Chapter-6

DISCUSSION

6.1 To study the effect of Sitagliptin on metabolic syndrome, fatty liver and hepatic oxidative stress in high-fat diet induced animal model of obesity (Objective I).

The consumption of a hypercaloric diet along with a sedentary lifestyle precipitates obesity and metabolic complications, including insulin resistance, hepatic steatosis, and cardiovascular disorders in humans [Bays et al. 2013]. The intake of high-fat and fructose diet for prolonged periods is correlated with the induction of obesity and associated metabolic syndrome in preclinical models [Hariri et al. 2010; Saravanan et al. 2014]. Fat enriched diets induce obesity in humans as well as in animals. A positive co-relationship is established between the amount of fat and body weight gain in both rats and mice [Ghibaudi et al. 2002; Hira et al. 2017]. The earlier studies have also shown that the weight gained in animals is dependent on the proportion of fat consumed by them [Hariri et al. 2010]. Literature provides evidence of the induction of obesity with a high-fat diet having more than 30% of fat content [Hill et al. 2000]. The high-fat diet induced animal model of obesity is a simple model, which closely mimics the obesity phenotype in humans. In the present investigation, animal models of obesity and metabolic complications were developed by feeding them the HFFW diet for sixteen weeks, where the animals developed significantly increased body weight, impaired glucose tolerance, hyperinsulinemia, insulin resistance, and dyslipidemia in Swiss albino mice. Moreover, metabolic syndrome parameters such as serum free fatty acids, uric acid, ALT, AST were altered in HFFW-fed mice, along with impaired leptin

and adiponectin levels. Hence, from these results, it can be inferred that this experimental animal model represents all the pathological features mimicking obesity and associated metabolic syndrome complications. Our findings reiterate earlier reports on the induction of obesity and metabolic syndrome in animal models by chronic supplementation with a high-fat diet [Pang et al. 2008; Morakinyo et al. 2015].

High-fat diets are associated with weight gain [Bettaieb et al. 2016]. Similarly, we observed a significant enhancement in the body weight of the Swiss albino mice by 1±0.5 folds between control and HFFW-fed mice, after eight weeks. When the increased calorie intake overrides the energy expenditure, positive energy gets deposited in the fat stores in the body and is reflected as increased fat content [Hill et al. 2012; Cui et al. 2015; Morakinyo et al. 2015; Zhou et al. 2015]. As increased adiposity influences glucose metabolism and insulin sensitivity, exacerbating insulin resistance [Kahn et al. 2006; Wang et al. 2012; Zhou et al. 2015; Jung et al. 2017], here also, we observed a significant increase in the FBG levels by 1.5±0.5 fold and impaired glucose tolerance in the HFFW-fed Swiss albino mice at the end of eight weeks [Lozano et al. 2016]. Therefore, we initiated the drug administration from the ninth week onwards till the end of the sixteenth week in the treatment groups.

At the end of sixteen weeks, the energy-rich fatty diet elicited a significant increase in the body weight of the Swiss albino mice in the metabolic syndrome group, along with a significant increase in the FBG levels. Some studies have shown that body weight is not affected by sitagliptin [Kim et al. 2008; Sharma et al. 2012]. However, in contrast to this, others have demonstrated a considerable decrease in body weight with

sitagliptin in both the clinical [Soliman et al. 2013; Katsuyama et al. 2015] and preclinical studies [Goldsmith et al. 2015; Karabulut et al. 2015; Magdy et al. 2017]. Although there is a substantial debate on the regulation of body weight with sitagliptin, our results support the body weight reduction at higher doses of sitagliptin, i.e., 20mg/kg and 30mg/kg, with a reduction potential of 16.3% and 16.4%, respectively, similar to that of metformin. The up-regulated hepatic cannabinoid-1 and GLP-1 receptor mRNA expression, might have contributed in decreasing weight gain according to Coskun et al. [Coskun et al. 2017]. FBG levels were reduced significantly at all three doses of sitagliptin.

We also evaluated the cumulative food intake at the end of the experimental study by evaluating the total food consumed every three days throughout the entire sixteen weeks. There was a significant increase in the cumulative food intake in the metabolic syndrome group, with respect to the control group, similar to the earlier studies [Cui et al. 2015; Zhong et al. 2017; Wang et al. 2019]. However, no difference was observed among the treatment groups, which indicated that body weight reduction with sitagliptin was independent of the food intake behaviour, similar to the previous studies [Tahara et al. 2011]. In contrast to this, Kushwaha et al. reported that sitagliptin decreased body weight by decreasing the food intake behaviour [Khushwaha et al. 2019]. To determine the fat deposition in the liver and visceral adipose locations, liver weight index and visceral fat index were measured at the end of the study. It was found that both the organs had significantly increased fat depositions in the metabolic syndrome group, in contrast to the control group. Earlier studies have also observed

increased fat deposits in adipose and non-adipose locations with the high-fat diet [Tahara et al. 2011; Magdy et al. 2017; Wang et al. 2019]. However, at the higher doses of sitagliptin, i.e. 20mg/kg and 30mg/kg, both the liver weight index and visceral fat index were significantly reduced. Thus, suggesting that sitagliptin reduced fat deposits in the liver and visceral adipose locations, similar to Magdy et al. [Magdy et al. 2017], without affecting the food intake.

Insulin resistance is a common denominator which gets induced in obesity and associated metabolic complications. The risk of DM is high in obesity as it is often accompanied by excess body weight, besides genetic predisposition and age [Pedersen 2013]. The increased fat depots in the adipose tissue induce resistance to insulin-mediated mechanisms [Cooke et al. 2016]. Insulin resistance develops in the peripheral organs besides adipose tissue, such as liver, skeletal muscle, pancreas, etc. due to the ectopic lipid deposition [Korenblat et al. 2008; Lê et al. 2009; Ouchi et al. 2011; Zabielski et al. 2018]. The low-grade inflammation developed in the adipose tissues due to persistent fat accumulation prompts the saturated adipocytes to release the pro-inflammatory mediators, inducing insulin resistance. Expansion of the adipose tissue is followed by the infiltration of the immune cells around the necrotizing adipocytes for removal of the cellular debris, thereby developing persistent immune responses, which also precipitates insulin resistance. The free fatty acids released from the inflamed adipose tissue activates the IKK-β, PKR, JNK signaling cascades intracellularly, which also drives the inflammatory responses. These signaling pathways inhibit the insulin

signaling pathway exacerbating insulin resistance in obesity [Aguirre et al. 2002; Nishimura et al. 2009; Nakamura et al. 2010].

Henceforth, FSI and HOMA-IR estimation performed at the end of the HFFW diet supplementation revealed a significant increase in their levels in the MS group, relative to the control group [Zhou et al. 2015; Jung et al. 2017]. However, in the treatment groups, the FSI level was observed to be significantly reduced in a dose-dependent manner, whereas reduction in HOMA-IR was similar at higher doses of sitagliptin, i.e., 20mg/kg and 30mg/kg, similar to metformin. To assess glucose tolerance and insulin tolerance in the Swiss albino mice, OGTT and ITT were performed at the end of the study. There was a significant decrease in glucose tolerance and insulin sensitivity in the HFFW-fed MS group, as observed from the impaired blood glucose levels at different time points from the OGTT and ITT. Also, we evaluated the AUG and found a significant increase in MS group, relative to the control group [Morakinyo et al. 2015; Nagai et al. 2016; Balakumar et al. 2018; Hu et al. 2020]. However, there was a significant improvement in the glucose and insulin tolerance tests in the sitagliptin treated groups at 20mg/kg and 30mg/kg. Our findings corroborate with the earlier studies of Giannocco et al., and Karabulut et al., who suggested that improved insulin sensitivity with sitagliptin is attributed to glucose-dependent inhibition of DPP-IV, GLUT-4 upregulation in heart and skeletal muscles, and improvement in islet cell mass function [Giannocco et al. 2013; Karabulut et al. 2015]. Sitagliptin inhibits DPP-IV in a glucose-dependent manner, thereby preventing the breakdown of incretins, and eventually mediating the insulin release in response to prolonged GLP-1

levels in the circulation. Moreover, improved glucose tolerance with sitagliptin might also be attributed to the decreased glucagon production and reduced hepatic glucose production [Duez et al. 2009]

Due to the prolonged high-fat diet consumption, adipocytes get saturated with the increased calorie supplies, inducing insulin resistance in the adipocytes [Garg 2011]. This increases the lipolysis in the adipocytes leading to increased intracellular and systemic free fatty acids levels, which migrate to the liver. Also, free fatty acids are supplied from the diet as the chylomicron remnants. The clearance of free fatty acids also decreases in obesity due to high-fat diet intake. This together increases the pool of free fatty acids in the circulation, which moves to the liver and increases the *de novo* lipogenesis [Panchal et al. 2010; Cooke et al. 2016]. Similarly, we also noticed a significant increase in the free fatty acids level in the MS group after sixteen weeks of HFFW diet [Yu et al. 2005; Pang et al. 2008; Cho et al. 2010], which were found to be reduced significantly in the treatment groups. Decreased insulin resistance in the treatment groups, as observed from the decreased HOMA-IR and improved OGTT and ITT, would have mediated the lipolysis in the adipose tissues, directing the energy mobilization and decreasing the circulatory free fatty acids levels [Tahara et al. 2011].

During significantly raised free fatty acids levels, they get re-esterified in the hepatocytes as triglycerides, which is further released in the circulation as VLDL. In case of insufficient amounts of lipid, the apolipoprotein B100 particles get degraded, which are required for VLDL secretion and regulation of LDL uptake by the LDL receptor. Therefore, VLDL, LDL, and HDL production and release depend on the level

of lipids present in the liver. When there is increased hepatic lipid content, VLDL, LDL increases, and HDL decreases, indicating dyslipidemia. Decreased glucose tolerance and hyperinsulinemia are also related to the increased lipid disorder [Blüher 2009; Bayly 2014; Taher et al. 2014]. In the present study, sixteen weeks of HFFW induced dyslipidemia, as evidenced by significantly increased TC, TG, LDL-C, VLDL-C, and decreased HDL-C in the MS group [Morakinyo et al. 2015; Dong et al. 2016; Magdy et al. 2017]. Also, an increase in the hepatic TC and TG levels were observed after extended high-fat diet supplementation in the MS group, which reflected an increased lipid deposition in the liver [Santiago et al. 2012; Choi et al. 2016; Randy et al. 2016; Jeong et al. 2017]. The hepatic lipid accumulation in the MS group was also evident from the H&E stained histopathological sections, which revealed a higher number of lipid droplets compared with the control group. This was probably due to the increased dietary load of free fatty acids from the HFFW diet [Lieber et al. 2004; Fakhoury-Sayegh et al. 2015]. Moreover, previous studies also indicate the reduced degeneration of the hepatocytes in the liver of neonatal diabetic rats with sitagliptin [Coskun et al. 2017]. *De novo* lipogenesis is increased by almost three-fold in non-alcoholic fatty liver conditions [Ameer et al. 2014]. It is interesting that since *de novo* lipogenesis is stimulated by both, i.e., insulin through sterol regulatory element binding-protein 1c and glucose through carbohydrate response element-binding protein, and therefore it is up-regulated in states of both hyperinsulinemia and fat-enriched diets [Roden 2006]. However, sitagliptin significantly reversed the serum lipid profile and the hepatic fat deposits at the higher doses. Besides hyperglycemia, hyperlipidemia is also related to the increased insulin secretion from the pancreas, due to the reduced sensitivity of

insulin receptors. However, with the improved lipid profile in the treatment groups, insulin sensitivity was also increased.

Due to the increased metabolic load on the liver, mitochondria get overwhelmed precipitating oxidative stress [Rajwal et al. 2017]. An imbalance occurs between reactive oxygen species generation and antioxidant status of cells [Houstis et al. 2006; Pintana et al. 2013]. The increased free fatty acids flux in obesity overwhelms the mitochondrial capacity inducing the oxidative stress. MDA reflects the lipid peroxidation of the cell membrane by the free radicals, whereas SOD and GSH are the intracellular enzymatic and non-enzymatic antioxidant defences, respectively, protecting from the reactive oxygen species-induced damage [Pessayre et al. 2001]. Similarly, in the present study, we also observed an increase in the hepatic lipid peroxidation and decrease in the antioxidant enzymes SOD and GSH in the MS group at the end of the HFFW diet [Randy et al. 2016; Braud et al. 2017]. Reduced SOD activity could be responsible for the damaging effects of the free radicals, along with their contribution in increasing the superoxide radicals. The superoxide radicals reduce the glutathione peroxidase activity, which is responsible for the catalysis of the lipid peroxides at the expense of GSH, contributing to the oxidative stress [Blum et al. 1985]. However, the administration of sitagliptin significantly restored the level of antioxidant enzymes, mitigating the hepatic oxidative stress compared with the HFFW-fed group. Decreased free fatty acids load on the liver, due to the improved insulin sensitivity would have also contributed in improving the oxidative stress in the liver via decreasing the lipid peroxidation and up-regulating the SOD and GSH levels in the obese mice.

ALT and AST enzymes are well-documented indicators of liver health, and their elevated levels signify the hepatocellular injury [Poudyal et al. 2010]. During the increased metabolic states of high-fat diet feeding in animal models, the plasma levels of ALT and AST are found to be elevated [Zheng et al. 2008; Kang et al. 2016]. As expected, here also, we observed a significant elevation in the levels of ALT and AST in the MS group after sixteen weeks of HFFW diet, relative to the control group [Randy et al. 2016]. However, their levels were improved significantly at higher doses in the treatment groups [Magdy et al. 2017]. Ameliorated ALT and AST levels indicate the healthy status of the liver in the treatment groups, thus suggesting reduced hepatic stress.

In states of persistent positive energy supply, the adipocytes are facilitated to store the excess calories, which induces morphological and functional changes in the adipocytes. Adipose tissue remodelling occurs, causing hyperplasia or hypertrophy, or both, predisposing them towards a pro-inflammatory state [Choi et al. 2014]. The chronic inflammatory state affects the insulin sensitivity of the adipocytes and also the release of the adipokines. As adipokines exert a significant role in regulating the different cellular functions related to energy intake, satiety, insulin sensitivity, lipid homeostasis, inflammation, fat distribution, etc., and therefore disturbed adipokine profile also contributes in the precipitation of metabolic complications [Vázquez-Vela et al. 2008; Jo et al. 2009; Unamuno et al. 2018]. Leptin levels were significantly increased, while the levels of adiponectin were reduced in the MS group after a fat-enriched diet [Cui et al. 2015; Morakinyo et al. 2015]. However, we observed

significantly improved levels of leptin and adiponectin in the treatment groups similar to the previous studies [Lamont et al. 2008; Tahara et al. 2011; Hibuse et al. 2014; Wu et al. 2016], where Wu et al, suggested increased leptin and adiponectin levels is linked with the regulation of phosphatidylcholine 3-kinase (PI3K)-protein kinase B (PKB/ AKT) pathway. Sitagliptin mediated improved adipocytokine levels would have also contributed in the improved insulin sensitivity in the Swiss albino mice in the treatment groups.

Elevated uric acid levels are linked as a risk factor for the development of obesity, insulin resistance, metabolic syndrome, and fatty liver by adversely affecting the synthesis and oxidation of fatty acids, accumulation of triglycerides, and also the synthesis of adiponectin. It inhibits AMPK and reduces the β-oxidation of fatty acids [Kanbay et al. 2016]. Earlier studies on diet pattern indicate hyperuricemia after high-fat and fructose diet intake. There is an increase in the activity of the enzyme adenosine monophosphate (AMP) deaminase that increases the degradation of nucleotides to uric acid. Fructose also inhibits its excretion inducing hyperuricemia [Ekpenyong et al. 2015]. Postulated mechanisms for uric acid-induced metabolic syndrome includes: inhibition of endothelial nitric oxide bioavailability, activation of the renin-angiotensin system, or direct actions on vascular smooth muscle cells by stimulating the expression of inflammatory mediators [Tran et al. 2009]. Lowering of uric acid reduces diet-induced fatty liver and associated metabolic complications in the animal models [Kanbay et al. 2016]. Similarly, in the present study also, we observed increased uric acid levels in the MS group in comparison with the control group [Kim et al. 2011].

However, chronic administration of sitagliptin was able to suppress the increased uric acid levels significantly.

GLP-1 is responsible for the post-prandial insulin increase by 50-70%. Due to the breakdown of active incretins (insulinotropic) to their inactive (non-insulinotropic) forms by the DPP-IV enzyme through the N-terminal dipeptide cleavage, the release of insulin and glucagon from the pancreatic cells is inhibited mediating enhanced glucose levels. GLP-1 ameliorates hyperglycemia, glucose tolerance, and insulin resistance [Migoya et al. 2010; Picatoste et al. 2013]. Sitagliptin mediates glucose-dependent glycaemic control through inhibiting the DPP-IV enzyme, without inducing hypoglycemia. After sixteen weeks of the HFFW diet, GLP-1 levels were found to be significantly decreased in the MS group, which were restored in the treatment groups in MS+SGN20 and MS+SGN30. Besides exhibiting the direct insulin-dependent effects, it also exerts extra-pancreatic actions, such as in the liver, it exerts glucoregulatory actions through decreasing glycogenolysis and increasing glucose metabolism (Baggio et al. 2007). These results suggest that sitagliptin ameliorates fatty liver and associated metabolic syndrome parameters.

Earlier studies show a remarkable difference observed between the metabolic parameters in the male and female animals, both fed high-fat diet, where the female animals depicted more resistance to the diet induced complications, thereby decreasing the onset of metabolic abnormalities as compared to the male animals. This is linked with the high estrogen levels in the females, which improves hepatic lipid accumulation in addition to the metabolic parameters. Along with this, sex-dependent differences in

the energy metabolism is also responsible for the delay in weight gain in females, which is mediated by leptin, and fibroblast growth factor 21 [Toth et al. 2021].

6.2 To analyze the potency of Sitagliptin on white adipose tissue inflammation, adiponectin expression and hepatic fatty acid metabolism in experimentally induced obese mice with focus on AMPK signaling (Objective II).

Obesity is associated with chronic low-grade inflammation, where the adipocytes and macrophages are the key players in the process [Johnson et al. 2012]. WAT expansion precipitates increased macrophage infiltration inside, further affecting the production of inflammatory molecules [Klionsky et al. 2012]. The dysregulated production of the pro-inflammatory cytokines and anti-inflammatory cytokines in WAT in obesity, is also referred to as metaflammation, i.e., metabolically triggered inflammation [Torres-Leal et al. 2010]. The prolonged high-fat diet leads to an increased level of pro-inflammatory molecules and a decreased level of anti-inflammatory molecules in the adipose tissues [Bettaieb et al. 2016; Ying-Ying et al. 2016]. Similarly, in the present study, we observed elevated levels of the cytokines IL-6 and TNF-α and the chemokine MCP-1 in the eWAT in the MS group, after sixteen weeks of HFFW diet, compared with the control group [Cui et al. 2015]. However, we observed a reduction in their levels in the treatment groups at the higher doses of sitagliptin. A similar effect of sitagliptin is reported in the previous studies in the liver and cardiomyocytes [Lee et al. 2013; Zheng et al. 2018]. It reduced inflammation in a dose-dependent manner in *in vivo* allyl-isothiocyanate and freund's complete adjuvant mouse models of inflammation, which is attributed to its direct effect and not GLP-1

mediated effect [Újhelyi et al. 2014]. It also reduced inflammation in mouse models of trinitrobenzene sulfonic acid and dextran sulfate sodium induced colitis [Salaga et al. 2018]. Sitagliptin mediated inhibition of NFκB and TNFα is also reported by Youssef et al, in rat model of remote myocardial injury induced by renal ischemia/reperfusion [Youssef et al. 2015].

Adiponectin has a vital role in energy metabolism in the liver, skeletal muscles, and adipose tissues. Moreover, the synthesis and release of adiponectin is negatively correlated with the expansion of adipocytes [Drolet et al. 2009]. The reduced expression of adiponectin is associated with obesity [Nigro et al. 2014]. It regulates the insulin sensitivity, glucose uptake, gluconeogenesis, fatty acid synthesis, and oxidation, reducing the lipid accumulation in the liver and skeletal muscle, which is triggered through the AMPK phosphorylation mechanisms [Yamauchi et al. 2002; Yamauchi et al. 2007]. Adiponectin has a strong inverse correlation with the fatty liver and metabolic complications, suggesting its role in their regulation [Haque et al. 2002]. The high-fat diet-induced animal models of NAFLD showed reduced mRNA and protein expression of adiponectin in the liver [Yao et al. 2011]. Also, adiponectin knock-out models developed severe steatosis in high-fat diet feeding [Kamada et al. 2007]. Here also, we observed significantly reduced mRNA expression of adiponectin in the liver in the MS group, compared with the control group. Moreover, reduced adiponectin mRNA expression was observed in the WAT of mice fed a high-fat diet [Yamauchi et al. 2001]. Similarly, in the adipose tissues also, we observed reduced adiponectin expression in the

MS group. However, adiponectin expression was significantly improved, in the liver and eWAT, with the higher doses of sitagliptin after the eight weeks of treatment.

The histological examination of the adipose tissue indicated that sixteen weeks of the HFFW diet caused the hypertrophy of the adipocytes in the MS group, as evidenced by the hypertrophic adipocytes. Whereas the relative number of the large hypertrophic adipocytes was found to be reduced with sitagliptin administration in the treatment groups compared with the MS group. The adipocyte hypertrophy was also reduced in earlier studies on high-fat diet fed C57Bl/6J mice [Souza-Mello et al. 2010]. The functional feature of adipose tissue is dependent on the adipose cell distribution, where the hypertrophic adipocytes facilitate chronic low-grade inflammation marked by increased pro-inflammatory cytokines and reduced insulin sensitivity [Jo et al. 2009; Zhou et al. 2015; Jung et al. 2017].

The intracellular energy balance is regulated by AMPK after getting phosphorylation on the α subunit [Kemp et al. 2007]. In the tissues with high metabolic activity, AMPK regulates the critical enzymes involved in the lipid and glucose metabolism [Viollet et al. 2009; Woods et al. 2017]. Phosphorylated AMPK in the liver diminishes the fatty acid synthesis through directly phosphorylating the ACC enzyme and inactivating it. ACC enzyme is the direct downstream target of phosphorylated AMPK and a rate-limiting enzyme in the synthesis and oxidation of fatty acids. AMPK is also involved in inhibiting the enzyme sterol regulatory element-binding protein -1c (SREBP-1c), which is responsible for fatty acid synthesis [Zhou et al. 2001; Li et al. 2011]. Phosphorylation of ACC enzyme diminishes the levels of malonyl Co-A that is

involved in CPT-1A inhibition, thereby helping in the mitochondrial oxidation of fatty acids [Abu-Elheiga et al. 2003; Zhou et al. 2015]. PPARα also promotes fatty acid oxidation, where the studies in the rat hepatocytes showed its expression to be AMPK dependent [Kang et al. 2016]. Animal models of high-fat diet-induced obesity and fatty liver have demonstrated diminished hepatic AMPK activity [Son et al. 2013; Woo et al. 2014; Randy et al. 2016]. Similarly, in the present study also, we observed significantly reduced hepatic expression of P-AMPK in the liver. However, investigation of the hepatic phosphorylated AMPK in the treatment groups showed significantly higher expression of the active AMPK than the MS group. Thus, suggesting that sitagliptin regulates the hepatic fatty acid metabolism via inducing AMPK phosphorylation. On measuring the mRNA expression of the markers of fatty acid synthesis and oxidation, i.e., CPT-1A, FASN, and PPARα, it was found that the expression of CPT-1A and FASN was significantly increased, while PPARα was reduced in the MS group, with respect to the control group, following the previous models of high-fat diet feeding [Choi et al. 2016; Jeong et al. 2017]. FASN, a key enzyme in the *de novo* lipogenesis, is involved in the regulating triglyceride synthesis in the cells by converting the malonyl Co-A to palmitate. Fatty acids are converted to fatty-acyl-CoA with the enzyme fatty-acyl-CoA synthase, which is transported into the mitochondria for β-oxidation through CPT-1 [Bartlett et al. 2004]. Hence, FASN and ACC enzymes are closely linked with the lipid accumulation in the hepatocytes. In the treatment groups, sitagliptin significantly reduced the mRNA expression of CPT-1A and FASN, whereas significantly increased the expression of PPARα. However, the observed effects of sitagliptin were only at the higher doses, i.e., 20mg/kg and 30mg/kg. As adiponectin is a

potent activator of AMPK, its upregulation in the hepatic tissues mediated AMPK phosphorylation, bringing down the fatty acid synthesis and increasing the fatty acid oxidation. Sitagliptin significantly inhibited hepatic lipid accumulation by inducing AMPK phosphorylation and reducing the mRNA expression of key enzymes involved in the lipogenesis. Therefore, manipulation of AMPK is suggested to be a potential therapeutic strategy for the treatment of obesity and NAFLD.

6.3 To investigate the effect of Sitagliptin on oxidative stress and mitochondrial biogenesis markers in white and brown adipose tissues in metabolically compromised obese mice (Objective III).

Prolonged intake of a high-fat diet increases the free fatty acids supplies, which migrates to the adipose tissues for fat storage, eventually increasing the lipid accumulation in the adipose depots and contributing to the increased mass of the WAT [Morakinyo et al. 2015]. With obesogenic diets, an increase in BAT mass is also observed in the previous studies, which might be due to decreased thermogenesis in obesity [So et al. 2011; Bargut et al. 2019]. Similarly, we observed a significant increase in the weight of eWAT and iBAT in the MS group, after sixteen weeks of HFFW diet in the MS group, relative to the control group. However, the adipose tissue weights were reduced significantly in the sitagliptin treated groups at the higher doses. Decreased lipogenesis and decreased fatty acids in the circulation might be linked to the reduced eWAT weight, whereas increased thermogenesis contributed to reduced iBAT weight.

The consumption of a high-fat diet is linked with the increased reactive oxygen species production and declined antioxidant defence systems in the enlarged adipocytes

generating oxidative stress [Charradi et al. 2013]. The inflammation in the fat accumulated adipocytes also promotes oxidative stress. The oxidative stress induced in the hypertrophied adipocytes causes oxidative damage inducing dysfunction in the mitochondrial components through protein modifications, lipid peroxidation, relating the pathogenesis of the obesity-associated metabolic complications [Le Lay et al. 2014; Furukawa et al. 2017]. Reactive oxygen species overproduction occurs in obesity due to increased mitochondrial oxidation of free fatty acids, which generates the electron donors in the electron transport chain [Brownlee 2005]. In the obese animal models, eWAT also secretes uric acid, which is responsible for increasing the activity of the enzyme xanthine oxidase. Interestingly, xanthine oxidase acts as an oxidoreductase, increasing the production of reactive oxygen species [Tsushima et al. 2013]. NADPH oxidase converts the molecular oxygen to free radicals and thus acts as an important source of reactive oxygen species in the adipose tissues [Furukawa et al. 2017]. Reducing oxidative stress in the adipocytes modulates the secretion of the adipokines, and hence they can be a therapeutic target for the treatment of obesity and related complications [Day et al. 2017]. Providing high-fat diet to the animals have demonstrated an increase in the levels of the reactive oxygen species generation, in both the white and brown adipocytes, accompanied with oxidative damage, than their lean counterparts [Alcalá et al. 2017; Furukawa et al. 2017]. In the present study also, we observed significantly increased levels of MDA and reduced levels of SOD and GSH in the MS group after prolonged HFFW diet in both the adipose tissues, i.e., eWAT and iBAT. However, in the treatment groups, sitagliptin significantly reduced the MDA levels and restored the SOD and GSH levels at the higher dose.

AMPK regulates multiple functions in the metabolic tissues such as liver, skeletal muscle, adipose tissues, and hence its activation holds the potential for the treatment of obesity and related disorders. In WAT, it is involved in the regulation of glucose and lipid metabolism, insulin sensitivity, lipolysis, fatty acid oxidation, and inflammation [Bijland et al. 2013]. While in BAT, it induces fatty acid oxidation, lipolysis, adipocyte differentiation, improves the metabolic activity, redox state and restores the mitochondrial function hence, the thermogenesis [Rong et al. 2007; Vila-Bedmar et al. 2010; van Dam et al. 2015; Zou et al. 2018]. Previous studies suggest the regulation of BAT activity and the energy expenditure via the activation of hypothalamic AMPK [López et al. 2010; Whittle et al. 2012]. Moreover, recent studies have confirmed AMPK to be a potent target for anti-obesity therapy because of its involvement in the regulation of the browning process, thermogenesis, and the energy balance in the WAT and BAT [Wu et al. 2018]. A decrease in the AMPK activity in the white and brown adipocytes is observed in diet-induced animal models of obesity [Lindholm et al. 2013; Prieto-Hontoria et al. 2013; Ruderman et al. 2013]. Similarly, in the present study also, we observed significantly decreased P-AMPK protein expression in the WAT and BAT in the MS group, which was improved significantly at the higher dose of sitagliptin.

Phosphorylated AMPK regulates the cellular energy content by increasing the ATP production through increasing the mitochondrial pool via the upregulation of mitochondrial biogenesis [Winder et al. 2000; Bergeron et al. 2001]. Mitochondrial dysfunction increases the production of free radicals, which causes oxidative stress in the liver [Lama et al. 2017] and adipose tissues [Wilson-Fritch et al. 2004] in obese

animals. High-fat diet feeding for extended duration leads to mitochondrial impairment, mitochondrial DNA damage, reduced mitochondrial DNA repairing capacity and decreased mitochondrial biogenesis in the liver [Araújo et al. 2016], skeletal muscles [Oh et al. 2017], and the white and brown adipose tissues [Araújo et al. 2016; Lee et al. 2017] in the animal models of obesity. Reduced mitochondrial biogenesis is observed in obesity, fatty liver, and metabolic syndrome. Mitochondrial biogenesis is essential for cellular health and is regulated by PGC-1α, NRF1, and TFAM genes. PGC-1α, a co-transcriptional regulation factor, activates the NRF1 gene, which induces the expression of TFAM gene, which regulates the mitochondrial DNA content and transcriptional activity [Lehman et al. 2000].

Targeting energy expenditure is an attractive concept for the treatment of obesity [Tseng et al. 2010]. Inducing thermogenesis in BAT or WAT might be a therapeutic strategy for combating obesity and its associated metabolic complications [Cypess et al. 2010; Kiefer et al. 2012]. The non-shivering thermogenesis in BAT is extremely mitochondrial dependent [Bal et al. 2017]. Thermogenesis activity in WAT and BAT was enhanced by berberine in db/db mice, through increased mitochondrial DNA copy number and increased PGC-1α and NRF1 mRNA expression, thus providing a defence against obesity [Zhang et al. 2014]. Epigallocatechin-3-gallate reduced the body weight via inducing thermogenesis and mitochondrial biogenesis, and phosphorylation of AMPK in BAT, as evidenced by increased mitochondrial DNA content and increased expression of PGC-1α, NRF-1, and TFAM, in diet-induced obese mice [Lee et al. 2017].

In consensus with the other studies, we also observed reduced mitochondrial activity, as evidenced by the reduced thermogenesis and mitochondrial biogenesis markers in the eWAT and iBAT after sixteen weeks of the HFFW diet. The mRNA expressions of PPARα, PGC-1α, NRF-1, TFAM, and UCP-1 was reduced significantly in eWAT and iBAT in the MS group. However, PPARα, PGC-1α, NRF-1, and TFAM mRNA expressions were improved significantly in both the eWAT and iBAT, in the treatment groups, at the higher dose. While the UCP-1 mRNA expression was significantly improved by the higher dose of sitagliptin in iBAT only.

The potential side effects of sitagliptin includes nasopharyngitis, upper respiratory tract infection, and headache. However, in an open-label multi centre-trial, sitagliptin administration once daily for twelve months improved the quality of life and reduced the Pittsburgh sleep quality index (Sakamoto et al. 2013). Studies of sitagliptin on high-fat diet fed mice show a reverse in memory impairment thereby improving cognition (Gault et al. 2015).

From the mechanism-based approach, the current study lacks the involvement of the specific inhibitors of AMPK and adiponectin. Although, the inclusion of the metformin as the positive control group has provided evidence for the efficacy of sitagliptin as an effective therapeutic agent for combating obesity and its associated metabolic complications. Moreover, the results suggest that the effects of sitagliptin in the present study are produced at a lower dose than that of metformin. Furthermore, the inclusion of an anti-obesity agent would have strengthened the results.