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IDENTIFICATION AND CHARACTERIZATION OF NOVEL SIGNALS REGULATING FEEDING BEHAVIOR AND ENERGY BALANCE

Evidences indicating TFF2 as a novel potential therapeutic target for diet-induced obesity treatment

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« Parfois, du reste, il parait beaucoup plus "scientifique" de poser une question que de vouloir donner d'emblée et à tout prix une réponse ».

Eugène Minkowski

Résumé

La recherche dans le domaine de l'obésité a énormément progressé pendant les dernières décennies et a apporté une contribution fondamentale à la compréhension des mécanismes biologiques et physiologiques impliqués, de même que leurs interactions avec l'environnement obésogène. Les études génétiques et génomiques ont mis en évidence les traits héréditaires majeurs qui peuvent causer ou prédisposer à l'accumulation excessive de gras corporel et ils ont stimulé la caractérisation de nombreux gènes codant pour des protéines impliquées dans la physiologie du bilan énergétique. Malgré le progrès considérable des connaissances, les pharmacothérapies actuelles ne démontrent pas d'effets suffisamment efficaces sur la perte persistante de poids, et sont souvent suivies par des effets secondaires importants. Le travail présenté dans cette thèse a comme objectif principal d'identifier et de caractériser de nouvelles cibles thérapeutiques pour le traitement et la prévention de l'obésité et des maladies métaboliques associées. Plus spécifiquement, nous avons concentré nos études sur les mécanismes précoces régulant la prise alimentaire et le metabolisme énergétique.

Nous nous sommes premièrement intéressés aux changements métaboliques précoces qui surviennent avec la menopause et qui peuvent predisposer au développement de l'obésité. Nous avons ainsi analysé les effets aigus de la prévalence androgénique sur l'expression génique du tissu adipeux rétro-péritonéal, chez un modèle murin de menopause. Nos résultats demontrent qu'une seule injection de dihydrotestostérone induit des changements significatifs dans le profil transcriptionnel du tissu adipeux. Enfin, l'expression augmentée de plusieurs transcrits myogéniques dans ce tissu témoigne de sa plasticité exceptionnelle, une qualité qui pourrait être exploitée à des fins thérapeutiques.

Nous avons ensuite analysé les effets rapides que la consommation de repas à haute teneur lipidique cause sur la perception de la satiété chez la souris. Selon plusieurs évidences, obtenues chez des sujets humains de même que chez des modèles animaux, les repas riches en gras ont des effets réduits et retardés sur la perception de la satiété, comparativement aux glucides ou aux protéines. Ils peuvent donc favoriser la surconsommation passive d'énergie et, à long terme, l'accumulation de poids corporel. Nous avons utilisé la méthode de l'analyse sérielle de l'expression génique (SAGE) et

étudié les changements transcriptionnels induits par un seul repas dans des tissus clés de la souris, comme l'estomac et l'hypothalamus. Nous avons ainsi identifié plusieurs nouveaux transcrits qui avaient été spécifiquement et rapidement régulés par le repas riche en gras. Un certain nombre de ces gènes a été sélectionné pour caractériser ultérieurement leur potentiel dans le développement de l'obésité induite par la diète (OID). Cette thèse présente la première caractérisation *in vivo* des rôles du gène trefoil factor family member 2 (*Tff2*) dans la régulation du bilan énergétique et l'OID. Chez les souris, la déficience du gène *Tff2* a altéré significativement le comportement alimentaire, ainsi que la prise énergétique et la dépense d'énergie après douze semaines de diète riche en gras. En conclusion, les souris *Tff2* KO étaient moins efficaces dans l'accumulation de l'énergie ingérée et, par conséquent, plus résistantes à l'OID par rapport aux souris normales. Les résultats obtenus dévoilent des rôles totalement nouveaux pour *Tff2* et indiquent pour la première fois son implication dans la régulation du bilan énergétique. Les évidences ici décrites suggèrent que *Tff2* pourrait être une cible optimale pour la conception de molécules pharmacologiques, qui contrôleraient simultanément plusieurs points critiques pour la régulation du poids corporel et le traitement de l'obésité.

Abstract

Research in the domain of obesity has greatly evolved in the last decades and offered a fundamental contribution for understanding the biological and physiological mechanisms involved, and their interactions with the obesogenic environment. Genetic and genomic studies have pointed out the major hereditary traits that may cause or predispose to excessive fat accumulation, and stimulated the characterization of their products' action in the physiology of energy balance. The homeostatic and hedonic models have been introduced and intensely scrutinized to explain the reciprocal interaction between feeding behavior and energy utilization. However, despite the considerable advances in knowledge, current pharmacotherapies are not sufficiently efficacious in determining and maintaining a significant weight loss, and only bariatric surgery is presently considered a resolutive though invasive intervention. The work presented in this thesis has the long-term objective to identify and characterize novel therapeutic targets for the treatment and prevention of obesity and related metabolic diseases.

With this aim, we firstly investigated the acute metabolic changes induced by menopause transition and responsible for the augmented risk of obesity observed in post-menopausal women. Therefore, we analyzed the acute effects of androgenic prevalence on retroperitoneal adipose tissue gene expression, in a mouse model of menopause. Our results demonstrated that one single injection of dihydrotestosterone could induce significant changes in the transcriptional profile of adipose tissue. Finally, the overexpression of several myogenic transcripts in this tissue further proves its high plasticity, a feature that could be used for a variety of therapeutic interventions.

In the second place, we have focused the attention on the short-term mechanisms regulating food intake and, particularly, the early effects of high-fat (HF) consumption on satiety perception, or satiation, during the consumption of a meal. Several evidences in humans and animal models have demonstrated that HF foods have reduced and delayed effects on satiety compared to carbohydrates and proteins, and therefore may more easily lead to passive overconsumption and weight gain. We used the serial analysis of gene expression (SAGE) to analyze the differential transcription profiles induced by a single meal in key tissues of fasted mice, such as the hypothalamus and stomach, and identify

novel transcripts specifically and rapidly regulated by HF food. Among the genes whose expression was differentially modulated by a HF meal, a number of transcripts were selected to further characterize their potential in the development of diet-induced obesity (DIO). This thesis presents the first *in vivo* investigation of trefoil factor family member 2 (*Tff2*) roles in energy balance and DIO. Feeding behavior, energy intake, and energy utilization and dissipation were altered in *Tff2* knock-out (KO) compared to wild type (WT) mice challenged with HF diet. Finally, *Tff2* KO mice were less able to accumulate the energy ingested and thus more resistant to DIO compared to WT controls. These results unveiled totally novel roles for *Tff2* in mice, and indicated its significant involvement in the regulation of feeding behavior and energy metabolism. Moreover, the evidences here described suggest that *Tff2* might be a valuable target for the design of pharmacological tools that can simultaneously affect multiple critical control points for the regulation of body weight and the therapy of obesity.

Avant-propos

Le travail présenté dans cette thèse a été une opportunité extraordinaire tant dans mon cheminement professionnel que personnel. Faire de la recherche dans le domaine de l'obésité à l'Université Laval a été un grand honneur pour moi et je ne l'oublierai jamais. De plus, je désire remercier mon directeur de recherche, Dr. Jonny St-Amand, pour sa détermination, son soutien constant de même que pour m'avoir offert l'opportunité de faire de la recherche de haut niveau dans un autre pays. Je respecte énormément sa méthode d'enseignement et je souhaite à tous les étudiants passionnés de retrouver sur le chemin un tel mentor. Je remercie également Dr. Mayumi Yoshioka, dont le professionnalisme et la capacité extraordinaire d'organisation auront grandement influencé mon attitude au travail. J'aimerais aussi la remercier pour ces heures passées à discuter de molécules et de fonctions métaboliques inconnues, de même que pour celles passées à analyser les données presque toujours surprenantes, à imaginer des explications logiques et à les tester en laboratoire de façon simple et rapide. Je tiens aussi à remercier Dr. Ping Ye, qui m'a accueillie dans le laboratoire, ainsi que tous les étudiants que j'ai rencontrés pendant mon parcours d'études et qui m'ont soutenue, particulièrement Isabelle Riedl, Olivier Moreault, Rose-Guerline Cherizol et Carole-Anne Potvin. De plus, le CHUL et le CREMOGH ont représenté pour moi, un milieu exceptionnel et stimulant pour le travail en recherche et je souhaite ainsi remercier tout le personnel pour leur professionnalisme. J'apprécie aussi grandement la possibilité que j'ai eue à travailler en collaboration avec l'équipe du Dr. Denis Richard au CRIUPCQ et d'apprendre de nouvelles techniques. J'en profite aussi pour remercier le Dr. Richard et tous les professionnels de recherche ainsi que les étudiants qui ont précieusement contribué et aidé à la recherche.

Pendant mes études, j'ai rédigé quatre articles scientifiques qui sont rapportés dans les chapitres 2 à 5.

Le Chapitre 2 contient l'article « A single dose of dihydrotestosterone induced a myogenic transcriptional program in female intra-abdominal adipose tissue», qui a été publié en 2010 dans le *Journal of Steroid Biochemistry and Molecular Biology*. Dr. Jonny

St-Amand et Dr. Mayumi Yoshioka ont conçu et planifié le projet de recherche. Ma contribution était la suivante: analyse et interprétation des données; rédaction de l'article.

Le Chapitre 3 présente l'article « Feeding induced changes in the hypothalamic transcriptome », publié en 2009 dans *Clinica Chimica Acta*, et le Chapitre 4 présente l'article « Feeding regulates the expression of pancreatic genes in gastric mucosa », publié en 2010 dans le *Journal of Obesity*. Les travaux décrits dans les deux articles ont été conçus par les Drs. Jonny St-Amand et Mayumi Yoshioka. J'ai participé aux procédures expérimentales (SAGE), analysé et interprété les données et j'ai rédigé le manuscrit.

Le Chapitre 5 présente l'article « *Tff2* acts as a mastermind to control lipid absorption, energy expenditure and feeding behavior », qui a été récemment soumis à *EMBO Reports*. Le travail illustré dans le manuscrit a été produit en collaboration avec Dr. Denis Richard du CRIUCPQ, Université Laval, et avec Dr. Nikolaus Blin et Dr. Aftab Ali Shah de l'Université de Tübingen. Le projet de caractérisation in vivo du gène *Tff2* a été conçu et planifié par les Drs. Jonny St-Amand et Mayumi Yoshioka. J'ai effectué les procédures expérimentales, analysé et interprété les données, et j'ai contribué au développement des hypothèses. Enfin, j'ai rédigé le manuscrit.

En conclusion, je désire remercier le Québec et tous les Québécois, qui m'ont si chaleureusement accueillie dès le premier instant. J'adore votre accent et votre « Bonjour » à toutes les heures du jour et de la nuit. Je remercie aussi l'Italie, qui m'a poussée à quitter le pays en 2008 et puis à rentrer pour l'aimer encore plus en 2011, grâce à mon mari, le phénoménologue le plus brillant que je connaisse. De plus, je tiens à remercier ma famille, qui n'a jamais hésité à me soutenir moralement, même si j'étais si loin de ma maison. Anche il Québec é casa mia adesso.

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5-HT, serotonin

α -MSH, α-melanocyte stimulating hormone

ACTH, corticotropin

AgRP, agouti-related peptide

AMPK, AMP-activated protein kinase

ApoA4, apolipoprotein A4

APPL1, adaptor protein of adiponectin receptor

ARC, arcuate nucleus

BAT, brown adipose tissue

BDNF, brain derived neurotrophic factor

BMI, body mass index

BMR, basal metabolic rate

CART, cocaine and amphetamine regulated transcript

CB1, endocannabinoid receptor

CCK, cholecystokinin

CRBP2, cellular retinol binding protein type 2

CREB, cAMP response element-binding

CRH, corticotropin releasing hormone

CXCL5, CXC-chemokine ligand 5

CXCR4, CXC chemokine receptor 4

DA, dopamine

DHT, dihydrotestosterone

DIO, diet-induced obesity

DIT, diet-induced thermogenesis

DMH, dorsomedial hypothalamic area

DPP-IV, dipeptidyl peptidase-IV

DR, dopamine receptor

EAT, exercise activity thermogenesis

EEA, energy expenditure of activity

ER, estrogen receptor

ERK-1/2, extracellular signal-regulated kinase-1/2

FTO, fat mass and obesity associated

GAD, glutamic acid carboxylase

GHS-R, growth hormone segretagogue receptor

GI, gastro-intestinal

GLP-1, glucagon-like peptide 1

GLP-1R, GLP-1 receptor

GRP, gastrin-releasing peptide

HF, high-fat

HPA, hypothalamic-pituitary-adrenal axis

JNK, c-Jun N-terminal kinase

Ig, immunoglobulin

IL, interleukin

IKK-beta, inhibitor of kappa B kinase-beta

IRS-2, insulin receptor substrate-2

LCFA, long-chain fatty acids

LDL, low-density lipoproteins

LF, low-fat

LHA, lateral hypothalamic area

LIF, leukemia inhibitory factor

LPL, lipoprotein lipase

LPS, lipo-polysaccharides

MAPK, p38 mitogen-activated protein kinase

MC, melanocortin

MC4R, melanocortin 4 receptor

MCH-1, melanin concentrating hormone-1

MCP-1, monocyte chemoattractant protein-1

MRAP, melanocortin receptor accessory protein

mTOR, mammalian target of rapamycin

MUP1, major urinary protein 1

NEAT, non-exercise activity thermogenesis

NFkB, nuclear factor kappa-B

NPY, neuropeptide Y

NST, non-shivering thermogenesis

NTS, nucleus of the solitary tract

ObRs, leptin receptors

OEA, acylethanol-amide oleoylethanolamide

PAR4, protease-activated receptor 4

PCOS, polycystic ovarian syndrome

PKC, protein kinase C

POMC, pro-opiomelanocortin

PP, pancreatic polypeptide

PPAR-α, peroxisome proliferator-activated receptor-α

PVN, paraventricular nucleus

PYY, peptide YY

RBP4, retinol-binding protein 4

SAGE, serial analysis of gene expression

SERT, serotonin transporter

SF-1, steroid factor-1

SH2B1, Src-homology-2 domain containing putative adaptor protein 1

SNS, sympathetic nervous system

SOCS-3, suppressor of cytokine signaling-3

Src, proto-oncogene tyrosine-protein

TDEE, total daily energy expenditure

TFF2, trefoil factor family member 2

TG, triglycerides

TGF-β, transforming growth factor-β

TLR-4, toll-like receptor 4

TNF-α, tumor necrosis factor-α

TRH, thyrotropin releasing hormone

TSH, thyroid stimulating hormone

UACL, ulcer-associated cell lineage

UCP1, uncoupling protein 1

VMH, ventromedial hypothalamic area

VTA, ventral tegmental area

WAT, white adipose tissue

WC, waist circumference

WHO, World Health Organization

CHAPTER 1: INTRODUCTION

1.1 OBESITY AS A DISEASE

Defining obesity

Obesity is a complex condition caused by a broad constellation of factors, including genetic and environmental contributors, which are beyond individuals' control. By definition, obesity is characterized by an excess of body fat, either total or a particular depot of body fat. The adverse consequences of this condition include ill health [1], functional impairment [2], poor quality of life [3], serious morbidities [4] and increased mortality [5]. Is obesity a disease? Eminent researchers and experts in the field have tried over time to answer this question. In 2008, a panel of experts was invited by the Obesity Society (TOS) to analyze pertinent arguments and evidences about the opportunity and scientific relevance of labeling obesity as a disease [6]. The conclusions reached by the panel are of crucial interest for a global approach to the obesity issue. Given the lack of a clear and shared definition of "disease", the direct answer was judged not attainable. However, the experts unanimously chose the utilitarian approach, according to which obesity should indeed be considered a disease, as this is likely to generate far more positive than negative consequences, in terms of: 1) greater resources for prevention, treatment and research; 2) surge of high-quality caring professionals specifically dealing with obesity; 3) reduction of weight bias and discrimination against obese persons.

Diagnosing obesity

The operational definition of obesity is primarily based on body mass index (BMI, weight divided by height squared) and waist circumference (WC) assessments. However, the effects of adipose tissue accumulation may vary as a function of age, ethnicity, sex and other factors. Therefore, the operational definitions need to be adjusted depending on these factors and following the progress of knowledge about obesity. According to the World Health Organization (WHO), an individual should be classified as overweight when his BMI is higher than 25 kg/m^2, and obese when his BMI equals or overcomes 30 kg/m^2. Patients with BMI above 30 kg/m^2 are further sub-classified based on the severity of the

condition. Finally, a BMI value higher than 40 kg/m^2 indicates patients with severe or extreme obesity, for which a surgical intervention may be necessary [7, 8]. In the clinical practice, however, the mere calculation of BMI is not sufficiently accurate, particularly since the true body composition and fat distribution are not taken into account. The increased knowledge about the endocrine and metabolic negative consequences of intra-abdominal fat accumulation [9] led to the use of WC assessment as an important, though still not exhaustive, indicator of health status. WC represents, indeed, a reliable index of the absolute amount of abdominal fat, but it cannot discriminate between visceral and subcutaneous depots. The healthy limits of WC are generally fixed at 102 cm in men and 88 cm in women, as recommended by several guidelines [7, 8]. As extensively reported, overweight/obese patients with central or abdominal obesity constitute a particular subgroup presenting an increased risk of type-2 diabetes and cardiovascular disease [10-12]. The evaluation of abdominal fat accumulation is now considered of crucial importance, especially since its clinical relevance can widely vary among people. For instance, post-menopausal women may present selective deposition of intra-abdominal adipose tissue and a concomitant loss of muscle mass [13, 14], but these modifications would not sensibly affect body weight and BMI. Furthermore, changes in abdominal fat accumulation are frequent with age and more common in some ethnic group, regardless of BMI values [8]. Together with BMI and WC, the clinical assessment of obesity also requires an accurate analysis of metabolic indices, firstly glucose tolerance/insulin sensitivity as well as lipid and inflammatory profiles [15]. In addition, Sharma & Padwal recently suggested that the clinical assessment should also address the aetiological determinants of overweight/obesity, since they may greatly vary among individuals but are necessary elements for successful obesity management [16].

Since the first look at the issue, the crucial concept to acknowledge about obesity appears to be variability: in any individual, a unique combination of interconnected factors influences body weight and fat accumulation, and therefore contributes to obesity aetiology, clinical presentation as well as social and health consequences (Figure 1).

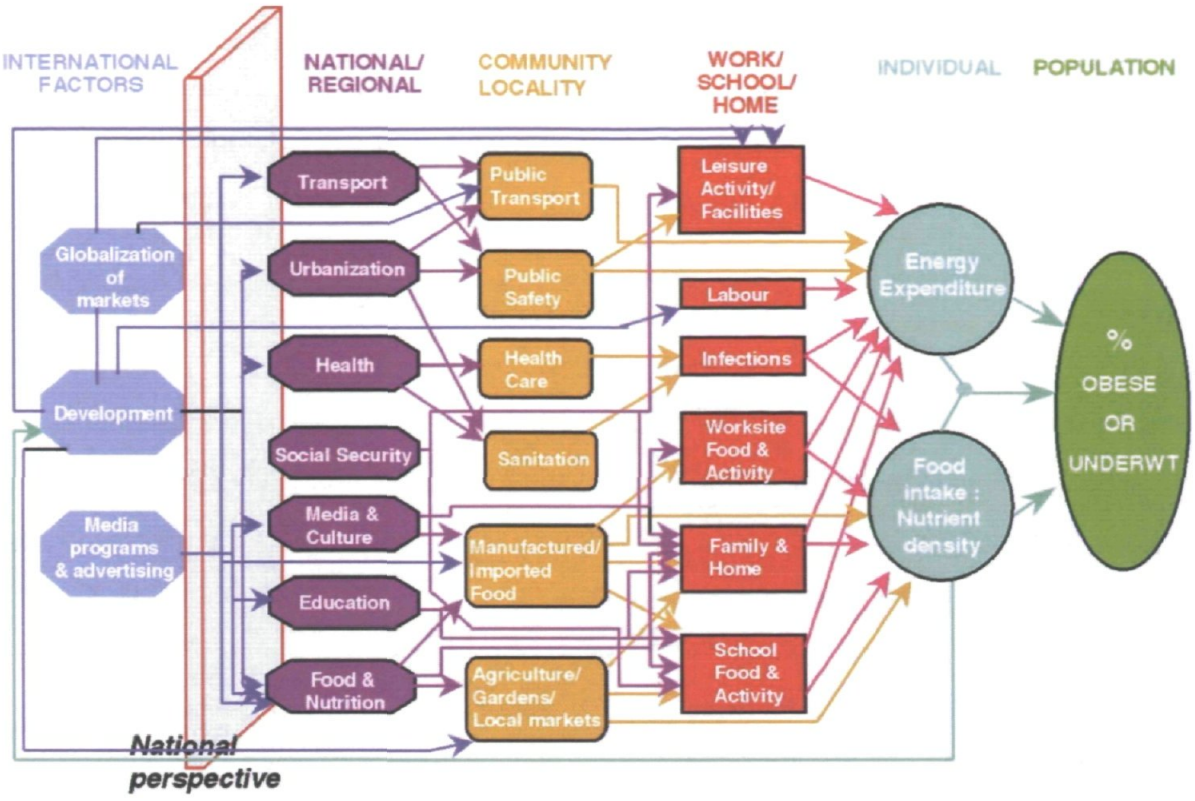

Figure 1. Causal web of societal influences on obesity prevalence. Adapted from: Ritenbaugh C, Kumanyika S, Morabia A, Jeffery R, Antipatis V. of the International Obesity Task Force, 1999, http://www.iotf.org

Sex differences in obesity epidemiology

Significant sex, as well as gender (related to social behavior), differences exist between men and women in terms of obesity. Scrutinizing the differences may be crucial in order to guide both clinical practice and therapeutic approaches. According to WHO 2008 estimates, in most countries the prevalence of obesity has been higher in women than men. In United States, two thirds of women 40 to 60 years of age are overweight or obese [17], and women are more likely to suffer from extreme obesity than men [18]. In particular, menopause is now considered an indipendent risk factor for body weight gain, abdominal obesity, metabolic syndrome and cardiovascular disease [19]. Several studies have concluded that menopause causally contributes to deteriorate the metabolic profile of women independently of aging [19], and a great deal of attention has been recently devoted to the significant physiologic events occurring during the menopausal transition and perimenopausal phases. These phases cover the early changes in hormonal secretion and

menstrual regularity that culminate in the final menstrual period and menopause [19]. From the hormonal perspective, in the transition phase, the progressive decline of estrogen secretion is accompanied by a less rapid drop of androgen levels, which originates a temporary state of androgenicity [20, 21]. It has been hypothesized that most deleterious metabolic changes occurring in post-menopausal women may stem during the transitional phase from the effects of acute androgenicity, and further investigations are being conducted to better elucidate the role of androgenism in women [22, 23]. This is also of high interest for other pathologic conditions involving a chronic state of hyperandrogenism in women, such as those included in the polycystic ovarian syndrome (PCOS) [23], which is again strongly correlated to obesity. It should also be noticed that psychiatric eating disorders more prevalently affect women than men, and women are nine times more vulnerable to anorexia nervosa and bulimia nervosa [18]. Taken together, this rich line of epidemiological evidences, as well as the indications showing that the negative clinical implications of central obesity may be greater in women than men, have led researchers and clinicians to give top priority to women in both research investigations and medically assisted weight loss programs [22]. The largest part of weight loss commercial programs have been set up on women, while a limited number of studies has analyzed sex differences of pharmacokinetics and clinical response following anti-obesity drugs treatment. It has been postulated that studies in this direction would be greatly beneficial for improving the management and therapy of obesity [23].

Health concerns of excess body fat

Over the second half of the 20th century, overweight and obesity prevalence has reached epidemic proportions, involving developed and developing countries. Obesity is now estimated to be the most prevalent nutritional problem in the world, whose contribution to ill health and mortality has overcome that of undernutrition and infectious diseases [24]. Unfortunately, this condition is now affecting a rising number of children and adolescents [25], causing major concerns for the future generations and underlining the need for urgent global interventions. As Olshansky and colleagues reported in 2005, the health consequences of the obesity epidemic are expected to reverse the 20th century gains in life expectancy [26]. These data are mostly due to the endocrine, metabolic and cardiovascular

consequences engendered by the excess of body fat. Furthermore, obese persons have a higher risk to develop certain cancers and to experience a reduced quality of life due to non-fatal but still invalidating disorders [4]. Table 1 shows the prevalent health risks potentially associated with excess body weight. The pertinent discussion of the relationship between obesity and any of these health concerns is beyond the scope of this thesis. However, some of those connections need to be described since they pertain to the deleterious consequences of obesity/fat excess on the endocrine regulation of energy metabolism.

Table 1 **Potential health concerns associated with obesity**

Organ System/ Disease State	Health Effects
Cancers	Men: esophageal cancer, stomach cancer, colorectal cancer, liver cancer, gallbladder cancer, pancreatic cancer, prostate cancer, kidney cancer, non-Hodgkin's lymphoma, multiple myeloma, leukemia.
	Women: uterine cancer, cervical cancer, ovarian cancer, breast cancer, colorectal cancer, liver cancer, gallbladder cancer, kidney cancer, non-Hodgkin's lymphoma, multiple myeloma.
Cardiovascular	Atherosclerosis, myocardial infarction, stroke
Dermatologic	Acanthosis nigricans, skin tags, acne, boils, hirsutism, pathologies of augmented folds, plantar hyperkeratosis, cellulite, stretch marks, varicose veins.
Endocrine	Insulin resistance, type 2 diabetes mellitus
Gastrointestinal	Nonalcoholic fatty liver disease, gallbladder disease
Musculoskeletal	Osteoarthritis and degenerative joint disease, changes in foot anatomy due to excess load
Pulmonary	Obstructive sleep apnea
Reproductive	Men: premature testosterone decline, erectile dysfunction
	Women: polycystic ovary syndrome

Adapted from Brown VW, Fujioka K, Wilson PW, and Woodworth KA (2009) Am. J. Med., vol. 122: pag S9

Adipose tissue and metabolic dysfunctions

A recurrent state of positive energy balance (energy intake overcoming energy expenditure) enhances fat accumulation and body weight gain. The way energy is stored as fat, or differently employed, may greatly vary among individuals, mainly as a function of genetic background and environmental stimuli. The adipose tissue, however, is not only a functional store, which protects the body from variations in energy availability. A great wave of research has revealed that adipose tissue is a key endocrine organ, producing and releasing a relevant panel of different factors, and thus influencing metabolic and immune homeostasis [27-29]. In addition, different fat depots display distinct endocrine potentials, and this led researchers to highlight the importance of fat topology in determining the health status in a condition of excess fat deposition [30]. While subcutaneous fat is considered a useful and not harmful "metabolic entrepôt", numerous epidemiological and physiologic studies have evidenced that intra-abdominal/visceral fat is particularly detrimental, due to its correlation with metabolic and cardiovascular disorders [11, 12, 31-33]. Menopause may represent a useful clarifying example. Pre-menopausal women generally tend to accumulate more subcutaneous than visceral fat compared to men, and this may contribute to lower their risk of cardiovascular accidents. Menopause, however, is associated with substantial changes in hormonal balance and fat distribution, typically causing a selective deposition of visceral fat that may enhance the cardio-metabolic health risks [13, 14, 34]. An elevated abdominal fat depot is primarily predictive of insulin resistance [35], a condition in which the increased insulin production is counterbalanced by decreased insulin sensitivity in key metabolic tissues, such as liver, skeletal muscle and adipose tissue. Individuals presenting insulin resistance are at higher risk to develop type-2 diabetes, and a threatening atherogenic and prothrombotic profile [36]. Abdominal obesity together with a specific atherogenic dyslipidemia, insulin resistance, impaired fibrinolysis and a low-grade pro-inflammatory profile mainly characterizes the condition defined as metabolic syndrome [15]. Though it is clear that insulin resistance is commonly present in abdominal obese persons, it is still uncertain if the visceral fat has to be considered the initial culprit of the dysmetabolic state. A number of interesting hypotheses have been generated over time. Several mechanisms may be involved, distinctly or, most probably, in concert: 1) as the excessive energy intake and a permissive neuro-endocrine environment

[33] cause the subcutaneous "good" fat to become dysfunctional, the energy surplus is stored in visceral "bad" fat or in other unfavorable ectopic sites, such as the liver, heart, skeletal muscle or pancreas, thus compromising their normal metabolic functions [37]; 2) since lipolysis is substantially increased in insulin-tolerant visceral adipose tissue, the higher output of free fatty acids into the portal circulation directly affects liver physiology, and causes enhanced hepatic glucose production, decreased insulin clearance and elevated release of triglycerides (TG) in circulation [38, 39]; 3) the endocrine potential of adipose tissue includes a large number of adipokines (such as resistin and adiponectin) [27] and inflammatory cytokines (such as interleukins and tumor necrosis factor (TNF)-α) [29], whose signaling finally impacts the inflammatory system and its cross-talk with energy metabolism regulation, thus leading to insulin resistance and to the pro-inflammatory, pro-thrombotic and hypertensive state characterizing visceral obesity [40, 41].

Links between metabolic dysfunctions and inflammation in obesity: cellular level

The studies exploring metabolic and inflammatory features of adipose tissue in obesity have evidenced the extraordinary interaction between the physiologic control of energy balance and immunity. Ultimately, metabolic and inflammatory pathways are interdependent, and converge to one finely regulated system that preserves life through protection of the homeostasis. In recent years, this intriguing subject has been enriched with a plethora of studies, introducing the concept of obesity (or diabetes) as a chronic low-grade inflammatory disorder [42].

The adipocyte is a highly complex cell, in which the flux of energy is regulated as a function of global energy availability. In turn, to preserve homeostasis, the levels of peripheral energy stores proportionally influence intake and expenditure mechanisms. These roles are accomplished by a specialized network of sensing signals and hormonal messengers, which allow short-term and long-term communication between adipose tissue and distant organs such as muscle, liver, gut and brain [27, 28]. While the system is prepared to balance occasional perturbations, the regular and persistent over-nutrition is likely to compromise the homeostasis, and shift it toward a new energy set point. From a cellular point of view, when the flux of energy into the adipocyte is in unbearable excess, the stress caused to its organelles, particularly the endoplasmic reticulum and mitochondria,

may switch on important inflammatory pathways [42, 43]. Indeed, the activation of c-Jun N-terminal kinase (JNK) and nuclear factor kappa-B (NF-kB) downstream signaling leads to increased expression of pro-inflammatory cytokines and chemokines such as inteurleukin (IL)-6, TNF-α, and monocyte chemoattractant protein-1 (MCP-1) [41, 43]. These molecular events might explain the subsequent macrophage recruitment into the expanding adipose tissue [44, 45]. Macrophage activation is likely to amplify the pro-inflammatory cascade and further impair cell insulin sensitivity, thus alimenting a vicious cycle. The resulting state of chronic inflammation, hypoxia, oxidative stress and mechanical stress of hypertrophied adipocytes has a deleterious impact on systemic energy balance, by affecting glucose and lipid metabolism in other key sites such as muscle, liver and pancreatic β cells [35]. As mentioned above, the overloaded insulin-tolerant adipose tissue may also present increased levels of lipolysis [38, 39, 46]. Lipids are then re-directed toward ectopic sites of accumulation, further perturbing the whole-body metabolic and inflammatory equilibria [37, 46].

High-fat consumption and inflammatory response

The regular over-consumption of high fat (HF) and energy dense food, often present in Western diets, has been indicated as the main culprit for the obesity epidemic and its deleterious consequences [47, 48]. HF food is likely to promote over-consumption because of its delayed and reduced effects on satiety perception (compared to high-carbohydrate or high-protein intake), though the molecular events responsible for this phenomenon are still far from being completely elucidated. Furthermore, it is interesting to note that HF food consumption has been shown to promote tissue and systemic inflammation in various models of diet-induced obesity [49-53]. Studies in both rodents and humans have evidenced altered blood circulating levels of inflammatory markers following HF feeding. Longer HF diets may entail a rise of inflammation markers not only in adipose tissue [50, 51], but also in the gut [53, 54], liver, muscle [49, 53], and hypothalamus [52]. Interestingly, de la Serre and colleagues recently showed in rats that the HF-induced inflammation of the intestinal epithelium might promote food intake and lead to obesity [54]. In addition, several reports have evidenced that HF-induced obesity causes the activation of inflammatory pathways in the hypothalamus, thus impairing key hypothalamic regulatory signals, such as leptin [55,

56] and insulin receptor downstream events [52, 57]. Since inflammatory pathways may substantially influence the mechanisms controlling energy intake, it is possible to hypothesize that HF-induced inflammation may directly contribute to the pathogenesis and development of obesity, in genetically vulnerable subjects. Recent evidences appear to further this idea. In a study conducted by Posey and colleagues, the ability of insulin to reduce food intake and activate hypothalamic signal transduction was reduced in HF vs. low-fat (LF) pair-fed rats, with HF-fed rats showing increased hypothalamic levels of inhibitor of kappa B kinase-β (IKK-β), i.e. a marker of inflammatory signaling activation [57]. Increase of IKK-β signaling had previously been shown to promote HF food intake and susceptibility to diet-induced obesity in mice [58]. Therefore, HF intake may be a sufficient stimulus to induce inflammation and impair insulin effects in the hypothalamic circuitries controlling food intake [57]. In addition, the HF-induced activation of inflammatory pathways in the hypothalamus may eventually lead to neuronal apoptosis [59]. Interestingly, neurons expressing pro-opiomelanocortin (POMC, a satiety signal) and those producing agouti-related peptide (AgRP, an appetite trigger) showed comparable levels of apoptosis in rats with a higher resistance to diet-induced obesity, whereas POMC neurons were specifically depleted in obesity-prone mice following HF feeding. The authors finally suggested that diet composition, and not caloric intake, may be directly responsible for activating apoptotic proteins in the hypothalamus; and that, depending on genetic background and on different environmental factors, changes in genesis and survival rates of hypothalamic neurons can influence food intake and body adiposity [59].

The evidences so far acquired suggest that the dysfunction of key tissues for the regulation of energy balance may originally be induced by excess lipid intake, but locally transduced by inflammatory reactions. The possibility to modulate the impact of inflammatory pathways on energy metabolism, without affecting the immune homeostasis, has opened novel interesting avenues for the research of potential therapeutic targets to prevent or treat obesity.

The case of metabolically healthy obese persons

To conclude this section, which attempted to give a comprehensive view of the most prevalent metabolic health concerns for overweight and obese patients, it appears pertinent

to add some comments about the metabolically healthy obese phenotype. Indeed, although the greatest part of obese patients experience ill health, it is well known that obesity is not always automatically associated with metabolic and cardiovascular disorders. Despite the lack of clear consensus about the definition of "metabolic normality", a recent review on the topic has reported that approximately one in three obese individuals remains metabolically healthy despite the excess of body fat [60]. These subjects generally present normal blood levels of glucose and lipids, regular blood pressure and a healthy cytokine profile. In addition, they have equivalent cardio-metabolic risk factors compared to lean counterparts, and their good health might even suffer from weight loss [61]. Notably, compared to the "unhealthy" patients, the metabolically healthy individual is a rare phenotype [62], estimated for instance at around 1.3 % of the US population, and frequently characterized by limited intra-abdominal fat accumulation, high levels of physical activity and an earlier age of onset (< 20 years) [60, 62]. However, in a recent report, Kuk & Arden have pointed out that metabolically normal obese subjects are still at higher risk for all-cause mortality compared to non-obese counterparts [62]. In fact, regardless of whether they present insulin resistance or other common metabolic risk factors, obese patients are more likely to die for traumas, and advanced-stage cancers (because of late diagnosis). In addition, obese patients tend to avoid seeking health assistance, and this is probably to ascribe in the context of weight-bias and discriminatory attitudes frequently showed by health-care providers [63].

Beyond the clinical aspects and from a strictly physiologic point of view, it is interesting to recognize that: 1) in certain subjects, the large accumulation of fat may be the counterbalance to a novel metabolic stability [64]; 2) increased levels of physical activity are often associated with reduced visceral fat depots and improvement of cardio-metabolic risks, despite small changes in total body weight [65]; 3) in a number of obese subjects, weight loss may even have disadvantageous effects, for instance on immune parameters [64, 66] and stress vulnerability [67, 68]. This has led physiologists to describe body fat gain as an important biological adaptation to complex environmental stimuli, and to suggest the prevailing therapeutic importance of a healthy lifestyle compared to the general focus on body weight loss [64]. As the following paragraph will briefly illustrate, the current strategies employed to induce weight loss and treat obesity have encountered only a modest

and limited success. A better understanding of the physiologic mechanisms underlying feeding behavior and promoting the healthy attitude for physical exercise would be strategically helpful in designing new successful treatment approaches.

A look into the current therapeutic options for obesity

The main targets of obesity treatment are the reduction of body weight/adiposity and the improvement of cardio-metabolic risk factors. Diet and lifestyle interventions currently represent the keystones of obesity management and therapy. However, the weight loss achieved is often small and hardly maintained. In fact, though various options have been used for dietary interventions, the long-term preservation of a clinically significant weight loss, i.e. 5-10% reduction of initial body weight, is rarely attained [69]. On the other hand, increased levels of physical activity have been associated with reduced visceral fat depots and improvement of cardio-metabolic risks, despite small changes in total body weight [65, 70].

The regulation of body weight involves important physiologic systems that tend to protect the organism against starvation and energy store depletion. As a consequence, the optimal treatment strategy needs to embrace a combination of therapies and requires the a priori evaluation of the aetiological determinants characterizing each patient [16]. The 2006 Canadian clinical practice guidelines in the management and prevention of obesity in adults and children [24] suggested that a "comprehensive lifestyle intervention" is necessary and should combine the behavioral therapy with activity enhancement and dietary counseling. The use of a selected pharmacologic agent should be considered for those overweight or obese adults who are unable to attain or maintain clinically important weight loss with dietary and exercise therapy, and to prevent obesity-related symptoms. Ultimately, bariatric surgery may be a necessary option for adults with clinically severe obesity (BMI $\geq$ 40 kg/m2 or $\geq$ 35 kg/m2 with severe co-morbid disease), when lifestyle interventions prove inadequate to achieve healthy weight goals.

As the current therapeutic options, except for bariatric surgery, still prove largely unsatisfactory in the attempt to reach and maintain a healthy weight, the research work described in this thesis has the long-term objective to identify and characterize novel pharmacological targets for the prevention and therapy of obesity. In particular, the drugs

presently available for treatment are hardly successful in weight-maintenance, while they often present unpleasant when not risky side effects. Before proceeding to the presentation of my research project, it is interesting to describe the current pharmacological options and some of the potential new molecules presently under trial.

The pharmacologic approach to obesity: works in progress

As shown in Figure 2, the pharmacological approaches so far designed mainly act to: 1) reduce appetite / increase satiety; 2) reduce the absorption of nutrients; 3) increase energy expenditure. Globally, the weight loss achieved by pharmacotherapies has been generally modest, being 2 to 7,9 kg greater than that obtained with placebo treatments [71, 72].

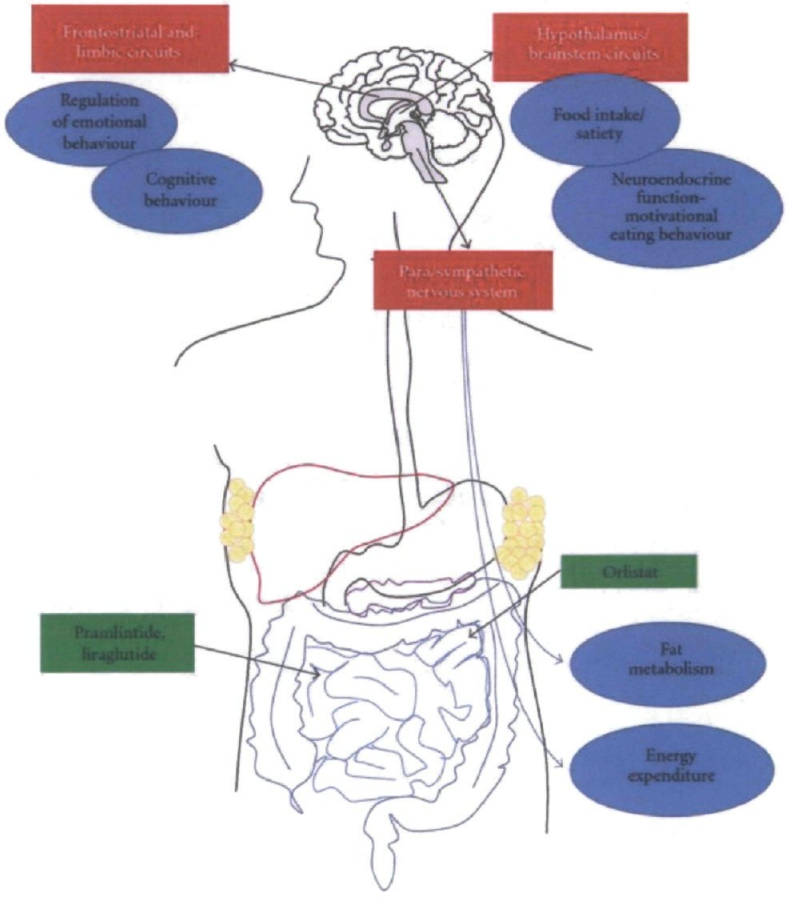

Figure 2. Overview of central and peripheral functional targets of anti-obesity pharmacotherapies. Adapted from: Ioannides-Demos LL, Piccenna L, and McNeil JJ. (2011) J. Obes.

In the past, various drugs have been used to treat obesity, including thyroid hormone, dinitrophenol and amphetamines, as well as amphetamine analogues, aminorex, fenfluramines, and rimonabant [71]. However, the serious adverse events associated with these drugs have usually led to their withdrawal from American and European markets, or to severe restriction of their use (such as for amphetamines) [73-75]. More recently, orlistat and sibutramine had been approved for long-term use (more than 3 months), though sibutramine has been finally pulled from the American market in 2010, because of its potentially dangerous sympatho-mimetic effects on heart-rate and blood pressure [76]. Therefore, the only drug presently available to treat obese patients is orlistat. While sibutramine was acting centrally, by inhibiting the serotonin and norepinephrine reuptake, and thus affecting appetite, orlistat functions as a gastrointestinal lipase inhibitor [69, 72]. Consequently, orlistat limits fat absorption in the small intestine, leading approximately to a 30% reduction of lipid uptake [69]. The weight loss achieved is modest (2.7 kg or 2.9% greater reduction compared to placebo-treated patients), but accompanied by a reduction of low-density lipoproteins (LDL) and cholesterol levels, as well as a better glycemic control, and a significant decrease of systolic and diastolic blood pressures [77-79]. The most common adverse effects of orlistat are gastro-intestinal and include diarrhea, flatulence, bloating, abdominal cramping, as well as dyspepsia and lipid-soluble vitamin deficiency [78, 80]. In addition, recent reports have pointed out the risk of liver injury associated with this drug consumption [81], thus caution is now warranted in selected patients.

In the recent past, the endocannabinoid receptor (CB1) antagonist rimonabant had generated great expectations for its capacity to reduce appetite and increase energy expenditure, leading to a significant weight loss [82]. However, since CB1 is widely expressed in the brain, rimonabant may present undesirable central effects [72, 83]. In particular, the higher risk of psychiatric and nervous system adverse events have caused its withdrawal from the European market in 2008, while the manufacturer decided to stop all the clinical research studies still in progress that same year [72]. Recently, a new wave of optimism about the pharmacological potential of CB1 blockers has been generated by the work of Quarta and colleagues in animal models, showing that functional endocannabinoid receptors are expressed in peripheral organs and significantly involved in the regulation of energy balance [84]. This study suggested that the design of CB1 antagonist molecules

unable to pass the blood brain barrier and selectively acting in periphery may be a promising strategy in the attempt to improve the beneficial metabolic effects (promoting energy oxidation), without causing the adverse events related to CB1 blockade in neural circuitries of mood and anxiety [84]. Among other molecules currently under trial, glucagon-like peptide 1 (GLP-1) analogues, namely liraglutide and exenatide, have recently shown promising results. These drugs have already been approved for the treatment of type 2 diabetes and have also demonstrated to be efficacious in promoting weight loss. The GLP-1 analogues, indeed, act on both peripheral and central targets, slowing down gastric emptying and promoting satiety, as well as inducing long-term anorexigenic effects that finally reduce appetite and energy intake [72, 85]. Phase III trials for obesity have shown beneficial effects of these two molecules on weight loss, glycemic control, and blood pressure without significant side effects compared to placebo controls [86, 87]. Finally, a combination of bupronion and naltrexone (named "Contrave") is currently under clinical trial and shows an interesting mechanism of action, thought to tackle the motivation/reinforcement that food brings (dopamine effect) and the pleasure/palatability of eating (opioid effect) [72, 88]. In brief, the two molecules act to promote α-melanocyte stimulating hormone (α-MSH) release, which is a potent anorexigenic factor with a wide range of targets [89].

In summary, this first section has introduced general concepts about obesity and the potentially detrimental consequences of excess fat accumulation for health. In particular, much attention has been paid to the physiological and molecular mechanisms associated with the cardio-metabolic risks in overweight and obese patients. Several studies in both animal models and humans have been cited, showing that the metabolic dysfunctions engendered by excess body fat in obesity are thought to be often initiated by the activation of inflammatory responses, and perpetuated by the creation of a vicious circuit between energy metabolism and innate immunity. One interesting aspect illustrating the overlap of inflammatory and metabolic components of homeostasis is the potential pro-inflammatory impact of HF consumption on gastro-intestinal and hypothalamic functions, since this might importantly contribute to the poor effects of HF intake on satiety perception, and favor energy overconsumption and body fat gain. In conclusion, the main pharmacological

approaches to treat obesity have been presented. In most cases, the modest efficacy of these drugs is associated with unpleasant if not risky adverse side effects. Hence, the identification of novel pharmacological targets and the development of new valuable molecules represent a vast and urgent area of research.

1.2 ORIGINS OF OBESITY

Interplay between genes and environment

Obesity represents an excellent model of a multifactorial phenotype disease. Obesity scientists are challenged to unravel how the complex interactions between individual genetic backgrounds and evolving environmental stimuli occur to influence physiologic adaptations and originate a person's behavior. In principle, the physiologic systems regulating energy balance function to preserve energy homeostasis, by finely reacting to periodic fluctuations in energy intake and utilization. For instance, the consumption of one meal can trigger different short-term reactions that depend on its composition, palatability and size. In simplified terms, if the energy ingested has overcome the actual energy need, the system is able to decipher it and to react proportionally, likely by reducing appetite and increasing energy expenditure in the following hours. However, what happens over the long term when the excess of energy intake occurs regularly? Is the system prepared to cope with it? Several evidences support the concept according to which individuals are differently equipped (in biological terms) when reacting to an obesogenic environment [90-92]. Energy balance is controlled by long-term reactions balancing: 1) energy intake; 2) energy expenditure; 3) nutrient partitioning and 4) adipogenesis. The genetic predisposition might substantially affect all 4 major points of regulation, and with variable magnitude. The genomic studies attempt to identify and characterize the genes involved in these metabolic pathways; to dissect their relative importance in the regulation of energy balance and the development of obesity; and to evaluate their interaction with behavioral and environmental factors. The work presented in this thesis endeavors to participate in this rich and fascinating domain of obesity research.

Genetics and genomics of obesity

The genetic issue can firstly be approached from an "evolutionary" point of view. In 1962 James Neel introduced the "thrifty genotype" (low metabolic rate and insufficient thermogenesis) hypothesis [93, 94], which has been further supported and revisited by obesity scientists and clinicians over time [94-97]. In essence, it has been hypothesized that, since human beings have evolved to efficiently store energy and successfully resist to

periods of famine, they presently result genetically unprepared to cope with an obesogenic environment. By definition, the expression "obesogenic environment" basically designates a condition in which, for a large variety of reasons, the excess of energy availability coincides with the decreased levels of physical effort required to get food [98, 99]. However, it has also been claimed that the "thrifty genotype" hypothesis is inadequate to account for the global biological predisposition to obesity, and to explain the current epidemic in all populations [97, 100]. In a commentary on the topic, Claude Bouchard proposed to open the genetic scenario to other categories, such as the hyperphagic genotype (poor regulation of appetite and satiety and propensity to overfeed), or the "low lipid oxidation" genotype; and to consider that the genetic predisposition to accumulate weight may be caused by many genes and by the combination of more than one genotype [97]. Moreover, recent studies have pointed out to the possibility that a portion of the biological predisposition does not directly derive from DNA sequence heterogeneity, but is rather entrained by epigenetic events occurring during fetal life and perinatal/early postnatal periods [101, 102]. These fascinating hypotheses further support the role of biological predisposition in determining commensurate behavioral phenotypes. Moreover, there is large agreement on the point that, since the obesity epidemic occurred only over the last four decades, it cannot be explained by selection-driven changes in our genome. Rather, in developed as well as developing areas of the world, the shift toward an obesogenic environment has modified behaviors and led to body weight gain in genetically vulnerable portions of the population. Interestingly, several epidemiologic studies in twins and families have demonstrated the importance of genetic traits in determining eating behaviors and body weight. In 2003, Loos and Bouchard reviewed the most important epidemiologic studies and concluded that heritability (intended as a trait due to genetic factors, but not necessarily to single genes) of human adiposity is between 30% and 70%, with the highest values coming from twin studies; and that the risk of obesity is two to three times higher for a person with a family history of obesity [103]. In contrast, only 1% to 5% of obesity cases can be explained by a single-gene mutation. Therefore, for most people affected, obesity is a complex disorder caused by multiple genes and multiple gene variants. To simplify, four levels of genetic contribution to obesity have been proposed: 1) genetic obesity: mutations in one gene lead to obesity regardless of the environment; 2) strong predisposition:

overweight in non-obesogenic environment and obesity in obesogenic environment; 3) slight predisposition: normal weight in non-obesogenic environment and overweight in obesogenic environment; 4) genetically resistant: normal weight in obesogenic environment [103].

As mentioned above, the genomic perspective considers the effects of all genes as well as the interaction of those genes with each other and with the environment, and may be very helpful in the attempt to unravel the critical pathways and design better strategies for obesity prevention and intervention. In general, different approaches have been used to identify genes affecting the regulation of energy balance and the accumulation of fat tissue, ranging from single candidate-gene studies to more powerful genome-wide association studies. To date, more than 400 genes or markers have been associated with obesity [104]. Interestingly, many of the gene variants associated with high BMI and body weight are near genes expressed in the brain and involved in neuronal development and activity, and might affect eating behavior [105, 106]. For instance, melanocortin 4 receptor (*MC4R*) and Src-homology-2 domain containing putative adaptor protein 1 (*SH2B1*) are involved in neuronal signaling [107, 108]; brain derived neurotrophic factor (*BDNF*) is involved in neural development and may have a role in eating behavior [109]; fat mass and obesity associated (*FTO*) is expressed in regions of the brain affecting feeding regulation [110]. Moreover, heritability and linkage studies have proved that at least three patterns of eating behavior, namely restraint, disinhibition and hunger, are heritable [111]. Disinhibition has been genetically associated to neuromedin, a satiety factor, in a French-Canadian cohort [112] and to TAS2R38, a bitter taste receptor in a cohort of Amish women [113]. Recently, two specific variants of glutamic acid carboxylase (*GAD*) have been associated with disinhibition and disordered food intake, specifically increased carbohydrate consumption, in women [114]. On the other hand, physical activity is an important contributor to daily energy expenditure and, therefore, to body weight regulation. Epidemiologic studies have reported that 30% to 70% of the variation in physical activity is also inherited [115-118]; and a number of linkage and association studies have pointed out some candidate genes that might explain small, but significant, portions of this variation [119]. Some of these candidate genes have been selected for study because they are involved in known pathways affecting energy balance, such as leptin receptor, *AgRP*, and *MC4R* [119-121]. Other genes

may influence the motivational aspects of both feeding and physical activity, and include dopamine receptor (*DR*) D2/4, serotonin transporter (*SERT*), serotonin (*5-HT*) 2A/C, orexin A [122]. Interestingly, mesolimbic dopamine (DA) and opioid circuitries (including hypothalamus, ventral tegmental area and nucleus accumbens) appear to have an important role in motivation of voluntary physical activity and food-seeking behaviors [122]. In particular, orexin A-containing neurons of the ventral tegmental area (VTA) may be directly involved in opioid-dependent appetitive behavior and increased locomotion, as evidenced in rats [123].

The works presented in Chapters 2-4 are three transcriptomic studies, in which the serial analysis of gene expression (SAGE) method was used to compare the differential effects of physiologic and experimental conditions on the transcription profile of a chosen tissue. Although mRNA is not the ultimate product of a gene, transcription represents the first step in gene regulation, and can unveil preliminary elements about functional regulatory networks. Remarkably, gene expression profiling provides so far the most global picture possible of what a system is doing in a single experiment [124]. Techniques for the evaluation of gene expression have progressed from methods developed for the analysis of single, specific genes (e.g., Northern, slot, and dot blotting; semiquantitative and quantitative real-time PCR; and nuclease protection assays) to techniques focused on identifying all genes that differ in expression among experimental samples (e.g., subtractive hybridization, differential display, sequencing of expressed sequence tags, SAGE, and microarrays). Although microarray is the most commonly used technique for large-scale analysis of transcriptomes, the SAGE method is preferable when a discovery potential is needed [125, 126]. A few studies have so far given a global picture of gene expression in key tissues for energy homeostasis [127, 128], while often focusing on the overt obesity condition [129]. However, the early molecular mechanisms responsible for the de-regulation of energy balance controls and development of obesity have not been clarified, most probably because such mechanisms can be blunted after chronic feeding of lipids and long-term obesity [130]. As described later, our works aimed at contributing to fill this gap of knowledge, and specifically to identify the early signals triggered during the consumption of a HF meal.

Although increased food intake and decreased physical activity are largely considered the two most important factors in determining fat accumulation and higher body weight, and thus the two greatest contributors to obesity epidemic, other behavioral factors may have a significant impact. Interestingly, recent evidences have highlighted that short sleep duration and low dietary calcium intake are also, and even more, important predictors of excess body weight [131]. Given the complex network of factors that influence human behavior, the independent study of one single component may be complex, and biased by co-effects introduced by other contributors that importantly affect the balance between intake and expenditure. For this reason, studies in animal models are very important in helping scientists to dissect crucial physiological pathways before proving their importance in humans. This is also possible because, in animal studies, the investigator can strictly control the experimental conditions, in order to get the closest observation of single phenomena. The work presented in Chapter 5 of this thesis is a candidate-gene study conducted in mice, in which we attempted to characterize the role of a particular gene by studying the phenotypic consequences of its deletion from mouse genome, after controlling for important environmental conditions, such as the light-dark cycle and food availability.

Animal models for the study of obesity

Studies made in animal models have been fundamental for obesity research, in order to shed light on the genetic and physiological bases of energy balance regulation, and the impact of environmental factors. Furthermore, the development of obesity animal models allows researchers to test dietary interventions and/or pharmacological treatments that may be eventually used to control human obesity epidemic [132, 133]. To date, mice have represented the most extensively used and advantageous model for studying obesity, for several reasons. The mouse genetic map has been completely determined, with over 6500 PCR-based microsatellite markers recorded [134]. Furthermore, there are several different mouse strains, which provide a wide range of alternative genetic variances, and generally combine a short breeding cycle with a large litter size. This facilitates rapid proliferation of generations, and thus is very useful for inbreeding and analyzing selected genotypes/phenotypes [132]. Classical models of genetic obesity are those generated by spontaneous single-gene loss-of-function mutations, as occurs in the Agouti lethal yellow

mutant mouse (A^Y), and *ob/ob* and *db/db* mice. While the first is caused by the ubiquitous expression of Agouti protein (due to genomic loss of the tissue-specific control promoter element) [135, 136], the latter are characterized by defective leptin signaling. The *ob* mutation is a deletion of a single base pair in the gene coding for leptin, which results in a frame-shift and a premature stop-codon [137], causing mutant mice to develop hyperphagia, obesity, insulin resistance, type-2 diabetes and hyperinsulinemia. The *db* mutation is an autosomal recessive trait characterized by a point mutation in the leptin receptor gene [138, 139]. Consequently, the defective leptin signaling in the hypothalamus causes mutant mice to be hyperphagic and obese, and show hyperleptinemia, hyperinsulinemia and insulin resistance. In particular, *db/db* mice are frankly hyperglycemic, starting from 8 weeks of age, and thus represent an optimal model for studying type-2 diabetes [138, 139]. Currently, at least 10 known spontaneous single-gene loss-of-function defects that generate massive obesity have been described and genetically characterized. In some cases, the period between discovery of the original phenotype and its subsequent genetic and physiological characterization has been very long, as for leptin (50 years) [132]. The need for accelerating the progress of knowledge has led to the artificial generation of genetic mutations, and a great number of transgenic models with the obese (or lean) phenotype has been created [140, 141]. The current sophisticated gene-targeting strategies allow to introduce virtually any desired change in the genome, and also to specifically target a tissue or cell type [142]. The two most widely used techniques to generate a transgenic model are: 1) the global or tissue-specific over-expression of a target gene in an animal model; 2) the total ablation, or knock out, of the target gene in all tissues. While the over-expressing transgenic model may be not completely predictable and, in some cases, poorly informative, the knock out models have often produced unexpected effects that have added novel insights into the physiological actions of the target genes [132]. Recently, knock-in models have also been developed, in which the endogenous gene is replaced with a mutated form. Knock-in models are advantageous in that they allow to specifically address the effects of changes in protein structure or function [143]. However, it should be considered that, depending on the gene involved, the artificial manipulation of the genome to create a transgenic animal may directly result in embryonic death; or, in contrast, may elicit significant compensational events during the development of modified

animals, so that the direct effects of the gene studied may be missed or misinterpreted. A classical example is the case of neuropeptide Y (*Npy*) KO mice, which present no major phenotypic defects in energy regulation, despite the recognized pivotal importance of brain NPY signaling for energy balance [144]. In this model, the apparent lack of functional effects would, on the contrary, testify of the crucial importance of the physiologic pathways controlled by NPY, given that the system is prepared to overcome its loss with redundant compensational signals. In recent years, the Cre/loxP system strategy has innovated the technical procedures for transgenesis and allowed to produce the tissue-specific and time-specific knock out of target genes [145].

Given the importance of environmental, especially dietary, factors for influencing feeding behavior and body weight, the manipulation of diet has been successfully used in obesity research to study the effects of specific nutrients and/or energy loads that affect the control of energy homeostasis. In rodents, a plethora of studies has analyzed the impact of long-term HF consumption on body weight and energy metabolism, and thus specifically studied what has been defined as the HF diet-induced obesity (DIO) in genetically normal models. Interestingly, in both rats and mice, there are strain-specific responses to HF consumption. C57BL/6J mouse strain is the most widely used in HF-DIO studies, as these mice generally develop obesity, hyperinsulinemia and insulin resistance, and thus closely represent the progression of human DIO [146, 147]. On the other hand, the 129Sv strain is more resistant to DIO compared to C57BL/6J and show higher glucose tolerance and lower insulin circulating levels compared to other mouse strains [148]; the A/J strain mice present low glucose levels and are resistant to obesity and diabetes even on HF diet [149]; the CAST/Ei mice are lean after 12 weeks of HF diet [150]. Though the study of obesity-susceptible models has been of high importance for understanding the development of obesity and related diseases, the analysis of resistant models may also be fruitful in order to elucidate which are the biological factors that might be protective against fat accumulation as well as the environmental or physiological mechanisms that may eventually override that protection. In the work of Levin and colleagues, for instance, outbred Sprague-Dawley rats that show opposite responses to HF diet have been selected and inbred lines have been derived from those most resistant and those most susceptible to become obese [151]. The authors have subsequently investigated the physiological and genetic factors that

differentiate the two lines and thus contributed to highlight potential mechanisms leading to obesity [152].

1.3 PHYSIOLOGY OF ENERGY BALANCE AND OBESITY

Energy balance represents the result of a simple and yet elaborate equation combining food intake and energy expenditure. Basically, the energy ingested is metabolized to fuel the basal metabolism, as well as thermogenesis and muscle action (the three terms of energy expenditure); the fuels in excess are then efficiently stored as fat, to be readily used in times of need [153]. To maintain energy balance, three coordinated systems work in a feedback loop: 1) a nutrient and energy sensing system; 2) a central control hub receiving, integrating and originating redundant signals; 3) an effector system capable to adjust, through diverse pathways, energy intake and expenditure. As described in the previous section, this highly complex network is subject to inter-individual variability, due to genetic predisposition and early-life events. Environments and lifestyles interact with biological predisposition and substantially influence both food intake and energy expenditure. In a condition of *ad libitum* intake, the obesogenic environment is more likely to override the physiologic control of homeostasis and induce long-term adaptation to excess energy flow. As a matter of fact, most obese people do reach energy balance. In particular, the changes in body composition are likely to alter and refashion nutrient oxidation and total energy expenditure, in order to reach nutrient balances. The enlarged body weight normally causes an increase in energy expenditure, which is proportional to energy intake. Therefore, in obese individuals, body weight and composition can remain relatively stable for long periods of time. As explained by Tremblay A., fat mass accumulation can be considered a fundamental mechanism of protection through which the body responds to the elevated flow of energy that de-stabilizes homeostasis [64]. In this perspective, adipose tissue should be regarded as a functional vehicle of defense. Moreover, weight regulation may appear an asymmetrical system, which strongly resists to a reduction in energy stores -once a proper energy set point is reached- whereas weight gain can be a much easier adaptation [64].

The purpose of this section is to describe the current knowledge about the major central and peripheral mechanisms regulating energy balance. In the brain, numerous structures are involved in this regulatory system, but three are mainly concerned: the caudal brainstem; the hypothalamus; and the cortex and limbic systems. In the periphery, the gustatory system, gut, liver, pancreas, adipose tissue, and muscle generate a complex

network of signals that sustain bidirectional avenues of communication with the brain. These signals include neural connections of the autonomic nervous system, as well as hormones and metabolites. A particular attention will be addressed to gut nutrient-sensing molecules with a role in the perception of satiety during the consumption of a meal, as this issue is directly related to the research work presented in the thesis.

Nutrient sensing

Nutrient sensing starts in the oral cavity and the gut, from where taste buds and gastro-intestinal (GI) sensors capture and immediately transmit the relevant information to the caudal brainstem. The communication to the brainstem is rapid as well as crucial for the control of ingestion, and takes place by the activation of neural pathways in the gustatory and vagal afferents. In the oral cavity, an efficient system of taste receptors is responsible for the early detection of essential nutrients [154]. This system works to discriminate between beneficial or harmful foods, and thus influences food intake. The tastes of sweets and aminoacids (or umami) are considered beneficial, and intercepted by a family of three G-protein-coupled receptors, namely T1R1, T1R2, T1R3. On the other hand, the bitter taste of potentially toxic food is recognized by the activation of the T2R family of about thirty receptors. The salt taste is also captured, and mediated by the amiloride sensitive sodium channels. Interestingly, the presence of fat taste receptors has been debated, and not yet fully clarified. A number of candidate receptors able to detect fatty acids in the oral cavity have been studied, including the fatty acid translocator CD36, the potassium channel KV 1.5 and, more recently, the transient receptor potential type m5 [155-157]. It has been proposed that fat taste receptors may significantly influence ingestive behaviors in rodents and humans, and be responsible for high or low fat preference in specific phenotypes [156, 158, 159]. In the gut, chemo- and mechano-sensory mechanisms mediate to the brainstem a quantitative estimation of the food temporarily stored in the stomach, and communicate the presence of nutrients, and energy, that will be soon available for metabolism. In the brainstem, the information received through vagal sensors is integrated with the signaling generated by various gut-secreted hormones. The GI tract releases more than twenty different regulatory peptide hormones, which are sensitive to the nutrient content, and able to influence food intake and various physiological processes. The coordinated changes in

the levels of these hormones modulate the satiety/appetite balance, and thus affect the short-term regulation of energy homeostasis. The greatest part of these signals is released post-prandially and has a satiating effect, while the secretion of the only "hunger" peptide so far discovered, ghrelin, is rapidly suppressed by meal ingestion. Gut hormones act by binding their specific receptors on vagal afferents, and thus triggering a message rapidly transmitted to the brain. However, these molecules can also access the blood circulation and directly reach the brain, particularly the hypothalamic structures important for the regulation of energy balance. In addition, a number of these gut peptides are also expressed in brain structures, where they act as neurotransmitters, not rarely serving different functions from those exerted in the periphery [160]. A growing number of studies have confirmed that gut-secreted hormones are not only crucial for the short-term regulation of food intake and energy homeostasis, but can also be key activators of long-term physiologic adjustments of energy balance. As a consequence, gut-secreted hormones are considered optimal targets for anti-obesity drug designing. Furthermore, it is expected that much more gut-derived hormones exist and will be discovered in future days [161].

Gastric peptides influencing energy homeostasis

As mentioned, ghrelin is the only GI hormone so far discovered that enhances appetite. It is mainly secreted by the oxyntic glands of the gastric mucosa, and was first identified as an endogenous ligand of the growth hormone segretagogue receptor (GHS-R) [162, 163]. This receptor is expressed on vagal afferents innervating the gastric walls, and also abundantly present in the hypothalamus, particularly on NPY/AgRP and POMC expressing neurons of the arcuate nucleus (ARC). Ghrelin secretion follows a basic circadian rhythmicity [164] and is negatively influenced by food ingestion. High circulating levels of ghrelin are reached during fasting periods, and stimulate appetite and meal initiation in rodents and humans, while having no direct effects on meal size [165]. It has been reported that ghrelin is also capable to stimulate gastric secretion and motility, and to protect gastric mucosa from epithelial damage. Together with short-term actions, ghrelin also displays long-term effects on body weight and energy metabolism, as its chronic administration can induce weight gain and enhance fat storage in animal models [166, 167]. In particular, it has been reported that ghrelin can modulate glucose and lipid metabolism, by acting on various

target tissues in antagonism with insulin physiologic actions. Moreover, animal models with disrupted ghrelin signaling were resistant to HF-DIO, and showed a preference for fat as a metabolic fuel [168]. Interestingly, the number of physiologic pathways affected by ghrelin has been substantially extended in recent years, going from the modulation of blood pressure to potential immuno-regulatory properties [169]. As ghrelin is expressed in brain structures other than the hypothalamus, new hypotheses have also been done about its extra-hypothalamic functions. In a recent review, Andrews ZB describes the last findings about the neuro-protective and neuro-modulatory properties of this hormone [170]. Ghrelin is thus an interesting example of a gut-secreted hormone with multiple and differential effects, that could be selectively targeted to modulate satiety and body weight.

Though the stomach represents a crucial site for the modulation of satiety during the consumption of a meal, the list of gastric secreted peptides involved in these mechanisms is still exiguous. Among these, gastrin-releasing peptide (GRP) is expressed by the endocrine cells of the stomach, and stimulates gastrin release while potently delaying gastric emptying [171], thus inducing satiety. It has been reported that peripheral administration in mice and intravenous infusion of GRP in humans can inhibit appetite and food intake [172, 173]. Following studies have demonstrated that this peptide is also expressed in the brain and displays a wide array of physiologic functions potentially affecting energy balance [174].

Major intestinal and pancreatic peptides influencing energy homeostasis

A larger number of powerful hormonal peptides regulating food intake is found in the proximal and distal sections of the intestine. Among these, cholecystokin (CCK), peptide YY (PYY) and GLP-1 have been extensively studied in recent years, leading to important and advantageous findings [160]. Globally, the secretion of gut-derived signals is finely regulated in response to nutrient intake, so that the amount of each peptide does not become disproportionate, and its activity is temporally limited, often having a very short life. It has been proposed that the effects of the regulatory hormones released in circulation are additive, while signal redundancy should allow the system to efficiently work even in the absence of one or more actors. Furthermore, intestinal hormones have also shown important long-term influences on a wide range of biological and physiological targets, and

thus have been evaluated as potential therapeutic tools. In at least one case, that of the GLP-1 agonists, the works so far conducted have been successful and will probably lead to new pharmacological approaches for obesity as well as diabetes.

CCK was the first gut hormone proved to influence food intake [175]. Starting from those studies and following the discoveries of recent years, it has become more and more evident that the GI tract is to be considered the largest endocrine organ of the body. CCK is released postprandially by the enteroendocrine cells (I cells) located in the mucosa of the duodenum, jejunum and proximal ileum [176, 177]. Its secretion is specifically induced by lipids, and by the peptides and aminoacids derived from protein digestion [171, 177]. Several evidences have confirmed that CCK acutely inhibits food intake through the activation of CCK1 receptors on the vagal nerve [178, 179]. The action of CCK is, indeed, prevalently paracrine and neurocrine, driven by the activation of neural pathways on the vagus nerve [180], and also exerted by the down-regulation of G-coupled receptors and neurotransmitters on vagal afferents [181]. Specifically, high levels of CCK down-regulate the expression of two important appetite-mediating receptors, the endocannabinoid receptor CB1 and melanin concentrating hormone-1 (MCH-1), while promoting the expression of Y2 receptors, targets for satiety signaling, on neurons projecting to the gut [181]. In addition, CCK acts to influence gastric motility and, particularly, to delay gastric emptying, and these effects certainly contribute to the suppression of feeding [180]. In humans, intravenous infusion of CCK causes an acute reduction of the meal size and duration [182], but not when administered more than 30 minutes before the start of a meal [183]. It has become clear that CCK effects on food intake have a strong acute but not chronic impact, since they do not persist following chronic infusion, and fail to be valuable after 24 hours [184]. It should be added that intermittent administration of CCK acutely reduces food intake, but this effect is compensated by the subsequent increase in the number of meals, and in appetite between meals [185, 186]. Finally, studies in animal models with disrupted CCK signaling have shown controversial results, failing to clearly demonstrate a role for CCK in the regulation of energy homeostasis [160]. However, both CCK and CCK1/2 receptors are expressed by specialized neurons in the myenteric plexum and in the brain, where they play a role in learning and memory, anxiogenesis, nociception as well as satiation [177]. Interestingly, it has also been reported that CCK may mediate the appetite-

inhibiting effects of inflammation, and exert immunosuppressive actions in gut inflammatory reactions [187, 188].

PYY is a member of the PP fold family, which also includes NPY and pancreatic polypeptide (PP). The proteins belonging to this family share a common tertiary structural motif known as the PP fold, and bind the Y family of receptors. The three PP fold peptides present distinct levels of affinity for the five Y receptors, namely Y_1, Y_2, Y_4, Y_5, Y_6 [180]. Furthermore, the Y receptors are differently distributed in the body and coupled to a variety of downstream signals, thus covering a wide range of biological and physiological actions. The gut hormone PYY is released by the L cells of the intestine, more abundantly in the distal sections, and also by the pancreas [160]. Its secretion follows food ingestion and is proportionally related to the caloric intake, while being suppressed by fasting [180]. Interestingly, it has recently been shown that exercise can also increase PYY release and prolong its activity [189]. The full-length peptide is cleaved to remove the N-terminus residues, and release the truncated form named PYY_{3-36}. Both forms are finally found in circulation after feeding, and acutely influence gastric emptying and food intake [190]. While PYY_{3-36} is known to selectively bind the Y_2 receptor, the full-length peptide can bind with high affinity the Y_1, Y_2 and Y_5 receptors, and this explains the differential range of peripheral and central effects that PYY may exert. Peripheral injection of PYY_{3-36} shows potent short-term satiating effects in rodents and humans, effects that specifically correlate to the activation of Y_2 receptors on vagal afferents [191, 192]. The acute effects on food intake may be accentuated by the delaying of gastric emptying, as PYY can significantly modify intestinal motility and the speed at which food is digested, though mainly through Y_1 rather than Y_2 receptors [190]. In contrast, the central administration of either form of PYY stimulates appetite-triggering pathways, switched on by Y_1 and Y_5 brain receptors [193, 194]. However, when the injection of PYY_{3-36} specifically targets the ARC, it results in a potent suppression of food intake in rodents [191]. Coherently, Y_2 receptor is highly expressed on NPY producing neurons of the ARC, where it is thought to auto-limit NPY release and promote POMC-derived signaling [180, 195]. In addition, the recent finding of direct PYY expression in the brain [196], together with previous works in PYY-deficient animals showing dysfunctional energy homeostasis, suggest that PYY may also have long-term physiological effects on body weight [197, 198]. Belonging to the same protein

family, PP is released by the endocrine pancreas after a meal, and also suppresses food intake. Its circulating levels are proportional to the calories ingested and remain high for up to 6 hours [180]. PP demonstrates higher affinity for the Y_4 and Y_5 receptors, and also acts to relax the gall bladder and inhibit pancreatic secretion. Some authors have suggested that its satiating effects specifically depend on its ability to delay gastric emptying, but other mechanisms may be involved [180]. Acute peripheral administration reduces food intake in rodents and humans [199, 200], acting through brainstem and hypothalamic pathways [201], and its anorexigenic effects persist for 24 hours after injection [200]. Furthermore, chronic administration of PP in *ob/ob* obese mice improved weight gain as well as the glucose and lipid profile, while transgenic mice over-expressing PP presented lower appetite and fat mass [201]. These studies demonstrated potential long-term functions for PP on energy homeostasis, and interesting therapeutic possibilities.

GLP-1 is one of the preproglucagon gene products (also including glucagon, GLP2 and oxyntomodulin), and is released postprandially by L cells of the ileum and colon. This peptide is a potent incretin, whose secretion follows carbohydrate and fat intake and stimulates insulin release from the pancreas. However, GLP-1 rapidly reduces the sugar rise in the bloodstream long before insulin release, most probably through its capacity to delay gastric emptying [202]. Its actions follow the peptide binding to the GLP-1 receptor (GLP-1R), expressed on both the brainstem-vagus axis and the hypothalamus. Functional studies conducted after vagotomy in rats have proved that the vagal communication to the brainstem represents the crucial avenue for short-term GLP-1 signaling [203]. Peripheral GLP-1 acutely suppresses appetite and food intake in rodents and humans [204]. However, the satiating effects may not have an immediate impact on meal size during food consumption, but rather prolong the period of satiety to the following meals [202]. Nonetheless, chronic administration of GLP-1 inhibits food intake and also reduces body weight in humans [205, 206], suggesting further central regulatory roles for this peptide. However, gut-secreted GLP-1 has a very short half-life and is rapidly inactivated by dipeptidyl peptidase-IV (DPP-IV) cleavage. The discovery that exendin 4, deriving from the saliva of the Gila monster lizard, is a potent GLP-1R agonist has opened new strategies for the study of the physiological and therapeutic relevance of this receptor [207]. Interestingly, exendin-4 has a half-life approximately 80-fold longer in the blood compared

to endogenous GLP-1. Promising findings in rodents and humans have shown that exendin 4 treatments have beneficial effects on glucose homeostasis, while also promoting body weight loss [208, 209]. Therefore, GLP-1 agonists (together with DPP-IV inhibitors) currently represent a rising class of therapeutic agents able to treat diabetes and possibly obesity, and numerous clinical trials have been completed or are presently under progress [210]. Not unlike the other gut hormones, GLP-1 is also expressed in various brain structures, where it displays specialized neuro-protective functions, as well as classic metabolic actions modulating energy homeostasis [211-213]. Another preproglucagon cleavage product, oxyntomodulin, also acts by binding GLP-1 receptor but with 50-fold lower affinity than GLP1. However, oxyntomodulin acutely inhibits food intake with the same potency [214], while chronic central administration in rats reduces body weight and possibly affects energy expenditure [215]. Unlike GLP-1, it has been proposed that the major oxyntomodulin actions involve hypothalamic pathways rather than the vagus-brainstem axis [216, 217]. Its therapeutic potential, alone or in combination with other agents, is currently under evaluation.

In the pancreas, β cells have sensing capabilities and signal glucose availability to the brain, through the secretion of insulin and amylin. Amylin peptide acts rapidly to reduce food intake, specifically meal size, and delay gastric emptying, while down-regulating glucagon expression [218]. Amylin primary target is the area postrema in the hindbrain, which lacks of a functional blood-brain barrier and is easily reached through circulation. On the other hand, insulin is a complex regulatory hormone with a wider range of action. The central targets for insulin signals are the hypothalamic nuclei controlling energy balance, but also various other brain structures, such as the limbic system [219]. In the periphery, liver, muscles and adipose tissues are privileged targets, but virtually all tissues are affected by its signaling. Furthermore, insulin is released by adipose tissue and, together with leptin, constitutes an important signal communicating the size of adipose mass to the brain [220].

The contribution of gut microbiota to energy metabolism

Discussing the gut and the mechanisms that control energy sensing and processing, interesting novel findings have recently pointed out the role of gut microbiota. The

population of germs colonizing the mucosa of the distal gut constitutes an extra genetic pool outnumbering that of the host, and may substantially affect the host biology [221]. In particular, gut microbes are able to harvest energy from the food ingested, and thus significantly modify the digestive and absorptive mechanisms [222]. It has been demonstrated that fermentation processes occurring in the microbial colonies may digest components of the diet, such as dietary polysaccharides, otherwise indigestible for the host. The subsequent increase in the absorption of energy may alter the mechanisms regulating energy utilization and the deposition of fat [223]. Some authors have proposed that the microbiota of obese individuals can be more efficient at extracting energy from the diet compared to the microbiota of lean subjects, and a number of studies have well described these changes in rodents and humans [222, 224]. As already ascertained, the germ populations are different in lean and obese individuals, having dissimilar proportions of the two major bacterial divisions, Bacteroidetes and Firmicutes. Moreover, recent investigations have pointed out the role of gut microbiota in the origin of metabolic endotoxemia and low-grade inflammation that characterize obese states and insulin resistance. HF diets have been associated to a rise in bacterial lipo-polysaccharides (LPS) plasma levels, and elegant works conducted by Cani & Delzenne have verified that gut bacteria may be directly responsible for this phenomenon [225-227]. Furthermore, changes in the gut microbiome, and subsequent alterations of the gut barrier functions, can alter the response to a HF diet and confer resistance (or susceptibility) to obesity and diabetes [228]. Therefore, the gut microbiome represents another important environmental contributor to energy balance and obesity development, and needs to be studied in detail. The therapeutic modulation of gut bacterial colonies through prebiotic/probiotic treatments has been successfully tested in rodents, and could represent a novel strategy for the prevention and cure of metabolic diseases [228, 229]. Interestingly, changes induced in mouse gut microbiota by the administration of prebiotics (inulin-type fructan and oligofructose) led to increased circulating levels of GLP-1 and PYY, while reducing ghrelin release [230].

Portal vein and liver sensing

All absorbed nutrients, except for long chain fatty acids, firstly reach the hepatic portal vein, to be received by the liver and processed by hepatic enzymes. The vagal afferent

fibers innervating the portal vein act as glucosensors [231], and have been involved in feeding responses, as well as the satiating properties of glucose induced by insulin signaling [232]. The portal vein glucosensors have also been shown to respond to GLP-1, and to mediate the protein-induced suppression of food intake [233]. Moreover, the liver itself represents an important sensing system that communicates to the brain the presence of fuels. The vagal afferents are again considered key actors of liver-brain cross talking, but other mechanisms could be involved [234]. In addition, it has been shown in rodents that the brain also presents lipid-sensing capabilities, similar in the molecular mechanisms to liver lipid-sensing pathways, but distinct in the physiological outcomes. In particular, the short-term accumulation of long-chain fatty acids (LCFAs, that can easily cross the blood-brain barrier) in the hypothalamus is sensed by the brain, and results in the suppression of glucose production, whereas the overflow of LCFAs in the liver due to HF ingestion promotes glucose production and insulin resistance [235]. This liver-brain balance model is thought to guarantee glucose homeostasis in normal conditions, and to be altered by HF-induced obesity toward a prevalent glucose production and, consequently, an increased risk of type-2 diabetes [235, 236].

Energy-sensing long-term signals: the central role of adipose tissue

The short-term signals described in the previous paragraphs trigger rapid responses to food intake, and promptly influence meal size and duration, as well as meal composition, during food consumption. However, these signals have to be integrated with long-term acting circuitries, which monitor global fuel availability and needs, and basically act by enhancing or reducing the sensitivity to short-term factors. Furthermore, long-term factors significantly influence energy utilization mechanisms and body weight, having a larger impact on homeostasis. White adipose tissue (WAT) has to be considered a central player of the system controlling energy balance [237]. The primary buffering of energy intake and expenditure occurs in the WAT, through the regulated fluctuations between triglyceride deposition and release. In recent years, it has become clear that WAT is a true endocrine organ whose hormones, particularly leptin and adiponectin, are crucial for energy homeostasis and influence several physiologic conditions. In addition, WAT is a source of numerous secreted factors, defined as adipokines, which impact a wide range of biological

and physiological functions, and that are often involved in the pathological consequences associated with fat mass accumulation. The important corollary is that WAT sustains extensive and complex ways of communications with several other tissues, such as the skeletal muscle, adrenal cortex and, particularly, the brain, through a fascinating though intricate cross-talk which has not been completely elucidated. The following paragraphs will focus on the endocrine role of the adiposity signals leptin and insulin, and describe their long-term impacts in the regulation of energy balance. Other important adipokines will be treated, with a particular attention to adiponectin and some of the pro-inflammatory cytokines released by the WAT. Finally, the communication between the WAT and skeletal muscle will be briefly analyzed.

Adiposity signals: leptin and insulin

Adipose mass is sensed by the brain, and induces commensurate changes in energy intake and energy expenditure in order to preserve homeostasis. A new era of obesity research was inaugurated by the discovery of leptin, the cytokine-like hormonal product of the *ob* gene. Leptin is released by fat tissues proportionally to their size, and signals the amount of adipose energy stores directly to the brain [137, 238]. Moreover, a quota of leptin secretion is independent of body fatness, but influenced by gender (with women having higher levels of circulating leptin compared to men) or hormonal regulation (such as by insulin and glucocorticoids) [239-241]. The activation of leptin receptors (ObRs, encoded by the *db* gene) on key hypothalamic nuclei reduces food intake and promote energy expenditure. In particular, the privileged targets of leptin signal are two sets of neurons in the ARC: 1) NPY and AgRP-expressing neurons, whose activation triggers appetite but suppresses energy expenditure; 2) POMC and cocaine and amphetamine regulated transcript (CART)-expressing neurons, whose products α-MSH and CART potently induce satiety and thermogenesis. Leptin acts simultaneously in the two directions, by inhibiting the release of the orexigenic NPY and AgRP, and promoting the activation of catabolic pathways switched on by POMC signaling. However, leptin is not a satiety factor in humans, since food ingestion does not acutely influence its levels [242]. In contrast, the fasting-induced drop of leptin secretion is rapid, and has potent effects on appetite and the preservation of energy stores, thus early protecting the organism from starvation and fuel depletion. It is

now accepted knowledge that leptin signal is not evolved to protect from excess fat accumulation, but rather to avoid energy exhaustion in case of famine [242]. Coherently, obese individuals have higher leptin levels but fail to respond to its effects, even when the levels are further increased by long-term leptin treatments [243]. As extensively shown, chronically high leptin circulating levels severely impair the saturable leptin transport through the blood-brain barrier, mediated by the short form of leptin receptors (ObRa) [244, 245]. In addition, the prolonged activation of brain leptin receptors (the long form, ObRb) down-regulates their expression and compromises the efficacy of down-stream intracellular signals [242]. Interestingly, it has recently been reported that endoplasmic reticulum stress can play a significant role in the development of leptin resistance, mainly by inhibiting leptin receptor signaling [243, 246]. In conclusion, except for the rare case of monogenic leptin deficiency, this hormone cannot be exploited as a therapeutic tool for obesity, as it was firstly suggested.

Leptin is mainly produced by adipose tissue, but also found in several other sites including the brain, lymphoid tissues, ovary, placenta and mammary epithelium, as well as the bone marrow [247]. Moreover, ObRs are located throughout the central nervous system and peripheral tissues, including the liver, pancreas and skeletal muscle. Coherently, this hormone is involved in a variety of physiologic functions influenced by the energy status, such as the neuroendocrine regulation of reproductive system; the hypothalamus-pituitary thyroid axis; glucose metabolism and insulin sensitivity; immune functions; and bone metabolism [243].

Insulin is primarily released by pancreatic beta-cells following food intake. Plasma insulin levels are also correlated with the adipose mass though, unlike leptin, specifically associated with the visceral better than subcutaneous fat mass levels [220]. Furthermore, insulin-circulating levels rapidly follow the changes in energy balance and signal any variation to the brain. Insulin also uses a receptor-mediated transport system to cross the blood-brain barrier and reach the key hypothalamic nuclei involved in the regulation of energy balance. The insulin receptor is highly expressed in the ARC, where its activation dampens the NPY signaling and enhances POMC-triggered pathways [248, 249]. In contrast with leptin, insulin levels are acutely influenced by food intake. In particular, the main trigger of insulin release is the rise of local glucose concentration in the pancreas.

However, the grade of glucose-induced insulin release is a direct function of adipose mass. Therefore, insulin and leptin signals work together in order to inform the central nervous system about the energy status, the size of fat mass and its distribution. While insulin levels represent the minute-to-minute interaction of ongoing metabolic processes and body adiposity, leptin more specifically indicates the activity of adipocytes [220]. In addition, insulin is a key modulator of glucose and lipid utilization and storage. Its signal is essential for the uptake of glucose and lipid by most tissues.

The regulatory potential of adipokines: focus on adiponectin

As previously anticipated, adipose tissues secrete hundreds of signals with endocrine, paracrine and autocrine actions, in different combinations that depend on fat topology and metabolism. These "adipokines" [250] can highly differ in terms of protein structure and physiologic effects, but most of them are associated with immuno-regulatory pathways. Besides multi-task hormones such as leptin, this copious group of factors includes classical cytokines, such as TNF-α and IL-6; chemokines, such as MCP-1; growth factors, such as transforming growth factor (TGF)-β; and also proteins involved in lipid metabolism (retinol binding protein and CD36), glucose homeostasis (adiponectin), angiogenesis (vascular endothelial growth factor), vascular haemostasis (plasminogen activator inhibitor 1), as well as the regulation of blood pressure and coagulation pathways (angiotensinogen and adipsin) [237]. It is now accepted knowledge that fat represents a fundamental actor for preserving the delicate equilibrium of different physiologic functions. It is therefore expected that perturbations of systemic or local metabolic pathways, such as the excess flow and accumulation of fatty acids in adipocytes, impact on adipose tissues by significantly altering the expression and secretion of different adipokines, and thus exposing the physiologic homeostasis to important modifications. Among these signaling molecules, adiponectin and inflammatory cytokines have been extensively studied in recent years for their role in the pathogenesis and development of insulin resistance and obesity. Their analysis allowed researchers to elucidate some of the crucial mechanisms switched on by fat accumulation, and highlight potential markers and targets for future obesity and diabetes therapies.

Adiponectin is the most abundant protein signal secreted by adipose tissue, showing anti-diabetic, anti-inflammatory and anti-atherogenic properties [251]. Its role is predominantly insulino-sensitizing, basically leading to higher glucose utilization and lipid oxidation in a variety of tissues and physiologic conditions, from skeletal muscle to pancreas and brain, from reproduction to liver disease [251]. In addition, recent findings have also highlighted interesting protective functions of adiponectin in carcinogenesis [252]. The insulin-sensitizing effects of adiponectin are mainly mediated by the sequential activation of AMP-activated protein kinase (AMPK), p38 mitogen-activated protein kinase (MAPK) and peroxisome proliferator-activated receptor (PPAR)-α, all involved in energy sensing and glucose/lipid metabolism pathways. Furthermore, it has been recently demonstrated that the efficacy of adiponectin signaling cascade depends on the correct activation of the adaptor protein APPL1 by adiponectin receptors [251]. The wide distribution of adiponectin receptors, namely AdipoR1, AdipoR2 and T-cadherin, in peripheral tissues and central structures contribute to prove the importance of this hormone for the whole-body metabolism [253]. Among the many hormonal and nutritional stimuli regulating adiponectin release, insulin as well as glucocorticoids, TNF-α and HF diet represent negative modulators, whereas PPAR agonists, such as thiazolidinediones and fibrates, promote its secretion [251]. In contrast with most other adipokines, adiponectin circulating levels are lower in obese and diabetic subjects, and are inversely correlated to BMI and, particularly, visceral fat [254, 255]. For these reasons, adiponectin has been considered a reliable diagnostic marker of pathologic metabolic conditions, such as the metabolic syndrome. Adiponectin-deficient mice develop clear symptoms characterizing the metabolic syndrome, particularly insulin resistance, glucose intolerance and hypertension [256-258]. On the other hand, studies in mice lacking AdipoR1 or AdipoR2 expression have shown that both receptors have important roles in energy metabolism, though with opposite outcomes. While AdipoR1 KO mice are obese, glucose intolerant and present low energy expenditure, AdipoR2-deficient animals are lean and resistant to DIO, with elevated energy oxidation levels [259]. As mentioned above, adiponectin also presents anti-inflammatory actions, mainly exerted by inhibiting the release of pro-inflammatory cytokines (such as IL-6, TNFα) and dampening the activation of NFkB-related pathways; and also by promoting the activity of anti-inflammatory cytokines (such as IL-10 and IL-

1A), in macrophages and monocyte-derived cells as well as adipocytes [251]. On the other hand, inflammatory cytokines are able to modulate adiponectin expression. The elevated circulating levels of adipose-TNFα observed in obesity and diabetes states are thought to be the main factor responsible for the drop of adiponectin release characterizing these conditions [260].

Have inflammatory mediators an impact on energy sensing?

Obesity is currently being described as a state of chronic mild inflammation, characterized by the enhanced expression and release of pro-inflammatory adipokines, which further progress as adipose tissue expands. Among the molecules involved, TNFα, IL-1β, IL-6, and MCP-1 have been the most extensively studied in recent years [261]. The pioneering works of Hotamisligil and colleagues were the first to show that adipose tissue is an important source of TNFα, and that this cytokine is up-regulated in obese states and can cause insulin resistance [262, 263]. Following findings have further corroborated the so-called "inflammatory hypothesis" [261, 264]. This model places inflammatory mechanisms at the origin of metabolic dysfunctions, and the adipose tissue at the center of a vicious circuit, in which pro-inflammatory signals further promote the recruitment of macrophages, and considerably affects the communication with and between numerous organs, including liver, skeletal muscle, pancreas, and brain. The high circulating levels of pro-inflammatory factors are likely to alter energy sensing mechanisms in both peripheral (as treated in the following paragraph) and central regulatory circuitries, principally by impairing leptin- and insulin-sensitivity in key tissues [265]. Besides the classical pro-inflammatory adipokines, other factors have recently been studied for their role in inflammation and the development of insulin resistance. These include resistin, retinol-binding protein 4 (RBP4), IL-18, MCP-1, CXC-chemokine ligand 5 (CXCL5), visfatin, and others [265]. Furthermore, a number of recent works have demonstrated that HF consumption and obesity can cause the activation of inflammatory pathways and the development of insulin resistance directly in key regulatory structures of the hypothalamus [52]. In a study conducted by De Souza and colleagues, the hypothalamus of HF diet-fed rats showed the increased expression of several pro-inflammatory cytokines (such as IL-6, TNF-α and IL-1β) and inflammation-responsive molecules, compared to lean animals. As a consequence, insulin-associated

pathways were substantially affected in both functional and molecular outcomes, following the serine phosphorylation of insulin receptor and insulin receptor substrate (IRS-2) [52]. Later, Posey et al. showed that the ability of intracerebroventricular injection of insulin to inhibit food intake was suppressed in HF fed rats, and that this effect was associated with the hypothalamic accumulation of pro-inflammatory lipids (palmitoyl- and stearoyl-CoA) and the activation of IKK-β, a marker of inflammation switch [57]. The altered equilibrium of immuno-modulatory factors is therefore thought to cause major modifications in the central balance of orexigenic and anorexinegic pathways, as also suggested by other works showing the inflammation-induced perturbation of POMC and AgRP signaling [266-268].

Clues about the intense cross talk between adipose tissue and muscle

Skeletal muscle represents the most important sink for glucose uptake and thus a key regulatory site for the control of glucose homeostasis. Furthermore, skeletal muscle has been recently highlighted as an endocrine organ, as it secretes a large variety of bioactive molecules, defined as "myokines", which also include cytokines, such as IL-6 and TNF-α [269, 270]. Myokines have an autocrine/paracrine range of action but also present endocrine activity. In particular, the bi-directional communication between skeletal muscle and adipose tissue is intense, and principally mediated by leptin and adiponectin, but also cytokine signaling [269, 270]. The major pathways affected by adipokines and myokines are those impacting glucose and lipid metabolism, and particularly those regulating insulin-dependent glucose uptake through the glucose receptor GLUT4, and lipolysis. The activation of leptin receptors in skeletal muscle triggers beneficial AMPK pathways and causes a reduction of intra-muscular lipid stores, through the decline of fatty acid uptake and their esterification to TG; and the stimulation of lipolysis and fatty acid oxidation [271, 272]. Adiponectin signaling also contributes to promote fatty acid oxidation and increase myocyte mitochondrial content [273, 274]. However, in obese and diabetes states, the muscle sensitivity to leptin and adiponectin signals is substantially impaired. It has been reported that the consumption of a HF diet causes the reduction of leptin receptor protein expression in human skeletal muscle [275], whereas the HF diet-induced up-regulation of the suppressor of cytokine signaling-3 (SOCS-3) in rodent muscle fibers prevents leptin-induced activation of AMPK [269]. On the other hand, aerobic training has been shown to

prevent, at least partially, the HF diet-induced development of leptin resistance [269]. Recent evidences disclosing the role and interactions of adipokines and myokines support the idea that myokines represent crucial elements of a muscle-to-fat regulatory axis which controls the ratio lean/fat mass as well as insulin and leptin sensitivity [270]. IL-6 signaling represents an interesting example. IL-6 is chronically produced by adipose tissue in states of excessive lipid accumulation and insulin resistance, and contributes to impair glucose homeostasis. However, IL-6 is also acutely released by the working skeletal muscle, in direct proportion to the contracting muscle mass and the exercise duration [276, 277]. Interestingly, the acute exercise-stimulated rise in IL-6 levels promotes glucose uptake and fatty acid oxidation, mainly through the phosphorylation of AMPK and Akt substrate-160, and thus demonstrates leptin- and insulino-mimetic outcomes in both skeletal muscle and adipose tissue [277-279]. In addition, the acute injection of IL-6 specifically induced lipolysis in the skeletal muscle, but not adipose tissue, of human volunteers [280]. In contrast, the chronic exposure to high IL-6 levels characterizing obesity de-sensitizes the insulin-related signaling in both tissues, altering IRS-1 activity and increasing SOCS-3 expression [279, 281]. In summary, IL-6 coordinated signaling within and between adipose tissue and skeletal muscle has opposite acute and chronic outcomes, being of critical importance for muscle performance during contraction, but leading to insulin resistance in both tissues when chronically elevated.

Integration and origin of response: the hypothalamus

The brain represents the master coordinator of eating behavior and body weight regulation. The brain regions responsible for the long-term control of energy balance act by monitoring the amount of available fuels in the body and consequently adjusting/refining food intake and energy expenditure. The hypothalamus is the primary site where integration of sensory, endocrine and learning/memory inputs produces a commensurate response in terms of behavioral, autonomic and endocrine outflows aiming to preserve energy homeostasis [153]. In the hypothalamus, ARC is the chief neuronal district involved in the regulation of energy balance. Neurons within the ARC are located near the fenestrated capillaries at the base of hypothalamus, thus being in a strategic position to receive various metabolic and hormonal circulating signals with normally no access to other brain areas [282, 283].

Furthermore, the ARC is innervated by a wide range of projections releasing all major neurotransmitters, and present metabolic-sensing neurons that are capable to sense and directly respond to nutritional stimuli, such as glucose and fatty acids [284]. Nevertheless, ARC neurons express the receptors for most important metabolic hormones, including leptin, insulin, ghrelin and adiponectin [282, 285]. The homeostatic model for the control of energy balance places the melanocortin (MC) system at the origin of complex regulatory pathways. In this model, two subsets of neurons within the ARC are primarily involved and have been extensively studied: POMC/CART and NPY/AgRP producing neurons [286, 287]. The activation of POMC/CART neurons has anorectic effects, while the stimulation of NPY/AgRP circuitries promotes food intake and has energy-conservative outcomes. POMC-derived peptide α-MSH acts by activating MC3/4R receptors, abundantly expressed in the ARC, paraventricular nucleus (PVN), lateral hypothalamic area (LHA) and dorsomedial area (DMH) of the hypothalamus, and causes the decrease of food intake and body weight, while promoting energy expenditure. AgRP exerts its anorexigenic effect mainly as a natural antagonist of MC3/4R, and potently inhibits POMC signaling. NPY acts on a different family of receptors (Y1-5), with comparable wide central expression, and positively influences energy intake. Interestingly, NPY neurons contact nearby POMC cells through inhibitory GABA-releasing projections, thus negatively influencing anorectic outflow. Since this inhibitory input that finally enhances appetite signaling is unidirectional, it has been indicated as one of the potential factors that promote overfeeding [288, 289]. Leptin-sensitive POMC/CART and AgRP/NPY cells are considered "first-order" neurons, whose ability to regulate food intake and energy expenditure depends on down-stream "second-order" neuronal targets, within hypothalamic and extra-hypothalamic sites [290]. The key downstream targets for the regulation of food intake and autonomic inputs are the PVN and the LHA, as well as the ventromedial hypothalamic area (VMH), DMH and brainstem areas. The homeostatic MC model suggests that, in second-order neuronal circuits, inputs from POMC neurons is opposed by inputs from NPY/AgRP cells, and further integrated with metabolic information derived from other brain areas. These second-order neurons in turn project to third and higher order neurons located in many areas of the brain and the spinal cord, finally modulating the complex responses driving eating behavior [290].

Hypothalamic down-stream targets of the ARC signaling

ARC projections to the PVN are classically associated with the regulation of neuroendocrine functions via the hypothalamic-pituitary axis, and with the modulation of the autonomic nervous system [291-293]. In particular, the PVN includes corticotropin releasing hormone (CRH) and thyrotropin releasing hormone (TRH) producing neurons, and therefore ARC-derived signaling may influence the adrenal and thyroid functions. The PVN is a critical site for MC4R signaling. Since MC4R is abundantly expressed in CRH neurons, it has been proposed that CRH, whose signal has anorexigenic effects, may act downstream of MC4R for the regulation of food intake [294]. In addition, other neuropeptides have been identified that can mediate POMC/MC4R signaling in the PVN: the transcription factor Sim1; BDNF; nesfatin; and oxytocin, whose release is enhanced by MC4R activation [295].

The importance of LHA for the control of energy balance was firstly evidenced when selective lesions of this hypothalamic region caused hypophagia in rats [296]. Following those studies, LHA was defined as the "feeding center", as it includes two subsets of neurons that release key appetite-stimulating peptides, namely orexin and MCH. These neurons receive direct inputs from the ARC and are regulated by leptin and ghrelin signaling [286]. Furthermore, LHA receives inputs from, and in turn projects to, a large variety of brain sites, including the nucleus accumbens, VTA, amygdala, orbitofrontal cortex, insular and olfactory cortices, as well as brainstem areas (such as the nucleus of the solitary tract), and is therefore critical for the modulation of different behavioral responses [287]. These are related to emotion, reward, learning, memory, motivation and motor responses in association with changes in energy state, and strongly influence feeding behavior. Though orexin and MCH projections significantly overlap, their signals are independent, and their targets and effects are distinct [297, 298]. Firstly, orexin shows excitatory neuronal activity, while MCH neurotransmission works via GABA inhibitory contacts. Orexin release is activated by fasting and depends on NPY Y1 and Y5 receptors to promote food intake. Since orexin neurons massively project to NPY neurons in the ARC, it has been proposed that orexin signaling may be upstream of the NPY system, thus also explaining their synergistic effects on feeding and metabolism [299]. Interestingly,

orexin action is strongly associated with the activation of arousal circuits in response to short-term changes in energy homeostasis, mainly resulting in food-seeking behaviors [300, 301]. Furthermore, it has been shown in rodents that sensitivity to orexin signal is subjected to circadian rhythms, as intracerebroventricular injection of orexin in rats promotes food intake in the light but not in the dark phase. In addition, chronic administration of orexin in rats does not influence body weight, since the increase of food intake observed in the light phase is compensated by a reduction of feeding in the dark phase [302]. MCH predominantly acts through competing with the α-MSH signaling, while it shows little or no interaction with NPY or orexin in promoting food intake [286]. Moreover, MCH gene activation negatively influences energy expenditure, and one mechanism of action includes the down-regulation of thyroid stimulating hormone (TSH) expression [303]. In obese *ob/ob* mice, MCH is over-expressed, but its levels decrease after leptin injection [304]. *Mch* KO mice are resistant to DIO, and present increased energy expenditure and locomotor activity [305].

The VMH also represents a key hypothalamic nucleus mediating leptin effects, and thus controlling food intake and energy metabolism [286]. It is a site of high leptin receptor expression, but also presents elevated expression of MC4R, and NPY Y1, Y2 and Y3 receptors. VMH receives afferent projections from the ARC and in turn increases the activity of POMC cells through microcircuits conveying excitatory inputs, which decline during fasting [306]. This hypothalamic region produces two important regulatory factors, namely steroid factor-1 (SF-1) and BDNF. SF-1 is a transcription factor that is co-expressed with leptin receptor and necessary for VMH development [307]. BDNF is highly and specifically produced by VMH neurons, and its expression is up-regulated by high leptin levels while being inhibited by fasting. BDNF is considered another important regulatory mediator of leptin signaling, and has been reported to impact glucose and lipid metabolism. Lack of BDNF, or deficiency of its receptor TrkB, has been associated with obesity in both rodents and humans [308, 309]. A key aspect of its action is the modulation of synaptic morphology and function, thus highlighting a role for synaptic plasticity changes in the regulation of energy balance [308]. VMH represents, together with the ARC, one of leptin hypothalamic targets showing a high degree of synaptic remodeling. Interestingly, it has been demonstrated that leptin can influence energy homeostasis by

actually remodeling the balance between excitatory and inhibitory inputs to the different cell groups [310], and thus regulate food intake.

Other hypothalamic nuclei are targets of leptin and ghrelin signals and receive inputs from the ARC and the brainstem. Among these, the DMH plays a key role for the entrainment of circadian rhythms to feeding schedules [311] and locomotor activity [312]. Selective lesions of this region in rats cause hypophagia and reduce locomotor activity, while the physiological rise of body temperature entrained by food intake is inhibited in these rats, and no change is observed in their final body composition [313].

Extra-hypothalamic down-stream targets of the ARC signaling: the brainstem

The caudal brainstem constitutes a critical site for the autonomic control of ingestion, digestion and absorption of food [314]. In particular, it is responsible for most parasympathetic pathways controlling ingestive and digestive processes via the vagus nerve, while it also generates the sympathetic responses associated with food intake and energy expenditure. As a consequence, the brainstem directly influences the functions of the locomotor and oromotor apparati that drive the approach to food, and the ingestion of foods and fluids in the oral cavity. Complete ingestive behavior and energy balance cannot be achieved if brainstem circuits are not functional [315]. ARC neurons directly project to key brainstem areas related to satiety signals and autonomic outflow, thus engendering the fundamental coordination between food intake and autonomic nervous system [314, 316]. In the caudal brainstem, feeding and body weight are mainly under the control of the dorsal motor nucleus of the vagus nerve. The latter is a region of high MC4R density that receives afferent projections from the mouth and GI tract, thus mediating chemical and mechanical sensing signals to the brain [295]. Moreover, POMC/MC4R expressing neurons within the brainstem are crucial for CCK- and GLP1-triggered satiety signaling, among other gut peptides. As a matter of fact, the brainstem can sufficiently regulate meal size and also efficiently respond to glucoprivation, though it cannot adequately govern long-term mechanisms for energy homeostasis. Interestingly, the brainstem houses glucose-sensitive neurons, and presents an elevated density of leptin receptors, particularly in the area postrema, the nucleus of the solitary tract and the dorsal motor nucleus of the vagus nerve; and ghrelin receptors, in the area postrema and nucleus of the solitary tract [314-316].

Focus on the MC system: POMC, MC3/4R, AgRP

The MC system is needed to receive and integrate the metabolic and hormonal information transmitted by a vast complexity of peripheral modulators, and finally generates commensurate responses that preserve energy homeostasis. Important subpopulations of hypothalamic neurons are directly sensitive to glucose, fatty acids and aminoacids. The crucial role exerted in the hypothalamus by specialized energy sensors, such as AMPK, mammalian target of rapamycin (mTOR) and cAMP response element-binding (CREB), has been extensively studied in recent years, and demonstrated as essential for energy metabolism. Furthermore, leptin and insulin represent the two major hormones that modulate energy balance through the MC system. The stimulation of leptin receptors promotes POMC neuronal activity and enhances the sensitivity of ARC neurons, and also nucleus of the solitary tract (NTS) neurons, to satiety signals, while it suppresses AgRP release [295]. Insulin also targets POMC and AgRP neurons to regulate satiety, energy expenditure and glucose metabolism [317, 318]. However, insulin stimulation of AgRP cells results in lower hepatic glucose production, whereas the activation of insulin receptor signaling on POMC neurons enhances glucose production in the liver and also promotes energy expenditure through MC pathways. Interestingly, the ability of cytokines to regulate important aspects of energy metabolism and feeding behavior also depends on POMC-related circuitries. A number of cytokines are expressed in the hypothalamus, including IL-1β, leukemia inhibitory factor (LIF), IL-18, and may substantially affect POMC/AgRP gene expression and secretion in order to influence appetite and hypothalamic-pituitary-adrenal axis (HPA)-regulated pathways, specifically as a response to emotional and inflammatory stress [295].

The inhibitory effects of POMC-derived peptides on food intake were first shown in the 1980s, following their brain administration in rats [319], and have been reproduced and finely investigated over the years. The MC system is now recognized as a primary player in the regulation of energy balance, and includes several components, among endogenous agonists and antagonists, a number of MC receptors (MC1/5R) and their accessory proteins (MRAPs). POMC-derived peptides, namely α-MSH, β-MSH, corticotropin (ACTH) and β-endorphin, are produced in differential combinations within the ARC neurons, but also in the NTS of the brainstem, in the pituitary as well as in a wide range of peripheral tissues

[295]. The major POMC-end product in the ARC is the desacetyl-α-MSH, more abundant than the acetylated form. However, the acetylation to the active α-MSH peptide is dynamically controlled by leptin [320] and dopamine [321, 322], and thus represents an important regulatory step that modulates the impact of the MC system on energy homeostasis. Evidences that α-MSH is a critical actor in energy balance control have been collected in rodent studies as well as in humans. *Pomc*-null mice are obese (the homozygous more than the heterozygous animals, in a gene-dose dependent manner) [323], and the genetic background significantly influenced the severity of obesity, the C57BL6 strain being more susceptible than the 129Sv [323-325]. However, central α-MSH administration is able to reverse the obese phenotype in *Pomc*-deficient mice, though the lack of all POMC-derived peptides and glucocorticoids in this model made it difficult to achieve clear conclusions. Similarly, in humans a rare *POMC*-null mutation has been identified that is lethal, unless subjects are supplemented with glucocorticoids. Their phenotype is also characterized by hyperphagia and obesity [326, 327]. Two major MC receptors have been characterized, namely MC3R and MC4R. While the roles of MC3R are still less clearly understood, the critical functions of MC4R have been successfully elucidated and include the regulation of appetite and body weight [328], as well as glucose homeostasis (at least in mice) [329], hepatic lipid metabolism and nutrient partitioning [330]. Basically, the studies have suggested that the central MC system is the key actor to orchestrate chronic energy balance [295]. MC-triggered regulatory pathways prepare glucose and lipid metabolism processes in peripheral organs for more efficient storage of incoming nutrients [331, 332]. In particular, MC system directly acts on liver, muscle and fat cell metabolism to promote fat storage, through modifying the balance among cell glucose uptake, TG synthesis, lipid deposition and lipolysis [330]. *Mc4r* KO mice are obese and also show defective regulation of insulin secretion and sensitivity, and sympathetic nervous system-dependent responses [329, 333, 334]. Again, the severity of the phenotype is strain-related, and C57BL6 mice become more obese than 129Sv models [325, 335]. In humans, *MC4R* mutations account for nearly 5% of morbidly obese subjects. The analysis of these cases demonstrates that functional MC4Rs are needed for the physiological regulation of appetite, body weight, height, bone mineral content and cardiovascular work but not for glucose homeostasis [295]. Interestingly, *Mc4r* deficiency significantly affects

energy expenditure regulation in mice, but not in humans [336]. The pathways involved have been studied in rats and target the brown adipose tissue (BAT), a key site for thermogenesis. These operate via the activation of the sympathetic nervous system, particularly through neuronal projections travelling from the hypothalamus (ventromedial preoptic nucleus, PVN, LHA) and the brainstem to the postganglionic neurons innervating the BAT [337, 338]. In addition, the sympathetic nervous system outflow to the WAT is also important for the regulation of lipid mobilization and thermogenesis [295].

AgRP is the endogenous antagonist of both MC3R and MC4R, and consequently promotes body weight gain through enhancing food intake and inhibiting energy expenditure. In addition, AgRP can also act as an inverse agonist, and even agonist, of POMC signaling [339-341]. It has been suggested that the physiologic modulation of MC receptor signaling might be exerted mainly by changes in AgRP levels rather than changes in POMC-derived peptides, since starvation and leptin deficiency mainly promote the release of AgRP [342]. In rodents and humans, the hypothalamus and the subthalamic nucleus are the major sites of AgRP expression [343]. However, a shorter AgRP transcript is also detectable in the periphery, more abundantly in the adrenal glands, testis, lung and dorsal root ganglia. Its expression is negatively regulated by leptin and insulin, but potently enhanced by fasting and ghrelin [343]. In particular, leptin and insulin can acutely influence the membrane potential, and depress neuronal firing in AgRP/NPY neurons [344], while loss of leptin or insulin receptors in the brain causes a rise in AgRP secretion [343]. Hypothalamic AgRP expression is particularly elevated in obese and diabetic mice [345], and transgenic mice over-expressing this peptide are hyperphagic and obese [346]. However, *Agrp* deficiency in rodents has produced variable phenotypes. Globally, *Agrp* null mice showed no changes in food intake and body weight [347-349], at least until 6 months of age when a rise in the metabolic rate, body temperature and locomotor activity negatively affected body weight [348, 349]. Interestingly, it has been reported that *Agrp* KO mice challenged with HF diet live significantly longer than WT mice even if no main metabolic difference is evidenced between the two genotypes, and both develop obesity [350]. Among its important functions, AgRP significantly influences neuroendocrine pathways through modulating the HPA axis. For instance, AgRP has been reported to inhibit the HP thyroid axis, and to lower TRH release as well as thyroid hormone

circulating levels [351]. In addition, the role of glucocorticoids in the modulation of energy homeostasis may substantially rely on their action in AgRP/NPY neurons [352, 353]. As a matter of fact, loss of glucocorticoids (as in adrenalectomy) profoundly affects AgRP activity, as it suppresses the fasting-induced increase in AgRP levels and abolishes this peptide's diurnal rhythms [352, 353]. In line with this, AgRP is considered a key actor in the modulation of neuroendocrine responses to stress and inflammation, and has a role in promoting IL1β-dependent up-regulation of ACTH [354, 355]. Furthermore, AgRP has been associated with disease-related changes in food intake and energy metabolism, showing beneficial effects in anorexia nervosa and tumors [343].

Feeding behavior in the environment: corticolimbic pathways

The metabolic mechanisms that control eating behaviors are rooted in a greater system housed within the brain, which enables complex interactions between mammals and the food-providing environment to occur. This higher system associates the internal regulatory processes governing the metabolic pathways with the externally oriented cognitive and hedonic circuitries [287, 356]. The neural representations of food experiences, gathered with information about the environment and the social context (including religious and ethnic habits) are constantly updated with memory and learning traces embedded in a neural network that comprises the orbitofrontal, prefrontal, anterior cingulated and insular cortices, as well as the hippocampus [287, 356]. The corticolimbic pathways are responsible for the integration of cognitive/affective representations of foods with sensory inputs, and drive the processes leading to motivational mechanisms and decision-making [310]. In this context, emotions are particularly important contributors, as they have most likely evolved to make animals engage in behaviors with beneficial effects and, on the other hand, to make them avoid potentially noxious actions [357]. As a matter of fact, tasting beneficial foods promptly elicits a feeling of pleasure, and functionally involves the reward system and its corticolimbic processes known as learning, liking, wanting [358]. In particular, the "liking" aspect of consciously experiencing the pleasure of a palatable food is under the control of specialized neuronal districts, including the midbrain, the caudal shell of the nucleus accumbens and the ventral pallidum, while it also involves the signaling cascades triggered by the mu-opioid receptor and the endocannabinoid CB1

receptor. In contrast, the "wanting" motivation driving the motor behaviors necessary to obtain the pleasure from feeding is more directly associated with the mesolimbic DA system, specifically with the dopaminergic projections from the VTA to the nucleus accumbens [287, 356]. In light of this, dopaminergic pathways appear essential for a functional eating behavior. Consistently, DA deficient mice present a severe hypophagia, which is comparable with the phenotype induced by lesions in the LHA [359]. These mice are unable to increase food intake in response to acute glucose deprivation, PYY administration or leptin deficiency. Therefore, DA is thought to be a critical signal downstream of the MC system, in order to promote feeding [286]. Interestingly, the hedonic/cognitive aspects of feeding behavior have long been considered subordinated compared to the homeostatic mechanisms controlling energy balance, but the knowledge brought about in the last decade has significantly modified this hierarchy and disclosed novel physiologic perspectives. According to the basic hypothesis, the ARC is the chief coordinator that communicates the metabolic information to the mesolimbic dopaminergic circuits via diverse routes: 1) direct ARC projections to the dopaminergic neurons in the nucleus accumbens; and 2) ARC-mediated activation of orexin and MCH neurons in the LHA, that in turn project to the VTA and the nucleus accumbens [290, 310]. However, the VTA and, globally, dopaminergic neurons have been shown to directly respond to important metabolic signals such as leptin, ghrelin and insulin [360, 361]. Therefore, together with the ARC funnel hypothesis, it is now largely accepted that metabolic signals can directly influence the reward and motivational controls of feeding, in a finely regulated coordination with the homeostatic system [287]. Finally, the ability to make decisions and choices in terms of ingestive behavior depends on the executive potential of the prefrontal cortex. The prefrontal cortex, which is connected to cortical areas involved in motor planning and execution, collects sensory inputs from inside and outside the body, as well as the emotional and cognitive information from the limbic system, and finally translates all available homeostatic and environmental information into adaptive behavioral responses [362, 363]. However, the obesogenic environment and the large availability of energy-dense highly palatable foods is likely to constantly force and finally impair the coordination between the homeostatic needs and the pleasure-inducing stimuli, with the latter often

prevailing and, at long-term, potentially causing obesity in genetically susceptible individuals.

The fuel oxidation contribution to energy balance

In the previous paragraphs, obesity has been mostly described as the result of maladaptive food choices, mainly mediated by dysfunctional appetite and satiety mechanisms that lead to overconsumption. It can be argued that excessive fat mass accumulation should also be regarded as the result of inadequate oxidation of fuels and failure of metabolic responses to balance (fat) dietary intake [364]. However, the two perspectives are intimately correlated, and emphasize the impact of fat consumption as a leading modifier of body weight control. The different macronutrient impact on appetite and satiety mechanisms will be analyzed in the following section. Here, the relationship between macronutrient intake and utilization that leads to fuel balance will be briefly illustrated, with a particular attention to fat balance.

Basically, the mechanisms responsible for fuel (macronutrient) balance directly affect appetite control and thus modulate energy intake. However, it is well known that carbohydrate and protein balances are tightly controlled, whereas the balance between fat intake and fat oxidation is less strictly regulated, and more easily leads to fat deposition in case of positive energy balance [365]. Carbohydrates are the main contributors to energy intake, and carbohydrate balance represents a major determinant of fuel and energy balance. Consequently, carbohydrate balance is the most rapidly and precisely regulated, through complex mechanisms that preserve a minimum carbohydrate supply while preventing the excessive storage. Moreover, when excessive carbohydrate flux cannot be compensated by the enhanced oxidation, the surplus is directed to *de novo* lipogenesis pathways [366]. On the other hand, as mentioned, lipid balance is the weak component of fuel balance. Unlike proteins and carbohydrates, the increase of lipid intake can only be compensated by the activation of oxidative pathways, while no other lipid overflow metabolic mechanism can be turned on. In addition, changes in lipid oxidation are not acutely regulated, and cannot rapidly and potently respond to lipid intake [365]. Since fat intake is not efficiently coupled to fat oxidation, this represents a major predisposing factor leading to fat accumulation and, ultimately, to obesity. Coherently, studies of lipid oxidation in lean and obese (or obesity-predisposed individuals) have demonstrated that

subjects more susceptible to gain weight have also a reduced capacity to oxidize fat, and thus respond less efficiently to increased lipid intake, such as in the case of a prolonged HF consumption [364]. In these individuals, fat deposition is the necessary route to restore lipid balance as, when body fat stores expand, lipid mobilization intensifies and finally promotes fat oxidation [367, 368]. Therefore, fat gain can be high enough to determine obesity even before a new lipid balance is reached [369]. On the other hand, exercise training is the only factor proved to induce fat oxidation even when fat stores are small, through the activation of the sympathetic nervous system [370]. Though muscle tissue constitutes up to 40% of body weight, it provides most of the energy for locomotor activity (a component of adaptive energy expenditure) by using ATP and stored creatine phosphate high-energy bonds as energy sources. Furthermore, muscles use blood glucose and glycogen stores as sources for acute energy needs and finally requires fatty acid beta oxidation to sustain them [371].

Components, inducers and effectors of energy expenditure

Energy expenditure represents a critical contributor to the regulation of energy homeostasis in normal individuals, and a balance component whose deregulation can promote weight gain and lead to obesity. The regulation of energy use entails the coordination of key lipid and carbohydrate metabolic pathways, through which glucose and fatty acids are distributed and employed as energy substrate for ATP and heat production [371]. Importantly, energy expenditure is regulated in accurate coordination with energy intake but also energy processing and storing operations. Basically, total daily energy expenditure (TDEE) in rodents and humans comprises: 1) the basal metabolic rate (BMR), which represents the major obligatory component of energy expenditure and involves all metabolic reactions that use energy to sustain life; 2) thermogenesis, that can be further subdivided in diet-induced thermogenesis (DIT) and non-shivering thermogenesis (NST), depending on the two main contributing factors, food intake and cold exposure respectively; 3) energy expenditure of activity (EEA), that in humans further comprises exercise activity thermogenesis (EAT), which implies volitional physical exercise, and non-exercise activity thermogenesis (NEAT), that comprises all other activities and is generally called "fidgeting" [372-374]. In terms of kcal/day and variability, the contribution of

thermogenesis to TDEE is small, but has the potential to be modulated by the environment, and thus to be therapeutically targeted. Besides the obligatory component of thermogenesis, which reflects the thermic efforts required for digesting and absorbing food, thermogenesis is mostly an adaptive contributor. The external exposure of mammals to cold or overfeeding triggers complex metabolic responses that influence oxygen consumption, food intake and heat generation, and specifically occur in the skeletal muscle and BAT in the form of shivering or non-shivering thermogenesis [373, 374]. EEA is also a key contributor to energy expenditure that can be significantly modified by changes in body weight and also influences weight regulation. Even small changes in the EEA can at long-term have a significant impact on energy balance, and it has been already reported that EEA can be directly related to obesity development [375-377]. Interestingly, Novak & Levine have pointed out the importance of NEAT in humans and rodents (in which EAT equally corresponds to NEAT) for energy expenditure and energy balance regulation, as NEAT shows great variability among individuals; it can be influenced by specific genetic traits as well as complex hormonal and biological regulations; and can predispose subjects to resistance or susceptibility in an obesogenic environment [374]. It is important to note that many of the molecular, neural and endocrine factors that affect feeding behavior also influence NEAT. Among these, it is worth mentioning fatty acids, POMC-derived peptides, MCH, ghrelin, CCK, leptin, amylin, and thyroid hormones [374].

An interesting and ever-expanding field in the study of energy expenditure is represented by research on thermogenesis and BAT. As mentioned, the leading – though sometime controversial – drive is the concept that adaptive changes in thermogenesis can be a major causal factor for the development of obesity and thus a critical target for obesity therapeutic interventions. Pivotal studies conducted at the end of the 1970s demonstrated for the first time that BAT was the key site for DIT and NST [378-380], and were followed by the discovery that a specific protein, uncoupling protein 1 (UCP1), is responsible for the generation of heat through the uncoupling of oxidative phosphorylation in the mitochondria [381]. UCP1 is uniquely found in the mitochondrial inner membrane of BAT and thus represents a signature differentiating brown from white adipocytes [382]. Noteworthy, both WAT and BAT contribute to energy expenditure, and are regulated by both central and autonomic nervous systems, in order to achieve a balanced energy homeostasis depending

on actual physiologic needs. In particular, the sympathetic nervous system (SNS) is the critical regulator of BAT activity. BAT presents extensive SNS innervations and β3 adrenergic receptor expression. Direct or hypothalamus-mediated stimulation of noradrenergic fibers acutely increases blood-flow through the BAT depots and promotes thermogenesis [383]. At the cellular level, norepinephrine binding to membrane receptors acutely induces lipid mobilization, through promoting lipolysis pathways and thus increasing fatty acid release. High levels of fatty acids represent the key signal needed to trigger the uncoupling activity in brown fat mitochondria, and consequently promote heat generation through dissipating the electrochemical gradient required for oxidative phosphorylation. Furthermore, the noradrenergic stimulation induces brown adipocyte proliferation, up-regulation of UCP1 and mitochondrial biogenesis [384]. Although several evidences have confirmed the importance of BAT and thermogenesis for energy metabolism and obesity development in rodents, the importance of thermogenesis and, especially, of BAT in humans remains dubious because still unclear. This reticence has been mostly due to the presumed absence of BAT depots in adults, and accompanied by the view that adaptive heat production is not a substantial component of normal human thermogenesis. However, recent reports have demonstrated that BAT depots and brown adipocytes are unambiguously present and active in adult humans, even though only in a minority of subjects [385-387]. UCP1 expression has been proved and the activation following cold exposure has also been shown [388, 389]. Interestingly, these studies also evidenced that women present a larger proportion of brown fat than men, and that the amount of BAT depots is inversely correlated with BMI [386]. The recent discoveries enhanced the research in this field, and the potential of modulating thermogenesis in humans to therapeutically tackle energy expenditure. Though BAT thermogenesis contribution is likely to be physiologically modest in humans, this does not exclude that pharmacological interventions which target BAT activity could represent valuable tools for the modulation of energy expenditure and the treatment of obesity.

Influence of gonadal steroid hormones on energy balance

In both mammals and humans, there are evident important sex differences in the regulation of energy homeostasis and obesity. Major differences have been demonstrated in eating behavior (treated later), body fat distribution and energy expenditure between males and females. Men and estrogen-deficient post-menopausal women tend to store fat in the abdominal and visceral adipose depots, whereas premenopausal women tend to accumulate more gluteo-femoral fat. As mentioned earlier in the text, this distinct fat topography can have major health outcomes [30]. Visceral fat has been clearly associated with increased cardiometabolic risk, while recent evidences show that larger gluteo-femoral fat stores are protective against cardiovascular diseases and metabolic risk [23]. In male mice, transplantation experiments in which inguinal subcutaneous fat was transferred into the visceral compartment of HF-fed recipient animals demonstrated the beneficial effects of subcutaneous adipose tissue, in terms of adiposity, insulin sensitivity and glucose tolerance [390]. In addition, women tend to have less fat-free mass compared to men, and this can contribute to the lower daily energy expenditure rates observed in women [391, 392]. Since the age-related decline in energy expenditure has been reported to be steeper in women, this would represent another risk factor explaining why obesity prevalence is globally higher in women [23]. Several evidences also demonstrated that energy expenditure of physical activity is specifically lower in women than men. While physical activity programs in men induced a significant reduction of body weight and body fat percentage without affecting energy intake, the same programs had weaker or no impact in women, in which a lower increase of energy expenditure was also associated with increased energy intake [393, 394].

Though behavioral and social differences between men and women may also significantly contribute to these differences and should be better elucidated, it is clear that the influence of gonadal steroid hormones has a major impact on peripheral and central controls of body weight. The greatest part of studies has analyzed the role of estrogen and has consequently focused on females, whereas less research has been committed to androgen effects. Animal studies have given crucial contributions, allowing gonadectomy and hormone replacement experiments to disclose the main effects specifically engendered by sex hormones. In rodents, ovariectomy leads to increased body weight and visceral fat mass while estrogen administration reverses these effects [395, 396]. Exogenous estrogen

administration in male rats also decreases visceral fat while increasing subcutaneous adipose depots [396]. Both male and female aromatase-KO mice, which are estrogen deficient but have increased testosterone levels, present larger intra-abdominal fat depots [397, 398]. In the periphery, estrogens have key regulatory effects on adipose tissue lipolysis and fat uptake. As shown in rodents, estrogen may act by modulating the alpha-2 adrenergic receptor and lipoprotein lipase (LPL) activity in adipocytes, finally influencing their lypolitic responsiveness [399, 400]. Moreover, it has been shown in premenopausal, but not post-menopausal, women that femoral fat cells present higher LPL activity compared to abdominal adipocytes [401]. Estradiol also displays a major impact on central mechanisms regulating body weight and energy balance, as it modulates leptin sensitivity in the hypothalamus. Ovariectomy has been shown to induce central leptin resistance, while leptin sensitivity is recuperated in ovariectomized mice and enhanced in male mice following estradiol administration [396, 402]. Interestingly, studies in estrogen receptor (ER) KO mice have demonstrated that the estradiol-associated influences on body fat distribution and central mechanisms controlling food intake and energy metabolism are mainly due to ERα signaling in the brain [403-405].

Though less extensively studied, androgens also have important effects on body weight regulation. Orchiectomy has been reported to reduce food intake and body weight in male rodents [18]. In men, while aging is associated with a decline in androgen levels and higher accumulation of adipose tissue in the visceral compartment, the administration of testosterone, but not dehydrotestosterone, to older men caused a reduction of total as well as abdominal fat [406, 407]. However, androgen treatment of ovariectomized mice leads to increased body weight and enlarged visceral fat depots, associated with reduced fatty acid oxidation [408]. In women, elevated endogenous androgen levels are associated with PCOS and menopause transition phase, and have recently gained great attention. A permanent, as in the case of PCOS, or even a temporary rise, as for menopause transition, in relative androgen circulating levels can significantly affect body fat distribution in women, by promoting visceral fat accumulation and thus increasing the risk for metabolic and cardiovascular diseases [23]. During the 2008 Stock Conference, Lovejoy and other eminent researchers in the field introduced a novel hypothesis on which they recommended further investigations: the increased obesogenic risks observed in post-menopausal women

can more markedly depend on the acute effects of androgenicity in the menopause transition phase, rather than uniquely stem from longer-term estrogen deficiency [23]. Studies in this direction would be beneficial not only for the clinical care of menopause but also for the treatment of PCOS, which is a condition strongly connected to obesity.

Chapter 2 of my thesis presents our contribution to this line of research. In particular, our work analyzed the acute transcriptional changes induced by a single dose of dihydrotestosterone in the adipose tissue of ovariectomized mice, and finally evidenced significant differences between acute and chronic effects of androgens on adipose tissue gene expression.

1.4 SHORT-TERM REGULATION OF ENERGY BALANCE

The regulation of body weight does not simply result as a function of energy intake or energy expenditure taken separately, but rather pertains the reciprocal adjustment of intake to expenditure, considered in a condition of *ad libitum* food intake. Energy balance is finely regulated by complex systems, and fuel balance more strictly coincides with lipid balance. Though body weight control seems to be asymmetrical, in that underfeeding generally evokes stronger and more efficient physiologic counteractive responses than overfeeding does, fat mass accumulation that follows excess lipid intake is not a threatening miscalculation of the system, but should rather be seen as the most convenient biological adaptation to re-equilibrate the balance [64]. Although still controversial in debates, variations in food intake are generally considered to have a greater influence on energy balance than the smaller changes in metabolic rates and energy expenditure that generally follow altered dietary patterns [242]. In particular, a great deal of research has been committed to the understanding of appetite and satiety, in order to point out the regulatory mechanisms of ingestion as well as the differential effects of macronutrients on food intake. The latter aspect is significantly related to the obesity epidemics, as several reports have evidenced the close specific relationship between fat intake and body weight gain. It is now accepted knowledge that lipid intake has slower and smaller effects on satiety perception, and thus positively influences meal size, leading fat consumers to over-consume energy in a single meal [242]. The suppression of hunger that occurs during the consumption of meals, and thus limits meal size while leading to meal termination, is referred to as "satiation". "Satiety" is more appropriately the state of hunger-absence causally engendered by food consumption, and represents the capacity to suppress eating in post-ingestive intervals, before next hunger resurging and meal. In considering the dietary fat as a risk factor for overconsumption and obesity, the effects of satiation are likely to be even more important than those exerted on post-ingestive satiety [47]. In light of this, investigating the molecular and physiologic short-term effects of lipid intake on the regulation of meal intake and feeding behavior can help to better understand and describe overconsumption, and may also disclose potential pharmacological targets for its prevention. The work presented in this thesis essentially relies on the latter assumption, and endeavors to contribute with novel

findings to this field. Before presenting the experimental results of my work, I would like to better describe the concept of passive overconsumption introduced by John E. Blundell in the 1990s, as well as the several evidences accumulated over the years demonstrating how fat intake modifies appetite, satiation and satiety regulatory mechanisms in animals and humans, potentially leading to obesity.

Influence of macronutrient intake on appetite and satiety

Feeding behavior responses may subtly or more markedly vary depending on the dietary components. More specifically, the physiological responses engendered in the GI tract depend not only on the energy content and macronutrient composition but also rely on the chemical structure, physical properties, and receptor affinities of different fats, carbohydrates and proteins [409]. Globally, it is well known that carbohydrates and proteins have a greater impact on satiation compared to lipids, and that proteins represent the most satiating of macronutrients, as reported in several short-term studies. It has been experimentally proved that subjects allowed to choose foods and eat freely until comfortable fullness finally consume greater energy from a range of HF foods than from foods high in carbohydrates or proteins [410, 411]. In the normality of everyday life, the elevated diffusion and ready availability (as well as the generally convenient costs) of HF foods are likely to influence food choices, and thus cause a rise in fat consumption, which has been actually characterizing the last decades [48]. HF hyperphagia has several explanations, including the high energy density of HF foods, as well as their pronounced palatability and the slower and disproportionate induction of satiety signals, and thus cannot be regarded as deliberate but rather as a passive form of overconsumption [47]. As mentioned, satiation and satiety mechanisms are easily overwhelmed when subjects (and animals) consume HF foods foods that have: 1) small volume and weight, despite high-energy density, and thus poorly induce the rapid mechanosensory gastric and intestinal feedbacks to the brain; 2) slower GI transit, and take longer time to be digested and reach the intestine, where most important satiety-regulating peptides are released; 3) enhanced palatability, and thus induce potent oral stimulation that rapidly activates reward pathways and facilitates intake. The impact of lipids on satiation and food intake has been extensively studied, leading to point out a number of significant alterations in the function of key

regulatory points for the control of feeding behavior and energy balance. In particular, fat has been shown to influence ingestive behavior at all stages and levels, from taste and other sensory qualities of HF foods, to digestion, absorption by the gut, as well as generation of (and sensitivity to) meal-induced signals, adiposity-sensing signals, and brain neurotransmitter signals with final substantial repercussions on energy metabolism [409, 412].

Most studies dealing with the impact of HF consumption on satiation and satiety mechanisms have mainly described the effects engendered by adaptation to chronic HF feeding. Several findings in both animal models (often rodents) and humans have contributed to highlight major changes occurring in gut morphology, regulatory peptide secretion and down-stream effects, as well as neuronal responses to long-term fat intake [412]. For instance, adaptation to a HF diet is accompanied by significant changes in: brush-border morphology, showing shorter and thicker intestinal microvilli, and a higher proportion of enterocytes; pancreatic secretion, with altered amylase and lipase release; and expression of ileal enzymes, that further increase fat absorption. Interestingly, obesity and long-term HF consumption in non-obese subjects have been specifically associated with reduced satiation responses to lipid, but not carbohydrate, intestinal infusion in both animal and human studies [412]. Moreover, both lean and obese men and women have been shown to ingest more energy when eating HF meals, thereby demonstrating weaker satiation stimuli in response to fat [411, 413]. In addition, HF diet-fed rats showed reduced satiety and shorter inter-meal intervals after 4 weeks of diet compared to LF-fed controls [414]. These results have been at least partially clarified by the study of the potential causing mechanisms. Firstly, chronic HF consumption engenders significant changes in GI functions, including gastric emptying and GI transit. Alterations of gastric emptying rates may be particularly relevant, as the delay of gastric emptying induced by nutrients and gut hormone signaling, such as CCK for instance, represents a major satiation stimulus. Adaptation to a HF diet has been associated in both rodents and humans with more rapid gastric emptying rates in response to a fatty, but not high-carbohydrate, meal [415-417]. Moreover, gastric emptying and GI motility strongly depend on vagal sensory neurons as well as myenteric neuronal system activation. Coherently, it has been shown in rats that chronic exposure to HF food selectively compromises vagal and enteric neuronal sensitivity

to lipid (oleate) intestinal infusion [414, 418]. In particular, the reduced sensitivity of vagal afferents to gut peptides signaling the presence of nutrients, specially fats, may represent a key contributor to dysfunctional satiation mechanisms. Among these signals, CCK is one of the most important short-term modulators of meal intake and has consequently received much attention. Several studies have reported that HF-fed mice and rats are less responsive to the suppression of food intake following exogenous CCK administration, and these results were significantly correlated with the lipid content of the diet rather than body weight or energy intake differences [419-421]. In humans, HF-food consumption, which is known to alter GI functions and promote energy intake, has been also associated with higher circulating levels of CCK [422]. However, it has not been possible to experimentally clarify if humans, not unlike rodents, are also less sensitive to CCK-induced satiation effects. Studies in animal models have reported the significant reduction of CCK1R expression and sensitivity on vagal afferents of obese Zucker rats [423, 424] and HF-fed mice [419]. Apolipoprotein A4 (ApoA4) is another important intestinal peptide, which is released following lipid intake and contributes to suppress food intake [425], presumably through a CCK1R-dependent mechanism [426]. Interestingly, chronic HF feeding has a blunting effect on ApoA4 secretion, which results hampered in both central and peripheral tissues after only few days of HF diet [130, 427]. Similarly, GLP-1 plasma levels are decreased in mice maintained on a HF diet, while its secretion is augmented in both ileum and colon of HF-fed obese rodents [428]. Among the signals induced by lipid intake in the GI tract, endocannabinoids, as well the related acylethanol-amide oleoylethanolamide (OEA, the monounsaturated analogue of anandamide) have recently gained attention for their potential effects on food intake and energy balance regulation. OEA is a lipid messenger whose release is specifically induced by lipid, but not glucose and aminoacid, ingestion (or intestinal infusion) [429], and substantially inhibits food intake via PPAR-α activation [430]. Furthermore, OEA action has been associated with prolonged inter-meal intervals [430, 431], delay of gastric emptying [429, 432], and enhanced fatty acid absorption by enterocytes [433]. It has been reported that HF-fed obese mice present higher gastric levels of OEA [432], while in rats small intestinal OEA levels drop following the chronic exposure to an oleic acid-enriched diet [434]. These evidences pointed out a further potential mechanism whose deregulation can significantly affect satiation and satiety

regulatory mechanisms in response to HF diet. Moreover, larger gut endocannabinoid pathways are presently under investigation for their role in the control of food intake as well as several GI functions in direct association with HF consumption [435]. Finally, it is worth noticing that HF-associated overconsumption may be further promoted by the increasing sensitivity to orexigenic peptides. NPY expression is enhanced in the hypothalamus of HF-fed rodents [436], and it can also be stimulated by a single HF meal in rats, though in pre-load type experiments [437]. In addition, these rats presented increased hypothalamic levels of orexin and galanin. Taken together, all these evidences indicate that overconsumption is more likely to be caused by a disrupted equilibrium between positive and negative feedbacks signals, in which the potentiation of orexigenic stimuli accompanies the blunting of anorexigenic satiatory signals. However, most experiments dealing with this issue have considered the changes induced by the chronic consumption and consequent adaptation to HF diet upon the short-term regulation of food intake. Lean and obese HF consumers have been considered, though it has not always been possible to dissociate the effects directly associated with fat consumption from those more likely engendered by fat accumulation and body weight gain, with all the related physiologic changes. In light of this, and as I will point out below, one of the main purposes of the work presented in this thesis was to elucidate which are the molecular signals that are rapidly and specifically modulated during the consumption of a single HF meal in chow-fed mice.

Influence of gonadal steroid hormones on eating behavior

As mentioned earlier, there are substantial evidences showing that eating behavior is sexually differentiated [18]. Studies focusing on estrogen-regulated pathways are more copious compared to those examining the effects of testosterone and androgens on eating behavior. Gonadectomy experiments have disclosed marked sex differences, in that orchiectomy reduces eating and body weight in males, whereas ovariectomy increases feeding and fat gain in females. These changes are reversed by testosterone in males and estradiol treatments in females, therefore suggesting that testosterone induces, while estradiol inhibits eating. However, the influence of these gonadal steroid hormones on meal patterns is again dimorphic, since changes caused by ovariectomy and estradiol replacement are associated with altered meal size, while the effects of testosterone and

orchiectomy significantly influence meal frequency [438, 439]. The distinct effects of estradiol and testosterone are likely to depend on post-pubertal maturation of receptor and post-receptor signaling, as shown in rats [18]. In particular, deletion studies of ER genes in mice have contributed to disclose the prevalent role of ER-α, compared to ERβ, in mediating the effects of estradiol on feeding [440], though this issue still remains controversial [18]. It is well known that the pattern of estradiol secretion in female rodent and human ovarian cycles can substantially modify feeding behavior, specifically by decreasing food intake during the peri-ovulatory phase [18]. The reduction of intake is mainly due to decreased meal size, and the drop of energy intake (estimated in 190 kcal/day less than other phases of the cycle in humans) may be significant over energy balance and body weight control [441, 442]. A few hypotheses have been tested in animal models, which indicated that estrogen variations might negatively affect food intake by enhancing the satiating power of CCK, as well as attenuating the acute effects of ghrelin [18, 443]. Furthermore, in women, increased preference for and higher consumption of sweet foods have been reported during the lutheal phase [444], and this study highlighted the importance of investigating the impact of palatability of foods and the eventual influence of gonadal steroid hormones on orosensory stimulation. Though a plethora of studies have been produced in recent years, the signals affecting appetite and satiety in post-menopausal women, and their relative contribution to increased obesity rates in menopause, have not yet been completely elucidated.

1.5 THE MANY ROLES OF TFF2, A GASTRO-INTESTINAL PEPTIDE

Gut mucosal growth, functions and repair mechanisms are highly dependent on nutrient availability. It is therefore expected that most gut mucosal signals might be modulated by feeding and/or may directly or indirectly influence eating behavior and energy metabolism. Trefoil factor family member 2 (TFF2) is a gut peptide already known for its protective functions and immuno-regulatory properties, though it had never been directly related to feeding behavior or energy metabolism regulation. However, we had previously observed that *Tff2* expression could be significantly regulated by food intake in the duodenum mucosa [11] as well as the mesenteric adipose tissue [476] of mice. In this work, I will report the first evidences demonstrating that TFF2 is also a modulator of feeding behavior and can significantly influence energy metabolism. Before proceeding to the following chapters, this section will describe the current knowledge about TFF2 and its physiologic functions, with special interest for the roles exerted by this peptide in the GI mucosa and as a modulator of the immune system.

Genomic organization and protein structure of trefoil peptides

Trefoil factors constitute a family of three small secreted peptides that are structurally characterized by one or more trefoil domains. By definition, a trefoil domain is a sequence of 38 or 39 amino acid residues in which 6 cysteines are disulphide-linked in a 1-5, 2-4, and 3-6 configuration. The conserved amino acid sequence, together with the disulphide bonds, forms the peculiar three-leaved structure that defines the "trefoil" peptide family [445]. The disulphide arrangement and the peptide length of the three loops are highly conserved among the trefoil domain-containing peptides. Interestingly, their protein structure is highly resistant to digestion by pancreatic and GI enzymes. All TFFs work as dimers, and are expressed in several tissues of the body where they exert sometimes comparable but nonetheless distinct functions. However, TFFs are mostly found in the GI tract (TFF1/2 mainly in the stomach, while TFF3 in the small and large intestines), and work in cooperation with mucins to the formation and stabilization of protective gel complexes, which are highly resistant to proteases and mechanical stress [445, 446]. In humans, the three *TFF* genes are found on chromosome 21 (21q22.3) in a head to tail organization that

suggests a coordinated transcriptional regulation. The same clustered organization is found for mouse *Tff* genes on chromosome 17, where they cover a 40 kb DNA segment and are oriented head to tail in the order: *Tff1*, *Tff2*, *Tff3*. It has been reported that *Tff* expression is strongly regulated by epigenetic mechanisms (through methylation) in both physiological and pathological conditions [445, 447].

TFF2 is a small stable secreted protein of 129 amino acids (in humans) and contains 2 trefoil domains separated by a short sequence of 7 residues. The molecule appears to be highly conserved among the species and, in particular, there is an 80% human-mouse homology for *TFF2* mRNA and an 86% homology for the TFF2 protein [446]. TFF2 domain structure is even more compact compared to TFF1/3, since it also contains: two extra-domain cysteine residues that form an intermolecular disulphide bond in the peptide [445, 446]; and a peptide chain which further connects the two trefoil domains [446]. Structurally, the domain is composed of a central short anti-parallel beta shift with one short helix above and one below it. The two domains are related by a two-fold symmetry and each domain contains a cleft, which is thought to accommodate a polysaccharide chain and thus be necessary for binding mucin glycoproteins [448].

Main physiologic functions of TFF2 in the gastro-intestinal mucosa

At first, TFF2 was considered mostly a structural rather than a regulatory peptide, essential for cross-linking mucin glycoproteins and stabilize the mucous layer. However, it was soon recognized that TFF2 could also significantly affect the healing rate of mucosal epithelia. TFF2 is expressed in different mucosal epithelia of the body, from the respiratory tract and uterus to salivary glands and conjunctiva epithelia [449]. However, this peptide is predominantly secreted in the stomach and duodenum, where its effects have been more extensively studied. In the stomach, TFF2 is principally released by neck mucous cells of the antral and pyloric glands, and can be found as a component of the gastric juice, mainly in the glycosilated form [446, 450]. Analyses in human subjects have shown that daily TFF2 concentration in the gastric juice can markedly vary, showing a 40-fold difference between day and night levels [450, 451]. In particular, TFF2 levels are lower in the afternoon and early evening, but dramatically rise during the night to reach a peak in the early morning (5 am) [450, 451]. In addition, the authors observed the sleep deprivation-

and age-associated reduction in TFF2 concentration [451], and also noticed that there might be a slight decrease in TFF2 gastric juice levels following food intake [450]. In normal conditions, TFF2 intestinal secretion is restricted to duodenal Brunner's glands. However, in case of injury, TFF2 can be rapidly up-regulated all along the enteric tube, including the colon and rectum [452]. Tff2 is recognized as an early-immediate gene, whose protein product can be released as soon as, and according to some authors even earlier than, 30 minutes after damage occurs [450]. In particular, TFF2 up-regulation is detectable even before that of TGF-α and epidermal growth factor (EGF), which are two key factors also early involved in restitution events [453]. Its elevated expression has been associated with pathological conditions such as peptic ulceration and inflammation bowel disease, and has been strictly correlated to a specific gland-like structure, namely the ulcer-associated cell lineage (UACL), which develops from glands/crypts adjacent to the ulcer during a variety of chronic inflammation conditions [454]. The UACL is a mucin- and TFF-producing structure that also contains a proliferative zone and is therefore thought to represent a natural repair kit activated after mucosal damage [455]. In terms of protection and repair of mucosa, TFF2 displays multiple functions. As mentioned, TFF2 primarily acts to maintain the normal resistance of mucosa to damage, and cooperates with mucins (specifically mucin 6) to the formation and stabilization of the mucosal layer. In case of internal or external aggressions and damages to the epithelia, TFF2 potently mediates several points of the coordinated repair processes, which include epithelial restitution, proliferation and differentiation [453, 454, 456]. The proper regulation of these processes is of pivotal importance for preventing mucosal inflammation and potential progression to cancer, as it has been noted that the largest part of lethal cancer malignancies arises from epithelia [456]. Epithelial "restitution" represents the rapid reconstitution of the epithelial continuity, and is a complex energy-demanding process that is achieved through two phases: 1) cell migration of neighboring cells to the damaged area (within minutes); 2) structural and functional reconstitution of a tight mucosal barrier (within hours or days) [450, 456]. TFF2 participates to both phases, primarily by promoting cell migration but also by influencing cell apoptosis, proliferation, differentiation as well as angiogenesis. The motogenic effects of TFF2 are dependent on signaling cascades downstream extracellular signal-regulated kinase (ERK)-1/2, protein kinase C (PKC)-α and the proto-oncogene tyrosine-protein (Src)

family of kinases, and finally enhance the efficient and rapid migration of cells to cover the denuded basal lamina. The chemotactic functions of TFF2 also include the recruitment and regulation of immune cells on the damaged area [456]. Once constituted, the monolayer of cells would re-establish tight junctions and cell polarity (morphological restitution), and restore the transmucosal epithelial resistance (functional restitution). When the damage extends deeper than the superficial epithelium, proliferation as well as differentiation and angiogenesis steps are further needed. In vivo restitution is actually a high energy-demanding process and requires an uninterrupted mucosal blood-flow. Interestingly, trefoil peptides have also shown pro-angiogenic properties in vivo and in vitro, being sensitive to local hypoxia [457], and thus stimulating the formation of new blood vessels during normal but also patho-physiological processes [458]. The TFF potential of regulating such crucial processes as apoptosis, proliferation and angiogenesis may have threatening consequences in case of aberrant TFF expression, and might contribute to cancer development and/or progression. TFF2 expression is actually altered in gastric cancer and gastric epithelial dysplasia that precedes the initiation of gastric carcinoma [459, 460]. Though TFF2 has never been directly associated with neoplastic transformation or cancer development, these aspects are highly important and should be taken into account in view of the pharmacological use of TFF2. Interestingly, the potential pharmacological properties of trefoil peptides, and TFF2 in particular, have already been analyzed in rodent models, and tests have been conducted to prove their cytoprotective and anti-inflammatory effects in the GI mucosa. In a rat model of colitis, luminal application of recombinant human TFF2 reduced inflammation and enhanced the rate of colonic epithelial repair [454]. In another rat model in which gastric and duodenal ulcers were induced by indomethacin and mercaptamin treatments, both oral and parenteral porcine TFF2 administrations accelerated healing of gastric ulceration, whereas they aggravated duodenal ulcers [461]. Interestingly, in the same study, oral labeled-TFF2 reached and bound the mucus layer of the stomach and small intestine, but was then degraded in the caecum and thus did not reach the colon. Finally, intravenous administration of human TFF2 has also been shown to actively protect rat gastric mucosa from ethanol-induced damage [462]. Therefore, TFF2 and the other trefoil peptides are considered greatly promising for therapeutic use in GI inflammatory pathologies, like inflammatory bowel disease or Chron's disease [463].

The importance of TFF2 for the stability and protection of GI mucosa has been further proved by the analysis of *Tff2* deficient mice. Farrell and colleagues found that *Tff2* KO mice are viable and fertile and show no gastric, duodenal or small intestine ulceration. However, lack of TFF2 significantly reduced gastric mucosa proliferation and also affected acid secretion, leading to: 1) decreased mucosal thickness, mainly characterized by reduced pit (surface mucous cells) size and gland height, but increased total endocrine cell count; 2) two-fold increase in acid secretion and enhanced parietal cell activation [452]. Furthermore, *Tff2* KO mice present increased susceptibility to develop ulcers following non-steroid anti-inflammatory drugs administration, especially at higher doses and longer time points (24 hours) [452, 464].

Immuno-regulatory properties of TFF2

TFF2 also presents important anti-inflammatory and immuno-modulatory functions in a variety of tissues. It was first discovered in rats that major sites of immune regulation can also produce TFF2, including the spleen and lymphoid tissues, at concentrations between 20% and 61% of those found in the stomach [465]. Furthermore, in the same model, TFF2 expression was significantly induced by bacterial endotoxins, and directly stimulated monocyte migration in vitro. It was later confirmed that TFF2 secretion can be regulated by both pro-inflammatory and anti-inflammatory cytokines [466], including TNF-α, IL-4 and IL-13, and in turn influence cytokine release and activation (i.e. IL-1, IL-6) as well as immune cell recruitment [464]. Interestingly, the transcriptomic analysis of *Tff2* KO mice showed that the lack of this gene significantly altered the expression of diverse crucial genes involved in innate and adaptive immunity [466]: several members of the cryptidin family, which are non-specific antimicrobial peptides and mouse orthologues of human defensins, were up-regulated; the expression of several genes involved in major histocompatibility complex (MHC) class I antigen presentation was differentially modulated, indicating the elevated formation of immunoproteasomes and improved antigen presentation; genes coding for various immunoglobulins (Ig) chain were also up-regulated. Furthermore, the same study also observed that cellular retinol binding protein type 2 (*Crbp2*) and *ApoA4* genes were significantly up-regulated in *Tff2* KO mice, whereas members of the major urinary protein (*Mup*) family were down-regulated [466]. While

CRBP2 plays a crucial role in absorption and metabolism of vitamin A (retinol) and beta-carotene [467], ApoA4 is involved in fat metabolism, showing important satiety effects following lipid intake [425]; and MUPs have been recently reported as novel modulators of glucose and lipid metabolism [468, 469]. If the first analyses of *Tff2* KO mice had suggested an indirect interplay between TFF2 and the immune response, following works confirmed and better clarified the relevant functional correlation between TFF2-modulated pathways and the immune system. In particular, Kurt-Jones et al. demonstrated that TFF2 can be directly produced by macrophages as well as lymphocytes, and that TFF2 deficiency significantly affects IL-1R signaling in mature T lymphocytes [464]. Both macrophages and T-lymphocytes harvested from *Tff2* KO mice were actually hyper-responsive to IL-1β but not LPS/toll-receptor 4 (TLR-4) stimulation in vitro, showing increased cytokine release and enhanced proliferation. The authors concluded that TFF2 is to be considered a novel cytokine with important anti-inflammatory properties, specifically associated with the negative regulation of IL-1R signaling. In addition, they also suggested that *Tff2*-deficient mice may closely resemble a model of constitutive IL-1R activation [464]. This has relevant implications, as IL-1R signaling induces the expression and secretion of multiple inflammatory cytokines and chemokines, including IL-6, MCP-1 and TNF-α, and can also significantly affect the regulation of energy metabolism, as it has been shown in rodents and humans [470, 471]. Though a specific TFF2 cell surface receptor has not yet been identified, the capacity of TFF2 to bind, even if not with high affinity, and activate the CXC chemokine receptor 4 (CXCR4) was recently demonstrated [472]. In that study, CXCR4, which is one of the most widely expressed and physiologically relevant chemokine receptors, was highlighted as a primary target of TFF2 in both lymphocytic and epithelial cancer cells, and its stimulation induced the activation of cell migration, proliferation and survival signaling cascades. In conclusion, TFF2 is presently considered a chemokine-like molecule with multiple and distinct functions [473], many of which are still to be disclosed. Though great efforts have been made to fully characterize the many roles of this molecule, further studies are therefore needed to verify its regulatory targets as well as its adequacy for a safe and efficacious pharmacological use.

1.6 RESEARCH OBJECTIVES

The main long-term objective of the research efforts presented in this thesis is to point out novel potential therapeutic targets for the pharmacological approach to obesity. Given the paucity and limited efficacy of presently available pharmacotherapies, the search for more largely effective therapeutic approaches is needed, but complex, as obesity is a multi-factorial condition that, in each individual, involves the unique interaction of the genetic background/biological predisposition with the environment. This interaction significantly influences the physiology of energy balance, which in turn governs behavioral adaptations. When considering obesity, feeding behavior and physical activity are the main indicated behavioral culprits, and consequently the most studied. More recently, other behavioral factors have been taken into consideration for their potential impact on energy homeostasis, such as stress intensity and sleep duration, but they ultimately become important only when they directly impact food intake and/or energy expenditure. The distinction between long-term and short-term regulating events should also be considered. In particular, some authors have focused their attention on the early mechanisms that control satiation and meal size, and whose de-regulation contributes to overconsumption, especially in an obesogenic environment.

The present work is part of a larger project that aims to point out novel regulatory signals that acutely affect food intake and modify energy balance, leading to weight gain and obesity. The term "novel" includes: not sequenced genes; sequenced but still not characterized genes; characterized genes which have not been considered yet as energy balance modulators. In particular, 1) the differential impact of gonadal steroid hormone changes on menopause obesity risk, and 2) the specific effects of HF intake on feeding behavior have been firstly investigated, with diverse significant outcomes. Finally, the greatest part of the research work described in this thesis has been committed to the identification and characterization of early signals that are responsible for the weaker satiation/satiety effects specifically engendered by HF food consumption. Acute changes in gene expression have been analyzed in key tissues for the regulation of energy balance, including the hypothalamus, stomach and, previously, duodenum and adipose tissue of

mice. Among the novel genes specifically modulated by HF intake in the duodenum, *Tff2* was selected for further in vivo characterization studies.

Figure 3 schematizes the general and specific objectives guiding the sequential research steps of this thesis:

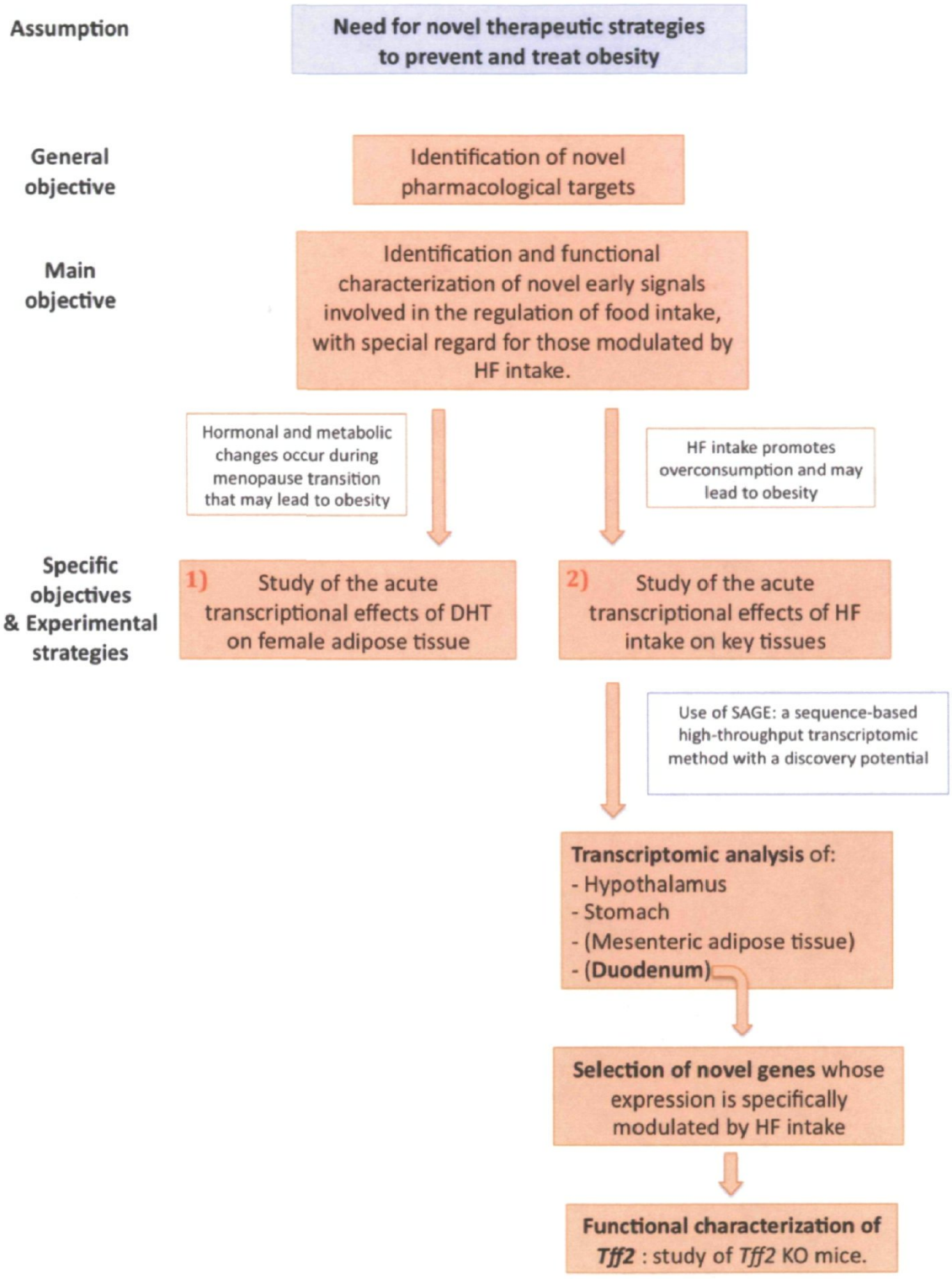

Figure 3. Schematic representation of research objectives and experimental steps.

CHAPTER 2:

A single dose of dihydrotestosterone induced a myogenic transcriptional program in female intra-abdominal adipose tissue.

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Résumé

Les stéroïdes sexuels sont des modulateurs clés de la masse adipeuse et peuvent déterminer des différences importantes en ce qui concerne sa distribution et son accumulation. Avec l'objectif d'étudier les spécificités de l'action androgénique au niveau du tissu adipeux (TA) abdominal féminin, nous avons utilisé la méthode de l'analyse sérielle de l'expression génique (SAGE) pour analyser le transcriptome du TA chez 4 groupes de souris femelles: intactes, ovariectomisées (OVX), OVX plus injection de dé-hydrotestostérone (DHT) à 3h ou 24h avant le sacrifice (DHT3h, DHT24h). Une moyenne de 19555 espèces de transcrits dérivant du TA rétropéritonéal a été analysée. Nous avons retrouvé un total de 321 transcrits modulés de façon différentielle par le DHT ou l'OVX, incluant 125 nouveaux gènes. Plusieurs gènes impliqués dans le métabolisme énergétique/production d'ATP étaient surexprimés suite à l'injection de DHT, tandis que des régulateurs importants du métabolisme lipidique étaient réduits. Le DHT a ainsi modulé de façon différentielle plusieurs transcrits impliqués dans la captation/relâchement du calcium, signalisation cellulaire, défense cellulaire et expression protéique. Un nombre surprenant de gènes myogéniques à été surexprimé, incluant les polypeptides légers et lourd de la myosine, les troponines, ainsi que plusieurs protéines liant l'actine. Ces résultats suggèrent que la condition DHT24h aurait induit un programme transcriptionnel de type myogénique dans le TA analysé. La présente étude clarifie plus à fond la spécificité du profil transcriptionnel aigu induit par les androgènes dans le TA intra-abdominal femelle, et pourrait contribuer de façon importante à la compréhension de l'endocrinologie en ménopause et son association avec l'obésité intra-abdominale.

A Single Dose of Dihydrotestosterone Induced a Myogenic Transcriptional Program in Female Intra-Abdominal Adipose Tissue.

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Short title: DHT-modulated transcripts in female mouse fat tissue.

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ABSTRACT

Sex steroids are key regulators of adipose tissue (AT) mass, determining gender-specific differences in fat distribution and accumulation. With the aim of exploring the relevance and peculiarities of androgen action in female intra-abdominal AT, we used the serial analysis of gene expression (SAGE) method to analyze the AT transcriptome in four groups of female mice: intact, ovariectomized (OVX), OVX plus dihydrotestosterone (DHT) injection at 3h or 24h before sacrifice (DHT3h, DHT24h). An average of 19555 transcript species was examined in retroperitoneal fat. We found a total of 321 transcripts differentially modulated by DHT and OVX, including 125 novel genes. Several genes involved in energy metabolism/ATP production were up-regulated by DHT, whereas important regulators of lipid metabolism were reduced. Transcripts involved in Ca^{2+} uptake/release, cell signalling, cell defence and protein expression were differentially modulated by DHT. A surprising number of myogenic genes were up-regulated, including myosin light and heavy polypeptides, troponins, as well as several actin-binding proteins. These results suggest that DHT24h may have induced a myogenic-like transcriptional program in adipocytes. The present study sheds light on the distinctive female transcriptional pattern acutely induced by androgens in intra-abdominal fat, and may add new insights into the global understanding of menopausal endocrinology and its association to intra-abdominal obesity.

Keywords: retroperitoneal fat tissue, androgens, SAGE, myogenic-like transcriptome.

1. INTRODUCTION

During menopause, and particularly during the transition period also called interphase [1, 2], women's hormone profile undergoes crucial changes which may profoundly affect their life and health conditions. The key event is the shift of sex hormone balance, which essentially alters the relative proportions of estrogen and androgen secretion, and their circulating levels. Specifically, in the early menopausal phases, the progressive decline of estrogen levels is accompanied by a more gradual fall of androgen ones, leading to a prolonged condition of androgenicity [1, 2]. This has an important impact on adipose tissue (AT) regulation and distribution, with an increase in total and central adiposity, and a simultaneous loss in fat-free mass [3]. Moreover, androgen excess has been associated to an increased risk of type 2 diabetes/metabolic syndrome, obesity [4, 5] and cardiovascular diseases (CVD), and a correlation has been suggested with the duration of exposure [1, 6]. If we consider that CVD still represent the leading cause of death among postmenopausal women [7], the study of fine endocrine changes occurring in this phase appears of primary importance, and should be oriented to clarify the specific role of androgen-regulated pathways and their potential as therapeutic targets.

We have already analyzed the short- and long-term effects of dihydrotestosterone (DHT) in male mice AT, finding that various essential pathways were modulated [8, 9]. In summary, our data from male AT have shown that DHT acutely stimulates glycolysis, fatty acids (FA) and triacylglycerol (TG) production, lypolisis and cell shape reorganization, influencing cell proliferation and differentiation [8]. The acute differential effects exerted by ovariectomy (OVX) and estradiol (E_2) on female mice AT have also been studied, showing a limited number of regulated transcripts [10].

The current study intended to identify the androgen-mediated molecular mechanisms which play a specific role in the regulation of female fat tissue. In particular, we were interested in identifying the transcriptional changes acutely induced by androgen in female intra-abdominal fat. In this attempt, we used the serial analysis of gene expression (SAGE) method and analyzed the retroperitoneal AT transcriptome of OVX mice, at 3 and 24 hours after DHT administration (DHT3h, DHT24h). We found that major changes on gene expression occurred 24h following DHT treatment. Surprisingly, lipid metabolism has been poorly affected by androgen treatment, whereas an unexpected myogenic-like transcriptional program has been induced.

2. MATERIALS and METHODS

2.1 Sample preparation

For these experiments, the retroperitoneal fat depot was chosen, as component of the intra-abdominal AT [11]. The tissue was obtained from C57BL6 female mice (14 per group), 14-15 weeks of age, purchased from Charles River Canada (St-Constant, Québec, Canada). The experiments were conducted in accordance with the requirements of the Canadian Council on Animal Care, and approved by the animal protection Committee of Laval University.

Animals were provided Laboratory Rodent Diet No. 5002 (PMI, St. Louis, MO) and water *ad libitum*. Intact mice were sham-operated. The other mice were ovariectomized 7 days before death (OVX), in order to have a reliable model of early menopause [12]. Vehicle (0.4% w/v Methocel A15LV Premium/ 5% ethanol) for the intact and OVX groups was injected 24 hours before death. In the two treated groups, DHT (0.1 mg) was injected 3 hours (DHT3h) and 24 hours (DHT24h) prior to sacrifice. Time points were decided on the basis of previous transcriptomic studies [13, 14], in which 3h and 24h time points had shown the highest transcriptional activity. The dose of DHT chosen, 0.1 mg, is the minimum dose needed to restore prostate size following castration in males [15], and has been previously administered as a physiological dose in other studies on both male and female mice [14, 16, 17]. However, further specifications are needed in the case of female mice, in which the DHT dose considered may result in supraphysiological circulating levels, at least in the first 6 hours after the injection. In fact, as shown by Zhang et al. [18], at 3 hours, DHT serum concentration should reach 5 to 6 ng/ml, and then gradually decrease to more physiological levels [19, 20], being finally lower than 500 pg/ml (18-24h). Immediately following the sacrifice, the retroperitoneal fat was dissected (between

09:00 and 12:15 h). The samples from all mice of the same group were pooled to eliminate inter-individual variations and to extract sufficient amount of mRNA. The tissues were frozen in liquid nitrogen, and stored at –80°C until analysis.

2.2 Transcriptomic analysis

The four SAGE libraries were constructed as previously described [21, 22]. Total RNA was isolated from pooled AT for each group (n = 14) by Trizol (Invitrogen Canada Inc., Burlington, ON). The quality of total RNA was monitored by micro-capillary electrophoresis (Bioanalizer 2100, Agilent Technologies, Mississauga, ON). Polyadenylated RNA was extracted with the Oligotex mRNA Mini Kit (Qiagen Inc., Mississauga, ON), annealed with the biotin-5'-T_{18}-3' primer and converted to cDNA using the cDNA synthesis kit (Invitrogen Canada Inc.). The resulting cDNAs were digested with *NlaIII* (New England BioLabs Ltd., Pickering, ON), and the 3' restriction fragments were captured using streptavidin-coated magnetic beads (Dynal Biotech LLC, Brown Deer, WI) and separated into two populations. Each population was ligated to one of two annealed linkers and extensively washed to remove unligated linkers [22]. The tag beside the most 3' *NlaIII* restriction site (CATG) of each transcript was digested with *BsmFI* (New England BioLabs Ltd.), thereby releasing cDNA fragments including the short 15-bp tags. The blunting kit from Takara Bio Inc. (Otsu, Japan) was used for the blunting and ligation of the two tag populations. The resulting ligation products containing the ditags were amplified by PCR and digested with *NlaIII.* The band containing the ditags was extracted from the 12% polyacrylamide gel with Spin-X microcentrifuge tube (Fisher, Pittsburgh, PA) and the purified ditags were self-ligated to form concatemers using T4 ligase

(Invitrogen Canada Inc.). The concatemers ranging from 500 bp to 1800 bp were isolated by agarose gel and extracted with Gene-Clean Spin (Qbiogene, Montreal, QC). The resulting DNA fragments were ligated into the *SphI* site of pUC19 and cloned into OmniMAX 2T1 competent cells (Invitrogen Canada Inc.). White colonies were picked up, and concatemer inserts were sequenced by the Applied Biosystems 3730 (Foster City, CA).

2.3 Bioinformatic analysis

Sequence files were analyzed using the SAGEana program, a modification of SAGEparser [23]. In brief, SAGE tags corresponding to linker sequences were discarded and replicate concatemers were counted only once. Identification of the transcripts was obtained by matching the 15 bp (sequence at the last CATG + 11bp tags) with SAGEmap, UniGene and GenBank databases. Classification of the transcripts was based upon the updated information of the genome directory [24] found at the TIGR web site (http://www.tigr.org/), the SOURCE (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch) and the OMIM (http://www.ncbi.nlm.nih.gov/), as well as upon previously published literature. We have previously shown that the SAGE method is very reproducible with $r^2 = 0.96$ between two SAGE libraries generated from two cDNA libraries constructed from the same total RNA pool [23].

2.4 Validation by quantitative real-time PCR (Q_RT-PCR)

First strand cDNA was synthesized using 5 μg of pooled RNA of each experimental group in a reaction containing 200 U of Superscript III Rnase H-RT (Invitrogen Canada Inc.), 300 ng of oligo-dT_{18}, 500 μM deoxynucleotides triphosphate, 10 mM dithiothreitol

and 34 U of human RNase inhibitor (Amersham Pharmacia, Piscataway, NJ) in a final volume of 50 µl. The resulting products were then treated with 1 µg of Rnase A for 30 minutes at 37°C and purified thereafter with Qiaquick PCR purification kits (Qiagen). The cDNA corresponding to 20 ng of total RNA was used to perform fluorescent-based real-time PCR quantification using the LightCycler real-time PCR apparatus (Roche Inc., Nutley, NJ) and the FastStart DNA Master SYBR green kit (Roche Diagnostics). Reading of the fluorescence signal was taken at the end of the heating to avoid non-specific signal. A melting curve was performed to assess non-specific signals. Annealing temperature was selected based on contamination levels and melting curve results. Prior to mRNA quantification, RNA samples were also verified for genomic DNA contamination. Oligoprimer pairs that allow the amplification of approximately 250 bp were designed by GeneTools software (Biotools Inc., Edmonton, AB) and their specificity was verified by blast in GenBank database. Gene name, GenBank accession numbers and regions used for the primer pairs were the following: ATPase, Ca^{++} transporting, cardiac muscle, fast twitch 1 (*Atp2a1*), ***NM_007504***, 82-366; metallothionein 2 (*Mt2*), ***NM_016673***, 1435-1724; myosin heavy polypeptide 1 (*Myh1*), ***AJ293626***, 1108-1356; myosin heavy polypeptide 4 (*Myh4*), ***XM_126119***, 1571-1693; troponin C, fast skeletal (*Tnnc2*), ***AV083137***, 41-227; troponin T3, fast skeletal (*Tnnt3*), ***L48989***, 417-544; tropomyosin 2, beta (*Tpm2*), ***M81086***, 1720-1944. The mRNA levels were calculated using a standard curve of crossing point (Cp) versus logarithm of the quantity, and expressed as the number of copies per microgram of total RNA [25]. The LightCycler 3.5 program provided by the manufacturer (Roche Inc.) was used to calculate the Cp according to the second derivative and double correction method previously described by Luu-The et al. [25]. The standard curve with

efficiency coefficient E=2 was established using known cDNA amounts of 0, 10^2, 10^3, 10^4, 10^5 and 10^6 copies of ATP synthase O subunit.

2.5 Statistical analysis

The significant difference was set at $P \leq 0.05$. For the SAGE data, the comparative count display (CCD) test was used to identify the transcripts which were significantly differentially expressed ($P < 0.02$, after Bonferroni adjustment) between the groups with more than a two-fold change. As previously described by Lash et al. [26], the CCD test makes a key-by-key comparison of two key-count distributions by generating a probability that the frequency of any key in the distribution differs by more than a given fold factor from the other distribution. The data were normalized to 100 000 tags for presentation.

3. RESULTS

The SAGE libraries revealed an average of 44181 SAGE tags per group, corresponding to 19555 transcript species. Among these, only 14 transcripts were found to be differentially modulated by OVX, whereas 307 transcripts were regulated by DHT. In particular, 224 genes were up-regulated and 83 were down-regulated following DHT treatment. In total, 125 novel transcripts were significantly modulated.

3.1 DHT-modulated transcripts involved in lipid metabolism.

Our data showed a general down-regulation of lipid metabolism-related transcripts following DHT24h treatment, including both anabolic and catabolic genes (Table 1). However, at DHT3h, only a few transcripts were modulated, including the up-regulation of malic enzyme 1 as well as fatty acid binding protein (*Fabp*) 4 and *Cd36*. At DHT24h we observed a reduced expression of biosynthetic genes such as stearoyl CoA desaturase 1, as well as a decreased transcription of genes involved in beta-oxidation/lipogenesis processes, such as acyl-CoA synthetase, desnutrin, expression sequenced tag (EST) *Lpl*. When we considered the regulatory transcripts which affect adipocyte metabolism, we found a diminished level of resistin and adiponectin receptor 2 gene expression, and an increase in caveolin 2, *Cd36* and EST *Ampk* β subunit levels. Interestingly, we found the DHT24h up-regulation of *Fabp3*, a transcript generally known to be expressed in muscle tissues.

3.2 DHT-modulated transcripts involved in glucose metabolism and energy production.

DHT treatment positively influenced glucose metabolism, tricarboxylic acid cycle (TCA) and ATP synthesis processes, and these effects are prominent at DHT24h when 59 transcripts were found to be differentially modulated compared to OVX control (Table 2). Numerous of these transcripts were mitochondrial, confirming that mitochondrial RNAs are polyadenylated [27]. Glycogen metabolism (protein phosphatase 1, glycogen phosphorylase and phosphoglucomutase 2) and glycolysis (phosphofructokinase, aldolase 1A, EST glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate mutase 2) transcripts were up-regulated. Lactate dehydrogenase 2 β chain gene expression was increased, as well as the transcription of many genes involved in TCA cycle reactions, such as aconitase 2, succinate-CoA ligase, and malate dehydrogenase 2. Overall, complex I (NADH dehydrogenase subunits 1α, 1β, 2, 3, 4), complex II (succinate dehydrogenase complex, subunit B), complex III (ubiquinol-cytochrome c reductase) and complex IV (cytochrome c oxidase subunit 1, 2, 3, 4a, 7a) of the mitochondrial electron transport chain were up-regulated. Consistently, the transcription of mitochondrial ATP synthase F0 and F1 complexes, and the ADP/ATP transporter solute carrier family 25 member 4 (*Slc25a4*), were enhanced. This increased expression of oxidative phosphorylation-related transcripts is accompanied by an elevated expression of myoglobin as well as creatine kinase and adenylate kinase, showing a considerable up-regulation of ATP production genes.

3.3 DHT-modulated transcripts involved in Ca^{2+} release/uptake.

Ca^{2+} release/uptake system represents a key intracellular regulatory mechanism which is well known to control numerous pathways. Therefore, it was interesting to find that several genes coding for Ca^{2+} channels and endoplasmic reticulum (ER)-associated Ca^{2+} binding proteins were up-regulated following DHT24h (Table 3). Ca^{2+} ATPase, voltage–dependent L-type Ca^{2+} channel as well as histidine-rich calcium binding protein, triadin, junctin and ryanodine receptor 1 are the main transcripts whose expressions were induced. Only the expression of calreticulin was dramatically reduced by DHT. It is important to mention that all the up-regulated genes cited above are usually expressed at high levels in muscle tissues in association with sarcoplasmic reticulum (SR).

3.4 DHT-modulated transcripts involved in cell structure/motility.

Cell structure associated genes, particularly those involved in motility, were found to be highly influenced by DHT treatment. In fact, 43 out of 44 transcripts were up-regulated following DHT24h, and only gelsolin gene expression was decreased. Among the transcripts listed in Table 4, we observed many genes usually associated to muscular contraction, such as myosin light and heavy chains, actin and several actin-binding proteins, troponin I, C, and T, as well as tropomodulin, tropomyosin 1α and 2β, and myomesin. Moreover, we observed the up-regulation of transcripts in microtubule systems, such as tubulin α4, kinesin family member 1c, and ring finger protein 30. In contrast, 2 extracellular matrix (ECM)-associated transcripts were down-regulated (matrix metalloproteinase 14 and decorin).

3.5 Other differentially expressed genes following DHT treatment.

In addition, DHT treatment showed modulations of a number of different genes involved in cell signalling (n=6), gene-protein expression (n=19) and cell defence (n=15). Cyclin G1 and N-myc downstream regulated gene 2 were up-regulated, whereas protein kinase Cδ (PKCδ) and EST insulin-like growth factor binding protein 7 were down-regulated, suggesting that most cells are likely to be in a synthetic/growing phase of their cycle. Consistently, the expression of carboxypeptidase E, an important pro-hormone processing exopeptidase, as well as cold shock domain protein A, several ribosomal proteins (4 out of 6 six transcripts), transcription elongation factor A and 1α2, and crystallin αB were induced, underlining a condition of enhanced protein synthesis and processing (Table 5).

Genes involved in cell defence and immunity were mainly down-regulated, including EST lysozime and lysosomal-associated membrane glycoprotein 1, EST adipsin, as well as a macrophage transcript named tartrate resistant acid phosphatase 5 (*TRAP/Acp5*), which is known to induce proliferation and differentiation of mouse and human adipocyte precursor cells [28].

Moreover, we found 23 partially characterized and 125 novel transcripts to be differentially modulated following DHT3h or DHT24h. These mainly showed an increased expression compared to OVX control, except for 13 tags which were stably down-regulated both at DHT3h and DHT24h (see Appendix 1 and 2). Interestingly, the SAGE tag GACCAGCAGAC was up-regulated by OVX, but DHT treatment reversed the effect.

3.6 SAGE data confirmation by Q_RT-PCR and comparison between AT and skeletal muscle most expressed genes.

Since our DHT24h SAGE data seemed to suggest the induction of a myogenic transcriptional program in adipose cells, the expression of seven myogenic transcripts significantly modulated at that time point was verified using the Q_RT-PCR method [29]. Globally, the PCR results are in good agreement with SAGE data (Fig 1).

Moreover, we compared the ten most expressed of the known genes presently found in intact and DHT24h AT with the ten most expressed ones detected by a previous SAGE analysis [14] in intact skeletal muscle. As shown in Table 6, there is an 80% overlap between intact skeletal muscle and DHT24h genes versus a 30% between intact and DHT24 AT. Therefore, the panel of transcriptional changes induced by DHT in AT appears closer to the expected muscular expression profile.

4. DISCUSSION

4.1 OVX, E_2 and DHT differential effects on female mice AT.

The androgenicity observed in the early phases of menopause is likely to play a major role in endocrine modifications characterizing this period. We have already studied the effects of OVX and E_2 on female mice AT transcriptome [10]. We have found few effects induced by E_2, whereas OVX had mainly up-regulated ECM components, such as procollagen type 1α2, fibronectin 1 and secreted acidic cysteine rich glycoprotein (*Sparc*). Moreover, fatty acid synthase (*Fasn*) gene expression decreased compared to intact AT. The present study further confirmed those data, and additionally showed the DHT-induced down-regulation of genes highly expressed in intact female retroperitoneal AT, namely EST carbonic anhydrase III, EST adipsin, ATP synthase subunit 6, EST *Fabp4*, and stearoyl-coA desaturase 1 [10]. Interestingly, the SAGE tag GAAAATGAGAA, which has no match in the databases, was also confirmed among the ten most expressed genes in intact AT, and its expression was reduced following DHT3h and 24h. This tag may be a good target for further characterization studies.

4.2 DHT effects on lipid metabolism transcripts.

The present work aimed to analyze the effects of DHT on intra-abdominal AT gene expression of female mice. There is substantial evidence that intra-abdominal (retroperitoneal and visceral) fat depots possess distinct features compared to subcutaneous fat for instance [11], both at the cellular and molecular/metabolic level [30, 31]. In particular, the cytokine profile as well as the contribution to insulin resistance and cardiovascular risk represent some of the differential features most extensively reported in the last years [32, 33]. We have previously described the acute effects of DHT in male

retroperitoneal AT [8]. In males, when the same experimental conditions were applied, lipid metabolism was mostly affected, with de novo FA synthesis, TG synthesis as well as lipolysis being up-regulated. Surprisingly, in females, few lipid metabolism-related genes were modulated. However, important transcripts such as *Scd1*, acyl-CoA synthetase long-chain family member 1 (*Acsl1*), desnutrin, EST *Lpl* and resistin were down-regulated compared to OVX control. Therefore, both lipogenesis and lipolysis appeared to be reduced. Interestingly, two transcripts coding for distinct fatty acid binding proteins (*Fabps*) showed a particular pattern of expression following DHT treatment. The different members of this lipid chaperone family (liver, intestinal, heart, adipocyte, epidermal, ileal, brain, myelin and testis) show unique patterns of expression [34]. In this study, we found that the transcription of the adipocyte member *Fabp4* was comparable in intact and OVX mice AT, and enhanced at DHT3h. However, its level diminished to that of intact AT at DHT24h. Conversely, at this time point, transcription of *Fabp3*, which is mostly expressed in heart and skeletal muscle, but also found in brown and white AT, was significantly increased. *Fabp3* gene is known to be regulated by androgens in muscle cells [35], but to our knowledge this is the first time that this modulation is observed in AT.

4.3 Induction of energy metabolism transcripts by DHT.

In contrast with reduced lipid metabolism modulation, our data revealed a general enhancement of glucose metabolism and energy production machinery. Indeed, several mitochondrial transcripts were up-regulated, underlining a transcriptional drive to ATP production by the electron transport chain. Consistently, our DHT24h data showed the up-regulation of an important ADP/ATP carrier, *Slc25a4*, which shuttles ADP and ATP at high specificity between mitochondria and cytosol. It is mainly expressed in heart and skeletal

muscle, and plays the fundamental role to link mitochondrial energy metabolism requiring ADP with the cytosolic milieu, where ATP is consumed [36]. Moreover, myoglobin gene expression was augmented, concordant with the potentially enhanced oxidative phosphorylation and the consequent higher oxygen requirements. The cellular demand of ATP appeared to be substantial, as creatine kinase and adenylate kinase transcripts were also up-regulated. These two enzymes are usually expressed by cells rapidly consuming energy, in order to prevent the depletion of ATP stores.

4.4 Induction of calcium signalling transcripts by DHT.

Intracellular Ca^{2+} signalling system has also been modulated at DHT24h. Eleven transcripts were affected and only calreticulin, a Ca^{2+} binding chaperone which plays an important role for Ca^{2+} buffering in non-muscle cells [37] was down-regulated. Overall, the expression of several important genes for Ca^{2+} uptake/release was increased. Seven of these genes are known to code for important SR-associated molecules. In particular, ryanodine receptor 1, junctin, and triadin are part (together with calsequestrin) of a macromolecular SR Ca^{2+} regulating complex, which is a major actor for normal Ca^{2+} release and contractility in cardiac and skeletal muscle [38, 39]. Moreover, three other DHT24h up-regulated genes are important components of the SR Ca^{2+} cycling complex: histidine rich calcium binding protein, voltage-dependent L-type calcium channel alpha-1S subunit and Ca^{2+} ATPase.

These data are consistent with the increased expression we observed for the energy production-related transcripts and with the up-regulation of numerous motility/myogenic genes, showing an expression pattern which is normally expected in muscle cells.

Nevertheless, it is interesting to note that increases of intracellular Ca^{2+} concentration have been reported to inhibit the early stages of adipogenesis in mice [40, 41].

4.5 Myogenic genes induction by DHT.

Recently, Zhang Y. et al. have reported a microarray analysis of DHT acute effects on male and female retroperitoneal AT [18]. SAGE and microarray are two distinct techniques with different but complementary features, whose choice depends on the specific experimental design and its final goal. They both are reliable and sensitive high-throughput transcript profiling technologies. Compared to microarray, SAGE may show less sensitivity in detecting the differential expression of low abundance mRNAs with a statistical significance, and can miss shorter transcripts which do not contain an NlaIII restriction site. However, it is a sequencing-based method that does not require prior knowledge of sequences to be analyzed, and thus offers a gene discovery potential. Moreover, the gene expression levels detected by SAGE represent the proportion of actual expression levels in the cells or tissue. The data analysis is less complex, and results are more easily reproducible and comparable among different laboratories. Indeed, studies that have compared SAGE and Affymetrix data showed that they present similar levels of sensitivity [42, 43], although the SAGE method was found to be more quantitatively reproducible [44]. In their work, Zhang and his colleagues found that, in both male and female mice, DHT modulated several genes involved in the regulation of adipogenesis, with their results indicating a reduced adipogenic differentiation. They also observed that a number of genes which were significantly modulated in both sexes are myogenic in nature. Our SAGE data, obtained from the same female mice used by Zhang et al., are consistent with their conclusion and show a more extensive picture of the transcriptional myogenic

program induced by DHT in female AT. They found by Affymetrix that 116 probesets (112 genes) were differentially modulated by DHT in female mice, and validated these results by Q_RT-PCR analysis for 98 transcripts. Overlapping the results of the three distinct but complementary techniques gives us a global image of the transcriptional program induced by DHT. Microarray analysis indicated a negative modulation of adipogenesis and suggested a possible myogenic response induced by DHT in AT. The information related to myogenesis is not only confirmed but further extended by SAGE study, in which more than 30% (55 out of 182) of the tags are myogenic in nature or mainly expressed in muscular tissues. In addition, SAGE data showed a more global picture, in which the up-regulation of ATP production and calcium release related mRNAs, together with the reduced expression of important lipid metabolism genes concordantly suggest a myogenic-like transcriptional program. Table 6 compares the ten most abundant known transcripts found by SAGE in intact female mouse AT and skeletal muscle [14], with the ten most expressed known genes in female DHT24h AT. The 80% overlap between the intact skeletal muscle and DHT24h AT ten most expressed genes further supports that DHT may have induced a myogenic-like transcriptional program in retroperitoneal adipocytes. It should be noted that both adipocytes and muscular cells derive from the same precursors, the mesenchymal pluripotent cells [45]. Testosterone and DHT are well known to promote the myogenic commitment of pluripotent precursor cells, and to inhibit adipogenesis through an androgen receptor-mediated pathway [45, 46]. This is associated, at an early stage, with the activation of muscle-specific transcription factors (such as myo D, myogenin, and myf 5), and is followed by the expression of desmin and myosin heavy chain II (*Myh2*) in terminally differentiated cells. Our data showed no differential expression for the early markers, though we found a substantial up-regulation of at least five specific markers of

differentiated muscle cells, namely Desmin, *Myh2*, *Tnnt1*, Actinin alpha 2 (*Actn2*), Actin, hence further suggesting a substantial myogenic trend in transcription.

4.6 Distinct gender effects of DHT on AT transcription: acute and chronic studies

The present results appear in contradiction with our previous acute study of DHT effects in male mice [8]. In males, in fact, lipid metabolism was globally affected, and the up-regulation of transcripts involved in cell structure reorganization likely indicated the stimulation of adipogenetic differentiation. In a following work, however, when the physiological dose of DHT was administered chronically, the effects on lipid metabolism stabilized toward an increased lipid mobilization, whereas FA and TG synthesis appeared to be reduced [9]. Globally, we concluded that the chronic androgen treatment may help to improve the metabolic profile of intra-abdominal AT. A similar chronic approach in females is needed to verify the extent of the myogenic induction engendered by androgens in intra-abdominal AT. However, it should be noted that the androgenicity characterizing the transition phase to menopause is a transient heterogeneous state, whose extent and duration are still hardly predictable, mostly because of a marked inter-individual variability in both estrogen and androgen levels [2]. In the present study, indeed, we were especially interested in understanding the initial events triggered by androgen predominance in the early phases of menopause, and substantiating the hypothesis that changed androgen levels may acutely affect gene expression in AT and possibly engender central fat accumulation. Finally, though a distinct gender regulation is apparent, the hypothesis that androgen may induce the transdifferentiation of mature adipocytes into muscle-like energy-expending cells offers another potential direction for improving the metabolic profile, and should be verified in finely designed chronic studies.

4.7 Plasticity potential of AT.

Several studies have already been published which show the transdifferentiation potential of AT-derived cells, and that they can be committed into myogenic, chondrogenic, osteogenic cells, as well as other non-mesenchymal lineages [47]. In particular, the myogenic potential has already been demonstrated for mouse pluripotent [46], preadipose [48] and mature fat cells [49], and has been recently employed for successful muscle regeneration in human and mouse models of Duchenne muscular dystrophy [50-52]. These results have opened new scenarios in which it would be possible, for instance, to use AT as a source of stem cells therapies for muscular diseases. We do believe that the extensive plasticity shown by AT in this and other works [47, 49] is a topic of critical interest which should be better and sooner addressed as another promising key target to prevent and treat intra-abdominal fat accumulation and its deleterious consequences.

In conclusion, we have analyzed the acute effects of DHT on the intra-abdominal AT gene expression in OVX mice. We have observed the modulation of few genes involved in lipid metabolism, whereas several transcripts related to skeletal muscle and muscular contraction were up-regulated. Concordantly, the expression of genes involved in glucose metabolism, ATP production as well as Ca^{2+} uptake/release cycle increased. Remarkably, the first 4 most expressed genes in DHT24h AT exactly correspond to the 4 most expressed in female intact skeletal muscle (Table 6), as found by SAGE method. We concluded that, 24 hours after a single dose of DHT, the retroperitoneal AT gene expression may have changed toward a myogenic-like transcriptional program. These results represent an important first step toward a better understanding of gender influence

on intra-abdominal AT physiology. Further studies which would verify the effects of chronic androgen prevalence will help the comprehensive understanding of menopause endocrinology and verify their potential as therapeutic targets for preventing and/or treating the intra-abdominal obesity associated with this condition.

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AUTHOR'S CONTRIBUTIONS & DECLARATIONS OF INTEREST

The present article has been approved by all listed authors and there is no conflict of interest that would prejudice its impartiality. MRDG analyzed and interpreted the SAGE data, and drafted the manuscript. MY and JstA conceived the study, designed it and critically revised the manuscript. JstA gave the final approval of the version to be published.

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Table 1. Female mouse DHT-modulated transcripts involved in lipid metabolism.

Tag	Intact	OVX	OVX+DHT		Description (UniGene cluster, *GenBank accession no.*)
			3h	24h	
Fatty acid synthesis/Lipogenesis					
CTATGTATCCG	31	31	**3** ↓	**6** ↓	EST Carbonic anhydrase 3 (Mm.300, ***CB947072***)
ATGCAGGGCCA	277	**114** ↓	255	121	Fatty acid synthase (Mm.236443; ***AF127033***)
TTGTCAGGTAG	113	47	**142** ↑	86	Malic enzyme 1, NADP(+)-dependent, cytosolic, supernatant (Mm.148155; ***NM_008615***)
GGCAAGTGCTA	83	32	7	**2** ↓	Stearoyl-Coenzyme A desaturase 1 (Mm.267377, ***AF509570***)
Beta-oxidation/Lipolysis					
TGGGTGTCCAG	90	68	25	**6** ↓	Acyl-CoA synthetase long-chain family member 1 (Mm.210323; ***NM_007981***)
CTGTGGAATGA	69	38	29	**9** ↓	Desnutrin (Patatin-like phospholipase domain containing 2) (Mm.29998; ***AY731699***)
ACAAGTCTCTG	91	79	31	**13** ↓	EST Lipoprotein lipase (Mm.1514, ***AA537700***)
TTTCTCCTGCC	16	16	7	**2** ↓	EST Lipoprotein lipase (Mm.1514, ***BY490220***)

Transport/Regulation					
GGGTACAAGAC	32	24	8	**3** ↓	Adiponectin receptor 2 (Mm.291826, ***BC064109***)
AATTATTTTGT	3	2	15	**16** ↑	Caveolin 2 (Mm.396075, ***AB049604***)
CTGCATAGCTC	21	21	**73** ↑	**78** ↑	CD36 antigen (Mm.18628, ***AK052825***)
GTTTTCCCCTC	2	1	3	**44** ↑	Fatty acid binding protein 3, muscle and heart (Mm.22220, ***NM_010174***)
AGCCAAAGGAA	718	570	**1371** ↑	771	Fatty acid binding protein 4, adipocyte (Mm.582, ***AK088535***)
ATCATCAGCGT	63	43	**3** ↓	**3** ↓	EST Fatty acid binding protein 4, adipocyte (Mm.582, ***BG794981***)
CAGAGCTGCTT	1	1	3	**16** ↑	EST Protein kinase, AMP-activated, beta 2 non-catalytic subunit (Mm.31175, ***BG794097***)
CCACTGTGTCC	51	36	25	**6** ↓	Resistin (Mm.1181, ***AF290870***)
CAAAAATAGTA	10	5	**29** ↑	17	Sulfotransferase family 1A, phenol-preferring, member 1 (Mm.368982; ***BC005413***)

Abbreviations: OVX, ovariectomy; DHT, dihydrotestosterone.
Numbers indicate the count of serial analysis of gene expression (SAGE) tags per 100000.
SAGE tag numbers in bold indicate a statistical significance ($P \leq 0.05$; OVX vs. Intact, DHT vs. OVX).

Table 2. Female mouse DHT-modulated transcripts involved in glucose metabolism and energy production.

Tag	Intact	OVX	OVX+DHT		Description (UniGene cluster, *GenBank accession number*)
			3h	24h	
Glucose/Aminoacid metabolism					
CCTACTAACCA	117	68	83	**594** ↑	Aldolase 1, A isoform (Mm.275831, ***NM_007438***)
AGGTCCACCAC	4	2	3	**34** ↑	EST glyceraldehyde-3-phosphate dehydrogenase (Mm.339669, ***AV019029***)
ACCGGTTTAAA	5	5	3	**46** ↑	Muscle glycogen phosphorylase (Mm.27806, ***AF124787***)
TTGCTTTGTTG	88	39	**125** ↑	31	Phosphoenolpyruvate carboxykinase 1(Mm.266867, ***NM_011044***)
CCTGCAACCAG	3	5	4	**69** ↑	Phosphofructokinase, muscle (Mm.272582, ***BC005526***)
TAATCCAACAG	18	13	16	**71** ↑	Phosphoglucomutase 2 (Mm.217764; ***NM_028132***)
GAAGCTGTTGC	5	7	5	**321** ↑	Phosphoglycerate mutase 2 (Mm.219627, ***BC010750***)
CTGGAAAAACA	1	3	3	**20** ↑	Protein phosphatase 1, regulatory (inhibitor) subunit 3C (Mm.24724; ***AK078506***)

GCTTGTGACGA	35	22	**75** ↑	32	Transaldolase 1 (Mm.29182, ***NM_011528***)
TCA cycle/ATP production					
CAACTGTATTT	31	16	27	**57** ↑	Aconitase 2, mitochondrial (Mm.154581, ***BC004645***)
GCTGTCCCAGG	1	1	0	**35** ↑	Adenylate kinase 1(Ak1)(Mm.29189, ***BC014802***)
AACCCTAATAA	53	58	**11** ↓	48	ATP synthase F0 subunit 6 (***NC_005089***, Pos:8129)
TTGATGTATCT	43	40	**4** ↓	**5** ↓	ATP synthase F0 subunit 8 (***NC_005089***, Pos:7788)
GTTCTTTCGTG	12	10	14	**48** ↑	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 2 (Mm.10314, ***BC006813***) ESTs (Mm.146141, ***BI651446***; Mm.371910, ***W10656***; Mm.180356, ***AV009312***; Mm.29827, ***BB025380***)
GCCGAGCATAA	23	22	40	**71** ↑	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit f, isoform 2 (Mm.133551, ***NM_020582***)

CGGGAGATGCT	53	34	41	**109** ↑	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit (Mm.41, ***NM_138597***)
GCATACGGCGC	13	11	37	**51** ↑	ATP synthase, H+ transporting, mitochondrial F1F0 complex, subunit e (Mm.136093, ***NM_007507***)
CCCTGCCTTAA	44	47	43	**366** ↑	Creatine kinase, muscle (Mm.2375, ***AK009950***)
AGGACAAATAT	278	247	**38** ↓	143	Cytochrome b (***NC_005089***, Pos:14548)
GGCTAGATTTC	59	29	**2** ↓	11	Cytochrome c oxidase subunit I (***NC_005089***, Pos:5739)
TGGTGTAAGCA	14	14	6	**113** ↑	Cytochrome c oxidase subunit I (***NC_005089***, Pos:6673)
ATGAGAACAGC	3	1	14	**19** ↑	Cytochrome c oxidase subunit I (***NC_005089***, Pos:6728)
AAGTCATTCTA	25	10	**39** ↑	31	Cytochrome c oxidase subunit I (***NC_005089***, Pos:6813)
AGTGGAGGACG	34	45	39	**157** ↑	Cytochrome c oxidase subunit II (***NC_005089***, Pos:7497)
AGCAGTCCCCT	249	200	402	**696** ↑	Cytochrome c oxidase subunit II (***NC_005089***, Pos:7500)

CTGCGGCTTCA	9	7	**37** ↑	**53** ↑	Cytochrome c oxidase subunit III (***NC_005089***, Pos:9322)
GAGGGGCAGGA	3	3	2	**30** ↑	Cytochrome c oxidase, subunit VI a, polypeptide 2 (Mm.43824, ***U08439***)
AATATGTGTGG	45	56	**159** ↑	128	Cytochrome c oxidase, subunit VIc (Mm.548; ***AK013459***)
ACGCTGACTCT	1	2	1	**43** ↑	Cytochrome c oxidase, subunit VIIa 1 (Mm.12907, ***AK079950***)
TGATATGAGCT	7	3	9	**29** ↑	Lactate dehydrogenase 2, B chain (Mm.9745; ***NM_008492***)
TCTTTGGAACC	22	14	27	**70** ↑	Malate dehydrogenase 2, NAD (mitochondrial) (Mm.297096, ***NM_008617***)
GGCACTGCGGC	9	10	24	**37** ↑	NADH dehydrogenase 1 alpha subcomplex 11 (Mm.279823, ***XM_128696***)
AAGGATGTGCC	17	16	37	**63** ↑	NADH dehydrogenase 1 alpha subcomplex, 13 (Mm.21162, ***NM_023312***)
CAGAATGTGCT	21	16	32	**60** ↑	NADH dehydrogenase 1 alpha subcomplex, 2 (Mm.29867, ***NM_010885***)
GGAGCCATTGG	17	11	23	**52** ↑	NADH dehydrogenase 1 alpha subcomplex, 5 (Mm.275780, ***BC028633***)

ACTCGGATGCT	5	7	9	**31**↑	NADH dehydrogenase 1 beta subcomplex, 2 (Mm.29415, ***NM_026612***)
CTTGCAAGTGA	38	39	48	**117** ↑	NADH dehydrogenase 1 beta subcomplex, 9 (Mm.322294, ***NM_023172***)
CTATTTCAAAG	9	8	**35** ↑	19	EST NADH dehydrogenase 1 beta subcomplex, 1, 7kDa (Mm.345904, ***CD741351***)
AGGAGGACTTA	106	120	**42** ↓	143	NADH dehydrogenase subunit 2 (***NC_005089***, Pos:4413)
GTAGTGGAAGT	39	52	64	**312** ↑	NADH dehydrogenase subunit 3 (***NC_005089***, Pos:9685)
ATTATAGTACG	2	2	7	**22** ↑	NADH dehydrogenase subunit 4 (***NC_005089***, Pos:11195)
TGGCTATAAGT	1	1	3	**17** ↑	NADH dehydrogenase subunit 4 (***NC_005089***, Pos:11236)
ACTACCATCAG	83	42	**6** ↓	13	NADH dehydrogenase subunit 5 (***NC_005089***, Pos:12436)
ATGATGTGAAT	25	18	**1** ↓	4	NADH dehydrogenase subunit 5 (***NC_005089***, Pos:12853)

GGTGGTGCTTT	27	29	31	**212** ↑	Solute carrier family 25 (adenine nucleotide translocator), member 4 (***Mm.16228***, XM_134169)
CCTCTCAGTAC	1	0	2	**16** ↑	EST Solute carrier family 25 (adenine nucleotide translocator), member 4 (Mm.16228, ***BY681341***)
AACTGCACACA	12	8	11	**37** ↑	Succinate dehydrogenase complex, subunit B, iron sulfur (Mm.246965; ***AK003052***)
ATTCTGTGGTG	1	1	4	**17** ↑	Succinate-CoA ligase, ADP-forming, beta subunit (Mm.38951, ***BC057605***)
TTGACAGACAC	59	38	73	**119** ↑	Ubiquinol-cytochrome c reductase (6.4kD) subunit (Mm.43162, ***NM_025650***); EST (Mm.337888, ***BY694329***)
CACGGGACCAC	33	25	32	**87** ↑	Ubiquinol-cytochrome c reductase hinge protein (Mm.181721, ***BC011388***)
Others					
TCATAATAAAC	1	1	1	**36** ↑	Adenylosuccinate synthetase like 1(Mm.3440, ***BC039943***)

AGCAAGATGGT	8	2	**31** ↑	12	Aminolevulinic acid synthase 1 (Mm.290578, ***BC022110***)
GAAACTCTACT	88	62	24	**12** ↓	Cysteine dioxygenase 1, cytosolic (Mm.241056, ***NM_033037***)
CTCAGGTCTCC	5	5	4	**128** ↑	Myoglobin (Mm.201606, ***BC025172***)

Abbreviations: OVX, ovariectomy; DHT, dihydrotestosterone; Pos., position on the mitochondrial genome.

Numbers indicate the count of serial analysis of gene expression (SAGE) tags per 100000.

SAGE tag numbers in bold indicate a statistical significance ($P \leq 0.05$; OVX vs. Intact, DHT vs. OVX).

Table 3. Female mouse DHT-modulated transcripts involved in Ca^{2+} release/uptake.

Tag	Intact	OVX	OVX+DHT		Description (UniGene cluster, *GenBank accession no.*)
			3h	24h	
CTCACGTCGCC	0	0	3	**25** ↑	Aspartate-beta-hydroxylase (Mm.239247, ***AK009903***)
CATCTTCAGCC	14	18	19	**399** ↑	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1 (Mm.35134, ***BC036292***)
TCATCTTTAAC	15	16	**0** ↓	**0** ↓	Calreticulin (Mm.1971, ***AK075605***)
ATACGAACCCC	1	1	1	**16** ↑	EST Calcium channel, voltage-dependent, gamma subunit 1 (Mm.57093, ***AV084449***)
TTGGAGACTCC	1	3	3	**47** ↑	Histidine rich calcium binding protein (Mm.39968, ***NM_010473***)
TGGGCCACCTC	4	6	6	**239** ↑	Parvalbumin (Mm.2766, ***AK013561***)
TTTTTCCTGAT	3	3	4	**42** ↑	Ryanodine receptor 1, skeletal muscle (Mm.226037, ***NM_009109***)
GGATTCAGAGA	1	5	1	**51** ↑	Sarcalumenin (Mm.35811, ***NM_175347***)
GGGATTTAAAA	1	0	2	**29** ↑	Triadin (Mm.338508, ***XM_483889***)
GGTGGGCAGGG	1	0	2	**20** ↑	Voltage-dependent L-type calcium channel alpha-1S subunit (Mm.337571, ***L06234***)

Abbreviations: OVX, ovariectomy; DHT, dihydrotestosterone.

Numbers indicate the count of serial analysis of gene expression (SAGE) tags per 100000.

SAGE tag numbers in bold indicate a statistical significance ($P \leq 0.05$; OVX vs. Intact, DHT vs. OVX).

Table 4. Female mouse DHT-modulated transcripts involved in cell structure/motility.

Tag	Intact	OVX	OVX+DHT 3h	OVX+DHT 24h	Description (UniGene cluster, *GenBank accession no.*)
AAGATCAAGAT	89	160	77	**678** ↑	Actin (Mm.360115, ***AK014303***; Mm.292865, ***M26689***; Mm.297, ***AK088691***; Mm.214950, ***M12866***; Mm.686, ***BY760441***), EST (Mm.213025; ***AA710012***)
ACTAGAGAGAC	1	2	0	**20** ↑	Actinin alpha 2 (Mm.37638, ***AY036877***)
CTTCTGAATAA	6	18	11	**254** ↑	Actinin alpha 3 (Mm.5316, ***NM_013456***)
CCAGCCAGCGT	1	3	4	**124** ↑	Ankyrin repeat domain 23 (Mm.41421, ***BC022973***)
CCCCTTTGGAG	0	0	0	**12** ↑	EST Ankyrin repeat domain 23 (Mm.41421, ***BB138080***)
TTCAGGGCGGG	3	6	3	**33** ↑	Bridging integrator 1(Mm.4383; ***NM_009668***)
GCCCCTCTCTT	11	10	4	**86** ↑	Desmin (Mm.6712, ***BC031760***)
CTCCTGGACAC	257	185	88	**40** ↓	Gelsolin (Mm.21109, ***AK076156***)
GTGCGATGCTG	1	1	2	**17** ↑	Kinesin family member 1C (Mm.99996, ***BC016221***)
TGCCTGTAGGC	3	3	3	**59** ↑	Myomesin 1 (Mm.4103; ***NM_010867***)

GTGATGCTAAG	29	51	71	**1823** ↑	Myosin light chain, phosphorylatable, fast skeletal muscle (Mm.14526, ***NM_016754***)
TGGGCAGCCTT	1	0	1	**18** ↑	EST Myosin light chain, phosphorylatable, fast skeletal muscle (Mm.14526, ***BY453372***)
AGAGAAGAGTG	9	8	5	**52** ↑	Myosin, heavy polypeptide 1, skeletal muscle (Mm.340132, ***AJ293626***)
CGCCTGTGTGA	5	6	1	**45** ↑	Myosin, heavy polypeptide 2, skeletal muscle (Mm.34425, ***AJ002521***)
GAGCAGACCGT	37	55	21	**751** ↑	Myosin, heavy polypeptide 4, skeletal muscle (Mm.297382, ***AJ278733***)
CCTACAGTTGA	29	32	46	**1270** ↑	Myosin, light polypeptide 1 (Mm.1000; ***AK003182***) EST (Mm.342003, ***CB588154***)
GTGGTAAGCTG	1	0	1	**21** ↑	EST Myosin, light polypeptide 1 (Mm.1000, ***AV290771***)
GACCAGAACAG	1	0	2	**14** ↑	Myosin, light polypeptide 2, regulatory, cardiac, slow (Mm.1529, ***NM_010861***)
TCTGGAGCTTC	0	1	0	**39** ↑	Myosin, light polypeptide 3 (Mm.7353, ***NM_010859***)
AGACTACCCCA	0	2	0	**23** ↑	Myosin, light polypeptide kinase 2, skeletal muscle (Mm.250604, ***BC019408***)

TCCTAGCAGAT	1	0	1	**12** ↑	EST Myosin XVIIIb (Mm.332583, ***AK077135***)
TGCATCATTTC	3	3	4	**87** ↑	Myozenin 1 (Mm.439911; ***NM_021508***)
CCTCTGTGGCT	1	3	2	**24** ↑	Nebulin-related anchoring protein (Mm.6384, ***NM_008733***)
TAAAAATTTAC	0	2	1	**28** ↑	EST Nebulin (Mm.246828, ***CR759458***)
GCTGTAGGGTG	1	1	1	**21** ↑	Obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF (Mm.38029, ***BC044882***)
ATGTGCAGACT	2	3	2	**80** ↑	Phosphodiesterase 4D interacting protein (myomegalin) (Mm.129840, ***AJ290946***)
GAGAGTTACAG	0	1	1	**19** ↑	Ring finger protein 28 (Mm.331961, ***AJ278734***)
GCTTTGGATGG	1	1	0	**25** ↑	Ring finger protein 30 (Mm.143796, ***NM_021447***)
ACCTTGTAGCT	1	0	0	**12** ↑	Sarcoglycan, alpha (dystrophin-associated glycoprotein) (Mm.18709, ***AK075915***)
TGAGTTAGTGG	2	0	1	**27** ↑	EST Synaptopodin 2 (Mm.317009, ***AK004418***)
CTTGCTGTTTG	0	0	0	**12** ↑	EST Synaptopodin 2-like (Mm.35789, ***AK084541***)
CCAGTATATGT	4	5	4	**30** ↑	Titin (Mm.46242, ***AK003152***)
GATAGCTTGGG	2	5	3	**120** ↑	Titin (Mm.46242, ***BC025840***)

ACCCGGAACAA	1	1	1	**25** ↑	Tropomodulin 4 (Mm.71935, ***BC068020***)
AAAGTCATTGA	33	49	39	**534** ↑	Tropomyosin 1, alpha (Mm.121878, ***AK077713***)
GACGCCATCAA	0	1	0	**18** ↑	EST Tropomyosin 1, alpha (Mm.121878, ***AW229930***)
CACTGACCTCC	17	13	17	**86** ↑	Tropomyosin 2, beta (Mm.646, ***AK003186***)
TGACAGAAGAG	24	34	25	**902** ↑	Troponin C2, fast (Mm.1716, ***NM_009394***)
GAGGGCCGGAA	22	37	38	**1049** ↑	Troponin I, skeletal, fast 2 (Mm.39469, ***NM_009405***)
ACCTGCTGTGT	0	4	3	**29** ↑	Troponin T1, skeletal, slow (Mm.358643; ***AF020946***) EST (Mm.361853; ***BG795511***)
GGTGCCAACTA	21	42	12	**413** ↑	Troponin T3, skeletal, fast (Mm.389992, ***NM_011620***)
ACTGTCCGGGC	10	5	13	**527** ↑	Troponin T3, skeletal, fast, isoform FB1e16 (Mm.350054, ***L48990***)
CTGAGCAACAC	2	1	0	**16** ↑	Tubulin, alpha 4 (Mm.1155, ***M13444***)
TGTGTCAACCT	2	4	**23** ↑	5	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide (Mm.332314; ***NM_011738***) EST (Mm.354610; ***BB833023***)
Extracellular matrix					
TGTTCATCTTG	110	**325** ↑	261	155	Collagen, type III, alpha 1 (Mm.249555; ***NM_009930***)

AGAATGAGATC	45	48	**5 ↓**	**7 ↓**	Decorin (Mm.56769, ***X53929***)
CCAACGCTTTA	17	**59 ↑**	69	44	Fibronectin 1 (Mm.193099; ***BC004724***)
GGACGCCCAAG	8	20	**2 ↓**	**2 ↓**	Matrix metalloproteinase 14 (membrane-inserted) (Mm.280175, ***NM_008608***)
CAATGTGGGTT	14	**57 ↑**	47	30	Periostin, osteoblast specific factor (Mm.236067; ***BC031449***)
CGCCTGCTAGC	42	**155 ↑**	93	60	Procollagen, type I, alpha 2 (Mm.277792; ***BC042503***)
GTTCCAAAGAA	10	**52 ↑**	77	58	Procollagen, type I, alpha 2 (RIKEN full-length enriched library,clone:1200014H15)(Mm. 277792; ***AK075707***)
TCTTCTATGCA	7	**40 ↑**	44	50	EST Procollagen, type I, alpha 2 (Mm.277792; ***CB575147***)

Abbreviations: OVX, ovariectomy; DHT, dihydrotestosterone.

Numbers indicate the count of serial analysis of gene expression (SAGE) tags per 100000.

SAGE tag numbers in bold indicate a statistical significance ($P \leq 0.05$; OVX vs. Intact, DHT vs. OVX).

Table 5. Other transcripts differentially expressed following DHT treatment in female mice.

Tag	Intact	OVX	OVX+DHT		Description (UniGene cluster, *GenBank accession no.*)
			3h	24h	
Cell Signaling					
TAATGGATAAG	7	5	1	**25** ↑	Cyclin G1 (Mm.2103, ***BC005534***)
AGCCTCTGTAG	17	18	**1** ↓	5	EST Insulin-like growth factor binding protein 7 (Mm.233470, ***BE850065***)
TAAAAAGAAAG	8	8	5	**53** ↑	N-myc downstream regulated gene 2 (Ndrg2) (Mm.26722; ***NM_013864***)
CTGGATATTTC	2	1	0	**12** ↑	EST N-myc downstream regulated gene 2 (Mm.26722, ***BY614809***)
CTGGAGGCTCT	9	19	**1** ↓	**1** ↓	Protein kinase C, delta binding protein (Mm.3124, ***NM_028444***)
CCCAGCGCTTG	0	0	0	**11** ↑	EST Sodium channel, type IV, beta polypeptide (Mm.335112, ***BC050045***)
Gene-Protein Expression and Post-Transcriptional Processes					
ACCGTGGGCCA	0	2	0	**20** ↑	ADP-ribosyltransferase 1 (Mm.261071;

					AK003107) EST (Mm.261071; ***W41430***)
ATCCTGGTAAT	3	3	2	**21** ↑	Carboxypeptidase E (Mm.31395, ***NM_013494***)
CCCCAGGAGAG	4	3	4	**32** ↑	Chaperone, ABC1 activity of bc1 complex like (S. pombe) (Mm.38330, ***NM_023341***)
CAAATGAAGTG	1	5	19	**29** ↑	Cold shock domain containing E1, RNA binding (Mm.277713; ***BC062097***)
ATTTAAGAAGA	6	6	9	**34** ↑	Cold shock domain protein A (Mm.299604, ***AF248546***)
GCTTCATCTCC	9	3	4	**25** ↑	Crystallin, alpha B (Mm.178, ***NM_009964***)
CTCACAAGGGG	1	1	**13** ↑	6	EST Ewing sarcoma breakpoint region 1 (Mm.142822, ***AA915168***)
ATGGCCAGGGC	4	2	4	**32** ↑	EST Reproductive homeobox on X chromosome, 9 (Mm.156958, ***BM251052***)
GCTGCCCTCCG	5	5	10	**25** ↑	EST Ribosomal protein L32 (Mm.104368, ***CA467963***)

TCTGCACCTCC	3	1	3	**51** ↑	Eukaryotic translation elongation factor 1 alpha 2 (Mm.2645, ***NM_007906***)
GATTCCGTGAG	39	54	125	**144** ↑	Ribosomal protein L37 (Mm.10474; ***AK012544***) EST (Mm.361657; ***BU530511***)
TAAGATGTCTG	2	1	2	**57** ↑	Ribosomal protein L3-like (Mm.46198, ***NM_025425***)
TCTGTGCACCT	13	10	34	**40** ↑	Ribosomal protein S11 (Mm.196538; ***NM_013725***) ESTs (Mm.338250, ***AV153457***; Mm.4985, ***AV061118***; Mm.316362, ***U93864***; Mm.347690, ***AA269463***; Mm.325693, ***BY398653***)
GCAGAGTGCGC	35	51	**14** ↓	25	Ribosomal protein S6 (Mm.325584, ***AK028205***; Mm.361402, ***BY761808***)
TTCAGCTCGAG	13	22	**3** ↓	10	Ribosomal protein S7 (Mm.371579; ***NM_011300***)
GCCAGAAGGAT	1	0	1	**15** ↑	SET and MYND domain containing 1 (Mm.234274, ***BC076601***)

AGTTCACAGAA	1	1	4	**15** ↑	SET and MYND domain containing 2 (Mm.156895; ***NM_026796***) EST (Mm.18810; **BB190792**)
ACCACTTTTGT	1	1	0	**33** ↑	Transcription elongation factor A, 3 (Mm.112, ***AJ223472***)
Cell defence					
TGGAGATAAGC	21	19	18	**2** ↓	Acid phosphatase 5, tartrate resistant (Mm.46354, ***NM_007388***)
AGATCTGCCCC	13	3	8	**25** ↑	ATPase, H+ transporting, V1 subunit G isoform 1 (Mm.371615, ***AK010131***; EST Mm.29868, ***AA051794***)
CTAGTGGTGGT	11	12	**0** ↓	**0** ↓	CD1d1 antigen (Mm.1894, ***BC055902***)
ACCCTAGTATG	7	14	1	**0** ↓	Complement component 1, q subcomponent, alpha polypeptide (Mm.439957, ***AK002655***)
ACATCCTGAGG	63	31	18	**5** ↓	EST Adipsin (Mm.4407, ***AA450423***)
GCGAGCACACT	5	12	**0** ↓	**0** ↓	EST Lysozyme (Mm.45436, ***BI695557***)

GAAGCCAACCC	23	21	**3** ↓	**2** ↓	EST Selenoprotein P, plasma, 1 (Mm.392203, ***BF533736***)
CAGTGCAACTC	7	**36** ↑	27	**9** ↓	EST Selenoprotein P, plasma, 1 (Mm.392203; ***BB771510***)
GATTGAGAATA	1	0	**13** ↑	2	Histocompatibility 2, D region locus 1 (Mm.33263, ***X00246***)
TTGATCCCCAT	25	31	**0** ↓	**2** ↓	Lysosomal-associated membrane glycoprotein 1 (Mm.16716; ***AY069968***)
TAACTGACAAT	4	6	**31** ↑	17	Metallothionein 2 (Mm.147226, ***NM_008630***)
AATTAAAAACA	63	43	**125** ↑	46	Microsomal glutathione S-transferase 1 (Mm.14796, ***BC009155***)
ATTGGGGGAGG	9	11	**57** ↑	25	Osteoclast inhibitory lectin (Mm.197536, ***AY320031***)
CAGGAGGAGTT	16	20	5	**2** ↓	Protein disulfide isomerase associated 3 (Mm.263177, ***NM_007952***)
GGTCGTGTATA	20	24	12	**4** ↓	Serine (or cysteine) proteinase inhibitor, clade G, member 1 (Mm.38888, ***BC002026***)

Abbreviations: OVX, ovariectomy; DHT, dihydrotestosterone.

Numbers indicate the count of serial analysis of gene expression (SAGE) tags per 100000.

SAGE tag numbers in bold indicate a statistical significance ($P \leq 0.05$; OVX vs. Intact, DHT vs. OVX).

Table 6. The ten most expressed genes in female mouse intact adipose tissue (AT) and AT 24h after DHT administration (DHT24h), compared with the ten most expressed genes in intact skeletal muscle (SM).

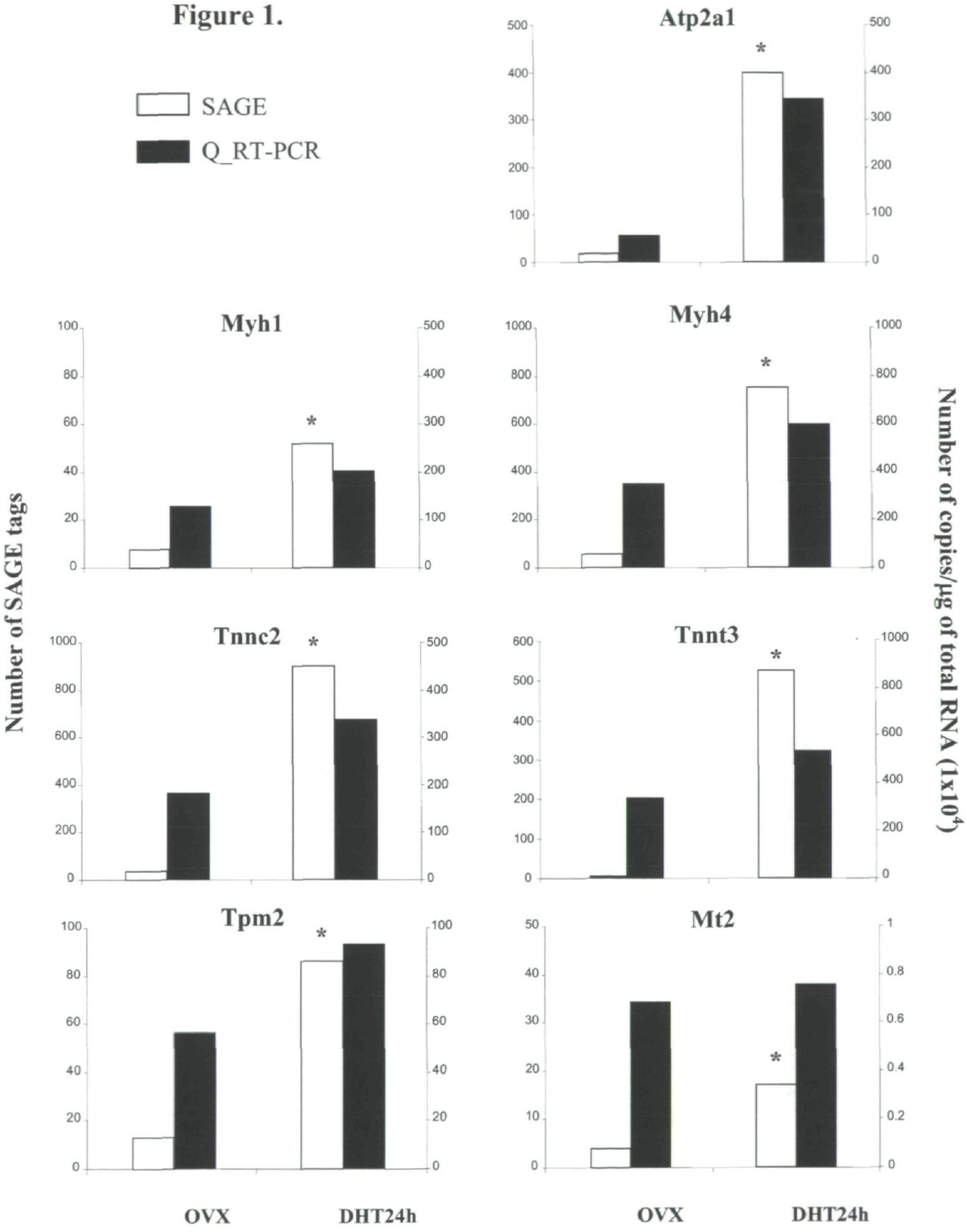
		Rank		
		AT		SM
SAGE tag	**Gene name**	**Intact**	**DHT24h**	**Intact**
AGCCAAAGGAA	Fatty acid binding protein 4	**1**	**5**	>100
AGGACAAATAT	Cytochrome b	**2**	26	>100
ATGCAGGGCCA	Fatty acid synthase	**3**	32	
CTCCTGGACAC	Gelsolin	**4**	97	>100
AGCAGTCCCCT	Cytochrome c oxidase subunit II	**5**	**7**	>100
CCTACTAACCA	Aldolase 1, A isoform	**6**	**9**	**7**
TTGTCAGGTAG	Malic enzyme 1	**7**	48	
TGTTCATCTTG	Collagen, type III, alpha 1	**8**	23	
AGGAGGACTTA	NADH dehydrogenase subunit 2	**9**	27	38
ACAAGTCTCTG	EST Lipoprotein lipase	**10**	>100	
GTGATGCTAAG	Myosin light chain, phosphorylatable, fast skeletal muscle	47	**1**	**1**
CCTACAGTTGA	Myosin, light polypeptide 1	46	**2**	**2**
GAGGGCCGGAA	Troponin I, skeletal, fast 2	83	**3**	**3**
TGACAGAAGAG	Troponin C2, fast	75	**4**	**4**
GAGCAGACCGT	Myosin, heavy polypeptide 4, skeletal muscle	35	**6**	**6**
AAGATCAAGAT	Actin	24	**8**	**5**
AAAGTCATTGA	Tropomyosin 1, alpha	40	**10**	**8**
ACTGTCCGGGC	Troponin T3 skeletal fast	>100	12	**9**
GGTGCCAACTA	Troponin T3 skeletal fast	84	13	**10**

FIGURE LEGEND

Figure 1 Confirmation of the serial analysis of gene expression (SAGE) data by the quantitative real-time PCR (Q_RT-PCR).

Asterisk represents a significant difference compared to the OVX group ($P \leq 0.05$). Abbreviations: *Atp2a1*, ATPase Ca^{++} transporting; *Myh1*, myosin heavy polypeptide 1, skeletal muscle; *Myh4*, myosin heavy polypetide 4, skeletal muscle; *Tnnc2*, troponin C, skeletal fast; *Tnnt3*, troponin T3, skeletal fast; *Tpm2*, tropomyosin 2, beta; *Mt2*, metallothionein 2.

Figure 1.


SAGE
Q_RT-PCR
Atp2a1
Myh1
Myh4
Tnnc2
Tnnt3
Tpm2
Mt2
*
Number of SAGE tags
Number of copies/μg of total RNA (1x10⁴)
OVX
DHT24h
OVX
DHT24h

CHAPTER 3

Feeding induced changes in the hypothalamic transcriptome

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Résumé

Contexte: L'obésité est un désordre complexe et multifactoriel qui nécessite une approche globale pour la prévention et le traitement. La présente étude a pour objectif d'identifier de nouveaux signaux hypothalamiques rapidement régulés par la consommation d'un repas riche (HF) ou faible en gras (LF), et qui peuvent influencer le bilan énergétique.

Méthodes: Après un jeûne de 12 heures, un groupe de souris a été sacrifié; les autres groupes ont consommé un repas HF ou LF *ad libitum* et ont été sacrifiés 3h après le début du repas. L'hypothalamus a été prélevé pour les procédures d'analyse sérielle de l'expression génique (SAGE).

Résultats: À partir des trois groupes expérimentaux de souris, nous avons isolé à l'aide de la SAGE environ 254588 tags, qui correspondent à 65548 espèces de transcrits. Les données montrent 12 transcrits régulés par la prise alimentaire. Parmi ces gènes, deux transcrits ont des fonctions mitochondriales (MtCo1, Ppid), trois sont impliqués dans le transport et la régulation des protéines (Ube2q2, Mup1, Sec13), un transcrit dans le contrôle du pH cellulaire (Slc4a3) et un autre dans le contrôle épigénétique de l'expression génique (Setd3). En plus, cinq nouveaux transcrits potentiels étaient modulés de façon différentielle.

Conclusion: La présente étude a identifié des gènes qui peuvent réguler les circuits hypothalamiques responsables des réponses précoces à la prise alimentaire. En plus, le repas HF a spécifiquement modulé l'expression de trois de ces gènes.

Feeding induced changes in the hypothalamic transcriptome

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Keywords: high-fat diet, hypothalamus, SAGE, mRNA regulation.

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ABSTRACT

Background: Obesity is a complex multifactorial disorder, which needs a comprehensive approach for prevention and treatment. The present study investigated the modifications in the hypothalamic gene expression induced by high fat (HF) and low fat (LF) meal ingestion in mice, in order to identify the signals rapidly mediating the hypothalamic control on energy intake.

Methods: After fasting, one group of mice was sacrificed and the others were fed *ad libitum* with HF or LF diet, and sacrificed 3 hours after the beginning of the meal. The hypothalamus was sampled and the serial analysis of gene expression method was performed.

Results: Approximately 254588 tags, which correspond to 65548 tag species were isolated from the three groups. The data showed twelve transcripts regulated by food intake. Among these, two transcripts have mitochondrial functions (MtCo1, Ppid), three are involved in protein transport and regulation (Ube2q2, Mup1, Sec13), one in cellular pH control (Slc4a3) and another one has a role in the epigenetic control of gene expression (Setd3). In addition, 5 potentially novel transcripts were differentially modulated.

Conclusion: The present study has identified genes that may regulate hypothalamic circuits governing the early response to food intake. Three genes were specifically modulated by high fat intake.

Introduction

Despite the recent advances in the understanding of the physiology of body weight regulation, the prevalence of obesity is increasing worldwide and is associated with type 2-diabetes, hypertension, dyslipidemia, as well as coronary artery diseases, stroke and certain cancers, causing a growing health concern in Western countries [1]. Obesity is a complex and multifactorial disorder that stems from a variety of dietary, lifestyle and genetic factors [2]. It is well-accepted that one important environmental factor predisposing to obesity is the amount of fat in the diet, worsened by decreased physical activity and energy expenditure and by the blunted satiety effect of fats. Indeed, several studies have reported that obese animals have an impairment of acute satiety signals accompanied by preference for high fat (HF) meals [1-3]. Noteworthy, energy homeostasis and body weight regulation are accomplished by a highly integrated, multidimensional and redundant neuro-humoral system, with overlapping pathways that prevent the effect of short-term fluctuations of energy balance on fat mass [2, 4]. The hypothalamus is the primary brain centre to which all these pathways converge and in which they are integrated in order to regulate feeding behaviour and energy intake, thus controlling short-term and long-term energy balance and body weight steady-state [5]. A very small imbalance in energy intake over a long period of time will have a large cumulative effect on weight gain. The resulting obesity overrides the energy regulatory system, so that the interactions between environmenntal factors, such as high-fat intake, and the homeostatic control of the elevated body weight become deleterious [6]. Therefore, it appears critical to clarify the mechanisms underlying appetite and satiety signals in the context of acute meal ingestion, with special attention to high fat meal ingestion. The hypothalamus becomes naturally one of the targets of such investigation, as

it is not only the primary site of convergence and integration for redundant energy status signals, but also the node within which the direction and magnitude of efferent responses are determined.

With the aim of identifying the rapid molecular mechanisms that impair energy homeostasis control consequently to a HF diet, we used the serial analysis of gene expression (SAGE) strategy to perform a transcriptomic analysis of hypothalamus in mice at 3 hours after low fat (LF3h) or high fat (HF3h) meal ingestion. Our objective was to identify differentially expressed transcripts significantly modulated by HF feeding, thus pointing out which are the mechanisms rapidly affecting hypothalamic circuits in response to fat intake. We used the SAGE method which is accepted to be a powerful strategy to analyze quantitatively, simultaneously and differentially the expression of all mRNAs of a tissue, while giving the advantageous opportunity of identifying still uncharacterized and novel transcripts [7, 8]. We had previously used this analytical method to study the transcriptome of the skeletal muscle [8, 9], adipose tissue [10], duodenum [11] and hypothalamus [12-14] in response to various stimuli, and had demonstrated that it represents a powerful and highly reproducible technology for transcriptomic studies [15]. Here, we present the results of the transcriptomic analysis of the hypothalamus in HF3h, LF3h and fasting mice.

Materials and Methods

Animals, diet and sample preparation

A total of 60 C57BL6 male mice (12 wk-old) were purchased from Charles River Canada Inc. and were acclimated for two weeks. They were single-housed at a temperature of 22 ± 3°C, with a 12 hours-light cycle (7:15 am lights on). LF diet (Research Diets # D12450B: 10% calories from fat, 70% from carbohydrate, and 20% from protein; 2.5% saturated fatty acids, 3.5% monounsaturated fatty acids, 4% polyunsaturated fatty acids, 0 trans fatty acids, 3.85 kcal/g) and tap water were served ad libitum. The last day of the acclimatization, the body weight paired mice were randomly distributed into three groups and fasted for 12 hours during darkness of the light cycle. On the experimental day, one group of fasted mice was sacrificed before the meal. The other mice were fed ad libitum with HF meal enriched with lard (Research Diets # D12492: 60% calories from fat, 20% from carbohydrate, and 20% from protein; 22% saturated fatty acids, 28% monounsaturated fatty acids, 10% polyunsaturated fatty acids, 0 trans fatty acids, 5.24 kcal/g) or LF meal (Research Diets # D12450B), and sacrificed 3 hours after the beginning of the meal. The amount of macronutrients and energy ingested was recorded. The mice of all the three groups (fasting, HF3h, LF3h) were alternatively sacrificed between 08:30 and 11:30 AM, by cervical dislocation under isoflurane anesthesia. The brain was removed from the skull, and the hypothalamus was immediately dissected, following the boundaries described by Paxinos and Franklin [16]. The whole hypothalamus was taken, using the optic chiasm as the rostal limit and the mammillary bodies as caudal reference. The hypothalamus was immediately frozen in liquid nitrogen, pooled together for each group and stored at -80°C until RNA extraction. All animal experimentation was conducted in accord with the

requirements of the Canadian Council on Animal Care and approved by the animal protection Committee of University Laval.

Transcriptome analysis

The three SAGE libraries were constructed as previously described [7, 8]. Total RNA was isolated from pooled hypothalamus for each group (n = 20) by Trizol (Invitrogen Canada Inc., Burlington, ON). The quality of total RNA was monitored by micro-capillary electrophoresis (Bioanalizer 2100, Agilent Technologies, Mississauga, ON). Polyadenylated RNA was extracted with the Oligotex mRNA Mini Kit (Qiagen Inc., Mississauga, ON), annealed with the biotin-5'-T_{18}-3' primer and converted to cDNA using the cDNA synthesis kit (Invitrogen Canada Inc.). The resulting cDNAs were digested with *NlaIII* (New England BioLabs Ltd., Pickering, ON), and the 3' restriction fragments were captured using streptavidin-coated magnetic beads (Dynal Biotech LLC, Brown Deer, WI) and separated into two populations. Each population was ligated to one of two annealed linkers and extensively washed to remove unligated linkers. The tag beside the most 3' *NlaIII* restriction site (CATG) of each transcript was digested with *BsmFI* (New England BioLabs Ltd.), thereby releasing cDNA fragments including the short 15-bp tags. The blunting kit from Takara Bio Inc. (Otsu, Japan) was used for the blunting and ligation of the two tag populations. The resulting ligation products containing the ditags were amplified by PCR and digested with *NlaIII.* The band containing the ditags was extracted from the 12% polyacrylamide gel with Spin-X microcentrifuge tube (Fisher, Pittsburgh, PA) and the purified ditags were self-ligated to form concatemers using T4 ligase (Invitrogen Canada Inc.). The concatemers ranging from 500 bp to 1800 bp were isolated by agarose gel and extracted with Gene-Clean Spin (Qbiogene, Montreal, QC). The

resulting DNA fragments were ligated into the *SphI* site of pUC19 and cloned into OmniMAX 2T1 competent cells (Invitrogen Canada Inc.). White colonies were picked up, and concatemer inserts were sequenced by the Applied Biosystems 3730 (Foster City, CA).

Bioinformatic analysis

Sequence files were analyzed using the SAGEana program, a modification of SAGEparser [15]. In brief, SAGE tags corresponding to linker sequences were discarded and replicate concatemers were counted only once. Identification of the transcripts was obtained by matching the 15 bp (sequence at the last CATG + 11bp tags) with SAGEmap, UniGene and GenBank databases on 4th October 2007. Classification of the transcripts was based upon the updated information of the genome directory [17] found at the TIGR web site (http://www.tigr.org/), the SOURCE (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch) and the OMIM (http://www.ncbi.nlm.nih.gov/), as well as upon previously published literature. We have previously shown that the SAGE method is very reproducible with $r^2 = 0.96$ between two SAGE libraries generated from two cDNA libraries constructed from the same total RNA pool [15].

Validation by quantitative real-time PCR (Q_RT-PCR)

First strand cDNA was synthesized using 5 μg of pooled RNA of each experimental group in a reaction containing 200 U of Superscript III Rnase H-RT (Invitrogen Canada Inc.), 300 ng of oligo-dT_{18}, 500 mM deoxynucleotides triphosphate, 5 mM dithiothreitol and 40 U of human RNase inhibitor (Amersham Pharmacia, Piscataway, NJ) in a final volume of 50 μl. The cDNA corresponding to 20 ng of total RNA was used to perform

fluorescent-based real-time PCR quantification using tha LightCycler real-time PCR apparatus (Roche Inc., Nutley, NJ). Reading of fluorescence signal was taken at the end of the heating to avoid non-specific signal. A melting curve was performed to assess non-specific signals. Oligoprimer pairs that allow the amplification of approximately 250 bp were designed by GeneTools software (Biotools Inc., Edmonton, AB) and their specificity was verified by blast in GenBank database. Gene name, GenBank accession numbers and regions used for the primer pairs were the following: solute carrier family 4 member 2 (Slc4a3), NM_009208, 3868-4113; peptidylprolyl isomerase D (Ppid), NM_026352, 445-590; ubiquitin-conjugating enzyme E2Q 2 (Ube2q2), NM_180600, 1975-2094; major urinary-protein 1 (Mup1), NM_031188, 127-405; mitochondrial cytochrome-c oxidase 1 (Mtco1), NC_005089, 1189-1347. The expression levels of mRNA (number of copies/μg total RNA) were calculated using a standard curve of crossing point (Cp) versus logarithm of the quantity. The standard curve was established using known cDNA amounts of 0, 10^2, 10^3, 10^4, 10^5 and 10^6 copies of hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1) and the LightCycler 3.5 program provided by the manufacturer (Roche Inc.). The Q_RT-PCR was performed in triplicates. Data were expressed as mRNA ratio to Hprt1 control.

Statistical analysis

Data of body weight, food intake and Q_RT-PCR were analyzed by the one-way ANOVA with the Tukey-Kramer test. Food and macronutrient intakes were analyzed by the Student's t-test. The significant difference was set at $P \leq 0.05$. For the SAGE data, the comparative count display (CCD) test was used to identify the transcripts which were significantly differentially expressed ($P \leq 0.05$) between the groups with more than a two-fold change [18]. The data were normalized to 100 000 tags for presentation.

Results

Food intake

The amounts of ingested food and macronutrients in each group are presented in Table 1. As expected, energy, protein and fat intake were higher in HF3h than in LF3h group, whereas carbohydrate intake was lower in HF3h group.

Transcriptomic changes in the hypothalamus after HF and LF meal ingestion

Three SAGE libraries were generated in order to identify the hypothalamic mRNAs differentially modulated after fasting, LF3h and HF3h. In total, we isolated 254588 SAGE tags, corresponding to globally 65548 tag species. Among these, only twelve transcripts were regulated by the LF and HF feeding (Table 2). Two of these transcripts have a role in mitochondrial functions, three are involved in the protein transport and regulation, one is involved in the cellular pH control and another one in the epigenetic regulation of gene expression. In addition, five novel transcripts were found to be differentially expressed in the LF and HF groups.

Three hours after meal initiation, the hypothalamus of HF group showed up-regulations of MtCo1 and of SEC 13 homolog (Sec13), whereas the Ube2q2 level was reduced compared to fasting mice. Furthermore, the solute carrier family 4 member 3 transcript (Slc4a3) showed higher expression in the HF mice when compared to the LF mice, while no difference was found between the fasting and HF groups. In addition, the current study revealed a subtle modulation induced by the LF meal on the hypothalamic mRNA levels. Indeed, MtCo1, Sec13, peptidylprolyl isomerase D (Ppid) and Mup 1/3 were

induced by the LF meal whereas Ube2q2 and SET domain containing 3 (Setd3) were down-regulated.

Confirmation of changes in gene expression by quantitative real-time PCR

We also performed Q_RT-PCR procedures in order to assess the expression levels of five genes identified as diet responsive by SAGE method and which may be involved in the regulation of feeding behaviour. MtCo1 and Ppid have mitochondrial functions, Slc4a3 is involved in pH regulation, whereas Ube2q2 and Mup1 have a role in protein metabolism and transport. Globally, Q_RT-PCR results were in good agreement with the SAGE analysis (Figure 1).

Discussion

Mitochondrial functions and feeding behaviour

The present study is consistent with previous reports on the hypothalamic transcriptome, in which several mitochondrial transcripts with distinct functions were listed among the most expressed and modulated mRNAs in various conditions [12, 19]. Indeed, neurons need high energy levels to maintain their functions and therefore possess an elevated density of mitochondria. Noteworthy, mitochondrial oxidative phosphorylation in the hypothalamus and consequent ATP status play a critical role in feeding behaviour control, constituting a main switch to regulate neuropeptide expression and food intake [20].

In the present study, at 3 hours after meal initiation, the expression of the mitochondrial transcript MtCo1 was highly induced both in the LF and HF mice groups, by 7- and 9-fold respectively. The corresponding protein is one terminal component of the mitochondrial electron transport chain, thus directly acting in the oxidative phosphorylation process. Interestingly, our previous work on the hypothalamic transcriptome of adrenalectomized (ADX) mice has shown a strong suppression of MtCo1 mRNA expression [14], whereas our study on the 10 most abundant hypothalamic transcripts has showed MtCo2 as the second most expressed transcript in this brain region [13]. These findings confirm the biological relevance of cytochrome c oxidase complex, and suggest that its prominent role in the hypothalamic control of energy balance merits further attention.

As key sensors of the cell energy status, mitochondria are not only able to switch a variety of pathways in case of energy scarceness, but also to finally trigger cell death

signals when the amount of damage is unsustainable for cell vital functions [21]. Our data showed that LF meal, significantly induced the expression of peptidylprolyl isomerase D (Cyclophilin D). This encodes the mitochondrial isoform of the peptidylprolyl cis-trans isomerases family, being an essential component of the permeability transition pore (PTP). The opening of PTP in response to low ATP levels and oxidative stress causes an abrupt increase of mitochondrial permeability to solutes, thus influencing neuronal susceptibility to cell death [22]. It would be, therefore, interesting to further investigate the role of this system in energy intake, and specifically to illuminate whether it may be related to the higher carbohydrate intake.

Transcripts involved in protein metabolism and feeding behaviour

In the hypothalamus of HF fed mice, the expression of Ube2q2 was reduced compared to the higher levels displayed in fasting mice, and a similar trend was found in LF fed mice ($P = 0.08$). The corresponding protein is a high molecular mass member of the E2 ubiquitin-conjugating enzyme superfamily, known to be responsible for the intermediate phase of the ubiquitination pathway. This pathway is fundamental in the regulation of protein stability and turnover, and has been implicated in potentially every aspect of cellular regulation [23]. Moreover, it is interesting to note that the ubiquitin-proteasome system, as well as the counteracting deubiquitinating enzymes play an important role in the generation of the circadian rhythm in mice [24]. Since the main regulator centre of circadian rhythm in mammals is the hypothalamic suprachiasmatic nucleus (SCN) [25] and the timing mechanism plays a relevant role in the generation of hunger sensation [26], it is plausible that changes in the hypothalamic levels of ubiquitin-system enzymes may affect feeding behaviour. Notably, the SCN and its projections to other hypothalamic nuclei (such

as the ventromedial and dorsomedial nuclei) have been indicated as main responsible for the circadian oscillations of orexigenic/anorexigenic signals [27, 28]. The dysregulation/loss of these mechanisms may lead to hyperphagia and obesity, as evidenced in rodents. [29]. Taken together, these insights contribute to focus our attention on the circadian regulation of feeding behaviour as another fundamental aspect that should be clarified in the attempt to identify good therapeutic targets for eating disorders.

The hypothalamus of LF fed mice has displayed an increase of Mup1/3 gene expression compared to fasting mice. To our knowledge, however, this is the first time that a member of MUP family is revealed to be expressed in this tissue and, generally, in the brain. MUP1 is a member of the rodent MUP multi-gene family coding for pheromone-binding proteins that function as carriers for volatile effectors of mouse physiology and behaviour [30]. As known so far, these proteins are mainly secreted by liver and filtered by the kidney into the urine, where the odorants bound are released in the air giving a long-lasting olfactory trace [31]. In addition, they have also been found to be excreted by a number of exocrine glands [32]. The presence of this transcript in the hypothalamus is actually unexpected. However, our previous study on the Glucocorticoid (GC)-regulated hypothalamic transcriptome [14] already detected the expression of a pheromone receptor, namely the vomeronasal 1 receptor D4, previously found only in the mice vomeronasal organ, whose sensory neurons receiving pheromones innervate to the accessory olfactory bulb [33]. This transcript was down-regulated in the hypothalamus of ADX mice and we suggested it may have nonpheromonal functions involved in the hypothalamic regulation of instinct behaviour and homeostasis [14]. The role of the olfactory bulb in the integration and modulation of feeding signals [34] and the evidence that MUPs levels are controlled by

energy homeostasis-regulating hormones, such as growth hormone and thyroxine [35], open a new field of interest for MUP-mediated pathways.

Other differentially expressed transcripts

We found that Sec13 mRNA is up-regulated by LF and HF feeding compared to fasting, whereas LF feeding represses Setd3 expressions. The protein coded by Sec 13, which is a structural component of the coat protein II (COPII) coated vesicles, is responsible for the protein transport from the endoplasmic reticulum to the Golgi apparatus [36]. Setd3 product performs the methylation of histone 3 on lysine 9 modification which notably characterizes silenced heterochromatin [37]. In addition, we have found that the Slc4a3 transcript was significantly up-regulated in HF mice when compared to LF group. The correspondent protein is an anion exchanger, precisely an acid loader that exchanges extracellular chloride for intracellular bicarbonate, thus contributing to the intracellular pH regulation [38]. The roles of these modulated transcripts in feeding behaviour are presently unknown. However, it is not unexpected to observe hypothalamic modulation of ion channels expression in response to a large variety of stimuli. Moreover, our previous study on the GC-modulated hypothalamic transcriptome in ADX mice have reported the suppression of the Slc4a3 by GC treatment [14], which is known to influence feeding behaviour [39]. Therefore, we suggest that the specific function of these transcripts within the hypothalamic circuits regulating feeding behaviour would merit further investigation.

Potentially novel transcripts differentially expressed

The power of SAGE method compared to other expression-profiling techniques is the possibility to identify unknown/uncharacterized transcripts among the sequenced tags.

This opportunity may be remarkable in the attempt of elucidating novel pathways and identifying potential therapeutic targets. In the present study, five novel transcripts differentially expressed by the HF3h and/or LF3h feeding have been found. Among these, 4 transcripts with the SAGE tags CATG plus AAAAATCATCG, TCGAATTGCAA, GCTGCCCTCCT, and AATATCACCTT were found to be significantly up-regulated by HF feeding. In particular, the tag with sequence CATG AAAAATCATCG displayed a more than 5-fold increase both in the LF and HF mice, appearing a good target for further characterization. Finally, the transcript with tag sequence CATG TCTGTGGCCTC was found to be significantly down-regulated in the LF mice.

Conclusions

The present study investigated the differential effects of LF and HF feeding on the hypothalamic transcriptome at 3 hours after meal initiation, and compared them to the mRNA expression in fasting mice. At this time point, twelve genes were globally regulated by feeding compared to fasting, and only two were differentially modulated between LF and HF. Totally, two transcripts involved in mitochondrial functions (MtCo1 and Ppid), three involved in protein transport and regulation (Ube2q2, Mup1 and Sec13), one implicated in the cellular pH control (Slc4a3) and a transcript with a role in the epigenetic control of gene expression (Setd3) were found to be differentially expressed. In addition, five potential novel genes were significantly modulated by LF and HF meals. Analyzing the transcriptome at three hours after feeding as unique time point may have given a partial picture of the numerous pathways that acutely modulate gene expression in the hypothalamus. Remarkably, the SAGE method detected the expression of several genes known to be involved in the regulation of energy balance, such as NPY, AgRP, POMC, and

CART. However, following the statistical analysis, the levels of these tags did not show a significant differential modulation 3h after LF or HF feeding (data not shown). This substantiates the ambiguity in precisely predicting when a neuronal/peptidergic or endocrine signal supposed to rise from the gastro-intestinal tract would induce a hypothalamic response and vice versa. In our study, we chose to conduct an acute 3h-analysis since the best therapeutic target will be the initial signal regulating the following cascade of changes. A later analysis of transcription risked to miss the early regulation [40]. Nevertheless, an earlier analysis (30 minutes or 1 hour) may not be helpful for the detection of HF-induced signals, which are known to be delayed compared to those specifically induced by carbohydrate or protein ingestion [41]. On the other hand, we didn't consider helpful to examine a longer dietary period or an obesity phenotype, since the early acute changes are frequently blunted in long-term obesity [42, 43].

Further investigations of the highlighted pathways within key hypothalamic nuclei, as well as the cloning and characterization of the novel genes detected by the SAGE method may help to complete the hypothalamic network of signals involved in appetite/satiety control. Finally, targeting these genes may be an advantageous strategy for the prevention and therapy of obesity.

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The manuscript has been approved by all listed authors and there is no conflict of interest that would prejudice its impartiality. MRDG analyzed and interpreted the SAGE data, and drafted the manuscript. MY and JstA conceived the study, designed it and critically revised the manuscript. JstA gave the final approval of the version to be published. We would like to thank Dr. Ping Ye and Mr. Mathieu LePage for their contribution to SAGE library construction, and Mr. Carl Bolduc for the informatic support.

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Table 1 Food and macronutrients intakes after the ingestion of low-fat or high-fat meal in mice.

	Fasting	LF3h	HF3h	
Number of mice	20	20	20	
Body weight (g)	26.7 (0.49)	26.7 (0.46)	26.9 (0.55)	
Energy intake (kcal)		6.10 (0.39)	9.65 (1.49)	*
Food intake (g)		1.59 (0.10)	1.84 (0.28)	
Protein intake (g)		0.31 (0.02)	0.48 (0.09)	*
Fat intake (g)		0.09 (0.004)	0.64 (0.099)	*
Carbohydrate intake (g)		1.09 (0.09)	0.49 (0.08)	

Data are mean (SEM).

* Significantly different from low-fat group ($P < 0.05$).

Table 2 The differentially expressed transcripts in hypothalamus at 3 hours after high-fat and low-fat meal initiation.

SAGE tags	F	Low-fat meal 3h		High-fat meal 3h		Description (UniGene cluster, GenBank Accession no.)	*Abbr*
AAAAATCATCG	**34**	**190**	↑	**187**	↑	NM	
GCTGCCCTCCA	**112**	**817**	↑	**1035**	↑	Cytochrome c oxidase subunit I (NC_005089, POS: 6816)	MtCo1
TCGAATTGCAA	**2**	**10**		**17**	↑	NM	
GCTGCCCTCCT	**1**	**12**	↑	**15**	↑	NM	
AATATCACCTT	**2**	**8**		**15**	↑	NM	
TAAGGAACAAA	**0**	**8**	↑	**8**	↑	SEC13 homolog (Mm.29296, NM_024206)	Sec13
CTTTGGGGTTG	**12**	**2**		**1**	↓	Ubiquitin-conjugating enzyme E2Q 2 (Mm.207894, NM_180600)	Ube2q2
GCTGTGTTTCA	**1**	**13**	↑	**6**		Peptidylprolyl isomerase D (Mm.295252, BC019778)	Ppid
TCTGTGGCCTC	**21**	**0**	↓	**9**	*	NM	
AATTTCTTCCT	**1**	**9**	↑	**3**		Major urinary protein 1 (Mm.335875, NM_03118); Major urinary protein 3 (Mm.250267, NM_001012323)	Mup1/ Mup3
CCAGGCCAGTG	**8**	**2**		**14**	*	Solute carrier family 4 member 3 (Mm.5053, NM_009208)	Slc4a3
TTCCTTGTTAA	**9**	**1**	↓	**7**		SET domain containing 3 (Mm.159185, NM_028262)	Setd3

↑ ↓ Significantly up- and down-regulated compared to fasting ($P \leq 0.05$).

* Significant difference between LF and HF fed mice ($P \leq 0.05$).

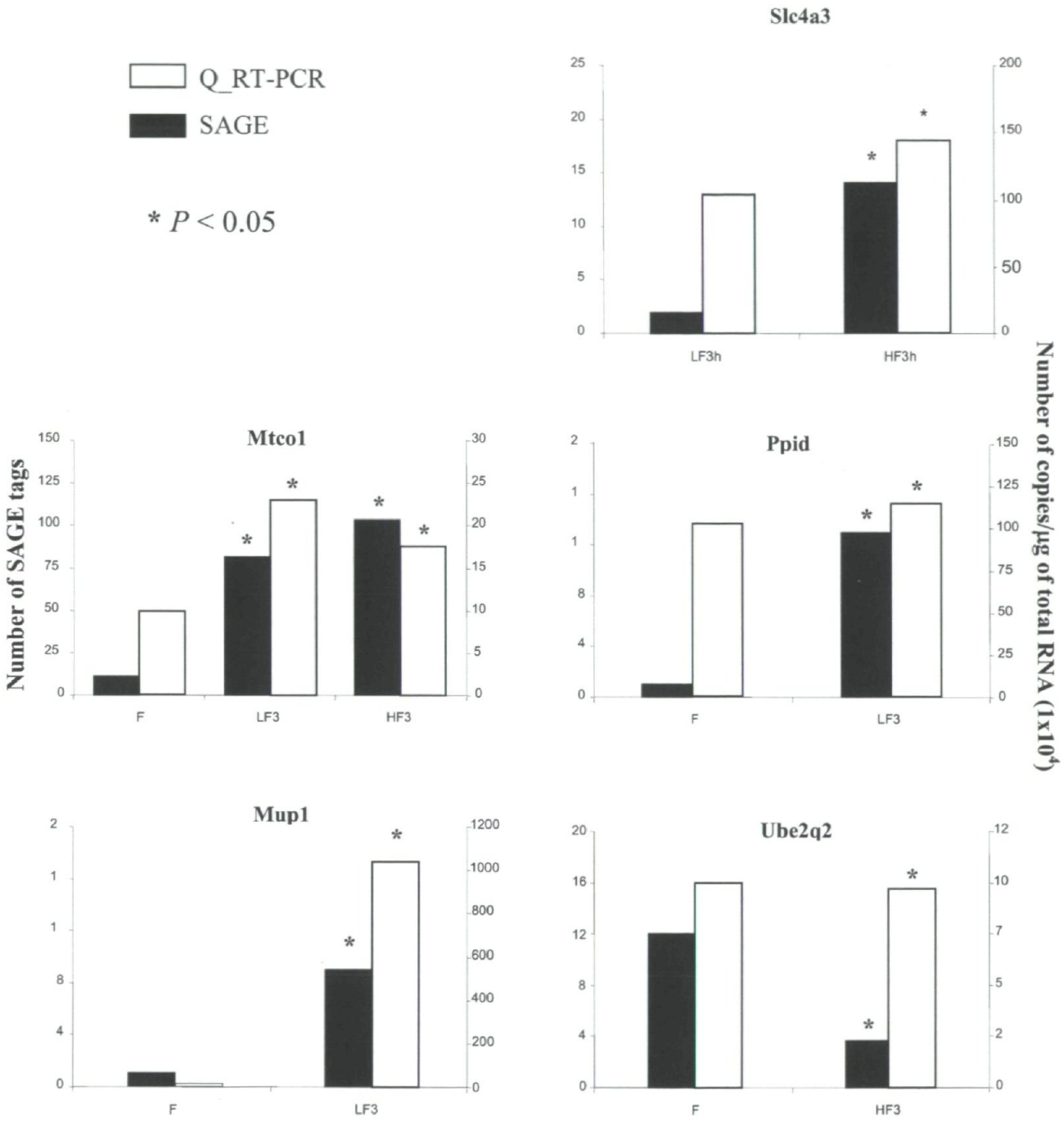
FIGURE LEGEND

Figure 1 Confirmation of the serial analysis of gene expression (SAGE) data by the quantitative real-time PCR (Q_RT-PCR).

Asterisk represents a significant difference compared to the fasting group ($P \leq 0.05$).

Abbreviations: F, fasting; LF3h, low-fat 3 hours; HF3h, high-fat 3 hours; MtCo1, mitochondrial cytochrome-c oxidase 1; Mup1, major urinary protein 1; Ppid, peptidylprolyl isomerase D; Ube2q2, ubiquitin-conjugating enzyme E2Q 2.

Figure 1.


Q_RT-PCR
SAGE
* $P < 0.05$
Slc4a3
LF3h
HF3h
Mtco1
F
LF3
HF3
Ppid
F
LF3
Mup1
F
LF3
Ube2q2
F
HF3
Number of SAGE tags
Number of copies/μg of total RNA (1x10⁴)

CHAPTER 4

Feeding regulates the expression of pancreatic genes in gastric mucosa

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Résumé

Le contrôle peu efficace, ou déficient, des mécanismes à court terme régulant la prise alimentaire peut compromettre l'homéostasie énergétique et conduire à l'obésité. Le tractus gastro-intestinal produit de nombreux peptides régulateurs. Toutefois, un nombre réduit de peptides gastriques est présentement reconnu pour leur rôle dans la régulation aigüe de la prise alimentaire. Dans le but d'identifier de nouveaux signaux gastriques, nous avons utilisé la méthode de l'analyse sérielle de l'expression génique (SAGE) et analysé le profil transcriptionnel de la muqueuse gastrique chez sept groupes de souris: à jeun; et sacrifiées 30 minutes, 1h et 3h après le début d'un repas faible en gras (LF) ou riche en gras (HF). Les repas LF ou HF ont modulé l'expression d'un total de 35 gènes comparativement au jeun, incluant 15 RNAs messagers qui codent pour des enzymes digestifs/protéines de sécrétion, et 15 nouveaux transcrits. Malgré que le profil d'expression basale n'ait pas subi des variations substantielles, les deux repas LF et HF ont significativement influencé la transcription. Cette étude représente la première analyse globale du transcriptome de l'estomac suite à des stimuli nutritionnels distincts. Les études futures ainsi que la caractérisation des gènes inconnus pourraient contribuer à l'identification de nouvelles cibles pour la thérapie et la prévention de l'obésité.

Feeding regulates the expression of pancreatic genes in gastric mucosa

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Keywords: digestive capacity; gastric mucosa gene expression; SAGE; short-term regulation of food intake.

ABSTRACT

The ineffective short-term control of feeding behaviour compromises energy homeostasis and can lead to obesity. The gastro-intestinal tract secretes several regulatory peptides. However, little is known about the stomach peptide contribution to the acute regulation of intake. In an attempt to identify new gastric signals, the serial analysis of gene expression (SAGE) method was used for the transcription profiling of stomach mucosa in 7 groups of mice: fasting; and sacrificed 30 minutes, 1 hour, 3 hours after a low-fat (LF) or high-fat (HF) *ad libitum* meal. In total, 35 genes were differentially modulated by LF and HF meals compared to fasting, including 15 mRNAs coding for digestive enzymes / secretory proteins, and 10 novel transcripts. Although the basic expression profile did not undergo substantial variations, both LF and HF meals influenced the transcription. This study represents the first global analysis of stomach transcriptome as induced by different nutritional stimuli. Further studies including the characterization of novel genes may help to identify new targets for the therapy and prevention of obesity.

INTRODUCTION

Obesity epidemic continues its worrying global progression, although significant advances have been achieved in the knowledge of its causes and consequences. This condition, in concert with glucose intolerance/type 2 diabetes, dyslipidemia and metabolic syndrome, widely contributes to what has been recently defined a "prosperity's plague" [1]. A complex system of social, psychological, as well as physiological and biological factors has to be considered in order to successfully control this "plague" and prevent from its further spread [2, 3]. From a physiological point of view, it is fundamental to comprehend the specific relative importance of long-term and short-term mechanisms involved in the regulation of energy balance [4]. Food intake and daily over-consumption may have a predominant impact on body weight regulation, and this led large interest to focus on appetite/satiety balance as one of the key potential therapeutic targets [5]. Multiple sites in the gastro-intestinal (GI) tract, including the stomach, proximal and distal small intestine, colon and pancreas are involved in the short-term regulation of energy homeostasis, which basically controls what, when and how much we eat within a single day or a single meal [6]. In addition to mechanoreceptors and chemoreceptors, which are activated during a meal and signal to the brainstem through the vagal nerve [4], several gut-derived peptides and lipid mediators have a role in the regulation of food intake and energy homeostasis [7]. The only recognized "hunger" gut-peptide so far discovered is ghrelin, which is mainly produced by the stomach and directly influences the number of meals consumed per day, though having probably no direct effect on meal size [8]. However, the stomach plays an essential mechanical role in the regulation of satiety perception and meal termination. It is, in fact, the first organ to receive the bolus of food, which is then rapidly homogenized and

partially digested before being delivered to the small intestine. Most importantly, gastric distension/accommodation and the finely controlled gastric emptying via the pylorus give a critical contribution for matching food delivery to the effective gut digestive and absorptive capacity [9]. As a consequence, gastric competence can be considered as the first limiting step of global ingestive and digestive capacity, and thus represents a relevant target for obesity prevention and treatment. Although motility and physical mechanisms involved in gastric-mediated satiety have been extensively studied and successfully targeted [10, 11], it is still unknown whether the gastric mucosa releases any biochemical signal which could directly or indirectly influence a rapid satiation response in the very short-term of meal consumption. In an attempt to explore this possibility, the serial analysis of gene expression (SAGE) method was used to identify the early transcriptional changes induced by a low-fat (LF) or high-fat (HF) meal in the gastric mucosa. The discovery potential offered by SAGE drove the choice to use this technology instead of other comparable transcriptomic methods, which would have limited the analysis to known genes. SAGE, indeed, is a powerful and reliable sequencing-based technique [12] which allows to detect the regulation of novel transcripts as well as characterized genes, as we have already shown in a number of previous studies [13, 14].

MATERIALS AND METHODS

Animals, diet and sample preparation

A total of 140 male C57BL6 mice (12 wk-old) were purchased from Charles River Canada Inc. and were acclimated for two weeks. Low-fat diet (LF, Research Diet # 12450B: 10% calories from fat, 70% from carbohydrate, and 20% from protein; 3.85 kcal/g) and tap water were served *ad libitum*. The last day of the acclimatization, the body weight paired mice were randomly distributed into seven groups and fasted for 12 hours during darkness of the light cycle. On the experimental day, one group of fasted mice was sacrificed before the meal. The other mice were fed *ad libitum* with high-fat (HF, Research Diet # 12492: 60% calories from fat, 20% from carbohydrate, and 20% from protein; 5.24 kcal/g) or LF meal, and sacrificed 30 min, 1 h and 3 h after the beginning of the meal. The amount of macronutrients and energy ingested was recorded. In total, seven groups of mice under isoflurane anesthesia (fasting, HF30min/1h/3h, and LF30min/1h/3h) were alternatively exsanguinated by cardiac puncture after cervical dislocation. Stomach was opened vertically, flushed clean with saline, and the mucosa was removed by scrapping with a glass microscope slide. The samples were rapidly frozen in liquid nitrogen and stored at -80°C until RNA extraction. All animal experimentation was conducted in accordance with the requirements of the Canadian Council on Animal Care, and approved by the animal protection Committee of Laval University.

Transcriptomic analysis

The seven serial analysis of gene expression (SAGE) libraries were constructed as previously described [15]. Total RNA was isolated from pooled stomach mucosa for each group (n = 20) by Trizol (Invitrogen Canada Inc., Burlington, ON). The quality of total RNA was monitored by micro-capillary electrophoresis (Bioanalizer 2100, Agilent Technologies, Mississauga, ON). Polyadenylated RNA was extracted (Oligotex mRNA Mini Kit, Qiagen Inc., Mississauga, ON), annealed with the biotin-5'-T_{18}-3' primer and converted to cDNA (cDNA synthesis kit, Invitrogen Canada Inc.). The resulting cDNAs were digested with *NlaIII* (New England BioLabs Ltd., Pickering, ON) and the 3' restriction fragments were isolated with streptavidin-coated magnetic beads (Dynal Biotech LLC, Brown Deer, WI) and separated into two populations. Each population was ligated to one of two annealed linkers and extensively washed to remove unligated linkers [15]. The tag beside the most 3' *NlaIII* restriction site (CATG) of each transcript was released by digestion with *BsmFI* (New England BioLabs Ltd.). The blunting kit from Takara Bio Inc. (Otsu, Japan) was used for the blunting and ligation of the two tag populations. The resulting ligation products containing the ditags were amplified by PCR and digested with *NlaIII.* The band containing the ditags was extracted from the 12% polyacrylamide gel. The purified ditags were self-ligated to form concatemers using T4 ligase (Invitrogen Canada Inc.). The isolated 500 bp to 1800 bp concatemers were isolated by agarose gel, and the resulting DNA fragments were ligated into the *SphI* site of pUC19 and cloned into OmniMAX 2T1 competent cells (Invitrogen Canada Inc.). White colonies were picked up, and the concatemer inserts were finally sequenced by the Applied Biosystems 3730 (Foster City, CA).

Bioinformatic analysis

Sequence files were analyzed using the SAGEana program, a modification of SAGEparser [12]. Identification of the transcripts was obtained by matching the 15 bp (sequence at the last CATG + 11bp tags) with SAGEmap, UniGene and GenBank databases. Classification of the transcripts was based upon the updated information of the genome directory [16] found at the TIGR web site (http,//www.tigr.org/), the SOURCE (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch) and the OMIM (http://www.ncbi.nlm.nih.gov/) as well as previously published literatures. We have previously shown that the SAGE method is very reproducible with $r^2 = 0.96$ between two SAGE libraries constructed from the same total RNA pool [12].

Validation by quantitative real-time PCR (Q_RT-PCR)

First strand cDNA was synthesized using 5 μg of pooled RNA of each experimental group in a reaction containing 200 U of Superscript III Rnase H-RT (Invitrogen Canada Inc.), 50 ng of random hexamers, 300 ng of oligo-dT_{18}, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM deoxynucleotides triphosphate and 40 U human RNase inhibitor (Roche) in a final volume of 50 μl. The resulting products were then treated with 1 μg of Rnase A for 30 minutes at 37°C and purified thereafter with Qiaquick PCR purification kits (Qiagen). The cDNA corresponding to 20 ng of total RNA was used to perform fluorescent-based real-time PCR quantification using the LightCycler real-time PCR apparatus (Roche Inc., Nutley, NJ) and the FastStart DNA Master SYBR green kit (Roche Diagnostics). Quantifications were conducted in triplicates. Reading of the fluorescence signal was taken at the end of the heating to avoid non-specific signal. A

melting curve was performed to assess non-specific signals. Annealing temperature was selected based on contamination levels and melting curve results. Prior to mRNA quantification, RNA samples were also verified for genomic DNA contamination. Oligoprimer pairs that allow the amplification of approximately 250 bp were designed by GeneTools software (Biotools Inc., Edmonton, AB) and their specificity was verified by BLAST in GenBank database. Gene name, GenBank accession numbers and regions used for the primer pairs were the following: chymotrypsin-like elastase family member 3B (elastase 3, Cela3b), *NM_026419*, 189-385; amylase 2a1, pancreatic (Amy2a1), *NM_001042712*, 848-1039; pancreatic lipase (Pnlip), *NM_026925*, 840-1067; major urinary protein 1 (Mup1), *NM_031188*, 127-405; protein disulfide isomerase associated 3 (Pdia3), *BC033439*, 994-1222; zymogen granule membrane protein 16 (Zg16), *NM_026918*, 348-522. The mRNA levels were calculated using a standard curve of crossing point (Cp) versus logarithm of the quantity, and expressed as the number of copies per microgram of total RNA [17]. The LightCycler 3.5 program provided by the manufacturer (Roche Inc.) was used to calculate the Cp according to the second derivative and double correction method previously described by Luu-The et al. [17]. The standard curve with efficiency coefficient E=2 was established using known cDNA amounts of 0, 10^2, 10^3, 10^4, 10^5 and 10^6 copies of ATP synthase O subunit.

Statistical analysis

Food and macronutrient intakes were analyzed by the two-way ANOVA. When the ANOVA revealed a significant interaction between diet and time, the contrast analysis was performed to identify the significant difference between the HF and LF groups from the same time points ($P < 0.05$). For the SAGE data, the comparative count display (CCD) test

was used to identify the transcripts which were significantly differentially expressed ($P \leq 0.05$) between the groups with more than a two-fold change, as previously described by Lash et al. [18]. The data were normalized to 50000 tags for presentation. Q_RT-PCR data were analyzed by the two-tailed Student's t-test ($P < 0.01$) for the time points formerly determined by the SAGE method.

RESULTS

Food intake

Food and energy intakes are presented in Figure 1 as cumulative and 30 minutes average consumption. As expected, cumulative energy as well as protein and fat intakes were higher, whereas carbohydrate intake was lower, in the HF groups compared to LF (Figure 2a). Interestingly, a distinct pattern of feeding behavior between LF- and HF-fed mice can be observed by comparing the average 30 minutes consumption of the two groups (Figure 1c). Mice assigned to LF meal ate a moderate amount of food during one hour, after which their consumption decreased to a minimum intake. In contrast, the HF group consumed a large amount of meal in the first 30 minutes, although the intake dropped in the following 30, reaching the minimum level at 1h. At that time point, in fact, LF consumption overcame HF one. However, the HF-group mice ingestion increased again in the last two hours.

Transcriptomic analysis

Seven SAGE libraries were generated to identify the transcripts differentially modulated in mouse gastric mucosa by the following experimental conditions: fasting; LF or HF meal at 30 min, 1 h and 3 h since the beginning of consumption (LF30m/1h/3h and HF30m/1h/3h, respectively). Among the 56382 SAGE tag species detected, a total of 35 transcripts were significantly regulated by LF and HF feeding compared to fasting, whereas 19 were specifically modulated by HF compared to LF.

Transcripts coding for digestive/secretory proteins. The most represented group includes transcripts coding for digestive enzymes and secretory pathway components

(Table 1). Among the modulated mRNAs were amylase 2a1, pancreatic lipase, carboxyl ester lipase, elastase 1 and 3, as well as carboxypeptidase A1 and B1. Globally, three of them have lipolytic functions, nine code for proteolytic enzymes and one gene is involved in carbohydrate digestion. In addition, two genes involved in zymogen granule secretion were regulated, namely syncollin and zymogen granule membrane protein 16. Interestingly, most of these genes showed a common pattern of regulation, since their expression was down-regulated at LF30m and 1h, as well as at HF1h and 3h, compared to fasting. Moreover, the expression of these transcripts tended to increase at LF3h, though the up-regulation was statistically significant only for six of them, such as amylase 2a1, chymotrypsinogen B1, and pancreatic lipase related protein 1. Remarkably, for 13 out of the 15 tags considered, the specific differential regulation between LF3h and HF3h achieved statistical significance. There is, therefore, a common down-regulation of these transcripts following both LF and HF feeding, although a temporal delay between the two groups can be observed. In addition, 3 hours after the beginning of the LF meal, their transcription tended to increase at higher levels than those observed at the fasting state.

Cellular defence-related and other transcripts differentially regulated by feeding. Eight transcripts with protective roles have been significantly regulated by feeding (Table 2). Among these, the mRNAs coding for the heat shock protein 70 (Hsp70) 1A and expressed sequence tag (EST) Hsp70 1B were both up-regulated by the LF meal, and the latter also by the HF meal, at 1 h following the beginning of ingestion. Gene expressions of cysteine-rich protein 1, metallothionein 2, and EST WD repeat domain 92 were reduced at LF3h compared to fasting, whereas onzin mRNA levels significantly decreased at HF3h. Moreover, HF specifically modulated the transcription of protein disulfide isomerase

associated 3 and EST elastase 2A/neutrophil elastase, respectively up- and down-regulated at 3 h.

The results also showed the differential regulation of two transcripts coding for proprotein convertase proteins (furin and kallikrein) and an interesting new candidate as potential regulator of energy metabolism, namely Mup 1 (Table 2). In addition, 12 novel transcripts with no match in public databases were significantly regulated by LF and HF feeding (Table 3). In particular, the tags GGAGAACAGCG and CTGACTCAAAT were specifically modulated by HF feeding compared to LF, and could represent potential targets for further characterization studies.

Q_RT-PCR confirmation of SAGE results.

To validate the SAGE results, the Q_RT-PCR analysis was also performed for some of the genes differentially regulated by feeding. The chosen genes are representative of the functional groups discussed. As presented in Figure 3, the Q_RT-PCR results globally confirmed the changes in expression level as well as the significant modulation evidenced by the SAGE method.

DISCUSSION

Fasting and feeding states modulated pancreas-related genes in the stomach.

The main regulated functional group is represented by digestive enzyme-coding genes, many of which are mostly expressed by the pancreas [19]. In addition, two transcripts involved in the secretory pathway, syncollin and zymogen granule membrane protein 16, were also modulated. Since many of the physiological changes induced by food intake should arise within minutes, the digestive enzymes and regulatory factors are normally synthesized and packed in secretory granules at rest, ready to be released when food ingestion and appropriate neurohumoral stimuli occur. Following the meal, another cycle of synthesis and packaging prepares the zymogenic cells to the next secretory events [9]. Concordantly, the present results showed a decreased transcription of digestive enzyme-coding and secretory genes following the start of ingestion, when the cells are more likely to invest energy in secretion. Moreover, in the LF-fed mice, the re-induction of these genes 3 hours after the beginning of intake seems a reasonable event, particularly if referred to their feeding pattern (Figure 2). Conversely, the digestion of a high-calorie and high-energy density meal, including its rate of emptying from the stomach, normally takes a longer time to be accomplished [20, 21]. The latter point may explain the delayed and prolonged down-regulation of digestive transcripts in the HF group, and also suggest that the re-induction of transcription observed at LF3h may have started later in the HF-fed mice. Interestingly, though all the mice had *ad libitum* access to food, feeding behavior and total ingestion were dissimilar between LF and HF groups (Figures 1 and 2), possibly explaining the differences in the transcriptional regulation of digestive enzymes. However, the levels of digestive enzymes and secretory proteins might as well influence the intake.

Actually, a physiological redundant excess of digestive capacity characterizes the GI system, and guarantees the effectiveness of nutrition. Moreover, the excess capacity for nutrient uptake, including the excess of surface, specialized cells, digestive enzymes and other secretory products, is proportionally related to body weight [9], and may plausibly contribute to compromise the efficient control of appetite/satiety balance in overweight and obese subjects. The stomach may represent a primary target for the control of meal size and satiation. Actually, surgical options which physically affect gastric capacity and emptying mechanisms successfully modify the eating behavior and metabolic profile of obese patients [10], though they still are invasive and life style-affecting methods [22]. Likewise, mechanisms other than mechanical may also affect gastric volume/emptying rates and thus regulate satiation during meal consumption. Given the present paucity of literature specifically regarding the effects of HF food intake on neuro-hormonal control of gastric capacity/motility and its potential short-term impact on satiation, the search for these pathways was the principal aim of the current study.

Plausible reasons for the expression of pancreatic genes in the stomach.

The observation that gastric mucosa could express pancreatic genes was surprising and raised many questions. In particular, it would be useful to explain the specificity of their role in the stomach and, most interestingly, their differential regulation in response to fasting and feeding. Actually, considering the high level of transcription that these genes reached in gastric mucosa, such as amylase 2a1 at fasting and LF3h, their expression should entail a needed role in this tissue. In contrast, it is hard to explain how the digestive enzymes, normally active at a neutral pH, could conceivably work in such an acidic milieu.

However, it should also be considered that these proteins are often released as precursors and eventually activated by specific signals or the appropriate pH. This is not the first time that the expression and activity of pancreas-related genes are detected in the stomach and other non-pancreatic components of the GI system. Terada and colleagues [19] had already showed the expression of alpha-amylase, trypsin, chymotrypsin and pancreatic lipase in normal and pathologic epithelial cells of gastric mucosa by immunohistochemistry and western blotting. They had also proved their enzymatic activity in stomach specimens, even if to a far lesser extent than in pancreas. In that report, the authors explained the presence of pancreatic enzymes in non-pancreatic tissues as a result of the common embryonic origin (foregut) shared by the gastro-enteric tissues. In addition, the mRNA expression levels of representative genes have been further confirmed by Q_RT-PCR in the present study. It can be hypothesized that the digestive enzymes expressed by the gastric mucosa would combine with the bolus of homogenized and partially digested food that finally reaches the small intestine. In normal conditions, the digestive enzymes would be in excess, but enough to guarantee the effectiveness of digestion in case of insufficient pancreatic secretion. Consistent with the redundant production of these proteins along the digestive tract was our previous study of the duodenum mucosa transcriptome, where analogue experimental conditions had been applied. In the intestine, the same pancreas-related transcripts were regulated (n=9) [13], but with an apparently opposite trend, being up-regulated at LF1h and HF3h. Interestingly, in the duodenal as well as gastric mucosa, a similar temporal delay of transcription between the LF and HF groups was observed, being limited to digestive enzyme-coding genes.

LF and HF modulated genes involved in the protection and defence of gastric mucosa.

Protection is one of the main functions and challenges of gastric mucosa. The mucosal epithelium is a primary barrier, which defends the whole organism from external dangerous agents eventually ingested. Moreover, the gastric mucosa has also to defend itself from uncontrolled acid secretion, inflammation, oxidative stress, and the epithelial damages that can be consequently engendered, thus compromising the physical and functional integrity of the barrier [23]. Among the transcripts differentially regulated in the gastric mucosa, seven were related to defence/protection. Metallothionein 2 [24, 25], Hsp70 (1A and 1B) [26, 27] and protein disulfide isomerase associated 3 genes [28, 29] code for multiple-task proteins, which can show chaperone activity, and/or be involved in cell redox homeostasis control and apoptosis regulation. The latter is a very important role, since gastric epithelium is subject to constant renewal by which the epithelial cells rapidly turnover (1-3 days in humans), undergoing a cycle of division and differentiation before succumbing to apoptosis [9, 30]. In this study, EST Hsp70 1B was up-regulated by both LF and HF at 1 h, and the same trend is recognizable for hsp70 1A. However, for the latter, the HF1h increase did not reach a statistical significance. Interestingly, a polymorphism of Hsp70 1B gene has been recently reported to associate with obesity-related traits [31], thus stimulating further questions about its acute modulation by food intake.

Mup1, already found in hypothalamus and duodenum, was expressed in the gastric mucosa and regulated by feeding.

In the present study, the gene coding for Mup1 was specifically down-regulated at HF30m compared to LF. In previous transcriptomic studies conducted with the SAGE

method, Mup1 gene was also significantly regulated by feeding in the duodenum mucosa and hypothalamus of mice [13, 14]. The corresponding protein is a pheromone transporter normally expressed by the liver. However, recent studies in mice have surprisingly revealed that Mup1 is also involved in glucose and lipid metabolism [32], and that it might play an important role in the regulation of energy expenditure [33]. These findings raise the interest about the specific role of this molecule at the tissue level but also as a potential modulator of energy balance.

Other interesting genes differentially regulated by feeding.

Globally, the most regulated class of genes was the one coding for proteolytic enzymes, particularly the serine-protease type. This group of proteases is highly represented in nature and shows numerous and functionally diverse functions, ranging from digestion and coagulation to apoptosis and immunity [34]. In addition to the digestion-related genes described above, feeding regulated two other transcripts coding for highly important serine-proteases, namely kallikrein and furin. EST kallikrein 1 was specifically regulated by HF3h compared to LF, whereas furin was down-regulated at LF1h. These two molecules act as pro-protein convertases in distinct regulatory pathways, the first cleaving kininogen to produce kinin peptide [35], and the latter processing the precursors of a large variety of proteins, including growth factors and receptors [36]. Interestingly, the specific pathways involving kallikrein-kinin [37, 38] and other proteins of furin family [39, 40] are presently being studied for their potential contribution to obesity and cardiovascular disorders.

CONCLUSION

The present was the first study to analyze the global transcriptional changes acutely induced in mouse stomach mucosa by feeding and, in particular, by different nutritional stimuli. The principal aim was to identify new signals specifically induced by HF intake, and which may represent potential pharmacological targets for the early modulation of appetite/satiety balance during the consumption of a meal. Both LF and HF regulated the gene expression in gastric mucosa, and 17 known genes were differentially modulated by HF compared to LF. In addition, a number of novel tags were significantly regulated, some of which may be good objects for future characterization studies. However, a lower number of genes was regulated in the stomach compared to duodenum, when the same experimental conditions have been applied [13]. This may suggest that gastric mucosa has a restricted role in the acute regulation of food intake, and mainly centered on meal initiation than meal size/termination control. Another plausible hypothesis is that satiation signals eventually raising from the mucosa could be induced earlier than 30 min after the beginning of the meal, at least at the transcriptional level. Although it is still uncertain whether gastric mucosa releases an early molecular signal specifically involved in satiation control, the present study contributed to highlight some potential mediators of this process. In addition, the characterization of novel regulated genes could stimulate future investigations. Since signals secreted by gastric mucosa may be the optimal targets for appetite control and obesity therapeutic strategies, further research efforts are deserved.

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AUTHOR'S CONTRIBUTIONS & DECLARATIONS OF INTEREST

The manuscript has been approved by all listed authors and there is no conflict of interest that would prejudice its impartiality. MRDG analyzed and interpreted the SAGE data, and drafted the manuscript. MY and JStA conceived the study, designed it and critically revised the manuscript. JStA gave the final approval of the version to be published.

FIGURE LEGENDS

Figure 1. Food and energy intake of low-fat and high-fat meals: cumulative (a, b) and 30 minutes average (c,d) measurements.

* Significantly different compared to low-fat (LF) meal at the same time point ($P < 0,05$).

§ Significant effect of the diet ($P < 0,05$).

Abbreviations: g, grams; kcal, kilocalories; LF, low-fat; HF, high-fat.

Figure 2. Thirty minutes average nutrient intake (1a, 2a, 3a) and the correspondent digestive enzyme-coding gene fold-change regulation by feeding (1b, 2b, 3b).

* Significantly different compared to low-fat (LF) meal at the same time point ($P < 0,05$).

§ Significant effect of the diet ($P < 0,05$).

Abbreviations: g, grams; LF, low-fat; HF, high-fat.

Figure 3. Serial analysis of gene expression (SAGE) and quantitative real-time PCR (Q_RT-PCR) results for some representative regulated genes.

* Significantly different compared to fasting ($P < 0,01$).

§ Significantly different compared to low-fat (LF) meal at the same time point ($P < 0,01$).

Abbreviations: LF, low-fat; HF, high-fat.

Table 1. Transcripts coding for digestive and secretory proteins differentially modulated by feeding in the gastric mucosa.

Tag	Fasting	Low-fat						High-fat						Description
		30min		1h		3h		30min		1h		3h		
Digestive enzymes														
CTGACTCAAAA	713	**86**	↓	**48**	↓	**2020**	↑	383	*	**106**	↓	**70**	↓ *	Amylase 2a1, pancreatic (Mm.439729; NM_001042712)
CCCTGGGTTCA	130	**4**	↓	**2**	↓	**353**	↑	72	*	**14**	↓	**0**	↓ *	Chymotrypsinogen B1 (Mm.34374; BC061083)
TTAGGAGGCTG	164	**11**	↓	**6**	↓	346		72	*	**12**	↓	**2**	↓ *	Pancreatic lipase (Mm.20407;NM_026925)
GGCTGTAATGT	65	**6**	↓	**6**	↓	175		52	*	**8**	↓	**7**	↓ *	Elastase 1, pancreatic (Mm.2131; NM_033612)
GTGTGCGCCGG	61	**4**	↓	**2**	↓	117		32		**2**	↓	**0**	↓ *	Elastase 3, pancreatic (Mm.297477;BC061066)
TTCTGTCTGGG	51	**0**	↓	**6**	↓	102		18		**2**	↓	**0**	↓ *	Trypsinogen 7 (RIKEN cDNA 2210010C04 gene; Mm.153729; BC061093)
CCCGGGTGCAA	0	4		4		**36**	↑	8		0		**2**	*	Pancreatic lipase related protein 1 (Mm.10753; BC068266).
GACCACACTGT	35	6		**0**	↓	109		28		10		**0**	↓ *	Carboxypeptidase A1

															(Mm.25377; BC052661)
GAGCACGTACG	14	0		0		36		4	0		**0**		*	Carboxypeptidase A2, pancreatic (Mm.268855; NM_001024698)
ATCTGTGTTGG	193	**27**	↓	**31**	↓	317		68	**37**	↓	**25**	↓	*	Protease serine 2 (Mm.276926;NM_009430)/ Trypsin 4 (Mm.383263;NM_011646)
GCCTACAACTA	30	**0**	↓	4		**113**	↑	16	**2**	↓	**0**	↓	*	Chymotrypsin-like (Mm.2745; NM_023182)
GCACCAAGTAC	26	**0**	↓	**0**	↓	**98**	↑	16	2		**2**		*	EST Carboxypeptidase B1 (Mm.34692; CF577795)
CAGACTAAGTA	24	4		2		**122**	↑	26	**0**	↓	2			Carboxyl ester lipase (Mm.236017; NM_009885)
Exocytosis/secretory pathways														
GCAAGACCCGC	26	2		**0**	↓	49		16	2		**5**		*	Syncollin (Mm.25210; NM_026716)

AAAGTATGCAA	122	**25**	↓	**37**	↓	98	72	**37**	↓	**34**	↓	Zymogen granule membrane protein 16 (RIKEN cDNA 1810010M01 gene; Mm.21835; BC031800)

Numbers indicate the count of serial analysis of gene expression (SAGE) tags per 50000.

↑↓ *indicate a significant differential regulation by LF or HF feeding compared to fasting ($P \leq 0.05$).*

* *indicates the significant modulation in the HF compared to the LF group at the corresponding time point ($P \leq 0.05$).*

Table 2. Transcripts with defence/protection roles and other genes differentially modulated by feeding in the gastric mucosa.

Tag	Fasting	Low-fat				High-fat				Description
		30min	1h	3h		30min	1h	3h		
Cellular defence/Protein modification										
CCAGGCCTTAC	53	23	44	**11**	↓	44	47	32		Cysteine-rich protein 1 (Mm.272368; NM_007763)
TGTTCAGTTTT	0	2	**23**	0	↑	0	8	0		Heat shock protein 1A-Hsp70 1A (Mm.6388; BC054782)
TCTACACTGCC	0	15	**56**	7	↑	10	**45**	0	↑	EST Heat shock protein 1B - Hsp70 1B (Mm.372314; BP766094)
TAACTGACAAT	91	38	37	**26**	↓	64	66	43		Metallothionein 2 (Mm.147226; BC031758)/EST Phosphatidylinositol-4-phosphate 5-kinase, type 1 alpha-Pip5k1a (Mm.296409; BU744153)
AACGCTTTCTA	81	27	46	33		34	45	**11**	↓	Placenta-specific 8 (onzin) (Mm.34609; BC010789)
CAGGAGGAGTT	28	13	21	18		22	4	**41**	*	Protein disulfide isomerase associated 3 (Mm.263177; BC033439)

GTGTGCGCTGG	10	0	0		38		6	0	**0**	*	EST Elastase 2A (Neutrophil elastase) (Mm.45316; BM730866)
TCGCTGCTTTA	75	44	40		**11**	↓	24	23	25		EST WD repeat domain 92 (Mm.298132; CO039767)
Regulatory mechanisms											
TATTTCAGTGA	0	6	8		**27**	↑	12	6	5		Adenylate cyclase 6 (Mm.157091; NM_007405)
GGCTGTCCTGT	0	2	15		13		2	6	**25**	↑	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17 (Mm.29644; BC096036)
TCCTGAAGAGG	2	8	**31**	↑	13		12	16	16		Furin (paired basic amino acid cleaving enzyme) (Mm.5241; BC048234)
AATTTCTTCCT	24	67	12		26		**8**	* 18	2		Major urinary protein 1/2 (Mm.335875; BC099597); (Mm.457981;AC:BC012259).
CAGCAAATGAA	6	6	0		33		4	2	**0**	*	EST Kallikrein 1 (Mm.142722; BG871914)
Others											
AAAAATCATCG	22	4	67		62		62	76	**93**	↑	NADH dehydrogenase 5, mt-Nd5 (CDNA clone IMAGE:4910858; Mm.455357;

									BC055066)
GACCTGGAGCC	30	23	8	**2**	↓	14	6	7	EST Ribosomal protein S14 (Mm.43778; AV212419)

Numbers indicate the count of serial analysis of gene expression (SAGE) tags per 50000.

↑↓ indicate a significant differential regulation by LF or HF feeding compared to fasting ($P \leq 0.05$).

** indicates the significant modulation in the HF compared to the LF group at the corresponding time point ($P \leq 0.05$).*

Table 3. Novel transcripts differentially modulated by feeding in the gastric mucosa.

Tag	Fasting	Low-fat			High-fat			
		30min	1h	3h	30min	1h	3h	
TTGTTGCTACT	26	13	19	13	16	**0** ↓	11	
GGAGAACAGCG	10	15	13	5	4	0	**29**	*
CTGACTCAAAT	4	0	4	26	4	0	**0**	*
TCCTATTAAGC	4	**38** ↑	**37** ↑	22	18	**35** ↑	23	
TTGGGGGAGGG	10	36	19	16	16	23	**61**	↑
CCTGCCCAGTA	10	29	42	40	**14** ↑	14	**61**	↑
TACCATATACT	22	25	4	**0** ↓	4	20	2	
TCTATGTCAGG	2	8	**29** ↑	9	6	12	2	
GTGTCTGGTAA	24	32	**89** ↑	62	56	35	43	
CACAAACATAT	0	13	8	**24** ↑	10	8	14	
CGAACAAAAGA	2	**29** ↑	19	18	24	18	14	
CCAGCAATCTT	18	67	**85** ↑	51	**82** ↑	64	45	

Numbers indicate the count of serial analysis of gene expression (SAGE) tags per 50000.

↑↓ indicate a significant differential regulation by LF or HF feeding compared to fasting ($P \leq 0.05$).

** indicates the significant modulation in the HF compared to the LF group at the corresponding time point ($P \leq 0.05$).*

Figure 1. Food and energy intake of low-fat and high-fat meals: cumulative (a, b) and 30 minutes average (c,d) measurements.

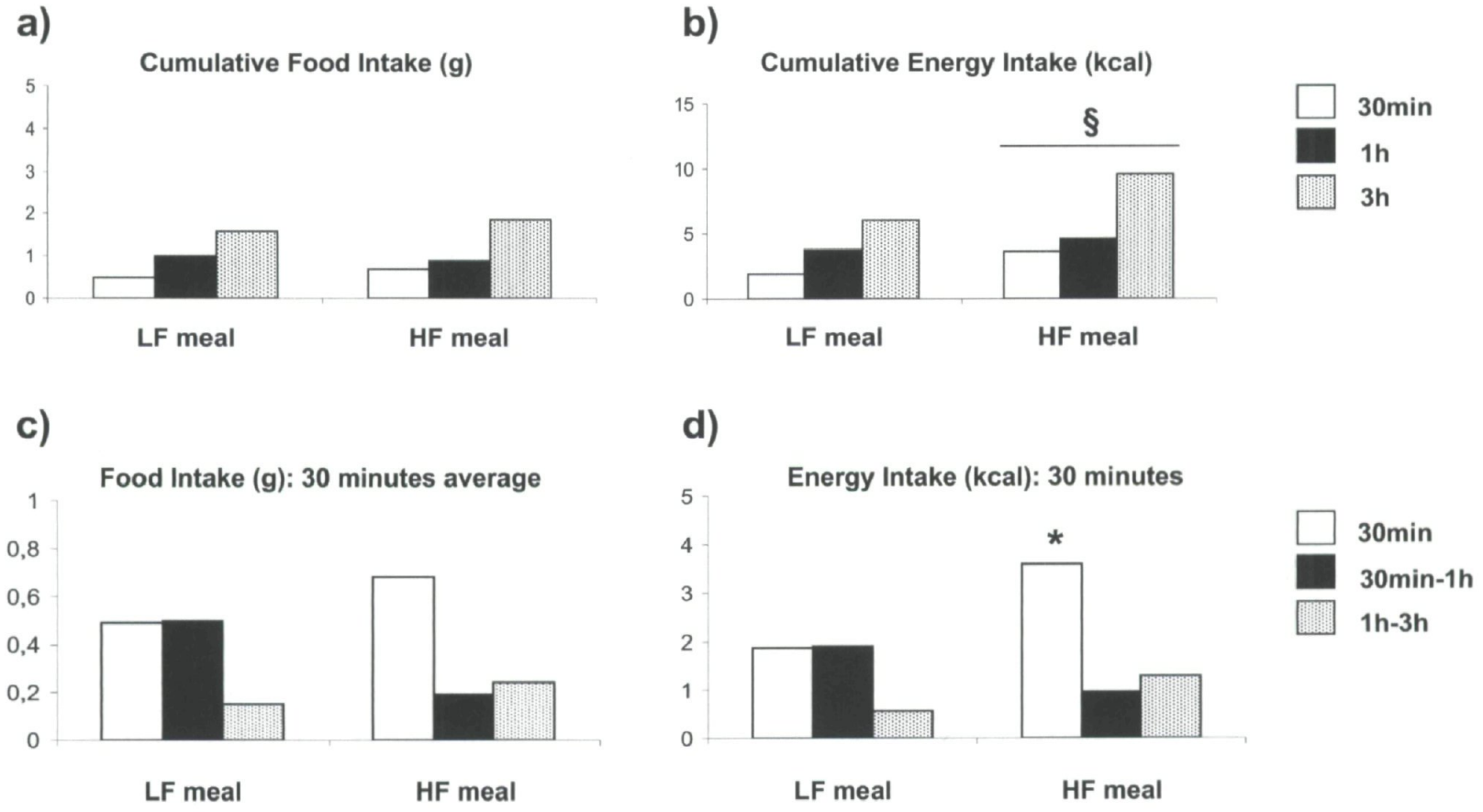


Figure 2. Thirty minutes average nutrient intake (1a, 2a, 3a) and the correspondent digestive enzyme-coding gene fold-change regulation by feeding (1b, 2b, 3b).

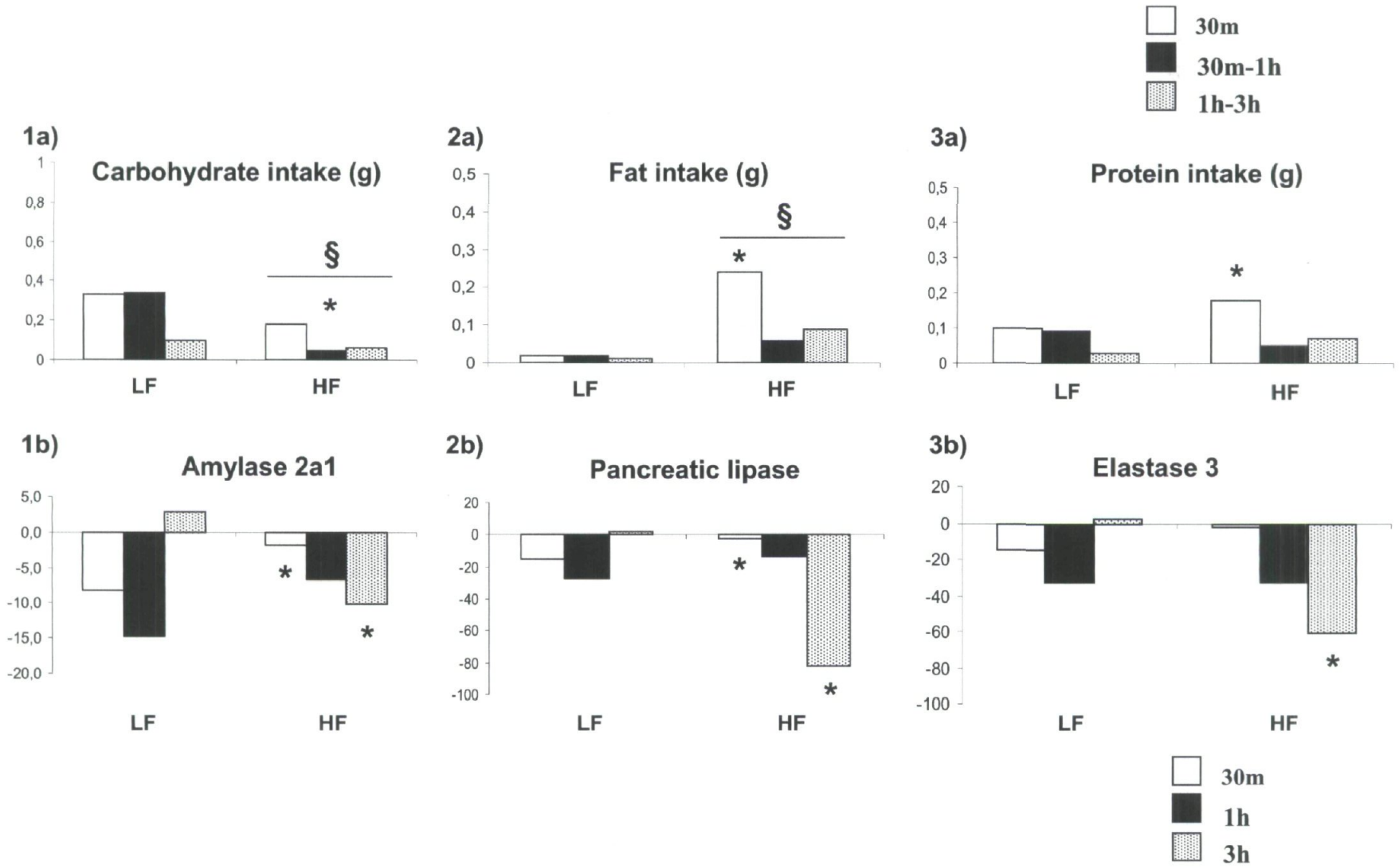
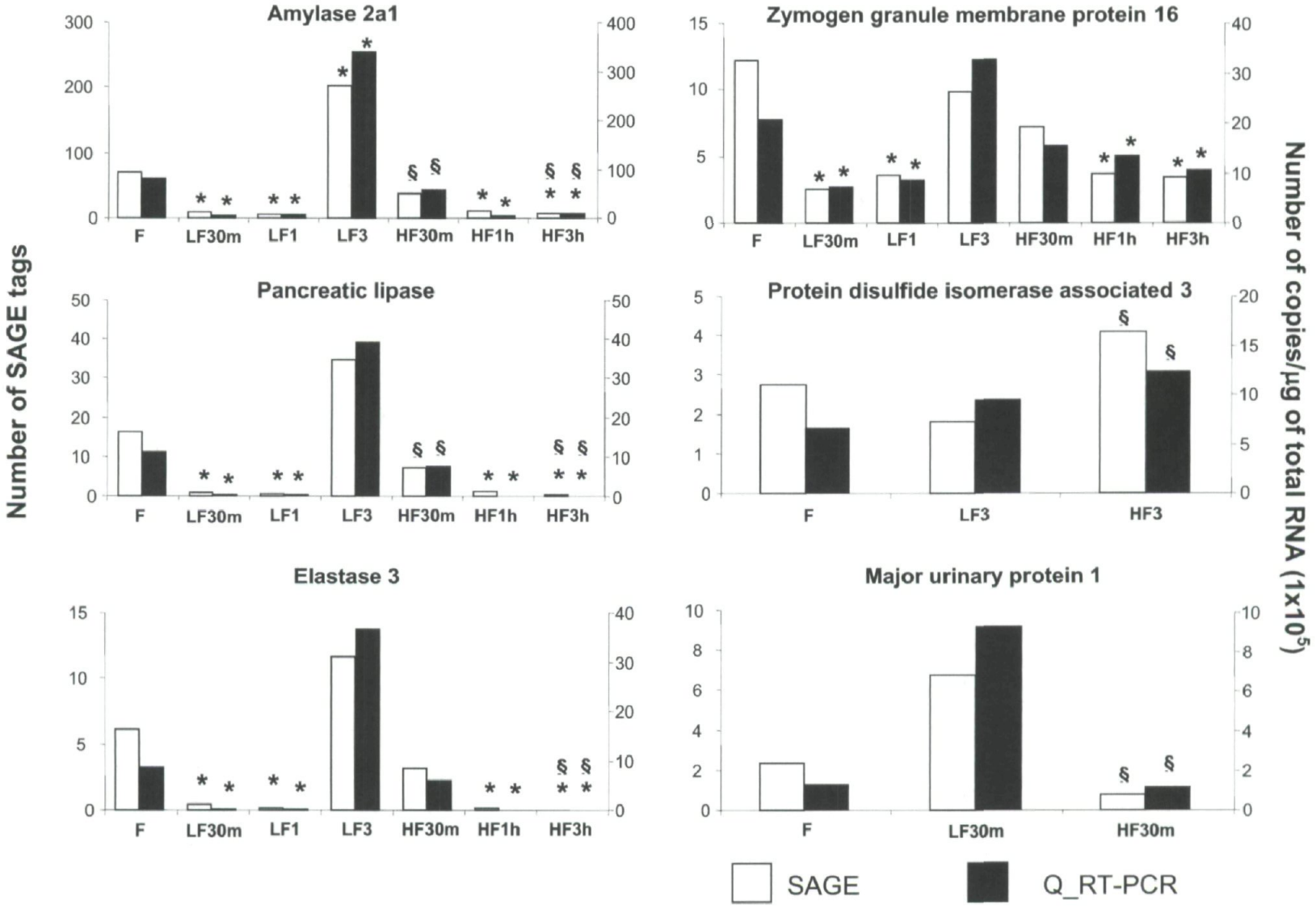


Figure 3. Serial analysis of gene expression (SAGE) and quantitative real-time PCR (Q_RT-PCR) results for some representative regulated genes.



CHAPTER 5

Trefoil factor family member 2 (*Tff2*) KO mice are protected from high-fat diet induced obesity

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Résumé

TFF2 est un peptide gastro-intestinal principalement connu pour ses fonctions de protection de la muqueuse. La consommation de repas riches en gras (HF) peut spécifiquement moduler la transcription de *Tff2* dans des tissus clés de la souris, ce qui indiquerait un rôle potentiel de ce gène dans le bilan énergétique. Les souris *Tff2* knock-out (KO) ont reçu une diète riche en gras et leur comportement alimentaire ainsi que leur métabolisme énergétique ont été analysés. Finalement, la déficience du gène *Tff2* a protégé les souris de l'obésité induite par la diète HF. Les souris *Tff2* KO ont montré un appétit plus élevé et une plus grande prise énergétique comparativement aux souris sauvages (WT), mais elles ont accumulé moins de poids et de dépôts adipeux, tout en préservant une masse maigre normale. Ces évidences suggèrent que TFF2 pourrait être une cible thérapeutique prometteuse pour le contrôle simultané de plusieurs mécanismes régulant le bilan énergétique.

Trefoil factor family member 2 (*Tff2*) KO mice are protected from high-fat diet induced obesity

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Running title: Tff2 deficiency alters energy balance in mice

Keywords: *Tff2* / HF intake / hypothalamus / energy metabolism

ABSTRACT

Trefoil factor family member 2 (TFF2) is a small gut peptide, mainly known for its protective and healing functions. High-fat (HF) feeding can specifically modulate *Tff2* transcription in key tissues of mice, suggesting a role in energy balance. *Tff2* knock-out (KO) mice were challenged with HF diet for 12 weeks. Food and energy intakes, body composition, as well as energy excretion and serum lipid and hormonal levels were analyzed. Finally, energy efficiency was estimated. *Tff2* KO mice showed a greater appetite and higher energy intake compared to wild-type (WT). Consistently, they presented lower levels of serum leptin, and increased transcription of agouti-related protein (*Agrp*) in the hypothalamus. Though energy and triglyceride fecal excretion were augmented in *Tff2* KO mice, digestible energy intake was superior. However, KO mice were finally protected from HF diet-induced obesity, and accumulated less weight and fat depots than WT animals, while keeping a normal lean mass. Energy efficiency was lower in HF-KO mice, while energy expenditure and locomotor activity were globally increased. The present work demonstrates previously unsuspected roles for *Tff2* and suggests it to be a mastermind in the control of energy balance and a promising therapeutic target for obesity.

INTRODUCTION

Several gut signals have important roles in modulating food intake, and therefore influence energy homeostasis [1, 2]. The trefoil factor family (TFF) in mammals comprises 3 peptides: TFF1, 2, and 3, predominantly expressed in the gastro-intestinal (GI) mucosa [3]. TFF2 is mainly secreted by gastric antral and pyloric glands, and by Brunner's glands of the duodenum [4]. The primary known role of TFF2 and the other members of the trefoil factor family is the protection of GI mucosa from internal and exogenous aggressions of the epithelium [5, 6]. In normal conditions, TFF2 works in association with mucins to the formation and stabilization of the mucus barrier, specifically monitoring gastric and duodenal sites [7, 8]. In response to injury, however, TFF2 can be rapidly expressed in the epithelia of the whole GI tract [6] and critically contribute to epithelial "restitution" and regeneration processes. In addition, TFF2 is able to modulate the immune response [9]. Previous reports have demonstrated that TFF2 is also expressed by lymphoid tissues and can influence immune cell chemotaxis and cytokine release [9, 10]. No study had previously associated TFF2 to the regulation of food intake and energy metabolism. We have recently demonstrated that *Tff2* expression is rapidly regulated by food intake in the mesenteric adipose tissue (AT) [11] and specifically modulated by high-fat (HF) intake in the duodenal mucosa of mice [12]. Moreover, *Tff2* modulation in the duodenum appeared to be temporally correlated to the regulation of apolipoprotein A4 (*ApoA4*), which is a well-known satiety factor associated with lipid intake-induced satiation [13]. We therefore selected *Tff2* as a novel candidate modulator of food intake and started to investigate its potential effects on feeding behavior in knock out (KO) mice. Previous studies have reported that *Tff2* KO mice are viable and fertile, though they present a reduced thickness

of the GI mucosa [6]. The current study is the first demonstrating that the lack of *Tff2* affects feeding behavior and energy metabolism in mice challenged with HF diet.

MATERIALS AND METHODS

Q_RT-PCR. Total RNA was extracted from the hypothalamus of 20 C57BL/6 male mice (12 week-old) / experimental group. The experimental design and diet specifications were previously described [14]. The gene name, GenBank accession number and region used for the primer pairs were the following: *Tff2*, NM_009363, region 136-302: TGACACCCCACAACAGAAAGAACT / CTTGCGAGCTGACACTTCCATG.

Food intake experiments in Tff2 KO mice. The 129/Sv-C57BL/6 *Tff2* KO mice were generated as previously described [15]. If not specified, a total of 32 KO (16 males and 16 females) and 26 WT mice (14 males and 12 females) participated in the experimental protocol. Of these, 29 mice were fed a LF diet (Research Diets # D12450CM) and other 29 received a HF diet (Research Diets # D08121503M) for a total of 12 weeks.

Short-term food intake experiments. After a 12h fasting, the mice had *ad libitum* access to a LF or HF meal, and their intake was measured at 30 minutes, 1h, 3h and 24h after the beginning of ingestion. For this set of experiments, a total of 40 KO (28 males and 12 females) and 37 WT (29 males and 8 females) mice were alternatively tested (12-15 week-old). All females were tested outside the periovulatory / ovulatory phase of the ovarian cycle, in order to control for the hormonal effects on food intake [16].

Long-term food intake experiments. The diets started when the mice were 16 week-old. During the 12 weeks of LF or HF diet, mice had *ad libitum* access to food and water. Each two or three days, food consumption was measured and fresh food was added; health status was also checked and body weight assessed. At the end of the diet, the 4h-fasted mice were randomly sacrificed starting at 1 p.m. (light cycle). Male mice were anesthetized with a commensurate dose of ketamyne-xylazine and perfused with ice-cold isotonic saline

before collecting the whole brain. Female mice were anesthetized under isoflurane and sacrificed by cervical dislocation. The following tissues were collected and weighed: mesenteric, retroperitoneal, gonadal ATs; liver; stomach and duodenum mucosa; BAT; right gastrocnemius skeletal muscle. The carcasses were kept and individually stored at -40°C for further analyses. The Q_RT-PCR method was used for the quantification of *Ucp1* mRNA in the BAT. Total RNA was extracted from the interscapular BAT depots of 16 *Tff2* KO and 14 WT male mice at the end of the diet. The technical specifications of the Q_RT-PCR procedures have been previously described [14, 17]. The gene name, GenBank accession number and region used for the primer pairs were the following: *Ucp1*, NM_009463, TGACGTCCCCTGCCATTTACTG / CGCAGAAAAGAAGCCACAAACC.

Initials. A total of 6 KO and 6 WT mice per diet and sex were kept as representative of the pre-diet condition and sacrificed when they were 16 week-old. These mice and their tissues were kept as pre-condition controls when needed.

Serum analyses. Leptin and insulin serum levels were measured by immunoassay ELISA kits (SpiBIO and ALPCO, respectively). Leptin levels were determined at weeks 0, 8 (in males, 16 KO and 13 WT) and 12. Serum insulin concentration was measured at week 12.

In situ hybridization. A total of 27 male mice (16 KO and 12 WT) were anesthetized with an intra-peritoneal injection of ketamine (150 mg/kg)-xylazine (10 mg/kg) and, without delay, intra-cardially perfused with 200 ml of ice-cold isotonic saline. The brains were removed and kept in paraformaldehyde (4%) for 7 days, transferred to a solution containing paraformaldehyde (4%) and sucrose (10%), and stored at -80°C before use. The brains were cut using a sliding microtome (Histoslide 2000, Reichert-Jung, Germany).

Brain sections (25 µm) were taken to include the whole hypothalamic ARC, and stored at -30°C in a cryoprotectant solution containing sodium phosphate buffer (50 mM), ethylene glycol (30%), and glycerol (20%). *In situ* hybridization histochemistry was used to localize *Pomc* and *Agrp* mRNA on ARC brain sections. The protocol was adapted from the technique described by Simmons [18] as previously reported [19]. *Pomc* and *Agrp* slides were exposed for 3 and 10 days respectively.

Fecal energy and digestible energy. Individual mouse feces were collected over 48 h at weeks 1, 4 and 12. The mean 24 h dry weight was determined. Fecal energy was measured by adiabatic bomb calorimetry, using the Parr 6100 bomb. The energy / gram of stools was determined, and the energy excretion / day was calculated. For each mouse, the total 12 weeks energy excretion was also derived, and subtracted to its total *gross* energy intake (gross energy / gram of LF food: 17.5 kJ; gross energy / gram of HF food: 24.8 kJ). The result estimated the global *digestible* energy attained by each individual in 12 weeks of diet. Serum triglyceride concentrations were measured in 16 KO and 13 WT male mice by a colorimetric enzymatic method, using the Triglyceride Quantification Kit (Biovision Inc., USA).

Body composition. At week 12 of diet, and a few days before the necropsy, body composition was analyzed using the Bruker's Minispec LF90II (Bruker Optics, Germany). Fat mass, lean mass and fluids were measured in grams by the analyzer, and the measurement was repeated three times for each mouse. The % of fat mass and lean mass / body weight were calculated.

Carcass analyses and energy gain. To estimate carcass energy, fat and protein contents, carcasses were processed as previously described [20]. To calculate energy gain,

initial contents were estimated from the live body weight of KO and WT mice with reference to a baseline group of initials (six per phenotype) sacrificed at the beginning of the experimental period.

Statistical analyses. Q_RT-PCR data were analyzed by the one-way ANOVA ($p < 0.05$). For all other tests, the data were analyzed by the two-way (genotype and diet) or three-way (genotype, diet and sex) ANOVA ($p < 0.05$). When the ANOVA revealed a significant interaction between two variables, the Tukey Kramer post-hoc test was performed to identify the significant difference between the two groups.

RESULTS & DISCUSSION

Tff2 gene is expressed in the hypothalamus and regulated by food intake. Various gut peptides with a role in modulating appetite and satiety are also expressed and/or have a specific receptor in the hypothalamus [21, 22]. Previous investigations have found no or very faint expression of *Tff2* in the hypothalamus of normal female mice that had been fed *ad libitum* [8, 23]. We used the quantitative real time PCR (Q_RT-PCR) method to further explore the expression of *Tff2* in hypothalamic samples of male mice, and verify the effects of fasting and feeding on its transcription. The mRNA of three groups of normal mice was analyzed: fasted, and sacrificed 3 hours after the beginning of a low-fat (LF3h) or HF (HF3h) meal (Figure 1). Interestingly, *Tff2* transcription was elevated in fasted state but substantially down-regulated by both LF3h and HF3h meals (6-fold and 27-fold reduction, respectively). Moreover, HF intake specifically down-regulated *Tff2* transcription compared to LF (4-fold reduction). To our knowledge, this represents the first evidence that *Tff2* is expressed and short-term modulated by different nutritional conditions in the hypothalamus.

Higher appetite and energy intake in Tff2 KO mice. We have studied the short-term and long-term feeding behavior, as well as energy metabolism in *Tff2* KO mice challenged with HF diet. Following 12 hours fasting, *Tff2* KO mice showed greater appetite and higher drive to consume a HF meal compared to controls (LF24h: WT, 4.3±0.1 g vs. KO, 4.9±0.1 g; HF24h: WT, 2.8±0.1 g vs. KO, 4.0±0.1 g). Energy intake results were also consistent, showing higher values at 24h for the HF-KO group (Figure 2A). In particular, while WT animals consumed more energy with the LF meal, the KO mice ingested a higher amount of energy from HF food. In long-term experiments, two distinct groups of 16-week-old

mice started a LF or HF diet, and were given *ad libitum* access to food for 12 weeks. In line with short-term evidences, 12-weeks food and gross energy intakes were significantly higher in *Tff2* KO mice compared to WT (Figure 2, B and C). However, the specific effect observed at 24 h in HF-KO vs. WT mice was blunted by the adaptation to long-term diet, and became not significant. Finally, gross energy intake was still more elevated in KO animals, yet both KO and WT mice consumed more energy from the LF than HF food. The latter aspect may appear in contradiction with other, but not all, models of diet-induced obesity (DIO) [24, 25], in which HF-derived energy intake normally prevails and leads to obesity. However, the hybrid 129Sv-C57BL6 strain used to generate the KO mice is more resistant to HF-DIO compared to pure obesity-prone C57BL6 strain [26], generally analyzed in DIO studies. In particular, 129Sv background has been already associated with increased LF vs. HF consumption, and reduced susceptibility to develop obesity and diabetes, compared to C57BL6 [26]. Therefore, it is of interest to observe the effects of *Tff2* deficiency in such a peculiar strain, when challenged with HF diet. Another important aspect to consider is that the prevalence of LF-derived energy intake was significantly associated with female groups (Figure 2C). The differential effects between male and female food intake became apparent in long-term but not short-term experiments, in which tests were conducted outside appetite-influencing phases of the ovarian cycle [27]. Estrogens are considered important determinants of gender dimorphism in feeding behavior, fat ingestion and body weight regulation [28]. In future studies, it would be useful to examine the potential functional relationships between *Tff2* and estrogens, as possible connections can be expected and have already been suggested in literature [29].

Altered levels of satiety/appetite signals in Tff2 KO mice. Since food intake was significantly different between *Tff2* KO and WT mice, we analyzed the hypothalamic expression of two key regulatory factors: proopiomelanocortin (*Pomc*) and agouti-related protein (*Agrp*). While *Pomc* product is known to negatively influence food intake, *Agrp* is a strong activator of appetite signals [30]. After 12 weeks of diet, we assessed by *in situ* hybridization the levels of these two transcripts in the hypothalamic arcuate nucleus (ARC) of male mice. While the levels of *Pomc* mRNA were comparable between the two genotypes (data not shown), we observed a significantly higher expression of *Agrp* transcript in the ARC of *Tff2* KO mice (in pixel density, LF: WT, 35.4±2.6 vs. KO, 44.8±3.5; HF: WT, 28.2±1.3 vs. KO, 47.6±3.0) (Supplementary figure S1). The latter findings are consistent with greater appetite and food intake in KO animals, and were further confirmed, as we detected substantially lower serum levels of the long-acting satiety hormones leptin (Table 1) and insulin (data not shown), compared to WT. In particular, leptin serum levels appeared to be constitutively lower in *Tff2* KO mice, before starting the diet as well as 8 and 12 weeks after LF or HF *ad libitum* consumption. Interestingly, circulating leptin was specifically reduced in HF-KO mice (8 and 12 weeks). Though leptin levels are known to proportionally correlate with adipose mass [31], pre-diet fat mass was comparable between KO and WT mice (data not shown), while fat stores were larger in HF vs. LF-consuming KO mice. This may suggest that other specific mechanisms affected the decreased leptin secretion in KO animals and, interestingly, in the HF-KO group of mice. Food deprivation is a prominent cause of leptin drop, which in turn is a rapid and powerful trigger of appetite and food intake [31]. As mentioned, 12h-fasting specifically stimulated

the short-term HF intake in *Tff2* KO mice, and this could represent another insight into a role for *Tff2* in the regulation of appetite / satiety mechanisms elicited by HF eating.

Tff2 KO mice excrete more energy. Regardless of the diet, *Tff2* KO mice excreted a higher quota of energy in the stools compared to WT controls (Supplemental figure S2). In particular, KO feces contained a greater fraction of triglycerides, suggesting an elevated loss of lipids. However, the deficit of fat absorption did not directly affect energy intake/efficiency in *Tff2* KO mice because, after adjusting for energy excretion, the overall level of energy available for utilization (or *digestible* energy intake) resulted still higher in *Tff2* KO than WT mice (Figure 2C). However, *Tff2* KO mice could not efficiently store the acquired energy.

Tff2 KO mice are leaner and have reduced fat mass. After 12 weeks of diet, *Tff2* KO mice were leaner, showing a lower body weight gain compared to WT (Figure 3). Furthermore, they accumulated less fat mass than WT controls. This was proved by analyzing the body composition in living animals, as well as by estimating the lipid-derived energy in the carcasses. A few days before the necropsy, at the 12^{th} week of diet, KO mice showed lower fat and lean masses (in grams) compared to WT. However, they presented less fat mass but higher lean mass / body weight percentage (%) compared to WT animals (Figure 3). After the necropsy, the energy content of mouse carcasses was evaluated, and the energy derived from fat and protein contents was calculated. The results demonstrated that *Tff2* KO mice gained less energy compared to WT animals (two-fold difference) (Figure 4). Remarkably, this effect became specifically significant when mice consumed a HF diet. Moreover, the decreased energy gain may be specifically due to lower fat accumulation, since we found no difference of protein gain between KO and WT animals

(Figure 4). Finally, *Tff2* KO mice presented reduced intra-abdominal fat depots (Supplementary figure S3). The latter point is of special interest, as we had previously found the expression of *Tff2* in the mesenteric AT, and its modulation by feeding [11]. In the future, it would be important to detect the cellular source of *Tff2* expression in white AT and the specific role of this gene in the physiology of such an important site for energy metabolism control and metabolic diseases [32]. As mentioned, *Tff2* is considered a cytokine-like molecule, which can be expressed by macrophages and other immune cells, and regulate chemotaxis [9]. Moreover, it cannot be excluded that TFF2 might be directly secreted by adipose cells [33]. It is relevant to highlight that, despite the impaired fat storing, KO mice were still able to accumulate lean mass (Figure 4); and the % of gastrocnemius skeletal muscle / body weight was specifically increased in HF-KO mice (data not shown). The latter elements would suggest that the reduced capacity to store energy could be specifically restricted to fat accumulation.

Increased energy expenditure and reduced energy efficiency in Tff2 KO mice. Tff2 KO animals showed higher energy intake, but they did not efficiently store the energy ingested. Coherently, energy efficiency was substantially lower in KO animals (two-fold difference; Figure 5A), and specifically reduced in HF-KO vs. HF-WT mice. We finally estimated energy expenditure by subtracting the accumulated energy from digestible energy intake, and found that *Tff2* KO mice expended significantly more energy than WT controls (Figure 5B). KO mice also showed enhanced spontaneous locomotor activity, as verified through behavioral tests in male mice (data not shown). In addition, while the brown AT (BAT) was smaller, the number of uncoupling protein 1 (*Ucp1*) mRNA copies / whole tissue was significantly higher in KO vs. WT mice (Supplementary figure S4). *Ucp1*

transcription is a key marker of enhanced thermogenesis and energy expenditure [34]. The smaller size of the BAT may therefore indicate an enhanced thermogenic activity, and future studies should verify this possibility.

The present work highlights previously unsuspected roles for *Tff2* in the regulation of energy balance. *Tff2* deficiency influenced food intake and substantially modified energy metabolism, finally protecting KO mice from DIO. Interestingly, *Tff2* transcription and its functions may be specifically related to HF intake, thus rendering it a novel optimal target for studying the impact of HF consumption on satiety mechanisms and energy metabolism. It is reasonable to suppose that the roles exerted by *Tff2* in the peripheral tissues may be distinct compared to its functions in the hypothalamus. As a matter of fact, the expression of this gene was early up-regulated after re-feeding in the duodenum [12] and mesenteric AT [11] of normal mice, but significantly down-regulated in the hypothalamus. The present lack of one clear receptor for TFF2 leads to presume that this secreted protein may interact with several molecules, even at low affinity, depending on the site of expression and presently unknown regulatory mechanisms [35-37]. Furthermore, it cannot be excluded that the potential role of *Tff2* in the modulation of energy balance may represent an important novel aspect of its immuno-regulatory functions. In line with that, Kurt-Jones et al. reported a potential functional association between *Tff2* and interleukin 1β receptor (IL1βR) signaling [9]. TFF2 is thought to be involved in the downstream signaling events following IL1βR activation, potentially having a relevant impact on important aspects of inflammation as well as energy metabolism.

In conclusion, this work uncovered the potential of *Tff2* deficiency to induce significant changes at multiple levels of energy balance control, namely food intake, lipid absorption and storing, and energy expenditure. The simultaneous regulation of these critical control points is not, or scarcely, attainable by presently available obesity pharmacotherapies [38]. However, it is well known that effective and long-lasting weight loss may be achievable only by a combined therapy that integrates the reciprocal adjustments of all energy balance components. Therefore, unraveling the mechanisms through which *Tff2* acts in both peripheral and central regulatory hubs may lead to design a unique pharmacologic tool that simultaneously targets lipid absorption, energy expenditure and feeding behavior to treat obesity and related complex diseases.

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DECLARATIONS OF INTEREST & AUTHOR'S CONTRIBUTIONS

The present article has been approved by all listed authors and there is no conflict of interest that would prejudice its impartiality.

MRDG collected, analyzed and interpreted the data, and drafted the manuscript. MY, IR, OM and RGC contributed to the collection, analysis and interpretation of the data. AAS and NB worked to the generation of *Tff2* KO mice and critically revised the manuscript. DR contributed to design the study, specifically carcass energy analyses and *in situ* hybridization procedures, and critically revised the manuscript. MY and JStA conceived the study, designed it and critically revised the manuscript. JStA gave the final approval of the version to be published.

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FIGURE LEGENDS

FIGURE 1. *Tff2* mRNA expression in the hypothalamus of male mice fed LF or HF meal for 3h after fasting.

The 1-way ANOVA with the Tukey-Kramer post-hoc test revealed effect of feeding. Significant difference from *fasting and $^{\#}$LF ($p<0.05$). N=3 per experimental condition.

FIGURE 2. Effects of *Tff2* deficiency on cumulative energy intake.

[A] Cumulative energy intake for 24h in *Tff2* KO and WT mice fed LF or HF meal.

The 4-way ANOVA with repeated measurements revealed effects of genotype[a] (KO>WT), time[b] (30min<1h<3h<24h), genotype × diet interaction (KO>WT in HF condition by a contrast analysis adjusted for multiple comparisons[c]), and genotype × diet × time interaction (KO>WT at 24h in HF condition by a contrast analysis adjusted for multiple comparisons[d]).3 Number of mice: Male WT (29), male KO (28), female WT (8), and female KO (12).

[B] Cumulative gross energy intake during 12 wks of LF or HF feeding in *Tff2* KO and WT mice.

The 3-way ANOVA revealed effects of genotype[a] (KO>WT), diet[e] (HF<LF), and diet × sex interaction[f] (However, the Tukey-Kramer post-hoc test did not reveal any statistical significance). Number of mice: Male LF-WT (7), male LF-KO (7), male HF-WT (7), male HF-KO (9), female LF-WT (7), female LF-KO (8), female HF-WT (5), and female HF-KO (8).

[C] Cumulative digestive energy intake during 12 wks of LF or HF feeding in *Tff2* KO and WT mice.

The 3-way ANOVA revealed effects of genotype[a] (KO>WT), diet[e] (HF<LF), and diet × sex interaction (HF<LF in female mice by the Tukey-Kramer post-hoc test[g]). Number of mice: Male LF-WT (7), male LF-KO (7), male HF-WT (7), male HF-KO (9), female LF-WT (7), female LF-KO (8), female HF-WT (5), and female HF-KO (8).

FIGURE 3. Body weight and body composition of *Tff2* KO mice.

Body weight: The 3-way ANOVA revealed effects ($p<0.05$) of genotype[a] (KO<WT), diet[b] (HF>LF), sex[c] (male>female), and genotype × sex interaction (KO<WT in male mice by the Tukey-Kramer post-hoc test[d]).

Fat mass: The 3-way ANOVA revealed effects of genotype[a] (KO<WT) and diet[b] (HF>LF).

Lean mass: The 3-way ANOVA revealed effects of genotype[a] (KO>WT) and diet[b] (HF<LF).

Data are mean ± SEM.Number of mice: Male LF-WT (7), male LF-KO (7), male HF-WT (7), male HF-KO (9), female LF-WT (7), female LF-KO (8), female HF-WT (5), and female HF-KO (8).

FIGURE 4. Changes of body weight, carcass fat and carcass protein during 12 wks of LF or HF feeding in *Tff2* KO and WT mice.

Body weight and carcass fat gains: the 3-way ANOVA revealed effects of genotype[a] (KO<WT), diet[b] (HF>LF), and genotype × sex interaction (KO<WT in male mice by the Tukey-Kramer post-hoc test[c]).

Carcass protein gain: the 3-way ANOVA revealed effect of sex (male>female).

FIGURE 5. Reduced energy efficiency and increased energy expenditure in *Tff2* KO mice.

[A] Gross and digestive energy efficiency during 12 wks of LF or HF feeding in *Tff2* KO and WT mice.

Energy efficiency was calculated from the following equation: carcass energy gain divided by cumulative gross/digestive-energy intake during the 12-wks period. The 3-way ANOVA revealed effects of genotype[a] (KO<WT), diet[a] (HF>LF), and genotype × sex interaction (KO<WT in male mice by the Tukey-Kramer post-hoc test[c]).

[B] Total energy expenditure in *Tff2* KO and WT mice fed LF or HF diet for 12 wks.

The 3-way ANOVA revealed effects of genotype[a] (KO>WT) and diet[b] (HF<LF), as well as trend ($p<0.06$) in diet × sex interaction (HF<LF in female mice by the Tukey-Kramer post-hoc test[c]).

Number of mice: Male LF-WT (7), male LF-KO (7), male HF-WT (7), male HF-KO (9), female LF-WT (7), female LF-KO (8), female HF-WT (5), and female HF-KO (8).

List of abbreviations in tables and figures

HF, high-fat; **LF**, low-fat; ***Tff2***, trefoil factor family member 2.

Table I. Leptin serum levels at weeks 0, 8 and 12 of LF or HF diet

		Leptin (ng/mL)		
		Week 0	**Week 8**	**Week 12**
Males	**WT-LF**	11,2 ± 2,2	24,7 ± 7,9	30,7 ± 6,1
	KO-LF	2,8 ± 0,7	3,7 ± 1,2	3,2 ± 0,6
	WT-HF	8,5 ± 2,3	59,4 ± 6,7	87,8 ± 7,2
	KO-HF	3,5 ± 0,5 [a, b, e]	8,9 ± 1,9 [a, b, e]	11,5 ± 2,6 [a, b, c, e, f]
Females	**WT-LF**	-	-	17,5 ± 4,8
	KO-LF	-	-	3,8 ± 0,9
	WT-HF	-	-	31,0 ± 14,6
	KO-HF	-	-	13,9 ± 8,4

Data are means ± SEM. Significant effect ($p < 0.05$) of: ***a****, Tff2 KO (Tff2 KO mice < WT mice);* ***b****, diet (HF diet > LF diet);* ***c****: sex (males > females);* ***e****, Tff2 KO* x *diet interaction (KO-HF < WT-HF);* ***f****, Tff2 KO* x *sex interaction (KO males < WT males). Statistical annotations referring to all genotype, diet and sex groups are displayed in HF-KO male cells.*

Figure 1

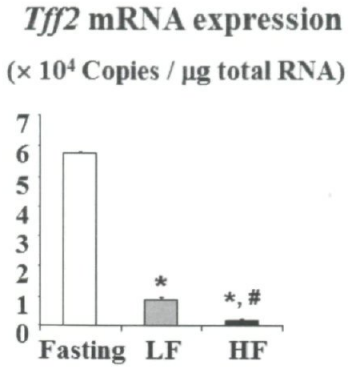
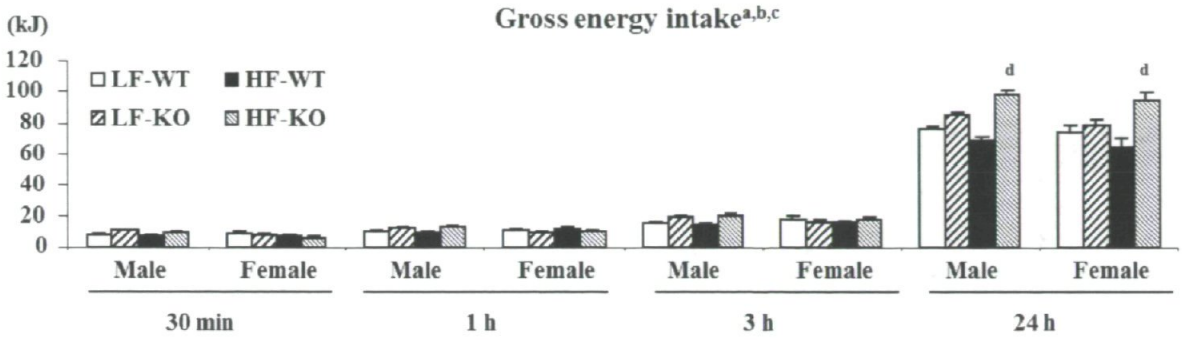
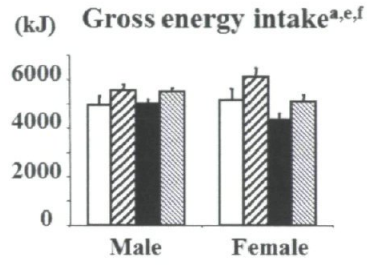

Tff2 mRNA expression
(× 10⁴ Copies / µg total RNA)
7
6
5
4
3
2
1
0
*
*, #
Fasting
LF
HF

Figure 2

[A]


Gross energy intake[a,b,c]
(kJ)
120
100
80
60
40
20
0
LF-WT
HF-WT
LF-KO
HF-KO
d
d
Male
Female
Male
Female
Male
Female
Male
Female
30 min
1 h
3 h
24 h

[B]


(kJ)
Gross energy intake[a,e,f]
6000
4000
2000
0
Male
Female

[C]

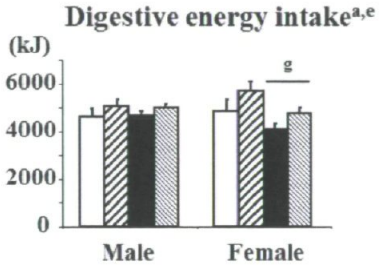

Digestive energy intake[a,e]
(kJ)
6000
4000
2000
0
g
Male
Female

Figure 3

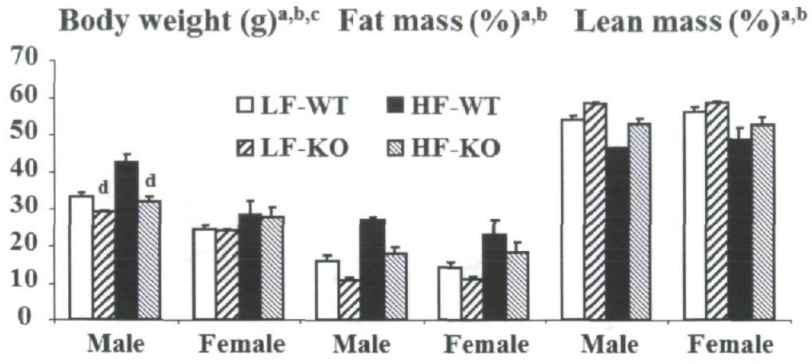

Body weight (g)a,b,c
Fat mass (%)a,b
Lean mass (%)a,b
70
60
50
40
30
20
10
0
LF-WT
HF-WT
LF-KO
HF-KO
d
d
Male
Female
Male
Female
Male
Female

Figure 4

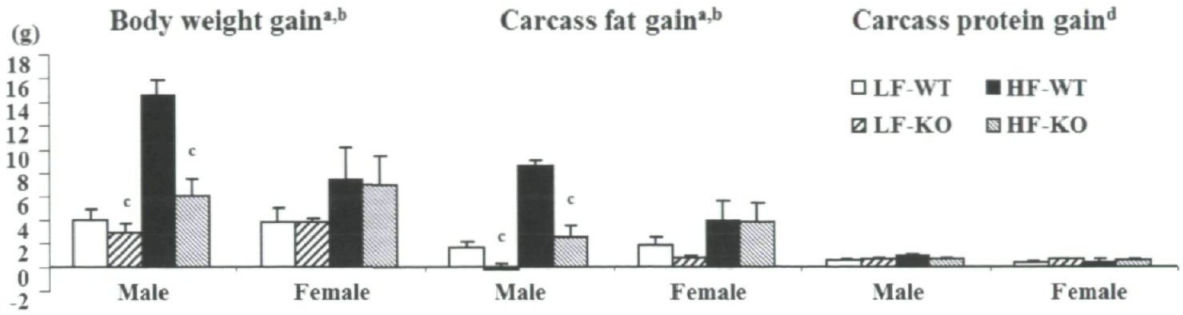
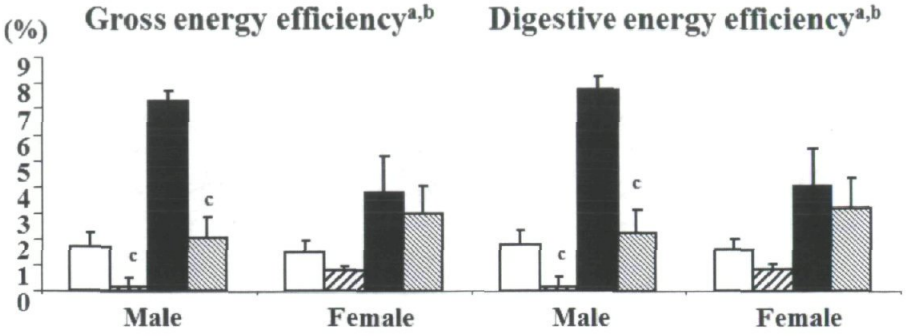
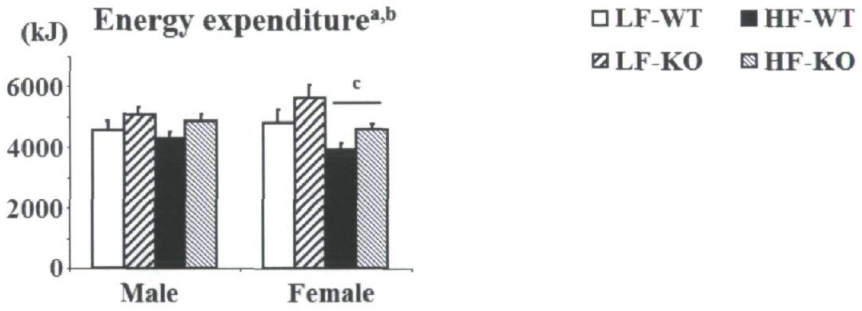

Body weight gain[a,b]
Carcass fat gain[a,b]
Carcass protein gain[d]
(g)
18
16
14
12
10
8
6
4
2
0
-2
LF-WT
HF-WT
LF-KO
HF-KO
c
c
c
c
Male
Female
Male
Female
Male
Female

Figure 5

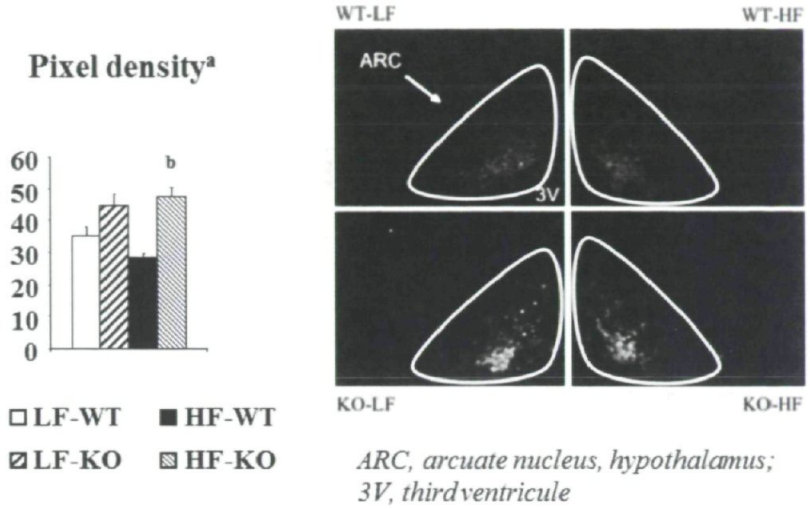
[A]


(%)
Gross energy efficiency[a,b]
Digestive energy efficiency[a,b]
9
8
7
6
5
4
3
2
1
0
c
c
c
c
Male
Female
Male
Female

[B]


(kJ)
Energy expenditure[a,b]
LF-WT
HF-WT
LF-KO
HF-KO
6000
4000
2000
0
c
Male
Female

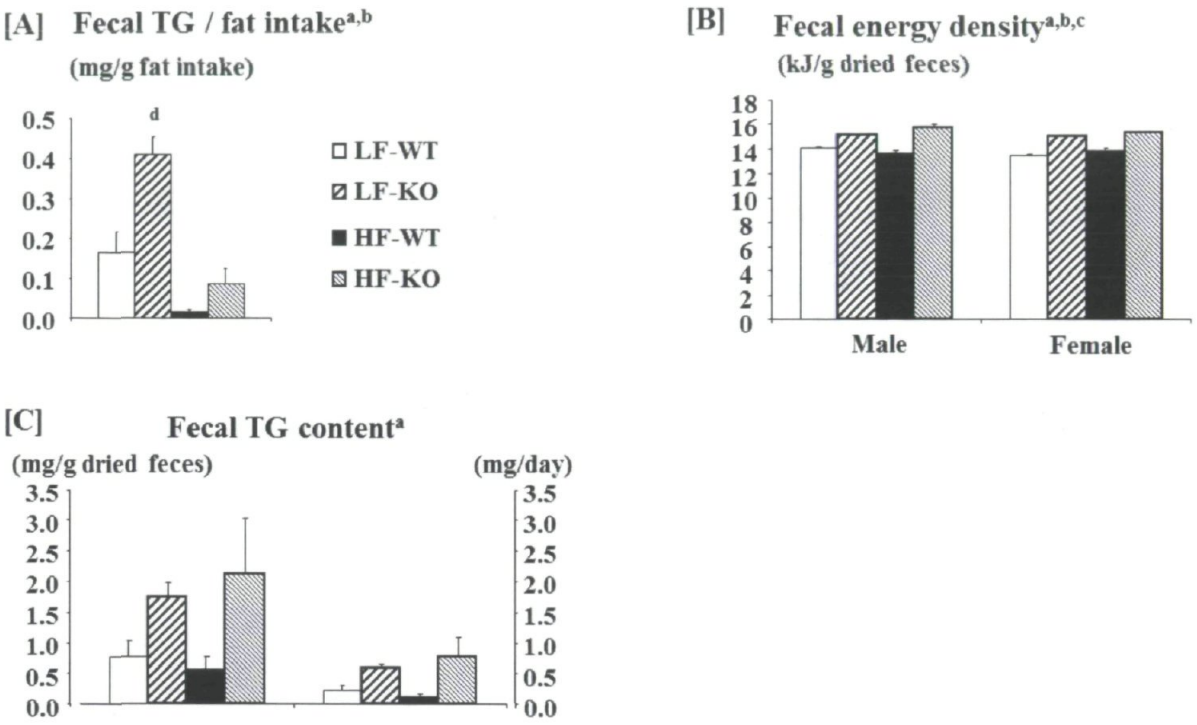
Supplementary figure S1. Agouti-related peptide (*Agrp)* mRNA expression in ARC of hypothalamus after 12 wks of LF or HF diet in *Tff2* KO and WT male mice.



ARC, arcuate nucleus, hypothalamus; 3V, third ventricule

The 2-way ANOVA revealed effect of genotype[a] (KO>WT), as well as trend ($p<0.09$) in effect of genotype × diet interaction (KO>WT in HF condition by the Tukey-Kramer post-hoc test[b]).
Number of mice: Male LF-WT (7), male LF-KO (7), male HF-WT (7), and male HF-KO (9).

Supplementary figure S2.



[A] **Fecal TG content / fat intake at 12th wk of LF or HF feeding in *Tff2* KO and WT male mice.**
The 2-way ANOVA revealed effects of genotype[a] (KO>WT), diet[b] (HF<LF), and genotype × diet interaction (KO>WT in LF condition by the Tukey-Kramer post-hoc test[d]).
Number of mice: Male LF-WT (7), male LF-KO (7), male HF-WT (7), and male HF-KO (9).

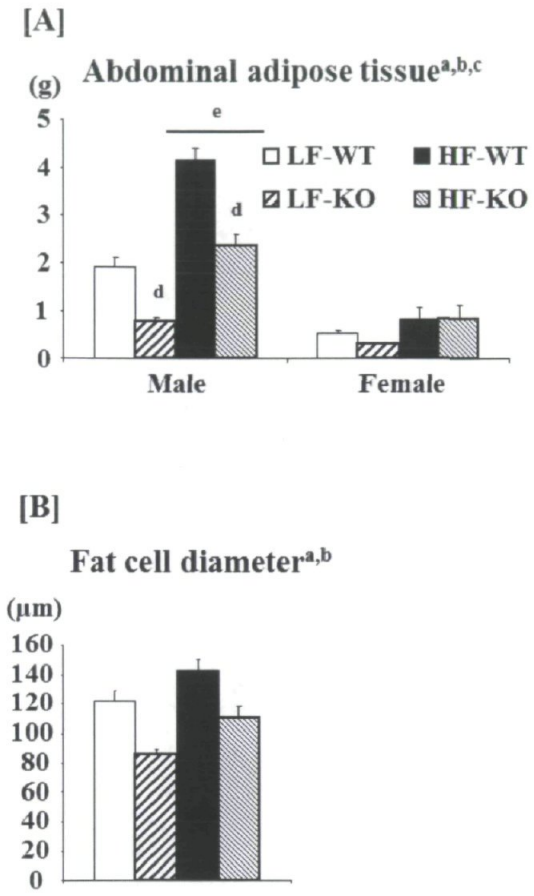
[B] **Mean fecal energy density during 12 wks of LF or HF feeding in *Tff2* KO and WT mice.**
The average of fecal energy density at 1st, 4th, 8th and 12th wks of HF or LF feeding was used for the statistical analysis. The 3-way ANOVA revealed effects of genotype[a] (KO>WT), diet[b] (HF>LF), and genotype × diet × sex interaction (KO>WT in HF condition of male, KO>WT in LF condition of male, KO>WT in HF condition of female, and KO>WT in LF condition of female mice by the Tukey-Kramer post-hoc test[c]).
Number of mice: Male LF-WT (7), male LF-KO (7), male HF-WT (7), male HF-KO (9), female LF-WT (7), female LF-KO (8), female HF-WT (5), and female HF-KO (8).

[C] **Fecal triglyceride (TG) content at 12th wk of LF or HF feeding in *Tff2* KO and WT male mice.**

The 2-way ANOVA revealed effect of genotype[a] (KO>WT).

Number of mice: Male LF-WT (7), male LF-KO (7), male HF-WT (7), and male HF-KO (9).

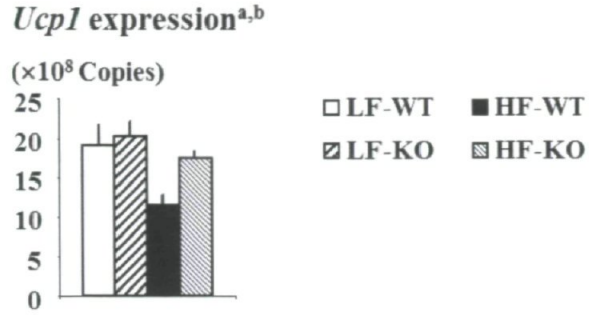
Supplementary figure S3.



[A] **Abdominal adipose tissue weight after 12 wks of LF or HF diet in *Tff2* KO and WT mice.**
The 3-way ANOVA revealed effects of genotype[a] (KO<WT), diet[b] (HF>LF), sex[c] (male>female), genotype × sex interaction (KO<WT in male mice by the Tukey-Kramer post-hoc test[d]), and diet × sex interaction (HF>LF in male mice by the Tukey-Kramer post-hoc test[e]).
Number of mice: Male LF-WT (7), male LF-KO (7), male HF-WT (7), male HF-KO (9), female LF-WT (7), female LF-KO (8), female HF-WT (5), and female HF-KO (8).

[B] **Retroperitoneal fat cell diameter after 12 wks of LF or HF diet in *Tff2* KO and WT male mice.**
The 2-way ANOVA revealed effects of genotype[a] (KO<WT) and diet[b] (HF>LF).
Number of mice: Male LF-WT (7), male LF-KO (7), male HF-WT (7), and male HF-KO (9).

Supplementary figure S4. Uncoupling protein 1 (*Ucp1*) mRNA expression in whole brown adipose tissue of *Tff2* KO and WT male mice fed LF or HF diet for 12 wks.



The 2-way ANOVA revealed effects of genotype[a] (KO>WT) and diet[b] (HF<LF). Number of mice: Male LF-WT (7), male LF-KO (7), male HF-WT (7), and male HF-KO (9).

CHAPTER 6: Conclusions

The search for novel therapeutic targets for the prevention and treatment of obesity led us to focus on the early events that acutely modulate food intake, and how these events affect the commensurate physiologic responses that preserve energy balance. The project presented in this thesis followed two experimental lines. The first line aimed at the identification of novel signals and mechanisms responsible for the augmented obesity risk observed in post-menopausal women. I contributed to this experimental line with one publication, and the results are described in Chapter 2. The largest contribution of my doctoral studies was committed to: 2a) the identification of novel transcripts specifically regulated by HF meal consumption in key tissues for the control of energy balance, namely hypothalamus and stomach (Chapters 3 and 4); and 2b) the first crucial experimental steps for the functional in vivo characterization of *Tff2*, a gene that had been specifically regulated by HF intake in the duodenum mucosa (Chapter 5).

DHT-induced early transcriptional changes in the adipose tissue of ovariectomized mice: main findings

Some authors have recently pointed out the role of the intermediate phase that leads to menopause, namely the menopause transition, in influencing women health status. During this heterogeneous transitional phase, significant changes in the relative equilibria between estrogens and androgens may be crucial. In particular, the relative though temporary prevalence of androgen levels may critically contribute to those changes in food intake, fat deposition and energy expenditure that progressively characterize menopausal women, and that increase their risk to develop central obesity and related metabolic diseases [23]. As described in Chapter 2, we were interested in elucidating the transcriptional variations that rapidly follow the rise in androgen levels in ovariectomized mice, through analyzing the effects of a single dose of DHT on the gene expression of retroperitoneal adipose tissue. Our results demonstrated for the first time that the acute androgen-induced regulation of gene expression in the adipose tissue can lead to a myogenic-like transcriptional program. We showed the up-regulation of several genes specifically associated to skeletal muscle as well as the over-expression of energy production-related transcripts and of important genes

for Ca^{2+} uptake/release, seven of which are known to code for important sarcoplasmatic reticulum-associated molecules. The evidence that the first 4 most expressed genes in the adipose tissue 24 hours after DHT injection exactly corresponded to the 4 most expressed mRNAs in female intact skeletal muscle further confirmed the extraordinary plasticity potential of adipose tissue. However, the results obtained from this study could not allow us to select significant androgen-regulated genes that may be responsible for the progressive shift toward post-menopausal metabolic profile and increased obesity risk. The differences between acute and chronic effects of androgens on male adipose tissue [474, 475] and the current paucity of knowledge about acute androgen effects in females led us to conclude that further studies are needed to define an experimental model that could be better representative of the menopause transitional phase.

HF intake-induced early transcriptional changes in the hypothalamus and gastric mucosa of mice: main findings

Studies in both animals and humans have unequivocally demonstrated that HF intake represents a weaker inducer of satiety and satiation signals, and thus may facilitate passive overconsumption and excessive energy intake, that progressively de-stabilize the metabolic controls of homeostasis [47]. We hypothesized that still unknown early signals differentially induced by LF and HF foods exist, and may not only explain their distinct impact on satiation but also be scrutinized as potential therapeutic targets to prevent overconsumption. We therefore analyzed the gene expression of key tissues in mice, and compared the differential effects engendered by LF or HF meal intake and fasted state. I contributed to the analyses of hypothalamus and stomach transcriptome, while mesenteric adipose tissue and duodenum mucosa had been previously studied [476, 477]. We finally selected a number of significant novel genes that had been specifically modulated by HF intake in these tissues, and chose to characterize the potential novel properties of *Tff2*.

In both hypothalamus and stomach, a small number of genes was differentially modulated by LF or HF feeding. In particular, we investigated the transcriptional changes occurring 3 hours after the beginning of the meal in the hypothalamus, and those induced 30 minutes, 1 hour and 3 hours after the start of meal intake in the gastric mucosa. In the hypothalamus, twelve genes were globally regulated by feeding compared to fasting, and

only two were differentially modulated between LF and HF. Totally, two transcripts involved in mitochondrial functions (*MtCo1* and *Ppid*), three involved in protein transport and regulation (*Ube2q2*, *Mup1* and *Sec13*), one implicated in the cellular pH control (*Slc4a3*) and a transcript with a role in the epigenetic regulation of gene expression (*Setd3*) were found to be differentially expressed. However, a reduced number of genes were modulated in such a key tissue for the control of food intake. This might have depended on the absence of significant transcriptional events at the chosen time point, or be related to the peculiar structural organization of the hypothalamus in distinct functional nuclei. The need for a large amount of mRNA as required by SAGE procedures led us to analyze a pool of whole mouse hypothalami, whereas the targeted analysis of single nuclei, such as the ARC or the PVN for instance, might have isolated and disclosed further specific changes in gene expression. In the stomach, both LF and HF meals modified the gene expression in gastric mucosa, and 17 known genes were differentially modulated by HF compared to LF. Among these, several pancreas-related transcripts coding for digestive enzymes were regulated. This study confirmed the possibility that genes generally considered to be specifically expressed in the pancreas can also be produced in other tissues, such as the stomach, but also the duodenum mucosa, as we had previously shown [477]. Furthermore, we demonstrated for the first time that their transcriptional levels are differentially regulated by distinct nutritional stimuli. The redundant and over-abundant expression of digestive-enzyme coding genes in the GI tract is not unexpected and may also suggest that the excess of nutrient uptake and digestive capacity may exert regulatory roles, as it is proportionally related to body weight and may affect food intake and satiety mechanisms.

Interestingly, in both the hypothalamus and stomach, food intake significantly regulated the expression of a pheromone receptor-coding gene, namely *Mup1*. In particular, *Mup1* was up-regulated by LF intake in the hypothalamus, while its transcription was specifically reduced by HF intake in the stomach (HF1h). Furthermore, the regulation of this gene had been also detected in the duodenum mucosa [477]. As mentioned, MUP1 is mainly known as a pheromone transporter in rodents though, according to recent studies in mice, it might also have important regulatory properties in energy metabolism [468, 469]. Evidences so far collected indicated that MUP1 may also participate to the regulation of glucose and lipid metabolism, and that it might be involved in the mechanisms controlling

energy expenditure. MUP1 may therefore represent a promising novel gene for future studies, and its potential influence on feeding behavior will be further investigated.

Finally, using the SAGE method gave us the possibility to discover previously unknown (not-sequenced and/or uncharacterized) genes in all the tissues analyzed. The SAGE is a sequencing-based technique that does not employ probes and therefore does not need prior knowledge of the genes to be analyzed. The results of SAGE offer a complete gene expression profile of the tissue under investigation, and allow comparing distinct experimentally-induced and physiologic transcription profiles in terms of absolute transcript number. Furthermore, SAGE makes it possible to detect the transcripts of genes that have not been previously sequenced or characterized, but are nonetheless significantly regulated by the experimental conditions [126]. In the hypothalamus and gastric mucosa, a total of 17 tags corresponding to no currently known gene have been modulated by meal intake, and 3 were specifically regulated by HF intake. In future procedures, the SAGE 15 bp tag sequence representing a novel transcript can be used to synthesize a probe, which will serve to isolate the correspondent full-length cDNA and subsequently clone it. Detailed computational analyses can be conducted to predict the function of novel gene sequences and ESTs on the basis of their homology to known genes, consensus sequences and key attributes such as signal peptides. Finally, the recombinant proteins can be expressed in *E. coli* cell culture, purified and then characterized *in vitro* and *in vivo* for their contribution to food intake and energy metabolism regulatory pathways.

Novel findings from *Tff2* KO mice study

The in vivo characterization study of *Tff2* in KO mice described in Chapter 5 has been highly prolific, as it has disclosed many novel functional implications for this peptide. TFF2 had previously showed multiple roles in mucosal protection, cell chemotaxis, cell proliferation, apoptosis regulation, immune cell and inflammatory response modulation. Our study has importantly contributed to further widen the knowledge about TFF2, and opened the way for the characterization of previously unexplored biological and physiological aspects of this peptide. Nonetheless, our findings globally suggested that TFF2 might be an optimal pharmacological target to simultaneously influence key districts of energy balance regulation.

The main novelties uncovered by our studies in normal and *Tff2* KO mice are the following:

- *Tff2* gene is expressed and rapidly regulated by food intake in the hypothalamus. *Tff2* hypothalamic expression was detected for the first time by Q_RT-PCR in fasted male mice, and its down-regulation was observed following LF and, especially, HF intake. The discovery of *Tff2* expression and early feeding-induced modulation in the hypothalamus of mice is particularly important, as it suggests regulatory functions. Furthermore, we had previously detected *Tff2* expression in the mesenteric adipose tissue, while it has long been know that the main sources of TFF2 production are the gastric and duodenal mucosa. Therefore, *Tff2* is expressed and regulated in key central and peripheral hubs for the short-term and long-term regulation of energy balance. An important line of future investigations will be certainly dedicated to identify the specific cells and districts that express *Tff2* in the hypothalamus/whole brain, and how it is involved in the central regulation of energy balance-related processes. An interesting approach would also be to map the expression of two recently discovered TFF2-activated receptors, namely CXCR4 [472] and protease-activated receptor 4 (PAR4) [478]. CXCR4, in particular, is a very well known chemokine receptor, whose activation potentially affects multiple functional areas of the central nervous system, with specific coverage of dopaminergic and opioid pathways [479, 480]. Once the specific hypothalamic sites of *Tff2* expression will be unveiled, interesting hypotheses might be tested about the potential role of TFF2 as a bridge molecule between hypothalamic homeostatic regulations and hedonic circuitries, within the VTA for instance, where CXCR4 is known to be expressed and active, and from where nerve fibers are known to connect mesolimbic dopaminergic pathways to hypothalamic nuclei [290].
- *Tff2* deficiency significantly altered appetite signals and food/energy intake in mice. *Tff2* KO mice ate more than WT controls, in both one meal and long-term diet experiments. It is important to notice that, in short-term experiments, HF meal consumption specifically enhanced the difference of food intake between KO and WT animals. This was coherent with the specific HF-induced regulation of *Tff2* gene expression observed in previously mentioned transcriptomic studies of the

duodenum and hypothalamus. Moreover, this would indicate that *Tff2* regulation may relevantly affect the differential impact of LF and HF meals on satiation and overconsumption, and thus further studies should better define the extent and modalities of TFF2 contribution. However, the specific "HF effect" was blunted by long-term HF consumption, and not detectable after 12 weeks of diet. This was not unexpected, as other lipid-induced signals, such ApoA4 for instance, are known to be actively regulated at short-term, but progressively tolerant and non-responding after long-term lipid stimulation [130, 427]. In addition, our study highlighted some of the molecular triggers of increased appetite in KO mice. Using in situ hybridization, we found that *Agrp* mRNA expression was significantly elevated in the ARC of *Tff2* KO mice, regardless of the diet consumed. Furthermore, leptin circulating levels were substantially reduced in *Tff2* KO mice, and specifically lower in HF-consuming KO mice. The functional relationship between TFF2 and leptin should be better scrutinized in the future, as the constitutively lower levels of leptin observed in KO mice cannot be fully explained by their smaller fat stores. For instance, HF-consuming KO mice had larger fat depots than LF-KO animals; nonetheless, leptin serum concentration was further reduced in HF vs. LF-KO mice.

- *Tff2* deficiency substantially affected energy excretion and increased fecal lipid loss. Regardless of the diet, Tff2 KO mice excreted significantly more energy in their feces compared to WT controls. The increased energy loss was associated with higher levels of TG fecal loss in KO versus WT mice. These findings may suggest that, in *Tff2* KO mice, the absorption mechanisms within the intestinal mucosa were substantially altered. As previous reports had evidenced a reduced mucosal thickness in the GI tract of *Tff2* KO mice, we also verified in histological specimens the presence of epithelial abnormalities, as well as the length of mucosal layer in the duodenum. We found no macroscopic evidence of epithelial damage in both gastric and duodenal mucosa of WT or KO mice. However, the thickness of the duodenal mucosa was significantly reduced in *Tff2* KO mice, mostly due to a reduction of villus length. Therefore, the reduced surface area for nutrient absorption in the duodenum might at least partially explain the augmented loss of energy in KO mice stools. Moreover, future analyses should clarify if the reduced mucosal thickness

also concerns other more distal tracts of the enteric tube; and if the altered absorptive and excretive mechanisms specifically concern lipids. It is well known that lipids mostly use distinct absorption routes compared to carbohydrates and proteins, which generally need active transportation through membrane receptors of enterocytes [481]. The effects of TFF2 on these mechanisms should be thoroughly characterized, as they may represent novel targets to therapeutically control fat absorption.

- *Tff2* deficiency made mice unable to efficiently store their energy surplus in adipose tissue. Though energy intake was superior in *Tff2* KO than WT mice, KO animals accumulated less weight and, most importantly, less fat mass compared to controls. Interestingly, intra-abdominal fat depots were reduced and retroperitoneal adipocytes were smaller in *Tff2* KO mice. The analyses of mouse carcasses also evidenced that KO mice had poorly gained energy from fat, while protein gain was comparable between KO and WT animals. The latter aspect further confirmed the evidences that Tff2 KO mice did not present a concomitant loss of lean mass and, in fact, the percentage of gastrocnemius skeletal muscle per body weight was higher in KO vs. WT mice, at the end of the HF diet. Therefore, *Tff2* deficiency selectively impaired fat mass accumulation. We had previously found *Tff2* gene expression in mesenteric adipose tissue, while its production in other adipose depots is presently uncertain and should be verified. According to up-to-date evidences, mostly coming from studies of TFF2 in immune system regulation [464], this peptide is more likely to be released by adipose tissue-resident macrophages than adipocytes. However, it has already been reported that macrophages and preadipocytes/adipocytes present overlapping transcription profiles [41], hence it cannot be excluded that adipose cells can also be a direct source of TFF2. This would further add to the complexity of TFF2 functions and networks; and to the accumulating evidences of tight connections and redundant overlaps between the pathways regulating energy metabolism and those associated with inflammatory and immune responses. The physiologic and patho-physiologic stimuli that regulate TFF2 release in adipose depots should be scrutinized, as it would open the way for characterizing the role of this novel potential adipocytokine.

- *Tff2* deficiency substantially compromised energy efficiency while enhancing energy expenditure. We could estimate energy expenditure levels from the difference between digestible energy intake and final energy gain in both WT and KO mouse groups. The results indicated that *Tff2* KO mice might have expended significantly more energy than WT controls. This was coherent with energy efficiency calculations, which confirmed that KO mice were less able to accumulate available energy; and thus suggested that other mechanisms exist through which energy was used, and lost, in these mice. The evidence of increased *Ucp1* expression in the BAT would support the hypothesis of enhanced energy expenditure. Further analyses of the many molecules involved in energy oxidation pathways, especially in the skeletal muscle, should be further considered. Moreover, *Tff2* KO mice appeared significantly more active than WT animals and, thus, increased locomotor activity may represent another route for energy utilization in KO mice. In the future, more accurate measurements of energy expenditure in individual calorimetric chambers should be conducted. Furthermore, it would be important to characterize if *Tff2* deficiency can differentially affect the activation of SNS pathways in mice, and to correlate these data with behavioral/locomotor activity parameters.

In conclusion, our study demonstrated for the first time that TFF2 might affect multiple regulatory hubs, acting as a mastermind coordinator of energy balance. Therefore, designing a therapeutic strategy that targets TFF2 might lead to simultaneously control feeding behavior, energy absorption, energy storing mechanisms and energy expenditure, and thus have a strong potential for the prevention and treatment of obesity. For these reasons, it will be critical to thoroughly clarify the molecular pathways directly or indirectly impacted by *Tff2* deficiency, as well as TFF2 exogenous administration, in animal models; and concurrently verify the degree of correspondence between rodent and human physiologic importance of TFF2 for energy balance. To date, only one study conducted in human subjects has correlated daily variations of TFF2 gastric juice levels (and its epithelial reparative potential) with satiety-regulating gut peptides, and found the reverse association with pancreatic polypeptide concentration in blood [451]. Our study in

transgenic mice suggested that TFF2 might be a satiety factor itself, but only further studies in normal mice and human subjects as well can allow us to unequivocally dissect direct TFF2-related effects from eventual compensatory events occurred in KO models.

Finally, the development of strategies for the therapeutic use of TFF2 should equally carefully verify the potential oncogenic implications of manipulating this peptide. While the strong anti-inflammatory properties of TFF2 may prevent the aberrant transformation of the damaged mucosa [482, 483], the TFF members are generally known to modulate key steps of the oncogenic process, most importantly by promoting cell survival [484]. The role of TFF2 appears to be distinct with respect to TFF1/3 and, to date, clear evidences about the direct oncogenic or pro-metastatic action of TFF2 have not been reported. However, the pro-angiogenic [458, 485] and anti-apoptotic [486] actions of TFF2, as well as the existence of a specific spasmolytic polypeptide (TFF2)-expressing gastric metaplasia [486] might indicate that the deregulation of this protein expression and functions could be potentially noxious. Therefore, any study with the aim to develop the therapeutic potentials of such a multitask peptide should scrupulously evaluate the safety of TFF2 manipulations, as well as the possibility to dissociate its beneficial metabolic and anti-inflammatory effects from the eventual oncogenic consequences.

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