Adiponectin regulation of AMPK on oleanolic acid treated insulin resistance Sprague Dawley rats

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Abstract

AMPK is the principal regulator of both glucose and lipid metabolic pathways, both critical in the etiology of type-2 diabetes (T2D). Various studies have demonstrated that AMPK can be activated by the adiponectin. Individuals suffering from T2D are known to have low adiponectin levels in their blood plasma. This study was aimed at assessing the effect of the anti-diabetic compound oleanolic acid (OA) on adiponectin levels and the subsequent regulation of AMPK. In addition, the study also assessed the influence of OA on the inflammatory cytokines, biomolecules that also play role in the development of T2D. In this study, Sprague Dawley rats were fed with a high fructose diet (HFD) to induce metabolic disturbance as a model for T2D. The rats that developed metabolic disturbances were considered as diseased, and were consequentially treated with OA. Analysis of adiponectin concentration in blood plasma, AMPK gene expression and subsequent genes that play vital roles in glucose and lipid metabolism (GLUT-4 & CPT-1) were studied using rat skeletal muscle. Lastly, inflammatory cytokine gene expression (IL-6 & IL-10) and inflammatory cytokine concentration levels (TNF-α, IL-6, IL-10, MCP-1 & VEGF) were also assessed. The results from these studies show a significant increase in blood plasma adiponectin concentration in OA treated rats compared to the untreated rats. Furthermore, OA significantly up-regulated AMPK gene expression with ~4-fold increase and GLUT-4 gene expression with ~1.5-fold. On the other hand, the CTP-1 gene expression was not significantly expressed. All inflammatory cytokines were significantly down-regulated by treatment with OA. However, when a HFD (high fructose diet) was fed to these rats, these cytokines were up-regulated. These results clearly indicate that OA produced desired effects in ameliorating insulin resistance or metabolic disturbance. In conclusion, this study further confirms that OA can be used as an effective therapeutic agent to ameliorate the symptoms of T2D. Furthermore, this study also suggest that OA's mechanism of action could be through AMPK pathway.

Declaration

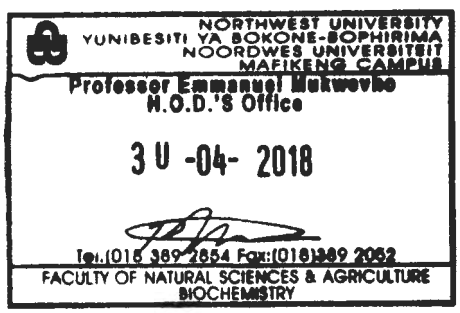
I, Matumba Mashudu Given hereby declare that the dissertation entitled

"Adiponectin regulation of AMPK on oleanolic acid treated Sprague Dawley rats"

Is entirely my own work. I have exercised reasonable care to ensure that the work is original and has not been taken from the work of others and where use of the work of others was made such work has been cited and acknowledged within the text of my work.

Student: **Matumba Mashudu Given** **Signature:** .....

Date: 30/04/2018



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List of abbreviations and symbols

ACC : acetyl-CoA carboxylase

AdipoR1 : adiponectin receptor 1

AdipoR2 : adiponectin receptor 2

AMP : adenine monophosphate

AMPK : AMP-activated protein kinase

ATP : adenosine 5‟ triphosphate

BSA : bovine serum albumin

CaMKK-β : calcium Calmodulin-Dependent Protein Kinase Beta

CCL 2 : chemokine (c-c motif) ligand 2

CCR 2 : c-c chemokine receptor type 2

cDNA : complementary DNA

CPT-1 : carnitine palmitoyltransferase 1

CON : control

DNA : deoxyribonucleic acid

DPP-4 : inhibitors of dipeptidyl peptidase 4

ECL : enhanced chemiluminescence

ELISA : enzyme-linked immunosorbent assay

gAD : globular adiponectin

GLP-1 : glucagon-like peptide-1

GLUT-4 : glucose transporter 4 protein

HFD : high fructose diet

HMW : high molecular weight

HRP : horse radish peroxidase

IL-1 : interleukin-1

IL-2 : interleukin-2

IL-6 : interleukin-6

IL-10 : interleukin-10

IRS-1 : insulin receptor substrate 1

JNK : jun n-terminal kinases

LKB-1 : liver kinase B1

MAPK : mitogen-activated protein kinases

MCP-1 : monocyte chemoattractant protein 1

MET : metformin

mRNA : messenger RNA

mTOR : mechanistic target of rapamycin

NFkB : nuclear factor kappa-light-chain-enhancer of activated B cells

ng/ml : nanogram per millilitre

ns : non-significant

OA : oleanolic acid

PAI-1 : plasminogen activator inhibitor-1

PBST : phosphate buffered saline tween-20

pg/ml : picograms per millilitre

PPAR-γ : peroxisome proliferator-activated receptor-γ

q-PCR : quantitative-polymerase chain reaction

RIPA : radioimmunoprecipitation assay buffer

RNA : ribonucleic acid

SDS-PAGE : sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM : standard error of the mean

STAT3 : signal transducer and activator of transcription 3

TNF-α : tumor necrosis factor alpha

T1D : type-1 diabetes

T2D : type-2 diabetes

TZD : thiazolidinedione

VEGF : vascular endothelial growth factor

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Chapter 1: Introduction and aims of the study

Type 2 diabetes (T2D) is an emerging health challenge all over the world as a result of high prevalence of obesity (Ghoshal & Bhattacharyya, 2015). It is important to note that obesity and T2D are causally linked through their association with the development of skeletal muscle insulin resistance (Steinberg & Beck Jorgensen, 2007). Insulin resistance is described as a condition in which circulating insulin becomes non-functional to target tissues, namely skeletal muscle, adipose tissue, and the liver (Shih *et al.*, 2009). This condition is well associated with T2D disease. Furthermore, insulin resistance is also known to be associated with reduced glucose uptake, reduced glucose utilization and inhibition of fatty acid oxidation by these tissues (Shih *et al.*, 2009). These result in the development of type-2 diabetes, a disease without a cure to date.

AMP-activated protein kinase (AMPK) is well a studied and described enzyme which is the master regulator of both lipid and glucose metabolic pathways. Repeatedly, literature has shown that dysregulation of AMPK plays a critical role in the etiology of T2D (Coughlan *et al.*, 2014). Once AMPK is activated it increases both fatty acid uptake and β-oxidation with concomitant increase in glucose uptake through the translocation of GLUT-4. GLUT4 is the major glucose transporter protein found intracellularly in the intercellular vesicles across the plasma membrane in the skeletal muscles (Jeon, 2016). In diabetes, the translocation is limited resulting in excess glucose in the blood resulting in hyperglycaemia. Furthermore, in diabetes, both lipid oxidation and glucose uptake are impaired. Owing to the fact that these two pathways are regulated by AMPK, this enzyme has now become a great target in the development of therapeutics much needed to ameliorate the symptoms of T2D (Coughlan *et al.*, 2014).

Apart from skeletal muscle as the major site of glucose disposal, the adipose tissue as well has now been shown to play a critical role as a site for the aetiology of diabetes. The adipose tissue

thus secretes various bioactive proteins and hormones into the circulation named adipocytokines essential in diabetes (Chandran *et al.*, 2003). One such hormone secreted by the adipose tissue is adiponectin. Adiponectin is a protein hormone that plays an important role in the modulation of glucose and lipid metabolism in insulin-sensitive tissues in both human and animals (Chandran *et al.*, 2003). Furthermore, literature has reported that adiponectin is associated with obesity-related insulin resistance and T2D (Haluzik *et al.*, 2004). In a study done by Ziemke & Mantzoros (2010), in this study adiponectin was knocked out in mice and the experiment resulted in glucose intolerance, insulin resistance and hyperlipidaemia especially in mice that were fed with high fat diets. In addition, literature has demonstrated that adiponectin's mechanism of action is through activation of AMPK enzyme which then regulates both glucose and lipid metabolism (Beylot *et al.*, 2006). Lastly, research has also demonstrated that patients suffering from T2D and obesity related to insulin resistance are associated with decreased adiponectin concentrations in blood circulation (Ziemke & Mantzoros, 2010). The fact that obesity and T2D are the results of adiponectin deficiency makes this hormone a very tempting target for possible therapeutic interventions focusing on the possibility that adiponectin treatment may improve T2D and obesity-related insulin resistance (Haluzik *et al.*, 2004).

Currently, there are limitations to treatment options for diabetes, such as compounds that reduce insulin resistance and hepatic glucose production and those that enhance insulin secretion and preserve β-cell function (Teodoro et al., 2008). Identification of compounds with anti-diabetic properties that possibly have therapeutic potential to diabetes are much encouraged in the quest for finding novel modalities for the treatment and management of the disease. Various plants and plants-derived foods can prove vital as sources of bioactive compounds in the treatment of diabetes. Recently, several plant-derived natural products have been identified to have glucose lowering effect (Osuna-Martínez *et al.*, 2014). One of the plant-

derived compound identified is oleanolic acid. Oleanolic acid (OA) is a bioactive pentacyclin triperpenoid belonging to the family *Oleaceae*. OA has been found to exert anti-diabetic effect although its actual mechanism of action is not well defined (Shanmugam et al., 2014). For example, one study in rats has shown that OA and oleanolic acid glycosides have glucose lowering effects after being administered as an oral glucose load (Teodoro et al., 2008). Furthermore, Oleanolic acid has also been shown to significantly lower blood glucose and weight loss in diabetic rats induced by streptozotocin (Wang et al., 2011). However, possible mechanism through which OA brings about this positive effect is not fully understood. Therefore, the aim of this study is to investigate the influence of OA on adiponectin regulation of AMPK on rats fed with high fructose diet.

Aim: To investigate the influence of OA on adiponectin regulation of AMPK in Sprague Dawley rats fed with HFD.

Objectives

1. To measure adiponectin concentration levels in Sprague Dawley rat's blood plasma using ELISA method.
2. To assess AMPK gene expression in skeletal muscle tissue using qPCR.
3. To analyse AMPK protein expression in skeletal muscle tissue using western blot.
4. To assess Glut-4 and CPT-1 gene expression in skeletal muscle tissue using qPCR.
5. To assess AdipoR1 and AdipoR2 gene expression in skeletal muscle tissue using qPCR.
6. To assess IL-6 and IL-10 gene expression in skeletal muscle tissue using qPCR.

   To measure concentration levels of inflammatory biomarkers (TNF-α, IL-6, MCP-1 and VEGF) in blood plasma using magnetic bead-based assays.

Chapter 2: Literature review

2.1 Type 2 diabetes (T2D) and lack of cure to date

Diabetes mellitus is a metabolic disease characterised by blood glucose level higher than normal resulting in hyperglycemia. This disease is associated with impaired insulin signalling pathway either through insulin insensitivity (insulin resistance) or autoimmune distraction of insulin production by the β-cells in the pancreas (Alismail & Jin, 2014). Furthermore, diabetes mellitus is a major public health concern with 382 million individuals being affected worldwide in 2013 (Balsan *et al.*, 2015). This number is expected to rise to 592 million by 2035 (Ali *et al.*, 2017). It was also estimated that diabetes caused 5.1 million deaths in 2013 (Coughlan *et al.*, 2014). There are two types of diabetes mellitus namely type 1 diabetes (T1D) also known as insulin-dependent and type 2 diabetes (T2D) also known as insulin-independent (Kim *et al.*, 2012). In addition, T1D is linked with low insulin production in the pancreas resulting in hyperglycemia, whereas T2D is associated with cellular insulin resistance even though insulin is produced normally by the pancreas (Kim *et al.*, 2012).

T2D constitutes more than 90% of all diabetes cases with T1D constituting less than 10% (Lago *et al.*, 2007). Statistics has demonstrated that over 350 million people worldwide have T2D, and the International Diabetes Federation projects that this number will increase to nearly 600 million by 2035 (Coughlan *et al.*, 2014). T2D is associated with a number of complications, including cardiovascular disease, blindness, kidney failure and lower limb amputation (Coughlan *et al.*, 2014). The main risk factor that causes T2D development is obesity. Obesity is a state of being excessively overweight or condition in which the number and size of adipocytes increase with further increase of the total fat mass (Balsan *et al.*, 2015). Obesity leads to insulin resistance, hyperglycemia and impaired metabolic function and all these are characteristics associated with T2D.

There is no cure available yet for T2D. Consequently. many studies are still being conducted to find a solution to this epidemic (Olokoba *et al.*, 2012). Although several medications are currently available to help manage or ameliorate T2D, there is an increasing need to find better or more effective treatments than those currently available (Coughlan *et al.*, 2014). Some of the therapeutic agents present for T2D treatment include; metformin, sulfonylureas, DPP-4 inhibitors, thiazolidinedione (TZD), α-glucosidase inhibitors, insulin and GLP-1 analogues (Zhang et al., 2009). Recent studies indicate that naturally occurring compounds can also be used for treatment of diabetes and obesity (Myung et al., 2012). This current research project intends to improve and develop the therapeutic modality much needed to cure or alleviate T2D.

2.2 AMP-activated protein kinase (AMPK) as the master regulator of metabolism

AMPK is a hetero-trimeric enzyme composed of three subunits namely the α-subunit, β-subunit and γ-subunit. The α-subunit is the catalytic subunit which contains a Ser/Thr protein domain and the γ-subunit contains sites for allosteric activation by AMP and inhibition by ATP (Zhang *et al.*, 2009). For this enzyme to work it must first be activated just like many other enzymes. Therefore, there are two ways that can activate the AMPK enzyme. The first one is an AMP-dependant pathway mediated by LKB1 while the second one is the Ca^{2+}-dependent pathway mediated by CaMKKβ (Zhang *et al.*, 2009). LKB1 is a hetero-trimer complex with regulatory proteins STRAD and MO25. Studies have shown that LKB1 is the major AMPK kinase in skeletal muscles, whereas CaMKKβ activates AMPK in response to increased intracellular Ca^{2-} (O'Neill *et al.*, 2013). Figure 2.1, shows all the components of the enzyme clearly and how the enzyme moves from active state to inactive state.

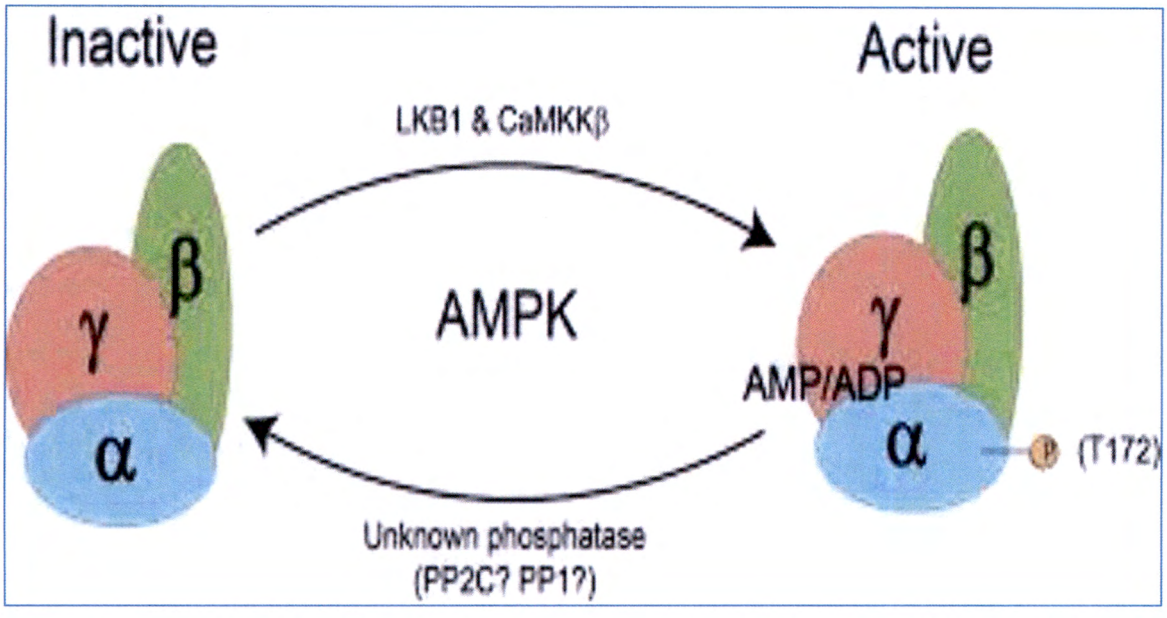

Figure 2.1: Three subunits of the AMPK, namely the α-subunit, β-subunit and γ-subunit. The α-subunit is the catalytic subunit hence it contain the Thr 172 protein domain and the γ-subunit contains sites for allosteric activation by AMP and inhibition by ATP. LKB1 and CaMKKβ activate the AMPK and unknown phosphatase inactivates the AMPK enzyme (Zhang et al., 2009).

AMPK is known as a master regulator of lipid and glucose metabolism (Coughlan *et al.*, 2014). Activation of the AMPK improves insulin sensitivity and glucose metabolism, making it a target to T2D. T2D is characterised by low glucose uptake and utilization by the skeletal muscles which leads to tissue starvation (Hayley et al., 2013). However activated AMPK in skeletal muscles stimulates glucose uptake, fatty acid oxidation, glucose transporter type (GLUT-4) translocation and mitochondrial biogenesis, while inhibiting protein and glycogen synthesis (Coughlan *et al.*, 2014). Furthermore, an increase in glucose uptake by the tissues results in positive effects in ameliorating diabetes symptoms hence it solves the case of insulin resistance (Steinberg & Beck Jorgensen, 2007). The major insulin-resistant organs in T2D are the liver, muscles and the adipose tissue (Zhang et al., 2009). Similarly in the liver tissue, activated AMPK stimulates glucose uptake, fatty acid oxidation, while inhibiting gluconeogenesis, as well as cholesterol, fatty acid and protein synthesis. In adipose tissue,

activated AMPK stimulates fatty acid oxidation and reduces fatty acid synthesis and lipolysis (Coughlan *et al.*, 2014).

AMPK activation plays a role in lipid metabolism by stimulating the phosphorylation of ACC2, which in turn increases fatty acid oxidation (Ghoshal & Bhattacharyya, 2015). ACC2 is a rate-controlling enzyme in the conversion of acetyl-CoA to malonyl-CoA. This phosphorylation of ACC2 inhibits the activity of ACC2, which results in decreased malonyl-CoA levels. Malonyl-CoA inhibits CPT-1, a rate-limiting step for the entry of long chain fatty acyl-CoA into mitochondria for oxidation (Kahn *et al.*, 2005). Therefore, increased fatty acid oxidation increases insulin sensitivity thus ameliorating insulin resistance related to T2D and obesity (Ghoshal & Bhattacharyya, 2015).

AMPK activation in the peripheral tissues or organs has been found to be beneficial for patients suffering from T2D. For this reason, the pharmacological activation of AMPK has been the main promising target for drug discovery and development during the past two decades (Coughlan *et al.*, 2014). AMPK is considered to be a crucial enzyme for diabetes treatment owing to its interaction with therapeutic drugs such as, metformin and TZDs that have long been used (Kim *et al.*, 2012). These drugs have been found to exert their beneficial effects through the indirect activation of AMPK, a mode which bypasses LKB phosphorylation. Hormones and several natural compounds and hormones including adiponectin can also activate AMPK. There is no direct AMPK activators that have reached clinical use for the treatment of metabolic disease, so further research on AMPK's regulation may lead to the development of compounds with better pharmacokinetics profiles and these will improve the potential for a clinically efficacious AMPK activator (Coughlan *et al.*, 2014).

2.3.1 Adiponectin

The adipose tissue is not simply an insert storage depot for lipids, but is also an important endocrine organ that plays a key role in the integration of endocrine, metabolic, and inflammatory signals for the control of energy homeostasis (Chandran *et al.*, 2003). The adipocyte secretes a variety of bioactive proteins into the circulation and exerts biological important reactions. These secretory proteins are termed adipocytokines, which include adiponectin, leptin, tumour necrosis factor (TNF-α), plasminogen activator inhibitor type 1 (PAI-1), adipsin, and resistin (Chandran *et al.*, 2003). Adiponectin is an adipocyte-specific, secreted protein/hormone with roles in glucose and lipid homeostasis (Meier & Gressner, 2004). This protein exhibits two major mechanisms of action by which it inhibits obesity and T2D, one by increasing insulin sensitivity and the other way is to increase fatty acid oxidation (Ghoshal & Bhattacharyya, 2015). Since AMPK regulates the crucial metabolism of lipids and glucose which are major factors in the development of type 2 diabetes, this study will therefore focus on adiponectin regulation of AMPK.

2.3.2 The adiponectin structure

Adiponectin is a 244-amino acid protein that was identified almost simultaneously by 4 different groups in the mid-1990s as an adipocyte-secreted hormone but remained unknown until the early 2000s (Ziemke & Mantzoros, 2010). This protein contains a modular structure that includes an N-terminal collagen-like domain and a C-terminal globular domain with significant sequence and structural similarities to the complement factor C1q (Meier & Gressner, 2004). Furthermore, X- ray crystallography of the globular fragment of adiponectin also reveals a striking structural homology to TNF-α, suggesting an evolution link between the TNF-α family members and adiponectin (Chandran *et al.*, 2003). The collagenous tail and a globular head form trimer-diamer, hexamer and high molecular weight (HMW) complexes which circulate at high concentration in blood (Vettor *et al.*, 2005). Figure 2.2 shows how the

trimer-diamer, hexamer and high molecular weight species appears in their structure. The HMW isoform was proposed to have a stronger association with insulin resistance, metabolic syndrome and cardiovascular disease as the biologically more active form of the hormone (Ziemke & Mantzoros, 2010).

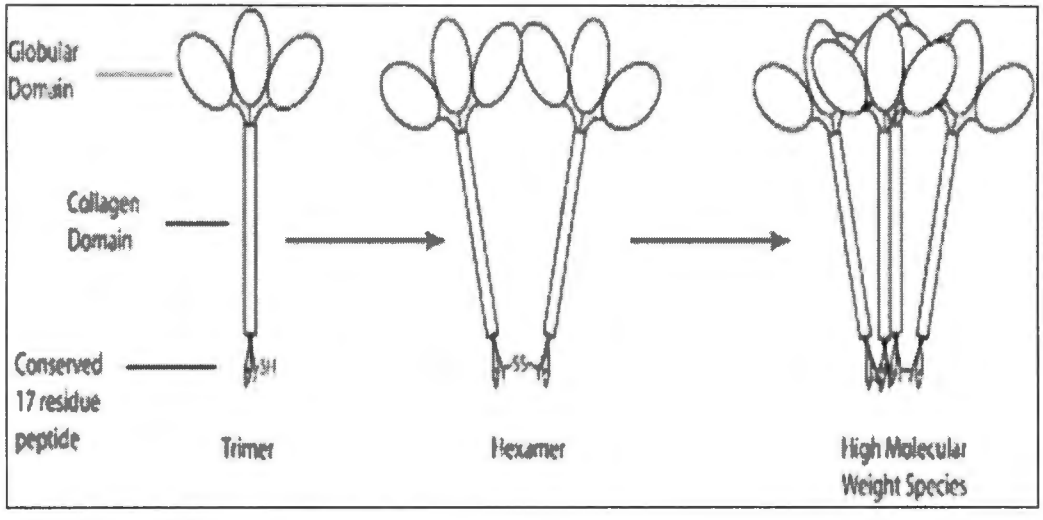

Figure 2.2: Model for assembly of adiponectin complexes. Three monomers made of globular and collagen domain form a trimer through association between their globular domains. Two trimers associate non-covalently through their collagenous domains to form a hexamer. Four to six trimers associate non-covalently through their collagenous domains to form High Molecular Weight (HMW) species, which circulate in the plasma (Chandran et al., 2003).

2.3.3 The adiponectin receptors

There are two receptors for adiponectin, termed AdipoR1 and AdipoR2 (Meier & Gressner, 2004). Both are 7-transmembrane proteins, and in contrast to the G-protein-coupled receptors family, these receptors have internal N-terminal and C-terminal regions as shown in figure 2.3 (Ziemke & Mantzoros, 2010). AdipoR1 is produced primarily in the skeletal muscles and endothelial cells among other tissues, whereas AdipoR2 is primarily found in hepatic tissues (Meier & Gressner, 2004). AdipoR1 has high affinity for globular adiponectin and a low affinity for full-length adiponectin, whereas AdipoR2 has intermediate affinity for both forms

of tissues. Both receptors are present in almost every tissue, including pancreatic β cells and malignant cells, one or the other receptor usually predominates (Ziemke & Mantzoros, 2010). Studies in mice link aging and high fat feeding to increased receptor expression in muscles and liver. AdipoR1 and AdipoR2 in muscles is inversely associated with circulating adiponectin concentrations whereas AdipoR2 in subcutaneous fat is positively associated with circulating adiponectin concentrations. Receptor expression in both muscles and fats is further increased in insulin-resistance states accompanied by hypoadiponectinemia. Physical activity up-regulates adiponectin receptors which suggest that the adiponectin hormone system may mediate exercise associated improvement in insulin resistance (Ziemke & Mantzoros, 2010). Overexpression of both receptors in liver of db/db mice improves insulin sensitivity. AdipoR1 overexpression decreases hepatic enzymes involved in gluconeogenesis whereas adipoR2 overexpression increases glucose uptake by stimulating glucokinase and PPAR-α (Ix & Sharma, 2010). Genes downstream of PPAR-α such as acyl-CoA oxidase 1 and uncoupling protein 2 are also stimulated by adipoR2 overexpression (Ix & Sharma, 2010).

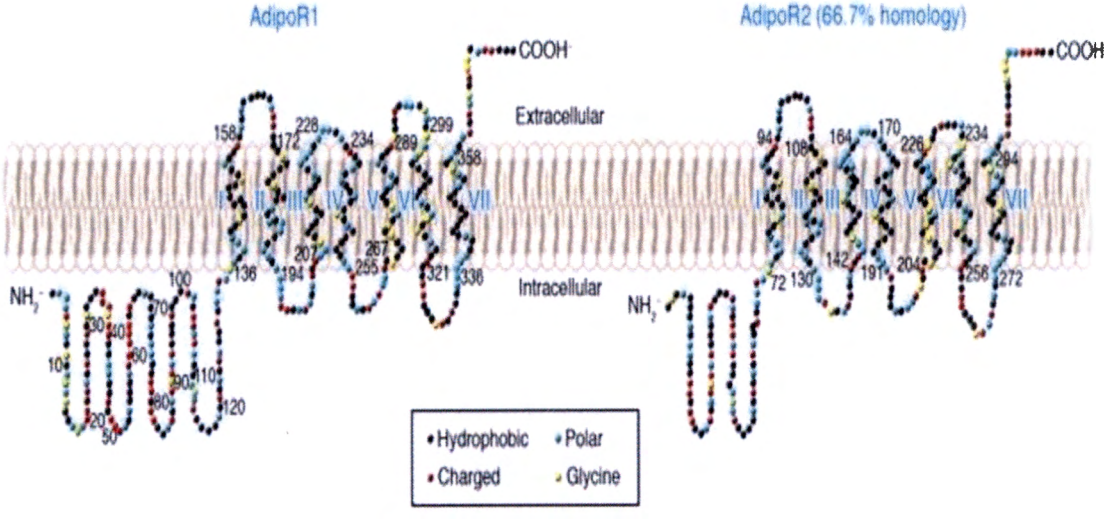

Figure 2.3: The structure of adipoR1 and adipoR2. AdipoR1 and AdipoR2 have 66.7% amino acid identity and both contain 7 transmembrane domains (I-VII) but structurally and topologically distinct from G-protein coupled receptors (Ghoshal & Bhattacharyya, 2015).

2.3.4 Adiponectin concentration vital in both T2D and obesity

It is well established that circulating adiponectin levels are reduced in patients who are T2D and obese (Bornard *et al.*, 2008). In addition, circulating concentrations of adiponectin are determined primarily by genetic factors, nutrition, exercise, and abdominal adiposity (Ziemke & Mantzoros, 2010). Several experimental studies have reported that both adipose adiponectin mRNA expression and plasma adiponectin levels are decreased in most rodents models of obesity, and adiponectin levels were also decreased by high-fat diet-induced obesity in monkeys. Reduction in adiponectin levels in this study of monkeys produced a decrease in insulin sensitivity and the development of T2D (Haluzik *et al.*, 2004). In individuals without T2D or obesity, the circulating adiponectin concentrations ranges between 500-30000 μg/L accounting for approximately 0.01% of total plasma protein (Meier & Gressner, 2004).

Adiponectin differ from other adipo-cytokines whose levels are increased in obesity in proportion to an increased total body mass. This is due to the fact that adiponectin expression is activated during adipogenesis, a feedback inhibition on its production may occur during the development of obesity (Chandran *et al.*, 2003). Research has demonstrated that woman have higher adiponectin concentrations than men, which is possibly linked to differences in estrogen or androgen concentrations (Ziemke & Mantzoros, 2010). Adiponectin levels are found to be lower in patients with essential hypertension and diabetic patients compared with non-diabetic subjects (Chandran *et al.*, 2003). Furthermore, increased serum adiponectin concentrations are associated with increased insulin sensitivity and glucose tolerance (Meier & Gressner, 2004). Therefore, reduced adiponectin expression and low concentration levels in blood circulation in T2D and obese patients provide promising results for the diagnostic and therapeutic role of adiponectin in obesity and T2D or insulin resistance (Ziemke & Mantzoros, 2010).

2.3.5 Adiponectin activates AMPK

Adiponectin mode of action is through binding to its receptors resulting in activation of several intracellular signalling pathways, but mainly AMP-activated protein kinase (AMPK). Moreover, it also activates other signalling molecules such as mTOR, nuclear transcription factor-kB (NF-kB), STAT3 and JNK (Ziemke & Mantzoros, 2010). Studies of specific deletions in AdipoR1 or AdipoR2 demonstrate that AdipoR1 predominantly mediates stimulation of AMPK, whereas AdipoR2 mediates stimulation of PPAR-α (Ix & Sharma, 2010). In muscles, the activation of AMPK is necessary for adiponectin's effects on fatty acid oxidation and glucose transport and is associated with increased AMP levels (Kahn *et al.*, 2005). Recent investigation showed the effects of adiponectin on AMPK signalling in cultured skeletal muscle from lean, obese and obese type 2 diabetic skeletal muscle and found that obese subjects display a blunted activation of AMPK in response to adiponectin treatment an effect that is exasperated in obese individuals with type T2D (Steinberg & Beck Jorgensen, 2007). Adiponectin induces fatty acids oxidation in muscles cells by sequential activation of AMPK, p38 MAPK (mitogen activated protein kinase) and PPAR-α. Adiponectin binds to its receptors which activate AMPK and stimulate the phosphorylation of ACC2 which in turn increase fatty acids oxidation (Ghoshal & Bhattacharyya, 2015). As previously discussed, adiponectin activates PPAR-α stimulating transcription of genes in the fatty acids oxidation and improving insulin sensitivity. Globular adiponectin (gAD) treatment has been shown to reverse skeletal muscles insulin resistance in the models of genetic and diet-induced obesity and these effects are linked to the activation of AMPK by adipoR1 (Steinberg & Beck Jorgensen, 2007). A recent study has shown that chronic activation of AMPK by adiponectin is a significant regulator of muscle mitochondrial biogenesis which suggests that a consequence of reduced adiponectin signalling may be impaired lipid oxidation and increased intramuscular lipid storage (Steinberg & Beck Jorgensen, 2007).

2.4 Cytokines involved in T2D

Cytokines are soluble glycoproteins, immunoglobulin in nature, and they are released by living cells of the host which acts non-enzymatically to regulate host cell function. This soluble glycoproteins make up the fourth major class of soluble intercellular signalling molecules, alongside neurotransmitters, endocrine hormones and autacoids (Nathan & Sporn, 1991). The central role of cytokine includes cell to cell communication, inflammatory response amplification and immune response regulation (Peters, 1996). Cytokines acts in concert with specific cytokine inhibitors and soluble cytokine receptors to regulate the human immune response (Opal & DePalo, 2000). For example, the local response to an infection or tissue injury involves the production of cytokines, which are released at the site of inflammation. These cytokines are usually referred to as anti-inflammatory or pro-inflammatory cytokines (Pedersen, 2000).The anti-inflammatory cytokines are a series of immune-regulatory molecules that control the pro-inflammatory cytokines receptors (Opal & DePalo, 2000). Research has demonstrated that inflammatory processes play a significant role in the etiology of diabetes and obesity (King, 2008). Furthermore, T2D is associated with increased circulating concentration of inflammatory markers (Pickup *et al.*, 2000). Example of inflammatory markers that were assessed in this study are tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1) and vascular endothelial growth factor (VEGF).

2.4.1 Tumour necrosis factor-α (TNF-α)

Visceral obesity and inflammation within white adipose tissue may be a crucial step contributing to the emergence of insulin resistance or T2D. Tumour necrosis factor-α (TNF-α) is an adipo-cytokines involved in inflammation and is found to be increased in the metabolic syndrome, T2D and obesity (Vettor *et al.*, 2005). Furthermore, TNF-α produced by white

adipose tissue is markedly up-regulated in obesity and contributes to insulin resistance by interfering with insulin receptor signalling (Meier & Gressner, 2004). TNF-α was the first pro-inflammatory cytokine implicated in pathogenesis of obesity-related insulin resistance and T2D (Esser *et al.*, 2014).

TNF-α suppresses adiponectin production in adipose tissue, whereas TZD prevent this inhibitory effect of TNF-α and high concentration of adiponectin also suppress secretion of TNF-α (Meier & Gressner, 2004). In another study, TNF-α dose dependently reduced the expression and secretion of adiponectin in 3T3-L1 adipocytes and the reducing effect of TNF-α on adiponectin mRNA was antagonised by co-incubation with troglitazone (Maeda *et al.*, 2001). In animal model study, adiponectin-knockout mice was found to have delayed clearance of free fatty acids in the plasma, low levels of fatty-acid transport protein 1 mRNA in muscle, high levels of TNF-α mRNA in adipose tissue and high plasma TNF-α concentrations (Haluzik *et al.*, 2004). Therefore, these studies indicate that TNF-α is significant indicator in the development of T2D and obesity and as such this study assesses its concentration in relation to oleonolic acid administration.

2.4.2 Interleukin-6 (IL-6)

Interleukin-6 (IL-6) is a pro-inflammatory cytokine produced in a variety of tissues, including activated leukocytes adipocyte and endothelial cells (Pradhan *et al.*, 2001). Recent studies have suggested that IL-6 could be involved in insulin resistance and its complications. It is now clearly established that a strong interaction occurs between cytokines and insulin signalling pathways, and generally lead to an impaired biological effect of insulin (Bastard *et al.*, 2006). Literature has indicated that IL-6 is found elevated in insulin resistance and obesity individuals (Coughlan *et al.*, 2014). Furthermore, IL-6 is secreted from adipose tissue and its plasma levels correlate with body mass and the degree of insulin resistance (Vettor *et al.*, 2005). In rodent

model of glucose metabolism, the in vivo infusion of human recombinant IL-6 has been shown to induce subsequent hyperglycemia, gluconeogenesis and compensatory hyperinsulinemia (Pradhan *et al.*, 2001).

2.4.3 Interleukin-10 (IL-10)

IL-10 is an anti-inflammatory cytokine that seems to act trying to block diabetes mellitus evolution. Its increase in T1D patients can be resulting from a compensatory mechanism to the increase of pro-inflammatory cytokines (Cardoso *et al.*, 2017). Again, IL-10 can inhibit the production of many other pro-inflammatory cytokines including TNF-α, IL-2 and IL-1 and impairs the phagocytic and all stimulatory capacity of macrophages (Emanuela *et al.*, 2012). One study in *vitro* demonstrated that adiponectin exerts potent immunosuppressive properties inducing the production of anti-inflammatory mediator IL-10 in a variety of myeloid cell types (Emanuela *et al.*, 2012).

2.4.4 Monocyte Chemoattractant protein-1 (MCP-1)

Monocyte chemoattractant protein-1 (MCP-1) is the first discovered and most extensively studied chemokine. Studies on its role in the cause of obesity and diabetes related disease have increased exponentially during the past two decades (Panee, 2012). Is also known as CCL2, which signals to macrophage through the CCR2 receptor, is strongly correlated with T2D and obesity (Daniele *et al.*, 2014). Circulating MCP-1 has been found significantly increased in patients with T2D. Furthermore, plasma level of MCP-1 has generally been found increased in obese individuals compared to the lean controls (Panee, 2012). Another possible reason why T2D and obesity are associated with inflammation is that the state of insulin resistance promote inflammation. This is due to the fact that insulin exerts an anti-inflammatory effect at the cellular and molecular level in *vitro* and in *vivo* (Dandona *et al.*, 2004). A number of studies

have demonstrated that subclinical inflammation likely play an important role in the pathogenesis of insulin resistance and T2D (Daniele *et al.*, 2014).

The high levels of circulating MCP-1 in obese patients were further increased by fructose consumption and reduced by low glycemic index diet (Panee, 2012). Systemic administration of MCP-1 in mice induced insulin resistance and this adverse effect was ameliorated by a CCR2 antagonist without affecting macrophage infiltration in adipose tissue (Panee, 2012). Efforts have been exerted to inhibit MCP-1 over production and ameliorate obesity related syndromes such as insulin resistance and T2D. Some of these inhibitors includes acarbose, rosiglitazone, troglitazone, capsaicin and bypass surgery exercise, etc. (Panee, 2012).

2.4.5 Vascular endothelial growth factor (VEGF)

VEGF is a cytokine that potentially stimulates angiogenesis, microvascular hyper-permeability and endothelium-dependent vasodilation effects that are largely mediated by endothelial nitric oxide synthase (De Vriese *et al.*, 2001). This cytokine was discovered to be a tumour secreted protein which rendered hyper-permeable to circulating macromolecules and small viens (Tsuchida *et al.*, 1999). Owing to the fact that it is also involved in small veins, VEGF is also one of the major growth factor that has an adverse effect on ocular neovascular disease such as diabetic retinopathy (retinopathy leads to blindness in diabetic patients) (Murata *et al.*, 1997). Research has demonstrated that, VEGF expression is upregulated in several cell types and tissue by high ambient glucose concentration and high glucose level in the culture medium increased VEGF expression in vascular smooth muscle cells, in retinal epithelial cells and in glomerular endothelial cells and increased renal expression of VEGF mRNA and protein was reported in experimental rat models of T1D and T2D (De Vriese *et al.*, 2001).

Plasma VEGF is elevated in patients with diabetes and the elevated levels correlate with endothelial damage or dysfunction and cardiovascular risk in hypertensive patients (Lim *et al.*,

2005). For example, one study showed that plasma VEGF concentration was high in diabetic patients who were hospitalized because of poor glycaemic control compared to the concentration in healthy subjects (Kakizawa *et al.*, 2004). Nonetheless, VEGF is usually raised in diabetes condition regardless of vascular disease (Lim *et al.*, 2005). Investigation of factors that promotes VEGF expression are important because their inhibitors are potential drugs for treatment of diabetic retinopathy and as such investigation such as this that seek to assess other potential modalities (oleanolic acid) in diabetes treatment are necessary (Murata *et al.*, 1997).

2.5 Oleanolic acid as the potential therapeutic plant derived compound

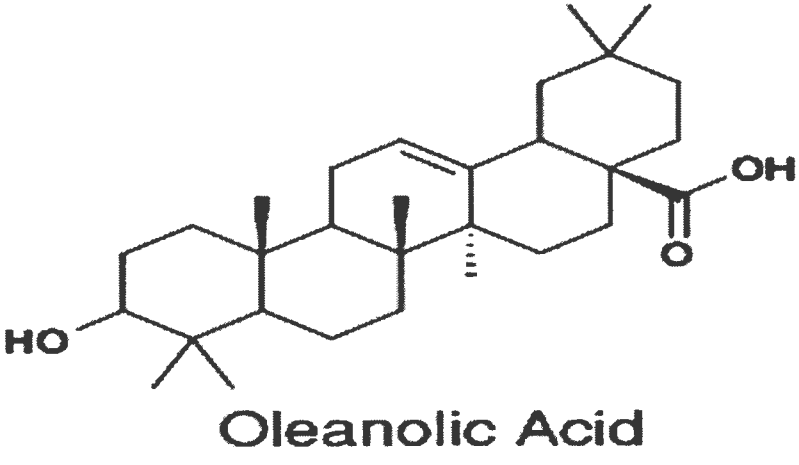

Figure 2.4: The chemical structure of Oleanolic acid (Liu, 1995).

Plant derived compounds that have anti-diabetic potential are crucial for the treatment and management of diabetes as they are affordable and non-toxic. One such compound is oleanolic acid shown in figure 2.4. Oleanolic acid is a pentacyclic triterpene present in may fruits and vegetables such as olive leaves, mistletoe sprouts, grapes, clovse and pomegranate flowers, that exhibits a wide range of pharmacological and biochemical effects including anti-inflammatory, anti-hypolipidemic, hypoglycemic effect and antioxidant effects (de Melo *et al.*, 2010). Oleanolic acid exists in nature as a free acid, but also serves as an aglycone of

triterpenoid saponins linked with one or more sugar moieties to form glycosides (Shanmugam *et al.*, 2014). Furthermore, oleanolic acid have very low toxicity (Somova *et al.*, 2003). Plants, fruits and vegetables are considered to be less toxic and have fewer side effects and these bioactive compounds present in food can alter gene expression and cellular events resulting in the modification of proteins and their functions (Jayaprakasam *et al.*, 2006). Therefore, oleanolic acid is considered as a potential therapeutic drug that can be used in T2D alleviation since is less toxic.

Oleanolic acid has been used as therapeutic agent in models of diabetes to improve insulin activity, to inhibit gluconeogenesis and to promote glucose utilization. Research has also indicated that OA has ability to inhibit gluconeogenesis and attenuate hepatic insulin resistance. Hepatic insulin resistance in obese condition is considered a major link between T2D and non-alcoholic fatty liver disease (Perry *et al.*, 2014). Furthermore, oleanolic acid lacks the adipogenic activity unlike the commonly used anti-diabetic therapeutics such as insulin or TZD that provides beneficial effects with the disadvantage of weight gain possibility (Sung *et al.*, 2010). Therefore, oleanolic acid has shown to be a promising therapeutic agent for diabetes and obesity alleviation. However, the mechanism of action through which oleanolic acid produces beneficial effect is not well understood.

Oleanolic acid ameliorates the obesity-associated insulin resistance and hyperlipidaemia (de Melo *et al.*, 2010). In addition, oleanolic acid and oleanolic acid glycosides have been identified that have glucose lowering effects. In one of the studies, oleanolic acid glycosides purified from plants, but not oleanolic acid itself reduced serum glucose levels in rats given an oral glucose load (Teodoro *et al.*, 2008). As mentioned previously, the potential anti-diabetic effects of oleanolic acid and derivatives and their mechanism of action have been less well studied. In this study, the influence of oleanolic acid on adiponectin concentration, AMPK expression and its subsequent genes involved in diabetes and, lastly, the inflammatory

cytokines will be assessed to investigate the beneficial effects of oleanolic acid against insulin resistance/T2D in Sprague Dawley rats fed with a high fructose diet.

2.6 High fructose diet (HFD) induce T2D and obesity-related insulin resistance

The model of animal treatment in this study includes rats fed with a high fructose diet. Research has demonstrated that, animals fed a diet high in fructose produce a model of insulin resistance, hyperlipidaemia, hyperinsulinemia with accompanying mild hypertension (Shih *et al.*, 2009). Furthermore, fructose intake has been recently linked to the epidemic of metabolic syndrome. Metabolic syndrome is a pathophysiological entity characterised by insulin resistance, obesity, hyperinsulinemia, dyslipidaemia, hypertension and T2D development (Sánchez-Lozada *et al.*, 2007). Soft drink intake with high fructose are associated with an increased risk for obesity in adolescents and for type-2 diabetes in young and middle-aged woman. Excess fruit juice also rich in fructose is associated with the development of obesity in children. Fructose–fed rats also develop features of metabolic syndrome (Nakagawa *et al.*, 2006). Fructose intake has greatly increased along with the incidence of T2D and more than 90% of ingested fructose is metabolised by the liver at first pass, were it stimulate lipid metabolism by increasing fatty acid triglyceride and this contributes to insulin resistance development (Glugliucci, 2017). Another study has indicated that, fructose-induced metabolic syndrome can be created experimentally either by feeding rats with a high-fructose diet or by adding fructose to drinking water (Sánchez-Lozada *et al.*, 2007).

Other studies showed that fructose consumption promotes a reduction of adiponectin expression and again fructose consumption enhances TNF-α expression in white adipose tissue (Carvalho *et al.*, 2010). Finally, one distinction between fructose and glucose is that fructose raises serum uric acid, which predicts the development of obesity and hypertension (Nakagawa *et al.*, 2006).

Chapter 3: Methods and Material

3.1 Animals

This study was conducted using male pups of Sprague Dawley rats obtained from WITS University, South Africa. Forty male Sprague Dawley pups were weighed on the third day following parturition and given a day for acclimatization before receiving treatments. On postnatal day 4, the male Sprague Dawley pups were randomly allocated into one of the five treatment groups described in Figure 3.1, each consisting of 8 pups. Treatment was given from 7 days old pups to 112 days old adult rats then followed by termination and collection of samples. All animal experiments were conducted in accordance with protocols approved by the Animal Ethics Screening Committee (AESC) of the University of Witwatersrand, Johannesburg, South Africa (AESC approval number 2014/47/D).

Study design and experimental conditions

Pups were divided into five groups of 8 rats each namely the Control (CON), Oleanolic acid (OA), High fructose diet (HFD), Oleanolic acid and High fructose diet (OA/HFD) and Metformin and High fructose diet (MET/HFD). Figure 3.1 further indicates how the rats were divided according to their experimental groups. The pups in CON group were given distilled water daily via orogastric gavage in addition to their suckling milk. The Pups in OA group were given 60 mg/kg of oleanolic acid administered via orogastric gavage daily. The pups in HFD group were given high fructose diet consisting of 25% fructose (w/v), in addition to their dietary milk. The pups in OA/HFD group were given high fructose diet consisted of 25% fructose (w/v) supplemented with 60mg/kg of oleanolic acid both administered via orogastric gavage daily. The pups in

MET/HFD group were given 500 mg/kg of metformin combined with high fructose diet consisted of 25% fructose (w/v) via orogastric gavage daily. All solutions were administered at a volume of 10 ml/kg.

The experiment began by feeding pups of 7 days old to 27 days old according to the five experimental groups illustrated on figure 3.1. HFD was used to induce insulin resistance to pups that were under the group that was given HFD. From day 28 to day 55, the rats were given normal diet-standard rat chow. From day 56 to day 112, the rats were finally fed again with HFD. At the end of the experiment on day 112, the rats were euthanized by an overdose of sodium pentobarbital (200 mg/kg body weight). Blood was collected by cardiac puncture and samples were dispensed into plain and heparin coated tubes, plasma was collected. Skeletal muscle tissues were collected and stored at -80℃.

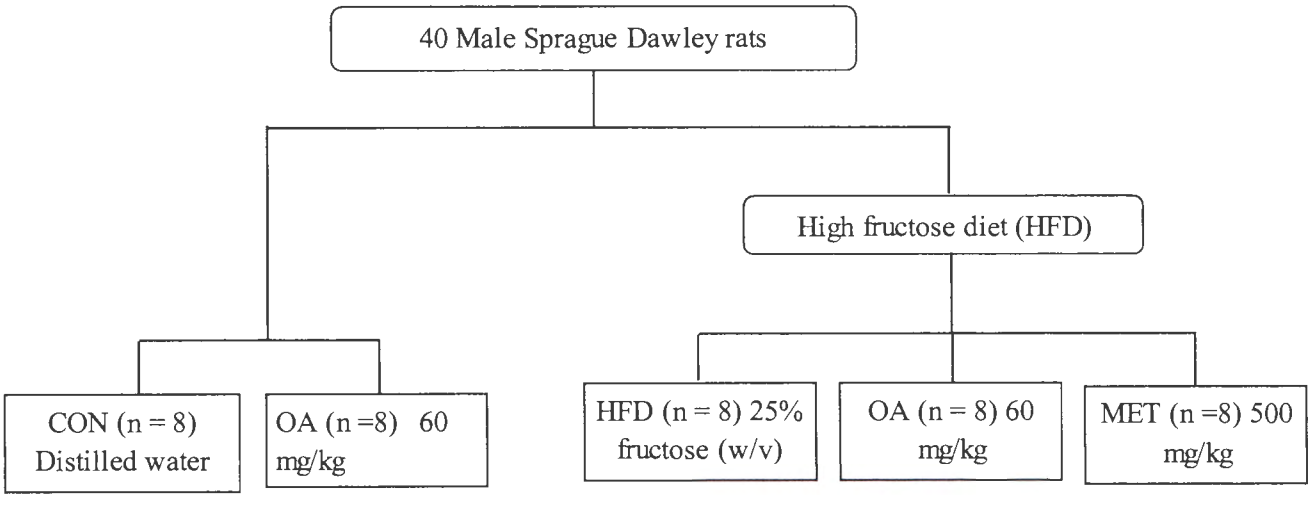

Figure 3.1: The diagram indicating arrangement of the five experimental groups of Sprague Dawley rats. CON = control, OA = oleanolic acid, HFD = High fructose diet and MET = metformin. n represent number of rats allocated per group.

3.2 Measurements of inflammatory markers concentration in Blood plasma

Bio-Plex Pro magnetic bead-based assays (Bio-Rad Laboratories, USA) on the Bio-Plex Platform (Bio-Rad Laboratories, USA) were used to measure plasma levels of inflammatory markers (TNF-α, IL-6, VEGF and MCP-1). Samples were evaluated undiluted. Samples were reacted with a mixture of fluorescent polystyrene beads bound with specific anticytokine primary antibodies, resulting in binding of the cytokines to the bead with the corresponding antibody. The biotinylated anticytokine secondary antibodies were then added and allowed to bind to the cytokine-bead complex followed by the addition of fluorescent phycoerythrin-conjugated streptavidin. All analytes levels in the quality control reagents of the kits were within the expected ranges. The standard curve for all the analysis ranged from 3 to 12000 pg/ml. Bio-Plex manager software version 6.0 was used for bead acquisition and analysis.

3.3 Measurement of gene expression using qPCR analysis

3.3.1 RNA extraction

Total RNA was isolated using Trizol (Life Technologies, USA). One sample of the frozen skeletal muscle tissue was randomly selected per group for RNA extraction. The frozen skeletal muscle tissue was crushed using a pestle and mortar in liquid nitrogen and 1ml of trizol was added to 200 mg of tissue sample. The mixture was homogenised for 3 minutes while working on ice. The homogenate was vortexed vigorously after addition of 200 µl of chloroform and then incubated on ice for 15 minutes. The homogenate was centrifuged at 12000 xg for 15 minutes at 4 ℃ to get the phase

spectrum. The aqueous phase was transferred to a new fresh tube. The total RNA was precipitated by incubating the solution for 10 minutes in ice after addition of 0.5 ml of isopropanol. After incubation the solution was centrifuged for 10 minutes at 12000 xg at 4 ℃ and the supernatant was removed. The pellet was washed with 70% ethanol and then centrifuged for 10 minutes at 7500 xg at 4 ℃. The supernatant was removed and the total RNA pellet was dissolved in 50µl of RNase free water.

Total RNA concentration and quality was measured using the NanoDrop Lite Spectrophotometer (Thermo Scientific). The integrity of the total RNA was analysed using 1% agarose gel electrophoresis as shown in figure 3.2. Briefly 1 g of agarose was dissolved in 100 ml of TAE buffer by heating the mixture with a microwave for 60 seconds. The mixture was allowed to cool down to at least 50 ℃, and then 1.5 µl of ethidium bromide was added. The solution was poured in the gel tray, the comb was inserted and the gel was allowed to solidify. The samples were loaded in the wells after being mixed with the loading dye. The electrophoresis was run for 45 minutes at 100 volts and TAE buffer was also used as a tank buffer. The gel was viewed under the doc machine (Bio-Rad Laboratories, USA).

Figure 3.2: The agarose gel showing the integrity of total RNA in all five groups the rats. The first lane was the molecular marker followed by the five experimental groups used in this study.

3.3.2 cDNA synthesis

The cDNA was synthesised using the SuperScript VILO cDNA Synthesis kit (Themofisher Scientific) according to the manufacturer's protocol. Briefly, master mix was prepared by mixing together the 5X VILO Reaction mix and the 10X SuperScript enzyme mix while working on ice. The volume of RNA required to make a concentration of 2.5 µg was calculated and DEPC-treated water was used to compensate the volume to a final of 20 µl in all reaction tubes. The tube contents were gently mixed and then incubated for 10 minutes at 25 ℃, then followed by 42 ℃ incubation for 60 minutes. The reaction was terminated by incubation at 85 ℃ for 5 minute. All incubations were done in the T100 Thermal Cycler (Bio-Rad, Singapore). The cDNA was stored at -20℃ until use.

3.3.3 Real-Time PCR

Real-Time PCR was performed using the PowerUp SYBR Green master mix (Applied Biosystems, Life Technologies) according to the manufacturer's protocol. The primers and the PowerUp SYBR Green master mix were mixed together appropriately according to the volumes given by the manufacturer. The cDNA template was mixed with RNase-free water. Solutions were transferred to appropriate wells in the 96 well plate and the template was added lastly. The plate was sealed with an optical adhesive cover. The step One plus Real-Time PCR system thermal cycling block instrument (Applied Biosystems, Life Technologies) was used. The reaction plate was placed in the instrument and the thermal cycling conditions were set then the run was started.

Table 3.1: List of primers used in the study.

Genes	Forward primer (F) and Reverse prime (R)
AMPK-α	F: 5′-GGCAAAGTGAAGATTGGAGAACA-3′ R: 5′-AACTGCCACTTTATGGCCTGT C-3′
AdipoR1	F: 3′-AAGCACCGGCAGACAAGAGC-5′ R: 3′-AGGAAGAACCAGCCCATCTG-5′
AdipoR2	F: 3′-CTGTGTGCTGGGCATTGCAG-5′ R: 3′-AGCCTATCTGCCCTATGGTG-5′
IL-6	F: 5′-GCCACTGCCTTCCCTACTTCA-3′ R: 5′-GACAGTGCATCATCGCTGTTCA-3′
IL-10	F: 5′-TGCCTTCAGTCAAGTGAAGACT-3′ R: 5′-AAACTCATTCATGGCCTTGTA-3′
GLUT4	F: 5′-GCAGCGAGTGACTGGACCA-3′ R: 5′-CCAGCCACGTTGCATTGTAG-3′
CPT-1	F: 5′-CGGTTCAAGAATGGCATCATC-3′ R: 5′-TCACACCCACCACCACGAT-3′
Actin	F: 5′-GACGAGGCCCAGAGCAAGAGA-3′ R: 5′-GGGTGTTGAAGGTCTCAAACA-3′
GAPDH	F: 5′-GAACATCATCCCTGCATCC-3′ R: 5′-CCTGCTTCACCACCTTCTT-3′

3.4 Measurement of AMPK phosphorylation using western blot

3.4.1 Protein extraction

Protein was extracted using the potent lysis buffer or RIPA, a 5 mg piece of tissue was weighed and 300 µl of ice cold lysis buffer was added to the Eppendorf tube. The mixture was homogenized with an electric homogenizer. The solution was centrifuged for 20 minutes at 12000 rpm at 4 °C. The supernatant with the protein was transferred into a new tube and the pellet was discarded. The protein was stored at -80 °C.

3.4.2 Protein concentration

The protein concentration was determined by the Bradford protein assay. Protein assay dye reagent concentrate (Bio-Rad Laboratories, Germany) was used. BSA was used as a protein standard, six dilutions of protein standard were prepared from 0.2 mg/ml to 1.2mg/ml. The protein assay dye reagent was prepared by diluting the 1 part of the dye reagent concentrate with 4 parts of distilled water and the solution was filtered through the whatman filter to remove particles. An amount of 100 µl of each standard and the sample solutions were pipetted into clean 15 ml tubes in duplicate and 5ml of the diluted dye reagent was added to each tube. The tubes were mixed by vortexing. The tubes were incubated for 15 minutes at room temperature, after incubation absorbance was measured at 595 nm using the spectrophotometer (SP-VIS 100, Spectrum Instruments). The BSA standard curve was generated as shown in figure 3.3 and concentration of the samples were measured. The concentration of the samples were diluted to one concentration of 0.686 mg/ml.

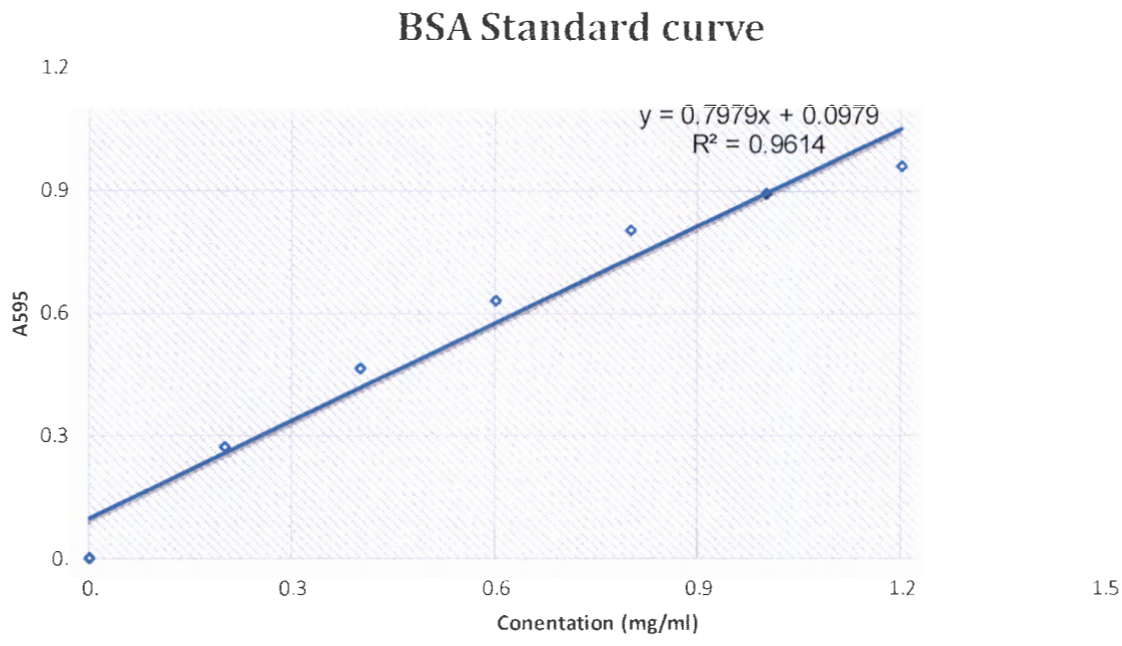

Figure 3.3: The figure showing BSA standard curve. Concentration of BSA is represented in mg/ml and the equation of the standard curve is shown is the figure.

3.4.3 SDS-PAGE

The two glass plates were clamped in the casting frames. Then 10% of the separating gel was prepared and appropriate amount of the separating gel solution was pipetted into the gap between the glass plates. The top of the separating gel was made horizontal by filling isopropanol on top of the separating solution until overflow. The separating gel solution was allowed to solidify. The isopropanol was discarded. Then 4% of the stacking gel was pipetted on top of the separating gel until overflow and the well-forming comb was inserted. The stacking gel solution was allowed to solidify and after solidification was complete the comb was removed. The glass plates were removed from the casting frame to the cell buffer dam. The electrophoresis buffer was poured into the inner chamber and the outer chamber. The samples were prepared by mixing 3 parts of the sample and 1 part of the sample buffer. Then 15 µl of the prepared samples with the concentration of 0.686 mg/ml were loaded in the wells starting with 2 µl of the

protein marker. The top part of the chamber was covered with the lid and then connected to the power pack. Electrophoresis was run at constant voltage of 120 volts for about an hour.

3.4.4 Western blot

At the end of the SDS-PAGE, the gel was marked for identification and the gel was placed in 50 ml of the transfer buffer for 30 minutes in a shaker. The membrane was cut to the appropriate size of the gel and the membrane was transferred to 100% methanol for 1 minute, followed by 1 minute in dH_2O. This was repeated 3 times and finally the membrane was transferred into the transfer buffer for 5 minutes before used. The open cassette was placed in a tray of transfer buffer with the black side in the buffer and the soaked pad was placed on top of the cassette. Filter paper was added on top of the pad followed by the gel on top and then the pre-soaked membrane was placed on top of the gel. Another filter paper was added on top of the membrane followed by the second soaked pad. The cassette was closed and then transferred to the tank. The tank was filled with transfer buffer to the appropriate required level. A small magnetic stirrer was added into the tank and the tank lid was closed. The apparatus were transferred to the cold room, the power pack and the magnetic stirrer were connected to the tank. The power pack was set to run for overnight at 30 V.

After the run, the membrane was transferred to 5% BSA in PBS-T for 1 hour at room temperature. The membrane was washed twice with PBS-T for 5 minutes each. The primary antibody was diluted 1:1000 with PBS-T and the membrane was incubated in the diluted antibody for overnight on a shaker in the cold room. The membrane was washed twice with PBS-T for 5 minutes each. The secondary antibody was diluted 1:5000 with PBS-T and the membrane was incubated in the diluted antibody for 1 hour

to each well and the plate was incubated for 30 minutes at room temperature in the dark room with gentle shaking. Lastly 50µl of stop solution was added to each well and absorbance was measured at 450nm immediately with a spectrophotometer (Matiskan Go, thermos Scientific). The protein standard curve was generated as shown in figure 3.4 and unknown concentration of the samples were determined.

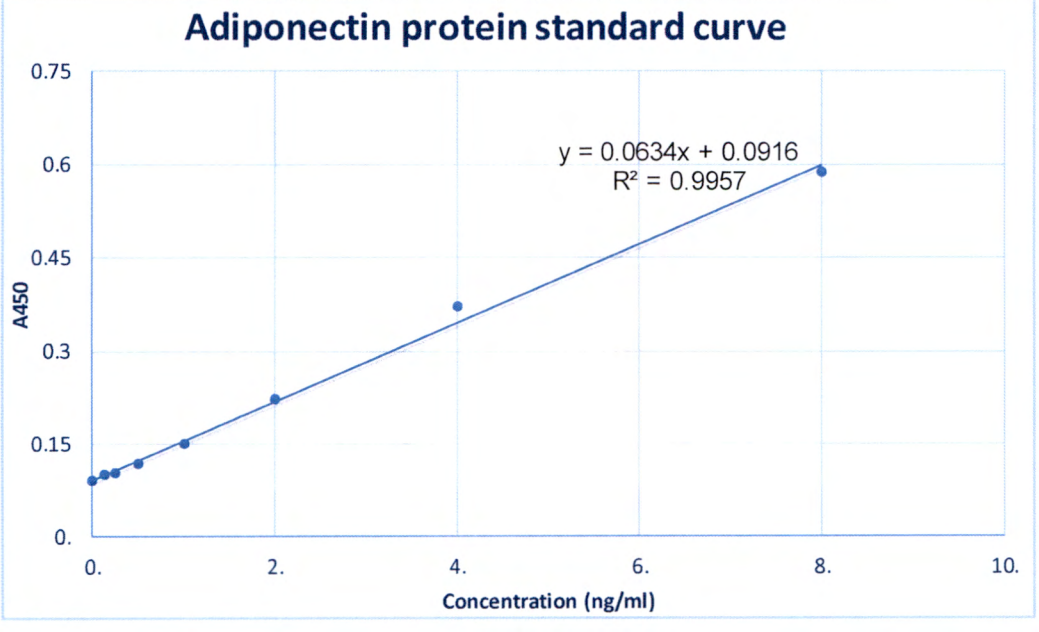

Figure 3.4: Figure showing adiponectin protein standard curve. Concentration of adiponectin standard is represented in ng/ml and the equation of the standard curve is shown in the figure.

on a shaker at room temperature. The membrane was washed twice with PBS-T for 5 minutes each. The ECL plus kit detection method was performed in the dark room according to the manufacturer's protocol (Bio-Rad Laboratories, United States). The membrane was viewed under the doc machine (Bio-Rad Laboratories, USA).

3.5 Measurement of adiponectin concentration using ELISA

The rat adiponectin ELISA kit (Sigma-Aldrich, United States) was used to measure adiponectin concentration in blood plasma according to the manufacturer's protocol. Briefly, 1X wash buffer was prepared by diluting the 20X wash buffer with deionized water and the 1X ELISA buffer was also prepared by diluting the 5X ELISA buffer with deionized water. The rat adiponectin protein standard was reconstituted with 1ml of deionized water and serial dilution of the standard were prepared. Then 1X biotinylated rat adiponectin detection antibody solution was prepared by diluting the detection antibody 1:1000 in 1X ELISA buffer and 1X HRP-Streptavidin solution was prepared by diluting HRP solution 1:100 in 1X ELISA buffer.

The protein standard and samples were added into appropriate wells 100 µl to each well and the plate was incubated at room temperature for 2.5 hours with gentle shaking. The solution was discarded and the wells were washed 4 times with 300µl 1X wash solution. Then 100 µl of 1X prepared biotinylated detection antibody was added to each well and the plate was incubated for 1 hour at room temperature with gentle shaking. The solution was discarded and the wells were washed 4 times with 300 µl 1X wash solution again. Then 100 µl of the prepared HRP-Streptavidin solution was added to each well and the plate was incubated for 45 minutes at room temperature with gentle shaking. The solution was discarded and the wells were washed 4 times with 300 µl 1X wash solution for the last time. Then 100 µl of ELISA colorimetric TMB reagent was added

Chapter 4: Results and Discussion

4.1 Concentration of adiponectin in blood plasma

Adiponectin is a protein secreted from the adipocytes, and has been reported to improve insulin sensitivity and reduce the risk of T2D (Goto *et al.*, 2014). Since adiponectin concentration levels are reduced in diabetes and obesity conditions, this study sought to assess if adiponectin levels are influenced by oleonolic acid.

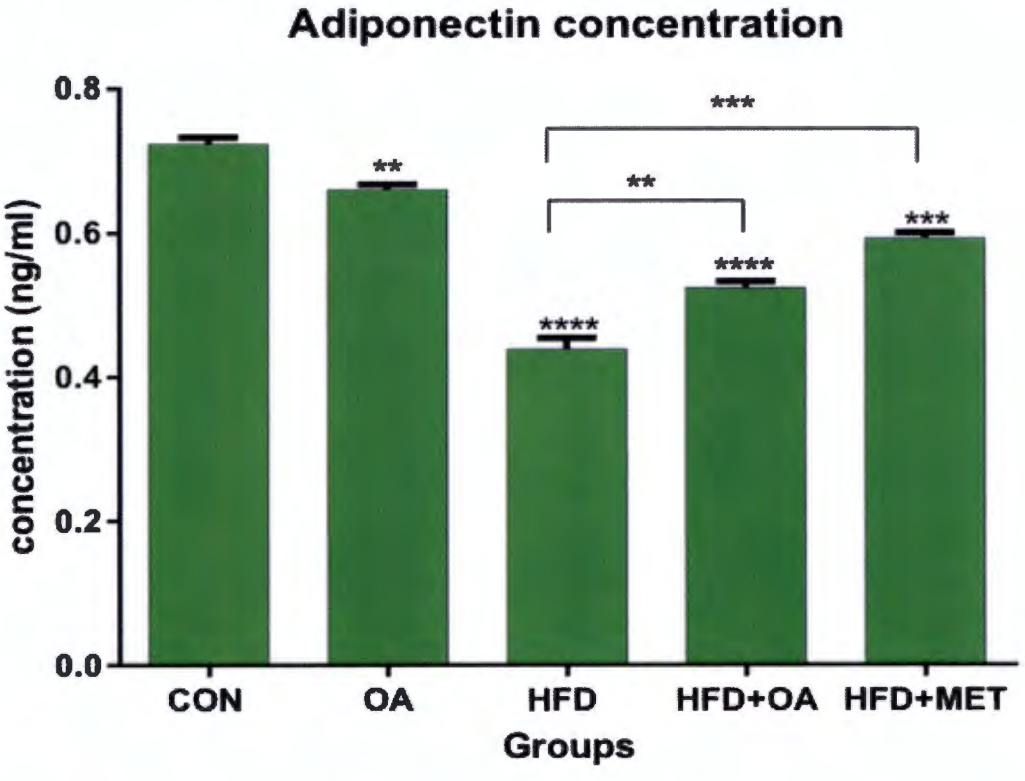

*Figure 4.1: Concentration of adiponectin in Sprague Dawley rat blood plasma amongst the five treatment groups. Concentration is represented in ng/ml and data is represented as mean ± SEM. The P value representing the degree of significance difference amongst the groups is indicated with stars (**<0.01; ***<0.001; ****<0.0001). CON (control), OA (oleanolic acid), HFD (high fructose diet), MET (Metformin).*

The results in figure 4.1 shows the concentration of adiponectin in blood plasma of Sprague Dawley rats in response to various treatments used. In this experiment the HFD group had the lowest adiponectin compared to all the other groups studied. The OA group had ~1.5-fold increase in adiponectin concentration compared to the HFD group which represent rats that were induced with metabolic dysfunction. The decrease of adiponectin in OA vs CON could be due to the reaction of rats against foreign molecule since oleanolic acid is not synthesised in the body. The HFD+OA group had ~1-fold increase in adiponectin concentration whereas the HFD+MET group had ~1.3-fold increase in adiponectin concentration compared to HFD group. These results indicate that adiponectin concentration was suppressed in the HFD group. However, this was reversed when HFD was used with oleonolic acid indicating promising influence of OA in metabolic dysfunction.

The high fructose diet fed rats serve as a model that mimics the human metabolic syndrome with its many aspects, including insulin resistance, hypertriglyceridemia, hypertension and compensatory hyperinsulinemia (Sharabi *et al.*, 2007). Results from this study confirmed that high fructose diet induced insulin resistance/metabolic dysfunction which correlated with the suppression of adiponectin concentration levels in the blood plasma as measured by ELISA. Furthermore, administration of oleanolic acid as the potential anti-diabetic plant derived compound showed some modest improvement in raising the concentration level of adiponectin. Compounds that can improve or upregulate the concentration of adiponectin in blood plasma are welcome as they provide alternative means for therapy needed in managing diabetes. This is due to the fact that adiponectin is known as the protein that stimulates insulin sensitivity and thus reducing the risks of T2D development.

This is the first study that has assessed the improvement of adiponectin concentration level in rats fed with high fructose diet when treated with oleanolic acid as the potential anti-diabetic drug. From a clinical perspective, the study may point to possible ways to treat T2D since

oleanolic acid could upregulate the adiponectin concentration although at modest levels. In addition, the exact mechanism through which oleanolic acid stimulates the upregulation of adiponectin concentration in blood plasma was beyond the scope of this study and thus require further elucidation in future.

The findings from this study, are in agreement with a study that was done by Mohammadi et al (2014), although they used *Zataria multiflora* extracts to evaluate insulin sensitivity in high fructose diet on streptozotocin induced diabetic rats (Mohammadi *et al.*, 2014). In that study, *Zataria multiflora* also increased the level of serum adiponectin concentration significantly.

4.2 Gene expression results using qPCR

4.2.1.1 AMPK expression in skeletal muscles tissue

As already alluded, AMPK enzyme is known as the main regulator of both lipid and glucose metabolic pathways. In this study we sought to investigate the influence of oleonolic acid on AMPK expression in HFD induced rats.

Rats were divided into five experimental groups according to the protocol explained in section 3.1 and muscle excised for analysis. RNA was then extracted in skeletal muscle of rats in the five different groups respectively. Real time qPCR was performed using sybergreen according to the protocol explained in section 3.3. Specific primers for AMPK gene were used and GAPDH primers were used as the reference gene, the sequence of primers used are listed in table 3.1.

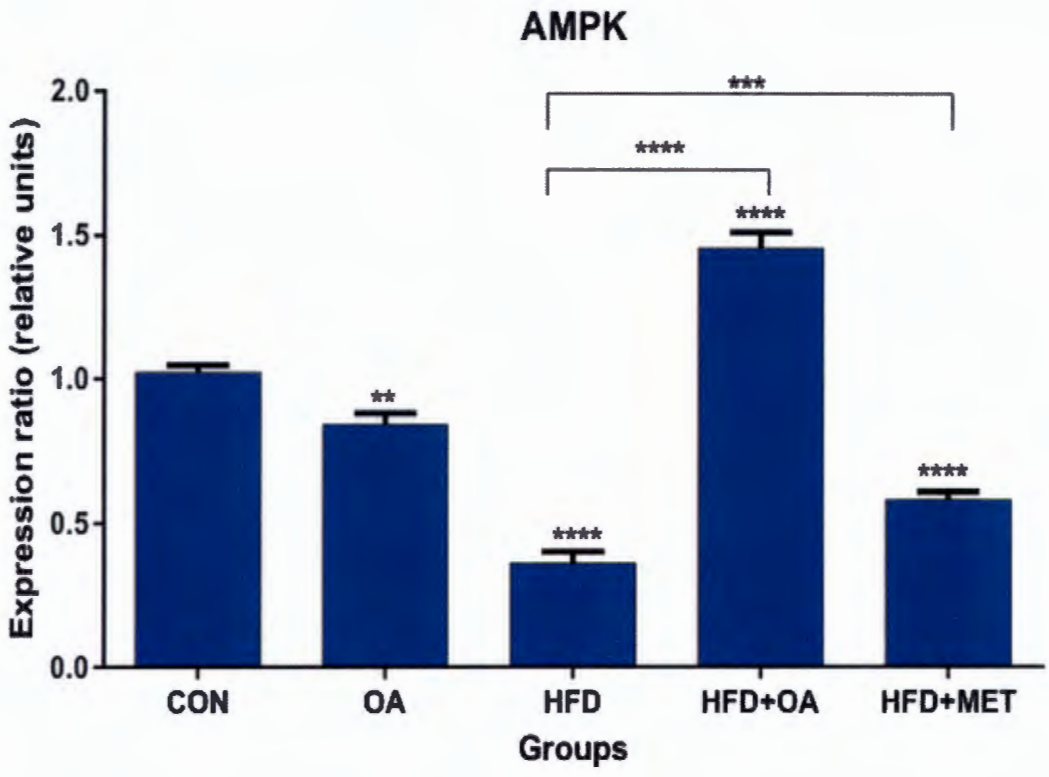

*Figure 4.2: AMPK gene expression of Sprague Dawley rats amongst the five treated groups. The expression ratio of the gene is represented in relative units. Data is represented as mean ± SEM. The P value representing the degree of significance difference amongst the groups is indicated with stars (**<0.01; ***<0.001; ****<0.0001). CON (control), OA (oleanolic acid), HFD (high fructose diet), MET (Metformin).*

The results in figure 4.2 show the gene expression of AMPK in the skeletal muscle tissue of Sprague Dawley rats. Rats fed with HFD showed a decrease of AMPK gene expression compared to the control group. However, the group of HFD+OA showed ~4-fold increase of AMPK gene expression compared to the HFD group. Furthermore, HFD+MET group showed ~1.6-fold increase of AMPK gene expression compared to HFD group. Metformin is used for its antidiabetic effect (Deschemin *et al.*, 2017). For many years metformin lowers blood glucose levels by inhibiting hepatic glucose production through activation of AMPK (Duca *et al.*, 2015). These results indicate that HFD had negative influence in the expression of AMPK gene, the main regulator of both glucose and lipid metabolism.

AMPK has been the focus of this study because of the role it plays in glucose and lipid metabolism, two main factors in the development of T2D. Activated AMPK stimulates glucose uptake and fatty acid oxidation in skeletal muscle. These results confirm or are agreement with other studies that have shown that excess fructose is deleterious in metabolic function of the cell and off course in the expression of genes known to confer protection against diabetes. In this study, one critical gene that confers protection against diabetes, AMPK, was significantly reduced in HFD fed rats. This has huge implications in the downstream influences of AMPK known to increase lipid oxidation in the mitochondria and increase in glucose transport.

Furthermore, research has demonstrated that oleanolic acid exerts beneficial effects against diabetes and metabolic syndrome (Castellano *et al.*, 2013). According to Castellano et al (2013), oleanolic acid stimulates AMPK in human HepG2 hepatoma cells and similarly maslinic acid a related natural triterpene to oleanolic acid with antidiabetic effects activates AMPK and improves insulin sensitization in mice. Results obtained showed that administration of OA significantly increases AMPK gene expression which proves that OA could be a promising drug on T2D alleviation.

4.2.1.2 AMPK protein expression using Western blot

Following the central dogma of molecular biology, we assessed AMPK protein expression following the assessment of its gene in the figure 4.2. Briefly, total protein was extracted from skeletal muscle tissue then run in SDS-PAGE. Western blot was then performed with specific antibody against phosphorylated AMPK.

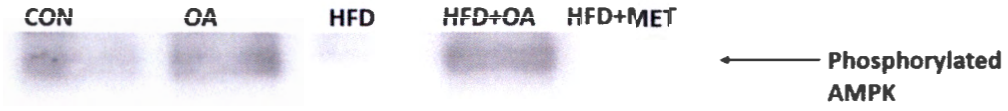

Figure 4.3: The figure showing phosphorylated AMPK protein expression in Sprague Dawley rat skeletal muscles, amongst the five groups. CON (control), OA (oleanolic acid), HFD (high fructose diet), MET (Metformin).

The results in figure 4.3 show the expression of phosphorylated AMPK protein in skeletal muscle tissue of the Sprague Dawley rats after Western blot assay was performed in the five treatment groups. The phosphorylated AMPK protein in the skeletal muscle tissue was decreased in the HFD and HFD+MET group compared to the CON group and all the other groups. Furthermore, the phosphorylated AMPK protein in the skeletal muscle tissue was more increased in the OA and the HFD+OA compared to the CON group. These results indicate that administration of oleanolic acid improved the phosphorylated AMPK protein expression in the skeletal muscle tissue.

AMPK activation occurs through phosphorylation in its 172 threonine and serine 108 residues. Results obtained demonstrated that phosphorylated AMPK protein was more expressed in rats that were given OA only and also in rats that were fed with high fructose diet but received oleanolic acid as treatment (HFD+OA). On the contrary, rats that were fed with high fructose diet to induce insulin resistance showed low phosphorylated AMPK protein expression as well as the rats that received metformin as treatment (HFD+MET). According to Shih et al (2009), activated AMPK/phosphorylated AMPK in muscle increases glucose uptake and fatty acid oxidation. Therefore, results obtained in oleanolic acid shows some promising effect on T2D amelioration since phosphorylated AMPK protein was more expressed in OA compared to the control.

4.2.2 AdipoR1 and AdipoR2 expression in skeletal muscles tissue

The metabolic effect of adiponectin are considered to be stimulated by two receptors, namely, AdipoR1 abundantly expressed in the skeletal muscle, and adipoR2 predominantly expressed in the livers (Chabrolle *et al.*, 2007). Here we studied their expression too to investigate the influence of oleanolic acid on these receptors using qPCR.

Rats were divided into five experimental groups according to the protocol explained in section 3.1. RNA was then extracted in skeletal muscle of rats in the five different groups respectively. Real time qPCR was performed using sybergreen according to the protocol explained in section 3.3.3. Specific primers for AdipoR1 and AdipoR2 gene were used and GAPDH primers were used as the reference gene. The sequence of primers used are listed in Table 3.1.

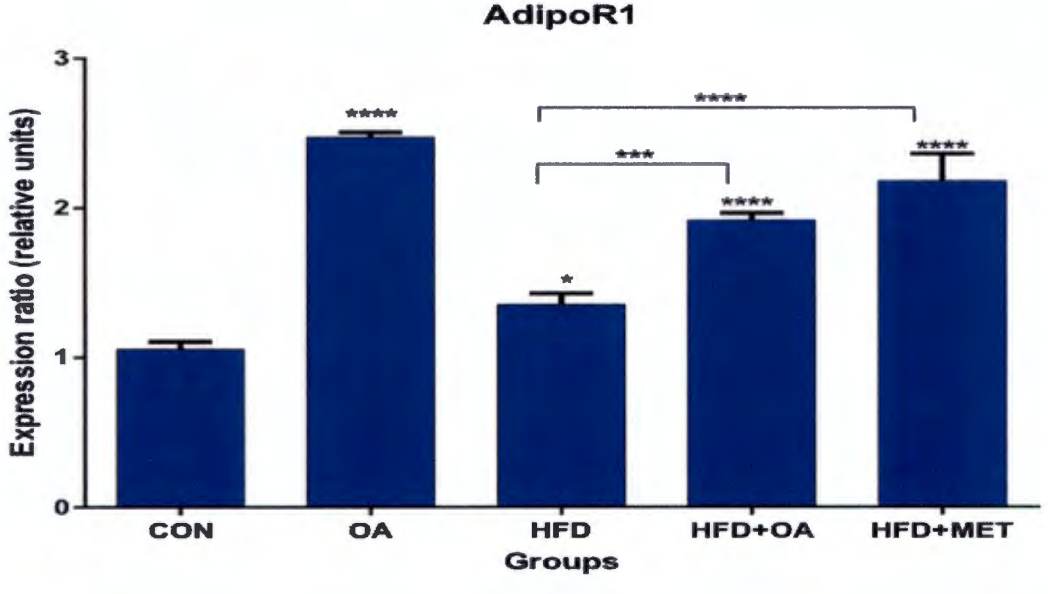

Figure 4.4: The figure showing gene expression of AdipoR1 amongst the five groups. The expression ratio of the gene is represented in relative units. Data is represented as mean ± SEM. The P value representing the degree of significance difference amongst the groups is indicated with stars (<0.1; ***<0.001; ****<0.0001) CON (control), OA (oleanolic acid), HFD (high fructose diet), MET (Metformin).*

The results of AdipoR1 gene expression in skeletal muscle tissue are shown in figure 4.4. The rats in HFD group had low AdipoR1 gene expression compared to all the other groups except for the CON group. The OA group showed ~2-fold increase in AdipoR1 gene expression compared to the CON group. In addition, the HFD+OA and HFD+MET groups showed ~1.4- and ~1.6 fold increases respectively in AdipoR1 gene expression compared to the HFD group. These results indicate that AdipoR1 gene was more expressed in the presence of oleanolic acid and Metformin whereas HFD negatively influenced it as shown in figure 4.4.

The adiponectin receptors are thought to transmit the insulin sensitizing effects of adiponectin (Bonnard *et al.*, 2008). This is due to the fact that adiponectin receptors play an important role in the mechanism through which adiponectin elicit response. If receptors are not available to bind adiponectin molecules, the good effects produced by adiponectin will be inhibited. According to Beylot et al (2006), adiponectin receptors are found to be suppressed in the case of diabetes. In this study the AdipoR1 gene expression was analysed in the insulin resistance induced rats treated with OA. The results showed a ~1.4-fold increase in AdipoR1 gene expression compared to the HFD group. From the results, rats that received treatment with OA showed a ~1.4-fold increase in AdipoR1 gene expression compared to the diseased rats. Based on the results obtained, OA was able to improve the AdipoR1 gene expression that is well known to be down regulated in the case of diabetes. In addition, AdipoR1 is abundantly expressed in the skeletal muscle (Chabrolle *et al.*, 2007). In this study AdipoR1 gene expression was accessed in rat skeletal muscle tissue were it is known to be in abundance. The results showed AdipoR1 gene expression amongst all the five groups in this study as shown in figure 4.4.

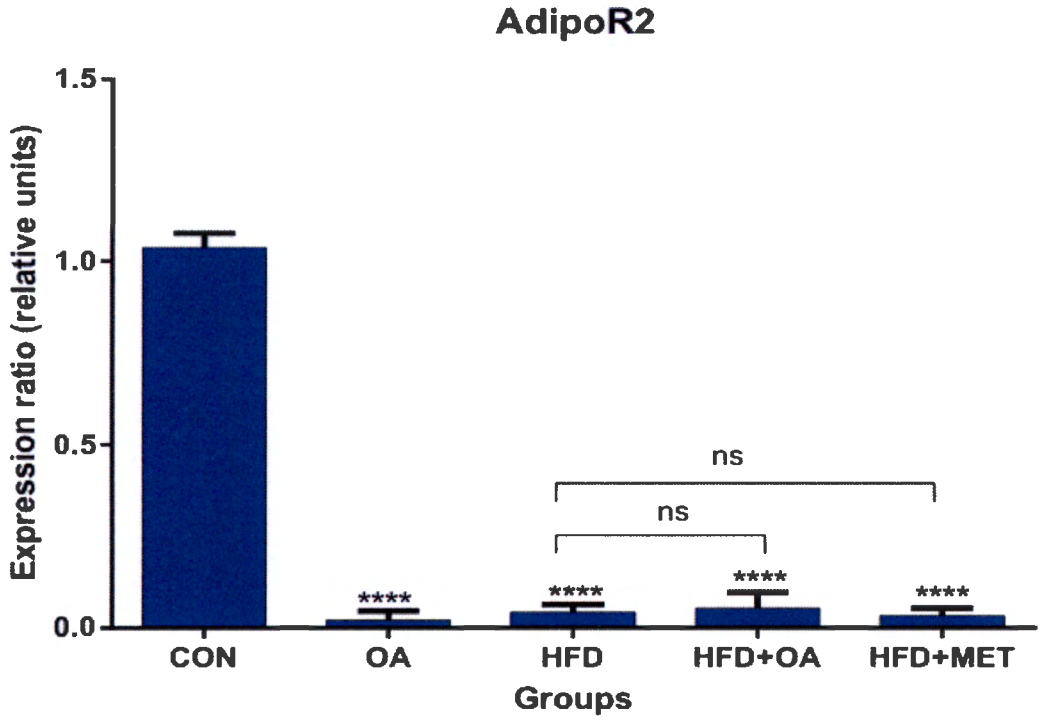

*Figure 4.5: The figure showing gene expression of AdipoR2 amongst the five groups. The expression ratio of the gene is represented in relative units. Data is represented as mean ± SEM. The P value representing the degree of significance difference amongst the groups is indicated with stars (****<0.0001; ns = no significant difference). CON (control), OA (oleanolic acid), HFD (high fructose diet), MET (Metformin).*

The results in figure 4.5 show the gene expression of AdipoR2 in rat skeletal muscle tissue. The AdipoR2 gene was low expressed in all the groups compared to the CON group. AdipoR2 is primarily found in hepatic tissues hence the results showed low expression of adipoR2 in the skeletal muscle tissue. These results indicate that AdipoR2 was not expressed in rat skeletal muscle tissue.

From the results obtained, AdipoR2 was not well expressed in all the groups compared to the CON group. Furthermore, there was no significance difference in AdipoR2 gene expression on rats induced with insulin resistance group (HFD) compared to rats in groups that received treatment (HFD+OA and HFD+MET). These results correspond to the reports that indicate that AdipoR2 is predominantly expressed in the livers not in the skeletal muscles (Chabrolle *et*

al., 2007). In this study the AdipoR2 gene expression was assessed in skeletal muscle where it is not predominantly expressed hence results obtained shown low AdipoR2 gene expression in all the groups compared to the control.

4.2.3 IL-6 and IL-10 expression in skeletal muscles tissue

The role of inflammation in the pathogenesis of T2D and associated complications has long been well established (Donath, 2014). Literature has shown that systemic inflammatory markers are risk factors for the development of T2D and metabolic dysfunction (Emanuela *et al.*, 2012). In this study gene expression of pro-inflammatory cytokine IL-6 and anti-inflammatory cytokine IL-10 were studied. This is due to the recent studies that have suggested that IL-6 is associated with insulin resistance, T2D and its complications (Bastard *et al.*, 2006). Furthermore, IL-6 has been reported to be up-regulated in insulin resistance and obesity individuals (Coughlan *et al.*, 2014), whereas IL-10 has been found to be elevated in diabetic patients.

In this study gene expression of IL-6 and IL-10 were analysed in skeletal muscles tissues of male Sprague Dawley rats. Rats were divided in five experimental group explained in section 3.1. RNA was then extracted in skeletal muscle of rats in the five different groups respectively. Real time qPCR was performed using sybergreen according to the protocol explained in section 3.3.3. Specific primers for IL-6 and IL-10 gene were used and GAPDH primers were used as the reference gene, the sequence of primers used are listed in table 3.1.

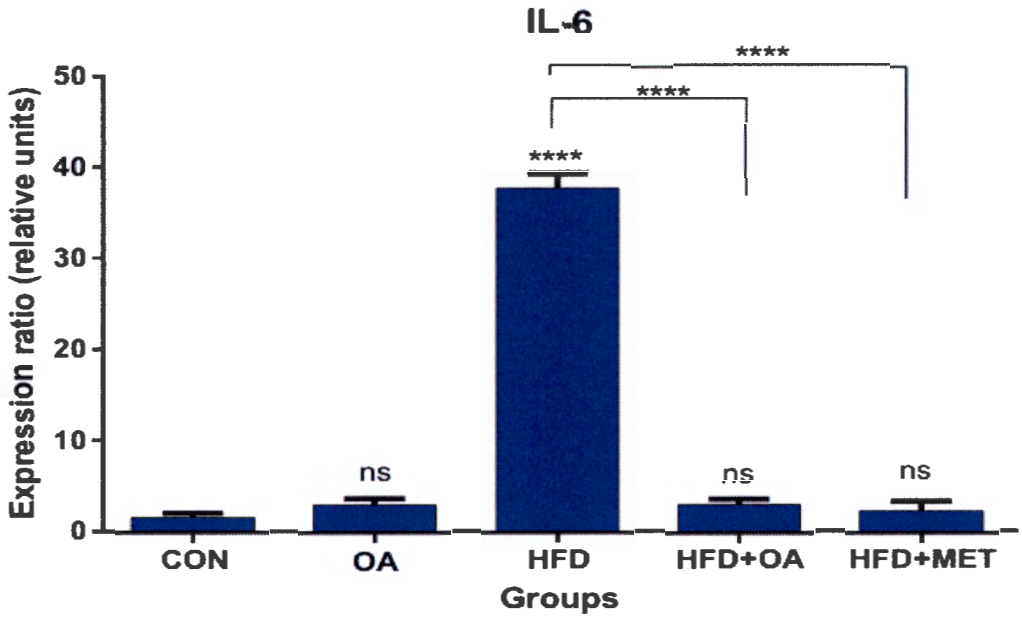

*Figure 4.6: The figure showing gene expression of IL-6 amongst the five groups. The expression ratio of the gene is represented in relative units. Data is represented as mean ± SEM. The P value representing the degree of significance difference amongst the groups is indicated with stars (****<0.0001; ns = no significant difference). CON (control), OA (oleanolic acid), HFD (high fructose diet), MET (Metformin).*

Results in figure 4.6 show that rats in HFD group showed an increase of ~24-fold increase of IL-6 gene expression compared to all other groups studied. These results indicate that IL-6 was expressed more in the group of HFD which represent rats induced with diabetes or insulin resistance. When oleanolic acid was used either with HFD or MET, the IL6 expression was not increased.

The results obtained here further indicates that HFD has negative and deleterious effects of diabetes. On the other hand oleonolic acid has consistently shown to have the opposite effects induced by HFD alone. These results agree with the reports that demonstrated that IL-6 was upregulated in insulin resistance and obesity individuals (Coughlan *et al.*, 2014). Furthermore there was no significance difference amongst the groups that were fed with high fructose diet and received treatment (HFD+OA and HFD+MET) compared to the CON group.

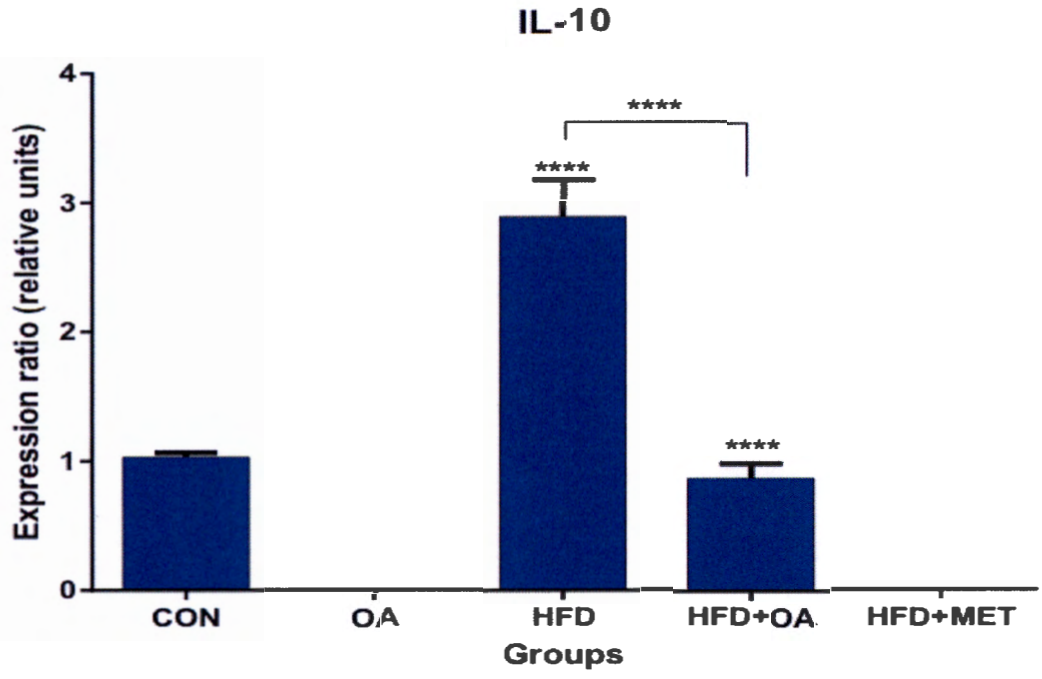

*Figure 4.7: The figure showing gene expression of IL-10 amongst the five groups. The expression ratio of the gene is represented in relative units. Data is represented as mean ± SEM. The P value representing the degree of significance difference amongst the groups is indicated with stars (****<0.0001). CON (control), OA (oleanolic acid), HFD (high fructose diet), MET (Metformin).*

The expression of IL-10 gene was higher by ~2.8-fold in the HFD group compared to the CON group. The HFD+OA group showed low IL-10 gene expression compared to the HFD and CON group. The OA and HFD+MET group were not detectable in response to IL-10 gene expression. The results in figure 4.8 indicate that IL-10 gene was more expressed in the rats that were induced with diabetes or insulin resistance. Again the HFD showed that it increased inflammatory expression known to increase diabetes.

Results in this study followed the previous figure 4.6, where IL6 was studied and found to be increased by HFD similarly as in this figure. The introduction of OA did not increase these inflammatories, indicating that it has desirable effects towards markers of diabetes or insulin resistance. Furthermore, results of rats fed with high fructose diet are in agreement with previous reports indicating that IL-10 is elevated in diabetes patients and the elevation is

believed to be caused by the compensatory mechanism to the increase in the pro-inflammatory cytokine (Cardoso *et al.*, 2017).

4.2.4 GLUT-4 expression in skeletal muscles tissue

GLUT-4 is the main glucose transporter encoded by the gene Glut-4. GLUT-4 is found on the skeletal muscle and adipose tissue cell membrane. Research has indicated that GLUT-4 is down-regulated in T2D or insulin resistance condition (Gibbs *et al.*, 1995). It with this reason that we sought to investigate the main glucose transporter expression in response to OA treatment.

RNA was then extracted from all different skeletal muscle tissues of rats in their distinct groups respectively. In this study GLUT-4 gene expression was analysed by real time qPCR, method explained in section 3.3.3. Specific primers for GLUT-4 gene were used and GAPDH gene as the reference gene, the sequence of primers used are listed in table 3.1.

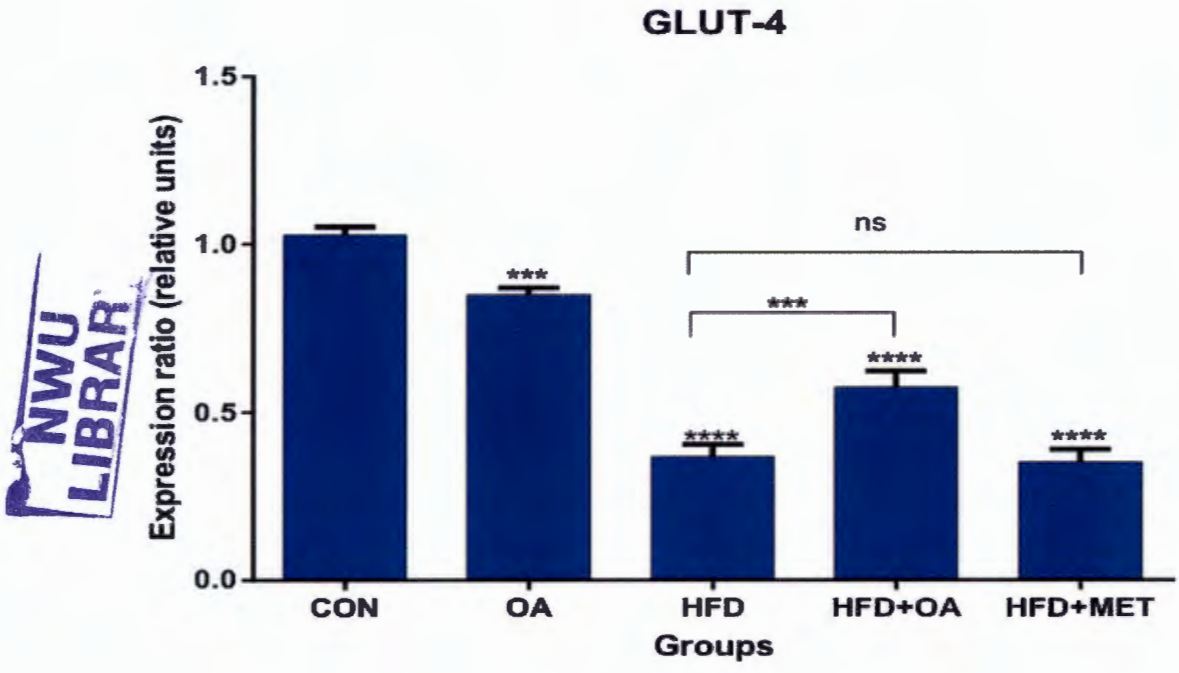

*Figure 4.8: The figure showing gene expression of GLUT-4 amongst the five groups. The expression ratio of the gene is represented in relative units. Data is represented as mean ± SEM. The P value representing the degree of significance difference amongst the groups is indicated with stars (***<0.001; ****<0.0001; ns = no significant difference). CON (control), OA (oleanolic acid), HFD (high fructose diet), MET (Metformin).*

The GLUT-4 gene was more expressed in the OA group compared to all the other groups except with the CON group (figure 4.8). The HFD+OA group showed ~1.5-fold GLUT-4 gene expression compared to the HFD group whereas there was a small difference in the GLUT-4 gene expression between the HFD and HFD+MET group. The results indicate that oleanolic acid enhanced the expression of GLUT-4 gene.

Results in this study demonstrated that GLUT-4 was suppressed in rats fed with high fructose diet. These results correspond to the literature that has reported that GLUT-4 is down regulated in insulin resistance condition (Gibbs *et al.*, 1995). Furthermore, OA increased GLUT-4 gene expression with ~1.5-fold in rats that were fed with high fructose diet and received OA as a treatment (HFD+OA). The increase in GLUT-4 gene expression is considered as positive effect because this will lead to increase in glucose uptake by the skeletal muscle and the adipose

tissue and this will result in amelioration of diabetes. In addition, rats that received metformin as a treatment (HFD+MET) were expected to also improve GLUT-4 gene expression compared to the rats that were fed with high fructose diet only without treatment (HFD). Results showed no significant difference between the two groups.

4.2.5 CPT-1 expression in skeletal muscles tissue

Lipid catabolism occurs mainly in the mitochondria and is controlled by a set of mitochondrial enzymes responsible for both lipid oxidation and synthesis. CPT-1 is the gate keeper for entry of fatty acids into the mitochondria (Winder & Hardie, 1999). The study investigated CPT-1 expression in response to various treatments especially influence of oleanolic acid.

Male Sprague Dawley rats were divided into five groups as indicated in figure 3.1 and the rats were subjected to the experiment explained in section 3.3.3. On the last day of the experiment, skeletal muscle tissues of rats from different groups were collected. RNA was then extracted from all different skeletal muscles tissues of rats in their distinct groups respectively. In this study CPT-1 gene expression was analysed by real time qPCR, method explained in section 3.3.3. Specific primers for CPT-1 gene were used and GAPDH gene as the reference gene, the sequence of primers used are listed in table 3.1.

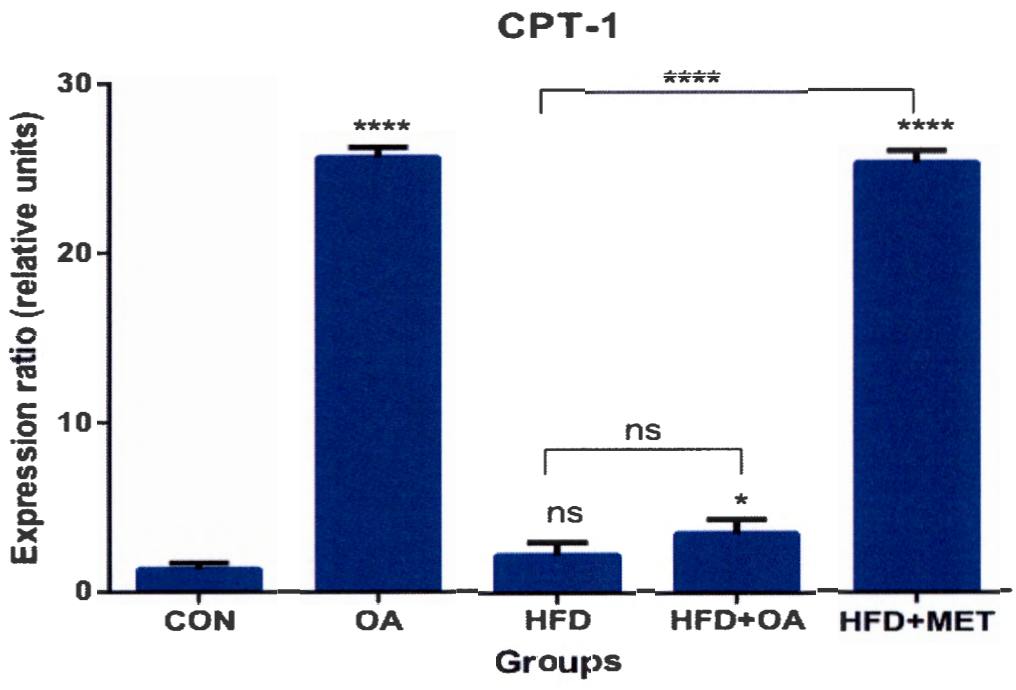

Figure 4.9: The figure showing gene expression of CPT-1 amongst the five groups. The expression ratio of the gene is represented in relative units. Data is represented as mean ± SEM. The P value representing the degree of significance difference amongst the groups is indicated with stars (<0.1; ****<0.0001; ns = no significant* difference). *CON (control), OA (oleanolic acid), HFD (high fructose diet), MET (Metformin).*

The results in figure 4.9 show the gene expression of CPT-1 in the rat muscle tissue. The OA group showed ~18.8-fold increase in CPT-1 gene expression compared to the CON group whereas the HFD+MET group had ~18.5-fold increase in CPT-1 gene expression compared to the CON group. The HFD group had the lowest CPT-1 gene expression compared to all the other groups except with CON group. These results indicate that both oleonolic acid and metformin were able to enhance the CPT-1 gene expression.

Results in this experiment demonstrated that CPT-1 gene was more expressed in rats that were given OA only and rats that were fed with high fructose diet and received metformin as treatment (HFD+MET). Whereas CPT-1 gene expression was low in rats that were given high fructose diet and received oleanolic acid as treatment (HFD+OA). Furthermore, there was no significant difference between these group and the rats that were given high fructose diet only

to induce insulin resistance (HFD). According to Bruce et al (2009), upregulation of CPT-1 result in increased lipid oxidation in the mitochondria at the same time reducing the lipid accumulation level and obesity. The effect of OA was expressed similarly to MET indicating further the potential of OA as modality that could be used in the treatment of diabetes.

4.3 Concentration of inflammatory biomarkers in blood plasma

4.3.1 Analysis of plasma TNF-α concentration

TNF-α is a pro-inflammatory cytokine that has been reported to be upregulated in obesity and it is believed that it contributes to the development of insulin resistance by interfering with the insulin receptor signalling pathway (Meier & Gressner, 2004). The study assessed this cytokine due to its negative effects and in the development of diabetes.

In this study, TNF-α concentration was measured in blood plasma of male Sprague Dawley rats. The rats were divided and treated according to the experimental groups indicated in figure 3.1. Briefly, the rats were fed with high fructose diet to induce insulin resistance, then OA was administered to rats that were fed with HFD to analyse the influence of OA on TNF-α concentration. Metformin was used as a positive control since is a drug that is currently used in amelioration of diabetes. Blood plasma was collected in all the rats on the last day of experiment and stored at -20℃. The method used to achieve this objective is well described in section 3.2.

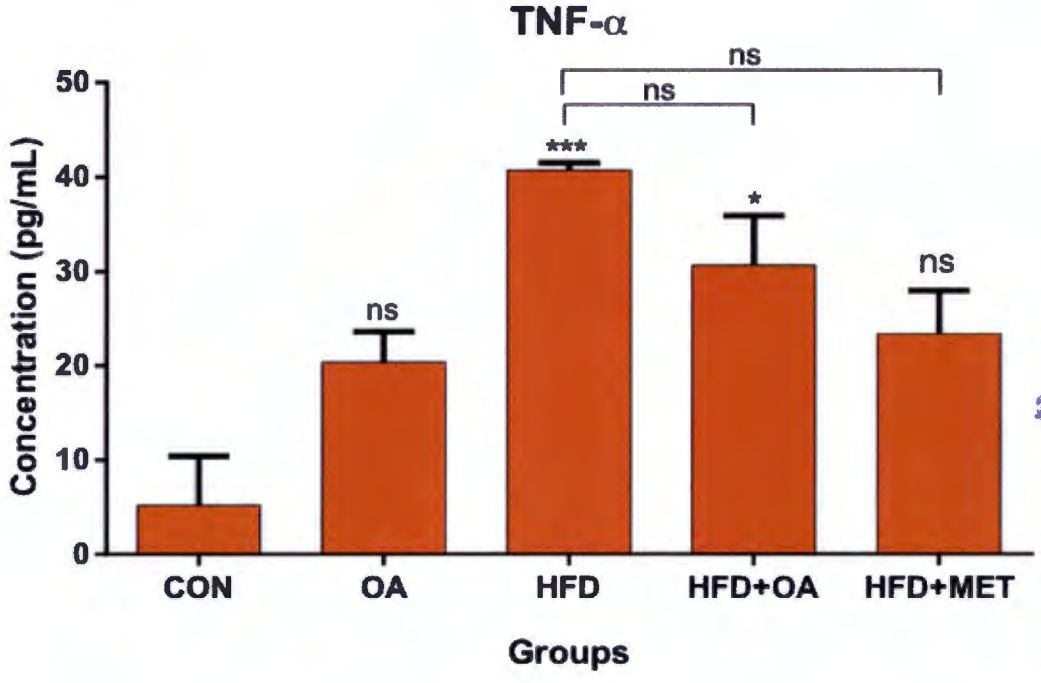

Figure 4.10: Concentration of TNF-α in Sprague Dawley rat blood plasma amongst the five treatment groups. Concentration is represented in pg/ml and data is represented as mean ± SEM. The P value representing the degree of significance difference amongst the groups is indicated with stars (<0.1; ***<0.001; ns = no significant difference). CON (control), OA (oleanolic acid), HFD (high fructose diet), MET (Metformin).*

The results in figure 4.10 shows the concentration of TNF-α in blood plasma of Sprague Dawley rats. The HFD group had ~78.4-fold increase in TNF-α concentration compared to the CON group. On the other hand, OA had the lowest TNF-α compared to other treatments. Furthermore, HFD+MET and HFD+OA group had lower TNF-α concentration compared to the HFD group. These results indicate that TNF-α was upregulated in groups that had high fructose diet and the groups that lack high fructose diet had suppressed TNF-α concentration in blood plasma.

Results obtained in this study are consistent with the previous studies which found that consumption of HFD upregulates TNF-α and consequently induce insulin resistance (Meier &

Gressner, 2004). Rats that were fed with HFD had the highest increase in TNF-α plasma concentration compared to the control group that had normal rat standard diet.

Since upregulation of TNF-α is associated with the development of insulin resistance, down-regulation of TNF-α concentration could be an alternative way of combating insulin resistance which characterise T2D (Meier & Gressner, 2004). In this study OA as a compound believed to have anti-diabetic effects was used to treat rats that were fed with HFD. Results showed that OA decreased TNF-α concentration in rats that were treated with OA failed to return TNF-α concentration to its normal state.

4.3.2 Analysis of plasma IL-6 concentration

There is now clear evidence supporting the interaction occurring between cytokines and insulin signalling pathways. IL-6 is a pro-inflammatory cytokine believed to be involved in insulin resistance, T2D and its complications after assessing gene expression of IL-6, we sought to also study its concentration in the blood of these rats.

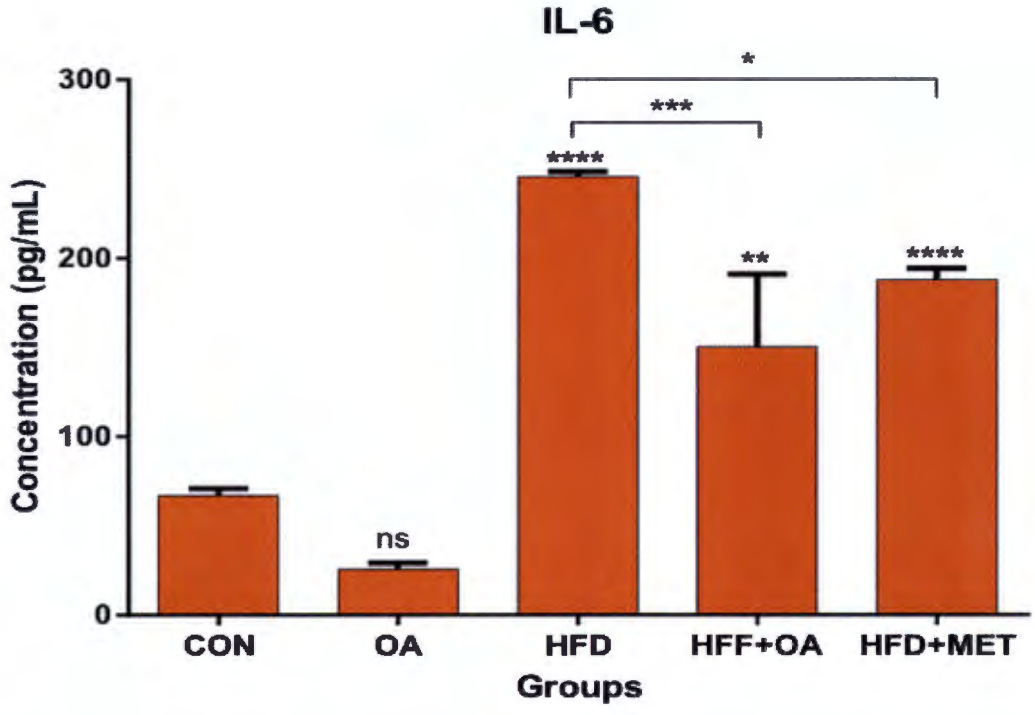

Figure 4.11: Concentration of IL-6 in Sprague Dawley rat blood plasma amongst the five treatment groups. Concentration is represented in pg/ml and data is represented as mean ± SEM. The P value representing the degree of significance difference amongst the groups is indicated with stars (<0.1; **<0.01; ***<0.001; ****<0.0001; ns = no significant difference). CON (control), OA (oleanolic acid), HFD (high fructose diet), MET (Metformin).*

Results in figure 4.11 shows that the HFD group had ~3.7-fold IL-6 concentration increase compared to the CON group. Both HFD+OA and HFD+MET groups had low IL-6 concentration compared to the HFD group. These results indicate that groups containing oleanolic acid suppressed the observed increased in the concentration of IL-6 in the rats fed with high fructose diet.

In this study, we found that IL-6 concentration was increased with HFD treated rats whereas decreased with oleanolic acid treatment. These results are in agreement with those of Hsieh et al (2013) who showed that concentration of IL-6 in adipose tissue which were elevated in high

fructose treatment were markedly decreased after *lactobacillus reuteri* feeding in male Sprague Dawley rats (Hsieh *et al.*, 2013).

Furthermore, this study shows that oleanolic acid decreased IL-6 concentration levels that were elevated in rats fed with high fructose diet. Level decrease of IL-6 concentration in blood plasma is considered as a positive effect in T2D due to the reports that indicate that IL-6 concentration in blood plasma is upregulated by obesity and in T2D (Guo *et al.*, 2012). This elevation is believed to be one of the factors that play a critical role in the etiology of T2D.

4.3.3 Analysis of plasma MCP-1 concentration

MCP-1 is a secretory protein which specifically attracts blood monocytes and tissue macrophage to its source through its cell surface receptor CCR2. These findings strongly support that suppression of MCP-1 in diabetic patients could be a potential target in ameliorating diabetes. In this study we sought to study MCP-1 response in relation to various treatments used in these rats.

In this study MCP-1 concentration was measured in blood plasma of male Sprague Dawley rats. The rats were divided and treated according to the experimental groups indicated in figure 3.1. Briefly, rats were fed with high fructose diet to induce insulin resistance, then OA was administered to rats that were fed with HFD to analyse the influence of OA on MCP-1 concentration. Metformin was used as a positive control since it is a drug that is currently used in the amelioration of diabetes. Blood plasma was collected in all the rats on the last day of experiment and stored at -20℃. The method used to achieve this objective is well described in section 3.2.

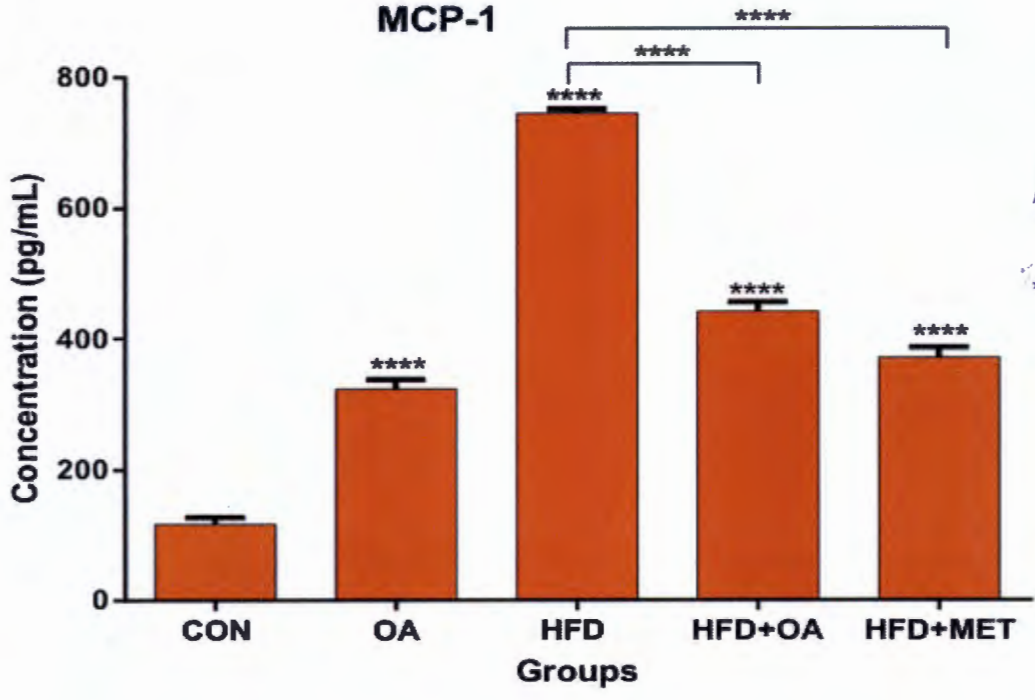

*Figure 4.12: Concentration of MCP-1 in Sprague Dawley rat blood plasma amongst the five treatment groups. Concentration is represented in pg/ml and data is represented as mean ± SEM. The P value representing the degree of significance difference amongst the groups is indicated with stars (****<0.0001). CON (control), OA (oleanolic acid), HFD (high fructose diet), MET (Metformin).*

The results in figure 4.12 show that rats in HFD group had ~6.4-fold MCP-1 concentration increase compared to CON. The OA, HFD+OA and HFD+MET groups had low MCP-1 concentration compared to the HFD group. These results indicate that MCP-1 was more expressed in the HFD group and the oleanolic acid and metformin showed suppression in the expression of MCP-1 concentration.

MCP-1 is known to be elevated in diabetic patients. According to the study done by Jain et al (2009), diabetic rats demonstrated elevated MCP-1 in blood plasma similar to those observed in diabetic patients (Jain *et al.*, 2009). In this study, rats fed with HFD to induce insulin resistance showed elevated MCP-1 with ~6.4-fold compared to the control. Literature supports that elevated MCP-1 plays a critical role in the etiology of T2D. In this study OA as an

antidiabetic drug was used to see if it will have an influence of suppressing MCP-1 levels in blood plasma. From the results obtained, OA reduced the MCP-1 levels in rats that were treated with OA (HFD+OA) compared to the rats that did not receive any treatment (HFD). Furthermore the OA was not able to return the MCP-1 level to its normal state since the HFD+OA group had higher MCP-1 level than the CON group as shown in figure 4.12.

4.3.4 Analysis of plasma VEGF concentration

Literature has demonstrated that VEGF is associated with the pathogenesis of diabetic retinopathy which is a leading cause of vision loss. Furthermore, research has also demonstrated that plasma VEGF is elevated in patients with diabetes and the elevated levels correlate with endothelial damage and cardiovascular risk in hypertension patients. These was the reasons we sought to assess the levels of VEGF in response to OA and HFD treatments in these rats.

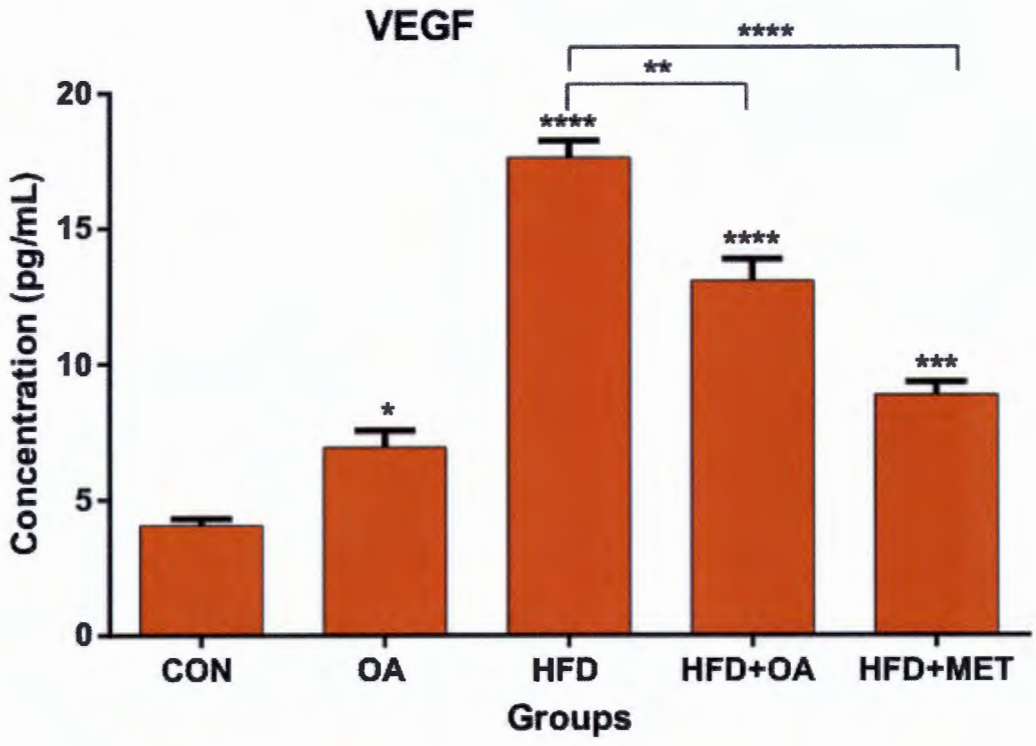

Figure 4.13: Concentration of VEGF in Sprague Dawley rat blood plasma amongst the five treatment groups. Concentration is represented in pg/ml and data is represented as mean ± SEM. The P value representing the degree of significance difference amongst the groups is indicated with stars (<0.1; **<0.01; ***<0.001; ****<0.0001). CON (control), OA (oleanolic acid), HFD (high fructose diet), MET (Metformin).*

The results in figure 4.13 shows the concentration of VEGF in blood plasma of Sprague Dawley rats. The rats in the HFD group had ~4.3-fold VEGF concentration increase compared to the control. The CON group had lowest VEGF concentration compared to all the other groups. The OA, HFD+OA and HFD+MET showed low VEGF concentration compared to the HFD. These results indicate that VEGF concentration was more upregulated in the HFD group and oleanolic acid as well as the metformin suppressed the expression of VEGF concentration.

From the results obtained, VEGF concentration levels in blood plasma were elevated with ~4.3-fold in rats fed with HFD to induce insulin resistance compared to the CON group. According to Lim et al (2005), plasma VEGF is known to be elevated in patients with diabetes and the

elevated levels are associated with endothelial damage and diabetic retinopathy. Reducing the VEGF concentration levels in blood plasma can be considered as a good effect in amelioration of diabetes. In this study, OA was able to reduce the level of VEGF concentration in the rats that were treated with OA (HFD+OA) compared to the rats that did not get treatment (HFD). Furthermore, the results also showed that the damage made by HFD was not reversed back to normal state since VEGF concentration level of group (HFD, HFD+OA and HFD+MET) were higher than the CON group as shown in figure 4.13.

Chapter 5: Discussion

This study was aimed to assess the influence of oleanolic acid on adiponectin regulation of AMPK as an alternative therapeutic pathway that could lead to T2D alleviation. The study was motivated by reports that demonstrated that adiponectin's mechanism of action is through AMPK activation (Ziemke & Mantzoros, 2010). Activated AMPK is known to play a beneficial role in T2D amelioration by regulating glucose and lipid metabolism gene. Therefore, genes associated with glucose and lipid metabolism as well as playing a crucial role in T2D (GLUT-4 & CPT-1) were assessed. Furthermore, this study also evaluated the effect of oleanolic acid on inflammatory cytokines (TNF-α, IL-6, IL-10, MCP-1 & VEGF). These experiments were done due to reports indicating that inflammatory cytokines are upregulated in obesity and they contribute to the development of insulin resistance. This study also indicated that some inflammatory cytokine play a role in adiponectin expression. The purpose of this why this study was to address the lack of a therapeutic much needed to combat T2D since is growing at an alarming rate with no cure to date.

Studies have shown that adiponectin plasma concentration is down-regulated in T2D and obesity related insulin resistance (Bonnard *et al.*, 2008). Adiponectin stimulates fatty acid oxidation and glucose transport increase in skeletal muscles which result in insulin sensitivity increase. As previously discussed, adiponectin exerts its response through activation of AMPK. In this study, oleanolic acid significantly up-regulated adiponectin concentration with ~1-fold in rats that were induced with insulin resistance. This is a positive effect exerted by oleanolic acid since an increase in adiponectin plasma concentration will lead to increase in insulin sensitivity.

Furthermore, gene expression of adiponectin receptors (AdipoR1 & AdipoR2) in skeletal muscles were assessed. This experiment was done due to the fact that, for adiponectin to elicit

its response it has to bind first to its receptors. Some studies have indicated that adiponectin receptors are suppressed in insulin resistance condition (Beylot *et al.*, 2006). In this study, oleanolic acid significantly increased AdipoR1 gene expression with ~1.4 fold in rats that were treated with oleanolic acid compared to the rats that were induced with insulin resistance. On the other hand, AdipoR2 was poorly expressed in all the groups compared to the control. These findings correspond to the reports that indicated that AdipoR2 predominates in the liver tissue and AdipoR1 dominates in skeletal muscle tissue. Again, these results suggest that oleanolic acid could be a potential compound in T2D alleviation.

Since AMPK is considered as the master regulator of both glucose and lipid metabolism and as a potential therapeutic target in alleviation of T2D. In this study, AMPK gene and phosphorylated AMPK protein expression were assessed. Oleanolic acid significantly increased AMPK gene expression with ~4-fold in rats that were treated with oleanolic acid compared to those that were induced with insulin resistance while phosphorylated AMPK protein was more expressed in rats that were treated with oleanolic acid. These results further confirm that oleanolic acid regulates AMPK and that it could be a potential therapeutic compound in T2D alleviation.

Genes that are involved in glucose transport and lipid oxidation (GLUT-4 & CPT-1) were also assessed. For glucose transport we assessed GLUT-4 gene expression in skeletal muscles tissue, since up-regulation of GLUT-4 gene is associated with increased glucose uptake by the skeletal muscle cells. In this study, oleanolic acid increased GLUT-4 gene expression with ~1.5-fold compared to the rats that were induced with insulin resistance. These findings suggest that oleanolic acid could be a potential therapeutic compound in T2D alleviation. For Lipid metabolism the study assessed CPT-1 gene expression, which plays a crucial role in lipid oxidation as a gate keeper for entry of fatty acids into the mitochondria (Winder & Hardie, 1999). In this study, there was no significance difference in CPT-1 gene expression between

rats that were treated with oleanolic acid and rats were induced with insulin resistance. However, oleanolic acid administered alone in rats that were not induced with insulin resistance was able to up-regulate CPT-1 gene expression. Since oleanolic acid did not manage to up-regulate CPT-1 gene expression in rats that were induced with insulin resistance, it could be said that oleanolic acid has no potential to improve lipid oxidation.

As mentioned before, T2D and obesity are associated with inflammation due to the state of insulin resistance promoting inflammation since insulin exerts anti-inflammatory effect at cellular and molecular level (Dandona *et al.*, 2004). Lastly, this study assessed the influence of oleanolic acid on inflammatory cytokines gene expression (IL-6 & IL-10) and inflammatory cytokines concentration levels in blood plasma (TNF-α, IL-6, IL-10, MCP-1 & VEGF). Rats fed with a high fructose diet to induce insulin resistance showed significant increased levels in all the cytokine concentration assessed in this study. These findings support the reports that indicated that inflammatory cytokines are found to be increased in insulin resistance and obesity individuals and the increase levels correspond with the degree of insulin resistance (Vettor *et al.*, 2005). Therefore, oleanolic acid was expected to decrease the concentration level of cytokines in blood plasma. Oleanolic acid significantly decreased the inflammatory cytokines concentration level in blood plasma. These findings again support that oleanolic acid could be a potential therapeutic compound in T2D alleviation.

Furthermore, high fructose diet up-regulated IL-6 and IL-10 gene expression in rats that were given a high fructose diet to induce insulin resistance. However, when oleanolic acid was administered to rats that were fed with high fructose diet there was a down-regulation of IL-6 and IL-10 gene expression. These results represent the correspondence between inflammatory cytokine gene expression and the inflammatory cytokine concentration levels in blood plasma. Again, this finding support that oleanolic acid could be a potential therapeutic compound in T2D alleviation.

Chapter 6: Conclusion and Recommendations

In conclusion, this study has shown that oleanolic acid significantly up-regulates adiponectin hormone known to stimulate glucose and lipid metabolism through activation of AMPK enzyme. Oleanolic acid also significantly up-regulated AMPK gene expression and phosphorylated AMPK protein in skeletal muscle, suggesting that oleanolic acid mechanism of action could be through AMPK activation.

Since diabetes is in part due to dysfunction in glucose and lipid metabolism, this study's investigation of oleonolic acid on genes involved in these two pathways showed that oleanolic acid up-regulated these genes whereas HFD decreased them. In addition, oleanolic acid significantly down-regulate inflammatory cytokines that were up-regulated by high fructose diet to induce insulin resistance. These results also show a success in the use of high fructose diet to produce an insulin resistance model for T2D study.

There were some limitation in this study. For example, the study could have been improved by assessing the influence of oleanolic acid on adiponectin gene expression in the adipose tissue in order to have comparison with results obtained from blood plasma. The study could have also assessed all the proteins related to the genes analysed in this study to further confirm that genes obtained correspond to the protein levels.

Oleanolic acid has shown significant up-regulation in GLUT-4 gene expression. However, in future obtaining glucose transport analysis as functional analysis would correlate well with the other studies conducted.

Chapter 7: References

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