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Metabolic characterization of the Ciliary Neurotrophic Factor knockout mouse: a preliminary study.

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SUMMARY

In humans and in experimental animals, administration of ciliary neurotrophic factor (CNTF) reduces food intake and body weight. Studies of animal models have consistently demonstrated that exogenous CNTF not only reduces food intake, by acting on hypothalamic and brainstem feeding centres, but also improves obesity-associated hyperglycaemia, hyperinsulinemia and hyperlipidaemia by exerting metabolic effects through actions on peripheral organs, including white adipose tissue, skeletal muscle and the liver. Very few data are yet available on a possible role of endogenous CNTF as a modulator of energy balance. In this project we tested whether endogenous CNTF might be involved in energy balance regulation by assessing the metabolic phenotype of CNTF ablated mice ($CNTF^{-/-}$) fed normal chow diet (NCD) or an high-fat diet (HFD). The main results obtained were: 1) adult male $CNTF^{-/-}$ mice challenged with an HFD for 13 weeks gained significant more body weight than the littermate controls fed the HFD (WT); 2) when compared with the respective WT animals, NCD fed $CNTF^{-/-}$ mice exhibited a higher adipocyte hypertrophy in mesenteric and epididymal visceral white adipose tissue (WAT) depots whereas the HFD fed $CNTF^{-/-}$ mice showed increased macrophage infiltration and an higher proneness of adipocyte to death at these visceral WAT depots; 3) visceral WAT cellular derangements in the $CNTF^{-/-}$ mice matched with the overexpression of the monocyte chemoattractant protein 1 and the proinflammatory cytokine tumour necrosis α, whereas adiponectin expression was found to be reduced in the epididymal depot of HFD fed mice; 4) lack of CNTF in both NCD and HFD fed mice involved an impairment of insulin signalling in the skeletal muscle and in the liver where, in addiction, a massive steatosis was especially evident in the HFD fed $CNTF^{-/-}$ mice in comparison with the WT mice kept under the same nutritional conditions; 5) HFD fed $CNTF^{-/-}$ mice exhibited higher circulating insulin and HOMA index in comparison with their HFD fed controls. Collectively, these data suggest that endogenous CNTF, possibly produced and secreted in the blood by a/some yet unidentified cellular source/s may reach peripheral metabolically relevant organs that bear its specific receptor and exert metabolic effects under distinctive physiological or pathophysiological conditions.

2. INTRODUCTION

2.1 OBESITY

The definition provided by the World Health Organization indicates obesity as a medical condition due to a chronic and sustained imbalance between calorie intake and energy expenditure, leading to an excess deposition of white adipose tissue (WAT) in the body and exerting a negative effect on health status (WHO, 2020).

The estimation of Body Mass Index (BMI, a parameter calculated by dividing an individual's weight in kilograms by the square of the body height in meters) is now recognized internationally as a unique measurement system to assess the degree of adiposity in humans and to establish whether a person is overweight or obese. The National Institute of Health defines the obesity condition in terms of BMI, establishing "threshold values" to be attributed to overweight and obesity conditions: the overweight subject has a BMI > 25 kg/m^2 whereas a value of BMI greater than 30 indicates an obesity condition. BMI, however, does not distinguish between weight associated with muscle and weight associated with the fat mass, therefore it is poorly informative in a large population of subjects. Another method to clinically assess obesity is the measurement of waist circumference which, notably, also provides information on the distribution of body fat. It is now known that people who carry excess fat centrally (inside the abdominal cavity) are more likely to suffer the morbid consequences of obesity including insulin resistance and diabetes, dyslipidaemia and cardiovascular disease, non-alcoholic steatohepatitis and even some cancers (Tchernof & Després, 2011)

The body catabolizes carbohydrates or sugars, proteins and fats, resulting from food intake, to produce energy, this can be used immediately to support normal daily body functions and physical activities, or store energy as lipids for future use by the body. Energy is measured in calories. Obesity results from an energy imbalance that means that the energy IN does not equal the energy OUT. Energy IN is the calories consumed with food and drinks. Energy OUT is the calories that are consumed for basic life functions as digesting, breathing, walking, and regulating body temperature. When the calories assumed are greater than the calorie expenditure, our body progressively store fat, leading to the condition of overweight and obesity.

2.1.1 Obesity, food consumption and mood

On the other hand, obesity is not only an anthropometric matter. Instead, It should be considered an extremely complex multifactorial disease, where excessive calorie intake associated with reduced physical activity - both conditions being primarily attributable to an incorrect lifestyle - matches with individual determinants such as hormonal and genetic imbalances, eating habits, how and where people live, attitudes and emotions, life habits (National Institutes of Health. 1998).
Specifically, mood and social life are crucial aspects affecting individual food behaviours and intake. Several studies show a strong correlation between food choices and mood, suggesting that people base their emotional state by changing both food quantities and food choices (Morris et al., 1987). (Morris *et al*., 1987). In certain psychological conditions, energy-dense and palatable food is preferred instead of others, and pre-clinical studies suggest this could be linked to the activation and the influence of foods on the activity of brain rewarding centres: food defined as "*palatable*" generate positive feedback on hunger centres, suppressing the satiety ones and leading over time to obesity (Volkow *et al*., 2011). Interestingly, high palatable foods have been shown to activate the dopaminergic transmission in the same brain regions also encoding reward and pleasure, similarly to what occurs in drug addiction. This suggests a possible role of the nervous system that controls "food addiction" behind obesity and high food intake. Other factors such as stress can influence feeding behaviour, that lead in an increase or decrease in food intake, determinate on the types of psychological or external factors: for example, the consumption of palatable foods may be able to reduce signs of stress and anxiety.. Macht, in 2008, showed that people suffering for depression prefer palatable "comforting foods" to alleviate their negative feelings, which in turn promote further vulnerability to depression and anxiety. In this context, Sharma *et al*., (2012) claimed that a high-fat diet leads to negative emotional states and alters basal corticosterone levels, thus inducing a vicious circle that promotes negative feelings, overeating and weight gain which results in depressed mood and obesity (Morris *et al*., 1987; Macht, 2008).

2.1.2 Endocrine disorders

The endocrine system plays an essential role in energy balance maintenance. Distinctive forms of obesity can be associated with specific endocrine diseases, among which hypothyroidism and

Cushing's syndrome are the most important (Weaver, 2008). Hypothyroidism is characterized by the storage of hyaluronic acid mainly in the dermis, but in other tissues too (Smith et al., 1989). (Smith *et al.,* 1989). Thyroid hormones are associated with growth hormone secretion, thus lack of triiodothyronine (T3) leads to reduced generation of insulin-like growth factor-1 (IGF-1). Hypothyroidism is also linked with raised leptin levels (Pinkney JH *et al*., 2000). It emerges that thyroxine (T4) and leptin are closely related, and they are controlled by a negative feedback mechanism. Administration of intracerebroventricular leptin alleviates the effects of hypothyroidism in animals which is characterized by a reduced reactivity to insulin, also due to an exaggerated cycle of glucose-fatty acids (Cettour-Rose *et al*., 2005). Finally, the hypothyroidism state lead to a diminished adrenergic activity and the combined reduction of catecholaminergic and thyroid hormone signalling to brown fat results in a reduction of mitochondrial UCP1 levels and reduced thermogenesis (de Jesus *et al*., 2001).

People with Cushing's syndrome are characterized by high amount of cortisol in the blood. When adipose precursor is exposed to glucocorticoids, is stimulated the differentiation and adipogenesis by activating the transcriptional factors of differentiation genes including leptin, lipoprotein lipase (LPS) and glycerol-3-phosphate dehydrogenase (Hauner *et al*., 1989). The chronic exposure to glucocorticoids on adipose tissue leads to enhancement deposition of visceral abdominal fat. Obesity develops in 52% (de Vile *et al*., 1996) of patients with craniopharyngioma, a benign suprasellar tumour.

2.1.3 Genetics of Obesity

Apart from obesity linked to endocrine disorders, the vast majority of patients suffer of a chronic condition of obesity that may be conceived as the consequence of an evolutive mismatch. In ancient times the man was a predator, and he adapted to periods of deprivation, when food was only periodically available, and famines were always present; at that time provision of food required a great physical effort. Over time our genome has evolved to define excellent physiological mechanisms to defend against body weight loss, whereas it has not evolved to face the great food availability typical of modern times. Adults of this era, despite living in industrialized and food-rich countries, still have a hunter-gatherer genome (Loos *et al*., 2003). So those genes that in the past could have provided survival benefits in times of famine may now be the genes that predispose to obesity. (Hill *et al*., 1998; French *et al*., 2001). Thus, susceptibility to obesity is established by genetic

determinants, but an 'obesogenic' environment is required for its phenotypic expression. The genetic predisposition to obesity is closely related to inappropriate lifestyle and unfavourable environmental conditions. When a 'restrictive' environment evolves towards an 'obesogenic' environment, individuals are most likely to gain weight. An example of this gene–environment relationship can be found in populations of Arizona: the prevalence of obesity and type 2 diabetes mellitus is lower in the Pima Indians living in Sierra Madre mountains - considered a 'restrictive' environment- than those that live in the south western -considered an 'obesogenic' environment (Ravussin *et al*., 1994).

Experimental studies conducted in pairs of identical twins offer one of the most powerful ways to obtain a reliable estimate of heritability. These studies show that the extent of a person's response to changes in lifestyle or environmental status (response to a positive -obesogenic- (Bouchard *et al*., 1990) or a negative -restrictive- (Bouchard *et al*., 1994) energy balance treatment) depends on a genetic predisposition considered largely hereditary. To study genetic epidemiology, we need to consider the level of heritability, identified as the fraction of the population variation in a trait (e.g., the BMI) that can be demonstrated by genetic transmission, in twins, adoption and family studies. Highest heritability rates, with values clustering around 70%, have been seen in monozygotic and dizygotic twins (Maes *et al*., 1997) or monozygotic twins reared apart (Allison *et al*., 1996; Stunkard *et al*., 1990) whereas only 30% of heritability was found in adoption studies. Through family studies it has been noted intermediate heritability levels between twin studies report and adoption studies (Rice *et al*., 1999).

There are some mendelian disorders in which obesity is a clinical manifestation, even if not a dominant feature, of the disease: for example, the Bardet–Biedl Syndrome (BBS), the Albright hereditary osteodystrophy (AHO) and the Prader–Willi Syndrome (PWS) (Loos *et al*., 2003). Recent research, however, has also stressed the existence of rare single-gene disorders in which obesity is the dominant characteristic and is widely independent of environmental factors (Loos *et al*., 2003). The most important mutation is carried on *Leptin* gene (LEP) (Loos *et al*., 2003). Leptin is a hormone that derive from adipose tissue, it causes satiety and circulate in proportion to fat mass (Schneeberger *et al*., 2014). It conveys information regarding energy status to the central nervous system by crossing the blood brain barrier via a saturable transport system and is strikingly involved in the regulation of food intake and glucose homeostasis (Considine *et al*., 1996). There are two kinds of mutation regarding LEP: the *ob* mutation that results in a lack of leptin production and the *db* mutation that involves the leptin receptor, which, lacking the normal intracellular C-terminus

motif, is ineffective (Tartaglia *et al*., 1995). In humans, only a few patients carrying a mutation in LEP have been described, but the phenotype of two rat strains carrying the mutations in leptin gene (*ob* and *db*) exhibit early obesity, hyperphagia, dyslipidaemia, low core temperature, hypertension, insulin resistance and consequent susceptibility to diabetes mellitus (Tartaglia *et al*., 1995; Zhang *et al*., 1994; Loos *et al*., 2003). Other rare mutations may affect the hypothalamic–pituitary–adrenal axis and involves the pro-opiomelanocortin (POMC) peptide that is post-transcriptionally processed to produce alpha-melanocyte-stimulating hormone (MSH) and the adrenocorticotropic hormone (ACTH). Leptin stimulates hypothalamic synthesis of α-MSH, that, after binding to the melanocortin 4 receptor (MC4R), generates signals that promote satiety and energy expenditure (Flier *et al*., 1998). Mutations in the POMC gene were found in two severely obese patients from unrelated families, displaying early-onset obesity associated with hyperphagia (Krude *et al*., 1998). Also, mutations that abolish the MC4R signalling are potentially able to lead to an increased BMI. However, the reported predominance of these mutations diversifies by a factor of 10, ranging from 0.5 to 5.8% (Kobayashi *et al*., 2002; Miraglia del Giudice *et al*., 2002; Yeo *et al*., 2003). The obesity, connected with a single-gene mutation, could be invert by administration of the human recombinant protein (Farooqi et al., 2002; Farooqi et al., 1999). (Farooqi *et al*., 2002; Farooqi *et al*., 1999). However, the magnitude of the obesity problem, that industrialized societies are facing today, cannot be the consequence of single-gene disorders or Mendelian obesity syndromes considering that this represent only small fraction of the whole obese population (Loos *et al*., 2003). Indeed, in most patients the obese phenotype depends to the combination of multifactorial agents such as genes and environmental (Loos *et al*., 2003). A lot of pre-clinical and clinical studies now offer candidate genes, selected by their function or role in biochemical pathways related to adipose tissue biology or regulation of energy balance at central level, that may be involved in the pathophysiology of obesity. Association studies have reported more than 70 candidate genes (Chagnon *et al*., 2003), some of these related to body fat and its distribution, whereas others connected to energy intake and expenditure mechanisms.

The mammalian adipose tissue, in addiction to white adipocyte, contains brown adipocyte which are characterized by many mitochondria that are larger that found in white adipocyte, where peculiar proteins allow use of the energy coming from fatty acid oxidation for heat generation: the uncoupling protein (UCPs) converting adenosine diphosphate (ADP) to adenosine triphosphate (ATP), phenomenon called “nonshivering thermogenesis”. UCPs are differently distributed: UCP1 predominantly in brown adipose tissue, UCP2 and UCP3 are widely expressed in skeletal muscle

(Kotzbeck *et al*., 2018). Only a few studies show a relationship between genetic polymorphisms of the UCPs proteins and obese phenotype (Oppert *et al*., 1994; Walder *et al*., 1998; Urhammer *et al*., 1997; Lanouette *et al*., 2001).

The adrenergic system stimulates thermogenesis and lipid mobilization: catecholamines (amongst other hormones) stimulate heat production by brown fat and lipolysis in WAT through β-adrenoceptors (β-ARs). Subcutaneous adipose tissues of obese individuals are characterized by a decrease of β-ARs. In some cases this receptor may be subject to polymorphisms that which are generally related to obese-phenotypes in several ethnic groups (Lin *et al*., 2001; Acho-Azcarate *et al*., 2002; Loos *et al*., 2003).The peroxisome proliferator-activated receptor gamma (PPARY), a transcription factor that stimulates the differentiation of adipocyte, is an interesting gene from an obesity point of view because it is targeted by thiazolidinedione, a novel class of insulin-sensitizing drugs. PPARY2, the most abundant in the adipose tissue, is increased in adipocytes from morbidly obese subjects (Vidal-Puig *et al*., 1997).

2.1.4 Epidemiological evidences

The obesity prevalence has been causing increasingly attention and concern in recent years due to its exponential and unstoppable spread. Obesity is considered a severe pathology because it represents a risk factor for the onset of other chronic pathologies which are responsible for 86% of the deaths in Europe (60% worldwide) with direct consequences for the affected individual and the society. For this reason, obesity is now defined as the most important problem of world public health (https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight).

According to the recent data provided by the World Health Organization (WHO), in 2016, more than 1.9 billion adults aged 18 years and older were overweight (https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight). Of these over 650 million adults were obese. Over the past 40 years, the worldwide prevalence of obesity has tripled, approximately 38.2 million children under the age of 5 were overweight or obese in 2019 (https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight).. Although obesity was considered a problem for rich countries, in Africa, the number of overweight children under 5 has increased by nearly 50 per cent since 2000, the number has doubled from 5.4 million in the 1990 to 10.6 million in 2014 (https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight). Nearly half of the children

under 5 who were overweight or obese in 2018 lived in Asia (https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight).

The prevalence of obesity was 18.5% among youth and 39.8% among adults in the United States in 2015–2016. Among youth, the prevalence of obesity among those aged 2–5 years was lower (13.9%) compared with older children (12–19 years, 20.6%; 6–11 years, 18.4%), and this pattern was seen in both boys and girls (Hales *et al*., 2017).

In Italy, according to the epidemiological inquiry "Rapporto Osservasalute 2016" (referred to ISTAT data collected in 2015), 35,3% of adult population is overweight, while 9,8% is obese. Overall, 45.1% of subjects aged ≥18 years is overweight. The territorial difference is considerable: Southern regions show higher percentage of obese adults, than those in the North. The percentage of overweight people increases with age: adults aged 18-24, 14% while adults aged 65-74, 46,0% (https://www.osservatoriosullasalute.it/wp-content/uploads/2017/05/ro-2016.pdf).

2.1.5 Consequences of Obesity

The risk factors due to overweight and obesity can be classified into two different pathophysiological categories. The first class of dysfunction is strictly linked to the gained mass of fat that, especially in massively obese subjects, cause sleep apnea and osteoarthritis (Bray, 2004). the other class of risk derives from the metabolic disorders related with excess fat (Bray, 2004). These patients are characterized by central obesity and other pathologies such as non-alcoholic fatty liver disease (NAFLD), hyperuricemia, dyslipidemia, hypertension and insulin resistance; the combination of these pathologies is classified as a metabolic syndrome (Tchernof & Després, 2011). The metabolic syndrome is a systemic inflammatory state widely related with type 2 diabetes mellitus (T2DM).

- Sleep apnea: the alteration of pulmonary activity is due to the decrease in residual lung volume associated with increased abdominal pressure on the diaphragm (Strohl *et al*., 2004)
- Osteoarthritis, predominantly developed in the knees and ankles, is closely connected to the degree of overweight (Felson *et al*.,1988). Nevertheless, the increased osteoarthritis in other nonweight-bearing joints propose that maybe there are some molecular pathways that alter bone metabolism and cartilage and it is not related to weight bearing (Bray, 2004).
- Analysing liver biopsies emerges that in obese individuals the prevalence of steatosis is 75%, while the incidence of steatohepatitis is 20%, and only 2% is classifiable as cirrhosis

(Bellentani *et al*., 2000). NAFLD (Non-alcoholic fatty liver disease) is characterized by a several liver deformities connected to obesity such as elevated liver enzymes, hepatomegaly, and abnormal liver histology, in particular steatosis (macro, and occasionally micro, vesicles of fat, predominately triglycerides, accumulate within hepatocytes) (Bray, 2004). Clinically, the disorder ranges from NAFLD to NASH, non-alcoholic steatohepatitis (focal hepatic inflammation and hepatocyte necrosis and death). In NASH patients chronic and sustained activation of some inflammatory pathways, leads to chronic inflammation of the liver parenchyma, massive lipid storage in hepatocytes, macrophage infiltration, liver fibrosis, and severe insulin resistance. In a substantial proportion of patients NASH evolves to cirrhosis and hepatocellular carcinoma.

- The dyslipidemic state is associated with visceral obesity and represents one of anomalies of the metabolic syndrome (Després *et al*., 2001; Després *et al*., 1990; Grundy *et al.*, 2005). It involves high concentration of triglycerides, low concentration of high-density lipoprotein (HDL) cholesterol, relatively normal total and low-density lipoprotein (LDL) cholesterol levels, but high levels of LDL particles that are smaller and denser than normal (Tchernof & Després 2011). This phenomenon is due to the remodelling of LDL and HDL by cholesteryl ester transfer protein and hepatic triglyceride lipase (Taskinen 2003; Taskinen2005). Lipid exchanges by cholesteryl ester transfer protein have been shown to be largely driven by the concentration of triglyceride-donor lipoproteins (Eisenberg 1984). Thus, when there is a state of hypertriglyceridemia, high levels of large VLDL1 particles promotes the transfer of triglyceride molecules to LDL and HDL in exchange for cholesteryl ester molecules (Tchernof & Després 2011). Therefore, both triglyceride-enriched LDL and HDL particles become good substrates for hepatic triglyceride lipase, leading to the deficiency of the lipid core of these lipoproteins, thereby forming small, dense LDL and HDL particles (Tchernof & Després 2011). Smaller HDL have reduced cholesteryl ester core content and becoming sensitive to degradation increasing clearance from the blood (Tchernof & Després 2011).
- Type 2 diabetes mellitus (T2DM) is strongly associated with overweight and obesity in both genders in all ethnic groups (Colditz *et al*., 1995; Chan *et al*., 1994). Insulin resistance, characterized by diminished responsiveness of the skeletal muscle, liver, and adipose tissue to insulin, and the subsequent hyperinsulinemia (the compensatory rise in insulin levels to maintain euglycemia) are key features of T2DM (Kaaks & Lukanova, 2001).

- There is a significant correlation between abdominal obesity, excess visceral adiposity and cardiovascular disease (CVD); Dagenais *et al*. showed that BMI was no longer an independent predictor of myocardial infarction after adjusting for abdominal obesity indexes such as WHR and waist circumference, in men and women with stable CVD. To confirm this finding, a large myocardial infarction case/control study, INTER HEART, conducted in a sample of 27.098 participants from 52 countries, showed that WHR was a stronger anthropometric correlate of myocardial infarction and could be significantly better than BMI in assessing the risk of myocardial infarction in the general population (Yusuf *et al*., 2005). Obviously, simple anthropometric measures of adiposity cannot replace blood pressure, history of diabetes, and the important lipid variables in CVD risk assessment (Wormser *et al*., 2011).
- One of the most important risk factors for an array of cardiovascular complications is hypertension. Insulin resistance and the metabolic syndrome (Després *et al*., 2006) potentiate the deleterious impact of hypertension on target organs and CVD risk. Some studies reveal that obese patients have higher rates of hypertension than normal-weight individuals (Chiang *et al*., 1969; Stamler *et al*.,1978), but not every obese patient is hypertensive, indicating the heterogeneity of obesity from the vascular standpoint (Poirier *et al*., 2003).

2.1.6 Pathophysiology of Obesity: molecular viewpoint

In a state of caloric excess white adipose tissue WAT plays a critical role by storing the surplus energy in form of triglycerides; as a result, white adipocytes increase in size and number therefore visceral and subcutaneous adipose depot expand (Giordano et al., 2013). The mitochondria of hypertrophic adipocytes are characterized by increased catabolism leading to oxidative stress and the production of free radicals (Giordano *et al*., 2013). Excess nutrients within the endoplasmic reticulum in adipocytes leads to "endoplasmic reticulum stress". Failure of the vasculature to expand as adipocytes enlarge induces adipose tissue hypoxia and activation of cell stress signalling programs, including transcription factor hypoxia-inducible factor 1 α (HIF1α), which eventually result in tissue fibrosis. Hypertrophic adipocytes are characterized by NLRP3 inflammasome activation that lead to formation of active caspase-1 that induces obese adipocyte death by pyroptosis, a proinflammatory programmed cell death (Giordano *et al*., 2013). These mechanisms strongly contribute to adipose tissue inflammation characterized by increased expression of C-reactive protein, interleukin 1,

interleukin 6, interleukin 1β, tumor necrosis factor α (TNFα) and leptin, activating resident macrophages. Furthermore, monocyte chemoattractant protein 1 (MCP1), a chemotactic adipokine, promote the infiltration and accumulation of circulating leucocytes to the adipose tissue. Activation of resident macrophages trigger the recruitment of circulating monocytes and $CD4^+$ Th1, $CD8^+$ cytotoxic T lymphocytes, NK T cells, B lymphocytes and mast cells (Anderson *et al*., 2010). All these cells are increased in obese adipose tissue and they are considered proinflammatory cells. Notably, anti-inflammatory cells, such as Tregs and Th2 cells, are decreased in obese adipose tissue (Feuerer *et al*., 2009; Winer *et al*., 2009); these cells are important because they can inhibit polarization of proinflammatory macrophages and can also prevent their further recruitment into tissues. Most macrophages infiltrating obese adipose tissue are found around adipocytes undergoing a necrotic-like death process and form distinctive structures that are called crown-like structures (CLSs) (Giordano *et al*., 2013). CLSs, through transmission electron microscopy, seems to be complex of activated macrophages, that are fused into syncytia (multinucleated giant cells), which surround individual dead adipocytes (Cinti *et al*., 2005; Giordano *et al*., 2013). Adipose tissue macrophages, (ATMs) increasing expression of a large set of proinflammatory genes (Lumeng *et al*., 2007; Nguyen *et al*., 2007) such as TNFα, directly cause insulin resistance by acting on insulin target cells (i.e. hepatocytes, myocytes, and adipocytes) in local tissues through paracrine mechanisms (Hotamisligil *et al*., 1993, 1996). In addition, these cytokines enter the systemic circulation and cause insulin resistance through endocrine mechanisms. This cascade of events leading to insulin resistance but also to a generalized inflammation of the adipose tissue that alters adipokine secretion profile, impairs insulin signalling, compromise triglyceride storage and increase basal lipolysis.

In addition to storing large amounts of lipids, adipocytes are also an important source of endocrine products, i.e. adipokines. The best characterized adipokines are adiponectin, an insulin sensitizer, and leptin. Therefore, the inflammatory process that takes place in obese fat induces not only adipocyte death and proinflammatory cytokine overproduction by adipocytes and infiltrating inflammatory cells, but also dysregulation of adipokine secretion.

Free Fatty acids (FFA) efflux from hypertrophic adipocytes into the circulation and altered lipoprotein synthesis and secretion by hepatocytes are the main pathophysiological mechanisms underlying dyslipidaemia, which is characterized by high levels of fasting FFA, triglycerides, and low-density lipoprotein cholesterol as well as low levels of high-density lipoprotein cholesterol.

Dyslipidaemia and the systemic proinflammatory milieu in turn favour abnormal lipid storage in several organs, a condition that has been called lipotoxicity, intended as an increase of circulating fatty acids and/or lipid accumulation in muscle and liver (Samuel and Shulman, 2012).

Lipids can interfere with insulin signalling mainly in two ways: circulating FFA interfere with normal transduction of insulin signalling (Glass and Olefsky, 2012), and the accumulation of lipid products, deriving from fats metabolism, lead to insulin resistance (Jornayvaz and Shulman, 2012).

In normal condition, insulin binds and activates the Insulin Receptor tyrosine kinase (IR), that lead to the phosphorylation of Insulin Receptor Subunits IRS-1, IRS-2, IRS-3 and IRS-4 and the transduction of the signal has as a consequence the glucose uptake into the cell and lipid store inside the adipocytes (Soleimani, 2015). In pathological condition, insulin receptor does not transduce the signal and insulin resistance occurs. Insulin resistance is defined as impaired insulin-mediated glucose clearance into target tissues and several mechanisms are involved in the establishment of this phenomenon. FFA -that remain in the blood vessels and are elevated in obesity- and saturated fatty acids (SFAs) bind to Toll-Like Receptor (TLR), activates intracellular IkB kinases (IKK), that causes degradation of IkB-α and consequent nuclear translocation and expression of NF-kB. Activation of NF-kB promotes local production of proinflammatory cytokines (IL-6, TNF-α) via SOCS3 and JNK (Kim, 2012). Activated JNK, promote serine phosphorylation of IRS that downregulates insulin signalling, associated with decrease tyrosine phosphorylation of PI3K, PDK, and Akt, resulting in reduced glucose transport into cells. Insulin resistance involves only IRS-1 and not IRS-2. IRS-1 mediates the effect of insulin on glucose uptake in adipocytes and skeletal muscles, while IRS-2 mediates the effect of insulin on kidney tubules (Kim, 2012).

In the liver, the storage of diacylglycerol (DAG) activates Protein Kinase Cε (PKCε), a member of PKC family, which inhibits the kinase activity of insulin receptor, this leads to a decrease in tyrosine phosphorylation of IRS1-2 and consequently a reduction of insulin activation of 1-phosphoinositol 3-kinase (PI3-K) and AKT2 (Jornayvaz and Shulman, 2012). Decrease of AKT2 phosphorylation result in reduces glycogen synthase (GS)-mediated glycogen synthesis and decreased suppression of gluconeogenesis (Jornayvaz and Shulman, 2012). These events drive glucose release through glucose transporter 2 (GLUT2) (Jornayvaz and Shulman, 2012). Hepatic insulin resistance, that is associated with hepatic steatosis, derive from the incapacity of insulin to activate the hepatic glycogen synthesis and to repress the hepatic glucose production. These are the key mechanisms that are involved in NAFLD.

Lipotoxicity not only affects the muscles and liver, but high levels of circulating FFA cause peripheral insulin resistance and impairment of β-cell insulin secretion (Poitout *et al*., 2008; Boden *et al*., 2002). In pancreatic β cells, lipids are used to supply energy through the β-oxidative pathway with low glucose concentration; on the contrary, with a high concentration of glucose the metabolism of FFA is oriented towards the esterification of lipids (Véret *et al.*, 2014). Accumulation and chronic elevation of FFA levels increase lipid synthesis, sphingolipids and ceramides (the precursors of many lipid messengers) which are important mediators of FFA-induced β cells dysfunction and apoptosis. Ceramide mediates dephosphorylation of protein kinase ERK 1/2 and PP2A resulting in decrease of insulin expression in islet of Langerhans and pancreatic β cells (Fontés *et al.*, 2009; Guo *et al.*, 2010). Yano and colleagues, shown that the accumulation of ceramides impaired mitochondria membrane integrity inducing an excessive production of ROS causing defect in insulin secretion in pancreatic β cells and their apoptosis (Yano *et al*., 2011; Kogot-Levin *et al*., 2014). These events drive to development of T2DM, that is associated with chronic hyperglycaemia and with abnormalities in lipid metabolism and excessive levels of circulating lipids (Butler *et al.*, 2003; Khan *et al.*, 2008).

2.2 Neuroendocrinology of the energy balance

The brain plays a central role in energy homeostasis by integrating multiple peripheral metabolic inputs, such as nutrients, gut-derived hormones, and adiposity-related signals (Rho *et al*., 2016). Central nervous system regulates diverse aspects of body metabolism and feeding behaviour, including hunger and satiety, food-seeking behaviour, gastric emptying, nutrient uptake in the gut, thermogenesis, pancreatic insulin secretion, as well as the effects of insulin in the liver, adipose tissue, and skeletal muscle (Morton *et al*., 2006; Broberger, 2005; Rho *et al*., 2016).

2.2.1 Historical perspective of neuroendocrinology of energy balance.

The brain research in feeding behaviour began in 1939, when Hetherington and Ranson demonstrated that bilateral damage to the ventromedial portion of the tuberal hypothalamus induced hyperphagia and obesity (Hetherington and Ranson, 1940). After few years, was shown that bilateral lesions in the adjacent lateral hypothalamus resulted in severe anorexia, weight loss, and even death by starvation (Anand and Brobeck, 1951). These investigations suggested the presence in the hypothalamus of two centres regulating food intake and energy balance and exerting opposite

actions (Giordano & Nisoli, 2018). Gordon Kennedy, in 1950, proposed that circulating agents secreted by peripheral organs in proportion to their energy stores could affect food intake and energy expenditure in a coordinated manner to regulate body weight (Kennedy, 1950; Coll *et al*., 2007). Through parabiosis experiments and hypothalamic lesions, in 1959 (Hervey, 1959), it was discovered that some, yet unidentified, blood-borne satiety factors, produced by the obese animal, affected food intake and that, for their action, was required an intact hypothalamus. In 1955, Mayer's glucostatic theory (Mayer, 1955) suggested that glucose metabolism in certain hypothalamic cells generates a signal to the brain areas controlling appetite and food intake; in 1960, stereotaxic studies demonstrated the role of the neurotransmitters acetylcholine and noradrenaline in the neural regulation of feeding (Giordano & Nisoli, 2018). Gibbs *et al.* in 1973 (Gibbs *et al*., 1973) discovered CCK (duodenal peptide cholecystokinin), the first gut hormone found to influence appetite.

Coleman in 1978, had identified a naturally genetically obese (*ob/ob*) and a naturally genetically diabetic (*db/db*) mouse strain (Giordano & Nisoli, 2018). Phenotypically were characterized by massive overeating, obesity, insulin resistance, and impaired sexual maturation leading to infertility (Giordano & Nisoli, 2018). Coleman and his colleagues deduced from parabiosis experiments, which establish cross-circulation between the two strains, that the *ob/ob* mouse lacked a circulating compound capable of preventing obesity, while the *db/db* mouse lacked the receptor for this factor (Coleman, 1978; Giordano & Nisoli, 2018). In the early 1990s, the advent of molecular genetic techniques allowed identifying the gene of Coleman's factor, whose product induced a strong satiety effect by acting on the central nervous system (CNS): the factor was named leptin (Zhang *et al*., 1994; Giordano & Nisoli, 2018).

2.2.2 The "Central Anatomy" of feeding behaviour and energy balance control

Lesioning studies performed in several species in the late 1930's through the 1950's highlighted the importance of hypothalamus in the regulation of body weight and demonstrated the presence in the hypothalamus of two opposite neuronal centres: the lateral (lateral hypothalamic area, LHA) and dorsal (paraventricular nucleus of hypothalamus, PVN) areas of the hypothalamus were described as "feeding centres", whereas more ventral structures (arcuate nucleus, ARC and ventromedial nucleus, VMN) were described as "satiety centres" (Hetherington and Ranson, 1939, 1940, 1942a,b). In the ARC, distinct and antagonistic neuronal populations coordinate various

peripheral and central signals as hormones, neuropeptides and neurotransmitters to control the hunger/satiety status (Schwartz *et al*., 2000). Two populations of neurons control appetite and energy expenditure: i) neurons that co-express the oroxygenic neuropeptide Y (NPY) and the aguti-related peptide (AGRP); ii) a population of neurons that co-express the anorexigenic neuropeptides cocaine-and amphetamine-regulated transcript (CART) and proopiomelanocortin (POMC) (Hahn *et al*., 1998). Both types of neurons (NPY/AGRP and POMC/CART) are regulated by leptin and insulin but in an opposing manner. POMC neurons produce the anorectic peptide α-melanocyte stimulating hormone (α-MSH) by post-transcriptional processing of POMC. To increase energy expenditure and reduce food intake, α-MSH binds to the melanocortin receptors 3 and 4 (MC3R and MC4R) on second-order neurons and activates catabolic pathways (Cowley *et al*., 1999). On the other hand, central administration of NPY increases food intake via Y1 or Y5 receptors, which are highly expressed in the ARC, PVN, and VMH (Raposinho *et al*., 2001). In the brainstem, three main structures are involved in regulating energy homeostasis: the area postrema (AP), the nucleus of solitary tract (NTS) and the dorsal motor nucleus of the vagus (DMX). These structures are strongly anatomically and functionally integrated to provide autonomic, behavioural and endocrine responses to energy-related peripheral cues and are collectively referred to as the dorsal vagal complex (DVC) (Young, 2012). Brainstem neurons contribute to the control of energy balance by elaborating energy status informations at different levels; by detecting circulating metabolites and hormones released in the bloodstream by peripheral organs; by receiving vagal inputs from gastrointestinal tract; by receiving neuronal inputs from midbrain and forebrain nuclei and by projecting into local brainstem circuits and other brain regions to provide information that will be integrated to control energy balance (Schneeberger *et al*., 2014).

2.2.3 Hormonal signals involved in the control of energy homeostasis

Researchers have discovered several circulating hormones, metabolites, and peptides that affect the energy balance, these factors exert a short- and/or long-term regulatory activity on feeding behavior through a concerted action on several distinct areas of the tuberal hypothalamus and/or the DVC (dorsal vagal complex). They come from at least three sites: the adipose organ (leptin, adiponectin, visfatin, omentin, and resistin), the gastrointestinal tract (ghrelin, cholecystokinin, peptide YY, pancreatic polypeptide, and glucagon-like peptide 1), and the endocrine pancreas (insulin, amylin). Their metabolic information get to the brain through four different way: interplay

with determined BBB transporters (insulin, leptin); propagation to adjacent brain areas through the circumventricular organs (ghrelin); direct action on neurons in the circumventricular organs (amylin); and, finally, stimulation of peripheral vagal sensory afferents (CCK, peptide YY, and GLP-1) and activation of gut-to-brain pathways (Giordano and Nisoli, 2018).

Leptin, the product of the *ob* gene, is a satiety hormone, derived from white adipocytes, that acting on central nervous system, especially on neurons located within the arcuate nucleus of the hypothalamus (ARH), determines a decrease of feeding and permits metabolic and neuroendocrine energy expenditure. Leptin interacts with its receptor, LepR, and the homodimerization of the receptor determines activation of Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, a mechanism of action in common with other cytokines (Cattaneo *et al*., 1999). Another pathway through which leptin acts is represented by phosphatidylinositol-3-OH kinase (PI3K) pathway, involved in both food intake and glucose homeostasis (Niswender *et al*., 2001). Among the different leptin receptor isoforms, LEPRb (the long isoform) is the most important. Indeed, its lack, or the lack of leptin, in both rodents and human causes a phenotype characterized by hyperphagia, reduced energy expenditure and severe obesity (Tartaglia *et al*., 1995; Lee *et al*., 1996; Montague *et al*., 1997). The leptin receptor is highly expressed in hypothalamic nuclei; in the ARC it's expressed in both AgRP and POMC neurons, where leptin produces opposite effects, stimulating POMC neurons and inhibiting AgRP neurons (Cowley *et al*., 2001). More recent studies demonstrate the presence of leptin receptor also in the DVC, in particular in a subpopulation of glial cells located in the AP/NTS border, suggesting the action on leptin also at the level of brainstem (Dallaporta *et al*., 2009).

Insulin is a hormone produced by beta (β)-cells of the pancreas; besides from its role in glucose metabolism it plays an important role in the CNS as an anorectic signal. Like leptin, insulin secretion induced by glucose into the bloodstream is in proportion to fat mass (Bagdade *et al*., 1967), and its receptor is expressed in hypothalamic areas important in the control of food intake (Benoit *et al*., 2002). Binding of insulin to its receptor leads to autophosphorylation of the receptor and recruitment of insulin receptor substrate (IRS) proteins, which coincides with the PI3K pathway activated by leptin (Xu *et al*., 2005). Activation of the insulin receptor in the hypothalamus leads to an increase in POMC expression and a decrease in NPY and AgRP expression in neurons of the ARC resulting in an anorexigenic effect (Schwartz *et al*., 1992).

Ghrelin is a hormone produced by the stomach and regulated by the ingestion of nutrients (Ariyasu *et al*., 2001). It has been described as a "hunger signal" because its secretion stimulate appetite

(Cone, 2005). Indeed, circulating ghrelin levels are increased under fasting conditions and reduced after refeeding. The orexigenic action of ghrelin is linked to the depolarization and activation of orexigenic NPY/AgRP neurons of the hypothalamic ARC by increasing GABA release onto them (Cowley *et al*., 2003; Zigman and Elmquist, 2003). A role for ghrelin in feeding behaviour has been documented by an action on the DVC of the brainstem too (Suzuki *et al*., 2010).

Cholecystokinin (CKK), a small peptide secreted from specific enteroendocrine cells of the duodenum, is postprandially secreted and its systemic delivery suppresses food intake in both animal models and humans (Gibbs and Smith, 1977; Kissileff *et al*., 1981). The anorectic effects of CCK are critically mediated by vagal sensory neurons that project into the NTS/AP, although CCK receptors are expressed in the brainstem and hypothalamus (Moran *et al*., 1997). CCK activates POMC neurons in the NTS, and MC4R signaling is required for CCK-induced suppression of appetite (Chi *et al*., 2004; Fan *et al*., 2004). Furthermore, it has been shown that leptin potentiates, and ghrelin attenuates the effect of CCK on appetite (Barrachina *et al*.,1997; Lee *et al.,*2011).

Amylin is a 37-amino acid peptide, an anorexigenic hormone co-secreted with insulin by pancreatic β-cells in response to meal-related stimuli (Young, 2005). It reduces food intake and it plays an important role in meal termination (Butler *et al*., 1990; Morley *et al*., 1991). Since AP-lesioned (APX) rats did not reduce food intake in response to amylin treatment (Lutz *et al*., 2001; 1995), the suppression of food intake by amylin seems to depend on a direct effect on neurons in the brainstems area postrema. Besides, direct AP application of low doses of amylin forbid food intake, while antagonism of AP receptors enhance food intake and nullify the anorexigenic effects of exogenous peripheral amylin (Mollet *et al*., 2004).

Glucagon-Like Peptide-1 (GLP-1) is secreted from intestinal mucosa and controls food intake, indeed GLP-1 levels are high following a meal and are low under fasting conditions. GLP-1 exert the satiety effect activating the vagal-NTS brainstem way. However, GLP-1 receptors are located in hypothalamus and brainstem, important brain areas for energy balance regulation (Merchenthaler *et al*., 1999).

Peptide YY similarly to GLP-1, is manly released from intestinal mucosa in response to nutrient ingestion (Adrian *et al*., 1985). It exerts anorexigenic effects in the ARC (Challis *et al*., 2003), but also in the brainstem and vagal-brainstem circuits (Koda *et al*., 2005). Neuronal activity in NTS and AP neurons increases with the peripheral release of this peptide (Blevins et al., 2008).

2.3 The Ciliary Neurotrophic Factor (CNTF)

The Ciliary Neurotrophic Factor (CNTF) was originally discovered as a neurotrophic factor contained in extracts of chick intraocular tissue where it was characterized for its ability to support the survival of ciliary ganglion neurons during a critical period of embryonic development (Adler et al., 1979). CNTF provides survival and differentiation to several neuronal cell types including sensory, sympathetic and motor neurons. Many other cell types, including oligodendrocytes, microglial cells, liver cells, and skeletal muscle cells also respond to exogenously administered CNTF, both *in vitro* and *in vivo* (Sendtner *et al*., 1994).

The CNTF is a 23kDa molecule of 200 amino acid. The CNTF protomer exhibit the four-helix bundle cytokine topology (Bazan, 1991), consisting of four helices, that are arranged in a left-handed, anti-parallel manner connected by two long crossover links and one short loop. The four copies of CNTF are arranged into two dimeric anti-parallel pairs. The low amounts of extracellular CNTF found *in vivo* and its potency both suggest that the active form of CNTF is monomeric (McDonald *et al*., 1995). However, given the higher concentrations of CNTF in the peripheral nerve (Williams *et al*., 1984), the CNTF dimer may serve as a storage form. Such CNTF dimers may provide additional stability to the intracellular stores of CNTF within Schwann cells and would be able to dissociate into active monomers following nerve injury and release of CNTF into an extracellular environment (McDonald *et al*., 1995). The CNTF is structurally and functionally related to members of a family of cytokines that includes leukaemia inhibitory factor (LIF), interleukin-6 (IL-6), interleukin11 (IL-11), oncostatin M (OSM), cardiotrophin (CT-1), leptin, cardiotrophin-like-cytokine (CLC) and neuropoietin (Bauer *et al*., 2007). These molecules can display overlapping biological activities because IL-6-type cytokines bind to plasma membrane receptor complexes containing the common signal transducing receptor chain gp130 (glycoprotein 130). Signal transduction of cytokines belonging to the IL-6 family involves the activation of JAK (Janus kinase) tyrosine kinase family members and transcription factors of the STAT (signal transducers and activators of transcription) family, but they also signal through the MAPK (mitogen-activated protein kinase) cascade or a cascade involving PI3K (Heinrich *et al*., 2003). The gp130 cytokines act on many different target cells (pleiotropism) and frequently induce redundant and partially overlapping effects on a variety of biological processes. Their actions via specific cell-surface receptors on their target cells can be auto-, para- or endo-crine, (Heinrich *et al*., 1998).

Signal transduction by CNTF requires that it bind first to CNTFR α; CNTFR α is anchored to the cell membrane by a glycosyl-phosphatidylinositol linkage. This binding allows the recruitment of gp130

and LIFR β subunits, forming a tripartite receptor complex. CNTF-induced heterodimerization of gp130 and LIFRβ leads to phosphorylation of JAKs (Janus tyrosine kinases), and the activated receptor provides docking sites for SH2-containing signalling molecules, such as STAT (signal transducers and activators of transcription) proteins. Once active, STATs dimerize and translocate to the nucleus where, by binding to specific DNA sequences, they determine a greater transcription of responsive genes (Sleeman et al., 2000). In mammals, activation of the JAK/STAT pathway stimulates cell proliferation, differentiation, cell migration and inhibits apoptosis. These cellular events are critical to hematopoiesis, immune development, mammary gland development and lactation, adipogenesis, sexually dimorphic growth and other processes (Rawlings *et al*., 2004). CNTF, unlike other growth factors that act on the nervous system, lacks a secretion sequence for release through vesicles, suggesting a cytosolic process (Lin *et al*., 1989) but not yet identified. Notably, CNTF is detectable in the nervous system only after birth, reaching adults levels during postnatal period, where it is not detectable during embryonic development (Stöckli *et al.*, 1991).

2.3.1 CNTF and CNTFRα distribution

As already mentioned, the CNTF was first identified in chick ciliary ganglion neuron (Adler *et al*., 1979) but later studies described the presence of CNTF and its bioactivity also in the sciatic nerve of the adult rat (Manthorpe *et al.,* 1986).

Subsequent studies confirmed the presence of CNTF mRNA especially in large motor peripheral nerves and not in target tissues such as skeletal muscle or skin (Stöckli *et al.*, 1989). Immunohistochemical studies showed that the expression of CNTF is confined to glial cells of central and peripheral nervous system: astrocytes (Stöckli *et al.*, 1991) and Schwann cells respectively (Stöckli *et al.*, 1991; Sendtner *et al.,* 1991; Rende *et al.,* 1992). Whereas CNTF expression is low in normal CNS grey areas, it is significantly upregulated in response to mechanical or ischemic lesions at the lesion site (Guthrie *et al.,* 1997; Lee *et al.,* 1997), indicating the involvement of CNTF in the protection of injured neurons and of axonal projections (Stöckli *et al.,* 1991; Dallner *et al.,* 2002).

The CNTFRα is widely expressed in the brain where is detectable in several areas including hypothalamus, thalamus, brainstem, cerebral cortex, and the olfactory bulb (Lee *et al*., 1997a). In both developing and adult nervous system the CNTFRα is expressed by neurons (Ip *et al*., 1993; Lee *et al*., 1996; MacLennan *et al*., 2000); however, it may be found also in cultured astrocytes suggesting that glial cells might also represent a CNTF target (Rudge *et al*., 1994). Besides its

expression in the central nervous system (CNS), CNTFRα is also found in peripheral organs, such as heart, skeletal muscle, kidney, liver, skin, lungs, adrenal gland, and testis (MacLennan *et al*., 2000). Recent immunohistochemistry studies provide evidence that tanycytes and ependymal cells of the third ventricle produce CNTF and contain its functional receptor. The close spatial relationship of cells that produce CNTF and cells that respond to CNTF in the ependymal layer is consistent with the possibility that the mouse ependyma is provided with CNTF-dependent paracrine and/or autocrine loops involved in the CNTF-mediated responses to physiologic or pathological stimuli (Severi *et al.,* 2012).

2.3.2 CNTF and energy balance: effect on CNS

The CNTF distinctively support the survival of brain motor neurons during the postnatal development (Sendtner et al. 1994). These considerations led to testing human recombinant CNTF in patients with amyotrophic lateral sclerosis where, although it failed to relieve symptoms and cure the disease completely, it unexpectedly resulted in anorexia and weight loss (ACTS, 1996; Miller et al. 1996). Importantly, the weight-reducing effect of exogenous CNTF was observed in leptin-resistant obese patients treated with subcutaneous Axokine, a human CNTF analogue with enhanced specificity and potency (Ettinger et al. 2003). These findings have raised considerable interest in the metabolic role of CNTF and have suggested the possibility of using this peptide, or its analogues, to treat human obesity and associated diseases (Findeisen et al. 2019). Clinical and preclinical studies have shown that administration of CNTF, or Axokine, to experimental animals led to significant weight loss and ameliorates obesity-related complications such as hyperinsulinemia, hyperglycaemia, and hyperlipidaemia (Gloaguen *et al*., 1997; Lambert *et al*., 2001; Watt *et al*., 2006). Unfortunately, 70% of obese subjects treated with Axokine developed antibodies against it, and the clinical trials were stopped (Ettinger *et al.,* 2003).

To understand the mechanisms by which CNTF induces weight loss, many research groups started to investigate its role in energy balance. They first hypothesized that CNTF mimics the ability of leptin to regulate the homeostasis of body weight. Indeed, similar to leptin, CNTF reduces appetite and body fat by providing a signal of energy intake and energy stores in the body and activating similar molecular pathways in the CNS (Anderson *et al*., 2003; Kelly *et al*., 2004; Vacher *et al*., 2011), especially in the Arcuate nucleus (ARC) of hypothalamus, a nucleus involved in hunger control

(Markus, 2005). Thus, CNTF shares signaling pathways with leptin in the ARC mostly via activation of Jak1/Jak2-STAT3 signaling (Lambert *et al*., 2001). Moreover, CNTF can act as a satiety factor in diet-induce obesity mice, *ob/ob* and *db/db* mice and in MC4R- deficient mice where leptin is ineffective (Gloaguen *et al*., 1997; Lambert *et al*., 2001; Xu *et al.,* 1998). Despite CNTFRα deletion in neurons that also express the leptin receptor, $CNTF_{Ax15}$ keeps its anoretic effect, suggesting that CNTF can act on different targets in the hypothalamus compared to leptin (Stefater *et al.,* 2012). Moreover, systemic administration of recombinant CNTF, or CNTF analogues, induced weight loss and this persisted after cessation of treatment (Lambert *et al.*, 2001). One intriguing explanation could be find in CNTF's effect on postnatal neurogenesis: it has been shown that CNTF could promote cell proliferation in the adult mouse hypothalamus and that newly formed cells are leptin-responsive neurons (Kokoeva *et al.,* 2005).

In normal condition, the distribution of CNTF in the mouse hypothalamus is limited to astrocytes and to tanycytes and ependymal cells lying the third ventricle (Severi *et al*., 2012). In mice rendered obese by a high fat diet (HFD), CNTF expression significantly increases in ependymal cells and tanycytes, whereas it decreases in mice kept in calorie restriction (CR) conditions. Interestingly, changes in CNTF expression correspond to changes in its receptor, CNTFRα. Taken together, these data propose that CNTF signaling increases in mice fed a HFD and conversely decreases in mice on CR (Severi *et al*., 2013).

Moreover, the administration of CNTF induces the activation not only of STAT3, but also of STAT1 and STAT5 in the ependymal cells and in tanycytes of the third ventricle and in glial cells of median eminence, in the area postrema and in the nucleus of the solitary tract (NTS, Severi *et al*., 2015, Senzacqua *et al*., 2016), suggesting a role for CNTF in these brain regions involved in energy balance regulation.

2.3.3 CNTF and energy balance: effect on peripheral tissues

CNTF is primarily known for its important effects within the nervous system, but it has metabolic effects also in peripheral tissues, such as muscle, liver, pancreas and adipose tissue. The expression of CNTF receptor in peripheral organs is also abundant and it has been detected in the heart, lungs, adrenal gland, skin, liver, kidney, testicles and pancreas.

Skeletal Muscle: a study indicates that CNTFRα can be highly expressed in denervated skeletal muscle (Weis *et al*., 1998). Administration of CNTF on skeletal myoblasts of adult human, induce the

differentiation into multipotent progenitor cells via p44/p42 MAPK pathway, also capable of differentiating into new phenotypes, mainly neurons, glial cells, smooth muscle cells, and adipocytes (Chen *et al*., 2015).

Furthermore, in the skeletal muscle, CNTF improves insulin sensitivity by controlling synthesis and expenditure of lipid and increasing fatty acid oxidation by activating AMP-activated protein kinase (AMPK) (Watt *et al*., 2006). CNTF is also able to stimulate muscle glucose uptake by PI3-Kinase/Akt pathway (Gregory R. *et al*., 2009) and to cause a significant overexpression of muscle differentiation-related genes and to downregulate atrophy-mediators in skeletal muscle (Tsompanidis *et al*., 2016).

Liver: CNTF is also able to act in the liver by reducing hepatic steatosis and by enhancing hepatic responsiveness to insulin (Sleeman *et al*., 2003). The hepatic triglyceride accumulation is weakened both *in vivo* and *in vitro* by treatments with recombinant CNTF for 24 hours with an increase in CPT-1 and PPARα expression, and a decrease in SREBP-1c, FAS and SCD-1 (Cui *et al.,* 2017).

Pancreas: Administration of CNTF to mice with alloxan-induced non-obese type 2 diabetes, improves insulin sensitivity and β-cell mass, reducing glucose-stimulated insulin secretion and insulin clearance in Swiss mice (Rezende *et al.*, 2012). CNTF protects mice against streptozotocin-induce diabetes (a model of type 1 diabetes) reducing amount of β-cells death and protecting pancreatic islets against cytokine-induce apoptosis (Rezende *et al.*, 2012).

Adipose tissue: The first study suggesting a role of CNTF on adipose tissue was performed by Henderson and colleagues in 1996 in genetically modified mice with implanted glioma cells secreting CNTF: the action of this neurotrophic factor resulted in fast and preferential loss of fat (Henderson *et al.*, 1996). In adipose tissue, the expression of CNTFRα (Zvonic *et al*., 2003) and the action of CNTF has been demonstrated: *db/db* (Liu *et al.,* 2007*)*, normal or obese mice (Blüher *et al.*, 2004) treated with recombinant CNTF showed an increase in β-3 adrenergic induction of mitochondrial UCP1 in brown adipose tissue*. In vitro*, in brown adipocytes, Ott and colleagues (2002) showed that recombinant CNTF lead to the phosphorylation of STAT3 and p42/44 MAP kinase and to the induction of UCP1 expression. Finally, CNTF activates the signaling pathway of AKT, a key element in the regulation of glucose transport within the cell. The response to the increase of p-AKT is blunted in the presence of PI3K and PKC inhibitors, suggesting a role of the PI3K and PKC pathways in the cascade effect of the CNTF (Ott, *et al*., 2002).

In vitro, CNTF also performs a direct function on white adipocytes. Experiments on 3T3-L1 cells (an immortalized cell line of rodent white adipocyte) have shown that the peptide stimulates the phosphorylation of STAT3, while AKT is phosphorylated only in pre-adipocytes (undifferentiated fat

cells), and not in mature adipocytes (Zvonic *et al*., 2003). Surprisingly, 3T3-L1 cells, during differentiation, do not express CNTFRα or LIFR (gp130 remains unchanged), but despite this, chronic treatments with CNTF have nevertheless stimulated the activation of STAT3. The activation of JAk-STAT3 pathway induced the reduction of Fatty Acid Synthase (FAS) and sterol regulatory element-binding protein-1 (SREBP-1), a transcription factor that activates the transcription of genes coding for proteins involved in the increase of lipogenic activity (Zvonic *et al*., 2003).

In human adipocytes differentiated from human multipotent adipose-stem (hMADS) cells, CNTF strongly activated the JAK-STAT3 pathway and acutely and transiently activated the AMPK and AKT pathways. Longer-term treatment with CNTF reduced the expression of lipogenic markers (FAS and SREBP-1) and increased the expression of lipolytic [hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL)] and mitochondrial (peroxisome proliferator-activated receptor g coactivator-1a and carnitine palmitoyltransferase 1) markers. In the TNFα-treated hMADS adipocytes, taken as an in *vitro* model of adipose tissue inflammation (Ruane *et al*., 2002), CNTF significantly reduced the expression of monocyte chemoattractant protein 1 and TNFα-induced AKT inhibition (Perugini *et al.*, 2019).

In conclusion, CNTF has a dual action: it acts at the level of hypothalamic feeding centers and on peripheral tissues, increasing insulin sensitivity on muscle and liver and stimulating thermogenesis in the adipose tissue (Matthews and Febbraio 2008).

Taking all data, it can be stated that the CNTF has a relevant role in the control of metabolic homeostasis and could be candidate as a novel satiety factor studied as a tool for therapeutic approach to obesity.

3. AIM OF THE PROJECT

The major aim of this project was to preliminary assess whether in mammals the presence and activity of ciliary neurotrophic factor is required for a proper regulation of body metabolism under physiological and/or pathological conditions.

To this end, morphological aspects and molecular parameters of lipid and carbohydrate metabolism were investigated in visceral white adipose tissue, skeletal muscle and liver of adult male $CNTF^{-/-}$ and WT mice fed a normal chow diet or a high-fat diet for 13 weeks.

4. MATERIALS AND METHODS

4.1. Animals and experimental conditions

$CNTF^{-/-}$ mice were generated as described by Masu et al. (1993). We analysed CNTF null mice (kindly provided by Prof Michael Sendtner, Institute of Clinical Neurobiology, University of Wurzburg, Germany) backcrossed into a C57BL/6J background for 10 generations. C57BL/6J mice were originally obtained from Charles River (Milano, Italy). Mice were housed individually in plastic cages under constant environmental conditions (12 h light/dark cycle at 22°C) with ad libitum access to food and water. All mice (CNTF null mice and C57BL/6J mice) initially received a normal chow diet (Charles River; 19 kJ% from fat, 50 kJ% from carbohydrates and 31 kJ% from proteins); when they were 4 weeks old, were divided into four subgroups a) C57BL/J6 mice (n=7) that received a standard normal chow diet (NCD) for 13 weeks, b) C57BL/J6 mice (n=7) that received a high-fat diet (HFD; Charles River; 50 kJ% from fat, 30 kJ% from carbohydrates and 20 kJ% from proteins) for 13 weeks, c) CNTF null mice (n=7) that received a standard normal chow diet (NCD) for 13 weeks and d) CNTF null mice (n=7) that received a high-fat diet (HFD; Charles River; 50 kJ% from fat, 30 kJ% from carbohydrates and 20 kJ% from proteins) for 13 weeks. Body weight was measured in live conscious animals 3 times a week and food intake were monitored daily. All experiments were conducted on male mice aged 12-14 weeks. Blood was collected through cardiac puncture, blood samples were centrifuged at 1000×g, 4°C for 15 minutes and plasma stored at −80°C until analysis. For morphological analysis, mice have been treated with an overdose of anesthetic (Avertin; Fluka Chemie, Buchs, Switzerland) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 5 min. Deposits of WAT, liver and skeletal muscle were dissected using a Zeiss OPI1 surgical microscope (Carl Zeiss, Oberkochen, Germany) and further fixed by immersion in 4% paraformaldehyde in PB overnight at 4°C, then they was dehydrated in ethanol, cleared in xylene, and embedded in paraffin. For molecular biology assays, animals were anesthetized with Avertin and euthanized by cervical dislocation. WAT, liver and skeletal muscle specimens were rapidly removed, snap-frozen in liquid nitrogen, and stored at -80°C until use. All efforts were done to minimize animal suffering and to reduce the number of animals used. The care of the animals was in compliance with the Council Directive 2010/63 / EU and all the experiments had been approved by the Italian Ministry of Health (authorization no. 405/2018-PR).

4.2. Plasma glucose, plasma Insulin, plasma CNTF

Plasma glucose levels was determined by standard commercial colorimetric enzymatic assay (BioMerieux, Marcy l'Etoile, France; Roche Diagnostics, Basel, Switzerland). Plasma insulin concentration was detected by ELISA (Ultrasensitive ELISA; DRG, Springfield, NJ). Plasma CNTF concentration was measured by ELISA (Cusabio Mouse CNTF ELISA Kit, distributed by CliniSciences, Guidonia Montecelio, Italy)

4.3. Cytokine profile and protein analyses: xMAP

Cytokine profile and insulin signaling protein phosphorylation (p) at insulin receptor IRS-1^{S312}, AKT^{S473}, GSK-3^{bS9}, and $P70S6K^{T421/S424}$ levels were measured by xMAP technology (MAGPIX; Luminex Corporation, Austin, TX) using commercial kits, validated by the manufacturer for multiplexing profiling (LRC0002M, LHO0001M, LHO0002; Life Technologies, Carlsbad, CA). The MILLIPLEX Analyst software (Millipore, Billerica, MA) was used for the analysis, interpolating the data on a standard curve. The phosphorylation of each protein is expressed as a total phosphorylated protein unit in picograms.

4.4. Quantitative real time-polymerase chain reaction

Total RNA was extracted from frozen pieces using TRIzol reagent (Invitrogen, Milano, Italy), purified, digested with ribonuclease-free deoxyribonuclease, and concentrated using RNeasy Micro kit (Qiagen, Milano, Italy) according to the respective manufacturer's instructions. For determination of messenger RNA (mRNA) levels, 1 µg RNA was reverse-transcribed with a high-capacity complementary DNA RT Kit with RNase inhibitor (Applied BioSystems, FosterCity, CA) in a total volume of 20 µl. Real time gene expression was examined in triplicate using TaqMan Gene Expression Assays (Applied BioSystems). Supporting Information Table S1 shows the list of the TaqMan probes. Reactions were carried out in a Step One Plus Real Time PCR System (Applied Biosystems) using 50 ng RNA in a final reaction volume of 20µl and the following thermal cycle protocol: initial incubation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 20 s. To avoid genomic contamination, a control reaction was included for each sample in which reverse transcriptase was omitted in the amplification mix. Relative mRNA expression was determined by the ΔC t method using TBP levels as the endogenous control. Data are presented as histograms + SEM.

Table S1

Gene	Assay ID
ADIPOQ	Mm00456425_m1
IL6	Mm00446190_m1
LEPTIN (LEP)	Mm00434759_m1
MCP1 (CCL2)	Mm00441242_m1
TBP	Mm01277042_m1
TNF	Mm00443258_m1
RESISTIN (RTN)	Mm00445641_m1

4.5. Light microscopy and morphometry

From each adipose depot have been cut serial paraffin sections (3 µm thick), posed on glass slides, and dried. For hematoxylin and eosin staining were needed to use an alternate section to assess morphology, and for immunohistochemical procedures to gauge tissue protein expression. Adipocyte size was defined as mean adipocyte area (in square micrometers) employing a drawing tablet and a morphometric program (Nikon LUCIA IMAGE, Laboratory Imaging, version 4.61; Praha, Czech Republic). To evaluate tissue sections was used a Nikon Eclipse E800 light microscope, and digital images were taken at 20× with a Nikon DXM 1200 camera (Nikon Instruments S.p.A, Calenzano, Italy). The count of total number of MAC-2-positive CLSs determined CLS density in each section contrasted with the total number of adipocytes and was indicated as CLS number/10,000 adipocytes. The CLS rate was calculated by dividing CLS density by mean adipocyte size.

4.6. Immunohistochemistry

For immunohistochemistry, 3 µm thick paraffin-embedded sections of the fat depots were dewaxed; they were then reacted with 0.3% H2O2 (in methanol; 30 min) to block endogenous peroxidase, rinsed with PBS, and incubated in 3% normal serum blocking solution (in PBS; 30 min). Sections have been put overnight at 4°C with rat monoclonal anti-MAC-2 antibody (dilution 1:1500; Cedarlane Laboratories, Burlington, Ontario, Canada). After rinsing in PBS, sections were incubated in 1:200 v/v horse anti-rat. IgG biotinylated HRPconjugated secondary antibody solution (Vector

Laboratories, Burlingame, CA) in PBS for 30 min. Histochemical reactions were executed by Vectastain ABC kit (Vector Laboratories) and Sigma Fast 3,3-diaminobenzidine (Sigma-Aldrich, Vienna, Austria) as the substrate. Sections were counterstained with hematoxylin, dehydrated in ethanol, and mounted in Eukitt® mounting medium (Sigma-Aldrich). For negative control the primary antibody was omitted, and staining was never observed in this sample.

4.7. Statistics

All assays were performed in triplicate. Data are reported as mean ± standard error of the mean (SEM). Comparisons between and among groups were performed, respectively, with Student's t-test and one-way analysis of variance (ANOVA). Group differences were considered significant for *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. All statistical analyses were carried out with Prism 6.0 (GraphPad Software Inc., La Jolla, CA).

5. RESULTS

5.1. CNTF null mice show an increase in body weight when subjected to HFD.

In normal chow diet (NCD) fed-condition, CNTF null ($CNTF^{-/-}$) and C57BL/6J (WT) male adult mice showed similar body weight (Fig. 1A), suggesting that the presence of CNTF is not required for body weight homeostasis under normal nutritional conditions. As expected, the body weight of WT mice receiving the HFD for 13 weeks significantly increased in comparison with NCD fed WT mice (Fig. 1A). Interestingly, the $CNTF^{-/-}$ mice exposed to the same dietetic treatment gained significant more body weight of about 25 % than the WT mice (Fig. 1A). Given that body weight increase can be due to increased food intake and/or decreased energy expenditure we preliminary evaluated the weekly amount of food intake for each experimental group. Results showed that CNTF deletion had no effect on the amount of food ingested (Fig. 1B and C). Therefore, the higher body weight seen in $CNTF^{-/-}$ mice exposed to the HFD is likely due to a reduction in energy expenditure.

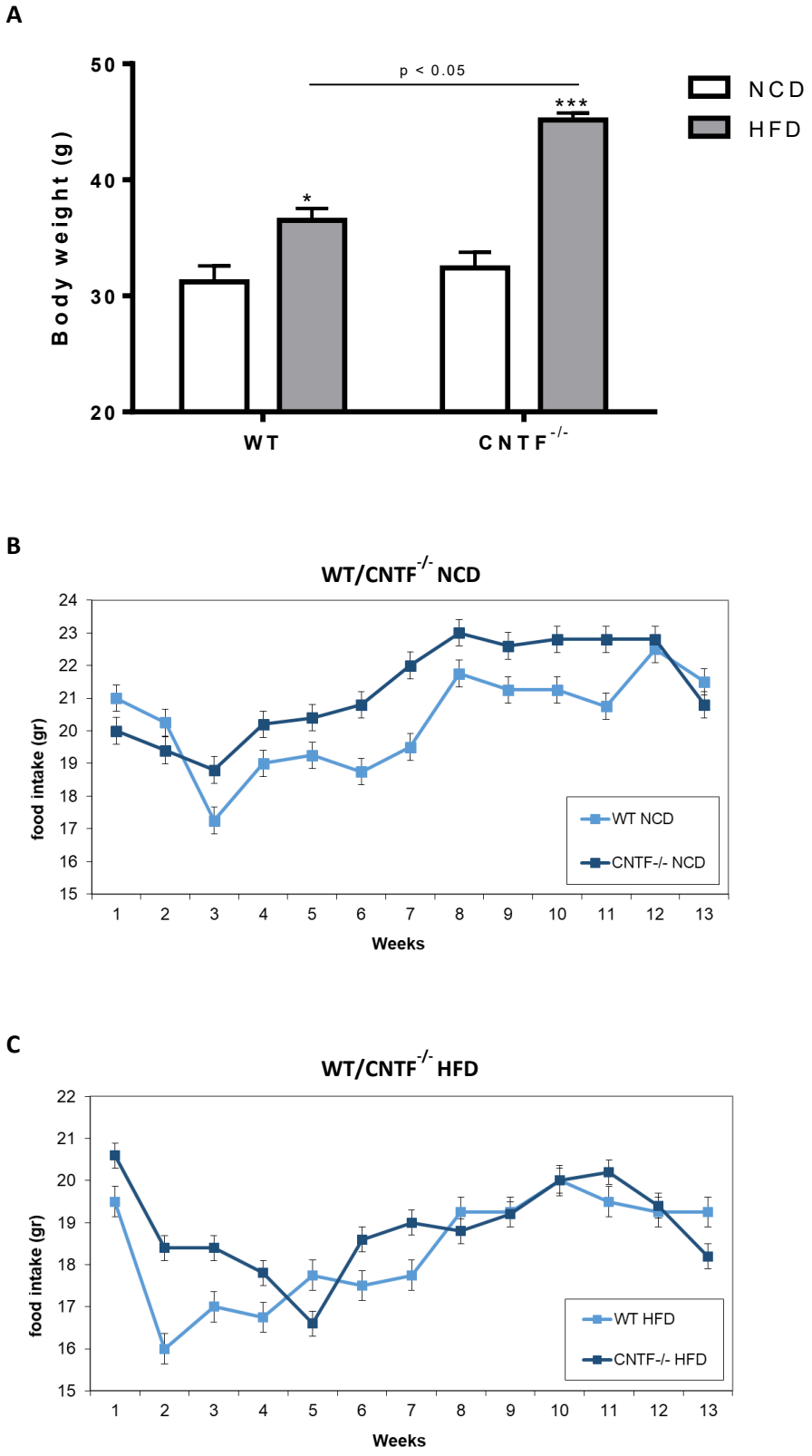

Figure 1: A*) Body weight of WT and CNTF$^{-/-}$mice fed NCD and HFD;* ***B****) food intake (gr/week) of WT and CNTF$^{-/-}$mice in NCD;* ***C****) food intake (gr/week) of WT and CNTF$^{-/-}$mice in HFD. Data are means ± SEM; *p < 0.05, ***p < 0.001 versus NCD.*

5.2. CNTF deficiency is associated with adipose tissue inflammation.

During obesity fat depots enlarge in the body, and WAT undergoes distinctive changes at histological and molecular levels. Thus, to characterize the adipose tissue of $CNTF^{-/-}$ mice we examined the histology of mesenteric (mWAT) and epididymal (eWAT) depots in NCD and HFD conditions. Mean adipocyte area and crown-like structures (CLS) density, detected by assessing the distribution of MAC2-positive macrophages, were evaluated in each visceral depot.

NCD $CNTF^{-/-}$ mice showed larger adipocytes both in mWAT and eWAT depots when compared to NCD WT mice, as shown in Fig. 2B (NCD WT) and Fig. 2C (NCD $CNTF^{-/-}$) where representative images from the mesenteric depots are shown. As expected, the mean adipocyte area in mWAT and eWAT depots was significantly higher both in WT and $CNTF^{-/-}$ mice exposed to the HFD when compared with the respective littermates kept in NCD conditions (Fig. 2A). In striking contrast, no differences in the mean adipocyte area were present in HFD conditions between WT and $CNTF^{-/-}$ mice (Fig. 2A). No differences were detected in CLS density between WT and $CNTF^{-/-}$ mice fed the NCD, whereas the density of MAC-2-positive CLS in both visceral depots was significantly higher in $CNTF^{-/-}$ mice than in WT mice fed the HFD (Fig. 3A). In Fig. 3B a representative picture from the mesenteric depot of a $CNTF^{-/-}$ mice in NCD condition showing a crown-like structure positive for MAC2 (arrow) is shown. Numerous MAC2 positive CLS (arrows) are evident in Fig. 3C and Fig. 3D, showing the mesenteric depots from a WT mouse exposed to HFD (Fig. 3C) and from a $CNTF^{-/-}$ mouse exposed to HFD (Fig. 3D), respectively.

Collectively, these morphological parameters suggest that lack of CNTF in mice exposed to HFD involves an obesity condition that is characterized by an increased adipocyte death and macrophage infiltration in fat depots. Thus, to better characterize the inflammatory state of the adipose tissue in $CNTF^{-/-}$ mice, we assessed the expression levels of specific inflammatory genes in mWAT and eWAT depots. qPCR analyses showed that the mRNA levels of the pro-inflammatory cytokines TNF-α and IL-6, and the gene encoding for the protein involved in macrophage infiltration, MCP1, were significantly higher in both WT and $CNTF^{-/-}$ mice fed the HFD compared to their respective controls in both depots (Fig. 4A-C). However, the mRNA levels of MCP1 and TNF-α increased significantly in $CNTF^{-/-}$ HFD mice compared to WT HFD mice in both depots (Fig.4 A and C), suggesting a higher inflammatory state in the visceral adipose tissues of $CNTF^{-/-}$ mice challenged by the HFD.

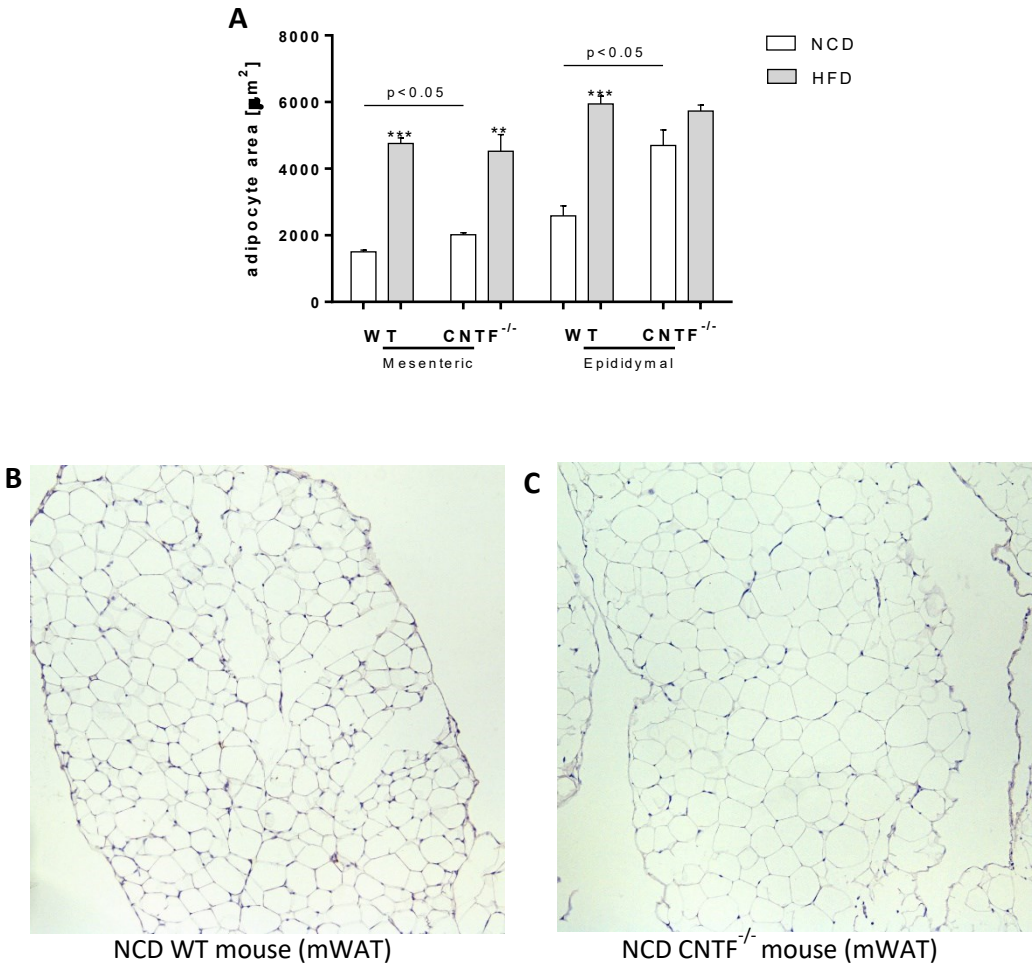

Figure 2: A*) Comparison of white adipocyte area in mWAT fat depot from WT and CNTF$^{-/-}$ mice kept on NCD and HFD;* ***B****) Representative microscopy pictures of adipocyte size of mWAT fat depots from WT mouse kept on NCD;* ***C****) Representative microscopy pictures of adipocyte size of mWAT fat depots from CNTF$^{-/-}$ mouse kept on NCD. Data are means ± SEM; *p < 0.05, ** p<0.01, ***p < 0.001 versus NCD.*

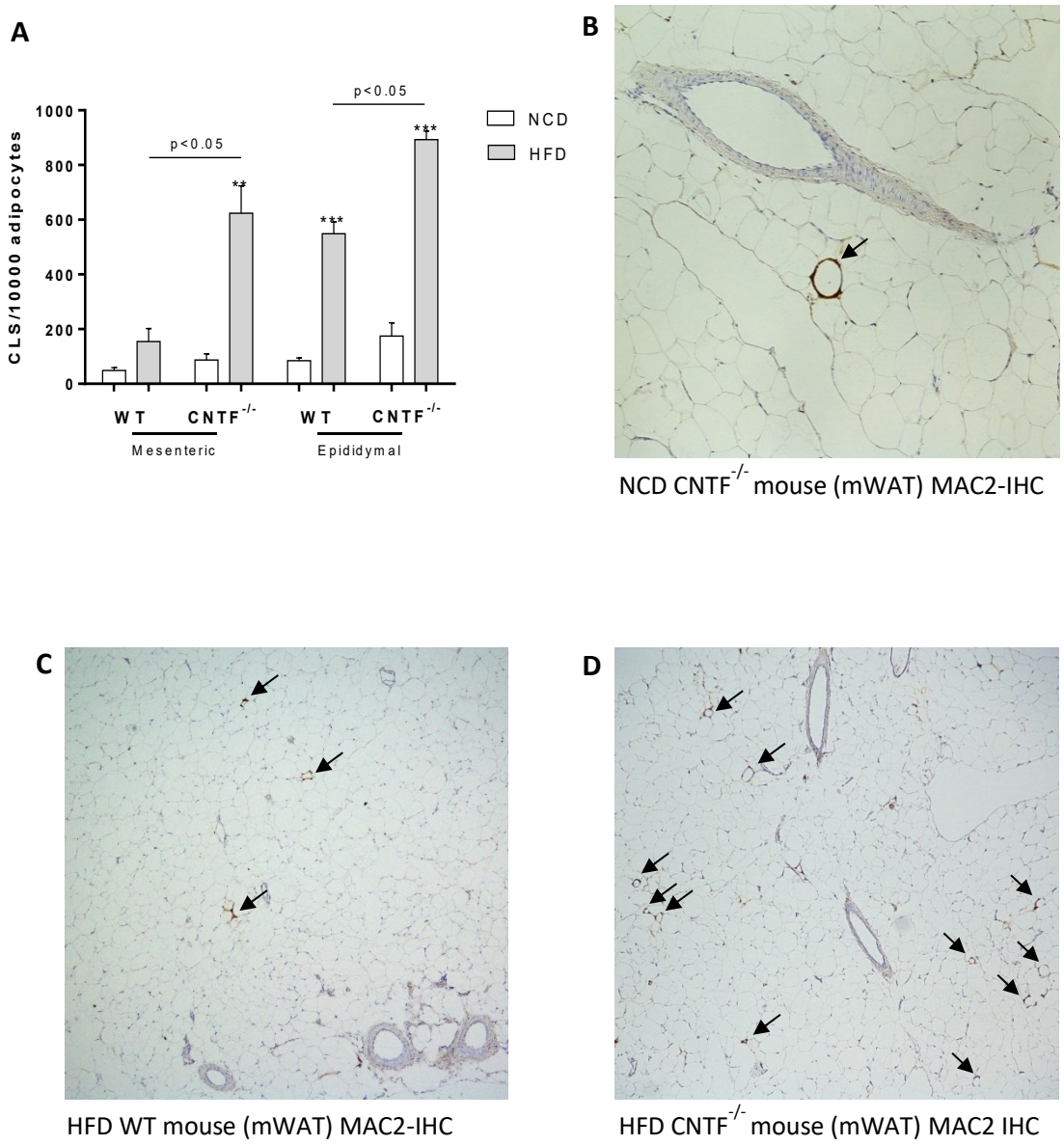

***Figure 3**: **A**) Comparison of CLS density in WT and CNTF$^{-/-}$ mice kept on NCD and HFD condition; **B**) Representative microscopy pictures of MAC-2-immunostained sections of mWAT fat depots from CNTF$^{-/-}$ mouse kept on NCD; **C**) Representative microscopy pictures of MAC-2-immunostained sections of mWAT fat depots from WT mouse kept on HFD; **D**) Representative microscopy pictures of MAC-2-immunostained sections of mWAT fat depots from CNTF$^{-/-}$ mouse kept on HFD; MAC2 positive CLS are identified with arrows; Data are means ± SEM; ** p<0.01, ***p < 0.001 versus NCD.*

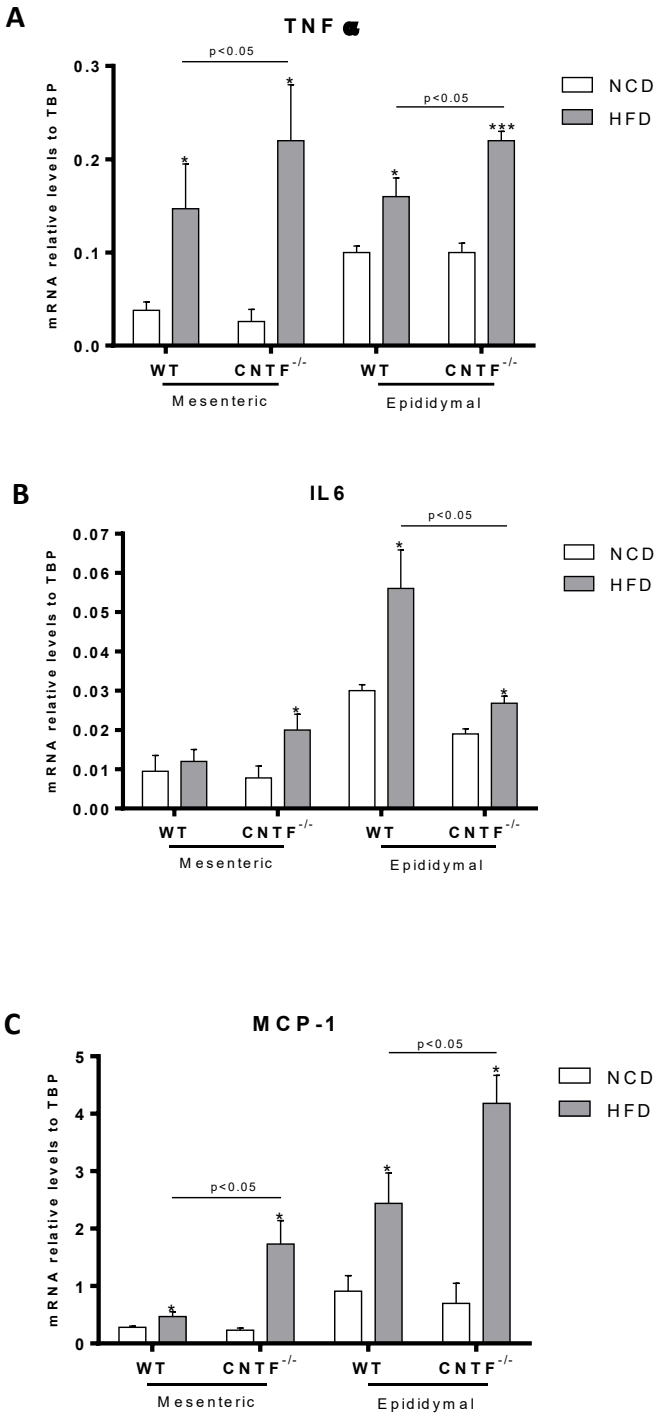

Figure 4: *qRT-PCR analysis of **A**) TNFα, **B**) IL-6, **C**) MCP-1 mRNA levels in mWAT and eWAT fat depots from WT and CNTF$^{-/-}$ mice kept on NCD and HFD; Data are means ± SEM; * p<0.05 versus NCD.*

5.3. CNTF deletion affects adipokines expression in visceral adipose tissue.

Leptin, adiponectin and resistin are hormones endowed with remarkable metabolic activities and produced by white adipocytes. Their dysregulation is associated with several aspects of the complications of obesity, including insulin resistance and T2DM. To assess whether CNTF deletion also involves impairment of the expression and, possibly, secretion of leptin, adiponectin and resistin, we measured by qPCR the levels of gene expression of these adipokines in mesenteric and epidydimal depots from CNTF$^{-/-}$ and WT mice kept in NCD and HFD.

In mWAT, leptin, adiponectin (AdipoQ) and resistin mRNA levels showed a decrease in WT mice fed with HFD compared to WT mice fed with NCD (Fig. 5A-C). Leptin mRNA levels were significantly higher in CNTF$^{-/-}$ mice fed the HFD compared to NCD CNTF$^{-/-}$ mice (Fig. 5A), whereas no differences were present in adiponectin and resistin mRNA levels (Fig. 5B and C).

In eWAT, leptin mRNA was significantly higher in WT and CNTF$^{-/-}$ mice fed the HFD compared to NCD mice (Fig. 5A). Conversely, adiponectin and resistin mRNA levels in CNTF$^{-/-}$ HFD mice were significantly decreased compared with CNTF$^{-/-}$ NCD mice and WT HFD mice (Fig. 5B and C).

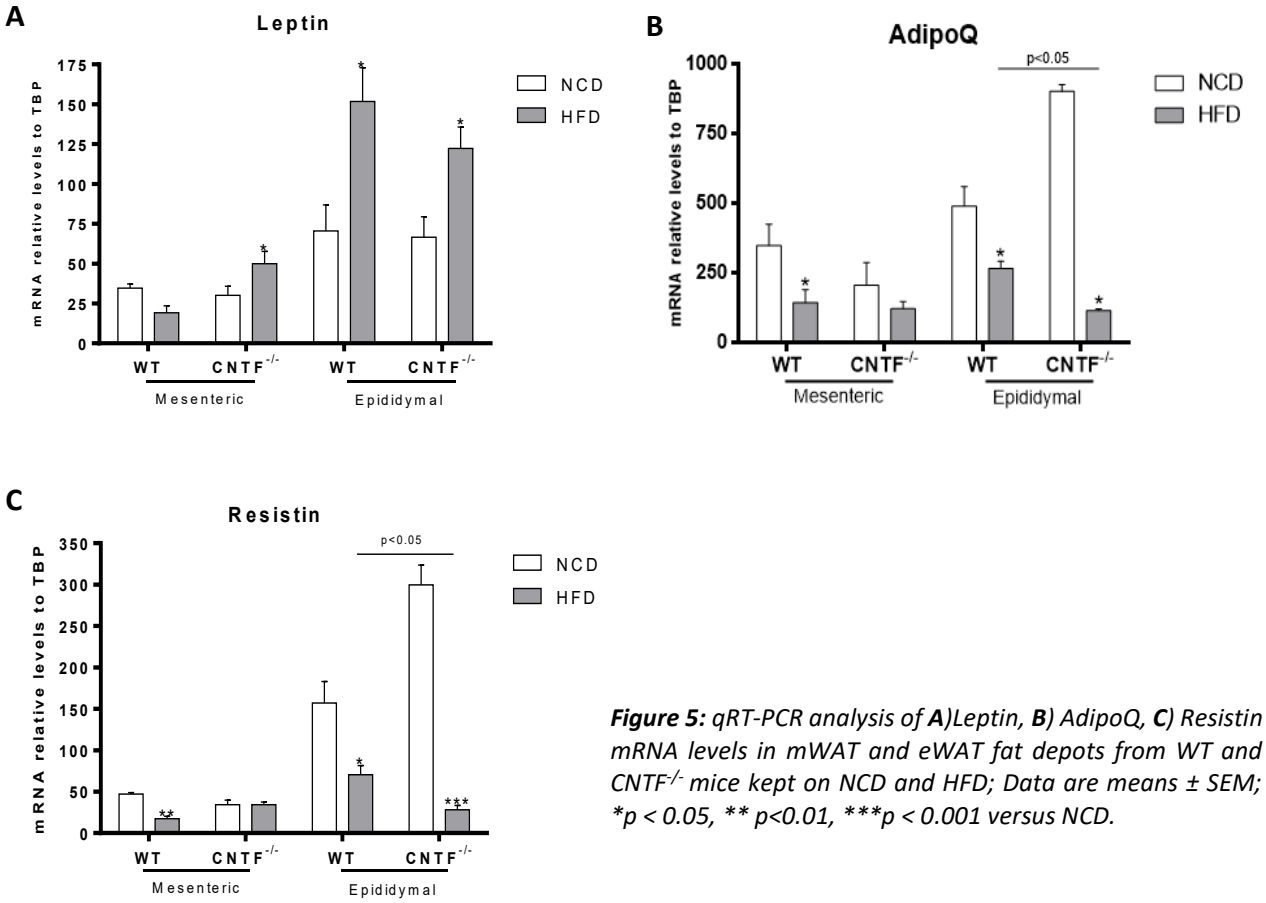

Figure 5: *qRT-PCR analysis of **A**)Leptin, **B**) AdipoQ, **C**) Resistin mRNA levels in mWAT and eWAT fat depots from WT and CNTF$^{-/-}$ mice kept on NCD and HFD; Data are means ± SEM; *p < 0.05, ** p<0.01, ***p < 0.001 versus NCD.*

5.4. CNTF null mice exhibit normal skeletal muscle morphology but have impaired insulin signalling.

To determine the involvement of CNTF on insulin signalling in skeletal muscle, the gastrocnemius muscle from WT and CNTF$^{-/-}$ mice fed NCD or HFD for 13 weeks was dissected and tissue morphology and insulin signalling were analysed.

In sections stained with haematoxylin and eosin, no evident morphological changes were observed in any of the experimental groups analysed (Fig. 6 A-D).

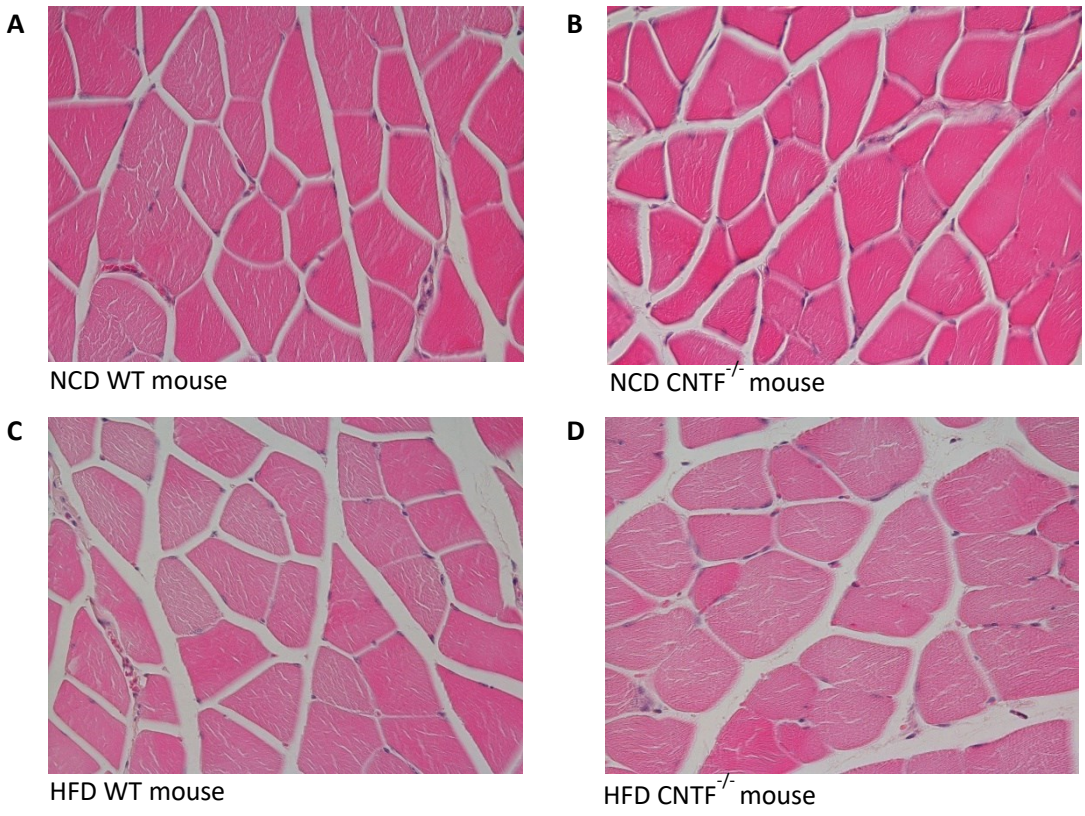

Figure 6: *Representative microscopy pictures of gastrocnemius muscle sections stained with haematoxylin and eosin from NCD WT mouse (**A**), NCD CNTF$^{-/-}$ mouse (**B**), HFD WT mouse (**C**), HFD CNTF$^{-/-}$ mouse (**D**).*

To assess insulin signalling, we evaluated phosphorylation of mammalian target of rapamycin (mTOR) complexes–dependent insulin signalling proteins AKT^{S473}, GSK-3^{bS9}, P70S6K$^{T421/S424}$ and IRS-1^{S312} involved in the insulin pathway by xMAP technology in gastrocnemius muscle biopsies. The mTOR complexes controls insulin signalling by regulating several downstream components involved in glucose homeostasis (AKT^{S473}), glycogenesis (GSK-3^{bS9}), and insulin-resistance (P70S6K$^{T421/S424}$, IRS-1^{S312}) (Yoon, 2017). Interestingly, phosphorylation of AKT^{S473} and P70S6K$^{T421/S424}$ were both reduced in NCD CNTF$^{-/-}$ mice compared to NCD WT mice, whereas only pAKT^{S473} was reduced in HFD CNTF$^{-/-}$ mice compared to HFD WT mice (Fig. 7A and B). pGSK3^{bS9} was significantly reduced in both HFD CNTF $^{-/-}$ mice and WT mice compared with NCD condition (Fig. 7C). In addition, phosphorylation of IRS-1^{S312,} was significantly decreased in CNTF$^{-/-}$ mice compared with WT when mice were exposed to the HFD (Fig. 7D).

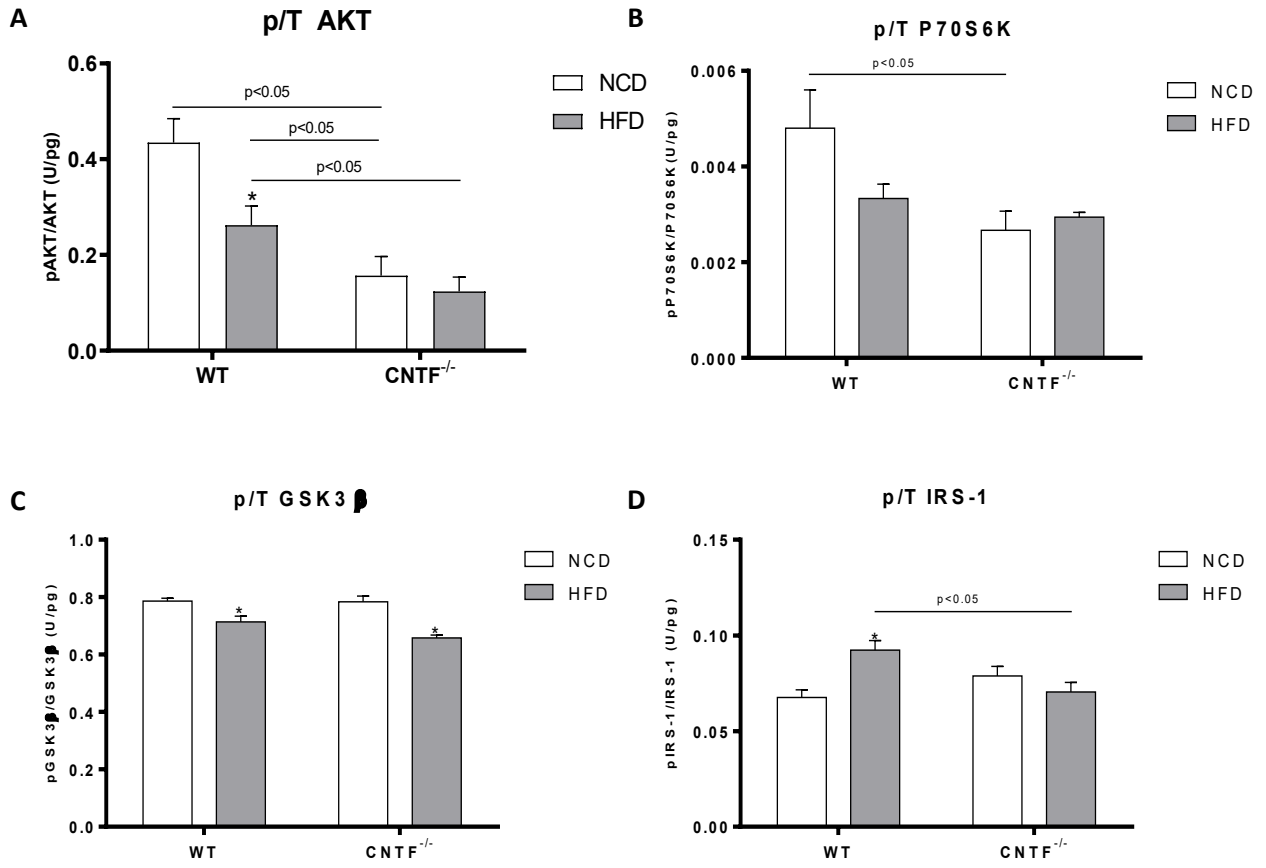

***Figure 7**: phosphorylation levels by xMAP technology of AKT^{S473} (**A**), P70S6K$^{T421/S424}$(**B**), GSK-3^{bS9} (**C**), IRS-1^{S312} (**D**), in gastrocnemius muscle from WT and CNTF$^{-/-}$ mice kept on NCD and HFD. Data are means ± SEM; *p < 0.05 versus NCD.*

5.5. CNTF deletion is associated with hepatic fat accumulation and steatosis.

In order to further investigate the metabolic effects of CNTF deletion on obesity-relevant organs, we examined histological sections of the liver of $CNTF^{-/-}$ mice, and of their control littermates, fed the NCD or the HFD for 13 weeks.

The results showed that, whereas hepatocytes from WT mice fed the NCD were apparently devoid of lipids (Fig. 8A), in the liver from the $CNTF^{-/-}$ mice fed the NCD a consistent number of hepatocytes mainly distributed around the centrolobular vein were filled with lipid vacuoles of different diameter (Fig. 8B). As expected, hepatocytes from HFD WT mice exhibited a consistent amount of intracellular lipids (Fig. 8C). However, a higher number of hepatocytes from HFD $CNTF^{-/-}$ mice contained more numerous and larger lipid droplets, suggesting a more advanced state of steatosis in these latter mice (Fig. 8D).

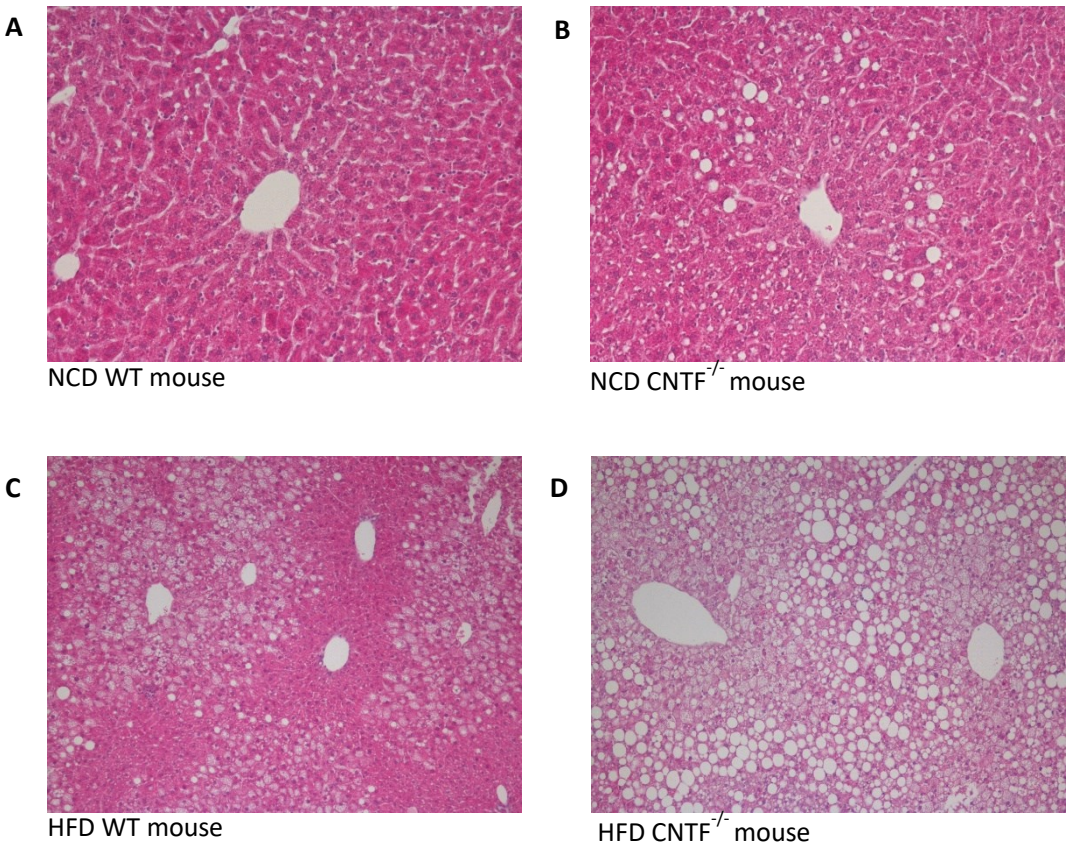

Figure 8: *Representative microscopy pictures of liver sections stained with haematoxylin and eosin from NCD WT mouse (**A**), NCD $CNTF^{-/-}$ mouse (**B**), HFD WT mouse (**C**), HFD $CNTF^{-/-}$ mouse (**D**).*

Evaluation of the phosphorylated proteins involved in insulin signalling by xMAP technology in hepatic biopsies revealed that the liver of NCD CNTF$^{-/-}$ exhibited reduced AKT^{S473} phosphorylation compared to NCD WT mice (Fig. 9A). A significant decrease of phosphorylation of AKT^{S473}, P70S6K$^{T421/S424}$ and GSK3^{bS9} was found in HFD WT and CNTF$^{-/-}$ mice compared to NCD WT and CNTF$^{-/-}$ mice (Fig. 9A-C). Interestingly, phosphorylation of P70S6K$^{T421/S424}$ was significantly increased in HFD CNTF$^{-/-}$ mice compared to HFD WT mice (Fig. 9B). Finally, no evident changes were observed for pIRS-1^{S312} except for a significant increase in the phosphorylation of IRS-1^{S312} in HFD CNTF$^{-/-}$ mice compared to HFD WT mice (Fig. 9D).

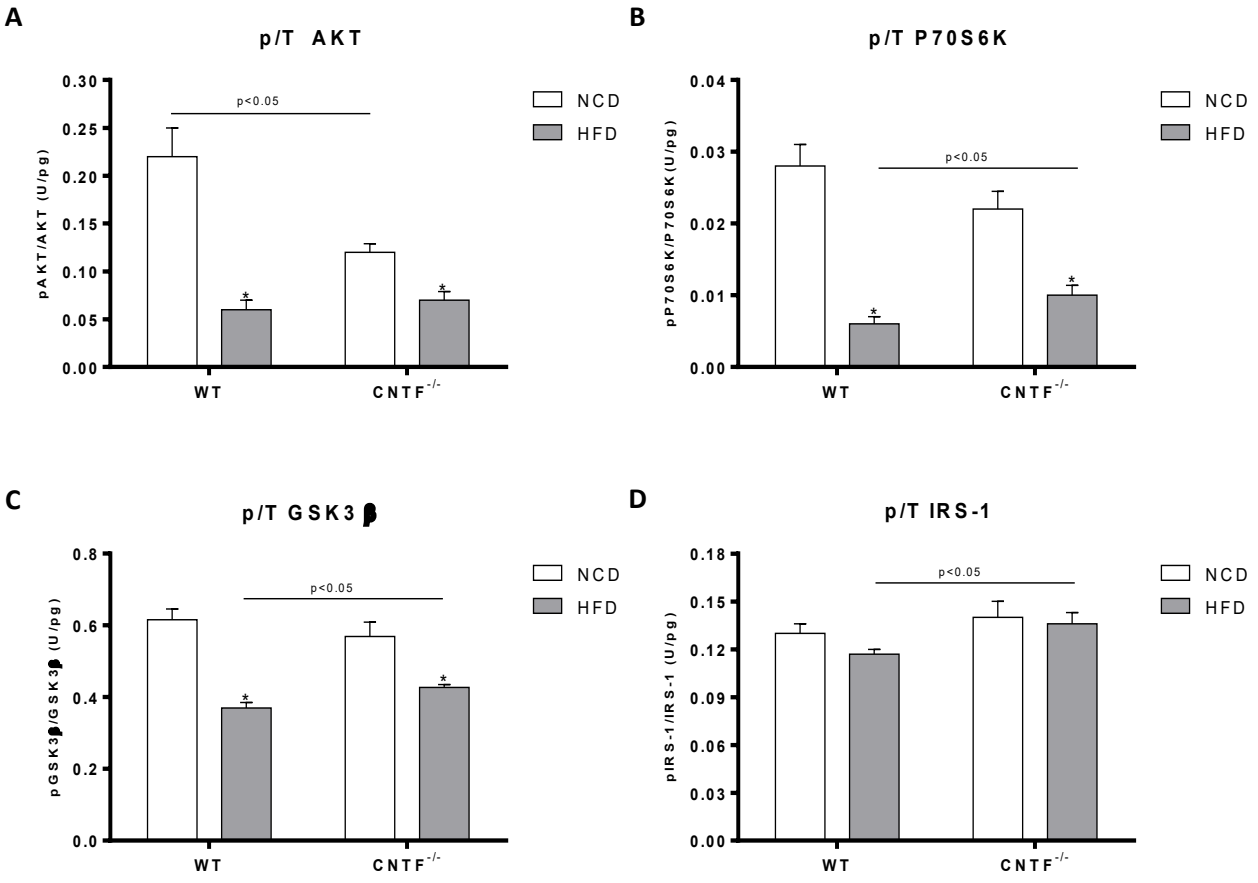

Figure 9: *phosphorylation levels by xMAP technology of AKT^{S473} (**A**), P70S6K$^{T421/S424}$(**B**), GSK-3^{bS9} (**C**), IRS-1^{S312} (**D**), in liver from WT and CNTF$^{-/-}$ mice kept on NCD and HFD. Data are means ± SEM; *p < 0.05 versus NCD.*

5.6. CNTF modulates plasma cytokines secretion and glucose homeostasis.

In order to further characterize the phenotype of $CNTF^{-/-}$ mice, we examined plasma cytokine profile by xMAP technology and plasma glucose and insulin concentration by ELISA. Plasma IL-1β and IL-6 were unchanged in $CNTF^{-/-}$ mice compared with WT mice in both NCD and HFD condition (Fig. 10A and B). As expected, TNF-α levels were significantly higher in HFD WT mice compared to NCD WT mice (Fig. 10C). Plasma IL-10 did not change among the groups except for a significant reduction in $CNTF^{-/-}$ mice compared to WT in NCD condition (Fig. 10D). Plasma levels of IL-1α were significantly higher in HFD $CNTF^{-/-}$ compared to HFD WT mice (Fig. 10E).

In order to assess the effect of CNTF deletion on glucose homeostasis, plasma glucose and insulin levels were examined. Plasma glucose and insulin levels were significantly increased in $CNTF^{-/-}$ mice and in WT mice when exposed to HFD compared with their respective littermates in NCD (Fig. 10F and G) but significant differences were found between KO and WT mice neither in the NCD fed animals nor in the HFD fed mice. As expected, plasma insulin levels were significantly higher in WT and $CNTF^{-/-}$ mice fed the HFD compared to NCD mice. Importantly, however, plasma insulin levels were significantly higher in HFD $CNTF^{-/-}$ mice compared with HFD WT mice (Fig. 10G). Accordingly, the HOMA-IR, a marker of insulin resistance, was significantly higher in $CNTF^{-/-}$ mice compared with WT mice under HDF feeding conditions (Fig. 10H).

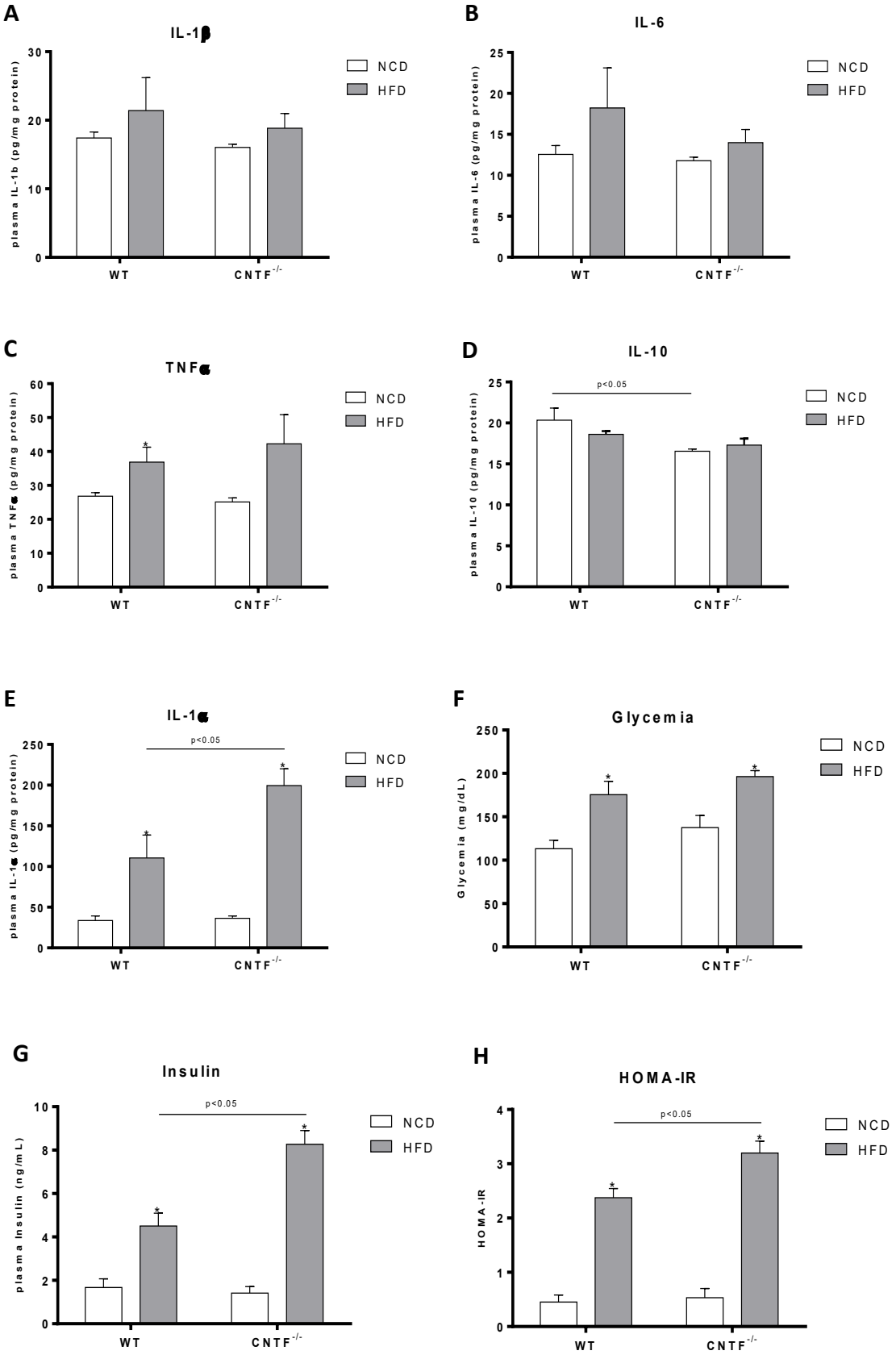

Figure 10: *plasma concentration levels by ELISA of IL-1β (**A**), IL-6 (**B**), TNF-α (**C**), IL-10 (**D**), IL-1α (**E**), glucose (**F**), Insulin (**G**) and HOMA-IR (**H**) from WT and CNTF$^{-/-}$ mice kept on NCD and HFD. Data are means ± SEM; *p < 0.05 versus NCD.*

5.7. CNTF is detectable in the plasma by ELISA.

The CNTF is produced and secreted by cells of the central and peripheral nervous system, it circulates in the blood at very low levels and through the bloodstream can reach target organs. In order to assess whether obesity is involved in a modification of blood CNTF concentration, plasma CNTF levels were analysed by ELISA. As expected, almost null amount of CNTF was detected in plasma from $CNTF^{-/-}$ mice both in NCD and HFD conditions (Fig. 11A). Interestingly, in WT mice fed an HFD for 13 weeks, CNTF plasma levels were higher, even whether not to a significant extent, than WT mice fed NCD (Fig. 11).

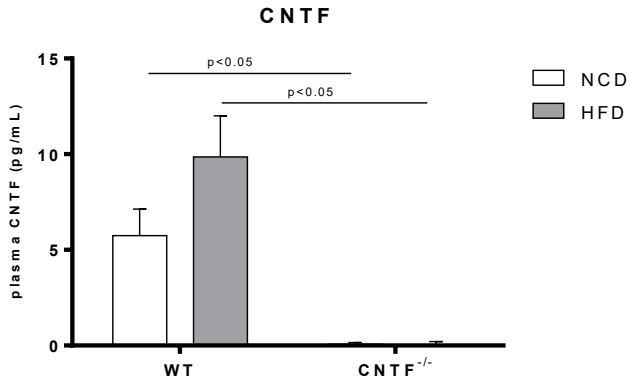

Figure 11: *plasma concentration levels by ELISA of CNTF from WT and $CNTF^{-/-}$ mice kept on NCD and HFD. Data are means ± SEM.*

6. DISCUSSION

As detailed in the Introduction, the CNTF was originally described as a growth factor supporting the survival and differentiation of chick ciliary ganglion neurons. Later, it was proved to be a trophic molecule for a wide variety of motor and sensory neurons in the central and the peripheral nervous system. In the central nervous system, CNTF is constitutively produced by glial cells, whereas in the peripheral nervous system it is produced by Schwann cells and is essential for the postnatal maintenance of motor neurons (Stöckli *et al.,* 1991). For this reason, human recombinant CNTF was tested as a treatment for the neurodegeneration involved in amyotrophic lateral sclerosis (ACTS, 1996; Miller *et al*., 1996). These therapeutic trials failed to provide satisfactory effects on motor performances, but, unexpectedly, CNTF administration led to anorexia and weight loss in these patients. To assess whether CNTF could be used as an anti-obesity drug, the *Regeneron Pharmaceuticals* created a recombinant version of human CNTF with improved potency and stability and coined it Axokine® ($CNTF_{Ax15}$). Subsequent studies confirmed that Axokine administration to humans and experimental animals resulted in decreased food intake, weight loss, and an improvement of obesity-associated hyperglycaemia, hyperinsulinaemia and dyslipidaemia (Gloaguen *et al*., 1997; Lambert *et al*., 2001; Bluher *et al*., 2004). Unfortunately, up to 87% of the Axokine treated patients became refractory to the drug's effects (Preti, 2003), producing neutralizing antibodies against Axokine (Ettinger *et al*., 2003). Therefore, the Food and Drug Administration stopped clinical trials on the possible use of CNTF, or analogues, as anti-obesity agents in humans.

Nevertheless, exogenously administered CNTF plays remarkable effects on energy balance and body weight regulation, holding interesting potentials for the treatment of obesity and its complications. The leptin discovery in 1994 by Friedman and colleagues (Zhang *et al*., 1994) was soon frustrated by the observation that most obese patients are high blood levels of leptin and are resistant to the leptin effects (Unger, 2003). Thus, recombinant human leptin cannot be used as a treatment to counteract obesity. Interestingly, CNTF and leptin receptors co-localize in the hypothalamus (Gloaguen *et al*. 1997) and systemic administration of both CNTF and leptin activates distinctive genes within the arcuate nucleus, suggesting that both cytokines can activate anorexigenic neuronal signalling (Gloaguen *et al*., 1997). In a series of elegant experiments, Gloaguen *et al*. showed that CNTF administration is able to overcome leptin resistance in mice (Gloaguen *et al*., 1997) Indeed, CNTF, differently from leptin, is able to reduce obesity-related phenotypes in *db/db* mice and in leptin resistant HFD-fed obese mice (Gloaguen *et al*., 1997). Recently, we have shown that in both

normally fed and HFD obese animals CNTF activates extracellular signal-regulated kinase signalling in median eminence β1- and β2-tanycytes and promotes leptin entry into and action on the hypothalamic feeding centres (Vanema *et al*., 2020).

Not all the metabolic effects of exogenously administered CNTF are due to its satiety action on brain centres. Indeed, the CNTF receptor α is not only present in the brain but also is widely expressed in peripheral organs and tissues, including white adipose tissue, skeletal muscle, liver and pancreas. Thus, beside a central satiety action, CNTF treatment has been reported to reduce fat accumulation, to improve insulin sensitivity in muscle, liver and fat and to increase energy expenditure by activating brown adipose tissue thermogenesis.

In early studies, mice treated with CNTF exhibited weight loss compared to pair-fed mice, suggesting that the metabolic actions of CNTF could be also related to an increase in energy expenditure (Lambert *et al*., 2001). Indeed, it was subsequently demonstrated that in adipose tissue CNTF increases energy expenditure enhancing fatty acid oxidation by activating the p38 MAPK and AMPK pathways (Crowe *et al*., 2008). We recently confirmed in an in vitro model of human adipocytes, the hMADS adipocytes, that CNTF treatment is able to reduce the expression of lipogenic markers (FAS and SREBP-1) and to increase the expression of lipolytic (HSL and ATGL) and mitochondrial (PGC-1a and CPT1) markers, driving their metabolism toward a less lipid-storing and more energy-consuming phenotype (Perugini *et al*., 2019). In addition to this, CNTF holds interesting therapeutic potentials in the inflamed obese fat. There is a close link between obesity and insulin resistance with the degree of fat inflammation that is strictly dependent on the number of macrophages infiltrating visceral fat (Giordano *et al.*, 2016). Thus, by applying in hMADS adipocytes a TNFα-induced metabolic stress condition, taken as *in vitro* model of adipocyte stress leading to adipose tissue inflammation, we also found that CNTF exerted a beneficial role decreasing the inflammatory state of adipocytes (Perugini *et al*., 2019). Finally, adipose tissue secretes hormones, such as leptin and adiponectin, that control glucose and lipid metabolism (Unger, 2003). Plasma leptin levels are positively correlated with an increase in total body fat mass as a result of more leptin being released from the large and hypertrophic adipocytes (Lonnqvist *et al*., 1997). Adiponectin is a protein secreted exclusively from adipose tissue with insulin sensitizing effect (Barrè *et al*., 2007). Serum adiponectin concentrations are inversely associated with obesity, insulin resistance and type 2 diabetes, whereas increased serum adiponectin concentrations are associated with improved insulin sensitivity (Kadowaki *et al*., 2005; Civitarese *et al*., 2004). Blüher and colleagues observed a significant increase in serum adiponectin in response to $CNTF_{Ax15}$ administration in diet-induced

obesity (DIO) mice, suggesting an involvement of CNTF in the pathophysiology of insulin resistance (Blüher *et al*., 2007). Collectively, these findings indicate that CNTF may have therapeutic properties on the enlarged, inflamed and dysfunctional adipose tissue present in obese patients.

Although CNTF is capable to induce the differentiation of skeletal myoblasts of adult human into multipotent progenitor cells (Chen *et al*., 2015), the interest of CNTF on skeletal muscle has been mainly focused on its insulin sensitizing action. Indeed, CNTF, by activating the AMPK pathway, significantly enhances fatty acids oxidation and reduces insulin resistance (Watt *et al*., 2006).

In the liver, exogenous CNTF has been shown to reduce steatosis and to enhance responsiveness to insulin in *db/db* mice (Sleeman *et al*., 2003). Moreover, plasma glucose and insulin levels were significantly reduced in *db/db* mice treated with $CNTF_{Ax15}$ (Sleeman *et al*., 2003). Finally, Watt and colleagues showed that rats infused with lipid and treated with $CNTF_{Ax15}$ displayed in the liver an increased insulin responsiveness and reduced JNK phosphorylation and NFκB expression (Watt *et al*., 2006).

In the endocrine pancreas, CNTF is able to protect mice against streptozotocin-induced diabetes (a model of type 1 diabetes), reducing amount of β-cells death and protecting pancreatic islets against cytokine-induce apoptosis (Rezende *et al.*, 2012).

Collectively, these experimental evidences show that CNTF administration exert significant anti-obesity and anti-diabetic metabolic effects through a concerted action on energy balance brain centres and metabolically relevant peripheral organs. It should be noted, however, that these data have been obtained mainly *in vitro*, a condition in which it is difficult to reproduce the complexity of *in vivo* biological systems; or in studies where animals were treated with exogenous CNTF, or Axokine, with supra-physiological levels that could cross-react with the receptors of other gp130 cytokine family members. A further major concern to be considered is that exogenous CNTF does not allow to identify whether any tissue-specific metabolic effect could result from a direct action of CNTF or from secondary CNTF-stimulated nervous and/or hormonal mechanisms. Recently, Findeisen and colleagues designed the gp130 IC7Fc ligand, in which a gp130 binding site is removed from IL-6 and replaced with the LIF receptor binding site of CNTF, fused with the Fc domain of immunoglobulin G, producing a cytokine with signal similar to CNTF, but dependent on the IL-6 receptor. IC7Fc has been shown to improve glucose tolerance and hyperglycemia and prevent weight gain and fatty liver disease in mice. In addition, IC7Fc, by activating the transcriptional regulator YAP1, either increases, or prevents, the loss of skeletal muscle mass, the typical pathophysiological condition of patients affected by sarcopenia (Findeisen *et al*., 2019). These

findings have renewed the interest in the therapeutic potentials of CNTF and opened the way to next-generation biological tools for the treatment of obesity and related complications, including type 2 diabetes, steatohepatitis and sarcopenia.

Until now, few studies have addressed the possible role of endogenous CNTF in energy balance regulation in different pathophysiological conditions. In this context, a few years ago we evaluated CNTF distribution and responsiveness in the hypothalamus of mice fed a high-fat diet (HFD), of *ob*/*ob* mice, and of mice fed a calorie restriction (CR) regimen. We found that CNTF expression significantly increased in the ependymal layer of the tuberal and mammillary regions of the hypothalamus of mice kept in HFD and that it significantly decreased in mice kept in CR conditions. Interestingly, changes in CNTF expression matched with changes in its specific receptor, the CNTFRα. As a result, hypothalamic responsiveness to CNTF was greater in HFD than in CR mice, suggesting that in mice an HFD was associated with increased CNTF signalling in the hypothalamus whereas CR was associated with reduced hypothalamic CNTF signalling (Severi *et al*. 2013). These data favour the hypothesis that endogenous CNTF may be involved in some aspects of energy balance regulation at the brain level.

The present study was undertaken to assess whether in mice the presence and activity of CNTF is required for a proper regulation of body metabolism under physiological and/or pathological conditions. To this end, we fed $CNTF^{-/-}$ mice and their corresponding WT mice with an NCD or with an HFD and evaluated some morphological and molecular aspects in peripheral metabolically relevant organs, including the visceral WAT, the skeletal muscle and the liver. First of all, our data show that CNTF deficiency makes mice most prone to obesity when they are subjected to HFD. Indeed, we observed that CNTF deletion results in increased body weight when mice were subjected to HFD but without any significant effect on food intake. Therefore, the higher body weight of $CNTF^{-/-}$ mice exposed to HFD is likely due to a reduction in energy expenditure processes.

CNTF deficiency in mice appears to exacerbate obesity-induced adipose tissue derangement and inflammation. The visceral WAT depots of CNTF null mice exposed to HFD were characterized by a higher degree of macrophage infiltration and fat inflammation as revealed by the increased number of CLSs, where death adipocytes are surrounded by aggregates of activated macrophages. On the other hand, subtle morphological adipocyte alterations even occur in NCD fed mice, where the $CNTF^{-/-}$ mice show larger adipocytes both in mWAT and eWAT depots when compared to NCD WT mice, highlighting a more lipid-storing prone phenotype in the $CNTF^{-/-}$ mice. The morphological changes observed in the HFD fed $CNTF^{-/-}$ mice matched with an increased expression of pro-

inflammatory genes, such as MCP1 and the TNF-α. Finally, CNTF deletion seems also to affect adiponectin expression in WAT, in accordance with the increase of serum adiponectin found in DIO mice after $CNTF_{Ax15}$ administration (Blüher *et al*., 2007). Indeed, we found that $CNTF^{-/-}$ mice exposed to HFD a significant decrease in adiponectin expression levels in visceral depots compared to their HFD WT littermates. Low adiponectin plasma levels are related to severe obesity and insulin resistance (Arita *et al*., 1999) and the reduced production of adiponectin by WAT can be involved in the condition of insulin resistance we found in the HFD fed $CNTF^{-/-}$ mice.

The skeletal muscle is the largest insulin-sensitive tissue that contributes up to 90% to the insulin-stimulated glucose disposal in healthy individuals (Kristiansen and Mandrup-Poulsen, 2005). CNTF null mice have impaired insulin signalling both in NCD condition and when subjected to the HFD. Indeed, phosphorylation of AKT^{S473} and $P70S6K^{T421/S424}$ were both reduced in NCD $CNTF^{-/-}$ mice compared to WT mice when fed chow diet. Notably, $pAKT^{S473}$ levels in NCD $CNTF^{-/-}$ mice are lower than in HFD WT mice indicating that a state of insulin resistance had occurred in $CNTF^{-/-}$ mice despite being in NCD. Moreover, $pAKT^{S473}$ was reduced in HFD $CNTF^{-/-}$ mice compared to HFD WT mice. In striking contrast, $pIRS\text{-}1^{S312}$ levels were significantly decreased in $CNTF^{-/-}$ mice compared with WT when mice were exposed to HFD. Serine phosphorylation of IRS proteins reduces the ability of IRS proteins to attract PI 3-kinase (AKT1), thereby minimizing its activation; excessive serine phosphorylation of proteins impairs the metabolic insulin signalling downstream, causing insulin resistance (Draznin, 2006). Collectively, these data show that the state of insulin resistance observed in CNTF null mice could be related to the reduction of AKT^{S473} and $P70S6K^{T421/S424}$ phosphorylation. This could suggest that the impairment of insulin signalling may occur downstream of mTORC complex but not at the IRS-1 level.

In the liver, CNTF deficiency resulted in an abnormal storage of lipids into hepatocytes both in NCD and HFD mice. Whereas hepatocytes from WT mice fed the NCD were normal, in the liver from the $CNTF^{-/-}$ mice fed the NCD numerous hepatocytes mainly distributed around the centrolobular vein were filled with differently sized lipid vacuoles. In the HFD fed mice, a higher number of hepatocytes from $CNTF^{-/-}$ mice contained more numerous and larger lipid droplets, suggesting a more advanced state of steatosis in comparison with the WT mice. In addition to this, CNTF null mice in HFD show an impaired insulin signalling in the liver due to a reduced phosphorylation of $P70S6K^{T421/S424}$ and $GSK3^{bS9}$ comparable to that observed in HFD WT mice. This data is confirmed by a significant increase of phosphorylation of $IRS\text{-}1^{S312}$ in HFD $CNTF^{-/-}$ mice compared to HFD WT mice.

Interestingly, phosphorylation of AKT^{S473} was reduced in NCD $CNTF^{-/-}$ mice compared to NCD WT mice.

The first link between an increase of proinflammatory cytokine plasma concentration and insulin resistance was provided by Hotamisligil *et al*. in 1993, which demonstrated that adipocytes express the proinflammatory cytokine TNFα and that its expression increases under obesity (Hotamisligil *et al*. 1993). Further studies have confirmed that obesity is a state of systemic chronic inflammation, as indicated by increased plasma concentrations of C-reactive protein (CRP), interleukin-6 (IL-6), and plasminogen activator inhibitor-1 (PAI-1). In our study it has been observed that, in HFD mice, the CNTF deficiency phenotype was related to a multi-organ inflammatory state, with numerous evidences in adipose tissue, skeletal muscle and liver. In order to understand whether this inflammation had become systemic, we evaluated IL-1β, IL-6, TNFα, IL-10 and IL-1α plasma concentrations. No relevant changes were noted except for the pro-inflammatory IL-1, that was significantly higher in HFD-CNTF null mice compared to HFD WT mice. This finding suggested that the lack of CNTF in mice, when subjected to a stress metabolic condition such as HFD, seems to make them prone to a state of blunt-generalized inflammation.

As expected, under HFD conditions, both $CNTF^{-/-}$ and WT mice were hyperglycaemic and insulin resistant. Interestingly, plasma insulin levels were significantly higher in HFD $CNTF^{-/-}$ mice compared to HFD WT mice. HOMA-IR, a marker of insulin resistance, was significantly higher in $CNTF^{-/-}$ mice compared with WT mice after HDF feeding, confirming again that obese $CNTF^{-/-}$ mice were more insulin-resistant than the obese littermates.

As far as is known, CNTF is produced and secreted by glial cells in the central and peripheral nervous system and it circulates in the blood at very low levels (Iłzecka, 2003) Accordingly, we detected CNTF in the blood of WT mice at very low concentrations (of the order of pg/ml). Interestingly, its concentration showed a tendency to increase in HFD-WT mice. Altogether, our data support a model where the CNTF is oversecreted under HFD fed conditions by some yet unidentified cellular sources and through the blood reaches target organs where promote energy metabolism and try to counteract the deleterious consequences of obesity.

In conclusion, our preliminary data show that CNTF is found in the circulation and exerts systemic metabolic effects. Its absence in $CNTF^{-/-}$ mice involves significant metabolic alterations in obesity-relevant organs such as white adipose tissue, skeletal muscle and liver. From a basic science standpoint, characterization of $CNTF^{-/-}$ mice could open new understandings of CNTF metabolic effects, its main target organs/cells and could also disclose novel aspects of the crosstalk among

peripheral organs/structures and between these and the energy balance neurocircuits. From a translational perspective, the demonstration of a metabolic role for endogenous CNTF would instigate fresh studies of the pathogenic mechanisms of obesity. The demonstration that CNTF is a circulating protein, whose levels change in specific pathological conditions such as obesity, could lead to its use as a novel, practical diagnostic and prognostic blood marker of obesity, diabetes, and related diseases.

7. REFERENCES

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