Also, adiponectin through AdipoR1 stimulates phosphorylation of Akt and activation of p38 MAPK which increases GLUT4 activity in the skeletal muscle and leads to better glucose metabolism [203, 209-211]. Additionally, adiponectin has been shown to activate AMPK through stimulation of its AdipoR1 (**Figure 10**).
This attenuates the stimulatory effects of AngII on NADPH oxidase (NOX) subunits oxidation of NADPH and decreases ROS formation [212] as well as prevents the impairment of GLUT4 translocation [213]. Moreover, this also activates malonyl-CoA decarboxylase and prevents further fatty acids synthesis. As a result, the increased malonyl-CoA level stimulates acetyl-CoA carboxylase (ACC) activity and promotes fatty acids beta-oxidation in the mitochondrion [88, 205, 214].

Adiponectin also participates in PPAR gamma stimulation effects on GLUT4 translocation which put altogether improves insulin sensitivity [186, 215]. Moreover, the anti-inflammatory effects of adiponectin are mediated via the inhibition of monocyte adhesion molecule and macrophage infiltration into adipose tissue, and decreased secretion of IL-6 and TNF-alpha [168, 203, 216, 217].

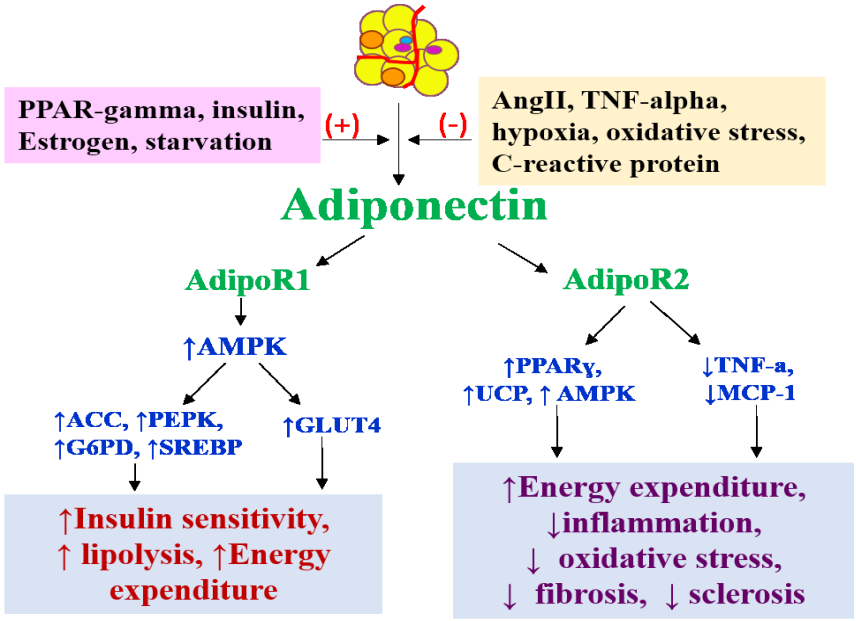


Figure 10. Adiponectin effects. Adiponectin signaling (in green and blue text) and effects (in blue boxes) shown in the picture. Elements which contribute to the stimulation (pink box) and inhibition (yellow box) are also presented.

Plasma adiponectin level is inversely correlated with obesity, insulin resistance, inflammation, and cardiovascular disease (CVD) [46, 103, 163, 165, 176]. In addition, adiponectin level has been shown to increase with weight loss and during fasting [218]. In clinical studies, high levels of circulating adiponectin were found to be protective against the development of type 2 Diabetes Mellitus independently of total fat mass and the degree of insulin sensitivity [179]. The mechanisms suggested to be implicated in this effect were through the stimulation of glucose uptake through AdipoR1 Akt phosphorylation and fat oxidation by an increased activity of AMPK [179, 219-221].

1.8 OBESITY AND INSULIN SENSITIVITY

Obesity is often associated with insulin resistance [7, 78]. Despite the enormous amount of research, the causes of insulin resistance associated with obesity continue to be vigorously debated. The main factors involved in insulin resistance linked to obesity are abnormal adipokine levels, inflammation, increased adipocyte size and dysfunction, as well as increased RAS activity [9, 163].

The profile of adipokine gene expression and production are altered with obesity and a HFD. What is observed is an increase of "anti-insulinic" factors, such as leptin, resistin, TNF-alpha, IL-1 and IL-6, and a decrease in "insulin-sensitivity" factors, for instance, adiponectin and irisin [9, 92, 93, 96, 103, 222].

Adipose tissue glucose uptake. Fat tissue is the main location for glucose consumption after the liver and skeletal muscle. After a meal is absorbed from the intestinal tract and nutrients are released into the blood stream, about half of the glucose is handled by the liver and the rest of the glucose is uptaken by tissue, mostly in an insulin dependent manner via the GLUT4 protein into skeletal muscle and adipose tissue [145, 223]. As an "insulin sensitive" tissue, adipose tissue can take up a large amount of glucose from blood after a meal and help to maintain normal glycemia [91, 146, 157]. Insulin resistance in adipose tissue alters systemic glucose distribution leading to hyperglycemia and compensatory insulin release [91, 121,22, 146, 156].

Adipose tissue regulates whole-body glucose homeostasis. Hyperinsulinemia stimulates the activity of plasma lipoprotein lipase and increases fatty acid uptake into adipocytes, and promotes an increase in lipid droplet size and as such, enlarges the adipocyte's volume [22, 81]. Enlarged adipocytes in turn become insulin resistant due to a loss of membrane glycoprotein structure and function [81]. This increased fat synthesis and lipolysis causes hyperlipidemia [157], which affects beta-cell function and whole body glucose homeostasis [136, 223, 224].

1.9 OBESITY MANAGEMENT, PREVENTION AND TREATMENT

Despite much success achieved in our understanding of the pathogenesis of obesity and intense studies on the development of new methods for the prevention and treatment of obesity, epidemiological studies and clinical trials indicate that the obesity problem remains unsolved and needs to be further studied.

Management of obesity involves correcting the patient's diet to avoid of excessive energy intake and balancing it with physical activity, when it is possible. However, as practice shows, they are not always effective at reducing body weight, and additional treatment with pharmacologic agents or surgical procedures are required when obesity is severe [53].

Intestinal inhibitors of the nutrients absorption, for instance alpha-lipase inhibitors and alpha-glycosidase inhibitors, are used widely in clinical practice and can provide about 5-15% weight loss in combination with increased physical activity [53, 56, 63, 225, 226]. These drugs however have side effects, such as intestinal colic, soiled clothing due to excessive fat release from the intestine, excessive gas production and impact the intestinal microbiota [53, 227, 228].

Surgical fat removal which is used in clinical practice, have long-term effects that are unclear and have contraindications such as heart failure, coronary artery disease, liver or/and kidney insufficiency, which has restrained there wide use [229].

Bariatric surgery, such as gastric bypass, laparoscopic gastrectomy or intestine removal is also used in patients with comorbid obesity to help decrease nutrient and fat absorption from the gastrointestinal system [229]. These procedures, like the surgeries mentioned above, also have contraindications such as a heart failure, coronary artery disease, liver or/and kidney insufficiency [230].

CHAPTER II. TYPE 2 DIABETES MELLITUS

2.1 DEFINITION AND EPIDEMIOLOGY OF TYPE 2 DIABETES MELLITUS (T2DM)

Blood glucose level is an important factor which is linked to life longevity and has a important impact on disease outcomes and mortality [139, 231-233].

Definition. Diabetes Mellitus (DM) is a group of metabolic diseases, characterized by chronic hyperglycemia and the development of complications [234]. DM is divided into two main types.

Type 1 diabetes Mellitus (T1DM) consist of about 5-10% of all cases of DM and develops suddenly, mostly at a young age. In this type of diabetes, hyperglycemia appears as a result of complete insulin deficiency due to the destruction/damage of pancreatic beta-cells which results from autoimmune or other altered responses. Consequently, these patients require exogenous insulin replacement [234].

Type 2 Diabetes Mellitus (T2DM) consists of the major portion of total cases of DM (90-95%). It develops mostly in obese people which are older than 40 years old, with a family history of T2DM [6, 235-238]. In this type of diabetes, hyperglycemia results from peripheral tissue insulin resistance and requires hypersecretion of insulin, which chronically leads to insufficient beta-cell function and insulin secretion [234, 239, 240]. Treatment of T2DM includes restoration of normal insulin level and enhancement of insulin sensitivity [234].

T2DM is an epidemic. T2DM is linked with obesity and was also announced by the World Health Organisation (WHO) as a worldwide epidemic, which has a great impact on the economy, quality of life, and leads to disability [47]. The cost related to the treatment of diabetes and its complications in the United States of America is very high and amounted to $132B USD in 2010, and this amount is estimated to increase to $192B USD in 2020 [5, 241]. The number of people with diabetes in the world has increased very quickly in the past decades and reached

285M people in 2010 [5]. However, the experts of the WHO have estimated even faster growth in the next few years which would lead to 439M individuals by 2030 [5, 242].

2.2 RISK FACTORS AND PATHOGENESIS OF TYPE 2 DIABETES MELLITUS

T2DM is a complex disease. Genetics, family history, overweight/obesity and age are common risk factors for T2DM. Gestational diabetes, metabolic syndrome, and smaller or excess body weight at birth have also been related to the development of T2DM [235, 236, 243-246].

The role of genetics and family history in the development of T2DM were shown in family studies of twins, siblings, and ethnicity [238, 247-251], as well as in animal models of diabetes [248, 252].

Body weight and obesity. While studies confirm that body weight management prevents the development of T2DM, even in people with impaired glucose tolerance [47, 233, 240, 253], the mechanism by which obesity affects insulin sensitivity continues to be studied. Since not all obese people develop insulin resistance, the "healthy fat" phenomena without insulin resistance as a variant of obese adipose tissue is widely discussed [148, 234, 240, 254-257].

Adipokines. The role of adipokines in the development of insulin resistance and T2DM was reviewed in the previous chapter. High levels of leptin, resistin, TNF-alpha and low levels of plasma adiponectin are all associated with insulin resistance and T2DM [103, 176, 222, 258]. The development of insulin resistance and T2DM are also observed in rodent models with leptin resistance [259, 260]. A low plasma adiponectin level is a risk factor for the development of both insulin resistance and T2DM [261, 262].

Blood lipid levels, especially cholesterol and triglycerides, are strongly elevated in obesity and T2DM, and are a risk factor for both. They are also risk factors for the development of diabetes complications [46, 259, 263]. Hyper- and dyslipidemia through a mechanism called

"glucolipotoxicity" [136, 141, 264, 265] leads to declining beta cell function, which has been shown to be a principal mechanism responsible for the pathogenesis of T2DM [223, 240].

Leptin, an adipose tissue derived hormone, through its effects on pro-opiomelanocortin (POMC) neurons can negatively regulate glucose homeostasis and locomotor activity, which explains its role in energy balance and development of obesity [266]. Interestingly, isolated suppression of leptin receptors specifically in POMC neurons reduces energy intake, body weight and improves insulin sensitivity [266].

Physical inactivity was found to be a risk factor for diabetes and cardiometabolic diseases [267]. Muscle activity leads to better insulin sensitivity by reducing lipid levels in plasma and in muscles [268, 269] as well as by regulating GLUT4 translocation and promoting glucose uptake [148].

Aging. Age is a strong risk factor for the development of T2DM and insulin resistance. This may in part be explained by the lower physical activity and excessive body weight gain, hormone imbalance, reduced muscle mass and lower ATP production capacity in mitochondria which is associated with aging [47, 240, 270-272].

2.3 WHOLE BODY GLUCOSE HOMEOSTASIS

Glucose homeostasis in mammals is a complex process which is tightly regulated by pancreatic islet cell hormones, but also by gastrointestinal peptides, brain hormones, and the sympathetic nervous system [273-275]. Glucose homeostasis is a highly dynamic process which depends on food intake, physical activity, stress, and other conditions (**Figure 11**) [88, 273, 276, 277]. Glucose homeostasis can be evaluated using a glucose tolerance test (GTT), for instance, after an oral administration of glucose (OGTT). An estimation method can be the insulin tolerance test (ITT), i.e. following an administration of insulin intraperitoneally or intravascularly [22, 146, 234, 273, 278]. The physiological glycemia response to a glucose load during an OGTT or after an insulin injection is shown on **Figure 11**. The results obtained from an OGTT (fasting and

postprandial glycemia i.e. after 2 hours of loading glucose) can be interpreted as (**Table 3**) normal glucose tolerance, prediabetic (impaired fasting glucose, impaired glucose tolerance) or diabetes mellitus [279].

Table 3. Glucose tolerance status according to the glycemia level during an OGTT

Glucose tolerance condition	Glycemia (Mmol/L)	
	Fasting	2h after a meal
Normal glucose tolerance	≤ 5.5	≤ 7.0
Impaired fasting glycemia	≥6.1-6.9≤	≤ 7.0
Impaired glucose tolerance	≤ 6.1-6.9	7.8 – 11.0
Diabetes Mellitus	≥ 7.0	≥ 11.1

Glucose homeostasis in the basal and stimulated state. Glucose homeostasis is usually determined by analysing glycemia in a basal (fasting) and stimulated (following the ingestion of a meal) state (**Figure 11**).

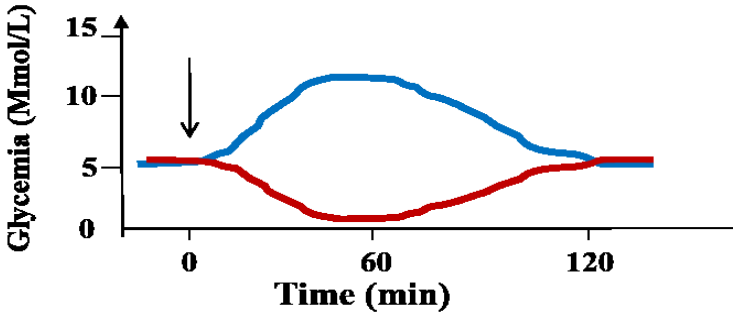


Figure 11. Glycemia measured during an OGTT or ITT. Glycemia response following an OGTT (blue line) and an ITT (red line) is shown. Arrow indicates when glucose or insulin was administered.

In the basal state, blood glucose levels are maintained between 2.2 and 5.5 mmol/L, while it can increase up to about 11 mmol/L after a meal. All ingested carbohydrates and lipids, are usually metabolized or stored as glycogen or as TG droplets, mainly in fat, liver and muscle cells 2-3 hours following a meal [273, 275, 276]. This physiological balance is lost in insulin resistance and

Diabetes Mellitus [240, 275, 280-282]. Physiological tests, such as the GTT or ITT, are widely used to evaluate glucose homeostasis [283].

Glucose entry into cells. Glucose as a water soluble substance cannot enter cells through the plasma membrane and as such, requires transporters, more specifically, non-energy-dependent facilitated membrane glucose transporters (GLUTs), and energy-dependent sodium glucose co-transporters (SLGTs). The different forms of GLUTs are tissue specific and some require stimulation by insulin to translocate to the cell membrane [145]. SGLTs are located in the brush border epithelium of the small intestine and in the kidney proximal tubule [284, 285].

Table 4. Glucose transporters, their tissue location and function.

GLUTs type	Location	Insulin dependent	Function	Sources
GLUT1	Ubiquitous	No	Facilitates glucose transport into cells according to its plasma concentration	[145]; [286]; [287]
GLUT2	Pancreas beta-cell	No	Glucose sensor in the pancreatic beta-cells, increases insulin secretion in response to glycemia	[144], [288] [289]
GLUT3	Brain neurones, neutrophils	No	Metabolic sensor in the brain, participates in the reward circuit	[290,291] [292]
GLUT4	Skeletal muscle, adipose tissue, heart, monocytes, leucocytes	Yes	Main glucose transporter after a meal	[286]
GLUT5	Intestine, gonadal cells	No	Fructose transporter	[293] [286], [294]

GLUTs. There are more than 15 types of GLUTs [286] that vary according to their tissue localization, glucose transport activity and dependence on insulin. Most of the transporters have low activity in both basal and stimulated states determined by plasma glucose concentration, and function independently of plasma insulin levels. [145, 286]. The type and location of the most common GLUTs and their function are listed in **Table 4**.

GLUT4 is the only glucose transporter with an activity that is mainly dependent on insulin [286]. GLUT4 is the main glucose transporter implicated after meals, i.e. stimulated state [156, 223, 276, 295]. The main defect in cells that are insulin resistant is insufficient translocation of GLUT4 to the plasma membrane [286]. Along with classical insulin dependent pathway, GLUT4 can also be activated without increasing insulin concentration, for example, during physical activity.
Details regarding the molecular mechanisms implicated in GLUT4 translocation are not clear yet. Some authors have suggested the involvement of the PKA-MAPK-p38 pathways through an activation of the AMP dependent kinase (AMPK), whereas others have implicated the inhibition of reactive oxygen species (ROS) formation which increase GLUT4 translocation through Akt/PKB phosphorylation [88, 148, 214, 296] or increased GLUT4 gene transcription by PPARɣ [149].

Glycated hemoglobin (HbA1c) is a fraction of hemoglobin, which non-enzymatically binds with amino-terminal valine in the glucose molecule according to its plasma concentration. It is used widely in clinical practice as a marker of mean blood glucose during the past 3 months in patients with DM [278, 297, 298]. Non-enzymatic glycation occurs with all proteins and lipids in the body. This changes their structure and function, as well as favors their oxidation. As a result, their degradation capability is also changed and they are accumulated in the body as advanced glycated end products (AGE) [297] and present with an accelerated ageing and increased risk of diabetes complications [244, 246, 298]. Hence, decreasing blood glucose level leads to a reduction in HbA1c and other glycated products level as well as risk of complications [244, 246, 299].

2.4 INSULIN AND INSULIN RECEPTOR SIGNALING PATHWAY

Insulin is a peptide hormone synthesized in the pancreatic islet beta-cells as a pre-proinsulin [274, 300]. It is processed in the endoplasmic reticulum to proinsulin which then travels to the golgi apparatus to be packaged into secretory granules. Proteases and carboxypeptidase E cleave the proinsulin molecule to produce C-peptide and mature insulin in equimolar concentration [274, 275, 301, 302]. Given the short half-life of circulating insulin due to its clearance uptake by liver and other organs, C-peptide is often used in clinical practice for diagnosis of diabetes and insulin secreting neoplasia [301-304].

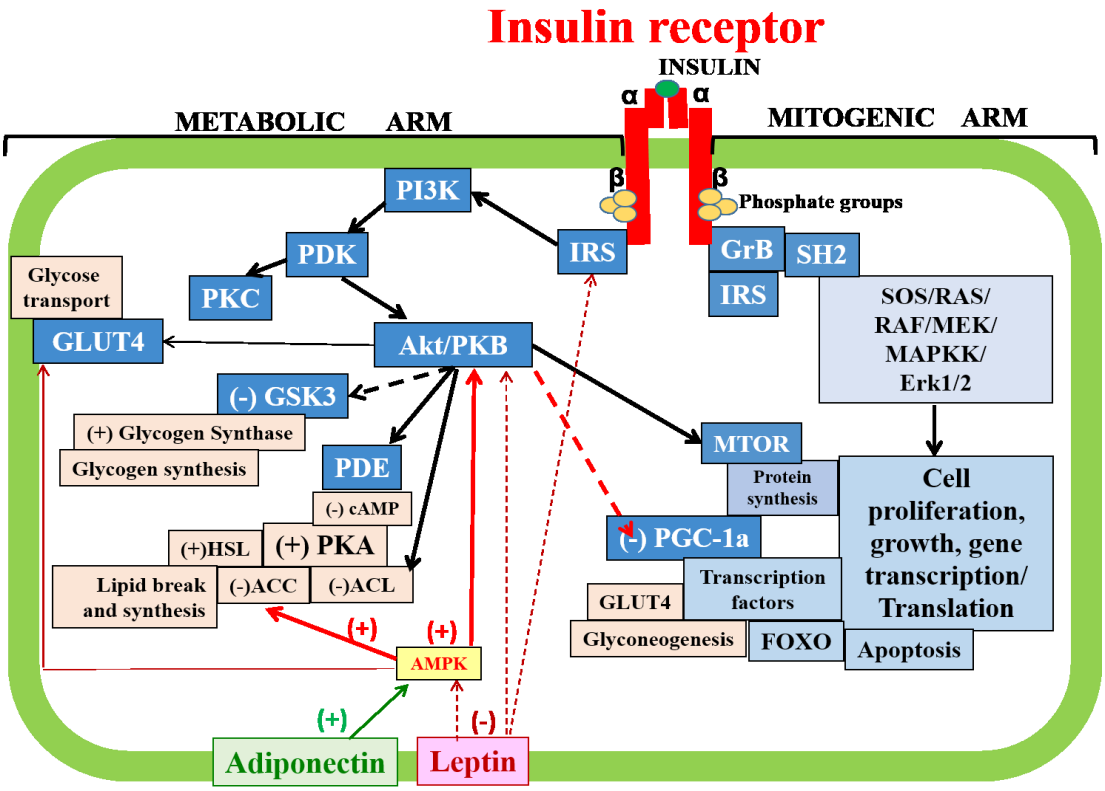


Figure 12. Insulin signaling pathways and crosstalk. On this figure are shown the first (blue boxes) and second (orange boxes) arms of the insulin pathway. Adipokine effects on insulin signaling are also shown in green for adiponectin and

The **insulin receptor** is a transmembrane tyrosine kinase receptor [305] which binds to insulin and the insulin-like growth factor (IGF). The structure of this receptor consists of 2 alpha and 2 beta

subunits [305, 306] (**Figure 12**). Insulin binds to both alpha subunits on the cell membrane, which provide signal to the beta subunits, where a series of phosphorylations are performed and then transmit the signal downstream towards both the insulin-receptor substrates (IRSs) and through the growth factor receptor bound protein (GrB) and to the Src Homology 2 (SH2) (**Figure 12**).

The first (metabolic) arm of the insulin pathway (**Figure 12**) provides signaling from the IRS to the phosphatidyl-inositol-3 kinases (PI3K) [307] which activates the PI3K dependent kinase (PDK1) and transduces through the protein kinase C (PKC) towards Akt/PKB as well as stimulates GLUT4 translocation to the plasma membrane [308, 309]. In turn, Akt/PKB passes this signal forward through different pathways, such as stimulation of glycogen synthesis through downregulation of the glycogen synthase kinase 3 (GSK3), decreasing of lipolysis by phosphorylating the hormone sensitive lipase (HSL) and stimulates lipid synthesis by increasing the activity of the acetyl-Co-A cocarboxylase (ACC) and ATP-citrate lyase [310]. Akt also downregulates the PPAR gamma coactivator 1 alpha (PCG-1) gene, a master regulator of energy metabolism through mitochondrial gene expression [311].

The second (mitogenic) arm of the insulin pathway (**Figure 12**) starts with the Src homology domain 2 (SH2) [312] and the adaptor protein GrB (growth factor receptor bound protein). It then involves serine/threonine-specific protein kinases such as son of sevenless (SOS), related proteins family (RAF) and superfamily (RAS), as well as mitogen activated protein kinases such as MEK, MAPKK, and the extracellular regulatory kinase 1/2 (Erk1/2). Thereafter, this activates protein synthesis through activation of transcription and translation factor genes to stimulate cell proliferation and growth [143, 307, 309, 313], where the forkhead box O (FOXO) transcription factor is involved, particularly regarding transcription of genes involved in neoglucogenesis and the glucose transporter GLUT4. Interestingly, GLUT4 translocation has also been proposed to be stimulated by muscle contraction through the stimulation of adaptor proteins, such as the insulin regulated aminopeptidase (IRAP) and the Akt substrate of 160 kDa (AS160) [150] (not shown in the Figure 12). Exercise and muscle contraction also improve glucose transport through the stimulation of AMPK [148].

Adipokines implicated in the regulation of insulin signaling. As shown in **Figure 12**, leptin has been suggested to negatively regulate Akt phosphorylation and IRS, and thus could contribute to the development of insulin resistance [69, 258, 314]. On the other hand, adiponectin through AdipoR1 and AdipoR2 receptors stimulates AMPK which leads to better insulin sensitivity [168, 261].

2.5 INSULIN RESISTANCE

Insulin resistance is the state where higher than physiological concentrations of insulin are needed to maintain normal blood glucose level [22, 315]. Insulin resistance is viewed as an adaptation to particular conditions such as pregnancy, growth, etc. The reason for insulin resistance in pathological states is unclear but it is the main cause of T2DM and also a major player in the pathogenesis of the metabolic syndrome [22, 272].

Insulin resistance is mainly due to impairment in insulin signaling, such as ineffective auto-phosphorylation of receptor subunits, IRS or other components. This can result from oxidative stress, nitrosylation of Akt and other factors that disable downstream signal transduction [11, 296, 316-319]. Insulin resistance can also result from abnormal receptor structure or its binding with antibodies or hormones such as insulin growth factor 1 (IGF-1) [319, 320]. This can be observed in patients with "acantosis nigricance", T2DM, and lipodystrophy [319, 321, 322].

Insulin resistance can be measured quantitatively using the euglycemic hyperinsulinemic clamp technique, which is considered to be the "gold standard" method [323, 324]. In this method, a high dose of insulin is infused so the pancreatic insulin secretion becomes negligible. Thereafter, glucose is infused in a continuous manner to maintain euglycemia. Insulin resistance can also be estimated mathematically through homeostasis assessment models, such as the homeostatic model of assessment (HOMA) index in the fasting state and also during an OGTT or ITT [325]. The other calculations, such as the glucose to insulin ratio or the HOMA-B, show a relationship between blood glucose and circulating insulin level [283, 326, 327].

2.6. ROLE OF ADIPOKINES IN THE PATHOGENESIS OF T2DM

The role of adipokines in the development of T2DM. One of the explanations for the correlation between obesity and insulin resistance are the effects of adipokines [9, 103, 176, 258, 328]. Indeed, the increased gene expression or plasma levels of adipokines which are associated with obesity such as leptin, resistin, TNF-a, NF-kB, IL-6, RBP-4, PAI-1 and MCP-1, can enhance the development of insulin resistance and T2DM [328]. For instance, the monocyte chemoattractant protein 1 (MCP-1) has been shown to reduce glucose uptake in adipocytes and plasma MCP-1 level is increased in patients with T2DM [11, 329]. In line with these data, MCP-1 gene knockout mice are insulin sensitive, while mice which overexpress this gene are insulin resistant [330, 331]. Also, TNF-alpha contributes to insulin resistance by affecting phosphorylation of p38 MAPK and p44/42 Erk1/2. As such, obese TNF-alpha knockout mice are protected from insulin resistance [104, 332].

2.7. THE ROLE OF PLASMA LIPIDS IN THE PATHOGENESIS OF T2DM

Increased FFA in plasma binds to specific G-protein coupled receptor (GPR40) and acts via the DAG-PKC pathway, which leads to ineffective phosphorylation of IRS and disables insulin signaling via Akt to GLUT4 translocation [107, 271, 333]. FFA also inhibits downstream insulin signaling through Akt, by blocking GSK3-dependent glycogen synthesis and via phosphorylation of FOXO1 increased gluconeogenesis [334]. As such, measurement of plasma lipid level is included into the standard treatment of T2DM to evaluate the risk of developing complications [159, 335].

GPR40. Plasma lipids, through binding to specific cell surface receptor GPR40, also indirectly reduce beta-cell function [140, 141, 336] by altering the expression of multiple genes, such as p27,

the pancreatic-duodenal hemobox 1 (PDX-1), PI3K, Akt and Erk1/2. Indeed, this impairs the beta cell's capacity to secrete insulin [136, 137, 337], and increases the incidence of T2DM.

High level of blood lipids and ectopic fat accumulation. The high plasma lipid levels due to insulin resistance in T2DM is a result of increased release of FA and TG by fat tissue and an accumulation by the liver and muscle [46, 159, 268, 269, 334] .

2.8 COMPLICATIONS OF T2DM

T2DM is characterized by the development of chronic complications, which are divided into macro- and microvascular.

Macrovascular complications of diabetes include atherosclerosis, coronary heart diseases, stroke and peripheral vascular diseases [244, 246, 338]. Cardiovascular incidents, such as a myocardial infarction and stroke are the main causes of death among people with T2DM, as there is a 4 times higher incidence than in the general population [263, 339, 340]. Amputations, due to peripheral vascular diseases, also occur at a higher rate among people with T2DM [241, 339].

Microvascular complications are more specific to diabetes and include nephropathy, retinopathy and neuropathy [263, 339, 340]. The development of these complications depends on disease duration, the level of hyperglycemia, high plasma lipids and blood pressure, gender and immunogenetics [82, 94, 241, 271, 338, 339, 341-344]. Diabetic nephropathy in T2DM occurs in 5-20% of patients, whereas in T1DM it develops in 40% of patients [335]. The risk factors for the development of nephropathy are the level of blood glucose, HbA1c and lipids, microalbuminuria and high blood pressure [335, 345]. High blood pressure has a strong impact on the progression of nephropathy and retinopathy [346].

Diabetes complications and RAS activity. The role of the RAS in the development and progression of nephropathy and retinopathy has been shown by many studies [347-350]. Moreover,

it is supported by the observed beneficial effects of RAS inhibitors which delay their progression and development [350, 351] [29, 352, 353]. Moreover, an ACE gene polymorphism has shown linkage with the development of nephropathy in T2DM [341, 354].

2.9 TREATMENT OF T2DM

Treatment of T2DM includes correction of two major pathogenic aspects, i.e. restore insulin sensitivity and replace the insulin deficiency [240].

Healthy life style and body weight correction. In 60% of obese people, the development of the T2DM was reversed by healthy lifestyle management, i.e. losing weight and eating a balanced diet [233, 253, 267, 340, 355]. Lifestyle managements have been shown to decrease T2DM incidence from 67 to 41% in the different studies [6, 225, 253], as it reduces blood sugar and body weight [238, 299, 338]. In the United Kingdom Prospective Diabetes Study [345], a healthy lifestyle led to lower blood sugar and to a decrease in HbA1c level which can be significant as different studies showed that a 1% decrease in HbA1c level lowered the risk of diabetes complications by 37% [237, 356-359]. However, adopting a healthy lifestyle is not easily accomplished by most people [53].

Secretagogues are pharmacologic agents that increase beta cell insulin secretion. Sulfonylureas and meglitinides bind with sulfonylurea receptors (SUR, a type of membrane potassium channel) on the beta-cells and increase insulin secretion. Sulfonylureas, such as glibenclamide, inhibit K_{ATP}-channels which lead to depolarization of the beta-cell plasma membrane followed by intracellular Ca-influx. This stimulates the secretion of insulin from intracellular granules [312, 360, 361]. However, these drugs have side effects such as hypoglycaemia and body weight gain [240, 362]. Incretins, such as the glucagon-like peptide 1 (GLP-1), stimulate insulin secretion from the pancreatic beta-cells through inhibition and closure of the ATP-sensitive K-channel followed by Ca+2 release [240]. The antidiabetic drugs dipeptidyl peptidase 4 (DPP4) inhibitors preserve GLP-1 from degradation and increases insulin secretion. These drugs can also promote hypoglycemia in T2DM [363]. Because inhibitors of DPP4 and GLP-1 analogs have additional

side effects on the gastrointestinal tract, such as nausea and vomiting, as well as dizziness and headache, this limits their wide administration [363].

Insulin sensitizers, such as biguanides (metformin), and agonists of PPARs (thiazolidinediones) help to restore insulin sensitivity [149, 364, 365]. Metformin acts through modulation of AMPK activity [158, 366] and promotes GLUT4 translocation [366, 367]. PPAR-ɣ agonists also act through modulation of AMPK activity and increase the synthesis of nuclear factors for gene expression, which stimulates translocation of GLUT4 and lipolysis, and increases plasma adiponectin level [149, 217]. TZD and metformin do not affect insulin secretion [240, 368, 369], and also have side effects, such as rhabdomyolysis and osteoporosis [363, 370]. Since biguanides restore insulin sensitivity, they were recommended as a first line T2DM treatment in combination with other drugs or insulin [240]. In contrast, TZD have been banned for clinical use in all countries because of side effects such as fluid retention, oedema, bladder cancer [371].

Regulators of glucose absorption are drugs which regulate blood sugar level by decreasing the absorption of glucose from the intestine or kidneys. The inhibitors of intestinal alpha-glucosidase, such as acarbose and glucobay, decrease glucose absorption in the intestine. These drugs have side effects on the digestive system, such as increased gases or modulation of the gut microbiota due to increased carbohydrate levels in the intestine. Sodium-glucose linked transporter-2 inhibitors (SGLT2 inhibitors) block glucose reabsorption in the kidney tubule to improve glycemic control in T2DM [284, 363]. They have lower hypoglycaemic effect, and have side effects, which mainly relate to excess of glucose level in the urine [372]. SGLT2 inhibitors have been shown to have side effects such as increased frequency of urination and urinary tract infection, risk of hypoglycemia, increasing of low density lipoproteins level and increased risk for cancer, thus there has been limited use of these drugs in clinical practice [372].

CHAPTER III. RENIN-ANGIOTENSIN SYSTEM

3.1 DEFINITION, CLASSIC AND MODERN THEORIES

RAS definition, function and components. The RAS includes components that participate in a cascade of reactions that converts angiotensinogen (Agt) to angiotensin I and II (AngI, AngII) (**Figure 13**). The main function of the RAS is to maintain blood pressure and fluid-sodium balance in the body [17, 373].

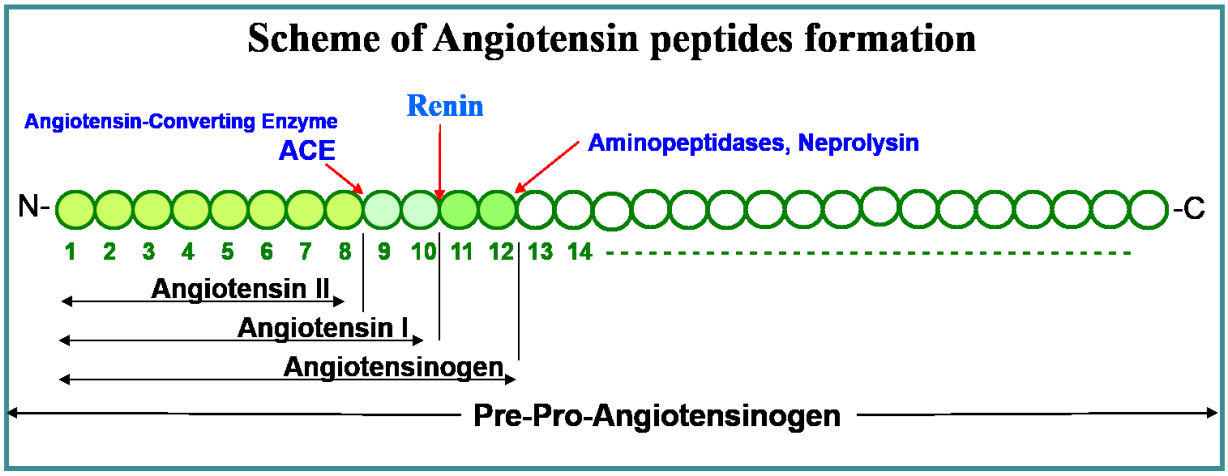


Figure 13. Scheme of angiotensin peptides formation. In black are shown the peptides used and generated in the RAS cascade. In blue are indicated the enzymes involved in the cleaving of the different amino acids.

Our knowledge about the RAS has expanded during the past decades, and the RAS is now known to be much more complex than originally thought. The RAS is involved in the development of many diseases (cardiovascular, endocrine, immune, inflammation and cancer) as well as tissue damage and disease complications [13, 373-375]. The RAS also has an effect on cell metabolism, proliferation and growth [9, 12, 351, 376, 377]. Clearly, the RAS has turned from a simple producer of angiotensin II to a complex multifunctional system of circulating and local components [119, 378-380].

The classical RAS pathway consists (**Figure 14**) of components which convert the non-active angiotensinogen (Agt) to angiotensin I (Ang I) through enzymatic cleavage by renin and then to angiotensin II (AngII) by the angiotensin-converting enzyme (ACE). AngII is considered to be the main physiologically active component of the system and acts mainly by binding to the AT1R which is present on cell plasma membranes, and leads to, for instance, vasoconstriction, cell proliferation, fibrosis and apoptosis [16, 381].

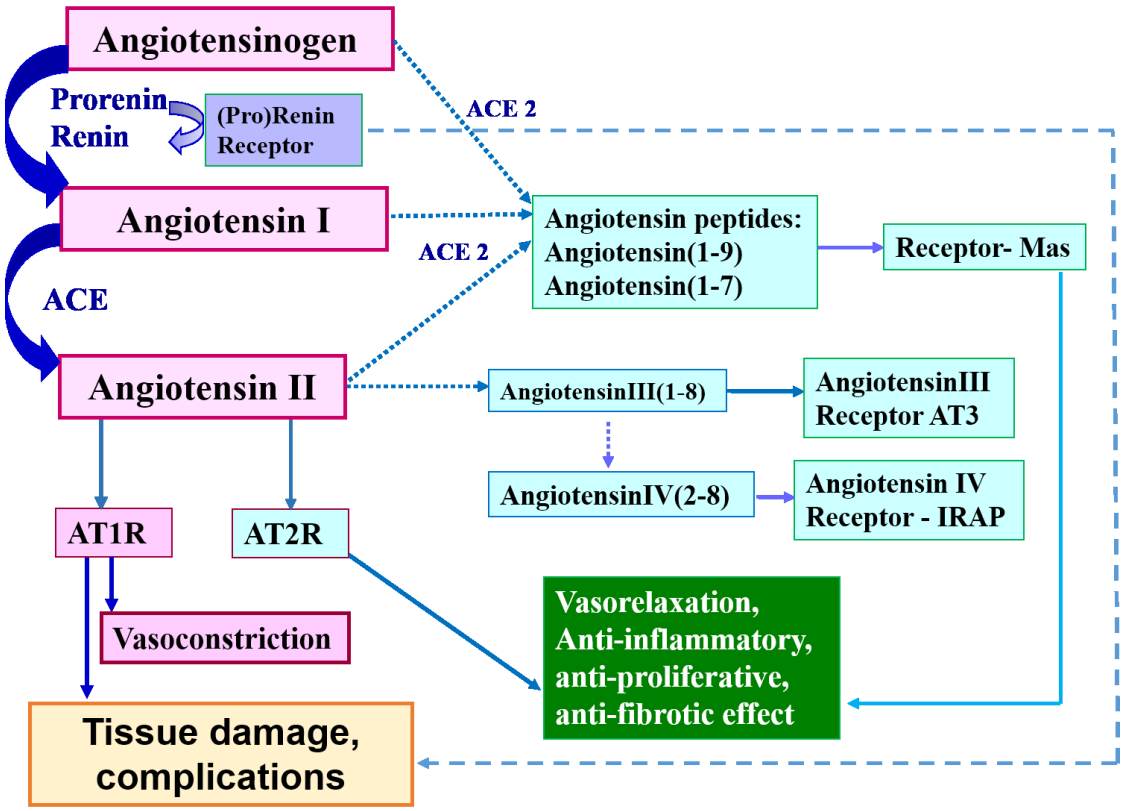


Figure 14. Renin-Angiotensin system components and general effects. Red boxes represent the classic RAS pathway whereas blue/green indicates new components and their known physiological impacts.

New components of RAS. Over the past decade, the RAS family was enriched [16, 381] by the detection of new members (**Figure 14**) such as 1) receptors, for instance AngIII receptor (AT3R) [382], AngIV receptor (AT4R)/ the insulin responsive aminopeptidase (IRAP), MAS receptor (MASR) [383], prorenin/renin receptor [(P)RR] [35] 2) enzymes, such as the angiotensin-converting

enzyme 2 (ACE2) [15]; 3) new angiotensin products, such as Ang(1-7)[384], alamandine [380] and Ang(1-9) [15, 380-383, 385].

3.2 RAS COMPONENTS

3.2.1 Angiotensinogen

Angiotensinogen (Agt) is an alpha-2 globulin peptide belonging to the serpin family, which is produced in the liver. The Agt molecule is present in two forms as an oxide and a redox. The oxide form of Agt has been shown to be more actively bound to renin [15]. Agt cleavage by renin was shown as a rate-limiting step in the RAS reaction cascade [14, 129, 386, 387].

3.2.2 Renin, Prorenin and their receptor

Renin also known as an angiotensinogenase is an enzyme which belongs to the aspartyl protease family. The renin gene has been shown to be expressed in many tissues, although in some at very low levels, for example in adipose tissue [19]. Renin is synthesized as a precursor molecule, preprorenin, which then matures to prorenin through glycosylation [388-390]. Renin is then produced from prorenin proteolytically by aminopeptidases, mainly in the kidney cells of the juxtaglomerular apparatus (JGA) [361, 390-393]. Renin release in the plasma is mainly regulated by : 1) baroreceptors which are sensitive to blood pressure 2) the macula densa's special cells located in the distal convoluted tubule of the kidney and connected with JGA cells which are sensitive to body liquid and salt volume, and 3) the sympathetic nervous system through beta1 adrenergic receptors and 4) the level of AngII in the plasma [181, 394, 395]. As such, after nephrectomy, only prorenin is detected in the blood [396] [397]. In addition, about 10% of renin is produced from prorenin via cleavage using alternative enzymes, such as peptidases [16].

Physiologically, the plasma concentration of renin is 10 times less than prorenin, due to wider production of prorenin by other tissues which cannot transform it into renin [396].

Prorenin can gain enzymatic activity by conformational changes at acidic pH [390]. Prorenin is synthesized in many tissues such as, the adrenal glands, gonads, placenta, and eyes [37, 396, 398]. High circulating soluble (P)RR levels have been related to preeclampsia, gestational diabetes and risk of abnormal fetus development [134, 399-401]. The increased plasma prorenin level seen in diabetes is thought to be due to its overproduction in the kidney collecting ducts [390, 402]. High plasma prorenin level is considered as a high risk for the development of diabetes complications, probably through the activation of (P)RR signaling [403]

The **renin/prorenin receptor [(P)RR],** is one of the newer members of the RAS which was discovered 14 years ago by Genevieve Nguyen [35]. After binding with the (P)RR, prorenin becomes non-proteolytically active, i.e. without breaking part of the molecule but by changing of its conformation and renin increases by 4-fold its activity [404], and as such, it can stimulate the RAS. Moreover, renin and prorenin binding to the (P)RR has been shown to activate AngII-independent pathways which may favor end-organ damage in pathological states. The (P)RR properties will be presented in more details in the next chapter as it is central to my thesis project.

3.2.3 Angiotensin peptides and their receptors

As shown above, the classic RAS pathway has 3 members, angiotensinogen, angiotensin I and II, as well as two enzymes (renin and ACE) [386]. In 1993, Campbell extracted additional components, such as Ang(1-7), Ang(1-9) and ACE2. Indeed, it was shown that Agt could be converted to additional molecules by serine proteases and peptidases in the presence of ACE-inhibitors [386].

Angiotensin I (Angiotensin 1-10) (**Figure 13**) is formed by cleaving two amino acids from Agt. AngI is viewed as a precursor peptide for angiotensins without any biological effects [405].

Angiotensin II (Angiotensin 1-8, Ang1-8) is an octapeptide which is formed by cleaving two amino acids from AngI by ACE. It is the main physiological peptide in the RAS and the most powerful hormonal vasoconstrictor in the body [16]. AngII has multiple roles, through binding to multiple cellular receptors, but its classic effects are mainly through its binding to AT1R [406]. Some authors have shown that circulating AngI can be used to produce AngII by tissues such as the brain [407].

Angiotensin-(1-9) (Ang1-9) is a nanopeptide formed from Agt [408]. It is formed through the action of ACE2 and its function was shown in experiments with endothelial cells of the kidney and heart [385, 408]. Ang(1-9) is cleaved by neprilysin to Ang(1-7) [408]. As such, Ang(1-9) is viewed as one of the possible targets for changing the direction of the RAS from AngII formation towards the Ang(1-7) pathway [408].

Angiotensin-(1-7) (Ang1-7) is a heptapeptide which can be produced through different mechanisms. It can be a product of the non-renin conversion of Agt, but may also be generated from Ang(1-9) and AngII by ACE2 and neprilysin [199, 409-411]. Ang(1-7) binds with its specific receptor, the MASR, and produces effects which are mainly oppose those of AngII [409, 410, 412]. Binding of Ang(1-7) to the MASR also alters metabolism by activating the PI3K and MAPK pathways [413]. In addition, it can activate phosphatases and cyclooxygenase 2, which leads to increased NOS activity [384, 414-416]. Moreover, it generates a signal to phosphorylate Akt which markedly improves glucose and lipid metabolism [417] and also downregulates PAI-1 and decreases thrombosis [418].

Angiotensin receptors. The different types of angiotensin peptides exert their effects predominantly through binding with theirs specific receptors [350, 415, 419-421]. Five types of receptors have been detected thus far: the AT1R and AT2R for AngII; the AngIII/AT3R for AngIII; the AT4R/IRAP for AngIV and the Mas-receptor for Ang(1-7)) [216, 414, 422 423-426].
The AT1R and AT2R are G-protein coupled receptors (GPCR) [416, 422, 427]. AT1R expression has been shown to be downregulated by statins, insulin and glucose [428]. Of note, AT1R is present in two isoforms in rodents: AT1RA which is expressed ubiquitously and AT1RB which is expressed only in the brain and adrenal glands [416]. AT1RA has been shown to be more important

for systemic vasoconstriction then AT1RB. Due to the large number and complexity of the signaling pathways involved when AngII binds with AT1R, not only does this cause vasoconstriction, but it also increases ROS formation [23, 416] and insulin resistance [429]. Moreover, hyperlipidemia and hypercholesterolemia have been shown to cause atherosclerotic lesion and tissue damage through an increase in AT1R gene expression [430, 431]. The AT2R effects are mostly produced via inhibition of protein kinases and stimulation of nitric oxide (NO) production [432]. This promotes vasodilatation which is beneficial in the treatment of hypertension [422]. In general, the AT2R produces effects that oppose the hypertensive and metabolic effects of the AT1R [432]. Of interest, the AT2R has been shown to be downregulated by the AT1R. [307, 427, 433].

3.2.4 Angiotensin-converting Enzymes

In humans, ACEs exist as two isoforms (gonadal and somatic), and are encoded by different genes. The ACE isoforms are transmembrane proteins, known as hydrolases, a type of carboxyl peptidase. ACE cleaves AngI into AngII and also cleaves bradykinin (a vasodilator) and the peptide Ac-SDKP (a regulator of hemopoiesis) [408, 434]. Of note, this effect on bradykinin inactivation is the cause for the side effects which are related to ACEs inhibitors such as cough and angioneurotic edema [406, 435]. ACE2 is a single-domain metalloprotease, expressed predominantly in the vascular epithelial cells of the heart, kidney and testis [434, 436]. ACE1 and ACE2 molecules have 42% homology in their enzymatic domain structure [434]. As mentioned earlier, ACE2 converts AngI to form alternative metabolites such as Ang(1-9) and Ang(1-7) [410, 437]. Due to the benefits associated with the activation of the ACE2/Ang(1-7)/Mas-receptor pathway for the prevention of hypertension related diseases and complications, specific activators for ACE2 have been developed [408, 436].

3.3 LOCAL RAS

Now it is well established that all RAS components are fully expressed and produced in many tissues in the body, and that they can act locally (paracrine/autocrine) but are also secreted into the blood, and as such, contribute to the circulating RAS [16, 119, 438, 439].

Role and function of local tissue RAS. The local RAS can function in a paracrine manner, as documented in the human normal tissue renin-angiotensin-aldosterone system (RAAS) atlas, which was compiled based on the data obtained from 23 tissues in 143 published papers [440]. The RAAS Atlas shows tissue-specific differences in gene expression of the different components of the RAAS. We will concentrate in this section on local RAS systems that can be implicated in the pathogenesis of obesity and diabetes although the adipose tissue RAS will be discussed in a separate section as it is closely related to my thesis project.

The kidney RAS is one of the most complex local RAS systems because : 1) it consists of local kidney intracellular RAS pathways; 2) it produces and contributes RAS components for circulating RAS, and 3) it is the only place where prorenin is converted to renin through proteolytic cleavage [16, 386]. The kidneys juxtaglomerular cells are specialized in the activation and secretion of renin into the blood [16]. The kidneys thus regulate systemic blood pressure by: 1) directly secreting renin into the blood; 2) producing and secreting RAS components into the circulation 3) regulating kidney tubular hemodynamics and reabsorption of water, ions and salt through stimulation of aldosterone secretion and AT1R stimulation [381, 427, 441-444]. The kidneys are target organs for the development of hypertension, diabetes, cardiovascular diseases and end-stage kidney diseases [445]. Improving kidney function by administration of RAS inhibitors are beneficial by lowering systemic blood pressure and also by reversing kidney damage through inhibition of local kidney RAS [392, 443].

Liver RAS. In the past, the liver was mentioned only as a place for synthesis of Agt. Recent studies have shown that the local liver RAS is important in the development of liver diseases, such as liver fibrosis. Indeed, AngII secretion stimulates collagen production and the development of fibrosis in stellate cells and cholangiocytes. As such, RAS inhibitors can treat liver fibrosis [441, 446]. AngII is also present in liver mitochondria and it is correlated with the level of oxidative stress and aging of those cells [406, 447, 448].

Pancreatic RAS is important for normal islet cell morphology and structure [128, 449-452]. Moreover, AngII, through regulation of local blood flow and oxidative stress, can modulate

beta-cell insulin production [450, 453, 454]. Indeed, increasing AngII signalling through AT1R stimulates ROS production and increases beta cell apoptosis [451, 455]. In line with this, inhibition of the RAS has been shown to have beneficial effects on islet morphology and insulin secretion [450, 451]. The RAS inhibitor perindopril prevents the development of HFD-induced impairment of glucose stimulated insulin secretion in the pancreas by decreasing the expression of inflammatory and oxidative genes (IL-1b, HIF-1 alpha) and proteins (NF-kB, UCP-2, and caspase-3 peptides) as well as by decreasing the number of the apoptotic cells [451, 452]. The ACE inhibitor enalapril increased islet cell mass and the expression of PDX1 and GLUT2 proteins in part by decreasing AT1R as well as by increasing AT2R proteins and the expression of the Mas-receptor gene expression [289, 450, 451] .

3.4 THE LOCAL ADIPOSE TISSUE RENIN-ANGIOTENSIN SYSTEM

Expression of RAS genes in adipose tissue. Adipose tissue fully expresses and synthesizes all the components of the RAS. Importantly, adipose tissue Agt can contribute to ~40% of the circulating concentration of this peptide [8, 9, 11, 46, 91, 97]. Indeed, the main source of plasma Agt after the liver is adipose tissue which explains the increased plasma RAS observed in obesity [9, 11]. Agt gene expression in adipose tissue is increased both by HFD induced obesity [8] and by high plasma glucose concentration [24], explaining the association between RAS activity and obesity and diabetes (**Table 5**).

In addition, feeding and refeeding increase Agt gene expression in rat adipose tissue by ~2 folds, while fasting decreases its expression by ~15% in acute experiments [9, 456, 457]. More specifically, the increased Agt production associated with obesity may be due to the increase in adipocyte size and fat masses through downregulation of PPAR gamma gene expression [11, 20, 457]. As such, plasma AngII level is not increased in tissue-specific Agt-KO mice with HFD induced obesity, indicating that adipose tissue RAS contributes significantly to these obesity-associated changes [458]. Moreover, this is accentuated by the fact that hyperglycemia also increases the RAS gene expression in different tissues, such as liver and fat [25, 91].

Differences have been reported in the RAS component gene expression when looking at different adipose tissue depots. For instance, higher expression has been observed in WAT compared to brown adipose tissue (BAT). It also differs within WAT depots, where higher levels are detected in visceral compared to subcutaneous fat pads which suggests that the local effects of the RAS are involved in the pathogenesis of obesity [129, 166, 459]. RAS components mRNA, such as Agt, ACE and AT1R, were also observed to be increased in perivascular white adipose tissue compared to BAT. Moreover, RAS components in ectopic fat depots, such as perivascular, intra-muscular and hepatic, have been implicated in local tissue remodelling, such as liver fibrosis [127-129]. The Agt gene expression in adipose tissue was also shown to be increased by insulin and androgen levels [109, 460-462] as well as downregulated by beta-adrenergic stimulation [461].

Table 5. Modulation of the RAS by obesity, insulin resistance/Diabetes Mellitus

Conditions / Adipose Tissue RAS	Obesity	Insulin resistance/ Diabetes Mellitus
Agt	↑protein and gene [14] ↑ gene [68]	↑ gene expression [11]
AT1R	↑mRNA [463]	↑ in KK-ay mice [464]
Circulating RAS	↑ Agt and ↑ AngII,[14] ↑increased PRA [465]	↑ AngII, [466]

Notes: ↑ (increased). RAS, renin-angiotensin system; Agt, angiotensinogen, AngII, angiotensin II, AT1R, angiotensin II type 1 receptor; KK-ay – diabetes mice model heterozygous for the yellow spontaneous mutation; PRA, plasma renin activity.

Local effects of adipose tissue RAS. Physiologically, AngII is essential for adipocyte differentiation and development [18, 20]. In addition, it has been shown to be important in the regulation of adipocyte cell size [81]. Indeed, while Mas-receptor deficient mice have no change in their adipocyte cell size despite an increase in fat mass and impaired lipid metabolism and insulin resistance [417], mice lacking the renin gene have smaller adipocytes in all fat pads [19]. Moreover, ACE inhibitors have been shown to improve insulin sensitivity due to their positive

effects on PPAR gamma adipocyte differentiation. Indeed, this leads to an increasing number of smaller adipocytes, while the total epididymal fat weight remains unchanged [27, 186].

The important role of the local RAS in adiposity was demonstrated in mice with Agt or renin systemic and Agt adipose tissue-specific gene suppression. Indeed, these mice presented with lower body weight and fat masses, better insulin sensitivity, lower plasma lipid levels and are resistant to HFD-induced obesity [9, 11, 18-20, 458, 467]. Interestingly, only renin deficient mice have decreased intestinal fat absorption, higher body heat production, and higher UCP-1 protein expression in adipose tissue suggesting that these effects may be through the (P)RR. In line with the RAS KO animal data, adipose tissue-specific Agt or ACE gene overexpression increases body weight and fat masses [119, 468]. Of interest, overexpression of the renin gene systemically or specifically in adipose tissue leads to obesity but also produces glucose intolerance [21]. Put together, these studies show that adipose tissue AngII can regulate body weight and fat masses. Interestingly, the abnormalities caused by whole body Agt deficiency can be reversed by re-expression of the Agt gene only in adipose tissue but not fully reversed by systemic infusion of AngII [20, 31, 458]. RAS inhibition was shown to stimulate adipocyte hyperplasia by favoring adipocyte differentiation in rodents[27, 186]. In addition, AngII regulates triglyceride level in Mouse (3T3-L1) and human adipocyte cell cultures by increasing the level and activity of key enzymes of lipid metabolism, such as fatty acid synthase and glycerol-3 phosphate dehydrogenase [462]. Moreover, animal studies showed that antagonism of the RAS could prevent the development of obesity [387].

Circulating RAS and insulin resistance. The plasma and local adipose tissue RAS activities can contribute to the development of insulin resistance [450, 452]. Indeed, AngII reduces the phosphorylation of insulin signaling pathway components, such as IRS and Akt, and impairs GLUT4 translocation, particularly in adipose tissue [22-24, 223, 426, 469, 470]. Moreover, AngII can increase the activity of the nicotinamide adenine dinucleotide phosphate oxidase (NOX) which leads to an increase in reactive oxygen species (ROS) formation [419]. This in turn affects Akt phosphorylation and inhibits GLUT4 translocation [23]. Circulating and local AngII binds to the AT1R and produces cell apoptosis, fibrosis as well as stimulates inflammation and inflammatory cytokines (IL-6 and TNF-alpha) secretion by adipose tissue, which can also contribute to insulin resistance [11, 166, 375, 376, 471]. In line with these results, inhibition of AngII decreases inflammation

and improves insulin sensitivity in animal models [19, 20, 83, 467] and in clinical studies [27, 29, 186, 472]. Hence, local adipose tissue RAS (**Figure 15**) may provide a link between the development of obesity, inflammation and insulin resistance.

3.5 EFFECTS OF RAS ON ADIPOKINES

Leptin and RAS. Interestingly, leptin has been shown to modulate RAS components, by enhancing Agt, AT1R gene expression and AngII level through activation of the Erk1/2 pathway [473]. In line with this study, leptin stimulated AngII effects were attenuated by treating cultured vascular smooth muscle cells with an ACEi [474]. In the brain, leptin has been shown to regulate the local RAS by enhancing the sympathetic nervous system activity [86]. Leptin also participates in the development of hypertension related to obesity [86] as it increases blood pressure by increasing plasma ACE activity through stimulation of lung ACE gene expression [199]. In addition, despite the fact that leptin stimulates insulin secretion through adrenergic stimulation of beta cells, it also affects insulin signalling via inhibition of IRS1 and GLUT4 activity and enhances AngII effect on ROS formation. Hence, put together, leptin can promote insulin resistance. Leptin resistance is associated with increased circulating leptin levels and decreases leptin receptor signalling that was found to participate in the development of cardiac and liver fibrosis by increasing AT1R and ACE expression. This in turn produces NOX, a NADPH complex activation independently of STAT3 signalling [194, 473]. AngII stimulates leptin production through AT1R stimulation [86].

Adiponectin and RAS. Angiotensin II through its AT1R effects has been shown to reduce adiponectin level in plasma and its gene expression in adipose tissue, which stimulates insulin resistance [217, 261, 314, 475]. As such, the beneficial effects of ACEi and ARBs on insulin resistance have been shown to be in part due to the resulting increased adiponectin levels [186, 212]. In contrast, as was mentioned before, adiponectin, through activation of AMPK, attenuates AngII stimulation of NADPH activity and ROS formation in renal tubular cells which prevents fibrosis and development of chronic kidney disease [212, 215, 261, 314, 475]. Evidence for the primary role of

RAS in the development of obesity is based on both animal and clinical studies, which showed the beneficial effects of inhibition of the systemic and adipose tissue RAS on body weight control [29, 42, 406, 421, 476, 477]. These treatments decreased body weight and fat masses, as well as improved glucose homeostasis and lipid levels in rodent studies with high-fat diet induced obesity and also in patients receiving ACEi [184, 406, 478].

3.6 RAS INHIBITION

Prevention of disease by RAS inhibition. Dysregulation of the RAS is a major cause not only of hypertension but also of tissue damage and complications. As such, RAS inhibition has proven to be very effective therapeutically for the prevention and delay of tissue and organ damage associated with AngII AT1R effects [29]. Indeed, authors have shown reduced hypertension-induced end-organ damage in mice which lack angiotensinogen in the heart and kidney [479, 480]. In addition, RAS inhibition has been shown to prevent T2DM [352] and its complications [26, 29, 347, 481].

Types of pharmacological inhibition of RAS. There are 3 types of pharmacological inhibition of the RAS used clinically: ACEi, ARB and renin inhibitors.

ACE inhibitors (ACEi). ACE inhibitors are the first RAS inhibitors synthesized in 1975 by Drs Ondetti and Cushman. There are now more than 10 ACEi commercially available and they are widely used in clinical practice for the treatment of hypertension, congestive heart failure, diabetic nephropathy, chronic kidney complications due to systemic sclerosis and to prevent stroke [477, 482, 483]. ACEi have also been shown to reduce body weight in humans [484] and rodents [478].

Angiotensin II receptor blockers (ARBs) are the second class of RAS inhibitors which prevent AngII from binding to the AT1R. ARBs are also widely used in clinical practice to treat diabetic

nephropathy, hypertension and congestive heart failure. ARBs, as with ACEi, have shown to be beneficial for people with obesity, as it decreases mortality and cardiovascular risk [437, 477, 481].

Direct renin inhibitors are the third type RAS inhibitors which block the catalytic activity of renin and as such, prevent the conversion of angiotensinogen to AngI [485, 486]. Aliskiren is the only drug of this type that was used in clinical practice to treat essential hypertension. Similarly to other RAS inhibitors, Aliskiren has been shown to produce a decrease in fat mass and body weight in both humans [487] and in mice [488]. Due to the harmful side effects which have been associated with its use, such as angioedema, high blood potassium level, high risk for cardiovascular events and death, the use of Aliskiren has been stopped in clinical practice [489].

Undesirable effect of RAS inhibition. ACE inhibitors, ARBs and renin inhibitors cause an increase in plasma renin/prorenin which is a well-known risk factor for diabetes complication potentially through binding with the (P)RR [41, 403, 490]. Thus, the development of therapy based on inhibition of many RAS pathways is important and will be discussed in the next chapter.

Protective effect of the RAS inhibition in obesity and Diabetes. The protective effect of ACE inhibitors in obesity and insulin resistance occurs by decreasing AngII formation as well as increasing bradykinin levels. They also stimulate the ACE2/Ang(1-7)-Mas- receptor axis, which increases the expression of Pdx1, GLUT2, ACE2 and the MASR proteins in the pancreas, heart and smooth vascular cells [15, 289]. Hence, changing the RAS pathway direction, through blocking AngII formation by ACE or signalling through AT1R, provides therapeutic advantages in addition to direct antihypertensive effects. Inhibition of the RAS also increases adiponectin level, which has beneficial effect on insulin resistance and cardioprotection [186, 475].

Clinical trials have shown that both ACEi and ARBs prevent the risk of new onset T2DM by 27% [491, 492]. As such, these drugs are now included into the algorithm for the treatment of obesity and diabetes as a first choice for antihypertensive agents [29, 53, 240].

CHAPTER IV. (PRO)RENIN RECEPTOR

4.1 STRUCTURE AND FUNCTION OF (P)RR

Prorenin/renin receptor [(P)RR] is a polypeptide molecule consisting of 350 amino acids [1]. This receptor is unique and does not have any analogy with any other known type of receptors.

Structure and location of the (P)RR molecule and gene. The (P)RR molecule consists of (**Figure 15**): a) *an extracellular domain*, *b) a transmembrane domain,* and c) *an intracellular domain*. The extracellular domain (marked as "a" in **Figure 15**) contains the renin/prorenin binding site and this part of molecule can be cleaved by furin and enters into the circulation as a soluble fraction of the (P)RR [493]. The transmembrane domain (marked "b" in **Figure 15**) has been shown to be important in signal transmission [494-496]. The intracellular domain (marked c in **Figure 15**) consists of a proton pump which can associate with a vacuolar type (V-) ATPase [38, 497, 498]. The (P)RR molecule is encoded by a single gene located on the X chromosome [1, 35, 499, 500].

4.2 (P)RR SUBSTRATE BINDING SITES

(P)RR binding activity is 2.5 times higher for prorenin than renin [501]. There are different binding sites on the receptor: the "handle" region where only prorenin binds and the "hinge" region where both renin and prorenin bind [32, 495, 496, 502, 503]. The (P)RR binding affinity with prorenin/renin is positively regulated by high temperature and acid pH [1, 35, 499, 500]. In all tissues, except the kidney juxtaglomerular cells, prorenin can only be activated non-proteolytically, that is without cleaving part of the molecule, by its binding to the (P)RR [43, 404, 504, 505].

4.3 (P)RR PATHWAYS

The unique property of this molecule is in its two different signalling pathway responses to a single substrate molecule binding (renin/prorenin). These pathways are shown in **Figure 15** as the angiotensin-dependent (Ang-D) and angiotensin-independent (Ang-ND) pathways [35].

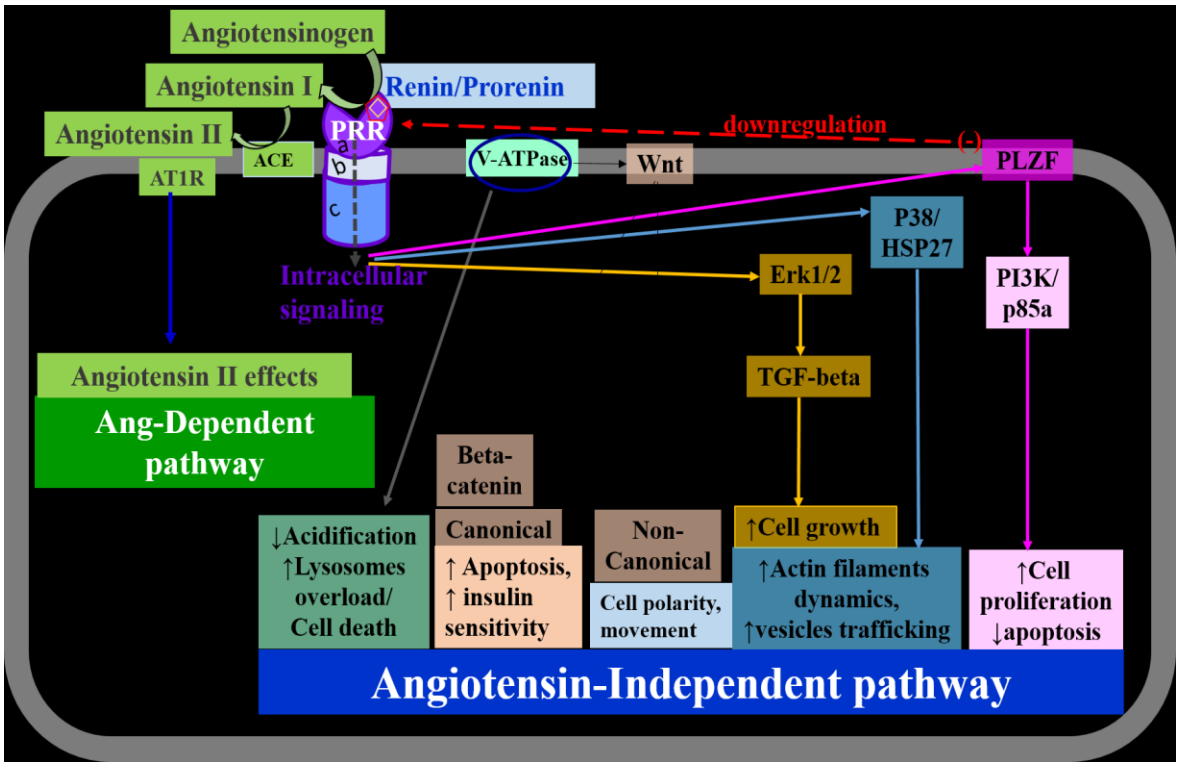


Figure 15. (P)RR Ang-D and Ang-ND pathways. Here are presented the Ang-D (green) and -ND (light green, orange, blue and pink) pathways. The different sections of the (P)RR are identified: the extra-cellular domain (a, purple), the transmembrane domain (b, light blue) and the intracellular domain (c, blue). PLZF downregulates the (P)RR gene (red doted arrow).

Ang-D pathway is related to the well-known classic pathway in which renin converts Agt to AngI. This process is stimulated by renin/prorenin binding to the (P)RR, as indicated previously, and occurs on the cell membrane with the participation of the extracellular domain of the (P)RR

or may occur in the circulation by the dissociated extracellular soluble domain of the receptor [401, 506].

The Ang-ND pathways occur first through intracellular signal transmission through the membrane bound domain of the receptor (**Figure 15**). These effects were classified as Ang-ND as they appear in response to binding renin/prorenin to the (P)RR, even when the RAS (Ang-D pathway) is blocked pharmacologically or genetically [2, 33, 35, 507, 508]. These involve activation of the mitogen activated protein kinase (MAPK) p38 and the heat shock protein 27 (HSP27), the external regulated kinase 1/2 (ERK1/2), the transforming growth factor beta (TGF-ß), and the promyelocytic leukemia zinc finger protein (PLZF), as a result of renin/prorenin binding to the (P)RR receptor [1, 40, 299, 404, 445, 508-511]. These pathways typically promote cell proliferation and growth as well as upregulate pro-fibrotic gene expression [512]. These different intracellular signaling pathways have been studied in many studies and are presented in **Figure 15** [43, 512-514]. For example, in cardiomyocyte cell culture, renin stimulated cell actin filament dynamics, even in the presence of an ARB, through phosphorylation of p38 and HSP27 proteins [510]. In another study, (P)RR blockade, either with the blocker peptide HRP or with (P)RR gene suppression using an siRNA, was shown in mesangial cells to decrease phosphorylation of Erk1/2, reduce the release of type IV collagen, TGF-ß and metalloproteinase 2 as well as promote or prevent fibrosis and proliferation [507]. In human vascular smooth muscle cells, activation of Erk1/2 by prorenin was independent from AngII generation as it occurred even in the presence of RAS inhibitors such as the ACEi imidaprilat and ARB candesartan [508]. Interestingly, (P)RR suppression using an siRNA was shown to inhibit prorenin-induced activation of Erk1/2 and MAPKK. However, both a tyrosine kinase inhibitor and a MAPKK inhibitor decreased prorenin-induced Erk1/2 activity which was not affected by ACEi imidaprilat and ARB candersartan which suggests that Erk1/2 activation results from an Ang-ND effects of the (P)RR [508]. In rat kidney medullary cells, the COX2 gene is upregulated by both the Ang-D and Ang-ND (P)RR pathways through Erk1/2 activation [515]. Indeed, the authors found that the increased COX2 upregulation after prorenin pre-treatment was present when the Ang-D pathway was inhibited by the ARB candesartan, as well as when AngII was administered alone [105, 516]. Binding of prorenin to the (P)RR also increases oxidative stress and ROS formation as well as upregulates peptides involved in apoptosis, such as bcl-2 and Bax [517]. These effects were

detected by blocking ROS formation with the NADPH inhibitor valsartan, an ARB, and as such, confirmed that this effect of the (P)RR was Ang-ND [517]. In another study, a novel Ang-ND pathway was identified where (P)RR, through stimulation of PLZF translocation, produced phosphorylation of PI3K p85a unit downstream. This was shown to promote cell proliferation and decrease apoptosis [512]. Therefore, if we block the RAS with the RAS inhibitors available in clinical practice (**Figure 16**), tissue damage and complications may occur due to the increased production of renin which would stimulate the (P)RR Ang-ND pathways [518]. Hence, inhibition of the RAS with the addition of a (P)RR blocker is viewed as a potentially more efficient treatment [499, 500, 502, 519].

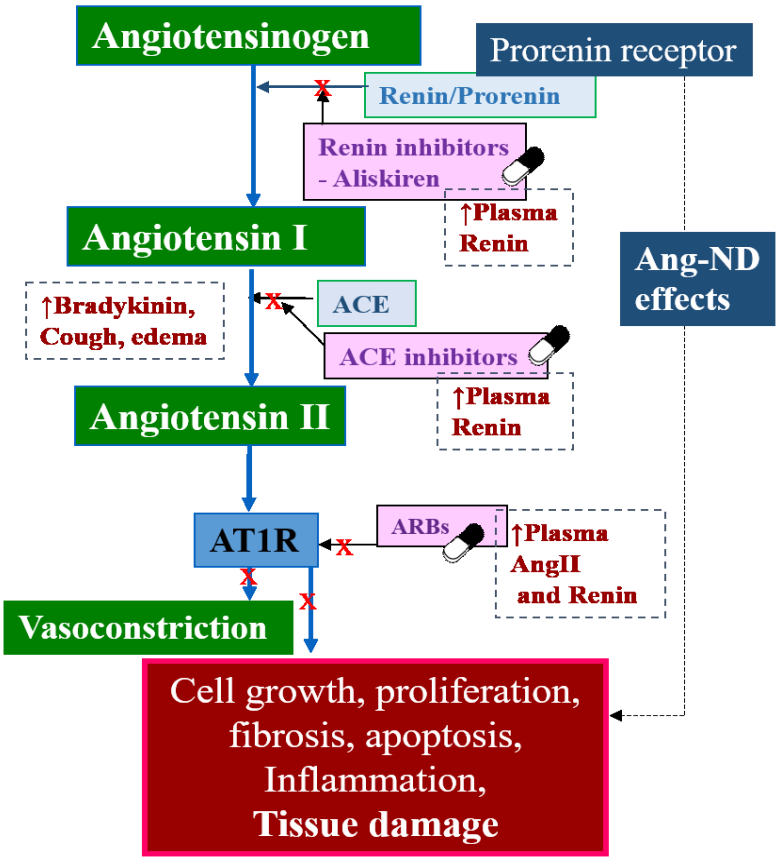


Figure 16. Renin Angiotensin system blockers (shown in the pink squares with pill image) and their undesirable effects (shown in red in the squares with dotted line). Ang-ND effects shown in dotted arrow. P.S. Aliskiren was stopped to use in clinical practice.

Renin-independent effects of the (P)RR have also been shown to be essential for organ development via Wnt/frizzled receptor signaling when the (P)RR associates with the V-ATPase [498, 511, 520-522]. Indeed, the (P)RR plays a role as an adaptor protein between the V-ATPase and the Frizzled/low-density lipoprotein receptor-related protein 5/6 (LRP5/6) complex in the Wnt/β-catenin pathway. This is important for both embryo tissue development and pathogenesis of degenerative diseases and cancer in adults [511, 523, 524]. In addition, an essential role of the (P)RR was also shown in tubule cells of mice with specific deletion of the (P)RR gene in the kidneys. Indeed, this produced a loss of acidification capacity and urine concentration due to the impairment of the proton-pump associated V-ATPase function and lead to death 2-3 weeks after birth [497, 509].

4.4 (P)RR ASSOCIATION WITH V-ATPASE

The (P)RR, also known as the ATPase H(+)-transporting lysosomal accessory protein 2 (ATP6AP2), associates at its C-terminal fragment with the vacuolar type H+-ATPase (V-ATPase) complex [38, 40, 525, 526]. The association of the (P)RR with the V-ATPase was discovered when the (P)RR gene and protein structure were first characterized [32, 35, 38, 40]. It was proposed that this association was the main reason for the lethality of (P)RR whole-body knock-out models. The V-ATPase is a ubiquitous enzyme that plays a role in the acidification of cell compartments which is essential for embryo tissue development [514].

The (P)RR/V-ATPase complex is involved (**Figure 15**) in the Wnt signaling through the canonical and non-canonical pathways [520]. The canonical pathway involves Wnt phosphorylation of GSK-3 and stabilization of beta-catenin, which prevents the destruction of the activated complex. The non-canonical pathway is related to planar cell polarity, and signals through the PKC and Ca2+/calmodulin-dependent protein kinase (CamK) pathways which activate Ca+2 dependent actin polymerization and cell movement. This pathway also produces JNK phosphorylation as well as stimulates cell microtubule and skeleton gene expression that regulate cell polarity [499, 500, 521, 527].

4.5 (P)RR GENE AND PROTEIN REGULATION

The level of (P)RR gene expression in different tissues has been shown in a few studies [35, 390]. The (P)RR mRNA level has been reported to be highest in the heart, brain, placenta, liver and pancreas [35, 38]. (P)RR negatively regulates its own expression by stimulating the expression of the promyelocytic leukaemia zinc finger protein (PLZF) which is a transcription factor [513, 528].This prevents excessive receptor activation [502, 529] and is hypothesized to be a physiological mechanism that would normally prevent obesity- and diabetes-associated complications, but is dysregulated when these pathologies develop [401, 438]. Similarly, the (P)RR gene expression in the kidney glomeruli was reported to be increased with low-salt intake via c-GMP protein kinase G (PKG) pathway as an adaptive mechanism to maintain fluid homeostasis, salt and blood pressure levels given its implication in the RAS [530]. High blood glucose level was shown to upregulate (P)RR expression in cultured mouse podocytes [527, 531]. This has been suggested to result, at least in part, from an increased (P)RR promoter activity as was demonstrated in cultured HeLa cells treated with high glucose [513]. Also, in rat mesangial cells, high glucose levels have been shown to upregulate (P)RR gene expression through activation and phosphorylation of the members of the PKC/ Raf /Erk and PKC/JNK-c-JUN pathways by enhancing the activity of the nuclear factor-kappa B (NF-kB) and the (P)RR promoter [532]. The (P)RR is expressed in adipose tissue and its expression has been shown to be increased by obesity [458, 533, 534].

4.6 (P)RR PHARMACOLOGIC BLOCKADE

A blocker peptide was characterized and named after the structure in the (P)RR domain, that is shaped like a handle, the handle-region peptide (HRP) or decoy peptide. This peptide consists of 10 amino acids corresponding to the amino acid sequence 10-19 in the prosegment of prorenin [32, 499, 500, 505]. Initially, HRP was selected and used by Ichihara's group in 2004 for its binding

affinity to the (P)RR and inhibition of renin/prorenin binding to the (P)RR [535]. Experimental studies have shown the beneficial effects of pharmacological blockade of the (P)RR with HRP [43, 134, 536-539]. In cultured kidney mesangial cells, HRP treatment reduced cell proliferation, production of the collagen type IV, Erk1/2 phosphorylation and TGF-beta gene expression [507], similarly to the results obtained in mesangial cells where (P)RR expression was knockdown by siRNA transfection [507]. Of interest, since the HRP prevents the binding of renin/prorenin to the (P)RR, it should not have any impact on the interaction of the (P)RR with the V-ATPase.

4.7 (P)RR IN HEALTH AND DISEASES

There are only two human diseases, X-linked mental retardation and hypertension which have been associated with mutations in the (P)RR gene [21, 404, 499, 500, 526]. In animal models with (P)RR gene suppression or by administration of the HRP, the (P)RR has been shown to be involved in the development of brain damage, glomerulosclerosis, cardiovascular and kidney diseases, as well as diabetes [502, 540-542]. To discriminate Ang-D from Ang-ND effects of the (P)RR, most of studies were done with the addition of RAS inhibitors [37, 42, 535].

4.7.1 Diabetes

High levels of circulating prorenin is well known as being a marker of diabetes complications although the reason for this was for a long time unknown as prorenin is inactive [42, 403, 442]. It is now thought that the (P)RR is implicated in this effect [502, 540]. In addition, insulin secretion can also be altered by the local pancreatic RAS as described in the previous chapter and as such, the (P)RR in the pancreas may be implicated in this effect as well [449]. In addition, (P)RR may also affect this parameter through its effects on V-ATPase as it is essential for maturation and secretion of insulin from beta cell granules [543]. Moreover, the (P)RR has been implicated in the development of diabetes complications [1, 74, 403, 530, 544-546] and the HRP was shown to be effective

for the treatment of these complications in animal models [43, 538, 539]. In addition, it was also shown to improve insulin sensitivity in fructose fed rats, a model of insulin resistance [134, 537].

4.7.2 Diabetic nephropathy

High glucose has been shown to stimulate tissue damage, such as podocyte injury, through an increase in podocin expression which is caused by an upregulation of the (P)RR and Wnt/beta-catenin signaling [527]. Pharmacological blockade of the (P)RR has been shown to prevent the development and even reverse diabetic nephropathy. For instance, HRP infusion was shown to prevent the development of diabetic nephropathy in streptozotocin-induced diabetic rats [40, 535]. In another study, (P)RR blockade with HRP even reversed diabetic nephropathy in hemi-nephrectomised streptozotocin-induced diabetic rat. Indeed, HRP markedly decreased proteinuria as a result of improved kidney morphology by decreasing the mesangial matrix expansion, lowering density of hyaline substances and reducing the thickness of the basal membrane [539]. Conversely, constitutive (P)RR gene deletion in the kidneys decreases podocin and nephrin protein production, [497] which produces renal structural and functional abnormalities. As a result, they lack the ability to concentrate their urine and have overt proteinuria which causes high lethality after birth although this is thought to result from the lack of V-ATPase function, a component that is not effected by the HRP [1, 404, 497, 500, 509, 547]. The well-known critical role of the RAS in the development of the urinary system and kidneys from the ureteric bud in the embryo was recently shown to also be related to Ang-ND effects of the (P)RR [497, 541, 548]. Indeed, abnormal development was shown in cultured kidney duct cells with (P)RR knockdown produced by siRNA. Moreover, prorenin treatment increased V-ATPase activity, which was not blocked by ibersartan, an ARB, but only by V-ATPase inhibitor bafilomycin [509]. In addition, conditional suppression of the (P)RR by Cre-loxP technology in the ureteric bud was shown to produce abnormal development of the urinary tract and kidneys, such as renal hypodysplasia and renal tubular acidosis, which were associated with decreased Ret/Wnt11 expression and Erk1/2 phosphorylation [548, 549]. Hence, similarly to AngII [550], (P)RR seems to be essential for embryonic development of the kidneys while in adults, dysregulation of the (P)RR seems to be

implicated in renal pathologies. This would explain why constitutive renal (P)RR KO models and (P)RR overexpression models have renal dysfunction.

4.7.3 Eye diseases

Both Ang-D and Ang-ND pathways of the (P)RR have been implicated in the development of tissue inflammation in the retina. In streptozotocin-induced diabetic mice, Erk1/2 phosphorylation in mouse retina was inhibited by HRP treatment and prevented the development of diabetes-induced retinopathy in these rats [43]. In AT1R-KO mice with streptozotocin-induced diabetes, HRP infusion also decreased adhesion of leukocytes by the suppression of the vascular endothelial growth factor (VEGF), but not the intracellular cell adhesion molecule (ICAM) in the retina [43]. In other studies, the (P)RR was shown to be involved in diabetic injury of the retina through Wnt signaling and association with V-ATPase [538] as well as through the activation of the VEGF [551]. Also, retinal neovascularisation after ischemia was shown to result from Ang-ND effects of the (P)RR through stimulation of ICAM and VEGF [552]. Moreover, the (P)RR was shown to be important in laminal formation of the retina in embryo through an impact on cell polarity and activation of par-atypical protein kinase C (aPCS3) [445].

4.7.4 Cardiovascular diseases

The (P)RR is involved in the development of the cardiovascular diseases [39, 40, 398, 510, 540, 553, 554]. For instance, an increased (P)RR gene expression was found in the heart of humans who died of heart failure and in rodent models with post-myocardial infarction which suggests an involvement of the (P)RR in cardiac pathophysiology [37, 555] In parallel, another group demonstrated that mice overexpressing (P)RR specifically in the heart had an increased Erk1/2 phosphorylation in the atrium, which triggered its dilation and fibrosis, as well as produced atrial fibrillation [556]. In another study, the authors demonstrated the role of the (P)RR in the

development of cardiomyopathy in spontaneously hypertensive rats through inhibition of the (P)RR using HRP which prevented heart remodelling [553, 554]. These authors suggested that fibrillation may occurred through an increased Erk1/2 phosphorylation which was observed in the atria of non-treated transgenic mice. Non-proteolytic activation of prorenin was shown to increase collagen IV expression in the heart, whereas HRP administration prevented cardiac fibrosis in rats with diabetes [37]. In rats with cardiac (P)RR gene overexpression, Erk1/2, TGF-ß and connective tissue growth factor gene expression was increased with an associated fibrosis. These changes were found to occur through Ang-ND pathways, whereas activation of the HSP27 and cell apoptosis were related to Ang-D effects of the (P)RR in the myocardium [557].

Interestingly, mice with specific vascular smooth muscle cells (VSMC) (P)RR gene deletion performed by Cre-loxP technology [558] exhibited increased mortality as they died at 20 weeks of age. They exhibited damaged and collapsed lung alveoli, showed fewer vacuolated VSMC and increased fibroblasts without plaques or inflammatory cells surrounding the VSMC in histological samples from the abdominal aorta. However, in KO mice, blood pressure was unchanged. Ablation of the (P)RR in cultured VSMC obtained from (P)RR floxed mice was done by transfection of recombinant adenovirus vector expressing CRE [558]. Interestingly, this induced cell death and apoptosis through V-ATPase related autophagy. The authors also showed that the treatment of VSMC with bafilomycin produced similar effects on the monocyte chemoattractant protein (MCP-1) and interleukin 6 (IL6) genes and suggested that the related (P)RR effects were most likely associated with the modulation of V-ATPase activity [558]. In contrast, overexpression of the (P)RR in VSMC in rats produced a cardiovascular phenotype characterized by an increase in blood pressure and aldosterone generation, while kidney function and plasma renin level were normal [559]. This suggests that, similarly to what is found in the kidney, although (P)RR seems to be essential for the embryonic development of smooth muscle cells because of its interaction with V-ATPase, its dysregulation is implicated in pathological states such as hypertension.

4.7.5 Central nervous system disorders

The (P)RR, via its association with V-ATPase function, participates in intracellular acidification, fusion of vesicle membranes, exocytosis and thus can regulate growth hormone release and secretion in the hypothalamus [528]. As such, treatment with a V-ATPase inhibitor, bafilomycin, or ablation of the (P)RR gene by siRNA both decreased growth hormone release in GH3 cells derived from growth hormone producing adenoma (GHoma) through destabilisation of V-ATPase function [528]. Interestingly, (P)RR gene suppression in the brain using intra-cerebroventricular injections of shRNA reduced blood pressure and improved heart function in hypertensive mice [542]. The authors proposed that this may result from the decreased cardiac and vasomotor sympathetic tone and improved baroreflex sensitivity measured in these mice. In addition, they also reported a reduction in AngII effects in the paraventricular nuclei [542]. The authors found that the AT1R and vasopressin protein expression in the brain were also reduced in these mice [542]. In line with those findings, the same group found that in neuron-specific (P)RR knockout mice, intracerebral infusion of prorenin did not increase blood pressure or AngII formation compared to wild-type mice which further supports the role of the brain (P)RR in the development of hypertension [560]. Another group suggested that the effects of the brain (P)RR in hypertension and heart function could be through an increase in plasma vasopressin level demonstrated in SHR and Wistar-Kyoto rats with suppression and overexpression, respectively, of human (P)RR gene in the supraoptic nucleus (SON) of the hypothalamus [561]. Indeed, in SHR, (P)RR knockdown in the SON attenuated hypertension and its harmful effects on the heart with an associated decrease in circulating vasopressin. Similarly, (P)RR overexpression in the SON of Wistar-Kyoto rats produced an increase in circulating vasopressin although without any effect on blood pressure and heart rate. This lack of cardiovascular effects may result from the modulation of redundant physiological mechanisms which may counter the effect of this increase in vasopressin [561]. Interestingly, prorenin pre-treatment of neuronal cells produced an increase in Erk1/2 phosphorylation, which was 50% greater in cells from SHR compared to those from Wistar-Kyoto rats [561]. Overexpression of human (P)RR in mouse neuroblastoma cells increased ROS level through increasing of NADPH activity and NOX2 mRNA level, which were not blocked by losartan, but reduced by inhibition of Ek1/2 and PI3K/Akt pathways which suggests that Ang-ND effects are implicated [562].

4.7.6 Gestational disorders

The extracellular fragment of the (P)RR molecule can be cleaved by furin and is called the soluble (P)RR. As such, its levels can be measured in body liquids, cord blood and urine and has been identified as an early predictor of gestational diabetes [400] and small for gestational age at birth [399]. Elevated circulating soluble (P)RR level and placental (P)RR expression were found in women with gestational diabetes and preeclampsia as well as in a rat model of preeclampsia [399, 400, 563, 564]. Moreover, increased level of placental (P)RR has been shown to be involved in the development of hypertension and insufficient kidney function present during preeclampsia [564]. High (P)RR level in cord blood is associated with small weight at birth, small placental weight and gestational diabetes [134, 399-401, 565].

4.8 (P)RR IN OBESITY

The role of the (P)RR in the development of obesity has been poorly studied and thus, needs further research. For instance, in human adipose tissue, (P)RR protein was visualised in the cell compartment, close to the cell membrane, and co-localised with renin and the plasminogen activator inhibitor type 1 (PAI-1) [533]. Interestingly, incubation of 3T3-L1 cells with renin produced Erk1/2 phosphorylation, and incubation of stromal-vascular fraction of human adipose tissue with renin increased AngI level, which suggested that (P)RR is functionally active and mainly expressed in the stromal-vascular fraction. These authors later showed that the (P)RR protein is also expressed in adipocytes and was present in higher levels in visceral compared to subcutaneous fat in both lean and obese patients while no effect of obesity was observed on (P)RR in these post-menopausal women [533]. This group also demonstrated that with overfeeding and HFD in adult rats a strong correlation between (P)RR expression in adipose tissue and adipocytes cell size could be observed [533, 566]. In another study, the (P)RR gene and protein expression were found to be increased with HFD in adipose tissue of both wild-type and adipose tissue specific angiotensinogen knockout mice [458]. In fructose fed rats, a model of insulin resistance without obesity, treated with HRP, body weight, fat mass, blood lipids and adipocyte

cells size were decreased and insulin sensitivity was increased compared to non-treated animals [134]. In this experiment, it was observed that the (P)RR gene and protein expression were increased in the soleus muscle but unchanged in the visceral, retroperitoneal and epididymal adipose tissue. Moreover, Wnt has been shown to inhibit adipocyte differentiation, their hypertrophy and development of obesity and insulin resistance [115, 567, 568]. As such, this may be another mechanism by which the (P)RR could have a role in the development of obesity.

CHAPTER V. AIM AND HYPOTHESIS OF THE PROJECT

5.1 OBJECTIVES AND HYPOTHESIS

5.1.1 Background

As described above, a review of the scientific literature has shown that obesity and T2DM are worldwide major epidemics, and the development of T2DM can be a complication of obesity (Chapter 1, in 1.6, 1.12). Also, the RAS plays an important role in the development of obesity and T2DM (Chapter 1, 2 and 3). RAS blockade/suppression, either pharmacological or genetic, has proved to be beneficial in the treatment and prevention of both obesity and T2DM in humans and animal models (Chapter 3, section 3.5 and 3.6).

Among the RAS components, the effects of the (P)RR have been less studied, particularly in adipose tissue. Importantly, this receptor has dual activity, as on binding of its substrates it promotes signaling along two pathways: Ang-D and Ang-ND. The Ang-D pathway stimulates the well-known classic RAS pathway, whereas the Ang-ND pathway effects enhance cell growth, proliferation, fibrosis, apoptosis, and play an independent role in the development of T2DM by triggering insulin resistance and diabetic complications (Chapter 2, section 2.5 and 2.6).

Based on this data, this **gives rise to the following questions that we attempted to answer using rodent models of obesity**:

- What is the role of the (P)RR, both systemically and specifically in adipose tissue, in the development of obesity and the associated T2DM?

- Could pharmacological blockade or genetic suppression of the (P)RR prevent the development of obesity and insulin resistance?

5.1.2 Hypothesis

We hypothesised that: 1. Pharmacological blockade of the (P)RR using HRP will prevent the development of HFD induced obesity and improve insulin sensitivity; 2. Adipose tissue specific (P)RR gene deletion would prevent the development of obesity and associated dysregulation of glucose homeostasis.

5.1.3 Objectives

Based on the above questions and hypothesis we formulated the following objectives. The **overall goal** of this project is to elucidate the mechanisms by which the (P)RR affects the development of obesity and T2DM.

Specific aims:

1. Study the effects of whole body pharmacological blockade of the (P)RR, using a blocker peptide (HRP), and a mouse model of obesity (mice fed ND vs HFD), on the development of obesity and insulin resistance. In this part, we studied the effects of HRP administration on:

a) (P)RR gene expression in adipose tissue and in other tissues;
b) Body weight, fat mass, distribution of fat pads, and cellular composition of fat tissue;
c) Glucose homeostasis and circulating metabolite levels;
d) Adipose tissue specific gene expression profile.

This part of my PhD work was done and presented as an equal contribution with PhD student Paul Tan, and our joint results were published in:

Tan, P., Shamansurova, Z., Bisotto, S., Michel, C., Gauthier, M.-S., Rabasa-Lhoret, R., Nguyen, T. M.-D., Schiller, P. W., Gutkowska, J. and Lavoie, J. L. (2014). **Impact of the prorenin/renin receptor on the development of obesity and associated cardiometabolic risk factors**. ***Obesity***, 22: 2201–2209. doi: 10.1002/oby.20844

2. Study the role of the (P)RR in the development of obesity and insulin resistance in mice with a gene deletion of the receptor, specifically in adipose tissue (KO). In this part we studied the effects of genetic (P)RR deletion in adult mice on:

   a) (P)RR gene expression in adipose tissue;
   b) Body weight, composition, fat mass and distribution;
   c) Glucose homeostasis and circulating metabolite levels;
   d) Locomotor activity and metabolic rate;
   e) Adipose tissue histology.

The results of this part were published in:
Shamansurova Z*, Tan P*, Ahmed B, Pepin E, Seda O, and Lavoie J.L. **Adipose tissue (P)RR regulates insulin sensitivity, fat mass and body weight.** ***Journal of Molecular Metabolism***.. August 2016. Volume 5, Issue 10, Pages 959–969.

CHAPTER VI. ARTICLE 1: IMPACT OF THE PRORENIN/RENIN RECEPTOR ON THE DEVELOPMENT OF OBESITY AND ASSOCIATED CARDIOMETABOLIC RISK FACTORS

Authors: Paul Tan*, Zulaykho Shamansurova*, Sandra Bisotto, Catherine Michel, Marie-Soleil Gauthier, Remi Rabasa-Lhoret, Thi M-D. Nguyen, Peter Schiller, Jolanta Gutkowska, and Julie L. Lavoie.

*These authors contributed equally to this work

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Problem: Could (P)RR inhibition prevent the development of obesity and insulin resistance?

Objective: Study the effects of the (P)RR in mice by administering a blocker peptide (HRP) subcutaneously during 10 weeks.

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Contribution of authors:

Zulaykho Shamansurova – second first author, did the mice assessment weekly, Echo-MRI, necropsy, sample collection, extraction of RNA, PCR, qPCR, data analysis, manuscript first draft, results shown in Figures 1, 2, 3, S5 and Tables 2, S4, S5, S7.

Paul Tan – extraction of RNA, protein, PCR, qPCR, calculations, manuscript revision. Results shown in Figures 1,2,3,4,5,6, S1, S2, S3, and Tables S1, S2, S3, S6.

Sandra Bisotto – coauthor, weekly measurement, necropsy, sample collection, measured circulating metabolites, plasma renin activity. Results shown in Figure S2 and in Table 2.

Catherine Michel – coauthor, worked with mice in animal facility, leads mice measurements, surgery, necropsy, sample collection. Results shown in Figure 1.

Marie-Soleil Gauthier – coauthor, worked on part of the human study, did qPCR, manuscript revision. Results shown in Table 1.

Remi Rabasa-Lhoret – coauthor, leaded the human study part, revised manuscript.

Peter Schiller – coauthor, development and synthesis of HRP.

Thu M.-D. Nguyen – coauthor, worked on HRP synthesis.

Jolanta Gutkowska – coauthor, revised manuscript.

Julie L. Lavoie – Supervisor, leader of the project, leaded all the steps, revised manuscript, worked on manuscript submission, communication with the journal and correspondences.

IMPACT OF THE PRORENIN/RENIN RECEPTOR ON THE DEVELOPMENT OF OBESITY AND ASSOCIATED CARDIOMETABOLIC RISK FACTORS

Paul Tan*[1,2,8], Zulaykho Shamansurova*[1,3,8], Sandra Bisotto[1], Catherine Michel[1], Marie-Soleil Gauthier[8,9], Rémi Rabasa-Lhoret[1,4,8,9], Thi M.-D. Nguyen[8], Peter W. Schiller[6,9], Jolanta Gutkowska[1,5] and Julie L. Lavoie[1,7,8]

[1]Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM) [2]Department of Biochemistry and molecular medicine, [3]Physiology, [4]Nutrition, [5]Medecine and, [6]Pharmacology and [7]Kinesiology of the Université de Montréal, [8]Montreal Diabetes Research Center and [9]Institut de Recherches Cliniques de Montréal, Montréal, Québec, Canada
*These authors contributed equally to this work

Keywords: (pro)renin receptor, handle region peptide, renin-angiotensin system, adipokines, insulin resistance, glucose homeostasis.

Short title: Preventing obesity and its risk factors

Word Count: 3419 words

Correspondence to:
Julie L. Lavoie, PhD
Centre de Recherche du CHUM (CRCHUM)
900, rue Saint-Denis, bureau R08.452
Montréal, Québec, Canada, H2X 0A9
Tel: (514) 890-8000 ext. 23612
Fax: (514) 412-7655

What is already known?

- Obesity is a world-wide growing epidemic and current treatments have limited success and are associated with important side effects
- The renin-angiotensin system has been implicated in obesity
- Few studies have investigated the implication of the prorenin/renin receptor in obesity

What this study adds?

- Prorenin/renin receptor is upregulated with obesity and insulin resistance in adipose tissue.
- We have found that obese mice treated with the handle region peptide have lower body weight and weight gain which was associated with a decrease in visceral adipose tissue weight and improved circulating metabolites such as triglycerides and insulin.
- We found that mice on normal diet also benefits from the handle region peptide treatment although there was no change in body weight.

Abstract

Objective: Obesity is a worldwide epidemic and current treatments have limited success thus, novel therapies are warranted. Our objective was to determine whether the prorenin/renin receptor [(P)RR] is implicated in obesity.

Design and Methods: Mice received a normal or high-fat/high-carbohydrate diet with the handle region peptide (HRP), a (P)RR blocker, or saline for 10 weeks. Post-menopausal non-diabetic obese women were enrolled in the Complication Associated with Obesity Study and were classified as insulin-resistant (IRO) or –sensitive (ISO) using a hyperinsulinemic-euglycemic clamp.

Results: In mice, obesity increased the (P)RR by 2-fold in adipose tissue. Likewise, renin increased by at least 2-fold. The HRP reduced weight gain in obese mice by 20% associated to a 19% decrease in visceral fat. This was accompanied by a 48% decrease in leptin mRNA in fat and 33% decrease in circulating leptin. Inflammatory markers were also decreased by the HRP treatment. HRP normalized triglyceridemia and reduced insulinemia by 34% in obese mice. Interestingly, we observed a 33% increase in (P)RR mRNA in the fat of IRO women compared to ISO.

Conclusions: This is the first report of a potential implication in obesity of the (P)RR which may be a novel therapeutic target.

Introduction

Although obesity is a major risk factor for many diseases such as cardiovascular diseases, diabetes and cancer, treatment of overweight and obese patients has had low success rate(1). In both mice and human, systemic and adipose tissue renin-angiotensin system (RAS) are upregulated with obesity(2). Moreover, angiotensin II (Ang II) increases lipid storage by reducing lipolysis and increasing lipogenesis(2).

The prorenin/renin receptor [(P)RR], an X chromosome-linked gene, binds both renin and prorenin. This increases by 3-5 fold renin's catalytic efficiency to convert angiotensinogen (AGT) into Ang I(3). It been detected in many tissues but only two groups have reported its presence in adipose tissue in women(4;5). Interestingly, the protective effect of the handle region peptide (HRP), a (P)RR blocker, has been demonstrated in animal models of many pathologies, such as cardiac diseases(6). Although controversial data have been reported concerning these beneficial effects, this is mostly due to methodological issues such as lower doses and shorter duration of treatment(7-10). Furthermore, tissue-specific (P)RR knock-out mice have increased mortality probably due to an impaired acidification of intracellular vesicles, a process implicated in cell survival(11). Indeed, the (P)RR can act as an adaptor between the vacuolar H+-ATPase and the receptor for Wnt molecules in a renin independent manner(6). As such, a defect in (P)RR can impair the Wnt signalling pathway conversely to blocking the receptor using HRP which is unlikely to interfere in this process.

The aim of our study was to determine the regulation of the (P)RR and renin in adipose tissue by obesity and to assess the implication of this receptor in the development of obesity and obesity-related risk factors. Our hypothesis was that the (P)RR and renin would both be increased with obesity and that the former contributes to the development of obesity and its associated risk factors.

Methods

Animals

All experiments were carried out on mice expressing the green fluorescent protein under the control of the renin promoter (REN-GFP)(12) (generous gift from Dr. Kenneth Gross at the Buffalo Cancer Institute). Mice were 12-15 weeks of age and were maintained on 12-h light/dark cycle with access to either normal (N) diet or high-fat/high-carbohydrate (HF/HC) diet and water *ad libitum*. All experiments were carried out in male mice. In addition, the impact of obesity on the (P)RR and renin were confirmed in females as previous studies looking at human adipose tissue (P)RR have been conducted only in women. Mice body weight (BW) and food weight were measured weekly. Care of the mice used in the 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.

HRP synthesis

The mouse peptide IPLKKMPS(13) was synthesized by the manual solid-phase technique(14). This peptide, consisting of part of the prosegment region (10P–19P) of prorenin, has been shown to bind directly to the (P)RR *in vitro* with a dissociation constant (K_D) of 3.5 nM(15;16). Furthermore, it was confirmed that HRP could prevent binding of both renin and prorenin to the receptor with a dissociation constant (K_I) of 16.7 nM and 15.1 nM respectively(15).

HRP administration

Mice were anesthetized and implanted subcutaneously with an osmotic mini-pump (model #1004; Alzet, Cupertino, CA) filled with the HRP or saline. The peptide was administered at a published effective dose of 0.1 mg/kg/day(6;13). This procedure was repeated after 4-5 weeks to ensure constant administration of the peptide as these are 28-42 days pump (length varies according to lot). Concomitantly, the mice received either a N or HF/HC diet for 10 weeks.

Human CAO cohort study

This was a secondary analysis of samples from post-menopausal non-diabetic obese women enrolled in the Complications Associated with Obesity (CAO) Study. The inclusion/exclusion criteria of this study have been reported(17). The study was conducted according to the

guidelines laid down in the Declaration of Helsinki and were approved by the University of Montreal ethics committee. All subjects gave a written informed consent before the study started. Women were subdivided into insulin-sensitive obese (ISO; N = 13) and insulin-resistant obese (IRO; N = 9), as measured by hyperinsulinemic euglycemic clamp and classified based on a cut-off proposed in a previous study(18). Before the clamp procedure, subcutaneous fat (SCF) samples were obtained from the periumbilical area by needle biopsy under local anesthesia as previously described(19).

Western Blot

Proteins were detected using a (P)RR(20), generous gift from Dr. Yumei Feng at Tulane University, renin (Molecular Innovations), GAPDH (Santa Cruz) and tubulin (Abcam) antibody. Each signal was normalized to its respective GAPDH or tubulin band.

Real-time PCR

Gene expression was evaluated using the primer sequences and conditions provided in Tables S1 and S2.

Statistical analysis

All values are expressed as means ± standard error (SE). A 2-way ANOVA was used to assess the effect of diet and the HRP. For parameters measured at many time points, a repeated measure 2-way ANOVA was applied. If interactions were detected, a Tukey post-hoc test was used. Differences in human gene expression were determined by non-parametric Mann–Whitney U tests. For renin mRNA in female mice, a Pearson's Chi-square was done. A $p < 0.05$ was considered statistically significant.

Results

Mouse adipose tissue (P)RR and renin

In HF/HC+Saline male mice, the (P)RR mRNA was increased by 2.1-fold in all fat pads (Figure 1A) and (P)RR protein was increased by 1.5-fold in peri-renal fat (PRF) and by 2.1-fold in

abdominal SCF with a tendency in peri-gonadal fat (PGF) ($p = 0.067$, Figure 1C) while it was unchanged in the heart, liver and kidney (Figure S1). Renin mRNA was increased by 1.7-fold in SCF and by 2.4-fold in visceral adipose tissue (VAT) (Figure 1B). The local protein produced, as assessed by the presence of GFP, was undetectable in all fat pads (data not shown) although total renin protein content was detected and was increased by 5.1-fold in PGF and decreased by 40% in SCF with obesity (Figure 1D). Likewise, circulating plasma renin activity (PRA) was increased by 1.9-fold in HF/HC+Saline mice (Figure S2). In PGF of obese mice, total renin protein was 84% of the kidney content (Figure 1D, S3 and Table S3). There was no effect of the HRP on both (P)RR and renin with the exception of the (P)RR mRNA in SCF which was decreased by 53% in obese animals (Figure 1A).

In obese female mice, the (P)RR protein increased by 1.4-fold in PGF while it tended to increase in PRF ($p = 0.084$, Figure S4). Also, the protein expression was 1.7-fold higher in PGF compared to SCF independently of the diet (Figure S4). Renin mRNA was significantly increased only in PGF and SCF with obesity (Table S4). Values presented were very small which may result from the smaller fat mass compared to males (Table S5).

RAS components in human SCF

IRO and ISO women were comparable for age, BMI, fat mass and waist to hip ratio as assessed by Dual X-Ray absorptiometry (Table S6)(17). We observed a 1.3-fold increase in (P)RR in the SCF of IRO compared to ISO subjects (Table 1). We found a 1.5-fold increase in AT1R similarly to previous reports(21). Conversely, there were no differences for ACE and AGT expression (Table 1). Moreover, renin could only be observed in 3 samples independently of insulin sensitivity (data not shown).

Mouse body and organ weight

HF/HC+Saline mice increased their BW (Figure 2A) and weight gain (Figure 2B) compared to the N+Saline group, mainly due to an increased fat mass (Figure 2C). HF/HC+HRP mice had decreased BW and weight gain by 11% and 20% respectively compared to HF/HC+Saline mice (Figure 2A-B), and both tended to be lower at week 9 ($p = 0.066$ for BW and $p = 0.073$ for weight gain). Nonetheless, these parameters were still elevated compared to the N group. Similarly to the BW, a 19% decrease in PRF was observed in HF/HC+HRP mice compared to

the HF/HC+Saline group with a tendency in SCF (p = 0.07) while the PGF was unaffected (Figure 2C).

Conversely, no weight changes were detected in N+HRP mice although a tendency could be observed (p = 0.08 for BW starting at week 5 and p = 0,064 for a general effect on weight gain, Figure 2A-B). Interestingly, a 31% decrease in PRF mass was observed in N+HRP mice compared to the N+Saline group , while the SCF tended to decrease (p = 0.07) (Figure 2C).

In contrast, no impact of the HRP on heart weight was observed in mice on both diets (data not shown). Moreover, the HRP had no effect on weekly food intake. As expected, the HF/HC groups had increased caloric intake compared to those on the N diet (Table S7).

Mouse adipokines

The HF/HC+Saline mice had increased adipose tissue leptin expression compared to their N diet controls (Figure 3A). Interestingly, HRP decreased leptin expression in all adipose tissue by around 48% and 77% in HF/HC+HRP and N+HRP mice respectively (Figure 3A). Similarly, circulating leptin was 2.2-fold higher in HF/HC+Saline mice compared to controls and was decreased by 33% and 54% in HF/HC+HRP and N+HRP mice respectively (Figure 3C). In SCF, resistin mRNA was increased by 2.8-fold in HF/HC+Saline mice compared to their controls and was decreased by 53% in the HF/HC+HRP mice without any changes in VAT (Figure 3B). Conversely, circulating resistin was 1.2-fold higher in HF/HC+Saline mice compared to the N diet controls without any effect of the HRP (Figure 3C). Tumor necrosis factor alpha (TNFα) mRNA expression was increased by around 2.1-fold in PGF and PRF of HF/HC+Saline mice while the HRP decreased it by 48% and 45% only in the PGF of HF/HC+HRP and N+HRP mice respectively (Figure 4A). Monocyte chemoattractant protein-1 (MCP-1) expression was increased by around 1.9-fold in PGF and PRF of HF/HC+Saline mice while the HRP decreased it by 68% and 59% only in the PGF of HF/HC+HRP and N+HRP mice respectively (Figure 4B). F4/80, a marker of macrophage, expression was increased by around 3.5-fold in PGF and PRF of HF/HC+Saline mice while the HRP decreased it by 38% and 36% only in the PGF of HF/HC+HRP and N+HRP mice respectively (Figure 4C).

Index of mouse adipocyte cell size

Mest/Peg1 mRNA, a marker of adipocyte size(22), was increased in all adipose tissue in HF/HC+Saline mice compared to their N diet controls (Figure 5). These values were decreased by around 33% and 57% in HF/HC+HRP and N+HRP mice respectively in all adipose tissue (Figure 5).

Mouse glucose and lipid homeostasis

Circulating triglycerides were increased by 1.4-fold in HF/HC+Saline mice compared to the N+Saline mice and were normalized in HF/HC+HRP mice (Table 2). Although hepatic triglycerides were also 1.4-fold higher in HF/HC+Saline mice, the HRP had no effect on this parameter (Table 2).

HF/HC+Saline mice had a 3.5-fold increase in circulating insulin and 1.4-fold rise in circulating glucose compared to N+Saline mice. Insulinemia was decreased by 34% and 27% in HF/HC+HRP and N+HRP mice respectively compared to their respective control groups (Table 2). This was not accompanied by a decrease in glycemia in HF/HC+HRP mice although a tendency could be observed (Table 2). Moreover, the G/I ratio was decreased by 55% with obesity whereas this was increased by 1.5-fold in both HF/HC+HRP and N+HRP mice (Table 2). In line with these results, we found that the GLUT1 mRNA was increased in all adipose tissue and in SCF for GLUT4 with obesity (Figure S5A-B) as reported by others in obese rats(23;24). Conversely, GLUT1 and GLUT4 expression were decreased in SCF by 29% and 45% respectively in HF/HC+HRP mice and by 51% and 31% in N+HRP mice. In PRF, 4-56% reduction was observed only in GLUT1 independently of diet (Figure S5A-B).

Mouse heart function

Ejection fraction and fractional shortening measured by echocardiography were decreased by 23% and 26% respectively compared to baseline in HF/HC+Saline mice (Figure 6A-B) as a result of a 1.9-fold increase in systolic and 1.5-fold in diastolic left ventricular volume (LVV) (Table S8). This effect on heart function was prevented by the HRP (Table S8) with a parallel decrease in systolic and diastolic LVV by 31% and 22% respectively.

Discussion

First, we observed that adipose tissue (P)RR was significantly increased by obesity in mice, both male and female, a novel finding that supports our hypothesis that the (P)RR is associated with the development of obesity. This observation seems specific to adipose tissue as the (P)RR was unchanged in other studied tissues. Previous studies comparing lean and obese women have not reported any differences in adipose tissue (P)RR expression although Achard *et al.* suggested its implication in the pathophysiology of obesity as the (P)RR in VAT was increased compared to SCF, similarly to our data in female mice (4;5). However, those studies considered obesity as a homogenous disease when it is well established that there is a wide variation in the prevalence of cardiometabolic risk factors among obese individuals due to differences in age, lifestyle and waist circumference whereas diet induced obesity mice have tightly controlled factors which make them easily comparable(25). As such, it is possible that the reported lack of difference in the (P)RR expression was due to variations in insulin sensitivity and cardiometabolic risk factors among obese subjects. Indeed, our human adipose tissue data showed a 33% increase in the (P)RR mRNA in IRO compared to age and BMI-matched ISO women demonstrating that the (P)RR is modulated by this parameter and could thus contribute to variability.

Renin protein synthesized locally in adipose tissue, assessed indirectly by measuring GFP, was undetectable while total renin content was increased with obesity. This implies that most of the renin in adipose tissue comes from the circulation as observed previously(26). This is further supported by the comparable increases in circulating renin activity detected in obese mice. Conversely, we found no effect of the HRP on circulating renin activity and gene expression similarly to what has been reported in other studies in different models(6). Given that renin and prorenin bind the (P)RR with a K_D in the nanomolar range, it is unclear whether adipose tissue renin levels are sufficient to induce physiological effects through the (P)RR. However, we have found that renin levels in PGF from obese animals increase to as much as 84% of the renal renin content suggesting that sufficient amounts are present to activate the (P)RR.

The effects of the HRP on BW in mice receiving the HF/HC diet are in contrast to a study in rats showing that a lower HRP dose did not affect the BW of obese transgenic rats

overexpresssing human renin even after 10 weeks of treatment on a standard chow (10). These divergent results may suggest that the BW phenotype may be specific to obesity induced by HF/HC feeding or that it requires the HRP dose used in our study. Similarly, no effect of the HRP on BW was reported in models of type 1 diabetic rats or in high-energy diet fed non-obese rats, again suggesting that diet-induced obesity is key to the phenotype we are reporting(27;28). Other *in vivo* studies have also questioned the beneficial effect of the HRP on different pathologies(6;7;29) although this may be due to methodological issues as lower doses of the peptide were administered and a shorter duration treatment was used compared to the studies reporting protective effects(6). The most convincing published data regarding the importance of treatment duration comes from 2 studies by the same group showing that a 4 weeks HRP treatment had no effect on cardiovascular damage in spontaneously hypertensive rats whereas 8 weeks of treatment improved cardiac function in the same animal model(6). In line with these results, a 20% reduction in weight gain in HF/HC+HRP mice could only be observed after 10 weeks of treatment in our study.

Interestingly, studies in obese rodents have shown that ACE inhibitors (ACEi) or Ang II receptor blockers (ARBs) decrease BW by 8 to 32% compared to non-treated groups (30-33). Given that the HRP only reduces local Ang II production without completely blocking its synthesis, the milder effects reported in our study suggest an important role of Ang II production in the HRP effect on BW. However, in contrast to studies with ACEi or ARBs which only observed a reduction in circulating triglycerides (31-33), we have noted a complete normalization implying that Ang II-independent pathways of the (P)RR are also involved in the beneficial effects of the HRP.

Alternatively, it is possible that the HRP reduced intestinal fat absorption. Although inactivation of RAS components such as AGT, renin and ACE in mice increases energy expenditure(34-36), only mice lacking renin have been reported to have decreased intestinal fat absorption(35). Conversely, this was not observed in AGT(34) and ACE(36) knock-out mice, suggesting that this renin effect may be Ang II-independent, potentially through an interaction with the (P)RR.

Similarly to the HRP effects on fat mass, leptin was decreased by the HRP in all adipose tissue and in the circulation independently of the diet. Given that leptin is known to correlate positively

with adipose tissue mass(37), our data suggest that the HRP targets fat mass probably due to higher expression of the (P)RR with obesity in adipose tissue. More specifically, it may decrease adipocyte cell size as we found that Mest/Peg, a marker of adipocytes cell size independently of obesity or diabetes(22), was also decreased in all adipose tissue by the HRP. Moreover, the HRP decreased inflammation and macrophage infiltration in VAT.

In addition, the HRP seems to improve insulin sensitivity as it decreased insulinemia and improved G/I ratio independently of the diet. In agreement with these results, we found an improved GLUT1 and GLUT4 expression pattern in adipose tissue with the administration of the HRP. We also found that the obesity-related increase in SCF resistin mRNA was decreased by the HRP although this was not paralleled by lower circulating levels. This supports the hypothesis that the correlation between insulin resistance and resistin may be due to an effect on the insulin signalling pathways specifically in adipocytes(38) and that regional differences exists between adipose tissue depots(39).

This improved glucose homeostasis is also in line with clinical trials which have reported that administration of ACEi or ARBs seems to reduce the risk of type 2 diabetes compared to other antihypertensive treatments. While HRP may have effects in skeletal muscle, as the (P)RR is also expressed in this tissue, given that others using type 1 diabetes rodent models do not see any changes in glucose homeostasis with the HRP(6), it is tempting to speculate that the observed effect is due to an action in adipose tissue and may be specific to obesity. Moreover, this hypothesis is further supported by the association we found between insulin sensitivity and human (P)RR where IRO women had increased (P)RR expression in their SCF.

Interestingly, the HRP had a similar effect on mice fed a N diet to those on HF/HC diet for PRF mass, leptin, Mest/Peg1, inflammation markers, circulating insulin levels and G/I ratio although no changes in BW or weight gain were observed. This suggests that metabolic effects might come before those on the BW and that insulin sensitivity might be improved by the HRP as a result of having less VAT coupled to potentially smaller adipocyte cell size.

Finally, we found that the HRP prevented the fall in cardiac function which is typically associated with the development of obesity(40). This is in line with other studies which have reported similar effects of the HRP on cardiac pathology related to hypertension without any

effect on blood pressure(6). This suggests that in addition to improving metabolic status of these animals, the HRP also ameliorates cardiovascular health.

Our data highlights the potential beneficial effects of (P)RR blockade on obesity and its related-cardiometabolic complications. As such, it suggests that it may be a novel preventive and therapeutic avenue for the metabolic syndrome in the future.

Conflict(s) of Interest/Disclosure

None.

Acknowledgments

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Table 1. Renin-angiotensin system components mRNA expression in human subcutaneous adipose tissue

	ISO (N = 13)	IRO (N = 9)
h(P)RR/18s	1.00 ± 0.08	1.33 ± 0.12*
hAT1R/18s	1.00 ± 0.11	1.49 ± 0.16*
hAGT/18s	1.00 ± 0.16	1.40 ± 0.28
hACE/18s	1.00 ± 0.09	1.27 ± 0.15

Values are normalized to 18s expression and are expressed as means ± SE of fold difference in IRO vs ISO group. * $p < 0.05$ compared to ISO. ISO, insulin-sensitive obese; IRO, insulin-resistant obese; h(P)RR, human (pro)renin receptor; hAT1R, human angiotensin II type 1 receptor; hAGT, human angiotensinogen; hACE, human angiotensin-converting enzyme.

Table 2. Effect of the HRP on mouse hepatic and circulating metabolites

	Normal diet		HF/HC diet	
	Saline	**HRP**	**Saline**	**HRP**
Hepatic triglycerides (mg/g liver)	65.48 ± 12.62	64.73 ± 16.06	89.44 ± 7.09*	82.55 ± 9.22*
Triglycerides (mmol/L)	2.43 ± 0.08	2.55 ± 0.16	3.48 ± 0.41*	2.22 ± 0.12†
Glucose (mmol/L)	13.33 ± 1.71	13.02 ± 1.34	18.00 ± 1.11*	15.37 ± 1.08*
Insulin (pmol/L)	42.96 ± 3.97	31.24 ± 6.41†	150.26 ± 20.19*	99.69 ± 18.01*†
Glucose/Insulin ratio	0.29 ± 0.03	0.42 ± 0.10†	0.13 ± 0.01*	0.20 ± 0.04*†

Concentrations are expressed as means ± SE. * $p < 0.05$ compared to N diet; † $p < 0.05$ compared to saline. N+Saline (N = 7), N+HRP (N = 7), HF/HC+Saline (N = 7) and HF/HC+HRP (N = 9) for triglycerides, glucose, insulin, Glucose/Insulin ratio. N+Saline (N = 11), N+HRP (N = 8), HF/HC+Saline (N = 10) and HF/HC+HRP (N = 10) for hepatic triglycerides.

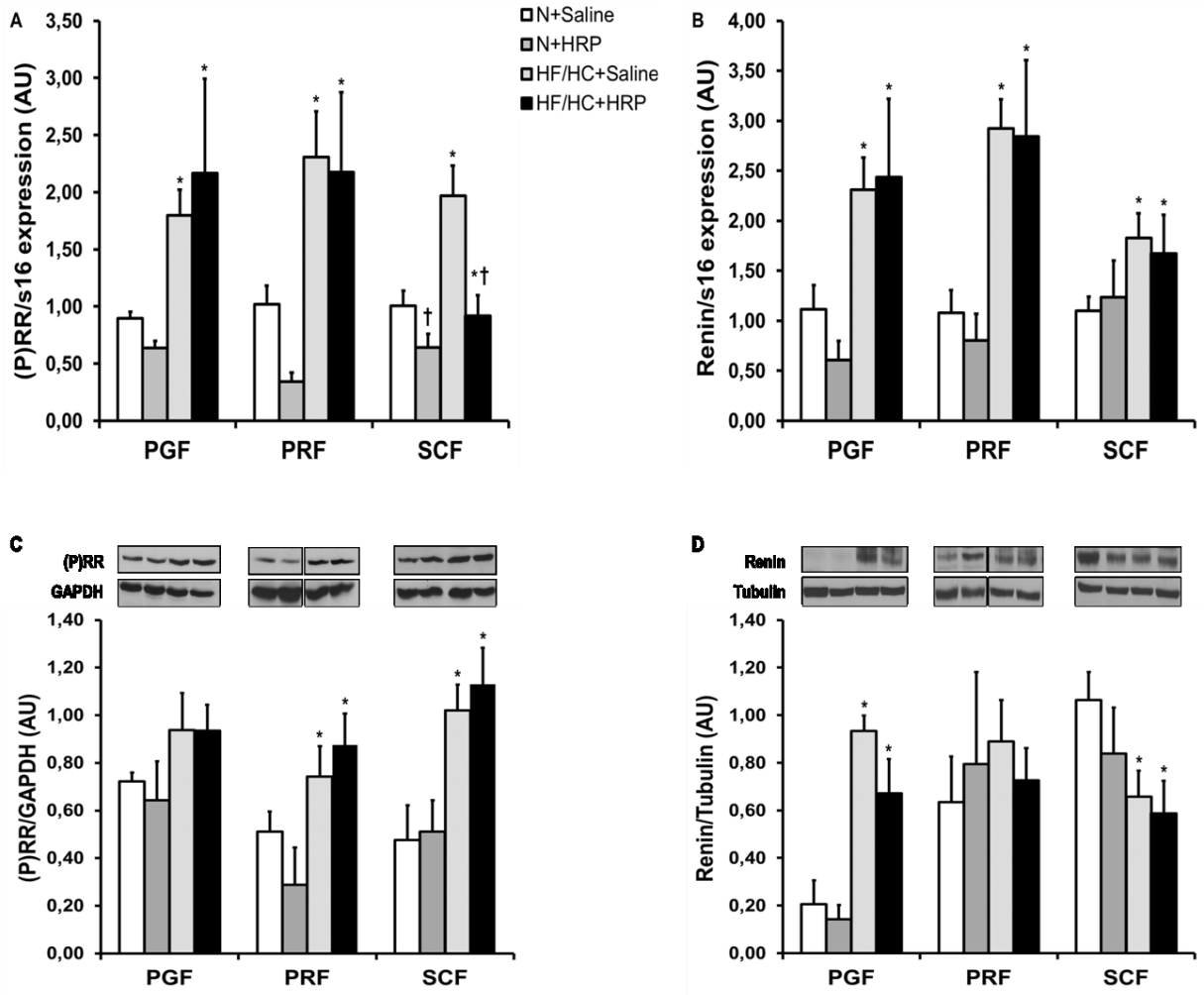

Figure 1. Effect of the HRP on mouse (P)RR and renin mRNA and protein in adipose tissues. **(**P)RR (A) and renin (B) mRNA data are presented as a ratio to s16 expression. (P)RR (C) and renin (D) protein data are expressed as a ratio to GAPDH and tubulin respectively. Images shown for (P)RR and renin in each tissues are from the same blot. Protein from the kidney (25 µg) was used as a positive control for (D).Values are expressed as means ± SE. * $p < 0.05$ compared to N diet; † $p < 0.05$ compared to saline. mRNA expression: N+Saline (N = 12), N+HRP (N = 10), HF/HC+Saline (N = 13) and HF/HC+HRP (N = 9). Protein expression: N+Saline (N = 4-5), N+HRP (N = 4-5), HF/HC+Saline (N = 6-7) and HF/HC+HRP (N = 6-7). PGF, peri-gonadal fat; PRF, peri-renal fat; SCF, abdominal subcutaneous fat.

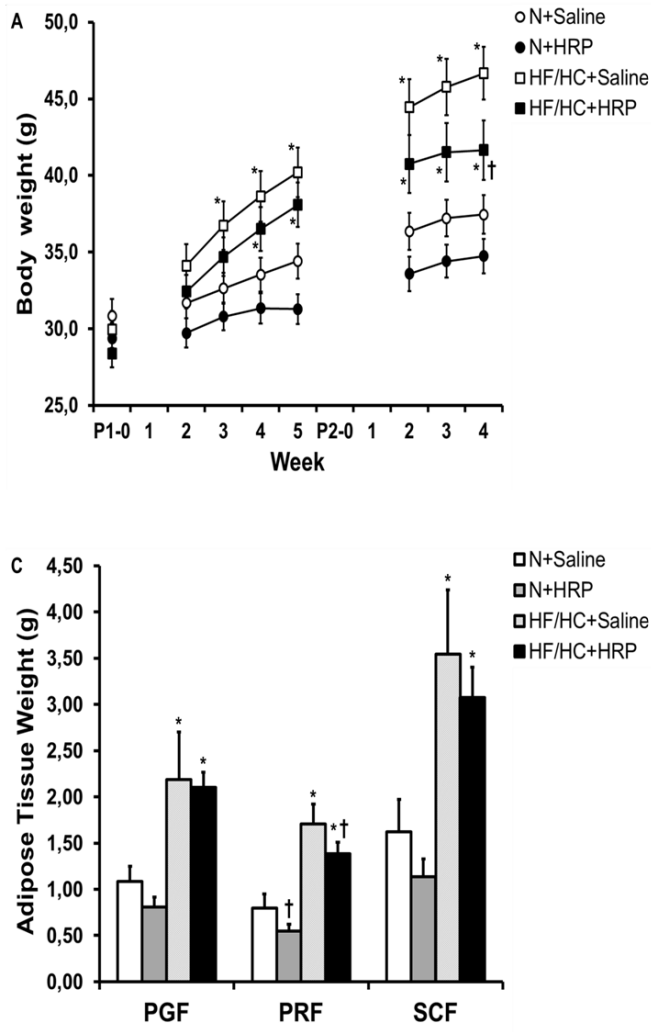

Figure 2. Effect of the HRP on mouse body weight, weight gain and adipose tissue weight. Body weight (A), weight gain (B) and adipose tissue weight (C) presented. Values are expressed as means ± SE. * $p < 0.05$ compared to N diet; † $p < 0.05$ compared to saline. N+Saline (N = 15), N+HRP (N = 10), HF/HC+Saline (N = 14) and HF/HC+HRP (N = 13). P1-0, pump 1-week 0; P2-0, pump 2-week 0. PGF, peri-gonadal fat; PRF, peri-renal fat; SCF, abdominal subcutaneous fat.

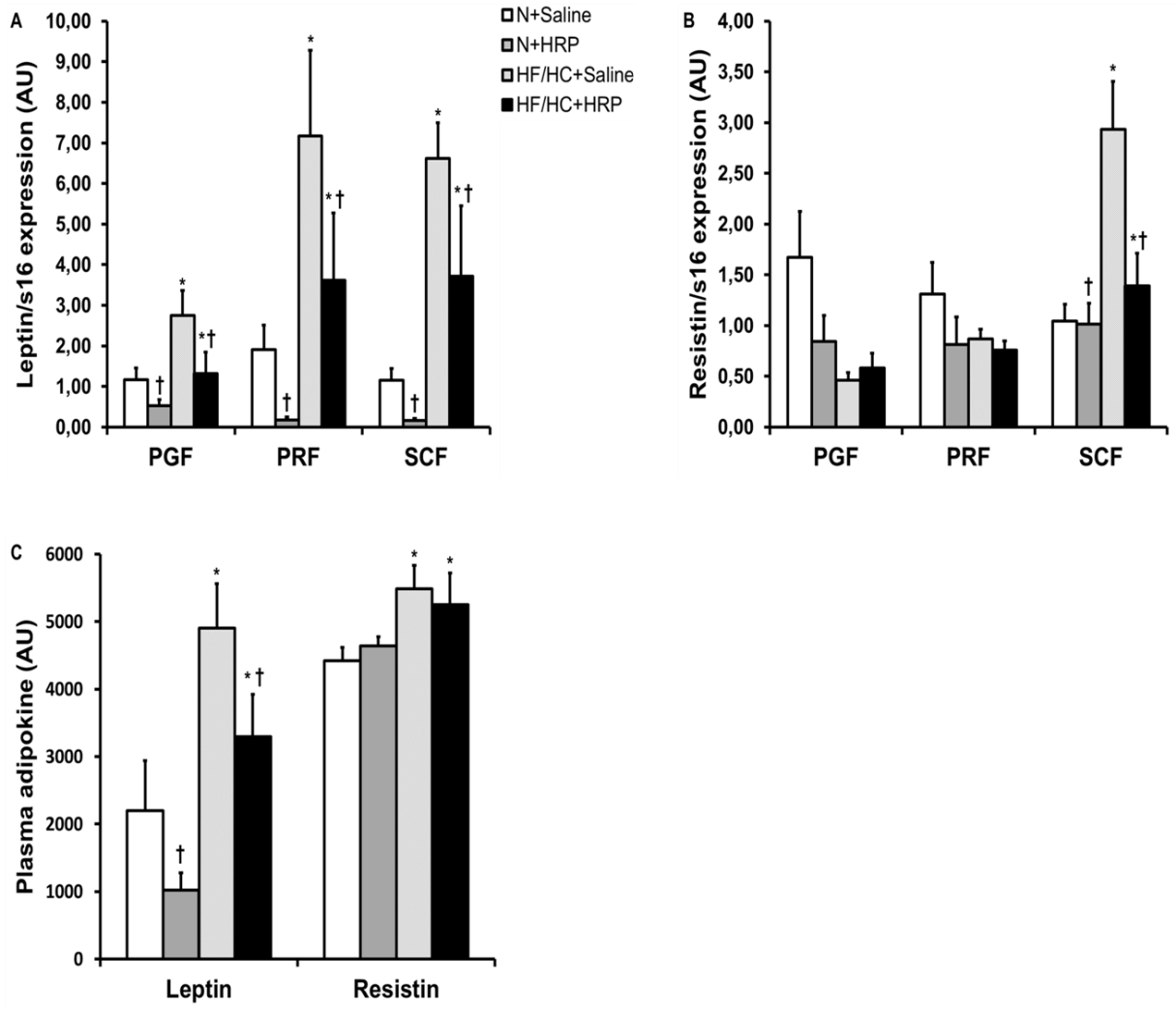

Figure 3. Effect of the HRP on mouse adipokine mRNA in adipose tissues and circulating levels. Leptin (A), resistin (B) mRNA and circulating levels (C). Expression data are presented as a ratio to s16 expression. Values are expressed as means ± SE. * $p < 0.05$ compared to N diet; † $p < 0.05$ compared to saline. mRNA expression : N+Saline (N = 12), N+HRP (N = 10), HF/HC+Saline (N = 13) and HF/HC+HRP (N = 9). Circulating levels: N+Saline (N = 4), N+HRP (N = 4), HF/HC+Saline (N = 4) and HF/HC+HRP (N = 4). PGF, peri-gonadal fat; PRF, peri-renal fat; SCF, abdominal subcutaneous fat.

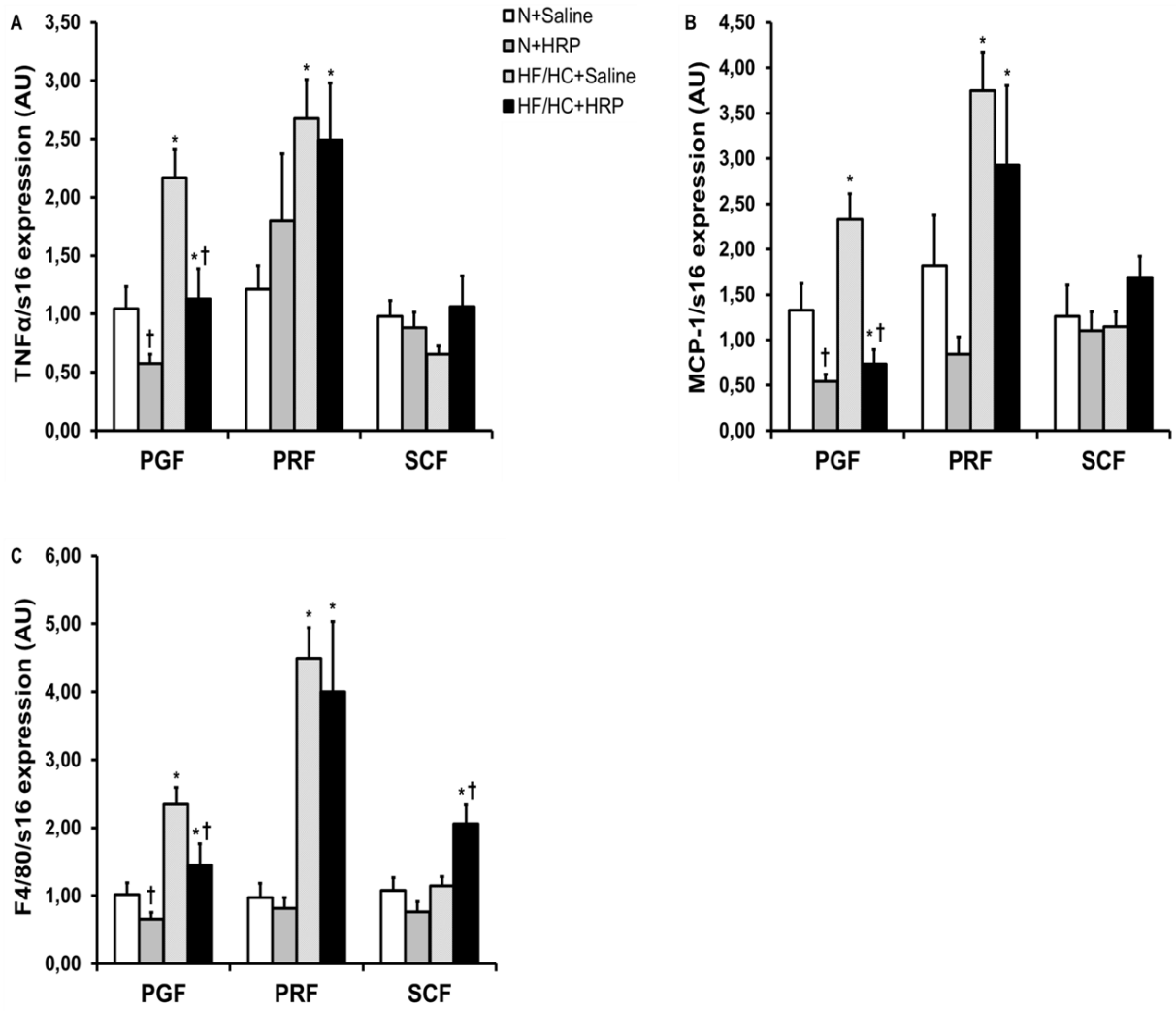

Figure 4. Effect of the HRP on mouse inflammation markers mRNA in adipose tissues. TNFα (A), MCP-1 (B) and F4/80 (C) mRNA. Expression data are presented as a ratio to s16 expression. Values are expressed as means ± SE. * $p < 0.05$ compared to N diet; † $p < 0.05$ compared to saline. N+Saline (N = 12), N+HRP (N = 10), HF/HC+Saline (N = 13) and HF/HC+HRP (N = 9). PGF, peri-gonadal fat; PRF, peri-renal fat; SCF, abdominal subcutaneous fat.

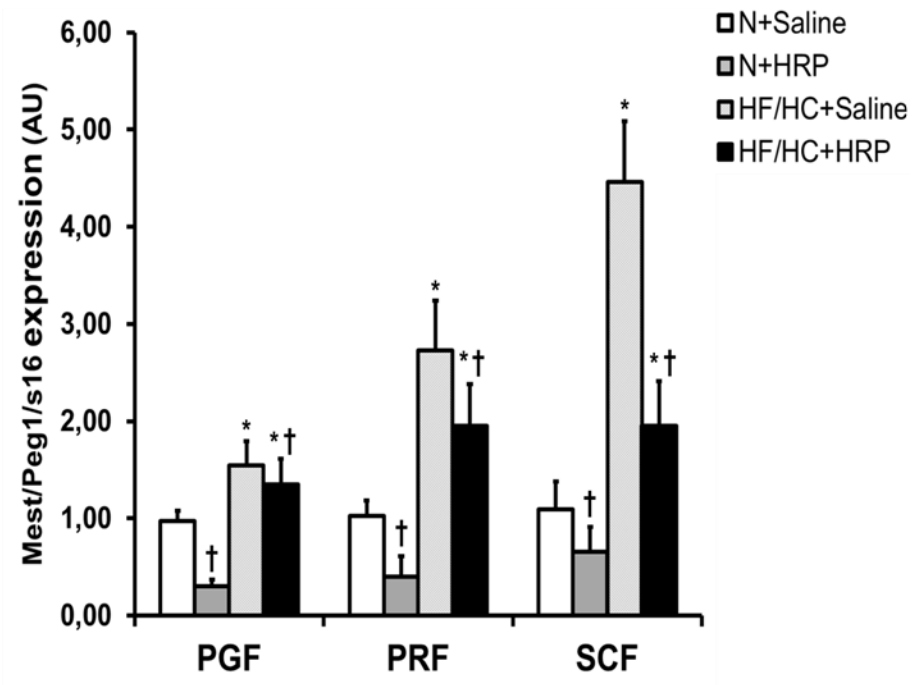

Figure 5. Effect of the HRP on mouse Mest/Peg1 mRNA in adipose tissues. Mest/Peg1 data are presented as a ratio s16 expression. Values are expressed as means ± SE. * $p < 0.05$ compared to N diet; † $p < 0.05$ compared to saline. N+Saline (N = 12), N+HRP (N = 10), HF/HC+Saline (N = 13) and HF/HC+HRP (N = 9). PGF, peri-gonadal fat; PRF, peri-renal fat; SCF, abdominal subcutaneous fat.

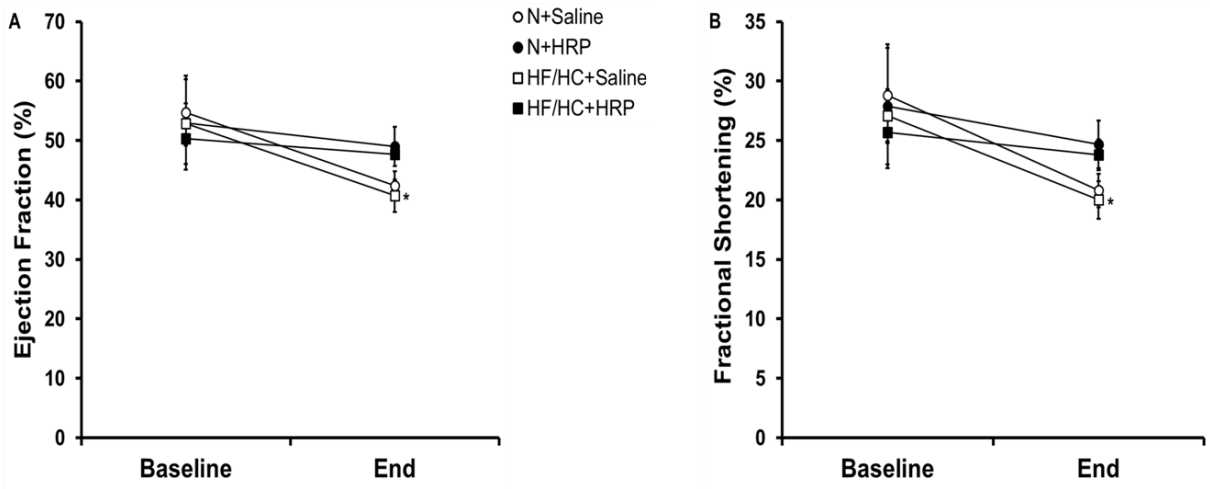

Figure 6. Effect of the HRP on mouse heart function. Ejection fraction (A) and fractional shortening (B). Values are expressed as means ± SE. * $p < 0.05$ compared to baseline. N+Saline (N = 6), N+HRP (N = 5), HF/HC+Saline (N = 7) and HF/HC+HRP (N = 8).

ONLINE SUPPORTING INFORMATION

IMPACT OF THE (PRO)RENIN RECEPTOR ON THE DEVELOPMENT OF OBESITY AND ASSOCIATED CARDIOMETABOLIC RISK FACTORS

Paul Tan*[1,2,8], Zulaykho Shamansurova*[1,3,8], Sandra Bisotto[1], Catherine Michel[1], Marie-Soleil Gauthier[8,9], Rémi Rabasa-Lhoret[1,4,8,9], Thi M.-D. Nguyen[8], Peter W. Schiller[6,9], Jolanta Gutkowska[1,5] and Julie L. Lavoie[1,7,8]

[1]Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM)
[2]Department of Biochemistry and molecular medicine, [3]Physiology, [4]Nutrition, [5]Medecine and, [6]Pharmacology and [7]Kinesiology of the Université de Montréal, [8]Montreal Diabetes Research Center and [9]Institut de Recherches Cliniques de Montréal, Montréal, Québec, Canada,
*These authors contributed equally to this work

Correspondence to:
Julie L. Lavoie, PhD
Centre de Recherche du CHUM (CRCHUM)
900, rue Saint-Denis, bureau R08.452
Montréal, Québec, Canada, H2X 0A9
Tel: (514) 890-8000 ext. 23612
Fax: (514) 412-7655

Expanded material and methods

Animals. All experiments were carried out on mice expressing green fluorescent protein under the control of the renin promoter (REN-GFP)(1) (generous gift from Dr. Kenneth Gross at the Buffalo Cancer Institute). Mice were maintained by backcross breeding to C57BL/6 (Charles River, St-Constant, QC, Canada). Mice were 12-15 weeks of age and were maintained on 12-h light/dark cycle with access to either normal (N) diet (2018; Harlan Laboratories, Madison, WI) or high-fat/high-carbohydrate (HF/HC) diet (F3282; Bio-Serve, Frenchtown, NJ) and water *ad libitum*. All experiments were carried out in male mice. In addition, the impact of obesity on the (P)RR and renin were confirmed in female mice as previous studies looking at human adipose tissue (P)RR have been conducted only in women. Mice body weight (BW) and food weight were measured weekly. Care of the mice used in the 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.

Handle region peptide (HRP) synthesis. The mouse peptide IPLKKMPS(2) was synthesized by the manual solid-phase technique(3). Its purity was established by thin-layer chromatography and analytical RP-HPLC. The structural identity was established by electrospray mass spectrometry: ES-MS [M+H]+ 913.49. This peptide, consisting of part of the prosegment region (10P–19P) of prorenin, has been shown to bind directly to the (P)RR *in vitro* with a dissociation constant (K_D) of 3.5 nM(4,5). Furthermore, it was confirmed that HRP could prevent binding of both renin and prorenin to the receptor with a dissociation constant (K_I) of 16.7 nM and 15.1 nM respectively(4).

HRP administration. To assess the impact of the (P)RR on obesity development, mice were anesthetized and implanted subcutaneously, under isoflurane anesthesia (induction at 4% and maintained at 2% in oxygen), with an osmotic mini-pump (model #1004; Alzet, Cupertino, CA) filled with either the HRP or saline. The peptide was administered at a published effective dose of 0.1 mg/kg/day(2,6). This procedure was repeated after 4-5 weeks to ensure constant

administration of the peptide as these are 28-42 days pump (length varies according to lot). Concomitantly, the mice received either a N or HF/HC diet for 10 weeks.

Mouse tissue collection. Mice were sacrificed by CO_2 asphyxiation and multiple tissues (visceral adipose tissue (VAT) from whole peri-renal (PRF), peri-gonadal (PGF), and abdominal subcutaneous fat (SCF), heart, liver and kidney) were collected, weighed and snap-frozen in liquid nitrogen until assayed. N varies for experiments with fat pads due to limited amount of adipose tissue. Blood was collected by intrathoracic cardiac puncture and placed in a chilled 1.5 ml tube containing 15µl of 500 mM EDTA (pH8.0) (EMD, Gibbstown, NJ, USA). Plasma was separated by centrifugation and samples were snap frozen in liquid nitrogen, and stored at -80ºC until assayed. For measurement of circulating glucose and insulin, mice were fasted overnight before tissue collection whereas triglycerides were assessed in non-fasted animals.

Human CAO cohort study. The cohort used to obtain the samples analysed in this study were from post-menopausal non-diabetic obese women enrolled from 2006 to 2007 in the Complications Associated with Obesity (CAO) Study. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and both studies were approved by the University of Montreal ethics committee. All subjects gave a written informed consent before the study started. The main objective of this study is to understand susceptibility to cardiometabolic complication in obese women. The inclusion/exclusion criteria of this study were reported(7). Briefly, post-menopausal women were included in the study if they 1) were obese with a body mass index (BMI) between 30 to 40 kg/m^2, 2) were aged between 55 to 70 years old, 3) had biological confirmation of the menopause status without taking hormone replacement therapy, 4) were non diabetic (fasting glucose <7.1 $mmol*l^{-1}$ or 2-h plasma glucose <11.1 $mmol*l^{-1}$ after a 75g OGTT), 5) non-smokers, and 6) were not taking medications known to interfere with metabolism except stable hypothyroidism replacement therapy. All participants were free of chronic or inflammatory diseases and no history of alcohol or drug abuse. Women were then subdivided into 2 groups, insulin-sensitive obese (ISO) and insulin-resistant obese (IRO), based on their insulin sensitivity as measured using by the 3-hour hyperinsulinemic euglycemic clamp and classified based on a cut-off proposed in a previous study(8). More specifically, patients who had a steady-state glucose disposal rate (GDR) ≤9.5 mg/min/kg fat

free mass (FFM) were classified as IRO and ≥12.0 mg/min/kg FFM as ISO. Before the clamp procedure subcutaneous adipose tissue biopsy samples were obtained after an overnight fast from the periumbilical level at both side of the body by needle biopsy under local anesthesia (20 mg xylocaine/mL) as previously described(9). Biopsy samples were washed from excess blood in saline, quickly frozen in liquid nitrogen and stored at −80°C until analysis. 59 subjects were included in the CAO study out of which 26 accepted the adipose tissue sub-study. For the present work adipose tissue biopsy samples were available for 22 patients (ISO: N = 13 and IRO: N = 9) for gene expression analysis.

Insulin sensitivity in human cohort. The study began at 07-30 h after a 12-h overnight fast. An antecubital vein was cannulated for the infusion of 20% dextrose and insulin (Actrapid. Novo-Nordisk, Toronto, Canada). The other arm was cannulated for sampling of blood. Plasma glucose was measured every 10 min with a glucose analyzer (Beckman Instruments, Fullerton, CA) and maintained at fasting level with a variable infusion rate of 20% dextrose. Insulin infusion was initiated at the rate of 75 mU/m2·min for 180 min. GDR was calculated as the mean rate of glucose infusion measured during the last 30 min of the clamp (steady state).

Real-time PCR. Gene expression was evaluated for (P)RR, renin, leptin, resistin, mesoderm-specific transcript/paternally expressed gene 1 (Mest/Peg1), glucose transporter 1 (GLUT1),GLUT4, TNFα, MCP-1 and F4/80 in mouse and for (P)RR, Ang II type 1 receptor (AT1R), AGT and angiotensin converting enzyme (ACE) in human using the primer sequences and conditions provided in Table S1 and S2. RNA was extracted from mouse tissues using Trizol (Invitrogen, Burlington, ON, Canada) and from human tissue with RNeasy total RNA Mini kit (Qiagen) according to the manufacturer's protocol. To remove genomic DNA, RNA samples were incubated with 2 U deoxyribonuclease I (DNase I; Invitrogen)/ug RNA for 30 min at 37°C. Single-stranded cDNA was synthesized by reverse-transcriptase reaction using SuperScript II Reverse Transcriptase (Invitrogen). The real-time PCR final volume was 10 µl and contained 0.3 µmol/l of the specific forward (F) and reverse (R) primers as well as 2.5 µl of single-stranded cDNA template in Rotor-Gene SYBR PCR Mastermix (Qiagen) a 1X final concentration. Primer sequences and Real Time PCR conditions for each gene are provided in Table S1 and S2, respectively. Real-time PCR for human renin and human angiotensinogen was done with

TaqMan Gene Expression Assay (Hs00982550_m1 and Hs01586213_m1; Applied Biosystems, Foster City, CA) with 2 µl of single-stranded cDNA template in a total volume of 20 µl. Each sample was run and analyzed in duplicate. Expression levels are expressed as relative values to s16 for mouse. In human, samples were normalized to 18s and are expressed as fold difference relative to gene expression in the ISO group.

Western Blot. Proteins were extracted from adipose tissues with lysis buffer and quantified by Bradford assay (Bio-Rad). Protein (50 µg) were separated by electrophoresis and transferred to nitrocellulose membranes (Amersham, Baie d'Urfe, QC). Membrane were blocked followed by overnight incubation at 4°C with primary antibody for (P)RR (1:750, generous gift from Dr. Yumei Feng at Tulane University), GFP (1:200, Chemicon International), renin (0,75µg/mL, Molecular Innovations), GAPDH (1:2000, Santa Cruz), β-actin (Abcam) and tubulin (1:30000, Abcam). Goat anti-rabbit (Santa Cruz, sc-2004) was used as secondary secondary. Bands were revealed using the ECL West Pico kit (Pierce, Rockford, IL). Each protein signal was normalized to its respective GAPDH or tubulin.

Metabolism. Mouse plasma was tested for quantitative determination of triglycerides (Sigma, Oakville, Canada), glucose (Wako) and insulin (Millipore [Linco]) according to the manufacturer's protocol. Insulin sensitivity was estimated by calculating the ratio of fasting blood glucose to insulin (G/I).

Circulating adipokines. 50 µl of plasma was used to measure the relative circulating adipokine level using the mouse adipokine array (R&D systems, Minneapolis, USA) following the manufacturer's protocol.

Renin activity measurement. RIA was performed on plasma with the Ang-I I^{125}-labeled RIA kit (Diasorin, Stillwater, MN) to assess mouse renin activity according to manufacturer's instructions as done previously(10,11).

Echocardiography. Transthoracic echocardiographic studies were performed before and at the end of treatment by high-resolution ultrasound biomicroscopy (Vevo660; Visualsonics,

Toronto, ON, Canada) equipped with 25-55 MHz probes that allow tracings of time-varying M-mode dimensions of the left ventricle (LV) as done previously(12).

Statistical analysis. All values are expressed as means ± standard error (SE). A 2-way ANOVA was used to assess the effect of diet and the HRP. For parameters measured at many time points, a repeated measure 2-way ANOVA was applied. If interactions were detected for these analyses, a Tukey post-hoc test was used. Differences in human gene expression were determined by non-parametric Mann–Whitney U tests. For renin mRNA in female mice, a Pearson's Chi-square was done. A $p < 0.05$ was considered statistically significant.

Supplementary Figures

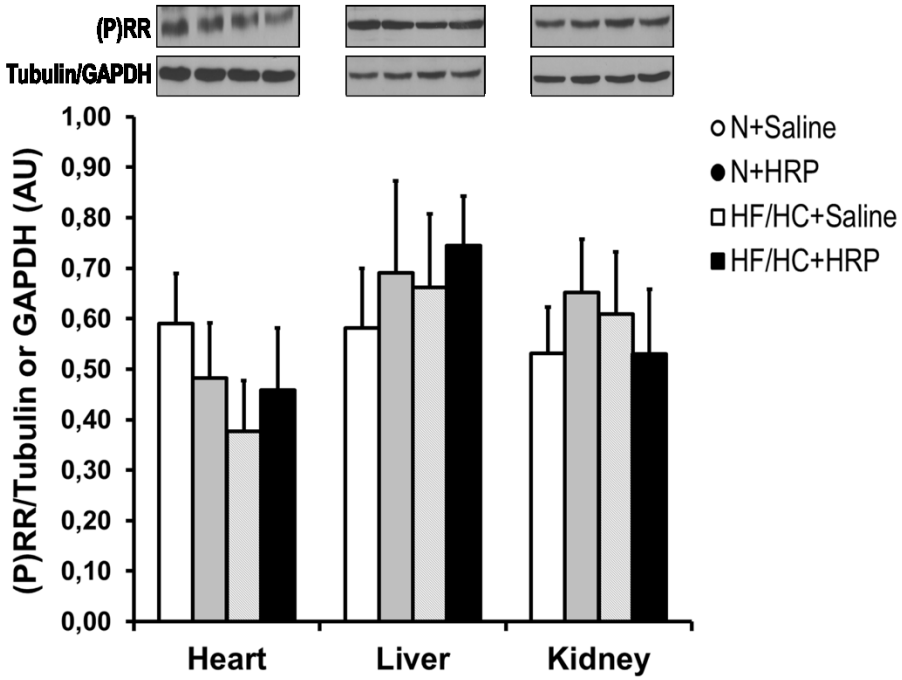

Figure S1. (P)RR protein in the heart, liver and kidney is not modified by diet and treatment. (P)RR protein data are expressed as a ratio to tubulin or GAPDH. Values are expressed as means ± SE. N+Saline (N = 6), N+HRP (N = 6), HF/HC+Saline (N = 6) and HF/HC+HRP (N = 6).

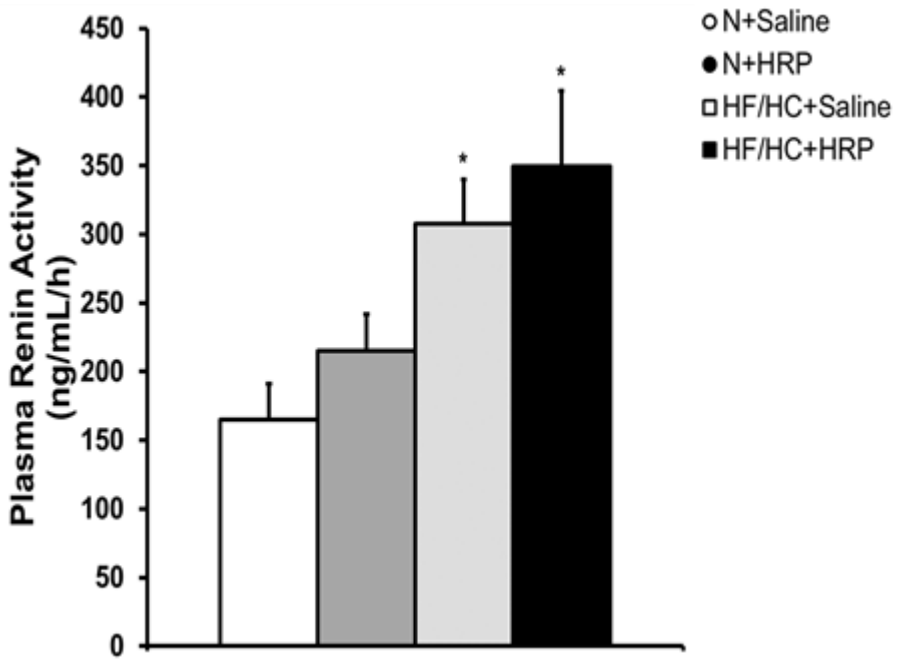

Figure S2. Effect of the HRP on mouse plasma renin activity. Values are expressed as means ± SE. * $p < 0.05$ compared to N diet group. N+Saline (N = 6), N+HRP (N = 11), HF/HC+Saline (N = 9) and HF/HC+HRP (N = 10).

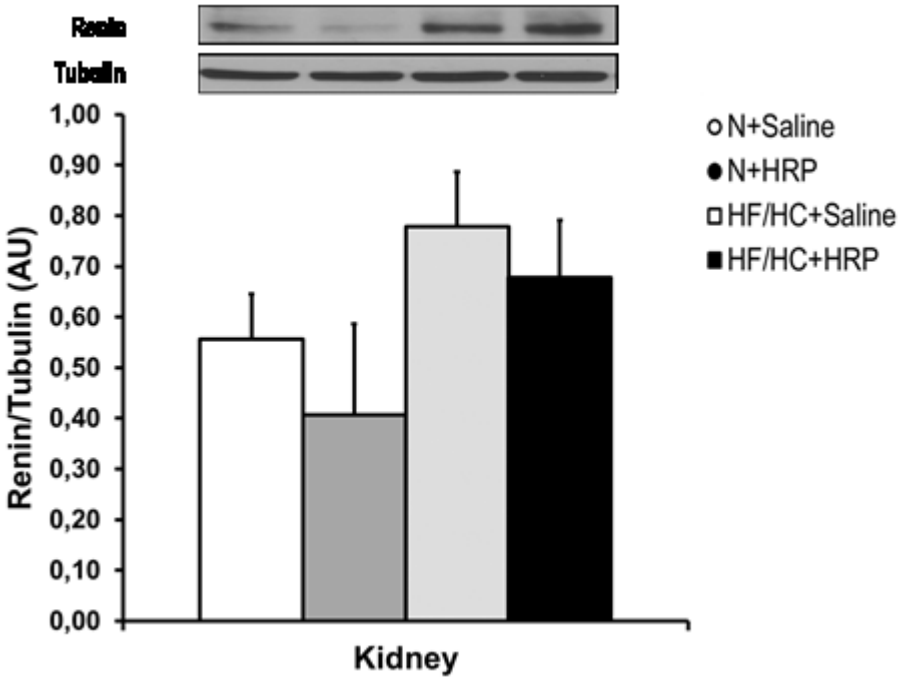

Figure S3. Renin protein in the kidney. Renin protein data are expressed as a ratio to tubulin. Values are expressed as means ± SE. N+Saline (N = 7), N+HRP (N = 7), HF/HC+Saline (N = 7) and HF/HC+HRP (N = 7).

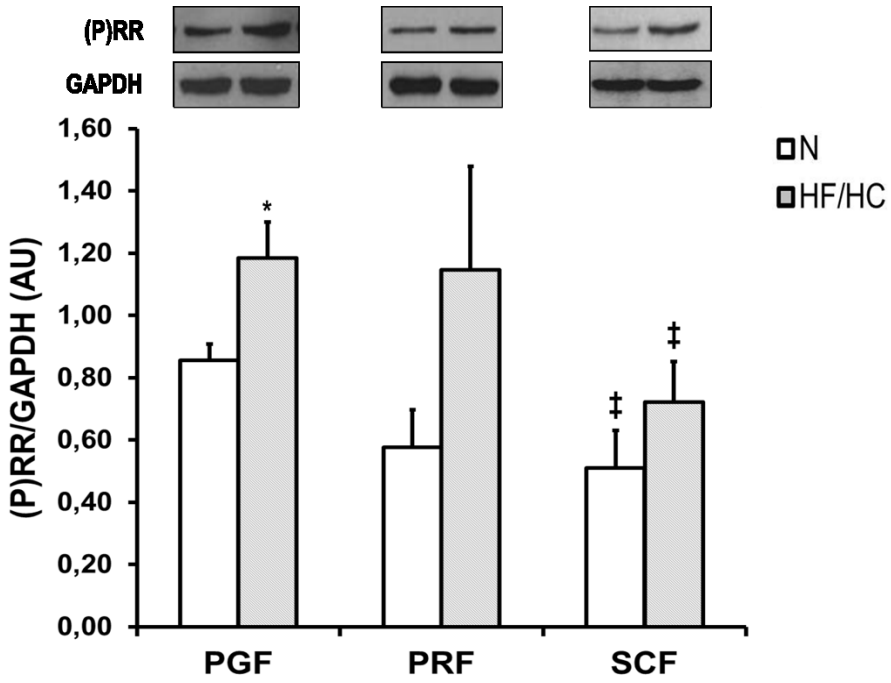

Figure S4. Effect of HF/HC diet on female mice (P)RR protein in adipose tissue. (P)RR protein data are expressed as a ratio to GAPDH. Values are expressed as means ± SE. * $p < 0.05$ compared to N diet; ‡ $p < 0.05$ compared to PGF. N diet (N = 8) and HF/HC diet (N = 8). PGF, peri-gonadal fat; PRF, peri-renal fat; SCF, abdominal subcutaneous fat.

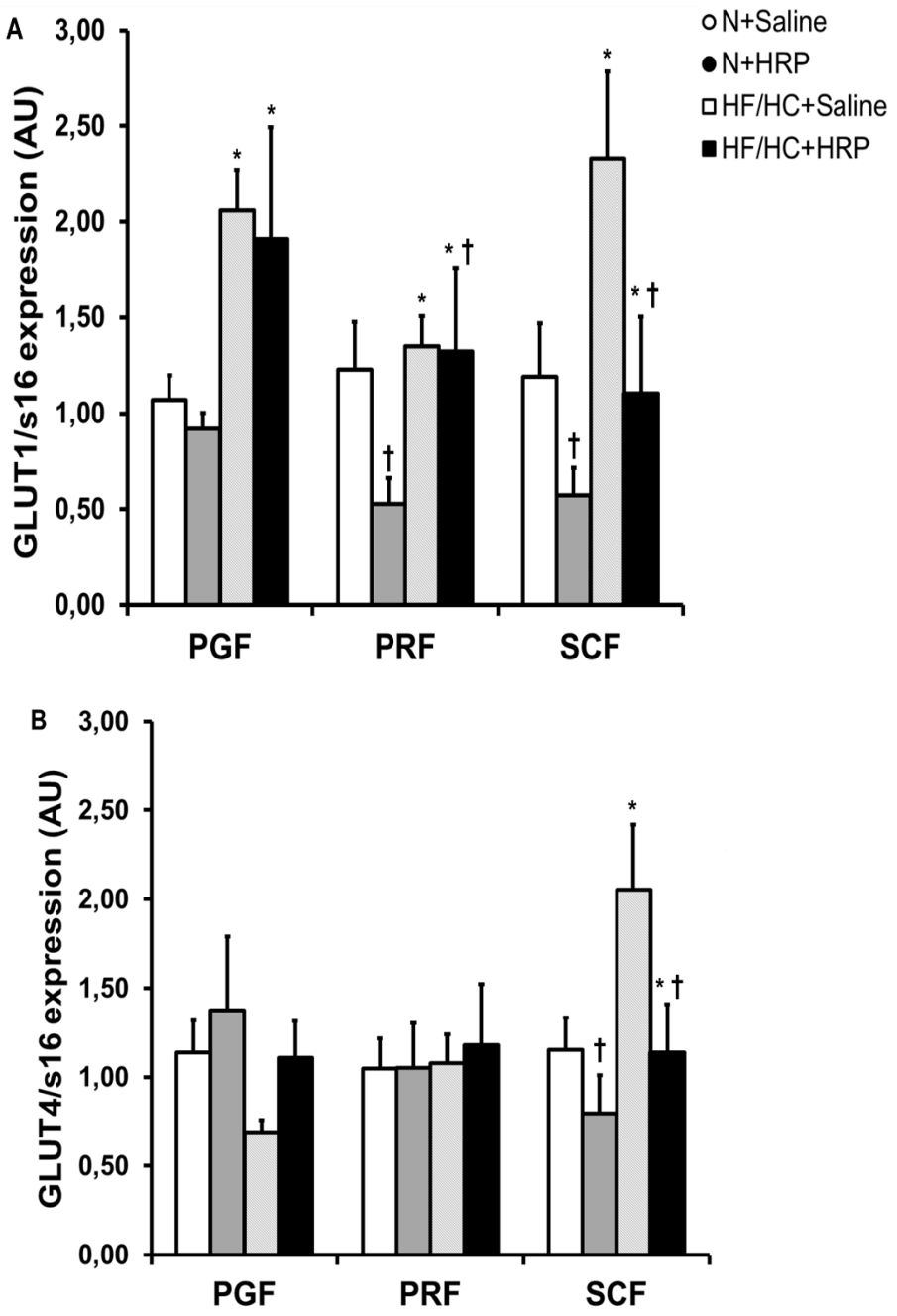

Figure S5. Effect of the HRP on mouse GLUT1 and GLUT4 mRNA expression in adipose tissues. GLUT1 (A) and GLUT4 (B) expression data are presented as a ratio to s16 expression. Values are expressed as means ± SE. * $p < 0.05$ compared to the N diet group; † $p < 0.05$ compared to saline. N+Saline (N = 12), N+HRP (N = 10), HF/HC+Saline (N = 13) and HF/HC+HRP (N = 9). PGF, peri-gonadal fat; PRF, peri-renal fat; SCF, abdominal subcutaneous fat.

Supplementary Tables

Table S1. Primer sequences for mice (m) and human (h) mRNA expression

Gene	Gene name	Forward primer sequence (5' → 3')	Reverse primer sequence (5' → 3')
m(P)RR	(pro)renin receptor	TTTGGATGAACTTGGGAAGC	CACAAGGGATGTGTCGAATG
mRen	Renin	AGCAAGGGCGAGGAACTGTTCACTG	GGTGGACAGGTAATGGTTGTCTGGG
mLep(13)	Leptin	GGGCTTCACCCCATTCTGA	GGCTATCTGCAGCACATTTTG
mRes(14)	Resistin	TCACTTTTCACCTCTGTGGATATGAT	TGCCCCAGGTGGTGTAAA
mMest/Peg1(15)	Mesoderm-specific transcript/paternally expressed gene 1	GTTTTTCACCTACAAAGGCCTACG	CACACCGACAGAATCTTGGTAGAA
mGlut1(16)	Glucose transporter 1	GCTGTGCTTATGGGCTTCTC	CACATACATGGGCACAAAGC
mGlut4(16)	Glucose transporter 4	ACATACCTGACAGGGCAAGG	CGCCCTTAGTTGGTCAGAAG
mTNFα	Tumor necrosis factor alpha	CACGCTCTTCTGTCTACTG	AAGATGATCTGAGTGTGAGG
mMCP-1	Monocyte chemoattractant protein-1	ATTGGGATCATCTTGCTGGT	CCTGCTGTTCACAGTTGCC
mF4/80	-	TGACAACCAGACGGCTTGTG	GCAGGCGAGGAAAAGATAGTGT

ms16(17)	40S ribosomal protein s16	ATCTCAAAGGCCCTGGTAGC	ACAAAGGTAAACCCCGATCG
hACE	Angiotensin converting enzyme	TGTGGAACGAGTATGCCGAGGC	GTGCCGTACTTCAGGGTGTGGTTG
hAT1R(18)	Angiotensin II type 1 receptor	ATGATTCCACCAGCGCCTGAC	GGTCCAGACGTCCTGCTGTCACT
h(P)RR	(pro)renin receptor	GCTCCCAGTGAGGAAAGAGTGTATAT	GCGCAAGGTGACTGAAAGG
h18s	40S ribosomal protein 18s	CTGAGAAACGGCTACCACATC	GGCCTCGAAAGAGTCCTGTAT

Table S2. Real Time PCR conditions for mice (m) and human (h) mRNA expression

Gene	Hold	Denaturation	Annealation	Elongation
m(P)RR, mRen	94°C 15 min	94°C 15 s	60°C 30 s	72°C 30 s
mLep	94°C 15 min	94°C 15 s	55°C 30 s	72°C 30 s
mRes	94°C 15 min	94°C 15 s	55°C 30 s	72°C 30 s
mGlut1, mGlut4	94°C 15 min	94°C 15 s	55°C 30 s	72°C 30 s
mMest/Peg1, ms16	95°C 5 min	95°C 5 s	60°C 10 s	-
mTNFα, mF4/80	95°C 10 min	95°C 15 s	58°C 30 s	72°C 30 s
mMCP-1	95°C 10 min	95°C 15 s	60°C 30 s	72°C 30 s
hREN, hAGT	50°C 2 min; 95°C 10 min	95°C 15 s	60°C 60 s	-
hACE, hAT1R and h(P)RR	94°C 15 min	94°C 15 s	60°C 30 s	72°C 30 s
h18s	94°C 15 min	94°C 15 s	59°C 30 s	72°C 30 s

Table S3. Renin level in adipose tissue compared to the kidney

	Normal diet		HF/HC diet	
	Saline	**HRP**	**Saline**	**HRP**
Ratio PGF/Kidney (%)	19 ± 9	13 ± 5	84 ± 6*	60 ± 13*
Ratio PRF/Kidney (%)	41 ± 16	50 ± 14	45 ± 8	31 ± 9
Ratio SCF/Kidney (%)	96 ± 11	75 ± 17	59 ± 10*	53 ± 12*

Values are expressed as means ± SE. * $p < 0.05$ compared to N diet. PGF, peri-gonadal fat; PRF, peri-renal fat; SCF, abdominal subcutaneous fat.

Table S4. Effect of HF/HC diet on female mice renin mRNA in adipose tissues.

	Normal diet (N = 8)	**HF/HC diet (N = 8)**
PGF (AU)	0.00 ± 0.00	$2.32 \times 10^{-1} \pm 1.06 \times 10^{-1}$*
PRF (AU)	$1.42 \times 10^{-1} \pm 1.42 \times 10^{-1}$	$2.51 \times 10^{-1} \pm 1.04 \times 10^{-1}$
SCF (AU)	0.00 ± 0.00	$5.04 \times 10^{-10} \pm 3.37 \times 10^{-10}$*

Values are expressed as means ± SE. * $p < 0.05$ compared to N diet. PGF, peri-gonadal fat; PRF, peri-renal fat; SCF, abdominal subcutaneous fat.

Table S5. Female mice body and adipose tissue weight.

	Normal diet (N = 8)	HF/HC diet (N = 8)
Initial body weight (g)	21.7 ± 0.6	21.5 ± 0.5
Final body weight (g)	25.2 ± 0.8†	34.2 ± 2.1§†
PGF (g)	0.76 ± 0.10	2.46 ± 0.40*
PRF (g)	0.43 ± 0.06	1.28 ± 0.16*
SCF (g)	0.57 ± 0.07	1.98 ± 0.17*

Values are expressed as means ± SE. * $p < 0.05$ compared to N diet; † $p < 0.05$ compared to initial weight. PGF, peri-gonadal fat; PRF, peri-renal fat; SCF, abdominal subcutaneous fat.

Table S6. Characteristics in human subjects for the CAO cohort study.

	IRO (N = 13)	ISO (N = 9)
Age (years)	56.0 ± 1.30	59.5 ± 0.94
BMI (kg/m^2)	34.06 ± 1.26	33.54 ± 0.69
Fat mass (g)	48.07 ± 0.71	47.06 ± 0.80
Waist to hip ratio	0.893 ± 0.020	0.870 ±0.012

Values are expressed as means ± SE. BMI, body mass index; IRO, insulin-resistant obese; ISO, insulin-sensitive obese.

Table S7. Weekly food consumption

	Normal Diet (kcal/week)		HF/HC Diet (kcal/week)	
Week	**Saline (N = 8)**	**HRP (N = 5)**	**Saline (N = 6)**	**HRP (N = 8)**
P-1-2	13.3 ± 0.8	12.2 ± 1.5	13.3 ± 1.5*	14.2 ± 0.6*
P-1-3	13.8 ± 0.7	13.7 ± 1.5	16.3 ± 1.3*	17.1 ± 0.5*
P-1-4	13.5 ± 0.9	13.0 ± 0.8	15.0 ± 1.2*	14.5 ± 0.4*
P-1-5	12.4 ± 0.5	13.5 ± 0.6	15.1 ± 1.1*	14.1 ± 0.4*
P-2-2	11.7 ± 1.5	11.7 ± 2.9	16.0 ± 1.4*	14.8 ± 0.5*
P-2-3	16.0 ± 1.3	16.5 ± 1.6	15.9 ± 1.1*	14.9 ± 0.4*
P-2-4	13.7 ± 0.7	13.1 ± 0.6	15.6 ± 1.4*	14.3 ± 0.5*
Total (kcal)	123.3 ± 3.8	121.0 ± 5.5	148.9 ± 9.9*	142.2 ± 3.8*

Values are expressed as means ± SE. * $p < 0.05$ compared to N diet.

Table S8. Effect of the HRP on left ventricular volume

	Normal Diet				HF/HC Diet			
	Saline (N = 6)		HRP (N = 5)		Saline (N = 7)		HRP (N = 8)	
Timeline	Baseline	End	Baseline	End	Baseline	End	Baseline	End
LVV;d (µl)	76.5 ± 5.3	87.4 ± 7.7	69.7 ± 10.7	89.3 ± 8.3	68.7 ± 3.8	102.4 ± 9.2*	70.7 ± 5.9	80.0 ± 7.3†
LVV;s (µl)	35.8 ± 6.0	50.3 ± 5.3*	33.2 ± 8.6	45.9 ± 5.8*	32.2 ± 2.5	60.1 ± 4.8*	35.2 ± 5.0	41.2 ± 3.2

Values are expressed as means ± SE. * $p < 0.05$ compared to Baseline; † $p < 0.05$ compared to saline. LVV, Left ventricular volume; d, diastole; s, systole.

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CHAPTER VII. ARTICLE 2: ADIPOSE TISSUE (P)RR REGULATES INSULIN SENSITIVITY, FAT MASS AND BODY WEIGHT

Authors: *Zulaykho Shamansurova, *Paul Tan, Basma Ahmed, Emilie Pepin, Ondrej Seda and Julie L. Lavoie

* These authors contributed equally to this work

Article type: Original article

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Problem: Could adipose tissue (P)RR regulate body weight, fat masses and insulin sensitivity.

Objective: Study the effects of the (P)RR through its gene deletion specifically in adipose tissue.

Citation:
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Contribution of authors:

Zulaykho Shamansurova – First author, did weekly assessments, Echo-MRI, Physioscan, metabolic cages, OGTT, IP-ITT, genotyping, necropsy, sample collection, extraction of RNA

and protein, PCR, qPCR, histology, data calculation and analysis, writing first version of manuscript. Results were shown in Figures 1, 2, 3, 5 and Supplementary Figure 1; in Table 1 and in Supplementary Tables – 1, 2, 3, 4, 5, 6, 7, 8.

Paul Tan – genotyping, necropsy, sample collection, Western Blot, plasma renin activity, manuscript revision. Results shown in Figures 4, Supplementary Figure 2, and in Table 1, Supplementary Tables 8.

Basma Ahmed – did qPCR for (P)RR gene tissue distribution. Results shown in Supplementary Figure 2.

Emilie Pepin – revision of manuscript with figures and tables, created Figure 6.

Ondrej Seda – coauthor, co-director of Basma Ahmed, revision of manuscript.

Julie L. Lavoie – Supervisor, leader of the project, leaded all the steps, revised manuscript, worked on manuscript submission, communication with the journal and correspondences.

Adipose tissue (P)RR regulates insulin sensitivity, fat mass and body weight

*Zulaykho Shamansurova[1,2,5], *Paul Tan[1,3,5], Basma Ahmed[1,2], Emilie Pepin [1,5], Ondrej Seda[1,6] and Julie L. Lavoie[1,4,5]

* These authors contributed equally to this work

[1]Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Departments of [2]Physiology, [3]Biochemistry and Molecular Medicine, and [4]Kinesiology of the Université de Montréal, [5]Montreal Diabetes Research Center, Quebec, Canada, [6]First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic.

Running title: Adipose (P)RR alters adipose tissue function

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Address for correspondence and reprint requests to:

Julie L. Lavoie, PhD

Centre de Recherche du CHUM

900 St-Denis Street, Tour Viger, R08.452

Montréal, Québec, H2X 0A9

Tel: (514) 890-8000 ext. 23612

Fax: (514) 412-7655

email: julie.lavoie.3@umontreal.ca

Abstract

Objective: We previously demonstrated that the handle-region peptide, a prorenin/renin receptor [(P)RR] blocker, reduces body weight and fat mass and may improve insulin sensitivity in high-fat fed mice. We hypothesized that knocking out the adipose tissue (P)RR gene would prevent weight gain and insulin resistance.

Methods: An adipose tissue-specific (P)RR knockout (KO) mouse was created by Cre-loxP technology using AP2-Cre recombinase mice. Because the (P)RR gene is located on the X chromosome, hemizygous males were complete KO and had a more pronounced phenotype on a normal diet (ND) diet compared to heterozygous KO females. Therefore, we challenged the female mice with a high-fat diet (HFD) to uncover certain phenotypes. Mice were maintained on either diet for 9 weeks.

Results: KO mice had lower body weights compared to wild-types (WT). Only hemizygous male KO mice presented with lower total fat mass, higher total lean mass as well as smaller adipocytes compared to WT mice. Although food intake was similar between genotypes, locomotor activity during the active period was increased in both male and female KO mice. Interestingly, only male KO mice had increased O_2 consumption and CO_2 production during the entire 24-hour period, suggesting an increased basal metabolic rate. Although glycemia during a glucose tolerance test was similar, KO males as well as HFD-fed females had lower plasma insulin and C-peptide levels compared to WT mice, suggesting improved insulin sensitivity. Remarkably, all KO animals exhibited higher circulating adiponectin levels, suggesting that this phenotype can occur even in the absence of a significant reduction in adipose tissue weight, as observed in females and, thus, may be a specific effect related to the (P)RR.

Conclusions: (P)RR may be an important therapeutic target for the treatment of obesity and its associated complications such as type 2 diabetes.

Keywords: (pro)renin receptor, renin-angiotensin system, adipose tissue knock-out mice, obesity, adipose tissue, insulin resistance

Abbreviations:

ANG, Angiotensin; BAT, brown adipose tissue; BB, beam break; HACT, horizontal activity; HFD, high-fat diet; HRP, handle-region peptide; KO, knock-out; ND, normal diet; OGTT, oral glucose tolerance test; PGF, perigonadal fat; PPAR-γ, peroxisome proliferator-activated receptor-γ; PRA, plasma renin activity; PRF, perirenal fat; (P)RR, prorenin/renin receptor; RAS, renin-angiotensin system; SE, standard error; SFC, abdominal subcutaneous fat; SM, skeletal muscle; SMG, submandibular gland; TG, triglycerides; V-ATPase, vacuolar proton pump H+-ATPase; VCO_2, carbon dioxide production; VO_2, oxygen consumption; WT, wild-type.

1. Introduction

Obesity and type 2 diabetes constitute worldwide pandemics [1,2]. Finding effective ways to prevent and treat these diseases is of major clinical and economic importance. The study of patients suffering from obesity and obese rodent models has shed light on the importance of the renin-angiotensin system (RAS) in the development of obesity [3,4]. In particular, adipose tissue RAS has been found to be increased in this condition, along with adipose tissue hypoxia and inflammation [5]. Those changes lead to compromised adipocyte function characterized by abnormal adipokine secretion and glucose and lipid metabolism, which together cause insulin resistance and increase the risk of developing type 2 diabetes [5]. Indeed, overexpression of angiotensinogen (Agt) in rodents, specifically in adipose tissue, causes obesity and insulin resistance, while systemic suppression of RAS genes protects from high-fat diet (HFD) induces obesity and improves insulin sensitivity [6]. In addition, clinical studies have shown that RAS inhibitors routinely used as anti-hypertensive treatments also improve insulin sensitivity and glucose homeostasis and prevent cardiovascular complications in diabetic patients [5].

The prorenin/renin receptor [(P)RR] has been shown to be a component of the RAS where its main role is to produce a non-proteolytic activation of prorenin and increase the catalytic activity of renin to cleave Agt into angiotensin I (Ang I) [7]. Ang I is then converted by the Ang converting enzyme to Ang II, the main physiologically active peptide of this system [8]. Ang II, via activation of many pathways, promotes cell proliferation, fibrosis, and apoptosis, which could lead to obesity-related complications, such as type 2 diabetes [9,10]. Simultaneously, binding of prorenin/renin to the (P)RR activates Ang II-independent pathways, including mitogen-activated protein kinases and tumor growth factor beta, which may also contributes to end-organ damage [11]. Unfortunately, the RAS inhibitors currently used clinically all cause a

compensatory increase in plasma renin concentrations and, as a result, may increase (P)RR Ang II-independent pathways as these drugs do not prevent renin from binding to the (P)RR [12,13]. Independently of prorenin/renin binding, the (P)RR (also known as ATP6AP2) acts as a adaptor to the cell membrane vacuolar proton pump H+-ATPase (V-ATPase), and consequently activates Wnt signaling [11]. Given that this signaling cascade is implicated in embryogenesis, whole-body suppression of the (P)RR is lethal [14,15]. Moreover, mice with specific (P)RR gene suppression in the heart or kidneys die at 2-4 weeks of age [16,17].

Our previous results showed that obese mice and insulin resistant obese women have increased adipose tissue (P)RR expression [18]. Furthermore, we demonstrated that systemic treatment of HFD-fed mice with the handle region peptide (HRP), a (P)RR blocker, reduces body weight gain and visceral fat masses and may improve insulin sensitivity [18], similarly to what is observed in models of RAS gene suppression [6]. The aim of the present study was to better understand the role of adipose tissue (P)RR in the metabolic effects induced by HRP treatment in the context of obesity using adipose tissue specific (P)RR KO mice.

2.0 Materials and Methods

2.1. Generation of adipose-tissue specific (P)RR KO mice

To produce mice with the (P)RR gene deleted specifically in adipose tissue, mice expressing the Cre-recombinase specifically in adipose tissue under the control of the AP2 promoter (AP2-Cre^{Salk} mice; [19,20]; #005069, Jackson Lab) were bred with mice with loxP sites flanking the (P)RR exon 2 between locus 2271-2276 ((P)RR-Loxp mice; a generous gift from Merck Frosst Canada) (**Supplemental Figure 1**). As the (P)RR gene is present on the X chromosome [11], male mice were homozygous KOs while female mice were heterozygous KOs.

2.2.Animals

Mice were placed in individual cages at 10 weeks of age and were maintained on a normal diet (ND; #2918, Teklad Global, Harlan Laboratories, Madison, WI, USA) and 12-h light/dark cycle. Starting at 12 weeks of age, mice were maintained on the ND or switched to a HFD (Bio-Serv F3282, Frenchtown, NJ, USA) for 9 weeks. Mice had access to water and food *ad libitum*. Body weight and food intake were measured weekly from 12 to 17 weeks of age. 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's Animal Care and Use Committee at the CHUM Research Center.

2.3.Mouse genotyping

Mouse genotypes were determined using genomic DNA from mouse tail snips extracted by hot-shot NaOH-EDTA protocol [21,22]. PCR were then performed using specific primers (IDT IL, USA) detailed in Supplementary Table 1. Each reaction contained 1µl 10X buffer, 0.2 µl 10mM dNTP, 0.1 µl of each primer, 6.4 µl of water, 0.5 µl Taq polymerase (Feldan, Bio-Basic, Markham, ON, Canada), and 2 µl of genomic DNA, as described previously [23]. PCR products were subsequently analyzed on 1% or 3 % agarose gels containing SYBR Green (Invitrogen by Life Technologies, Carlsbad, CA, USA).

2.4.Adipose tissue specificity of the KO model

The issue specificity of our KO model was confirmed by real-time PCR using the primers listed in **Supplementary Table 1**. For this purpose, 12 week-old male mice were sacrificed, and different white adipose tissue depots (abdominal subcutaneous fat (SCF) and visceral fat (perigonadal fat (PGF) and perirenal fat (PRF)) were collected, weighed, flash frozen, and

analyzed by real-time PCR. (P)RR gene expression was assessed in the different white adipose tissue depots as well as in other tissues such as, brown adipose tissue (BAT), heart, spleen, gonads, kidneys, submandibular gland, pancreas, liver, brain, adrenal glands, skeletal muscle, lung, diaphragm, and aorta.

2.5.Reverse transcription and qPCR gene expression

Tissue RNA was extracted using Trizol reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's protocol. To remove genomic DNA, RNA samples were incubated with deoxyribonuclease I (2U per µg RNA) for 30 min at 37°C. Single-stranded cDNA was synthesized by reverse-transcriptase reaction using SuperScript II Reverse Transcriptase (FisherScientific, Ottawa, ON, Canada). The RT-PCR final volume was 10 µl and contained 0.3 µmol/l of the specific forward (F) and reverse (R) primers [24] listed in Supplementary Table 1, as well as 2.5 µl of single-stranded cDNA template. The amplification was done using Faststart SYBR Green Master fluorescent dye (04 673 492 001; ROCHE, Mississauga, ON, Canada) using the Rotor Gene RG-3000 (Corbett Research).

2.6.Survival rate and fetal weight

We compared fetal weights in litters obtained from 8 pregnant mice (3-11 pups/litter), produced using the breeding protocol mentioned above, on the 17th day of gestation.

2.7.Body composition

Fat and lean body masses were analyzed using an Echo-MRI-100TM apparatus (Echo Medical Systems, Houston Scientific, Houston, TX, USA) both at the beginning and at the end of the protocol (at 12 and 20 weeks of age, respectively).

2.8.Mouse locomotor activity and indirect calorimetry

Mouse locomotor activity and metabolic parameters were studied in metabolic cages at 18 weeks of age (AccuScan Instruments, Columbus, OH, USA), and data were analyzed according to Ferrannini et al., 1988 [25]. After 3 days of acclimation, locomotor activity was evaluated by infrared beam interruptions by mouse movement in the horizontal and vertical axis, while metabolic parameters were assessed (oxygen consumption (VO_2) and carbon dioxide production (vCO_2)) for 24 hours. Data were summarized for both the light and dark cycles [26].

2.9.Oral glucose tolerance test (OGTT)

At 20 weeks of age, an OGTT was performed after overnight (16-17h) fasting. Dextrose (Hospira, Inc. Lake Forest, Il, USA) was administered orally by gavage at a dose of 2 g/kg for ND-fed mice or 5 g/kg for HFD-fed mice [27–29]. Blood was collected from the tail vein at baseline (T0) and 15, 30, 45, 60, and 120 minutes after gavage using a glucometer (Accu-Chek Performa, Roche, Indianapolis, IN, USA). Plasma was also collected at baseline (T0) and 30 minutes after gavage using glass capillaries (Precision, MO, USA), then transferred into BD vacutainer tube and separated by centrifugation (Becton, Dickenson, Mississauga, ON, Canada) for measurement of insulin and C-peptide levels, as described below.

2.10. Mouse necropsy and organ collection

At the end of the protocol (21 weeks of age), mice were anesthetized, blood was collected by intrathoracic cardiac puncture using the anticoagulant EDTA. Plasma was then isolated by centrifugation and stored at -80ºC. White adipose tissue (SCF, PGF and PRF) and other organs (BAT, liver, pancreas, and skeletal muscle) were collected and weighed. All samples were flash

frozen and kept at -80ºC until further analysis. Tibia was also collected and its length was measured using a caliper.

2.11. Circulating parameters

Plasma triglycerides (TG) and glycerol were measured using a Serum Triglyceride Determination Kit (Sigma, Oakville, Canada) following the manufacturer's protocol. Plasma insulin, C-peptide, adiponectin and leptin levels were measured using bead-based AlphaLISA immunoassay kits (Perkin Elmer, Waltham, MA) according to the manufacturer's instructions. Plasma renin activity was calculated by measuring Angiotensin I production by immunoassay kit (Diagnostic Biochem Canada, Dorchester, ON, Canada).

2.12. Tissue TG and glycerol content

Frozen liver samples were powdered, and 40-60 mg were used for the assay. TGs were extracted by homogenization and incubation with 0.5M KOH in ethanol for 20 min at 70ºC. Supernatant was separated with 0.15M $MgSO_4$ solution by centrifugation at 5000 rpm for 5 min. TGs were measured as described above for plasma TG. Glycerol and TG levels in skeletal muscle (soleus and plantaris combined) were measured as for the liver, with prior extraction in 20 mMol TRIS-HCL buffer containing protease inhibitors [30,31].

2.13. Total pancreatic insulin content

Pancreatic insulin content was measured by extraction of frozen whole organ powder in acidic ethanol as described [32]. Pancreatic acid–ethanol extracts were centrifuged at 3000 g for 15 min at 4°C and supernatants were collected. After an additional centrifugation, pancreas insulin

was measured as described above for insulin in plasma. Results were normalized by tissue weight.

2.14. Histology and immunohistochemistry

Adipose tissue was fixed in 10% formalin, embedded in paraffin, and sectioned using a microtome. Hematoxylin-Eosin stained slides were used for light microscopy. Embedding, sectioning, and staining were performed by the histology platform of the Research Institute in Immunology and Cancerology at the Université de Montréal. Adipocyte size was calculated in defined 2.33 x 1.73 μm square in 3 randomly selected digital photographic images taken for each fat pads per mouse under the 100X magnification using an Olympus 600 (MA, USA) light microscope and "Q-capture" software. Data were analyzed using Image-J software [33,34]. A subset of samples was deparaffinized, using 3 consecutive incubations in xylene for 5 minutes, and then rehydrated with 95% ethanol for 5 minutes. Slides were then heated in a microwave for 10 min in 10 mmol/L sodium citrate (pH 6.0) for antigen retrieval, incubated with 3% hydrogen peroxide for 20 min, blocked with 5% BSA (Roche Diagnostics, Germany) in 1.0 ml PBS for 30 min at room temperature (RT), and then incubated with goat anti-(P)RR antibody (#SAB2500134, Sigma Aldrich, Missouri, USA) at a 1:25 dilution in 1% BSA for 2 h at room temperature (RT). Finally, we used Alexa Fluorescent 555-Donkey anti-goat IgG (#AB150134, Life Technology, NY, USA) at a 1:200 dilution in 1% BSA for 1 h at RT. Signals were observed using an immunoluminescence microscope (Nikon eclipse TE2000-5) [35] and were digitalized using a QICAM imaging system.

2.15. Statistical analysis

All data are presented as mean ± standard error (SE). Differences between the groups for single measurements were evaluated by t-test. Two-way repeated measure ANOVA was used for analysis when comparing groups and different time points. When a significant interaction was detected, a Tukey post-hoc analysis was performed. Data were considered to be statistically significant when $p < 0.05$.

3. Results

3.1. Validation of AP2-Cre/(P)RR flox KO specificity

(P)RR gene deletion was first analyzed by real-time PCR in complete KO and WT ND-fed male mice in the various adipose depots and additional tissues (**Supplemental Figure 2a and b**). We found that the (P)RR gene expression was significantly decreased in KO mice compared to WT mice in all white adipose tissue: PGF (37%), PRF (49%) and SCF (51%) (**Supplemental Figure 2a**). Conversely, no differences were observed in BAT or in any other tissues studied (**Supplemental Figure 2a and b**). These results confirmed the tissue specificity of our KO model. The adipocyte specificity was then validated using immunofluorescent staining of histological sections. Indeed, both male and female KO mice had reduced fluorescent signals (close to no signal in males) in adipocyte membranes of all white adipose tissue analysed compared to WT (**Supplementary Figure 3**).

3.2. Male KO mouse survival rate is lower compared to females during development

The litter size was assessed in 8 ND-fed dams at day 17 of gestation. We found significantly more female than male pups (66.2 ± 3.7% vs 33.8 ± 3.7%), suggesting that complete deletion

of the (P)RR gene in adipose tissue can induce some lethality during embryogenesis. However, despite the reduced number of male pups per litter, male fetal body weights were, in accordance with the literature, in general 2 to 7% heavier than female pups without any effect of genotype (**Supplementary Table 2**) [36].

3.3. Adult KO mice weigh significantly less than WT mice

Despite a similar food intake (**Supplementary Table 3**), both male and female adult KO mice weighed significantly less than their WT littermates (**Figure 1A and B**). Indeed, body weight was 23% lower in males on ND and 10% lower in females on both ND and HDF diet at 17 weeks of age (**Figure 1B and C**). No other visible physical differences could be observed between the KO and WT groups.

3.4. Adult KO male mice are smaller than WT mice

We next investigated whether the (P)RR gene deletion might impact their growth at 21 weeks of age. First we compared mouse size and found that the tibia length of male KOs was 3% shorter than those of WT littermates (**Supplementary Table 5**). Next, we compared different organ weights and found that male KO mice had significantly lower liver weight expressed as a ratio to tibia length (13%) compared to WT, while heart weight was not different (**Supplementary Table 5**). Conversely, no differences in tibia length and organ weight could be observed in female mice on either ND or HFD (**Supplementary Table 5**).

3.5. Leaner body composition of adult male KO mice

ND-fed male KO mice were found to have significantly lower fat mass normalized to body weight at the beginning (37%; 12 weeks of age) and at the end (54%; 21 weeks of age) of the

experiment compared to WT littermates as assessed by EchoMRI (**Figure 1D**). We also found that lean body mass in ND-fed male KO mice was increased by 5% and 11% at the beginning and end of experiment respectively (**Figure 1G**). Conversely, no differences in total fat and lean body masses were observed in female mice, either on ND or HFD (**Figure 1E, F, H, I**).

3.6. Adult KO mice have lighter fat pad masses than WT mice

All fat pad masses were found to be significantly lower (32-53%) in male KO mice compared to WT mice fed ND at 21 weeks of age (**Figure 2A**). ND-fed female KO mice presented with a significant decrease in SCF (32%) and BAT (21%) weight, whereas PRF tended to be smaller ($p = 0.073$) but without reaching significance while PGF was unchanged (**Figure 2A**). HFD-fed KO female mice showed no significant decrease in any of the fat pad masses compared to WT (**Figure 2A**).

3.7. Adipocytes are smaller in adult male KO mice compared to WT

Interestingly, adipose tissue histology demonstrated that adipocytes from visceral fat pads of male KO were significantly smaller in size (40-47%) compared to those from male WT while only a trend was observed in adipocytes from SCF fat pads ($p = 0.097$) (**Figure 2B and C**). In contrast, no significant differences in adipocyte size were observed in all fat pads from female KO mice compared to WT fed either diet (**Supplementary Table 4**). As expected, female WT and KO mice fed a HFD had larger adipocytes compared to ND (**Supplementary Table 4**).

3.8. KO mice display greater locomotor activity and metabolic rate

Both male and female KO mice fed a ND were significantly more active during their active period (dark cycle), males by 37% and females by 24%, compared to WT mice as a result of

increased horizontal activity (**Figure 3A and D**), while there was no change in vertical activity (**Supplementary Table 6**). In contrast, we observed no changes in physical activity during their inactive period (light cycle) (**Figure 3A and D)**. In addition, the changes in physical activity observed in male KOs were associated with a significant increase in oxygen consumption (25 and 27%) and in carbon dioxide production (22 and 26%) during the both the dark and light cycles (**Figure 3B and C**). Conversely, these parameters were unchanged in female mice (**Figure 3E and F**).

3.9. ″Beiging″ of adipose tissue may occur in KO mice

″Beiging″ is the presence of brown-like adipocytes within white adipose tissue; these brown-like adipocytes are characterized by increased mitochondrial density and small lipid droplets [37]. In male KO mice compared to WT littermates, the mRNA expression of *Prdm16*, a marker of ″beiging″, was increased by 151% in PRF and decreased by 70% in SCF while it was unchanged in PGF (**Figure 4A**). *Prdm16* mRNA expression was similar in all fat pads in female mice fed either ND or HFD (**Figure 4B**).

3.10. KO mice have improved insulin sensitivity compared to WT

Since (P)RR KO mice are leaner, we hypothesized that they might have improved glucose tolerance and insulin sensitivity. We therefore performed an OGTT, and surprisingly, KO mice had similar glucose tolerance in response to an oral bolus of glucose (**Figure 5A - C**). However, male KO mice had markedly lower plasma insulin and C-peptide levels compared to their WT littermates, both at baseline and 30 minutes after gavage (40-45% and 29-51%, respectively) (**Figure 5D and G**), indicative of improved insulin sensitivity. Similar observations could be

made in HFD-fed female KO mice (**Figure 5C, F and I**). However, no changes in glucose tolerance, plasma insulin or C-peptide levels could be observed in ND-fed female KO mice (**Figure 5 B, E, H**). Given that the insulin/C-peptide ratios were comparable in all groups (**Supplementary Table 7**), this strongly suggests that (P)RR deletion in adipose tissue does not affect pancreatic insulin secretion. This is further supported by the fact that total pancreas insulin content was similar between KO and WT mice fed either diets (**Supplementary Table 8**).

3.11. KO mice have different circulating adipokine levels compared to WT

As expected given their important decrease in total fat masses (**Figure 1D**), leptin levels were significantly lower (3-fold) in male KO mice compared to WT males (**Table 1**). While no differences could be observed between female KO and WT mice fed either ND or HFD, plasma leptin levels were increased by HFD as expected. Interestingly, we found that circulating adiponectin levels were significantly 1.5-fold higher in male KO mice and 1.4-fold higher in ND and HFD-fed female KO mice compared to their WT littermates (**Table 1**), which correlates with the improved insulin sensitivity observed during the OGTT.

3.12. Adipose tissue specific (P)RR deletion does not affect circulating lipid levels

Since KO mice presented with lower total fat mass compared to WT mice, we evaluated whether this translated to altered plasma TG levels. However, plasma TG levels were similar in all KO and WT mice (**Table 1**).

3.13. KO mice have decreased TG content only in skeletal muscles

After adipose tissue, liver and skeletal muscles are two major lipid storage sites [38]. Thus, we verified if (P)RR deletion led to an aberrant storage of lipids in these tissues given their reduced

adipose tissue weights. We found that liver TG content was similar between KO and WT mice in both males and females (**Supplementary Table 8)**. In contrast, KO male mice were found to have lower skeletal muscle glycerol (25%) and TG content (61%), compared to WT males fed a ND while no differences could be observed in females fed either diets (**Supplementary Table 8**).

3.14. Adipose tissue specific (P)RR deletion does not affect plasma renin activity

We found no differences in plasma renin activity between WT and KO mice (**Table 1**), demonstrating that the (P)RR KO in adipose tissue does not affect circulating RAS activity and as such, systemic RAS modulation did not contribute to the phenotypes observed in KO mice.

4. Discussion

To study the role of adipose tissue (P)RR gene deletion on energy balance, we generated adipose tissue specific KO animals by Cre-loxP technology and confirmed the specificity of the model by evaluating (P)RR expression in different tissues as well as adipocyte specific protein expression in all adipose tissues studied**.** The rate of gene deletion observed are in line with previous studies which have shown that the AP2-Cre^{Salk} mice generated a 50-80% labeling when breeding with a fluorescent reporter gene line **[**19**]**. This moderate deletion, however, allows us to make a better link between the phenotypes observed and the effects of the (P)RR gene. **Figure 6** summarizes the metabolic alterations induced by adipose tissue (P)RR deletion. Interestingly, both male and female adipose tissue specific (P)RR KO mice had decreased body weights, which were associated with decreased fat masses without any change in food intake. As this phenotype was less pronounced in female mice, given that they are hemizygous KO, total fat

mass and adipocyte size were decreased significantly only in male mice. Importantly, this did not result in increased circulating or liver lipid levels but was associated with reduced skeletal muscle TG content but only in male mice. This leaner phenotype could be caused in part by the increased locomotor activity observed in both male and female KO mice during their active period. Furthermore, the more accentuated phenotype observed in males may result from an increase in basal metabolic rate shown by the elevated O_2 consumption and CO_2 production both during their active and inactive period while locomotor activity is unchanged during their inactive period.

It is know well known that the (P)RR has both Ang II-dependent (by increasing prorenin and renin catalytic activity and therefore Ang II production) and Ang II-independent effects (by its intracellular signaling and adaptor function to the V-ATPase). Of note, a decrease in (P)RR expression leads to a decrease in Ang II production while KOs of other components of the RAS (such as Agt KO) totally abrogate Ang II production. Our observations are in line with adipose tissue and systemic KOs of different components of the RAS and various RAS pharmacological inhibitors studied in rodents which reported decreased body weight and fat masses. In addition, although it has not been evaluated with RAS inhibitors, our data are similar to RAS KOs, which also present with increased physical activity and basal metabolic rate [6]. In line with this literature, overexpression of Agt specifically in adipose tissue produces an increase in weight gain along with a decrease in physical activity [6]. In contrast, adipose tissue-specific Agt KO mouse models do not have any metabolic alterations [39,40] although this may result from the uptake of circulating Agt or Ang II into the adipose tissue, which would compensate for the local deletion. Given that our deletion of the (P)RR gene induces similar but not identical

changes in body weight compared to other RAS-KO mouse models (where Ang II production is abrogated), our results strongly suggest that the (P)RR KO effects results from both Ang II-dependent and -independent signalling.

Adipose tissue-specific modulation of the RAS can directly affect fat mass development. Indeed, it has been shown that Ang II can favor adipogenesis and lipogenesis through the activation of the AT2R and inhibits lipolysis through its binding to the AT1R, therefore promoting lipid accumulation [41]. As such, by decreasing the expression of adipose tissue (P)RR, we may have caused a decrease in renin and prorenin catalytic activity, leading to a reduction in Ang II production, which consequently lowered lipid accumulation, as observed in our model. In addition to the reduction in fat mass, we are the first group, to our knowledge, to show that a reduction in (P)RR expression in adipose tissue can contribute to the browning of white adipose tissue, which is functionally reflected by the increased resting and active metabolic rate of our male KO animals in addition to the elevation in PRDM16 expression in visceral fat pads. Furthermore, the higher lean body mass observed in male KO mice may also contribute to this elevated metabolic rate. Indeed, the 29% higher total lean mass observed in these mice may result from an increased muscle mass due to their elevated locomotor activity, and increased muscle mass is typically associated with an increased metabolic rate [42]. In addition, this increased basal metabolic rate may be caused in part by white adipose beiging, which is suggested by the observed increase in Prdm16 in our study. These results are in line with previous results obtained by our group in which systemic (P)RR inhibitor administration caused an increase in the expression of 'beiging' markers in white adipose tissue potentially through the activation of PPARγ (**Figure 6**) [43]. It also confirms the implication of the RAS in

the regulation of thermogenesis initially demonstrated by Takahashi *et al* in which whole body renin KO mice demonstrated elevated metabolic rate associated with an increased expression of uncoupling protein 1 in adipose tissues and, as a result, increased thermogenesis [44]. Interestingly, beiging of white adipose tissue has not been observed in other RAS KO models. As such, the observations in the renin KO mice may result from a link with the (P)RR.

In addition to the smaller fat mass, lack of (P)RR leads to a "healthier" adipose tissue structure. Indeed, larger adipocyte size has been linked with insulin resistance and development of obesity in humans and animal models [45]. Also, increased Ang II has been associated with increased adipocyte size and number, which would contribute to the development of obesity [46–48]. In line with these data, many RAS KO animals have been reported to have smaller adipocytes [44,49,50]. Hence, the smaller adipocyte size observed in our male KO mice may result from reduced Ang II production given the absence of the (P)RR. However, this decreased adipocyte size was not observed in our female KO mice, not even in those fed a HFD. Thus, this may explain why differences in insulin responses and fat pad size are more pronounced in male compared to female KO mice.

In line with the 'healthier' adipose tissue structure, our data suggest that (P)RR KO mice (males fed a ND and females fed a HFD) have better insulin sensitivity than WT mice. Indeed, although we observe no change in the glucose tolerance during an OGTT, circulating insulin concentrations were reduced. The reduced fat mass, increased lean body mass, and plasma adiponectin levels found in the KO mice might have contributed to the increased insulin sensitivity. Interestingly, although we found no modification in many parameters, for instance

fat mass and glucose homeostasis, nonetheless, in female mice fed a ND, we observed a modification in circulating adiponectin. This suggests that the change may be an initiating factor in the modulation of glucose homeostasis by (P)RR that precedes the significant changes in fat pad mass. Furthermore, this is in line with our recent publication which reported that systemic HRP administration to HFD fed mice can improve circulating adiponectin concentration. This is also in agreement with previous publications showing that Ang II decreases adiponectin gene expression and plasma levels, whereas RAS inhibitors increase plasma adiponectin levels [51,52]. However, in other RAS suppression models, both glucose and insulin levels were decreased to produce an improved glucose homeostasis [42,44,49,50,53]. More recently, Wu *et al* reported that adipose tissue (P)RR KO mice have no change in glucose tolerance when fed a ND but show increased plasma insulin levels [54]. This change in insulin levels, which is different from our observations, could be attributed in part to the severe lipodystrophy and liver steatosis present in their model, leading to a decrease in insulin clearance [54,55]. Indeed, by using the adiponectin-Cre, known for its high efficiency in gene deletion and robust adipose-tissue specificity [19], Wu *et al* demonstrate the effect of a more severe (80% decrease) (P)RR deletion while our model shows a dose-dependent effect (as differences in phenotypes can be observed between the hemizygous KO females and the homozygous KO males) induced by a milder (between 30 and 50%, respectively) decrease in (P)RR gene expression as we used the AP2-Cre mouse. These levels of deletion are in line with a previous study, which demonstrated that the AP2-Cre^{Salk} mice generated a 50-80% labeling when breeding with a fluorescent reporter gene line while the Adiponectin-Cre generated a near 100% recombination [19].

In line with the developmental defects associated with (P)RR KOs described in the literature [14,15], we found that adipose-tissue specific (P)RR deletion led to a 50% reduction in the expected number of KO male pups born. Given that this effect has not been reported in adipose-specific RAS KO models, we propose that Ang II-independent effects mediated by the (P)RR may play a role in fetal development, due to its association with V-ATPase and its concomitant regulation of the Wnt-signaling cascade. Moreover, in addition to their lower adiposity, we have shown that male KO mice are markedly shorter and have reduced liver weight, despite a similar food intake, compared to WT male mice. We did not observe such differences in hemizygous female (P)RR KO mice possibly because the gene deficiency is only partial.

Interestingly, the data presented here are very much in line with those previously published by our group which have shown that the administration of HRP, a (P)RR blocker, to mice fed a HFD also reduces body weight gain through decreased fat mass and seems to improve insulin sensitivity by improving adiponectin profile [18]. Taken together, our results suggest that adipose tissue (P)RR is involved in the development of obesity and its associated complications. Indeed, suppression of the (P)RR specifically in adipose tissue produced a clear phenotype of reduced body weight and fat masses as well as improved adipose tissue structure and insulin sensitivity. This indicates that adipose tissue (P)RR may be an important target for the development of new drugs for the treatment of obesity and type 2 diabetes. For instance, a more effective approach for the treatment of diabetes and its complications could be to combine the classical RAS inhibitors with a (P)RR blocker such as the HRP. This would block the Ang II-independent pathways, further reduce Ang II-dependent pathways, and prevent associated end-organ damage. However, in line with the study from Wu *et al*, it would be of the upmost

importance to carefully monitor the downregulation of the (P)RR activity to prevent lipodystrophy and the concomitant liver steatosis.

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Conflict of Interest. The authors have no conflict of interest to declare.

Supplementary information is available on the Molecular Metabolism's website.

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Table 1. Effect of adipose tissue (P)RR gene deletion on circulating parameters.

Gender	**Male**		**Female**			
Diet	**ND**		**ND**		**HFD**	
Genotype	**WT**	**KO**	**WT**	**KO**	**WT**	**KO**
PRA (ng Ang/mL/h)	25.4 ± 3.0	21 ± 4.1	40.8 ± 6.7	39.0 ± 6.3	40.7 ± 11.4	37.1 ± 10.7
Leptin (ng/mL)	30.35 ± 6.55	10.26 ± 3.07†	10.17 ± 1.77	11.25 ± 2.07	131.89 ± 25.82	121.07 ± 20.61
Adiponectin (pg/mL)	1.98 ± 0.08	2.92 ± 0.13†	1.42 ± 0.05	1.82 ± 0.05†	1.07 ± 0.10	1.46 ± 0.05†
Triglycerides (mg/mL)	7.82 ± 1.71	7.82 ± 1.71	3.74 ± 0.43	2.73 ± 0.37	3.54 ± 0.47	4.62 ± 0.75

Data are presented as mean ± SE with n = 8-15 per group. † $p < 0.01$ compared to WT. HFD, high-fat diet; KO, knock-out; ND, normal diet; PRA, plasma renin activity; (P)RR, (pro)renin receptor; WT, wild-type.

Figures

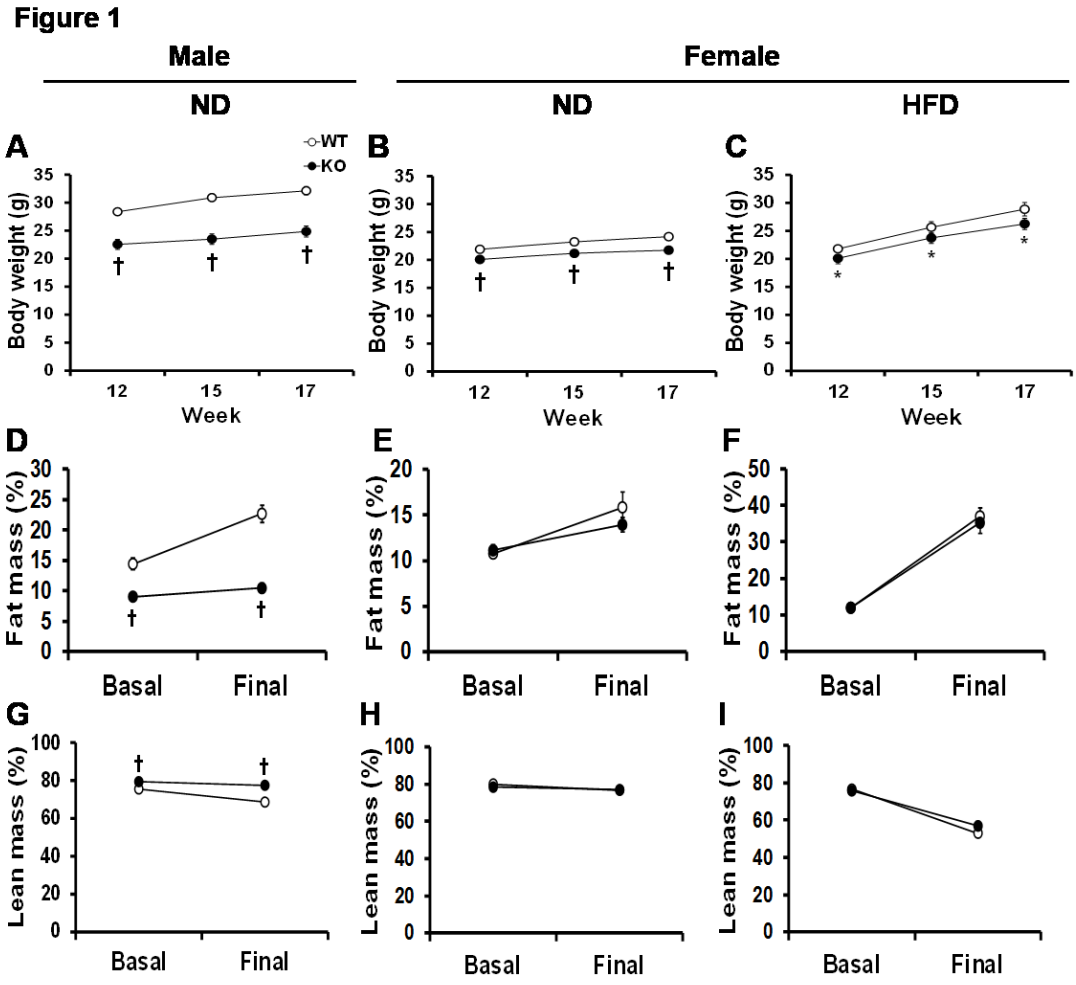


Figure 1. Adipose tissue (P)RR gene deletion decreases body weight and alters body composition in male mice. Body weight in male mice on ND (A) and female mice on ND (B) and HFD (C) at the beginning (12 weeks), in the middle (15 weeks) and at the end (17 weeks) diet period. Fat mass and lean mass in male mice on ND (D, G) and in female mice on ND (E and H) and HFD (F and I) at 12 (basal) and 20 (final) weeks of age. Data are presented as mean ± SE with n = 6-21. * $p < 0.05$ and † $p < 0.01$ compared to WT. HFD, high-fat diet; KO, knock-out; ND, normal diet; WT, wild-type.

Figure 2

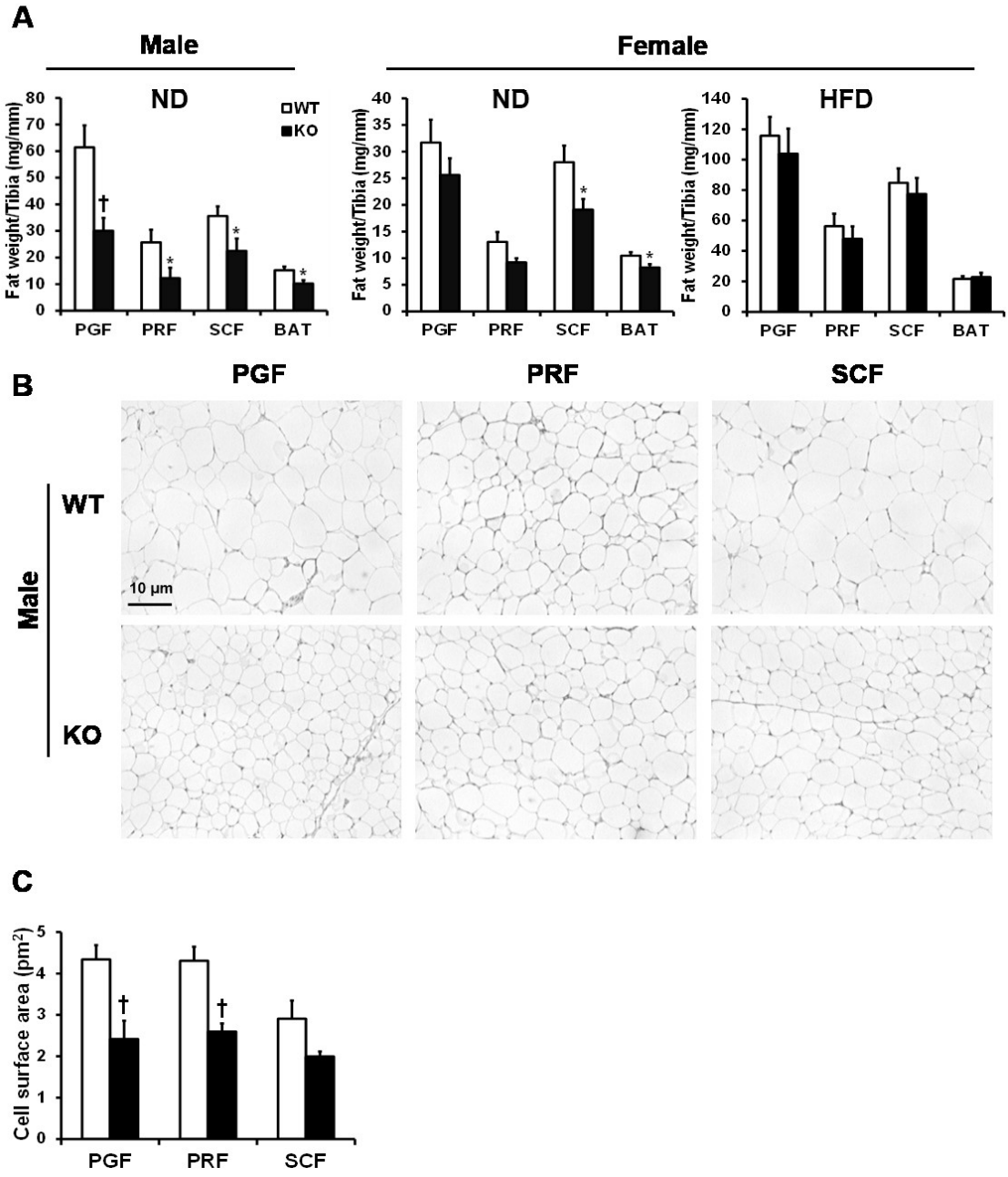


Figure 2. Adipose tissue (P)RR gene deletion decreases fat pad weight in male and female mice on ND and visceral adipocyte size in male mice. Weight of different fat pads in male and female mice on ND and female mice HFD (A). Representative histological sections of visceral and subcutaneous fat pads stained with Hematoxylin-Eosin (scale bar = 10 µm, magnification = x100) (B). Quantification of mean adipocyte size from histological sections

(C). Data are presented as mean ± SE with n = 7-16. * $p < 0.05$ and † $p < 0.01$ compared to WT. BAT, brown adipose tissue; HFD, high-fat diet; KO, knock-out; ND, normal diet; PGF, perigonadal fat; PRF, perirenal fat; SCF, abdominal subcutaneous fat; WT, wild-type.

Figure 3

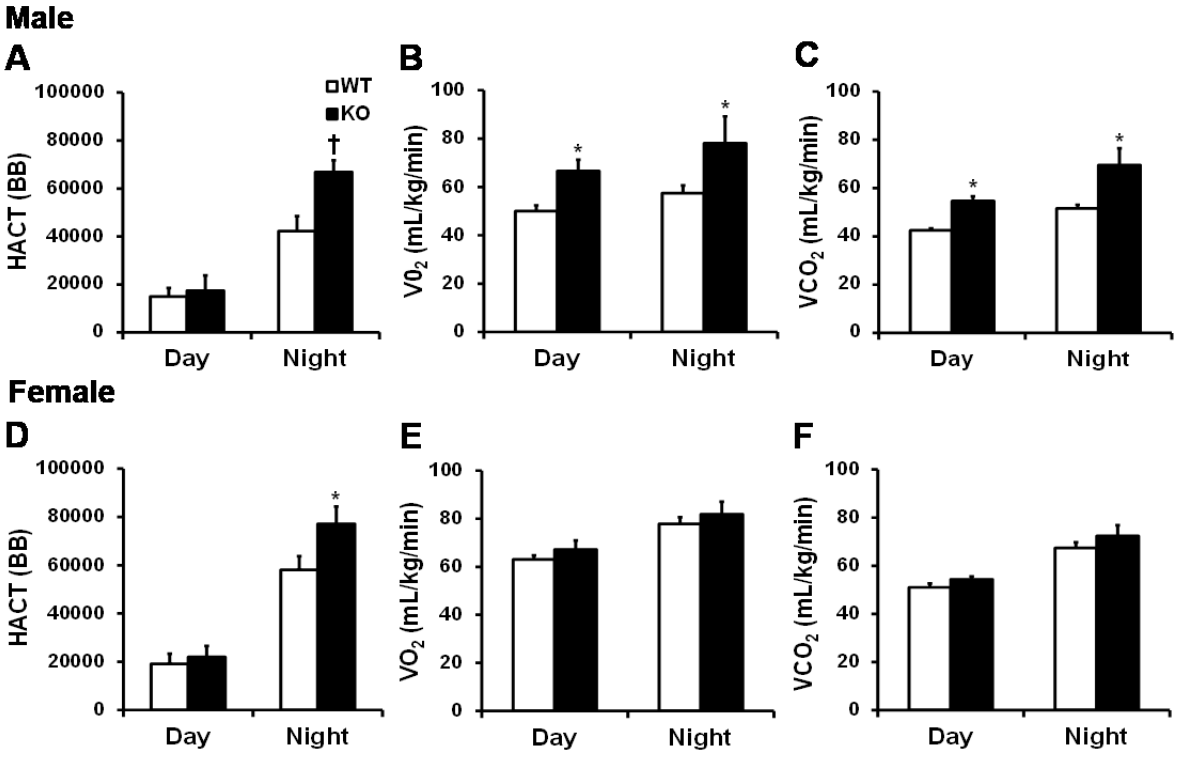


Figure 3. Adipose tissue (P)RR gene deletion increases energy expenditure in male mice. Locomotor activity and metabolic rate (depicted as O_2 consumption and CO_2 production) in male (A, B and C) and female (D, E, and F) mice in light (inactive) and dark (active) cycles. Data are presented as mean ± SE with n = 6-11 per group. * $p < 0.05$ and † $p < 0.01$ compared to WT. BB, beam break; HACT, horizontal activity; VCO_2, carbon dioxide production; VO_2, oxygen consumption; KO, knock-out; WT, wild-type.

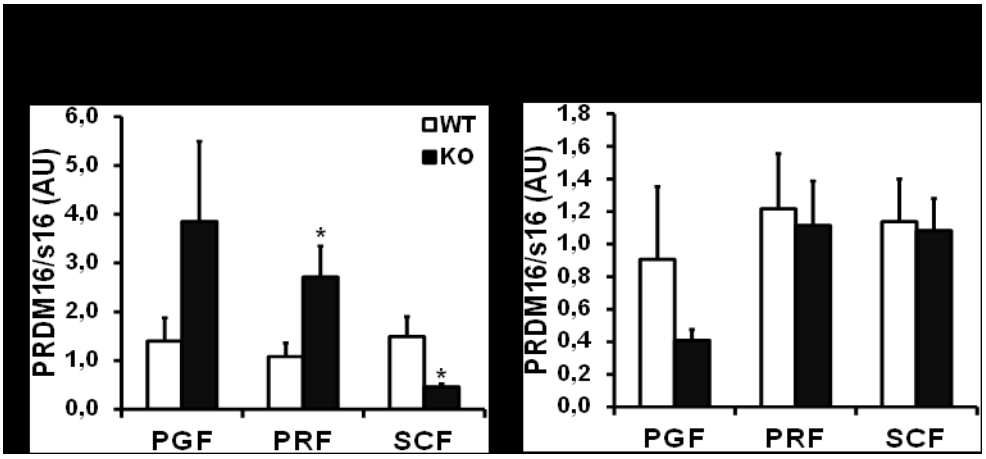


Figure 4. Adipose tissue (P)RR gene deletion increases a marker of ″beiging″ in PRF of male mice. PRDM16 mRNA levels in adipose tissue of male (A) and female (B) mice fed ND. Data are normalized to s16 mRNA levels and are presented as mean ± SE with *n* = 4-10 per group. * $p < 0.05$ compared to WT. KO, knock-out; PGF, perigonadal fat; PRDM16, PR domain containing 16; PRF, perirenal fat; SCF, abdominal subcutaneous fat; WT, wild-type.

Figure 5

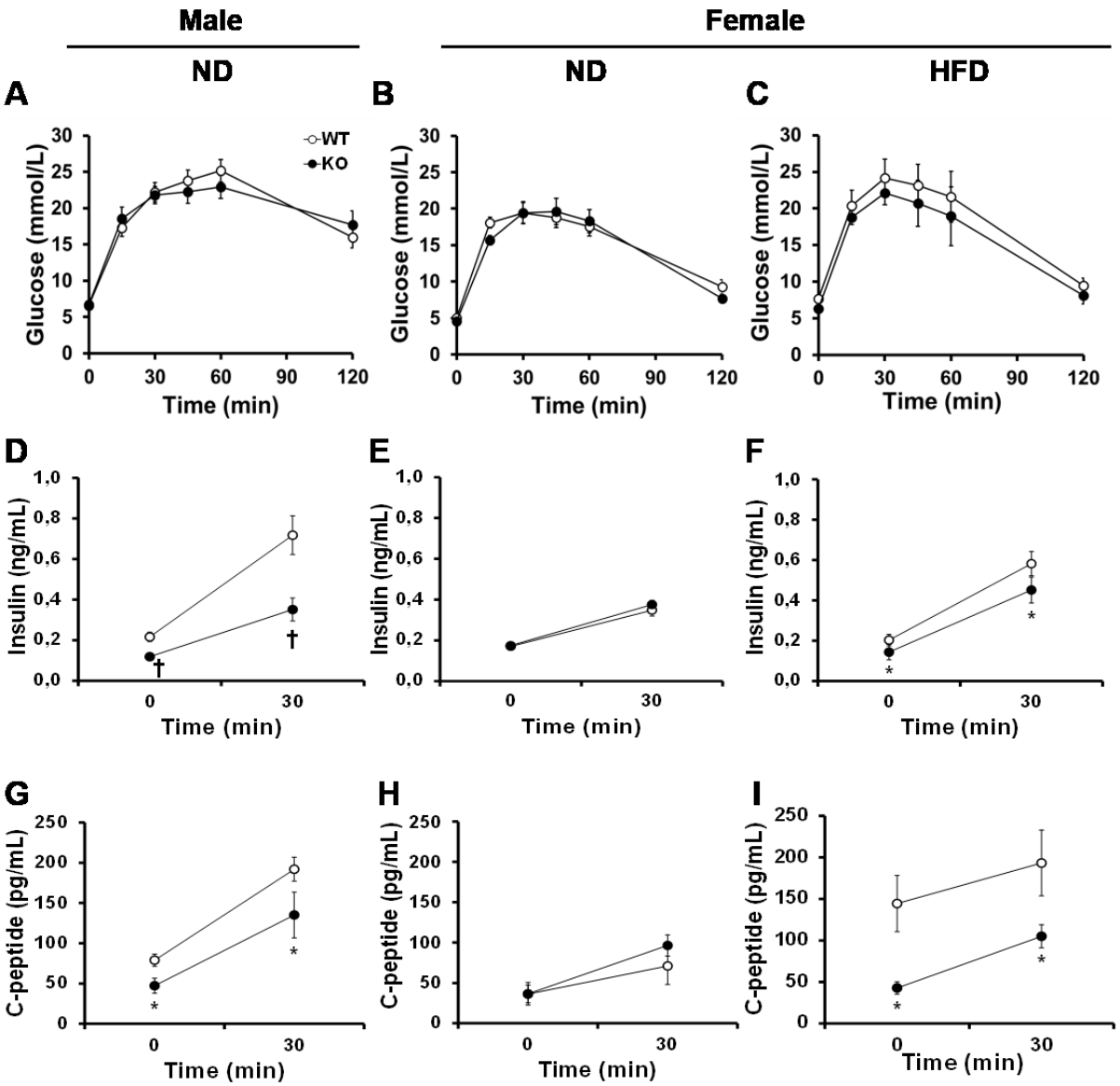


Figure 5. Adipose tissue (P)RR gene deletion leads to increased insulin sensitivity in male mice. Plasma glucose levels during an OGTT (A, B, C). Basal and stimulated plasma insulin (D, E, F) and C-peptide (G, H, I) levels. Data are presented as mean ± SE with *n* = 9-14 per group. * $p < 0.05$ and † $p < 0.01$ compared to WT. HFD, high-fat diet; KO, knock-out; ND, normal diet; WT, wild-type.

Figure 6

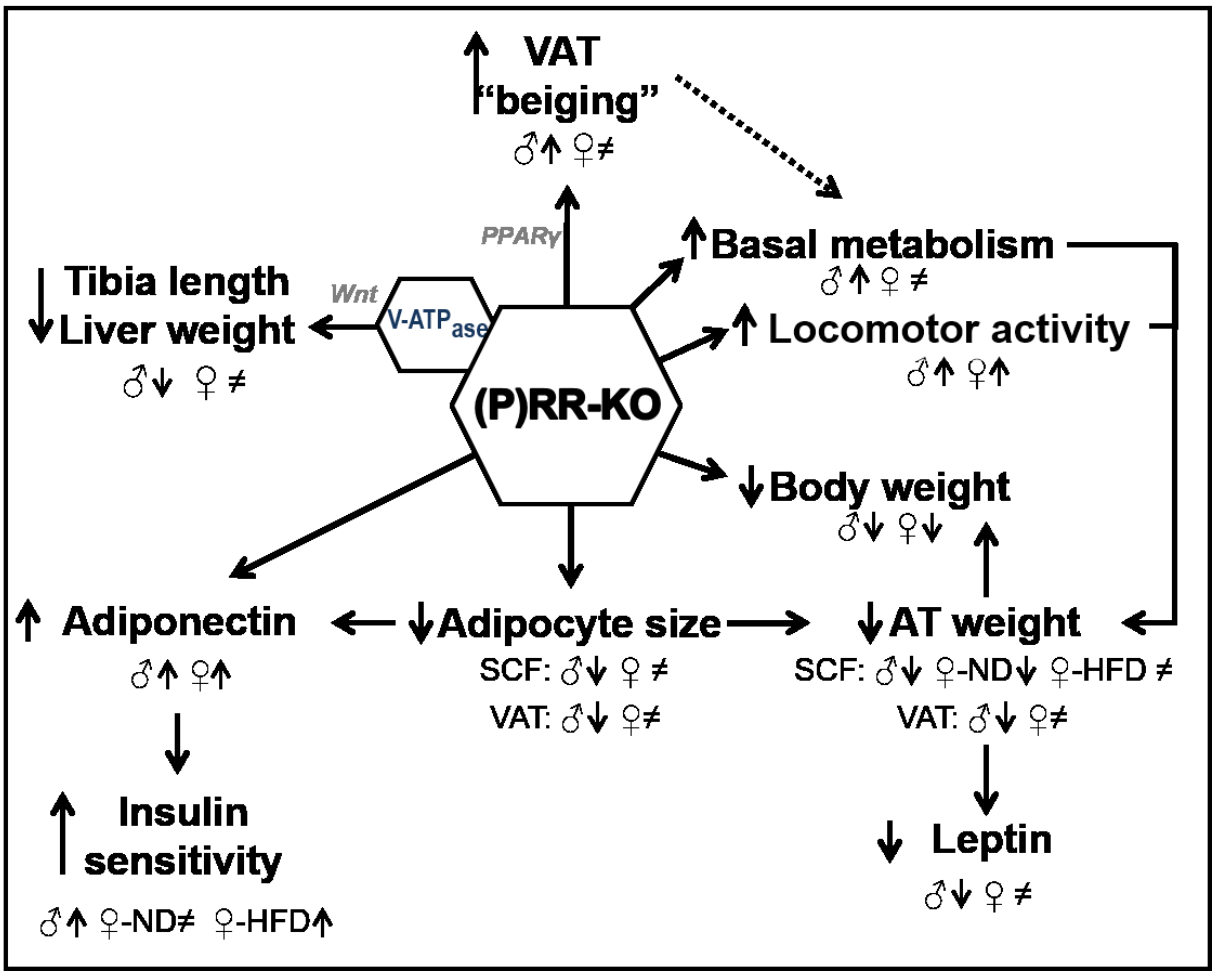
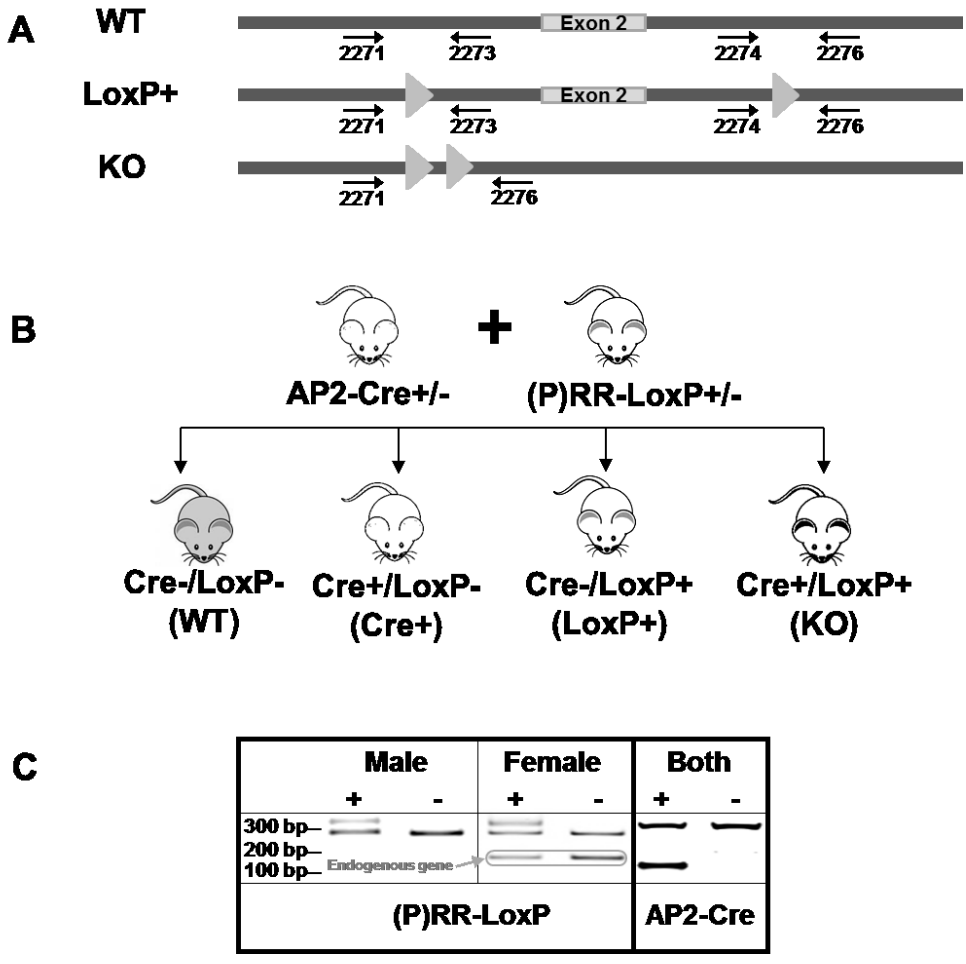
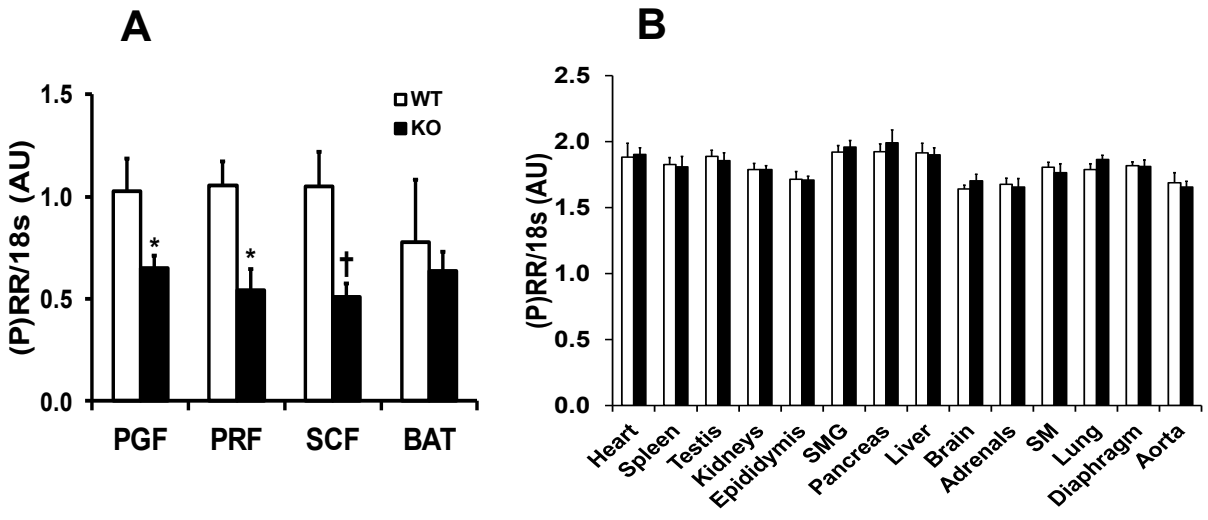


Figure 6. Summary of the metabolic alterations induced by adipose tissue (P)RR gene deletion. Dashed arrows indicate potential mechanism. The ♀ symbol alone is used when the effect is observed in female mice on either ND or HFD. When the effect is different, the signs ♀-ND and ♀-HFD are used.

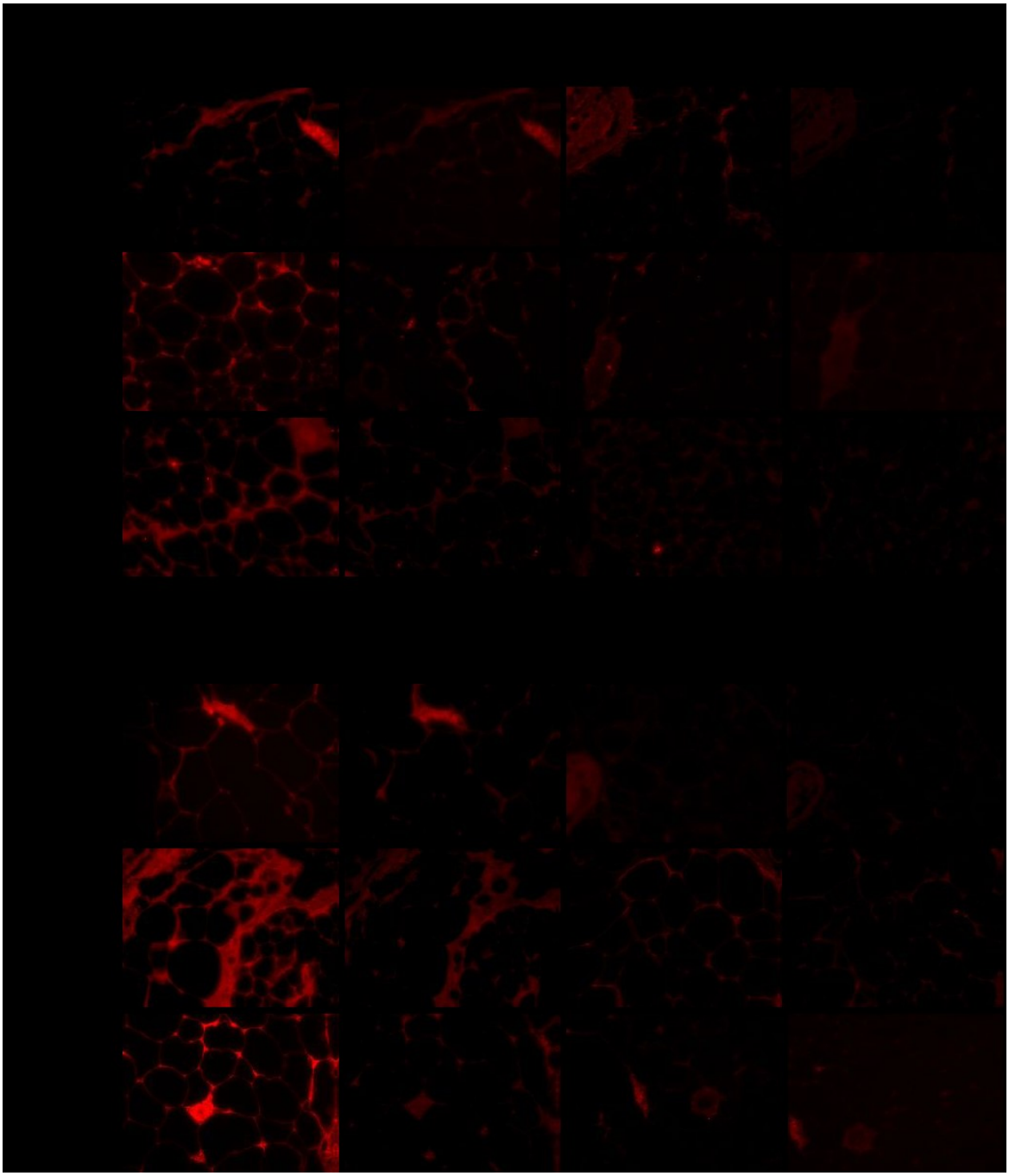
Supplementary Figures



Supplementary Figure 1. Adipose tissue specific (P)RR gene deletion. (A) Adipose tissue specific AP2-Cre/(P)RR-LoxP gene inactivation map. **(B)** Knock-out (KO) mice were obtained by breeding AP2-Cre+/- with (P)RR-LoxP+/- heterozygous mice. From this breeding, we obtained Cre-/-LoxP-/- (WT), Cre+/-LoxP-/- (Cre+), Cre-/-LoxP+/1 (LoxP+) and Cre+/-LoxP+/- (KO) mice. Because the (pro)renin receptor [(P)RR] gene is located on the X chromosome, male KO mice were homozygous and females were heterozygous. (C) Agarose gel showing genotyping bands patterns for Cre and LoxP.



Supplemental Figure 2. (P)RR gene deletion is specific to adipose tissue. (P)RR mRNA levels in adipose tissues (A) and other tissues (B) in male mice. Data are normalized to 18s mRNA levels and are presented as mean ± SE with n = 5-6 per group. * $p < 0.05$ and † $p < 0.01$ compared to WT. BAT, brown adipose tissue; KO, knock-out; PGF, perigonadal fat; PRF, perirenal fat; (P)RR, (pro)renin receptor; SCF, abdominal subcutaneous fat; SMG, submandibular gland; SM, skeletal muscle; WT, wild-type.



Supplementary Figure 3. (P)RR immunohistochemistry in adipose tissue in male (A) and female (B) mice. KO mice showed a significant decrease in (P)RR staining on adipocyte membranes both in male and female mice. KO, knock-out; PGF, peri-gonadal fat; PRF, peri-renal fat; (P)RR, (pro)renin receptor; SCF, abdominal subcutaneous fat; WT, wild-type.

Supplementary Tables

Supplementary Table 1. Primer sequences for PCR and qPCR

Primers	Oligonucleotide sequences	PCR conditions and cycles
(P)RR-LoxP	5'-CTGGATCCCGGAGCATGGGTAAAGG-3' 5'-CAGGTGTGCTGCTATTAATAGG-3' 5'-GCCCCTCTCTTACAGTTCTATCAGT-3' 5'-AGCACTCTCTTCCAGGTATGTTGTG-3'	H: 95 ºC, 5 min; D: 95 ºC, 30 sec; A: 60 ºC, 30 sec; E: 72ºC, 45 sec; Cycles : 40
Cre-recombinase	5'-CTAGGCCACAGAATTGAAAGATCT-3' 5'-GTAGGTGGAAATTCTAGCATCATCC-3' 5'-GCGGTCTGGCAGTAAAAACTATC-3' 5'-GTGAAACAGCATTGCTGTCACTT-3'	H: 95 ºC, 5 min; D: 95 ºC, 30 sec; A: 60 ºC, 30 sec; E: 72 ºC, 45 sec; Cycles : 40
X/Y chromosome	5'-GAAGTGAATTGAAGTTTTGGTCTAG-3' 5'-GGGACCTAACTGTTGGCTTTATCAG-3' 5'-CCTATGAAATCCTTTGCTGCACATGT-3' 5'-AAGATAAGCTTACATAATCACATGGA-3'	H: 95 ºC, 5 min; D: 95 ºC, 30 sec; A: 60 ºC, 30 sec; E: 72 ºC, 45 sec; Cycles : 40
(P)RR	F - 5'-TTTGGATGAACTTGGGAAGC-3' R - 3'-CACAAGGGATGTGTCGAATG-5'	H: 95 ºC, 15 min; D: 95 ºC 15 sec; A: 60 ºC 30 sec; E: 72 ºC 30 sec; Cycles : 40
18s	F - 5'-CTGAGAAACGGCTACCACATC-3' R - 3'-GGCCTCGAAAGAGTCCTGTAT-5'	H: 95 ºC 15 min; D: 95 ºC 15 sec; A: 60 ºC 30 sec; E: 72 ºC 30 sec; Cycles : 40
s16	F - 5'-ATCTCAAAGGCCCTGGTAGC-3' R - 5'ACAAAGGTAAACCCCGATCG-3'	H: 95 ºC, 10 min; D: 95 ºC 15 sec; A: 60 ºC 30

		sec; E: 72 ºC 30 sec; Cycles : 40
PRDM16	F - 5'-GCCATGTGTCAGATCAACGA-3'	H: 95 ºC, 10 min; D: 95 ºC 15 sec; A: 60 ºC 30 sec; E: 72 ºC 30 sec; Cycles : 40
	R - 5'-CCTTCTTTCACATGCACCAA-3'	

A, annealing; AP2, adipocyte protein 2; D, denaturation; E, elongation; F, forward; H, hold; (P)RR, (pro)renin receptor; R, reverse.

Supplementary Table 2. Foetal weight in AP2Cre/(P)RRLoxP strain

Genotype (male/female ratio)	Male weight (g)	Female weight (g)
WT (4/5)	0.90 ± 0.02	0.84 ± 0.03*
KO (7/7)	0.90 ± 0.03	0.88 ± 0.04*
Lox-p (6/9)	0.88 ± 0.03	0.87 ± 0.04
Cre (1/8)	0.93 ± 0.10	0.88 ± 0.02*

Data are presented as mean ± SE. * $p < 0.05$ compared to male pups. *n* per group is indicated in parenthesis. AP2, adipocyte protein 2; KO, knock-out; (P)RR, (pro)renin receptor; WT, wild-type.

Supplementary Table 3. Average daily calorie intake

Gender	Diet	Genotype	*n*	kcal/day
Male	ND	WT	14	14.08 ± 0.56
		KO	9	13.35 ± 0.33
Female	ND	WT	24	12.99 ± 0.57
		KO	18	12.21 ± 0.36
	HFD	WT	17	14.01 ± 0.46
		KO	16	13.16 ± 0.48

Data are presented as mean ± SE. HFD, high-fat diet; KO, knock-out; ND, normal diet; WT, wild-type.

Supplementary Table 4. Adipose tissue (P)RR gene deletion effect on adipocyte size in female mice.

Diet	**ND**		**HFD**	
Genotype	**WT**	**KO**	**WT**	**KO**
n	4	5	5	5
SCF (pm^2)	3.1 ± 0.9	3.5 ± 0.7	4.4 ± 0.3	4.9 ± 0.4
PGF (pm^2)	2.1 ± 0.1	2.8 ± 0.1	4.8 ± 0.4	4.5 ± 0.3
PRF (pm^2)	3.4 ± 1.0	3.4 ± 0.9	5.1 ± 0.5	4.4 ± 0.5

Data are presented as mean ± SE. HFD, high-fat diet; KO, knock-out; ND, normal diet; PGF, perigonadal fat; PRF, perirenal fat; (P)RR, (pro)renin receptor; SCF, abdominal subcutaneous fat; WT, wild-type.

Supplementary Table 5. Effect of adipose tissue (P)RR gene deletion on tissue weight and tibia length

Gender	Male		Female			
Diet	ND		ND		HFD	
Genotype	WT	KO	WT	KO	WT	KO
Liver/tibia (mg/mm)	87.1 ± 2.4	75.5 ± 3.7*	68.9 ± 2.9	65.5 ± 0.4	61.2 ± 2.5	61.3 ± 2.8
Heart/tibia (mg/mm)	8.8 ± 0.3	8.8 ± 0.4	7.9 ± 0.3	7.3 ± 0.2	8.2 ± 0.4	7.1 ± 0.7
Tibia length (mm)	17.9 ± 0.1	17.3 ± 0.1†	18.0 ± 0.1	17.7 ± 0.2	17.5 ± 0.2	17.4 ± 0.1

Data are presented as mean ± SE with n = 7-14 per group. * $p < 0.05$ and † $p < 0.01$ compared to WT. HFD, high-fat diet; KO, knock-out; ND, normal diet; (P)RR, (pro)renin receptor; WT, wild-type.

Supplementary Table 6. Vertical distance is not modified by adipose tissue (P)RR gene deletion.

Gender	Genotype (*n*)	Day (cm)	Night (cm)
Male	WT (7)	482.7 ± 72.5	4748 ± 857.4
	KO (7)	310.3 ± 48.7	5111 ± 426
Female	WT (19)	1598.9 ± 598.6	10292.8 ± 2062.5
	KO (16)	1826.7 ± 503.5	12582.7 ± 2190.4

Data are presented as mean ± SE. HFD, high-fat diet; KO, knock-out; ND, normal diet; (P)RR, (pro)renin receptor; WT, wild-type.

Supplementary Table 7. Effect of adipose tissue (P)RR gene deletion on glucose homeostasis during an OGTT

	Male		Female			
	ND		ND		HFD	
	WT	KO	WT	KO	WT	KO
C-peptide/Insulin (T0)	0.41 ± 0.06	0.52 ± 0.13	0.36 ± 0.08	0.30 ± 0.07	0.76 ± 0.23	0.56 ± 0.17
C-peptide/Insulin (T30)	0.29 ± 0.02	0.40 ± 0.06	0.30 ± 0.10	0.29 ± 0.05	0.39 ± 0.10	0.28 ± 0.04

Data are expressed as mean ± SE with *n* = 5-19 per group. KO, knock-out; (P)RR, (pro)renin receptor; WT, wild-type.

Supplementary Table 8. Effect of adipose tissue (P)RR gene deletion on tissue metabolites

Gender	Male		Female			
Diet	ND		ND		HFD	
Genotype	WT	KO	WT	KO	WT	KO
Total liver triglycerides (mg)	24.6 ± 6.4	18.7 ± 3.5	13.5 ± 2.1	22.9 ± 4.5	73.8 ± 13.5	61.1 ± 11.6
Total muscle triglycerides (μg)	317.0 ± 52.2	83.0 ± 27.1*	177.2 ± 39.5	120.8 ± 31.1	141.1 ± 41.9	163.2 ± 32.0
Total muscle glycerol (μg)	31.5 ± 2.3	22.9 ± 2.6*	27.2 ± 2.5	20.7 ± 2.1	25.3 ± 3.5	27.9 ± 2.9
Total pancreas insulin (μg)	19.4 ± 1.8	23.3 ± 1.5	26.1 ± 1.4	25.1 ± 2.1	25.8 ± 1.4	25.3 ± 1.5

Data are presented as mean ± SE. *n* = 6-14 per group. * $p < 0.05$ compared to WT. HFD, high-fat diet; KO, knock-out; ND, normal diet; (P)RR, (pro)renin receptor; WT, wild-type.

CHAPTER VIII. GENERAL DISCUSSION AND CONCLUSION

8.1 (P)RR IS INVOLVED IN THE DEVELOPMENT OF OBESITY IN MICE

Our first indicator that the (P)RR may be implicated in the development of obesity was in our first study where we demonstrated that the (P)RR gene and protein in adipose tissue were increased by obesity (**Article 1, Figure 1**). Interestingly, we also found that (P)RR gene expression was increased in SCF of insulin resistant obese woman compared with insulin sensitive obese woman suggesting that the (P)RR could be implicated in the development of insulin resistance associated with obesity (**Article 1, Table 1**). In our study, this increase in (P)RR gene expression in adipose tissue with obesity was accompanied by higher renin gene and protein expression as well as higher circulating renin (**Article 1, Figure 1 B and D**) which is consistent with the increases observed in other RAS components that have been reported in adipose tissue with obesity and insulin resistance [9, 11, 14]. In addition, HRP had no effect on these parameters with the exception of the SCF where a decrease in (P)RR expression could be observed. In contrast, in adipose tissue (P)RR KO mice fed a ND, plasma renin activity was found to be unaffected although they were leaner then the WT mice (**Article 2, Table 1**), which suggests that the receptor may not be implicated in adipose tissue production of renin, although renin protein and mRNA were not assessed in this study. Hence, it is possible that in our first study, obesity contributed to the regulation of the (P)RR and renin independently from one and other.

We then confirmed the implication of the (P)RR in obesity in our first paper by showing that increased body weight and fat masses were reduced by HRP administration in mice fed a HFD, whereas only PRF was decreased in mice on a ND without any significant changes in their body weights (**Article 1, Figure 2**). These results suggested that the (P)RR regulates fat mass independently of diet. In the literature, only one other study has evaluated the effect of HRP

treatment on body weight and fat masses. Indeed, in high-fructose diet fed rats, HRP treatment had no effect on total body weight, but significantly decreased fat mass and adipocyte cell size, which was increased in this model of insulin resistance [134].

Interestingly, in our study, similarly to HRP-treated mice, adipose tissue specific (P)RR KO mice had lower body weight and reduced fat mass, both in males and females (with the exception of females on HFD for the fat mass) (**Article 2, Figure 1**). This thus suggests that adipose tissue is central in the effects of the (P)RR on body weight and fat mass during HRP treatment.

Lower body weight and reduced fat mass have been observed (**Table 6**) in mice with genetic suppression of other RAS genes (i.e. angiotensinogen, renin and ACE), and the reduced fat mass in these cases were shown to be due to the reduced trophic effects of AngII on adipocytes [18, 19, 569]. Hence, it is more than likely that the Ang-D pathway of the (P)RR was implicated in the effects observed in our studies (**Chapter 4, Figure 15).** However, we were unable to establish if Ang-ND pathways were also implicated which is also likely given their implication in cell proliferation. Moreover, male hemizygous KO mice also had increased lean mass compared to WT males, which could contribute to their increased metabolic rate and as such, to their decreased weight (**Article 2, Figure 3**). Given that this type of effect has not been reported in other RAS models [18-20], this further supports that Ang-ND pathways may be involved.

A recently published study generated adipocyte specific (P)RR gene suppression in mice using the adiponectin-promoter [570]. They demonstrated that (**Table 6**), similarly to our animals, these KO mice were resistant to HFD induced obesity and had improved insulin sensitivity but associated with increased circulating insulin. However, these mice had marked liver steatosis as well as higher blood pressure which was suggested to be as a result of an increase in the circulating soluble fraction of the (P)RR [570]. These differences between our adipose specific (P)RR KO model and this recently published model most likely results from the different cre mice used to produce these KO models. Indeed, the adiponectin cre mice have been shown to produce an increased deletion compared to the AP2-cre [571, 572]. As such, this resulted in a more marked phenotype in the Wu *et al.* study which resembles lipodystrophy [570].

Table 6. Comparison between the different RAS KO animals

Mice models / Parameters	Whole body Agt KO mice (Massiera et al. 2001)	Adipose Agt KO mice (Yiannikouris et al. 2012; AP2-Cre^{Salk})	Whole body Renin-KO mice (Takahashi et al., 2005 and 2007)	Whole body ACE KO mice (Jayassooriya et al., 2008)	Whole body AT1R KO mice (Kouyama et al., 2005)	Adipose (P)RR KO mice (Adiponectin -Cre; Wu et al. 2016)	Adipose (P)RR KO mice (AP2-Cre^{Salk}; 2nd paper)
Body weight	↓[18]	↓[458]	↓[19]	↓[573]	↓[574]	↓[570]	↓
Fat mass	↓[18]	↓[458]	↓[19]	↓[573]	↓[574]	↓[570]	↓
Food intake	↑[18]	NA	~[19]	~[573]	~[574]	NA	~
Locomotor activity	↑[18]	NA	~[19]	~[573]	NA	NA	↑
Plasma Insulin	~[18]	~[458]	↓[19]	NA	~[574]	↑[570]	↓
Plasma glucose	~[18]	↓[458]	↓[19]	↓[573]	↓[574]	↓ [570]	~
Plasma lipids	↓[18]	NA	↓[19]	~[573]	~[574]	~[570]	~
Plasma leptin	~[18]	↓[458]	NA	↓[573]	↓[574]	↓ [570]	↓
Plasma Adiponectin	NA	NA	↑[19]	NA	~[574]	NA	↑

Intestinal fat absorption	~[18]	NA	↓[19]	~[573]	NA	NA	NA
Metabolic rate	~ body heat production[18]	NA	↑ O2 consumption[19]	NA	↑ O2 consumption [574]	NA	↑ O2 consumption
Thermogenic gene expression	~UCP1 mRNA in BAT [18]	NA	↑UCP2 mRNA in WAT[19]	↑ PCG-1a mRNA in the liver [573]	↑ UCP1 mRNA in BAT [574]	↓ PPAR-γ in adipocytes [570]	↑PRDM16 in PRF and ↓ in SCF
Adipocyte size	↓[18]	~[458]	↓[19]	NA	↓[574]	NA	↓
Plasma Renin Activity	NA	NA	↓[575]	NA	NA	~ [570]	~
Blood Pressure	↓[18]	↓[458]	↓[19]	NA	↓[574]	↑ [570]	NA

Notes: ↑, increasing; ↓, decreasing; ~, unchanged; NA, not assessed. Agt- Angiotensinogen; ACE – Angiotensin converting enzyme; AT1R – Angiotensin type 1 receptor KO – knockout; UCP – uncoupled protein; PPAR-γ, peroxisome proliferation activated receptor type gamma; PCG-1a, PPAR-γ coactivator 1-alpha; PRDM16 – PR domain containing 16.

8.2 ADIPOSE TISSUE (P)RR PARTICIPATES IN THE REGULATION OF LIPID METABOLISM

HRP treatment of GFP mice normalized circulating TG (**Article 1, Table 2**). Conversely, we found no differences in plasma TG or glycerol levels in the KO mice (**Article 2, Supplementary Table 8**), whereas muscle TG and glycerol levels were lower in male hemizygous KO mice (**Article 2, Supplementary Table 8**) suggesting that a more systemic reduction in (P)RR may be required to affect circulating parameters. Nonetheless, these results suggest that systemic (P)RR blockade with HRP or its gene deletion in adipose tissue improve lipid metabolism [406] and do not promote ectopic fat accumulation and lipodystrophy [94, 142, 224, 576]. Our recent study with HRP in HFD fed mice, the same animal model as in article 1, in fact suggests that HRP may promote a TG/FFA cycling by stimulating both lipolysis and lipogenesis [3].

Similarly to what we have shown with HRP, several reports from the literature have demonstrated that circulating TG are decreased with the administration of RAS inhibitors although not normalized [9, 26]. In addition, it has been demonstrated that increased plasma lipid levels in a model of insulin resistance, rats fed high-fructose diet, were decreased after HRP administration [134]. Also, mice with whole body Agt gene suppression showed decreased lipid content in the plasma, liver and muscle [18, 20, 467]. In these models, it was suggested that the mechanism involved was a reduction in AngII leading to a reduction in the gene expression and activity of lipogenic enzymes and an increase in lipolytic enzymes [18-20]. In addition, AngII, specifically in adipose tissue, was shown to promote "trophic" effect and increase adipocyte size and reduce insulin sensitivity [11, 20]. Interestingly, plasma lipid levels were unchanged in mice overexpressing AGT specifically in adipose tissue in whole body Agt KO mice, although adipose tissue fatty acid synthase (FAS) activity was lower in these mice suggesting that these local effects were not sufficient to have any systemic effect on circulating lipids [20]. Put together, these data suggest that (P)RR deletion/inhibition can improve circulating lipids by modifying adipose tissue lipid metabolism through AngII-D mechanisms. Moreover, these changes should contribute to the reduced adipose tissue weight (**Article 1, Figure 2, and Article 2, Figure 1**) observed in our studies.

8.3 POTENTIAL MECHANISMS IMPLICATED IN THE EFFECTS OF THE (P)RR IN THE DEVELOPMENT OF OBESITY

One of the mechanisms that may be implicated in the effect of the (P)RR on body weight may be through modulation of energy homeostasis. Indeed, we found that (P)RR KO mice had increased locomotor activity during their active period (**Article 2, Supplementary Table 6**). Interestingly, hemizygous male KOs also had increased O_2 consumption and CO_2 production during their inactive period, which suggest that they also have an increased metabolic rate (**Article 2, Supplementary Table 4**). This increased metabolism may result in part from white adipose tissue "beiging" as we observed higher PRDM16 gene expression in KO mice compared to WT mice (**Article 2, Figure 4**). Interestingly, similar results were recently reported by our laboratory using HRP in obese mice (the same animal model as in article 1) [3]. Hence, although we did not determine whether HRP can modulate these parameters similarly using metabolic cages, our recently published data suggests that HRP should modulate basal metabolism. In line with these results, whole body and adipose tissue-specific Agt deficiency as well as whole body renin deficiency have been reported to increase locomotor activity [18]. In whole body Agt deficient mice, this phenotype was shown to be associated with the AngII deficiency, more specifically in the brain [18]. Moreover, increased thermogenesis was also observed in mice with whole body renin and Agt gene suppression, characterised by an increase in UCP-1 gene expression in brown adipose tissue [18, 19]. Put together, this certainly suggests that Ang-D mechanisms may be involved in the metabolic phenotypes observed in both HRP treated mice and the (P)RR KO mice. Interestingly, the food consumption was unchanged by both HRP (**Article 1, Supplement data, Table S7**) and adipose tissue specific (P)RR deficiency (**Article 2, Supplementary Table 3**). As such, food intake is not implicated in the body weight phenotype associated with (P)RR blockade or deficiency observed in our studies. Similar observations were reported in mice with whole body renin [19], ACE[573], and AT1R[574] deficiency (**Table 6**). Interestingly, in whole body renin deficient mice, a reduced intestinal fat absorption was observed and was proposed to be a potential mechanism implicated in the decreased

adiposity observed in these mice [19]. Given that the latter was observed only in renin deficient mice, this suggests that this effect may be through an interaction with the (P)RR and may thus contribute to the decreased body weight observed with HRP in our obese mice.

8.4 ADIPOSE TISSUE (P)RR IS INVOLVED IN THE DEVELOPMENT OF INSULIN RESISTANCE

In mice treated with HRP, we found lower plasma insulin level in both the ND and HFD fed groups compared to their untreated controls despite a similar glycemia (**Article 1, Table 2**). As a result, glucose to insulin ratio was increased independently of diet in mice treated with HRP (**Article 1, Table 2**), suggesting a better insulin sensitivity. In line with these results, we observed lower plasma insulin level in male hemizygous (P)RR KO mice and in female heterozygous KO mice but only when challenged with a HFD (**Article 2, Figure 5**). In addition, we assessed circulating C-peptide levels in KO mice and found it to be lower, similarly to insulin (**Article 2, Figure 5**). However, we found no differences in the circulating insulin/C-peptide ratio (**Article 2, Supplementary Table 7**) or in total pancreatic insulin content (**Article 2, Supplementary Table 8**), indicating that there were no differences in insulin metabolism between the (P)RR KO and WT mice. Hence, similarly to HRP treated mice, we hypothesize that these lower plasma insulin levels observed in the (P)RR KO mice is likely due to a better insulin sensitivity, as described in other mouse models of RAS deficiency such as in whole body renin [19] and ACE deficiency [573] as well as in HRP treated rodents [134, 577]. In the latter studies, improved insulin sensitivity in mice treated with HRP with myocardial infarction has been reported to result from a decrease in NADPH oxidase activity and ROS formation, most likely through the reduction in local AngII production in skeletal muscle [577]. In a similar fashion, in fructose fed rats, a model of insulin resistance, it was suggested that the improved insulin sensitivity observed with the administration of HRP was through a decreased AngII formation in skeletal muscle [134]. Interestingly, in humans, the (P)RR was increased in SCF from obese women with lower insulin sensitivity compared to those with better insulin sensitivity (**Article 1, Table 2**). Similarly, as shown in **Table 6**, whole body renin deficient mice have reduced

plasma insulin with no change in glucose levels following an OGTT [19]. In contrast, plasma glucose and insulin levels were unchanged in whole body Agt deficient mice, while in ACE KO and AT1R KO mice glycemia was lower without any change in insulinemia [573,574]. Thus, given that the renin KO and (P)RR KO had similar responses to an OGTT compared to the other RAS KO models, we suggest that these effects on insulin sensitivity are through Ang-ND effects of the (P)RR. Similarly to our data from the (P)RR KO mice, the improved insulin sensitivity observed in whole body renin KO mice was associated with higher plasma adiponectin [19]. These authors however suggested that this may result from lower circulating AngII levels given that the administration of a low-dose of Ang II abolished the beneficial effects of the renin deficiency although this may have just stimulated parallel pathways [19]. In mice with whole body ACE deficiency, an increased glucose clearance was observed during an OGTT and it was suggested that the improved insulin sensitivity resulted from reduced AngII production [573]. However, in these mice it was proposed that additional favorable effects may have been obtained through the increased bradykinin level which would improve insulin signaling through IRS1 [573]. Hence, given that (P)RR inhibition/deletion does not alter Ang II circulating levels but only reduces tissue levels, this suggests that Ang-ND effects of the (P)RR mainly contributed to the improved insulin sensitivity observed in our studies with HRP and in adipose tissue (P)RR KO mice while Ang-D effects are probably also involved but to a lesser extent.

8.5 POTENTIAL MECHANISMS IMPLICATED IN THE EFFECTS OF THE (P)RR IN THE DEVELOPMENT OF INSULIN RESISTANCE

Based on our results and the literature, different mechanism may be involved in the effects of the (P)RR on insulin resistance. First, in article 1, we found that HRP treatment, in mice fed either HFD or ND, normalized glucose transporters GLUT1 (in SCF and in PRF) and GLUT4 (in SCF) gene expression in adipose tissue compared to untreated controls (**Article 1, Figure 5**). In addition, (P)RR inhibition/suppression may produce a healthier adipose tissue which may contribute to improve insulin sensitivity. Indeed, we found that HRP treatment, independently of diet, may favour the production of smaller adipocytes as assessed by lower expression of the

Mest/Peg1 mRNA (**Article 1, Figure 3**) and confirmed recently histologically [3]. This was also observed in male hemizygous KO mice, as indicated histologically by a reduced cell surface area of adipocytes (**Article 2, Figure 2**). Hence, modification of adipocyte size may contribute to improve insulin sensitivity as it has been shown that adipocytes size plays a role in their metabolism and function, particularly insulin sensitivity and apoptosis [186].

This modulation of adipocyte size by the (P)RR may in part be through its Ang-D effects. Indeed, overexpression of Agt, specifically in adipose tissue, has been shown to increase adipocyte size and, as a result, reduce insulin sensitivity [11, 20]. In contrast, in mice with adipose tissue specific Agt deficiency, authors didn't find any changes in adipocyte cell size [458], whereas in whole body ACE KO and AT1R KO mice smaller size adipocytes were observed [573,574]. These results suggest that circulating RAS may have compensated for the lack of adipose tissue Agt. Moreover, given that the (P)RR has been shown to reduce cell proliferation through Ang-ND effects [115, 507, 549], the smaller adipocyte size reported with (P)RR inhibition/suppression may be through both Ang-D and Ang-ND effects, as presented in Article 2 (**Article 2, Figure 2**) and in our recent paper [3]. Also, the inflammatory cytokine TNF-alpha gene expression was reduced in HRP treated GFP mice (**Article 1, Figure 4**) which may also contribute to the improved adipose tissue function.

Furthermore, GFP mice fed either diet and treated with HRP exhibited lower circulating leptin levels (**Article 1, Figure 3**) with an associated reduction in its adipose tissue gene expression compared to untreated mice. Similarly, in male KO mice fed ND circulating leptin levels were also lower compared to WT mice (**Article 2, Table 1**). Leptin secretion is correlated with adipose tissue mass [85] and high plasma leptin levels have been demonstrated to be a marker of the metabolic syndrome [170]. In addition, AngII has been shown to stimulate the production of leptin [578], hence, it is possible that these lower leptin levels after HRP administration (**Article 1, Figure 3**) and in the (P)RR KO mice (**Article 2, Table 1**) may also result from the reduced Ang-D effects of the (P)RR. Interestingly, plasma leptin levels were reduced in almost all RAS KO models except for the mouse model with whole body Agt deficiency (Table 6), further supporting the potential role of Ang-D pathways in these effects of the (P)RR.

We also showed that plasma adiponectin levels were increased in all KO groups (**Article 2, Table 1**) which suggests that this may be a mechanism by which adipose tissue (P)RR plays a role in regulating insulin sensitivity. Indeed, higher plasma adiponectin levels are associated with better insulin sensitivity and improved lipid metabolism [205, 211, 219]. Interestingly, despite the gender-dependent results regarding body weight and fat masses, circulating adiponectin levels were increased in all groups which suggests that this is due to a specific effect of adipose tissue (P)RR. A recent publication from our laboratory also demonstrated that HRP improved adiponectin modulation in obese mice [534]. Moreover, AngII has been shown to suppress adiponectin gene expression and its plasma levels [164, 168, 186]. In addition, suppression/deletion of the (P)RR in adipose tissue leads to the modulation of the PPAR gamma pathway by increasing the activity of PRDM16, which stimulates brown adipocyte gene expression during differentiation of white adipocytes and thus promotes its beiging with the associated beneficial effect on insulin sensitivity and metabolic rate [3, 92, 120].

8.6 LIMITATIONS OF OUR STUDY

As adipose tissue (P)RR gene deletion seems to have increased lethality in hemizygous male KO mice, the number of animals obtained was limited. Moreover, because the adipose tissue (P)RR gene deletion reduced fat mass substantially in the male KO mice, we had difficulty obtaining large amounts of samples to do detailed histological, RNA and protein analysis. This also limited the capabilities to extract adipocytes and investigate them *in vitro*. For future studies, the use of an inducible Cre-model would be of great use to avoid these issues. In addition, this would help to have a better perspective on the true effect of the (P)RR gene in adipose tissue although the use of the female heterozygous mice contributed to better evaluate this as they had a more modest phenotype and thus, less developmental issues.

In a clinical setting, patients arrive with an already established obesity and need to be treated to reduce the excess weight and not to prevent the development of obesity. However, to gain insight into whether (P)RR is involved in the development of the disease, we first studied the effect of HRP given at the same time as the HFD, hence, this would investigate the preventive

effect of this blocker. Unfortunately, our study was limited by time and by funding and we didn't have the chance to determine if HRP could be used as a treatment for obesity. These are studies that are planned for the future which would certainly further support the potential use of the HRP in clinical practice.

8.7. CONCLUSION

Based on our results and the literature, the (P)RR can be involved in the development of obesity and insulin resistance by the mechanisms presented on **Figure 17**.

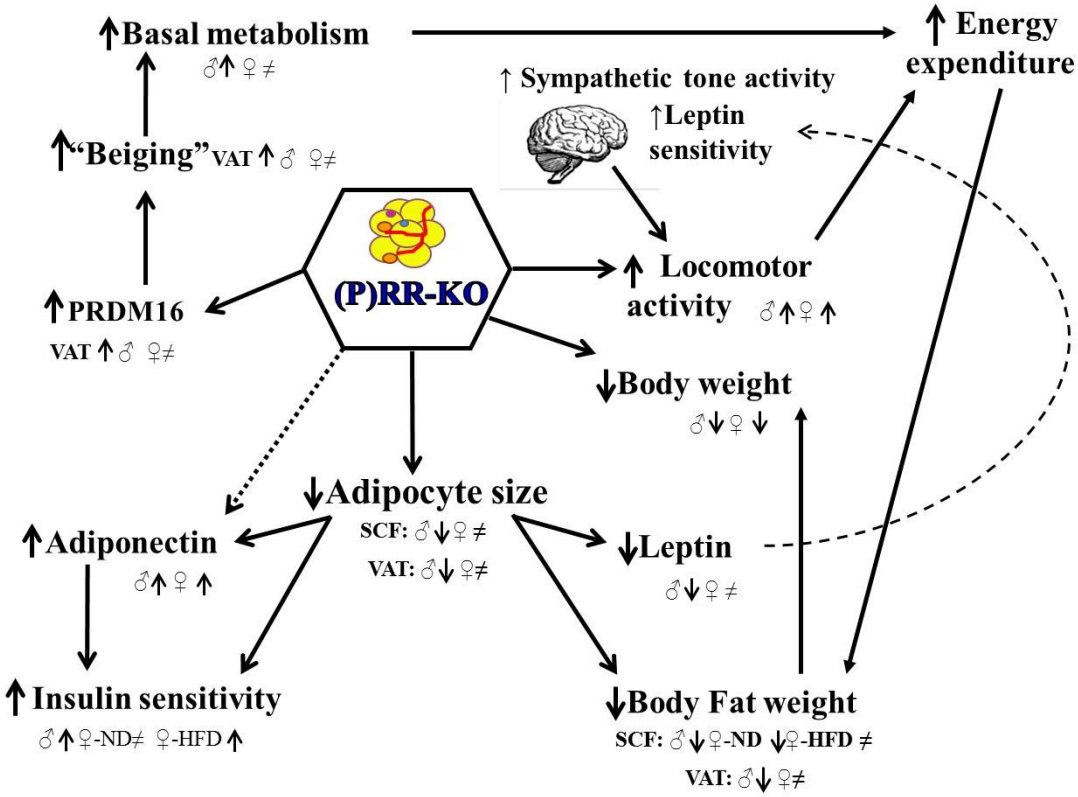


Figure 17. Effects of adipose tissue (P)RR in the development of obesity and insulin resistance. ♂ **- male mice, ♀, females; effects: ↑, increased; ↓ decreased; ≠ unchanged;** VAT, visceral adipose tissue; SCF, subcutaneous fat; AngII, Angiotensin II.

Indeed, the (P)RR seems to favour adipocyte hypertrophy through Ang-D [11, 14, 18], i.e. local

AngII production, and also Ang-ND, probably through increased Wnt signaling and suppression of the PPAR gamma pathways [110, 579]. Increasing adipocyte size leads to abnormal secretion of adipokines such as, TNF-a, leptin and adiponectin, and thus promote inflammation and insulin resistance [81, 91] (**Chapter 1, Table 1**). Moreover, there are central mechanisms which may be involved in the development of obesity which would produce the decrease in locomotor activity observed (**Figure 17**) and may increase sympathetic nervous system activity [182]. Based on the results obtained in (P)RR KO mice, we propose that there is a feedback regulation between adipose tissue and brain **(Figure 17),** which may act through the modulation of leptin secretion by adipose tissue and leptin receptor sensitivity in the brain. Feedback mechanism between WAT and the brain have also been proposed in mice with whole body Agt deletion but with Agt gene overexpressed specifically in adipose tissue [20]. Moreover, obesity may also be favoured by a decreased basal metabolic rate as inhibition/suppression of the (P)RR produces WAT beiging. Hence, put altogether, our results show the beneficial effect of suppression/deletion of the (P)RR to prevent the development of obesity and insulin resistance, where adipose tissue (P)RR seems to be a main target. As such, this supports the need for future studies to investigate new treatments of obesity and diabetes by targeting the (P)RR.

8.8 PERSPECTIVES

In order to better understand the mechanisms and function of the (P)RR in both the Ang-D and Ang-ND pathways, it would be interesting to study adipose tissue (P)RR KO mice treated with RAS inhibitors, such as ACEi or ARBs. This would allow us to differentiate Ang-ND effects from Ang-D effects, involved in the development of obesity and insulin resistance.

In addition, to further evaluate the impact of (P)RR inhibition/deletion on insulin sensitivity, it would be useful to conduct clamp studies as well as to do more detailed analysis of insulin signalling pathways and additional pathways which regulate GLUTs activity. We could for instance investigate GLUT1 and GLUT4 translocation by evaluating its content separately in intracellular and membrane fractions in cultured cells from isolated adipose tissue and muscle

following *in vivo* experiments with inhibition of the Ang-D effects with ACE inhibitor and AT1R blocker in combination with HRP.

Our results regarding (P)RR suppression leading to lower plasma leptin and higher adiponectin levels, together with the increased mouse locomotor activity, suggest a relationship between adipose tissue and the brain. This could be confirmed in future experiments through immunohistochemical measurements of the expression of dopamine, serotonin, neuropeptide Y, agouti related peptide and (P)RR in specific brain nuclei, such as in the lateral and ventromedial nuclei of hypothalamus as has been done in other studies [182].

The higher O_2 consumption and CO_2 production measured in our (P)RR KO mice suggests that they have a higher metabolic rate, which needs to be further investigated. For instance, we could measure UCPs expression and content in adipose tissue, both brown and white. Additional experiments need to be done to confirm the WAT "beiging" that we proposed in our 2nd paper. For example, since cold exposure is a trigger factor for "beiging" of WAT, we could measure basal metabolism and body temperature using metabolic cages during a cold exposure. Following this, we could also assess UCPs, PPAR gamma, PGC-1alpha and other "beiging" genes and proteins expression in white adipose tissue collected at the end of the experiment. We could also separate the Ang-D effects by using RAS inhibitors which would be administered before the experiment.

Appendix. List of current publications

Published papers:

1. Shamansurova Z*, Tan P*, Ahmed BM-A, Pepin E, Seda O. and Lavoie JL. **Adipose tissue (P)RR regulates insulin sensitivity, fat mass and body weight**. ***Molecular Metabolism***, August 2016. 23;5(10):959-69. doi: 10.1016/j.molmet.2016.08.009. (*equal contribution)
2. Tan P*, Shamansurova Z*, Bisotto S, Michel C, Gauthier M-S, Rabasa-Lhoret R, Nguyen TM-D, Schiller PW, Gutkowska J and Lavoie JL (2014): **Impact of the prorenin/rennin receptor on the development of obesity and associated cardiometabolic risk factors**. ***Obesity*** 22: 2201-2009. (*equal contribution)

Conference and Congress presentations:

Oral presentations:

1. Shamansurova Z, Tan P, Bisotto S, Michel C, Nguyen TM.-D, Schiller PW. and Lavoie JL. **Effects of (Pro)renin receptor blockade on glucose metabolism in mice on high fat high carbohydrate diet.** *2012* ***Canadian Hypertension Congress***. Toronto, October, 2012 (oral presentation).
2. Shamansurova Z, Ahmed BA.-M, Kajla S, Michel C, Seda O and Lavoie JL. **The (Pro)renin receptor in adipose tissue is important for body weight control and glucose homeostasis.** ***Canadian Hypertension Congress*** 2013, October, Montreal. (oral presentation)

Poster presentations:

1. Shamansurova Z, Tan P, Ahmed BM-A, Michel C, Kajla S, Ondrej S, and Lavoie JL. **Circulating RAS in Mice with Prorenin Receptor Gene Deletion Specifically in Adipose Tissue.** ***The FASEB J*** *April 2015 29:971.4*
2. Shamansurova Z, Basma A.-M. Ahmed, Kajla S, Michel S, Seda O and Lavoie JL. Impact of the (pro)renin receptor on adipose tissue structure and function. ***Canadian Hypertension Congress 2014***, Gatineau, October 16-19, 2014.
3. Shamansurova Z, Tan P, Bisotto S, Michel C, Nguyen TM-D, Schiller PW and Lavoie JL. **The (Pro)renin receptor blocker peptide effects on regulation of body weight and glucose**

homeostasis in mice with diet induced obesity. EB2014, April, San Diego. *April 2014* ***The FASEB Journal*** *vol. 28 no. 1 Supplement 1101.10*

4. Shamansurova Z, Ahmed BA-M, Kajla S, Michel C, Seda O and Lavoie JL. **Genetic blockade of (Pro)Renin receptor in adipose tissue modulate the mice body weight and glucose homeostasis**. EB2014, April, San Diego. 1173.6 *The FASEB Journal vol. 28 no. 1 Supplement 1173.6*
5. Shamansurova Z*, Ahmed BAM, Kajla [S], Michel C, Seda O and Lavoie JL. **Modulation of body weight and glucose homeostasis in mice lacking the (pro)renin receptor in adipose tissue**. In abstract book : ***16e Congrès des étudiants, stagiaires et résidents CR CHUM***. December, 2013. P84.
6. Shamansurova Z, Ahmed B.A.-M., Michel C., Kajla S. and Lavoie J.L. **Importance du récepteur de la rénine/(pro)rénine dans le tissu adipeux pour le contrôle du poids corporel et de l'homéostasie du glucose**. ***Congrès des étudiantes de Département de Physiologie UdeM****. October, 2013, Montreal. N12. (Présentation par affiche).*
7. Shamansurova Z.M., Tan P., Bisotto S., Michel C, Nguyen T.M.-D, Schiller P.W. and Lavoie J.L. **Rôle du récepteur à la rénine/prorénine dans la régulation de l'homéostasie de glucose et l'expression génique dans le tissu adipeux**. ***Cardiometabolic health research nethwork, Diabetes, Obesity,*** *May 10-11, 2013. Quebec city. (poster presentation).*
8. Shamansurova Z, Tan P, Bisotto S, Michel C, Nguyen Thi M.-D., Schiller PW., Gutkowska J and Lavoie JL. **Link between the prorenin/renin receptor and glucose homeostasis in mice fed a high-fat/high-carbohydrate diet**. ***Experimental Biology****, April, 2013. Boston, USA. (poster presentation).*
9. Shamansurova Z.M, Tan P, Bisotto S, Michel C, Nguyen T-M D, Schiller PW and Lavoie JL. **Effect of (pro)renin receptor blockade on adipose tissue gene expression and glucose homeostasis in obesity**. *21[e]* ***Congrès annuel des étudiants, stagiaires et résidents du Centre de recherche du CHUM.*** *2013, Montréal (poster presentation).*
10. Shamansurova Z.M, Tan P, Bisotto S, Michel1 C, Nguyen T-M D, Schiller PW and Lavoie JL. **The role of the PRR in the regulation of glucose homeostasis in mice fed with a high-fat/ high-carbohydrate diet**. ***7th Annual Retreat MDRC****. January, 2013, Montreal. (poster presentation).*

11. Shamansurova Z, Ahmed BA.-M., Kajla S and Lavoie JL. **The role of adipose tissue (Pro)Renin Receptor expression in glucose homeostasis**. *2012* ***Canadian Hypertension Congress***. *Toronto, October, 2012* (poster presentation).

12. Shamansurova Z, Ahmed BAM, Tan P, Bisotto S, Michel C, Nguen TMD, Schiller PW, and Lavoie JL**. Regulation of the (pro)renin receptor by obesity; a possible therapeutic avenue?** ***6th Annual Retreat MDRC****. 2012, Montreal. P48. (poster presentation).*

13. Shamansurova Z, Ahmed BMA, Bisotto S, Michel C, Nguyen TMD, Schiller PW and Lavoie JL. **(Pro)renin receptor implication in the pathogenesis of obesity**. **19e** ***Congrès annuel des étudiants, stagiaires et résidents du Centre de recherche du CHUM***. *Montreal, 2011. P142. (poster presentation).*

14. Shamansurova Z., Ahmed BM, Tan P., Bisotto S., Michel C., Nguyen TM-D., Schiller PW. And Lavoie JL. **Modulation Du Récepteur À La (Pro)rénine Par La Diète Riche En Gras; Un Nouveau Joueur Dans L'obésité Et Ses Complications**. ***20e Congrès SQHA****. 2011, Quebec city. (poster presentation)*

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