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# Extracellular vesicles as a therapeutic strategy to prevent or reverse obesity and its metabolic complications in the field of nanomedicine

Edward Milbank

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Edward Milbank. Extracellular vesicles as a therapeutic strategy to prevent or reverse obesity and its metabolic complications in the field of nanomedicine. Human health and pathology. Université d'Angers; Universidad de Santiago de Compostela, 2016. English. NNT : 2016ANGE0074 . tel-02613088

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Edward MILBANK

*Mémoire présenté en vue de l'obtention du*

***grade de Docteur de l'Université d'Angers et de Docteur de l'Université de Santiago de Compostela***

*sous le sceau de l'Université Bretagne Loire et de l'Université de Santiago de Compostela*

**École doctorale :** *Ecole Doctorale Biologie Santé*

**Discipline :** *Aspects moléculaires et cellulaires de la biologie*

**Spécialité :** *Biologie cellulaire*

**Unités de recherche :** *INSERM 1063 SOPAM : « Stress Oxydant et Pathologies Métaboliques »  
NeurObesity*

**Soutenue le** *09 Décembre 2016*

**Thèse N° :** *107864*

## Extracellular vesicles as a therapeutic strategy to prevent or reverse obesity and its metabolic complications in the field of nanomedicine

### JURY

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# Acknowledgments

First of all, I would like to dedicate my sincere gratitude to my research advisors.

**Dr. Ramaroson ANDRIANTSITOHAINA** has helped me through it all, and in addition to shaping my research skills, has provided a perfect balance between guidance and freedom, and has brought levity to many difficult situations. Thank you for supporting my scientific education for the last five years.

**Pr. Miguel LOPEZ** took a chance on me when he accepted to be part of this project. During my stay, he provided me advices, guidance, valuable comments and suggestions that benefited me much in the completion and success of this study. Thank you for all your support.

My gratitude is also addressed to the examiners, **Dr. Chantal BOULANGER**, **Pr. Anxo VIDAL**, **Dr. Serge LUQUET** and **Dr. Emmanuel GARCION**. Thank you for agreeing to take part in the evaluation of this work.

I would also like to thank my thesis committee members, **Pr. Bernard MULLER** and **Dr. Emmanuel GARCION**. The committee members have nonetheless provided invaluable feedback and suggestions that hugely shaped my projects, contributing to the successful completion of the thesis.

Furthermore, I am grateful for the support and advice of **Dr. Carmen MARTINEZ**. Thank you for the many constructive and inspiring discussions. Thank you for helping me solving all the everyday problems. All this would not have been possible without all your help.

I would also like to thank **Dr. Jacques NOIREAUD** and **Dr. Raffaella SOLETI**. Somehow, you were at the beginning of all this amazing experience. Thank you for shaping my research skills, teaching me all your knowledge and supporting me in my work. It was a pleasure sharing these years with you.

I would like to thank **Dr. Sylvain RECOQUILLON** and **Mehdi FRIFRA** for your support and friendship during these 3 years. Whether around a coffee or discussing science, it was always a truly appreciated moment. Thank you for all your advices that benefited me much in the completion and success of this study.

My gratitude is also addressed to **Dr. Cristina CONTRERAS**. Thank you for all your support during my stay in Santiago. Thank you for taking your time to share your knowledge and skills with me. I truly appreciated.

I would like to thank my office colleagues. **Mireille WERTHEIMER, Dr. Nadia BENABBOU, Camille KOFFI, Simon BOUSSEAU** and **Dr. Raffaella SOLETI**. Thank you for your daily good mood. Thank you for all your support and advices. I truly appreciated sharing this office with all of you for these three years. I will miss all of you.

My gratitude is also addressed to all my colleagues. **Dr. Luisa VERGORI, Dr. Audrey FLEURY, Grégory HILAIRET, Dr. Ousama DAYOUB, Vanessa BIRONNEAU, Catherine BRIAND, Catherine DOURGUIA, Dr. Soazig LELAY, Dr. Denis LEIBER, Maëva DURCIN, Mathieu NITIEMA, Patricia MALLEGOL, Dr. Zainab SAFIEDEEN** and **Marine MALLOCI**. I would like to thank you all for your advices, for your support and for your kindness. It was a real pleasure sharing with you these three years. I would also like to thank my USC colleagues for all the support and help you offered me during my stay in Santiago de Compostela. **Patricia Seoane Collazo, Ismael González-García, Ángela Fraga Varela, Tania López González, Ánxela María Estévez Salguero, Laura Liñares Pose** and **Eva Rial Pensado**. Thank you.

This work would not have been possible without the different helpful collaboration. Thus, I would like to thank the different platforms **SCIAM, Lentivec** and **PACEM**. I would also like to thank **Dr. Celine BEAUVILLAIN** for the numerous discussions we had.

I have been fortunate to receive fundings during my graduate experience. Thank you to: **University of Angers, University of Santiago de Compostela** and the **Erasmus Mundus Program NanoFar**.

In addition to graduate research, my life has been full of additional “balancing” experiences. I would like to thank all my friends. I feel immensely fortunate that our lives intertwined for these years.

My special thanks go to my whole family for always being there for me and providing me their unconditional support and their persistent confidence.

Finally, I would like to dedicate this thesis to my parents. You were the first persons to support my scientific career and always believed that I could reach all the goals I set for myself, even when no one else did. I am very proud to be your son.

# Communications and Publications

## Communications:

1. “EVs as a therapeutic strategy to prevent or reverse obesity and its metabolic complications in the field of nanomedicine”. MÉRIMÉE Project. March 2014. Santiago de Compostela. Oral communication.
2. “EVs as a therapeutic strategy to prevent or reverse obesity and its metabolic complications in the field of nanomedicine”. NanoFar Autumn School, October 2014, Bruxelles, Poster.
3. “EVs as a therapeutic strategy to prevent or reverse obesity and its metabolic complications in the field of nanomedicine”. Journées Scientifiques, Nantes, December 2014, Poster.
4. “EVs as a therapeutic strategy to prevent or reverse obesity and its metabolic complications in the field of nanomedicine”. Journée de la SFR, May 2015, Angers, Oral communication, 3<sup>rd</sup> price.
5. “EVs as a therapeutic strategy to prevent or reverse obesity and its metabolic complications in the field of nanomedicine”. NanoFar Autumn School, Octobre 2015, Nantes, Poster.
6. “EVs as a therapeutic strategy to prevent or reverse obesity and its metabolic complications in the field of nanomedicine”. CIMUS seminar, May 2016, Santiago de Compostela, Oral communication.

## Publications:

1. Edward Milbank, Raffaella Soleti, Emilie Martinez, Badreddine Lahouel, Grégory Hilairet, M. Carmen Martinez, Ramaroson Andriantsitohaina and Jacques Noireaud. *Microparticles from apoptotic RAW 264.7 macrophage cells carry tumor necrosis factor  $\alpha$  functionally active on cardiomyocytes from adult mice*. J Extracell Vesicles. 2015 Oct 23;4:28621
2. Edward Milbank, M. Carmen Martinez, Ramaroson Andriantsitohaina. *Extracellular vesicles: pharmacological modulators of the peripheral and central signals governing obesity*. Pharmacol Ther. 2016 Jan;157:65-83.



# Abbreviations

17 $\beta$ -HSD: 17 $\beta$ -hydroxysteroid dehydrogenase

$\alpha$ -MSH:  $\alpha$ -Melanocyte-stimulating hormone

A2MG: Alpha-2 macroglobulin

AgRP: Agouti-related protein

AMPA/KA: Carbonic anhydrase and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor/kainate

AMPK: AMP-activated protein kinase

AMPK-DN: AMPK dominant negative

ARC: Arcuate nucleus

BAT: Brown adipose tissue

BMDCs: Bone marrow derived dendritic cells

BMI: Body mass index

BMPs: Bone morphogenetic proteins

BMP8B: Bone morphogenetic protein 8B

BSX: Homeobox domain transcription factor

cAMP: Cyclic adenosine monophosphate

CART: Cocaine- and amphetamine-regulated-transcript

CCK: Cholecystokinin

CNS: Central nervous system

DMEM: Dulbecco's Modified Eagle Medium

DMH: Dorsomedial nucleus

DNA: Deoxyribonucleic acid

DPP-4: Dipeptidyl peptidase-4

EMA: European Medicines Agency

EPA: Eicosapentaeneic acid

ER: Endoplasmic reticulum

ERK: Extracellular signal-regulated kinase

ESCRT: Endosomal sorting complex responsible for transport

EVs: Extracellular vesicles

FDA: Food and Drug Administration

FGF1: Fibroblast growth factor 1

FGF2: Fibroblast growth factor 2

FoxO1: Forkhead box O1

GFP: Green fluorescent protein

GHS-R: Growth hormone secretagogue receptor

GLP-1: Glucagon-like peptide 1

GLP-1R: Glucagon-like peptide 1 receptor

GM-CSF: granulocyte macrophage colony-stimulating factor

GRP78: Glucose-regulated protein 78

HDL: High-density lipoprotein

HH: Hedgehog

HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A

IGF1: Insulin-like growth factor 1

IL-6: Interleukin-6

Lamp2b: Lysosome-associated membrane protein 2b

LDL: Low-density-lipoprotein

LHA: Lateral hypothalamic area

LPS: Lipopolysaccharide

MC3R: Melanocortin-3 receptor

MC4: Melanocortin-4

MC4R: Melanocortin-4 receptor

MCH: Melanin-concentrating hormone

MC-P1: Monocyte chemotactic protein-1

MIF: Macrophage migration inhibitory factor

MPs: Microparticles

MS: Metabolic Syndrome

MSH: Melanocyte-stimulating hormone

mTOR: Mammalian target of rapamycin

MVB: Multivesicular bodies

NO: Nitric oxide

NPY: Neuropeptide Y

ObR: Leptin receptor

OX-1R: Orexin receptor 1

OX-A: Orexin A

OXs: Orexins

pCREB: Phosphorylated cAMP response-element binding protein

POMC: Pro-opiomelanocortin

PP: Pancreatic polypeptide

PPAR: Peroxisome proliferator-activated receptor

PVH: Paraventricular nucleus

PYY: Peptide YY

ROCK-1: Rho-associated kinase

ROS: Reactive oxygen species

RVG: Rabies virus glycoprotein

Shh: Sonic Hedgehog

SNS: Sympathetic nervous system

T2DM: Type II diabetes mellitus

TGF-  $\beta$ : Transforming growth factor  $\beta$

TRH: Thyrotropin-releasing hormone

UCP 1: Uncoupling protein 1

UPR: Unfolded protein response

VLDL: Very low-density-lipoproteins

VMH: Ventromedial hypothalamus

WAT: White adipose tissue

WHO: World Health Organization

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# I. Introduction



## Obesity

Obesity, defined as an abnormal or excessive fat accumulation that may impair health, started to be considered as a pathological state 20 years ago by the World Health Organisation (WHO) owing to its increasing health impacts and worldwide prevalence. Obesity had an epidemic expansion worldwide, including in numerous emerging countries. As an example, France obesity trend lines showed an increase of 6.1 to 15 % of obese people between 1980 and 2012 (OBEPI-Roche). According to WHO 2015 latest report, 35% of worldwide adult population is considered overweighted or obese and the projections appeared to be alarming. By 2030, these statistics could be increased up to 57,8% highlighting the pandemic state of obesity.

In clinic, obesity is usually identified using the body mass index (BMI), calculated by dividing the body weight in kilograms (kg) by the square of the height in metres (m) defining an approximate measure of total body fat. WHO stated that a patient exhibiting a BMI value equal or exceeding  $30 \text{ kg/m}^2$  was considered obese. Although well accepted in clinical fields because of its easy and costless application, BMI accuracy is discussed. By not considering neither the fat mass amount variability between different patients (age, gender, activities...), nor its pattern of distribution, BMI index can generate misunderstandings in the severity of the obesity. Indeed, mainly 2 types of obesity depending on the fat distribution are described. An excess of visceral fat, commonly termed “apple-shaped” obesity is more likely associated to cardiovascular diseases than the “pear-shaped” one characterized by accumulation of fat around hips. Thus, it is important to define the exact phenotype of declared obesity to develop specific individual therapies. Therefore, a better understanding of the origins of the pathology is needed for the development of therapeutic alternatives.

Considered as a multifactorial pathology, obesity has many causes, including genetic, metabolic, behavioural and environmental.

### 1. Origins of obesity

#### i. Genetic causes

As observed in numerous pathologies, genetic predisposition is undeniable for obesity. The implication of genetics in obesity development is known for decades from now. Bouchard and colleagues showed the best illustration of the impact of



genetics on obesity on 1990. Persons exposed to the same alimentation during 3 months were differently impacted in term of body weight gain - variability of 2 to 10 kg -, while perfect homozygous twins exhibited exactly the same one (Bouchard et al., 1990). These startling observations confirmed the *Thrifty Gene Hypothesis* advanced by Neel in 1962 by which some genes could influence the metabolic efficiency between different individuals by modulating their capacities to store or contrary expend excesses (Neel, 1962). Genetic implications in obesity were later confirmed by the emergence of new technologies that enable the discovery of more than 250 genes directly implicated in weight gain, the severity of obesity and its complications (Rankinen & Bouchard, 2006). Moreover, clinical observations showed that 70% of the obese patients had one obese parent. This risk was multiplied by 5 in case of a BMI superior of 40 kg/m<sup>2</sup> (Drummond & Gibney, 2013). However, this latest observation has to be toned, as family members do not share only genetic information, but also a way of life, including alimentation and physical habits, which have been demonstrated critical for the development of obesity.

On the other hand, some severe cases of obesity may be caused by a mutation of one gene or even of an entire chromosome region. These mutations are mainly associated to pleiotropic syndromes in which body weight gain is not the unique pathological component (Huvenne et al., 2016). These monogenic obesities are mainly provoked by the mutation of genes encoding for the melacortin/leptin pathway implicated in the regulation of food intake and energy expenditure, but also in the adrenal function and pigmentation. These rare obesity forms represent less than 5% of all the obesity cases.

However, even if genetic plays an important role within the development of some case of obesity, its narrow relation with environmental parameters is unquestionably.

## ii. Obesogenic lifestyle of modern societies

The obesogenic environment of the actual industrialized societies is characterized by a combination of hypercaloric overnutrition and increasingly sedentary habits. Indeed, body weight depends on an accurate balance between food intake and energy expenditure, and when the former exceeds the latter appears an excess of fat accumulation within peripheral tissues.

Food intake, modulated by complex biological mechanisms (see Milbank et al., 2016), enables a constant adapted availability of nutrients within the organism. However, alterations of food intake habits and increased body weight gain are frequently correlated. The 21<sup>st</sup> century-increased access to unhealthy food related to the growing obesogenic social pressure encountered in modern societies creates new non-adapted alimentary habits. Thus, quantitative (hypercaloric nutrition) or qualitative (complex carbohydrates consumption decrease or lipid excess) alterations of food intake are frequently associated with an increased body weight (Bray & Popkin, 1998; Drewnowski, 2003).

On the other hand, energy expenditure tends to decrease over the years. The decreasing attraction for manual labours, which correlates to the development of office works observed in modern societies contributes to these increased sedentary habits. Medical field experts stated that a daily physical activity - 150 minutes of moderate exercise or 75 minutes of an intense one - could help improving individuals' welfare. Also called the "sitting disease", physical inactivity is a true sanitary issue. WHO stated that 60 to 85% of worldwide population does not engage in enough activity. Implicated in numerous pathologies (obesity, cancer, cardiovascular diseases or depression among others), physical inactivity appears to be the fourth leading risk factor for global mortality. The overall result is that an increased food intake associated to a decrease of energy expenditure induces an imbalance of input/output ratio, participating to obesity development.

### iii. Other factors implicated in obesity development

Apart from genetic and environmental causes, numerous other considerations have to be taken into account within the obesity development.

The adipose tissue is the main fat storage organ. In humans, 2 types of adipose tissue are encountered, the brown adipose tissue (BAT) and the white adipose tissue (WAT). While the BAT is mainly implicated in the thermogenic process, the WAT allows the storage of fatty acids as triglycerides. Through its metabolic and endocrine functions, the WAT appears as a central organ in energetic and metabolic homeostasis. The accumulation of fatty acids within the adipocytes - the main cell type constituting the WAT - will induce their hypertrophy (cell size increase). When adipocytes have

reached their maximal size, they recruit pre-adipocytes, which will be differentiated in mature adipocytes to increase fat storage capacity. These recruitment and maturation steps are known as adipogenesis. The differentiation of pre-adipocytes to a mature phenotype is governed by multiple signals, including extracellular factors such as the bone morphogenetic proteins (BMPs) (Huang et al., 2009), transforming growth factor  $\beta$  (TGF-  $\beta$ ) (Zamani & Brown, 2011), insulin-like growth factor 1 (IGF1) (Kawai & Rosen, 2010), fibroblast growth factor 1 and 2 (FGF1 and FGF2) (Widberg et al., 2009; Xiao et al., 2010). Aside these extracellular signals, WNT and Hedgehog (HH) pathways are also implicated within pre-adipocyte maturation. The inhibition of both Wnt and Hedgehog (HH) pathways is essential for adipogenesis to proceed (Christodoulides et al., 2009; Cousin et al., 2007). Thus, WAT can expand itself through a size increase (hypertrophy) but also through an increase of adipocytes number (hyperplasia) allowing an improved storage of fatty acids. Therefore, an alteration of adipogenesis may lead to ectopic accumulation of lipids leading to obesity complications. Moreover, stress (Kuo et al., 2007), viral infections (van Ginneken et al., 2009) or even pollution (Kim et al., 2012) have been described to induce obesity symptoms through an alteration of WAT adipogenesis.

Moreover, the WAT has an important endocrine role. Through the release of numerous adipokines and cytokines, WAT governs several metabolic functions. Released by the adipocytes, the leptin (i) activates central anorexigenic pathways (Klok et al., 2007), (ii) improves insulin sensitivity (Toyoshima et al., 2005) and (iii) participates in obesity related low-grade inflammation (Paz-Filho et al., 2012). The adiponectin is also released by WAT adipocytes and is implicated in insulin sensitivity (Awazawa et al., 2011) and hepatic gluconeogenesis (Zhou et al., 2005) among others. Moreover, resistin has been described to be involved in insulin resistance (Barnes & Miner, 2009). Finally, interleukin-6 (IL-6) - a pro-inflammatory cytokine - secretion is increased in an obesity context (Eder et al., 2009). This non-exhaustive list shows the implication of WAT in numerous energetic functions. In obese patients, the endocrine dialog between WAT and other regulatory organs is deregulated generating metabolic disorders.

Recent findings suggest also an important correlation between intestinal flora quality and obesity. Experimental studies showed that mice raised under conventional

methods had over 60% more total body fat compared with those raised under germ-free conditions (Bäckhed et al., 2004). Moreover, germ-free mice (devoid of intestinal flora) were protected from high fat diet induced obesity, but also from obesity associated metabolic disorders as type II diabetes (Diamant et al., 2011). The emergence of novel methods allowing the characterization of human intestinal flora brought some new evidences of the contribution of intestinal flora composition within the development and maintaining of obesity. It was shown that type II diabetes occurrence was partially related to the presence of bacterial species (compared to non-diabetic patients) (Burcelin et al., 2011). Cotillard et al. (2013) reported that non-diabetic overweighted or obese individuals with reduced microbial gene richness (40%) presented more pronounced dysmetabolism and low-grade inflammation at same BMI levels.

Psychological factors should also be taken in account within the development of obesity. Some psychological disorders can induce obesity due to an alteration of the feeding behaviour. Indeed, in some anxiety and depression cases, food is perceived as a reward or gratification inducing dopamine production activating pleasure centres within the brain. Thus, a gratification habit through a favourable food could lead to overeating and morbid obesity (Singh, 2014).

This non-exhaustive list of obesity origins highlights the complex and multifactorial aspect of this pathology characterized by an important heterogeneity between obese patients. Therefore, during obese patient medical cares, a precise evaluation of these different parameters must be realized in order to develop individually adapted therapeutic strategies.

## 2. Obesity and associated complications

Numerous studies showed a direct link between severe cases of obesity (BMI > 35 kg/m<sup>2</sup>) and increased rate of mortality. As an example, it has been described that 40 years old male and female non-smokers obese patients exhibited large decreases in life expectancy - 5.8 and 7.1 years respectively - compared to same aged non-obese patients (Peeters et al., 2003). A long-term study conducted in the United States revealed that the risk of mortality was increased by 20 to 40% among overweight

persons and by 2 or 3 times among obese patients (Adams et al., 2006). Another study lead by Flegal et al. (2005) demonstrated that underweight - BMI < 19 kg/m<sup>2</sup> - and obese patients - BMI > 35 kg/m<sup>2</sup> - had their mortality rates increased compared to the normal weight category. However, this increased rate of mortality in obese patients may mostly be due to associated pathologies than to obesity itself. Herein, we will describe the principal encountered obesity-associated complications.

i. Type II diabetes

The strong association between obesity and type II diabetes has been described in numerous studies giving birth to the “*diabesity*” term. In 1995, meta-analysis data obtained from a big cohort showed a relative increased risk of 40 to develop type II diabetes in women with a BMI > 31 kg/m<sup>2</sup>, compared with normal weighted women (BMI ≤ 22 kg/m<sup>2</sup>) (Colditz et al., 1995). A similar risk was observed for men patients. Indeed, obese men patients (BMI > 35 kg/m<sup>2</sup>) had relative risks increased to 42.1% compared to normal weighted men (BMI ≤ 23 kg/m<sup>2</sup>) (Chan et al., 1994). The 2 last described studies also demonstrated that this narrow link between obesity and type II diabetes does not depend on the gender of the patients. Moreover, 75% of diabetic patients are obese, and it has been described that obesity increases the risk of type II diabetes by a factor of 8 in female, and by a factor of 10 in male individuals.

The underlying mechanisms of obesity-induced type II diabetes are mostly due to insulin resistance. In obese patients, adipose tissue release, within the extracellular environment, increased amounts of non-esterified fatty acids, glycerol, hormone and pro-inflammatory cytokines among others that are implicated in the development of lipotoxicity insulin resistance inducing type II diabetes diseases (Kahn et al., 2006; Van Gaal et al., 2006).

ii. Dyslipidemia

Obesity is associated with inadequate lipid profile. Dyslipidemia is characterized by decreased concentrations of high-density lipoprotein (HDL) and increased cholesterol, triglycerides, low-density-lipoprotein (LDL) and very low-density-lipoproteins (VLDL) (Despres, 1991). The mechanisms inducing dyslipidemia are involving insulin resistance. In obese patients, insulin resistance is associated with impaired lipoprotein lipase, which is implicated in HDL formation, decreasing their

serum levels (Pouliot et al., 1991). As HDL major role is to capture circulating cholesterol, a decrease of their blood levels induce cholesterol deposit within arteries, leading to atherosclerosis development.

### iii. Cardiovascular diseases

Obesity, in particular the visceral or “apple-shaped” one, is a well-known risk factor of heart failures, arterial hypertension and coronary heart diseases leading to an increase of the cardiovascular induced mortality (Artham et al., 2009). Mechanisms underlying the deleterious effect of obesity on cardiovascular diseases are numerous.

Obesity induces an increase of total blood volume, cardiac flow and frequency caused by the augmented metabolic demand induced by body weight excess. Therefore, obese patients exhibit a greater cardiac workload compared to healthy subjects, increasing the risks of heart failure (Poirier et al., 2006). The risk of developing heart failure is 2 times higher in obese patients than in healthy individuals (Kenchiah et al., 2002).

Due to this increased cardiac output, obese patients have increased risks to suffer from arterial hypertension. Indeed, hypertension is about 6 times more frequent in obese subjects than in lean individuals (Stamler et al., 1978). Associated to this increased cardiac output that contributes to the establishment of hypertension, obesity-induced hyperinsulinemia may also play an important role within this development. Indeed, increased insulin blood concentrations modulate the sympathetic nervous system, which contributes to the development of hypertension. However, this hyperinsulinemia-induced hypertension would rather be linked to a decrease of peripheral vascular resistance than to an increase of blood pressure (Böner, 1994).

Moreover, obese patients exhibit high predisposition to suffer from coronary heart diseases (or atherosclerosis). Large-scaled studies indicated an increased risk of coronary heart diseases in obese patients. The relative risk is increased from 1.19 in healthy individuals (BMI of 21 kg/m<sup>2</sup>) to 3.56 in obese patients (BMI > 29 kg/m<sup>2</sup>) (Kenchiah et al., 2002). As described above, obese patients exhibit abnormal lipid profile that contributes dangerously to atherosclerosis progression. A low rate of HDL/LDL associated to high levels of circulating free fatty acids and cholesterol induce the expansion and inflammation of atheroma plaque within arteries. The increased release of pro-inflammatory cytokines by infiltrated macrophages maintains

the pro-inflammatory state within the plaque (Rocha & Libby, 2009). Atherosclerotic development associated with obesity is frequently associated with other diseases as strokes.

#### iv. Obesity and stroke

Evidences showing an increased risk of stroke in obese patients are strong. An increase in ischaemic stroke risk of 11% for each 1-point increase in BMI was observed in middle-aged obese men (Song et al., 2004). Another study showed that obese patients having a BMI > 30 kg/m<sup>2</sup> had a relative risk of 1.95 for ischaemic stroke and 2.25 for haemorrhagic stroke (Song et al., 2004). Experimental studies demonstrated that obese rats exhibited impaired endothelium-dependent vasodilation within cerebral circulation partly due to an increase in oxidative stress and impaired nitric oxide (NO)-dependent dilator responses inducing endothelial dysfunction, one major cause of stroke development (Erdös et al., 2004; Phillips et al., 2005).

However, recent findings have shown an inverse correlation between body weight and post stroke mortality, named as the *Obesity Paradox*. Venmos et al. (2011) observed a reduced mortality in overweight (-18%) and obese (-29%) patients when compared with the normal-weight group. More recently, Doehner et al. (2013) have shown among patients with stroke lower mortality risk in overweight (-14%) and obese (-24%) as compared with normal weight individuals. Mechanisms underlying this protective effect are still not fully determined. However, as mentioned previously, the limitations of BMI to discriminate fat and lean masses could partially explain this *Obesity paradox*. Indeed, increased muscle mass and muscular strength is associated with better cardiovascular prognosis and survival (Artero et al., 2012).

#### v. Obesity and cancer

WHO stated that from a quarter to a third of breast, colon, endometrium, kidney and oesophagus cancers were associated to obesity and inactivity (Bianchini et al., 2002). In 2004, a meta-analysis from the Comparative Risk Assessment project showed that on 7 million deaths due to cancer, 2.43 million were attributable to metabolic risk factors, including overweight and obesity (Danaei et al., 2005). The mechanisms underlying the strong association between obesity and cancer development are numerous and complex.

Insulin resistance observed in obese patients may play an important role within cancer development. To compensate this decreased insulin sensitivity, the pancreas increases insulin production and release creating a hyperinsulinemia state, which has been shown in numerous epidemiological and experimental studies to increase risk of cancer. In vitro studies clearly demonstrated that insulin could act as a growth factor to promote cell proliferation and to inhibit apoptosis (Ish-Shalom et al., 1997; Khandwala et al., 2000). Moreover, it has been shown that hyperinsulinemia could contribute to an increased growth of mammary tumours in vivo. Ferguson et al. (2013) have demonstrated that hyperinsulinemic breast cancer model mice exhibited larger primary tumours and more numerous pulmonary metastases compared to controls.

Increased levels of circulating oestrogens in obese patients are also associated to increased risks of cancer, in particular in breast and endometrium cancers. Within the adipose tissue, the enzyme  $17\beta$ -hydroxysteroid dehydrogenase ( $17\beta$ -HSD) converts estrone in a more active hormone, estradiol increasing its bioavailability. Binding of oestrogen on its  $\alpha$ -receptors has been demonstrated to promote cellular proliferation and apoptosis inhibition in breast and endometrial cells (Calle & Kaaks, 2004). This increased bioavailability of estradiol has been shown to increase cancer risk by a factor of 5 compared to healthy individuals (Lukanova et al., 2004).

Obesity induces numerous subsequent pathologies that may impair individuals' lifestyle and health. It appears to be a major health problem in actual societies trending to serious economic and social burdens, raising the need to find innovative accurate treatments.

In order to develop adapted treatments, a better understanding of the underlying mechanisms of obesity regulation leading to the maintenance of the balance between food intake and energy expenditure is necessary. Therefore, the next part, driven by a 2016 published review (Milbank et al., 2016), will provide an overview of the associated genes and molecules acting on pathways implicated in the regulation of feeding behaviour. After a preliminary outline of this complex regulation system, actual anti-obesity pharmacological treatments will be described. Next, we will focus on the implication of extracellular vesicles in the maintaining of obesity. And finally, a novel "nanobiomedicine" approach to correct obesity will be discussed.





## Extracellular vesicles: Pharmacological modulators of the peripheral and central signals governing obesity



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### ARTICLE INFO

Available online 23 November 2015

#### Keywords:

Obesity  
Peripheral/central regulation  
Extracellular vesicles  
Pharmacotherapy  
Targets  
Tools

### ABSTRACT

Obesity and its metabolic resultant dysfunctions such as insulin resistance, hyperglycemia, dyslipidemia and hypertension, grouped as the “metabolic syndrome”, are chronic inflammatory disorders that represent one of the most severe epidemic health problems. The imbalance between energy intake and expenditure, leading to an excess of body fat and an increase of cardiovascular and diabetes risks, is regulated by the interaction between central nervous system (CNS) and peripheral signals in order to regulate behavior and finally, the metabolism of peripheral organs. At present, pharmacological treatment of obesity comprises actions in both CNS and peripheral organs. In the last decades, the extracellular vesicles have emerged as participants in many pathophysiological regulation processes. Whether used as biomarkers, targets or even tools, extracellular vesicles provided some promising effects in the treatment of a large variety of diseases. Extracellular vesicles are released by cells from the plasma membrane (microvesicles) or from multivesicular bodies (exosomes) and contain lipids, proteins and nucleic acids, such as DNA, protein coding, and non-coding RNAs. Owing to their composition, extracellular vesicles can (i) activate receptors at the target cell and then, the subsequent intracellular pathway associated to the specific receptor; (ii) transfer molecules to the target cells and thereby change their phenotype and (iii) be used as shuttle of drugs and, thus, to carry specific molecules towards specific cells. Herein, we review the impact of extracellular vesicles in modulating the central and peripheral signals governing obesity.

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**Abbreviations:** A2MG, alpha-2 macroglobulin; AgRP, agouti-related protein; AMPA/KA, carbonic anhydrase and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor/kainate; AMPK, AMP-activated protein kinase; ARC, arcuate nucleus; BMI, Body Mass Index; BSX, homeobox domain transcription factor; cAMP, cyclic adenosine monophosphate; CART, cocaine- and amphetamine-regulated-transcript; CCK, cholecystokinin; CNS, central nervous system; DPP-4, dipeptidyl peptidase-4; EMA, European Medicines Agency; EPA, eicosapentaenoic acid; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; ESCRT, endosomal sorting complex responsible for transport; EVs, extracellular vesicles; FDA, Food and Drug Administration; FoxO1, Forkhead box O1; GHS-R, growth hormone secretagogue receptor; GLP-1, glucagon-like peptide 1; GLP-1R, glucagon-like peptide 1 receptor; GRP78, glucose-regulated protein 78; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MC4, melanocortin-4; MCP-1, monocyte chemoattractant protein-1; MIF, macrophage migration inhibitory factor; MPs, microparticles; MS, Metabolic Syndrome; MSH, melanocyte-stimulating hormone; mTOR, mammalian target of rapamycin; MVB, multivesicular bodies; NO, nitric oxide; NPY, neuropeptide Y; ObR, leptin receptor; pCREB, phosphorylated cAMP response-element binding protein; POMC, pro-opiomelanocortin; PP, pancreatic polypeptide; PPAR, peroxisome proliferator-activated receptor; PYY, peptide YY; ROCK-1, rho-associated kinase; ROS, reactive oxygen species; Shh, Sonic Hedgehog; T2DM, type II diabetes mellitus; TRH, thyrotropin-releasing hormone; UPR, unfolded protein response; VMH, ventromedial hypothalamus.

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## 1. Introduction

Defined as an abnormal or excessive fat accumulation that may impair health, obesity has reached epidemic proportions worldwide. Latest 2015 World Health Organization projections appear to be alarming. Since by 2030, up to 57.8% of the world's adult population – 3.3 billion people – could be either overweight or obese. The close relationship between obesity and modern lifestyles could explain these startling trends. Undeniably, the obesogenic environment of the actual industrialized societies is the combination of both hypercaloric overnutrition and increasingly sedentary habits. Indeed, body weight depends on an accurate balance between energy intake and energy expenditure, and when the former exceeds the latter appears an excess fat accumulation in peripheral tissues. When located into misfit organs, such as skeletal muscles or liver instead of storage-specialized adipose tissue, this fat accumulation may induce metabolic disorders. The obesity-related metabolic dysfunctions are related with an increased prevalence of others associated pathologies such as type II diabetes mellitus (T2DM), cardiovascular diseases, or even cancer. These correlations make the obesity to appear as a major health problem in actual societies trending to serious economic and social burdens, raising the need to find innovative accurate treatments.

So far, the most effective available treatment of obesity is bariatric surgery. It has been shown that bariatric surgery not only decreases body weight but also improves T2DM. Nevertheless, mostly due to harmful and risky side effects, and because its consequences on metabolism are still not completely understood, the needs to find very effective pharmacological treatments are crucial.

Despite the medical research growing efforts made on our knowledge on the pathology of obesity, the development of anti-obesity drugs remains elusive. Even if some therapeutic strategies display some beneficial effects on decreasing body weight, most of them do not reach the sufficient level of acceptability required by regulatory authorities such as the US Food and Drug Administration (FDA) due to numerous associated side effects. In this respect, the complexity of this multifactorial pathology – environment, genetic and individual-dependent – renders difficult the development of fully effective anti-obesity treatments.

The treatments developed by pharmaceutical companies up to now are based on the control of the principles of obesity – accurate balance between food intake and energy expenditure – by acting on satiety pathways with a balanced consumption of available nutrients. However, due to the lack of specificity of these drugs and to the narrow relationship between alimentary and psychiatric pathways, the majority of the developed treatments display negative effects on mood disorders. One way to counteract these non-desired actions is to increase the specificity of the pathways targeting through a new “nanobiomedicine” approach. Indeed starting at what has been conducted in other diseases such as neurodegenerative disorders or even cancer, using cell-derived nano/microvesicles as “cargos” of specific therapeutic molecules could increase the efficiency of the actions concomitantly with the decrease of the unwanted effects.

This review will provide an overview of the associated genes and molecules acting on pathways implicated in the regulation of feeding behavior. After a preliminary outline of this complex regulation system, we will attach the discussion on actual anti-obesity treatments. Then, we will focus on the implication of extracellular vesicles (EVs) in the maintaining of obesity. And lastly, a novel “nanobiomedicine” approach to correct obesity will be discussed.

## 2. Regulation of obesity

A better understanding of the underlying mechanisms of regulation of obesity leading to the maintenance of the balance between food intake and energy expenditure helps to find novel therapeutic targets of

anti-obesity drugs. Central and peripheral regulations of these processes help to maintain the balance (Fig. 1).

### 2.1. Peripheral regulation

The gastrointestinal tract is the first site of interaction between the ingested nutrients and the body, and it represents a vital actor controlling food intake and, in consequence, the regulation of energy balance. The initial signal detected by the stomach is mechanosensitive, mainly perceived by the vagal stretch, and followed by changes in the release of different hormonal regulators all along the gastrointestinal tract from the stomach to the colon.

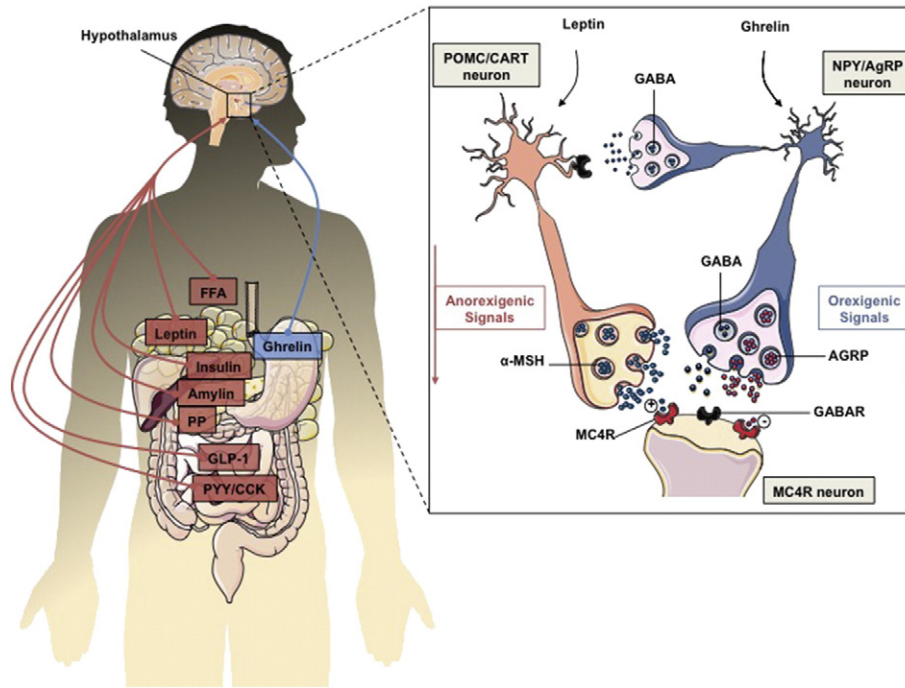
- *Ghrelin*

Ghrelin is produced by the gastric oxyntic cells but also by other cells of the gastrointestinal tract. Ghrelin exerts its orexigenic action by binding to the growth hormone secretagogue receptor. Secretion of ghrelin, mainly by mucosa of the empty stomach, is rapidly abolished after eating. Indeed, in humans, plasma levels of ghrelin rise before each meal and fall to basal levels within 1 h after ingestion of food suggesting a physiological role for ghrelin in initiating individual meals (Cumplings et al., 2001). In this context, ghrelin has approximately equal potency than neuropeptide Y (NPY) in the duration and magnitude of the feeding stimulation (Wren et al., 2000) (see below). Ghrelin secretion induces the activation of c-Fos in both NPY and agouti-related protein (AgRP) neurons, two regions of crucial importance in the regulation of feeding (Nakazato et al., 2001). Although these findings suggest that ghrelin antagonists could be good candidates to reduce food intake, the use of such drugs as anti-obesity agents is unlikely since ghrelin knock-out mice has been shown to have normal body composition and show a normal growth rate, appetite, and feeding behavior (Sun et al., 2003).

- *Cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1) and peptide YY (PYY)*

When food leaves the stomach, CCK, GLP-1 and PYY are released by the upper, the lower small intestine, and the more distant portions of the gastrointestinal tract, respectively.

- a. CCK: CCK, synthesized and secreted by I-cells from the upper intestine, functions as a satiation signal in humans. CCK binds type 1 and 2 CCK receptors (CCK1R and CCK2R, respectively) but the effects of CCK to elicit satiety after a meal are mediated by CCK1R. The natural ligand for CCK1R is the sulfated octapeptide of CCK (CCK-8), although other natural variants such as CCK-33, CCK-39 and CCK-58 can also bind to CCK1R. Briefly, both types of receptors activate phospholipase C and the subsequent inositol-3-phosphate production responsible to the release of calcium from intracellular stores. Also, activation of both adenylate and guanylate cyclase has been described in CCK1R-transfected CHO cells. Several actions have been described for CCK; thus, CCK can function as an insulin secretagogue (Lo et al., 2011), it participates in the regulation of gastrointestinal motility, and it also induces satiety through actions in the brain. Vagal afferent mechanisms have been implicated in the control of gastrointestinal motility, enzyme secretion and satiety signals by CCK (for review see Dufresne et al., 2006).
- b. GLP-1: GLP-1 is an incretin hormone produced by intestinal L-cells that promotes meal-stimulated satiety and insulin secretion (Drucker, 2006). GLP-1 exerts its effects through the activation of GLP-1 receptors (GLP-1R) mainly localized at the pancreas, heart, blood vessels, gastrointestinal tract and CNS. Since GLP-1 leads to stimulation of insulin secretion when glucose concentration is elevated and protects pancreatic  $\beta$ -cells from apoptosis, GLP-1R agonists or inhibitors of dipeptidyl peptidase-4 (DPP-4), the enzyme responsible for N-terminal cleavage and inactivation of GLP-1, have been clinically used for the treatment of T2DM (Campbell & Drucker, 2013). At least at the pancreatic level, GLP-1 signals through GLP-1R induce cyclic adenosine monophosphate (cAMP)



**Fig. 1.** Regulation of food intake. Crosstalk between peripheral and central signals (Adapted from [Dietrich & Horvath, 2012](#)). Multiple peripheral signals – leptin, insulin, glucagon-like peptide 1, amylin, pancreatic polypeptide (PP), peptide YY (PYY), cholecystokinin (CCK), ghrelin and nutrients as free fatty acids (FFA) – have been shown to modulate food intake through a direct action on the central nervous system (anorexigenic signals: red arrow / orexigenic signals: blue arrow). Through their role of sensor, they will provide information about energy availability that will be integrated by the hypothalamic neurons. Within the hypothalamus (right box), two subsets of neurons are expressed: (i) pro-opiomelanocortin (POMC)/cocaine- and amphetamine- regulated transcript (CART) neurons implicated in satiety promotion, and (ii) neuropeptide Y (NPY)/agouti-related protein (AgRP) associated with hunger and appetite. Anorexigenic signals are driven through the activation of POMC/CART neurons following leptin fixation on its receptor, inducing neuronal firing and release of  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) within the synapse made with melanocortin neurons expressing melanocortin 4 receptors (MC4R).  $\alpha$ -MSH binds MC4R inducing a subsequent reduction of food intake. On another side, ghrelin activates NPY/AgRP neurons, which release AgRP within the synaptic cleft. AgRP antagonizes MC4R promoting food consumption. NPY/AgRP neurons also release gamma-aminobutyric acid (GABA) that (i) bind gamma-aminobutyric acid receptors (GABAR) on MC4R neurons, and (ii) suppress POMC/CART neurons activity promoting hunger pathways.

production by a mechanism sensitive to AKT and ERK1/2 inhibition ([Liu & Habener, 2008](#)).

- c. Peptide tyrosine tyrosine (PYY): PYY is a peptide hormone belonging to the pancreatic polypeptide (PP) family of peptides. L-cells on intestinal tract release PYY<sub>1–36</sub> which is rapidly converted to PYY<sub>3–36</sub> after cleavage by the enzyme DPP-4 (for review see [Stadlbauer et al., 2015](#)). Locally, PYY<sub>3–36</sub> controls secretion and motility of the gastrointestinal tract; recent data indicate that PYY<sub>3–36</sub> can also regulate food intake and subsequently energy homeostasis by acting directly on CNS (see below). Interestingly, whereas circulating levels of PYY<sub>3–36</sub> are lower in obese subjects, food intake is reduced in both obese and non-obese individuals after PYY<sub>3–36</sub> administration, suggesting that PYY<sub>3–36</sub> may be pharmacotherapeutically interesting against obesity ([Batterham et al., 2003](#)).

• Leptin

Leptin, a protein mainly released by adipocytes, exerts its actions by binding to the leptin receptor (ObR) in CNS and in peripheral tissues such as heart, vessels, and pancreas. Although leptin is specially reported as an adipokine able to enhance metabolism and reduce appetite, recently, it has been shown that leptin displays a wide range of pleiotropic effects by acting on the cardiovascular, nervous, immune and reproductive systems (for review see [Ghantous et al., 2015](#)). Activation of ObR induces JAK-STAT pathways, insulin receptor substrate and MAPK ([Ahima & Osei, 2004](#)).

Leptin actions on energy metabolism are related to its ability to cross the blood brain barrier through a specific transport system to achieve hypothalamic arcuate nucleus (ARC) neurons ([Banks et al., 1996](#)). In this area, leptin modulates the activity of two ARC populations of neurons: the pro-opiomelanocortin (POMC) and the AgRP neurons. In response to leptin, POMC neurons secrete anorexigenic neuropeptides (cocaine- and amphetamine-regulated-transcript, (CART), and

POMC) and the precursor of  $\alpha$ -melanocyte-stimulating hormone (MSH), which reduces body weight. In addition, leptin inhibits NPY/AgRP neurons and subsequently reduces the release of orexigenic neuropeptides, including NPY and AgRP, which exerts its orexigenic effects acting as an  $\alpha$ -MSH antagonist through its binding on melanocortin-4 (MC4)-receptor.

Circulating leptin levels are positively correlated with the adipose mass and, although obese subjects are hyperleptinemic compared with lean persons ([Maffei et al., 1995](#)), they appear to be resistant to the central hypothalamic effects of leptin. In this respect, association of leptin with medications that could decrease leptin resistance may provide better weight-loss outcomes ([Blüher & Mantzoros, 2009](#)).

• Insulin

Insulin is a pancreatic hormone rapidly secreted after a meal intake and acts as an anorectic signal. Indeed, insulin decreases food intake in rodents ([Air et al., 2002](#)) and non-rodent mammals ([Foster et al., 1991](#)). Stimulation of insulin receptor, which possesses an intrinsic tyrosine kinase activity, via insulin receptor substrates activates PI3K pathway, mainly through the activation of the Akt/PKB and the PKC $\zeta$  cascades allowing modulation of insulin-induced appetite-regulating actions. Also, insulin can mediate central actions by acting on POMC and NPY/AgRP neurons. Intracerebral administration of insulin in rats inhibits the fasting-induced increase in NPY mRNA expression in the ARC ([Schwartz et al., 1992](#)), whereas NPY expression is enhanced in insulin-deficient rats ([White et al., 1990](#)).

• Amylin

Amylin, a 37-amino-acid hormone secreted from pancreatic  $\beta$ -cells after feeding, circulates in the blood to activate specific G protein-coupled receptors in the brainstem leading to an acute increase in cAMP. The major effects induced by amylin are related to the suppression of glucagon release from the pancreas, to a reduction in food

intake by inducing satiety (Lutz, 2010) and to gastric emptying. The net effect of these actions is to decrease blood glucose, associated with longer-term reductions in body weight (Hay et al., 2015). All these effects indicate that amylin and their analogues may be encouraging therapeutic opportunities for obesity.

- Pancreatic polypeptide (PP)

In addition to PYY (see above) and NPY (see next paragraph), PP is the third member of PP-fold family of peptides. PP is secreted by F-cells of pancreatic islets and acts on Y4 receptors at the CNS. Peripheral administration of PP induces anorectic effects. Indeed, it decreases food intake, reduces body weight and improves insulin resistance and dyslipidemia in obese rodents (Asakawa et al., 2003). Since circulating levels of PP are inversely proportional to adiposity, development of agonists to Y4 receptors in order to mimic PP effects is growing and may represent new candidates for therapies against obesity. However, precaution needs to be taken because central PP administration stimulates food intake probably by acting on another type of receptors (Campbell et al., 2003).

## 2.2. Central regulation of energy homeostasis

The regulation of the energy homeostasis by the hypothalamus is dependent on the balance between orexigenic factors – NPY and AgRP – and anorexigenic factors such as POMC and CART. Hypothalamus dysfunction seems to be a key player in the development of obesity. In this context, a positive correlation between volume of hypothalamus and body mass index (BMI) as well as leptin plasma concentration has been described (Horstmann et al., 2011). In addition, in rodents, early inflammation of hypothalamus characterized by overexpression of IL-6 and TNF- $\alpha$  is observed within 24 h of high-fat diet feeding versus late peripheral inflammation in liver and adipose tissue. Since the main affected areas are hypothalamic arcuate nucleus and adjacent median eminence, these results suggest that injury of a key brain area for energy homeostasis is associated with obesity (Thaler et al., 2012).

It has been shown that central injection of either NPY or AgRP increases food intake whereas overexpression of AgRP causes obesity by blocking the MC4 receptor. Conversely, inducible ablation of AgRP-expressing neurons causes acute anorexia in adult mice (Gropp et al., 2005; Luquet et al., 2005). In the same lane, inducible ablation of POMC neurons leads to hyperphagia and obesity.

Interestingly, peripheral hormones – insulin, leptin and ghrelin, see above – can regulate food intake via the control of hypothalamic activity since this region of hypothalamus is less protected by blood brain barrier than other brain areas (Meister, 2007). In particular, the maintenance of homeostasis is in part regulated by the bidirectional signaling between the gastrointestinal tract and the brain via neural – both central and enteric nervous systems – hormonal, and immunological mediators. Indeed, a tight link between peripheral factors and their integration at the central level accounts for this regulation. Hunger signals mediated by an increase in ghrelin and a decrease in insulin, glucose, leptin and CCK promote the activity of NPY/AgRP neurons, which in turn leads to a reduced activity of the MC4 system yielding a marked orexigenic effect. In contrast, following a meal with high levels of glucose, insulin, PYY<sub>3–36</sub>, CCK and reduced ghrelin levels have been measured. Altogether, these changes in hormone levels lead to an increase in POMC/CART neuronal activity and subsequently  $\alpha$ -MSH release and then, satiety.

Interactions between NPY/AgRP and POMC/CART neurons occur mainly via GABA production. GABA inhibits POMC/CART neurons increasing food consumption (Berthoud & Morrison, 2008). Indeed, pharmacological drugs such as the lipid-derived mediators, endocannabinoids, activate cannabinoid – CB<sub>1</sub> and CB<sub>2</sub> – receptors and inhibit GABA-mediated to reduce feeding (Bellocchio et al., 2010).

The potential involvement of gut microbiota in the development of obesity has been investigated and is now a growing area of research.

Indeed, gut microbiota regulate the communication between the brain and the gastrointestinal tract mainly by controlling food intake and satiety (Cryan & O'Mahony, 2011; Davey et al., 2013; Sam et al., 2012). Data obtained from *ob/ob* mice and from germ-free mice demonstrate that the former present different gut microbiota composition than that their wild type littermates (Ley et al., 2005), while the latter do not develop diet-induced obesity (Bäckhed et al., 2007). The role of gut microbiota in the regulation of obesity has been extensively reviewed elsewhere (Burokas et al., 2015; Duca & Lam, 2014). Briefly, this resistance to weight gain in germ-free mice is attributed to an increase in colonic epithelial AMP-activated protein kinase (AMPK), a sensor for cellular energy status. Furthermore, AMPK is also increased in the liver of germ-free mice, and play a role in the decrease in hepatic de novo lipogenesis.

### 2.2.1. Molecular integrators of eating/satiety stimulations in the hypothalamus

The mechanisms by which peripheral signals regulate activity of hypothalamic neurons involve changes in gene expression regulating intracellular signaling cascades. The main pathways implicated regarding the hormone actions are JAK/Stat – Stat3 and Stat5 – PI3K, AMPK and the mammalian target of rapamycin (mTOR).

The JAK/Stat3 pathway is activated by leptin in the hypothalamus, mainly in POMC/CART neurons. Specific deletion of Stat3 on CNS generates obese and hyperphagic mice (Gao et al., 2004). Activation of JAK/Stat pathway induces transcription of SOCS3, POMC and thyrotropin-releasing hormone (TRH). Also, Stat5 deficient mice display obesity and hyperphagy (Lee et al., 2008), but in contrast to Stat3, no changes on expression of POMC have been observed.

Both leptin and insulin activate PI3K signaling within hypothalamic neurons to suppress food intake (Niswender et al., 2001; Niswender et al., 2003). Although the molecular mechanism implicated in the effects of the two hormones in hypothalamus is not completely elucidated, hyperpolarization and inactivation of ARC neurons as a consequence of the activation of K<sub>ATP</sub> channels via PI3K might explain the central effects of leptin and/or insulin on the regulation of food intake and body weight (Spanswick et al., 2000).

AMPK is a sensor of the AMP/ATP ratio, such that depletion of cellular energy stores activates AMPK signaling. In the ARC, activation of AMPK by ghrelin increases food intake (Andersson et al., 2004), while glucose, leptin, and insulin inhibit hypothalamic AMPK activity (Lee et al., 2005; Minokoshi et al., 2004). In NPY/AgRP neurons, ghrelin stimulates AMPK and inactivates acetyl-CoA carboxylase leading to the decrease of cytoplasmic pool of malonyl-CoA resulting in an increase of fatty acid  $\beta$ -oxidation, which promotes the generation of reactive oxygen species (ROS). The upregulation of AgRP and NPY genes is mediated via a mechanism involving hypothalamic homeobox domain transcription factor (BSX), forkhead box O1 (FoxO1) and the phosphorylated cAMP response-element binding protein (pCREB) (de Morentin et al., 2011). In contrast, both leptin and insulin decrease the activation of AMPK and subsequently up-regulate malonyl-CoA leading to reduced food intake (Gao et al., 2007).

The serine-threonine protein kinase TOR, a target of rapamycin antibiotic, forms two distinct complexes, TORC1 and TORC2. The former regulates the activity of the translational initiation machinery when activated by nutrients – glucose and aminoacids – and the later modulates activation of Akt mainly by insulin (Inoki, 2008). Interestingly, mTOR levels vary inversely with those of AMPK and the activity of mTOR is regulated by AMPK. In ARC neurons, mTOR activity decreases before feeding, and increases after feeding (Cota et al., 2006).

## 3. Pharmacological treatments. The pharmacotherapy arsenal against obesity: past, present and future

Modifications in lifestyle towards remoteness from obesogenic environments and behavior patterns remain the primary prescribed aid to



effectively challenge obesity. Sustained restricted-caloric diets – 1500 and 1800 kcal/day for women and men, respectively – associated with enhanced physical activity have demonstrated effects – loss of 3 to 5 kg on the first 2 years (Shai et al., 2008) – during the first step of this alimentary behavior modification. However, lifestyle interventions have shown a low rate of success in most obese subjects generally due to the high-cost medical monitoring. In this respect, one-third of patients who had experienced substantial weight loss regained more than 5% of their body weight in the first year (Weiss et al., 2007). The lack of efficiency of this long-term preventive strategy uncovered a surgery approach. Bariatric surgery represents an additional therapy with a high and stable success rate in reduction of body weight. Bariatric surgery reduces the size of the stomach, increasing the fullness sensation, thus reducing the amount of ingested food (for full review, see Frühbeck, 2015). Although depending on the serious risks of surgery and metabolic complications, and due to the high cost of the operation, bariatric surgery remains considered as a last-resort treatment, and not as the currently large-scaled therapy.

Given the failure of behavior modifications and bariatric surgery to represent viable and stable anti-obesity therapies, research focused on the development of pharmacological drugs acting on molecular targets known to regulate the balance between food intake and expenditure. However, mostly due to the complex interactions between the multiple pathways regulating food intake and energy expenditure, anti-obesity drugs currently available are limited in number and efficacy. Most of the pharmacological agents developed to counter obesity attempted to decrease food intake to restore the energetic balance towards consumption-expenditure stability. The basic principle behind the development of anti-obesity drugs has been to target the pathways that stimulate satiety.

At the very beginning, in the early 70s, of this anti-obesity pharmacotherapy investigation, centrally targeted and sympathetic-like agents were the main given treatments (see below). They showed very promising effects on decreasing body weight. While activating the sympathetic nervous system inducing the release of catecholamines in the hypothalamus, these drugs promoted satiety, subsequently suppressing appetite (Motycka et al., 2011). However, these encouraging outcomes were associated with important deleterious side effects. One of these most infamous safety disasters is the combine pharmacological therapy fenfluramine-phentermine, also known as Fen-Phen. Approved in 1973 by the US FDA, Fen-Phen therapy was based on amphetamine properties of the both analogues, fenfluramine and phentermine. Very popular in the 1990s owing to its demonstrated effectiveness on reducing body weight, Fen-Phen was withdrawn from the market in 1997 following numerous declared side effects linked to life-threatening such as pulmonary hypertension, and heart diseases (Connolly et al., 1997). Despite this safety and financial disaster, other amphetamines-like drugs were later developed. Sibutramine, a serotonin or 5-hydroxytryptamine (5-HT)-norepinephrine reuptake inhibitor, also acting on central  $\alpha_1$ - and  $\beta_1$ -adrenergic receptors, and peripheral  $\beta_3$ -adrenergic receptors (Ledonne et al., 2009), was considered as an approved anti-obesity drug regarding its effects on body weight. However, as observed for the Fen-Phen, deleterious effects appeared after long-term use. An increased risk of cardiovascular events following sibutramine treatment made the FDA to pull it out from the market in 2010 (AFDA, 2010). Distributed all over the central and peripheral nervous system, the ubiquitous location of 5-HT receptors could explain the deleterious unwanted effects observed after amphetamine-like drugs uptake.

Looking forward on specific central mechanisms, inverse agonists of cannabinoid ( $CB_1$ ) receptors were developed. When administrated in both humans and animals, the  $CB_1$  receptors inverse agonists decreased food intake while increasing energy expenditure, leading to a subsequent body weight decrease (de Kloet & Woods, 2009). A synthetic  $CB_1$  receptor inverse agonist, the rimonabant, was developed and accepted by the pharmacological instances – FDA and EMA (European

Medicines Agency) – in 2006. However, once more, the patients included in the clinical-trials developed severe side effects. Narrowly, linked to psychiatric pathways, rimonabant-treated patients exhibited increased depression and suicide risks (Moreira & Crippa, 2009).

Due to these adverse deleterious effects, the above mentioned anti-obesity drugs did not provide the stable warranties needed for a long-term pharmacotherapy that requires efficiency, safety and durability. However, the phentermine – a sympathomimetic amphetamine-like drug modulating catecholamines levels within the hypothalamus – is actually still used as a short-term treatment. Combined with restricted-caloric diets and enhanced physical activity, phentermine has demonstrated significant effects on decreasing body weight – 12.2 kg loss related to the 4.8 kg loss in placebo group (Haddock et al., 2002). As a short-term treatment, phentermine side effects – insomnia and anxiety – are usually transient, providing an efficient pharmacological approach.

However, considering that the body needs to be regulated in a chronic manner to avoid accurate undesirable modification of the energy balance, and since the previously described therapies showed promising effects only during the first steps of the treatment before reaching plateaus, the needs to uncover long-term pharmacotherapies that could be included in pluritherapy is urgently needed. Certainly, the “magic bullet” drug is, at the present, considered more as a medical dream than a suitable therapy, although some potential promising drugs acting on metabolic pathways within the CNS to reduce body weight are now under development (see future directions). Currently, the FDA approved three drugs as long-term therapies. These drugs can be extensively classified regarding their central or peripheral action.

### 3.1. Peripherally targeted therapies

As most of the currently developed therapies focus on decreasing food intake through satiety pathways, these peripherally acting approaches do not act directly on appetite, but they attempt to decrease fat absorption at an intestinal level.

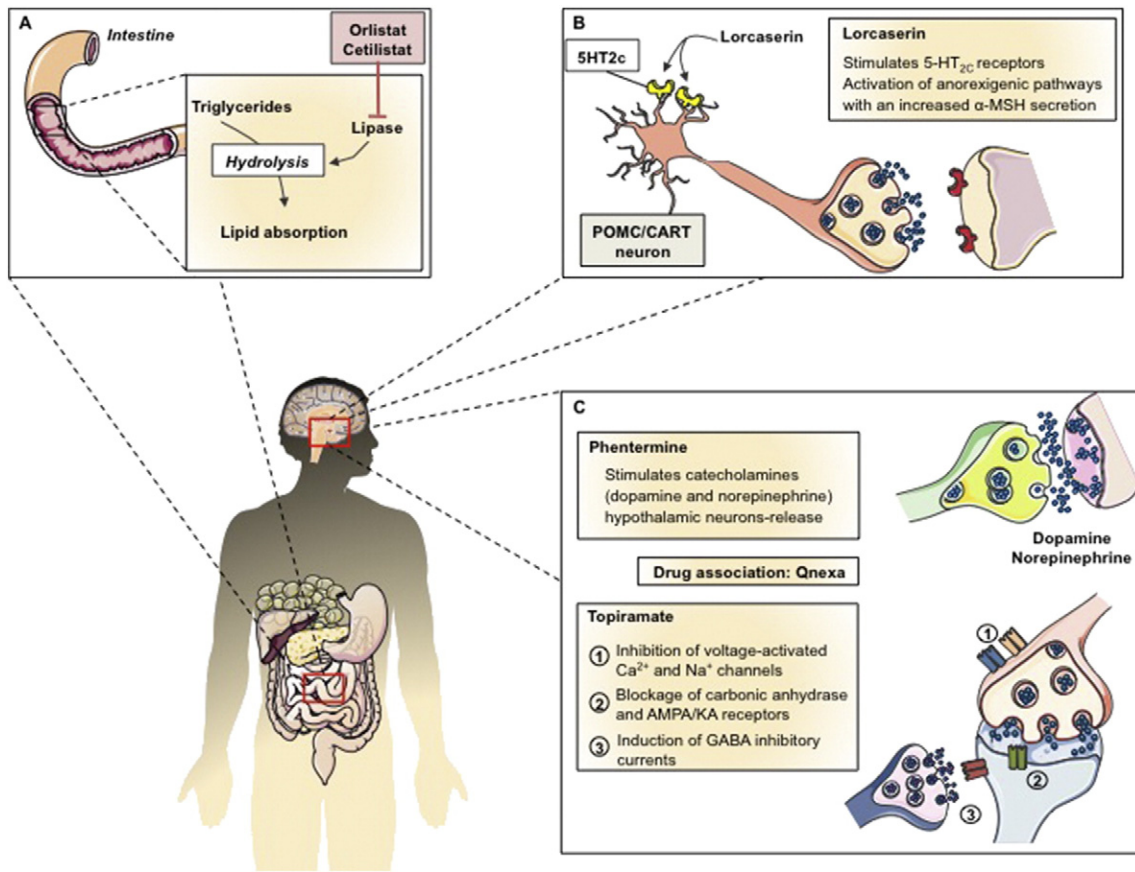
Approved in 1999 by the FDA, Orlistat remained for more than 10 years the only accepted obesity treatment. Orlistat is a gastrointestinal lipase inhibitor that decreases fat absorption through its inhibitory binding activity directly on pancreas and stomach produced-lipases. Physiologically active in the small intestine, these lipases degrade triglycerides in free fatty acids that can be latter absorbed by the intestinal epithelium. Therefore, through the inhibition of lipases, Orlistat will decrease intestinal fat absorption resulting on a calorie intake reduction (Fig. 2A). However, several side effects have been reported after the use of this drug. Diarrhea, flatulence, abdominal pains are the most common encountered adverse effects. Moreover, Orlistat uses recently attracted FDA and EMA consideration with 13 cases of serious liver injury following this peripheral medication (Ioannides-Demos et al., 2010). These side effects associated with a modest efficiency – a weight loss of 2.9% compare to a placebo group after one year (Hutton & Fergusson, 2004) – underscored the urgent needs to find new anti-obesity options.

Still looking on this fat absorption decrease strategy, Cetilistat is still under pharmacological research and clinical trials. Acting on the same pathway as Orlistat through the inhibition of pancreatic lipase, Cetilistat displays comparable efficiency as Orlistat but in an interesting manner, with less restraining side effects (Bryson et al., 2009) (Fig. 2A).

Thereby, associated with a low-fat and reduced-calorie diet, Orlistat and Cetilistat remain nowadays-possible anti-obesity pharmacological options. However, their moderate efficiencies and their associated side effects highlight the necessities to develop new anti-obesity strategies targeted on the master regulator of this regulation, the CNS.

### 3.2. Centrally targeted therapies

As discussed previously, endocannabinoids strategy, in particular using  $CB_1$  receptor antagonists provided severe psychiatric and



**Fig. 2.** Mechanism of action of the current anti-obesity drugs. One current anti-obesity drug is driven by a peripheral approach (A) and two others acting on the central nervous system (B, C). (A) Orlistat and Cetilistat decrease fat intestinal absorption through their inhibitory binding activity directly on pancreas and stomach produced-lipases. (B) Lorcaserin, a serotonin receptor agonist, binds and activates 5-HT<sub>2c</sub> serotonin receptors on pro-opiomelanocortin (POMC)/cocaine- and amphetamine- regulated transcript (CART) neurons followed by an increased  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) secretion. (C) Phentermine stimulates catecholamines – dopamine and norepinephrine – hypothalamic neurons-release that act as appetite suppressants. Topiramate suppressant appetite effects are mediated through the (i) inhibition of voltage-activated calcium and sodium channels, (ii) the blockage of carbonic anhydrase and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor/kainate (AMPA/KA) receptors and (iii) the induction of gamma-aminobutyric acid (GABA) inhibitory currents. The associated drug therapy (Qnexa) leads to food intake decrease.

neurologic side effects underscoring the needs to trigger other central food intake regulating pathways. The implication of 5-HT in this regulatory pathway opened a new avenue in anti-obesity drug development. 5-HT is an essential neurotransmitter implicated in numerous central processes within the CNS such as modulation of the circadian rhythms, regulation of mood and is also implicated in cognitive functions as memory and learning (El-Merahbi et al., 2015). In an interesting manner, 5-HT is also involved in the regulation of appetite. The pleiotropic effects of 5-HT results of its action on the multiple 5-HT receptors described. Actually, 5-HT are grouped in 7 different families including 14 different receptors (Fink & Göthert, 2007). Located all over the brain, the serotonergic neurons are also found in the hypothalamus, and 5-HT participates in the regulation of food intake. Hypothalamic serotonin 5-HT<sub>2c</sub> receptor drives the 5-HT-mediated appetite regulation. In 2008, Xu et al. showed that 5-HT<sub>2c</sub> receptor  $-/-$  mice had an obese phenotype associated with a hyperphagic behavior (Xu et al., 2008). Following its secretion by serotonergic neurons projections into the hypothalamus, 5-HT will bind 5-HT<sub>2c</sub> receptors on MC4R neurons. Ensues an activation of anorexigenic pathways with an increased  $\alpha$ -MSH secretion associated with a reduced AgRP expression (Heisler et al., 2006). Throughout this neuromodulation, 5-HT will promote satiety and body weight loss. Following this strategy, 5-HT agonists were developed during the early 1990s. At the very beginning, two 5-HT agonists – fenfluramine and dexfenfluramine – known to act as an appetite suppressant were developed. However, due to their non-selectivity, these treatments provoked valvular heart diseases through

their binding to 5-HT<sub>2B</sub> receptors (Thomsen et al., 2008). Thus, due to the numerous and complex pathways in which 5-HT is involved, the needs to increase the specificity of the 5-HT-like drugs is one of the principal matter considering this anti-obesity therapeutics accomplishment.

Approved by the FDA in 2012, lorcaserin is a 5-HT receptor agonist with a high selectivity for the 5-HT<sub>2c</sub> receptors – with an affinity 104 fold superior other than that for 5-HT<sub>2B</sub> receptor (Smith et al., 2008). This selectivity for 5-HT<sub>2c</sub> receptor subtype limits the threats due to the other receptors subunits such as hallucinations for 5-HT<sub>2A</sub> receptors or cardiac risks for 5-HT<sub>2B</sub> receptors. Acting as a 5-HT<sub>2c</sub> receptor agonist on POMC hypothalamic neurons, lorcaserin offers the needed efficiency without numerous side effects (Fig. 2B). Early studies demonstrated that chronic injections of lorcaserin in high-fat diet obese-induced rats provide promising effects by decreasing food intake and body weight (Fleming et al., 2013). Now engaged in phase III clinical trials such as BLOOM (Behavioural modification and lorcaserin for overweight and obesity management in diabetes mellitus) or BLOSSOM (behavioral modification and lorcaserin second study for obesity management), lorcaserin displays the expected promising effects. Results following the first year of treatment show that 47.5% of the lorcaserin-treated group lose at least 5% of their body weight – 20.3% on the placebo group – and that 22.6% lose at least 10% of their body weight – 7.7% on the placebo group – (Smith et al., 2010). One of the limiting factors on the previous strategies was the frequent occurrence of severe side effects. Interestingly, following lorcaserin treatment, only very rare cases of valvular heart diseases have been reported. These results underscore

the necessity to increase the specificity of pharmacological drugs for the future therapies. However, the use of lorcaserin still exhibit some uncomfortable side effects such as headache, weakness, nausea, dry mouth, cognitive impairment, priapism and bradycardia (Fleming et al., 2013). Given the multiplicity of adverse effects, some safety concerns should be settled when lorcaserin is prescribed. And like for every anti-obesity pharmacological approach, physical activities and restricted-caloric-diets should be persistent.

However, as seen in other pathologies, monotherapies give only few convincing results. Moreover, obesity is characterized among others by a complex regulation involving lots of partners such as sensory facts, mechanosensitive and hormonal signals, all these converging to the CNS through different connected pathways. Given these complex interactions, combination therapies have been developed throughout the past years.

Approved in 2012 by the FDA Endocrinologic and Metabolic Drug Advisory Committee, the combination of phentermine and topiramate – Qnexa – revealed promising results on treating obesity. As discussed earlier, phentermine is used since 1959 as an obesity short-term treatment. Owing amphetamine-like properties, phentermine stimulates catecholamines – dopamine and norepinephrine – hypothalamic neurons-release that act as appetite suppressants (Fig. 2C). Phentermine is also known to modulate leptin and NPY levels within the brain leading to food intake decrease. However because of its severe side effects, phentermine was associated with an anti-convulsing drug, topiramate. Initially prescribed for epilepsy and migraines, it appeared that patients treated with topiramate lose weight, suggesting a potential role as anti-obesity drug. These observations have been confirmed when topiramate-treated obese rats lose weight following both the decrease of food consumption and surprisingly the increase of energy expenditure (Richard et al., 2000). The exact mechanism of how topiramate is acting on food intake and bodyweight decrease remains currently unknown. However, it has been suggested that its suppressant appetite effect could be mediated through the inhibition of voltage-activated calcium and sodium channels; through the blockage of carbonic anhydrase and  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor/kainate (AMPA/KA) receptors; and also through the induction of GABA inhibitory currents (Perucca, 1997) (Fig. 2C). Providing promising results on small animals, phentermine and topiramate combined therapy has been included in phase III human clinical trials titled CONQUER, EQUATE and EQUIP (Allison et al., 2012; Gadde et al., 2011). Results provided by these 3 trials confirm the preliminaries expected results and convince the FDA to approve their market release. The analysis of the results provided by the 3 clinical studies show that approximately 75% of the Qnexa-treated subjects display a body weight decreased of 5%, and that nearly 50% exhibit a 10% body weight loss following a one year treatment (Garvey et al., 2012). To ensure of the desired stability and efficiency of the anti-obesity Qnexa pharmacotherapy, a subsequent clinical trial – SEQUEL – has been conducted to obtain 2 years suitable parameters. The results show a maintained weight loss following 108 weeks of treatment (Garvey et al., 2012). Interestingly, these clinical trials show that Qnexa treatment also improves obesity-related metabolic dysfunctions such as waist circumference, blood pressure, triglycerides and HDL/LDL cholesterol levels. However, despite providing encouraging effects on body weight loss and associated constants, phentermine/topiramate drug combination is also coupled to adverse effects such as paresthesias, dizziness, insomnia, constipation, depression and anxiety (Garvey et al., 2012). Furthermore, as described for the previous drugs, cardiovascular safety concerns need to be investigated. From this point and due to the deficiency of long-term data on Qnexa-induced cardiovascular side effects, EMA does not approve the proposal market authorization, waiting for convincing clinical trials results.

Alternative therapies are now developed to increase the specificity of the actions of pharmacological drugs trying to target in an exclusive manner the endocrine circuits components regulating appetite and/or energy expenditure.

### 3.3. Anti-obesity pharmacological approaches in development

Food intake is defined through the accurate balance between anorexigenic and orexigenic signals in hypothalamus. To establish new pharmacotherapies, companies focused on hypothalamic pathways attempting to hamper orexigenic signals while stimulating anorexigenic ones. To increase the efficiency of these developed drugs, an improved specificity towards hypothalamic master regulators appeared to be the leading target considering their upstream actions. Therefore, apart from the above-described pharmacological drugs accepted by FDA but still under investigation through long-term clinical trials, other strategies are emerging.

Following the hypothalamic appetite regulators targeting desire, RM-493 was developed. As discussed previously,  $\alpha$ -MSH, a derived POMC hormone, binds MC3R and MC4R to stimulate anorexigenic signals leading to the reduction of food consumption. Interestingly, RM-493, through its MC4R agonist function, mediates the same appetite inhibitory effects as  $\alpha$ -MSH does. Preliminary studies demonstrated that obese primate treated with this MC4R agonist during eight weeks showed a body weight decrease of 13.5% (Chen et al., 2015). This non-human trial results showed that the body weight reduction evoked by RM-493 was mostly due to a loss of body fat, but in an interesting manner, also to an increase of energy expenditure (Chen et al., 2015). However, these results were not verified on obese human patients. The RM-493 treated patients did not show any significant body weight decrease. Although an additional human trial has been performed in parallel regarding the energy expenditure component, these obese patients had their resting energy expenditure increased by 6.4% compared to placebo. Despite the fact that the low efficacy of this drug on body weight-decrease on obese primates, RM-493 has been included in a phase II trial in 2012.

As the MC receptors have a central role in inhibiting food consumption, they remain a chiefly studied target. Recently the combination of bupropion and naltrexone – Contrave – was included in phase III clinical trials. This combined pharmacotherapy decreases body weight by modulating the hypothalamic melanocortin system. Bupropion – a dopamine and norepinephrine reuptake inhibitor – stimulates the release of  $\alpha$ -MSH leading to a food consumption reduction. Concurrently, Bupropion also induces  $\beta$ -endorphin release initiating a negative feedback loop on  $\alpha$ -MSH-producing neurons decreasing  $\alpha$ -MSH release. Therefore, bupropion is associated with naltrexone, an opioid receptor antagonist, which prevents this negative feedback loop, and consequently increases  $\alpha$ -MSH anorectic effect (Ornellas & Chavez, 2011). This drug association provides promising results, 6.7% to 8.1% body weight loss – depending on the prescribed dose – compared to placebo group (Greenway et al., 2010). However, recurring adverse effects remain such as headache, nausea, and vomiting. As described for the other anti-obesity drugs, cardio-vascular warranties are requested for the drug to be approved. Therefore, a long-term clinical trial has been initiated in June 2012 with a completion date in 2017 to assess of these cardiovascular risks.

The S-2367 or Velneperit drug acts an antagonist of the Y5 receptor. Velneperit prevents binding of NPY to the hypothalamic Y5 receptor to reduce food intake. Recently, Velneperit-treated obese patients showed a 5% bodyweight loss compared to placebo group – phase II and III clinical trials – (Omori et al., 2012). Because this treatment is associated with a calorie-reduced diet, these results are only acceptable starting a later-associated combined therapy.

Drugs acting on leptin are developed due to its central action as an appetite-suppressant hormone. As described above, leptin binds its receptors (Ob-R), mostly located in the ARC nucleus within the hypothalamus, activating POMC neurons while inhibiting NPY/AgRP ones, generating anorexigenic signals. However, the suggested leptin monotherapy might not be the best option due to obesity-induced leptin resistance (Ozcan et al., 2009). Therefore, a combined therapy has been conducted associating a leptin analogue, metreleptin to an amylin



analogue, pramlintide thought to restore leptin sensitivity in an obesity context. Phase II trials provide promising results – 12.7% body weight loss after 20 weeks – (Ravussin et al., 2009). As observed with the others drugs, nausea, headache and injection site events are the main adverse effects encountered.

Regarding the latest strategies in term of developing anti-obesity drugs, it appears that either the lack of efficiency, the needs to combine them or the adverse effects encountered, encourage the research and medical fields to think about new targets or tools to increase the efficiency and tolerance of these needed treatments.

#### 4. Extracellular vesicles

As discussed previously, one of the restraining factors concerning the efficiency of anti-obesity drugs development is their specificity of action. Indeed, narrowly close to psychiatric and cardiovascular pathways, these active molecules induce negative undesirable side effects. Staring at the complexity of this pathology, it appears as necessary to develop specific treatments.

Drawing from how cells can intercommunicate with complex physiological or pathological biosystems, novative “nanobiomedicine” has been developed the past decades. The strategy is to use cell-derived membrane vesicles as shuttles of active molecules in order to increase the specificity and efficiency of the treatments. Herein, we will review the impact of EVs, as targets and tools, in an obesity context.

##### 4.1. Extracellular vesicles, biogenesis, composition and fate

There are multiple ways for the cells to communicate within their surrounding environment. Firstly described as either simple secretion of soluble factors or direct cell-to-cell interaction, this communication actually involves additional partners. Indeed, decades ago, it appears that mammalian cells (Trams et al., 1981), especially tumor cells (Dvorak et al., 1981) or platelets (George et al., 1982) could release membrane-shed vesicles, named microparticles (MPs), into solid tissue such as cartilage (Anderson, 1969), or even in biological fluids as blood or semen (Colombo et al., 2014; Crawford, 1971; Stegmayr & Ronquist, 1982). Furthermore, it has been shown that MPs are not the only actors of this vesicle-dependent intercellular communication. Indeed, nano-scaled vesicles could be released by cells through the fusion of the internal multivesicular bodies (MVB) with the plasma membrane. Regarding the fact they are formed by exocytosis, the term exosome has been suggested (Johnstone et al., 1987).

EVs term also contains another category of shed-vesicles, the apoptotic bodies, released by apoptotic cells (Hristov et al., 2004), but less significant in a therapeutic way, they will not be later discussed in this review.

Assumed to be biologically non-significant at the very beginning of their discoveries (Wolf, 1967), the two former types of EVs actually possess multiple important roles in numerous communication processes. Either in physiological responses – immune surveillance (Raposo et al., 1996), blood coagulation (del Conde et al., 2005), tissue repair (Martinez & Andriantsitohaina, 2011)... or in pathological disorders – cancer (De Toro et al., 2015) and cardiovascular diseases mostly (Gaceb et al., 2014), the EVs will carry and deliver multiple information through the transfer of lipids, proteins or nucleic acids.

Released by almost all cell types, MPs and exosomes differ in term of biogenesis, morphology, size, and secretion pathway (for review see Gaceb et al., 2014; Tual-Chalot et al., 2011). Recently, the International Society for Extracellular Vesicles (ISEV) proposes recommendations based on standardization of sample collection, isolation and analysis methods in EV research (Witwer et al., 2013). However, although efforts have been made to homogenize protocols, confusion between different types of EVs are still observable in the literature. In addition, the development of new technologies, mainly associated to flow cytometry, has allowed the characterization of new proprieties of EVs. The purpose of

the following part is to understand the mechanisms through which EVs are formed; how they interact with their recipient cells; and how they can sustain pathophysiological information; all this driven in an obesity context.

- MPs

Initially considered as “cell dust” (Wolf, 1967), MPs are now considered as small bioactive vesicles with a diameter included between 0.1 to 1  $\mu\text{m}$ . Even though the mechanisms driving the MP formation are not completely understood, several studies provide strong evidences that MPs are released through the blubbing of the membrane following chemical and physical cell activation or apoptosis (Fig. 3). These activation stimuli induce a maintained increase of intracellular calcium concentration leading to (i) a calcium-dependent proteolysis of cytoskeletal proteins (Miyoshi et al., 1996), and (ii) to kinase activation and phosphatase inhibition (Yan et al., 2009), both contributing to cytoskeleton disruption, essential step for the later membrane howling and MPs releasing. In addition to these observations, increased intracellular calcium concentration induces changes on activity of phospholipid transporters, inducing the externalization of the negatively charged aminophospholipid phosphatidylserine – naturally located at the inner leaflet of the plasma membrane (Panatala et al., 2015). As a result of the phosphatidylserine outer-leaflet exposure, an excess negative charge at the plasma membrane is observed, consequently leading to its disruption and later-blubbing. However, approximately 50% of MPs do not expose phosphatidylserine at their surface (Arraud et al., 2014), indicating that further studies are needed to deciphering the mechanisms implicated in the generation of MPs. Moreover, several pathways leading to an increase of intracellular calcium concentration are implicated in MP release mechanisms. Thereby, the ROS (Burger et al., 2011), Rho-associated kinase (ROCK-1) (Sebbagh et al., 2001) and the extracellular signal-regulated kinase (ERK) (Kunzelmann-Marche et al., 2002) are ones of the possible molecular actors inducing MP release.

On the other hand, apoptosis induction also triggers the release of MPs. Following activation of characteristic death pathways – ROCK-1 and Caspase 3 (Coleman et al., 2001) – the phospholipid bilayer membrane is disorganized through disruption of the subsequent cytoskeleton leading definitely to MPs releasing. However, these calcium-dependent MP release mechanisms are not exclusive.

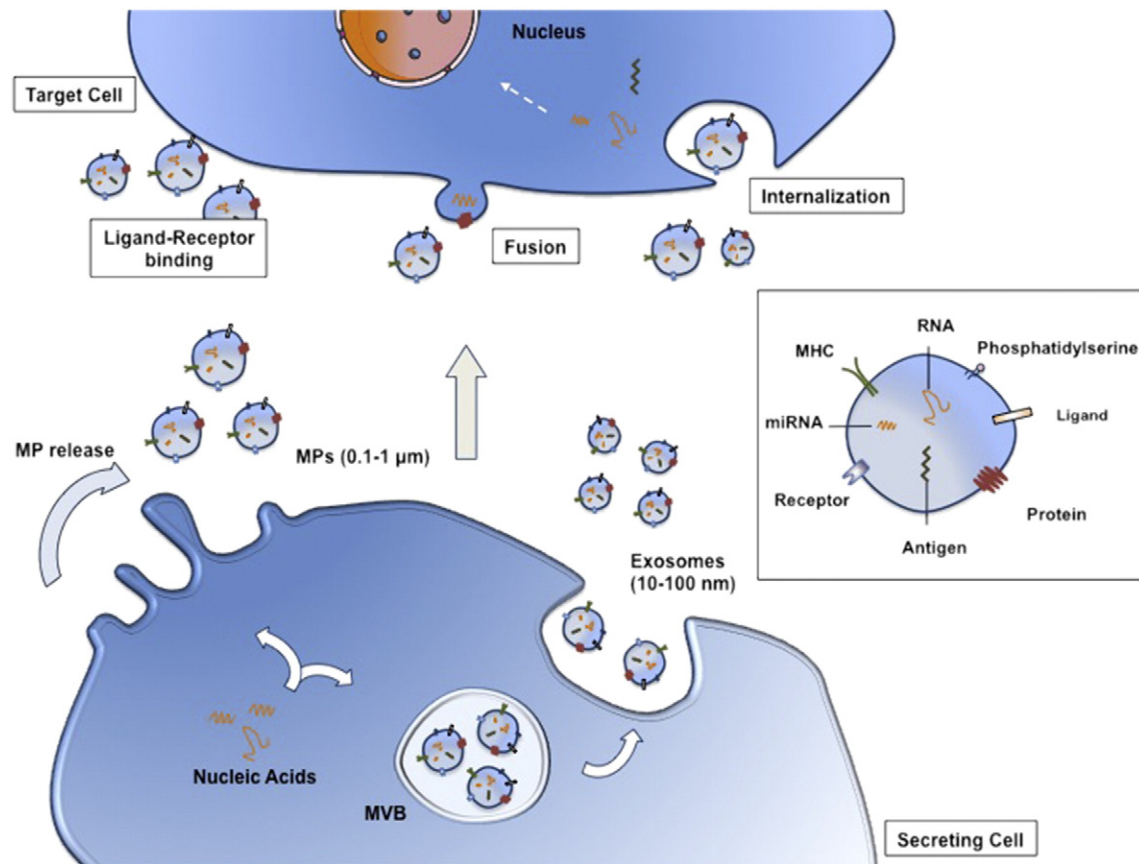
- Exosomes

While MPs take origin through the membrane blubbing, the exosomes, from 30 to 100 nm in size, are originated from the endosomal membrane cell compartment. Exosomes are formed through the endocytosis-mediated invagination of membrane fragments that fuse to form MVB (Raposo & Stoorvogel, 2013). The formation of MVB involves molecular mechanisms grouped into four multiprotein complex, the endosomal sorting complex responsible for transport (ESCRT) – 0, I, II and -III. These complexes are involved in recognition of proteins at the endosomal membrane and membrane blubbing (Raposo & Stoorvogel, 2013). The latter formed MVB have subsequently two potential fates regarding their cholesterol content. Low concentrations of cholesterol within the membrane of the fashioned MVB induce their degradation lysosomal-mediated pathway through their fusion with lysosomes (Möbius et al., 2002). Contrasting, an enrichment of cholesterol within MVB is markedly associated with the release of proper-formed exosomes, through the fusion of MVB with the plasma membrane inducing the exocytosis of these endosomes, calling them in, an interesting manner, exosomes (for full review, see Colombo et al., 2014) (Fig. 3).

- Messages carried by EVs

Interestingly, EVs – either MPs or exosomes – can naturally carry distinct signals carried by proteins, lipids and nucleic acids (Fig. 3). Due to their complex release mechanisms (different from MPs to exosomes) depending on stimulus and/or cell origin, the exact composition of these vesicles remains difficult to determine. However,





**Fig. 3.** Extracellular vesicles: Biogenesis, composition and fate (adapted from Raposo & Stoorvogel, 2013). Microparticles (MPs) are formed through the blebbing of the plasma membrane while exosomes are generated by the fusion of multivesicular bodies with the membrane inducing their release in the environment. MPs and exosomes may target the recipient cells through (i) direct interaction by ligand-receptor binding, (ii) fusion with the plasma membrane, and (iii) internalization processes. These pathways result in the delivery of proteins and nucleic acids into the membrane, cytoplasm or nucleus of the target cells. Extracellular vesicles as a schematic representation (right box) can carry either membrane proteins such as ligands, receptors, proteins, phospholipids or major histocompatibility complex proteins, and internalized components as nucleic acids – miRNA and RNA – and antigens.

data emerging from the literature improved our comprehension on their biochemical features. Through proteomic studies, initial reports demonstrated that all EV types carried shared surface proteins such as endosomal, plasma membrane and cytosolic ones, and this regardless of the origin cell (Théry et al., 2001). But as it is also known, EVs can also bear specific proteins that differ depending on the origin cell type as well as on the stimuli used to their generation. As an example, endothelial cell-derived MPs carry mostly metabolic enzymes, adhesion proteins and cytoskeleton-associated proteins whereas tumor-derived exosomes promote the transfer of oncogenes and oncomiRNAs (Kharaziha et al., 2012). Concerning the lipid composition little is known. However, studies showed that EVs were enriched in sphingomyelin, phosphatidylserine and cholesterol depending on their sorting mechanisms (Carayon et al., 2011). In a more interesting manner, EVs can contain nucleic acids, especially small RNAs including mRNAs and miRNAs (Valadi et al., 2007), conferring them the ability to transfer genetic material to their target cells.

Once released, through previously described mechanisms, the EVs assume their cell-to-cell-communication functions through interactions with their recipient cells (Fig. 3). EVs can interact with target cells and then modify their phenotype and/or function by different mechanisms (Andriantsitohaina et al., 2012; Gaceb et al., 2014; Martinez & Andriantsitohaina, 2011; Tual-Chalot et al., 2011): through direct interaction with the receptors present at the surface of their target cells and subsequent activation of cascade signalisation, or by transferring lipids, proteins, nucleic acids by fusion or internalization, EVs are able to transfer material to their target cells (Fig. 3).

The current interest in EVs derives not only from their great potential as novel biomarkers but also as a new way to deliver innovative

therapies to specific target cells. Indeed, research on EVs has exploded in the past decade, since these microstructures seem endowed with multiple roles, from blood coagulation to inter-cellular communication in pathophysiology.

Recent studies have reported that EVs possess therapeutic potential through reprogramming of target cells, affording modulation of cellular processes and secretomes – the molecules secreted by cells – and eventually favoring tissue repair after reprogramming of target cells. Thus, enhanced levels of circulating EVs are not always accompanied by a deleterious effect; indeed, some populations of EVs could deliver protective biological messages, preserving endothelial function, vascular integrity and tissue repair. Indeed, EVs from septic patients are able to stimulate the release of anti-inflammatory cytokine such as interleukin (IL)-10 that likely contributes to the protective effect of EVs (Mostefai et al., 2013). Moreover, EVs from human mesenchymal stem cells protect against cisplatin-induced acute kidney injury in the mouse, with tissue survival achieved through increased expression of anti-apoptotic genes (Bruno et al., 2012). Also, mesenchymal stem cell-derived exosomes can elicit hepatoprotective effects against toxicants-induced injury, mainly through activation of proliferative and regenerative responses (Tan et al., 2014). In a model of mouse myocardial ischemia/reperfusion injury, mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling (Arslan et al., 2013). Herein, EV composition is driven by pathophysiological processes conferring them specific capacities. However, new developed engineering methods allow determining exactly their composition making the EVs a potential new delivery tool (see below).

Taken together, these observations corroborate the important role of EVs as a new communication way that may be involved in the sustainability of pathophysiological information (Harding et al., 2013). EV implication in immunology and cancer has been well characterized during the past years; however, their role in obesity-induced complications is not fully developed. Herein, we try to understand how EVs can participate in the development and maintaining of secondary obesity-induced disorders.

However, recent advances in the field demonstrated overlaps in the biogenesis mechanisms of MPs and exosomes disabling the actual supporting idea of discerning them by their specific markers (Gould & Raposo, 2013). Therefore, within this review, some studies and subsequent results will not discriminate either MPs or exosomes but will be focused on EVs as a whole.

#### 4.2. Extracellular vesicles and metabolic diseases

Surely driven by classic processes including endocrine and paracrine communication, the subsequent disorders associated with obesity development – T2DM, cardiovascular diseases, hepatic steatosis, chronic inflammation and cancer – are also under the control of several circulating factors. In particular, the initiation and progression phases of these disorders are governed by metabolites and cytokines (Cui et al., 2011; de Luca & Olefsky, 2008; Reilly et al., 2005;), or miRNAs (Bonauer et al., 2010). However, very recent experimental evidences demonstrated that the EVs held a crucial role in the establishment of these secondary obesity-complications.

- a. Correlation between EV rate and severity of metabolic disorders. Accumulating hints showed that the plasmatic levels of EVs were correlated with the severity of the diseases conferring EVs a potential role of biomarkers. For instance, long-term high-fat diet fed rats exhibit a substantial increase of the level of MPs within the plasma compared to chow-fed rats (Heinrich et al., 2015). These observations are confirmed through human preclinical studies including obese, metabolic syndrome and T2DM patients in whom numbers of MPs are increased (Agouni et al., 2008; Campello et al., 2015; Diamant et al., 2002; Goichot et al., 2006; Noci et al., 2015). Regarding the correlation between exosomes and metabolic dysfunctions, less is known. However, two recent studies have shown a positive relation between exosomes rate and metabolic disorders development. Phoonsawat et al. (2014) showed that *ob/ob* mice display elevated rates of exosomes compared to wild-type mice. Moreover, Kranendonk's team demonstrated that exosomes levels bearing cystatin C were positively related to metabolic complications of obesity in patients with clinically vascular diseases (Kranendonk et al., 2014a). These recent findings uncover a potential role of EVs in the pathogenesis of metabolic diseases. Initially considered as simple biomarkers, EVs are now believed to participate in an active manner in the development and the maintaining of metabolic subsequent obesity-induced disorders.
- b. Involvement of EVs in the development and maintenance of obesity and obesity subsequent diseases
  - a. *Type II diabetes*  
Adipose tissue is the principal site of energy storage. When food consumption is in excess compare to the body needs, it is converted in fat and stored into the white adipose tissue. As the obesity is defined as a long-term excess of calorie intake, it will finally lead to an exceed storage in the white adipose tissue inducing its hypertrophy and hyperplasia. Presently, both adipose hypertrophy and hyperplasia are considered to be associated with intracellular abnormalities of adipocyte function leading to notably abnormal cytokines secretion, adipose tissue-mediated insulin resistance and inflammation, resulting in obesity clinical appearances and subsequent metabolic disorders such as T2DM.

Although the role of adipokines such as TNF- $\alpha$ , IL-6 or macrophage migration inhibitory factor (MIF) in obesity-associated insulin resistance is well established (Hotamisligil et al., 1994; Senn et al., 2003; Verschuren et al., 2009), the exact mechanisms through which inflamed adipose tissue leads to T2DM may also encompass the EVs.

The first evidence showing a possible implication of adipose tissue-derived EVs in intercellular communication is demonstrated using 3T3–L1 precursor adipocyte cell line which could actively release EVs (Lancaster & Febbraio, 2005). These preliminary results are confirmed using either primary rat adipocytes or adipose tissue in which EV release is also observed (Deng et al., 2009; Müller et al., 2009). However, upon this active secretion, it is also demonstrated that adipocyte-released EVs could also induce secondary obesity-associated disorders such as inflammation and insulin-resistance.

Interestingly, Deng et al. (2009) have shown that exosome-like vesicles released from adipocytes from *ob/ob* mice could induce deleterious effects. Indeed, when injected into wild type C57BL/6 mice, EVs from *ob/ob* mice are taken up by peripheral monocytes and strikingly induced their differentiation into macrophages, leading to an increase in pro-inflammatory cytokine – TNF- $\alpha$  and IL-6 – release. Besides this inflammatory context induction, they also demonstrated that the injection of *ob/ob* EVs induces systemic insulin-resistance (Deng et al., 2009). The authors suggest that these effects are dependent of a TLR4 pathway. Indeed, when injected in TLR4-deficient mice, *ob/ob* EVs are inefficient in inducing pro-inflammatory cytokines and the subsequent insulin-resistance.

These preliminary results obtained on rodent models are confirmed through human pre-clinical studies. In 2014, Kranendonk and colleagues have shown for the first time that human adipocyte-derived EVs could impair insulin signaling in both hepatocytes and skeletal muscle contributing to systemic insulin-resistance. However, they have also shown that human adipose tissue-derived EVs induce differential effects depending on the type of target cells. Thus, the effects are more pronounced in hepatic cells, where EVs induce a deregulation of gluconeogenesis and glucose, lipids and glycogen-storage, than in skeletal muscle, where a sole impairment of glucose uptake is observed (Kranendonk et al., 2014b).

Taken together, these results suggest that adipose tissue-derived EVs could participate in the inflammation encountered in obese adipose tissue enhancing the inflammatory context inducing subsequent local and systemic insulin-resistance.

Moreover, besides this insulin resistance disorder, it has been shown that EV-mediated communication could also participate in the elaboration of hypertrophic adipocytes that contribute to the deleterious effects of adipose tissue in an obesity context. In response to weight gain, white adipose tissue, and the adipocytes composing it, will expand inducing deregulations in cytokine and chemokine secretion, hypoxia, cell death, immune cell infiltration, and impairment in fatty acid metabolism and storage (McArdle et al., 2013). Accumulating evidences gained from the past years demonstrated that EVs released by adipose tissue could also be implicated in adipocyte function deregulation. Following stimuli found in obese adipose tissue such as fatty acids or ROS, large multilocular primary rat adipocytes or even differentiated human adipocytes release exosomes into the interstitial space. These exosomes participate in pathophysiological processes through the induction of an increase of fatty acid esterification and a decrease of lipolysis within the target small unilocular adipocytes (Müller et al., 2011). Considering that hypertrophic adipose tissue provides more unfavorable metabolic profile than smaller one (Arner et al., 2010), these results suggest there could be a deleterious EV-mediated communication within obese tissues contributing to the

inflammatory insulin-resistance context encountered in obese patients.

b. *Cardiovascular diseases*

Even though several studies demonstrate the implication of EVs in obesity-induced diabetes development, EVs are also involved in other complications including cardiovascular diseases.

Endothelial dysfunction is usually coupled with the initiation and development of cardiovascular diseases, such as atherosclerosis (Shimokawa, 1999). Interestingly, because of its location, endothelium is one of the principal targets of EVs. Under pathological conditions, it has been shown that EVs could contribute to the establishment of a proinflammatory phenotype that leads to endothelial dysfunction (Diamant et al., 2004). It has been shown that the molecular mechanisms underlying EV-induced endothelial dysfunction are related with a reduction of nitric oxide (NO) production from endothelial cells. We have demonstrated that MPs from metabolic syndrome (MS) patients decrease in vitro NO and O<sub>2</sub><sup>-</sup> production and eNOS activity in endothelial cells. Also in vivo injection in mice of MPs derived from MS patients impaired endothelium-dependent relaxation and decreased eNOS expression (Agouni et al., 2008). Moreover, MPs from MS patients induce an ex vivo vascular dysfunction through another mechanisms involving Fas/Fas ligand pathway increasing NO and ROS release and altering cyclooxygenase metabolites and monocyte chemotactic protein-1 (MCP-1) production (Agouni et al., 2011). These data suggest a role of MPs from MS in promoting endothelial dysfunctions that could lead to atherosclerosis development.

Moreover, data from the literature also show that EVs released by monocytes can participate in the obesity-induced vascular dysfunctions. Aharon et al. (2008) have shown that monocyte cell line (THP-1)-derived EVs adhere and penetrate endothelial cells and induce their apoptosis associated to cell thrombogenicity. As obesity is characterized by an inflammatory state and that endothelial dysfunction, thrombogenicity and apoptosis are associated with cardiovascular diseases; these data suggest that immune cell-derived EVs can also participate in obesity-induced disorders.

Considering the deleterious effects mediated by EVs in the development of metabolic complications in an obesity context, pharmacological strategies were developed focusing in decreasing EV release or by inhibiting their functions.

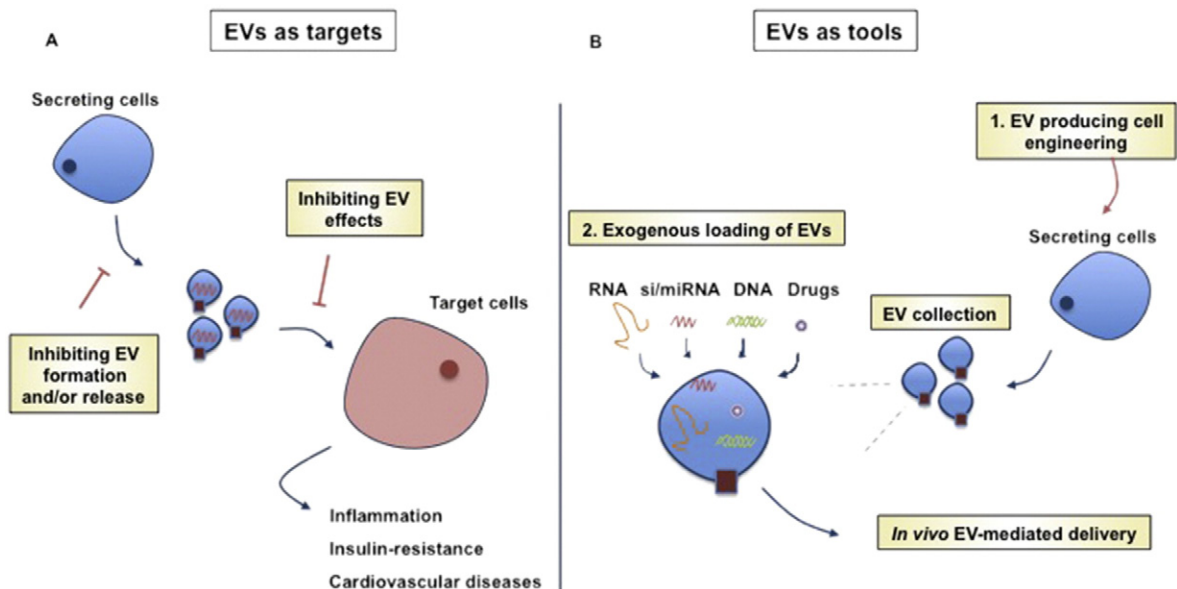
4.3. *Extracellular vesicles as a therapeutic target*

Because of the demonstrated positive correlation between EVs and occurrence and/or severity of metabolic diseases, therapeutic approaches decreasing their release and subsequent associated effects are raising (Fig. 4A). As the precise mechanisms governing the EV formation are not completely elucidated, the following strategies may raise non-specific limitations, however they showed promising effects.

Nutritional interventions can correct circulating levels of MPs and then, the subsequent deleterious effects that they evoke. Indeed, supplementation of red wine polyphenols reduces the increase in MP levels in hypertensive rats (López Andrés et al., 2012). Moreover, in the same model, red wine polyphenols prevent macrovascular inflammation and oxidative stress, and microvascular endothelial dysfunction. These results suggest that red wine polyphenols may represent new therapeutic strategies by correcting the production of MPs and their associated effects. Also, in humans, chronic consumption of oat-enriched diet for 8 weeks reduces both circulating levels of platelet-derived MPs and the early stage vascular inflammation in T2DM patients (Zhang et al., 2014), suggesting that as for red wine polyphenols, dietary intervention with oat supplementation can reduce deleterious effects of high levels of MPs found in pathological conditions.

As described above, stable success rate in reduction of body weight is described when bariatric surgery is used in obese patients. Cheng et al. have shown that at one month after bariatric surgery, the decrease in BMI is associated with the improvement of glycaemia and reduction in endothelial-, platelet- and monocyte-derived MPs reflecting a diminished inflammation (Cheng et al., 2013).

Regarding pharmacological treatments, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are used in the purpose to decrease EV release. The associated



**Fig. 4.** Extracellular vesicles as targets and tools. Extracellular vesicles (EVs) can either be seen as targets and tools in pathological states. (A) EV implication in obesity-induced metabolic diseases has been well characterized inducing inflammation, insulin-resistance and cardiovascular diseases. Even if the exact mechanisms governing the EV formation and release remain unknown, targeting their production could be an attractive lead to decrease the associated deleterious effects. Another approach could be to inhibit the EV effects through direct or indirect ways. (B) However, through their delivery properties, it is suitable to perceive EVs as therapeutic tools. 1) Through the modulation of the content of the EV producing cell, EV composition can be driven. 2) As exogenous drug or nucleic acids loading is enabled through electroporation methods, the design of specific loaded EVs open new avenue in drug discovery. RNA, miRNA, siRNA, DNA or even pharmacological drugs can be loaded into EVs enhancing their in vivo delivery.



medication between pitavastatin and eicosapentaenoic acid (EPA), an omega-3 fatty acid, significantly decreases platelet-derived MP levels compared to monotherapy with EPA. Moreover, adiponectin levels – decreased in obese patients – are increased following the pitavastatin-EPA treatment. Taken together these data indicate that this associated therapy could be beneficial in the prevention of vascular complications in hyperlipidemic T2DM patients (Nomura et al., 2009). Another statin family member, pravastatin, modifies the MPs release. Although eight-week treatment with pravastatin does not change MP levels in T2DM patients, a significant reduction in platelet activation markers is observed. Indeed, glycoprotein IIIa exposure on platelet-derived MPs is decreased, probably resulting from a decrease in platelet activation (Sommeijer et al., 2005). Moreover, Mobarrez and colleagues demonstrated that patients with peripheral arterial occlusive disease treated for eight weeks with atorvastatin have (i) reduced thrombin generation, and (ii) decreased expression of tissue factor, glycoprotein IIIa and P-selectin on MPs originating from platelets (Mobarrez et al., 2011). Thus, atorvastatin treatment could have beneficial effects on hemostatic variables, which are expected to be beneficial in terms of atherothrombotic complications. Furthermore, it has been demonstrated that endothelial and monocyte-derived MPs release could also be affected by statin pharmacotherapy. For instance, fluvastatin prevents MP release from TNF- $\alpha$ -activated endothelial cells in a Rho/Rho kinase dependent pathway (Tramontano et al., 2004). Moreover, combined therapy with simvastatin and losartan, an angiotensin II type I receptor antagonist, decreases monocyte-derived MPs in a more efficient way than monotherapy indicating valuable anti-atherosclerotic therapy in patients with T2DM (Nomura et al., 2004a, 2004b).

Besides this statin-mediated MP releasing, calcium antagonists are also able to significantly reduce circulating levels of MPs thus decreasing their deleterious effects in metabolic diseases. For instance, T2DM patients treated with nifedipine for six months exhibit reduced levels of endothelial-, monocyte- and platelet-derived MPs (Nomura et al., 2007). Benidipine has the same effects on decreasing levels of MPs. Following six months benidipine treatment, hypertensive patients with T2DM show reduced quantities of endothelial- and monocyte-derived MPs (Nomura et al., 2005). Furthermore, the administration of probucol associated with ticlopidine to hyperlipidemic T2DM patients induces a decrease in levels of monocyte- and platelet-derived MPs (Nomura et al., 2004a, 2004b). Taken together, these results raise new strategies facing T2DM using calcium antagonists to decrease MP formation and/or release, thus inhibiting the MP-evoked effects.

Following this desire to decrease MP release, peroxisome proliferator-activated receptor (PPAR) agonists are used as potential pharmacological agents to treat diabetes and hyperlipidemia. For instance, patients with metabolic syndrome treated with pioglitazone, a PPAR- $\gamma$  agonist, have reduced circulating levels of endothelial-derived MPs (Goldberg & Dansky, 2006). Also, sulphonylureas such as glibenclamide used to stimulate insulin secretion from  $\beta$  pancreatic cells could also decrease the number of released MPs (Henriksson et al., 2011). Moreover, alpha-glucoside inhibitors – miglitol and acarbose – showed promising effects on decreasing platelet-derived MPs in T2DM patients following a three months-treatment (Nomura et al., 2011; Shimazu et al., 2009).

Other strategies trying to inhibit MP-mediated effects are still emerging. Tesse et al. demonstrate that treatment of mice with rosiglitazone, another PPAR- $\gamma$  agonist, decrease both hyporeactivity and inflammation induced by lymphocyte-derived MPs (Tesse et al., 2008). Thus, these data suggest that PPAR agonists could be a potential therapeutic approach to fight against vascular dysfunction mediated by MPs associated with inflammatory diseases.

Although these different approaches targeting MP release decreasing provide encouraging results in limiting MP-mediated metabolic effects, the exact mechanism by which these drugs act are currently not completely elucidated. Indeed, it should be noticed that the changes in the release of the quantity of MPs could result both from a direct effect

of the drug on MP formation, but also from drug-induced change in MP clearance (Martinez et al., 2011). Moreover, because of the wild varieties of EVs encountered within the organism, such therapeutic believes require the drug delivery systems that enable the targeting of specific EV-producing cell type.

In addition, at the present, less is known about pharmacological approaches demonstrated effects in decreasing exosomes release and they are generally focused on tumor development (for full review, see Andaloussi et al., 2013b). Thus, unlike MPs that can be seen as pharmacological targets in an obesity context, new paradigm is raising using EVs, in particular exosomes, as a potential tool exploiting their cargos properties to deliver drugs or even nucleic acids to their target cells.

## 5. Extracellular vesicles, a bio-inspired innovative way to treat numerous diseases

Since the early 1960s and the first-time aroused “gene therapy concept”, a lot of progress is achieved. At first thought to treat only genetic disorders by replacement of the defective DNA by “good DNA” (Roger, 1972), gene therapy has reached unexpected heights. Indeed, through the development of modified DNA sequence to induce protein inactivation – targeted silencing drugs, antisense oligodeoxynucleotides, ribozymes and RNA interference – it opened a new avenue in drug development. However, their therapeutic potential is limited due to (i) their low stability within body fluids such as blood, inducing their rapid degradation following systemic injections, (ii) to inadequate protein inactivation mostly because of their limited tissue-specificity actions inducing numerous deleterious side effects.

To counter these undesired consequences following non-specific delivery, last decades provide some stimulating breakthrough through the expansion of nanomedicine strategies. Adenovirus, liposomes, or even synthetic nanoparticles delivery strategies are developed in regenerative medicine (Chaudhury et al., 2014) or against several pathologies such as cancer (Stylianopoulos & Jain, 2015). However, two main issues limited their uses. Firstly, due to their membrane composition – solely composed of lipids – the crossing of the recipient-cell membrane was limited (Andaloussi et al., 2013a; Subra et al., 2007). Secondly, their repeated injections induced inflammation-undesired responses. These two latter matters enhance the desire to develop new bio-inspired nanovesicles, by taking into account the intrinsic property of the EVs. EV-associated therapeutic effects are due to their capacity of carrying proteins and nucleic acids, in particular mRNA and miRNA, and in consequence, they are thought to be used as delivery vehicles (Ratajczak et al., 2006). As mentioned previously, their composition can be physiologically determined (see above), or, in an interesting manner, driven by exogenous modulations either on origin cells or directly on purified EV populations.

- EV produced by cell engineering

Thus, EVs can be engineered to over-express different therapeutic players – proteins, mRNA or miRNA – by driving their synthesis of from the relevant EV-producing cells (Fig. 4B). For instance, physiological shear stress induce MP release from endothelial cells carrying atheroprotective miRNAs – miR143/145 – which induce an atheroprotective smooth muscle cell phenotype suggesting that the enrichment of miR143/145 into MPs may provide a promising strategy to combat atherosclerosis (Hergenreider et al., 2012). Also, EVs derived from apoptotic endothelial cells generated during atherosclerosis contain miR-126, which controls endothelial cell signaling and affords atheroprotection in vivo (Zernecke et al., 2009). EVs from activated/apoptotic T lymphocytes harboring the morphogen Sonic hedgehog (Shh) also have therapeutic potential. As an example, endothelial dysfunction in mouse coronary artery after ischemia/reperfusion is prevented by treatment with Shh-carrying EVs. Moreover, EVs expressing Shh favor in vitro angiogenesis (Soleti et al., 2009) and develop a positive impact

on the recovery of hind limb flow after peripheral ischemia (Benamer et al., 2010). Very recently, we report in a rat model of ischemia–reperfusion that stimulation prior to reperfusion of Shh pathway by EVs reduces both infarct size and subsequent arrhythmias by preventing ventricular repolarization abnormalities. Besides its effect on both angiogenesis and endothelial dysfunction, we demonstrate a novel cardio-protective effect of EVs harboring Shh acting directly on the cardiomyocytes (Paulis et al., 2015). Thus, EVs can be engineered to over-express different therapeutic players – proteins, mRNA or miRNA – by driving the synthesis of the relevant EV-producing cells and that can be exploited as biocompatible specific vehicles for anti-obesity drugs. However, as the exact secretion EV mechanisms are not fully understood, new strategies are developed to accurately control their composition.

- Direct EV exogenous loading

Therefore, another strategy consists to load EVs with pharmacological drugs able to act on target cells. Isolated tumor-derived MPs loaded with a package of chemotherapeutic drugs are able to kill hepatocarcinoma-tumor cells in a specific way without unwanted side effects (Tang et al., 2012). Furthermore, Alpha-2 macroglobulin (A2MG) incorporated into MPs activates distinct host protective responses in murine sepsis, when compared to soluble A2MG, leading to enhanced bacterial containment and clearance ultimately resulting in improved survival. A2MG incorporation into MPs induces an active form of the protein, which counteracts the immune paresis typical of certain stages of sepsis (Dalli et al., 2014). Thus, A2MG enrichment in MPs represents an important host protective mechanism in sepsis and could be harnessed for therapeutic purposes. This property is also displayed by exosomes. Human tumor cell-derived exosomes deliver exogenous RAD DNA repair proteins directed siRNA to recipient cancer cells inducing their massive reproductive cell death (Shtam et al., 2013). However, using tumor cells, as producer of EVs – both MPs and exosomes – do not completely resolve the inflammation process encountered in delivery strategies. Indeed, even thought to be biological material, these produced EVs can bear immunogenic components at their surface.

To counter this efficiency-limiting process, recent studies use dendritic cells at their immature state to produce EVs, mainly exosomes, deprived in immunogenic capacities. After several injections, no evidence for immunogenicity or toxicity is detected (Alvarez-Erviti et al., 2011).

An additional problem to overcome is the lack, or relative low, of specificity of the EV treatment. One approach to increase this tissue targeting is using non-traditional systemic administration routes. A study led by Zhuang in 2011 used exosomes to deliver anti-inflammatory drugs to the brain through intranasal administration. The exosomes, loaded with curcumin or with a Stat-3 inhibitor – JSI-124, are intranasally injected. Under these conditions, up to 60% of loaded-exosomes are taken up by microglial cells, and surprisingly induce their apoptosis, demonstrating the still-active effect of these incorporated substances (Zhuang et al., 2011). Regarding the lack of efficiency of the previous strategies to cross the blood-brain barrier, this study shows an efficient innovative way to improve brain-located deliveries. Driven by the same motivation of improving the tissue specificity of the delivery, one breakthrough study shows, for the first time, that engineered exosomes can be addressed to target in a specific way the CNS. Indeed in 2011, Alvarez-Erviti and colleagues exposed that immature dendritic cells transfected with a plasmid coding for an exosomal protein, Lamp2b, fused with a specific glycoprotein derived from the neurotropic rabies virus that will enable the blood brain barrier crossing, RVG, produce neuronal-targeted exosomes (Alvarez-Erviti et al., 2011). These exosomes, loaded with a specific siRNA inhibiting Beta-secretase 1 (Bace1) expression, are intravenously injected into mice. This study obtains remarkable results with a 62% reduction of Bace1 expression throughout the brain, with, very interestingly,

minimal liver and spleen targeting – major detoxification organs – (Alvarez-Erviti et al., 2011). The same research group also suggests that this strategy could be used to target other tissue, employing other conjugated tissue-specific peptides (Andaloussi et al., 2013a), opening the field to a wide variety of pathologies.

Quite the same strategy was used in 2013 when Ohno's group engineered the exosomes surface making them carrying a specific GE11 peptide that bears specifically the EGFR – epidermal growth factor receptor – widely expressed by the tumor cells. Intravenously injected, let-7a miRNA-loaded exosomes inhibit the breast cancer development in RAG2<sup>−/−</sup> mice (Ohno et al., 2013).

Another possibility is to load EVs with iron oxide particles and to exploit the superparamagnetic properties of generated particles in order to control their sorting or their distal manipulation in a precise area (Faraj et al., 2012). These authors have generated endothelial MPs loaded with iron oxide nanoparticles enabling their noninvasive monitoring with magnetic resonance imaging in mice, but this approach may allow to delivery drugs loaded into EVs in a confined area reducing collateral effects.

Taken together, these different studies underline the capacity of EVs to be used as natural bio-carriers. Due to their intrinsic biological properties, EVs appear as a suitable alternative at traditional delivery systems actually in development limiting inflammation processes and enhancing specific delivery. Moreover, they provided promising results on brain targeting enhancing the chance of developing hypothalamic-targeted drugs to counter obesity.

## 6. Extracellular vesicles in an obesity-targeted therapeutic development context

### 6.1. Peripheral and central molecular targets implicated in energetic metabolism

The growing interest of worldwide research group for obesity results in an identification of upstream specific actors in peripheral tissues regulating metabolism as well as within the master center of its regulation, the hypothalamus. However, modulation of activity of these actors into specific – peripheral or central – tissues avoiding undesired effects remains difficult due to their ubiquitously localisation. Underlining the increasing craving of the past decade to develop specific treatment to counter the epidemic spread of obesity, novel nanobiomedicine strategies based on EV use could be attractive.

#### 6.1.1. AMPK

Through its “fuel gauge” function by sensing the cellular energy status (Hardie et al., 2003), the AMPK has an important role in both peripheral tissue- and the hypothalamic-mediated regulation of metabolism.

At the peripheral level, AMPK regulates fat accumulation by inhibiting white adipogenesis and by favoring fatty acid oxidation and brown adipogenesis, and also reduces cholesterol synthesis and inflammatory cytokine production (Fernández-Veledo et al., 2013). For instance, activation of AMPK by either resveratrol or AICAR reduces metabolic disturbances in several animal models of obesity. Polyphenols stimulate AMP-activated protein kinase, lower lipids, and inhibit accelerated atherosclerosis in diabetic LDL receptor-deficient mice (Iglesias et al., 2002; Zang et al., 2006) and in humans (Cuthbertson et al., 2007).

Concerning the AMPK implication in the central regulation of food intake, a leader study showed that modulation of AMPK activity within the ventromedial hypothalamus, a distinct hypothalamic nucleus involved in feeding behavior, was sufficient to modulate food intake and body weight. Through the inhibition of AMPK directly into the ventromedial hypothalamus, using recombinant adenoviruses carrying dominant negative isoforms of the protein, the stereotaxic-injected mice showed a significant decrease of their body weight within the 2 following days (López et al., 2010). Firstly explained by the alteration of food

intake, it appears lately that this phenomenon was more linked to an increase of energy expenditure through thermogenesis program in the brown adipose tissue rather than an inhibition of food consumption (López et al., 2010). However, still learning from what was discovered regarding AMPK central role in the regulation of obesity, other researches were lead studying subsequent activated secondary's actors.

Altogether, these data illustrate that the enhancement and the inhibition of AMPK activity at the peripheral and central level, respectively, can bring beneficial effects regarding obesity. Thus, controlling this subtle balance may represent therapeutic challenge using EVs to deliver protective biological messages.

#### 6.1.2. Mammalian target of rapamycin (mTOR)

As described above, the protein-synthesis implicated protein mTOR plays a crucial role in the regulation of food intake and body weight (Cota et al., 2006). It has been shown that, in liver and adipose tissue, mTOR activation augments de novo lipogenesis and inhibits lipolysis leading to the expansion of adipose tissue. Interestingly, in insulin resistance state, mTOR activity is decreased resulting in increased lipolysis and decreased mitochondrial combustion of free fatty acids inducing ectopic lipid accumulation in skeletal muscle, heart, liver, and pancreas, exacerbating insulin resistance and other metabolic disorders (for review see Chakrabarti & Kandror, 2015).

Interestingly, localized directly within the hypothalamic neurons implicated in the regulation of food intake, NPY/AgRP and POMC/CART, mTOR is inhibited under AMPK activation (Kudchodkar et al., 2007). Indeed, intracerebral rapamycin infusion enhanced POMC neurons firing, thereby causing a reduction of food intake and body weight (Yang et al., 2012). Similarly at what was described for the AMPK, mTOR has a crucial role in the central regulation of food intake and is a potential target for EVs therapeutic strategies.

#### 6.1.3. Sirt 1

This increasing interest on how food intake is regulated uncovered another potential peripheral and hypothalamic therapeutic target, Sirt1. At first described as an actor promoting longevity in yeast species such as *Saccharomyces cerevisiae* through its homolog Sir2, Sirt1 is implicated in energy balance regulation (Milne et al., 2007). Its implication was firstly demonstrated in the peripheral pathways of this regulation by modifying insulin signaling. Different studies revealed that Sirt1 could increase insulin-induced tyrosine phosphorylation of insulin receptor substrate 2 – IRS 2 – through its deacetylation in hepatocytes, a vital step in insulin signaling pathway (Zhang, 2007). Interesting similar results showed that Sirt1 could decrease the transcription of the protein tyrosine phosphatase PTP1B in hepatocytes and myocytes enhancing phosphorylation of IRS2 allowing insulin pathway signalisation (Sun et al., 2007). Lately was also discovered Sirt1 implication in leptin signaling in hepatocytes. Through the inhibition of STAT3 transcription by deacetylation, Nie et al. showed that Sirt1 was also implicated in leptin-regulated glucose homeostasis (Nie et al., 2009).

Nonetheless, throughout its localization in AgRP and POMC neurons, Sirt1 implication was also studied in the central regulation of food consumption regulation. As an interesting point, hypothalamic levels of Sirt1 were decreased with fasting corroborating the probable implication of Sirt1 in food intake regulation (Sasaki et al., 2010). Moreover, intracerebroventricular injections of a pharmacological Sirt1 inhibitor (Ex-527) had demonstrated anorexigenic effects on mice models. In addition, they advanced that Sirt1 inhibition could reduce NPY/AgRP neuronal firing but also NPY/AgRP inhibition on POMC neurons (Dietrich et al., 2010). However, Ramadori et al. (2010) showed that mice with a specific POMC neuron-specific Sirt1 knockout had unchanged food intake rising that the specific targeting of only NPY/AgRP Sirt1 expression is essential due to Sirt1 different food intake regulation depending on its location.

#### 6.1.4. GRP78

It was recently demonstrated that obesity occurrence could be linked to endoplasmic reticulum (ER) stress in different tissues including adipose tissue, liver and hypothalamus. Indeed, growing evidences showed that disruption of ER homeostasis leads to a feedback mechanism that prevents the accumulation of misfolded proteins in the ER lumen. This response, called unfolded protein response (UPR), tries to restore normal cell function, including the increase in the production of chaperone proteins, such as glucose-regulated protein 78 (GRP78) (Lee, 2005).

Markers of ER stress including GRP78 are significantly increased in cultured adipocytes treated with oxidized-LDL (Chen et al., 2013), in adipose tissue (Ye et al., 2010) and liver of obese mice (Li et al., 2011) as well as in adipose tissue of obese pregnant women (Liong & Lappas, 2015). Furthermore, recent data show the complexity on the regulation of the signals regulating energetic metabolic implicating a SIRT1/mTORC/ER stress axis. Indeed, SIRT1-dependent suppression of mTORC functionally inhibits hepatocellular ER stress leading to the improvement of hepatic steatosis and body insulin resistance and restores glucose homeostasis (Li et al., 2011).

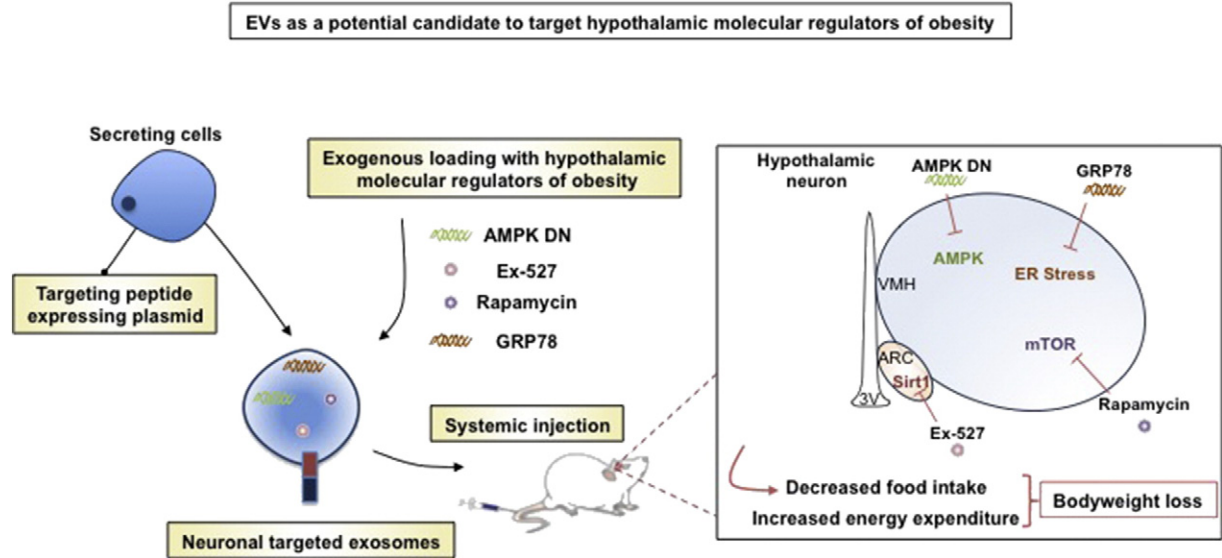
On the other hand, obesity induces ER stress within the hypothalamus leading to the development of insulin and leptin resistance and weight gain (Won et al., 2009; Zhang et al., 2008). Interestingly, these findings also demonstrate that chemical chaperone-mediated ER stress restores insulin and leptin sensitivity but also stabilizes body weight. Following this ER stress-obesity association, Contreras et al. (2014) have demonstrated that overexpression through stereotaxical microinjection of GRP78-coding adenovirus within the ventromedial hypothalamus (VMH) of obese Zucker rats reduces body weight by increasing thermogenesis, but also restores insulin and leptin sensitivity. Interestingly, maternal consumption of high fat-diet during pregnancy produces ER stress into the hypothalamus of recently weaned – 28 days – mice but not in new-borns – 0 days – suggesting that lactation period is a maternal trigger for metabolic changes in the offspring (Melo et al., 2014).

This non-exhaustive list of molecular target implicated in hypothalamic regulation of food intake could be attractive leads to counter the epidemic development of obesity.

#### 6.2. Future directions using EVs

Although these strategies have major effects on reducing mice body weight throughout an inhibition of regulatory proteins – AMPK, mTOR, Sirt1... – at peripheral and central levels, they also raise a currently unsolved limit. Indeed, together the carrier – adeno- or retrovirus – and the way of administration – stereotaxic microinjection – do not entirely suit to the therapeutic demand. As discussed previously, even if the use of viruses is shown to be effective facing gene delivery, there are risks associated with it as well, like immunogenicity, viruses replication possibility, and they also have been described to cause brain and heart damages. Moreover, the needs to develop less invasive-delivery strategies are becoming necessary. Facing these issues, EVs appear as an alternative viable delivery candidate (Fig. 5). As described above, uses of EVs as delivery tools provide remarkable results facing numerous complex diseases such as cancer and neurodegenerative disorders. Indeed, EVs possess numerous characteristics that confer them ideal drug-delivery aptitudes. They are stable within the body and produced at high concentrations. Even after repeated injections, EVs do not induce (i) inflammation processes, (ii) tumor generation (Thirabanjasak et al., 2010), or (iii) immune rejection after allogenic administration (Bruno et al., 2009). In addition, EVs carry proteins and nucleic acids that can be easily modulate to confer them specific pharmacological functions. And lastly, compare to other synthetic particles, EVs possess intrinsic homing capacities that can be modified to increase cell-type specific targeting. Therefore thinking about delivering anti-obesity drugs designed to positively modulate the expression of food intake-implicated





**Fig. 5.** Future directions using extracellular vesicles as a vector in an obesity-driven context. Through their delivery properties and regarding their pharmacological uses in other pathologies, EVs appear to be a valuable candidate to increase the specificity of anti-obesity drugs. As recently demonstrated, EVs can be engineered to express targeting peptides at their membrane increasing the specificity of their delivery following a systemic injection (Alvarez-Erviti et al., 2011). Moreover, electroporation mediated loading enables the encapsulation of nucleic acids within the EVs. Following the upstream hypothalamic targets governing food intake regulation, the hypothetical drugs could be used: (i) AMPK dominant negative (AMPK DN) to inhibit AMPK expression within the ventromedial hypothalamus, (ii) Ex-527 to inhibit Sirt1 expression within the arcuate nucleus (ARC), (iii) Rapamycin, a mTOR inhibitor and (iv) GRP78 as an inhibitor as endoplasmic reticulum (ER) stress. All these hypothalamic targets showed promising effects on reducing food intake and increasing energy expenditure, but they were hampered by non-human suitable stereotaxic microinjections. Therefore, EVs appear as a potential candidate to deliver these pharmacological designed drugs in a specific hypothalamic manner.

actors such as AMPK, mTOR or Sirt1 appear as a new strategy to counter this worldwide complex disease.

## 7. Conclusion

Throughout this review, we highlighted many supporting ideas that EVs could play a central role in maintaining and developing obesity and its metabolic subsequent induced disorders. Therefore, they appear as a potential pharmacological target. The inhibition of EV release could decrease their mediated deleterious effects improving obese patients' health. However, due to the intrinsic properties, they can also be seen as delivery tools. As mentioned all long throughout this review, the main issue characterizing anti-obesity drug development is the lack of specificity of the delivered active substances. Regarding their crucial role in establishing accurate modulation of energy balance facing wide daily variations in food consumption, upstream hypothalamic molecular actors and the subsequent involved pathways are now suggested as major targets to develop anti-obesity wonder drugs. Drawing from what was done in other complex diseases and trusting their delivery aptitudes, EVs appear as a novel nanobiomedicine approach increasing specificity of the delivery while reducing unwanted side effects. Therefore, the idea of exploiting them in an anti-obesity designed manner is opening a new avenue in pharmacological treatment of obesity.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

## Acknowledgments

EM is recipient of a doctoral fellowship from Nanofar Erasmus Mundus Program.

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Throughout this review, the difficulty of developing accurate and efficient therapeutic strategies against obesity pandemic expansion was highlighted. As the mechanisms underlying its central regulation are complex and often interconnected to other molecular pathways (for example, governing other functions within the organism), central anti-obesity drug development is difficult to achieve. The latest strategies in term of developing anti-obesity drugs are limited either by the lack of efficiency or due to numerous associated adverse side effects encountered. As mentioned all long during this review, the main issue characterizing anti-obesity drug development is the lack of specificity of the delivered active substances.

Obesity regulation is mainly driven within the central nervous, in particular in the hypothalamus. In order to define novel tissue-specific therapeutic strategies, a better understanding of hypothalamus organization and mediated-functions is crucial.

### 3. The hypothalamus, a central actor in feeding behaviour regulation

The importance of hypothalamus in body weight regulation is known for decades. In the 1940s, hypothalamic lesions conducted in small animals induced increased food intake and hyperphagia (Hetherington & Ranson, 1940, 1942). Since then, strong experimental evidences and clinical observations confirmed the crucial role of hypothalamus in feeding behaviour.

The hypothalamus is a region located below the thalamus and is involved in the modulation of numerous functions. As a master centre for the integration of peripheral signals, the hypothalamus participates among others in (i) endocrine regulation, (ii) temperature regulation, (iii) sexual behaviour and reproduction, (iv) control of daily cycles in physiological state and behaviour, (v) sleep, (vi) emotional feelings, (vii) and the control of water and food intake. Herein, we will focus on the implication of hypothalamus organization in the control of energy balance regulation.

The hypothalamus is organized in anatomically distinct interconnected hypothalamic nuclei including the arcuate nucleus (ARC), the ventromedial nucleus (VMH), the paraventricular nucleus (PVH), the lateral hypothalamic area (LHA) and the dorsomedial nucleus (DMH) among others, that participate in the regulation of energy metabolism.

**Arcuate nucleus of the hypothalamus.** One of the most important nuclei involved in the control of food intake is the ARC. Its implication in feeding behaviour regulation is known for decades. In 1969, Olney exhibited that lesions within the ARC induced obesity development and hyperphagia (Olney, 1969). The ARC is anatomically localized in the ventral part of the hypothalamus at both sides of the third ventricle. Interestingly, the blood brain barrier surrounding the ARC has been described to be semi-permeable facilitating (i) the sensing of bloodstream peripheral signal and nutrient fluctuations, and (ii) the release of hypothalamic hormones (Rodríguez et al., 2010). There are 2 major neuronal populations modulating the energy balance within the ARC. One population, which stimulates food intake and coexpresses neuropeptide Y (NPY) and Agouti-related protein (AgRP). The second subset of neurons is implicated in the inhibition of food intake and coexpresses cocaine- and amphetamine-related transcript (CART) and pro-opiomelanocortin (POMC). Neuronal projections from the 2 populations communicate with the other hypothalamic nuclei implicated in the regulation of appetite such as the PVH, the LHA and the DMH (Bouret et al., 2004). With the subsequent melanocortin-3 receptor (MC3R) and melanocortin-4 receptor (MC4R) neurons, they constitute the central melanocortin system (discussed above). Thus, ARC participates intensely in the regulation of feeding behaviour.

**Ventromedial nucleus of the hypothalamus.** Localized on both sides of the third ventricle, above and laterally from the ARC, the VMH is known for long time as involved in feeding behaviour. In the 1940s, experimental evidences showed that lesions in this particular region induced deregulations in energetic metabolism leading to obese phenotype (Hetherington & Ranson, 1940, 1942). The VMH is related to other nuclei through axonal projections. NPY, AgRP and POMC neurons from the ARC project into the VMH. On the other hand, VMH neurons project into hypothalamic and extrahypothalamic areas such as brainstem (Cheung et al., 2013). Moreover, evidences exhibit the important role of VMH within the energy expenditure regulation via the BAT thermogenesis. Perkins and colleagues showed that an electric stimulation of VMH increased the BAT temperature. This BAT temperature increase was mediated by BAT  $\beta$ -adrenergic receptors (Perkins et al., 1981). More recently, it was demonstrated that an inhibition of AMPK signalling within the VMH induced an increase of BAT thermogenesis associated to a body weight decrease with unmodified

food intake, highlighting the crucial role of thermogenic energy expenditure in energy balance (López et al., 2010; Whittle et al., 2012; Morentin et al., 2012; Martínez de Morentin et al., 2014; Beiroa et al., 2014).

**Paraventricular nucleus of the hypothalamus.** Localized within the anterior hypothalamus above the third ventricle, the PVH is implicated in the control of both appetite and endocrine function. Indeed, experimental evidences have demonstrated that lesions in the VMH induced an increase of food intake and body weight (Aravich & Sclafani, 1983). The PVH receives many afferent NPY/AgRP and POMC/CART projections originating from the ARC. For example, Sutton et al. (2014) have shown that leptin sensitive neurons from the ARC projected their axons within the PVH. Moreover, the PVH is enriched in melanocortin receptors (Kishi et al., 2003). Among others, Fekete and colleagues demonstrated the implication of PVH in the energetic balance regulation. Indeed, they showed an interaction between ARC neurons and thyrotropin releasing hormone (TRH) neurons localized in the PVH that could positively modulate food intake (Fekete et al., 2000).

**Lateral hypothalamic area.** The LHA has a major role in orexigenic responses mainly through orexins (OXs) and melanin-concentrating hormone (MCH). It has been described that orexin A (OX-A) could enhance food intake when centrally injected (Sakurai et al., 1998; López et al., 2002). OX-A receptive sites for stimulation of food intake are located in particular nuclei as PVH, DMH and so, LHA. Indeed, central injections of OX-A within other hypothalamic nuclei did not induce any modulation of food intake (Dube et al., 1999). Moreover, Martins et al. (2016) have demonstrated that VMH and LHA were functionally interconnected to modulate BAT thermogenesis. They have shown that the thermogenic effect of bone morphogenetic protein 8B (BMP8B) is mediated by (i) the inhibition of AMPK in the VMH and (ii) the increase of OX signalling via the OX receptor 1 (OX-1R) in the LHA. Thus, LHA has a central role in the regulation of energetic balance by modulating food intake and energy expenditure.

**Dorsomedial nucleus of the hypothalamus.** The DMH implication in several metabolic processes such as food intake and thermogenesis is well described. Indeed,



lesions within the DMH result in hyperphagia and obesity (Bernardis & Bellinger, 1998). Other evidences appeared to corroborate the implication of DMH with food intake regulation. In obese mice models, NPY mRNA expression is increased in DMH. Chen et al. (2004) have shown that  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) fibres are in close apposition to NPY neurons. They have postulated that  $\alpha$ -MSH may suppress NPY gene expression in the DMH indirectly via inhibitory interneurons, possibly through GABAergic pathways. Moreover, as observed for the ARC, DMH also projects  $\alpha$ -MSH fibres to the TRH neurons localized in the PVH increasing the hypothesis that DMH participates in food intake regulation (Guan et al., 1998).

As discussed here, energetic metabolism - food intake/energy expenditure - regulation appears to be a complex interconnected system. Either the anatomic organization of hypothalamus, the specific function of each nuclei or the multitude of axonal projections linking one area to another render the development of tissue specific therapeutic strategies challenging. However some recent studies exhibit some really promising effects on decreasing body weight. Novel therapeutic approaches arise, focusing on hypothalamic actors involved in obesity regulation, such as AMPK or glucose-regulated protein 78 (GRP78) - an endoplasmic reticulum (ER)-resident chaperone that can prevent accumulation of unfolded/misfolded proteins within the ER - (López et al., 2010; Contreras et al., 2014).

As already discussed, an inhibition of AMPK signalling within the VMH induced significant decrease of body weight in obese rats associated to an increase of BAT thermogenesis (López et al., 2010; Whittle et al., 2012; Morentin et al., 2012; Martínez de Morentin et al., 2014; Beiroa et al., 2014). However, the way of administration - hypothalamic stereotaxic microinjection - is not suitable for human therapies. In order to translate this strategy to the bedside, administration methods need to be improved using less-invasive ways. Using a novel “nanobiomedicine” approach could help to increase the tissue specificity of the delivery. However, the hypothalamus, and more over of the VMH, specific targeting remains unachieved at the moment, increasing the difficulty of developing suitable treatments.

## II. Objectives





The general objective of this study was to investigate the effect of neuronal-targeted exosomes loaded with a dominant negative isoform of AMPK on the central regulation of energy balance as a therapeutic strategy to prevent or revert obesity and its metabolic complications. The goal of this project was to translate the knowledge on exosomes to the bedside in order to improve the management and treatment of obese patients. This would allow using exosomes silenced with the deleterious targets as potential tools to fight against the diseases in the field of nanomedicine.

To achieve this study, specific aims were developed (Fig. 6).

Firstly, a neuronal-targeted exosome model loaded with a dominant negative isoform of AMPK was developed. Its ability to deliver nucleic information was assessed (i) in vitro on a neuronal cell line and (ii) in vivo on an animal model.

Secondly, we wanted to determine whether these exosomes harbouring dominant negative isoforms of AMPK could decrease body weight gain in animal models of diet-induced obesity.

Finally, the central mechanisms leading to the decrease of body weight gain in animal models of diet-induced obesity were characterized. Molecular analysis of hypothalamic AMPK pathway associated with an increase of BAT thermogenesis process was achieved.

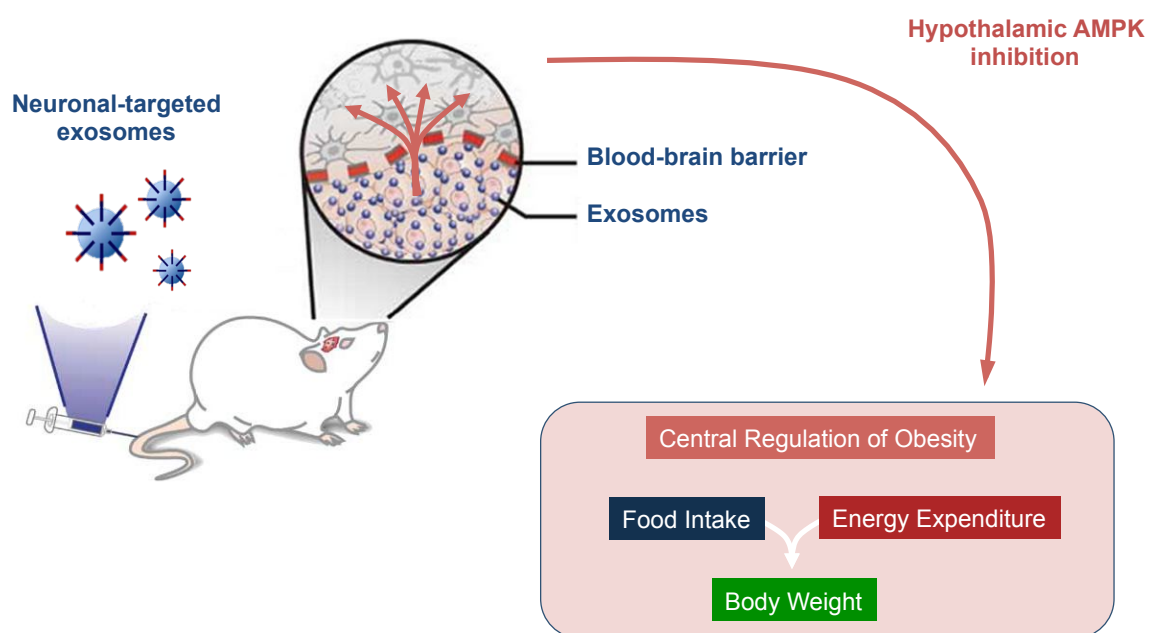


Figure 6: Schematic representation of the principal objectives

## III. Strategy



The use of modified DNA sequence to induce modifications in host genome - most commonly called gene therapy - has opened new insights in drug discovery. However, gene therapy therapeutic potential is hampered by inadequate tissue-specific delivery following systemic injections inducing numerous side effects. To increase this specificity of the delivery and to guarantee the integrity of the nucleic acid in the organism, the use of carriers/vectors is essential. However, the use of these vectors includes some disadvantages. The use of non-adapted vectors may induce unwanted immune system reactions, inducing inflammation leading, in rare cases, to organ failures. Cases of infections were also reported in gene therapy clinical trials. Moreover, the use of pleiotropic vectors such as viruses may induce non-specific delivery within healthy cells modifying their genome, inducing numerous diseases such as cancer. As described above (Milbank et al., 2016), new insights about using exosomes as a vehicle has emerged the last decades. As a mimic of nature's delivery systems, exosomes appears as a potential candidate in delivery strategies, overcoming the limitations observed within the majority of actual nanomedicine delivery approaches. Indeed, exosomes exhibit many of the required features of an ideal drug delivery system, such as a high stability within biological fluids, biocompatibility, and minimal toxicity concerns.

In order to increase the specificity of drug delivery that is one of the most important limiting factors within numerous actual therapies including obesity, the following strategy was thought and designed (Fig. 7). Following the novative and promising strategy exposed by Alvarez-Erviti and colleagues in 2011 in which they conferred neuronal targeting properties to exosomes (Alvarez-Erviti et al., 2011), we wanted to apply this strategy to a metabolic context.

To limit as possible host immune reactions, immunologically inert vehicles had to be designed. In this regard, exosome appeared as a good candidate as their main role within the organism is the intercellular communication. Moreover, as their composition is dependent of the originating cells, controlling it was credible. Therefore, as a source of non-inflammatory exosomes, immature dendritic cells described to be immunologically inert, were used. Next, as the specificity of the delivery in gene therapy is crucial, immature dendritic cells were engineered to

express a brain targeting peptide - composed of an exosomal protein Lamp2b (Lysosome-associated membrane protein 2b) fused to the neuron-specific-targeting RVG (rabies virus glycoprotein) peptide - that will be carried at the surface of the later-produced exosomes, conferring them brain targeting capacities. Once brain-targeted, exosomes were loaded with a plasmid encoding for a dominant negative isoform of AMPK (AMPK-DN) - an inactive form of the protein by modification of its active site-. The aptitude of Lamp2b-RVG exosomes to cross the blood brain barrier and to reach the brain was evaluated using entire animal fluorescence imaging. Their efficiency to deliver their nucleic content was assessed in vitro and in vivo. Next, AMPK-DN loaded neuronal-targeted exosomes were injected intravenously in high fat diet induced obese mice model and their functional effects on decreasing body weight were evaluated.

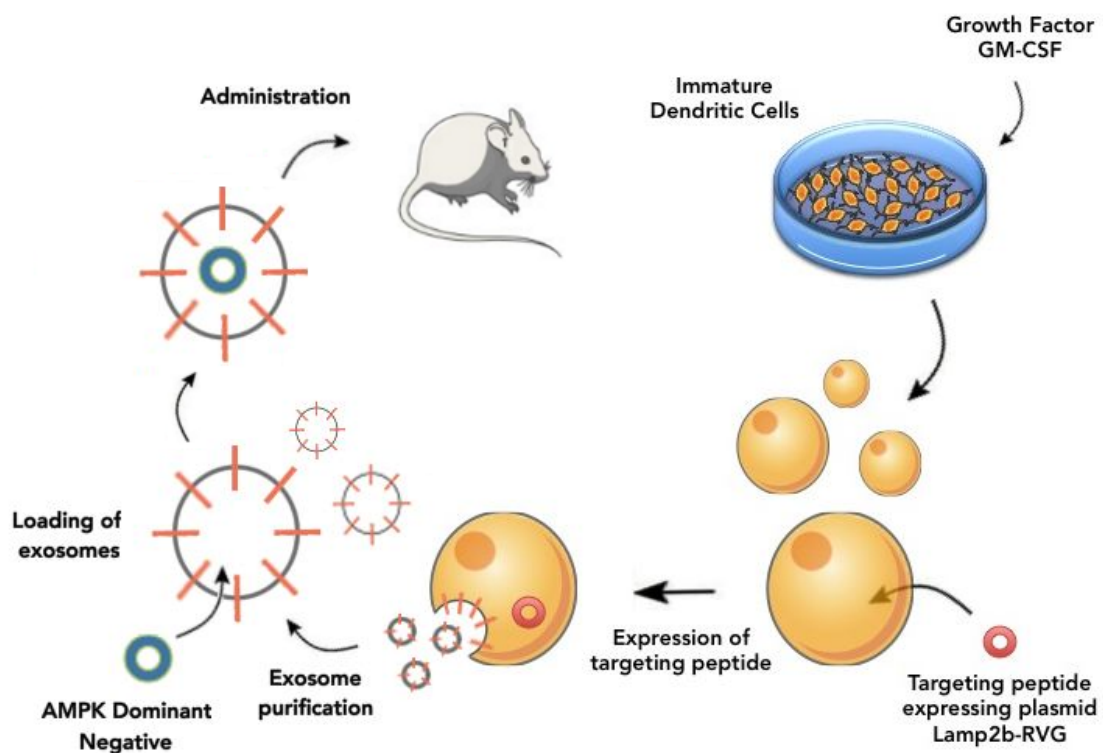


Figure 7: Schematic representation of production, harvest and administration of hypothalamic targeted exosomes for drug delivery (Adapted from Alvarez-Erviti et al., 2011).

# IV. Methods





## 1. Cell culture

### i. Bone marrow derived dendritic cells (BMDCs)

#### a. Isolation

Tibias and femurs from CO<sub>2</sub> euthanized C57BL/6 mice were removed and placed in conical tubes on ice. The bones were cleaned removing excess muscles and ligaments and placed in a clean dish. The epiphyses were cut off and the diaphysis was transferred to a separate Petri dish containing fresh Dulbecco's Modified Eagle Medium (DMEM) medium. The bones were flushed using a 10 mL syringe with a 27-gauge needle containing fresh DMEM. The supernatant was collected without taking any clumps and transferred to a 50 mL conical tube. The cells were separated using a syringe by pipetting them up and down. The cells were centrifuged for 10 min at 300 g at room temperature (RT). The supernatant was discarded and the pellet was incubated 5 min with a sterile red blood cell lysis buffer (8.26 g of NH<sub>4</sub>Cl, 1 g of KHCO<sub>3</sub> and 37 mg of EDTA for 1 L of buffer) to lyse the red blood cells. 15 mL of DMEM with serum was added to neutralize the reaction. The mixture was centrifuged 10 min at 300 g at RT. The pellet was collected and washed with DMEM and centrifuged 10 min at 300 g at RT. The pellet was harvested and the cells were cultured in BMDCs growth medium composed of DMEM containing Glutamax, 10% (vol/vol) foetal bovine serum (FBS), 10 U/mL of penicillin and 100 µg/mL of streptomycin, 1mM of sodium pyruvate and supplemented with 10 ng/mL of mouse recombinant granulocyte macrophage colony-stimulating factor (GM-CSF).

#### b. Culture

The BMDCs were seeded in six-well plates and cultured in 2 mL of BMDCs growth medium. BMDCs were incubated at 37°C and 5% CO<sub>2</sub> for 4 days. To induce the maturation of the BMDCs, they were incubated with lipopolysaccharide (LPS) for 24 h (1µg/mL).

### ii. JAWS II

An immortalised dendritic cell line established from the bone marrow of a p53-knockout C57BL/6 mouse was used for this study. Also known as JAWS II, this cell

line was acquired from the American Type Culture Collection (CRL-11904; ATCC Manassas, VA). JAWS II were grown in an incubator at 37°C and 5% CO<sub>2</sub> in a growth medium composed of alpha minimum essential medium with ribonucleosides, desoxyribonucleosides, and supplemented with 20% (vol/vol) of FBS, 4mM of L-glutamine, 1 mM of sodium pyruvate, 10 U/mL of penicillin, 100 µg/mL of streptomycin and 5 ng/mL of GM-CSF. The cell cultures were maintained by transferring the non-adherent cells into a centrifuge tube. The remaining adherent cells were washed with phosphate-buffered saline (PBS) 1X then treated with a mixture of 0,25% trypsin - 0,02% EDTA for 7 min at 37°C. The two obtained cell populations were pooled and centrifuged at 1000 rpm for 10 min. The pellet was collected and resuspended in fresh medium and dispensed into new flasks.

### iii. Neuro-2A

The Neuro-2A cells were generously provided by the Lentivec platform (Université d'Angers, Angers, France). They were grown in an incubator at 37°C and 5% CO<sub>2</sub> in a growth medium composed of DMEM supplemented with 10% (vol/vol) of FBS, 10 U/mL of penicillin, 100 µg/mL of streptomycin, 2mM of L-glutamine and 1mM of sodium pyruvate. When the confluence achieved, the cells were treated with a mixture of 0,25% trypsin - 0,02% EDTA for 3 min at 37°C. The collected cells were centrifuged at 500 g for 5 min and the pellet was suspended in fresh growth medium and divided in new flasks at a 1/6-subculture ratio.

## 2. Cell characterization

Cell surface markers of BMDCs were evaluated using APC-conjugated antibodies to CD11c, PE-Vio770-conjugated antibodies to CD80, FITC-conjugated antibodies to CD86 and Vioblue-conjugated antibodies to MHC Class II (Miltenyi Biotec). Briefly, cells were centrifuged at 500 g for 5 min, washed with PBS 1X and then incubated with optimal concentrations of conjugated antibodies for 45 min on ice. The respective fluorescence were then analysed by a MacsQuant cytometer (Miltenyi Biotec). Live cells were gated on their forward and side light scatter properties. Flow cytometric analysis was performed using FlowJo software.

### 3. Lamp2b-RVG plasmid engineering

The plasmid encoding for the Lamp2b sequence was gently provided by Seow Yiqi (Molecular Engineering Laboratory, Proteos, Agency for Science Technology and Research, Singapore). Lamp2b plasmid was digested using XhoI (5 U/ $\mu$ L) and BspEI (10 U/ $\mu$ L) restriction enzymes (New England Biolabs), and dephosphorylated with calf intestinal phosphatase (10 U/ $\mu$ L) (New England Biolabs). In the same time, RVG forward and reverse primers were designed to contain XhoI and BspEI corresponding overhangs (see Table 1 for detailed sequences). These RVG primers were phosphorylated with T4 polynucleotide kinase (10 U/mL) (New England Biolabs) and inserted at the N-terminus of digested Lamp2b plasmid using T4 polynucleotide ligase (10 U/mL) (New England Biolabs). Using the base complementarity and phosphorylation-dephosphorylation reactions to recreate phosphodiester links ensure the accuracy of the engineering.

Targeting peptide	
RVG F	5'-TCGATACACCATTTGGATGCCCGAGAATCCGAGACCAGGGACACCTTGTGACATTTTACC AATAGCAGAGGGAAGAG AGCATCCAACGGGT-3'
RVG R	5'-CCGGACCCGTTGGATGCTCTCTCCCTCTGCTATTGGTAAAAATGTCACAAGGTGTCCCTGG TCTCGGATTCTCGGGCATC CAAATGGTGTA-3'

Table 1: Sequence of RVG primers

### 4. CMV and SF1-AMPK-DN plasmids

To induce AMPK inhibition, dominant negative plasmids were designed. CMV- and SF1-AMPK-DN plasmids were provided by Viraquest (North Liberty).

### 5. DNA polyacrylamide gel electrophoresis

To validate the accuracy of the Lamp2b-RVG plasmid engineering, a polyacrylamide gel electrophoresis was performed to separate the DNA fragments regarding their sizes. Briefly, Lamp2b and Lamp2b-RVG plasmids were digested using HindIII and NHEI restriction enzymes, and the resultant products were set to migrate on a 20% polyacrylamide gel electrophoresis. Once the migration completed, the gel is immersed into a 0,5  $\mu$ g/mL ethidium bromide solution for 10-30 minutes.

After two washes with water, the gel was visualized using UV on the imager Infinity VX2 (Vilber Lourmat).

#### 6. DNA sequencing

To further the investigation on the accuracy of Lamp2b-RVG sequence, DNA sequencing was realized using the GenomeLab Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, Fullerton, CA) following manufacturer's instructions and analysed on a Beckman CEQ 8000 DNA sequencer (Beckman Coulter).

#### 7. Cell transfection

To evaluate the efficiency of the different transfection protocols (described below), a green fluorescent protein (GFP) encoding plasmid was used as a positive transfection probe. Once optimized, the transfections were realized using the plasmid of interest, Lamp2b-RVG.

##### i. JetPei Macrophage

On the day before the transfection, BMDCs or JAWS II cells were seeded in 6-well plates at a density of  $4 \times 10^5$  cells in complete growth medium (described above). On the day of transfection, cells were transiently transfected with GFP plasmids (1,5  $\mu\text{g}$ ) using 3  $\mu\text{l}$  of JetPei-Macrophage (Polyplus-transfection, France) for each well. The transfected medium was replaced by complete growth medium after 6 h. The respective fluorescence was analysed 48 h after by flow cytometry.

##### ii. Lipofectamine 2000

BMDCs or JAWS II cells were seeded in 6-well plates at a density of  $5 \times 10^5$  cells in complete growth medium (described above) on the day before transfection. On the day of transfection, cells were transiently transfected with a mixture composed of (i) GFP plasmids (1  $\mu\text{g}$ ) and (ii) 2  $\mu\text{l}$  of Lipofectamine (Life Technologies) for each well. The transfected medium was replaced by complete growth medium after 6 h. The respective fluorescence was analysed 48 h after by flow cytometry.

### iii. MacsFectin

Following manufacturer's instructions, JAWS II cells were seeded at a density of  $4 \times 10^5$  cells in 6-well plates in complete growth medium on the day before transfection to reach a density of 60%. On the day of transfection, the transfection complexes composed of 2 or 4  $\mu\text{g}$  of GFP and 4, 8 or 12  $\mu\text{l}$  of MacsFectin (Miltenyi Biotec) were added to each well. The respective fluorescence was analysed 48 h later by flow cytometry .

For the transfection of Lamp2b-RVG encoding plasmid, cells were seeded in T75 flask on the day before transfection in complete growth medium (described above) at a density of  $5 \times 10^6$  cells. On the day of transfection, cells were transiently transfected with Lamp2b-RVG plasmids (20  $\mu\text{g}$ ) using 40  $\mu\text{l}$  of MacsFectin. After one day, the medium was replaced by FBS-exosome-free medium (growth medium with 200 000 g ultracentrifuged-FBS). Forty-eight hours later, the medium was collected and the exosomes were isolated (see below).

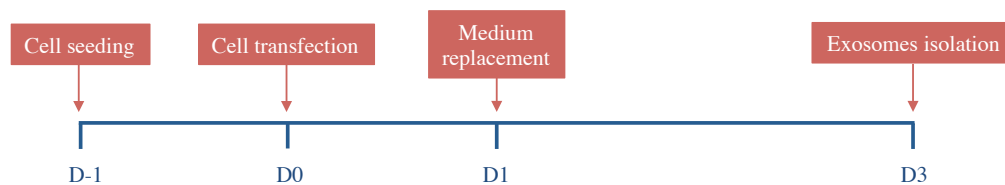


Figure 8: Timeline representation of Lamp2-RVG transfection and isolation of Lamp2b-RVG exosomes

### 8. Efficiency of transient plasmid expression

For flow cytometric analysis of GFP fluorescence, cells were isolated, washed and resuspended in 200  $\mu\text{l}$  of PBS 1X. The cells were then flow cytometrically analysed with a flow cytometer 500 MPL System (Bekman Coulter) using the MXP software (Beckman Coulter). Live cells were gated on their forward and side light scatter properties.

## 9. Determination of hypodiploid DNA

After the cell transfection process, culture medium was collected, and adherent cells were trypsinized, combined with floating cells from the original culture medium, and centrifuged. Cells were then fixed in 70% ethanol for at least 4 h at 4 °C and washed in PBS 1X. Then, they were incubated for 10 min in a solution containing type I-A RNase A (0.05 mg/mL, Sigma Aldrich) in PBS 1X at 37 °C. Propidium iodide (PI, Sigma Aldrich) was then added at a final concentration of 12.5 µg/ml. After 15 min in the dark at room temperature, samples were analysed by a MacsQuant cytometer (Milenyi Biotec). Flow cytometric analysis was performed using FlowJo software.

## 10. Isolation and purification of exosomes

Culture medium from transfected JAWS II cells was collected and centrifuged twice 10 min at 300 g and 2 000 g to remove cells and cell debris. The resultant supernatant was centrifuged at 10 000 g for 30 min to exclude microparticles (MPs). Exosome pellets were further isolated from MP-depleted supernatants by a 200 000 g centrifugation step for 2 h at 4°C using a MLA-50 rotor in an Optima Max-XP ultracentrifuge (Beckman Coulter, USA). The exosome pellet was washed once with PBS 1X using the same previously ultracentrifuge process before being resuspended in PBS 1X. The exosome samples were kept at -80°C until use.

## 11. Exosomes quantification

Determination of the corresponding amount of exosomes was carried out by measuring exosome-associated proteins determined against bovine serum albumin standards (Lowry method). Briefly, 5 µL of each exosome suspension were loaded in duplicate in 96-well plates. 25 µL of reagent A (BioRad) and 200 µl of reagent B (BioRad) were added to the exosome suspension and incubated 10 min in the dark at RT. The absorbance was analysed at 570 nm in a plate reader (Berthold).

## 12. Electron microscope analysis of exosomes

Exosomes were visualised using electron microscopy. Purified exosomes were fixed overnight in a freshly prepared 2,5 % paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Exosomes were pelleted using previously described ultracentrifugation process and resuspended in 2,5 % glutaraldehyde solution.

Exosomes were deposit on copper grids and negatively stained with phosphotungstic acid and observed with a JEM1400, JEOL at 200kV.

### 13. Size distribution of JAWS II-derived exosomes

Purified exosomes (50  $\mu\text{g}$ ) derived from JAWS II dendritic cells were diluted in 1 mL of PBS 1X, and size distribution was analysed at 37°C with the Zetasizer Nano ZS 90 (Malvern Instruments, Orsay, France) or the NanoSight NS 300 (Malvern Instruments, Orsay, France) according to the manufacturer's instructions.

### 14. Exosomes loading

Purified exosomes were loaded with nucleic acids - either siRNA Texas Red, GFP, CMV-AMPK-DN or SF1-AMPK-DN - using Exo-Fect (System Biosciences) following the manufacturer's protocols. Briefly, exosomes (50-300  $\mu\text{g}$ ) were incubated with 10  $\mu\text{l}$  of Exo-Fect solution, 20 pmol siRNA Texas Red or 5  $\mu\text{g}$  of plasmid (GFP, CMV- or SF1-AMPK DN), and 70  $\mu\text{l}$  of PBS 1X for 10 min at 37°C. The reaction was stopped by placing the tube on ice. The solution was ultracentrifuged as previously described to pellet the exosomes. Exosomes were resuspended in PBS 1X and stored at -80°C until use.



Figure 9: Schematic representation of exosome loading

Exosomes (black lines) were exogenously loaded with nucleic acids (yellow rounds) using Exo-Fect reagent through the poration of exosomal membrane.

### 15. Applications of exosomes in cell culture

Twenty four hours before treatments with exosomes, (i) JAWS II were plated at a density of  $2 \times 10^4$  cells in  $\mu$ -Slide 8 Well (Ibidi, Munich, Germany) with 300  $\mu\text{L}$  of complete growth medium, and (ii) Neuro-2A were seeded in 24-well plates at a

density of  $1 \times 10^5$  cells in 1 mL of complete growth medium. On the day of the treatment, siRNA Texas Red ( $1 \mu\text{g}$ ), GFP ( $1 \mu\text{g}$ ) or CMV-AMPK-DN ( $0,5, 1$  or  $2 \mu\text{g}$ ) loaded exosomes were added to the pre-cultured cells. The “exo-transfected” cells were collected at different times and analysed either by confocal microscopy or western blot (see corresponding sections).

#### 16. Confocal microscopy

One  $\mu\text{g}$  of siRNA Texas Red- or GFP- loaded exosomes were added to the pre-cultured JAWS II cells. The respective fluorescence - siRNA Texas Red (excitation max: 596 nm and emission max: 615 nm) and GFP (excitation max: 488 nm and emission max: 509 nm) - were analysed at different times (2, 6 and 24 h) by confocal microscopy (CLMS 700, Zeiss, ZEN fluorescence, Jena, Germany).

#### 17. Animals

##### i. Obese mice model

Eight to twelve weeks old obese male C57BL/6 mice (Animalario General University of Santiago de Compostela (USC), Santiago de Compostela, Spain and Charles River Laboratories) were used for the experiments. All animals were housed on a 12-hour light (8:00 to 20:00), 12-hour dark cycle (20:00 to 8:00), in a temperature- and humidity- controlled room. The animals were allowed free access to standard laboratory pellets of 6-7% high fat diet chow and tap water. In all the experimental settings, animals and their food were daily weighted at the end of the light phase. The experiments were performed in agreement with the International Law on Animal Experimentation and were approved by the USC Local Ethical Committee, the Xunta de Galicia (Project ID 15010/14/006), and the University of Iowa Institutional Animal Care and Use Committee.

##### ii. Nude mice model

Twelve weeks old male nude mice were used for the analysis of the bio-distribution of exosomes to get rid of hair auto fluorescence. The animals were housed at the animal facilities of Angers University Medical School (SCAHU) and received human care according to the guidelines of the French Agriculture Ministry. All animals were housed on a 12-hour light (8:00 to 20:00), 12-hour dark cycle (20:00 to



8:00), in a temperature- and humidity- controlled room. The animals had free access to normal diet all along the experiments.

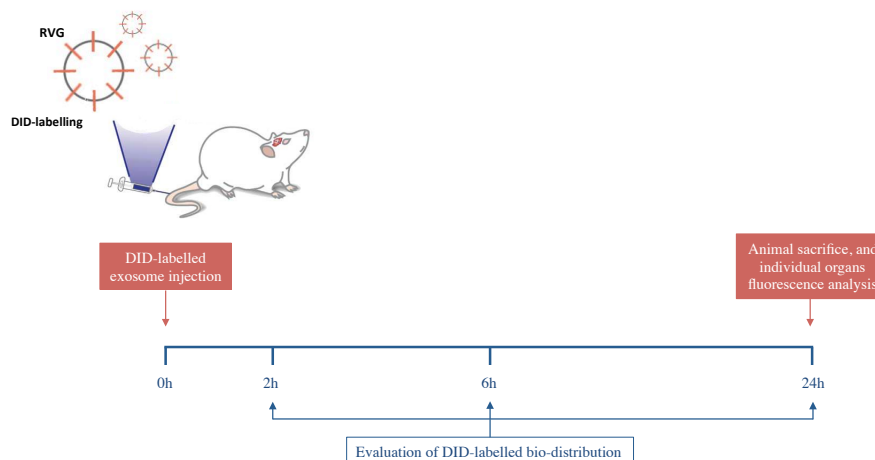
## 18. Exosomes labelling

Exosomes were stained with DID solution (excitation max: 644 nm; emission max: 665 nm) (Vybrant Cell Tracers, Molecular Probes, Eugene, OR) following the manufacturer's protocols. Briefly, exosomes were incubated with 5  $\mu\text{g}/\text{mL}$  of Vybrant Cell Tracers DID in PBS 1X for 10 min at RT, then washed two times in PBS 1X with a 200 000 g ultracentrifuge step for 2 h. The resultant DID-labelled exosomes were recovered in PBS 1X and directly used.

## 19. Animal experimental design

### i. DID-labelled exosome injection

100  $\mu\text{g}$  of DID-labelled exosomes or an equal volume of PBS 1X were injected intravenously in nude mice as previously described. For analysis of DID-labelled exosome bio-distribution, the multispectral imaging system MAESTRO *In-Vivo* Fluorescence Imaging System (CRI, Cambridge Research & Instrumentation, USA) was used. DID-labelled exosomes bio-distribution was analysed at different times (2, 6 and 24 h) on isoflurane-sedated mice. Isolated organs (liver, spleen, lungs, heart, brain and skeletal muscle) were also analysed following the sacrifice of the animal. The data were analysed with the MAESTRO *In-Vivo* Fluorescence Imaging System software (CRI, Cambridge Research & Instrumentation, USA).



**Figure 10: Timeline representation of DID-labelled exosome bio-distribution**

ii. GFP-loaded exosome injection

Hundred  $\mu\text{g}$  of non- or GFP-loaded exosomes (as previously described) were injected either (i) intravenously into the tail vein of the mice using an appropriate insulin syringe, or (ii) administrated intranasally on anesthetized animals placed in supine position. Two  $\mu\text{L}$  of exosomes suspension was delivered in each nostril respecting 2 min between 2 inhalations. A total of 20  $\mu\text{L}$  was inhaled. Forty-eight hours following the injection/administration, mice were deeply anesthetised with ketamine and transcardially perfused with 25 mL of 10 % sucrose in  $\text{H}_2\text{O}$  to eliminate the blood from the animal. Mice were then transcardially perfused with 30 mL of 4 % paraformaldehyde (PFA) in PBS 1X. Brain were removed, and post fixed in 4% PFA overnight at 4°C. Brains were then immersed in 30% sucrose solution for 48 h at 4°C before being frozen in Tissue-Tek (Sakura, Tokyo, Japan). Next, they were cut in section of 20  $\mu\text{m}$  using a cryostat CM3050S (Leica Biosystems, France) and placed on slides.

The slides were then treated with a solution of 0,1 % Sudan Black B (Allied Chemical; New York, NY) in 70% ethanol for 5 min to eliminate the auto fluorescence due to the presence of lipofuscin.

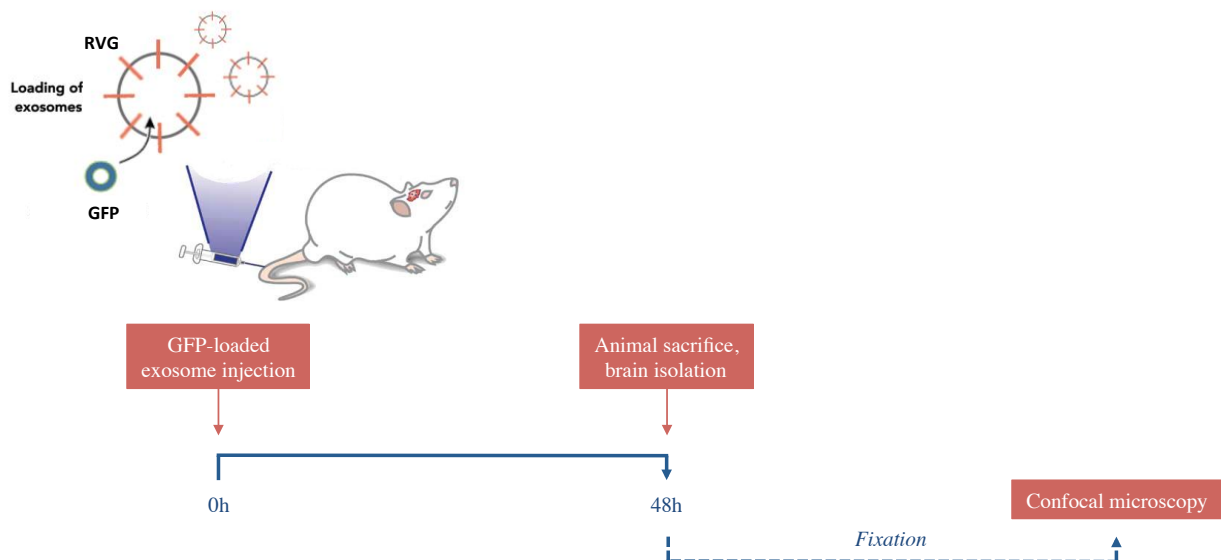


Figure 11: Timeline representation of GFP loaded exosome bio-distribution

iii. CMV-AMPK-DN and SF1-AMPK-DN loaded exosome injection

Hundred  $\mu\text{g}$  of non-, CMV-AMPK-DN- or SF1-AMPK-DN-loaded exosomes were injected intravenously in the tail vein of the mice at day 0 and 3. The duration of treatment was chosen as designed plasmids had a maximal expression within the cells for the first 72 h. We repeated the injection twice to observe if chronic injections could maintain the therapeutic effects. Daily measurements of body weight, food intake, rectal and BAT temperature (see below) were performed. At day 6, an insulin tolerance test was achieved (described below). Then, the animal was sacrificed and the organs were collected for further analysis (see below). Each group (control, CMV-AMPK-DN and SF1-AMPK-DN) was composed of 10 mice.

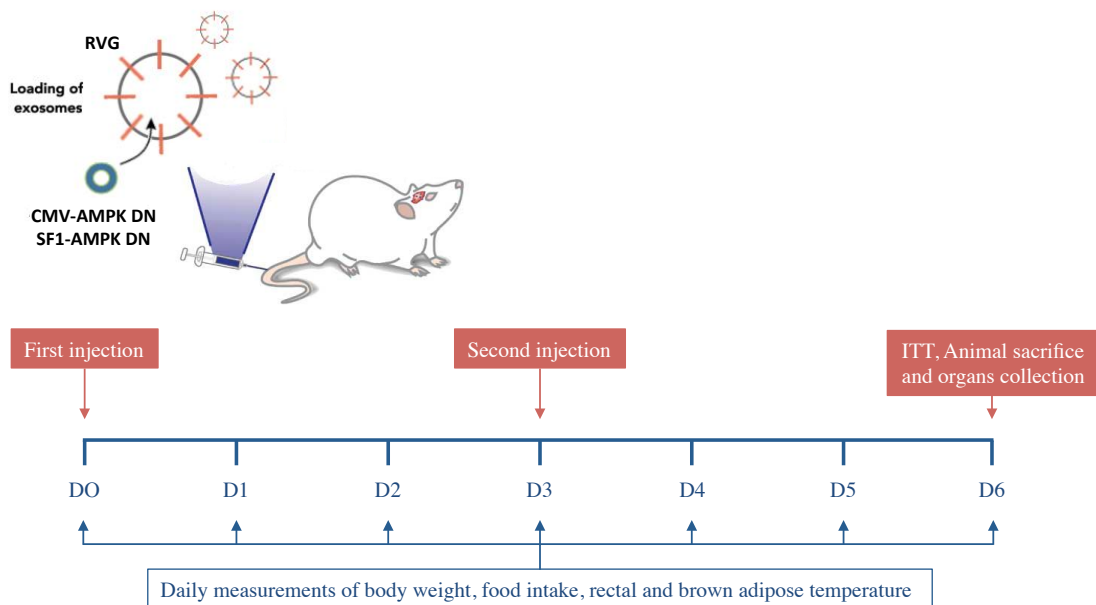


Figure 12: Timeline representation of AMPK-DN loaded exosome injection

## 20. Temperature measurements

Body temperature was recorded daily with a rectal probe connected to digital thermometer (*BAT-12 Microprobe-Thermometer; Physitemp; NJ, US*). Skin temperature surrounding BAT was daily recorded with an infrared camera (*E60bx: Compact-Infrared-Thermal-Imaging-Camera; FLIR; West Malling, Kent, UK*) and analyzed with a specific software package (*FLIR-Tools-Software; FLIR; West Malling, Kent, UK*). Two types of BAT temperature were represented: (i) the average BAT temperature corresponding to the average of the temperatures of all BAT tissue,

and (ii) the maximum BAT temperature corresponding to the highest temperature detected within the BAT.

### 21. Insulin Tolerance Test

At the end of the 6 days of treatments, an intraperitoneal injection of 0.75 U/kg insulin (Actrapid, Novonordisk) was performed and blood glucose levels were measured at 0, 15, 30 and 60 min with a glucometer (Accucheck, Roche).

### 22. Sample processing

Mice were killed by cervical dislocation. From each animal, the hypothalamic dissections (ARC or VMH) for western blotting assays were harvested and immediately homogenized on ice to preserve phosphorylated protein levels. BAT, scWAT, gWAT, liver, skeletal muscle and serum were also collected. Samples were stored at -80°C until further processing. Dissection of the ARC and the VMH was performed by micropliers.

### 23. Western Blot

#### i. Characterization of exosomes

Ten  $\mu\text{g}$  of exosomes were separated on 4-12% polyacrylamide gel electrophoresis. After migration, proteins were transferred to nitrocellulose membranes and incubated with the following antibodies: Lamp2 (1/1000, Abcam), Alix (1/1000, Cell Signalling) and CD81 (1/1000, Cell Signalling).

#### ii. Exosome-treated cell analysis

After treatment with exosomes, JAWS II cells were collected and lysed to extract protein content. Ten  $\mu\text{g}$  of protein lysates were separated on 4-12% polyacrylamide gel electrophoresis, and were electrotransferred to nitrocellulose membranes and incubated with the following antibodies: p-ACC (1/1000, Cell Signalling), ACC (1/1000, Millipore) and  $\beta$ -actin (1/5000, Sigma).

#### iii. Sample analysis

ARC, VMH, BAT and liver tissues were lysed to extract protein content, subjected to a 8% polyacrylamide gel electrophoresis, electrotransferred to a PDVF

membrane and incubated with the following antibodies: p-ACC (1/1000, Cell Signalling), ACC (1/1000, Millipore), SF-1 (1/1000, Santa Cruz) and  $\beta$ -actin (1/5000, Sigma).

#### 24. Serum Analyses

The serum was collected after centrifugation of the blood for 10 min at 4°C. IL-6 levels were quantified using Quantikine Elisa kits (R&D systems) following manufacturer's instructions.

#### 25. Statistics

The analysis of the obtained experimental data was performed by Excel (Microsoft) and Prism (GraphPad Prism). Statistical analysis was achieved using Mann-Whitney non-parametric tests for the comparison of non-paired samples. Results are expressed as mean  $\pm$  SEM. The differences were considered significant when  $p$  value  $<0.05$  and stated as follow: \* for  $p<0.05$  and \*\* for  $p<0.01$ .

# V. Results



## **1. BMDCs exhibit an immature dendritic phenotype but with a low rate of transfection efficiency**

As described by Quah and O'Neill in 2005, dendritic cells are able to produce large quantities of exosomes (Quah & O'Neill, 2005). Therefore, bone marrow progenitors were isolated from C57BL/6 mice and differentiated in dendritic cells. To ensure that BMDCs expressed the desired dendritic phenotype, the expression of specific surface markers was analysed through FACS analysis. Firstly, the expression of the specific dendritic marker CD11c was evaluated between non- and GM-CSF-stimulated cells. As shown on figure 13, a shift of the fluorescence was observed on the cells treated with the growth factor GM-CSF traducing an increase of CD11c expression (Fig. 13A). Moreover, as the produced exosomes must be immunologically inert, the BMDCs must hold an immature state. As immunostimulatory molecules such as CD80, CD86 and MHC II are important activation markers for BMDCs, their expressions were evaluated. Compared to 24 h LPS-stimulated activated mature BMDCs in which CD80, CD86 and MHC II were increased, the BMDCs obtained under the experimental protocols used exhibited low expression of these immunostimulatory molecules (Fig. 13B, 13C and 13D). According to these results, the BMDCs produced displayed an immature dendritic phenotype.

We further investigated the transfectability of BMDCs using the GFP encoding plasmid as a positive transfection probe. BMDCs were transiently transfected with GFP using two different commercialized liposome-based transfection reagents, JetPei-Macrophage and Lipofectamine 2000. Whatever the reagent used, the GFP fluorescence detected by FACS analysis was not increased compared to the non-transfected control condition traducing an inefficiency of the protocols (Fig. 13E). This inefficiency of the cells to express the GFP was associated to a high increase of cell death (data not shown).

As BMDCs are primary cells, the obtained results are not surprising. Described to be more sensitive to the environmental stress due to the toxicity of the transfection reagent and to the exogenous introduction of DNA, primary cells are hardly transfectable - low rate of efficiency associated to a high rate of cell death -. Therefore, using more resistant cells, an immature dendritic cell line, emerged.



## **2. JAWS II cell line, a highly transfectable suitable exosome-producer model**

In order to obtain a suitable source of neuronal-targeted exosomes, a high ratio of cell transfection was necessary. Therefore, JAWS II cell line - an immortalized immature DC line established from the bone marrow cultures of p53<sup>-/-</sup> C57BL/6 mice - was used. As previously investigated with the BMDCs, we evaluated the transfectability of JAWS II using the GFP encoding plasmid as a positive transfection probe. JAWS II were transfected using the two same transfection reagents - JetPei Macrophage and Lipofectamine 2000 - and interestingly, compared to a control non-transfected condition, GFP fluorescence was increasing - 33.9% and 28.3% respectively - within these cells as shown by FACS analysis (Fig. 14A). Moreover, no increase of cell death was observed following these protocols (data not shown). However, regarding our desire to produce high quantity of neuronal-targeted exosomes, these slight increases of the efficiency of the transfection appear to be insufficient. Thus, we continue our investigation with the MacsFectin transfection reagent. Still using the GFP plasmid as a positive transfection probe, we could notice an increase of nearly 70% of GFP signal within JAWS II cells as determined by FACS analysis (Fig. 14A). An additional mortality assay was realized on the MacsFectin-transfected cells and no increase of cell death was observed under our experimental conditions (data not shown). Moreover, as often described, we tried to modulate the DNA/transfection reagent ratio - 1/2, 1/3 and 1/4 - to increase the efficiency of the transfection. However, no significant difference was noticed between these protocols (Fig. 14B). According to the high percentage of transfection and low rate of mortality obtained with this transfection reagent, we continue our investigation following this experimental procedure.

## **3. Neuronal targeting plasmid engineering**

The major breakthrough of this study was the specific delivery of AMPK-DN in neuronal cells. Previous studies showed that native radiolabelled extracellular vesicles injected intravenously were mostly located in lungs, liver, spleen and kidneys with a really small amount in the brain (Hwang et al., 2015). Our aim was to target AMPK within the VMH. Thus, increasing exosome localization within the brain was one of

the restraining factors of this study. Following the studies of Alvarez-Erviti and colleagues, the RVG peptide was fused to the extra-exosomal N-terminus of murine Lamp2b, a protein found abundantly in exosomal membranes (Alvarez-Erviti et al., 2011). This specific cloning of RVG at the N-terminus part of Lamp2b just after the signal peptide ensured RVG to be located at the outer surface of the membrane (Fig. 15A and 15B).

The accuracy of the Lamp2b-RVG plasmid construction was evaluated by Northern Blot. Lamp2b and Lamp2b-RVG plasmids were digested using HindIII and NHEI restriction enzymes between AAGCTT and GCTAGC respectively. The respective fragments were differentiated depending on their sizes in kilobases (kb). As shown on figure 15C, some Lamp2b-RVG digested sequences migrated less far compared to native Lamp2b plasmid' ones, traducing an insertion of 87 pair of bases within their sequences (Fig. 15C). To confirm the accuracy of the insertion, DNA sequencing was performed on Lamp2b-RVG. As shown on figure 15D, a 100% homology was observed between the theoretical sequence and the engineered one (Fig. 16D).

#### **4. Nano-scaled vesicles produced by JAWS II cells are physically homogenous and possess exosomal markers**

Vesicles released by JAWS II cells were collected by ultracentrifuge methods. To confirm of their exosomal origins, they were morphologically analysed. The first analysed parameter was the size of the isolated vesicles. As determined by Zetasizer and Nanotracking analysis, all samples had a normal distribution around a mean diameter of 70-80 nm, corresponding to what previously reported for exosome size distribution (Fig. 16A and 16B). The isolated nano-scaled vesicles were then analysed by electron microscopy giving an average size slightly lower - 60 to 70 nm - than the one observed through the previously physic methods due to the dehydration of the samples (Fig. 16C and 16D). Moreover, a protein analysis of the vesicles was performed through Western Blot. The vesicles expressed 3 characteristic exosomal markers - Alix, CD81 and Lamp2b - (Fig. 17E). As determined by their sizes and the expression of specific markers, the isolated nano-scaled shared the same characteristics of exosomal vesicles.

## 5. Loaded exosomes can deliver their content in vitro

The capacity of exosomes to deliver nucleic acids was firstly assessed in vitro. Exosomes were loaded with 2 different fluorescent nucleic acids, a siRNA Texas Red and a GFP encoding plasmid. Loaded exosomes were incubated with dendritic JAWS II cells during 2, 6 and 24 h. The respective fluorescence was then analysed directly on these cells by confocal microscopy. As observed on figure 17, siRNA Texas Red loaded exosomes induced an increase of red fluorescence within the cells after 2 h of incubation compared to control condition - cells incubated with non-loaded exosomes - (Fig. 17A). In the same way, GFP-loaded exosomes induced an increase of green fluorescence within the dendritic cells compared to the control condition after 6 and 24h of incubation (Fig. 17A). The duration of the incubation differs following the used nucleic acid as the GFP encoding plasmid has to be taken in charge by the cells (transcription/translation processes), while the siRNA Texas Red is already labelled with a fluorescent dye.

To evaluate if this delivery strategy could be used in an obesity driven context, we wanted to assess whether exosomes could deliver CMV-AMPK-DN in vitro. Therefore, we used a neuronal cell line model, Neuro-2A cells, believed to be targeted by RVG exosomes. Indeed, RVG peptide will specifically bind to acetylcholine receptors expressed on neuro-endothelial and neuronal cells. Neuro-2A cells were treated for 36 h with CMV-AMPK-DN alone (AMPK-DN 1  $\mu$ g) and Lamp2b-RVG exosomes loaded with increasing quantities of CMV-AMPK-DN plasmids (exosomes 0.5; 1 and 2  $\mu$ g). Herein, increasing quantities of plasmids were used to find out which condition could deliver the highest amount of AMPK-DN within Neuro-2A cells. We evaluated the phosphorylation of acetyl CoA carboxylase (ACC) as AMPK is the main regulatory kinase of this enzyme. CMV-AMPK-DN loaded RVG exosomes induced a decrease of ACC phosphorylation levels compared to non-treated cells, without any difference between the 3 different growing plasmid concentrations (Fig. 17B). Compared to what was shown by Alvarez-Erviti and colleagues in 2011 in which BACE1 siRNA loaded exosomes (50, 100 and 200 nM) induced a concentration dependent knockdown, we were expecting that decrease of ACC phosphorylation levels would be associated with increased plasmid concentrations. Nevertheless, the chosen plasmid quantities herein were certainly too comparable to induce such an

effect. Another unexpected element within these results was the decrease of ACC phosphorylation induced by CMV-AMPK-DN plasmid alone (Fig. 17B). However, some studies demonstrated that DNA plasmid could be uptake directly by cells. Sramkova and colleagues demonstrated that non-encapsulated DNA plasmid could be internalized from the apical plasma membrane of the salivary gland epithelium in live animals (Sramkova et al., 2012).

Taken together, these *in vitro* results indicate that nucleic acids encapsulated within the exosomes are not damaged and are able to induce functional effects, confirming exosomes' potential role as a delivery tool. However, as Neuro-2A cells are not isolated specifically from the hypothalamus, they are not specialized in energy balance regulation. Thus, further investigations on hypothalamic cells would be needed to confirm the potency of hypothalamic release.

## **6. Brain targeting of Lamp2b-RVG exosomes**

In order to evaluate the impact of RVG targeting strategy on the bio-distribution of exosomes systemically injected, they were labelled with the near-infrared dye DID. DID is a lipophilic dye that only fluoresces when incorporated in lipid membrane, such as the exosome one. To avoid any non-specific fluorescence following intravenous injection due to free dye that could bind proteins, cells or tissues, DID-labelled exosomes were washed 2 times before all injections. 100  $\mu$ g of DID-labelled native (Exo Mock) or RVG exosomes (Exo RVG) were injected into the tail vein of nude mice. Anesthetized live mice were imaged using DID fluorescence spectra (excitation max: 644 nm; emission max: 665 nm) at 2, 6 and 24 h using the *in vivo* fluorescence imaging system MAESTRO. As shown on figure 18A, the obtained entire animal images did not enable to determine accurately the specific fluorescence of each organ and tissue (Fig. 18A). Therefore, the animals were sacrificed at the end of the 24 h and the lungs, the liver, the spleen, the skeletal muscle, the heart and the brain were collected and the specific fluorescence was analysed *ex vivo*. Native and RVG exosomes were mostly localized in the lungs, the spleen and the liver, 3 major detoxification organs (Fig. 18B). Interestingly, RVG exosome brain localization appeared to be increased of 35 % compared to the native ones (Fig. 18C). As RVG peptide targets the alpha-7-subunit of the nicotinic acetylcholine receptor, also

expressed by cardiac cells, we were expecting to observe an increase in RVG exosome heart localization (Wiklander et al., 2015). Surprisingly, no differences between the 2 different exosomal populations were detected. Herein, to harmonize the results, some more live mice imaging analysis would be needed to sleek the differences.

### **7. Neither intravenous injections nor intranasal administrations of GFP-loaded exosomes induced a significant increase of GFP within the brain**

Whether the RVG strategy increased exosome brain localization following a systemic injection, we had to evaluate if exosomes were able to release their nucleic content within the hypothalamic neurons. Recent publications have described that different routes of administrations could target efficiently the brain.

As already described, Alvarez-Erviti et al. (2011) demonstrated that neuronal targeted exosomes could reach the brain and induce a specific knockdown of Bace1 expression following an intravenous injection. Moreover, Cooper and colleagues systemically injected RVG exosomes loaded with a siRNA inhibiting Alpha-synuclein ( $\alpha$ -Syn) expression, and they observed a reduced  $\alpha$ -Syn mRNA and protein levels throughout the brain after 3 days (Cooper et al., 2014). Even if intravenous injections have shown promising results in brain targeting and delivery, recent publications have described the nasal system as a novel direct route to the brain. Danielyan and colleagues showed that injected mesenchymal stem cells within the nasal cavity could migrate through the cribriform plate to reach the brain tissue via the olfactory and trigeminal pathways (Danielyan, 2009). Moreover, it was described that neural stem cells injected in the nasal cavity could reach the mouse brain and target glioma models (Reitz, 2012). This nasal route was also used with exosomal nanoparticles. Indeed, a study leaded by Zhuang in 2011 used exosomes to deliver anti-inflammatory drugs to the brain through intranasal administration. The exosomes, loaded with curcumin or with a Stat-3 inhibitor, JSI- 124, were intranasally injected. Under these conditions, up to 60% of loaded-exosomes were taken up by microglial cells, and surprisingly induce their apoptosis, demonstrating the still-active effect of these incorporated substances (Zhuang et al. 2011).

Thus, intravenous and nasal routes appear as good candidates to increase the delivery in the mice brain.

The efficiency of exosomal nucleic acid delivery *in vivo* was evaluated using a GFP encoding plasmid and confocal microscopy. One of the major restrictions in fluorescence imaging is tissue autofluorescence. Decades ago, it has been described that autofluorescence could be caused by Lipofuscin. Lipofuscin is an auto fluorescent pigment composed of residues of lysosomal digestion and is found in high quantity in nerve cells (Goyal, 1982). Lipofuscin spectrum overlaps those of all commonly used fluorophores, including GFP, making difficult to distinguish specific signals from auto fluorescence. In order to decrease autofluorescence within tissues from the central nervous system, numerous studies have demonstrated that staining with Sudan Black B could reduce lipofuscin fluorescence (Schnell et al., 1999; Belichenko et al., 1996; Oliveira et al., 2010).

Therefore, mice were treated either by 100  $\mu$ g of non- or GFP-loaded exosomes through 2 different routes, intravenously and intranasally. 48 hours after the injection, mice were sacrificed and brain removed as described in the methods. The duration of 48 h of treatment was chosen as the GFP plasmid has a transitory expression. Transiently transfected cells express the introduced GFP gene without integrating it into their genome. The peak of the transient expression occurs 48 to 60 h after transfection and then decreases rapidly (Skala et al., 1986). Once fixed, included and cut, the brain slices were treated with a Sudan Black B solution to avoid lipofuscin auto fluorescence.

Sudan Black B treated brain slices were analysed by confocal microscopy. As the VMH is the major centre of regulation of energy balance, the fluorescence analysis was focused on this particular region using phase contrast microscopy (Fig. 19). Surprisingly, no significant difference of GFP signals within the neurons between control, intravenously injected or intranasally administrated was detected (Fig. 19). This absence of specific GFP signals could be due to a degradation of the GFP encoding plasmid within the exosomes. However, the obtained *in vitro* results (Fig. 17) demonstrated that nucleic acids encapsulated within the exosomes were not damaged and were able to induce functional effects. Thus, this absence of GFP signals could be either due to (i) a too small amount of GFP-loaded exosomes crossing the blood brain barrier and reaching the VMH or (ii) to a limit of the confocal microscopy resolution.

To inhibit AMPK signalling within the VMH to induce body weight decrease, adenoviral vectors were used (López et al., 2010; Whittle et al., 2012; Morentin et al., 2012; Martínez de Morentin et al., 2014; Beiroa et al., 2014). However, any studies used plasmid construction to deliver AMPK DN within the VMH. Moreover, any equivalence is known between virus and plasmid quantity to inject to induce the desired effects. Thus, we followed our first hypothesis, and continue our investigation on functional effects despite the negative results described above.

### **8. SF1-AMPK-DN loaded exosomes induced a body weight decrease without modifying food intake**

Next, we investigated the potential of AMPK-DN loaded exosomes to decrease body weight following systemic injections. As discussed before, the VMH plays a major role in the modulation of both feeding and thermogenesis, with AMPK playing a key role in the last effect (López et al., 2010; Whittle et al., 2012; Morentin et al., 2012; Martínez de Morentin et al., 2014; Beiroa et al., 2014). However, staring at the previously described results on exosomes bio-distribution (Fig. 18), the release of AMPK-DN will not only be localized within the VMH following the same protocols and strategy. Due to this predicted non-specific delivery of AMPK-DN and due to the involvement of AMPK in lot of metabolic processes at a whole body level (Hardie et al., 2012), this could lead to undesirable side effects and so, on a decrease of the efficiency of the treatment. Thus, needs to increase at a molecular level the specificity of the delivery were required. The idea of using a new targeting peptide was thought. However, no VMH extracellular specific receptor was identified until now limiting this approach.

Therefore, a novel strategy to limit the expression of AMPK-DN to the VMH was thought and designed. Indeed, the first thought AMPK-DN plasmid was designed under the control of cytomegalovirus promoter (CMV). However, CMV promoter has the characteristic to have a high expression potential within all the cells of the organism. So, the idea of designing an AMPK-DN plasmid under the control of a specific promoter of the VMH emerged. One of the earliest identified molecular markers expressed specifically within the VMH is the nuclear receptor steroidogenic factor-1 (SF-1). SF-1 is a transcription factor essential for terminal differentiation and

maintenance of VMH neuronal populations (Cheung et al., 2013), which appears during the embryonic development. Thus, designing a plasmid encoding for AMPK-DN under the control of SF-1 will allow the AMPK-DN to be expressed only in the VMH. SF1-AMPK-DN encoding plasmids will be delivered all over the organs but they will not be translated limiting the unwanted side effects.

Therefore, 3 different mice groups were treated: (i) non-loaded exosome control group (Control), (ii) CMV-AMPK-DN loaded exosome group (CMV-AMPK-DN) and (iii) SF-1-AMPK-DN loaded exosome group (SF1-AMPK-DN). As described in the experimental procedure, all 3 groups were injected twice (every 3 days) with 100  $\mu$ g of exosomes in the tail vein and the effects on body weight and food intake was evaluated. As shown on figure 21, SF-1 AMPK-DN loaded exosomes induced a significant decrease of body weight ( $1.98 \pm 0.52$  g) compared to control and CMV-AMPK-DN groups (Fig. 20A). Moreover, this effect is induced directly after the first day of injection and is maintained and increased all over the 6 days of treatment traducing the efficiency of this strategy on decreasing body weight.

Well-described by Lopez and colleagues in 2010, AMPK-DN induced body weight decrease is rather linked to an increase of BAT thermogenesis than to anorexia (López et al., 2010). Therefore, food intake was analysed all along the 6 days of treatment in parallel of body weight analysis. Although a slight decrease of food intake ( $-0,53 \text{ g} \pm 0,051$ ) within the SF1-AMPK-DN group compared to control and CMV-AMPK-DN groups can be observe during the first 3 days, it cannot explain the body weight loss observed in these mice (Fig. 20B). Indeed, staring at the food intake following the second injection (day 3 to day 6), we can observe that the 3 curves fit indicating an equal food intake while the body weight continue to decrease (Fig. 20B). Moreover, comparing the cumulative food intake on the 6 days of experiment indicate no difference between the 3 mice groups traducing an equal alimentation for all the mice (Fig. 20C) confirming that body weight loss is not associated with anorexia but rather to an increase of BAT thermogenesis (see below).



## **9. SF1-AMPK-DN loaded exosomes did not induce discrete increases of rectal and BAT temperatures**

As previously mentioned, AMPK-DN induced body weight decrease is frequently associated to an increase of BAT thermogenesis. BAT thermogenesis is an essential component of the energy balance equation. It has been recognized for a long time that the hypothalamus, in particular the VMH, plays a crucial role in the regulation of BAT activity (Cannon & Nedergaard, 2004). More recently, numerous studies provided strong evidences that hypothalamic AMPK had a crucial role in BAT activity regulation through an increased activity of sympathetic nervous system (SNS). Indeed, the inhibition of AMPK within rats' VMH induced body weight loss and increased expression of thermogenic markers in BAT (López et al., 2010; Whittle et al., 2012; Morentin et al., 2012; Martínez de Morentin et al., 2014; Beiroa et al., 2014). Following these described mechanisms, rectal and BAT temperatures were analysed on the 3 groups of animals - Control, CMV-AMPK-DN and SF1-AMPK-DN -. Rectal temperature was also analysed, as an increase of BAT thermogenesis will be echoed with an increase of the global temperature of the animal. Surprisingly, we did not observe any increase of rectal temperature following our treatments (Fig. 21A). Identically, no modifications of BAT temperatures were observed, neither in the average BAT temperature nor on the maximal BAT temperature (Fig. 21B, 21C and 21D). In any case, these results should be taken with care and, as discussed afterward, a proper analysis of energy expenditure (by indirect calorimetry) must be performed, because molecular evidences (increased UCP1 expression) account for a higher thermogenic activity in the BAT (see below).

These results were not the ones expected; body weight decrease is frequently associated with an increase of BAT activity. However, staring at the standard deviations obtained within each group of animal, the accuracy of the measures appears to be insufficient. Indeed, on a same measure, a standard deviation of 0,5 to 1 °C can be observed in the same group of animals. As described by Gilmore et al. (2008), performing stressful procedures (rectal temperature recording) in view of cage mates may cause stress in observer animals. They showed that heart rate and core body temperature were significantly increased in observers during handling procedures.

These results may indicate that such procedures should be done in isolation from other mice with an adaptation period.

#### **10. SF1-AMPK-DN loaded exosomes improved sensitivity to insulin**

Obesity induces insulin resistance leading to an increase of the prevalence of type 2 diabetes. Therefore, to determine whether AMPK-DN loaded exosomes could increase insulin sensitivity, we measured glucose levels at different times following an intraperitoneal injection of insulin. SF1-AMPK-DN loaded exosomes induced a decrease of blood glucose ( $p=0,052$ ) compared to control group (Fig. 22A and 22B). Thus, insulin sensitivity seems to be increased following SF1-AMPK-DN loaded exosomes injection.

#### **11. SF1-AMPK-DN loaded exosomes did not induce modulation in hypothalamic AMPK pathway at the end of the treatment period**

After the 6 days of treatment, the mice were sacrificed and the VMH isolated. AMPK-DN is an inactive mutated form of AMPK by modification of the active site through the substitution of the threonine 172 to an aspartic acid. Due to this mutation, AMPK-DN will not be able to play its kinase role of the subsequent actors, such as ACC.

Thus, to evaluate the effects of AMPK-DN, we observed the phosphorylation levels of ACC, and this for the 3 groups of mice. Neither CMV- nor SF1-AMPK-DN loaded exosomes induced decrease of the levels of ACC phosphorylation compared to control group as shown on the western blot quantification (Fig. 23A and 23B). This was probably related to the timing, since AMPK effects on ACC are normally very quick (15-30 minutes) and transient. In addition, this lack of effect may be related to the fact that AMPK action was restricted to SF-1 expressing cells and here we performed western analyses of VMH micro-punches, that contained SF-1 and non-SF-1 populations. To test this hypothesis, we analysed the expression of SF1 directly on isolated VMH samples and also on the other harvested hypothalamic nuclei. As shown on figures 23C and D, SF1 expression was not exclusive to the VMH samples (Fig. 23C and 23D). SF1 is also expressed within the other hypothalamic material

following the VMH isolation traducing that the isolation was not executed in the best conditions. A further and proper anatomical analysis will be required in subsequent experiments for a proper validation of the method. Optimization of the conditions will be discussed later on (see discussion).

## **12. SF1-AMPK DN loaded exosomes induced a increase of BAT UCP-1**

As previously mentioned, AMPK inhibition in the VMH mediates body weight decrease associated with increased BAT thermogenesis. Indeed, it has been well described that an inhibition of hypothalamic AMPK activity will increase the SNS activity resulting in norepinephrine release and stimulation of  $\beta$ 3-adrenergic receptors on the brown adipocyte membrane (López et al., 2010; Whittle et al., 2012; Morentin et al., 2012; Martínez de Morentin et al., 2014; Beiroa et al., 2014). Within the BAT tissue, an increase of cAMP induces the activation of the protein kinase A leading to heat production through the unique presence of uncoupling protein 1 (UCP 1) (López et al., 2016).

Following the uncertain obtained results on core body and BAT temperatures, we continue our investigation through a molecular analysis of BAT UCP 1 following the 6 days of treatment. Interestingly, SF1-AMPK-DN loaded exosomes induce an increase of UCP 1 expression compared to the control and CMV-AMPK-DN conditions (Fig. 24A and 24B). This result is indicative of increased thermogenic capacity in the BAT of SF1-AMPK-DN treated mice.

## **13. Loaded exosomes intravenous injections did not induce any immune response**

Moreover, we investigated whether CMV- or SF1-AMPK-DN loaded RVG exosomes induced immune responses in vivo following a systemic injection. Therefore, we evaluated the levels of IL-6 within the serum. IL-6 a pro-inflammatory cytokine implicated in the acute phase of inflammation (Eder et al., 2009). Whether non-loaded, CMV-AMPK-DN or SF1-AMPK-DN loaded exosomes did not induce any increase of IL-6 serum concentrations as shown on figure 25. These results might

indicate and confirm the potential of using exosomes as a novel immunologically inert delivery vehicle.

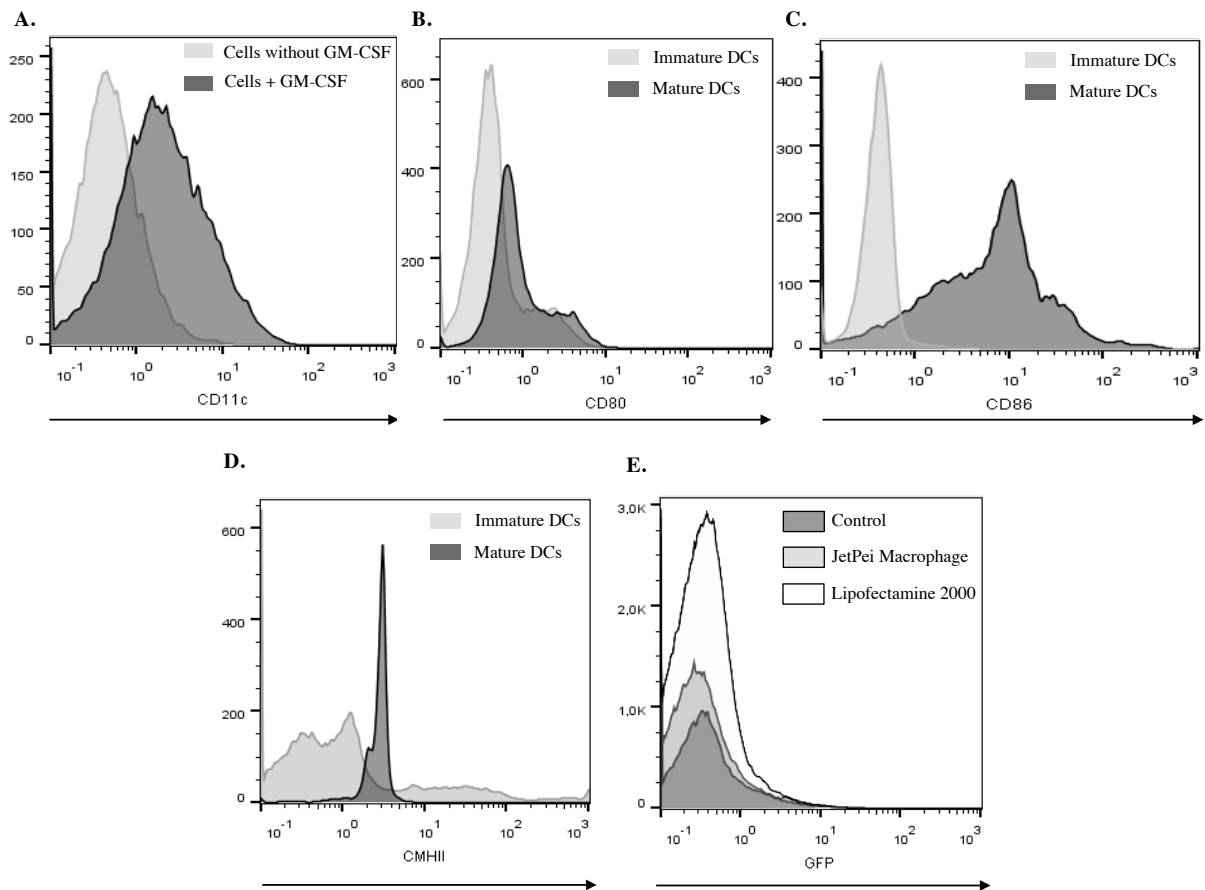


Figure 13: BMDCs phenotype and transfectability. Expression of specific immature dendritic cells markers: CD11c, CD80, CD86 and CMHII (A, B, C and D). Efficiency of transfection of BMDCs (E).

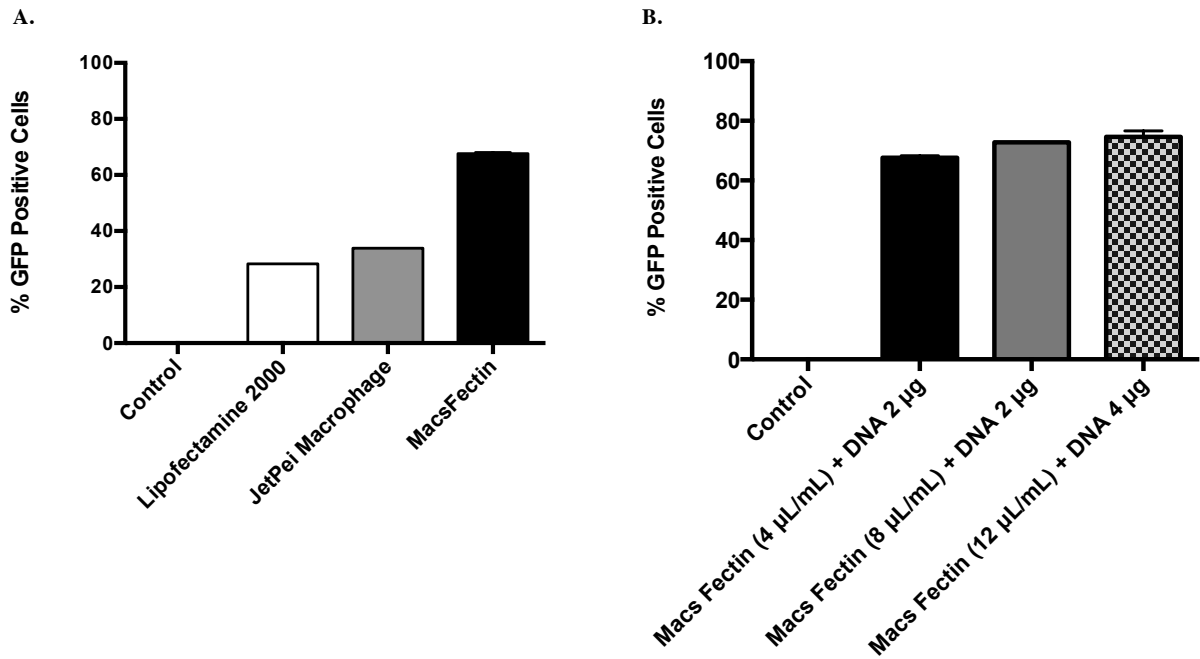
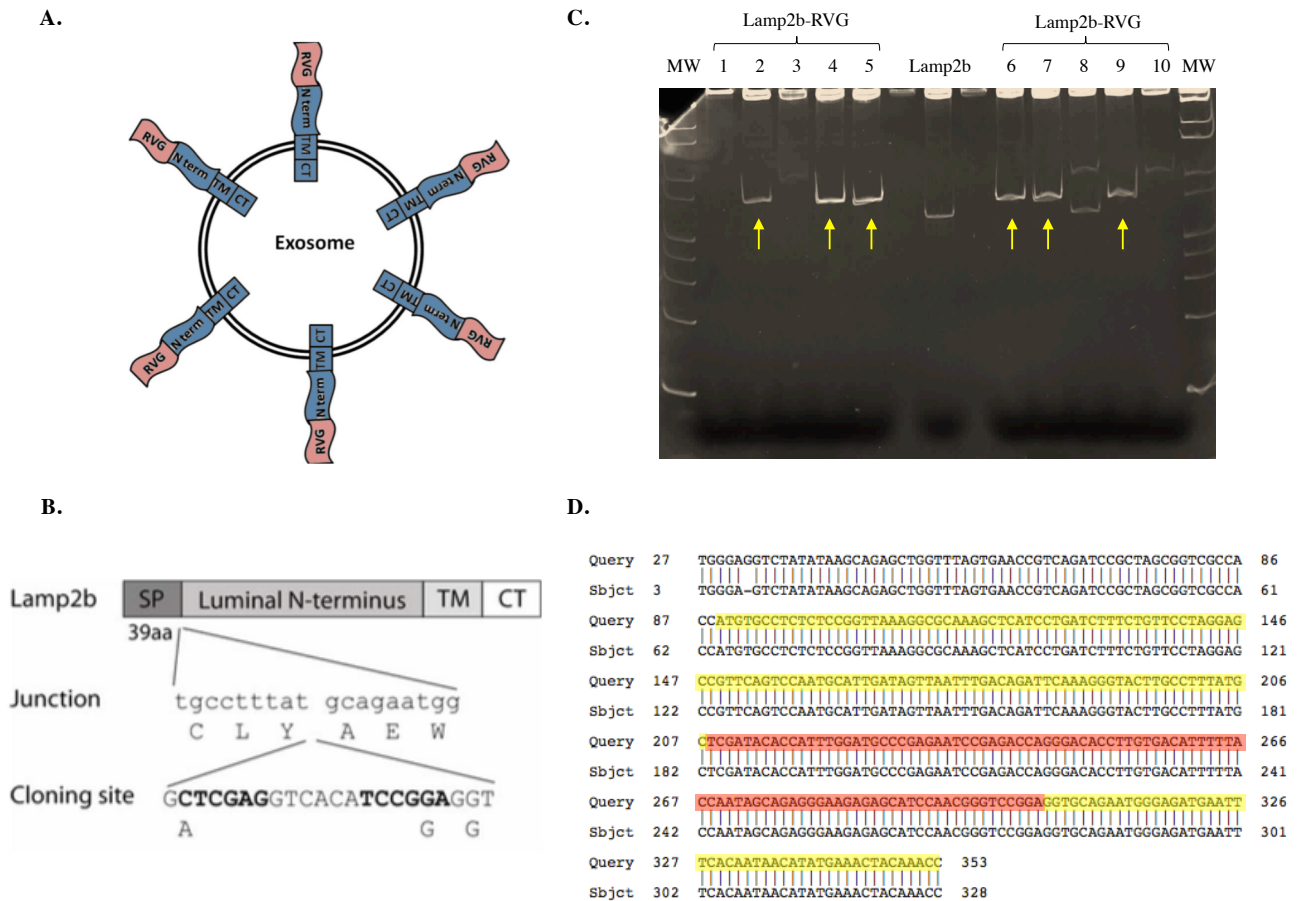


Figure 14: JAWS II cells transfection efficiency. Percentage of GFP positive JAWS II cells using Lipofectamine, JetPei Macrophage and MacsFectin transfection reagents (A). Percentage of GFP positive JAWS II cells using different DNA/MacsFectin ratios (B).



**Figure 15: Lamp2b-RVG plasmid engineering.** Schematic representation of Lamp2b-RVG engineered exosome (A). Representation of the cloning of RVG into Lamp2b after the signal peptide at N-terminus (Adapted from Alvarez-Erviti et al. 2011) (B). Northern Blot of digested Lamp2b and Lamp2b-RVG plasmid products. The yellow arrows show the Lamp2b plasmids having an insertion within their sequences (C). NCBI blast result obtained between theoretical and engineered Lamp2B-RVG sequences. Yellow represents Lamp2B sequence and Red represents RVG (D).

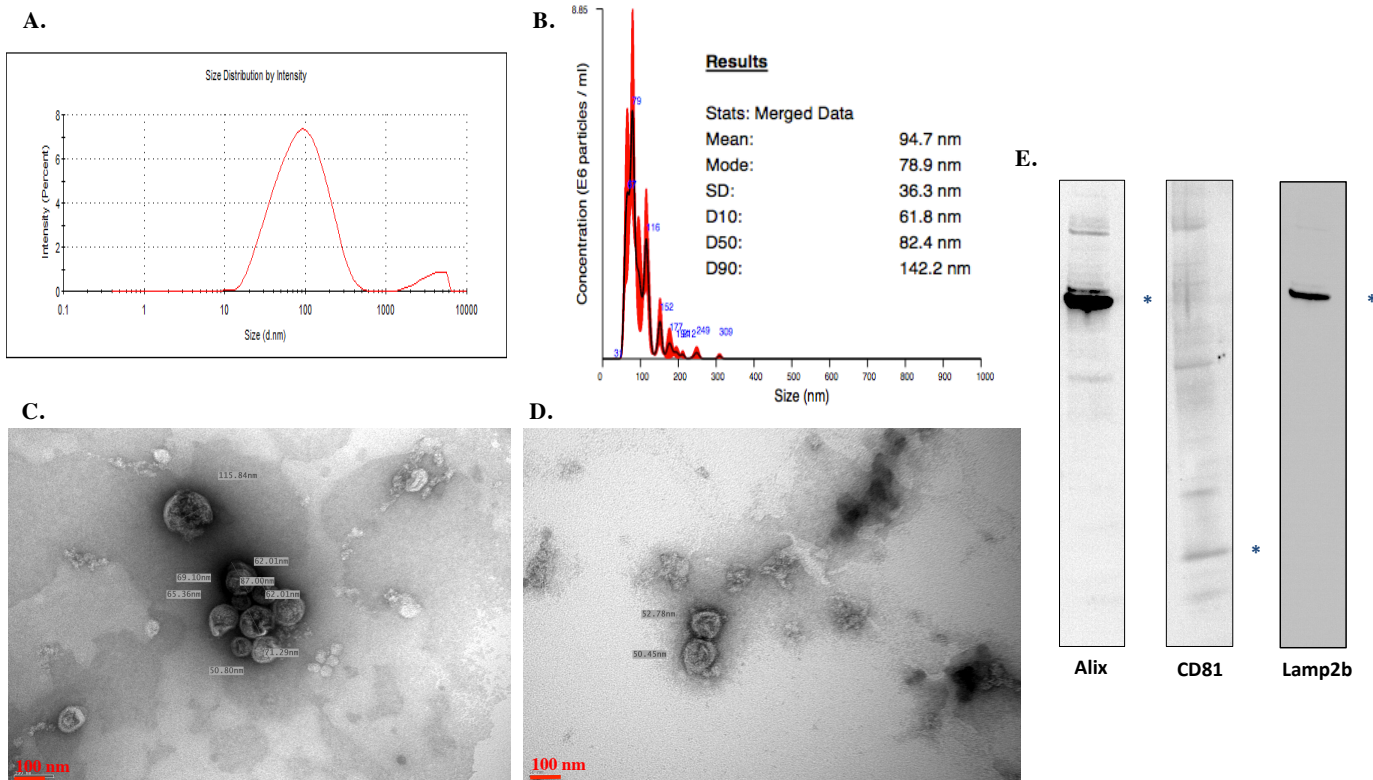


Figure 16: Morphological analysis of JAWS II cell produced exosomes. Size distribution analysis of exosomes by Nanosizer (A) and Nanotracking analysis (B). Electron microscopy of exosomes (C, D). Bars = 100 nm. Western Blots of Lamp2, Alix and CD81 (E). Stars indicate the immunoblotted protein.



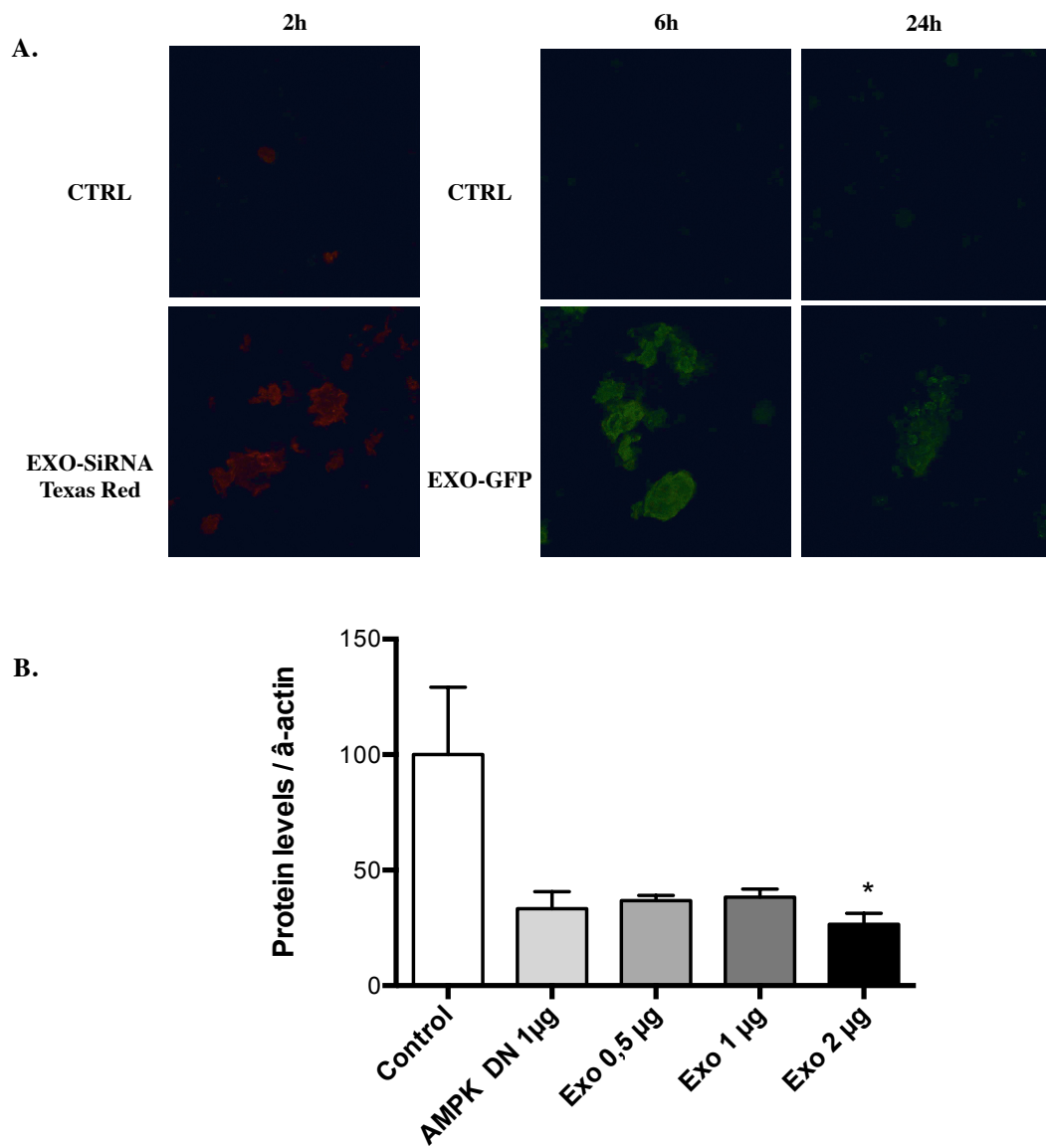


Figure 17: Evaluation of exosome delivery in vitro. Confocal microscopy of JAWS cells treated with siRNA Texas Red- or GFP- loaded at 2, 6 and 24 h (A). Levels of ACC phosphorylation on JAWS II cells following treatments with AMPK-DN plasmid or exosomes (Exo 0,5  $\mu$ g, Exo 1  $\mu$ g and Exo 2  $\mu$ g) (B). \* for  $p < 0,05$

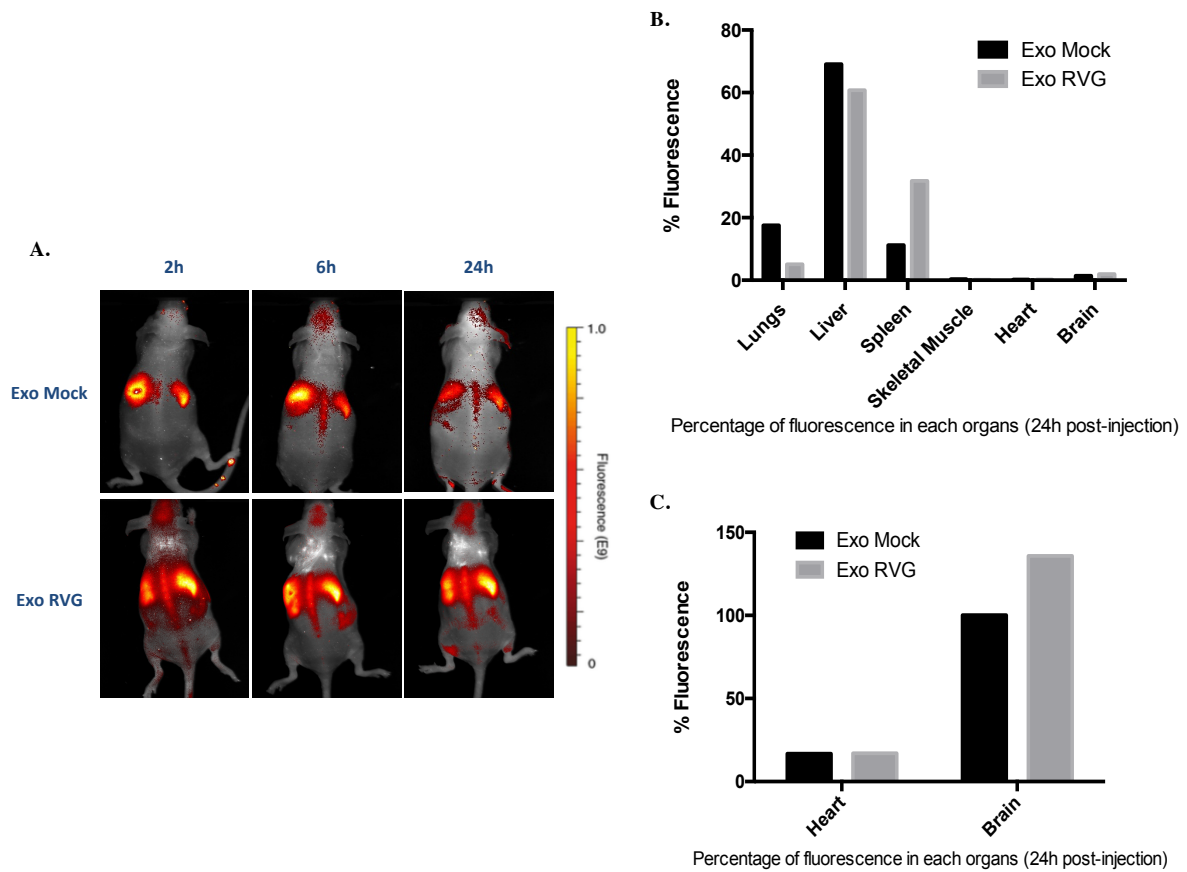


Figure 18: Bio distribution of Lamp2b-RVG exosomes. Entire animal live images of DID labelled exosomes bio distribution (A). Ex vivo analysis of DID labelled exosomes fluorescence on isolated organs (B). Ex vivo analysis of DID labelled exosomes fluorescence on heart and brain (C).

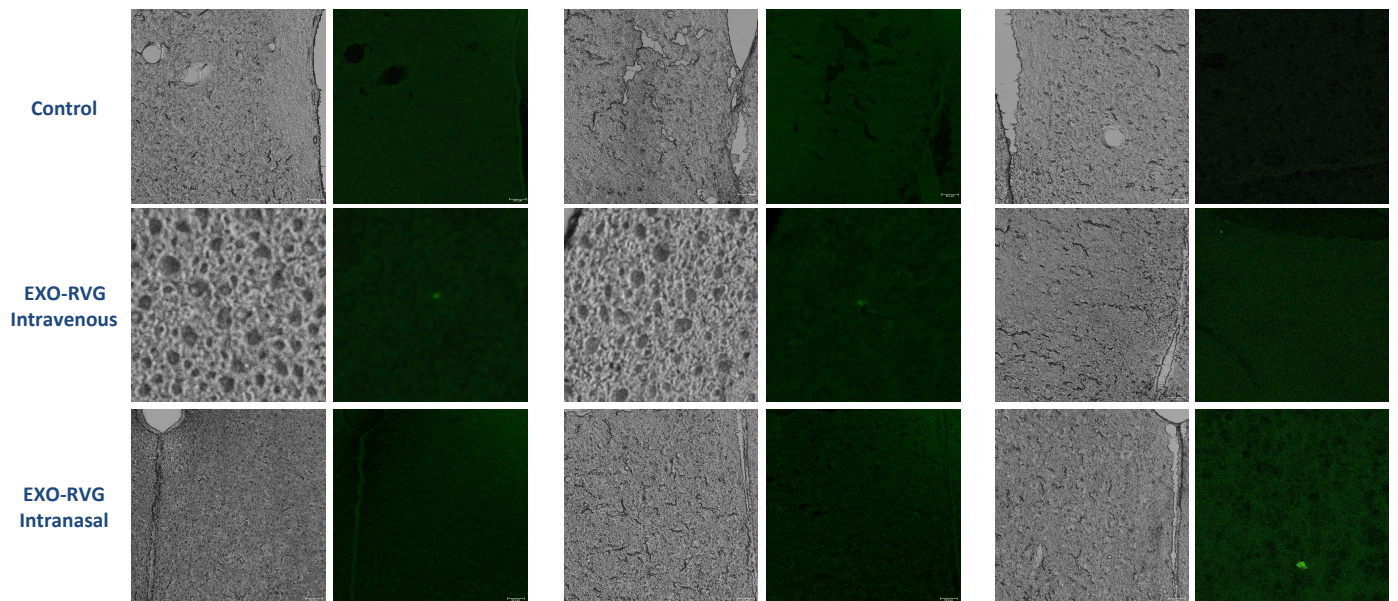


Figure 19: Hypothalamic delivery of GFP. Confocal microscopy of mice brain following GFP-loaded exosomes intravenously injected or intranasally administrated.

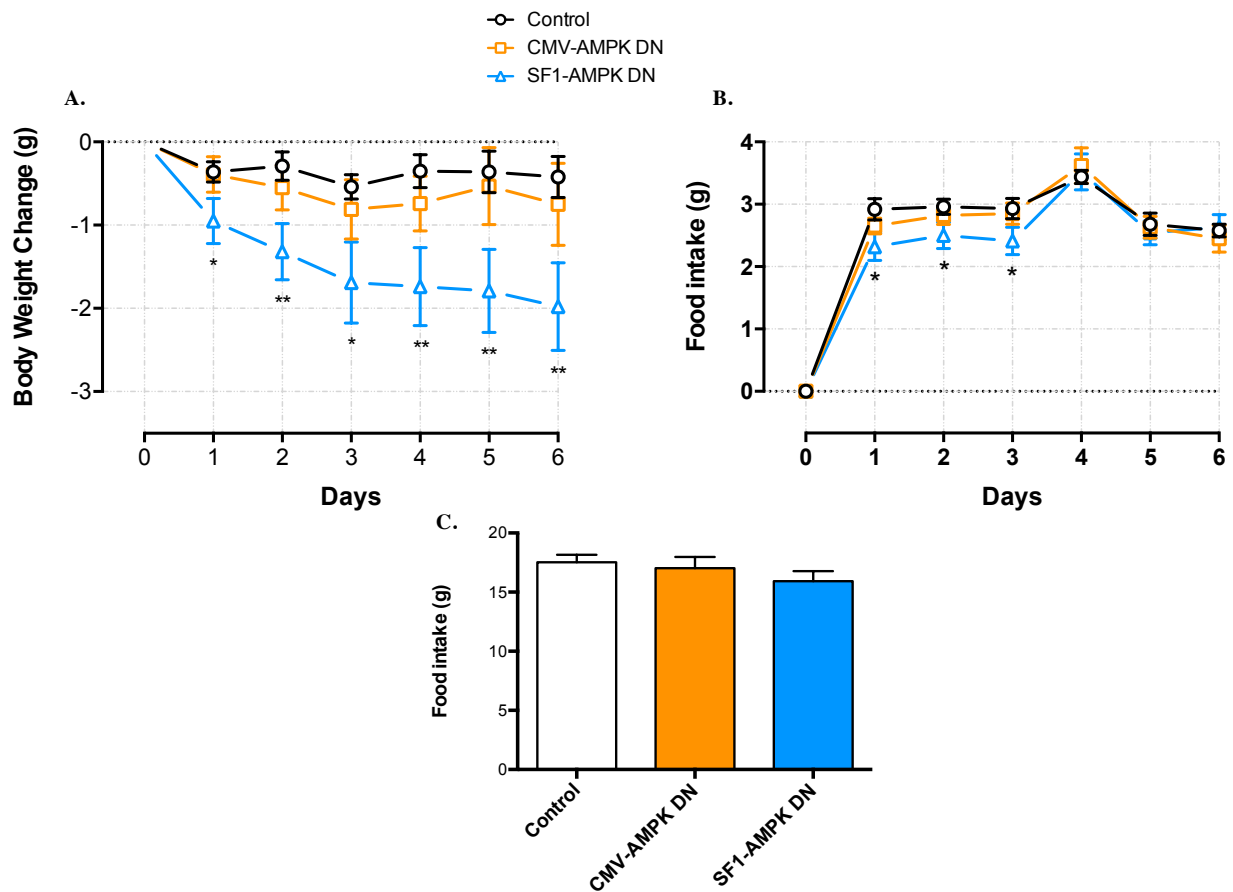


Figure 20: Body weight and food intake analysis. Body weight (A) and food intake (B) changes following non-loaded, CMV-AMPK-DN or SF1-AMPK-DN intravenous injection. Cumulative food intake (C). \* for  $p < 0,05$ , \*\* for  $p < 0,01$ .

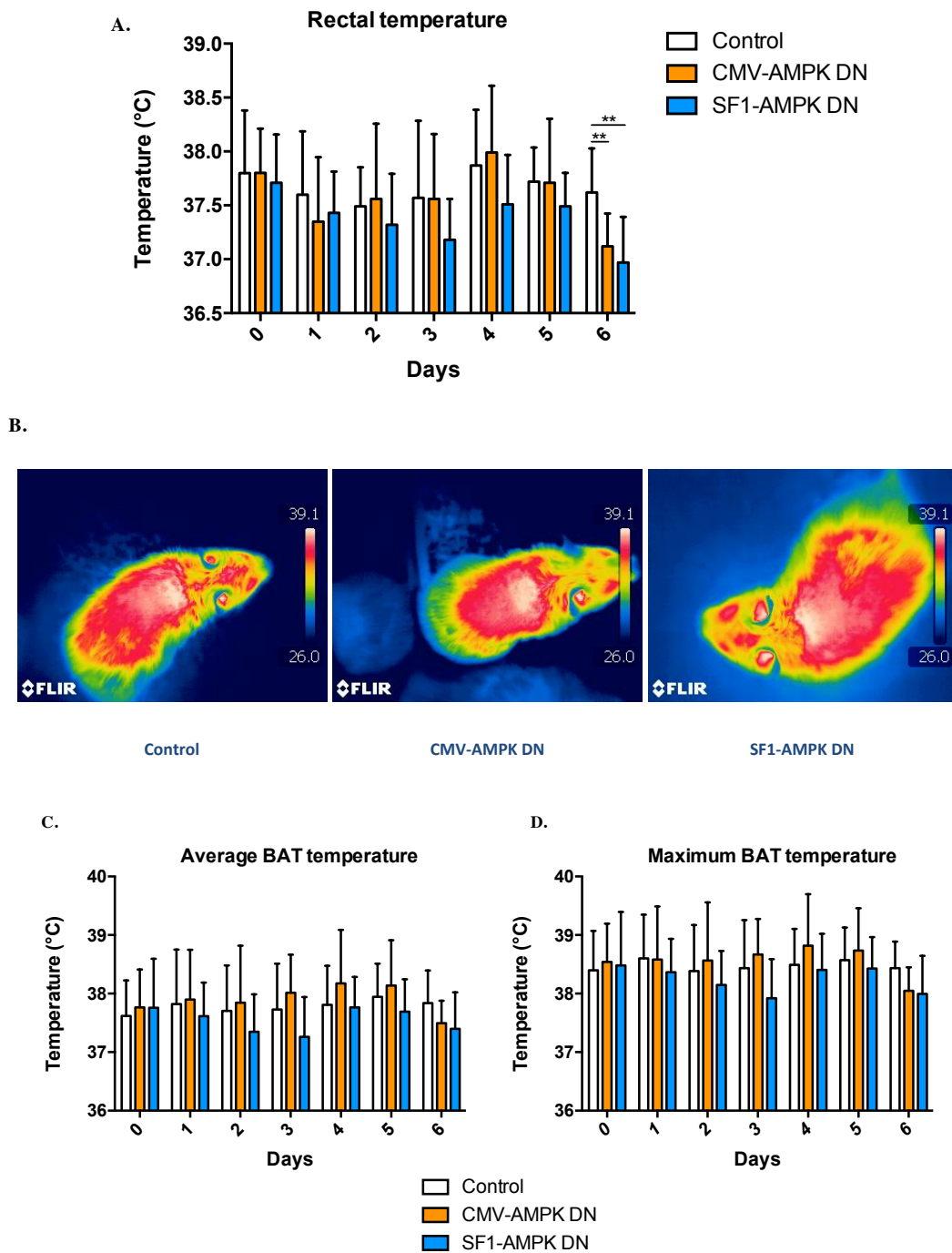
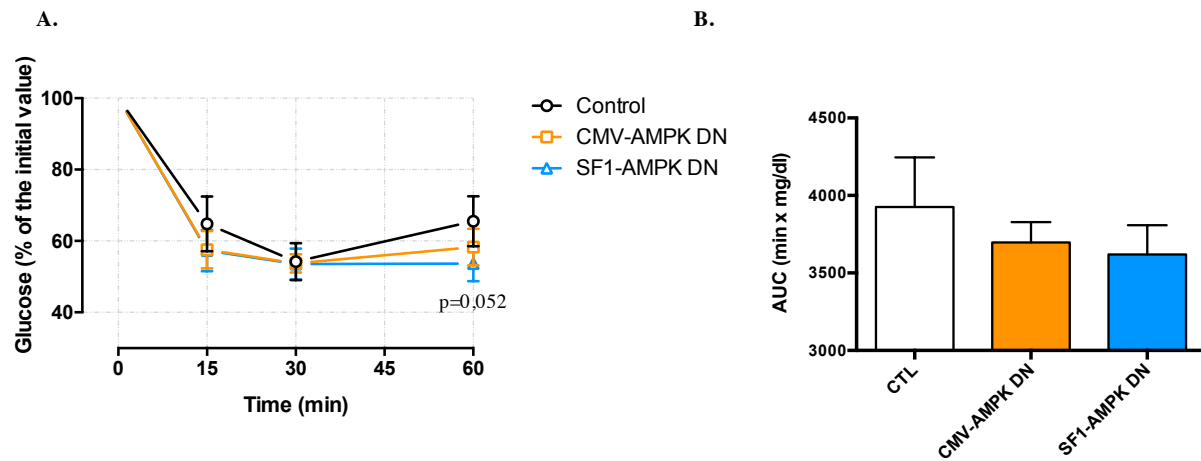


Figure 21: Core body temperature and BAT temperature changes. Core body (A) and BAT (C, D) temperature changes following non-loaded, CMV-AMPK-DN or SF1-AMPK-DN intravenous injection. Representative BAT thermal images of mice (B). \*\* for  $p < 0,01$ .



**Figure 22: Insulin Tolerance Test.** Glucose concentration evolution following an insulin injection (A). Area under the curve from ITT (B).

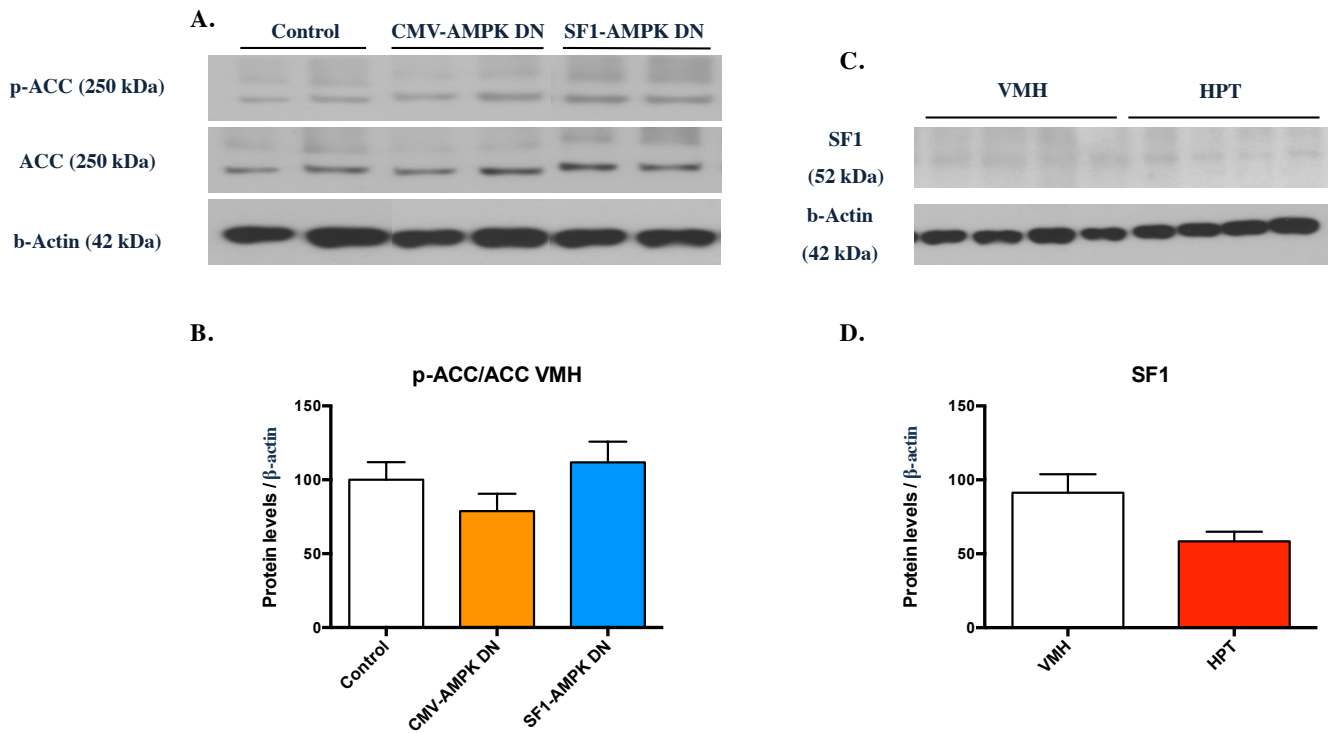


Figure 23: Western Blots of p-ACC and SF1 in the VMH. Evaluation of levels of ACC phosphorylation in the VMH following non-loaded, CMV-AMPK-DN or SF1-AMPK-DN intravenous injection (A and B). SF1 expression within the VMH and other hypothalamic regions (C and D).

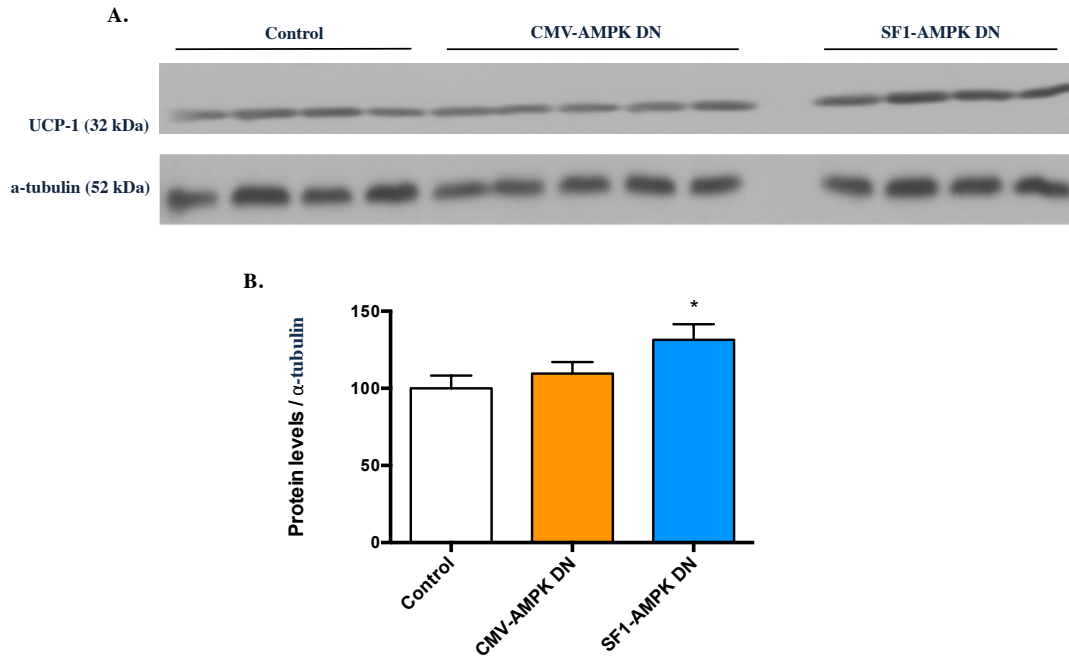


Figure 24: Western Blots of UCP-1 in BAT. Evaluation of levels of UCP-1 in the BAT following non-loaded, CMV-AMPK-DN or SF1-AMPK-DN intravenous injection (A and B). \* for  $p < 0,05$



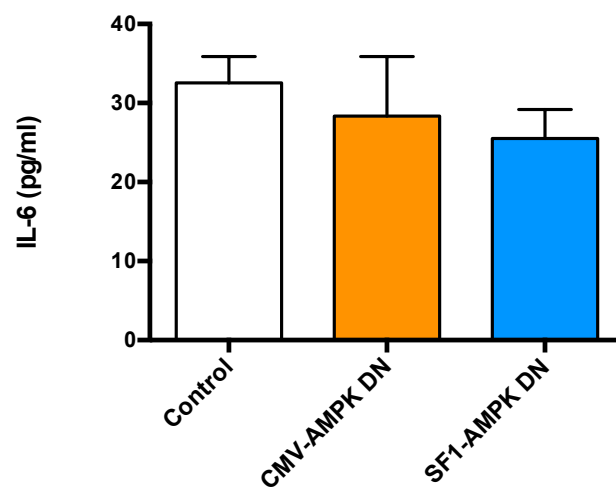


Figure 25: IL-6 serum levels following intravenous injection of exosomes. Evaluation of IL-6 serum following non-loaded, CMV-AMPK-DN or SF1-AMPK-DN intravenous injection.

## VI. Discussion



Although, anti-obesity drugs - FDA-approved or in development - are numerous, none of them exhibit the expected effectiveness mostly due to the lack of specificity of the treatments. As obesity regulation is complex and interconnected to other organism functions, increasing the specificity of the treatment appears as a crucial. Allowing the crossing of the BBB is a major challenge in delivering novel designed anti-obesity therapies to the brain in general and to the hypothalamus in particular. Herein, we demonstrated that neuronal-targeted exosomes could deliver a dominant negative isoform of AMPK specifically in the hypothalamic VMH inducing a body weight decrease of obese mice model.

The toxicity of viruses or synthetic nanoparticles (PEI-nanoparticles, liposomes or lipid nanoparticles) led us to use exosomes as tool delivery. Engineered to express the neuronal RVG peptide at their surface, exosomes exhibited an increased capacity to target the brain. The size and morphological appearance and composition of the neuronal Lamp2b-RVG targeted exosomes were consistent with previous studies (Schneider & Simons, 2013; Simons & Raposo, 2009).

However, loaded with a GFP encoding plasmid, intravenously injected exosomes did not induce any green fluorescence increase within neurons composing the hypothalamus. Moreover, intranasal administrations were performed. Although considered as a non-invasive method facilitating the targeting of the CNS and enhancing the drug absorption due to the rich vasculature and highly permeable structure of the nasal mucosa, intranasal administration did not increase the GFP signals within the VMH. Indeed, intranasal administration includes some disadvantages that could explain this lack of GFP signals. Firstly, the delivery efficiency is decreased with increasing molecular weight of the therapeutic agent. Moreover, the concentration achievable in the different regions of the brain varies following the nature of the delivered agent (Talegaonkar & Mishra, 2004). Thus, by non-controlling specifically the target of delivery, a nasal administration appears as inadequate.

Compared to the *in vitro* delivery efficiency observed, the *in vivo* results appeared as contradictory. However, as demonstrated, most of the exosomes are localized within the lungs, the spleen and the liver following a systemic injection, with a small amount within the brain, reducing hypothalamic GFP signals. Due to this small amount of GFP within the brain, confocal microscopy resolution could be too weak to

detect GFP fluorescence. Therefore, using other molecular detection methods such as Western Blot or FACS analysis using anti-GFP antibodies could increase the observed signals, corroborating the one observed in cellular assays (Zhang et al., 2015; Cui et al., 2014). However, when loaded with dominant negative isoform of AMPK under the control of the specific promoter SF1, exosomes induce functional effects on mice body weight. As these effects are mediated in the VMH (López et al., 2010), this functionally confirms that exosomes are probably able to cross the BBB, to reach the VMH and to deliver their content within the desired neurons.

Herein, it would be interesting to confirm the presence of the exosomes within the VMH to confirm the specificity of the observed effects. To distinguish the engineered and loaded-injected exosomes from the endogenously produced ones, analysis of specific exosomal markers could be realized. Currently, the identification of exosome-specific markers that could distinguish a subset of exosomes from another remains incomplete and controversial. However, owing to the fact that the engineered exosomes bear the neuronal peptide RVG at their surface, a RVG driven molecular analysis of the VMH treated mice either by western blot or immunohistochemistry could be realized (Bu et al., 2015).

AMPK has been demonstrated to participate in energetic balance regulation for decades. Firstly investigated in the 1980s as a central regulator of lipid metabolism (Hardie et al., 1989), AMPK is now considered to be the main energy sensor in eukaryotic cells (López et al., 2016). Recent evidences demonstrated that an inactivation of AMPK specifically in the VMH using adenoviral AMPK-DN induced weight loss independently of food intake (López et al., 2010; Martínez de Morentin et al., 2014). This body weight decrease is explained through the “Malonyl-CoA Hypothesis” in which an accumulation of malonyl-CoA within the hypothalamus is sensed as a nutrient abundance signal. Indeed, an inhibition of hypothalamic AMPK increases ACC activity, resulting in increased production of malonyl-CoA (Diéguez et al., 2009). As the phosphorylation of ACC by AMPK is inhibitory, we were expecting to observe a decrease of ACC phosphorylation levels. However, in our experiments, no significant differences were observed. As mentioned previously, the homogeneity of the VMH isolation must be investigated. In this matter, Moerch et al. (2007) developed an innovating method to increase the homogeneity of thymus samples. Using specific thymus markers recognized by antibodies, they were able to

differentiate the different populations of cells composing the thymus by FACS analysis. The idea of using this method on hypothalamus to collect only the VMH part has emerged. Immunoblotting the VMH cells with specific SF1 antibodies would allow to obtain 100 % homogeneity, possibly revealing ACC phosphorylation levels decreasing. Moreover, as already mentioned, the kinetics of the molecular analysis may appear non-adapted. As demonstrated, ACC phosphorylation appears within minutes after activation by either citrate (Park et al., 2002) or AMPK (Rubink & Winder, 2005). Thus, realizing the molecular analysis at the end of the 6 days of treatment may hide the decrease of ACC phosphorylation within the VMH samples. Therefore, performing immunohistochemistry analysis at earlier times could help to reveal the ACC phosphorylation decrease following the injection of AMPK-DN loaded exosomes.

The functional effect associating the inhibition of hypothalamic AMPK to a decrease of the body weight is mediated by an increase of BAT thermogenesis. Lots of evidences demonstrated that hypothalamic AMPK was acting as a central regulator of BAT thermogenesis. Interestingly, anatomic observations showed a connection between the VMH and other brain regions, including neurons in raphe pallidus (Rpa) and inferior olive (IO), implicated in the control of thermogenesis in BAT (Shibata, et al., 1999). Moreover, electric stimulations of the VMH enhanced BAT temperature in rat models (Holt et al., 1987). Furthermore, electric stimulation of VMH increased the rate of noradrenaline turnover within the BAT (Saito et al., 1987). Also, SF-1 knockout mice exhibiting a defective VMH displayed an impaired thermogenesis regulation within the BAT (Kim et al., 2011). Finally, treatments with antagonists of  $\beta$ 3-adrenergic receptors abrogate BAT thermogenesis increasing (López et al., 2010; Martínez de Morentin et al., 2014). All together, these data demonstrate that BAT thermogenic activity is increased by enhanced activity of the sympathetic nervous system, and is mediated by the release of noradrenaline on BAT  $\beta$ 3-adrenergic receptors, leading to an increased expression of UCP-1. Our data recapitulate the increased UCP 1 protein levels in the BAT after treatment with SF1-AMPK-DN loaded exosomes but we fail to detect any effect in the BAT temperature of the animals. This may look contradictory but the sampling of the BAT temperatures and also the timing (one point per day) were probably confounding factors. Also, stressful procedures in view of cage mates may cause stress in observer animals. Therefore,

using a telemetry approach - that would allow a constant recording of temperature - could help obtaining more accurate results without inducing any stress to the animals, as the measure is automatically recorded. Moreover, it would be interesting to record mice activity at the same time, as it is a crucial component of energy expenditure (Clement et al., 1989). In this sense, analysis of the energy expenditure by indirect calorimetry will also provide interesting and complementary information.

One limiting factor within the development of novel therapeutic strategies using innovative vectors is the immune character of its delivery. Repeated virus or synthetic nanoparticles injections were demonstrated to increase the activation of the complement and of coagulation factors (Waehler et al., 2007; De Jong et al., 2008). Moreover, delivery of nucleic acids by synthetic nanoparticles is mediated by the “sponge effect” characterized by endosomal rupture and leakage of Cathepsin B inducing inflammation and apoptosis (Hornung et al., 2008). Herein, following 2 injections of exosomes, pro-inflammatory IL-6 levels were not increased between the different groups highlighting the non-inflammatory character of such exosomal vectors. The same analysis was observed with injections of siRNA-loaded exosomes within mice model (Alvarez-Erviti et al., 2011). This escape from the host immune system is due to multiple factors. The exosome small size allows them to avoid phagocytosis by macrophages. Moreover, the exosome high stability within the blood mostly due to CD 55 and CD 59 confers them the ability to avoid opsonins, coagulation factors and complement (Clayton et al., 2003). And lastly, the presence of tetraspanin CD 9 at the surface on the exosome facilitates direct membrane fusion and release within the cytosol bypassing the endosomal-lysosomal pathway, decreasing inflammation risks (Théry et al., 1999). All this together highlights the high potential of exosomes as delivery tools.

Even if some results have to be analysed carefully, and that some more experiments need to be realized to confirm the preliminary ones, we were able to demonstrate that neuronal targeted exosomes were able to deliver in a specific way a dominant negative isoform of AMPK within the VMH of mice inducing their body weight decrease associated with increased UCP1 expression in BAT.

The growing interest of worldwide research group for obesity results in an identification of upstream specific actors in peripheral tissues regulating metabolism as well as within the master centre of its regulation, the hypothalamus. However, modulation of activity of these actors into specific – peripheral or central – tissues avoiding undesired effects remains difficult due to their ubiquitously localisation. Taken together, these results underline the capacity of exosomes to be used as natural bio-carriers. Due to their intrinsic biological properties, exosomes appear as a suitable alternative at traditional delivery systems actually in development limiting inflammation processes and enhancing specific brain targeting increasing the chance of developing hypothalamic-targeted drugs to counter obesity. Therefore, the idea of exploiting exosomes in an anti-obesity designed manner is opening a new avenue in pharmacological treatment of obesity.



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## Annex

This second project has been started during my master degree and finished during my PhD studies without any relation with the above-described project.

**Tumour necrosis factor- $\alpha$  carried by microparticles from apoptotic RAW 264.7 macrophage cells triggers deleterious effects on cardiomyocytes from adult mice.**

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*Introduction:* After ischaemic injury and in patients with atherosclerosis, the pool of inflammatory macrophages is enlarged in the heart and in atherosclerotic plaques. Monocyte/macrophage-derived microparticles (MPs) are part of the pathological process of unstable atherosclerotic plaques. The hypothesis that these MPs could induce deleterious effects in adult myocardial cells was evaluated.

*Methods:* MPs were isolated through differential centrifugation from the supernatant of RAW 264.7 macrophages. MPs were characterized through flow cytometry and Western Blot. Their effects on cardiac cell shortening were evaluated in presence or absence of specific inhibitors. Interaction between MPs and cardiomyocytes was detected through confocal microscopy. MP pro-apoptotic effects were assessed through Western Blot.

*Results:* We showed that these MPs contained the soluble form of tumour necrosis factor alpha (TNF- $\alpha$ ). Furthermore, cardiomyocyte sarcomere shortening amplitudes and kinetics were reduced within 5 min of exposure to MPs without any modifications in Ca<sup>2+</sup> transients. The contractile effects of MPs were completely prevented after

pre-treatment with nitric oxide synthase, guanylate cyclase or TNF- $\alpha$  inhibitors as well as blocking TNF- $\alpha$  receptor 1 with neutralizing antibody. Moreover, confocal microscopy showed that, after 1 h, MPs were clearly surrounding rod-shaped cardiomyocytes, and after 2 h they were internalized into cardiomyocytes undergoing apoptosis. Indeed, after 4 h of treatment with MPs, cardiomyocytes expressed increased apoptotic markers expression (caspase-3, caspase-8, Bax and cytochrome C) as demonstrated by Western Blot.

*Summary/Conclusion:* The present study showed that these MPs carry functional TNF- $\alpha$  and activate TNFR1 in isolated adult murine ventricular cardiomyocytes, inducing a negative inotropic effect via a mechanism sensitive to NO synthase inhibitor and that the mitochondrial intrinsic pathway was implicated in their proapoptotic effects.

ORIGINAL RESEARCH ARTICLE

## Microparticles from apoptotic RAW 264.7 macrophage cells carry tumour necrosis factor- $\alpha$ functionally active on cardiomyocytes from adult mice

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After ischaemic injury and in patients with atherosclerosis, the pool of inflammatory macrophages is enlarged in the heart and in atherosclerotic plaques. Monocyte/macrophage-derived microparticles (MPs) are part of the pathological process of unstable atherosclerotic plaques. The present study focused on effects of MPs, produced by apoptotic murine RAW 264.7 macrophage cell line, in adult murine cardiomyocytes. Flow cytometry and western blot analysis showed that these MPs contained the soluble form of tumour necrosis factor alpha (TNF- $\alpha$ ). Cardiomyocyte sarcomere shortening amplitudes and kinetics were reduced within 5 min of exposure to these MPs. Conversely, Ca<sup>2+</sup> transient amplitude and kinetics were not modified. The contractile effects of MPs were completely prevented after pretreatment with nitric oxide synthase, guanylate cyclase or TNF- $\alpha$  inhibitors as well as blocking TNF- $\alpha$  receptor 1 with neutralizing antibody. Microscopy showed that, after 1 h, MPs were clearly surrounding rod-shaped cardiomyocytes, and after 2 h they were internalized into cardiomyocytes undergoing apoptosis. After 4 h of treatment with MPs, cardiomyocytes expressed increased caspase-3, caspase-8, Bax and cytochrome C. Thus, MPs from apoptotic macrophages induced a negative inotropic effect and slowing of both contraction and relaxation, similar to that observed in the presence of TNF- $\alpha$ . The use of specific inhibitors strongly suggests that TNF- $\alpha$  receptors and the guanylate cyclase/cGMP/PKG pathway were involved in the functional responses to these MPs and that the mitochondrial intrinsic pathway was implicated in their proapoptotic effects. These data suggest that MPs issued from activated macrophages carrying TNF- $\alpha$  could contribute to propagation of inflammatory signals leading to myocardial infarction.

Keywords: *microvesicle; atherosclerosis; inflammatory cytokine; isolated myocardial cells; adult mice*

Responsible Editor: Elena Aikawa, Harvard Medical School, USA.

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Received: 22 May 2015; Revised: 26 August 2015; Accepted: 30 September 2015; Published: 23 October 2015

The healthy myocardium hosts a large number of macrophages that are among the largest cardiac resident cell populations, with fibroblasts, myocytes and endothelial cells (for recent review, see Ref. 1). Heart macrophages deserve attention because they perform numerous tasks in wound healing, regeneration and tissue remodelling. They are recognized as central actors in cancer, infection, rheumatoid arthritis, diabetes, obesity, atherosclerosis and stroke. Furthermore, after ischaemic injury and in patients with atherosclerosis, an increased production and supply of inflammatory monocytes likely

enlarge the pool of inflammatory macrophages in the heart and in atherosclerotic plaques. These inflammatory macrophages may directly harm cardiomyocytes due to their ability to produce the tumour necrosis factor alpha (TNF- $\alpha$ ), a pro-inflammatory cytokine that induces myocyte apoptosis (2) and a negative inotropic effect (3,4), cardiac  $\beta$ -adrenergic dysfunction (5), sphingosine production (6), phospholipase A2 activation/increased arachidonic acid (7) and inducible nitric oxide (NO)-synthase (NOS) activity (3). Elevated expression levels of TNF- $\alpha$  in cardiomyocytes and increased plasma concentrations of TNF- $\alpha$  have been

reported in patients with end-stage heart failure (8). Experimental animal studies also showed that cardiac-specific overexpression of TNF- $\alpha$  results in the development of a dilated cardiomyopathy with ventricular hypertrophy, ventricular dilation and other cardiomyopathy-like phenotype (9). Furthermore, high plasma levels of TNF- $\alpha$  are found in cardiac diseases in which left ventricular dysfunction develops, such as septic shock, reperfusion injury, myocardial infarction, myocarditis, cardiac allograft rejection and congestive heart failure (10). TNF- $\alpha$  induces its biological effects by binding to 2 distinct sarcolemmal receptors, TNF receptors 1 and 2 (TNFR 1 and 2) (11). Upon binding to TNF- $\alpha$ , the TNFRs recruit a number of molecules to form the TNFR complex, which in turn initiates the activation of downstream signalling cascade (12). Both TNFR1 and TNFR2 are expressed in murine heart, but deleterious effects of TNF- $\alpha$  seem mainly initiated by activation of TNFR1 (reviewed in Ref. 13). Thus, TNF- $\alpha$ /TNFR1 signalling induces mitochondrial dysfunction, reactive oxygen species (ROS) production, contractile abnormalities and cell death (4,11).

Microparticles (MPs) are extracellular vesicles of 0.1–1  $\mu\text{m}$  in diameter, with pro-coagulant and pro-inflammatory properties (14). Briefly, according to source cell type and triggering stimuli specificity (i.e. chemical/physical activation resulting in sustained high  $[\text{Ca}^{2+}]_i$  or apoptosis), MP formation occurs due to cytoskeleton disruption and subsequent plasma membrane blebbing. MPs contain nucleic acids, proteins and antigens from their cells of origin. Circulating MPs are present in the blood of healthy individuals, but MP formation increases with some diseased states, such as metabolic syndrome, sepsis, pre-eclampsia, rheumatoid arthritis, diabetes and in various clinical situations associated with thrombosis. Thus, several types of MPs including leukocyte-derived MPs are present in atherosclerotic plaques (15,16). Leukocyte-derived MPs are part of the pathological process of unstable atherosclerotic plaques and are thought to contribute to plaque rupture and subsequent myocardial infarction or stroke (17). As shown by Leroyer et al. (18), the majority of plaque MPs originates actually from leukocytes (more than 50%) of which almost 30% were from macrophages. Subsequently, it was shown that a large proportion (>90%) of these MPs are CD14+ indicating their monocyte/macrophage origin (15).

In the present study, we have focused on MP released by apoptotic macrophages, testing the hypothesis that they could carry TNF- $\alpha$  and thus induce deleterious effects in adult myocardial cells. The murine RAW 264.7 cell line was used as a model for macrophages (19) and we investigated the effects of MPs released *via* apoptosis induced by actinomycin D upon contractility in cardiomyocytes isolated from hearts of adult C57BL/6 mice.

## Material and methods

### Ethical approval

All experimental procedures were conducted in compliance with local guidelines for the care and use of laboratory animals (authorizations n°D49007002 on 17/07/2012). Furthermore, the study complied with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication, 8th edition, Washington (DC): National Academies Press (US); 2011. 2, Animal Care and Use Program).

### Preparation of MPs

The murine RAW 264.7 macrophage-like cell line was obtained from BALB/c mice (American Type Culture Collection, Manassas, VA). Cells were maintained at 37°C in 5% CO<sub>2</sub>/95% air in DMEM (Lonza, Basel, Switzerland) supplemented with 10% decomplexed foetal calf serum (FCS, Invitrogen, Cergy Pontoise, France), 1% penicillin/streptomycin (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and 1% L-glutamine (Lonza). Cells were seeded in culture plates ( $2 \times 10^6/\text{mL}$ ) and then treated with actinomycin D (1  $\mu\text{g}/\text{mL}$ ) for 24 h. Following treatment, supernatants were harvested and centrifuged twice at 1,500 g for 15 min and 5 min in order to eliminate cells and large debris, respectively. Remaining supernatant was subjected to 3 series of centrifugation (13,000 g for 1 h, then twice at 13,000 g for 45 min), to pellet MPs and the supernatant was replaced by 200  $\mu\text{L}$  of 0.9% saline salt solution and stored at 4°C until subsequent use. Preliminary studies analyzing the concentration-dependent MP effects showed that 1  $\mu\text{g}/\text{mL}$  corresponds to the maximally effective concentration in inducing functional effects and thus we used this concentration to analyze the impact of MPs upon contractility. With regard to apoptosis, we used 10  $\mu\text{g}/\text{mL}$ , a concentration in accordance with that used to study apoptosis triggered by MPs from lymphocytes in bronchial epithelial cells (20). Determination of the corresponding amount of MPs was carried out by measuring MP-associated proteins determined against bovine serum albumin standards (Lowry method).

### Characterization of MPs

To determine MP concentration and the localization of TNF- $\alpha$  either on plasma membrane or inside, the MP labelling with TNF- $\alpha$  was carried out. Two microlitres of MPs were incubated 45 min with either 0.5  $\mu\text{L}$  of anti-TNF- $\alpha$  alexa fluor® 488 (0.5 mg/mL) or anti-rat IgG1 kappa alexa fluor® 488 (0.5 mg/mL) (eBioscience, San Diego, CA) as an isotype-matched negative control. The presence of intraMP TNF- $\alpha$  was assessed after MP fixation (2% paraformaldehyde, 15 min, Electron Microscopy Sciences, Hatfield, PA) and permeabilization (0.1% saponin, 5 min). After incubation with antibodies, samples were diluted in 200  $\mu\text{L}$  of 0.9% saline salt solution.

Then, Flow-count fluorospheres (2  $\mu$ L, equal to volume of MPs) (Beckman Coulter, Villepinte, France) were added in order to calculate MP count. The samples were analyzed in a flow cytometer 500 MPL System (Beckman Coulter) using the MXP software (Beckman Coulter). Sample analysis was stopped after the count of 10,000 events.

#### *Isolated cardiomyocyte preparation*

Ventricular cardiomyocytes were isolated from 8- to 10-week-old male adult C57BL/6 mice. Mice were pretreated with heparin (i.p. 100 U, 5 min, Heparine Choay, Sanofi Aventis, Paris). Then, mice were euthanized by CO<sub>2</sub>. The heart was rapidly excised, rinsed in ice-cold Hanks-HEPES buffer [in mM: NaCl 113, KCl 4.7, NaHCO<sub>3</sub> 12, KHCO<sub>3</sub> 10, Na<sub>2</sub>HPO<sub>4</sub> 0.6, KH<sub>2</sub>PO<sub>4</sub> 0.6, MgCl<sub>2</sub> 1.2, HEPES 10, glucose 5.5, taurine 29, supplemented with 2,3-butanedione monoxime (BDM) 10, pH 7.4], and then mounted on a Langendorff perfusion system and perfused (3 mL/min at 37°C) first with Hanks-HEPES buffer for 5 min to be cleared from blood and then with buffer supplemented with 10 mg/mL liberase TH (Roche Diagnostics GmbH, Mannheim, Germany) for 6–9 min. The heart was placed into Hanks-HEPES buffer supplemented with BDM (10 mM) and FCS (10%). The atria were removed and the ventricles were gently dissociated with scissors and pipettes in the same medium. The suspension was filtered through a nylon mesh (200  $\mu$ m) and incubated for decantation of the cells for 10 min. CaCl<sub>2</sub> was progressively added in the Hanks-HEPES buffer in 4 steps of 5 min (0.125, 0.25, 0.50, 1 mM).

#### *Ca<sup>2+</sup> transient and cell shortening*

Isolated cardiomyocytes were placed on laminin (Sigma-Aldrich)-precoated glass coverslips (Corning, NY) and loaded with Fura-2 AM (1  $\mu$ M, Molecular Probes, Eugene, OR) at room temperature for 20 min and then washed with HEPES-buffered solution (in mM: NaCl 116, KCl 4.7, NaHCO<sub>3</sub> 4.4, Na<sub>2</sub>HPO<sub>4</sub> 0.6, KH<sub>2</sub>PO<sub>4</sub> 1.5, MgCl<sub>2</sub> 1.2, HEPES 20, glucose 10, taurine 20, pH 7.3) containing 1.25 mM CaCl<sub>2</sub>. Sarcomere shortenings and Ca<sup>2+</sup> transients were studied using field stimulation (0.5 Hz, 0.1 ms, 7–15 V, 22°C) in the absence or in the presence of MPs from 5 min to 1 h. Sarcomere length and fluorescence (excitation at 340 and 380 nm, measured at 510 nm) were simultaneously recorded (IonOptix Myocyte Calcium and Contractility Recording System, Dublin, Ireland).

#### *Inhibitors and chemicals*

In some experiments, cardiomyocytes were preincubated for 30 min with different pharmacological inhibitors: L-NAME (100  $\mu$ M, NOS inhibitor), 1*H*-[1.2.4]oxadiazolo [4.3-*a*]quinoxalin-1-one [ODQ, 25  $\mu$ M, soluble guanylate cyclase (sGC) inhibitor], 6,7-dimethyl-3-[(methyl{2-[methyl ({1-[3-(trifluoromethyl)phenyl]-1*H*-indol-3-yl} methyl) amino]ethyl} amino)methyl]-4*H*-chromen-4-one, -diHCl (SPD 304, 10  $\mu$ M, TNF-inhibitor, Calbiochem, Darmstadt,

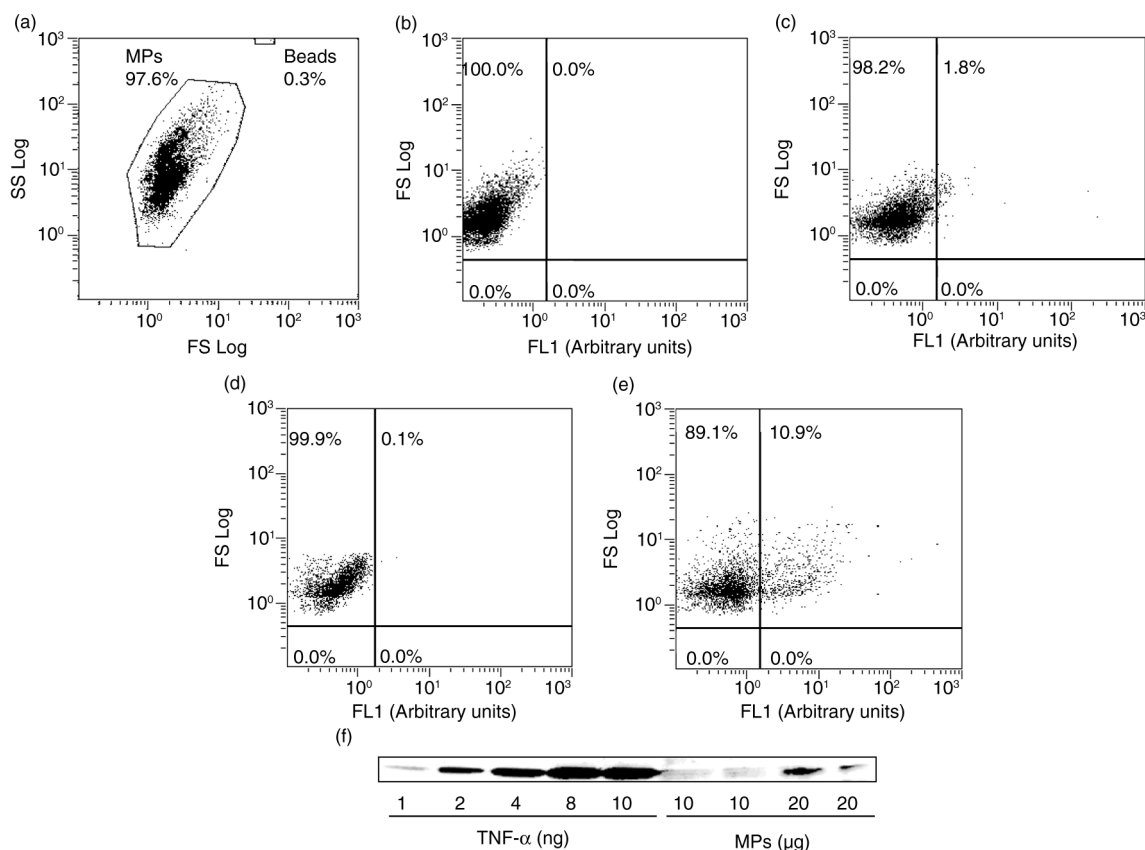
Germany). A specific antibody to TNFR1 (purified anti-mouse TNFR type 1/p55, 20  $\mu$ g/mL, BioLegend, San Diego, CA), a leaf purified Armenian hamster IgG (20  $\mu$ g/mL, BioLegend) or TNF- $\alpha$  (50 ng/mL, Miltenyi Biotec, Bisley, UK) were also used. All drugs were prepared as a stock solution in appropriate solvents. All the drugs were used at already reported maximally active concentrations. On the day of the experiment, stock solutions were diluted to the desired concentration and the final concentration of DMSO was <0.1%. For the treatment with MPs (4 h) of cardiomyocytes further used in western blot, M199 culture medium was supplemented with creatine (5 mM), carnitine (5 mM), taurine (5 mM), HEPES (25 mM) and penicillin/streptomycin (1%). Unless stated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

#### *Western blot*

After treatment with MPs for 4 h, cardiomyocytes were homogenized and lysed. Proteins (20  $\mu$ g) were separated on 4–12% SDS-polyacrylamide gel (Invitrogen) electrophoresis (150 V, 90 min). Blots were probed with polyclonal rabbit anti-cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), mouse anti-caspase-8 (Cell Signaling), anti-Bax (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-cytochrome C (Santa Cruz). In another set of experiments, total proteins from MPs (10 and 20  $\mu$ g) were also probed with a polyclonal goat anti-TNF- $\alpha$  (Santa Cruz). Mouse TNF- $\alpha$  recombinant protein was also used as a positive control (1, 2, 4, 8 and 10 ng) (Miltenyi Biotec). A polyclonal goat anti- $\alpha$ -tubulin (Santa Cruz) was used as a loading control. Membranes were washed 3 times in Tris-buffer solution (20 mM Tris, 61.5 mM NaCl) containing 0.1% Tween and incubated for 90 min at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz). Protein bands were detected by enhanced chemiluminescence plus (Amersham Biosciences, Piscataway, NJ). Immunoblots were quantified by densitometric analysis (Bio1D, Scientific Software Group, Provo, UT).

#### *Interaction between MPs and cardiomyocytes*

MPs were loaded with 2  $\mu$ M PKH67 dye stock (Sigma-Aldrich) in NaCl (0.9%) solution, incubated for 2 min at room temperature and then added to an equal volume of FCS to stop the labelling. MPs were centrifugated (13,000 g, 45 min) and the pellet resuspended in NaCl (0.9%) solution. Fluorescence was checked by flow cytometry (Beckman Coulter). Isolated cardiomyocytes were then stimulated with MPs (10  $\mu$ g/mL) labelled with PKH67 during 1, 2 and 4 h at room temperature. At the end of stimulations, cardiomyocytes were fixed with paraformaldehyde (2%, 20 min), washed and diluted with phalloidin-tetramethylrhodamine isothiocyanate (50  $\mu$ g/mL, Sigma-Aldrich) during 90 min at room temperature, in order to label F-actin filaments. After washing with



**Fig. 1.** Tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) expression in macrophage MPs. (a) Representative images of cytometer analysis showing MPs and counting beads represented on a forward light scatter (FS)/side light scatter (SS) dot-plot; dot plots of MP gated region are representations of FS vs. alexa fluor<sup>®</sup> 488 fluorescence signals from (b) unlabelled MPs, (c) intact MPs incubated in the presence of anti-TNF- $\alpha$  alexa fluor<sup>®</sup> 488, permeabilized MPs incubated (d) with isotype IgG1 alexa fluor<sup>®</sup> 488 or (e) with anti-TNF- $\alpha$  alexa fluor<sup>®</sup> 488. (f) Western blot showing TNF- $\alpha$  expression in MPs (10 and 20  $\mu$ g) compared to a mouse recombinant TNF- $\alpha$  (1, 2, 4, 8 and 10 ng).

phosphate buffer solution, cells were observed by confocal microscopy (CLMS 700, Zeiss, ZEN fluorescence, Jena, Germany).

### Statistics

Results were expressed as mean  $\pm$  SEM and n represents the number of cells or mice for functional and western blot experiments, respectively. Statistical analysis was performed using ANOVA and subsequent Bonferroni post-hoc test for functional experiments, or ANOVA and Mann–Whitney U-test for western blot data (Prism<sup>®</sup> V5.0 software).

## Results

### Expression of TNF- $\alpha$ in MPs from apoptotic macrophages

Flow cytometry analysis of MPs (Fig. 1a) showed that 1  $\mu$ g of MP proteins, the concentration used for the present functional study, corresponds to  $52.14 \pm 5.4 \times 10^3$  MPs.

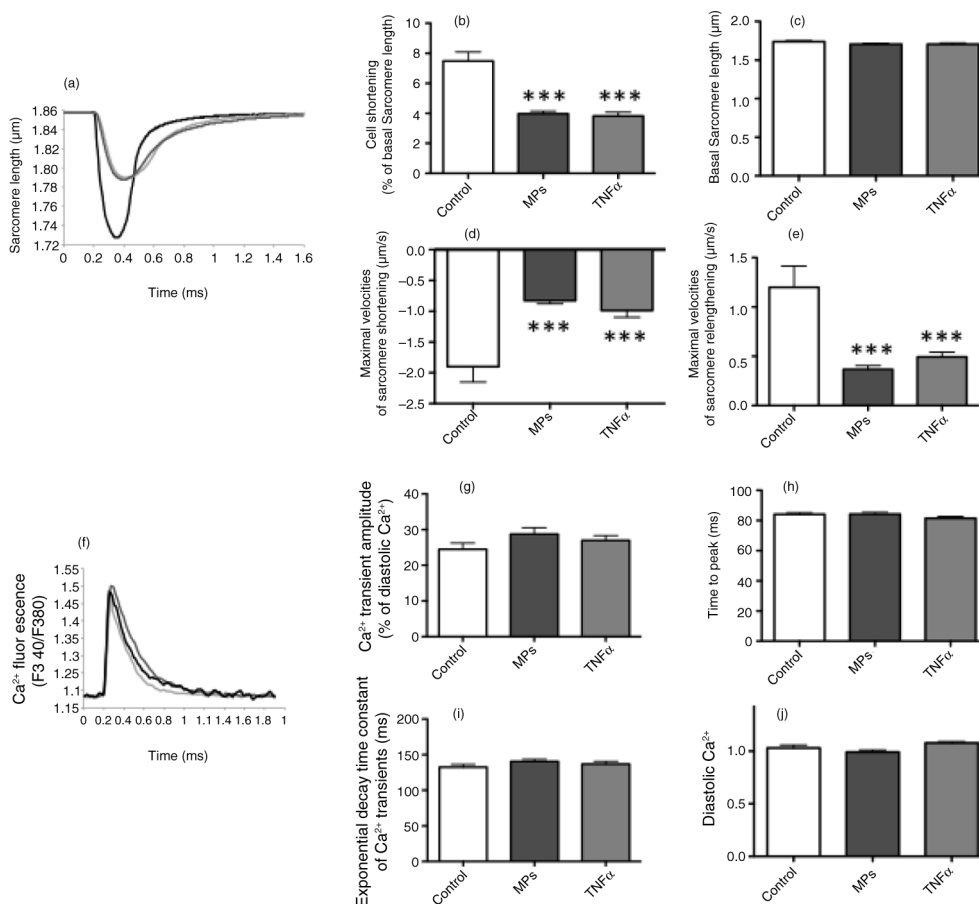
As shown in Fig. 1b, no fluorescence was observed in MPs without TNF- $\alpha$  labelling. Upon labelling, only 1.8% of MPs present TNF- $\alpha$  on surface (Fig. 1c). Interestingly, we found that TNF- $\alpha$  is inside 11% of MPs as evidenced after permeabilization labelling (Fig. 1d and e).

This expression was also confirmed by western blot analysis (Fig. 1f). Using the recombinant protein TNF- $\alpha$  at different growing concentrations as a positive control, these data indicated that MPs from apoptotic macrophages contain the soluble form of TNF- $\alpha$ . However, under our experimental conditions, we cannot exclude the presence of MPs bearing TNF- $\alpha$  at their membrane. Furthermore, the concentration of MPs used has been found in human atherosclerotic plaques (see Ref. 21).

### Ca<sup>2+</sup> transient and cell shortening

Isolated cardiomyocytes were loaded with Fura-2 (1  $\mu$ M for 20 min) and paced at 0.5 Hz. Cell shortenings and changes in cytosolic Ca<sup>2+</sup> were simultaneously recorded. The sarcomere shortening triggered by the electrical





**Fig. 2.** Effects of macrophage MPs (1  $\mu\text{g}/\text{mL}$ ) and TNF- $\alpha$  (50 ng/mL) upon sarcomeric shortenings and Ca<sup>2+</sup> transients of adult murine cardiomyocytes. (a) Representative traces of sarcomere shortenings of control and MP-treated cardiomyocytes (digital detection and fast Fourier transformation). (b) Sarcomere shortening (% of resting sarcomere length), (c) resting sarcomere length, maximal velocities of (d) sarcomere shortening and (e) relengthening, (f) representative traces of Ca<sup>2+</sup> transients, (g) Ca<sup>2+</sup> transient amplitude (F340/380), (h) time to peak and (i) exponential decay time constant of Ca<sup>2+</sup> transients and (j) diastolic Ca<sup>2+</sup> levels in control, MP- and TNF- $\alpha$ -treated cardiomyocytes. Data are expressed as mean  $\pm$  SEM ( $n=28-50$ ). \*\*\* $P < 0.001$  vs. Control (statistical analysis performed using ANOVA and subsequent Bonferroni post-hoc test).

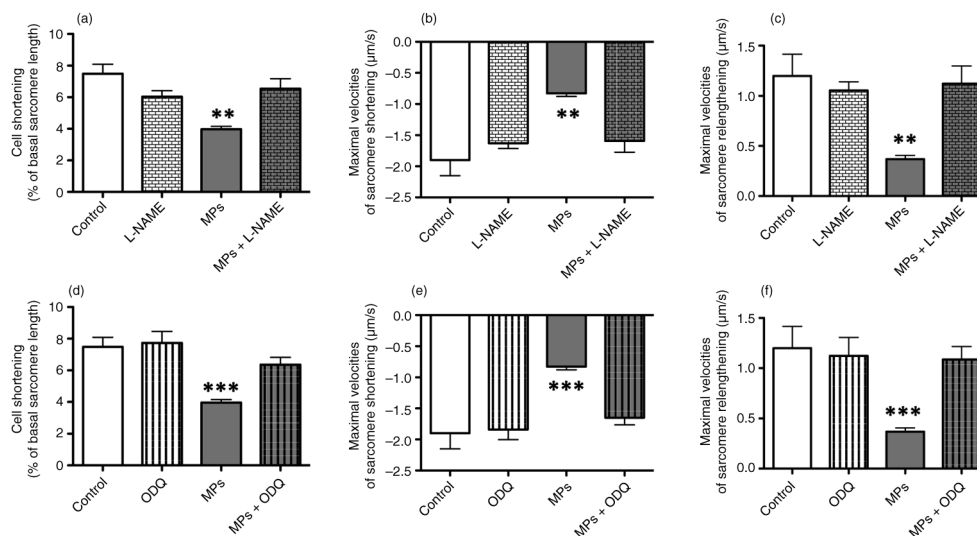
stimulation was reduced by MPs (1  $\mu\text{g}/\text{mL}$ ) and TNF- $\alpha$  (50 ng/mL) (Fig. 2a and b), without affecting the resting sarcomere length (Fig. 2c). The effect was apparent, maximal within 5 min of exposure to the MPs and TNF- $\alpha$ . The maximal velocities of sarcomere shortening and relengthening were also significantly reduced in the presence of MPs and TNF- $\alpha$  (Fig. 2d and e). Conversely, Ca<sup>2+</sup> transient amplitude (Fig. 2f and g) and kinetics (Fig. 2h and i) were not significantly modified by MPs and TNF- $\alpha$ , and the diastolic Ca<sup>2+</sup> level remained unchanged (Fig. 2j).

NO pathway is well documented as a negative modulator of cardiac inotropy (22,23). Thus, experiments were carried out to evaluate the impact of inhibitors of this pathway. The reduction of cell shortening amplitude and slowing of contraction kinetics observed when cells were perfused with 1  $\mu\text{g}/\text{mL}$  MPs was completely prevented after pretreatment (30 min) of the NOS inhibitor,

L-NAME (Fig. 3a, b and c) or of the sGC inhibitor, ODQ (Fig. 3d, e and f).

Because of the presence of TNF- $\alpha$  revealed by western blotting of MPs (see above), we also tested the effect of (a) SPD304, known to prevent TNF- $\alpha$  binding to its receptor (24) and of (b) a specific antibody to TNFR1. In some control experiments, we also used a leaf purified Armenian hamster IgG (20  $\mu\text{g}/\text{mL}$ ) which has no effect on cardiomyocyte contractility (data not shown). Interestingly, the effects of MPs (1  $\mu\text{g}/\text{mL}$ ) upon cell shortening were completely prevented after pretreatment with SPD304 (10  $\mu\text{M}$ , 30 min) (Fig. 4a, b and c) or the antibody to TNFR1 (20  $\mu\text{g}/\text{mL}$ , 1 h) (Fig. 4d, e and f).

The 3 inhibitors (L-NAME, ODQ, SPD 304) and TNFR1 antibody alone have no significant effects upon Ca<sup>2+</sup> transients (data not shown) and cell shortening (Figs. 3 and 4) concomitantly measured.



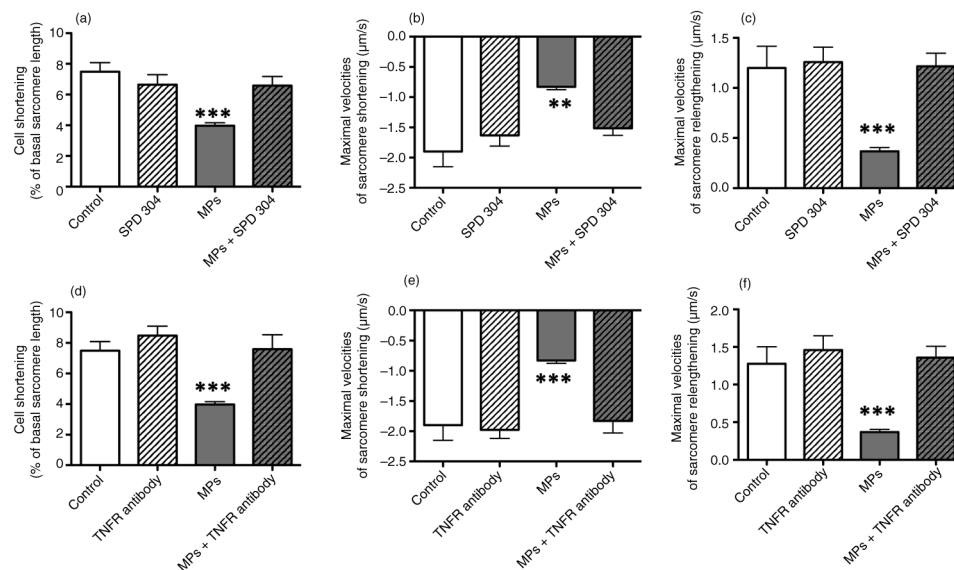
**Fig. 3.** Effects of macrophage MPs (1 µg/mL) upon sarcomeric shortenings of adult murine cardiomyocytes in the absence or in the presence of different inhibitors. (a) Sarcomere shortening (% of resting sarcomere length) and maximal velocities of (b) sarcomere shortening and (c) relengthening, in control, L-NAME (100 µM) alone-treated and MP-treated cardiomyocytes in the absence or in the presence of L-NAME. (d) Sarcomere shortening (% of resting sarcomere length) and maximal velocities of (e) sarcomere shortening and (f) relengthening, in control, ODQ (25 µM) alone-treated, and MP-treated cardiomyocytes in the absence or in the presence of ODQ. Data are expressed as mean ± SEM (n = 17–50). \*\*P < 0.01 vs. L-NAME and MPs + L-NAME. \*\*\*P < 0.001 vs. ODQ and MPs + ODQ. Statistical analyses were performed using ANOVA and subsequent Bonferroni post-hoc test.

#### Interaction between MPs and cardiomyocytes

Firstly, using flow cytometer, we checked whether MPs from apoptotic murine RAW 264.7 macrophage cell line, were effectively labelled with the probe PKH67. A right

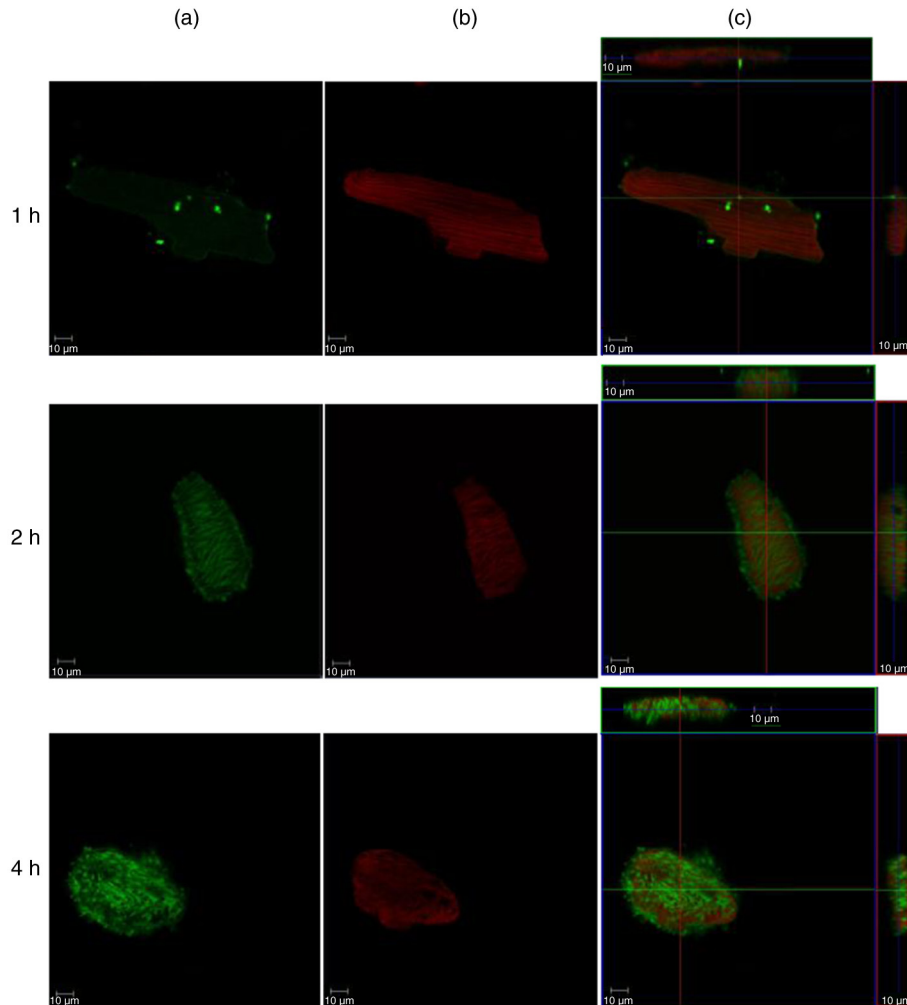
shift of fluorescence peaks clearly confirmed that MPs were efficiently labelled (data not shown).

Then, cardiomyocytes from adult mice were treated with labelled MPs (10 µg/mL) during different durations



**Fig. 4.** Effects of macrophage MPs (1 µg/mL) upon sarcomeric shortenings of adult murine cardiomyocytes in the absence or the presence of different TNF-α blockers. (a) Sarcomere shortening (% of resting sarcomere length), maximal velocities of (b) sarcomere shortening and (c) relengthening in control, SPD 304 (10 µM) alone-treated, and MP-treated cardiomyocytes in the absence or in the presence of SPD 304. (d) Sarcomere shortening (% of resting sarcomere length), maximal velocities of (e) sarcomere shortening and (f) relengthening in control, TNFR1 antibody (20 µg/mL) alone-treated, and MP-treated cardiomyocytes in the absence or in the presence of TNFR1 antibody. Data are expressed as mean ± SEM (n = 12–50). \*\*P < 0.01, \*\*\*P < 0.001 vs. SPD 304 or TNFR1 antibody and MPs + SPD 304 or TNFR1 antibody. Statistical analyses were performed using ANOVA and subsequent Bonferroni post-hoc test.





**Fig. 5.** Interactions between macrophage MPs and adult murine cardiomyocytes. Confocal images of (a) PKH67-labelled MPs (green), (b) phalloidin-labelled adult ventricular cardiomyocytes and (c) merge images after 1, 2 and 4 h of incubation. For each merge image, orthogonal sections are represented in the  $XY$  plane (central panel), in the  $XZ$  plane (upper panel) and in the  $YZ$  plane (right panel). White bars: 10  $\mu\text{m}$ .

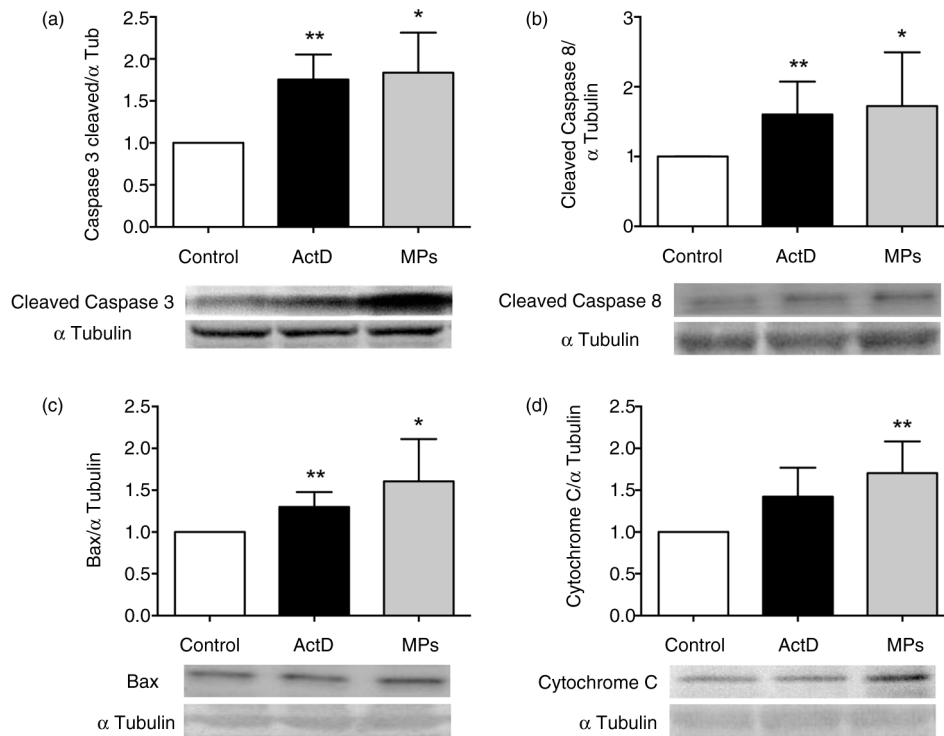
of time (1, 2 and 4 h) and observed in confocal microscopy in a z-stack of serial imaging of 2  $\mu\text{m}$ . After 1 h, MPs were clearly surrounding rod-shaped (i.e. alive) cardiomyocytes (Fig. 5). After 2 and 4 h of treatment, as illustrated on orthogonal sections in  $XZ$  and  $YZ$  planes, MPs were internalized into cardiomyocytes undergoing to the early phase of apoptosis (Fig. 5). Thus, cardiomyocyte apoptosis was also studied after 4 h of pretreatment with MPs (10  $\mu\text{g}/\text{mL}$ ) in terms of caspase-3, caspase-8, Bax and cytochrome C expressions. The caspase family of aspartate-specific cysteine proteases is the central executor of apoptosis. The expressions of 17 kDa-cleaved caspase-3 and of 18 kDa-cleaved caspase-8 fragments were similarly increased in MP-exposed murine adult cardiomyocytes (caspases vs.  $\alpha$ -tubulin densitometric ratios increased, Fig. 6a and b). The expression of the proapoptotic protein Bax, which determines the ability of cells to undergo apoptosis, was also significantly increased in the presence

of MPs and the proapoptotic effect of MPs was further confirmed by an increase of cytochrome C on the cytosolic extract probably by an increase of cytochrome C release from mitochondria (Fig. 6c and d).

## Discussion

In the present work, we used the murine RAW 264.7 cell line induced in apoptosis using actinomycin D to promote MP release. The present study showed that these MPs carry functional TNF- $\alpha$  and activate TNFR1 in isolated adult murine ventricular cardiomyocytes, leading to cell shortening without affecting calcium signalling *via* a mechanism sensitive to NO synthase inhibitor.

Thus, in the present study, MPs from apoptotic macrophages induced a negative inotropic effect and slowing of both contraction and relaxation, similar to the effects observed in the presence of TNF- $\alpha$ . It should already be noted that such effects were not observed in the presence



**Fig. 6.** Apoptosis of adult murine cardiomyocytes in the presence of macrophage MPs. Western blot showing increased expression of (a) 17 kDa-cleaved caspase-3, (b) 18 kDa-cleaved caspase-8, (c) Bax and (d) cytochrome C in MPs (10  $\mu$ g/mL) or actinomycin D (ActD) (1  $\mu$ g/mL)-exposed adult murine cardiomyocytes. Data are mean  $\pm$  SEM (n = 5–12). \*P < 0.05 and \*\*P < 0.01 vs. control (statistical analyses were performed using ANOVA and Mann–Whitney U-test).

of MPs obtained from apoptotic human lymphoid CEM T cell line where apoptosis was also induced by treatment with actinomycin D (25). Furthermore, the negative inotropic effect of MPs from apoptotic macrophages was absent after pretreatment with a TNF- $\alpha$  inhibitor, suggesting that receptors to the cytokine were involved in the responses to MPs. Reduced contractility by cytokines has been described in whole heart, multicellular preparations and isolated ventricular cardiomyocytes, and has been correlated with NO induction by constitutive NOS in numerous studies (3,4,26). However, the findings are contradictory in the time frame of effect of TNF- $\alpha$ . Most report effects occurring progressively, over 10 min (4,7) though relatively acute effects (1 min), have been reported (27,28). Furthermore, some studies have shown a biphasic effect of TNF- $\alpha$ , with an initial increase of systolic  $[Ca^{2+}]_i$  preceding an ultimate decrease (7,29). Furthermore, although most studies have reported a reduction in systolic  $[Ca^{2+}]_i$  and shortening (4,7,28,29), others have found no effect on systolic  $[Ca^{2+}]_i$  (4) and even an increase (7). As pointed out by Greensmith and Nirmalan (28), the lack of homogeneity regarding the effects of TNF- $\alpha$  on contractility in cardiomyocytes may be as a result of the diverse models, temperatures and techniques used. In our study, the maximal effects of MPs from apoptotic murine RAW 264.7 macrophage cell line carrying TNF- $\alpha$  occurred within 5 min and

we also showed that within 1 h of treatment, these MPs were still surrounding cardiomyocytes which strongly supports the view that the effects were most likely through receptor binding, that is, TNFRs. This point was reinforced by the complete blockade of the effect in the presence of the TNF- $\alpha$  inhibitor or TNFR1 antibody. Furthermore, the rapid effect of TNF- $\alpha$  by MPs containing the soluble form of the cytokine is not surprising. Indeed, MacKenzie et al. (30) showed that shed microvesicles from human THP-1 monocytes were capable of releasing bioactive interleukin-1 $\beta$  (IL-1 $\beta$ ) from their contents to activate IL-1 receptors at target cells (HeLa cells) within 5 min.

Reduction in contractile amplitude can result either from depressed  $Ca^{2+}$  transients or from altered  $Ca^{2+}$  myofilament sensitivity. In the present study, we never observed any change in the amplitude of  $Ca^{2+}$  transients. Our study also showed that the negative inotropic effect of MPs from apoptotic macrophages could be completely blocked by the NOS inhibitor L-NAME and the sGC inhibitor ODQ, suggesting that NO pathway is implicated in the observed effect. It also strongly suggests that the sGC/cGMP/PKG pathway is the sole mechanism for this MP-induced negative inotropy.

TNF- $\alpha$  induces cardiomyocyte apoptosis and this effect is mediated by an increase in oxidative stress (31,32).

The pro-apoptotic role of TNF- $\alpha$  has been extensively investigated in various *in vitro* and *in vivo* models (31–33). TNF- $\alpha$  causes apoptotic cell death by the two different but interrelated pathways, so-called extrinsic (TNFR binding and caspase-3 activation) and intrinsic (overproduction of ROS and mitochondrial injury) pathways (32). In the present study, chronic exposure to MPs from apoptotic macrophages induced apoptosis and increased the expression of proapoptotic proteins, such as cleaved caspase-3 and -8, Bax and cytochrome C, suggesting that the intrinsic pathway, involving the mitochondria, was particularly involved in the proapoptotic effects of these MPs. Human atherosclerotic plaques harbour MPs released from apoptotic cell death within the lesion (18). Canault et al. (21) also showed that these MPs carry catalytically active metalloprotease TNF- $\alpha$  converting enzyme (TACE/ADAM17), which significantly enhances the cell surface processing of the TACE/ADAM17 substrates TNF- $\alpha$ , and TNFR1. Present study suggests that MPs from apoptotic macrophages carry also *per se* TNF- $\alpha$  that would potentiate their proapoptotic property. Altogether, these data support the interaction between MPs bearing TNF- $\alpha$  and TNFR1 on the cardiomyocytes for the rapid cell shortening and most likely the internalization for the induction apoptosis.

During the acute phase of myocardial infarction, resident macrophages in the myocardial tissue are activated following neutrophil infiltration into the infarcted region. In addition, monocytes, induced by the expression of chemotactic factors, infiltrate the infarcted myocardium, differentiate to macrophages and secrete a variety of inflammatory cytokines participating to the evolution towards cardiac failure. Even if further works need to be carried out in Langendorff perfused hearts in order to add substantially to the physiological relevance of the present study, our report suggests that MPs issued from activated macrophages could carry TNF- $\alpha$  and then contribute to propagation of inflammatory signals leading to myocardial infarction. Thus, the present study allows supporting the hypothesis that MPs from human carotid atherosclerotic plaques may contain active TNF- $\alpha$ , which could contribute to MP-induced inflammatory signals in human atherosclerotic lesions.

## Acknowledgements

The authors are grateful to the ITMO “Physiopathologie, Métabolisme, Nutrition” Inserm, and the “Collectivités Territoriales Angevines” for financial support. The authors thank the “Plateforme de Métabolomique” of University of Angers for technical assistance and SCAHU staff (University of Angers) for animal handling. EM is recipient of a doctoral fellowship from Nanofar Erasmus Mundus Program.

## Conflict of interest and funding

The authors have not received any funding or benefits from industry or elsewhere to conduct this study.

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# Thèse de Doctorat

Edward MILBANK

## Extracellular vesicles as a therapeutic strategy to prevent or reverse obesity and its metabolic complications in the field of nanomedicine

### Résumé

A ce jour, les thérapies anti-obésité restent limitées. De récentes études ont fourni des résultats prometteurs en démontrant une diminution du poids de la souris via une injection stéréotaxique d'une forme dominante négative de l'AMPK (AMPK DN) directement dans le noyau ventromédial hypothalamique (VMH). Cependant, le potentiel thérapeutique de cette thérapie génique se voit entravé par une libération non spécifique de l'AMPK suite à une injection intraveineuse, plus adaptée à une approche clinique. Nous avons donc développé une approche de « nanobiomédecine » en utilisant des exosomes - nanovésicules contenant des lipides, des protéines et des acides nucléiques - pour délivrer l'AMPK DN spécifiquement au niveau du VMH. Des cellules dendritiques immatures ont été utilisées pour produire des exosomes non-inflammatoires. Pour permettre le ciblage spécifique du VMH par les exosomes, les cellules dendritiques ont été transfectées pour exprimer Lamp2b, une protéine exosomale, fusionnée au peptide de ciblage neuronal RVG. De façon intéressante, les exosomes Lamp2b-RVG ont été localisés au niveau du cerveau suite à une injection intraveineuse. Les exosomes Lamp2b-RVG ont ensuite été chargés par l'AMPK DN sous le contrôle d'un promoteur spécifique du VMH, apportant une double spécificité tissulaire aux exosomes. Les exosomes Lamp2b-RVG chargés avec l'AMPK DN induisaient une diminution de la phosphorylation de l'acetyl-CoA carboxylase dans des cellules Neu2A *in vitro*. De plus, l'injection intraveineuse d'exosomes Lamp2b-RVG chargés avec l'AMPK DN induisait une perte de poids de l'animal après 6 jours de traitement, démontrant le potentiel de cette approche de « nanobiomédecine ».

### Mots clés

Obésité - Régulation centrale - Exosomes - Libération - Ciblage neuronal - AMPK DN

### Abstract

Actual pharmacological therapies for treating obesity are limited. Promising results on decreasing mice body weight were obtained using a ventromedial nucleus hypothalamic (VMH) stereotaxic injection of a dominant negative isoform of AMPK (AMPK DN). However, DNA-mediated therapeutic potential is hampered by inadequate tissue-specific delivery following a systemic injection - more adapted to a bedside approach -. Herein, we developed a nanobiomedicine approach using exosomes - nano-scaled endogenous vesicles containing lipids, proteins and nucleic acids - to deliver DNA in a hypothalamic specific way. Immature dendritic cells were used to generate non-inflammatory exosomes. Exosome neuronal targeting aptitudes were achieved by constraining the dendritic cells to express Lamp2b, an exosomal protein, fused to the neuron-specific RVG peptide. Interestingly, DID-labelled Lamp2b-RVG exosomes were found into the mice brain following an intravenous injection. Isolated Lamp2b-RVG exosomes were then loaded by transfection-mediated techniques with AMPK DN under the control of a VMH specific promoter conferring double tissue expression specificity to the exosomes. AMPK DN-loaded exosomes induced a decrease of acetyl-CoA carboxylase phosphorylation in Neu2a neuronal cells *in vitro*. Furthermore, intravenously injected AMPK DN loaded exosomes induced a decrease of mice body weight following 6 days of treatment, demonstrating the potential of this nanobiomedicine approach.

### Key Words

Obesity - Central Regulation - Exosomes - Delivery - Neuronal Targeting - AMPK DN