UNIVERSITA' DEGLI STUDI DI PARMA

Dottorato di ricerca in Biologia del Comportamento

Ciclo: XXIV

Social stress, obesity and glucose tolerance: a psychobiological investigation

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A Michela

L'amore non svanisce mai.
La morte non è niente,
io sono solo andato nella stanza accanto.

Io sono io. Voi siete voi.
Ciò che ero per voi lo sono sempre.
Datemi il nome che mi avete sempre dato.
parlatemi come mi avete sempre parlato.
Non usate un tono diverso.
Non abbiate un'aria solenne o triste.
Continuate a ridere di ciò che ci faceva ridere insieme.
Sorridete, pensate a me, pregate per me.
Che il mio nome sia pronunciato in casa come lo è sempre stato,
senza alcuna enfasi, senza alcuna ombra di tristezza.
La vita ha il significato di sempre.
Il filo non si è spezzato.

Perchè dovrei essere fuori dai vostri pensieri?
Semplicemente perchè sono fuori dalla vostra vista?
Io non sono lontano, sono solo dall'altro lato del cammino.

C. Peguy

Index

Introduction

Assumption

Stress is a part of daily life. The social and physical environments in which we live have an enormous impact on our physiology and behavior and influence the process of adaptation . Interpersonal conflicts, a hostile work environment, low socioeconomic status, or lack of social support can all have a negative effect on health. Exposure to chronic stress is associated with a higher risk for affective disorders, impaired immune and reproductive function, and cardio metabolic dysfunction (McEwen and Wingfield 2003, McEwen 2001). It is important, therefore, to identify mechanisms by which social stress can contribute to the development of these disorders, and this may ultimately lead to new interventions to prevent or limit the severity of stress-related disease (Scott, Tamashiro and Sakai 2012)

Stress

All living organisms strive towards a dynamic equilibrium, called *homeostasis*, that is constantly challenged by certain internal or external events termed as *stressors* (Chrousos and Gold 1992, McEwen 2000, Sapolsky, Romero and Munck 2000). The basic concept of stress in physiology and biomedical research was originally introduced by Selye (Selye 1950) described as a non-specific response of the body to an external noxious stimulus. Later in time the concept of stress was redefined by distinguishing between *stressor* and *stress response*. The *stressor,* is defined as the causative event real or perceived threats the homeostasis (Goldstein and Kopin 2007). Stressor may be classified in terms of is nature as physical (e.g. cold, heat, radiation, noise, vibration, chemical

stressors, pain, and immobilization), psychological (e.g. animals handling, restrain) and social stressors (e.g. unemployment, marital separation, death of partner, defeat). In terms of duration, stressors may be either acute (single, intermittent, time-limited exposure) intermittent (continuous long-term prolonged exposure, intermittent long-term exposure) or chronic (Kvetnansky, Sabban and Palkovits 2009).

The response to an acute stress activates the stress response system, (also called the *fight or flight* response (Cannon 1926)) consisting in physiological (increase respiratory and cardiovascular activity, decrease no-vegetative functions) and behavioral mechanisms that allow the body to adapt and respond to challenges of homeostasis (Cannon 1926, Cannon 1929, Romero 2004) (**Fig. 1**). Individual differences in appraisal of a stressor may also determine the degree of perceive stress and how the individual cope with it (Weiner, Freedheim and Schinka 2003).

The adaptive physiological response to acute stress involves a process called allostasis (Sterling 1988), in which the internal milieu is maintained in a process of achieving stability trough changes in anticipation of physiological set-points of the homeostatic mechanism (Chrousos 2009). The allostatic response is necessary for an organism to cope with stress and be adaptive in the short run. However, if recovery from the acute event is not accompanied by an adequate homeostatic response, the deleterious effects of stress-mediator on psychological and physiological function, termed the "allostatic load," will occur (**Fig. 2**) (McEwen and Stellar 1993, McEwen 1998b, Chrousos and Gold 1992).

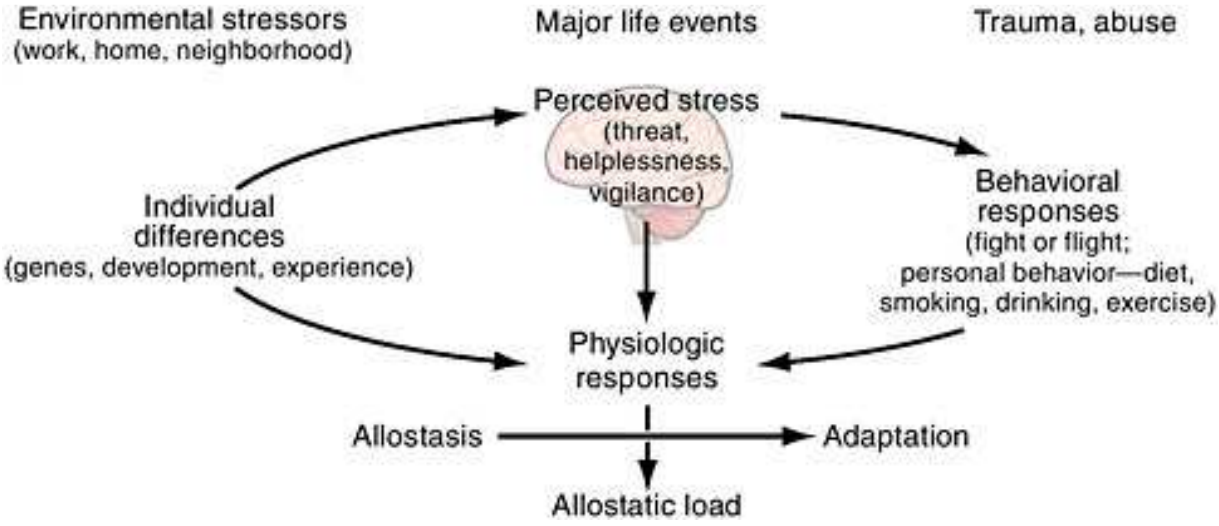

Figure 1 The stress response and development of allostatic load. The perception of stress is influenced by one's experiences, genetics, and behavior. When the brain perceives an experience as stressful, physiologic and behavioral responses are initiated, leading to allostasis and adaptation. Over time, allostatic load can accumulate, and the overexposure to mediators of neural, endocrine, and immune stress can have adverse effects on various organ systems, leading to disease. (McEwen 1998a)

A recent re-conceptualization of stress terminology led Koolhaas and colleagues (Koolhaas et al. 2011) toemphasize that physiological response to stressor by itself does not always indicate a stress event. They propose that the use of *stressor* and *stress response* should be restricted of a condition of unpredictability and uncontrollability. The unpredictable situation should be characterized by the absence of an anticipitatory response while the time for recovery, rather than the amplitude of response, can determine an uncontrollability event.

Stress response

The allostatic system involves a interrelationship between endocrine and nervous system to activate stress response, or prototypical stress response that determines changes in hormones secretory patterns (Sapolsky et al. 2000) (**Fig. 3**). The principal effectors are glucocorticoids (GCs) and cathecholamines.

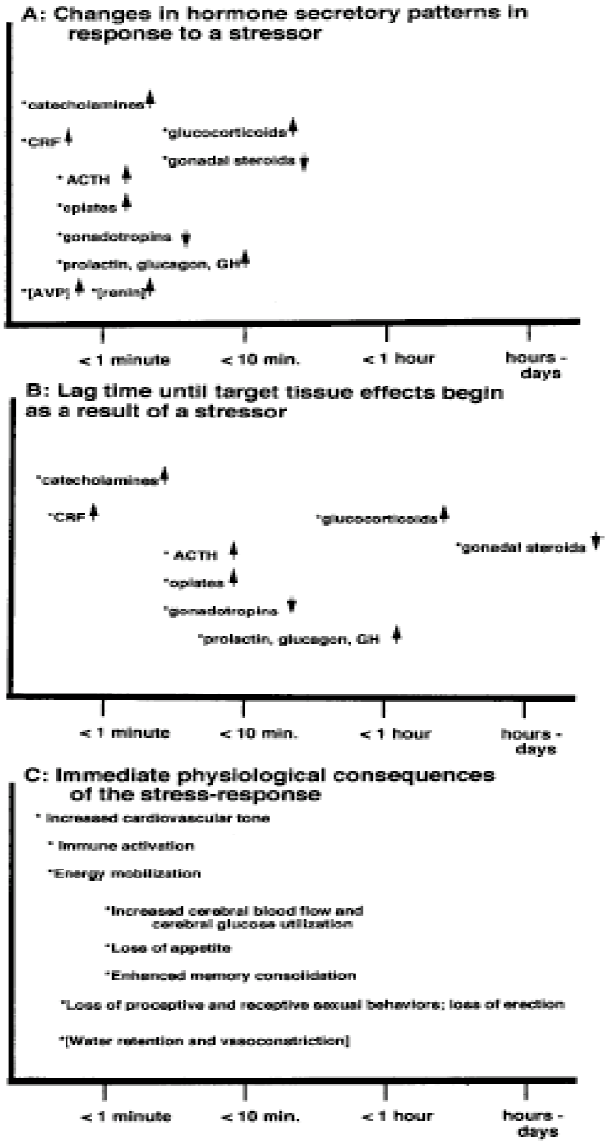

Figure 3: Prototypical stress response

The Sympathoadrenal Axis (SAM) is a part of sympathetic nervous system and is responsible for initiating the *flight or fight* response while the Hypothalamic Pituitary Adrenals axis (HPA) describes the second part of the stress response. Both these two systems have a crucial function in the metabolic and cardiovascular preparation of the body to perform behavior.

Regarding SAM system, stress activates the *locus coeruleus* which results in the release of catecholamines, norepinephrine (NE) and epinephrine (Epi), from the adrenal medulla (**Fig.4**) (Charney 2004). As the medulla is stimulated by nervous impulses the SAM axis serves as a general alarm system (Weiner et al. 2003). Activation of *locus coeruleus* inhibits parasympathetic outflow and neurovegetative function, including eating and sleep (Goldstein 2003).

Several hypothalamic nuclei participate in the organization of responses to different stressors. Stress activates neurons in the paraventricular nucleus (PVN) of the hypothalamus that mainly activate the parvocellular conticotropin-releasing hormone (CHR) and the arginine-vasopressin neurons of the paraventricular nucleus (AVP) (Chrousos and Gold 1992). During an acute stress the paraventricular nucleus (PVN) of the hypothalamus regulates the anterior pituitary secretion of adrenocorticotropin (ACTH), mainly via CHR, while AVP contributes synergistically (Vale et al. 1981, Lamberts et al. 1984, Kyrou and Tsigos 2009). In turn ACTH triggers release of GCs from adrenal cortex (**Fig.4**).

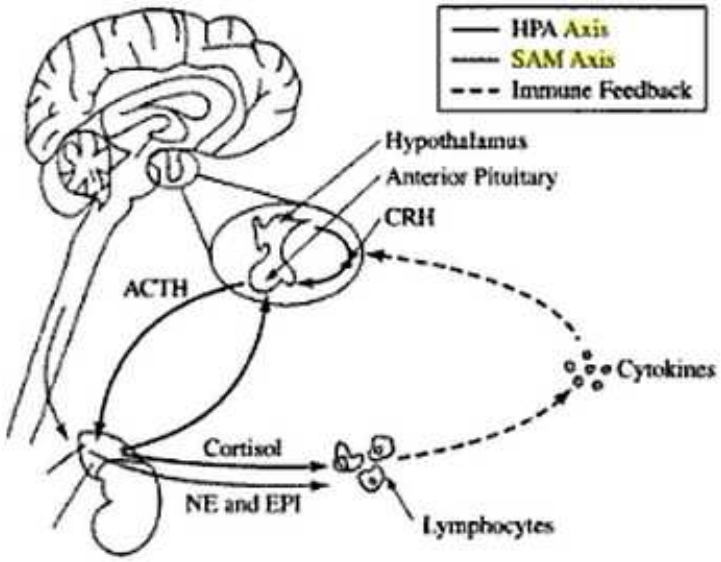

Figure 4: HPA axis. Stress activates the CNS, causing the release of CRH from hypothalamus. CRH stimulates the release of ACTH from anterior pituitary, which in turn stimulates the release of cortisol from adrenals. Cortisol also feedback the anterior pituitary, suppressing the release of more ACTH.
SAM axis. Stress stimulates nerve that directly innervate the adrenal medulla, which in turn release norepinephrine (NE) and epinephrine (EPI) in the bloodstream (Weiner et al. 2003)

Once secreted GCs are transported from corticosteroid-binding globulin (CBG) to target tissues, it will be converted to the active form though the activity of 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) enzyme. Another isoform , 11β-HSD2, is present to convert active cortisol (Corticosterone in rodents) (Chrousos and Gold 1992) to inactivate cortisone. Cortisol binds his receptors and exerts a negative feedback on PVN by inhibiting CHR and ACTH secretion (Charmandari, Tsigos and Chrousos 2005). GCs potentially affect overall body metabolism activating or repressing genes involved in catabolic or anabolic pathways to allow the body to cope with stress (Lowell and Spiegelman 2000, Kyrou and Tsigos 2009). GCs increase hepatic gluconeogenesis and elevate plasmatic glucose concentration , induce lipolysis and degradation of

proteins to provide an immediate energy (Pasquali et al. 2006). Furthermore GCs antagonize the anabolic action of grow hormone (GH), sex steroids and insulin (counter-regolatory effect) (Tsigos and Chrousos 2002). GCs secretion in response to an acute stress has also been related with increase of high palatable (sweet, high fat) food intake (Dallman et al. 2004).

As a part of an adaptive response to stress, the activation of the HPA axis and the SAM are programmed to be activate for a limited duration. When the stress response system is chronically activated, pathophysiological states can arise in susceptible individuals (McEwen and Wingfield 2003, de Kloet, Joëls and Holsboer 2005, Koolhaas et al. 2011).

Stress and obesity

Obesity is a pathology characterized by an excessive accumulation of fat depots with a multi-factorial etiology (Bouchard 1991). Failure of chronic stress adaptation, has been associated with metabolic disease pathogenesis such as obesity and metabolic syndrome (Björntorp 2001).

HPA hyper-activity is considered a predictor factor for obesity and comorbid pathologies (Pasquali et al. 2006, Rosmond and Björntorp 2000, Björntorp 2001). As described previously during an acute stress response GCs are characterized by having a lipolytic action, while excessive endogenous production are associated to increase adiposity. The link between GCs and increased visceral adiposity is clearly demonstrated in patience with Cushing syndrome (Cushing 1932) and patients treated with corticosteroids (Hollifield 1968) that display body weight gain, hypertension and are at the increased risk factor for developing diabetes (Friedman et al. 1996). Moreover subjects suffering depression show hypercortisolemia and increased food intake, leading to an obese phenotype (Gold, Goodwin and Chrousos 1988). Thus, the anabolic effects of elevated GCs result from combination of increase food intake and increased sensitivity to GCs in different metabolic tissues (Peckett, Wright and Riddell 2011) .

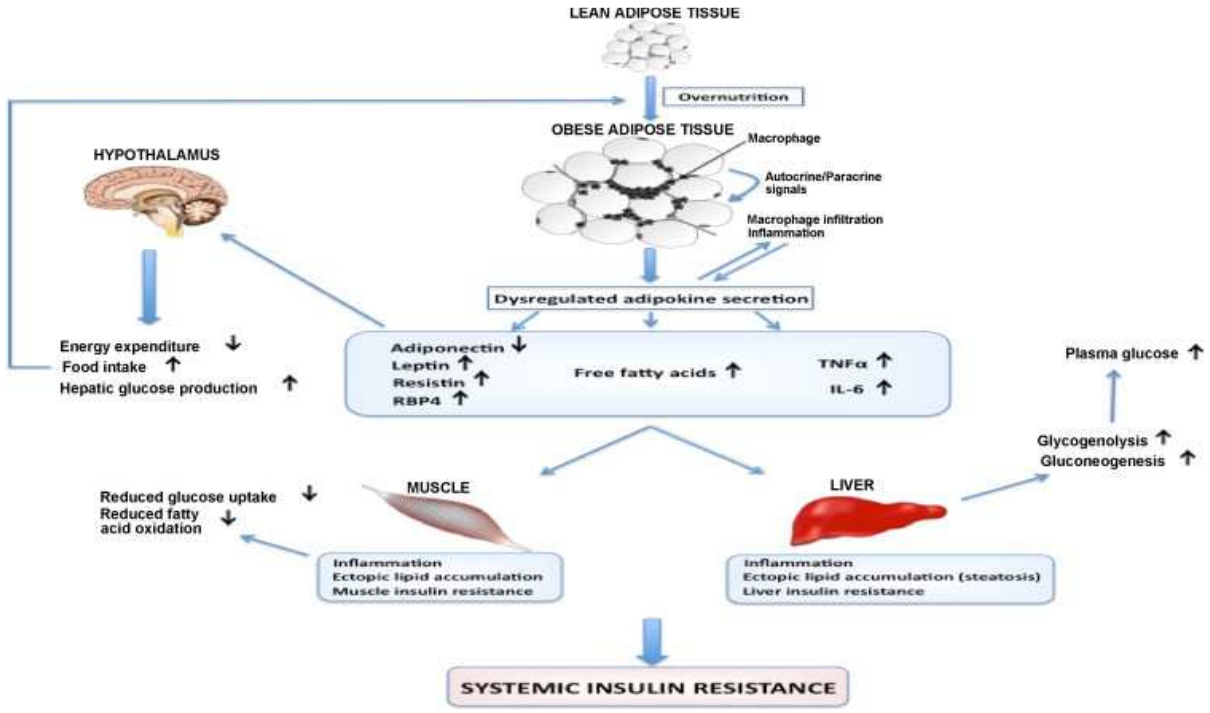

Figure 5: Obesity-induced changes in adipokine secretion and the development of insulin resistance. Expansion of adipose tissue in obesity leads to increased macrophage infiltration and inflammation with enhanced production of pro-inflammatory cytokines such as TNF and IL-6. This is accompanied by an increased release of free fatty acids and dysregulated secretion of leptin, adiponectin, resistin and retinol binding protein-4 (RBP4). Together, these adipocyte- and macrophage-derived substances can act in a paracrine or autocrine fashion to further exacerbate adipose tissue inflammation. On the systemic level, altered adipokine secretion can lead to increased food intake and reduced energy expenditure through actions in the hypothalamus and to decreased muscle and liver insulin sensitivity through enhanced ectopic lipid deposition and inflammation. (Galic, Oakhill and Steinberg 2010)

Increased levels of 11β-HSD1 are found in the adipose tissue of obese subjects. 11β-HSD1 enzyme leads to an increase in local GCs concentration and result in obesity as demonstrated in transgenic animal models (Masuzaki et al. 2001) and in humans (Paulsen et al. 2007). Beside induce adipose cells hypertrophy GCs stimulate adipogenesis (Rosen and Spiegelman 2000) promoting the differentiation of preadipocytes into mature adipocytes. In the liver GCs increase the activities of

enzymes involved in fatty acid synthesis promote the secretion of lipoproteins and induce the hepatic gluconeogenic pathway (Ottosson et al. 1994, Wang 2005) (**Fig. 5**). The overall result being hyperglycemia.

Stress induced GCs secretion enhance NPY (Zukowska-Grojec 1995) which can explain the increased food intake in obese patients. Recent work from Kuo et al (Kuo et al. 2007) has demonstrated the presence of NPY/Y2-receptor pathway in adipose tissue (most on white adipose tissue, WAT). During chronic stress NPY is released from neurons where induce angiogenesis, pre-adipocyte proliferation and maturation.

Elevated GCs concentration can led to obesity also by regulating appetite. Increased GCs induce rodents (Dallman, Pecoraro and la Fleur 2005) and human (Dallman et al. 2004) to ingest more food, especially high caloric food. Both diet and stress are etiological factors for the development of eating disorders such as binge eating disorders (BED) (Hagan et al. 2003). GCs effects on food intake are mediated trough the arcuate nucleus (ARC) of the hypothalamus. ARC neurons involved in orexigenic (propiomelanocortin (POMC)-containing neurons) and anorexigenic (neuropeptide Y (NPY)/Agouti related protein (AGRP) neurons) regulation express leptin, ghrelin and insulin receptors (Charmandari et al. 2005).

All these findings appear to account for the idea that stress cortisol levels may inhibit catabolism and be responsible for the frequently observed relationship between hypercortisolemia and central (abdominal, visceral) fat deposition.

Adipose tissue is not only important as a store tissue but can be considered a endocrine organ (Galic et al. 2010). Adipose tissue secretes circulating factors called adipokines such as leptin, adiponectin, resistin, TNFα and IL-6, that play an important role in obesity and T2D pathogenesis.

Leptin: Leptin is potent satiety-stimulating hormone (anorectic effect) able to increase energy expenditure and prevent weight gain. Elevated level of GCs stimulate leptin secretion (Zakrzewska et al. 1997, Zakrzewska et al. 1999). Plasma leptin increase in obese patient and decrease with weight loss consistent with his role as signal of adipose tissue stores (Havel et al. 1996, Lönnqvist, Wennlund and Arner 1997). The leptin receptor ObR is found in high concentration in hypothalamic areas regulating food intake and also in peripheral tissue with more abundance in adipose tissue (Tartaglia 1997). Leptin binds his receptors in the hypothalamus where it inhibits the secretion of hypothalamic NPY and CRH, while it stimulates arcuate nucleus POMC neurons that secrete α-MSH, a potent anorexic and thermogenic peptide (Heiman et al. 1997) (**Fig. 6**).

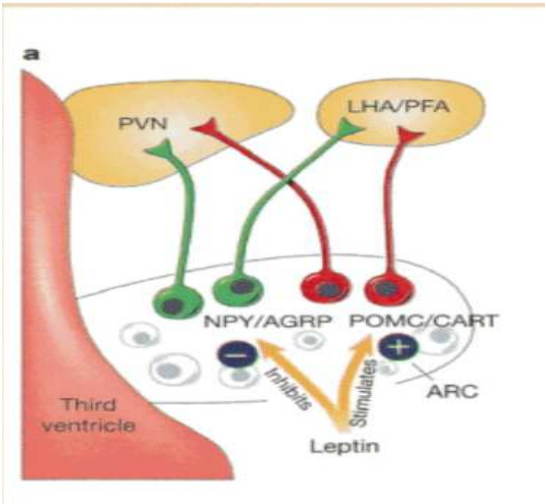

Figure 6: NPY/AGRP and POMC/CART neurons in the arcuate nucle, are first-order neurons in the hypothalamic response to the circulating adiposity signals insulin and leptin. (Schwartz et al. 2000)

The absence of leptin found in Ob/Ob mice (INGALLS, DICKIE and SNELL 1950) or ObR, found in db/db mice (Chen et al. 1996) leads to uncontrolled food intake and obesity. Leptin effects on metabolism is not only limited to hypothalamus but other tissues express ObR (Tartaglia 1997), it acts directly on skeletal muscle to increase FA oxidation (Muoio et al. 1997). In humans monogenic form of obesity caused by a deficit in leptin is rare (Farooqi and O'Rahilly 2005), instead the higher percentage of population show elevated leptin concentration that lead to an impaired sensitive and resistance to this hormone a syndrome called “leptin resistance”.

Adiponectin:

Adiponectin improved insulin sensitivity in model of genetic and diet-induced obesity decreasing plasma FA, TGs content in muscle and liver and enhance insulin-stimulate tyrosine phosphorylation of insulin receptor and insulin receptor substrate 1 (Díez and Iglesias 2003). Adiponectin has an anti-inflammatory effect reducing TNF-α secretion, indeed experiments with adiponectin knock-out show that these mice have high plasma TNF-α and injection of viral-mediated adiponectin reverses this effect (Maeda et al. 2002). Kubota et al. (Kubota et al. 2007) have shown that adiponectin regulates energy expenditure through activation of AMPK in the hypothalamus. They show that in the hypothalamus, AdipoR1 and AdipoR2 colocalize with the leptin receptor ObR (Kubota et al. 2007). Plasma Adiponectin, contrary to leptin, is decreased in obesity and increased with weight loss (Ukkola and Santaniemi 2002).

TNF-α and IL-6. Tumor necrosis factor- α and IL-6 are cytokines cytokines found to be increased in WAT and plasma of obese subjects. Increased visceral mass in obesity pathology is also considered a chronic low-grade inflammation condition (Monteiro and Azevedo 2010) associated with macrophages infiltration (Coenen et al. 2007). TNF- α is primary secrete by M1 (classically activated) macrophage, impaired insulin signaling, glucose uptake and reduce FA oxidation in hepatocyte and skeletal muscle cells (Moller 2000). TNF-α induces also insulin resistance in vitro and in vivo both in animals and humans (Hotamisligil 1999). IL-6 concentration is positively correlated with body mass and FA. IL-6 has been demonstrated to inhibit the insulin signaling pathway, determine a reduction in insulin-induced insulin receptor and IRS-1 phosphorylation Furthermore, IL-6 can promote fatty acid oxidation and glucose uptake in skeletal muscle (Galic et al. 2010).

The role of social stress in obesity

The most commonly used species to study chronic stress are rodents and non-human primates, as they share many of the same neuroendocrine systems and anatomical structures (construct validity) with human. They also share many similar behavioral and physiological responses to chronic stress, and often respond in the same way to pharmacological compounds (Scott et al. 2012). Like humans, many rodents and non-humans primates are social animals, and interactions with conspecifics are common and can be considered a source of both support and stress in their natural environments.

In humans (Chandola, Brunner and Marmot 2006, McKittrick 2001) and animals (Koolhaas et al. 1997, Sapolsky 2005) among the major advantages of living in social groups, social support and cooperation stand out, although there are also significant disadvantages arising from social conflict and competition. Thus the development of appropriate animal models may allow investigating the relationship between chronic social stress and neuroendocrine dysfunction.

In social species, agonistic behavior displayed during social interactions plays a fundamental role in determining and/or maintaining the social position or dominance of an individual within a group (Koolhaas, Schuurman and Wiepkema 1980, Koolhaas et al. 1997). Because different cost of social status (Creel 2001) during and after social stress, dominant animals show different behavioral (Blanchard, McKittrick and Blanchard 2001), endocrine (Henry, Stephens and Ely 1986, Henry and Stephens 1977) and physiological (Bartolomucci et al. 2001, Sgoifo et al. 1994) responses in comparison with subordinate animals.

In contrast to many of the stressful manipulations used in laboratory studies, social stress is a recurring factor in the lives of animals and humans (Blanchard et al. 2001). In the laboratory animals social defeat is considered the main model for studying social stress in rodents due to its ecological and ethological validity (Miczek K.A. 1991). Sensory contact model proposed by Kudryavtseva et

al (Kudryavtseva 2000, Kudryavtseva 1991) is based on the development of an aggressive/submissive behaviors through repeated experiences of a daily social encounter. A recent variant, called chronic psychosocial stress, has included the natural behavior in most of the animal to defending a territory, adding to social interaction a chronically sensory contact (Bartolomucci et al. 2001).

Single or repeated acute social defeats have been demonstrated to be a potent activator of hormonal and cardiovascular stress response (Koolhaas et al. 2011, Sgoifo et al. 1994) leading to decreased weight gain and food intake in subordinates animals (S. Bhatnagar 2005, Koolhaas et al. 1997, Meerlo et al. 1996). In contrast chronic social defeat leads to long-term changes in HPA activity including persistent elevations in basal glucocorticoids. Subordination stress is associated with elevated corticosterone (Blanchard et al. 1995, Sapolsky 2005, Bartolomucci et al. 2001). Is important to note that dominants animals are not “stress-free”. In some models dominants showed even higher levels of GCs, depending on hierarchy stability, aggression levels and group composition (Bartolomucci et al. 2001, Sapolsky 1992). Besides the hyperactivation of stress response in both subordinates and dominants the effects of the social stress on body weight may completely diverge, depending on the social status of the subject. Moreover food composition and food availability can constitute an environmental challenge by itself with a different, social rank and stress-dependent, impact on intake and food choice (Coccurello, D'Amato and Moles 2009). Many works demonstrated that as consequence of the loss of the territory ownership, subordinates animals are more prone to develop a complex metabolic stress syndrome which is characterized by hyperphagia, fat deposition and body weight gain while dominant, despite equal amount of Kcal ingested resulted resistant to obesity (Bartolomucci et al. 2001, Bartolomucci et al. 2004b, Bartolomucci et al. 2005, Dadomo et al. 2011, Moles et al. 2006). Finally, despite subordination

stress is commonly associated with the development of the stress disorders, including obesity and metabolic syndrome, the correlation between subordination and obesity is not always manifested depending of animal species used (Finger, Dinan and Cryan 2011) and protocols (Tamashiro, Hegeman and Sakai 2006, Coccurello et al. 2009).

In conclusion determining the relationships between social factors (dominance and subordination, (Creel 2001) and individual vulnerability to stress exposure is a productive way to shed light on the factors determining individual disease susceptibility (Bartolomucci et al. 2005).

Diabetes

Diabetes mellitus is a chronic disease characterized by an elevated concentration of glucose in bloodstream. Depending from the etiology and from insulin action, diabetes can be classified in Type 1 or insulin dependent and Type 2 (T2D) or non-insulin-dependent diabetes mellitus. T2D is not a single, but rather heterogeneous disease characterized by disruption of glucose homeostasis caused by an imbalance in glucose production and utilization (DeFronzo 2004) and insulin resistance. Insulin is an hormone produce by pancreatic β cells involved in regulation of carbohydrate and fat metabolism. In a normal condition insulin is secreted in a biphasic pattern to promote glucose uptake, inhibit glucagon secretion and inhibit lipid catabolism in peripheral tissue as adipose, liver and muscle to restore normo-glycemia (DeFronzo, Tobin and Andres 1979).

The risk of an individual to develop diabetes involves a complex interaction between genetic (individual predisposition) and environmental factors among which elevated GCs and obesity are the most important

Stress and insulin resistance

GCs interfere at several levels with insulin action (Amatruda, Livingston and Lockwood 1985). GCs inhibits insulin secretion from pancreatic b-cells in vitro (Lambillotte, Gilon and Henquin 1997) and in vivo (Delaunay et al. 1997) but enhance vagal stimulation of insulin secretion (Das 2011). Synthetic glucocorticoids (dexamethasone) induce progressive insulin resistance by impairing insulin's ability to translocate intracellular glucose transporters (GLUT 4) to the cell surface (Coderre et al. 1996) and by modulating post-binding and degradation of insulin (Caro and

Amatruda 1982). Furthermore GCs impaired insulin-dependent glucose uptake in the peripheral tissues and increase hepatic gluconeogenesis (Rooney et al. 1993). In addition GCs exert their diabetogenic effect opposing the action of insulin to reduce central appetite (Dallman et al. 2007)

Obesity and T2D

One of the primary risk factors for T2D disease is obesity. In T2D patinets manifest hyperglycemia, impaired insulin secretion, insulin resistance in muscle, liver and adipose tissue and abnormal glucose uptake (DeFronzo 2004). Both glucotoxicity and lipotoxicity are involved (Del Prato 2009).

Chronic elevation of plasma glucose impaired insulin action, these glucotoxic effects were demonstrated in partially pancreatectomized rats, where, upon improvement of hyperglycemia by drugs administration, insulin secretion and insulin action were restored to normal (Ehrenkranz et al. 2005, Rossetti et al. 1987). Lipotoxicity exerts is function through a toxic effect of elevated FFA, commonly elevated in obese individuals (Björntorp et al. 1969b, Björntorp, Bergman and Varnauskas 1969a).

FFA seems to produce a defects in insulin stimulated glucose transport and phosphorylation which is caused by a defect in insulin signaling (Boden and Shulman 2002). For reasons that are not well understood, raising plasma FFA levels also results in accumulation of several metabolites involved in FFA re-esterification including longchain acyl-CoA and diacylglycerol (DAG). DAG is a potent activator of kinases cascade such as PKC (protein C kinase), JNK (c-Jun N-terminal kinase) and IKK-β (I kappa B kinase-β), which phosphorylate the protein IRS-1, preventing their tyrosine phosphorylation and thus impairing the insulin signaling. FA may also contribute to insulin

resistance through activation of TLR (Toll-like receptors), which have been hypothesized to be involved in adipocytes and macrophages inflammation. The defect in the response insulin results in a further accumulation of FA, thus creating a vicious circle (Boden 2008)

Obesity is associated with hypertrophy of adipocyte due to an increased accumulation of TGs. These cells overproduce adipokines, and inflammatory cytokines, such as TNF-α, IL-6 some of which appear to cause cellular resistance to insulin (Rabe et al. 2008). Cytokines are also involved in activation of HPA (Dunn 2000). At the same time, the hypertrophic adipocytes decrease synthesis of adiponectin, which appear to enhance insulin responsiveness (Rabe et al. 2008).

Animal model of T2D

Due to the complex interaction between genes and environment factors etiology of diabetes is difficult to understand and study in humans. Because many of the mouse models have characteristics similar to those of the human condition, mouse models provide a unique opportunity to study the onset, development, and course of the disease as well as a unique opportunity to study the molecular mechanisms that lead to diabetes and possible treatment (McMurray and Cox 2011). Rodents have been used for many years to study obesity (Speakman et al. 2007) and T2D (Chen and Wang 2005).

Type of animal models	Obese	Non-obese
Spontaneous or genetically derived models	*ob/ob* mouse *db/db* mouse Zucker (fa/fa) fatty rat KK (Kuo Kondo) mouse KK/A^y (yellow KK obese) mouse NZO (New Zealand obese) mouse NONcNZO10 mouse OLETF (Otsuka Long Evans Tokushima fatty) rat ZDF (Zucker diabetic fatty) rat JCR/LA-cp (James C Russel/LA corpulent) rat M16 mouse SHR/N-cp (spontaneously hypertensive rat/NIH-corpulent) rat TSOD (Tsumara Suzuki obese diabetes) mouse Obese rhesus monkey female Yucatan minipigs	Cohen diabetic rat GK (Goto-Kakizaki) rat Torri rat Non-obese C57BL/6 (Akita) mutant mouse ALS(alloxan sensitive)/Lt mouse
Diet/nutrition induced models	Israeli Sand rat (Psammomys obesus) Spiny mouse (Acomys calirinus) C57/BL 6J mouse Ctenomis talarum (Tucotuco) Gottingen minipigs	--
Chemically induced	GTG (goldthioglucose) treated obese mice	ALX or STZ adult models Neonatal STZ rat
Surgically induced	VMH (ventromedial hypothalamus) lesioned dietary obese rat	Partial pancreatectomized animals
Genetically modified animals (transgenic/knockout)	β_3 receptor knockout mouse Uncoupling protein (UCP1) knock-out mouse	Transgenic or knockout animals (mainly mice) of genes implicated in insulin resistanse (*e.g. IRS-1, IRS-2, GLUT-4*), lipid and glucose metabolism (*e.g. PPARs*) and insulin secretion (*GLUT-2, Glukokinase, IGF-1R*) human islet amyloid polypeptide (hIAPP) transgenic rodents

Table 1: Animal models of T2D (Chatzigeorgiou et al. 2009)

Human can develop T2D without be obese, so the non-obese T2D animal models allow us to dissociate obesity with diabetic syndrome (**Table.1**).

The obese/T2D models are the most used and common models, they can be classified in others subclasses. We will take in exam the most important.

Spontaneous type 2 diabetic animals:

The classic, spontaneously arising mouse mutant *ob/ob* and *db/db* result from defects in the adipose-tissue-derived hormone leptin and its receptor (ObR) respectively (INGALLS et al. 1950, Chen et al. 1996).The involvement of leptin and its receptor in these two phenotypes was highlighted by suturing different mice together as shown in **Fig. 4** so that both animals share a common circulatory system (parabiopsis experiments, Fig.4 (Coleman 1973).

Leptin or ObR deficiency leads to obesity hyperglycemia, insulin resistance and impaired glucose tolerance in *ob/ob* and *db/db* mice. Heterozygous mutations result in moderate obesity. The clinical symptoms and course of the disease is more severe in *db/db* than in *ob/ob* mouse, which explains the shorter life-span of the *db/db* mouse. In *ob/ob* mice chronically elevated insulin levels are associated with impaired insulin receptor autophosphorylation (Gannon 2001).

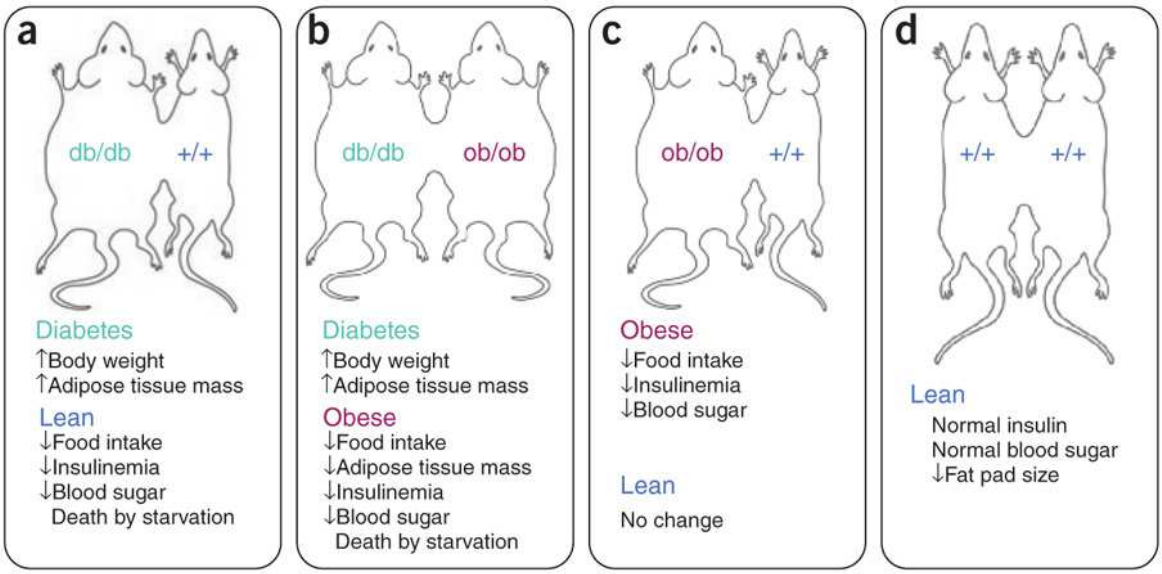

Figure.4. Parabiotic mice. In this experiment, two mice were sutured together to form a shared circulatory system. (Coleman 2010)

Both *ob/ob* and *db/db* mice showed similar hypotalamic NPY overexpression , possible cause of increased food intake. Lack of leptin or ObR induced also hypercorticosteroidism that contribute with hyperinsulinemia to muscle insulin resistance (Srinivasan et al. 2005).

The Zucker diabetic fatty (fa/fa) presents also a mutation on leptin receptor. They show a similar glucose and insulin profile to ob/ob and db/db mice. Down regulation of β-cells GLUT-2 transporter couple to impaired insulin synthesis has been reported to be responsible for hyperglycemia. Other glucose transporter, GLUT-4, is found to decrease in adipose tissue. Moreover they show a hyperlipidemia and hypertension (Panchal and Brown 2011)

Type 2 diabetes is polygenic and may involve polymorphisms in multiple genes encoding the proteins involved in insulin signalling, insulin secretion and intermediary metabolism. KK mouse (Kuo Kondo) and KK/A^y nouse (Yellow KK obese) are two poligenic model for obesity and

diabetes. KK mice are hyperphagic, hyperinsulinemic, insulin resistant and show moderate obesity that occur after insulin resistance onset. They show an increase number and size of pancreatic cells with failure of insulin to suppress gluconeogenesis while induce glycolysis and lipogenesis.

KK/A^y mice carried both lethal yellow (A^y) obese and diabetic KK genes. They manifest severe obesity, hyperglycemia, hyperinsulinemia and glucose intolerance. Studies using isolated adipocytes indicate that tissue responsiveness to insulin is decreased. Histological and immunocytochemical studies show that the pancreatic islets are hypertrophied and the β-cells are degranulated. These findings suggest that the principal cause of diabetes in these mice is insulin resistance (Srinivasan et al. 2005).

Otsuka Long-Evans Tokushima Fatty Rats (OLETF) despite impairment inglucose and insulin pathway, show hypertrigliceridemia and high cholesterol (Kawano et al. 1994).

Diet-induced diabetic animals

High fat diet is associated with increased weight gain, elevated circulating TG levels, and insulin resistance (IR) in humans (Winzell and Ahrén 2004). The response to high fat diet (HFD) is strain-dependent in the mouse (Sumiyoshi, Sakanaka and Kimura 2006). C57BL/6 mice are characterized by a marked obesity, hyperinsulinemia, insulin resistance, glucose intolerance and develop a peripheral leptin resistance (Surwit et al. 1988). The disadvantage of this model is that mice develop T2D syndrome only with a prolonged HF alimentation. While after two weeks of HFD body weight increased leading to a obese phenotype, diet-induced metabolic changes became evident later on time (Panchal and Brown 2011).

Transgenic and knock-out animals

Transgenic technique provide opportunity to investigate the role of specific gene on peripheral insulin action or secretion (Insulin receptor -IR, insulin receptor substrates -IRS1, IRS2, GLUT2-4, TNF-α, glucose kinase-GK etc) (Gannon 2001, Plum et al. 2005) All these mice show key features of T2D. Recently tissue specific KO models have been created allowing a further insight into insulin action in target tissues. An example are IRKO mice (ko for Insulin receptor IR) where IR can be silenced in different tissues:β-IRKO (pancreatic β-cells), MIRKO (muscle), LIRKO (liver), NIRKO (brain) and FIRKO (adipose tissue) (Saltiel and Kahn 2001).

Chemical compounds induced diabetic animals

Alloxan (ALX) and streptozotocin (STZ) are the most prominent diabetogenic compounds, through their use is possible to verify the role of environmental factors, especially chemicals on T2D onset, they exert their diabetogenic action by a direct citotoxic action on pancreatic β-cells. ALX and STZ are structural analogues of glucose and eneter pancreatic β-cells via GLUT2 transporter. Single injection induce Type 1 diabetes (Lenzen 2008). Because the citotoxic action of SZT on insulin-secreting cells, a better treatment to induced T2D is the injection of a low-dose STZ in presence of high energy diet (Srinivasan et al. 2005).

Aims

Stress has been associated with changes in eating behavior and food preferences (Dallman et al. 2003). In humans psychosocial and socio-economical challenges have been related with neuroendocrine-autonomic dysregulation followed by visceral obesity and increased in body mass index (BMI) (Van Strien et al. 1986, Rosmond, Dallman and Björntorp 1998). The chronic activation of stress response system, leads to an increase in food intake, especially high palatable food (Dallman et al. 2004, Dallman et al. 2005), obesity and metabolic syndrome (Rosmond et al. 1998, Björntorp 1993, Björntorp 1996a, Tsigos and Chrousos 2002). Altogether these metabolic disorders result in a pre-diabetic state, which may turn in type 2 diabetes (T2D) in susceptible individuals in a nutrional rich environment (Björntorp 1996b, Boden 2002, Chan et al. 1994, Colditz et al. 1990, DeFronzo 2004). In addition, comorbid pathology such as “atypical depression” linked to stress (DSM-IV, American Psychiatric Association, 2000) has been associated to eating disorders-induced obesity.(Mitchell and Mussell 1995, Stein et al. 2007, Stunkard 2011). While several genetic or pharmacological animal models of metabolic syndrome and T2D have been developed, so far there is a paucity of models in which the diseases are triggered by psychogenic stimuli.

Aim of the present study is:

Chapter One: the original characterization of a mouse models of early metabolic syndrome/T2D onset induced by exposing mice to chronic subordination stress in the presence of high fat diet.

Chapter Two: determine if vulnerability to stress induced metabolic disease is status dependent. Specifically we will directly compare the metabolic consequences of being high in rank (dominant) and low in rank (subordinate) in mice exposed to our model of chronic psychosocial stress CPS and HFD

Chapter Three: characterized if CPS may be considered a model of stress- induced binge eating disorders (BED) and understand the role of hyperphagia in stressed-induced obesity and T2D using a Pair-feeding protocol.

Chapter One

A mouse model of stress-induced type 2 diabetes

Abstract

A chronic exposure to stress has been associated with neuroendocrine changes and metabolic disorders including obesity, glucose intolerance and insulin resistance. While these disorders have been identified as underlying causes in the development of pre-diabetes, the extent to which chronic psychological stress might represent a causal factor for the etiology of type2 diabetes has not been well investigated so far. We developed and validated a naturalistic model of chronic subordination stress (CSS). Subordinate mice develop a complex syndrome characterized by up-regulated hypothalamus pituitary adrenocortical (HPA)-axis functioning, behavioral depression-like disorders as well as autonomic and immune-endocrine changes. Importantly, a robust phenotype in stressed mice is the development of hyperphagia that in mice fed a high fat diet (HFD) is associated with vulnerability to obesity. The present study aimed to extend validity of our model to a condition of pre-diabetes onset and identify underlying molecular mechanisms. Subordinate mice showed an increased Corticosterone level compare to Con mice. When fed HFD they developed a severe hyperglycemia, hyperinsulinemia, hyperleptinemia and dyslipidemia, all considered risk factors for T2D. Moreover they showed a remarkable β-cells dysfunction (hyperplasia and increased insulin secretion), hepatic insulin signaling (IRS1, IRS2 reduction),ketoacidosis and impaired HPA axis functionality as observed in diabetic patients.

All these results taking together suggested that CPS at HFD can be considered a valid animal model of glucocorticoids-induced T2D. Moreover compare to other animal models the physiological and

metabolic profile typical of a metabolic/T2D syndrome is not induced by genetic or pharmacological treatment by only used a natural behavior that occur in a social group such as psychosocial interaction.

Matherials and methods

Overview

3-months old male mice were group housed in groups of 3 siblings, considered as controls group (CON) or were submitted a chronic psychosocial stress and identified as subordinates (SUB) or dominants (DOM) by behavioural observation(Bartolomucci et al. 2004b). All experiment consisted of a 5-days basal period followed by 4 weeks chronic stress experimental phase in which animals cohabitated in the same cage divided by means of a perforated polystyrene-metal partition, which allows a continuous sensory contact but no aggressive interaction. Body Weight and Food Intake were monitored daily. During baseline and 1st week of stress all mice were fed by a chow standard diet, while starting from 2nd week we assigned mice to standard chow diet or high fat diet group (both groups were balanced for body weight, food intake and aggression) (see below for diet details). Between day 21 and 23 animals underwent a glucose tolerance test (GTT). At sacrifice, different metabolic tissues and plasma blood were collected to measured lipid and hormone profile. In this study we have considered only control and subordinate mice

Animals and diet

Male Swiss CD1 mice where derived from an outbred stock originally obtain by Charles River Italia. Mice were reared in a 12-hour light-dark cycle (light on at 7am) and at constant temperature

of 22 ± 2 °C. At weaning in postnatal day 28, groups of siblings males were housed in plexiglass cages (38 X 20 X 18) and bedding was changed weekly to avoid excessive manipulation.

In vivo experiments were conducted at University of Parma (Italy) and University of Minnesota (USA). All animal experiments were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/EEC) and approve by ethical committees of University of Parma and the Italian Institute of Health or by IACUC UMN.

In both laboratories mice were fed with Standard (SD) and High Fat (HF) comparable diets (**Table. 1**).

		Cat. #	Kcal/g	% Kcal from fat
Univ. Of Parma	**SD Diet** (Mucedola SRL, Milano)	4RF21	3,9	6,5
	HF Diet (Mucedola SRL, Milano)	modified 4RF21	5,2	45
UMN	**SD Diet** (Harlan Lab, USA)	2018 Teklad global	3,1	18
	HF Diet (research Diets Inc., USA))	D12451	4,73	45

Table 1: Diet characteristics

Chronic Subordination Stress (CSS) protocol

We characterize an ethological model of chronic subordination stress, which is based on a natural behaviour of male mice, i.e. possessing and defending a territory (Bartolomucci et al. 2001, Bartolomucci et al. 2005).The protocol followed in this study was a modified version of our standard procedure for CSS paradigm (Moles et al. 2006, Bartolomucci et al. 2009b). Swiss CD1 male mice to be used as residents or intruders, were individually housed in cages (38 X 20 X 18) for a 5-days baseline period. At the end of baseline period, 4 weeks of stress procedure started. At the

beginning of the stress phase each resident received an unfamiliar weight matched intruder mouse and the two animals were allowed to freely interact for 10 min. After the interaction, the two animals were separated by a perforated partition, which allowed continuous visual, auditory and olfactory sensory contact but no aggressive interaction.The partition was removed daily, always at same time of the day, for a maximum of 10 min to let mice interact and establish a social status. During baseline period and first week of stress phase all mice were fed SD, while starting from 2nd week, experimental dyads and controls were assigned to two different groups matched by weight, food intake and aggression. One group was fed with standard diet while the second with HF diet until the end of the experiment (**Fig. 1A**). Throughout the stress phase body weight was monitored every other day while food intake daily. Food and water were available ad libitum to all experimental mice. During the social interaction offensive behaviors of the animals were manually recorded and mice social status was determined as described (Bartolomucci et al. 2009b). Age and weight-matched mice, housed in groups of 3 siblings, were included as the non-stressed control group (Con) according to our standard protocol which is based on the observations that no metabolic, immune-endocrine and behavioral evidence of stress activation or anxiety exist in group-housed siblings (Bartolomucci et al. 2001, Bartolomucci, Palanza and Parmigiani 2002, Bartolomucci et al. 2003b).

Glucose and insulin tolerance tests (GTT)

Glucose Tolerance Test was performed following an overnight fast (12h). Blood glucose levels from tail bleeding were monitored at 0, 30, 60 and 120 min after intra-peritoneal injection of 0.1cc/10gr body weight of D-glucose at 10%. All blood glucose measurements were done using Accucheck Aviva glucometer (Roche Diagnostics, Indianapolis, IN, USA).

Echo-MRI (Fat and Lean mass composition)

Total body composition was measured in vivo using a 3-in-1 QMR body composition analyzer (Echo medical system, Houston, TX). Mice were introduced into a plexiglass cylinder for a maximum of 1 min to measure the fat and lean tissue in vivo.

Indirect calorimetry

Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured for each mouse for 24h after a 6 hours acclimatization period by using the Oxymax/Comprehensive Lab Animal Monitoring System (Columbus Instruments, Ohio). All oxygen consumption measurements were normalized to lean mass (ml/hour/kg lean mass) since lean tissues contribute more to the total energy expenditure compared to fat mass (Butler and Kozak 2010).

Serum analysis

At sacrifice plasma was collected from trunk blood after an overnight fasting using heparinized tubes (Sarstedt, s.r.l., IT). Levels of circulating lipids and hormones were detected using different techniques. Corticosterone was measured in duplicate with a commercially available RIA kit according to published protocol (Bartolomucci et al. 2009b). Insulin was measured by RIA using rat insulin standards (Biotrack RPA-547, Amersham, Milan, Italy) according to manufacture instructions. Leptin, ghrelin and glucagon were determined using the Bio-Plex Pro^{TM}mouse diabetes 8-plex and Adiponectin assays and measured with Bio-Plex® system, luminex technology (Bio-Rad Laboratories, Inc., USA) according kit protocol. Quantitative analyses of plasmatic lipids and glycemia were assessed through a computerized chemical analyzer Hitachi 911 (Roche Diagnostic Systems, USA) using Trider colorimetric enzyme methods and homogeneous enzymatic test (Reading at: Tot cholesterol 510 nm, TGs 546 nm, HDL 600 nm). LDL has been calculated with the following formula: Tot cholesterol - (TGs/5) - HDL.

Real-Time PCR analysis

Total RNA was isolated from perigonadal fat pad, liver and muscleusingSTAT60 isolation reagent (Tel-Test, Inc., USA) according to the manufacturer's instruction and quantified by absorbance at 260 nm in a spectrophotometer (Nanodrop, Thermo Scientific). Integrity was assessed with electrophoresis agarose gel by Sybr-safe stain (Invitrogen). Real-time quantitative PCR was performed using TaqMan or SybrGreen sequence detection system on ABI 7900 instrument

(Applied Biosystem) as described (Medina-Gomez et al. 2005). Expression of target genes were corrected by the geometrical average of 4 different housekeeping genes: 18S, β2-microglobulin, β-actin and 36B4 using Best-keeper tool (Pfaffl et al. 2004).

Liver triglycerides content

Hepatic triglycerides content was assessed in frozen liver tissue (100-300 mg) by ethanolic KOH saponification, followed by assay of glycerol content (Sigma Diagnostics, USA) after neutralization in MgCl2. After incubation at 30 °C for 15 min, the samples was read in spectrophotometer at 540 nm and hepatic triglycerides content calculated (Norris et al. 2003).

Light microscopy and immunohistochemical analysis

Pancreas tissue for morphological and immunohistochemical analysis was prepare according to protocols published by (De Matteis, Ricquier and Cinti 1998). We stained sections with H&E for light microscopy analysis and for immunohistochemistry co-localization with primary monoclonal mouse anti-Insulin (Sigma I-2018) and anti-glucagon (Sigma G-2654) following by secondary biotinylated anti mouse immunoglobulinrise in sheep (Amersham RPN1001). Sections were acquired using a digital camera and microscope (Olympus IX70) and Image-Pro® plus software (MediaCybernetics, MD, USA) was used to quantify insulin and glucagon staining.

Thermal nociceptive threshold

The hyperalgesic response on the hot-plate is considered to result from a combination of central and peripheral mechanisms. Mice, only HF Con and Sub, were individually placed on a hot-plate with the temperature adjusted to 50 ±0.2 C. The latency to the first sign of paw licking response to avoid the heat was taken as an index of the pain threshold. The cut-off time was 30 s in order to avoid damage to the paw (no animals reach the cut-off time). The test was performed at the end of 3rd week of CSS protocol, a couple of days before sacrifice.

Quantitative analysis of neuroactive steroids

At sacrifice cerebral cortex cerebellum and spinal cord were collected and store at -80C. Sample were extracted and purified as described by Caruso and collaborators (Caruso et al. 2008). Quantitative analysis was performed using on the basis of calibration curves daily prepared and analyzed. The limit of quantification (LOQ) was calculated as the lowest amount of steroid measured with a minimum error of ±20% in triplicate, as described by Vallée and collaborators (Vallée et al. 2000).

Inter-assay accuracy and reproducibility of the method were calculated over a series of blank samples spiked with 0.5, 2.5 and 5 ng/sample and estimated on the basis of calibration curves. Accuracy was calculated by the ratio (obtained value/true value × 100) in five samples prepared and injected in duplicate in different days. Precision was determined as coefficient of variation (CV%) calculated on the basis of five samples prepared and injected in different days (Caruso et al. 2008).

Statistical analysis

Data were checked for agreement with parametric assumption and analyzed with General linear models followed by Tukey's HSD post hoc test (Statsoft, Inc. Tulsa, OK). Independent data set were analyzed by two way ANOVA using social rank (controls or subordinates) and diet (SD, HF) as between-subject. Dependent data set (weekly food intake and glucose tolerance test, etc) were analyzed by two-way ANOVA for repeated measures with social rank and diet as between –factors and repetition as within- factor. Hepatic triglycerides content was analyzed with unpaired t-test.

Results

Vulnerability to obesity induces by CSS and HFD

We previously validated a mouse model of chronic subordination stress (CSS)-induced obesity (Bartolomucci et al. 2009). In this study we aimed to extend this basic finding by providing a detailed metabolic characterization and more importantly to understand if increased vulnerability to obesity might increases the risk to develop glucose intolerance and the metabolic syndrome. Consisted with previous findings chronic subordination stress determined a significant increase in BW gain, food intake and perigonadal fat pad weight in mice fed HF diet (**Fig 1B,C,D**). Furthermore HFD Sub mice also showed increased fat mass while they did not show a decrease in lean mass when compared to control HFD mice (**Fig. 1E,F**), nor any changes in energy expenditure (data not shown) as determined with indirect calorimetry (Moles et al., 2006). As expected, fasting basal plasma corticosterone, was elevated in Sub mice as compared with controls (**Table. 2**). Overall HFD resulted in a blunted plasma corticosterone which is in line with previous studies (Dallman et al. 2005, Dallman et al. 2006, Dallman et al. 2007, Dallman et al. 2003).

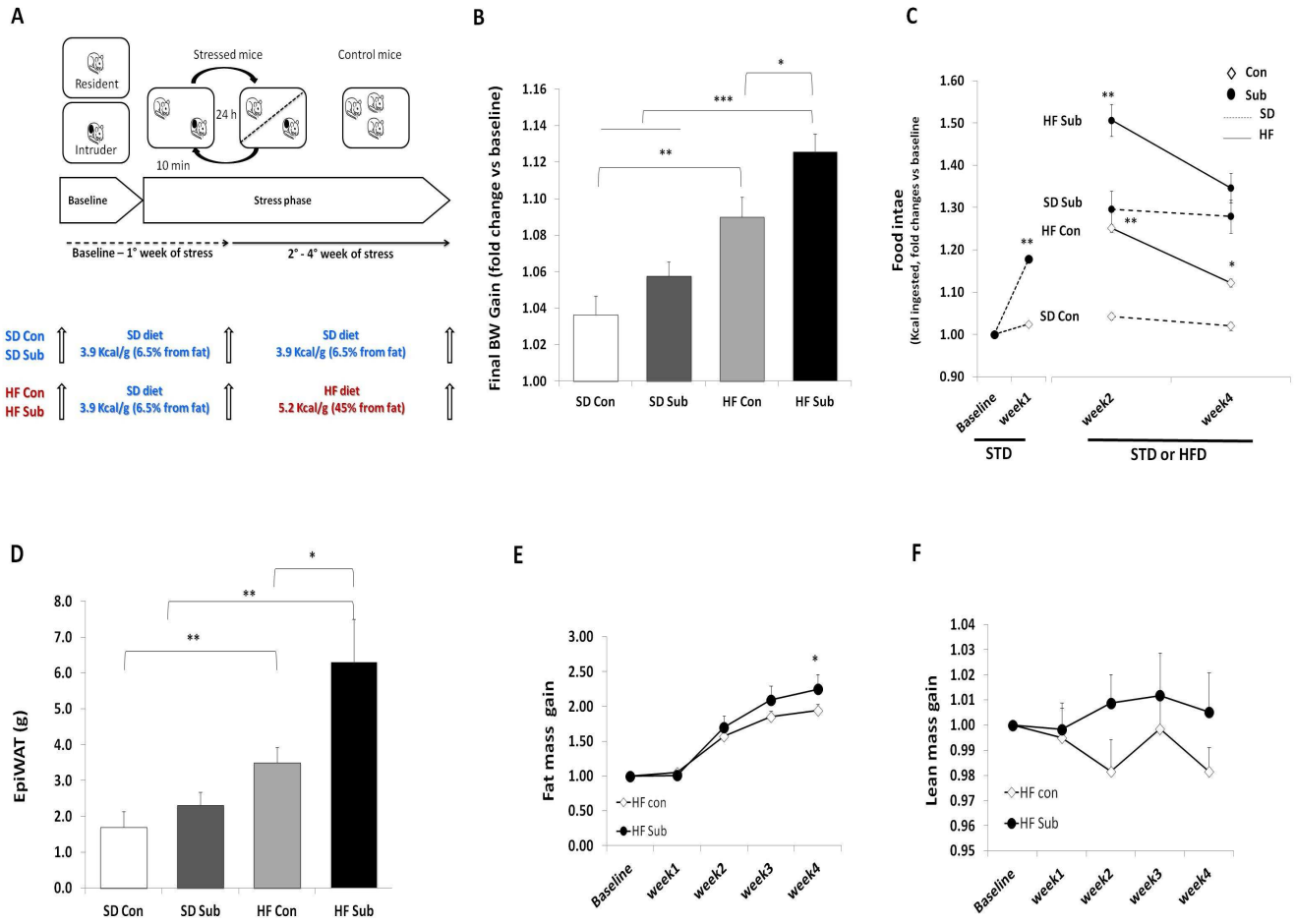

Figure 1: **Metabolic consequences of subordination stress and high fat diet.**

A) Body weight gain. Subordinate mice fed high fat diet (HF Sub) showed the larger body weight gain when compare to all other groups. As expected, diet (HF vs SD) and subordination stress (vs controls) independently affected body weight gain (Diet $F(1,94)= 35.65$ $p<0.01$; Stress $F(1,94)= 7.86$, $p<0.001$).

SD con= 18; SD sub= 23; HFD con= 23; HFD sub= 34.

B) Food Intake. Subordinate mice were always hyperphagic when compared to control animal. The effect being particularly evident when mice were fed HFD. Control mice fed HFD where hyperphagic when compared to control mice fed SD (Diet $F(1,90)= 52,34$ $p<0.001$; Stress, $F(1,90)= 8,60$ $p<0.005$, Stress x week $F(3,270)= 4.19$, $p<0.01$; stress x diet $F(3,270)= 16,58$, $p<0.001$).

SD con= 16; SD sub= 23; HF con= 21; HF sub= 34.

C) Epidydymal fat pad weight after overnight fasting. HF Sub showed a larger increased in epididimal fat pad (EpiWAT) weight when compare to all other groups, wile as expected HFD per se increased massively the weight of the same fat pad (Diet $F(1,77)= 77.1843$, $p<0.001$; Stress $F(1,77)= 5.4646$, $p<0.05$).

SD con= 15; SD sub= 20; HF con= 21; HF sub= 25.

D) Body composition in mice fed HFD. Fat (left) and lean (right) mass gain vs baseline was greater in HF Sub when compared to HFD Con. Lean mass did not differ between HFD Sub and HFD Con even thought HF Con showed a more pronounced trand toward a reduction by week 4 when compared to HF Sub.
HF con= 9, HF sub= 11. *p< 0.05. **p< 0,01, ***p< 0.005

A metabolic like-syndrome in HFD Sub mice

Having characterized a mouse model of stress + high fat diet induced obesity we asked if these mice also showed key features of the metabolic like syndrome and associated hormonal changes. In line with an obese phenotype, leptin was increased while adiponectin and ghrelin were decreased in Sub mice compare to controls (**Table. 2**).

		Standard Diet				High Fat Diet				Effects	
		Controls	n	Subordinates	n	Controls	n	Subordinates	n	Diet	Stress
Corticosterone	ng/ml	76.78 ± 6.23	(9)	112.29 ± 15.64	(19)	51.14 ± 10.02	(15)	79.25 ± 13.81	(22)	p=0,054	p<0,05
Leptin	mg/dl	773.25 ± 61.81	(6)	1123.15 ± 224.53	(8)	3446.56 ± 2463.92	(5)	10156.26 ± 4031,60*	(6)	p<0,005	
Ghrelin	mg/dl	209.21 ± 89.14	(6)	60.43 ± 15.60	(8)	769.99 ± 387.97	(6)	100.45 ± 52.39	(6)		p<0,05
Glucagon	mg/dl	20.66 ± 3.78	(4)	19.93 ± 3.08	(7)	34.05 ± 6.98	(6)	26.99 ± 0.92	(6)	p<0,05	
Adiponectin	mg/dl	8.98E+06 ± 2.30E+06	(4)	2.40E+06 ± 6,58E+05*	(7)	6.08E+06 ± 1.35E+06	(5)	4.23E+06 ± 1.05E+06	(5)		p<0,005

Table 2: Hormones: Mice fed high fat diet (HF) showed higher fasting leptin (Diet $F(1,21)= 7,14$ $p<0.05$) and glucagon (Diet $F(1,19)= 4.4257$, $p<0.05$), compare to mice fed standard diet (SD). Chronic subordination stress induced a decrease in fasting ghrelin (Stress $F(1, 22)=4.424815$, $p<0.05$) and insulin ($F(1,45)= 4.37906$ $p< 0.05$) with Sub mice showing lower levels compare to controls.
Adiponectin ($F(1,17)=11.32651$, $p<0.005$), and plasma corticosterone ($F(1,61)=4.5764$, $p<0.05$). were increased by stress. Corticosterone resulted to be lower at HF compare to SD ($F(1,61)= 3.8918$, $p=0.053$)
*p<0.05

As showed in **Table 3**, HFD Con and SD Sub mice showed increased fasting total cholesterol, HDL, and nonesterified fatty acid (NEFA) levels. The combination of chronic stress and hypercaloric diet in HF Sub, determined a further increase in total cholesterol, LDL and NEFA when compared to all other groups. A common finding in obesity is increased plasma TGs and liver steatosis. On the contrary, plasma TGs were increased in SD Sub mice while where normalized in HF Sub mice which argue for an altered lipid trafficking in HF Sub mice.

		Standard Diet		High Fat Diet		Effects	
		Controls (14)	Subordinates (16)	Controls (19)	Subordinates (26)	Diet	Stress
Tot Cholesterol	mg/dl	105,21 ± 4,28	118,56 ± 5,24	135,05 ± 3,83*	155,69 ± 6,68* **	p<0,001	p<0,01
HDL	mg/dl	57,86 ± 3,23	60,94 ± 3,87	67,79 ± 2,45	75,62 ± 3,93*	p<0,005	
LDL	mg/dl	21,07 ± 1,40	24,50 ± 2,79	41,05 ± 2,28*	52,73 ± 2,96* **	p<0,001	p<0,01
Tryglicerides (TG)	mg/dl	131,93 ± 4,88	164,81 ± 10,52	130,74 ± 10,69	136,38 ± 7,18		p<0,05
NEFA	mg/dl	0,87 ± 0,06	1,22 ± 0,07*	1,10 ± 0,05	1,52 ± 0,06* **	p<0,001	

Table 3 Lipidic profile measured at sacrifice showed a main effect of diet on Tot Cholesterol ($F(1,71)=32.214$, $p<0.001$), LDL ($F(1,71)=75.7264$, $p<0.001$), HDL ($F(1,71)=10.869$, $p<0.005$), glycemia ($F(1,71)= 36.562$, $p<0.001$) and NEFA ($F(1,71)=18.034$, $p<0.001$) with mice at HFD showing an increased in lipids level. Tot Cholesterol ($F(1, 71)=8.298$, $p<0.01$), LDL ($F(1,71)=7.6794$, $p<0.01$), NEFA ($F(1,71)=37.145$, $p<0.001$) and TGs ($F(1,71)=4.5270$, $p<0.05$) were increased also by subordination stress at STD and HFD. Only glycemia showed an interaction between diet and stress ($F(1,71)= 8.44$, $p< 0.005$). *$p<0.05$, **$p<0.01$

Fasting liver TGs content was lower in Sub mice independently from the diet (Fig. 2). Arguing for increased metabolic cost of chronic stress, FFA were actively converted into ketones in liver. Indeed b-hydroxybutirrate showed a trend to be higher in a subgroup of HF Sub mice when compared to HF Con (Fig. 2). Taking these findings altogether our hypothesis is that stress and high fat diet resulted

in increased beta oxidation in the liver of stressed mice along with a lower capacity to mobilize lipids from the adipose tissue. Increased plasma NEFA would, in absence of increased energy expenditure, accumulate in plasma and stored again as TGs in adipose tissue and skeletal muscle. Overall subordinate mice fed high fat diet showed a metabolic-like syndrome . Biochemical changes characteristic of the metabolic syndrome and increased FFA in particular are recognized risk factors for type 2 diabetes. Accordingly we set out to determine if stress and high fat diet might affect glucose tolerance in mice.

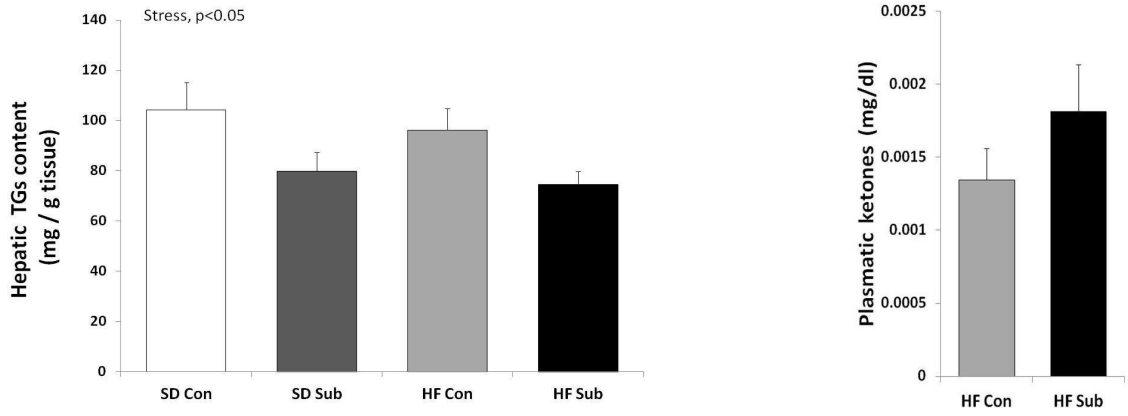

Figure 2 Hepatic TGs content was decreased in stress mice (Stress $F(1, 85)= 8.3642$, $p<0.005$) independently from diet. SD con= 17; SD sub= 22; HF con= 24; HF sub= 26

Moreover, plasma ketones (b-hydroxybutirrate) showed a trend to be higher in a subgroup of HF Sub

HF con= 4; HF sub= 5

Decreased hepatic insulin signaling and glucose intolerance in mice exposed to CSS and HFD

Obesity is a recognized risk factor for type 2 diabetes (Kahn, Hull and Utzschneider 2006). After 3 weeks of stress there was no significative change in basal glucose or glucose tolerance in SD Sub mice. This suggests that stress might not be sufficient to alter glucose tolerance. However, when subordinates were fed a HF we were able to demonstrate a significant increase in fasting glucose as well as glucose intolerance when compared to all the other experimental groups (**Fig. 3 A**).

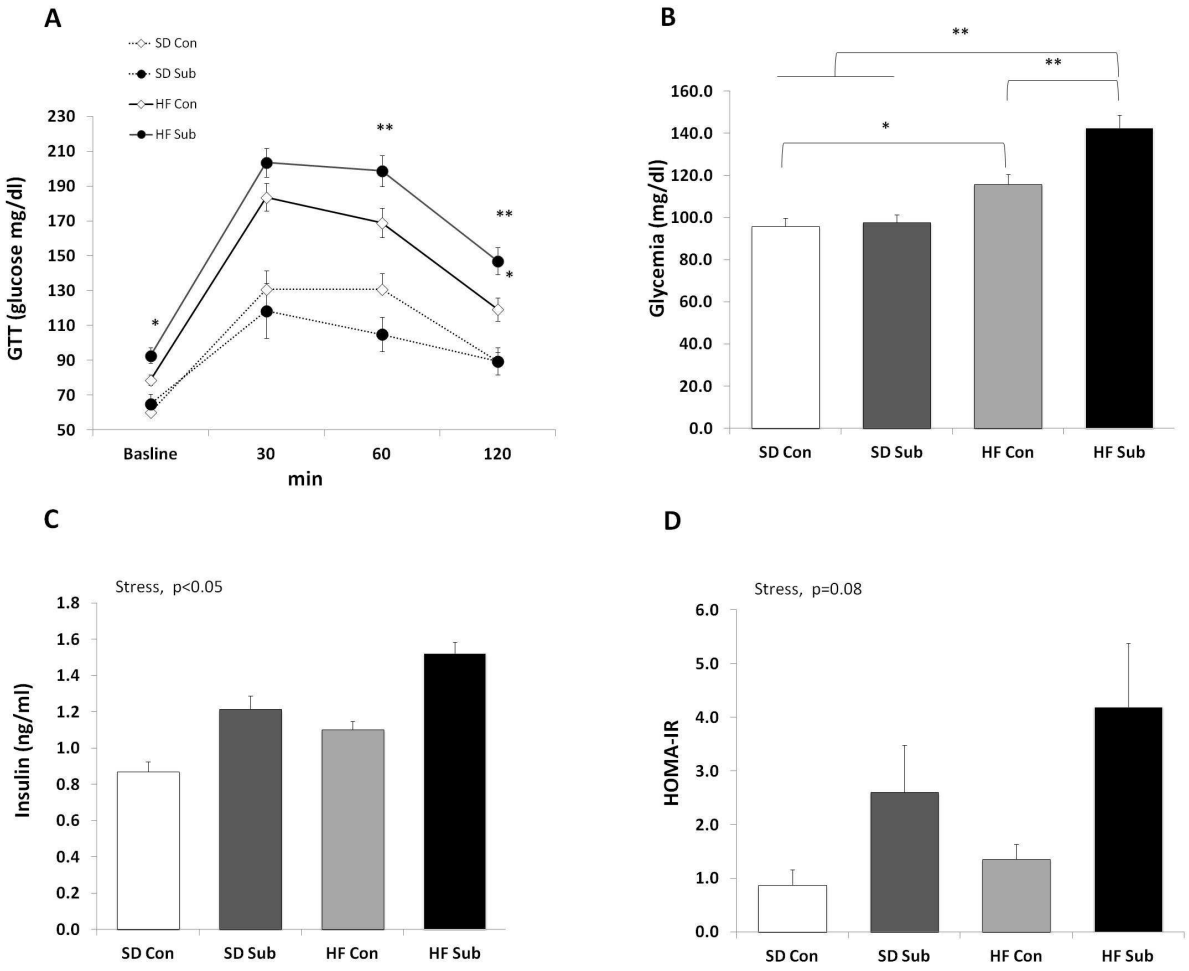

Figure 3: A) Glucose tolerance test (GTT). Mice at HFD showed higher levels of glucose at baseline (Diet $F(1,52)= 24.0565$, $p<0.001$) and responded with higher glucose concentration after injection (Diet x Time

F(3,156)= 12.075, p<0.001). We saw also a significant interaction between stress and diet (Stress x Diet F(1,52)= 4.916, p<0.05) with HF Sub showing higher glucose level compare to other groups. SD con= 9; SD sub= 8; HF con= 20; HF sub= 19

B) Glycemia: At sacrifice glucose level was increased as main effect of HFD (Diet F(1,71)= 36,56 p<0,001). HF Sub showed the highest glucose concentration compare to SD, HF Con (Diet x Stress F(1,71)= 8,44 p<0.005). SD con= 14; SD sub= 16; HF con= 19; HF sub= 26

C) Insulin. Subordination stress induced an increase of insulin concentration both at SD and HF (Stress F(1,45)=4,38, p<0,05). SD con= 9; SD sub= 9; HF con= 12; HF sub= 19

D) HOMA-IR: Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting insulin and glucose levels as (insulin X glucose)/22.5 where insulin where reported as mU/l and glucose as mmol concentration (Matthews et al. 1985). HFD induced a raise in HOMA-IR only in Sub mice and not in Con. SD con= 6; SD sub= 6; HF con= 11; HF sub= 19

*p< 0.05, **p< 0.01

HFD also resulted in a lower degree of glucose tolerance in control mice. At sacrifice (i.e. 1 week after the GTT with stress and diet continuing through), HF Sub mice showed further increased fasting glucose and hyperinsulinemia which resulted in an overall increase in insulin resistance based on the significant increase in the Homa-IR index (Matthews et al. 1985) (**Fig. 3 B-C-D**).

In line with increased plasma insulin, insulin immunostaining was increased as well in pancreatic islets (**Fig. 4 A**). Despite increased Homa-IR HFD Sub mice did not differ from controls in the Insulin Tolerance Test (ITT) (not shown). However, HFD Sub mice showed a blunted increase response to insulin-stimulated hypoglycemia-induced corticosterone release which is interpreted as HPA-axis dysfunction and observed in diabetic patients and animal models (**Fig. 4 B**) (Chan et al. 2002, Sapolsky et al. 2000, Dallman et al. 2007)

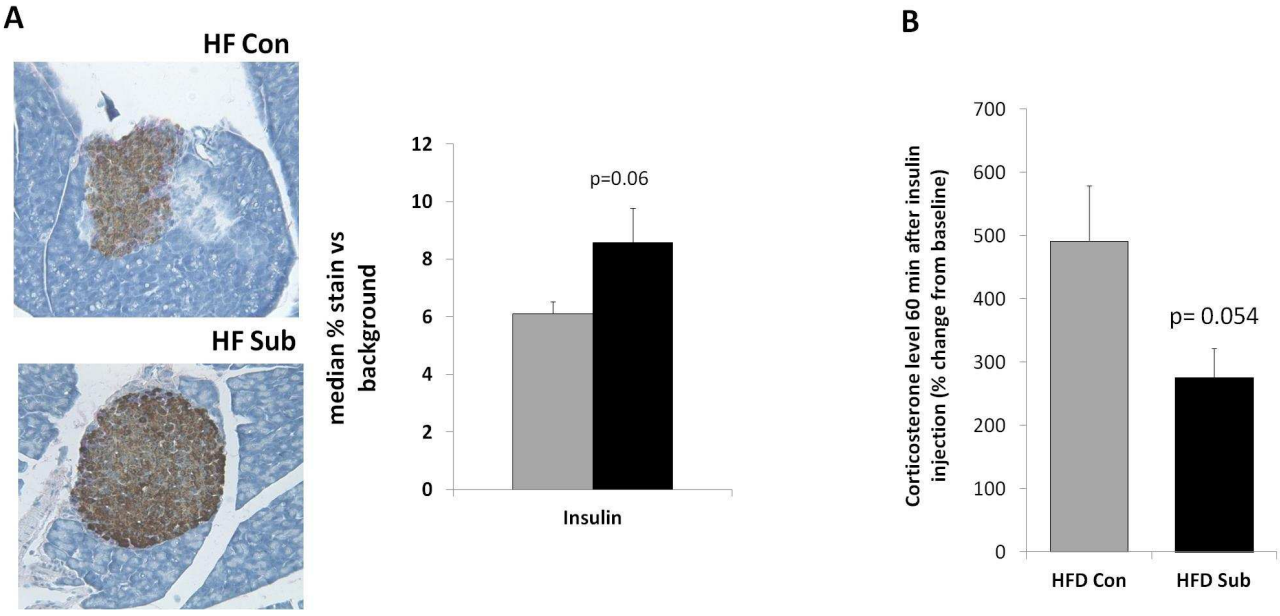

Figure 4: A) Immunohistochemical (20X) analysis of insulin in pancreas. Insulin staining demonstrated a higher number of insulin-positive cells (β cells) in HF Sub compare to Con by trend (t-test$_{2,24}$= 1,95 p=0.06). HF con= 13; HF sub= 13

B) Corticosterone release after 60 min acute stress exposure (ITT test) was reduced by trend in HF Sub mice compare to HF Con (t-test$_{2,10}$= 2,17 p=0.054). HF con= 6; HF sub= 6

After having characterized a new naturalistic model of pre-diabetes we attempted to Identify key molecular signatures in metabolic tissues involved in glucose homeostasis, i.e. the liver and skeletal muscle (**Fig. 5A-B**). Social stress in absence of HFD (SD Sub) and HFD per se in control mice did not induce hyperglycemia or insulin resistance (**Fig. 3B-C**). Interestingly SD Sub mice also showed a compensatory upregulation of insulin receptor in hepatocytes which suggest increased glucose clearance (**Fig. 5 A**, IRS1 and IRS2). Importanty, SD Sub and HF Con mice showed a significative change in hepatic and skeletal muscle gene associated with insulin resistance and diabetes such as IRS1, IRS2, CPT1a (Fig 5 A-B) (Withers et al. 1998, Previs et al. 2000, Park et al. 1995)and an increase in insulin-independent GLUT2 in the liver (**Fig. 5 A**). Accordingly these moelcular changes

can be interpreted as potential risk factors not as pathological changes. When on the contrary, stress and high fat diet where combined in the HF Sub group, mice showed a further significative decrease in the same genes as well as with a significative decrease in GLUT4 in skeletal muscle (**Fig. 5 B**).. Importantly HF Sub mice are hyperglycemic, glucose intolerant and insulin resistance thus suggesting that a further decrease in gene expression would trigger a pathological condition.

In conclusion, our data demonstrate that high fat diet and stress are synergistic risk factor for the development of glucose intolerance, hyperglycemia and hyperinsulinemia. The underlying mechanisms likely involves a downregulation of insulin-mediated signaling in hetapocytes and skeletal muscle and insulin-induced glucose uptake.

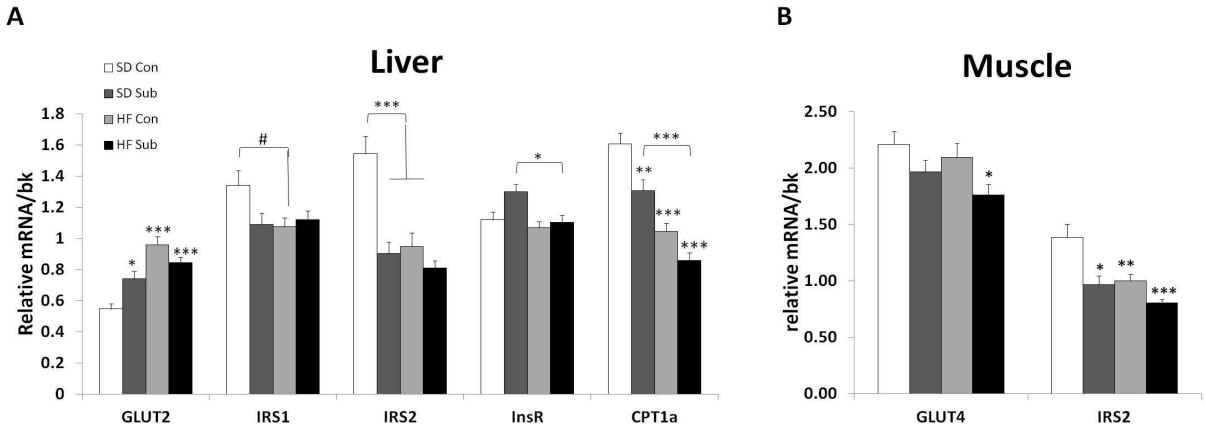

Figure 5: Gene expression analysis. **A) Liver genes analysis** showed that HF diet induced an increased in GLUT 2 receptor ($F(1,77)= 31,90$ $p<0.001$) whit Con significantly increased more than Sub at HF (Stress x Diet, $F(1,77)= 10,97$ $p<0.005$). IRs2 and CPT1a were decreased by HF diet (IRS2, $F(1,78)=16.8013$ $p<0.001$; CPT1a, ($F(1,78)= 5,02$ $p<0.001$) and by stress (IRS2, $F(1,78)= 25.0544$ $p<0.001$; CPT1a, $F(1,78)= 1,22$ $p<0.001$). Both IRS1 and IRS2 showed a significant interaction between stress and diet with HFSub showing the lower gene expression level (IRS1, $F(1,79)= 4.327$ $p<0.05$; IRS2, diet $F(1,77)=8.6150$ $p<0.005$). InsR was decreased only by HF ($F(1,79)= 6.169$ $p<0.05$).

B) **Muscle gene analysis** showed a main effect of HF on IRS2 ($F(1,77)= 11,69$ $p<0.01$) with mice at HF diet showing a decrese expression of this gene. Both IRS2 and GLUT4 were decreased in Sub mice regardless diet (IRS2, $F(1,77)=18,73$ $p<0.0001$; GLUT4 $F(1,77)= 6,98$ $p<0.01$).

SD con= 15-17; SD sub= 19-20; HF con= 18-21; HF sub= 24-26 *p<0.05, **p<0.01, ***p<0.005 vs SD Con

Subordination stress at HFD induces thermal hyperalgesia

Stress and diabetes have been associated with altered response to painful stimuli (Miczek, Thompson and Shuster 1982, Courteix, Eschalier and Lavarenne 1993, Calcutt 2002). Latency to lick the plantar forepaw in the hot plate test (**Fig. 6**) was measured in a subgroup of control and subordinate mice fed HFD. Subordinate mice showed a lower latency to licking suggesting a reduction in the thermal threshold and hyperalgesia.

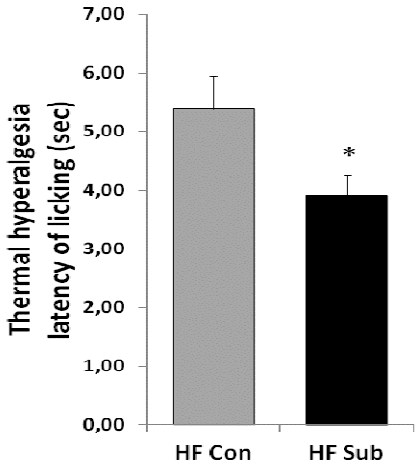

Figure 6: Thermal hyperalgesia following 3 weeks of HFD feeding in Con and Sub mice. HF Sub mice showed a reduced latency to first licking in hotplate test when compared to HF Con ($t\text{-test}_{2,24}= 2.14$ $p<0.05$)

Role of subordination stress and HFD on neuroactive steroids

Neurosteroids are important neuroactive molecules involved in neurodegenerative processes (Lapchak and Araujo 2001, Wojtal, Trojnar and Czuczwar 2006). Neurosteroids are synthesized from cholesterol in the central nervous system (CNS) by means of a series of enzymatic processes as showed in **Fig 7**.

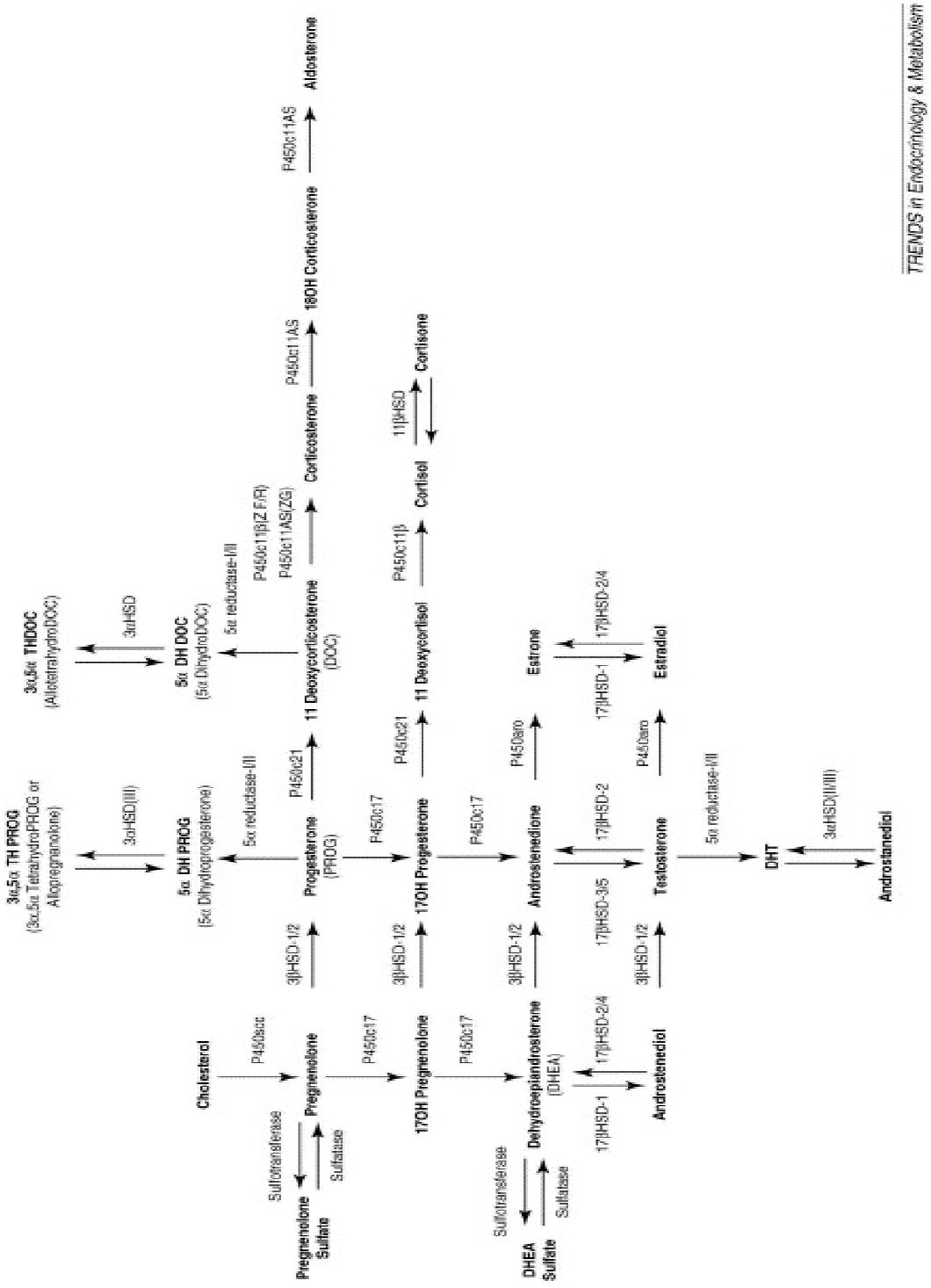

Figure 7: Neurosteroid metabolic pathway. (Mellon and Griffin 2002)

Neuroactive steroids were measure in spinal cord (**Fig 8**), cerebellum (**Fig 9**) and cerebral cortex (**Fig 10**) of controls and subordinate mice fed both standard and high fat diet. Sciatic nerve were also collect from animals but the of neurosteroids concentration was not detectable due to the low amount of sample. In the brain, neurosteroids have been shown to act directly on membrane receptors for neurotransmitters. Besides these effects, neurosteroids also regulate important glial functions, such as the synthesis of myelin proteins (Baulieu, Robel and Schumacher 2001). Detection limit for each neurosteroid are listed in **Table 4**.

Limit of quantification

		pg/mg sample
PREG	Pregnanolone	0,05
PROG	Progesterone	0,05
DHP	Dihydroprogesterone	0,25
THP	Tetrahydroprogesterone/allopregnanolone	0,1
THPi	Isopregnagnolone	0,1
DHEA	Deidroepiandrosterone	0,05
T	Testosterone	0,02
DHT	Dihydrotestosterone	0,05
3α DIOL	Androstanediol	0,05
17α-E	17α-Estradiol	0,02
17β-E	17β-Estradiol	0,02

	Spinal cord				**Cerebellum**				**Cerebral cortex**			
n	SD Con	SD Sub	HF Con	HF Sub	SD Con	SD Sub	HF Con	HF Sub	SD Con	SD Sub	HF Con	HF Sub
PREG	4	4	3	3	9	8	8	8	9	8	8	8
PROG	4	4	3	3	9	8	8	8	9	8	8	7
DHP	4	4	3	3	9	8	7	8	9	8	8	8
THP	4	4	3	3	9	8	4	5	7	6	6	4
THPi	4	4	3	3	9	8	8	7	9	8	8	8
DHEA	4	4	3	1	9	8	5	3	4	2	7	2
T	4	4	3	1	9	8	7	6	9	8	7	6
DHT	4	4	3	2	2	< DT	3	3	4	4	5	6
3α-DIOL	4	4	3	3	9	8	8	8	9	8	7	8
17α-E		< Detectability			8	8	1	2	2	3	1	1
17β-E	4	4	2	3	9	8	2	3	9	7	2	6

Table 4: Detection limits of neuroactive steroids and sample size in different areas

Subordination stress exerted minor or no changes in mice fed standard diet while HFD had a major impact on neuroactive steroid level.

As showed in **Fig.8** neuroactive steroids were evaluated in spinal cord of experimental animals. Due to a small amount of tissue collectable form mice, spinal cord of animals coming from same group were pulled to a minimum quantity of tissue required to obtain an accurate reading. All neurosteroids were detectable with the exception of 17α-E. Data showed a major impact of HFD. HFD induced a decrease in PREG and an increase in PROG and their metabolites, DHP and THP, while THPi was decreased when compared to SD Con mice. In subordinate mice fed HFD we showed a similar overall trend with several notable exceptions. First of all while DHP and THP were similarly increased in CON and SUB HFD, while PROG was only slightly but not significantly increased in SUB HFD when compared to SUB and control mice fed SD. This suggests a stress-induced increase in the activity of 5-α-reductase. Furthermore, HF Con mice showed an increased in 3α-diol production when compare to SD Con, while subordinate mice showed no change in this neuroactive steroid. DHEA and T could be not analyzed because all sample in HF Sub were sub-threshold except for one.

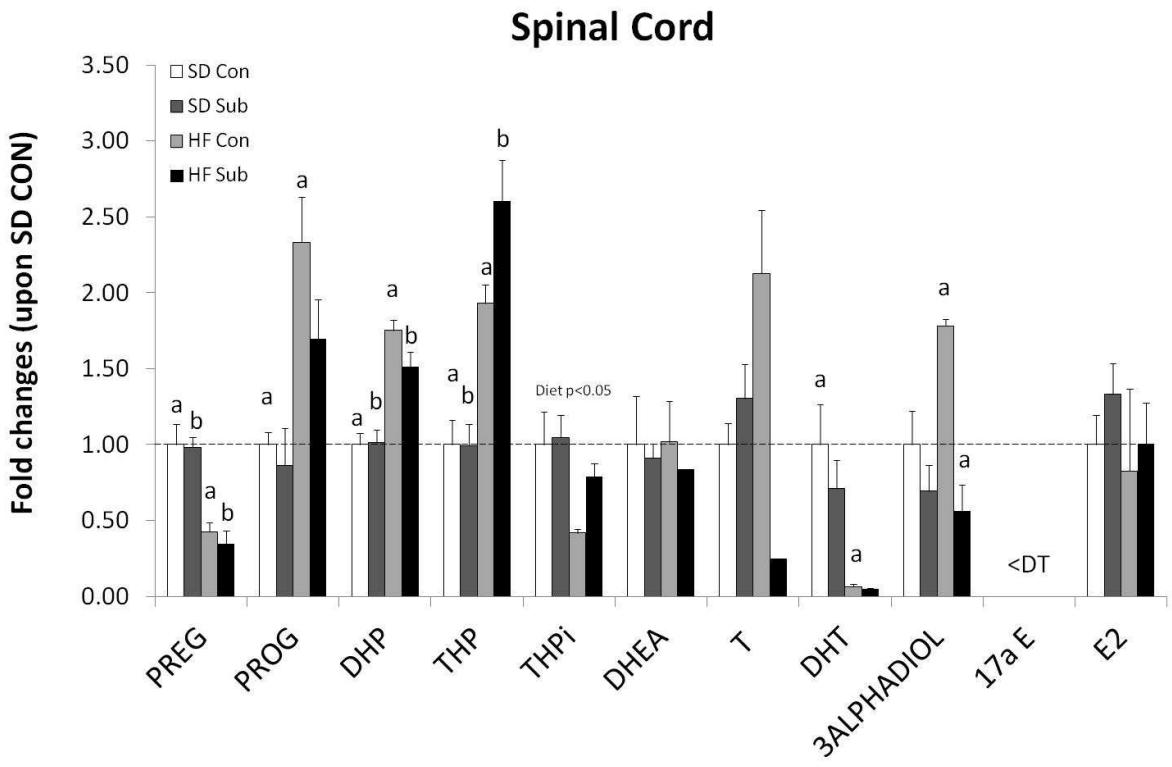

Figure 8: Effect of diet and social status on neuroactive steroids in spinal cord. Analysis of neurosteroids in SC showed a major effect of HFD on PREG ($F(1,10)= 37.52$ $p<0.001$), PROG ($F(1,10)= 23.17$ $p<0.001$), DHP ($F(1,10)= 58.31$ $p<0.001$), THP ($F(1,10)= 49.85$ $p<0.001$), THPi ($F(1,10)= 1.67$ $p<0.05$), and DHT ($F(1,9)= 13.93$ $p<0.005$). 3α-Diol was increased only in Controls as effect of HFD (Stress $(1,10)= 17.77$ $p<0.01$, Diet x Stress $F(1,10)= 6.39$ $p<0.05$)

In cerebellum (**Fig 9**) HFD induced very similar changes in CON and SUB mice. Specifically we observed a decrease in PREG but only minor changes in PROG and its metabolites. On the contrary, DHEA and T were lower in HF fed mice compare to SD. 3α-Diol was increased by HFD with Sub mice showing the highest concentration when compare to all other groups. 17α-Diol could be not analyzed because all sample in HF Con were sub-threshold except for one.

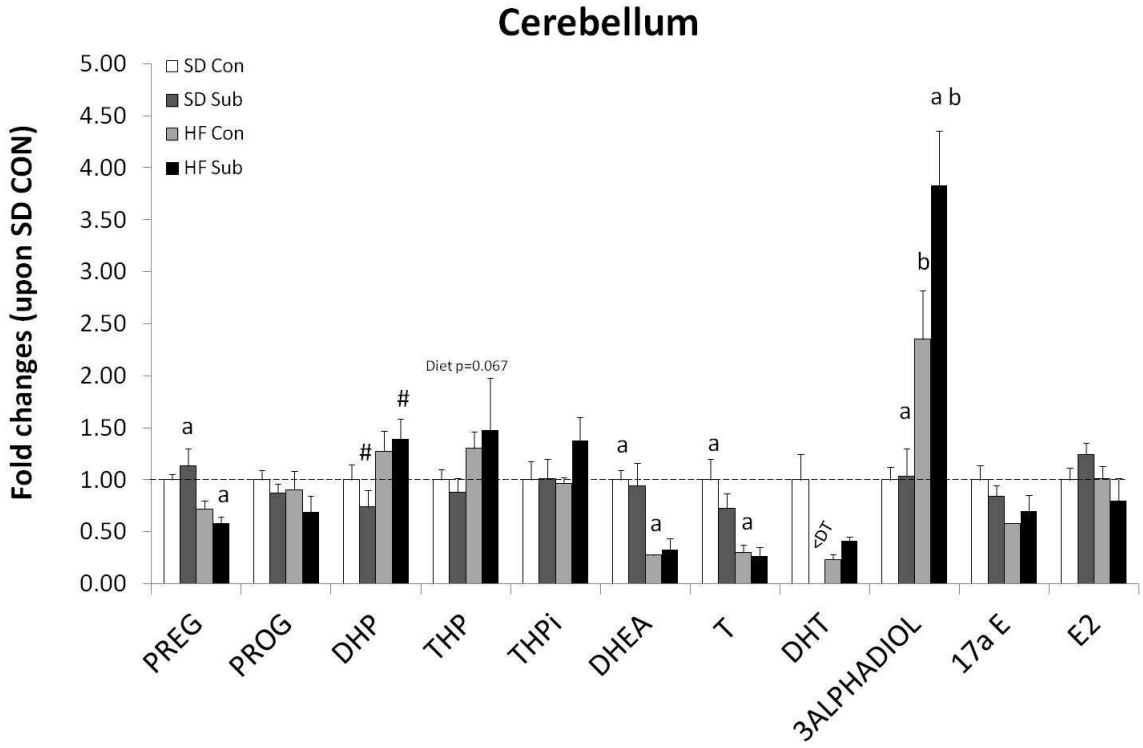

Figure 9: Effect of diet and social status on neuroactive steroids in cerebelllum. Analysis of neurosteroids in cerebellum showed a main effect of HFD on PREG ($F(1,29)= 17.33$ $p<0.001$), DHP ($F(1,28)= 7.11$ $p<0.05$), THP ($F(1,22)= 3.69$ $p=0.067$), DHEA ($F(1,21)= 13.57$ $p<0.01$), T ($F(1,26)= 14.17$ $p<0.001$) and 3α-Diol ($F(1,29)= 31.77$. $p<0.001$). 3α-Diol was also increased as effect of subordination with the highest rise in HF Sub (Stress $(1,29)= 4.24$ $p<0.05$, Diet x Stress $F(1,29)= 3.84$ $p=0.059$)

Finally, in the cerebral cortex (**Fig. 10**) PREG, PROG and DHT were significantly decreased in HF mice compare to SD. On the other and DHP and THP were increased as effect of HF diet.

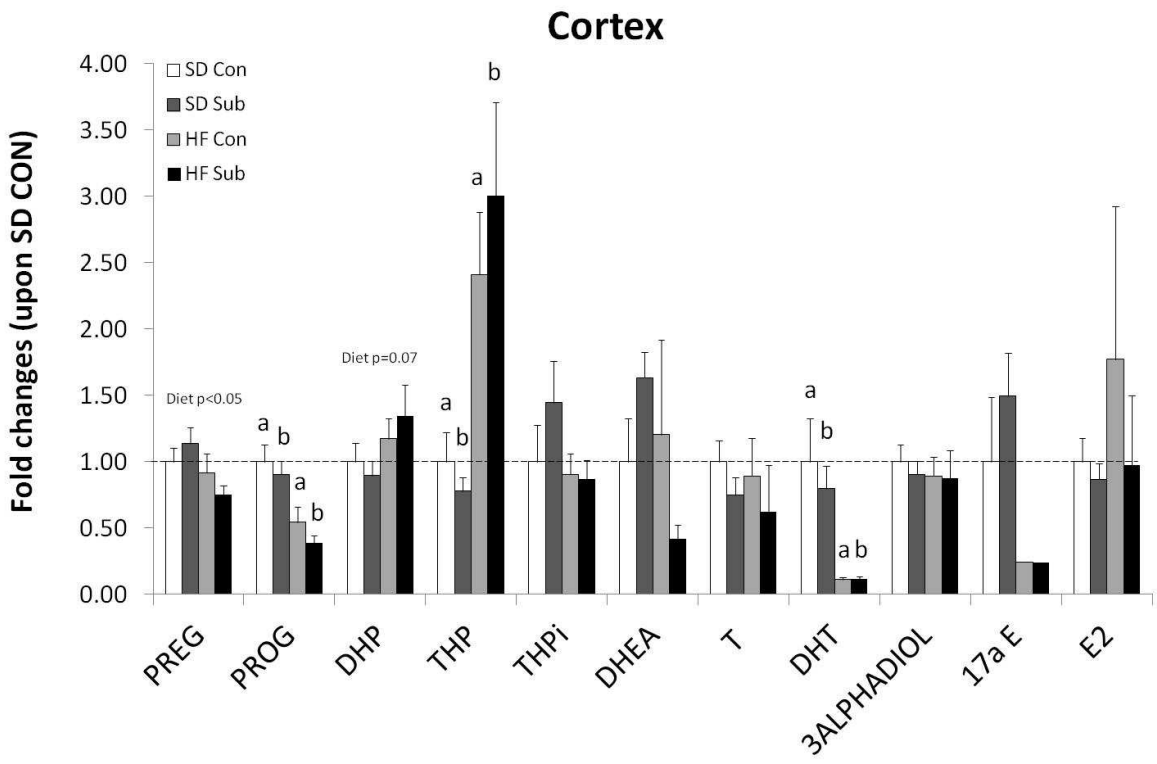

Figure 10: Effect of diet and social status on neuroactive steroids in cerebral cortex. Analysis of neurosteroids in cerebral cortex showed a main effect of HFD on PREG ($F(1,29)= 4.23$ $p<0.05$), PROG ($F(1,28)= 20.55$ $p<0.001$), DHP ($F(1,29)= 3.46$ $p=0.07$), THP ($F(1,19)= 2303$ $p<0.001$), DHT ($F(1,15)= 25.88$ $p<0.001$).

Discussion

Obesity is a major risk factor for T2D (Surwit et al. 1988, Winzell and Ahrén 2004, Srinivasan et al. 2005, Buettner et al. 2006, Karasawa et al. 2009). Similarly, psychosocial stress-induced metabolic disorders has been established in humans (Dallman et al. 2006, Bose, Oliván and Laferrère 2009) and mice (Bartolomucci et al. 2004b, Moles et al. 2006, Bartolomucci et al. 2009b, Coccurello et al. 2009, Kuo et al. 2007, Finger et al. 2011). Despite extensive investigation no naturalistic models of psychosocial stress has recapitulated glucose intolerance and a diabetic state. Here we characterized an animal model in which chronic exposure to a psychosocial stress, using social defeat paradigm, and obesogenic nutritional environment, induce a T2D-like state.

Obesity induced by chronic stress and HFD

Fat storage in visceral adipose tissue, following a high fat high calorie diet, is commonly associated with insulin resistance and T2D (Björntorp and Rosmond 2000, Boden 2002, DeFronzo 2004). Our subordination stress paradigm induced increased weight gain with both STD and HFD (Bartolomucci et al. 2004b, Foster et al. 2006a, Kuo et al. 2007, Bartolomucci et al. 2009a, Bartolomucci et al. 2010, Dadomo et al. 2011). BW gain is associated with an increase in food intake and resulted in increased visceral adiposity and total fat mass only when mice are fed HFD. Subordinate mice showed increased circulating cort when compared to controls. Cort levels are otherwise lower in mice fed HFD as previously demonstrated (Dallman et al. 2003, Dallman et al. 2005).

CSS and HFD induce a metabolic-like syndrome

Chronic subordinations stress in a nutritionally rich environment induced a metabolic-like syndrome in mice. . In line with increased adiposity leptin level raised (Friedman and Halaas 1998). Elevated visceral fat pad, hyperphagia and hyperleptinemia showed by HF Sub suggested a state of leptin resistance (Halaas et al. 1995, Halaas et al. 1997, Levin and Dunn-Meynell 2002)

Adiponectin and ghrelin levels were down-regulated. Adipocyte-derived adiponectin is reduced obese patients (Aguilera et al. 2008) and is implicated in the development of insulin resistance (Maeda et al. 2002, Hotta et al. 2001). Administration of adiponectin inhibits endogenous glucose production in the liver and improves obesity-associated insulin resistance (Yamauchi et al. 2001). Ghrelin is a 28 aminoacid gut-derived hormone that induces food intake and has been associated with obesity (Heiman and Witcher 2006, Maffei et al. 1995). HFD induced a rise in gherlin concentration only in control mice. Under stress mice showed a downregulation of ghrelin, in line with the hypothesis suggested that plasma ghrelin concentration might be decrease in obese subjects as a consequence of chronic positive energy balance resulting also by elevated leptin and insulin concentration (Ravussin et al. 2001, Tschöp et al. 2001).

Dyslipidemia is recognized as one of the principal components of metabolic syndrome (Alberti, Zimmet and Shaw 2006). In our model only 3 weeks of HFD was sufficient to increase cholesterol, LDL, NEFA. Moreover stress-induced increase of TGs concentration only at standard diet, can be explained with increased circulating NEFA mostly in CSS mice at HFD. In a insulin sensitive state, insulin has the capacity to suppress HSL and stimulate LPL to regulate triglycerides, NEFA and LDL concentration. Despite the decreased TGs and increased NEFA, LDL increase can be considered an effect of impairment of insulin sensitivity (Aguilera et al. 2008). Arguing for increased metabolic cost of chronic stress, FFA were actively converted into ketones. Indeed b-

hydroxybutirrate (β-OHB) showed a trend to be higher in a subgroup of HF Sub mice when compared to HF Con. Ketoacidosis normally develop in patient with Type 1 diabetes (lack of insulin) however T2D patients are also a risk (Kitabchi et al. 2001). Diabetic ketoacidosis (DKA) is characterized by hyperglycemia and increased circulating ketone bodies. In results as a ineffectiveness of insulin sensitivity with elevation of counter-regulatory hormones as glucagone and cortisol (Gerich et al. 1976, McGarry et al. 1989). Both, lack of insulin sensitivity and increased in counter-regulatory hormones activates hydrolysis of TGs in glycerol and FA. The elevated concentration of FFA released in blood stream induced an increase of production of ketone bodies in liver (Umpierrez, Khajavi and Kitabchi 1996, Umpierrez and Kitabchi 2003).

Hyperglycemia, hyperinsulinemia, decreased TGs concentration and elevated NEFA and ketone bodies altogether suggested that stress and high fat diet resulted in increased beta oxidation in the liver of stressed mice along with a lower capacity to mobilize lipids from the adipose tissue.. Overall subordinate mice fed high fat diet showed a metabolic-like syndrome

Stress and HFD induced insulin resistance and glucose intolerance

Stress and HFD interacted to induce glucose intolerance and insulin resistance. As for metabolic and lipidic parameters, three weeks of HFD was able to exert some effect on glucose homeostasis but only the interaction with stress resulted in a overt glucose intolerance and insulin resistance state. Moreover the role of stress in glycemic control seemed extremely important inasmuch we demonstrated that stress per se could induce an increase of plasmatic insulin independently from the nutritional environment. Furthermore according to the “bihormonal abnormality hypothesis” proposed by (Unger and Orci 1975) plasmatic glucagon was also increased at HFD, acting as a

insulin counter-regulatory hormone. Compared to other groups HF Sub developed insulin resistant as measured in GTT. They exhibited the highest basal glycemia with a 2-fold elevation in glucose after injection. Stress at HFD increased basal blood glucose as well as blood insulin and insulin resistance index (HOMA-IR). Importantly, in subordinate mice fed standard diet, high insulin was paralleled by normal fasting glucose and glucose tolerance thus suggesting a compensatory increase in insulin signaling. Indeed, while muscle and liver insulin secondary messengers IRS1, IRS2 and CPT1a decrease in SD Sub may be considered a risk factor to develop T2D (Dong et al. 2006, Withers et al. 1998, Park et al. 1995) InsR was over-expressed in liver as possible compensatory mechanism. Thus resulted in a complete insulin sensitivity in Sub SD where insulin was still able to normalize glycemic . On the other hand when fed HF diet subordinates showed a down-regulation of all genes involved in insuling signaling in peripheral tissue (InsR, IRS1, IRS2 and CPT1a). Furthermore plasmatic hyperinsulinemia was associated with pancreatic Langherans cells hyperplasia and increased number of insulin-positive cells (β cells). As demonstrated in LIRKO animal models (Michael et al. 2000, Escribano et al. 2009), liver specific InsR knock-out developed impaired glucose tolerance with fasting hyperglycemia, hyperinsulinemia and β-cells hyperplasia. Besides insulin signaling disruption HFD and stress induced an increased in GLUT2, no-insulin dependent glucose transporter in liver while is reported from Shafrir et al. (Shafrir, Ziv and Kalman 2006) high level of insulin was not sufficient to exceed muscle insulin resistance as shown by a down regulation of GLUT4 with a significant effect only in CSS mice at HFD. As further confirmation of insulin resistant state, HFD Sub mice showed a blunted increase response to insulin-stimulated hypoglycemia-induced corticosterone release which is interpreted as HPA-axis dysfunction and observed in diabetic patients and animal models (Chan et al. 2002, Sapolsky et al. 2000, Dallman et al. 2007)

Subordination stress induced thermal hyperalgesia in mice

Neuropathic pain is a common phenomenon resulting from injury to central or peripheral nervous system (Kapur 2003) and considered an important late complication of diabetes (Brown and Asbury 1984). Neuropathic pain has been associated with impaired glucose tolerance (Smith and Singleton 2008, Russell et al. 2008) and chronic hyperglycemia typical of T2D (Shaikh and Somani 2010, Ueta et al. 2005, Greene et al. 1992, Tavakoli et al. 2008). On the contrary, the early stages of T2D is characterized by the development of thermal hyperalgesia in humans (Dyck et al. 2000) and animal models (Courteix et al. 1993, Calcutt 2002). As reported from Sullivan and colleagues (Sullivan et al. 2007), degree of hyperglycemia may considered primary risk factor in the development of neuropathic pain. Indeed has been reported that neuropathy and other complications associated to diabetes can be dramatically reduced or delayed by glycemic control (Duby et al. 2004, Cardone and Dyck 1990, Romanovsky et al. 2004) In our experimental model subordinate mice fed HF diet showed a decreased latency threshold in response to a painful thermal stimulus. These data are opposite to the classical observation of social-defeat induced analgesia (Mickzec et al., 1982) therefore suggesting that the hyperalgesic response might be considered a biomarker of early stage T2D rather than a pure stress-induced effect. Thermal hyperalgesia further confirm the validity of our model of pre-T2D and suggest that a longer exposure to to stress and HFD are required to observe the development of neuropatic pain.

Stress and HFD affect CNS Neuroactive steroids

Recent findings demonstrate an important role of neurosteroids in control of nervous system activity (Melcangi et al. 2001, Melcangi and Panzica 2009, Panzica et al. 2012) and their protective effects in experimental model of diabetic neuropathy (Leonelli et al. 2007, Roglio et al. 2007). Acute stress is known to modulate neurosterogenesys pathway, in particular by increase THP and allotetrahydro-deoxycorticosterone (THDOC) to level able to modulate positively $GABA_A$ receptors reducing anxiety (Mellon and Griffin 2002).

Starting from this evidence we characterized different areas of nervous system involved in neurosteroidogenesis (Baulieu et al. 2001).

The preliminary analysis described above showed that neurosteroids in spinal cord, cerebellum and cerebral cortex are affected primarily by HFD while stress in absence of HFD does not exert a major effect. More importantly subordinate mice fed HFD showed the more dramatic. . PREG was generally reduced in all brain areas in mice fed HFD. While a general trend for increased PROG and of its metabolites DHP and THP, was observed.

Cholesterol derived neurosteroids play also a beneficial role in diabetic neuropathic pain. Streptozotocyn induced diabetes induce a pheripheral sensory axon damage leading to a impaired nociception. STZ treated rats show a dramatic drop in the level of several neurosteroids (Caruso et al. 2008, Saredi et al. 2005) while treatment with PROG, DHP and THP significantly improved hypoalgesia showed by diabetic rats by reducing heat sensitivity threshold (Leonelli et al. 2007, Kawano et al. 2011). (Leonelli et al. 2007, Kawano et al. 2011)Our data showed an opposite scenario. While neuropathic pain is associated to hypoalgesia and lower level of neurosterogenesis, in our model we observed hyperalgesia and increased level of PROG and their metabolites.

Remarkably however PREG was signlificantly lower in mice fed HFD which suggest that pre-T2D might be associated with early onset deficit in P450scc enzyme that converts cholesterol to pregnenolone while the enzymes downstream of PREG can still be functionally upregulated to compensate for the lower PREG level (Saredi et al. 2005). If our hypothesis is correct extending the duration of stress+HFD should lead to a progressive decrease in all neurosteroids as observed in STZ rats. Further studies are required to confirm this hypothesis.

In conclusion the present study describes a innovative animal model of social stress induced T2D. Several animal models of stress and HFD induced obesity and metabolic syndrome have been characterized so far (Tamashiro et al. 2007, Tamashiro et al. 2011, Finger et al. 2011, Bartolomucci et al. 2009a, Chuang et al. 2010). However that was only limited evidence that T2D might develop in those animal models. In our model of CSS, mice developed spontaneously hypercortisolemia, and showed all the physiological and metabolic alterations typical of T2D only after 3 weeks of experiment allowed us to conclude that CSS can be considered a valid behavioral model.

Chapter Two

Vulnerability to obesity, plasma lipids and glucose tolerance in male mice under chronic psychosocial stress: modulation by social status

Abstract

The chronic activation of stress response has been associated with metabolic disorders. Stress-induced neuroendocrine changes, in particular hyperactivity of the HPA-axis, may alter food intake and oppose glucostatic insulin action thus resulting in increased abdominal fat, glucose tolerance and the metabolic syndrome. Overall these metabolic disorders result in a pre-diabetic state which may turn into type-2 diabetes in susceptible individuals or in a nutritional rich environment. Genetic and endocrine factors responsible for development of pre-diabetes have been well characterized, while psychogenic influences received less interest. Data discussed in Chapter 1 demonstrated that subordinate mice under chronic stress and fed a high fat diet develop a metabolic like and a pre-type 2 diabetes like syndrome. Here we set out to determine if vulnerability to stress induced metabolic disease is status dependent. Specifically we will directly compare the metabolic consequences of being high in rank (dominant) and low in rank (subordinate) in mice exposed to our model of chronic psychosocial stress CPS (Bartolomucci et al. 2004b, Moles et al. 2006) . Previous studies from our laboratory showed in male mice (CD1

strain) an opposite influence of social status in CPS-induced metabolic disorders (Bartolomucci et al., 2009). Subordinates (SUB) showed weight gain and vulnerability to high fat diet (HFD)-induced obesity. Dominants (DOM) showed decreased weight gain and resistance to HFD-induced obesity. In the present study we confirmed and extended these observations by characterizing lipid profile and glucose tolerance which are hallmarks of pre-diabetes. In line with previous findings, SUB were hyperphagic, obesity-vulnerable and showed increased fat pad weight; DOM were hyperphagic, obesity-resistant and showed decreased fat pad weight. The protective effects on body weight gain of being high in rank also resulted in a normal fasting glycemia, insulinemia and glucose tolerance and a normal plasma lipid profile when compared to SUB. Moreover Dom showed a decrease in IRS2, gluconeogenesis and glicolysis marker genes

Exposure to HFD exerted minimal effects on DOM metabolic profile. These data demonstrates a remarkable difference in the effects of social status on stress-induced metabolic like disorders and further demonstrates that individuals high in rank are remarkably resilient to metabolic-like disorders.

Materials and methods

Animals

Adult males Swiss CD1 were derived from an outbred stock originally obtain from Charles River Italia (Calco, Italy). Mice were born and reared in a colony room at University of Parma at 20 ± 2 °C and 12 hr light/dark cycle (7am – 7pm). After weaning (PND 25-28) they were housed in same sex-groups of siblings (max 8 per cage) in Plexiglass cages (38 X 20 X 18) with wood shaving bedding that was changed weekly to avoid excessive manipulation. The colony was fed with a pellet standard chow diet (STD) (Mucedola SRL, Milano, Italy). All animal experiments were conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/EEC) and approved by ethical committees of University of Parma and the Italian Institute of Health.

Diets

For this study we use two diets produced by Mucedola SRL (Milano, Italy). During baseline and first week of stress all mice were fed using a Standard Diet (SD), 4RF21 with a caloric intake of 3.9 Kcal/gr, 6.5% from fat, while starting from 2nd week of stress a group of mice were fed with a special diet derived from chow with a caloric intake of 4.5 Kcal/gr, 45% from fat (High Fat Diet - HF).

Chronic psychosocial stress (CPS)

The procedure, originally described by Bartolomucci et al. (Bartolomucci et al. 2001), consisted of a 5-day baseline period followed by 4 weeks of chronic stress experimental phase in which stressed animals cohabitated in the same cage divided by means of a perforated polystyrene-metal partition, which allows a continuous sensory contact but no aggressive interaction. For control animals, 3 month-old male mice were group housed in groups of 3 siblings.

To be used as resident animals, mice were individually housed in plexiglass cages (38 X 20 X 18) for 5 days baseline period to allow the establishment of individual territory. At the beginning of stress phase each resident mouse received an unfamiliar weight matched intruder mouse, who was previously individually housed, and the two animals were allowed to interact freely for 10 min. In order to prevent injuries, social interaction was interrupted if fighting escalated (where the dominant mouse persistently bit the opponent). After the interaction, the animals were separated by perforated partition to avoid aggressive behavior but maintained sensory contact. The partition was removed daily, always at the same time of the day, for a maximum of 10 min to let mice interact and establish a social status. During social interaction offensive and defensive behaviors were recorded and social status was determined as followed: the chasing and biting mouse was defined as “Dominant” (DOM), while the mouse displaying upright posture, flight behavior and squeaking vocalization was defined as “Subordinate” (SUB) according to methods previously described (Bartolomucci et al. 2001, Bartolomucci et al. 2004b). To be considered in the analysis, dyads developed the dominance/subordination relationship between 2^{nd} and 4^{th} day of stress and maintained a stable hierarchy over all experiments.

Procedure

During baseline period and first week of stress phase all mice were fed SD, while starting from 2nd week, experimental dyads and controls were assigned to two different groups matched by weight, food intake and aggression. One group was fed with SD while the second with HF until the end of the experiment. Throughout the stress phase body weight was monitored every other day while food intake daily. Food and water were available *ad libitum* to all experimental mice.

Serum analysis

At sacrifice plasma was collected from trunk blood after an overnight fasting using heparinized tubes (Sarstedt, s.r.l., IT), centrifuged at 4000 RPM for 10 min and plasma was frozen at -20 °C for later analysis. Levels of circulating lipids and hormones were detected using different techniques. Trunk blood was collected in heparinized tubes, centrifuged at 4,000 RPM for 10 min and plasma was frozen at −20°C for later analysis. Corticosterone was measured in duplicate with a commercially available RIA kit (Diagnostic Systems Laboratories, Inc., USA) with a sensitivity of 0.06 ng/ml. The interassay variability was 3.4%. Insulin was measured by RIA using rat insulin standards (Biotrack RPA-547, Amersham, Milan, Italy) according to manufacture instructions. Leptin, was determined using the Bio-Plex Pro™mouse diabetes 8-plex and analyzed with Bio-Plex® system, luminex technology (Bio-Rad Laboratories, Inc., USA) according to kit protocol. Quantitative analyses of plasmatic lipids, NEFA and glycemia were assessed through a computerized chemical analyzer Hitachi 911 (Roche Diagnostic Systems, USA) using Trider colorimetric enzyme methods and homogeneous enzymatic test (Reading at:

Tot cholesterol 510 nm, TGs 546 nm, HDL 600 nm). LDL has been calculated with the following formula Tot cholesterol - (TGs/5) - HDL.

Glucose tolerance tests (GTT)

Glucose Tolerance Test was performed following an overnight fast (12h). Blood glucose levels from tail bleeding were monitored at 0, 30, 60 and 120 min after intra-peritoneal injection of 0.1cc/10gr body weight of D-glucose at 10%. All blood glucose measurements were done using Accucheck Aviva glucometer (Roche Diagnostics, Indianapolis, IN, USA).

Echo-MRI (Fat and Lean mass composition)

Total body composition was measured in vivo using a 3-in-1 QMR body composition analyzer (Echo medical system, Houston, TX). Mice were introduced into a plexiglass cylinder for a maximum of 1 min to measure the fat and lean tissue in vivo.

Indirect calorimetry

Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured for each mouse for 24h after a 6 hour acclimatization period by using the Oxymax/Comprehensive Lab Animal Monitoring System (Columbus Instruments, Ohio). All oxygen consumption measurements were normalized to lean mass (ml/hour/kg lean mass) since lean tissues contribute more to the total energy expenditure compared to fat mass (Butler and Kozak 2010).

Real-Time PCR analysis

Total RNA was isolated from perigonadal fat pad, liver and muscleusingSTAT60 isolation reagent (Tel-Test, Inc., USA) according to the manufacturer's instruction and quantified by absorbance at 260 nm in a spectrophotometer (Nanodrop, Thermo Scientific). Integrity was assessed with electrophoresis agarose gel by Sybr-safe stain (Invitrogen). Real-time quantitative PCR was performed using TaqMan or SybrGreen sequence detection system on ABI 7900 instrument (Applied Biosystem) as described (Medina-Gomez et al. 2005). Expression of target genes were corrected by the geometrical average of 4 different housekeeping genes: 18S, β2-microglobulin, β-actin and 36B4 using Best-keeper tool (Pfaffl et al. 2004).

Statistical analysis

Data were checked for agreement with parametric assumption and analyzed with General linear models followed by Tukey's HSD post hoc test (Statsoft, Inc. Tulsa, OK, USA). Independent data set were analyzed by two way ANOVA using treatment (CON, SUB and DOM) and diet (STD or HFD) as between-subject factors. Dependent data set (weekly food intake and glucose tolerance test) were analyzed by two-way ANOVA for repeated measures with social rank and diet as between –factors and repetition as within-subject factor. Correlation was performed with Pearson's test. All analyses were performed using Statistica software (Statsoft, Inc. Tulsa, OK) and considered statistically significant when $p < 0.05$.

Results

Social status modulates the metabolic consequences of chronic psychosocial stress

We previously demonstrated that exposure to chronic psychosocial stress in male mice (CD1 strain) induced opposite status-dependent effect on metabolic functions (Moles et al. 2006, Bartolomucci et al. 2009b). In the present study we confirmed and extended these observations by characterizing hallmarks of metabolic syndrome and type 2 diabetes.

In line with previous findings (Bartolomucci et al. 2009b), fasting plasma Corticosterone level was increased in stressed mice after 28 days of stress exposure which is not substantially affected by social status despite the higher level was reached by DOM STD. Overall HFD resulted in a decrease in plasma Corticosterone levels (**Fig. 1A**) in line with previous findings (Dallman et al. 2003, Dallman et al. 2005, Dallman et al. 2006, Dallman et al. 2007).

DOM SUB mice showed a remarkable stress-induced hyperphagia when compared to controls both when fed standard and high fat diet. When mice are fed HF diet (Week 2) stressed mice and controls showed an overall increase in Kcal ingested compared to SD with normalization at the 4^{th} week (**Fig. 1B**). When combining food intake and body weight change, SUB and DOM mice showed an opposite effect on food efficiency. Food efficiency, calculated as BW gain per gram of food ingested, was significantly higher in SUB when compared to control and DOM fed HFD (**Fig. 1D**). SUB gained more weight and perigonadal adipose tissue while DOM gained less weight and perigonadal fat compare to controls at STD. This effect became more pronounced at HFD (**Fig. 1C-E**). Furthermore, we demonstrated an increase in total fat mass in HFD SUB mice compare to HFD CON and to HFD DOM (**Fig. 1F**).

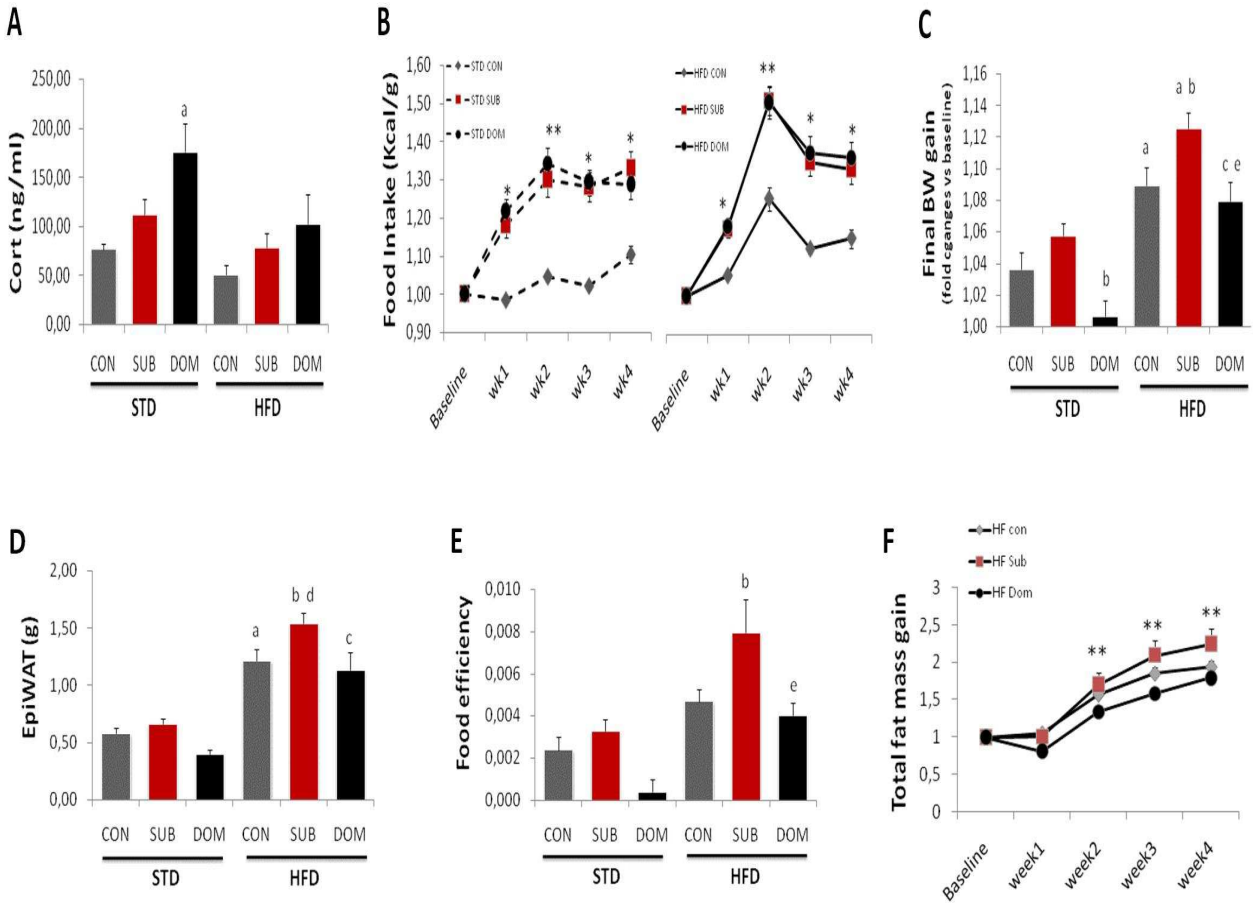

Figure 1*:* **A) Corticosterone level.** Fasting basal plasma Corticosterone collected at sacrifice was increased in SUB and DOM when compare to Controls ($F(2,81)= 5,49$ $p<0,001$). HFD induced a decrease in cort concentration in all groups (Diet $F(1,81)= 6,64$ $p<0,05$) *$p<0.05$.

STD Con, n=9; STD Sub, n=19; STD Dom, n=16; HFD Con, n=14; HFD Sub 22; HFD Dom, n=7.

B) Food Intake. Mice were always hyperphagic when compared to controls. The effect was particularly evident when mice were fed HFD (Stress $F(2,142)= 28,83$ $p<0,01$, Stress x week $F(6,426)= 2.61$ $p<0.05$). HFD induced a further increase in Kcal ingested (Diet $F(1,142)= 9,09$ $p< 0.005$, Diet x week $F(3,426)= 19,44$ $p<0.001$) * $p<0.05$, **$p<0.01$.

C) Body weight gain. . HFD induced a significant increase in body weight in all animals (Diet $F(1,150)= 52,84$ $p<0,001$). Subordinate mice fed high fat diet (HFD SUB) showed a larger body weight gain when

compared to all other groups. Dominants always showed a significant lower BW gain when compare to Subordinate mice. **a,b,c,d,e** $p<0.05$.

STD Con, n=18; STD Sub, n=23; STD Dom, n=24; HFD Con, n=23; HFD Sub, n=34; HFD Dom, n=34.

D) Food efficiency. Food efficiency calculated as BW gain (g) / total amount of food ingested (g) increased with HFD in all groups (Diet $F(1,151)= 19,11$ $p<0,001$). DOM displayed always lower while SUB always higher compare to CON with a significant increase in food efficiency at HFD (Stress $F(2,151)= 6,28$ $p<0,005$). $*p<0.05$, $**p<0.01$.

STD Con 17; STD Sub 23; STD Dom 24; HFD Con 25; HFD Sub 34; HFD Dom 34.

E**) Epididymal fat pad weight after overnight fasting.** No significant difference was found in weight of epididimal adipose tissue at STD. At HFD all groups showed an increase in visceral adiposity (Diet $F(1,105)= 108,46$ $p<0,001$) compare to STD. SUB showed a larger increase in epididimal fat pad (EpiWAT) weight when compared to all other groups (Stress $F(2,105)= 7,52$ $p<0,001$) while DOM did not differ from CON. **a,b,c,d** $p<0.05$.

STD Con, n=15; STD Sub, n=20; STD Dom, n=22; HFD Con, n=21; HFD Sub, n=25; HFD Dom, n=8.

F) Total fat mass gain (normalized on BW). Total fat mass gain vs baseline was greater in HFD Sub when compared to HFD Con and DOM starting from the 2^{nd} week of stress (Stress $F(2,28)= 3,10$ $p=0.06$) , $**p<0.01$

HFD Con, n=9; HFD Sub, n=11; HFD Dom, n=11.

In line with different vulnerability to DIO, leptin increased in SUB mice. DOM did not show any differences when compared to their Controls both STD and HFD. We found also a positive correlation between adipose tissue and leptin concentration (**Fig.2A**). We saw also a decrease in Pparg2 gene expression in WAT in DOM mice compare to their CON and SUB at STD, while HFD induced a decrease in all groups (**Table 1**).

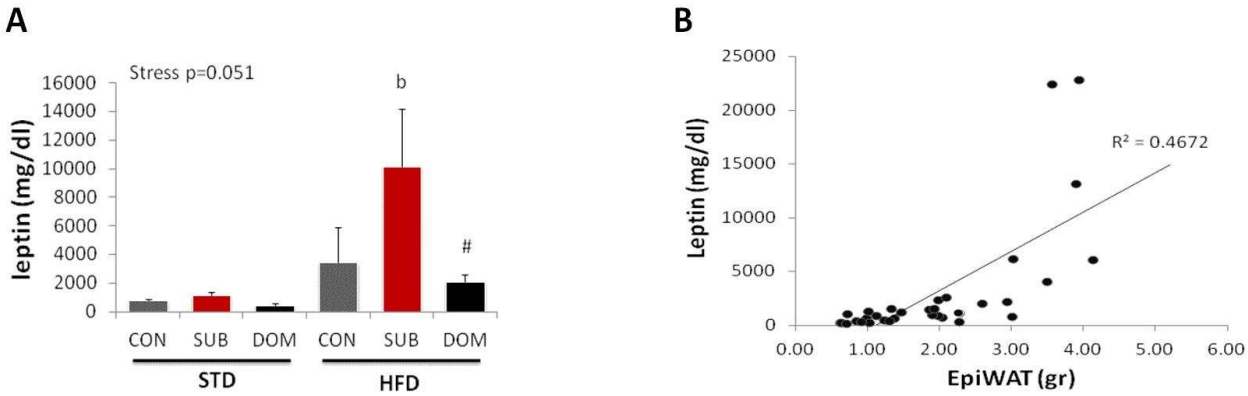

Figure 3*: A) Leptin*: Concentration of leptin was strongly regulated by HFD (Diet F(1,30)= 8.45, p<0,01) while stress induced an opposite trend effect between SUB and DOM (Stress F(2,30)=3.29, p=0.051). Subordinates at HFD showed higher levels compared to CON and SUB at STD and HFD DOM *p<0.05 , #p=0.06.

STD Con 6; STD Sub 8; STD Dom 6; HFD Con 5; HFD Sub 6; HFD Dom 5 .

B) There was a positive correlation between the amount of EpiWAT and leptin plasma concentration (r= 0.69, p<0.05)

Subordination or Dominance-associated metabolic changes

Tot cholesterol, LDL and HDL were increased as an effect of HFD. However the effect was status dependent. Dominant mice (both STD and HFD), showed no change in lipid concentration when compare to CON. Remarkably and despite DOM showed increase energy expenditure (Moles et al., 2006; Bartolomucci et al., 2009), DOM showed a complete resistance to HFD-induced FFA increased. Subordination stress on the contrary resulted in increased fasting cholesterol, LDL and NEFA, with the effect being more pronounced at HFD

Interestingly, HFD had no effect on plasma TGs levels which suggest that TG were either stored in metabolic tissues (SUB and CON) or oxidized (DOM). Only subordination stress at STD induced a raise in TGs levels when compared to all others groups, while this effect was lost at HFD. Unlike SUB mice, dominants showed same TGs level at STD and HFD and always presented lower levels when compared to SUB (**Fig. 3 A-B**). As demostrated in Chapter one, preliminary data showed an effect of subordination stress on b-hydroxybutirrate (ketone), while DOM presented lower concentration when compared to Con and SUB (**Fig. 3 C**).

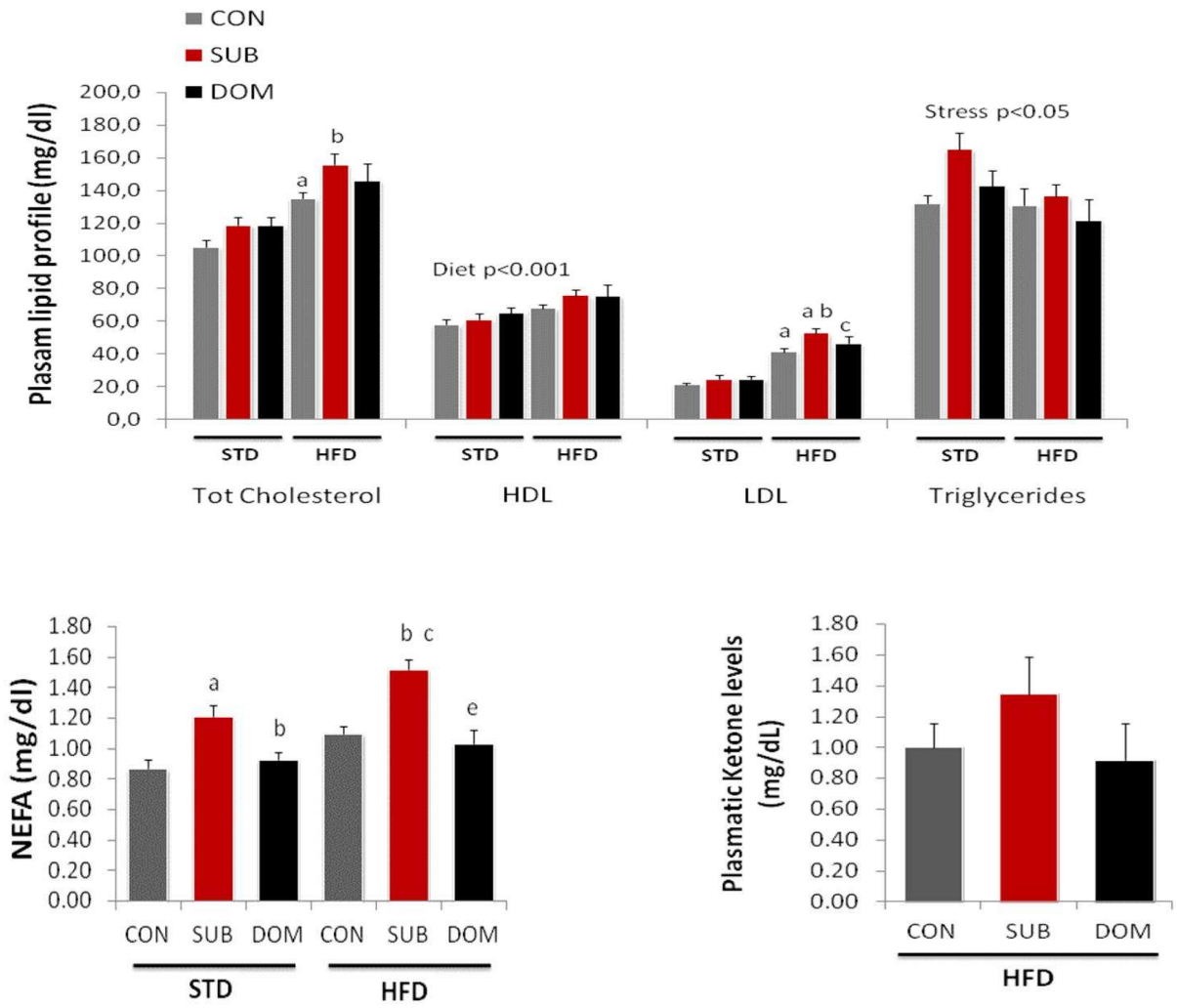

Figura 3: Lipidic profile determined at sacrifice showed: **A-B)** pronounced effect of diet on Tot Cholesterol (Diet F(1,97)= 37,04, p<0,001), LDL (Diet F(1,97)= 96,08, p<0,001), HDL (Diet F(1,97)= 12,81, p<0,001), glycemia (Diet F(1,97)= 35,73, p<0,001) and NEFA (Diet F(1,97)= 15,67, p<0,001) with mice at HFD showing an increased in lipids level. Tot Cholesterol (Stress F(2,97)= 4,01, p<0,05), LDL (Stress F(2,97)= 4,19, p<0,05), NEFA (Stress F(2,97)= 26,64, p<0,001), glycemia (Stress F(2,97)= 4,88, p<0,01) and TGs (Stress F(2,97)= 3,31, p<0,05) were increased also by subordination stress at STD and HFD. Only glycemia showed an interaction between diet and stress (Stress x Diet F(2,97)= 7,31, p<0,005) with SUB showing always higher, while DOM lower glucose concentration when compared to their CON.

C) Preliminary data showed an increase in ketones in Sub mice at HFD while DOM are similar when compare to CON. **a,b,c,d,e** p<0.05.

STD Con 14; STD Sub 16; STD Dom 20; HFD Con 19; HFD Sub 26; HFD Dom 8.

Glucose tolerance and insulin sensitivity are modulated by social status

Because obesity has been associated with metabolic syndrome, insulin resistance and diabetes (Gallagher, Leroith and Karnieli 2011) we focused our attention on key features considered risk factors for developing these pathologies.

After 3 weeks of stress, mice fed STD did not show any significant change in basal glucose or glucose tolerance compare to CON. HFD induced a marked glucose intolerance that was different in stressed and Control mice. SUB showed basal hyperglycemia and higher glucose intolerance while DOM responded similarly to CON mice upon glucose injection (**Fig. 4A).**

At sacrifice (1 week after GTT), HFD-SUB showed an even higher fasting glycemia when compared to all other groups (**Fig. 4B)**. Moreover, Subordination stress induced an increase in insulin concentration. This effect was more pronounced when animals were fed HFD, suggesting an insulin resistance as demonstrated by an increase in HOMA-IR index in HFD SUB (**Fig. 4C-D**). Meanwhile DOM seemed to be protected against stress-induced hyperglycemia and hyperinsulinemia, with DOM showing normal glucose and insulin levels when compare to controls at STD.

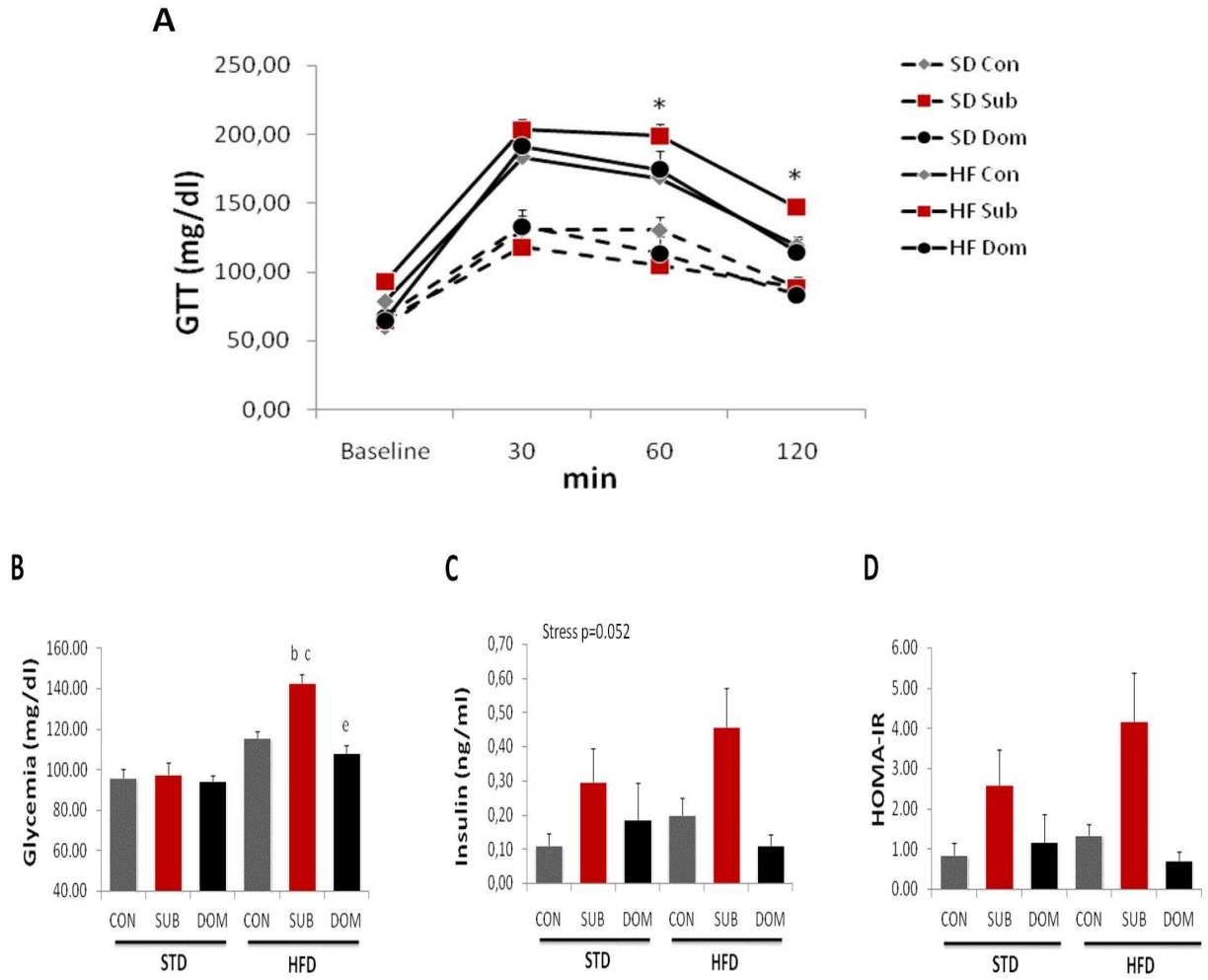

Figure 4*:* A) Glucose tolerance test (GTT). Mice at HFD showed higher levels of glucose at baseline (Diet $F(1,66)= 55,01$ $p<0,001$) and responded with higher glucose concentration after injection (Diet x Time $F(3,198)= 19,24$, $p<0,001$) We saw also a trend interaction between stress and diet (Stress x Diet $F(2,66)= 2,94$, $p=0.059$). At STD mice showed the same glucose tolerance profile. HFD induced only in SUB mice a higher glucose intolerance compare to CON and DOM *$p< 0.05$.

STD Con 9; STD Sub 8; STD Dom 10; HFD Con 20; HFD Sub 19; HFD Dom 6.

B) Glycemia. glucose level was increased as main effect of HFD (Diet $F(1,97)= 35,73$ $p<0,001$). HFD SUB showed the highest glucose concentration compare to STD, HFD Con and Dom (Stress $F(2,97)= 4,88$ $p<0,01$, Stress x Diet $F(2,97)= 7,31$, $p<0,005$) **a,b,c** $p<0.05$, #$p=0.054$

STD Con 14; STD Sub 16; STD Dom 20; HFD Con 19; HFD Sub 26; HFD Dom 8.

C) Insulin. Subordination stress induced an increase by trend of insulin concentration both at STD and HFD (Stress F(2,54)03,12, p=0,52). DOM mice showed the same insulin concentration at both diets. STD Con 9; STD Sub 9; STD Dom 6; HFD Con 12; HFD Sub 19; HFD Dom 5.

D) HOMA-IR. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting insulin and glucose levels as (insulin X glucose)/22.5 where insulin where reported as mU/l and glucose as mmol concentration (Matthews et al. 1985). HFD induced a raise in HOMA-IR only in SUB mice while DOM and CON did not change. Moreover subordination stress determined an increase in insulin resistance index compare to CON and DOM at STD and HFD.

STD Con 6; STD Sub 6; STD Dom 5; HFD Con 11; HFD Sub 19; HFD Dom 4.

In previous chapter we demonstrated that insulin resistance in SUB animals can be explained at least in part with a decrease in gene expression of gene associated with insulin signaling in metabolic tissues, among them a decrease in IRS2 in muscle and liver. At variance with the downregulation of IRS2 observed in SUB mice, DOM showed no differences when compared to their controls, suggesting a normal functionality of insulin signaling. HFD induced a decrease in IRS2 expression in CON and DOM but they always remained higher compare to SUB mice (**Fig. 5).**

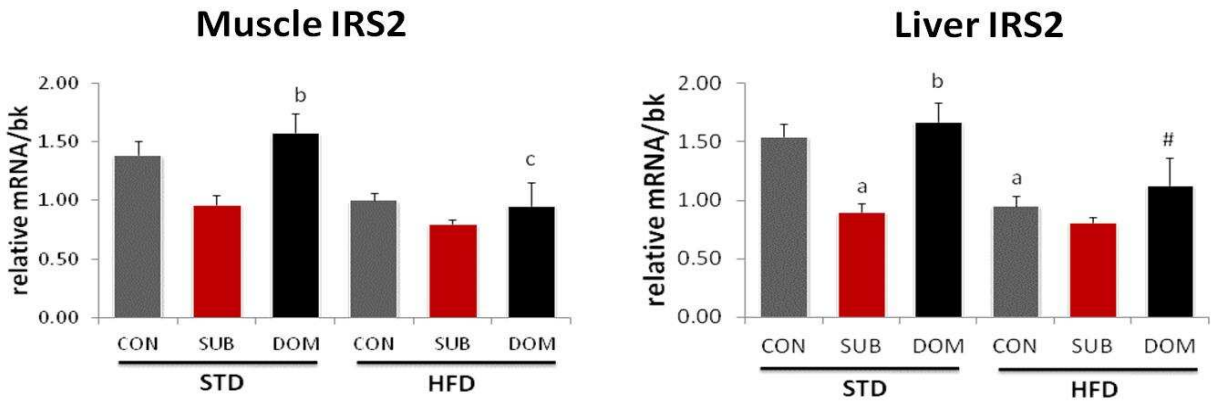

Fig.5 IRS2 in muscle and liver was down-regulated by HFD (muscle: F(1,100)= 19.03, p<0,05); Liver: F(1,102)= 17.89, p<0,001). In muscle we saw also an effect of stress (F(2,100)=8.72, p<0.05). SUB mice

always showed a down-regulation when compare to CON and in particular with DOM at STD and HFD. **a,b,c** p<0.05, #p=0.06 vs STD Dom.
STD Con 17; STD Sub 19; STD Dom 18; HFD Con 20; HFD Sub 26; HFD Dom 8.

Moreover, DOM mice showed an decrease in gluconeogenesis genes, such as Phosphoenol pyruvate carboxykinase (PEPCK) and Pyruvate carboxylase (PC), and a decrease in glicolisys with a reduction of Glucose kinase (GK), when compare to their CON and SUB. The overall effect of HFD was a decrease decreasing PEPCK and PC expression in DOM. (**Table 4)**.

Table4

													Effects	
Liver	**SD Con**		**SD Sub**		**SD Dom**		**HF Con**		**HF Sub**		**HF Dom**		**Diet**	**Stress** **D x S**
PEPCK	0,98 ± 0,05 **a**		0,90 ± 0,05 **b**		1,28 ± 0,08 **a,b**		0,86 ± 0,07		0,68 ± 0,06		0,95 ± 0,13		p<0.00	p<0.001
PC	1,23 ± 0,06		1,18 ± 0,06		1,28 ± 0,05 **a**		1,09 ± 0,06		1,04 ± 0,04		0,94 ± 0,09 **a**		p<0.001	
GK	0,45 ± 0,06 **a**		0,47 ± 0,05 **b**		0,48 ± 0,08		1,45 ± 0.21 **a,c**		1,34 ± 0,16 **b,d**		0,47 ± 0,11 **c,d**		p<0.00	p< 0.05 p< 0.0
WAT														
PPARg2	1,13 ± 0,08 **a**		1,47 ± 0,16		1,04 ± 0,09		1,11 ± 0,11		1,01 ± 0,09 **a**		1,06 ± 0,16			p=0.08
Muscle														
GLUT4	2,21 ± 0,12		1,97 ± 0,10		2,26 ± 0,13		2,09 ± 0,13		1,76 ± 0,09		1,88 ± 0,17		p<0.05	p<0.05

SD Con 17, SD Sub 19-20, SD Dom 17-19, HF Con 20-22, HF Sub 24-26, HF Dom 7-8

Level of gene expression in all groups. mRNA level of above genes were measured using qRT PCR and corrected using best-keeper value (mean of 18S, 36B4, b2M, b-actin). An overall effect of diet was found in PEPCK (F(1,104)=12.60,=), PC (F(1,104)=18.04), GK (F(1,99)=21.89) and GLUT4 (F(1,100)=4.12).
A significant effect of stress was found in PEPCK (F(2,104)=8.32), GK (F(2,99)=4.31) and GLUT4 (F(2,100)=3.66), while only GK showed also a significant interaction between diet and stress (F(2,99)=4.58).
a,b,c,d, p<0.05

Discussion

In the present study we provide a characterization of social status–related physiological and metabolic effects in mice undergoing a chronic-psychosocial stress interaction. Up to now different studies have demonstrated the role of social status on energy balance in different rodent models (Foster et al. 2006b, Bartolomucci et al. 2004b, Moles et al. 2006, Bartolomucci et al. 2005) but little work has been done on the characterization of metabolic parameters. Here we focused our attention primarily on the role of dominance in energy balance and metabolic dysfunction.

Subordinates and dominants show effects of chronic social stress

Animals under chronic social stress, regardless of social status, showed an increase in glucocorticoids level (Sapolsky 2005, Bartolomucci et al. 2009b, Bartolomucci et al. 2001, Tamashiro, Nguyen and Sakai 2005) contrary to the report by Finger et colleagues (Finger et al. 2011) which showed no changes in corticosterone level under chronic stress, but only when chronic stress was followed by an acute stress stimulation. As reported in chapter one, HFD induced a reduction in Corticosterone concentration in animal fed high fat diet in agreement with previous studies by Mary Dallman`s group (Dallman et al. 2003, Dallman et al. 2005, Dallman et al. 2006, Dallman et al. 2007). In line with this hypothesis HFD SUB and DOM mice showed an increased food efficiency and weak release of circulating Corticosterone at HFD compare to STD (Pecoraro et al. 2004).

Dominant mice are resistant to DIO

Obesity and visceral adiposity are highly correlated with glucocorticoids levels in human (Dallman et al. 2004, Strain et al. 1980) and animal models (Rosmond et al. 1998, Dallman et al. 2006, Dallman et al. 2007, Sapolsky et al. 2000). However social status and individual differences in corticosterone-obesity connection have not received a great attention. In our model we consistently demonstrated that, despite stress-induced hyperactivation in HPA axis, SUB and DOM mice showed opposite metabolic consequences (Bartolomucci et al. 2004b, Bartolomucci et al. 2009b) . In the present study we confirmed and extended previous data published by our group (Bartolomucci et al. 2009b). When fed STD subordinate showed hyperphagia, slight increase in body weight and no change in adiposity. However, when fed HFD SUB mice showing a remarkable vulnerability to obesity. On the contrary, despite hyperphagia, DOM showed a lower increase in BW and adiposity at STD and HFD compare to both controls and SUB mice. As shown by Bartolomucci et al., (2009), chronic dominance stress has an effect on reducing the diameters of perigonadal adipose tissue cells, hyperthermia and increased sympathetic tone in adipose tissue. Peroxisome profilerator-activated receptors, particulary PPARg-2, are key regulators of adipocyte differentiation and energy storage in WAT (Vidal-Puig et al. 1997, Medina-Gomez et al. 2007) and have been associated with obesity development (Flier 1995). The reduction of adipogenic Pparg2 gene in WAT in DOM mice compare to SUB could be considered one of the possible mechanisms explained by a lower fat pad increase due to dominance. Interestingly preliminary data showed that baseline fat mass percentage predicts social rank contrary to what assessed by Kellie and colleagues (Kellie et al, 2007). Our result was in line with what has been previously demonstrated concerning energy expenditure (Moles et al. 2006). These data are however preliminary and have not been discussed in the present thesis.

In accordance with described change in BW and adipose tissue circulating leptin was increased primarily as a consequence of subordination stress at HFD while DOM showed a slight increase in proportion to the amount of adipose tissue (Halaas et al. 1995, Halaas et al. 1997, Friedman and Halaas 1998). The slight increase in BW, adipose tissue and leptin at HFD suggested negative energy balance leading to a resistance to DIO in DOM.

The negative energy balance shown by DOM was the cause of a decrease in food efficiency compared to SUB and is in accordance with published data showing a locomotor hyperactivity , hyperthermia, tachycardia and increased symphatetic tone in dominant mice under CPS (Bartolomucci et al. 2003a, Bartolomucci et al. 2005, Bartolomucci 2007, Bartolomucci et al. 2009b, Moles et al. 2006, Bartolomucci et al. 2004a). Taken togheter, these results suggest a higher energetic cost in determining and maintainig dominant status (Sapolsky 2005, Bartolomucci et al. 2005, Moles et al. 2006).

In humans, it has been demostrated that life events such as social stress and socio-economics challenges, are associated with increased cortisol secretion, compulsive eating behavior, metabolic syndrome and T2D (Björntorp 2001, Rosmond et al. 1998, Van Strien et al. 1986). As demostrated in the present study, SUB and DOM mice showed an increase in corticosterone and hyerphagia, but resulted in opposite metabolic effects on vulnerability to DIO. As described in chapter one SUB mice showed a vulnerability to obesity, increased adiposity (Kuo et al. 2007, Bartolomucci et al. 2009b) and insulin and leptin resistance (Halaas et al. 1997, Friedman and Halaas 1998) , considered risk factors of metabolic and T2D onset, especcially if fed HFD.

Dominant mice are less vulnerable of metabolic consequences of DIO

Chronic HPA axis hyperactivation is been associated with pathogenesis of metabolic syndrome (Chrousos 2000, Rosmond 2005, Anagnostis et al. 2009) and dyslipidemia is recognized as one of the principal components of metabolic syndrome (Alberti et al. 2006). As reported by Moles and colleagues (2006) DOM mice, subjected to a similar social stress protocol, showed a lower increase in FFA as well as TGs when compare to SUB. Here we confirmed these data reporting as well as a resistance to diet induced raise in FFA and TGs in DOM also a lower tot cholesterol and LDL levels when animals are fed HFD.

Not only dominant mice, showed resistance to obesity and absence of a metabolic-like syndrome (despite hyperphagia and upregulated HPA-axis) they also showed a remarkable resistance to the development of diabetes. DOM mice, showed comparable if not increased HPA axis activation when compared to SUB, showed normal fasting, glucose and insulin concentration. Indeed lower HOMA-IR index suggested a complete insulin sensitivity in dominant mice. Contrary to the common findings that GC-induced hyperglycemia (Lansang and Hustak 2011), our data demonstrate a normal glucose balance despite increased plasma corticosterone, which is in line with recent data (Shpilberg et al. 2012, in press). Moreover it should be noted that both corticosterone and insulin are necessary in higher concentrations to induce an increase in visceral adipose tissue (la Fleur et al. 2004).

As discussed in Chapter one SUB showed a decrease in IRS2 in liver and muscle, associated with T2D (Withers et al. 1998). On the contrary, DOM showed completely normal IRS2 level in both liver and muscle. These findings corfirmed a profile of normal glucose homeostasis in dominant stress animal.

In conclusion we demonstrate that the social status has a main impact on the metabolic consequences of chronic stress. Dominance in particular protects mice from obesity and diabetes

Chapter Three

The role of hyperphagia in stress-induced metabolic disorders in mice: modulation by social status

Abstract

As reported from Bartolomucci et al, mice subjected to chronic psychosocial stress spontaneously develop hyperphagia. This behavior add to a depressed-like phenotype showed by SUB mice, induced an higher vulnerability to DIO compare to dominant mice. To better understand the role of hyperphagia in subordination stressed-induced obesity and T2D (as previously described) we designed a Pair-feeding (PF) protocol in which stressed mice, both SUB and DOM, were fed with a controlled amount of food (for details see protocol below). Results showed that limit spontaneous hyperphagia was able to abrogate subordination stress-induced obesity with a severe effect in DOM. Despite losing weight most of the metabolic consequences of stress were not reverse. Stressed mice, both SUB and DOM, showed hyperglycemia and dyslipidemia despite molecular signatures of insulin resistant state were partially reverse by PF. Only Triglycerides level were improved as a consequence of PF. As last evidence we postulate the existence of a Stress Induced-Binging-Obesity (SIBO) syndrome. Binge Eating Disorders (BED) is a pattern of eating disorders characterized by an uncontrolled and compulsive eating in brief period of time and is associated with obesity (Stunkard 2011, de Zwaan 2001) and present higher co-morbidity with psychopathologies as depression (Dingemans and van Furth 2012). Several line of evidence suggest also connection between stress

and BED in humans (Van Strien et al. 1986, Stein et al. 2007) Behavior animal model (tail pinch, restrain test, shock test) but only few underling the role of social stress in the eating disorder onset.

Starting from this evidences we postulated that Subordinate mice can be considered a valid model to study BED because presenting hyperphagia, obesity and depression (Bartolomucci et al. 2001, Bartolomucci et al. 2004b, Bartolomucci et al. 2005, Bartolomucci et al. 2009a, Dadomo et al. 2011). Moreover we demonstrated also an effect of acute stress-induce hyperphagia and hunger-induced hyperphagia

Base on these evidences we performed a study using a Pair-Feeding (PF) protocol to test the hypothesis if CPS-induced BED was required to induced obesity and metabolic-like syndrome.

Data suggested that limiting hyperphagia was able to reverse the effect of DIO in Sub and Dom mice but not the metabolic consequences induced by Stress at HFD.

Material and methods

Animals

Adult males Swiss CD1 were derived from an outbreed stock originally obtain from Charles River Italia (Calco, Italy). Mice were born and reared in a colony room at University of Parma at 20 ± 2 °C and 12 hr light/dark cycle (7am – 7pm). After weaning (PND 25-28) they were housed in same sex-groups of siblings (max 8 per cage) in Plexiglass cages (38 X 20 X 18) with wood shaving bedding that was changes weekly to avoid excessive manipulation. The colony was fed with a pellet standard chow diet (STD) (Mucedola SRL, Milano, Italy). All animal experiments were conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/EEC) and approve by ethical committees of University of Parma and the Italian Institute of Health.

Diets

For this study we use two diets produced by Mucedola SRL (Milano, Italy). During baseline and first week of stress all mice were fed using a Standard Diet (SD), 4RF21 with a caloric intake of 3.9 Kcal/gr, 6.5% from fat, while starting from 2nd week of stress a group of mice were fed with a special diet derived from chow with a caloric intake of 4.5 Kcal/gr, 45% from fat (High Fat Diet - HF).

Chronic psychosocial stress (CPS)

The procedure, originally described by Bartolomucci et al. (Bartolomucci et al. 2001), consisted of a 5-days baseline period followed by 4 weeks of chronic stress experimental phase in which stressed animals cohabitated in the same cage divided by means of a perforated polystyrene-metal partition, which allows a continuous sensory contact but no aggressive interaction. As control animals, 3 months-old male mice were group housed in group of 3 siblings.

To be used as resident animals, mice were individually housed in plexiglass cages (38 X 20 X 18) for 5 days baseline period to allow the establishment of individual territory. At the beginning of stress phase each resident mouse received a unfamiliar weight matched intruder mouse, who was previously individually housed too, and the two animals were allowed to interact freely for 10 min. In order to prevent injuries, social interaction was interrupted if fighting escalated (with dominant mouse persistently bit the opponent). After the interaction, the animals were separated by perforated partition to avoid aggressive behaviors but maintained sensory contact. The partition was removed daily, always at same time of the day, for a maximum of 10 min to let mice interact and establish a social status. During social interaction offensive and defensive behaviors were recorded and social status was determined as followed: the chasing and biting mouse was defined as “Dominant” (Dom), while the mouse displaying upright posture, flight behavior and squeaking vocalization was defined as “Subordinate” (SUB) according to previously described (Bartolomucci et al. 2001, Bartolomucci et al. 2004b, Bartolomucci et al. 2009a). To be considered in the analysis dyads developed the dominance/subordination relationship between 2^{nd} and 4^{th} day of stress and maintained a stable hierarchy over all experiment.

Pair feeding protocol

Mice, subjected to a CPS stress were exposed to STD during baseline and first week of stress (to induce dominance and induce depression-like effect). HFD was provided from 2nd week until the end of the experiment (e weeks in total). To limit spontaneous hyperphagia and investigate its mechanistic role in stress-induced obesity we will use a pair feeding protocol (PF) (Ellacott et al. 2010). At baseline all mice received STD food *ad libitum.* During 1st week of stress, SUB and DOM mice were pair fed to their baseline food intake, while during the 3 weeks of HFD they were pair fed to food ingested by the CON-group how started HFD one day before (**Fig. 1**). Using this protocol, the normal transient hyperphagic response to palatable diet shown by control mice, as showed in Chapter one, will not be prevented (Bartolomucci et al. 2009a).

Serum analysis

At sacrifice plasma was collected from trunk blood after an overnight fasting using heparinized tubes (Sarstedt, s.r.l., IT), centrifuged at 4000 RPM for 10 min and plasma was frozen at -20 °C for later analysis. Levels of circulating lipids and hormones were detected using different techniques. Trunk blood was collected in heparinized tubes, centrifuged at 4,000 RPM for 10 min and plasma was frozen at −20°C for later analysis. Corticosterone was measured in duplicate with a commercially available RIA kit (Diagnostic Systems Laboratories, Inc., USA) with a sensitivity of 0.06 ng/ml. The interassay variability was 3.4%. Insulin was measured by RIA using rat insulin standards (Biotrack RPA-547, Amersham, Milan, Italy) according to manufacture instructions.

Leptin, ghrelin and glucagone were determined using the Bio-Plex Pro™mouse diabetes 8-plex and measured with Bio-Plex® system, luminex technology (Bio-Rad Laboratories, Inc., USA) according to kit protocol. Quantitative analyses of plasmatic lipids, were assessed through a computerized chemical analyzer Hitachi 911 (Roche Diagnostic Systems, USA) using Trider colorimetric enzyme methods and homogeneous enzymatic test (Reading at: Tot cholesterol 510 nm, TGs 546 nm, HDL 600 nm). LDL has been calculated with the following formula Tot cholesterol - (TGs/5) - HDL.

Glucose tolerance tests (GTT)

Glucose Tolerance Test was performed following an overnight fast (12h). Blood glucose levels from tail bleeding were monitored at 0, 30, 60 and 120 min after intra-peritoneal injection of 0.1cc/10gr body weight of D-glucose at 10%. All blood glucose measurements were done using Accucheck Aviva glucometer (Roche Diagnostics, Indianapolis, IN, USA).

Real-Time PCR analysis

Total RNA was isolated from perigonadal fat pad, liver and muscleusingSTAT60 isolation reagent (Tel-Test, Inc., USA) according to the manufacturer's instruction and quantified by absorbance at 260 nm in a spectrophotometer (Nanodrop, Thermo Scientific). Integrity was assessed with electrophoresis agarose gel by Sybr-safe stain (Invitrogen). Real-time quantitative PCR was performed using TaqMan or SybrGreen sequence detection system on ABI 7900 instrument (Applied Biosystem) as described (Medina-Gomez et al. 2005). Expression of target

genes were corrected by the geometrical average of 2 different housekeeping genes: 18S, β2-microglobulin, using Best-keeper tool (Pfaffl et al. 2004).

Statistical analysis

Two different analysis were performed with these set of data. In the first analysis we compared CON (fed *ad libitum*) *vs* pair-fed stressed mice (SUB-PF and DOM-PF) using a one-way ANOVA with Social rank (stress effect) as between factor. In the second analysis we compared stressed mice fed *ad libitum* (SUB ad lib and DOM ad lib) *vs* Pair-fed stressed mice (SUB PF and DOM PF) with social rank (stress effect) and diet (ad lib or PF) as between-factors. Dependent data set (BW gain, weekly food intake and glucose tolerance test) were analyzed with a two-way ANOVA for repeated measures wirh, social rank (1^{st} comparison) or social rank and diet (2^{nd} comparison) as between-factors and repetition as within-subject factor. All analysis were followed by Tukey's HSD post hoc test (Statsoft, Inc. Tulsa, OK).

Results

Hyperphagia is required for stress-induced obesity at HFD

As showed in Chapter One and Two, animal subjected to a chronic psychosocial stress especially if at HFD developed a remarkable hyperphagia. Despite this behavior was present in both SUB and DOM mice the prodiabetic/metabolic like syndrome occurred only in Subordinates who showed a higher vulnerability to DIO, hyperglycemia and dislipidemia. To better understand the importance of stress add HFD, in relation to the metabolic syndrome onset, we determined if hyperphagia is required for stress-induced metabolic changes. Mice exposed to CPS were subjected to a Pair-fed protocol (SUB PF, DOM PF) and compared with stressed mice coming from experiment with ad lib food (Chapter two, SUB ad lib, DOM ad lib). The controls were group house siblings with no food restriction. In detail the PF protocol consisted in a baseline phase with standard diet food ad libitum. During the 1st week of stress, SUB and DOM ate the amount of food they were eating during baseline (STD). Starting from 2nd week of stress until the end of experiment, stressed mice were fed at HFD with the amount of food eating by controls (normalized per BW) (**Table 1**).

Eperimental groups	Baseline	Stress week 1	Stress week 2-4
CON ad lib	STD	STD ad lib	HFD diet ad lib
SUB PF	STD	STD Pair fed vs Baseline	HFD Pair fed vs CON ad lib
SUB ad lib	STD	STD ad lib	HFD diet ad lib
DOM PF	STD	STD Pair fed vs Baseline	HFD Pair fed vs CON ad lib
DOM ad lib	STD	STD ad lib	HFD ad lib

Table 5: Experimental time line for Pair-feeding protocol.

Using a PF protocol we were able to limit the spontaneous hyperphagia showed by stressed mice fed ad lib but not the normal transient hyperphagic response to high palatable fat diet shown by control mice, as well when the HFD food was first present on week 2(**Fig. 1A**) (see Chapter One and Two, (Bartolomucci et al. 2009a). Preventing hyperphagia completely abrogated diet-induced obesity in SUB PF. Indeed SUB PF mice showed no difference in BW and epiWAT compared to CON ad lib fed mice and were drastically leaner then SUB ad lib. As shown in Chapter two, at sacrifice SUB and DOM, regardless feeding protocol, showed opposite effect on BW gain and epididimal weight (**Fig. 1B-C**). In line with this predictable effect of social status on energy balance, DOM PF mice, showed a drastic reduction in BW and epiWAT compared to all other groups. Remarkably, fasting plasma corticosterone was increased in PF SUB mice when compared with ad lib fed SUB and CON. On the contrary PF DOM only showed a slight and not significant increase in corticosterone when compared to ad lib fed DOM (**Fig. 1D**).

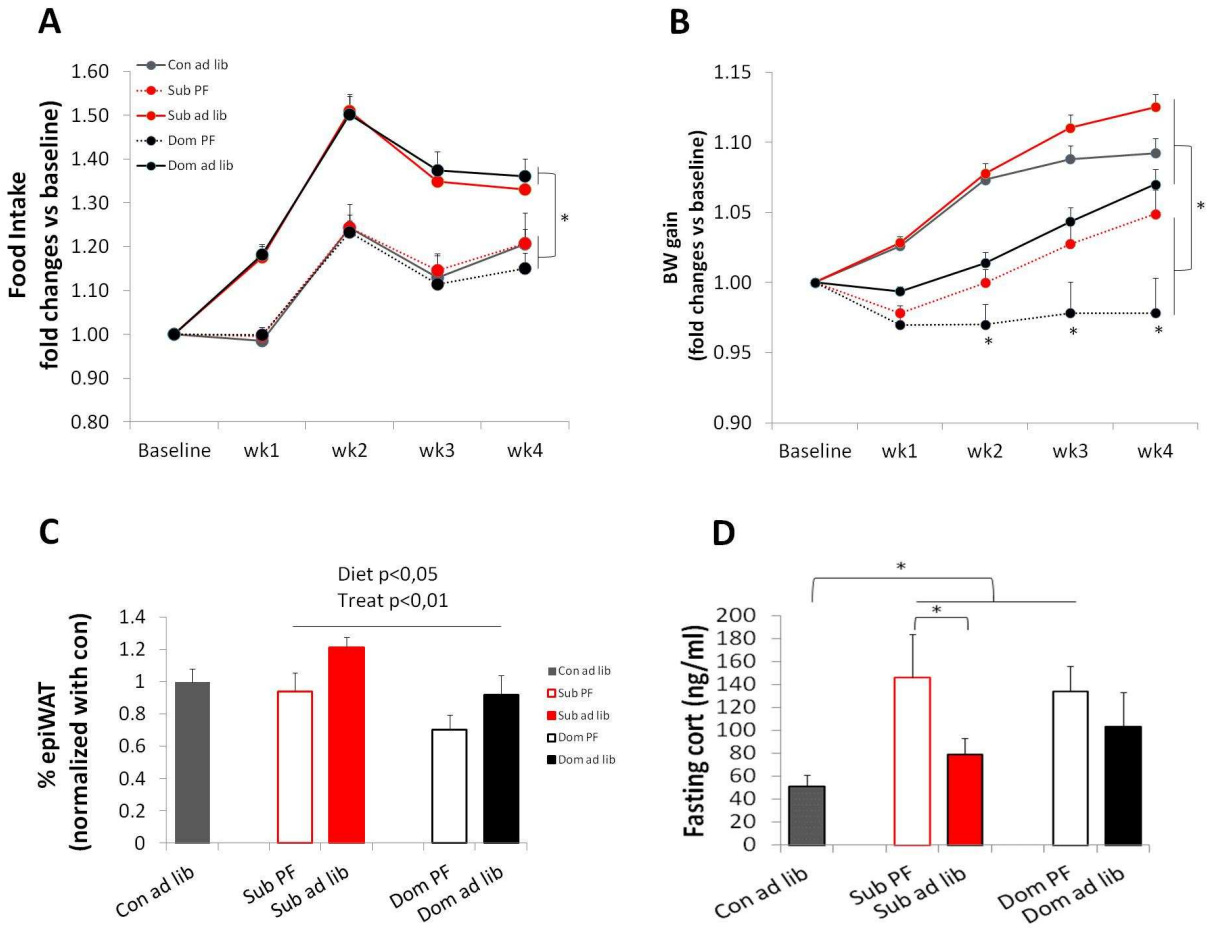

Figure 1: A) Stressed mice pair-fed (SUB PF, DOM PF) showed a decrease food intake when compare to ad lib SUB and DOM (Diet $F(1,89)=33,03$ $p<0.05$). Regardless diet (PF, ad lib) and treatment (CON, SUB and DOM) all animals showed the hyperphagic response to HFD (Diet x week $F(3,297)= 3,13$ $p<0.001$). *$p<0.05$ CON ad lib, n=12; SUB PF, n=11; DOM PF, n=11; SUB ad lib, n=34; DOM ad lib, n= 32.

B) Limiting hyperphagia prevent the effect of stress induce changes in BW gain compare to CON ($F(2,31)=5,40$ $p<0.01$). Mice PF showed a reduction in BW gain when compare to ad lib counterpart (Diet $F(1,84)=37,29$ $p<0.001$, Diet x week $F(3,252)= 10,18$ $p<0.001$). Stressed mice, both PF or ad lib fed, showed social-status effect, with SUB always weighing more the DOM (Treatment $F(1,84)=19,79$ $p<0.001$, treatment x week $F(3,252)= 9,95$ $p<0.001$). *$p<0.0$

CON ad lib, n=12; SUB PF, n=11; DOM PF, n=11; SUB ad lib, n=34; DOM ad lib, n= 32.

C) Limiting hyperphagia was also able to prevent the effect on adiposity with PF mice showing a lower increase in epididimal fat pad weight (normalized on Con) when compare to ad lib mice (Diet, $F(1,48)=6,08$ $p<0,05$; Treatment ($F(1,48)=7,20$ $p<0.01$)

CON ad lib, n=21; SUB PF, n=9; DOM PF, n=10; SUB ad lib, n=25; DOM ad lib, n= 8.

D) Circulating Corticosterone was increase as effect of pair-feeding (Diet F(1,39)= 3,87 p=0.056). SUB PF showed a significant increase when compare to SUB ad lib ($t\text{-test}_{1,20}$=-4,64 p<0.001). *p<0.05
CON ad lib, n=7; SUB PF, n=7; DOM PF, n=7; SUB ad lib, n=22; DOM ad lib, n= 7.

In line with a reduction in BW and epiWAT circulating leptin was decreased while ghrelin was increased in SUB PF compare to SUB ad lib. DOM PF showed no changes when compared to DOM ad lib (**Figure 2**)

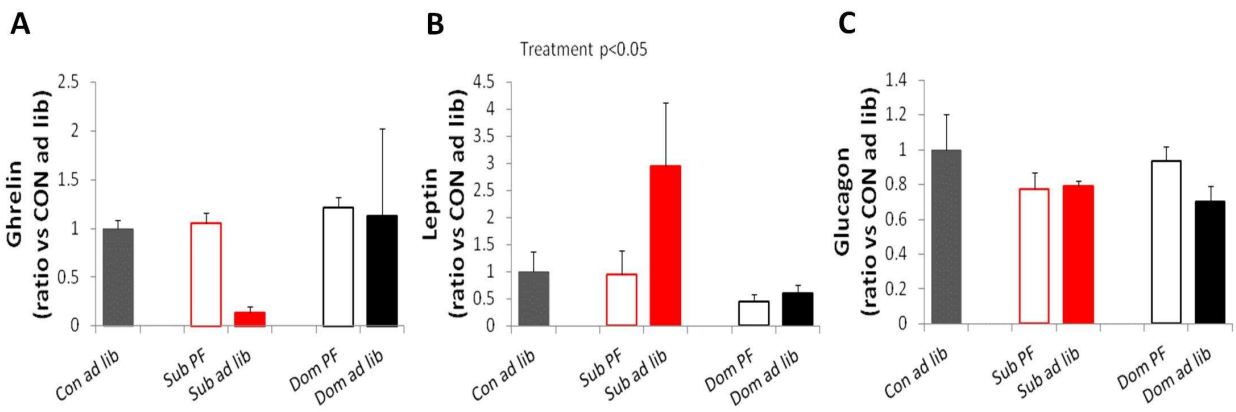

Figure 2: Hormones level (express as ration upon CON ad lib) A) Ghrelin level were reduced in SUB PF but not in DOM PF when compare to their counterpart fed ad lib. **B)** On the other side Leptin was decreased in SUB PF but not in DOM PF (Treatment F(1,25) = 6,27 p<0.05). **C)** No changes in Glucagon level between PF and ad lib mice. *p<0.05
SUB PF, n=8-9; DOM PF, n=7-10; SUB ad lib, n=6; DOM ad lib, n= 5-6

Prevent stress-induced hyperphagia is not sufficient to normalize glucose tolerance in subordinate mice

Subordinate but not dominant mice induced glucose intolerance and a metabolic-like syndrome in mice ad libitum fed HFD. To determine if a pair feeding protocol could normalize glucose tolerance as well obesity PF mice underwent the GTT. The same. Despite a reduction in BW and epiWAT in PF mice, both SUB PF and DOM PF showed a higher fasting glucose when compared to CON, SUB and DOM fed *ad libitum*. Overall PF groups showed improved glucose tolerance as demonstrated by similar peak glucose level at 30 to 120 minutes when compared to ad lib fed groups. (**Fig. 3A-B**). Expression of IRS2 in liver, considered a target gene in insulin resistance, showed same profile in PF and ad lib animals. IRS2 (expressed as ration upon CON) was slightly decreased in SUB PF and increased in DOM PF compare to CON as for ad lib mice fed HFD (**Fig. 3C**).

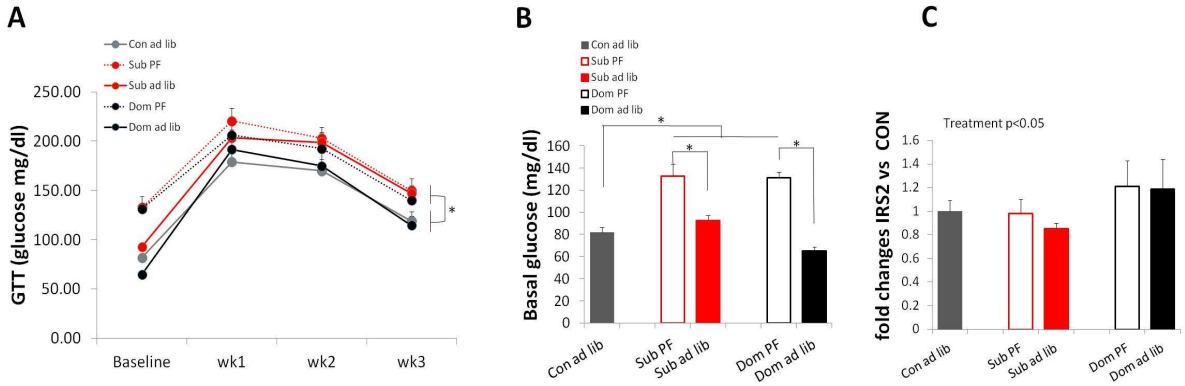

Figure 3: A) Glucose tolerance test: mice pair-fed showed an higher basal glycemia compare to CON (treatment, $F(2,34)=13,44$ $p<0.001$) and mice fed ad libitum (diet, $F(1,47)=47,29$ $p<0.001$; Treatment, $F(1,47)=4,43$ $p<0.05$) as shown in panel **B).** Pair-feeding induced an overall increase of glucose level during GTT (diet, $F(1,47)=5,58$ $p<0.05$), *$p<0.05$.

CON ad lib, n=11; SUB PF, n=13; DOM PF, n=13; SUB ad lib, n=19; DOM ad lib, n= 6.

C) Liver IRS2 mRNA level (express as variation upon control) showed an overall effect of treatment ($F(1,48)=4,54$ $p<0.05$) with DOM mice always showing higher expression when compare with SUB and CON. PF had no effect on IRS2 expression.

CON ad lib, n=12; SUB PF, n=9; DOM PF, n=9; SUB ad lib, n=26; DOM ad lib, n= 8.

Furthermore pair-feeding alone was not sufficient to correct completely the dyslipidemia observed in Subordinate mice fed ad libitum HFD (see Chapter two). Tot cholesterol, LDL and HDL were not different between PF mice and their ad lib fed counterpart. On the other hand plasma TGs were significantly reduced in PF DOM and SUB mice suggesting that PF induced a remarkable increase in lipolysis (**Fig. 4**). The increased in lipolysis were supported by a higher concentration of ketone b-hydroxybutirrate in DOM PF compared to DOM ad lib fed and controls. As showed in Chapter One, ketones concentration was increased in SUB ad lib fed compare to control. Pair feeding did not induced a further increase in SUB.

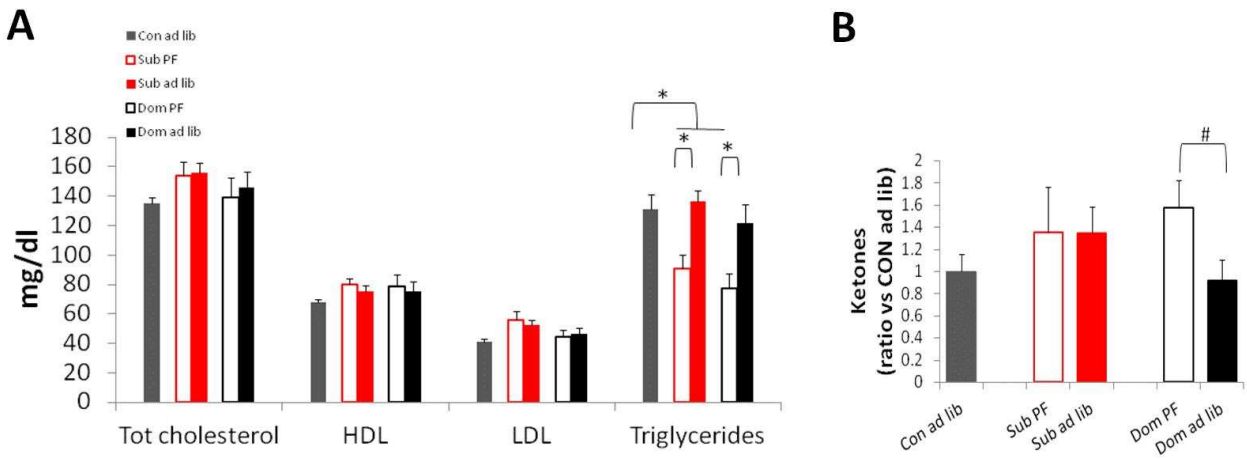

Figure 4: Lipid profile and Ketones A) at sacrifice showed pronounced effect of PF only on circulating TGs level. PF mice showed lower TGs concentration when compare to CON (treatment $F(2,28)=7,33$ $p<0.005$) and stressed mice ad lib fed (diet $F(1,46)=18,14$ $p<0.001$). *$p<0.05$

CON ad lib, n=12; SUB PF, n=9; DOM PF, n=10; SUB ad lib, n=26; DOM ad lib, n= 8.

B) Ketones (b-hydroxybutirrate) were higher in SUB mice compared to controls while only DOM PF showed an increase by trend when compare to controls and DOM ad lib fed (t-test $_{1,10}$ #$p=0.064$)

CON ad lib, n=4; SUB PF, n=8; DOM PF, n=7; SUB ad lib, n=5; DOM ad lib, n= 5.

Chronic subordination stress-induced binge eating disorder (BED)

Results presented in chapter one and two showed that stress-induced hyperphagia is associated with an increase in BW and adipose tissue in subordinate mice. Data discussed in the present chapter demonstrate that hyperphagia is necessary and sufficient to explain obesity in subordinate mice. Accordingly, to gain insight into the feeding behavior of subordinate mice we performed a preliminary study in a subset of subordinate mice ad libitum fed high fat diet. Subordinate mice showed key features of BED i.e. acute stress-induced hyperphagia (Levine and Morley 1981, Gluck et al. 2004) and hunger-induced hyperphagia (Hagan et al. 2003). Indeed, subordinate mice showed an increase in food intake in the 6h following a daily defeat episode (light phase) (**Fig.5A**). Moreover following overnight fasting (12h), stress mice showed hyperphagia in the subsequent 24h, measured as fold change % on 24h pre-fasting, when compare to CON (**Fig. 5B**).

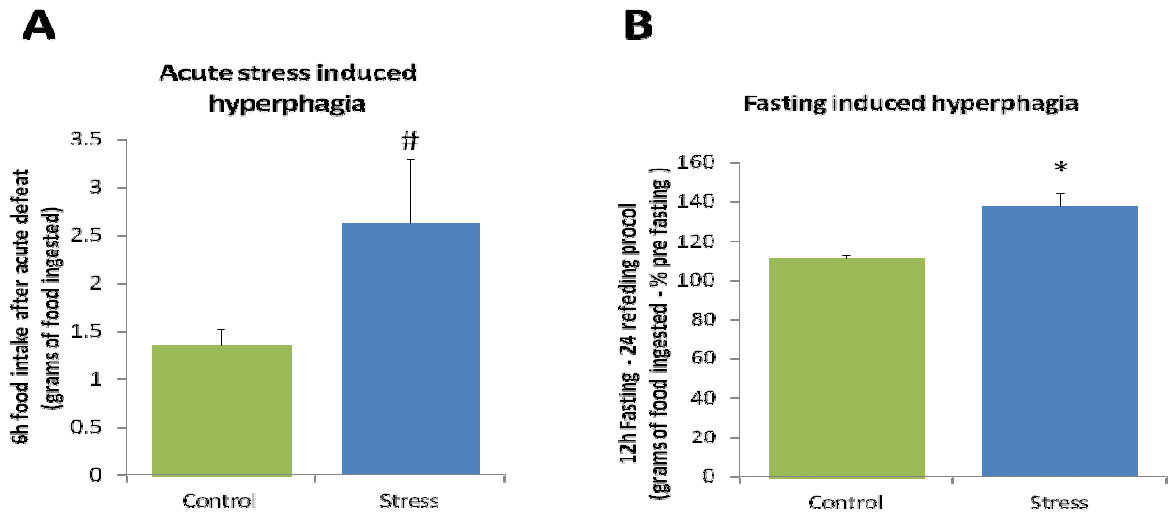

Figure 5. Analysis obtained reanalyzed data collected during the glucose tolerance test suggested that stressed mice fed HFD showed **A)** acute stress (defeat for 10 min) induced increase in food ingested in the subsequent 6h (light phase) ($t\text{-test}_{1,25}$= 2.05, $^{\#}p$=0.051).

B) increased 24h food intake following overnight fasting (preparation for GTT, mice were fasted 12h overnight and injected with 1g/Kg glucose during test) ($t\text{-test}_{1,23}=3.40$, $^{*}p<0.005$). n=10-15

Discussion

As described in the previous chapters chronic subordination stress is able to induce metabolic disorders such as obesity and Type 2 diabetes. Stress-induced hyperphagia is one of the most robust phenotype we observe in our model (Bartolomucci et al. 2004b, Dadomo et al. 2011, Bartolomucci et al. 2009b, Bartolomucci et al. 2010) as well as in similar other animal models of social stress (Finger et al. 2011, Solomon et al. 2007). In dominant mice hyperphagia can be considered an adaptation to sustained metabolic cost of hyper-arousal associated with maintaining a high rank (Bartolomucci et al. 2009a, Bartolomucci et al. 2003a, Bartolomucci et al. 2004a, Sapolsky 2005). On the other hand in subordinate mice, hyperphagia is more likely a specific eating disorder associated with their depression-like phenotype (Bartolomucci et al, 2004, 2005, 2010, Dadomo et al, 2011). Using a pair feeding protocol we aimed to test the hypothesis that hyperphagia is required for stress-induced physiological end metabolic changes observed in dominant and subordinate mice.

Preventing hyperphagia protects subordinate mice from DIO but not hyperglycemia and hypercorticolesterolemia

Because obesity is a major risk factor for the development of the metabolic syndrome and T2D (Björntorp 1996b, Björntorp and Rosmond 2000, Boden 2002, Chan et al. 1994) a food restriction regimen promoting weight loss should normalize the metabolic syndrome as well (Aude, Mego and Mehta 2004). Previous report showed that reducing calories intake attenuated but did not prevent of insulin resistance and obesity in mice, thus indicating a role of HFD diet independent from caloric intake (Petro et al. 2004). In our model, 20-25% of caloric restriction compared to *ad libitum* intake

prevented the risk to develop obesity in subordinate mice. In line with a lower increase in body weight and epididymal fat, SUB PF mice showed a reduction in plasmatic leptin levels (Maffei et al. 1995). Ghrelin, a gastric orexigenic hormone inducing hunger in rodents and humans (Wren et al. 2001b, Wren et al. 2001a), decreased in SUB ad lib mice when compared to CON, was normalized by food restriction in SUB PF mice. Our results are in line with recent published data (Chuang et al. 2011) using model of chronic social defeat stress (CSDS) where stress-induced BW decrease at HFD (Chuang et al. 2010) is associated with higher ghrelin concentration. These data support the hypothesis that hyperghrelinemia plays a potential role as adaptive mechanism regulating food intake during caloric restriction (Barazzoni et al. 2003, Hansen et al. 2002, Tschöp, Smiley and Heiman 2000) and stress-induced food reward (Chuang et al. 2011, Perello et al. 2010). In addition, ghrelin seems also involved in regulation of glucose metabolism (Nass et al. 2010). Zhao and colleagues (Zhao et al. 2010) demonstrated that knock out for ghrelin O-acyltransferase (GOAT), an enzyme essential for ghrelin processing, showed a severe hypoglycemia. They suggested that ghrelin is essential for the maintenance of blood-glucose levels through his stimulation of hepatic glucose production and decreases glucose uptake in skeletal muscle and fat cells (Nass et al. 2010).

Besides up-regulation of hormones involved in food intake control, such as leptin and ghrelin (Zigman and Elmquist 2003), SUB PF showed an increase in basal corticosterone suggesting an higher stress sensitivity in subordinate mice under caloric restriction diet (Pankevich et al. 2010). Corticosterone is released in response to many types of stressors including food restriction. Both caloric restriction and hypercorticosteronemia play an important role in the mobilization of energy reserves during energy deficit by stimulating lipolysis and glucose synthesis (gluconeogenesis) and inhibiting glucose catabolism (glycolysis) (Hagopian, Ramsey and Weindruch 2003, Dallman et al. 2007). We argued that to prevent hypoglycemia in a severe caloric restricted diet, both ghrelin and corticosterone act to increase endogenous production of glucose. As a result SUB mice under PF

developed hyperglycemia compared to other mice. As showed in Chapters one and two, in a normal feeding condition, elevated glycemic level in subordinate mice at HFD was associated with insulin resistance and glucose intolerance. Despite increased basal glycemia SUB PF showed improved insulin sensitivity during glucose tolerance test resulting from glucose level at 120 min after injection comparable to basal level. Notwithstanding an improved insulin sensitivity we could not conclude that 3 weeks of caloric restriction completely abolish effects of stress and HFD on glucose intolerance/insulin resistance. As an evidence IRS2 in liver was not different compare to SUB and CON ad libitum fed.

While glucose tolerance was improved after 3 weeks of PF, total cholesterol, HDL and LDL were still higher in SUB PF when compared to CON, with no changes compared to Sub ad libitum fed mice. Only TGs showed a decrease in PF mice. While obesity is been associated with elevated TGs concentration, weight loss reduces TGs levels (Tonstad and Després 2011). Moreover according to our hypothesis suggesting that pair feeding would induce an increase in gluconeogenesis, hydrolysis of TGs, a necessary step to produce free glycerol which is one of the primary substrate for gluconeogenesis.

Stress promotes binge eating in subordinates mice

Stress has been associated with increased preference for palatable food (Dallman et al. 2003, Dallman et al. 2004, Dallman et al. 2005, Gibson 2006). Altered feeding behavior is a frequent finding in individuals with atypical depressive disorder characterized by reversed vegetative symptoms as hyperphagia and hyperinsomnia (APA 2000). Furthermore neuropsychiatric eating disorders, in particular Binge Eating Disorder (BED), has been associated to obesity and increased

risk factors to develop T2D, dyslipidemia and cardiovascular diseases (Mitchell and Mussell 1995, Wilfley, Wilson and Agras 2003), DSM-IV). BED is characterized by recurrent episodes of large ingestion of food in a short period of time in absence of a detrimental behavior. Stress is increasingly recognized as a risk factor for BED (Van Strien et al. 1986, Stein et al. 2007, Striegel-Moore et al. 2007). In humans psychological (e.g., interpersonal, ego-threatening, and work related) stressors have been linked with an increase in food intake whereas stress induced from the threat of physical harm or discomfort and indexed as physiological symptoms of anxiety, illness, and fear of injury decreases food intake (Heatherton, Herman and Polivy 1991).

Several animal models of BED have been developed (Corwin and Buda-Levin 2004, Mathes et al. 2009). While acute stress typically reduces food intake in rodents, the combination of acute stress and deprivation-refeeding cycles with palatable food cause binge-like behavior (Hagan et al. 2002, Hagan et al. 2003, Boggiano et al. 2005). Starting from these evidences we aimed to validated chronic subordination stress model accordingly to criteria described by Corwin et al. (Corwin and Buda-Levin 2004): 1) The behavior should occur repeatedly over an extended period of time; 2) Bingeing animals should consume more food in brief, discrete, periods of time than controls do under similar circumstances. 3) If compensatory behavior is present, it should be initiated by the animal rather than imposed by the investigator.

Indeed Subordinate mice developed hyperphagia as a consequence of stress throughout experimental protocol (criteria 1). Furthermore SUB subjected to an acute stress such as acute defeat and to 12h fasting consumed more food when compared to controls (criteria 2) and manifested this altered eating behavior without manipulation by the investigator (criteria 3).

Moreover crucial differences between animal and human binge eating include the fact that subjective feelings of distress or loss of control, which have been found in humans (Stein et al. 2007), are not easily assessed in animals (Corwin and Buda-Levin 2004). Besides binging episodes, in our model

of CSS SUB mice developed a depressive-like behavior as showed by a reduction of locomotor activity and increased social avoidance (Meerlo et al. 1996, Bartolomucci et al. 2010, Bartolomucci et al. 2001, Bartolomucci et al. 2004b, Bartolomucci et al. 2005, Dadomo et al. 2011), further supporting the conclusion that CSS can be considered a valid animal model to study BED.

In conclusion, preliminary evidence demonstrated that chronic subordination stress may be considered a good model of BED. While several other behavioral animal models for BED subjected the animals to repeated food restriction/refeeding cycles to induce the disorder (Hagan et al. 2002, Corwin and Buda-Levin 2004), in chronic subordination stress paradigm, subordinate mice spontaneously developed binge eating-like disorder. Furthermore these data suggested that subordination stress-induced BED (hyperphagia and binge episodes), might be a causal factor in subordination stress induced obesity. Indeed preventing hyperphagia with a pair feeding protocol protect subordinate mice from obesity.

Preventing hyperphagia in dominant mice resulted in a severe weight loss

As previous discussed in dominant mice under chronic stress hyperphagia is a result of increased metabolic cost of acquired and maintained high rank (Sapolsky 2005), increased locomotor activity, sympathetic tone (norepinephrine) to adipose tissue and hyperthermia (Bartolomucci et al. 2009a, Bartolomucci et al. 2004b, Bartolomucci et al. 2004a, Moles et al. 2006). As demonstrated in previous studies using different social stress models such as CPS (Bartolomucci et al. 2009a) and visible burrow system (Tamashiro et al. 2006), dominant mice showed a decrease in BW despite hyperphagia at HFD when compare to SUB.

Caloric restriction resulted in a severe reduction in final BW gain and epidydymal mass when compared to ad libitum fed DOM mice and SUB PF. Surprisingly, despite body weight loss, fat pad weight, ghrelin and leptin plasma level showed same level concentration when compare to DOM ad libitum fed. Moreover while in SUB caloric restriction-induced hypercolecorticosteronemia this effect was not present in dominants mice. While we discussed previously a possible role of hyperghrelinemia and hypercorticosteronemia in inducing hyperglycemia in subordinates, dominant mice showed same basal glucose concentration when compare to SUB PF, despite absence of elevated hormones. This data suggested a different mechanism involved in glucose raised. Glucagone is known to regulate glucose homeostasis, acting in response to hypoglycemia to elevate blood glucose concentration (Jones, Tan and Bloom 2012). As a possible mechanism we hypothesized that the slight increased in glucagone in DOM PF compare to DOM ad lib was able to activate glycogenolysis and gluconeogenesis and increase basal glycemic level. Basal hyperglycemia did not prelude to impair insulin sensitivity. Indeed DOM PF showed a normal response to glucose injection during GTT comparable to that shown by DOM ad lib with an amelioration in IRS2 gene expression in both group compare to CON.

As for subordinate mice, total cholesterol, HDL, LDL were higher in DOM PF when compared to CON and with no changes compared to DOM ad libitum fed mice. On the contrary TGs showed a decrease similarly to what observed for SUB PF. The decreased in TGs levels were followed by a trend for increased plasma ketone bodies. Caloric restriction in animals induce a larger increase in blood levels of the ketone body β-hydroxybutyrate (Mattson, Duan and Guo 2003). During conditions of increased fasting on high energy demand, ketogenesys is activated to supply cells and mainly neurons with ketone bodies, such as β-hydroxybutyrate (Laffel 1999). In conclusion we argue that metabolic cost of dominance adding to a caloric restriction exacerbated lipolysis leading

to decrease in TGs a limited ketoacidosis and increased glycemic trough glycogenolysis/gluconeogenesis.

General conclusions

The use of animals in research is essential to study the pathophysiology of diseases and develop new and more efficacious treatments for humans. Type 2 diabetes, specifically obesity-associated T2D, is predicted to become the disease showing the highest increase in mortality by the year 2030 (Mathers and Loncar 2006). Up to now several rodent models (Chatzigeorgiou et al. 2009, LeRoith and Gavrilova 2006, McMurray and Cox 2011) were developed to study T2D. However these models, suffer from low face and construct validity because the experimental manipulations (genetic manipulation, pharmacological injection, long term HFD feeding) necessary to induce T2D do not reflect what typically occurs in humans, where the vast majority of T2D are disease is triggered by different risk factors.

Both in humans (Rosmond 2005, Anagnostis et al. 2009) and animal models (Chan et al. 2002, Shpilberg et al. 2012), evidences suggest that elevation in GCs concentration is closely associated with diabetes onset. In humans, it has been demostrated that life events such as social stress and socio-economics challenges, are associated with increased cortisol secretion, compulsive eating behavior and changes in food preferences (Dallman et al. 2004, Dallman et al. 2005), increased visceral fat pad depots (Björntorp 1993, Björntorp 1996b, Björntorp 2001, Bose et al. 2009, Dallman et al. 2007), neuroendocrine-autonomic dysregulation and metabolic disorders leading to obesity (Björntorp 1993, Björntorp 1996b, Björntorp 2001, Bose et al. 2009, Dallman et al. 2007) and metabolic syndrome (Anagnostis et al. 2009, Björntorp and Rosmond 2000). While obesity and metabolic syndrome have been identified as risk factors in the development of T2D in susceptible individuals (Boden 2002, Chan et al. 1994), the extent to which chronic psychosocial stress might represent a common causal factor has not been well investigated yet. The main objective of this thesis was the novel characterization of a naturalistic rodent model of T2D onset by exposure of male mice to chronic psychosocial stress (CPS) associated to a high caloric diet (HFD).

In laboratory rodents social defeat is considered the main model for social stress due to its ecological and ethological validity (Miczek et al 2001). Moreover, as demonstrated by Koolhaas (Koolhaas et al. 1997), social defeat induces the largest increase in corticosterone and adrenaline when compared with conventional animal models of stress.

In our CPS model we consistently demonstrated that, despite a general stress-induced hyperactivation in the HPA axis, subordinate and dominant mice showed opposite metabolic consequences (Bartolomucci et al. 2004b, Bartolomucci et al. 2009b). While injection of exogenous GCs (as mimicking an hyperactivation of the stress response system) associated to HFD has been reported to induce a rapid onset diabetes (ROD model (Shpilberg et al. 2012)), in our CPS model elevated GCs *per se* were not sufficient to explain the development of T2D. Dominant and subordinate mice showed similarly elevated GCs levels (if anything higher in the dominant) but indeed, only subordinate mice developed most of the symptoms associated with T2D. Without any genetic or pharmacological manipulation, only 3 weeks of chronic social stress and HFD were able to induce obesity, increased visceral adiposity and leptin and determine insulin resistance. Metabolic changes were paralleled by molecular changes in metabolic tissues including down-regulation of gene gene associated with insulin resistance and diabetes such as InsR, IRS1, IRS2, CPT1a and glucose transport GLUT4.

These results suggest that our model of chronic social subordination stress represents a new and valid model for T2D.

We also attempted to identify the behavioural mechanism underlying the stress-induced obesity-diabetes phenotype by 1) investigating the role played by social status and 2) by investigating the role of hyperphagia. Individual rank in the social hierarchy had a dramatic impact on stress-induced metabolic consequences and development of T2D. While subordinate mice are vulnerable to obesity and diabetes, dominant mice are resistant and show a lower increased in weight gain and adiposity at

HFD. The effects of resistance to obesity and being high in rank also resulted in a normal fasting glycemia, insulinemia and glucose tolerance and a normal plasma lipid profile when compared to SUB and CON mice. Moreover as showed in previous studies (Bartolomucci et al. 2001, Bartolomucci et al. 2003a, Bartolomucci et al. 2004a, Moles et al. 2006, Sapolsky 1992) the hyper-locomotor activation, increased sympathetic tone, tachycardia and hyperthermia reported in dominant animals, together with our results showed a phenotype of resistance to develop T2D.

Blocking hyperphagia in subordinate mice prevented diet-induced obesity. Furthermore, subordination stress in a condition of caloric restriction induced a larger increase in corticosterone and glucose levels, suggesting higher stress sensitivity in these mice. Limit spontaneous hyperphagia was able to abrogate subordination stress-induced obesity with a severe effect in DOM. Despite losing weight most of the metabolic consequences of stress were not reverse. Stressed mice, both SUB and DOM, showed hyperglycemia and dyslipidemia despite molecular signatures of insulin resistant state were partially reverse by PF. Only Triglycerides level were improved as a consequence of PF.

Based on these evidences we postulate the existence of a Stress-Induced- Binging- syndrome. Binge Eating Disorders (BED) is a pattern of eating disorders characterized by an uncontrolled and compulsive eating in a brief period of time. BED has been associated with obesity (Stunkard 2011, de Zwaan 2001) and presents high co-morbidity with depression disorders (Dingemans and van Furth 2012). Several lines of evidence also suggest a connection between stress and binge eating in humans (Van Strien et al. 1986, Stein et al. 2007). Animal models of BED commonly use a combination of food restriction and acute stress (tail pinch, restrain test, shock test) are used to study BED but only a few consider the role of social stress in the eating disorder onset. Based on our previous evidences of subordination stress-induced hyperphagia, vulnerability to obesity and

depression-like behaviors (Bartolomucci et al. 2001, Bartolomucci et al. 2004b, Bartolomucci et al. 2005, Bartolomucci et al. 2009a, Dadomo et al. 2011), we demonstrated that subordinate mice fed HF diet showed key features of BED, such as acute stress-induced hyperphagia and hunger-induced hyperphagia.

Altogether these findings allow to recognize the key role of social factors in the development of metabolic (obesity, metabolic syndrome, T2D) and psychopathologic (depressive-like behaviors) disorders. We were able to validate a new naturalistic model of rapid pre-diabetes onset without external genetic or pharmacological manipulations and to demonstrate the relevance of individual vulnerability to disease as based on social role and experience (subordination vs dominance).

Acknowledgments

Finally done!!!

These last three years have been filled with events that indelibly marked my life. I realize maybe for the first time that bad things unfortunately happen, but, as my best friend has taught me, we need to keep your head up and smile for the good things. My first thanks goes to Michela, for the beautiful moments we had together, I am always smiling thinking of you. I miss you.

Back to science, I would to thank Alessandro Bartolomucci, Toni Vidal-Puig and Paola Palanza to encourage me to give always the best in science and even beyond.

Thanks to Stefania, Meghana and Sergio for helping in the unknown world of…RT-PCR… but above all for the great time spent together in lab and out. how to forget the rest of the TVP "big family" …Iris, Crystal, Antonio, Nuria, Ko, Alberto, Mark, Agnes, Andrew, Martin, Sam, Chong.

Thanks to all, past and present, students at the "small rodents lab" and to ASSB .... Thanks to Riccardo, Morris and Pilo for the great Sunday lunches after experiments (nap included ... I'm sorry Ricky, but you were always the first). Thanks to Laura for being patient in the last period (finally I have done), to Francesco to make me always smile, no matter what .... to Gaia (the " desaparecida" is back), to Shanis for sharing with me a lot in these years of PhD, to Harold my favorite psychologist (along with Betta), to Paola-Geppo and Pimpa (now I consider you a only thing), Mrco and Romano.).

Thanks to all MInneapolis group, in primis Maria, Cheryl and Ivana (yours was the best cake ever) for helping me in the last months.

Thanks to my PT... I stopped cry

At the end I would thank my family, without them, nothing would be done!!!

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