ALTERED MACROPHAGE DIFFERENTIATION AND LEPTIN EXPRESSION IN VISCERAL WHITE ADIPOSE TISSUE FOLLOWING CHRONIC SLEEP RESTRICTION IN MICE

A Thesis

presented to

the Faculty of Natural and Applied Sciences

at Notre Dame University-Louaize

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biology

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Dedication

This thesis is dedicated to my mother, the woman who taught me to perform all of life's tasks, no matter how big or small, to the best of my ability and without complaint. She is the woman I will always aspires to be.

Acknowledgements

I would like to acknowledge and give my warmest thanks to my supervisor Dr. Colette Kabrita at Notre Dame University-Louaize, who made this work possible. Her guidance and advice carried me through all the steps of writing my thesis and finishing this research. Her vision, sincerity, and motivation have deeply inspired me. She has taught me the methodology to carry out this research and to present the research work as clearly as possible. It was a great privilege and honor to work and study under her guidance.

My sincere thanks also goes to Mrs Nada Maalouf and Elizabeth Saliba, lab. Assistants for the inestimable help given to me. I cannot begin to list all the benefits of having the professional assistance like them.

Finally, I would also like to give a special thanks to my husband and my parents for their continuous support and understanding when undertaking my research and writing my thesis. Your prayer for me was what sustained me this far.

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List of abreviations

BS	Blood Sugar
BW	Body Weight
C	Control
CD38	Pro-inflammatory macrophage
CD163	Anti-inflammatory macrophage
IHC	Immunohistochemistry
IR	Insulin Resistance
FBS	Fasting Blood Sugar
Lep	Leptin
M1	Pro-inlammatory Macrophage
M2	Anti-inflammatory Macrophage
RT	Room Temperature
SNS	Sympathetic Nervous System
SR	Sleep Restriction
T2D	Type 2 Diabetes
vWAT	Visceral White Adipose Tissue

Abstract

Chronic sleep restriction (CSR) has been implicated in higher risk of insulin resistance (IR) and immune-metabolic dysfunction. No studies investigated the effect of CSR on macrophage differentiation in vWAT in relation to leptin synthesis and glucose homeostasis. Therefore, this study aimed at assessing blood glucose levels and vWAT expression of leptin, CD38, and CD163 following CSR.

Adult male C57BL/6 mice were housed under 12:12 L/D cycle (lights on at 0700 hrs) with free access to food. Following a two-week acclimatization period, animals were divided into 2 groups: 1) control (C, N=8) with normal sleep and 2) experimental/sleep restricted (SR, N=7) subjected to 11 days of sleep restriction. All animals underwent timed weekly measurements of body weight (BW), food consumption, and fasting blood sugar (BS) for the duration of the experiment. They were sacrificed at the end of the study (at 2400 hrs): BW, vWAT weight, and BS were measured; vWAT was processed for leptin, CD38, and CD163 immunocytochemistry.

Compared to C, SR mice had a significantly lower BW (21.42±1.72 g vs 19.77±1.14 g, respectively; P=0.006) and vWAT weight (4.25±0.77 % vs 2.35±0.39 %, respectively; P=0.000), and higher BS (105±2 mg/dl vs 150±4 mg/dl, respectively; P=0.000) at the end of the experiment. There was no significant difference in mean food consumption between the 2 groups, even though SR ate more. Qualitative protein analysis of experimental vWAT revealed the presence of CD38-immunoreactive cells, but not leptin or CD163. In contrast, control vWAT showed both leptin- and CD163-, but not CD38-, immunoreactivity.

These findings suggest that 1) CSR is associated with a rise in BS, BW loss, absence of leptin synthesis, and expression of M1 (inflammatory) macrophages in vWAT, 2) the elevation

in BS and decrease in BW are probably related to disrupted insulin signaling rather than leptin dysregulation, and 3) the possible metabolic dysfunction accompanying CSR is probably related to leptin inhibition and/or inflammation-dependent pathways in vWAT. Although this study could not establish a cause-effect relationship between inflammation and adipoinsular physiology in CSR, its basic findings may lend useful information to prospective studies in this area that may have important medical implications.

Keywords: CD163, CD38, hyperglycemia, insulin resistance, macrophage differentiation.

I. INTRODUCTION

Sleep is a basic biological need that, like any other, is indispensable for the maintenance of overall health and well-being. The fact that humans spend about one-third of their life sleeping (or, at least, attempting to) reflects the neurophysiological significance of adequate sleep on the central pathways implicated in immune-endocrine regulation and energy homeostasis. Yet, sleep problems, manifest as altered quality and/or quantity, remain a real culprit and major public health concern of modern societies that have traded off sleep to `accommodate social and work schedules. Studies have shown that the worldwide nocturnal sleep duration has decreased by 1-2 hours during the second half of the 20th century (Van Cauter et al., 2008) and the incidence of obesity has concurrently doubled (Flegal et al., 2002). Furthermore, compelling evidence in the scientific literature links sleep disturbances in both quality and quantity (for example poor and/or shortened sleep duration) to increased morbidity and mortality from cardiometabolic complications and immune disturbances (Irwin et al., 2016). Associated metabolic deragement includes impaired glucose and lipid metabolism (hyperglyemia and dyslipidemia, respectively) secondary to insulin resistance (IR), thus increasing the risk of developing diabetes mellitus type II (T2D), obesity, and metabolic syndrome (MetS) (Trouwborst et al., 2018; Grundy et al., 2005). The prevalence of MetS is progressively rising such that, comorbid with sleep deprivation, it has become the major cause of mortality in developed and underdeveloped countries; studies show that the main driver of MetS is inflammation (Kassi et al., 2011; Chan et al., 2019). Altered immune responses; on the other hand, implicate both the innate and adaptive pathways that mediate inflammation and T cell activity, respectively (Ghanem et al., 2019; Janeway et al., 2001). Examples of impaired immune cell activity include increased infiltration of macrophages into tissues, such as white

adipose tissue (WAT), and their differentiation into the inflammatory phenotype, as well as elevated levels of inflammatory cytokines (e.g. Il-1 β and IFN-γ), and acute phase proteins all of which play a major role in the modulation of sleep (O'Rourke et al., 2011; Fujisaka et al., 2013; Janciauskiene et al., 2011). Noteworthy, the functional relationship between sleep and immunity is bi-directionally, that is inflammation causes sleep loss and vice versa.

Visceral white adipose tissue (vWAT) plays a significant role in the pathogenesis of MetS and its physiology appears to be highly affected by sleep. The functional significance of vWAT lies in its leptin-mediated endocrine control of the metabolic pathways that underlie energy homeostasis, such as regulation of insulin sensitivity and glucose homeostasis, as well as the secretion of pro- and anti-inflammatory mediators and complement proteins that mediate immune responses (Coelho et al., 2013; Paz-Filho et al., 2012). In obese individuals and those exhibiting abnormal glucose metabolism, such as T2D, the cellular cross-talk between adipocytes and macrophages in vWAT is disrupted. Obesity is associated with enlargement of adipocytes and resultant increase in the distance between these cells and vWAT vasculature, thereby inducing a state of tissue hypoxia (Liu, and Nikolajczyk, 2019; Lee et al., 2014; Trayhurn, 2013). This hypoxic condition induces the expression of hypoxia-inducible factor 1 (HIF-1) by adipocytes that, on long term, further intensify the metabolic and immune complications associated with obesity (Lee et al., 2014). In other words, chronic hypoxia has two major effects on vWAT: 1) it causes fibrosis of adipose tissues (Sun, K., et al., 2013) and 2) it results in increased infiltration of macrophages into adipose tissues in order to remove necrotic adipocytes, thus releasing in the process inflammatory cytokines (e.g. IL-6 and TNF-α) and increasing their inflammatory responses (O'Rourke et al., 2011; Fujisaka et al., 2013). A remarkable shift or, in other words, differentiation of these macrophages into the inflammatory

(M1) rather than anti-inflammatory (M2) phenotype (Lumeng et al., 2007; Wentworth et al., 2010). M1 phenotype is the most contributor to adipocyte inflammation in obese, because the numbers and frequencies of this phenotype correlate with (IR) (Lumeng et al., 2007). In summary, obese individuals and those exhibiting abnormal glucose metabolism, such as T2D, the cellular cross-talk between adipocytes and macrophages in vWAT is disrupted due, in part, to increased macrophage infiltration into adipose tissue and biased differentiation into the inflammatory (M1) rather than anti-inflammatory (M2) phenotype.This, in turn, is believed to be linked to IR and abnormal carbohydrate metabolism. While findings link a potential role of altered macrophage differentiation to IR in metabolic pathologies, the implications of this in sleep restriction (SR) and, thus, the cellular mechanisms underlying the sleep-adipocyte axis are understudied. Whether insufficient sleep induces physiolgoical changes in the cellular activity of vWAT remains elusive.

Several factors affect leptin synthesis and secretion by vWAT, including insulin and sleep. There is an adipo-insular axis between adipose tissues and pancreatic β-cells via leptin and insulin, respectively: leptin inhibits insulin release while insulin stimulates adipocyte production of leptin (Kieffer and Habener, 2000; Kieffer et al., 1996). This physiological axis appears to be disrupted in metabolic pathologies characterized by IR and inflammation. Furthermore, circulating leptin levels are decreased following acute SR in both humans and rodents (Spiegel et al., 2004), an effect that is partly attributed to the associated inhibitory effects of enhanced sympathetic outflow on adipocytes (Rayner and Trayhurn, 2001; Caron et al., 2018; Trayhurn et al., 1996). Sympathetic activation of adipocytes also results in increased release of free fatty acids (FFA) into the bloodstream, eventually leading to dyslipidemia and subsequent

development of peripheral IR and increased morbidity from cardiovascular complications (Caron et al., 2018).

vWAT constitutes an important physiological link between immune and endocrine factors. Both immune and endocrine factors appear to interact with inadequate sleep to bring about metabolic dysfunction; however, the exact mechanisms by which sleep quantity affects adipocyte physiology is unclear. Noteworthy, most of the studies done in this area considered acute, rather than chronic, SR paradigms that carried for 5 days at most and which did not examine the physiological interlink between macrophage phenotypic differentiation and adipokine expression. For example, in healthy men, one night of acute sleep deprivation caused a reduction in the daily energy expenditure and increased postprandial plasma glucose which was attributed to impaired insulin-mediated glucose uptake by tissues following sleep deprivation (Benedict et al., 2011). The reduction in energy expenditure observed following one or 2 nights of sleep restriction was explained as due to an aleration in the body's sensitivity to catabolic signals, such as increased sympathetic outflow (Kato et al., 2000). Decreased insulin sensitivity of tissues, including subcutaneous adipocytes, was also reported in non-obese individuals following 3 and 4 nights of restricted sleep (Klingenberg, et al., 2013; Broussard et al., 2012). In addition, acute and chronic sleep restriction were shown to be associated with reduced leptin levels in girls and adolescent males, respectively, as well as elevated free fatty acid concentrations (Boeke et al., 2014; Spiegel et al., 2004; Donga et al., 2010).

As such and to our knowledge, no studies have investigated the potential involvement of vWAT macrophage phenotypic differentiation and/or the cellular expression of leptin in relation to IR and aberrant glucose metabolism following chronic SR. Therefore, the present study aimed at gaining insight into the effect of chronic SR on adipose tissue physiology by assessing the

relative expression of M1 and M2 type macrophages in relation to intracellular leptin expression and blood glucose levels. This stems from our speculation that the same vWAT factors implicated in obesity may also underlie the metabolic and immune complications seen in chronic SR. It is plausible that, in the presence of IR, SR may push macrophage differentiation into the inflammatory pathways in visceral WAT which may negatively affect leptin synthesis, further exacerbating pancreatic insulin release and precipitating peripheral IR. Visceral WAT was chosen because it is closely linked wih IR and cardiometaolic complications. Therefore, test 2 hypotheses: 1) that chronic sleep restriction (CSR) may interact with IR (or may favor a state of IR) to favor macrophage differentiation toward the inflammatory (M1) phenotype in vWAT, 2) that macrophages polarization may be associated with reduced leptin expression by adipocytes. Gaining insight into the physiological mechanisms interlinking sleep, adipocyte physiology, and IR may have valuable clinical implications in the management and treatment of sleep-related metabolic disorders, such as cardiovascular diseases, obesity, and MetS.

II. LITERATURE REVIEW

A. An Overview of Sleep: Architecture and Neuroendocrine Regulation

Sleep is part of a daily biological rhythm that is indispensable for survival. It is an active state during which central neural networks are remodeled and constituted and the body conserves energy. Several brain regions interact to regulate the many aspects of sleep, such as its timing, duration (amount), and depth, and these include the hypothalamus, thalamus, forebrain, and brainstem (Telias and Wilcox, 2019; Brown et al., 2012). A two-process model was put forth to explain how the sleep/wake rhythm is regulated, namely through the intimate interaction between a circadian process and a homeostatic one (Borbely and Achermann, 1999; Saper et al., 2005). The circadian process is regulated by a central time-keeping system which determines the arousal state, that is the timing of sleep; the homeostatic process is linked to metabolism (and accumulation of metabolites during activity period) and controls sleep need or propensity (i.e. sleep driver) that usually builds up with prolonged wakefulness (Koh et al., 2008). Conversely, the latter decreases in sleep.

Just like any other endogenously-generated biological rhythm, the circadian (near 24-hour) control of the sleep/wake rhythm is carried by a master oscillator which, in mammals, resides in the suprachiasmatic nuclei (SCN), also refered to as the master clock or circadian pacemaker (Kwon et al., 2011). The SCN is a group of neurons and glia located in the anterior hypothalamus that, by keeping track of time, entrain the various endogenous rhythms to the 24-hour light/dark cycle of the external environment. This is made possible because a subpopulation of SCN neurons receives photic input from the retina via the retinohypothalamic tract; light information is then transduced by other SCN neurons which project to various brain regions/nuclei, one being the pineal gland, to control neuroendocrine activity (e.g. melatonin secretion) (Grivas and Savvidou, 2007; Brzezinski, 1997). Furthermore, the SCN, via the

autonomic nervous system and hormones, synchronizes (i.e. temporally couples) the activity of the various peripheral oscillators or clocks distributed in peripheral tissues, such as the liver, kidney, skin, and heart, as well as non-SCN brain regions (Astiz et al., 2019; Mohawk et al., 2012; Ueyama et al., 1999; Kalsbeek et al., 2010). This allows the temporal alignment of the entire circadian clockwork (or biological time-keeping system) to the external light/dark cycle.

The arousal system is turned off in sleep and on in wakefulness. This is explained by the flip-flop model which is composed of two components: reticular activating system (RAS) and ventrolateral preoptic area (VLPO). RAS is a component of the reticular formation located anteriorly in the brainstem that is implicated in the regulation of the sleep-wake cycle and wakefulness (Arguinchona and Tadi, 2020). It is composed of 4 main components, each containing groups of serotonergic, cholinergic, histaminergic, and noradrinergic neurons that are activated by the lateral hypothalamus (LH). The latter releases the neurotransmitter orexin in response to light hitting the retina and this allows the transition from sleep to wakefulness (Nishino, 2011). Further regulation of sleep/wake states is brought about by the VLPO: inhibition of VLPO neurons by GABA and galanin activate wakefulness; whereas activation of these neurons by adenosine that acculmulates over the course of a day promotes sleep (Brennan and Charles, 2009). Therefore, RAS promotes waking, whereas VLPO promotes sleep.

During sleep the brain cycles through two phases that are distinguished by the differential pattern and intensity of neuronal activity in the different brain regions: rapid eye movement (REM), also known as paradoxical or dream sleep, and non-REM which is quiet or deep sleep. Each sleep phase is regulated by a specific region in the brain, with non-REM and REM being controlled by the homeostatic and circadian systems, respectively (Besedovsky et al., 2019). Unlike REM, non-REM is composed of 4 stages: N1, N2, N3, and N4, with N1 and N2 being

referred to as light sleep, whereas N3 and N4 are deep sleep stages (Medic et al., 2017). In addition to the distinctive pattern of neuronal activity characterizing each sleep phase, the activity of some biological processes are also different between REM and non-REM. For example, the heart and breath rates are decreased during non-REM as compared to REM (Medic et al., 2017; Purves et al., 2001).

A non-dialyzable substance that has a control over sleep was first elucidated by Legendre and Pieron (1913) through their study on sleep-deprived dogs. This study gained much interest and was later reinvestigated by Schnedorf and Ivy (1939). They stated that their trials gained 9 positive tests for the substance when they transfused cerebrospinal fluid (CSF) from sleep-deprived dogs into normal recipients. Monnier et al. (1964,1965) showed electrical stimulation in the intralaminary thalamus of rabbits resulted in the release of a dialyzable nonapeptidic sleep-promoting factor of about 849 kD into the cerebral venous blood. Later, they synthetized it and named it delta-inducing peptide (DSIP). Pappenheimer et al. (1967) did further research on this factor; they showed that the infusion of 1-3 ml of CSF of sleep-deprived goats into cats improved sleep in those cats to about 12 hours. However, because cats sleep most of time, they were not suitable models for this laboratory assessment. For this reason, pappenheimer et al. (1967) repeated their experiment on rats and goats because the sleep/wake cycle could be easily altered in rats and the shape and thickness of the occipital bone in goats are suitable for frequent withdrawal of CSF in the absence of anesthesia. The infusion of CSF from normal goats into rats showed slight changes in rodent activity, whereas the infusion of CSF from sleep-deprived goats reduced the rats' motor activity for several hours. The researchers concluded that there was a humoral factor that improved sleep in those rats and reduced their motor activity. In addition, another study also showed that the infusion of 0.1 ml of CSF from sleep-deprived goats into the

cerebral ventricle of rats resulted in an increase in slow wave sleep (SWS) of the latter (Fencl et al., 1971). Fencl et al. (1971) isolated a dialyzable low molecular weight molecule from CSF, which he named factor S, that was shown to induce sleep in rats by depressing their motor activities and increasing SWS. This humoral factor S also induced sleep in cats (Pappenheimer et al.,1967). Noteworthy, factor S was shown to be only induced by sleep and none of the other CSF constituents, such as serotonin, GABA, and glutamic acid (Fencl et al., 1971). Furthermore, Krueger et al. [1980] extracted from human urine a substance that resembled factor S and which they named urinary sleep-promoting factor (SPU). SPU is an endogenous factor S, known as sleep-promoting factor (SPF), that presents in the cerebrospinal fluid as a low molecular weight bacterial cell wall peptidoglycan known as muramyl peptide (Pappenheimer et al., 1967; Pappenheimer, 1983).

1. Sleep Restriction: Causes and Physiological Consequences

The worldwide prevalence of sleep restriction (SR) among the different age groups is attributed to several factors, both socioeconomic and biological. Studies based on sleep quality and efficiency in children and adolescents have reported that these tend to have delayed bedtimes and less total sleep time a night, particularly on weekdays, than what is recommended for their age groups (Carskadon, 1990). Changes in adolescent sleep patterns are attributed to an alteration in the circadian timing system that occurs at puberty (Carskadon et al., 1993). In addition to the developmental changes, the absence of parental awareness about the importance of regular bedtiming and nocturnal sleep adequacy, as well as the presence of electronic devices in the bedroom at night are shown to alter both sleep quality and quantity (Fuller et al., 2017). Similarly, sleep studies in adults showed that the percentage of those who sleep less than 6 hours

a day, as opposed to the recommended 7-8 hours on average (Brooks et al., 2012; Chin, 2017; Church, 2012), is increasing with time (Knutson et al., 2010) . SR in adults is partly linked to lifestyle behaviors, such as the consumption of caffeine, smoking, long work hours, work shifts, and increased accessibility to media, as well as an individual's chronotype (Kabrita et al., 2014; Caruso, 2014; Levenson et al., 2016). Furthermore, patients suffering from certain psychiatric disorders, such as depression, have impaired sleep continuity and disturbed REM and non-REM sleep (Armitage, 2007; Reynolds and Kupfer, 1987; Benca et al., 1997). Noteworthy, the aforementioned behaviors result in misalignment between biological (circadian) and social time, also termed social jetlag (Erin, 2011), which have adverse consequences on daytime performance and overall health in humans, particularly young adults (Nobis, 2016) .

Among the adverse health consequences of restricted sleep is the increased risk of chronic diseases such as obesity, diabetes T2D, hypertension, and heart disease (Wu et al., 2014; Shan et al., 2015; Wang et al., 2015; Wang et al., 2016). Some studies found an association between short sleep duration and the development and severity of T2D, along with the prevalence of cardiovascular diseases and mortality (Gangwisch et al., 2007; Cappuccio et al., 2011; Gangwisch et al., 2007; Knutson et al., 2006). This association; however, cannot be reported as cause-effect relationship (Gangwisch et al., 2007; Knutson et al., 2006). In addition, both animal and human studies show that long-term sleep deprivation or restriction weakens the immune system, thereby rendering the body more prone to infections and low-grade inflammation (Besedovsky et al., 2019). For example, chronic sleep restriction in rats led to systemic bacterial invasion (Everson and Toth, 2000). The disruption of intestinal defenses and systemic invasion by the intestinal flora caused fatal sepsis in sleep-deprived rats (Besedovsky et al., 2019; Everson et al.. 1993). SR has been also associated with mortality caused by an elevation of certain pro-

inflammatory markers, such as IL-6, TNF α, IFN γ (Yehuda et al., 2009; Zager et al., 2007; Hurtado-Alvarado et al., 2013).

2. Sleep Restriction is a Potential Risk Factor for Metabolic Syndrome

Metabolic syndrome, named earlier Syndrome X by Raven [1988], is defined by a group of interconnected factors that immediately augment the risk of heart disease and T2D by resistance of target tissues to insulin action (Kassi et al., 2011; Reaven**,** 1988). It is characterized by dyslipidemia, such as high triglyceride (TG) and apo lipoprotein B (apoB) and low high-density lipoprotein (HDL) levels, in addition to hypertension and dysregulation in glucose homeostasis. As such, there are two main components which are considered as core clinical manifestations of MetS: visceral obesity and IR (Kassi et al., 2011). Because of differences in the definitions and components of MetS that were elucidated by various organizations, along with associated diseases and disorders, no exact definition and accepted pathogenic mechanism are set for this disorder. Pro-inflammatory diseases and sleep problems are shown to be associated with MetS, making the description even more sophisticated (Kassi et al., 2011). Whether MetS appears as a particular syndrome or a surrogate of combined risk factors is still unclear.

The prevalence of MetS is progressively rising such that it has become the major cause of morbidity and mortality in developed and underdeveloped countries, especially with comorbid sleep deprivation. As MetS is shown to be associated with many diseases, such as polycystic ovarian syndrome (PCOS), fatty liver, cardiovascular diseases, skin conditions (psoriasis), and others (Stefanadi et al., 2018; Mikolasevic et al., 2016; Yao et al., 2017; Amato et al., 2015;

Diamanti-Kandarakis and Dunaif, 2012; Qiao et al., 2007), it appears that a common denominator to these pathologies, and thus the major driver of MetS, is inflammation (Chan et al., 2019). Both constitutional (non-modifable) and environmental lifestyle (modifiable) factors contribute to the pathogenesis of MetS. These include genetic predisposition, age, sex, over and/or under nutrition, unhealthy diet, obesity, family history of diabetes, sedentary lifestyle, inflammation, stress, smoking, alcohol abuse, and sleep deprivation (Cameron et al., 2004; Gupta and Gupta, 2010; Swarup et al., 2014; Raikkonen et al., 2007; Chan et al., 2019).

B. Low-Grade Inflammation is a Major Physiological Culprit in MetS

Low-grade inflammation is characteristic of many diseases and disorders including neuropsychiatric, neurological, degenerative, cardiovascular, metabolic, T2D, sleep disorders, and chronic pain conditions (Donath and Shoelson, 2011; Libby et al., 2013; Bauer and Teixeira, 2019; Mullington et al., 2010; McGeer and McGeer, 2004). Inflammation, which is an immediate innate immune response, is induced by pathogen-associated molecular patterns (PAMPs) from bacteria or viruses, damage-associated molecular patterns (DAMPs) which are released from stressed or injured cells, autoreactive antibodies against self, normal flora, stressor under central nervous system control, cellular dysfunction, obesity, and/or some nutrients and metabolites (Besedovsky et al., 2019; Bianchi, 2007; Chu and Mazmanian, 2013; Matzinger, 2007; Mackey and McFall, 2006). Accumulating body of evidence links an altered immune system activity to the pathology of metabolic disease. That is, chronic inflammation is shown to be the most significant factor in the occurrence of MetS (Sharma, 2011), as well as in the development of diabetes and atherosclerosis (Kanter et al., 2008; Maiti and Agrawal, 2007). In such pathologies, the clinical features of the acute phase (systemic) response to inflammation

includes altered hepatic production of acute-phase proteins and leukocyte activity. Leukocyte activity is skewed toward more inflammatory cytokine and tumor necrosis factor release. That is, there is a decrease in the level of anti-inflammatory cytokines (e.g. IL-10 and IL-4) and an increase in the pro-inflammatory ones (e.g. IL-1, IL-6, TNF-α, and β, IFN γ) (Xie et al., 2006; Gabay, 2006; Kushner, 1993). Importantly, low circulating levels of IL-10, a strong anti-inflammatory cytokine that antagonizes IL-6 and TNF-α, are strongly associated with MetS, especially T2D in elderly, obese, and non-obese women (van Exel et al., 2002; Esposito et al., 2003). Some studies reported elevated blood levels of IL-6 to be associated with IR in obese males and females and hyperandrogenic females (Xu et al., 2014; Peng et al., 2016; Bruun et al., 2003; Lin et al., 2011; Escobar-Morreale et al., 2003). In addition, high levels of TNF-α are associated with IR via a direct action on the insulin receptor substrate 1 (IRS-1); binding of TNF-α to IRS-1 diminishes its intrinsic tyrosine kinase activity and, hence, the associated intracellular signaling pathways (Plomgaard et al., 2005). Furthermore, increased hepatic production of acute phase proteins in response to high levels of pro-inflammatory cytokines, such as C-reactive protein (CRP), is also shown to be associated with low-grade inflammation in MetS (Jain et al., 2011; Devaraj et al., 2009; Ridker et al., 2000). This can be attributed, in part, to the resistance of hepatocytes to insulin's normal action in suppressing the synthesis and release of acute phase proteins which highlights its anti-inflammatory role (Tessari et al., 2006; Festa et al., 2000). This is supported by a cohort of the Women Health Study which showed that CRP levels were powerful biomarkers of T2D and inflammation (Donath and Shoelson, 2011; Wang et al., 2013).

1. Sleep Restriction, Inflammation, and Metabolic Syndrome are Physiologically Interlinked by Complex Neuroendocrine and Metabolic Pathways

The functional relationship between sleep and immunity is bi-directionally, that is inflammation causes sleep loss and vice versa. Sleep loss causes inflammation by modifying both the innate and adaptive immune responses and, thus, the synthesis and release of circulating cytokines (Besedovsky et al., 2019). The imbalance in the proportion of inflammatory to anti-inflammatory cytokines, coupled with endocrine dysfunction, is believed to be among the root causes of the increased risk of developing metabolic disorders upon SR.

Systemic inflammation affects sleep and tissue metabolism via several physiolgocial pathways implicating the neuro-endocrine-immune axis. Modulation in immune cell activity, such as leukocytosis, increased infiltration of macrophages into tissues (e.g. adipose tissue) and altered differentiation into the inflammatory phenotype, elevated levels of pro-inflammatory mediators, and acute phase proteins play a major role in the modulation of sleep. Studies in both humans and laboratory animals show that inflammatory cytokines, such as IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α/β, IFN-α/β, and IFN-γ, affect sleep (Imeri and Opp, 2009). Pro-inflammatory cytokines (e.g. IL-6,IL-1β, TNF-α, IL-2, and IFN-γ,) induce sleep, whereas anti-inflammatory cytokines (e.g. IL-4 and IL-10) inhibit sleep or do not alter its regulation (Weschenfelder et al., 2012). These cytokines interact with neurochemical systems, such as serotoninergic, cholinergic, and glutamatergic pathways, to regulate normal sleep (Imeri and Opp, 2009; Grazia de Simoni et al., 1995). Various studies emphasized the involvement of IL-1 β and TNF-α in the regulation of sleep because the receptors for these cytokines are present in the hypothalamus, brainstem, hippocampus, and cerebral cortex that involved in sleep regulation (Imeri and Opp, 2009; Gemma et al., 1997). Microinjections of IL-1β and TNF-α increased the

duration of NREMS in many species including humans (Obal and Krueger, 2003). Whether IL-1β promoted or inhibited NREMs depended on the time of day, dose, and route of administration (Susic and Totic, 1989; Opp et al., 1992; Gaykema et al., 2000**).** For exapmle, IL1-β injection is shown to enhance sleep in humans, mice, rats, rabbits, monkeys, and cats (Obal and Krueger, 2003; Opp and Krueger, 1994; Susić and Totić, 1989; Friedman et al., 1995; Fang et al., 1998; Tobler et al., 1984; Dinarello, 1991). In rats and cats, low doses of IL1-β promoted sleep, whereas relatively higher doses inhibited it. Furtheremore, a microinjection of this cytokine at night enhaced non-REM sleep, while the same dose given during the day inhibited it (Obal and Krueger, 2003; Opp et al., 1991; Krueger et al., 1984). Along with the enhacement of the duration of non-REMs, it also improved SWS during this phase depending on the route of administarion (Obal and Krueger, 2003; Hansen and Krueger, 1997). That is, the microinjection of IL-1β intracerebroventricularly (i.c.v.) or intravenously (i.v.) improved EEG delta waves, while the microinjection of this cytokine intraperitoneally (ip) in rats and mice decreased EEG delta waves (Obal and Krueger, 2003; Hansen and Krueger, 1997). These cytokines enter the brain through the blood brain barrier (BBB) and regulate sleep (Besedovsky et al,. 2012).

Furthermore, inflammation and sleep problems, such as insomnia, REM sleep behavior disorder, hypersomnia, and somniloquy, have been observed in individuals with autoimmune conditions as a result of auto-reactive antibodies produced against neuronal voltage-gated potassium channel complexes (VGKC) (Cornelius et al., 2011; Iranzo et al., 2006). This includes human immunodeficiency virus (HIV)-infected individuals who experience sleep problems, such as insomnia and obstructive sleep apnea (OSA) (Taibi, 2013). Immunotherapy in some autoimmue cases has been shown to improve sleep (Cornelius et al., 2011).

There is no clear cause-effect relationship between inflammation and sleep problems. It is a vicious cycle whereby inflammation is shown to cause sleep disturbance which, in turn, causes further inflammation that develops into a chronic condition, leading to the development of some components of MetS. In other words, inadequate sleep can worsen inflammation, thus leading to deranged metabolism. Many laboratory studies reported that SR alters the levels of hormones implicated in metabolism and energy homeostatis, such as decreased leptin and increased ghrelin and cortisol levels, in addition to increasing sympathetic tone, increasing IR, and reducing glucose clearance (Chaput et al., 2007; Spiegel et al., 1999). These neuroendocrine changes appear to be interconnected because, based on some studies, the intravenous administration of glucocorticoids (e.g. dexamethasone) to human subjects caused an increase in the level of leptin, suggesting a regulatory role of glucocorticoids on leptin secretion (Udden et al., 2003; Laferrère et al., 2000, 2002). This is supported by the observation that glucocorticoid deficiency resulting from adrenalectomy in leptin-deficient mice caused reduction of body weight which implicated the role of glucocorticoids in regulating leptin synthesis and secretion (Udden et al., 2003; Dubuc and Wilden, 1986).

a. Sleep Loss Causes Low-Grade Inflammation and Metabolic Disturbances

Sleep loss induces low-grade inflammation which increases the risk of developing MetS and vulnerability to infectious diseases in both human and experimental animals. Several measures have been undertaken to assess low-grade inflammation following sleep disturbance in humans and animas, such as levels of inflammatory markers (CRP, IL-6, IL-1β, and TNFα), white blood cell counts (especially neutrophil counts), and platelet counts. SR for one week leads to an increase in IL-6 levels in healthy males (Pejovic et al., 2013). In addition, SR for 5

nights in humans is also shown to result in an elevation in the heart rate along with variation in pro-inflammatory cytokines, such as IL-1β, IL-6, IL-17A, and TNFα (Irwin et al., 2006; Van Leeuwen et al., 2009). The upregulation of pro-inflammatory cytokines following SR was linked to NF-kB activation. Furthermore, sleep deprivation for a whole night results in increased high-sensitivity C-reactive protein (hsCRP) levels, which is a stable marker of inflammation (Meier-Ewert et al., 2004), probably reminiscent to increased levels of IL-6 which stimulates hepatic CRP production (Van Leeuwen et al., 2009). Similarly, Besedovsky et al. (2019) reported an elevation in IL-6 mRNA levels following 36 hours of sleep deprivation in mice and chronic SR for several days in humans. In addition, TNF-α levels increased after several days of restricted sleep in C57BL6 mice (Besedovsky et al., 2019; Hurtado-Alvarado et al., 2018).

Epidemiologic studies show that insufficient sleep is a risk factor for obesity, diabetes, cardiovascular disease, and metabolic syndrome (Grandner et al., 2016; Beccuti and Pannain, 2011; Kim et al., 2018). For example, chronic SR is associated with high blood pressure in children and adolescents (Wells et al., 2008; Archbold et al., 2012). Poor sleep quality is also shown to be associated with the development of hypertension in obese adolescents (Hannon et al. , 2014). Furthermore, a strong association between partial SR and impaired glucose tolerance has been reported. This is supported by human studies that showed that individuals who slept 4 hours per night for one week exhibited impaired glucose tolerance (Spiegel et al., 1999; Buxton et al., 2010). One of the plausible mechanisms underlying SR's cardiometabolic effects are linked to autonomic neural control, namely the associated increase in sympathetic nervous system (SNS) activity a subsequent increase in catecholamine release (Spiegel et al., 1999; Irwin et al., 1999; Gottlieb et al., 2006; Vgontzas et al., 2009). Catecholamines bind to β3-adrenergic

receptors on adipocytes and stimulate these cells to initiate lipolysis and, thus, the release of free fatty acids (FFA) and glycerol into the bloodstream (Xu et al., 2009; Londos et al., 1999; Arner, 2002). This results in the accumulation of FFA and glycerol in other cell types, such as the liver and skeletal muscles, which become resistant to insulin action as well as impair insulin production by pancreatic β cells (Sears and Perry, 2015; Arner, 2002). This, with comorbid inflammation, increases the risk of developing obesity, namely visceral obesity. The latter is correlated with excessive release of FA by adipocytes and their accumulation in other cell types, including the liver, which become resistant to insulin action and production (Sears et al., 2015). Thus, the local reduction of insulin signaling in adipocytes, along with their increased susceptibility to inflammation and increased production of inflammatory cytokines, can exert huge impact on the global energy metabolism through the disruption of insulin signaling in other peripheral tissues (Broussard and Brady, 2010).

b. Sleep Restriction and Neuroendocrine Regulation of Metabolism: The Effect on Leptin Secretion by White Adipose Tissue

Visceral adipose tissue plays a significant role in the pathogenesis of MetS. Adipose tissue is classified as white, brown, and beige , with the last two types being morphologically and functionally different from white adipose tissue (WAT) (Zoico et al., 2019). The physiological significance of WAT lies in its endocrine role; it is the source of hormones, known as adipokines (e.g. leptin and adiponectin), that play a key role in the metabolic pathways implicated in energy homeostasis (Coelho et al., 2013). Adipokines affect appetite, satiety, and energy balance which, in turn, have implications on body fat mass and overall body weight. For example, leptin suppresses appetite and its absence causes obesity, while adiponectin has the opposite effect to

that of leptin. Furthermore, in addition to adipocytes, adipose tissue is composed of many other cell populations, such as pre-adipocytes, fibroblasts, vascular endothelial cells, mast cells, eosinophils, B cells, T cells, and adipose tissue macrophages (ATMs) (Sorisky et al., 2013; van Harmelen et al., 2005; Gesta et al., 2007). This cellular heterogeneity accounts for the different secretions of, adipose tissue , such as growth factors, pro- and anti-inflammatory mediators, and complement proteins, in addition to adipokines (Coelho et al., 2013).

Leptin is a peptide hormone which belongs to the cytokine family and is primarily produced by WAT. It plays a key role in the metabolic pathways that regulate food intake and energy expenditure, as well as the circadian rhythmic activity of the gonadotropic, thyrotropic and adrenal axes (Paz-Filho et al., 2012; Berglund et al., 2012). It is also involved in bone formation, tissue remodeling, inflammation, insulin sensitivity, and the regulation of glucose homeostasis (Paz-Filho et al., 2012; Kelesidis et al., 2010). The central effect of leptin is mediated by its receptors-B (LepR-B) expressed in two hypothalamic neuronal populations: the medially-located nuclei coexpress agouti-related peptide (AgRP) and neuropeptide Y (NPY), while the laterally-located nuclei coexpress cocaine and amphetamine-related transcript (CART) and pro-opiomelanocortin (POMC). The latter neurons comprise a subpopulation of the arcuate nucleus (ARC) of the hypothalamus that play a role in regulating food intake and energy expenditure (Friedman and Halaas, 1998; Paz-Filho et al., 2012). The high expression of NPY/AgRP and the low expression of POMC/CART promote hunger (in energy deficient states), whereas the low expression of NPY/AgRP and the high expression of POMC/CART promote satiety (in energy sufficient/abundant states) (Pinto et al., 2004; Ziotopoulou et al., 2000). Therefore, the NPY/AgRP neurons are orexigenic (i.e. they stimulate appetite);whereas, the POMC/CART neurons are anorexigenic (i.e.they suppress appetite) (Pinto, S., et al., 2004).

In one study on mice, animals which were leptin-deficient or leptin-resistant showed excessive eating behavior and developed obesity (Paz-Filho et al., 2012). Other similar studies showed that the administration of leptin reversed obesity in the leptin-deficient mice (Myers et al., 2008). Similar observations have been reported in humans, whereby low leptin levels were correlated with higher body weights over a short period of time (Ravussin et al., 1997). Thus, leptin plays a pivotal role in energy homeostasis.

Leptin and insulin interact to regulate metabolism and maintain energy balance. Insulin plays a chronic role in the regulation of leptin gene expression and production by white adipose tissue (Rechtschaffen and Bergmann, 1995). For example, increased plasma insulin concentration, such as in insulinoma, is shown to increase circulating leptin levels and gene expression in WAT in both rodents and humans (Kieffer and Habener, 2000; Vidal et al., 1996; Utriainen et al., 1996; Saladin et al., 1995; Saad et al., 1998; Malmström et al., 1996; Kolaczynski et al., 1996). Conversely, leptin has a negative feedback on insulin gene expression, thereby decreasing its synthesis and secretion by pancreatic beta cells (Fu et al.. 2013). This suppressive effect is mediated by the autonomic nervous system (ANS) and by the direct actions of leptin on its membrane receptors present on β-cells (Kieffer and Habener, 2000). There is an adipo-insular axis between adipose tissues and pancreatic β-cells via leptin and insulin, respectively (Kieffer and Habener, 2000; Kieffer et al., 1996). Moreover, cytokines, cortisol, catecholamines, fatty acids, and glucose affect leptin synthesis and secretion by WAT (Fried et al., 2000). Insulin also acts in synergy with cortisol to regulate leptin production (Spiegel et al., 2004).

In addition to the peripheral neural and endocrine control on leptin synthesis and release, the suprachiasmatic nucleus plays a central role in regulating the daily timing of leptin release.

Circulating leptin has a circadian profile with a characteristic nocturnal peak in both humans and rodents in a response to daytime meal ingestion (Schoeller et al., 1997; Simon et al., 1998). At night (during sleep), leptin levels increase to signal to the brain the abundance of energy stores during this phase, thus suppressing hunger (feeding behavior) (Sinha et al., 1996; Schoeller et al., 1997). Its levels in the blood are proportional to adipose tissue mass (Maffei et al.. 1995). Its elevation during daytime persists in sleeping patients receiving continuous enteral nutrition, indicating that sleep itself affects leptin regulation (Simon et al., 1998). Low leptin levels reminiscent to insufficient sleep result in wrong signaling to central (hypothalamic) regulators of energy hemeostasis about the body's energy status, thus promoting feeding behaviors at wrong times of the day (or circadian phase), slowing down metabolism, and favoring energy storage. In rats and mice, food deficiency and/or starvation cause sleep problems; whereas, total sleep deprivation leads to excessive eating behavior (Knutson et al., 2007; Danguir and Nicolaidis, 1979; Rechtschaffen and Bergmann, 1995). Since SR causes reduction in leptin, this reflects a normal adaptation to the increased caloric need during extended wakefulness. These observations confirm that the regulation of leptin by sleep is paralleled by changes in appetite regulation.

The effect of sleep loss on leptin levels involve several mechanisms, such as an elevation in sympathetic nervous system outflow (Akerstedt and Froberg, 1979). Adipocytes receive SNS innervation which activation has broad effects on leptin synthesis, lipolysis, and macrophage infiltration into adipose tissue (Caron et al., 2018). First, activation of sympathetic nerves inhibits the transcription and secretion of leptin from adipocytes (Caron et al., 2018; Li et al., 1997; Mantzoros et al., 1996). Several studies on sleep loss reported an increase in plasma catecholamine levels in both animals and humans (Everson, 1995; Muller et al., 1993; Irwin et al., 1999; Lusardi et al., 1999). Therefore, the decrease in leptin levels upon sleep loss are

attributed, at least in part, to the inhibitory effects of enhanced SNS outflow (Rayner and Trayhurn, 2001). In other words, the activation of SNS releases catecholamines which bind to their β3-adrenergic receptors on adipocytes to reduce leptin release (Caron et al., 2018; Li et al., 1997; Trayhurn et al., 1996; Gettys et al., 1996) . Therefore, low leptin relieves the inhibition on insulin synthesis, thus leading to hyperinsulinemia. Second, SNS activation of adipocytes enhances lipolysis and the release of FFA and glycerol into the bloodstream, eventually leading to accumulation of fat in other cells and the subsequent development of insulin resistance (Caron et al., 2018). Decreased insulin sensitivity of peripheral tissues, including WAT, leads to hyperglycemia and increased lipolysis by adipose tissue that increases the risk of developing cardiovascular complications.

2. Visceral Adiposity, Insulin Resistance, and Macrophage Differentiation in White Adipose Tissue

Macrophages have gained an attention as important contributors to adipose tissue functioning. Macrophages constitute about 5% of the cells in adipose tissues of lean mice and humans (Boutens and Stienstra, 2016). Macrophages have the capacity to adapt to a changing environment, causing them to differentiate into either anti-inflammatory or pro-inflammatory phenotypes. Their role in adipose tissue is to maintain tissue homeostasis by clearing debris, participate in tissue immune surveillance, and resolve inflammation (Boutens and Stienstra, 2016; Murray and Wynn, 2011). The bone marrow-derived monocytes and yolk-sac progenitors give rise to macrophages (Russo and Lumeng, 2018). Studies in mice have shown that macrophages differentiate into either M1 or M2 depending on the stimulus (Russo and Lumeng, 2018). M1 or CAM is a classical/Pro-inflammatory macrophage that enhances hepatic steatosis

and adipogenesis, while alternative/anti-inflammatory macrophages (M2 or AAM) do the opposite (Saklayen, 2018). M1 macrophages secrete TNFα, IL-6, IL-1β, and reactive oxygen species (ROS), while M2 cells secrete IL-10, IL-4, and TGF-β (Atri et al., 2018).

Inflammation causes the infiltration of M1 macrophages into WAT (Sears and Perry, 2015). Studies show a strong association between adipocyte size and adipose tissue macrophage (ATM) accumulation in obese and non-obese humans and rodents (Kosteli et al., 2010; Ortega Martinez de Victoria et al., 2009). In addition, visceral adiposity has been shown to increase the rate of lipolysis and infiltration of ATMs into adipose tissues (Kosteli et al., 2010). Since obesity and visceral fat increase adipocyte size and, therefore, the rate of lipolysis and ATM accumulation, it could be possible that the increase in SNS activity and resultant increase in the rate of lipolysis may favor accumulation of ATMs in adipose tissues.

Despite the growing number of studies that have investigated the physiological interplay between the adipo-insular signaling pathways implicated in energy homeostasis, their role in SR when extended over more than a week (the latter being the maximal experimental period considered by studies in this area) and how they may interact to bring about metabolic and immune dysfunction remains elusive. To our knowledge, no study has looked at macrophage activity in vWAT as a potential mediator of disrupted leptin synthesis by adipocytes under chronic SR paradigms, knowing that inadequate sleep is associated with elevated levels of inflammatory cytokines that lead to inflammation. Therefore, the present study aimed at gaining insight into the effect of chronic SR on adipose tissue physiology by assessing the relative expression of M1 and M2 type macrophages in relation to intracellular leptin expression and blood glucose levels. This stems from our speculation that the same vWAT factors implicated in

obesity may also underlie the metabolic and immune complications seen in chronic SR. It is plausible that, in the presence of IR, SR may push macrophage differntiation into the inflammatory pathways in visceral WAT which may negatively affect leptin synthesis, further exacerbating pancreatic insulin release and precipitating peripheral IR. Visceral WAT was chosen because it is closely linked wih IR and cardiometaolic complications. This was to test 2 hypotheses: 1) that chronic sleep restriction (CSR) may interact with IR (or may favor a state of IR) to favor macrophage differentiation toward the inflammatory (M1) phenotype in vWAT, 2) that macrophages polarization may be associated with reduced leptin expression by adipocytes. Gaining insight into the physiological mechanisms interlinking sleep, adipocyte physiology and IR may have valuable clinical implications in the management and treatment of sleep-related metabolic disorders, such as cardiovascular diseases, obesity, and MetS.

III. Materials & Methods

1. Animals

Fifteen male C57BL/6 mice were purchased from Lebanese American University. They were aged 3 months (upon arrival) to 4 months (at the end of experiment), and had an average weight of 2.12 g during the course of the experiment. Animals were acclimatized to the housing conditions in the Science Lab. animal facility for 2 weeks before the start of the experiment and were housed in individual cages under constant environmental conditions [23 ± 2°C, 50% humidity, 12:12 light/dark cycle with lights on at 07:00 hrs [correspondimg to *Zeitgeber time* (ZT) 0)] with *ad libitum* access to food and water.

The study was approved by NDU-University Institutional Review Board (UIRB). Mice were treated in accordance with the guidelines dictated by the "Guide for the care and Use of Laboratory Animals (National Research Council of the National Academies, 2011).

2. Experimental Procedure

a. Animal Groups and Sleep Restriction

Following the acclimatization period, mice were randomly distributed into two groups: control (C, n=8) and experimental (sleep restricted, SR; n=8). Sleep restriction was carried for 18 consecutive hours (starting at ZT9) using the multiple platform method, the experimental design is shown in figure 1. The body weight, fasting blood sugar, and food consumption were measured weekly throughout the duration of the experiment (including the acclimatization period).

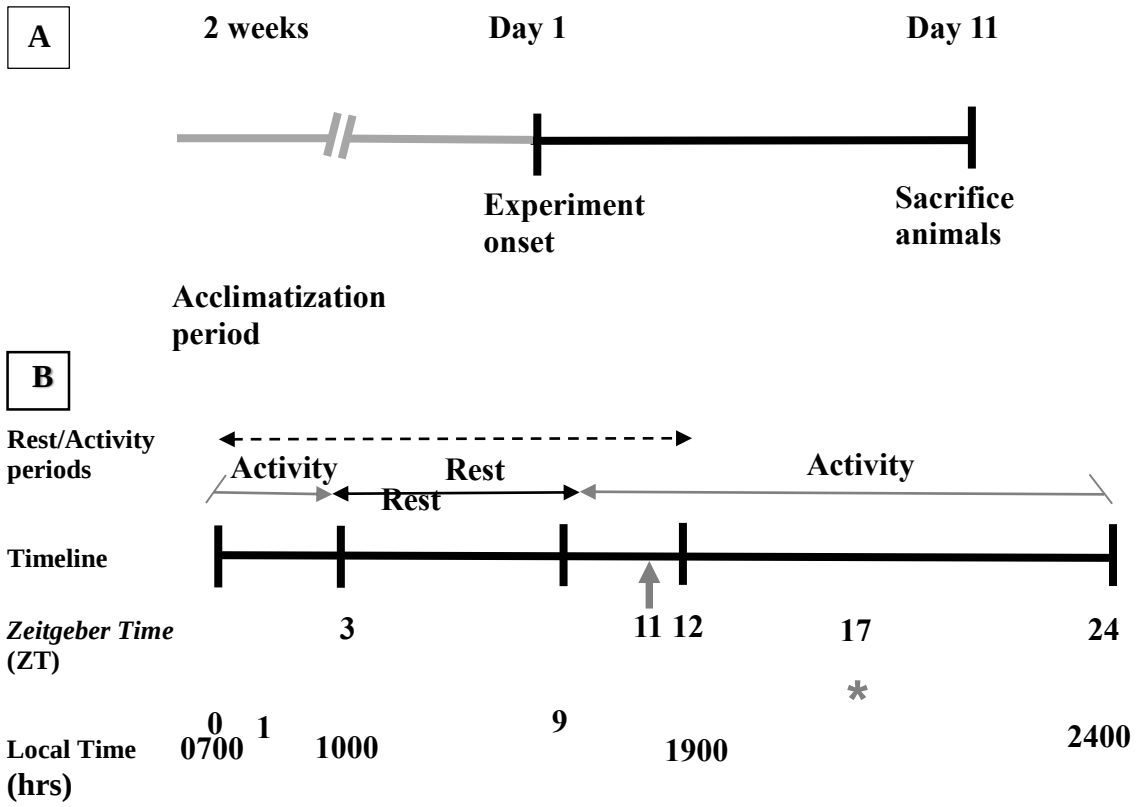

Figure 1. Schematic representation of the experimental procedure.

A) Shows both control and experimental groups that were acclimated to housing conditions for 2 weeks prior to the experimental period; C and SR mice were sacrificed on day 11 (at ZT17) at the end of the experiment. B) Shows the daily rest-activity schedule in C (horizontal dashed arrow; rest period ZT0 - ZT12) and SR groups (horizontal solid arrows; rest period, ZT9– ZT3). The asterisk denotes the time point when both C and SR mice were sacrificed. The blue arrow (B) shows the time point of BW and BS measurements that were measured weekly at the same time (ZT11) in both groups.

Abbreviations: C, control; SR, sleep restricted; BS, blood sugar; BW, body weight.

b. Tissue Collection and Histological Processing

Mice of both groups were anesthetized with isoflurane and sacrificed on week 4 around 2400 hrs (ZT17- ZT18). Prior to tissue collection, the bodies were fixed with 10% formalin through intracardial perfusion. Visceral white adipose tissues and livers were removed and weighed before storing then in the same fixative overnight at 4^{o}C. This was followed by dehydrating tissues in ascending ethanol concentrations (2 washes each) and clearing with 3 washes of xylene, with the last xylene immersion carried in a 60^{o}C oven for 30 minutes. Tissue infiltration with paraffin was done gradually, over 30-minute intervals, in the oven until a 1:1 xylene-to-paraffin ratio was attained. At this point, the tissues were transferred to pure paraffin and incubated in the oven overnight. Tissues were embedded in paraffin blocks using a histoembedder (Medite TES 99, Germany) and cut into thin 10μm sections using a microtome (Leica, Germany).

c. Immunohistochemical Analysis of Collected Tissues

Tissue sections were deparaffinized 2 times in xylene and subsequently rehydrated in decreasing concentrations of ethanol (100%, 95%, and 70%; 10 minutes each). Sections were incubated 3 times for 5 minutes in 1X TBS (50mM Tris-Cl, 150mMNaCl, pH 7.6). Heat induced epitope retrieval (HIER) was performed by placing the sections horizontally in coplin jars, containing 100 ml of Tris-EDTA buffer (10mM Tris base, 1mM EDTA solutions, 0.05% Tween 20, pH 9) at 60^{o}C in a water bath overnight. Slides were removed from antigen retriever solution and placed in sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6) for more than 20 minutes to cool at room temperature. Sections were incubated 3 times for 5 minutes in TBS. Endogenous peroxidase activity was then blocked with 0.3% H_2O_2 for 15 minutes. Sections were incubated 3 times for 5 minutes in TBS. Subsequently the sections were incubated in normal

blocking serum for one hour at room temperature to block non-specific antibody binding. This was followed by separating the slides into 3 batches and incubating each batch in one of the following primary antibody solutions (containing the specific antibody diluted in 1X TBS containing 0.3% Triton X-100): anti-leptin (ab16227, diluted 1:500), anti-CD38 (ab216343, diluted 1:1000), and anti-CD-163 (ab182422, diluted 1:20000). All tissues were incubated in the primary antibody solution overnight at 4°C.

The following day, the sections were allowed to reach room temperature before they were washed 3 times in TBS followed by incubation with horseradish peroxidase secondary antibodies (ab205718) diluted at 1:20000 in TBS with 0.3% Triton X-100 for 1h at RT. Sections were incubated 3 times for 5 minutes in TBS. Peroxidase activity was detected (15 min incubation at RT) using diaminobenzidine (DAB) substrate kit (ab64238), resulting in brown color. Afterwards, sections were rinsed in TBS for 45 minutes, and then slides were counterstained in Harris Hematoxylin for 2 minutes. After that, slides were washed with acid alcohol differentiator (HCl-ethanol solution, 0.1 % HCl in 70% ethanol) for 5 seconds to remove excess stain and to improve contrast, followed by rinsing with distilled water for 2-3 seconds. Slides were washed with bluing agent (ammonium water, 5 ml ammonium hydroxide, 1000 ml distilled water) for 2 seconds to enable fast and accurate bluing of the nucleus, then with distilled water for 2-3 seconds. Dehydration was performed by 2 times 5 min incubation in 70%, 95% and 100% ethanol followed by 2 times 5 min in xylene before coverslips were finally mounted using DPX.

For each C and SR mouse, the paraffin-embbeded mesenteric fat depot was sectioned at 10 μm and three consecutive sections of this depot were examined for tissue expression of each of leptin-, CD38-, and CD163- immunoreactivity using the light microscope (Leica DM500).

Regarding the intensity of the stain, labelled cells for either antibody showed variability in the intensity of staining in both animal groups, ranging from strong and easy to identify in some tissues while extremey weak and barely visible in some others. That is, for a number of tissue examined, some cells were weakly labelled and it was difficult to get a count of. Therefore, tissues in which weakly stained cells were difficult to depict were considered as "absent" for the signal; only cells which showed strong immunoreactivity and were clearly visible were referred to as "present" for the signal.

Adipose tissue weight was expressed as percentage of body weight. For the identification of leptin-, CD38-, and CD163-immunoreactive cells in vWAT, the respective liver from each mouse was used as a positive control in comparison. For each protein marker, data was reported as the percentage of animals within the group which showed the presence of the signal in their vWAT.

3. Statistical analysis

Data was analyzed using the Statistical Package for Social Sciences version 26 for windows (SPSS Inc., Chicago, II, USA). Independent t-test was used to compare the means of BW and BS between control and SR groups, one-way ANOVA was used to compare the means of BW and BS within groups, and paired t-test was used to compare if there is a statistical difference in means of BW and BS between the ends of the baseline and experimental periods in each group. Independent t-test was also used to compare the means of adipose tissue weight (actual and as percentage of body weight) between C and SR groups; and to compare the means of food consumption between C and SR groups. In addition, Chi-square test was used to compare protein tissue expression between C and experimental groups.

All values were reported as means ± standard deviation. P-values < 0.05 were considered significant.

IV. Results

1. **Body Weight, Blood Sugar, Adipose Tissue Weight, and Food Consumption**

Body weight, blood sugar, and adipose tissue weight were measured in control and experimental groups. The mean body weight of control was 21.42±1.72 g at the end of the experiment. In comparison to C, the mean body weight in SR was significantly lower (19.77±1.14 g, P=0.006) following 11 days of sleep restriction (table 1). As shown in table 1, when compared to baseline values, the mean BW of C did not change at the end of the experiment, while that of SR showed a significant decline of 12.9% (p=0.001) post experimentation.

Regarding blood sugar measurements, the mean BS of control mice at the end of the experiment was 105±2 mg/dl (refer to table 1). This value was significantly lower than that in SR following 11 days of restricted sleep (105±2 mg/dl vs 150±4 mg/dl , respectively, P=0.000). As reported in table 1, there was no change in the mean BS of C at the end of the experiment relative to baseline values, while the SR group showed a significant increase of 36.4% (p=0.01) in BS levels at the end of the study priod when compared to pre-experimental values.

Figure 2 is a graphical representation of BW and BS levels in both animal groups as measured throughout the course of the experiment. In C, there was no significant change in either BW or BS throughout the study (one-way ANOVA: $F_{(3,28)}$=0.036, P=0.991 and $F_{(2,21)}$=1.673, P=0.212, respectively) (figure 2.A and 2.B). SR animals; however, had a significant decrease in BW and increase in BS in the last two weeks (one-way ANOVA: $F_{(3,24)}$=22.145, P=0.000 and $F_{(2,18)}$=97.400, P=0.000,l respectively) (figure 2.A and 2.B).

Regarding adipose tissue (refer to table 2), the mean adipose tissue weight of control was 4.25±0.77 % of BW at the end of the experiment. In comparison to control, the mean adipose

tissue weight in SR was significantly lower (2.35±0.39 %, P=0.000) following 11 days of sleep restriction.

As shown in table 3, there was no significant difference in the mean weekly food consumption between C and SR mice (31.41±0.11 g vs 37.82±7.66 g , respectively; P=0.145). The weekly food consumption is also graphically shown in figure 3.

2. Leptin, CD38, and CD163 Immunoreactivity

The liver was used as positive control to detect cellular immunoreactivity to anti-leptin, anti-CD38, and anti-CD163 in both C group (fig.4 A & B) and SR groups (fig.5 A-D). Qualitative analysis of the expression of leptin in vWAT revealed the presence of leptin-labelled cells in control group (fig.4, C, D) and their absence in SR group (fig.4.E & F). A significant difference was observed in anti-leptin immunoreactivity between C and SR group, in which leptin present in each individual mouse in C group and absent in all mice of SR group (refer to table 4).

Qualitative analysis of the expression of CD38, a marker of M1 macrophages, in vWAT revealed its presence in the SR (fig. 5.G), but not in the control group (fig. 5.E). As shown in table 4, a significant difference was also observed in anti-CD38 immunoreactivity between C and SR group, in which M1 was absent in each individual mouse in the C group, but present in all mice of SR group.

As for M2 phenoptype, qualitative analysis of the expression of CD163, a marker of M2 macrophage, in vWAT revealed its overall presence in C group (fig.5.F) but its absence in SR group (fig.5.H). A significant difference was observed in anti-CD-163 immunoreactivity between C and SR group, in which M2 present in each individual mouse in C group, but absent in all mice of SR group (as reported in table 4).

Table 1. Table showing mean body weight and blood sugar of control and experimental mice at the end of each of baseline and experimental periods.

	Body Weight (g)			Blood Sugar (mg/dl)		
Animals	**Before**	**After**	**[2]P-value**	**Before**	**After**	**[2]P-value**
C	21.46±1.52	21.42±1.72	0.732	109±5	105±2	0.159
SR	22.71±0.70	19.77±1.14	0.001	110±7	150±4	0.010
[1]P-value		0.006			0.000	

The reported values show means ± standard deviation; $p<0.05$ denotes statistical significance.

[1]Independent sample t-test.

[2]Paired t-test.

Abbreviations: C, control; SR, sleep restriction.

Table 2. Measurement of adipose tissue weight at the end of the experiment in control and SR mice.

Animals	Adipose Tissue Weight (g)	Adipose Tissue Weight (% of BW)
C	0.90±0.13	4.25±0.77
SR	0.44±0.08*	2.35±0.39*
P-value	0.000	0.000

The reported values represent mean ± standard deviation; $P<0.05$ denotes statistical difference between the means.

Abbreviations: C, control; SR, sleep restriction; BW, body weight.

Table 3. table showing mean weekly food consumption of control and experimental mice throughout the experiment.

Animals	Food Consumption (g)
C	31.41±0.11
SR	37.82±7.66
P-value	0.145

The reported values show means ± standard deviation; $p<0.05$ denotes statistical significance.

Abbreviations: C, control; SR, sleep restriction.

Table 4. Table showing the presence or absence of each of letpin, CD38-, and CD163-immunoreactive cells in white adipose tissue of control and sleep restricted mice.

Protein Signal	C	SR	P-value
% Leptin **Yes** **NO**	100 (8) 0 (80)	0 (7) 100 (7)	0.000
% CD-38 **Yes** **No**	0 (8) 100 (8)	100 (7) 0 (7)	0.000
% CD-163 **Yes** **No**	100 (8) 0 (8)	0 (7) 100 (8)	0.000

Chi-square test was used to compare the presence of protein-labeled cells between control and experimental groups.

Abbreviations: C, control; SR, sleep restriction.

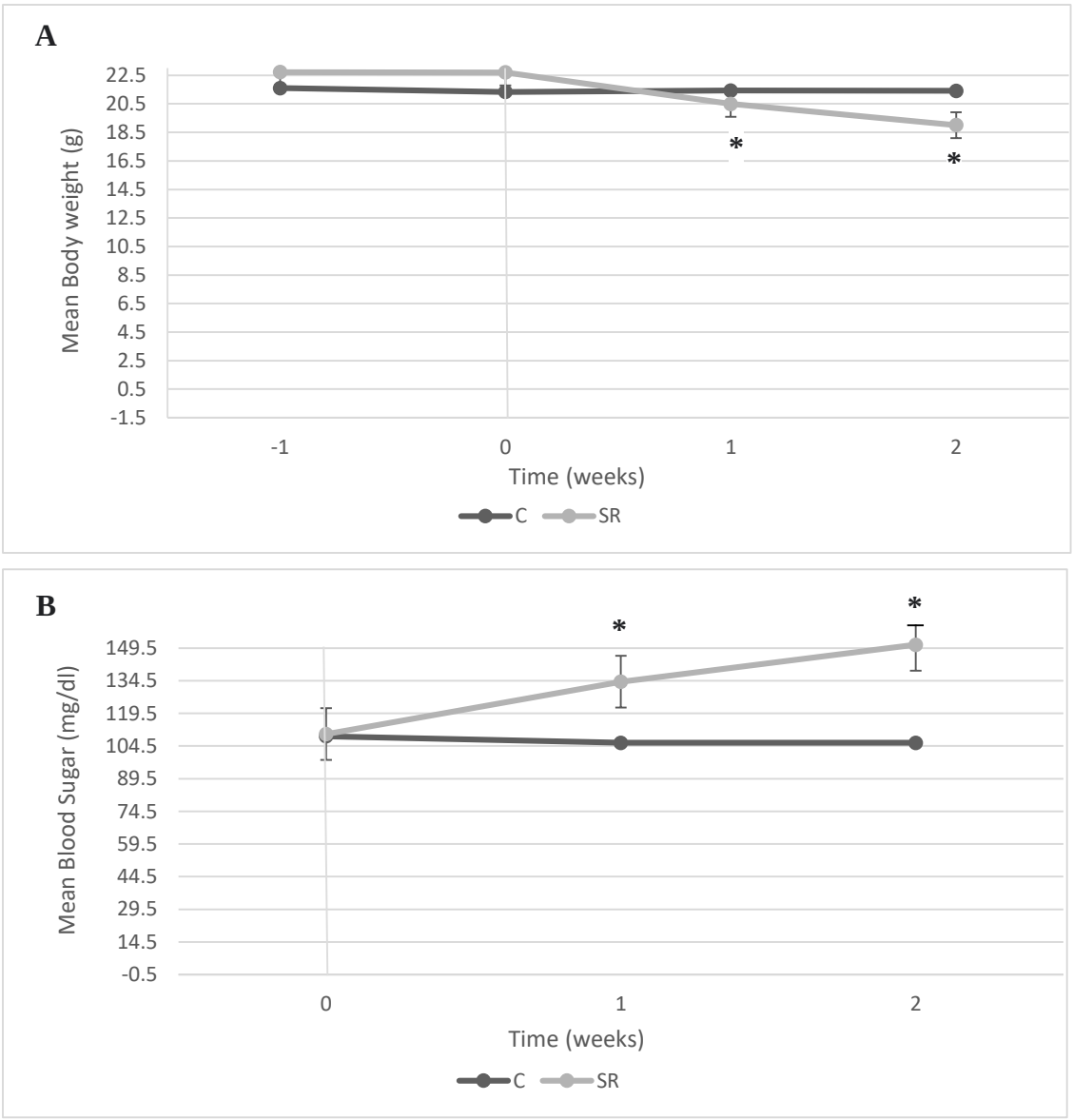

Figure 2. Graph showing variations of body weight and blood sugar in mice. A) the body weight in control mice remained the same throughout the course of the experiment, while the SR mice showed a significant decline in mean body weight. B) The blood sugar in control the remained same throughout the course of the experiment, while SR mice showed a significant increase in blood sugar.

Values show means±SD

*P<0.05 shows significant difference.

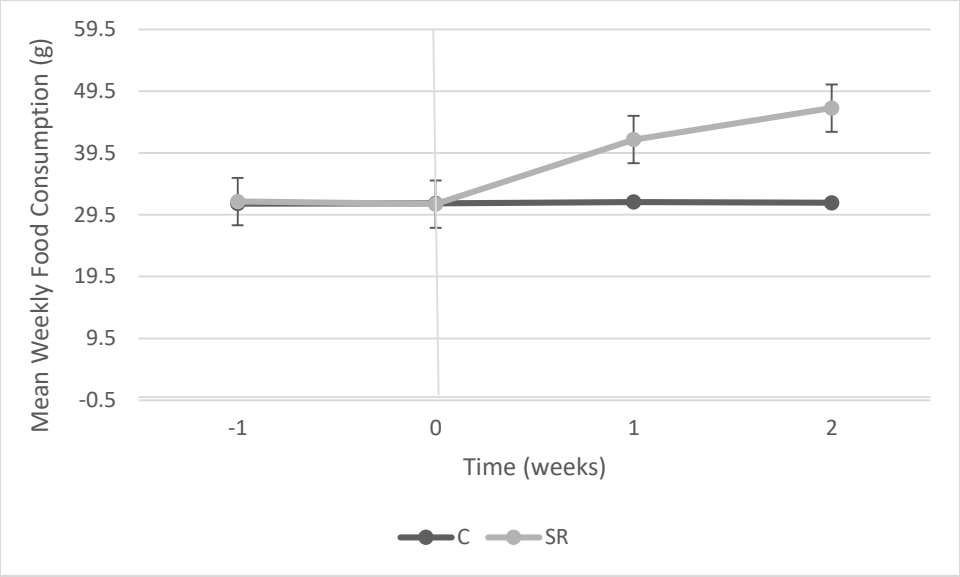

Figure 3. Graph showing variations of weekly food consumption in mice. The weekly food consumption in control mice remained the same throughout the course of the experiment, while the SR mice showed an elevation in mean weekly food consumption.

Values show means±SD

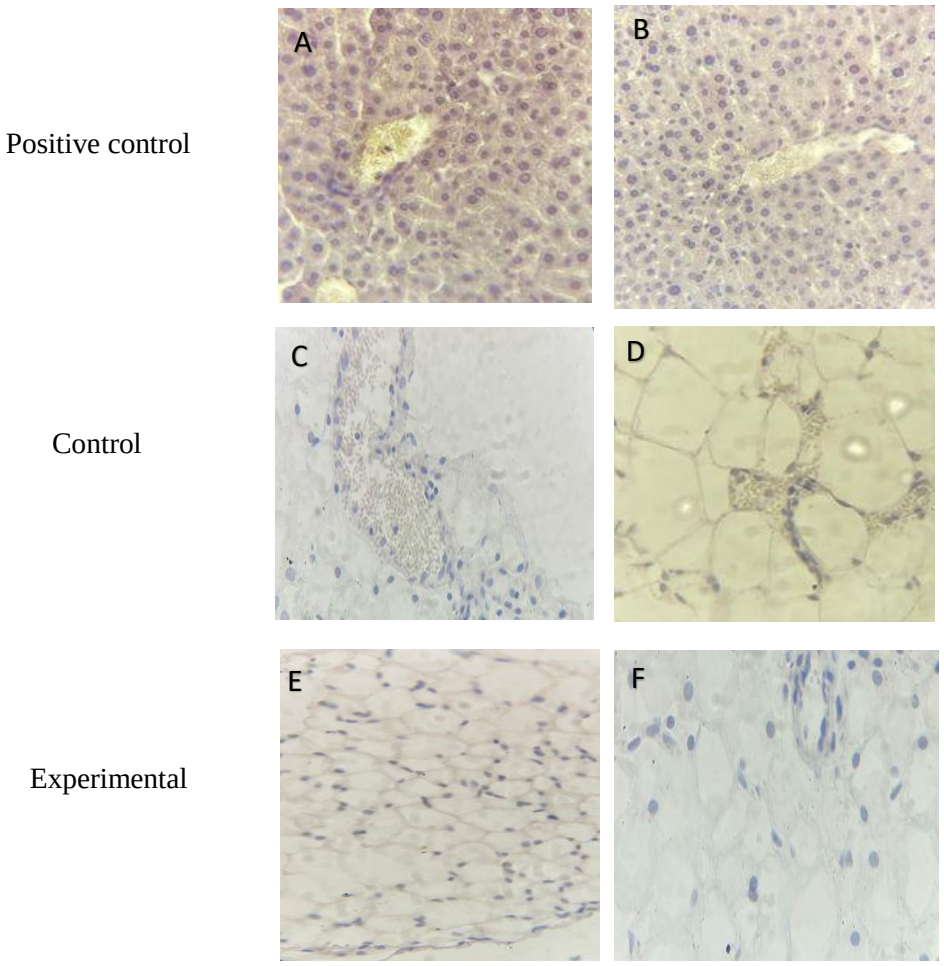

Figure 4. Light photomicrographs showing the localization of anti-leptin immunoreactivity in adipose tissue and liver in controlled and SR mice. A) positive control of liver showing anti-leptin localization in controlled mice and B) positive control of liver showing anti-leptin localization in sleep-restricted mice.**; (**C & D) showing anti-leptin localization in adipose tissues in cotrolled mice; **(**E & F) showing absence of anti-leptin localization in adipose tissues in sleep-restricted mice. All photomicrographs were captured at 40X.

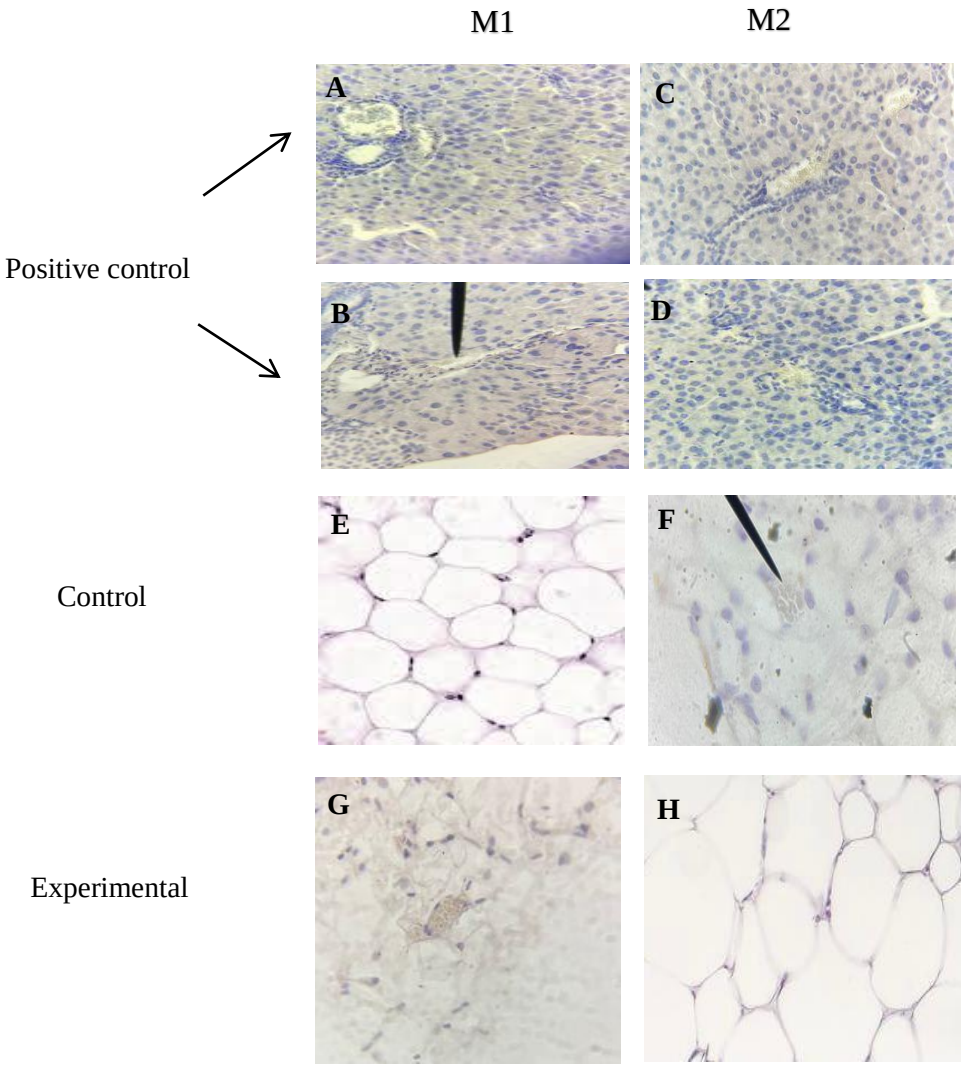

Figure 5. Light photomicrographs showing the localization of anti-CD38 and anti-CD163 immunoreactivity in adipose tissue and liver in control and SR mice. (A & B) positive control (liver tissue) showing anti-CD38 and anti-CD163 localization in control mice, respectively; (C & D) positive control (liver tissue) showing the localization of anti-CD38 and anti-CD163 in sleep-restricted mice, respectively**; (**E & F) depict the vWAT that show the absence of anti-CD38 and the presence of anti-CD163 immunoreactive cells in adipose tissues in control mice, respectively; **(**G & H) depict the vWAT that show the presence of anti-CD38 and the absence of anti-CD163 immunoreactive cells in adipose tissues in sleep-restricted mice, respectively. All photomicrographs were captured at 40X.

V. Discussion and Conclusion

This study is the first to investigate the link between leptin expression in vWAT and possible skeweness of macrophages toward M1 (inflammatory) phenotype following chronic sleep restriction. To our knowledge, most of the studies conducted in this area considered acute SR paradigms an did not investigate the effect of sleep restriction in general, and chronic disruption in particular, on macrophage differentiation and leptin expression in vWAT. In the present study, animals were restricted from sleeping for 18 consecutive hrs, beginning day 1 after the acclimatization period, in order to determine whether BS, BW, adipose tissue weight, leptin, M1, and M2 expression were significantly modified after 11 days of sleep restriction. At the end of the experiment, SR mice exhibited a higher BS (by 36 %) and vWAT weight (by 45 %), but had lower BW (by 13%), than control. Unlike control, SR showed only M1, but not M2 or leptin, expression in vWAT.

Consequently, subjecting animals to weeks of sleep restriction appeared to result in elevated blood glucose as shown in the present study. Noteworthy, the level of circulating insulin was not measured to further assess the possibility of insulin insensitivity of tissues that might have characterized the SR group. However, the reported rises in fasting blood sugar in the SR group may be explained by the possible disruption in insulin secretion and/or tissue insensitivity to insulin. Shigiyama et al. (2018) reported that 6 h of sleep deprivation in male C57BL/6J mice significantly resulted in higher plasma glucose level than that of control, thereby suffering from impaired glucose tolerance. In addition to hyperglycemia, an elevated hepatic glucose production was observed which indicated a state of hepatic insulin resistance. Another study in humans also showed that individuals who slept 4 hours per night for one week exhibited impaired glucose tolerance (Spiegel et al., 1999; Buxton et al., 2010). According to related studies, CSR activates SNS outflow to vWAT thus leading to a reduction in leptin synthesis and secretion; a decrease in

circulating leptin subsequently relieves the inhibition on insulin synthesis, thus leading to hyperinsulinemia (Rayner and Trayhurn, 2001). Comparatively, in this study the absence of leptin expression in vWAT of SR may possibly link to the respective observations on hyperglycemia (and potential impact on insulin secretion) that are documented in the scientific literature (Chaput et al., 2007; Spiegel et al., 1999; Buxton et al., 2010; Caron et al., 2018). In the absence of quantitative data; however, such conclusions cannot be drawn and further quantitative-type of investigations are required.

Regarding body weight changes, sleep restriction for about 11 days revealed significant alterations in the mean BW between C and SR groups at the end of the experiment. A significant decrease in the mean BW was also observed in the SR group before and after the experimental period, but not in control. Furthermuore, a significant decline of 44.65 % was observed in the mean adipose tissue weight in SR group relative to control. The unexpected weight loss in SR mice might be possibly due to the abnormal metabolic milieu and associated physiological complications, as manifest by the elevated blood sugar, which might predispose to a prediatbetic or diabetic state with prolonged sleep disturbance. The metabolic disturbances associated with sleep restriction have been explained by many studies. For example, sleep restriction is associated with sympathetic activation of adipocytes which enhance lipolysis and, therefore, the release of free fatty acids and glycerol into the bloodstream, thus leading to dyslipidemia and subsequent development of IR in peripheral tissues; IR, in parallel, causes or precipitatres hyperglycemia (Caron et al., 2018; Xu et al., 2009; Londos et al., 1999; Arner, 2002). It should be noted; however, that the exact cause-effect relationship between IR and abnormal lipid and carbohydrate metabolism seen following sleep restricrion is still unclear. Other studies reported by researchers stated that sleep restriction causes insulin resistance and impaired glucose

tolerance (Van Cauter, 2011; Van Cauter et al., 2008; Wilcox, 2005). Therefore, it is plausible to think that the alteration in glucose homeostasis following CSR in this present research may be attributed to the possible development of tissue resistance to insulin and that, if prolonged, CSR can eventually lead to hyperglycemia and diabetes-like complications. Despite the absence of leptin expression in SR group, paradoxically both BW and adipose tissue weight were decreased in this study. This contrasts other studies which reported that the absence of leptin caused an increase in body weight and adiposity (Paz-Filho et al., 2012; Ravussin et al., 1997). Thus, leptin dysregulation seen in SR animals in this study cannot explain the concomitant decrease in BW loss and adipose tissue weight in this group. Rather, the abnormal metabolic milieu created by insulin resistance, namely the disrupted glucose homeostasis and increased fat breakdown by peripheral tissues, may contribute, in part, to the observed weight loss in SR animals. Furtheremore, the experimental mice had 20.4 % higher consumption of food than that of control during the experimental period even though it was not statistically significant. As well, the experimental mice that ate more showed significant decrease in BW at the end of the experimental period. This observation could be supported by Rechtschaffen et al. 1983] who stated that sleep deprivation in rats resulted in a greater weight loss in sleep-deprived compared to control animals even though the former ate more. They reported that the weight loss in deprived rats could be attributed to an increased ratio of catabolism to anabolism and an impaired fluid retention. Moreover, Kant et al. (1984) reported that 72 h of sleep deprivation in humans caused an increase in urea excretion (an end product of protein catabolism) that could be caused by high consumption of proteins during the study. In the same study, a decrease in urinary glucose levels were observed in sleep-deprived humans. Another study conducted by Lamon et al. (2021) showed that a single night of total sleep deprivation in healthy adults was sufficient to

induce anabolic resistance by reducing postprandial muscle protein synthesis and a procatabolic environment. Thus, the unexpected weight loss in this present research might be possibly attributed to dehydration and increased catabolism of proteins.

Leptin, often referred to as the satiety hormone, is primarily secreted by WAT, with the main role being to balance energy expenditure, and caloric intake of the body. Qualitative analysis of the expression of leptin in vWAT revealed its overall presence in C group (see fig.3.C & D) and its absence in SR group (see fig.3.E & F). These results show that SR may possibly negatively affect leptin expression in vWAT, at least at the time point that were selected in this study. Therefore, SR may possibly have an effect over the expression of leptin in vWAT. Some studies reported that the increase in the sympathetic nervous system activity elevates urinary and plasma catecholamine levels which, in turn, decrease leptin release in both sustained sleep-deprived rats and partial sleep-deprived human, respectively (Everson, 1995; Müller et al., 1993; Rayner and Trayhurn, 2001; Spiegel et al., 2004). Other scientific studies revealed that acute and chronic sleep deprivation in healthy men dropped leptin levels in the blood (Mullington et al., 2003; Spiegel et al., 2004). However, in this present study, CSR resulted in total absence of leptin expression in vWAT. Furthermore, the absence of leptin expression in SR mice could be attributed to a change in the circadian rhythm of leptin from chronic forced arousals during the supposedly rest period (light phase).

Noteworthy, in order to maintain a normal adipose tissue function, an anti-inflammatory milieu provided by M2 macrophages is indispensible. In this study, CSR seemed to disrupt the normal function of adipose tissue by probably favoring pro-inflammatory phenotype M1 over anti-inflammatory phenotype M2 (seen in SR mice; figure 3). ATMs, derived from circulating monocytes or renewal of tissue-resident macrophages (yolk-sac progenitors), offer this anti-

inflammatory milieu through M2 phenotype expression that was seen in C group and not in SR group in this research (Hassnain Waqas et al., 2017; Liang et al., 2020). In other words, CSR might drive the expression of M1 phenotype which indicates inflammatory responses in vWAT of SR mice, whereas its absence in non-sleep deprived mice indicates no inflammatory responses in these tissues. Moreover, pro-inflammatory macrophage (M1) were previously reported in SR (Castoldi et al., 2016), so it is may be the macrophages in this present study has shifted toward M1 phenotype over M2 phenotype in SR group. Thus, CSR may have resulted in a negative effect on CD163 expression; ; however, with the limitations of the study this would require further investigation.

When leptin is expressed in vWAT in non-sleep restricted group the macrophage phenotype is M2, while it is M1 in SR group in the absence of leptin. These results might elucidate that expression of M2 phenotype and the absence of the expression of M1 phenotype in non-sleep restricted mice may play a role in maintaining normal vWAT function in the absence of inflammation and, thus, leptin expression in adipocytes remain intact. In contrast, the expression of M1 phenotypes and the absence of the expression of M2 phenotypes in sleep-restricted mice that has been caused by inflammation might disrupt vWAT function and block leptin expression in adipocytes. Thus, these interpretations possibly give an insight that macrophage phenotype expression or differentiation pathway might possibly have an effect on leptin expression in vWAT.

In summary, chronic sleep restriction appeared to result in elevated fasting blood sugar which may be explained by the possible disruption of insulin secretion and/or tissue sensitivity, in addition to the absence of leptin expression in vWAT and the reduction in BW and adipose tissue weight. The latter may be attributed to the abnormal metabolic milieu (i.e. elevated blood

glucose and the possible switch of peripheral tissues from glucose to fatty acid metabolism), as well as the effects on urea excretion and protein catabolism. In addition, it is possible that CSR, through modulating the WAT microenvironment, may affect the differentiation pathways of infiltrating macrophages, probably favoring pro-inflammatory phenotype M1 over anti-inflammatory phenotype M2 along with the absence of leptin expression. Thus, it is possible that macrophage polarization in vWAT puts an extra inhibitory effect on leptin, in addition to that exerted by SNS.

The reported glycemic rise in the SR group that showed no leptin signal in vWAT may be explained by the potential impact of inadequate sleep on insulin secretion and/or tissue insensitivity. In the latter case, one would expect the development of a state of hyperinsulinemia that would be attributed, at least in part, to the possible loss of the negative feedback control of leptin on insulin secretion. Such interpretations; however, cannot be drawn from this study since the levels of insulin in the blood were not measured and,therefore, the answers to these speculations need further quantitative type of investigations. Despite the results provided in this research that stated a possible skewness of macrophages into M1 phenotypes which, in turn, might exert an extra inhibitory effect on leptin expression in vWAT, the detailed role of macrophage polarization on leptin expression and insulin resistance in chronic sleep restriction require further investigations.

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