6

GENERAL DISCUSSION

GENERAL DISCUSSION

Today's Western society and work promotes a sedentary lifestyle, which from an evolutionary perspective, is a relatively recent development. This, coupled with the high availability of foods with high caloric content in Western cultures, superimposed on dated genotypes has given rise to the pandemic of obesity with its related increase in prevalence of the metabolic syndrome (MetS), type 2 diabetes (T2D) and cardiovascular diseases (CVD). At the same time, the perceived social stress in everyday's society has increased (1). Furthermore, lack of sleep and sleep disturbances have become increasingly common and today we are, even voluntarily, sleep restricting ourselves and sleep significantly less than thirty years ago (2).

The metabolic effects of perceived stress are mediated by glucocorticoids (GCs) that are secreted by the adrenals as a result of stressor-induced activation of the hypothalmus-pituitary-adrenal (HPA) axis. The effects of GCs include recruitment of energy storages from fat and muscle (3, 4), but also central activation with adjustment of feeding behavior resulting in an increase in the intake of palatable foods to compensate for the catabolic effects (loss of energy) in peripheral tissues. These metabolic adaptations occur during the stressful event, with the purpose to be enable the evolutionary "fight or flight" response (5). It is therefore not surprising that, in the presence of such an evolutionary 'pressure' of stress, the consumption of "comfort food" as well as "emotional eating" has increased (6), further facilitating the development of obesity, the MetS, T2D and CVD (7). Epidemiological data show a clear association between increased plasma levels of the GC cortisol and CVD and obesity, but animal models of the MetS are inconclusive with respect to whether the HPA axis is activated or not. Thus, up till now, it was unknown if, and to what extent an increased activity of the HPA axis plays a pathogenic role in the development of the MetS, and *vice versa*, whether development of MetS leads to increased activity of the HPA axis, if there would be a role of development of specific features of the MetS, *i.e.* obesity, on the activity of the HPA axis.

In this thesis we have chosen to evaluate both whether baseline, non-stressed, activity of the HPA axis is affected during the development of obesity, and *vice versa*, to what extent a period of exposure to increased endogenous GC levels would affect specific features of the MetS. The major conclusions and implications of our findings will be discussed in this chapter.

Impact of the development of obesity on baseline, non-stressed, activity of the HPA axis

As stated above, overweight and obesity have become prominent global health problems and are no longer a health issue that only affects adults but also children and adolescents. Overweight and obesity are often accompanied by other metabolic abnormalities, such as dyslipidemia and insulin resistance, that can be clustered to define a clinical syndrome that is associated with increased cardiovascular morbidity and mortality: the MetS (8).

The basal activity of the HPA axis, i.e. not induced by stress, has been reported only in a limited number of studies in obese individuals (9-12), and these results have been inconclusive. A key issue in this respect is the difficulty to assess the activity of the HPA axis in conditions were individuals are exposed to social stress, as is present in everyday life. In this respect, animal models can be very useful, since under laboratory conditions, in principle it is possible to control for many potential stressors that might affect the outcome.

In **chapter 2** we critically evaluated the current literature available reporting the effects of high fat diet (HFD) on the basal, non-stressed, activity of the HPA axis in mice by performing a structured, and systematic review of all the available literature on this topic using the main medical databases. We included only original mouse studies that reported parameters reflecting the activity of the HPA axis after a prolonged period of HFD feeding, because there is extensive documentation that HFD feeding *per se* is able to induce obesity and insulin resistance, especially in wild-type C57Bl/6 male mice (13-16). For inclusion in the review, at least one basal corticosterone (CORT) measurement and a proper control group was required. Studies with adrenalectomized mice, transgenic animals only, HFD for less than two weeks, or other interventions besides the HFD, were excluded, as they do not represent normal physiology. In addition, we excluded studies with an insufficient duration of exposure to the HFD to be able to induce significant features of the MetS. We found twenty studies that fulfilled our stringent inclusion criteria, but surprisingly, in only five studies the evaluation of the HPA axis was the primary research question. Plasma CORT levels in these twenty studies were found to be increased, unchanged, or even decreased. Additional parameters reflecting the activity of the HPA axis, such as 11β-dehydrogenase (11β-HSD-1) expression in peripheral tissues such as liver and fat, and corticotropin releasing hormone (CRH) and/or glucocrticoid receptor (GR) expression in the central nervous system, were evaluated only in five out of these 20 studies, and these data were also not consistent.

Importantly, there were many differences between the studies that precluded a reliable comparison of the effects of HFD on the HPA axis between studies. For instance, the relative energy contribution of fat in the diet varied between 32-65%, and different mouse strains were used, resulting in a large variation in weight gain. In addition, there were different housing and sampling conditions. All these factors are known to differentially affect the activity of the HPA axis, and therefore, all can contribute to the different results observed in the studies. Most importantly, different housing conditions, e.g. group-wise *vs*. individual housing, has shown to dramatically affect both basal and stress-induced HPA axis activity, as in a group a social order is about to form with dominant and subordinate individuals, that display different coping strategies when faced with social stress (17, 18). In addition, the majority of the studies failed to report on the sampling conditions for CORT (especially whether stress-free sampling was performed: at best, some reported on the elapsed time between opening of the cage and sample collection). Some studies even reported the use of anesthesia before sampling, which, by definition, requires more handling of the animal, which has an additional effect (besides the anesthetic *per se*) on the central nervous system.

Thus, the systematic review demonstrated that the effects of HFD on the basal activity of the HPA-axis, and its contribution in the propensity to become obese and develop other manifestations of the MetS on a HFD, remained unclear. For a proper and reliable evaluation of the basal activity of the HPA axis, we reasoned that an appropriate study design is therefore a prerequisite. We proposed that such an appropriate design should, at least, take the following factors into account: 1) choice of mouse strain (genetically modified or not, DIO-resistant or not, resistant to stress or not), 2) duration and content of the diet, 3) standardization of housing conditions with a possibility to acclimate in advance, 4) proper methods for stress free sampling of CORT, and 5) the timing of the sampling to reflect circadian rhythmicity.

Therefore, in **chapter 3** we applied the most appropriate study design as proposed in **chapter 2**. In this study, the aim was to document the effects of HFD on the basal activity of

the HPA axis, both peripherally and centrally, in C57BL/6 mice with previous extensive available documentation with respect to the development of obesity and insulin resistance when fed a HFD (12-15). We measured plasma CORT using a stress-free sampling method both at the circadian nadir and at the circadian peak (in the evening). HFD decreased diurnal peak CORT already at the first week of HFD feeding and the levels remained significantly lower when compared to controls up to twelve weeks (the end of the experiment). This finding is in line with a recent study reporting decreased basal plasma CORT values in a similar study design, but with a shorter duration of the diet (19). Furthermore, we observed that HFD induced complex changes in CRH and GR mRNA expression in the central nervous system areas responsible for feeding behavior and limbic functions, namely the paraventricular nucleus (PVN) of the hypothalamus, the amygdala, and hippocampus. In the peripheral white adipose tissues, HFD induced a profound down regulation of 11β-HSD-1 enzyme mRNA. Since this enzyme converts cortisone and 11-dehydrocorticosterone into their active forms cortisol and CORT, this reflects decreased CORT exposure at the tissue level. In agreement with these findings, we found no changes in 11β-HSD-2 enzyme mRNA expression, which converts cortisol and CORT into their inactive forms, thus leading to a reduced net effect of CORT exposure in the tissue level.

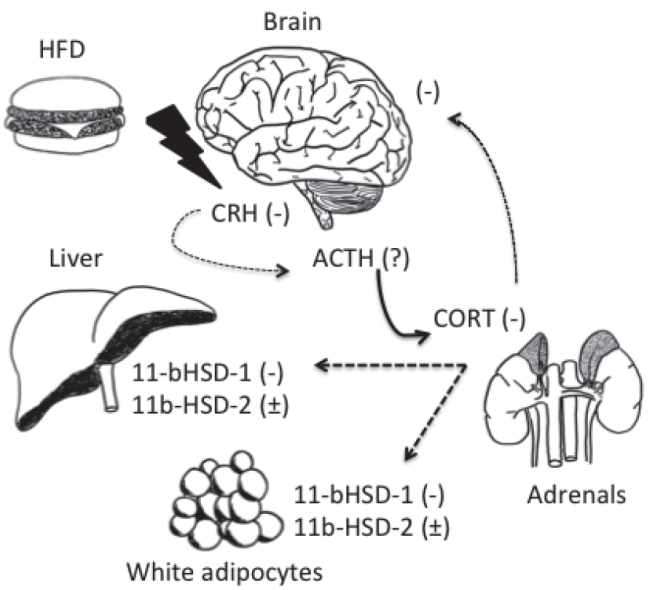

Figure 1. Tentative model of the effect of HFD on the HPA axis activity. See text for explanation

The results found in the first two studies, first of all indicate that a proper study design is crucial for reliably evaluating the HPA axis. In addition, it appeared that the development of obesity leads to secondary adaptive responses of the HPA axis that cannot be interpreted by just measuring

circulating CORT concentrations only. Fundamental in this respect is the fact that obesity *per se* increases body fat and thus the distribution volume for CORT (which is fat soluble), and therefore decreases circulating CORT levels and stimulates central activation of the HPA axis as discussed in the introduction. However, the net result of HPA axis activation in obesity is complex and is also influenced by central and peripheral interactions with orexigenic hormones (like leptin) (20-24), by changes in adrenal sensitivity for adrenocorticotropic hormone (ACTH) (25, 26), and by changes in enzymatic (de)activation of CORT in peripheral tissues (by the 11β-HSD type 1 and 2) (27, 28). Thus, it can be argued that the adaptation of the HPA axis to the HFD, at least in part, is secondary to the increasing body weight. Pair-feeding (29), in which less HFD is given to a caloric amount that is consumed in chow-fed animals, provides an experimental set up that eliminates the effect of energy intake and possible subsequent weight gain, thus allowing to observe the "true" effect of HFD on the HPA axis parameters. These results, however, could be hampered by the stress induced by the reduced feeding (30), and if so, periods of hunger experienced by the pair-fed group would also no longer represent a stress-free model.

The present study was performed in adult mice naive for HFD. Therefore the results should be interpreted with caution and cannot simply be extrapolated to neonates, pups, adolescent mice, or to the offspring of HFD-fed mothers. Previous studies have shown that maternal exposure to HFD during pregnancy and postnatally, even without developing obesity, resulted into increased weight and insulin as well as leptin resistance of the offspring, both in adolescence and adulthood (31-36). Exposure to maternal HFD resulted in increased plasma CORT in rat pups (37) but weaning of pups to HFD decreased 11β-HSD-1 activity in the liver and adipose tissues (38). Furthermore, maternal exposure to HFD led to changes in melanocortin expression and disturbances of the pro-opiomelanocortin (POMC) system in the fetal offspring of nonhuman primates (39). Finally, some studies have also demonstrated altered stress-induced responsiveness of the HPA axis: adult offspring of HFD-fed rats exhibited an increased reactivity to acute stress (40) and neonatal pups from HFD-fed mothers showed a decreased response to stress whereas in adulthood stress responsiveness was increased (41). These findings demonstrate that HFD exposure may result in multiple effects on both basal and stimulated activity of the HPA axis, and that these effects are dependent on the duration of the exposure as well as on the timing (age) of the exposure. Therefore, it should be noted that the results obtained from different studies reflect the particular experimental set up.

Thus, HFD, which is abundantly available in today's society, induces complex changes in the diurnal regulation of various components of the HPA axis. This is of paramount importance because activation of the HPA axis in the central nervous system, for instance, not only affects metabolic 'sensing' but is also a key modulator for the limbic system, facilitating learning, memory formation and retrieval (42), thereby affecting individual (psychological) well being.

For future studies, it would be interesting to investigate whether the effects of HFD on both the basal activity of the HPA axis and stress responsiveness are reversible. This is of importance since it has been shown that obesity is a risk factor for Alzheimer's disease (43) and depression (44), both of which are also associated with alterations of the HPA axis. Futher more, there is no awereness on whether development of obesity leads to secondary adaptive changes of the HPA axis which may, in turn, lead to altered set points of hormone release and tissue sensitivity as has already been extensively documented for pituitary-gonadal axis (45).

The effects of a period of endogenous GC excess on the MetS and atherosclerosis

The fundamental question that arises from our previous observations is whether the HPA axis is able to adapt sufficiently during the development of obesity in the presence of chronic (social) stress. From an evolutionary point of view, social stress is considered to be a chronic challenge, as cortisol, secreted during stress response, promotes feeding behavior (46) to compensate for the energy loss that takes place during the "fight or flight" responses. However, in the given context, such an energy loss might never take place and thus the individual, driven by the evolutionary drive orchestrated by the central nervous system, only further promotes a positive energy balance resulting in weight gain and insulin resistance, continuing the vicious cycle.

GCs are also potent anti-inflammatory agents that are widely used for their immunosuppressive properties but they also play a major role in glucose, lipid, and protein metabolism (47-49). In humans, an increased activity of the HPA axis has been linked to the development of the MetS (50). In addition, manipulation of cortisol exposure at the tissue level in mice, through stimulation or abrogation of 11β-HSD-1 and 11β-HSD-2 activity can increase, or regress, visceral fat accumulation, as well as other features of the MetS (27, 28). However, as discussed extensively in the previous paragraphs, these associations should be interpreted with caution and do not prove causality. In agreement, however, with these observations is the rare clinical syndrome of Cushing's syndrome (CS). CS in the human is the result of prolonged excessive exposure to GCs (in the majority of cases caused by ACTH secreting pituitary adenoma with subsequent adrenal overstimulation) and is typically associated with an increased prevalence of the MetS, albeit with a specific phenotype, with increased cardiovascular morbidity and mortality (51). Intriguingly, patients treated for CS remain at increased cardiovascular risk, even after long-term successful correction of GC excess (52). Although this is the best human model representing a (transient) period of GC excess, in the absence of other pathology or auto-immunity, the causal relation between the episode of cortisol overexposure and long-term changes in cardiovascular risk factors is not established and is difficult to assess because of the rarity and heterogeneity of CS in humans.

Therefore, in **chapter 4** we used a mouse model that has previous extensive documentation of development of certain features of the MetS, in this case obesity and insulin resistance, when exposed to HFD (13-16). In that study, we aimed to identify factors that modulate metabolic recovery from a period of overexposure to GCs. Male C57Bl/6J mice, fed a low fat diet (LFD) or HFD, received CORT or vehicle in the drinking water for 4 wks, followed by a washout period of 8 wks. CORT treatment increased plasma CORT, food intake and plasma insulin and lipids in both diets. Abrogation of CORT treatment normalized plasma CORT levels diet-dependently: mice fed LFD had normal circadian plasma CORT levels already after 4 weeks of washout whereas the CORT peak was still decreased in the HFD-fed mice when compared to their respective controls. Food intake and body weight normalized after removal of the CORT treatment. Intriguingly, at week 12 (i.e. after an eight weeks washout period and 6 weeks after normalization of body weight in the HFD-fed mice), plasma insulin levels were still significantly higher in CORT-treated mice on both diets, and HFD-fed CORT-treated mice had persistently decreased lean body mass and increased fat mass. Thus, in mice, a period of CORT excess induced long-lasting increase in plasma insulin levels and fat mass. However, the changes in body composition were present only in the presence of HFD. Interestingly, the recovery of the HPA axis, when measured as circadian plasma CORT levels, occurred earlier with LFD.

This can be of importance because CS patients, like all humans exposed to chronic stress, are likely to make dietary choices directed towards highly palatable foods (53) which would negatively affect the recovery of the HPA axis after treatment. In addition, it has been shown that HFD reduces the adrenal cortex sensitivity to ACTH (25, 26) and, therefore, could aggravate the withdrawal from, at least, synthetic steroids and postpone the recovery of endogenous adrenal cortisol production. Furthermore, slower recovery of the HPA axis secondary to HFD could also contribute to the psychopathologies as observed in CS patients even after long term correction from the supraphysiological cortisol levels (54). These observations are in complete agreement with the evolutionary drive of the stress response and points at a crucial role for dietary composition in the development of the MetS in conditions with periodic excessive GC exposure, like is the case in patients treated for CS, but possibly also in the general population exposed to chronic stress.

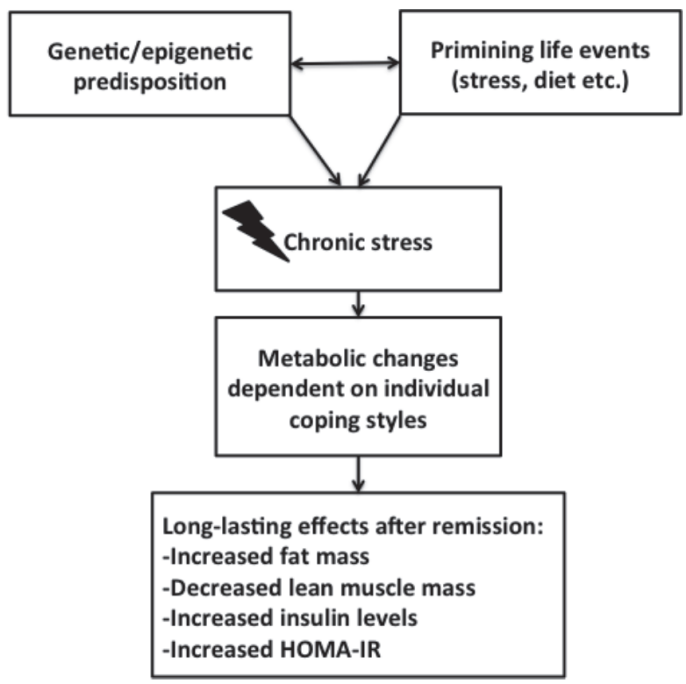

Figure 2. Individual experiencing chronic stress, like in CS, can, dependent on genetic/epigenetic predisposition, priming life events and individual coping styles, develop MetS and increase risk for CVD. Certain effects such as altered body composiotion, decreased insulin sensitivity and cardiovascular mortality can prevail long even after remission.

In **chapter 4** we chose to give CORT in the drinking water as opposed to supplementing the mice with e.g. subcutaneous (sc) CORT pellets. As expected, this non-invasive supplementation of CORT via the drinking water allowed the preservation of a certain degree of variability of circulating plasma CORT in the experiment, thereby preventing excessive tissue desensitation to CORT. Previous

studies that have used sc pellets for CORT supplementing resulted in abolishment of the ultradian rhythm, which restored after removal of the pellets in intact rats (55). In patients with CS, the diurnal variation in cortisol secretion is abolished in most patients. Cortisol secretion in these patients was further characterized by markedly amplified total daily hormone secretion secondary to an approximately 7-fold higher basal secretion rate, and increased secretory burst mass (ACTH and cortisol) and frequency (cortisol) (56). To be able to distinct between the preserved rhythm and continuously increased CORT levels and their long term effects, both methods of supplementation should be compared in one experiment. In the studies performed in **chapter 4**, the mice were given LFD or HFD with or without CORT treatment and no choice of diet was applied. We know, however, that GCs direct the choice of food toward more palatable, high energy foods rich in fat and sugar (53). Therefore, to truly estimate the effects of CORT treatment on body weight and composition as well as on insulin resistance, a study would be needed where choice of food is available during the tretament and washout.

In **chapter 5** we investigated the effects of a period of high GC exposure *vs* continuous overexposure on the development of atherosclerosis in a mouse model with human-like lipoprotein metabolism, namely ApoE*3-Leiden.CETP (E3L.CETP) female mice on a C57Bl/6J backtground. These mice represent a well-established model for the development of atherosclerosis when fed a cholesterol-rich diet (57). We induced high plasma CORT levels by adding CORT to the drinking water for either 5 wks (transient high exposure) or 17 wks (continuous high exposure). We found that CORT treatment increased body weight and food intake for the duration of the treatment and increased white adipose tissue weight in both treatment groups in the long-term. Both transient and continuous CORT treatment decreased total atherosclerotic lesion area to the same extent, without reducing plasma cholesterol levels. This was accompanied by a decrease in macrophage content of the plaque to a similar extent after both treatments.

The fact that CORT treatment reduced atherosclerotic lesion area, and tended to decrease lesion severity to a similar extent in transiently *vs* continuously exposed mice suggests that GCs are able to induce long-term effects in the preliminary processes of atherosclerotic plaque formation such as inhibiting the uptake of oxidized low-density lipoproteins (LDL) by macrophages (58, 59). Subsequent transformation of macrophages into foam cells that produce a variety of cytokines will further accelerate the process of plaque formation (60, 61) as well as the regulation of the expression of adhesion molecules in the vascular endothelium, thereby restricting the number of neutrophils entering the vessel wall (62, 63). The macrophage content of the plaque was significantly reduced in the continuously exposed group, and a similar trend was also observed in the transiently exposed group. We did not observe any differences in the number of monocytes adhering to endothelium between the groups. More likely, CORT excess stimulated the inhibition of macrophage growth and/or maturation instead of monocyte recruitment. GCs have been shown to inhibit oxidized LDL-induced macrophage growth by suppression of granulocyte/macrophage colony-stimulating factor (M-CSF) (64), thereby inhibiting atherosclerotic plaque formation.

It is difficult to compare our data with the limited data available from other mouse studies that evaluated the effects of GC on atherosclerosis. For instance, many of these studies used chronic stress as a model to increase endogenous GC but this will also induce other endocrine and metabolic changes (65) that may affect atherosclerosis development. Other studies used ApoE-deficient mice that harbor a different pro-inflammatory state (66) in contrast to the low-grade inflammation model of E3L.CETP mice used in our study (67, 68).

Whereas our observations in mice in **chapter 4**, i.e. insulin resistance, dyslipidemia and changes in body composition, are in a good agreement with the phenotype found in CS patients (49, 69-71), our findings in **chapter 5** are striking because in humans, increased GC secretion, like in patients with CS, is associated with CVD, although the exact role of GC in the development of atherosclerosis is not yet clearly established. Intriguingly, patients with CS remain at increased CVD risk, even after long-term successful correction of GC excess (52). Although limited, data in patients with CS indicate that carotic intima media thickness (IMT) is increased and vessel wall plaques are more common (69, 72, 73). Apart from atherosclerosis, CS patients have abnormal fat distribution, coagulopathy, and osteoporosis. Recent data indicate that remission of CS improved some but not all cardiovascular risk markers (74, 75).

Thus, increased CORT exposure in mice with human-like lipoprotein metabolism has long-lasting, beneficial effects on atherosclerosis, although it negatively affects body fat distribution and insulin sensitivity, by promoting fat accumulation in the long-term. This indicates that the increased atherosclerosis observed in humans in states of GC excess may not be related to cortisol *per se*, but may be the result of circulating GC (endogenous or synthetic) concentrations and of complex effects of GCs on the endothelium and/or coagulation (74, 75), and, finally, of epigenetic mechanisms (76, 77).

The results in **chapter 5** were obtained in adult mice and therefore age can be a factor determining the outcome. In the present study, the mice were allowed to age to adulthood without developing atherosclerosis and then were given a cholesterol trigger to start the development of the atherosclerotic plaques simultaneoulsy with the CORT treatment. Therefore, the timing of the treatment is fundamentally directed to the initiation of the atherosclerosis development. However, in humans the development of the plaques and thickening of the intima start already earlier in life (adolescence) (78) and thus the period of high GC exposure, like seen in CS, takes place in the later stages of the development of the atheroslerosis rather than during the initiation of the process. Therefore, it would be interesting to investigate the effects of CORT treatment on atherosclerotic plaque development in a model where the development would be initiated earlier in life and the CORT treatment would take place at a later stage in plaque development. It must be, however, considered that the processes triggering the development of atherosclerosis in mice can be crucially different from humans, in which it is thought to be a complication of dyslipidemia in combination with insulin resistance. Thus the timing of the GC excess should be critically applied in the investigation of the relationship between the developent of atherosclerosis and dyslipidemia and insulin resistance. Since E3L.CETP on a cholesterol-rich diet become hyperlipidemic without developing insulin resistance, the mouse model could be further improved i.e. by adjusting the fat content of the diet. Finally, it is possible that the immunosuppressive potency of cortisol is not exactly the same as that of CORT, precluding perfect comparisons.

GCs have potent immunomodulatory properties, and synthetic GCs are widely used in the treatment of auto-immune diseases, like rheumatoid arthritis and inflammatory bowel disease. Conversely, inflammatory cytokines like TNF-alpha (TNF-α) and interleukin-6 (IL-6), can modulate pituitary hormone secretion, in particular ACTH secretion (79). This implicates that the neuroendocrine (central nervous system and hormones) and immune systems communicate bi-directionally. Recent data also showed that receptors belonging to the native immune system, the toll-like receptors (TLR), are stimulated by fat containing substances. Fatty acids are able to do so by mimicing the fatty acid moieties of the lipid A-moiety of bacterial lipopolysaccharide (LPS).

This moiety is a high affinity ligand for TLR4. Stimulation of these TLRs with saturated fatty acids appeared to evoke a pro-inflammatory response, that eventually resulted in insulin resistance and atherosclerosis (80). In agreement, a population-based study recently reported increased TLR type 2 and -4 expression and activity in the monocytes of patients with MetS, but without diabetes or CVD, *vs* controls (81). This novel observation in the human clearly indicates that indeed increased TLR activity could contribute to an increased risk for diabetes and cardiovascular disease. Thus, the purpose for the bi-directional communication between the neuroendocrine (central nervous system and hormones) and immune systems is evident from an evolutionary point of view: it is crucial for survival to have an integrated system that informs the individual about threats, but also about opportunities. As a consequence, nutritional status and infectious pressure are integrated and lead to autonomous decisions to fight or flight, on reproduction or ageing, and on sleep or vigilance. In case of HFD, apparently we can simulate a bacterial attack, and mislead the body with unnecessary reactions and undesired effects (82).

To evaluate the effects, long lasting or otherwise, of HFD and/or CORT, on the functionality of the HPA axis more detailed than what has been described in **chapters 3**, **4** and **5**, it is necessary that future studies evaluate, and control for, stress induction, recovery from it and behavioral changes which they may induce. This is imperative in order to understand the behavior and choices of the individual induced by the adaptation of the HPA axis. Based on this knowledge, advice can be given to counteract the metabolic and psychological pathologies, which may help the individual to cope better and recover faster even in the presence of altered set points of hormone production and tissue sensitivity.

Implications and future perspectives

Chapters 2 and **3** described in this thesis clearly demonstrate that studies with the aim to evaluate the effects of an intervention on the HPA axis have to fulfill certain methodological criteria to enable a reliable evaluation. Although such a statement seems obvious, our studies indicate that this was clearly not evident to researchers involved in metabolic studies and illustrates that the metabolic and behavioural effects of the stress response cannot be easily separated. Thus, such an appropriate design should, at least, take the following factors into account: choice of mouse strain, duration and content of the diet, standardization of housing conditions with a possibility to acclimate in advance and methods for stress-free sampling of CORT, and the timing of the sampling. This standardization and correction for parameters, which might otherwise hamper the interpretation of the results, is of importance since measures of the circulating plasma CORT only might not be sufficient enough when evaluating HPA axis activity since secondary effects might mask the "true" effects. This is the case in human obesity where increased cortisol levels are not present, whereas cortisol secretion is increased, primarily because of increased clearance and increased distribution volume of the circulating cortisol resulting in secondary central activation of the HPA axis (10, 11). In the same lines, diurnal CORT levels may reflect counteracting mechanisms directed towards regaining homeostasis both centrally and peripherally (18, 19-23).

As we showed in **chapter 3**, HFD alone is able to modulate basal HPA axis activity and CORT metabolism in the central nervous system and in peripheral tissues. This might be the case also in the presence of a period of increased GCs (**chapter 4**), and could affect metabolic recovery and cardiovascular risk. Thus, it would be of interest to further elucidate the contribution of various diets during regular treatment of patients with CS, both before and after remission, to

metabolic abnormalities and behavior. These observations then could serve as a framework for guidelines for referral of patients who are prescribed steroid for (preventive) dietary advice. The profound inhibitory effect of high CORT on atherosclerotic plaque formation found in **Chapter 5** was remarkable. Increased CORT exposure in mice with human-like lipoprotein metabolism had beneficial, long-lasting effects on atherosclerosis, but negatively affected body fat distribution and insulin sensitivity, by promoting fat accumulation in the long-term. This indicates that the increased atherosclerosis observed in humans in states of GC excess may not be related to cortisol *per se*, but may be the result of complex and perhaps indirect, effects of cortisol on the cardiovascular system. Given the widespread distribution of GCs and their immunosuppressive indications, these complex effects that include the effects on the endothelium and coagulation, apparently outweigh the immunosuppressive effects on plaque formation and merit further research.

REFERENCES

1. Marmot MG, Bosma H, Hemingway H, Brunner E, Stansfeld S. Contribution of job control and other risk factors to social variations in coronary heart disease incidence. *Lancet* 1997; 350: 235-9
2. Sharma S, Kavuru M. Sleep and metabolism: an overview. *International Journal of Endocrinology* 2010; 2010: 270832
3. McMahon M, Gerich J, Rizza R. Effects of glucocorticoids on carbohydrate metabolism. *Diabetes / Metabolism Reviews* 1988 ; 4: 17-30
4. Andrews RC, Walker BR. Glucocorticoids and insulin resistance: old hormones, new targets. Clinical Science (Lond) 1999; 96: 513-23
5. Rutters F, Nieuwenhuizen AG, Lemmens SG, Born JM, Westerterp-Plantenga MS. Acute stress-related changes in eating in the absence of hunger. *Obesity (Silver Spring)* 2009; 17: 72-7
6. Greeno CG, Wing RR. Stress-induced eating. *Psychological Bulletin* 1994; 115: 444-64
7. Kuo LE, Czarnecka M, Kitlinska JB, Tilan JU, Kvetnanský R, Zukowska Z. Chronic stress, combined with a high-fat/high-sugar diet, shifts sympathetic signaling toward neuropeptide Y and leads to obesity and the metabolic syndrome. *Annals of the New York Academy of Sciences* 2008; 1148: 232-7
8. Executive summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *Journal of the American Medical Association* 2001; 285: 2486-2497
9. Pasquali R, Cantobelli S, Casimirri F, Capelli M, Bortoluzzi L, Flamia R, Labate AM, Barbara L. The hypothalamic-pituitary-adrenal axis in obese women with different patterns of body fat distribution. *Journal of Clinical Endocrinology & Metabolism* 1993; 77: 341-6
10. Rosmond R, Dallman MF, Björntorp P. Stress-related cortisol secretion in men: relationships with abdominal obesity and endocrine, metabolic and hemodynamic abnormalities. *Journal of Clinical Endocrinology & Metabolism* 1998; 83: 1853-9
11. Roelfsema F, Kok P, Frolich M, Pereira AM, Pijl H. Disordered and increased adrenocorticotropin secretion with diminished adrenocorticotropin potency in obese in premenopausal women. *Journal of Clinical Endocrinology & Metabolism* 2009; 94: 2991-7
12. Roelfsema F, Kok P, Pereira AM, Pijl H. Cortisol production rate is similarly elevated in obese women with or without the polycystic ovary syndrome. *Journal of Clinical Endocrinology & Metabolism* 2010; 95: 3318-24
13. Surwit RS, Kuhn CM, Cochrane C, McCubbin JA, Feinglos MN. Diet-induced type II diabetes in C57BL/6J mice. *Diabetes* 1988; 37: 1163-7
14. West DB, Boozer CN, Moody DL, Atkinson RL. Dietary obesity in nine inbred mouse strains. *American Journal of Physiology* 1992; 262: R1025-32
15. Parekh PI, Petro AE, Tiller JM, Feinglos MN, Surwit RS. Reversal of diet-induced obesity and diabetes in C57BL/6J mice. *Metabolism* 1998; 47: 1089-96

16. Kleinridders A, Schenten D, Könner AC, Belgardt BF, Mauer J, Okamura T, Wunderlich FT, Medzhitov R, Brüning JC. MyD88 signaling in the CNS is required for development of fatty acid-induced leptin resistance and diet-induced obesity. *Cell Metabolism* 2009; 10: 249-59

17. van Dijk G, Buwalda B. Neurobiology of the metabolic syndrome: an allostatic perspective. *European Journal of Pharmacology* 2008; 585: 137-46

18. Bartolomucci A, Cabassi A, Govoni P, Ceresini G, Cero C, Berra D, Dadomo H, Franceschini P, Dell'Omo G, Parmigiani S, Palanza P. Metabolic consequences and vulnerability to diet-induced obesity in male mice under chronic social stress *PLoS One* 2009; 4: 4331

19. Man TY, Michailidou Z, Gokcel A, Ramage L, Chapman KE, Kenyon CJ, Seckl JR, Morton NM. Dietary manipulation reveals an unexpected inverse relationship between fat mass and adipose 11β-hydroxysteroid dehydrogenase type 1. *American Journal of Physiology - Endocrinology and Metabolism* 2011; 300: E1076-84.]

20. Malcher-Lopes R, Di S, Marcheselli VS, Weng FJ, Stuart CT, Bazan NG, Tasker JG. Opposing crosstalk between leptin and glucocorticoids rapidly modulates synaptic excitation via endocannabinoid release. *Journal of Neuroscience* 2006; 26: 6643-50

21. Tasker JG. Rapid glucocorticoid actions in the hypothalamus as a mechanism of homeostatic integration. *Obesity (Silver Spring)* 2006; 14 Suppl 5: 259S-265S

22. Lin S, Thomas TC, Storlien LH, Huang XF. Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice. *International Journal of Obesity and Related Disorders* 2000; 24: 639-46

23. Banks WA, Coon AB, Robinson SM, Moinuddin A, Shultz JM, Nakaoke R, Morley JE. Triglycerides induce leptin resistance at the blood-brain barrier. *Diabetes* 2004; 53: 1253-60

24. Van Heek M, Compton DS, France CF, Tedesco RP, Fawzi AB, Graziano MP, Sybertz EJ, Strader CD, Davis HR Jr. Diet-induced obese mice develop peripheral, but not central, resistance to leptin. *Journal of Clinical Investigation* 1997; 99: 385-90

25. Kruse M, Bornstein SR, Uhlmann K, Paeth G, Scherbaum WA. Leptin down-regulates the steroid producing system in the adrenal. *Endocrine Research* 1998; 24: 587-90

26. Hsu HT, Chang YC, Chiu YN, Liu CL, Chang KJ, Guo IC. Leptin interferes with adrenocorticotropin/3',5'-cyclic adenosine monophosphate (cAMP) signaling, possibly through a Janus kinase 2-phosphatidylinositol 3-kinase/Akt-phosphodiesterase 3-cAMP pathway, to down-regulate cholesterol side-chain cleavage cytochrome P450 enzyme in human adrenocortical NCI-H295 cell line. *Journal of Clinical Endocrinology & Metabolism* 2006; 91: 2761-9

27. Masuzaki H, Paterson J, Shinyama H, Morton NM, Mullins JJ, Seckl JR, Flier JS. A transgenic model of visceral obesity and the metabolic syndrome. *Science* 2001; 294: 2166-70

28. Cooper MS, Stewart PM. 11Beta-hydroxysteroid dehydrogenase type 1 and its role in the hypothalamus-pituitary-adrenal axis, metabolic syndrome, and inflammation. *Journal of Clinical Endocrinology & Metabolism* 2009; 94: 4645-54

29. Bi S. Role of dorsomedial hypothalamic neuropeptide Y in energy homeostasis. *Peptides* 2007; 28: 352-6

30. Morrison CD, Berthoud HR. Neurobiology of nutrition and obesity. *Nutrition Reviews* 2007; 65: 517-34.

31. Bayol SA, Farrington SJ, Stickland NC. A maternal 'junk food' diet in pregnancy and lactation promotes an exacerbated taste for 'junk food' and a greater propensity for obesity in rat offspring. *British Journal of Nutrition* 2007; 98: 843–851

32. White CL, Purpera MN, Morrison CD. Maternal obesity is necessary for programming effect of a high-fat diet on offspring. *American Journal of Physiology – Regulatory, Integrative and Compartive Physiology* 2009; 296: R1464–1472

33. Nivoit P, Morens C, Van Assche FA, Jansen E, Poston L, et al. Established diet-induced obesity in female rats leads to offspring hyperphagia, adiposity and insulin resistance. *Diabetologia* 2009; 52: 1133–1142

34. Samuelsson AM, Morris A, Igosheva N, Kirk SL, Pombo JM, et al. Evidence for sympathetic origins of hypertension in juvenile offspring of obese rats. *Hypertension* 2010; 55: 76–82

35. Franco JG, Fernandes TP, Rocha CP, Calviño CM, Pazos-Moura CC, Lisboa PC, Moura EG, Trevenzoli IH. Maternal high-fat diet induces obesity and adrenal and thyroid dysfunction

in male rat offspring at weaning *Journal of Physiology* 2012 Aug 6. [Epub ahead of print]

36. Sun B, Purcell RH, Terrillion CE, Yan J, Moran TH, Tamashiro KL. Maternal High-Fat Diet During Gestation or Suckling Differentially Affects Offspring Leptin Sensitivity and Obesity. *Diabetes* 2012; 61: 2833-41
37. D'Asti E, Long H, Tremblay-Mercier J, Grajzer M, Cunnane SC, Di Marzo V, Walker CD. Maternal dietary fat determines metabolic profile and the magnitude of endocannabinoid inhibition of the stress response in neonatal rat offspring. *Endocrinology* 2010; 151: 1685-94
38. Drake AJ, Livingstone DE, Andrew R, Seckl JR, Morton NM, Walker BR. Reduced adipose glucocorticoid reactivation and increased hepatic glucocorticoid clearance as an early adaptation to high-fat feeding in Wistar rats. *Endocrinology* 2005; 146: 913-9
39. Grayson BE, Levasseur PR, Williams SM, Smith MS, Marks DL, Grove KL. Changes in melanocortin expression and inflammatory pathways in fetal offspring of nonhuman primates fed a high-fat diet. *Endocrinology* 2010; 151: 1622-32
40. Rudyk O, Makra P, Jansen E, Shattock MJ, Poston L, Taylor PD. Increased cardiovascular reactivity to acute stress and salt-loading in adult male offspring of fat fed non-obese rats. *PLoS One* 2011; 6: e25250
41. Trottier G, Koski KG, Brun T, Toufexis DJ, Richard D, Walker CD. Increased fat intake during lactation modifies hypothalamic-pituitary-adrenal responsiveness in developing rat pups: a possible role for leptin. *Endocrinology* 1998; 139: 3704-11
42. Sonino N, Fava GA. Psychiatric disorders associated with Cushing's syndrome. Epidemiology, pathophysiology and treatment. *CNS Drugs* 2001; 15: 361-73
43. Martins IJ, Hone E, Foster JK, Sünram-Lea SI, Gnjec A, Fuller SJ, Nolan D, Gandy SE, Martins RN. Apolipoprotein E, cholesterol metabolism, diabetes, and the convergence of risk factors for Alzheimer's disease and cardiovascular disease. *Molecular Psychiatry* 2006; 11: 721-36
44. Cizza G. Major depressive disorder is a risk factor for low bone mass, central obesity, and other medical conditions. *Dialogues in Clinical Neuroscience* 2011; 13: 73-87
45. Dandona P, Dhindsa S. Update: Hypogonadotropic hypogonadism in type 2 diabetes and obesity. *Journal of Clinical Endocrinology & Metabolism* 2011; 96: 2643-51
46. Rutters F, Nieuwenhuizen AG, Lemmens SG, Born JM, Westerterp-Plantenga MS. Acute stress-related changes in eating in the absence of hunger. *Obesity (Silver Spring)* 2009; 17: 72-7
47. van Raalte DH Ouwens DM, Diamant M. Novel insights into glucocorticoid-mediated diabetogenic effects: towards expansion of therapeutic options? *European Journal of Clinical Investigation* 2009; 39: 81-93
48. Chanson P, Salenave S. Metabolic syndrome in Cushing's syndrome. *Neuroendocrinology* 2010; 92 Suppl 1: 96-101
49. Arnaldi G, Scandali VM, Trementino L, Cardinaletti M, Appolloni G, Boscaro M. Pathophysiology of dyslipidemia in Cushing's syndrome. *Neuroendocrinology* 2010; 92 Suppl 1: 86-90
50. Pasquali R, Vicennati V, Cacciari M, Pagotto U. The hypothalamic-pituitary-adrenal axis activity in obesity and the metabolic syndrome. *Annals of the New York Academy of Sciences* 2006; 1083: 111-28
51. Newell-Price J, Bertagna X, Grossman AB, Nieman LK. Cushing's syndrome. *Lancet* 2006; 13; 367: 1605-17
52. Dekkers OM, Biermasz NR, Pereira AM, Roelfsema F, van Aken MO, Voormolen JH, Romijn JA. Mortality in patients treated for Cushing's disease is increased, compared with patients treated for nonfunctioning pituitary macroadenoma. *Journal of Clinical Endocrinology & Metabolism* 2007; 92: 976-81
53. Rutters F, Nieuwenhuizen AG, Lemmens SG, Born JM, Westerterp-Plantenga MS. Acute stress-related changes in eating in the absence of hunger. *Obesity (Silver Spring)* 2009; 17: 72-7
54. Tiemensma J, Kaptein AA, Pereira AM, Smit JW, Romijn JA, Biermasz NR. Negative illness perceptions are associated with impaired quality of life in patients after long-term remission of Cushing's syndrome. *European Journal of Endocrinology* 2011; 165: 527-35
55. Sarabdjitsingh RA, Spiga F, Oitzl MS, Kershaw Y, Meijer OC, Lightman SL, de Kloet ER. Recovery from disrupted ultradian glucocorticoid rhythmicity reveals a dissociation between hormonal and behavioural stress responsiveness. *Journal of Neuroendocrinology* 2010; 22: 862-71

56. van den Berg G, Frölich M, Veldhuis JD, Roelfsema F. Combined amplification of the pulsatile and basal modes of adrenocorticotropin and cortisol secretion in patients with Cushing's disease: evidence for decreased responsiveness of the adrenal glands. *Journal of Clinical Endocrinology & Metabolism* 1995; 80: 3750-7

57. de Haan W, de Vries-van der Weij J, van der Hoorn JW, Gautier T, van der Hoogt CC, Westerterp M, Romijn JA, Jukema JW, Havekes LM, Princen HM, Rensen PC. Torcetrapib does not reduce atherosclerosis beyond atorvastatin and induces more proinflammatory lesions than atorvastatin. *Circulation* 2008; 117: 2515-22

58. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993; 362: 801-809

59. McNeill E, Channon KM, Greaves DR. Inflammatory cell recruitment in cardiovascular disease: murine models and potential clinical applications. *Clinical Science (Lond)* 2010; 118: 641-55

60. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *New England Journal of Medicine* 2005; 352: 1685-95

61. Frostegård J, Ulfgren AK, Nyberg P, Hedin U, Swedenborg J, Andersson U, Hansson GK. Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis* 1999; 145: 33-43

62. Wheller SK, Perretti M. Dexamethasone inhibits cytokine-induced intercellular adhesion molecule-1 up-regulation on endothelial cell lines. *European Journal of Pharmacology* 1997; 331: 65-71

63. Caprio M, Newfell BG, la Sala A, Baur W, Fabbri A, Rosano G, Mendelsohn ME, Jaffe IZ. Functional mineralocorticoid receptors in human vascular endothelial cells regulate intercellular adhesion molecule-1 expression and promote leukocyte adhesion. *Circulation Research* 2008; 102: 1359-67

64. Sakai M, Biwa T, Matsumura T, Takemura T, Matsuda H, Anami Y, Sasahara T, Kobori S, Shichiri M. Glucocorticoid inhibits oxidized LDL-induced macrophage growth by suppressing the expression of granulocyte/macrophage colony-stimulating factor. *Arteriosclerosis, Thrombosis, and Vascular Biology* 1999; 19: 1726-33

65. de Kloet ER, Joëls M and Holsboer F. Stress and the brain: from adaptation to disease. *Nature Reviews Neuroscience* 2005; 6: 463-475

66. Laskowitz DT, Lee DM, Schmechel D, Staats HF. Altered immune responses in apolipoprotein E-deficient mice. *Journal of Lipid Research* 2000; 41: 613-20

67. Kleemann R, Verschuren L, van Erk MJ, Nikolsky Y, Cnubben NH, Verheij ER, Smilde AK, Hendriks HF, Zadelaar S, Smith GJ, Kaznacheev V, Nikolskaya T, Melnikov A, Hurt-Camejo E, van der Greef J, van Ommen B, Kooistra T. Atherosclerosis and liver inflammation induced by increased dietary cholesterol intake: a combined transcriptomics and metabolomics analysis. *Genome Biology* 2007; 8: R200

68. Kühnast S, van der Hoorn JW, van den Hoek AM, Havekes LM, Liau G, Jukema JW, Princen HM. Aliskiren inhibits atherosclerosis development and improves plaque stability in APOE*3Leiden.CETP transgenic mice with or without treatment with atorvastatin. *Journal of Hypertension* 2012; 30: 107-16

69. Faggiano A, Pivonello R, Spiezia S, De Martino MC, Filippella M, Di Somma C, Lombardi G, Colao A. Cardiovascular risk factors and common carotid artery caliberand stiffness in patients with Cushing's disease during active disease and 1 year after disease remission. *Journal of Clinical Endocrinology & Metabolism* 2003; 88: 2527-33

70. Giordano R, Picu A, Marinazzo E, D'Angelo V, Berardelli R, Karamouzis I, Forno D, Zinnà D, Maccario M, Ghigo E, Arvat E. Metabolic and cardiovascular outcomes in patients with Cushing's syndrome of different aetiologies during active disease and 1 year after remission. *Clinical Endocrinology (Oxf)* 2011; 75: 354-60

71. Barahona MJ, Sucunza N, Resmini E, Fernández-Real JM, Ricart W, Moreno-Navarrete JM, Puig T, Farrerons J, Webb SM. Persistent body fat mass and inflammatory marker increases after long-term cure of Cushing's syndrome. *Journal of Clinical Endocrinology & Metabolism* 2009; 94: 3365-71

72. Colao A, Pivonello R, Spiezia S, Faggiano A, Ferone D, Filippella M, Marzullo P, Cerbone G, Siciliani M, Lombardi G. Persistence of increased cardiovascular risk in patients with Cushing's disease after five years of successful cure. *Journal of Clinical Endocrinology & Metabolism* 1999; 84: 2664-72

73. Albiger N, Testa RM, Almoto B, Ferrari M, Bilora F, Petrobelli F, Pagnan A, Mantero F, Scaroni C. Patients with Cushing's syndrome have increased intimal media thickness at different vascular levels: comparison with a population matched for similar cardiovascular risk factors. *Hormone and Metabolic Research* 2006; 38: 405-10

74. Geer EB, Shen W, Strohmayer E, Post KD, Freda PU. Body composition and cardiovascular risk markers after remission of Cushing's disease: a prospective study using whole-body MRI. *Journal of Clinical Endocrinology & Metabolism* 2012; 97: 1702-11

75. van der Pas R, de Bruin C, Leebeek FW, de Maat MP, Rijken DC, Pereira AM, Romijn JA, Netea-Maier RT, Hermus AR, Zelissen PM, de Jong FH, van der Lely AJ, de Herder WW, Lamberts SW, Hofland LJ, Feelders RA. The hypercoagulable state in Cushing's disease is associated with increased levels of procoagulant factors and impaired fibrinolysis, but is not reversible after short-term biochemical remission induced by medical therapy. J Clin Endocrinol Metab. 2012 Apr;97(4):1303-10.

76. Biddie SC, John S, Hager GL. Genome-wide mechanisms of nuclear receptor action. *Trends in Endocrinology and Metabolism* 2010; 21: 3-9

77. Lee RS, Tamashiro KL, Yang X, Purcell RH, Harvey A, Willour VL, Huo Y, Rongione M, Wand GS, Potash JB. Chronic corticosterone exposure increases expression and decreases deoxyribonucleic acid methylation of Fkbp5 in mice. *Endocrinology* 2010; 151: 4332-43

78. Stary HC. Lipid and macrophage accumulations in arteries of children and the development of atherosclerosis. *American Journal of Clinical Nutrition* 2000; 72: 1297S-1306S

79. Chesnokova V, Melmed S. Minireview: Neuro-immuno-endocrine modulation of the hypothalamic-pituitary-adrenal (HPA) axis by gp130 signaling molecules. *Endocrinology* 2002; 143: 1571-4

80. Holland WL, Bikman BT, Wang LP, Yuguang G, Sargent KM, Bulchand S, Knotts TA, Shui G, Clegg DJ, Wenk MR, Pagliassotti MJ, Scherer PE, Summers SA. Lipid-induced insulin resistance mediated by the proinflammatory receptor TLR4 requires saturated fatty acid-induced ceramide biosynthesis in mice. *Journal of Clinical Investigation* 201; 121: 1858-70

81. Jialal I, Huet BA, Kaur H, Chien A, Devaraj S. Increased toll-like receptor activity in patients with metabolic syndrome. *Diabetes Care* 2012; 35: 900-4

82. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. *Journal of Clinical Investigation* 2006; 116: 3015-25