The impact of human adipose tissue on metabolic dysfunction in obesity and type 2 diabetes mellitus

By

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DECLARATION

I declare that this thesis is a accurate record of my results obtained by myself within the labs at University of Warwick, Clinical Science Research Institute and, the data that has arisen is detailed in this thesis. All sources of support and technical assistance have been stated in the text of the acknowledgments. None of the work has been previously submitted for a higher degree. All sources have been specifically acknowledged by means of reference.

Synopsis

This thesis sought to investigate how systemic lipids may contribute to the adipocyte derived inflammatory response and highlight how the adipocyte's function can alter in different metabolic states which could contribute to the pathogenesis of type 2 diabetes mellitus (T2DM) and cardiovascular risk. Specifically, this thesis firstly examined the inflammatory nature of lipoprotein-associated phospholipase A2 (LpPLA2), a member of the phospholipase A2 super family of enzymes which previously has been shown to enhance Ox-LDL production in foam cells during arterial inflammation contributing to coronary artery disease. Therefore initial studies sought to (1) characterise PLA2 isoforms in lean, obese, T2DM abdominal subcutaneous (AbdSc) and omental (Om) in human adipose tissue (AT); (2) evaluate the role of lipids and inflammatory markers on circulating LpPLA2, and (3) determine the *in vitro* regulation of LpPLA2 in human adipocytes by its influence on LDL and Ox-LDL.

AT and sera from lean, overweight, obese and T2DM subjects were taken. PLA2 gene expression was determined by microarray, RT-PCR and Western Blot. Associations between circulating LpPLA2 and metabolic parameters were investigated. The human adipocyte cell line, Chub-S7, was used to assess the effects of oxidized LDL (Ox-LDL) and LDL on PLA2 expression.

LpPLA2 mRNA levels were higher in AbdSc AT than Om AT in obesity by 2-fold ($P<0.05$). The cPLA2 protein expression increased with obesity in AbdSc AT ($P<0.01$). T2DM showed increased LpPLA2 mRNA levels in AbdSc ($P<0.001$) and Om AT ($P<0.01$). Serum LpPLA2 showed positive correlations with cholesterol, TG, LDL, endotoxin and Ox-LDL ($P<0.001$) in non-diabetic subjects and with Ox-

LDL (*P<0.001*), LDL (*P<0.01*) and cholesterol (*P<0.05*) in T2DM. In differentiated pre-adipocytes, activation of LpPLA2 protein expression was noted in response to LDL and Ox-LDL (*P<0.001*). The adipocyte appeared to be an active source of LpPLA2, altered by fat depot and metabolic state, with LpPLA2 protein expression induced by LDL and Ox-LDL, *in vitro*. Increased LpPLA2 protein from the adipocyte in obesity and/or T2DM could contribute to raise circulating Ox-LDL, as noted in other studies as well with increasing adiposity, which promotes further inflammation and atherosclerotic risk.

Through the development of these current studies it appeared that how the adipocyte managed lipids was important to how the adipocyte may induce an inflammatory response and pathogenic factors. Therefore subsequent studies investigated how the change in metabolic state such as those derived in T2DM patients that undergo bariatric surgery may not necessarily reverse their inflammatory response. Previous studies from the team have shown that lipids may induce an innate immune response via toll like receptor (TLR) activation therefore subsequent investigations sought to consider the potential role of triglycerides (TG) as another mediator of inflammation. As such studies examined the specific impact of TG changes, pre- and post-bariatric surgery, on TLR expression in *ex vivo* AT and the *in vitro* effects of triglyceride rich lipoprotein (VLDL), on TLR expression in isolated human differentiated pre-adipocytes. Serum and AT was taken from a cohort of Obese, T2DM, female subjects prior to bariatric surgery and 6 months post-surgery. Human differentiated pre-adipocyte Chub S7 cells were again used to examine transcriptional effects of VLDL on TLR expression.

Following surgical intervention, BMI (*P<0.001*), blood glucose (*P<0.001*), insulin (*P<0.001*), HOMA-IR (*P<0.001*), TG (*P<0.05*), Cholesterol (*P<0.001*) and

LDL-cholesterol ($P<0.05$) were significantly improved. There was a significant reduction in TLR-4 mRNA post-surgery ($P<0.01$) irrespective of surgery type. It was also noted that subjects with the greatest drop (55.5% reduction) in TGs post-surgery ($P<0.001$) showed a significant correlated reduction in TLR4 mRNA expression ($P<0.001$). Whilst the *in vitro* treatment of differentiated Chub S7 cells highlighted VLDL induced TLR 4 mRNA expression ($P<0.05$) suggesting the inflammatory impact of lipids on adipocytes. These studies further highlighted that the reduction in AT inflammation appears dependent on how successfully subjects reduce their serum triglyceride, which appears supported by the *in vitro* findings. These studies suggest that bariatric surgery lead to metabolic improvement with weight loss, whilst dietary intervention is still required to ensure TGs reduce to reduce inflammation.

Taken together, these studies and thesis highlight the diverse nature of lipids and their interaction with the adipocyte to impact on their inflammatory response. These data also highlight the importance to maintain a good systemic lipid profile low in TG to reduce adipocyte induced inflammation and that AT may represent an important therapeutic target to reduce inflammation, atherosclerotic risk and development of metabolic complications.

ABBREVIATIONS

µg	Microgram
µl	Microlitre
3T3-L1	Mouse Embryonic Fibroblast Cell Line
AbdSc AT	Abdominal Subcutaneous Adipose Tissue
AbdSc	Abdominal Subcutaneous
adPLA2	Adipose specific PLA2
aiPLA2	acidic $Ca2^{+}$ independent PLA2
ANCOVA	Analysis of Covariance
AT	Adipose Tissue
BAT	Brown Adipose Tissue
BMI	Body Mass Index
BPD	biliopancreatic diversion
BPD-DS	Biliopancreatic diversion with duodenal switch
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
cDNA	Complementary (to mRNA) Deoxyribonucleic acid
ChREBP	Carbohydrate Response Element Binding Protein
CO_2	Carbon Dioxide
COX	Cyclooxygenase
cPLA2	Calcium dependent Phospholipase A2
CRP	C-reactive Protein
Ct	Cycle Threshold

CV	Coefficient of Variance
CVD	Cardiovascular Disease
CYP450	Cytochrome P450
Da	Daltons
DC	Detergent Compatible
dH_2O	Distilled Water
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DN*ase*	Deoxyribonuclease
dNTPs	Deoxynucleotides Triphosphates
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EETs	epoxyeicosatrienoic acid
ELISA	Enzyme-linked Immunosorbant Assay
ER	Endoplasmic Reticulum
FAM	RT-PCR Reporter Fluorochrome/Dye Label
FATP	Fatty acid transporter protein
FFAs	Free Fatty Acids
FGF	Fibroblast Growth Factor
g	Force of Gravity
g	Gram
GI	Gastrointestinal
GIP	Gastric Inhibitory Peptide

GLUT-4	Glucose-transporter-4
GLUTs	Glucose Transporters
H_2O	Water
HBSS	Hank's Balanced Salt Solution
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HETEs	Hydroxyeicosatetraenoic acid
HMW	Higher Molecular Weight
HOMA	Homeostasis Model Assessment
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
HPETEs	Hydroperoxyeicosatetraenoic acid
hr	Hour
HRP	Horseradish Peroxidase
HSL	Hormone-sensitive lipase
ICAM-1	Intercellular Adhesion Molecule-1
IFNβ	Interferon β
IGF-1	Insulin-like Growth Factor-1
IGT	Impaired Glucose Tolerance
IKK	Inhibitor of NF-κB Kinase
IKKα	Inhibitor of NF-κB Kinase-α
IKKβ	Inhibitor of NF-κB Kinase-β
IL	Interleukin
iPLA2	Calcium independent phospholipase A2
IR	Insulin Receptor
IRS	Insulin Receptor Substrate

IκB	Inhibitor of NF-κB
JNK	c-Jun N-terminal Kinase
kDa	Kilodaltons
L	Litre
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low density lipoprotein
LMW	Low Molecular Weight
LOX	Lipoxygenases
LPL	Lipoprotein lipase
LPLA2	Lysosomal PLA2
LpPLA2	Lipopeotein associated phospholipase A2
LPS	Lipopolysaccharide
LysoPC	Lysophosphatidylcholine
lysoPL	Lysophospholipids
M	Molar
MAPK	Mitogen-activated Protein Kinase
MCP-1	Monocyte Chemotactic Protein-1
MEKK1	Mitogen-activated Kinase Kinase-1
mg	Milligram
$MgCl_2$	Magnesium Chloride
MHO	Metabolic Healthy Obesity
min	Minute (time)
mL	Millilitre
mM	Millimolar
mQH_2O	Milli Q water (ultra-filtered water)

M_r	Molecular Weight
mRNA	Messenger Ribonucleic acid
MS	Metabolic Syndrome
MyD88	Myeloid Differentiation Primary Response Gene-88
NaCl	Sodium Chloride
NCEP-ATP III	National Cholesterol Education Program-Adult Treatment Panel III
NEFA	Non-esterified Fatty Acid
NEFAs	Non esterified fatty acids
NF-κB	Nuclear Factor-κB
NFκB	Nuclear factor kappa B
ng	Nanogram
NIK	NF-κB-inducing Kinase
nm	Nanometre
NPY	Neuropeptide Y
OB-Rb	Leptin Receptor
OD	Optical Density
Om AT	Omental Adipose Tissue
Om	Omental
ox-LDL	oxidized LDL
OXM	Oxyntomodulin
p	Phosphorylated
PAF-Ahs	Platelet-activating factor acetylhydrolases
PAI-1	Plasminogen Activator Inhibitor-1
PBMCs	Peripheral Blood Mononuclear Cells

PBS	Phosphate-buffered Saline
PBS-T	Phosphate-buffered Saline containing 0.1% Tween 20
PCR	Polymerase Chain Reaction
PI3K	Phosphoinositide-3 Kinase
PKA	cAMP dependent protein kinase
PKC	Protein Kinase C
PLA1	Phospholipase A 1
PLA2	Phospholipase A2
PLB	Phospholipase B
PLC	Phospholipase C
PLD	Phospholipase D
PNPLAs	Patatin like phospholipase domain containing lipases
PPAR-γ	Peroxisome Proliferator Activated Receptor-γ
PPRE2	PPAR-γ Response Element-2
PYY	Peptide tyrosine-tyrosine
RAGE	Receptor for Advanced Glycation End-products
RELM	Resistin-like Molecule
RIA	Radioimmunoassay
RIPA	Radio-Immunoprecipitation Assay
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT	Room Temperature
RT-PCR	Real-time PCR
RYGB	Roux-en-Y gastric bypass
s	Second (time)

Sc AT	Subcutaneous Adipose Tissue
Sc	Subcutaneous
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SG	Sleeve gastractomy
sPLA2	secreted PLA2
SR-B1	Scavenger Receptor class B type 1
SREBP1c	Sterol Response Element Binding Protein 1c
T2DM	Type 2 Diabetes Mellitus
Taq	Thermus Aquaticus (DNA polymerase)
TBS	Tris-buffered Saline
TBS-T	Tris-buffered Saline containing 0.1% Tween 20
TCA	Trichloroacetic Acid
TEMED	N, N, N', N'-Tetramethylethelenediamine
TG	Triglyceride
TGF-β	Transforming Growth Factor-β
TLR	Toll-like Receptor
TLRs	Toll-like Receptors
TNF-α	Tumour Necrosis Factor-α
TRAF	TNF Receptor-associated Factor
TRAF-6	TNF Receptor-associated Factor-6
Tris	Tris (hydroxymethyl) Aminomethane
Tris-HCl	Tris Hydrochloride

TZD	Thiozoladinedione
TZDs	Thiozoladinediones
U	Units
UV	Ultraviolet
V	Volts
v/v	Ratio of Volume per Volume
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VIC	RT-PCR Fluorochrome/Dye Label
VLDL	Very low density lipoprotein
w/v	Ratio of Weight per Volume
WAT	White Adipose Tissue
WC	Waist circumference
WHO	World Health Organisation
WHR	waist to hip ratio
WPRO	The Regional Office for Western Pacific Region of WHO and the International Obesity Task Force of WHO
ΔCt	Delta Cycle Threshold

CHAPTER 1 : Introduction

1.1 Obesity

1.1.1 Definition and causes of obesity

Obesity is a condition of energy imbalance between energy intake and energy expenditure**,** leading to hypertrophy (increase of cell size) and hyperplasia (increase of cell number) of adipose tissue. The increased fat mass can be reflected by simple anthropometric measures such as Body Mass Index (BMI), waist circumference (WC) and waist to hip ratio (WHR). BMI is calculated through the division of weight in kilograms by the square of height in meters. BMI over 30 has been classified as obesity, according to World Health Organization (WHO) international of classification obesity (Table 1.1). BMI is a powerful gender independent predictor of obesity related metabolic diseases such as hypertension, lipid profiles abnormalities, T2DM and cardiovascular diseases (Gierach, Gierach, Ewertowska, Arndt, & Junik, 2014).

In addition, fat accumulation site is also one of the parameters that predict the risk of obesity complications. Increased fat expansion around abdomen (subcutaneous and omental AT), known as central obesity, showed stronger correlations with obesity related diseases compared to the fat accumulation ino the lower part of body (hip, tight). Abdominal or visceral fat accumulation can be estimated by waist circumference (WC) and waist to hip ration (WHR). With the same BMI, subjects who had higher WC showed the higher risk of obesity related metabolic diseases. Several studies suggested that WC and WHR measurements are better predictors for obesity related metabolic diseases than BMI (Elbassuoni, 2013; Jayawardana, Ranasinghe, Sheriff, Matthews, & Katulanda, 2013; Jing et al., 2012). However, some studies suggested that WHR was not better than BMI in CVD

prediction in Northern Europe population (Goh, Dhaliwal, Welborn, Lee, & Della, 2014), whist WC is a better predictor than WHR and BMI in Asian women (Choi, 2011; Singhal, Mathur, & Pathak, 2011). Thus, the WC and WHR cut points are not appropriated across worldwide. Unfortunately, to date, there are still no standardized criteria for each specific ethnics.

Besides ethnicity, fat distribution is also influenced by gender through androgen metabolism in adipose tissue, resulting in higher prevalence of central obesity in male than female (De Maddalena, Vodo, Petroni, & Aloisi, 2012; O'Reilly, House, & Tomlinson, 2014). Studies in menopause women confirmed that fat distribution was shifted to abdomen in subjects with decreased estrogen and increased androgens (Lizcano & Guzmán, 2014; Vaidya et al., 2012). Environmental factors such as physical activity, food consumption, medications and illnesses contribute to obesity as well. High carbohydrate and fat composition of diets induced adipose tissue expansion (Cummins et al., 2014). Some medications used in T2DM treatments such as insulin (Home et al., 2014) and sulfonylurea (Thulé & Umpierrez, 2014) have been reported to correlate with weight gain.

Although, anthropometric measures such as BMI, WC and WHR have limitations in obesity related disease prediction due to ethnicity and gender, they are simple and useful tools for both individuals and health care professionals to be aware of obesity in order to prevent the early onset of obesity related diseases such as T2DM, hypertension, dyslipidemia and cardiovascular diseases (Abdullah et al., 2012).

Table 1. 2 Obesity Classification. BMI criteria for obesity diagnosis according to WHO, NHLBI and WPRO.

WHO (2000) Classification	NHLBI (1998) Classification	BMI (kg/m^2)	WPRO (2000) Classification	BMI (Kg/m^2)
Underweight	Underweight	<18.5	Underweight	<18.5
Normal range	Normal range	18.5-24.9	Normal range	18.5-22.9
Pre-obese	overweight	25-29.5	Overweight at risk	23-24.9
Obese I	Obese I	30-34.9	Obese I	25-29.9
Obese II	Obese II	35-39.9	Obese II	≥30
Obese III	Obese III	≥40		

Adapted from (Anuurad et al., 2003)

1.1.2 Epidemic of Obesity

Overweight and obesity is the global epidemic which the prevalence has been dramatically increased by 27.5% for adults and 47.1% for children during the past few decades (Kassebaum et al., 2014; Ng et al., 2014). It has been estimated by WHO in 2008 that obesity reached 35 % of population worldwide. The prevalence of obesity is rising all across the world. The highest obesity prevalence is in America with 35.7% for adults and 16.9% of children and it has been forecasted to increase by 51% in 2030 (Finkelstein et al., 2012; Padwal, 2014). Interestingly, Asian populations have also shown the drastically increasing number of overweight and obese. Studies in Japanese and Chinese populations, the combined prevalence of overweight and obese dramatically rise by 46% in Japan and 414% in China within 20 years periods (Collaboration, 2007).

It must be noted that the increased risk of complications related to obesity is ethnic specific. Asian populations seem to develop obesity related diseases at much lower BMI than western populations (Shiwaku et al., 2004; J. J. Yang, Shiwaku,

Nabika, Masuda, & Kobayashi, 2007). Thus, the Regional Office for Western Pacific Region of WHO and the International Obesity Task Force of WHO (WPRO) has proposed in 2000 the new specific criteria for overweight and obesity classification for Asian populations. According to this guideline, BMI over 25 is classified as obesity (Table 1.1). Increasing numbers of studies have been supported that the WPRO criteria was more sensitive to reflect the obesity status in Asian populations (Anuurad et al., 2003; Jitnarin et al., 2011; Shiwaku et al., 2004). Thus, the numbers of overweight and obese patients were unsurprisingly much higher when the WPRO criteria were used as the cut points (Jitnarin et al., 2011).

1.1.3 Obesity interventions

The increasing prevalence of obesity closely associates with the high risk of chronic diseases such as T2DM, cardiovascular disease and cancer. Obesity is considered as a major causation of hypertension and T2DM and prevalence of T2DM is also associated with BMI (Colosia, Palencia, & Khan, 2013). Obesity with T2DM is associated with lipid profile abnormality (Brea et al., 2011). Data from large cohort study indicated that high BMI with increased WHR increases mortality as well as risk of cardiovascular diseases (Borrell & Samuel, 2014; Myint, Kwok, Luben, Wareham, & Khaw, 2014). Thus, weight managements are essential for the health care of obesity. The obesity interventions are mainly involved with two approaches; the reduction of energy intake and induction of energy expenditures (Clapham & Arch, 2011). However, it is also suggested that intensive treatment of T2DM by using drugs alone did not improved prognosis but eventually increased mortality (Gorgojo Martínez, 2011). Weight control including modification of diet, increasing physical activity has shown to significantly improve CVD risk. It has been

reported that 10% weight loss reduced prevalence of T2DM for 30% and CVD for 20% (McQuigg et al., 2008). However, life style changes are difficult. It has been shown that almost 50% of participants failed to maintain weight (Barte et al., 2010).

1.1.3.1 Bariatric surgery

Bariatric surgery is recommended in T2DM patients with BMI ≥ 35 Kg/m^2, especially for patients with insufficient glycemic control (Buchwald, Ikramuddin, Dorman, Schone, & Dixon, 2011). The bariatric operation is classified into three major techniques; (1) Restrictive techniques which reduce the stomach size in order to reduce food digestion *e.g*. adjustable gastric bands, vertical banded gastroplasty and sleeve gastractomy (SG). (2) Malabsorptive type surgery which reduces food intake and absorption by cutting off some part of stomach, intestine and pancreas and creating bypass between stomach and intestine *e.g.* biliopancreatic diversion (BPD) and jejunoileal bypass. (3) Mix technique *e.g.* biliopancreatic diversion with duodenal switch (BPD-DS) and Roux-en-Y gastric bypass (RYGB) (Figure1.1.3.1.1) (Ashrafian & le Roux, 2009).

Bariatric surgery generally reduced visceral fat mass. Since chronic enlarged adipocytes mediate inflammation and insulin resistance, acute reduction of pathogenic fat mass consequently reduces circulating inflammatory cytokines such as Il-6, TNFα and CRP (Lasselin et al., 2014; Viana et al., 2013) and increases insulin sensitivity (Mor, Tabone, Omotosho, & Torquati, 2014). Lipolysis is inhibited during weight loss, resulting in reduction of circulating free fatty acid (Campos et al., 2014; Mor et al., 2014). In addition, angiogenesis in AT is gradually reduces after surgery due to the reduction of vascular endothelial growth factor-A (García de la Torre et al., 2008; Ledoux et al., 2008). Acute caloric restriction directly effects lipid profile,

reducing cholesterol, triglycerides and LDL. It has been reported that several anti-inflammation adipokine such as adiponectin and IL-10 increase during weight loss (Hosseinzadeh-Attar, Golpaie, Janani, & Derakhshanian, 2013; McCormick, 2013).

Bariatric surgery directly affects gut hormones. Different types of surgery influence different gut hormone production. The gastric bypass which shortens the delivery of nutrients to distal bowel increases the production of peptide tyrosine-tyrosine (PYY), oxyntomodulin (OXM) and Gastric Inhibitory Peptide (GIP), reducing food intake.

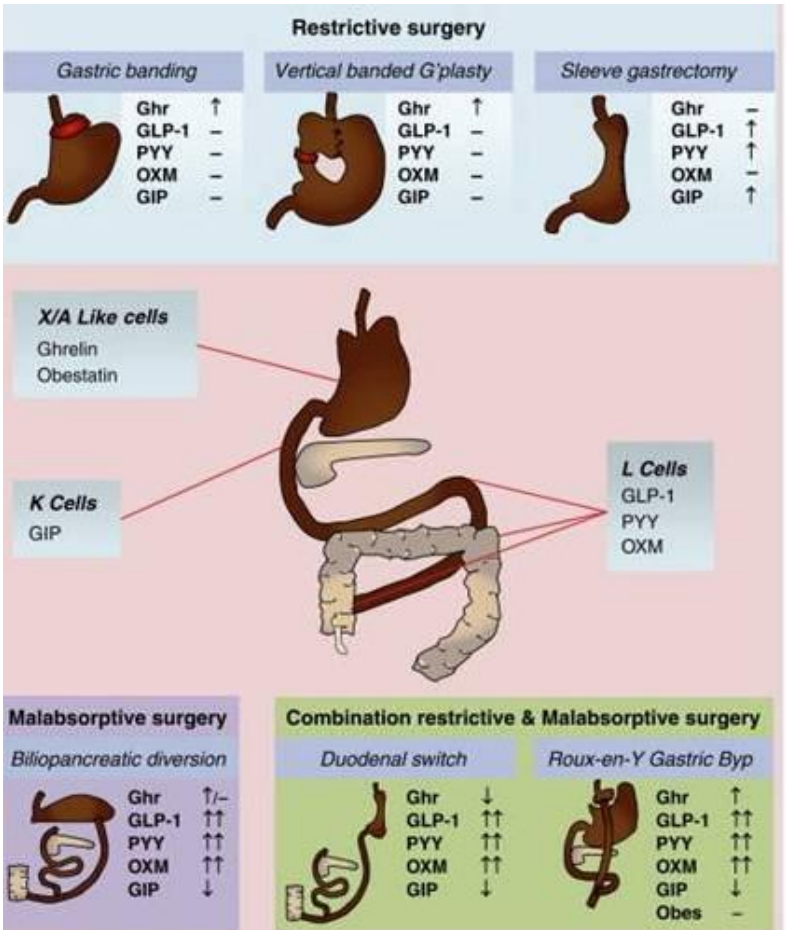

Figure 1.1.3.1.1 Bariatric procedures and their effects on gut hormones.
Ghr; Ghrelin (active appetite hormone), GLP-1; glucagon-like peptide-1 (promote energy storage in AT), PYY; peptide tyrosine-tyrosine (decrease food intake), OXM; oxyntomodulin (decrease food intake), GIP; Glucose dependent insulinotropic peptide or Gastric Inhibitory Peptide (decrease food intake). (Tharakan, Tan, & Bloom, 2011).

1.1.4 Metabolically healthy obesity

Although the strong association of obesity and its related diseases has been extensively reported in general population, numerous of studied reported that the development of obesity related chronic diseases does not always occur in some cases. This phenotype was termed as Metabolic Healthy Obesity (MHO) which is defined as an obesity (BMI>30 Kg/m^2) with no metabolic diseases such as hypertension, dyslipidemia and impaired glycemic control. With these criteria, increasing numbers of studies indicated that MHO phenotype was associated with the lower risk of T2DM compared to non-MHO phenotype (Hinnouho et al., 2014; Rhee et al., 2014). However, to date, there are no standardized criteria to distinguish between MHO and non-MHO and no evidence to state that MHO is not risky.

Interestingly, difference between MHO and non-MHO seems to strongly involve sub-clinical inflammation. MHO as well as non-obese showed the lower levels of complement component 3, C-reactive protein, TNF-α, IL-6, and plasminogen activator inhibitor-1and white blood cell count compared to non-MHO (Koster et al., 2010; Phillips & Perry, 2013). In addition, with the same BMI, non-MHO with high circulating inflammatory adipokines significantly associated with visceral obesity (Koster et al., 2010; Phillips & Perry, 2013; van Beek et al., 2014). Thus, development of obesity related metabolic diseases is most likely contributed by adipose tissue inflammation rather than BMI alone and routine measurement of anthropometry along with adipose tissue inflammatory might be helpful for diagnosis and treatment in obesity and metabolic diseases.

1.2 Adipose tissue as a contributor to metabolic dysfunction

1.2.1 Abdominal adipose tissue expansion in obesity

Abdominal adipose tissue (Abd AT) is categorized according to fat tissue compartment into 2 depots; abdominal subcutaneous AT and visceral or omental AT. AT is an important reversible storage pool which firstly responds to chronic excess energy. Excess energy stores mostly in the form of triglyceride (TG) via lipogenesis by adipocytes. Increased lipogenesis causes lipid accumulation, enlarging adipocyte size (AT hypertrophy). Besides triglyceride accumulation, pre-adipocyte also responds to energy excess via cell proliferation and differentiation, resulting in increasing numbers of mature adipocytes (AT hyperplasia) (Proença et al., 2014; Q. A. Wang, Tao, Gupta, & Scherer, 2013). Previous studies showed that obese subjects with BMI greater than 30 kg/m^2 have large fat cell sizes, compared to lean control subjects (Heinonen et al., 2014; van Harmelen et al., 2003). Excessive AT hypertrophy subsequently mediates adipocyte deregulation, increasing the production of non esterified fatty acids (NEFAs) and pro-inflammatory cytokines (*e.g*. TNFα, IL-6, IL-1β) release which were been reported as a cause of insulin resistance. In studies in human adipose tissue, TNFα expression was correlated with BMI, percentage of body fat and hyperinsulinaemia, whereas weight loss decreased TNFα expression (Rajkovic et al., 2014). Similarly, increased circulating IL-6 level is observed in obese and insulin resistant subjects (Bordon, 2014; Rajkovic et al., 2014). The significant correlation between serum free fatty acid and BMI as well as insulin resistance was also reported (Frohnert et al., 2013) and a study in C57Bl6/J mice indicated that high fat diet induced hepatic insulin resistance (Wiedemann, Wueest, Item, Schoenle, & Konrad, 2013).

Enlargement of adipocyte differs between AT depots and the enlarged size of the cell induced by excessive nutrition indicates cellular dysfunction. Large adipocytes were more profound in Om AT compared to Abd Sc AT. The different AT depots also exhibit differences in protein expression as well as cellular responsiveness. Expression of adipose genes such as adiponectin (Rahmouni, Mark, Haynes, & Sigmund, 2004) as well as pro-inflammatory cytokines (plasminogen activator inhibitor-1 (PAI-1) (Rahmouni et al., 2004), IL8 (Bruun et al., 2004) and IL-6 (Fried, Bunkin, & Greenberg, 1998) were higher in Om AT. Leptin is preferentially secreted in Sc AT (Schoof et al., 2004). Moreover, Sc AT is considered to be more sensitive to insulin than Om AT (Bolinder, Kager, Ostman, & Arner, 1983), whilst the Om AT suppresses about 50% of glucose uptake after insulin treatment (Stolic et al., 2002). Since the venous blood of Om AT flows through hepatic portal vain, the increasing secretions of pro-inflammatory adipokines are directly drained to circulation, leading to systemic inflammation.

Like other organ, AT contains not only adipocyte but also a population of cells of the innate immune system such as macrophage and lymphocyte. It is suggested that macrophage infiltration in AT is strongly associated with chronic, low grade inflammation in obesity (McNelis & Olefsky, 2014). Several studies reported that the number of macrophages was more in Om AT than Sc AT and induced insulin resistance (Harlev et al., 2014). Increasing macrophage infiltration is directly associated with the higher expression of calling signal, the monocyte attractant protein1 (MCP1), in Om AT (Harman-Boehm et al., 2007). In addition, impaired angiogenesis in disproportionate adipocyte hypertrophy in Om AT causes tissue hypoxia (Elias et al., 2012), leading to adipocyte apoptosis which potentially induces macrophage infiltration for death cell clearance.

1.2.2 Lipid metabolism and adiposity

Dietary fats are digested into monoglycerides, free fatty acids and cholesterols by gastric and pancreatic lipase. These digested insoluble products are emulsified by bile salt and absorbed into intestine. In enterocytes, triglycerides are re-synthesized and assembled with cholesterol ester and protein to form chylomicrons. Chylomicron particles are released into the circulation and hydrolyzed to release free fatty by lipoprotein lipase (LPL) in peripheral tissue such as AT and muscle and circulated until taken by the liver. Free fatty acids as well as cholesterol esters packed inside chylomicron remnant serves as precursors for liver to synthesize necessary lipids.

Liver re-synthesizes TG, using free fatty acids as precursors via hepatic lipogenesis, and delivers with cholesterol esters and other lipids by packing into very low density lipoprotein (VLDL) particles. Major lipids containing in VLDL particle is fatty acids, follows by cholesterol esters (Figure 1.2.2.1). Similar to chylomicrons, VLDL particles deliver fatty acids to adipose tissue, muscle and other tissue via LPL activity and become gradually smaller, resulting in a higher density lipoprotein compared to VLDL, termed low density lipoprotein (LDL). LDL particles are re-uptake via LDL receptors in liver. The remaining lipids (mostly is cholesterol) in LDL particle are stored in liver cells (Proença et al., 2014). In high fat dietary, liver control circulating LDL level and lipid storage by reducing synthesis of cholesterol as well as LDL receptor. Reduction of LDL receptor prolongs circulation of LDL, leading to high level of serum LDL. Prolonged LDL in circulation is susceptible for oxidation of both protein and lipid in LDL particle. Thus, long term high fat dietary as well as high serum LDL has been reported to correlate with increased oxidized

LDL (ox-LDL) (Figure 1.2.2.2). ox-LDL is recognized and uptake by macrophage via scavenging receptor.

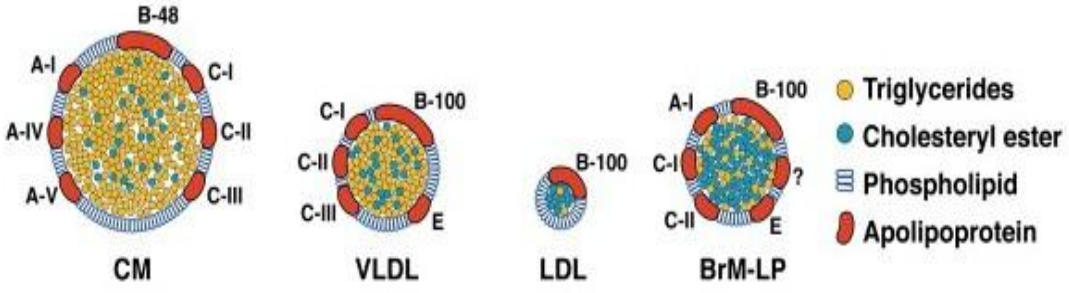

Figure 1.2.2.1 Classification of circulating lipoprotein. Lipoproteins are classified according to their density separated by ultracentrifugation into Chylomicron (CM), Very low density lipoprotein (VLDL), Low density lipoprotein (LDL). (Curcio, Johnson, Huang, & Rudolf, 2009)

HDL (High density lipoprotein) is originally synthesized in liver as a nascent HDL. It is considered as a good lipoprotein as it helps to “clean up” cholesterol from circulation. Circulating HDL uptake and esterifies cholesterol from peripheral tissue and other lipoproteins by lecithin-cholesterol acyltransferase (LCAT). HDL with cholerterol accumulation returns to liver via Scavenger Receptor class B type 1 (SR-B1). High serum HDL is associated with improvement of cardiovascular risk (Martin, Jones, & Toth, 2014).

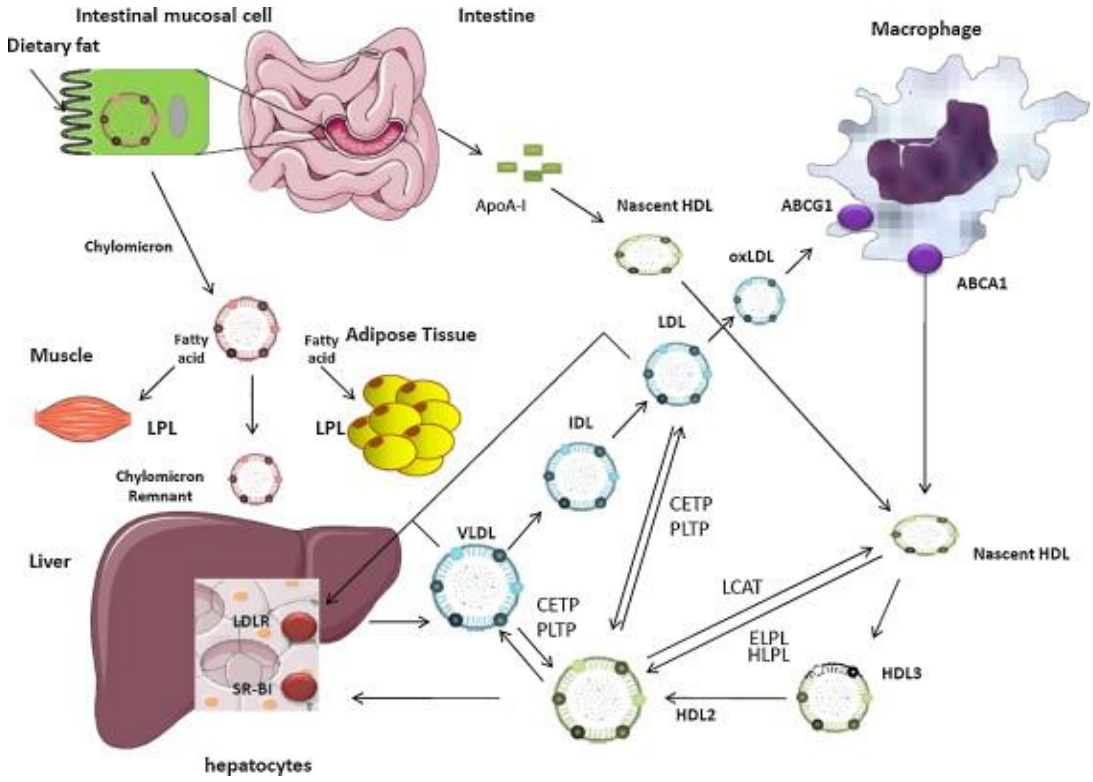

Figure 1.2.2.2 Overview of lipoprotein metabolism. Exogenous dietary fat is absorbed and deliver as chylomicron to liver. Liver is aprimary organ that synthesizes TG and cholesterol. Lipid productions are circulated in the form of Very low density lipoprotein (VLDL) which gradually deliver lipids to peripheral tissue by lipoprotein lipase (LPL) activity. VLDL becomes intermediate low density lipoprotein (IDL), low density lipoprotein (LDL) and uptake by liver via LDL receptor. Nascent HDL is synthesized by liver and delivered to obtain cholesterol and TG from tissue. Nascent HDL is increased its size because of lipid uptake, becoming HDL3 and HDL2. HDL is uptake by liver. (Giannarelli, Klein, & Badimon, 2011)

1.2.3 Lipogenesis and lipolysis in adipocyte

Free fatty acids delivered by both chylomicrons and lipoproteins; VLDL and LDL, are taken up by adipocytes by fatty acid transporter protein (FATP) and store as TG via TG re-synthesis (lipogenesis) in liver. In adipocytes, free fatty acids convert into fatty acyl-CoA and re-synthesize TG via lipogenesis (Figure 1.2.3.1). Key enzymes in lipogenesis are transcriptional controlled by Sterol Response

Element Binding Protein 1c (SREBP1c) and Carbohydrate Response Element Binding Protein (ChREBP) (Iizuka, 2013). Under insulin resistant state, high carbohydrate and fat diet induced SREBP1c expression, leading to fat deposition in responsive organs, especially AT and liver (Czech, Tencerova, Pedersen, & Aouadi, 2013). Up-regulation of ChREBP in liver is associated with insulin resistance, induces hepatic steatosis (Iizuka & Horikawa, 2008). Unlike liver, increased ChREBP in adipose tissue induces insulin sensitivity (Benhamed, Poupeau, & Postic, 2013). This data indicates that activation of lipogenesis in adipose tissue has metabolic benefits.

In contrast to lipogenesis, lipolysis is the process that intracellular TGs are hydrolyzed into NEFA and delivered to the circulation (Zaidi et al., 2013). This process is positively controlled by several hormones including, hormone-sensitive lipase (HSL) and catecholamine and negatively controlled by insulin (Frühbeck, Méndez-Giménez, Fernández-Formoso, Fernández, & Rodríguez, 2014). It is wildly known that high level of NEFA is observed in obese and T2DM subjects, indicating that lipolysis is increased in obesity and the increased rate of lipolysis is correlated with enlarged of adipocyte size (Lambert, Ramos-Roman, Browning, & Parks, 2014). In addition, hepertrophy of AT (enlarged cell size) is active in lipolysis (Michaud et al., 2014) and less susceptible to insulin (Zhai et al., 2010). Thus, increased fat mass in omental AT can be a season of increased free fatty acids in obesity.

In addition, several adipokines also affect lipid metabolism. Adiponectin increases NEFA uptake and β-oxidation in muscle cell, reducing circulating NEFA (Maeda et al., 2002; Terazawa-Watanabe, Tsuboi, Fukuo, & Kazumi, 2014). In contrast, TNFα reduced free fatty acid uptake and increases lipolysis, leading to

increased level of free fatty acid in circulation (Chen, Xun, Chen, & Wang, 2009; Ryden et al., 2002). IL-6 also has been reported to reduce the LPL activity in adipose tissue (Trujillo et al., 2004). Both circulation levels of TNFα and IL-6 correlates with free fatty acid in obesity.

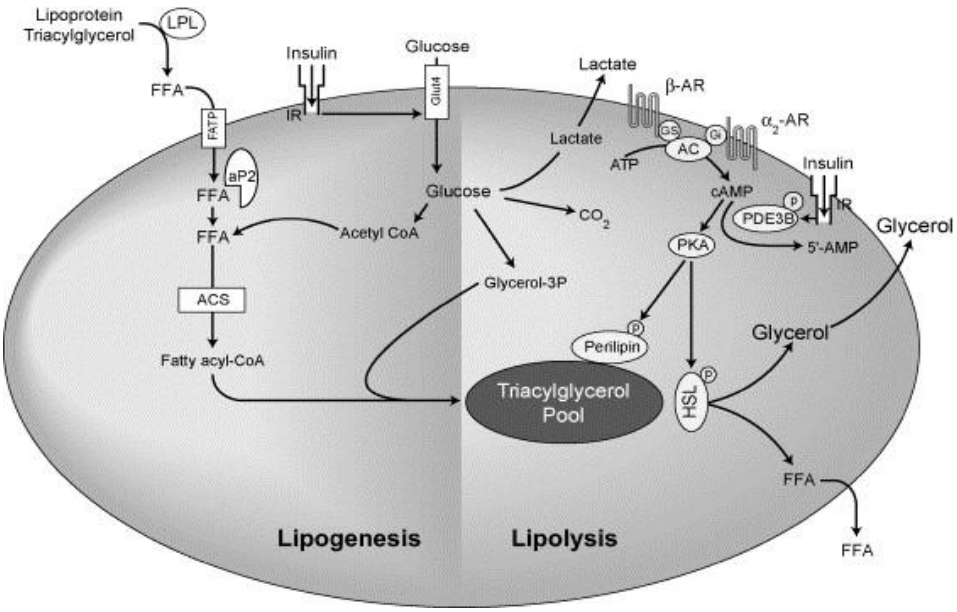

Figure 1.2.3.1 Overview of lipogenesis and lipolysis. In the fasting state, hormone sentitive lipase (HSL) is activated by insulin and hydrolyze intracellular TG to generate NEFA . NEFA is circulated to serve as an energy fuel of peripheral tissue. After meal, NEFA is uptake by LPL activity. The NEFA is re-synthesized into TG, stored in AT. (Avram, Avram, & James, 2005)

1.2.4 Inflammation in adipose tissue

The mechanism which turns on the inflammation in AT is not clear and progression of inflammation is complicated. Adipocyte itself has the ability to secret various inflammatory cytokines such as TNFα, MCP-1, IL-8and IL-6 as well as anti-inflammatory cytokines such as adiponectin, leptin and IL-10 (Ohashi, Shibata, Murohara, & Ouchi, 2014). Excessive AT hypertrophy and hyperplasia causes

adipocyte dysfunction, leading to overproduction of inflammatory and reduction of anti-inflammatory adipokines (Lafontan, 2014)(Figure 1.2.4.1).

Adiponectin is a hormone that is involved in many cellular activities, including adipocyte differentiation and muscle cell proliferation. It plays a protective role in atherosclerosis and T2DM. Serum level of adiponectin is decreased in obesity. Adiponectin treatment improves insulin sensitivity and glycemic control in animal models (Yamauchi, Iwabu, Okada-Iwabu, & Kadowaki, 2014).

Leptin is a satiety hormone that controls dietary intake and benefits to weight control by activating energy expenditure. Leptin is correlated with BMI and it is increased in obesity (Wilding, 2001).

Both IL-6 and TNFα are increased in obesity and exhibit pro-inflammatory as well as pro-atherogenic effects. TNFα and IL-6 activated inflammation pathway which further impaired the insulin signaling pathway (D. Cai et al., 2005; Makki, Froguel, & Wolowczuk, 2013)

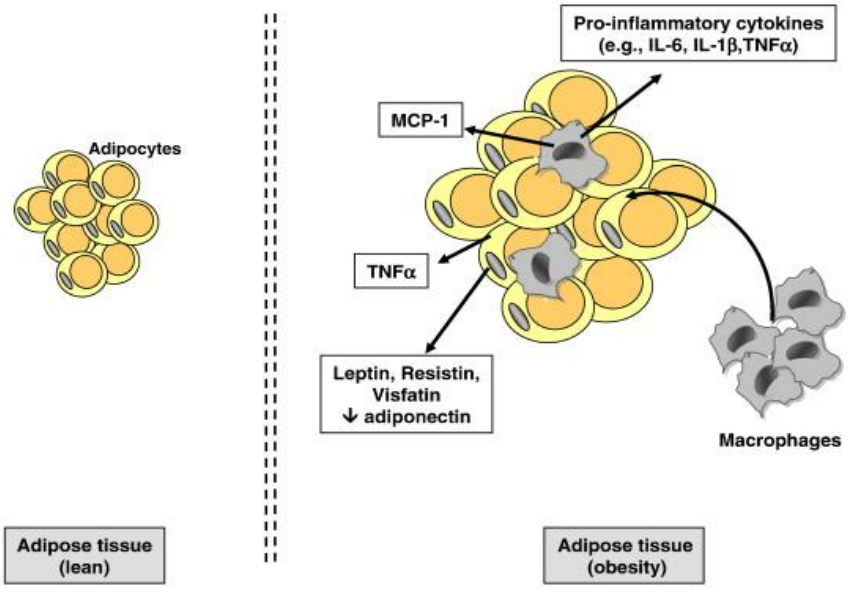

Figure 1.2.4.1 Adipose tissue secretion. In adiposity, enlarged adipocyte becomes dys-regulation, secreting inflammatory cytokines such as IL-6, TNFα ans MCP-1, reducing Anti-inflammatory adipokines production and increasing macrophage infiltration. (Capuron & Miller, 2011)

Adipocytes respond to those inflammatory cytokines and metabolic stress through various signal transduction pathway such as, the nuclear factor kappa B (NFκB) and Toll like receptors (TLRs) pathway. Following is the summary of NFκB and TLR pathway and their roles in adipocyte inflammation and obesity.

1.2.4.1 IκB/NFκB pathway

NFκB is a transcription factor protein family consists of 5 subunits member; RelA (p65), RelB, c-Rel, NFκB1 (p65) and NFκB2 (p52). The classical pathway of NFκB relies on the activation of RelA/p50 NFκB dimers, which activity is controlled by binding with its inhibitor, IκBα. Once the cells are activated by stimuli, IκBα subunit is phosphorylated by activated IκB kinase (IKK) to release an active RelA/p50 NFκB complex. The active NFκB translocates into nuclear and binds to its responsive element to start DNA transcription (Figure 1.2.4.1.1). The alternative pathway is to activate RelB/p52 complex which binds to p100 in un-activated cell (Razani, Reichardt, & Cheng, 2011).

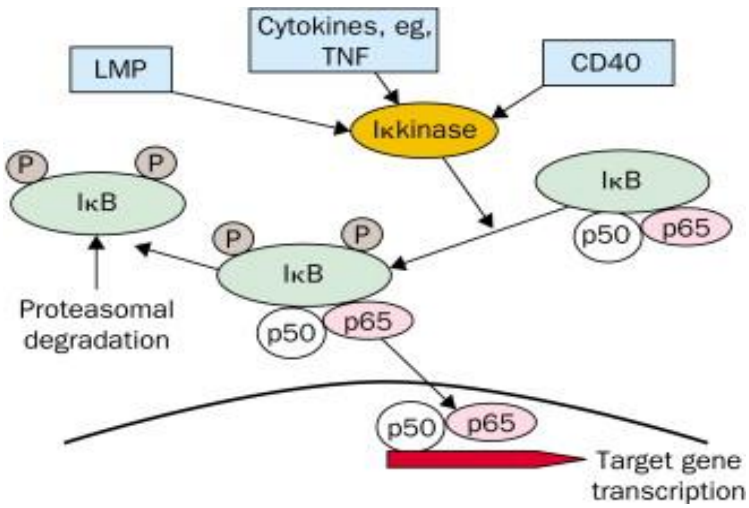

Figure 1.2.4.1.1 Activation of inflammation via IκB/NFκB cascade. NFκB is activated by a range of stimuli such as inflammatory cytokines, immunogenic antigen such as CD40 and LMP as well as FFA (Yung & Linch, 2003)

Activation of NFκB is induced by various stimuli. Cytokines such as TNFα, IL-6 activate NFκB via cytokine receptors, resulting in regulation of insulin signaling and cytokines productions (Lee & Lee, 2014). Activation of NFκB by high fat diet is an important factor that contributes to insulin resistance (Harte et al., 2013b). Study in IKKβ knockout mice shows the protective effect of insulin resistant developments induced by high fat diet (J. K. Kim et al., 2001). In vitro, incubation the L6 muscle cell with palmitate increased NFκB p65 and NFκB p50 expression and induced inflammatory cytokine production and insulin resistant phenotype (Barma et al., 2009).

Recently, increased serum level of CD40, the protein produced by a range of cell types such as T-cell, B-cell, granulocyte and macrophage, was observed in patients with obesity (Unek et al., 2010). It is involved in adipose tissue inflammation (Missiou et al., 2010). Incubation of CD40 with adipocytes activate pro-inflammatory cytokine productions (IL-6, MCP-1, IL-8, and PAI-) via NFκB activation (Chatzigeorgiou, Phieler, Gebler, Bornstein, & Chavakis, 2013; Missiou et al., 2010).

Lipopolysaccarides activate NFκB pathway via Toll like receptor (TLR), inducing the innate immune response in various tissue, including adipocyte. TLRs family has been reported to express in adipose tissue (Poulain-Godefroy et al., 2010). Under normal physiological condition, LPS derives from normal flora in gastrointestinal tract, is co-absorbed with fatty acids and release to circulation with chylomicrons (Manco, Putignani, & Bottazzo, 2010). In circulation, LPS binds to LPS binding protein (LPB), transporting LPS to lipoprotein such as VLDL and LDL. It has been reported that LPS level is increased in obesity and T2DM (Creely et al., 2007; Harte et al., 2010; Harte et al., 2012).

1.2.4.2 Toll like receptors

Toll like receptors (TLRs) are the membrane proteins that recognize antigens such as LPS and fatty acids and oxidized phospholipids, facilitating immune responses to infection and stress signals. To date, 20 TLRs have been identified. TLRs signaling consists of 2 pathways dependent on downstream adaptor proteins; MyD88-dependent and MyD88-independent pathways (Figure 1.2.4.2.1) (Rauta, Samanta, Dash, Nayak, & Das, 2014). MyD88 dependent pathway mainly involves in inflammatory cytokine production, while MyD88 independent pathway mediates cell proliferation and differentiation.

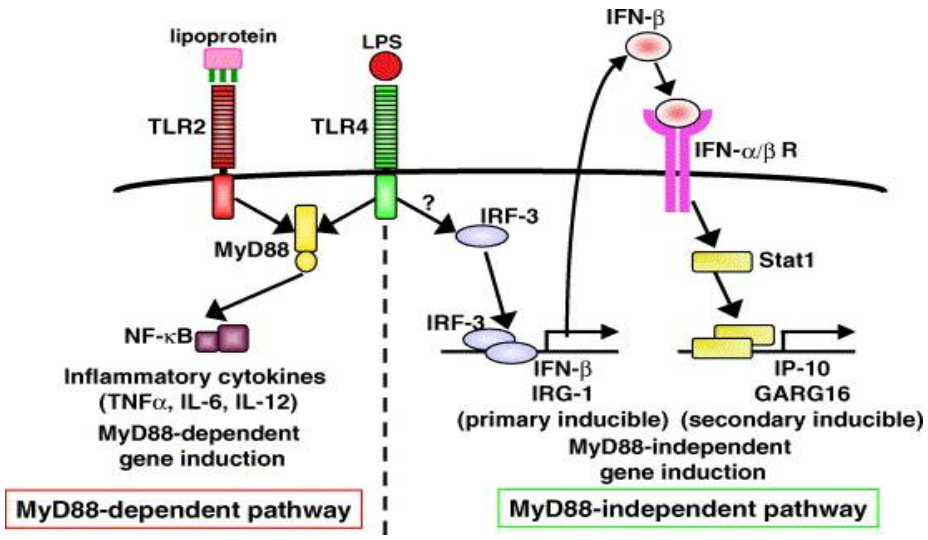

Figure 1.2.4.2.1 The activation of inflammatory pathway via TLR. A range of stimuli such as LPS, FFA and modified lipoprotein activate TLRs and induce inflammation via the activity of adaptor protein, MyD88. Activation of TLR by stimuli such as interferon β (IFNβ) also induces the cell response via MyD88 independent pathway. (Yamamoto, Takeda, & Akira, 2004)

TLR2 and TLR4 expression in adipose tissue associated with inflammation and insulin resistance. In obesity, TLR4 expression in Om AT is associated with AT

expansion (Fusaru et al., 2012). Increased of TLR 2 and TLR4 expression were observed in AT taken from obese subjects (S. J. Kim, Choi, Choi, & Park, 2012).

In addition, high fat diet increased the level of circulating LPS and increased of TLR4 expression was observed in C57BL/6 mice, suggesting that dietary fat absorption is associated with LPS (N. Wang et al., 2013). In human adipocyte primary cell culture, free fatty acid treatment induced TLR4 expression (Creely et al., 2007; Youssef-Elabd et al., 2012). LPS also induced TLR4 exprssion in adipose tissue (Creely et al., 2007; Kopp et al., 2009). Thus, increased lipolysis as well as endotoxinaemia in obesity mediates adipose tissue inflammation via TLR pathway.

1.3 Phospholipase enzymes

Phospholipases are enzymes that have the ability to hydrolyze phospholipids, especially glycerophospholipid in plasma membrane. These enzymes are divided depending on their type of reaction into two broad groups: (1) Phospholipases with acyl hydrolase activity. These enzymes hydrolyze the phospholipid at the *sn-1* acyl ester bond (Phospholipase A1) or at the *sn-2* acyl ester bond (Phospholipase A2), releasing arachidonic acid. (2) Phospholipase with phosphodiesterase activity. These enzyme cleaves the glycerol phosphate bond (Phospholipase C) and phospholipase D to remove the phosphate group from phospholipid (Capper & Marshall, 2001; Pniewska & Pawliczak, 2013; Wilton, 2005) (Figure 1.3.1).

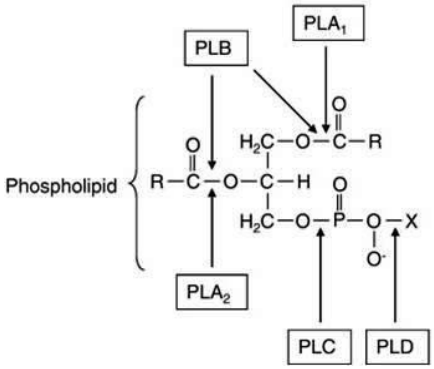

Figure 1.3.1 Phospholipases and their sites of action. Phospholipase A 1 (PLA1), Phospholipase A2 (PLA2), Phospholipase B (PLB), Phospholipase C (PLC), and Phospholipase D (PLD). Adapted from (Mortazavi, Keisary, Loh, Jung, & Khan, 2011)

They are involved in many cell activities including signal transduction, phospholipids membrane synthesis, lipid metabolism and immune activation. As the *sn-2* position of phospholipids of mammalian cells are enriched with arachidonic acid, the substrate of cyclooxygenases, lipoxygenases and P450 monooxygenase, that affects PLA2 activity has important implications for inflammation pathway (Balsinde, Winstead, & Dennis, 2002; Hui, 2012; Stephen B, 2006).

1.3.1 Phospholipase A2

The superfamily of phospholipase A2 (PLA2) is composed of a group of enzymes hydrolyzing glycerophospholipids at the sn-2 position. This hydrolysis reaction results in the release of free fatty acids such as arachidonic acid and lysophospholipids (lysoPL), and both products are important precursors for bioactive molecules that play role of various biological functions (Balsinde et al., 2002).

Based on the activity dependence on calcium ion and sequence homology, the mammalian PLA 2 superfamily are categorized into six principal enzymes, sPLA2, cPLA2, iPLA2, PAF AH, lysosomal PLA2 and a calcium-dependent intracellular

PLA2 being identified in white adipose tissue; denoted as adipose PLA2 (AdPLA2) (Capper & Marshall, 2001; Hui, 2012; Sajal, 2003).

1.3.2 Secreted Phospholipase A2

The secreted PLA2 (sPLA2) group is the largest and first discovered PLA 2 type. In mammalian system, 11 sPLA2s types (IB, IIA, IIC, IID, IIE, IIF, II, V, X, X12IIA and XIIB) have been identified. They are small protein (14-18 kDa) with multidisulfide bonds, containing Ca2+-binding domain. The action of sPLA2 is calcium dependent and their target sites are not only in cell membrane but also in microvesicles, pulmonary surfactant, lipoprotein, microbial membrane and dietary phospholipids (Balestrieri & Arm, 2006; Sajal, 2003; Wilton, 2005). One of the primary functions of sPLA2 is the release of arachidonic acid, the precursor of pro-inflammatory lipid mediators. Therefore, sPLA2s appear to be involved in many inflammatory diseases such as rheumatoid arthritis (Jamal et al., 1998), adult respiratory distress (Kitsiouli, Nakos, & Lekka, 2009), pancreatitis (S. Xu et al., 2010) and atherosclerosis (Quach, Arnold, & Cummings, 2014; Rosengren, Jönsson-Rylander, Peilot, Camejo, & Hurt-Camejo, 2006).

sPLA2 type I is considered as a digestive enzyme, mainly express in acinar cells of pancreas and secreted into intestinal lumen (Loweth, Scarpello, & Morgan, 1995; Peuravuori, Kollanus, & Nevalainen, 2014). The enzyme hydrolyses dietary phospholipid into free fatty acid and lysophospholipid which can be absorbed by enterocyte and then transport to liver (Lambeau & Gelb, 2008). In knockout mice model, the pla2g1b$^{-/-}$ mice were not developed to obesity when induced with the high fat/carbohydrate diet (Murakami et al., 2011).

sPLA2 type II is referred as an inflammatory PLA2. It was originally extracted from platelet and exudates from patients with rheumatoid arthritis (Jamal et al., 1998; Vadas, Stefanski, & Pruzanski, 1985). The Pla2g2a knockout mice also had a high risk of joint inflammation when induced with K/BxN autoantibody compared to the wild type mice (Eric Boilard, 2010). In additionally, sPLA2 IIA was high expression in synovial and chondrocytes in the joint of rheumatoid arthritis patients (Jamal et al., 1998) and epicardial adipose tissue of patients who have undergone cardiac surgery for coronary artery disease (Anne Dutour, 2010).

The role of sPLA2 in atherosclerosis is one of the major topics in this research field. Several type of sPLA2 have been reported to associated with LDL and HDL hydrolysis, leading to the development of atherosclerosis. For example, treatment of LDL with sPLA2 type III (Sato et al., 2008), V (Ohta, Giannattasio, Xing, Boyce, & Balestrieri, 2012), X (Kohji Hanasaki, 2002) generated modified LDL, enhancing facilitate macrophage form cell formation. Modified HDL by sPLA2 reduced cholesterol efflux from lipid loaded macrophages, leading to plague formation (Ishimoto, 2003).

In contrast to the role of sPLA2 in atherosclerosis and cardiovascular disease, their role in metabolic syndrome has not been fully explored. The single nucleotide polymorphism analysis showed the association of the human PLA2G5 with serum LDL and Oxidized LDL in subjects with type II diabetes (Wootton et al., 2007). In animal model, the $Pla2g10^{-/-}$ mice increased obesity (X. Li, Shridas, Forrest, Bailey, & Webb, 2010) while the another study failed to find the differences weight between the Pla2g10 knockout and wild type mice (Fujioka et al., 2008).

1.3.3 Cytosolic Calcium-Dependent Phospholipase A2

Calcium dependent Phospholipase A2 (cPLA2) family is the intracellular enzyme which consists of an N-terminal C2 which binds Ca2+, followed by a catalytic domain which has PLA2 and lysophospholipase activity. They have been classified into 6 types: cPLA2α, cPLA2β, cPLA2γ, cPLA2δ, cPLA2ε and cPLA2ζ with a wide range of molecular weight between 61-114 kD. cPLA2 requires Ca2+ for membrane binding rather than catalysis. In inactivated state, cPLA2 requires 50-110 mM for being stable at membrane (Clark, Schievella, Nalefski, & Lin, 1995). An increase intracellular Ca2+ causes the translocation of the enzyme to membrane and subsequently hydrolyzes the membrane phospholipase to release arachidonic acid. It is the only PLA2 that shows specificity for phospholipid substrate containing arachidonic acid.

cPLA2 has a consensus phosphorylation site for MAPK and different MAPKs are essential for cPLA2 mediated arachidonic release. cPLA2 can be phosphorylated by p42/44 MAPKs (Lin et al., 1993), p38 MAPK, JNK (X. Wang et al., 2008). Other kinases such as PKC and cAMP dependent protein kinase A (PKA) have been reported as a upper stream cascade of cPLA2 phosphorylation (Olivero & Ganey, 2000).

As arachidonic acid is an initial precursor of the inflammatory mediator, the association of cPLA2 expression with activity in various inflammatory diseases has been documented. Studies of cPLA2 knockout mice have a normal appearance with approximately 1.5 year longer life span (Bonventre & Sapirstein, 1999). However, then cPLA2 $^{-/-}$ mice showed a defect in reproduction. Pregnancy in the cPLA2 $^{-/-}$ mice was terminated after implantation due to the increase of vascular permeability in endometrium during implantation (Sapirstein & Bonventre, 2000). Under

pathophysiological state, the cPLA2 knockout mice showed different responses compared to wild type. In the LPS and zymosan induced adult respiratory distress syndrome, pulmonary edema, macrophage infiltration and deterioration of gas exchange are markedly reduced in cPLA2 knockout mice (Sapirstein & Bonventre, 2000). In 1-methyl-4-phenyl-1,2,3,6 –tetrahydropyridine induced brain injury model, cPLA2 knockout mice showed less reduction of brain striatail dopamine, the brain injury marker (Bonventre, 1998). These studies taken together suggest an important role of cPLA2 in various diseases.

1.3.4 Cytosolic Calcium-Independent Phospholipase A2

The calcium independent phospholipase A2 (iPLA2) family consists of a lipid hydrolase domain which was initially discovered in a potato tuber, patatin. They are also called patatin like phospholipase domain containing lipases (PNPLAs). Nine types of the human PNPLAs have been identified in human. The group of enzymes bearing an N-terminal region act mainly on phospholipase are called as phospholipase type which consists 4 enzymes member, iPLA2β, iPLA2γ, iPLA2δ and NRE, whereas the group of enzymes that lack of the N-terminal domain, mainly act of lipids, are so called Lipase type. There are 5 enzymes have been classified into the lipase type, iPLA2ε, iPLA2ζ, iPLA2η, PNPLA1 and PNPLA2. It is fully active in the absence of Ca2+ and the activity is not specific to the 2sn-fatty acid (Kudo & Murakami, 2002; Sajal, 2003; Schaloske & Dennis, 2006).

The classical iPLA2β plays a role in membrane remodeling to maintain the cell homoeostasis (Balgoma, Montero, Balboa, & Balsinde, 2010). Inhibition on iPLA2β via BEL treatment or antisense oligonucleotide lead to reduced AA incorporation into PLs and cellular LPC levels in P388D1 macrophages (Dennis,

1997). Similar results are also found in brain cell studies: it has been reported that BEL treatment inhibits the release of docosahexaenoic acid from the membrane phospholipids of astrocytes upon ATP stimulation (Green, Orr, & Bazinet, 2008). In contrast, overexpression of iPLA2 β and iPLA2γ in HEK293 cells results in the accumulation of free fatty acid and LPC, and this event can be blunted by BEL (Makoto Murakami, 2005). However, in Pla2g6 $^{-/-}$ mice did not show the different in phospholipid composition in testis, pancreatic β cell and peritoneal macrophage (Yoda et al., 2010), indicating that iPLA2 activity could be compensated by other phospholipase enzyme.

Recently, several studies established the roles of iPLA2 β in glucose-stimulated insulin secretion and glucose homeostasis. The Pla2g6 $^{-/-}$ mice showed no different blood glucose after feeding the chow diet, compared to wild type, but the knockout mice developed more severe hyperglycemia after admistration of streptozotocin, the β cell toxin (Bao et al., 2006; Song et al., 2010). In the overexpression mouse model, lower blood glucose and higher insulin in circulation at fasting were observed (Bao et al., 2008). These findings indicated that iPLA2 might be involved in insulin secretion. iPLA2β could be unregulated under high glucose condition. A high glucose treatment in vascular smooth muscle cell induced iPLA2 β expression via PKC activation, corresponding with the high expression of this gene in vascular tissue taken from patient with type II diabetes (Z. Xie et al., 2010).

Moreover, reactive oxygen species (ROS), including superoxide anion, hydroxyl radical and hydrogen peroxide have been known to potentiate iPLA2 activity (Sun et al., 2001; J. F. Xu, Yu, Sun, & Sun, 2003). When the uterine stromal cells were stimulated by hydrogen peroxide, a significant increase in iPLA2 was

detected via the increased calcium-independent arachidonic release (Birbes, Gothié, Pageaux, Lagarde, & Laugier, 2000). Similarly, in RAW 264.7 cells or mouse peritoneal macrophages, both hydrogen peroxide and superoxide anion treatments led to increases in iPLA2 activity (Martinez & Moreno, 2001).

1.3.5 Platelet-Activating Factor Acetylhydrolase

Platelet-activating factor acetylhydrolases (PAF-AHs) catalyze the acylhydrolysis at the sn-2 position of PAF to liberate acetate and lysoPAF. It also hydrolyzes the phospholipids with the oxidized fatty acid acyl group from the sn-2 position of PC and PE. In addition to the acylhydrolase activity, this enzyme also exhibits the transacetylase activity which transfers acetate or short chain fatty acid to lysoPAF and other lysophospholipases. Thereby, the two activities of this enzyme might help cells to control the level of PAF. These enzyme groups compose of four serine enzymes assigned to two numbering groups, group VII and group VIII PLA2 (Hui, 2012; Sajal, 2003; Wilton, 2005). The plasma type PAF AH or lipoprotein-associated PLA 2 is the only secretory protein while the other three are all cytosolic proteins. The major source of LpPLA2 in plasma is likely contributed by monocyte derived macrophage, T lymphocyte and mast cell (A. Cai, Zheng, Qiu, Mai, & Zhou, 2013; Rosenson & Hurt-Camejo, 2012). Recently, it LpPLA2 has been proposed as a new biomarker used for monitoring metabolic syndrome and CVD (Toth, McCullough, Wegner, & Colley, 2010).

1.3.6 Lysosomal PLA2

Lysosomal phosphoslipase A2 consists of two distinct enzymes, acidic $Ca2^{+}$ independent PLA2 (aiPLA2) or peroxiredoxin 6 and a macrophage lysosomal PLA2 (LPLA2) or group XV PLA2 (Sajal, 2003). The aiPLA2 is an anti-oxidant enzyme,

possessing glutathione peroxidease and phospholipase activity. The aiPLA2 knockout mice showed more sensitivity to oxidant agent (L. Li et al., 2007). It also involves in lung surfactant synthesis. The aiPLA2 knochout mice increased degradation of dipamitoyl-PC, lung surfactant, indicating its role in respiratory diseases (A. B. Fisher, Dodia, Yu, Manevich, & Feinstein, 2006; Manevich & Fisher, 2005). LPLA2 hydrolyzes PC and PE in Ca2+ independent manner. It is fully active in pH4.5 and preferentially localized in macrophage alveolar (Abe et al., 2004). The knockout of LPLA2 gene in mice showed foam cell formation in macrophage and increased the surfactant lipid accumulation (Hiraoka et al., 2006).

1.3.7 Adipose specific PLA2

Identification of adPLA2 or PLA2 group XVI first reported in 2008. It appears abundantly expressed in white adipose tissue and generates free fatty acid and lysophospholipid from phosphatidylcholine with a preference at the 2sn-position (Duncan, Sarkadi-Nagy, Jaworski, Ahmadian, & Sul, 2008). Pla2g16$^{-/-}$ mice are lean and have a reduced WAT mass with a low triglyceride and PEG2 content (Jaworski et al., 2009), indicated that AdPLA2 is crucial in the development of obesity.

1.4 Arachidonic acid pathway

Activation of PhospholipaseA2 provides the arachidonic acid release. The free arachidonic acid may subsequently metabolized by one of the three pathways; cyclooxygenase pathway, Lipoxygenase pathway and P450 monooxygenase, producing ecosanoids (Hyde & Missailidis, 2009). Due to the potent biological function of ecosanoids, cells keep the free arachidonic acid at a very low level by regulating phospholipaseA2 activity (Astudillo, Balgoma, Balboa, & Balsinde, 2012)

and arachidonic acid itself can induces cell apoptosis(Pompeia, Freitas, Kim, Zyngier, & Curi, 2002).

1.4.1 The role of Phospholipase A2 in arahidonic acid release.

Arachidonic acid is a polyunsaturated ω6 fatty acid, 20:4(ω6), esterified into the *2sn*-position of glycerolphospholipids located in the cell membrane. Body directly obtains this fatty acid from diet or alternately synthesizes from linoleic acid. The arachidonic acid release is restrictively control because it is a common precursor of eicosanoids which are the mediators of inflammatory reaction. Cells regulate the level of free arachidonic acid by two pathways depending on the intracellular level of the free fatty acid. Under physiological condition, the low concentration of arachidonic acid incorporates into lysophospholipids to form the glycerolphospholipids. This process generally takes several hours in primary cells and is considered to be the major pathway to control the arachidonic acid release (Pérez-Chacón, Astudillo, Balgoma, Balboa, & Balsinde, 2009). As lysophospholipid is necessary as an acceptor of free arachidonic acid in this process, the PLA2 activity may play a key role in arachidonic acid releases because it provides the availability of lysophopholipids. Under the pathological condition, the high arachidonic acid incorporates into triacylglycerol and dirachidonoyl phospholipids via the *de novo* pathway (Balsinde et al., 2002; Chilton, Fonteh, Surette, Triggiani, & Winkler, 1996).

1.4.2 Cyclooxygenase pathway

Cyclooxygenase (COX; Prostaglandin G/H synthase) exist of two isoforms, COX1 and COX2. COX1 is a housekeeping enzyme responsible to sustain the prostanoids level in order to maintain the cell homeostasis. COX2 is undetectable

under normal physiological condition but it can be induced in response to hypoxia, inflammation and cytokines (Cole, Kuhn, et al., 2012; Rizzo, 2011). These enzymes catalyze the stepwise conversion of arachidonic acid into the reactive intermediates PGG and PGH, which are the precursor of prostaglandins and thomboxanes (Simmons, Botting, & Hla, 2004; Vegiopoulos et al., 2010) (Figure 1.4.2.1). COX2 has been reported to be expressed in adipose tissue (Cao et al., 2011; Chu et al., 2010; Vegiopoulos et al., 2010) and the mRNA of COX2 and TNFα are noted to be increased in high fat diet induced obese rats (Cao et al., 2011; Chu et al., 2010). Stable transfection of antisense COX2 increased 3T3-L1 adipocyte differentiation, accompanying a decrease in PGE 2 and PGF 2α levels(Chu et al., 2010).

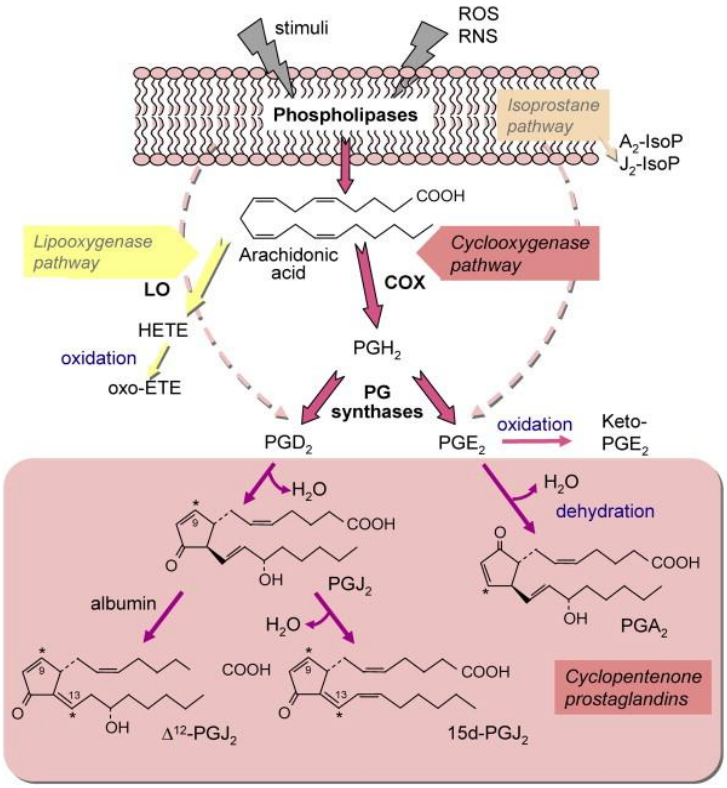

Figure 1.4.2.1 Cyclooxygenase pathway (Garzón, Oeste, Díez-Dacal, & Pérez-Sala, 2011)

1.4.3 Lipooxygenase pathway

In mammals, LOXs were categorized with respect to their positional specificity of arachidonic acid oxygenation into 4 major types, 5-LOXs, 8LOXs, 12LOXs and 15LOXs (Kühn & O'Donnell, 2006). Lipoxygenases oxygenate arachidonic acid in different position along the carbon chain, producing the corresponding 5S-, 12S- of 15S- hydroperoxides (hydroperoxyeicosatetraenoic acid, HPETEs) (Funk, Chen, Johnson, & Zhao, 2002). The hydroperoxides can further convert this into leukotrienes, hepoxylins and lipoxins which are important as mediators of inflammation in various cells (Borgeat & Naccache, 1990) (Figure 1.4.3.1).

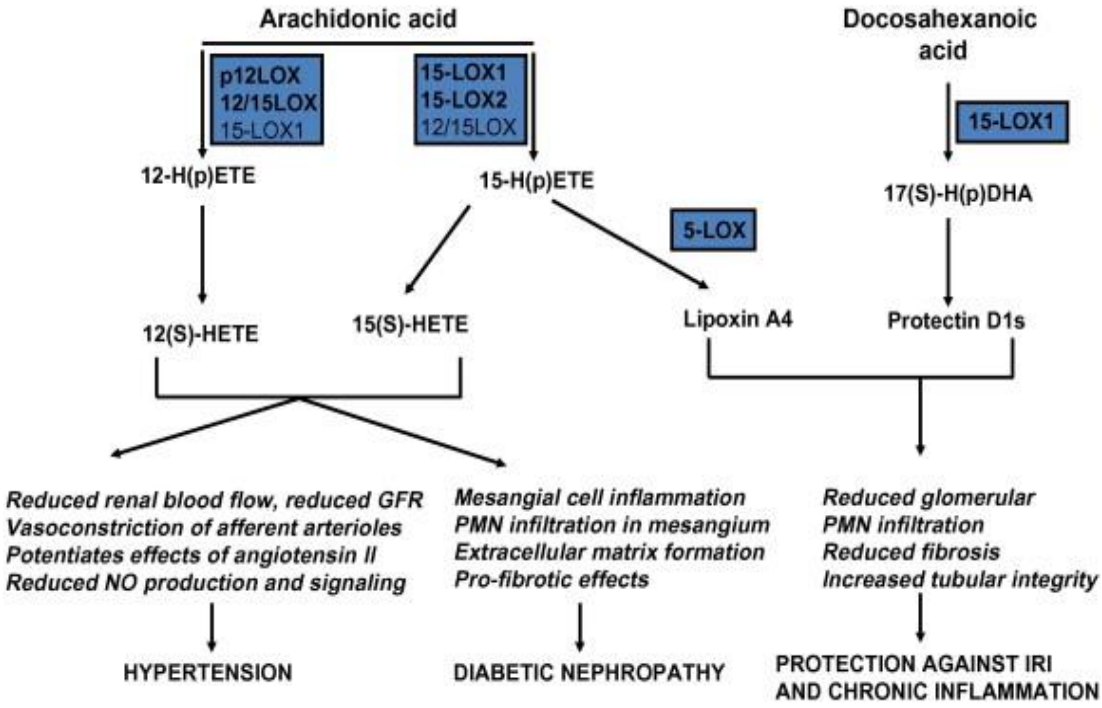

Figure 1.4.3.1. Lipoxygenases pathway (Dobrian et al., 2011)

The most interesting product in this pathway is LTB4. LTB4 is a ligand for peroxisome proliferator receptor alpha (PPARα) which involves in lipid metabolism as well as mitochondria β oxidation (Latruffe & Vamecq, 1997). In addition, LTB induced monocyte chemoattractant protein 1 (MCP1) in human monocyte, mediating allergic inflammation and asthma allergic inflammation and asthma (Ogawa & Calhoun, 2006).

1.4.4 P450 monooxygenase

Cytochrome P450 (CYP450) monooxygenases are the enzyme family that synthesizes epoxyeicosatrienoic acid (EETs) and hydroxyeicosatetraenoic acid (HETEs) from arachidonic acid. (X. Xu, Zhang, & Wang, 2011). Recently, the CYP pathway has wildly studied regarding its role in regulation of vascular tone (Rahman, Wright Jr, & Douglas, 1997; Sacerdoti et al., 2011). EETs are associated with vasorelaxation and ion transportation in kidney, leading to regulation of renal blood flow and Na+ reabsorption, resulting in blood pressure reduction (Rahman et al., 1997)

Several studies indicated EETs are involved as a key inflammatory mediator. Direct administration of 11,12EET attenuated TNFα induced NκB activation in HUVECs (Fleming et al., 2001). Similarly, incubation the cells with EETs and their specific inhibitor prevented IκB degradation, inhibiting NFκB activation (Y. Liu et al., 2005). Moreover, the anti-atherosclerotic effect has been reported. The inhibitor of sEH reduced the atherosclerotic lesion and the reduction of serum lipid, IL6, IL8, IL1α were also observed in a model of abdominal aortic aneurysm apolipoparotein E deficiency mice (Zhang et al., 2009).

1.5 LpPLA2 as a marker for monitoring cardiovascular diseases

Prevalence of cardiovascular diseases is strongly associated with obesity T2DM. The link between obesity and pathogenesis of cardiovascular diseases is complicate and involved by organs that play critical roles in energy metabolism. It is fundamentally associated with increased lipids in circulation, endothelial inflammation and accumulation of modified lipids within vascular wall (Douglas & Channon, 2010). Currently, subclinical atherosclerosis, namely as metabolic syndromes, is diagnosed in order to predict and prevent at high risk for CVD and T2DM in clinical practice. Currently, the National Cholesterol Education Program-Adult Treatment Panel III (NCEP-ATP III) has proposed a new criteria for screening CVD risks with the presence of at least three of five criteria; (1) hypertriglyceridnemia, (2) low HDL, (3) abnormal fasting blood glucose, (4) increased waist circumference, and (5) hypertension, is diagnosed as metabolic syndromes (MS) (Expert Panel on Detection, 2001).

1.5.1 Pathology of atherosclerosis

Increased production of circulating lipoprotein in liver together with reduction of fat storage capacity in AT are the factors that contribute to increased circulating LDL, TG and free fatty acid. Prolong LDL in circulation is susceptible to modification, especially by oxidation. Under the hypertension condition, ox-LDLs are penetrated into endothelial intima and subsequently induce macrophage infiltration into the plaque. Excessive macrophage uptakes of modified lipids create foam cells and deposit inside the vascular wall. Activated macrophages secret inflammatory cytokines and induce endothelial cell inflammation, which consequently increases the generation of reactive oxygen species (ROS). Cytokines

such as TNFα induced apoptosis of the foam cells, leading to accumulation of death cells which aggravate tissue damage (Douglas & Channon, 2010) (Figure 1.5.1.1).

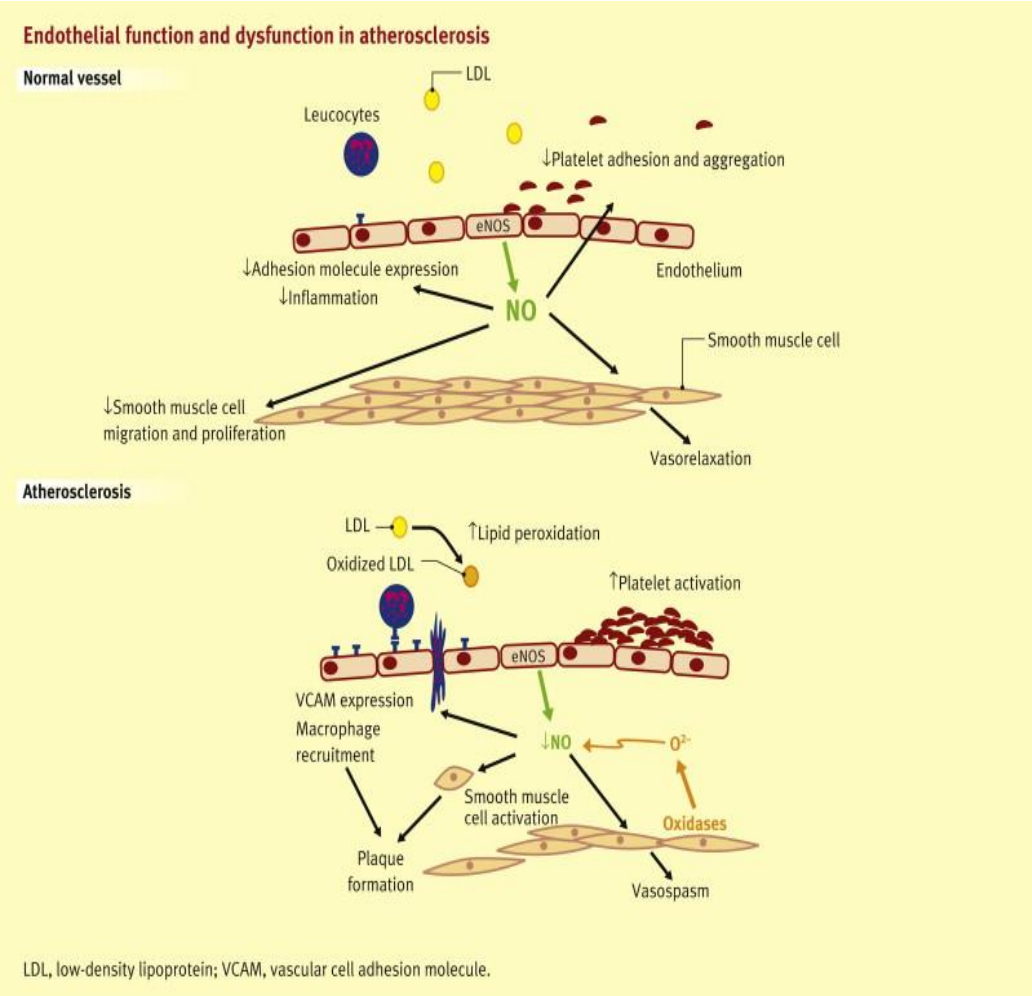

Figure 1.5.1.1 Pathogenesis of atherosclerosis (Douglas & Channon, 2010)

1.5.2 LpPLA2 and atherosclerosis

The majority of plasma LpPLA2 in healthy subjects bound to LDL and small proportion is associated with HDL. Plasma LpPLA2 is closely correlated with LDL and increased in obesity and T2DM. Currently, it has been proposed as a biomarker for cardiovascular diseases (A. Cai et al., 2013; Dallmeier & Koenig, 2014). Increased circulating LpPLA2 is associated with metabolic diseases such as T2DM

(Fortunato et al., 2014) and CVD (J. Liu et al., 2014). Study in patients undergoing carotid endarterectomy showed the high expression of LpPLA2 and lysophosphatidylcholine in plaques.

However, the role of LpPLA2 in atherosclerosis is controversial since its biological function mediates both anti atherogenic and pro atherogenic effects. The anti atherogenic primitively ascribed to its ability to degrade platelet activating factor (PAF) and hydrolyze ox-LDL, minimizing macrophage recognition. This process subsequently liberates lysophosphatidylcholine (LysoPC) and oxidized free fatty acids (ox-NEFA) which can induce apoptosis and further oxidize intact LDL. oxNEFA and LysoPC also induce various of inflammatory cytokine, worsen inflammation in the pathogenic lesion, exhibiting its pro-atherogenic effect (Figure 1.5.2.1).

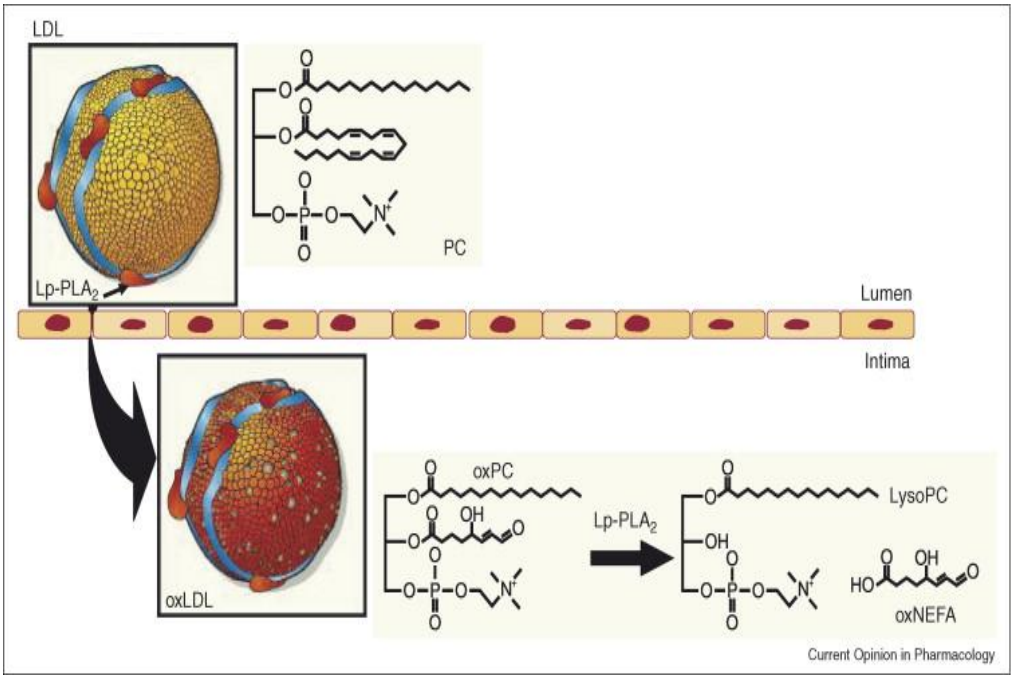

Figure 1.5.2.1 Hydrolysis of ox-LDL by LpPLA2. (Macphee, Nelson, & Zalewski, 2006)

However, the massive over-expression of LpPLA2 gene in apoliprotein E (apoE) $^{-/-}$ mice, which increased approximately 76-140 fold of plasma LpPLA2,

showed the protective effect of lipoprotein oxidation induced by copper ion. Whilst, diabetic/hypercholesterolemia animal model treated with LpPLA2 inhibitor markedly reduced the lysophosphatidylcholine in lesions and mitigated the development of atherosclerosis (Schmitz & Ruebsaamen, 2010). The controversial evidence demonstrated that LpPLA2 might be involved in complementary or opposite pathways in atherosclerosis, the understanding of expression, function and regulation of LpPLA2 in specific tissue and diseases state in different species might help to understand the CVD pathology.

1.6 Thesis Aim

Adiposity is a pathological condition leads to comordiality complications, including T2DM and cardiovascular diseases. Dysregulation of lipolysis and lipogenesis in AT expansion is a contributor of increased non-esterified fatty acid and circulating lipoproteins, especially LDL and oxLDL. Recently, LpPLA2 has been proposed as a new marker for CVD risk since extensive evident supported the strong association between prevalence of metabolic diseases and LpPLA2. It has been reported to express in immune cells and secreted to into circulation. In circulation, this enzyme mostly bound to LDL particles. LpPLA2 possesses both anti- and pro-atherogenic properties due to its biological activity. In LpPLA2 overexpression model, LpPLA2 exhibited the protective effects against lipoprotein oxidation, whilst in LpPLA2 inhibitor study, inhibition of LpPLA2 reduced the CVD progression. Thus, the understanding of expression, function and regulation of LpPLA2 in specific tissue and diseases state in different species might help to understand the link between adiposity and CVD pathology.

To date, there is no evident of role of LpPLA2 in AT. This thesis sought to (1) characterise PLA2 isoforms in lean, obese, T2DM abdominal subcutaneous (AbdSc) and omental (Om) in human adipose tissue (AT); (2) evaluate the role of lipids and inflammatory markers on circulating LpPLA2, and (3) determine the *in vitro* regulation of LpPLA2 in human adipocytes by its influence on LDL and Ox-LDL.

The mechanism underlying AT inflammation is mainly involved with NFκB pathway. Activation of TLRs potentiates NFκB activity. Previously, NEFA and ox-LDL have been reported to increase the TLR4 expression. Therefore, this study sort to investigate the impact of lipid changes in bariatric patients on TLRs expression as well as its adaptor protein.

CHAPTER 2 : General Methods and Materials

2. General Methods and Materials

ALL materials and methods used throughout this thesis were described in this chapter. The detail of reagent preparations and experiment protocols were in appendix A.

2.1 Adipose tissue collection

Paired human Sc and Om AT biopsies were taken from patients undergoing elective surgery. Subjects with a history of cancers as well as endocrine diseases or on treatment were excluded. None of the subjects was taking anti-inflammatory drugs or steroid drugs. Detailed medical drug histories were also taken. Ethical approval was obtained from the local ethics committee and all subjects gave written consent prior to enrolment. AT samples were frozen or processed for future use immediately after collection in order to inhibit the alteration of AT metabolism and cell lysis.

2.1.1 Adipose tissue freezing

AT sample were cut and aliquoted into a sterile centrifuge tube and immediately frozen in liquid nitrogen (N_2). After AT samples were completely frozen, the tubes were directly transferred into -80^{0}C freezer. The frozen samples can also be kept in N_2 for longer storages.

2.1.2 Mature and pre-adipocyte isolation from AT

AT composed not only of pre-adipocyte but also the connective tissue and immunological cells. To isolation pre-adipocyte, approximately 10-20 ml of AT was finely cut and transferred into 50 ml sterile centrifuge tube containing 10 ml of warm

collagenase (2mg/mL in HBSS, Worthington Biochemical Corporation, New Jersay, USA), then mixed vigorously under biological cabinet II. Subsequently, the AT/collagenase mixture was incubated in 37^0C shaking water bath for 30 minutes (min) with shaking at 10 mins interval to distribute collagenase evenly and digest the connective tissue.

After 30 min incubation, the centrifuge tubes were moved to a biological cabinet II and the mixture was filtered through a layer sterile cotton mesh into a new 50 mL sterile centrifuge tube, then centrifuge at 300-350 g for 5 mins. The mixture was then separated into three layers according to difference of density after centrifugation. The lowest density layer which consisted of mature adipocyte filled with lipid was on the top. The highest density, the stroma-vascular fraction containing several types of cells, together with pre-adipocyte, macrophage and leukocyte, was on the bottom. The middle layer was aqueous solution contain collagenase.

To collect the mature adipocytes, approximately 5 mL of the top creamy layer was gently transferred to a new sterile centrifuge tube. The cells were then carefully suspended with 10 mL of warmed serum free Dulbecco's modified Eagle's medium with high glucose (DMEM/F12) by manually inverting the tube. The tubes were then centrifuged at 300-350 g for 5 mins to remove contamination of collagenase and blood. The mature adipocytes were on the top layer and the aqueous layer underneath was carefully removed by using a sterile seropipette. This step was repeated until the adipocyte layer was appeared to free from blood. To keep the mature adipocytes for future works, the adipocyte layer was then aliquoted approximately 1 mL into sterile cryotube and quickly placed into dry ice to freeze the cells. The frozen cells were then kept in -80^0C until use.

To culture the cells, the pellet was carefully mixed and counted cell numbers by using heamatocytometer. Five thousand cells (100,000 cells/mL) were transferred into 25cm^2 culture flask containing completed media 10% fetal bovine (FBS), 20U/ml penicillin/Streptomycin and 10 µg/mL transferin in DMEM/F12 (phenol red free) and incubated at 37^{0}C, 5%CO_2 incubator for at least 16 hr to let the cells attached to the flask surface. After 16 hr, the media was removed to get rid of death cells and changed into fresh completed media.

2.1.3 Purity of adipocyte fraction isolated from AT

The contamination of monocyte/macrophage was observed by using immunohistochemistry with antibody against macrophage specific markers as described in the relevant chapters.

2.3 Human adipocyte culture

2.3.1 Human pre-adipocyte cell line

Chub-S7 was used as an *in vitro* experiment model throughout this thesis. It was firstly established in 2003. This cell line derived from subcutaneous abdominal white adipocyte taken from 33 years old women with BMI 52 Kg/m^2. The pre-adipocyte precursors were transfected with human telomerase reverse transcriptase and papillomavirus E7 oncoprotein in order to immortalize and conserve human pre adipocyte properties (Darimont, 2003). Similar to primary cell culture, Chub-S7 cells were maintained in Dulbecco's modified Eagle's medium with high glucose (DMEM/F12) supplement with 10% FBS, 20U/ml penicillin/Streptomycin and 10µg/mL transferrin in 37^{0}C and 5% CO_2 incubator.

2.3.2 Human pre adipocyte differentiation

The cells were grown until confluence. Then, cells were replaced the growth media with Differentiation media DMEM/F12 supplement with pre-adipocyte differentiation media supplement (Promocell) to promote cell differentiation. The media was changed every 48 hr for 4 days. At Day4, replace the Differentiation media with Nutrition media which contained DMEM/F12 supplement with adipocyte nutrition media supplement (Promocell) and fed the cells with fresh Nutrition media every 2-3 days for at least 14 days to increase adipogenesis during differentiation process. The cells were used for the relevant experiments at day 14.

2.4 Cell viability examination by Trypan Blue dye exclusion test

Cell viability was observed by visual method using light microscope. This method was based on cell membrane permeability of the living cells. Ten microlitters of adipocytes were re-suspended in 10µL of 0.4% trypan blue (Sigma, UK) in phosphate buffer saline (PBS, pH 7.6) for 5 min in room temperature (RT). The living cells are able to efflux the dye out of the cells, whist the death cells uptake the dye. Cell viability and numbers were counted using haemocytometer. The death cells stain blue.

2.5 Protein isolation

2.5.1 Protein isolation from AT

Approximately 50 mg of Human AT was quickly cut into tiny pieces, then transfers into sterile 15 mL tube. The sample was re-suspended in 1 mL of cold Phosphosafe™ Extraction buffer (Novogen ®, Merck, Germany) and homogenized

at 1,000 g for 30 secs by a Rotor-Stator Homogenizer (PowerGen125, Fisher Scientific, UK). The cell lysate was then transferred to 1.5 mL microcentrifuge tube and rapidly placed in liquid N_2 to freeze the cell lysate. Subsequently, samples were then removed and allowed to thaw at RT. After thawing, the microcentrifuge tubes were then spun at 18,000 g at 4^0C for 30 min. The samples were then separated into three layers after centrifugation. The cellular protein fraction was in the middle between the lipid layer and cell debris. The protein layer was carefully collected by using syringes and hypodermic needles, transferred to a sterile 1.5 mL microcentrifuge tube and kept in -80^0C freezer until use.

2.5.2 Cell trypsinization

The adherent adipocytes were detached from culture flask or plate by treatment of trypsin-EDTA solution (Gibco, UK). For this procedure, culture media was removed and the cells were washed three times with cold PBS. Then, the cells were incubated with 2.5 mL of 37^0C warmed 0.25% trypsin-EDTA solution for 3-5 mins (maximum 5 mins) at 37^0C. The trypsin-EDTA solution was then removed and quickly added 5 mL of completed media to stop trypsin activity. The mixture was gently re-suspened by using sterile seropipette and checked the detachment under light microscope. All cells should be separated nicely without any clump. The cell mixture was then transferred to 50 mL centrifuge tube and centrifuged at 2500 rpm for 10 min. The supernatant was then discarded. Cell pellet was washed for three times.

2.5.3 Protein isolation from cell culture.

After trypsinization, the cell pellets were lysed by adding 500 µL of cold Radio-Immunoprecipitation Assay (RIPA) buffer (Thermo Scientific, UK)

containing protease inhibitors cocktail and phosphatase inhibitors reagent. For the cells in 6 wells plate, 200 µL of RIPA were used. The samples were then incubated for 10 mins on ice with gentry shaking. After that, the samples were well mixed and collected by using pipette or cell scraper. The samples were centrifuged at 18,000 g for 10 mins, 4^0C. The supernatants were collected in 1.5 mL microcentrifuge tubes, rapidly frozen in liquid N_2 and kept in -80^0C until use.

2.6 Quantification of cellular protein concentration

2.6.1 Lowry method

Protein concentrations in samples that contained RIPA buffer were determined by using the Bio-Rad DC™ protein assay kit (Bio-Rad Laboratories, Hercules, CA). This method is based on the reduction of mixed acids in Folin reagent (phosphomolybdic-tungstic acids) by protein, producing blue colour of reduced forms of the acids. For this procedure, the protein samples in RIPA buffer were vortexed after allowed to thaw at RT. Working reagent was prepared by mixing 980 µL of reagent A (alkaline copper tartate solution) with 20µL of Reagent S (Surfactant) in glass test tubes. Five microliters of protein sample was added test tubes. One hundred and twenty microliters of working reagent was then added to the test tube, followed by 1 mL of reagent B (diluted folin reagent) and mixed by inversion. The mixture was then incubated for 15 mins at RT. The absorbance of the product was then quantified by spectrophotometer at a wavelength of 655nm.

2.6.2 Bradford assay

To quantify protein concentration in samples resuspended in non-detergent buffer, the BioRad Bradford protein assay kit was used (Bio-Rad Laboratories,

Hercules, CA). Bradford assay is based on the alteration of red cationic form of Coomassie brilliant blue G-250 to blue anionic form, induced by protein binding. In short, concentrate Bradford reagent was diluted 1:5 with distilled water (approximately absorbance was 0.500 at λ595 nm). One microliter of protein sample was mixed with 1 mL of working Bradford reagent and incubated for 5 mins at RT. The absorbance was measured at 595 nm.

2.7 Determination of protein expression by western blot

Western blot is an analytical technique used in identification and quantification of the certain protein in a protein mixture. In western blot, mixture of proteins was run through sodium dodecyl sulphate polyacrylamide gel which allowed proteins in the sample separated by size. Separated proteins in the gel were then transferred into PVDF^{TM} membrane by applying an electric current. The membrane was then immersed in blocking reagent in order to cover the area that not blotted on the membrane in order to prevent non specific binding of antibody. The protein of interest was probed by specific antibody at an appropriate dilution as described in the relevant chapters. The specific protein band was then quantified by using ECL.

2.7.1 Sample preparation

Protein samples were diluted into 20 or 40 µg/20µL (20 µL of 1 or 2 µg/µL, respectively) with deionized water and added 5µL of 5X loading buffer containing SDS, β-mercaptoethanol, glycerol and bromphenol blue (Geneflow, UK). The samples were boiled at 95^0C for 5 mins. In this step, proteins in the sample were denatured by heating and β-mercaptoethanol which was used to reduce the protein disulfide bond. The denatured peptides attached with SDS in a constant mass ratio

(1.4:1), creating rod shape with negative of polypeptides. Glycerol was added to prevent sample overflow and uneven sample loading. Anionic bromphenol blue dye was used for monitoring the protein migration progress. After 5 mins, the samples were the placed on ice for 2-5 mins.

2.7.2 Electropheresis

Each protein in the sample mixture was separated according to its molecular weight and the porous acrylamide gel. Percentage of acrylamide affected the porous sizes with higher percentage created smaller pore. Thus, 7% and 10% acraylamide were used for protein with MW < 50 and > 50 kilodalton (kDa), respectively.

For gel preparation, 1.5 mm glass plates were wiped with absolute methanol and allowed to dry. The clean glass plates and a rack of solidification were assembled according to manufacturer's instruction. Distilled water was poured between the glass plates to test leakiness. Fifty milliliter of 7.5 or 10% separating gel solution was prepared just before gel pouring. Once TEMED was added to the separating gel solution, the solution mixture was quickly and gentry mix and immediately poured into the gel casings until the level is equally at the top of the green grips of the rack. Distilled water or absolute of methanol was overlaid on the top of the separating gel solution and left approximately 30-45 mins at RT to complete gel polymerization. The 4% stacking gel was then prepared. Once the gels were set, the stacking gel solution was added by using pipette, after removing distilled water or methanol. The 1.5 mm thick, 10 wells comb was immediately inserted into the stacking gel to create wells, avoiding creating bubbles. Completely polymerization of the gel took approximately 1 hr at RT. After the gels were set, the comb was carefully removed and the wells were washed by distilled water to remove the excess un-polymerized

acrylamide. The glass plates with polymerized gels were then placed inside an electrophorator tank and filled the tank with 1L of running buffer, ensuring the gels were completely covered with the buffer. The sample tubes were briefly centrifuged and 25 µL of samples were loaded to each wells, followed by 5 µL of molecular weight protein markers well. An electrophorator was then connected with power supply using a fixed voltage of 100V. The electrophoresis was run approximately 1 hr and 1.30 hr for 7% and 10% separating gel respectively or until the dye fronts ran to the bottom of the gels.

2.7.3 Electroblotting

Electroblotting is the technique that transfers protein onto a membrane. Following electrophoresis, the glass plates with the gels were removed from the tank and carefully released the gel from the glass plate. The stacking gel layer was cut and discarded, and then the gel was washed in cold transfer buffer. To prepare a standard transfer stack, 2 sheets of filter papers and 2 fibre pads were soaked in cold transfer buffer at least 10 mins. Immobilon-P^{TM} PVDF membrane (0.45µm; Millipore, USA) was immersed in absolute methanol and subsequently soaked in cold transfer buffer at least for 10 mins. To set up a transfer stack from anode to cathode, a fiber pad was firstly laid over the black side of the transfer cassette, followed by a filter paper, gel, PVDF membrane, filter paper and a fiber pad. The stack then was placed in the electrotransfer unit, containing cold transfer buffer and ice pack. The cassette needed to be covered with the buffer and a magnetic stirrer was also put in the tank. The electrotransfer unit was then placed onto a magnetic stirrer in order to reduce the heat that produced by electric current. The peptides on the gels were transferred at 100 V,

1 hr for 1.5 mm thick gel. Overnight transfer with a constant voltage of 25 would be done for high molecular weight protein.

2.7.4 Antibody detection

After electroblotting, the membrane was removed from the cassette and then quick wash with PBS. The membrane was submerged in blocking reagent to reduce non-specific binding background on an orbital shaker for at least 4 hr at RT, or overnight at 4^0C. After incubation, the membrane was then quickly washed with PBS-Tween 20 (0.1% PBS-T) and incubated with primary antibody which was diluted with 5 mL of 0.2% I-block in PBS-T at a specific dilution, described in the relevant chapters. The incubation time varies depend on primary antibody with the short time incubation (1-2 hr) was done at RT, while the longer incubation (overnight) was done at 4^0C.The membrane was then washed to get rid of excess primary antibody for 5 times, 5 mins each, with PBS-T on orbital shaking and avoided to leave the membrane of 0.1% PBS-T for too long.

The binding of primary antibody with peptides on the membrane was detected by using secondary antibody conjugated with enzyme such as horseradish peroxidase. The secondary antibody was also diluted with 5 mL of 0.2% I-block in PBS-T. The membrane was incubated for 1 hr and then washed with PBS-T for 5 times, 5 mins each.

2.7.5 Quantification of protein bands

The horseradish peroxidase conjugated secondary antibody was detected by using a chemiluminescent detection system, ECL/ECL$^+$ (Amersham Pharmacia Biotech, Little Chalfont, UK). In chemiluminescent reaction, luminol was used as a substrate for peroxidase, creating emission of light at 428 nm. The light intensity

was increased up to 1,000 fold by p-iodophenol. The light emission was visualized using Hyperfilm ^{TM} (Amersham Pharmacia Biotech, Little Chalfont, UK) within 60 mins. For procedure, after secondary antibody incubations, the membrane was rinsed with PBS-T for 5 times, 5 mins each. Equal volume of reagent A and B were mixed just before use. The membrane was placed over the clear thin film in the orientation that allowed the peptide blotted site to expose the chemiluminescent reagent. Sufficient volume of the chemiluminescent reagent was poured to the membrane and incubated fir 1 min in dark. Then, the membrane was wrapped with the clear thin plastic wallet. The membrane in the wallet was placed inside the X-ray film cassette. To develop the film, a hyperfilm ^{TM} was placed over the membrane inside the cassette. This process needed to be done in the dark room. The exposure time of the film and the membrane depended on the light signal, which normally took 10-60 mins. The band density was then quantified by (Genesnap, Syngene, UK).

2.8 RNA isolation

To isolate total RNA from AT, approximately 50 mg of AT was cut and resuspended in 1 mL of QIAzol lysis reagent (QIAGEN, UK). The samples were then homogenized using a Rotor-Stator Homogenizer (PowerGen125, Fisher Scientific, UK) at 1000g for 1sec. For cell culture, adipocytes were rinsed with cold PBS and 250 µL of QIAzol lysis reagent was added directly to the culture plates. The plates were then incubated for 10 mins on ice tray with gentry shaking, and scraping the adipocytes with cell scraper.

Samples in QIAzol were extracted for total RNA by using a column based method (RNeasy lipid tissue kit, Qiagen, UK), according to the manufacturer's

instructions. Extraction was followed by a DNase digestion step (DNase I kit, Sigma, UK) to remove any contaminating genomic DNA.

2.8.1 RNA Quantification

Total RNA concentration was quantified by Nanodrop ND-1000 Spectrophotometer (LabTech, UK). The absorbance was measured at 260 (mainly absorbed by RNA) and 280 (absorbed by protein, solvents, salts) nm. The absorbance 260/280 ratio was assessed to estimate RNA purity with the ratio of 1.8-2.0 was acceptable for the next steps. Contamination by phenols was also estimated by measuring the absorbance at 230 and the 260/230 ratio of 1.8-2.0 was represented a highly purified RNA.

2.8.2 cDNA synthesis

Two hundreds nanograms of RNA from each sample was reverse transcribed using Bioscript Reverse Transcriptase (Bioline, UK) and random hexamers (Bioline, UK) and 10 mM dNTP mix (dATP, dGTP, dCTP and dTTP, Bioline, UK), according to the manufacturer's instructions.

Total mRNA in the sample was served as template in reverse transcription process. Random hexamers or oligo(dT) bound to its complementary region on mRNA and complementary DNA was synthesized by reverse transcriptase using dNTP as precursors (Figure 2.8.2.1).

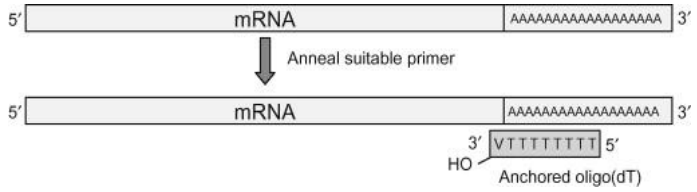

Figure 2.8.2.1 Diagram illustrating the primer annealing. Oligo dT anneals to the poly A tail of mRNA and the cDNA is reversed trancripted by reverse transcritase activity (Farrell Jr, 2010a)

2.9 Real-Time Polymerase Chain Reaction (RT-PCR)

RT-PCR is a fluorescent method that was used to monitor the primers used during each PCR cycle. In these studies, the TaqMan® assay was performed using a oligonucleotide probe which is labeled with 6-carboxyfluorescein (6-FAM) as a reporter dye at 5' end and a non-fluorescent quencher at 3' end. This probe binds in between the target gene template. The target gene PCR product was synthesized used the *Taq* DNA polymerase with specific primers. During the replication, the 5′ exonuclease activity associated with the *Taq* polymerase cleaves the dye probe, removed the 6-FAM from the quencher, resulting in a fluorescent signal. The accumulation signals from each cycle reflect gene expression at transcritption level (Figure 2.9.1).

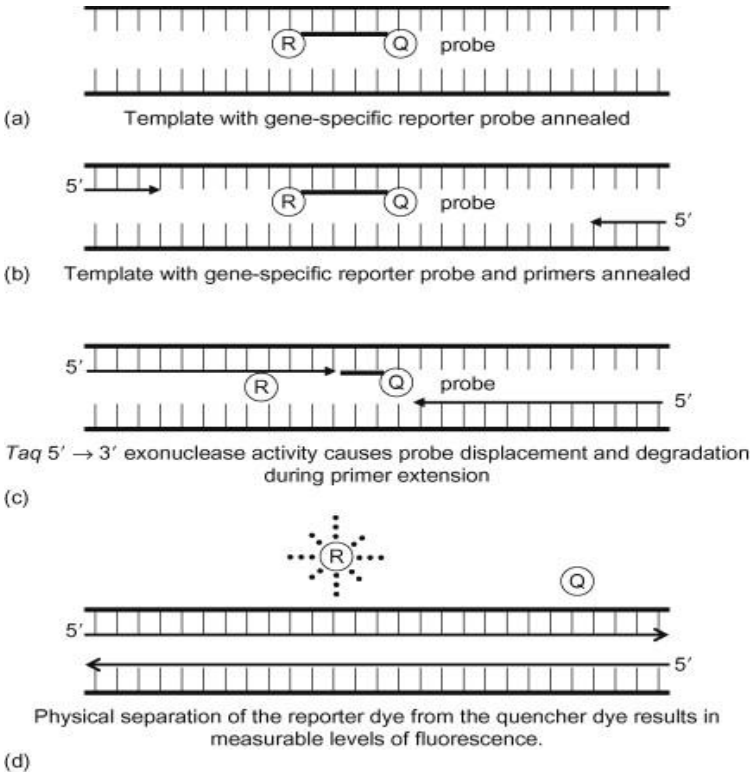

Figure 2.9.1 TaqMan® assay for RT-PCR. Reporter probe containing the fluorescent reporter and quencher dye is designed to anneal with specific region in mRNA. The probe is cleaves during DNA polymerization, allowing reporter dye to emit light.(Farrell Jr, 2010b)

For procedure, 1 µL of cDNA was mixed with 24 µL of a reaction buffer containing Taqman universal PCR mastermix (Applera, UK). All reactions were multiplexed with the housekeeping gene 18S, provided as a pre-optimised control probe (Applera, UK) enabling data to be expressed as delta threshold cycle (ΔCT) values (where ΔCT = CT of 18S subtracted from CT of gene of interest). The reactions of PCR were as follows; 50^0C for 2 mins, 95^0C for 10 mins, 44 cycles of 95^0C for 15 mins and 60^0C for 1 min. Measurements were carried out using an ABI 7500 Sequence Detection System (Applera, UK).

2.10 Microarray technique

Microarray is a technique used to detect expression of a set of genes. High quality of RNA was used to synthesize cDNA as previously described. cDNA was labeled with fluorescent day and hybridized with microarray, according to a standard Affymetrix protocols (Figure 2.10.1). PLA2 mRNA expression was investigated using the 219064_AT probe set and the Human Genome U133A DNA microarray (Affymetrix).

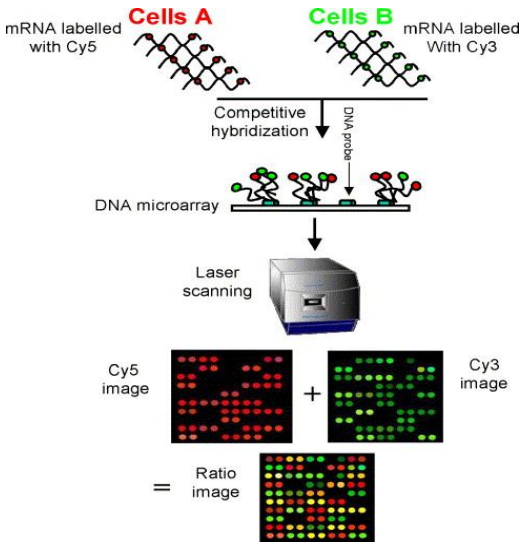

Figure 2.10.1Diagram illustrating DNA microarray technique (Lamartine, 2006)

2.11 Immunohistochemistry

Immunohistochemistry is a technique used to identify and localize the protein of interest within a tissue section.

2.11.1 Tissue section

Approximately 50 µg of fresh AT was fixed in formalin and embedded in paraffin. The tissue section were cut at 3µm thick and mounted on 3-Aminopropyltriethoxy-silane coated slides. The slides were incubated overnight at 60^0C. The sections were then de-paraffinized and rehydrated by soak in 100%Xylene for 3 mins twice, followed by 1:1 ratio of 100%Xylene and absolute ethanol for 3 mins, absolute ethanol 3 mins twice, 95% ethanol 3 mins, 70% ethanol 3 mins and 50% ethanol for 3 min, respectively. The slides were rinsed with tap water.

2.11.2 Section blocking

The slides were incubated in 1.5% hydrogen peroxide for 20 mins and rinsed with tap water. Then the slides were incubated in 1% horse serum (Vectastain Universal Elite ABC detection kit, Vector Laboratories, CA, USA)

2.11.3 Immunohistochemical staining

After blocking step, slides were rinsed with 0.05M, pH 7.6 PBS and incubated with primary antibody for 2 hr at RT. The slides were then rinsed 3 times with 0.05M, pH 7.6 PBS for 3 mins. Tissue sections were incubated with the biotinylated secondary antibody for 30 mins, RT and were washed with 0.05M, pH 7.6 PBS for 5 mins, 3 times. The slides were then incubated with an avidin-biotin complex (Vector Elite ABC reagent) for 30 mins and rinsed with PBS for 5 mins.

The DAB solution (Menarini Concentrated Substrate Cat. HK153-5K) was freshly prepared and applied to each tissue slide. The slides were incubated until a brown staining appeared.

2.11.4 Counter Staining of tissue sections

The slides were rinsed with deionized water and counter stained with Mayer's haematoxylin (BDH, Poole, UK) for 1 min and immediately washed with tap water. The slides were then dehydrated by immerging in IMS alcohol for 5 mins, followed by 100%Xylene for 2 mins and repeated this step with fresh 100%xylene. The slides were mounted with a xylene-based mounting agent (DPX; UK).

2.12 Plasma and serum collection

Whole blood was drawn into serum tube, plasma tube containing NaF for fasting blood glucose measurement and plasma tube containing EDTA tube for ELISA measurement (BD-vacutainer ®). Serum tube was left at RT for 30 mins to allow the blood to clot. All sample tubes were then centrifuged at 600g for 10 mins at RT. Supernatant was transfer to sterile 1.5 mL centrifuge tube and kept at -80^0C.

2.13 Enzyme Linked Immunosorbent Assay (ELISA)

Serum or plasma cytokines, LpPLA2 and ox-LDL were measured by using the commercial ELISA kits. ELISA analysis was carried out according to the manufacturer's instructions.

ELISA is based on antigen-antibody reaction. The antibody of the protein of interest was coated in the solid well plate. Samples were then pipetted into the well and incubated to allow the antigens in samples bound to the specific antibody. After

incubation, the reaction plates were washed with washing buffer, according to the relevant procedures in order to remove excess antigens. The peroxidase conjugated secondary antibody was added to detect the immobilized immune complex. After incubation, the plates were then washed with washing and subsequently added the substrate, TMB, to produce color. The color product was measured by microplate reader within 30 mins.

CHAPTER 3 : PLA2 Expression in Human Adipose Tissue

3.1 Introduction

Lipoprotein-associated phospholipase A2 (LpPLA2) is a member of the phospholipase A2 super family of enzymes. These enzymes hydrolyse polar phospholipids, such as those generated during the oxidation of low density lipoprotein (Ox-LDL), which results in the production of pro-inflammatory mediators, such as lysophosphatidylcholine and oxidised fatty acids (Rosenson & Stafforini, 2012; Toth et al., 2010; Zalewski, Nelson, Hegg, & Macphee, 2006). Whilst secreted soluble PLA2 (sPLA2) is known to increase arterial inflammation, other members of the family, such as cytosolic calcium dependent PLA2 (cPLA2) and calcium independent PLA2 (iPLA2), also appear to contribute to inflammatory processes through production of lipid mediators (Toth et al., 2010).

Recent interest in LpPLA2 has arisen due to a number of epidemiological studies that have shown circulating LpPLA2 to be up-regulated in conditions of inflammation and cardiovascular disease (CVD) (Khakpour & Frishman, 2009; Kinney et al., 2011; Oei et al., 2005; Winkler et al., 2007). In contrast, limited studies, to date, have examined circulating LpPLA2 in patients with T2DM, although a recent study has highlighted LpPLA2 levels as being positively correlated with body mass index (BMI), as well as triglycerides (TG), low density lipoprotein (LDL) and high density lipoprotein (HDL). Furthermore, elevated LpPLA2 levels have been associated with predicting T2DM (Maria Waegner et al., 2011; Nelson et al., 2012), although other reports have not been conclusive (Constantinides et al., 2011; Nelson et al., 2011). Much of the research, to date, has centred on CVD and the role of the macrophage as a major source of LpPLA2, although other cells types are also noted to express LpPLA2 including T lymphocytes, mast cells and hepatocytes (W.-Y. Wang et al., 2010; M. Yang et al., 2010; Zalewski et al., 2006). Previous *in vitro*

studies, using monocytes, have specifically examined the stimulatory effect of Ox-LDL on LpPLA2 activity, which indicates the potential influence of LpPLA2 on arterial inflammation (W.-Y. Wang et al., 2010); whilst in *vivo* studies have shown that Ox-LDL are significantly associated LpPLA2 in carotid endarterectomy (CEA) tissues (Vickers et al., 2009).

LpPLA2 appears to represent a biomarker of CVD and/or T2DM risk and previous LpPLA2 studies have considered the rationale for its correlation with metabolic disease. In particular, LpPLA2 activity may arise as a consequence of ectopic fat deposition, raising circulating lipids, with an increase in LpPLA2 activity triggering production of pro-inflammatory mediators (Gomes et al., 2009; Silva, Mello, & Damasceno, 2011). Alternatively, increased macrophage activity noted through raised souble-CD14 levels may arise in response to an inflammatory insult, such as metabolic endotoxaemia (Al-Attas et al., 2009; Harte et al., 2010). Clinical studies have implicated gut-derived endotoxin as a "primary insult" to activate the inflammatory state, contributing to metabolic disease, with current cross-sectional and longitudinal data showing elevated systemic endotoxin levels in conditions of obesity, T2DM, coronary artery disease, fatty liver disease and high fat diet (Baker et al., 2009; Creely et al., 2007). Taken together, both circulating endotoxin and lipids may contribute to circulating LpPLA2 activity. However, whilst it seems clear that LpPLA2 studies implicate the interrelationships between AT, inflammation and insulin resistance as important, no study to date has examined human AT and the role of the adipocyte as an additional factor influencing the regulation of PLA2 isoforms. Therefore the aim of this chapter was to examine the influence of metabolic state and AT depot on PLA2 expression and its isoforms.

3.2 Materials and Methods

3.2.1 Subjects: Tissue collection

For this study, a female cohort was utilised, consisting of lean, (Age: 44.4±6.2yr; BMI: 22.1±0.2 kg/m^2, n=26) overweight (Age: 45.4±12.3yr; BMI: 26.9±0.2 kg/m^2, n=22), obese (Age: 49.0±9.1yr; BMI: 33.7±0.7 kg/m^2, n=24) and T2DM subjects (Age: 53.0±6.13yr; BMI: 44.2±1.8 kg/m^2, n=14). Paired human abdominal subcutaneous (Abd Sc) and omental (Om) AT biopsies were taken from a sub-group of patients undergoing elective surgery who were either lean (age 43.6±6.2yr; BMI; 22.5±0.8 kg/m^2; n=9), overweight (age; 47.5±11.5yr; BMI: 27.4±0.4 kg/m^2; n=10) or obese (age; 48.1±8.5yr; BMI: 34.0±1.1 kg/m^2; n=5) and the tissues flash frozen immediately. All subjects had their height, weight and BMI measurements taken using standard equipment. Detailed medical drug histories were taken and those subjects on medication considered to alter inflammatory status were excluded, including the thiazolidinediones. Ethical approval was obtained from the local research ethics committee and all patients gave written consent. Fasted blood samples were taken prior to surgery and serum levels were analysed as detailed below.

3.2.2 RNA extraction and semi-quantitative RT-PCR

RNA was extracted from samples using RNeasy lipid tissue kit (Qiagen,West Sussex, UK) according to the manufacturer's instructions. Extraction was followed by a DNase digestion step to remove any contaminating genomic DNA. RNA was quantitated using the Nanodrop ND-1000 Spectrophotometer (LabTech, East Sussex, UK) and 200 ng of RNA from each sample was reverse transcribed using Bioscript

Reverse Transcriptase (Bioline, London, UK) and random hexamers, according to the manufacturer's instructions. Quantitative real-time PCR was performed with pre-designed gene specific Taqman probes and primers (Applied Biosystems, Paisley, UK, LpPLA2; Hs00173726_m1, cPLA2; Hs00233352_m1, iPLA2; Hs00185926_m1 and EMR1; Hs00173562_m1) in a reaction mix containing TaqMan universal PCR master mix (Applied Biosystems, Paisley, UK). All reactions were multiplexed with the housekeeping gene 18S, provided as a pre-optimised control probe (Applied Biosystems, Paisley, UK) enabling data to be expressed as delta threshold cycle (ΔCt) values (where ΔCt = Ct of 18S subtracted from Ct of gene of interest). Measurements were carried out in triplicate for each sample.

3.2.3 Microarray analysis

RNA from the AT samples was used for gene expression analysis with the Human Genome U133A plus 2.0 DNA microarrays (Affymetrix, Santa Clara, CA). A detailed methodology of the principles behind microarray is cited in Chapter 2. In brief for this study, gene expression was analyzed using the Human Genome U133A DNA microarray (Affymetrix). Preparation of cRNA and hybridization to DNA microarrays was performed according to standard Affymetrix protocols, as previously described (Saiki et al., 2009, Anveden et al., 2012). PLA2 mRNA expression was investigated using the 219064_AT probe set.

3.2.4 Protein determination and Western blot analysis

Human AT was homogenized and suspended in Phosphosafe™ extraction buffer (Novogen ®, Merck, Germany). Protein concentrations were determined using the Bio-Rad Detergent Compatible protein assay kit (Bio-Rad Laboratories, Hercules, CA) (Bradford, 1976). Western blot analysis was performed using a

method previously described (Towbin, Staehelin, & Gordon, 1979). In brief, 40 µg of protein were loaded on to a denaturing polyacrylamide gel (7.5%) (GeneFlow, Leicestershire, UK). Protein expression of cPLA2 (1:100, Cell Signaling) and iPLA2 (1:500, Sigma) was assessed using rabbit monoclonal antibodies. Equal protein loading was confirmed by examining β actin protein expression. No statistical difference was observed in β actin expression for all samples analyzed. A chemiluminescent detection system, ECL/ECL^{+} (Amersham Pharmacia Biotech, Little Chalfont, UK), enabled visualization of bands, whereas intensity was determined using densitometry (Genesnap, Syngene, UK).

3.2.5 Immunohistochemistry

Tissue samples were obtained from Medical Solutions and incubated with primary polyclonal LpPLA2 antibody (R&D Systems, UK) in a dilution of 1:100. Sections were developed using peroxidase substrate kit VIP® (Vector Laboratories Ltd. UK) for LpPLA2. In order to demonstrate specific binding, the primary antibody was omitted for negative control for LpPLA2 independently. A detailed methodology of immunohistochemistry is outlined in Chapter 2.

3.2.6 Cell cultures and human lipoprotein treatment

Abdominal subcutaneous adipose tissue (AbdSc AT) was digested, as previously described and detailed in Chapter 2, to isolate pre-adipocytes (McTernan et al., 2003). In brief, approximately 50-100 mg of AbdSc AT was finely chopped and incubated in collagenase (2 mg/ml; Worthington Biochemical, USA) for 30 min at 37^{o}C. Following centrifugation and several washing stages, the stromal vascular pellet was obtained.

The AbdSc pre-adipocytes were grown in 6-well plates to confluence in phenol red-free Dulbecco's modified Eagle's medium (PRF-DMEM F-12) containing 10% bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and transferrin (5 μg/ml) at 37^0C, 5%CO_2 incubation until confluent. To undertake cell differentiation, AbdSc pre-adipocytes were maintained in promocell pre-adipocyte differentiation media (PromoCell, Germany) for 48 hr and, following this time period, the cells were maintained in the promocell adipocyte nutrition media (PromoCell, Germany) for fourteen days, with media changes every two days.

3.2.7 Statistical Analysis

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 18.0 for Windows (SPSS UK Ltd, UK). Student t-test was performed comparing PLA2 gene expression among lean versus obese subjects. Analysis of variance (ANOVA) with Bonferroni correction controlling for type 1 error was used to compare mRNA and protein expression among groups with varying degrees of adiposity and T2DM status (adjusted *P*-value < *0.017*, considered as statistically significant).

Significance of gene expression between AbdSc and Om AT were determined by paired T-test. Determination of correlations in gene expression analysis and variables of interest were performed using Pearson's correlation coefficient for normally distributed variables and Spearman's rank correlation coefficient for variables that were non-Gaussian (LpPLA2). Significance for these tests was set at $P<0.05$.

3.3 Results

3.3.1 PLA2 microarray expression profile in adipose tissue.

The microarray was performed using 20 probes corresponding to 4 isoenzymes in the platelet activating factor acylhydrolase family (PLA2G7, PAF AH2, PAF AH 1B1and PAH 1B2), 3 isoenzymes in the Ca^{2+} dependent PLA2 family (PLA2 G4A, PLA2 G4C and PLA2 G4D), 4 isoenzymes in Ca^{2+} independent PLA2 family (PLA2 G6, PNPLA2, PNPLA4 and PNPLA5), 9 isoenzymes in secretory PLA2 family (PLA2 G1B, PLA2 G2A, PLA2 G2D, PLA2 G2E, PLA2 G2F, PLA2 G5, PLA2 G10, PLA2 G12A and PLA2 G12B).

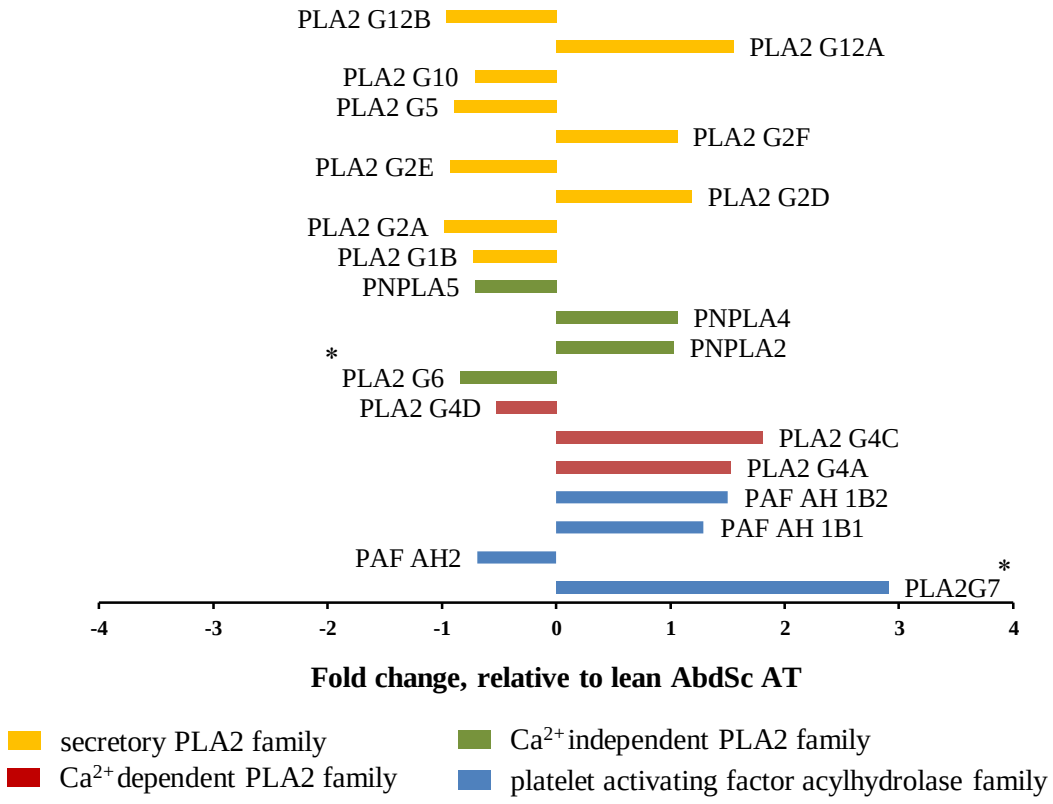

Figure 3.3.1.1 Microarray data analysis of PLA2 gene family in AbdSc AT. The bars represent a ratio between intensity signal in AbdSc AT taken from lean and obese. The significant fold change was assessed by unpaired *t* test (*$P<0.05$)

Significant changes in expression were noted for PLA2 G7 (LpPLA2) and PLA2 G6 (iPLA2) in AbdSc AT taken from obese subjects compared with AbdSc AT taken from lean individuals. PLA2 G6 showed decreased expression ($P<0.05$) in contrast to PLA2 G7, which showed upregulation ($P<0.05$) (Fig 3.4.1.1). No difference in expression of gene tested in this experiment were observed in Om AT (Fig 3.3.1.2). The significant changes in microarray data were further verified by RT-PCR and Western blot analysis.

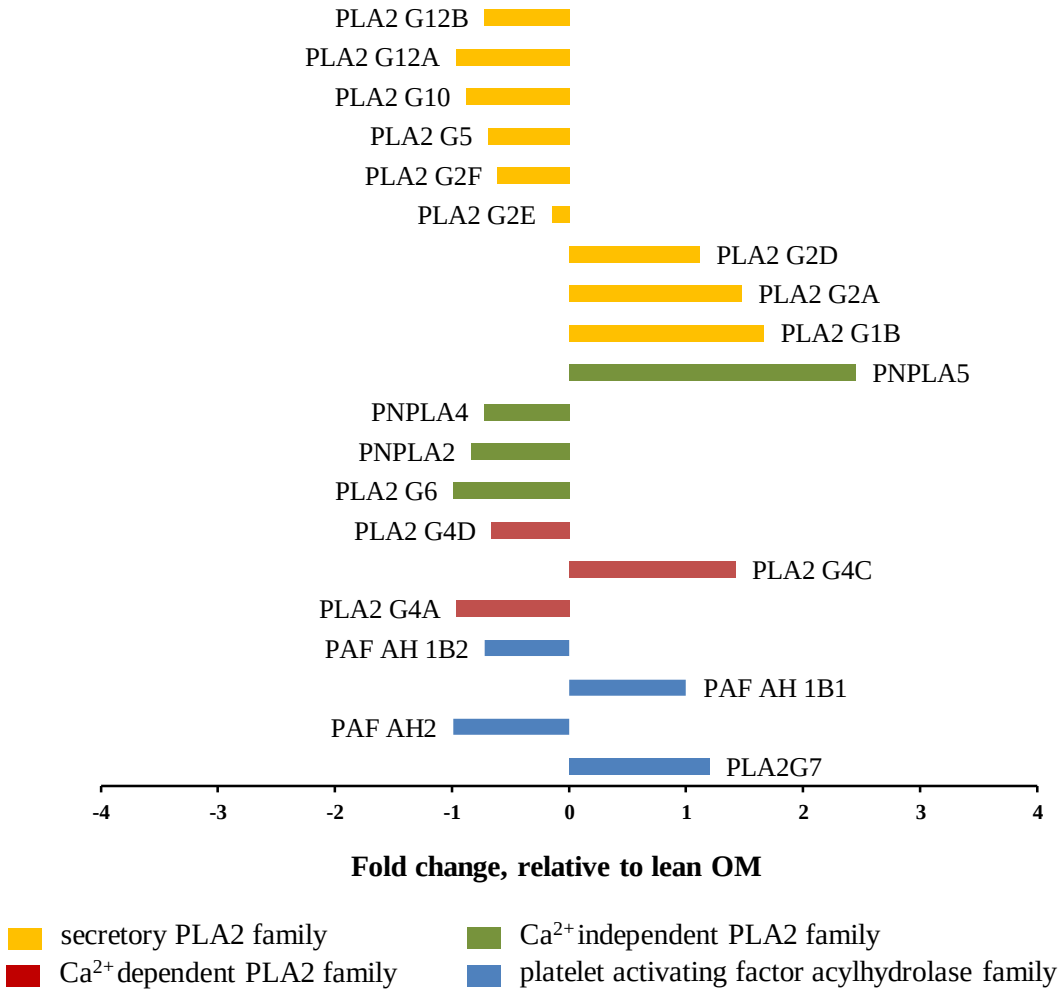

Figure 3.3.1.2 Microarray data analysis of PLA2 gene family in Om AT. The bars represent a ratio between intensity signal in Om AT taken from lean and obese. Student *t* test was used to test the significant difference between lean and obese.

3.3.2 Comparative gene and protein expression of PLA2 between AbdSc and Om AT taken from lean, overweight and obese subjects; microarray data verification.

The mRNA and protein level of LpPLA2 (gene PLA2G7) was significantly higher in the AbdSc AT than the Om AT in obese subjects (*P<0.05*). The expression of LpPLA2 was raised, but not significantly, in the overweight or obese AT samples (Fig 3.3.2.1A). Analysis of LpPLA2 western blot indicated that increasing adiposity alone did not increase LpPLA2 protein level (Fig 3.3.2.1B).

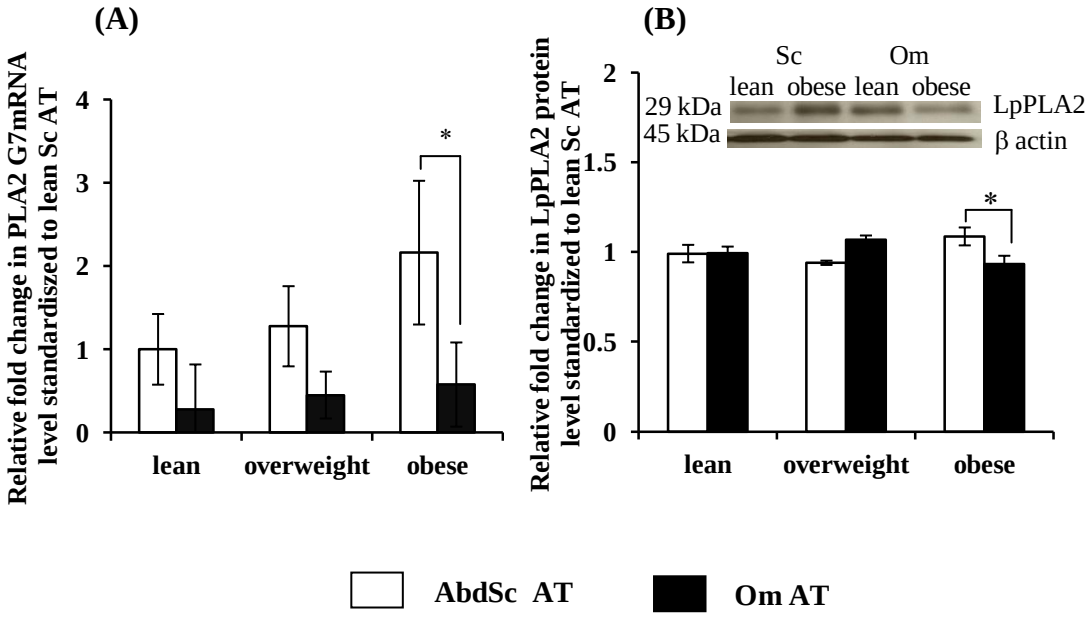

Figure 3.3.2.1. mRNA and Protein expression of LpPLA2 in AbdSc and Om AT from lean (n=9), overweight (n=10) and obese (n=5) non-diabetic subjects: (A) AbdSc and Om AT LpPLA2 mRNA expression (PLA2G7). (B) LpPLA2 protein levels. All RT-PCR and Western blot results were standardized to lean AbdSc AT. The difference in expression between AbdSc and Om AT was assessed by paired *t* test (*, *P<0.05*). Unpaired *t* test with bonferroni adjustment (correction for significant *P<0.017*) was performed to compare expression levels across adiposity.The mean of expression level of lean AbdSc AT was calculated and individual values measured in lean overweight and obese are normalized to this value.

The mRNA level of cPLA2 (gene PLA2G4A) was significantly higher in Om AT compared with AbdSc AT taken from lean subjects (*P<0.001*); whilst there was no significant difference in cPLA2 gene expression between Abd Sc and Om AT depots in either overweight or obese subjects. However within the Abd Sc AT depot, cPLA2 mRNA level was significantly increased in both the overweight (*P<0.05*) and obese subjects (*P<0.05*). In contrast, in the Om AT depot, cPLA2 mRNA level was significantly decreased in the obese subjects (*P<0.001*; Fig 3.3.2.2 A). Subsequent cPLA2 protein expression analysis confirmed the noted gene expression findings within Abd Sc AT, increased in overweight and obese subjects (*P<0.001* and *P<0.01*, respectively), whilst, again, Om AT decreased in the obese subjects (*P<0.01*; Fig 3.3.2.2 B

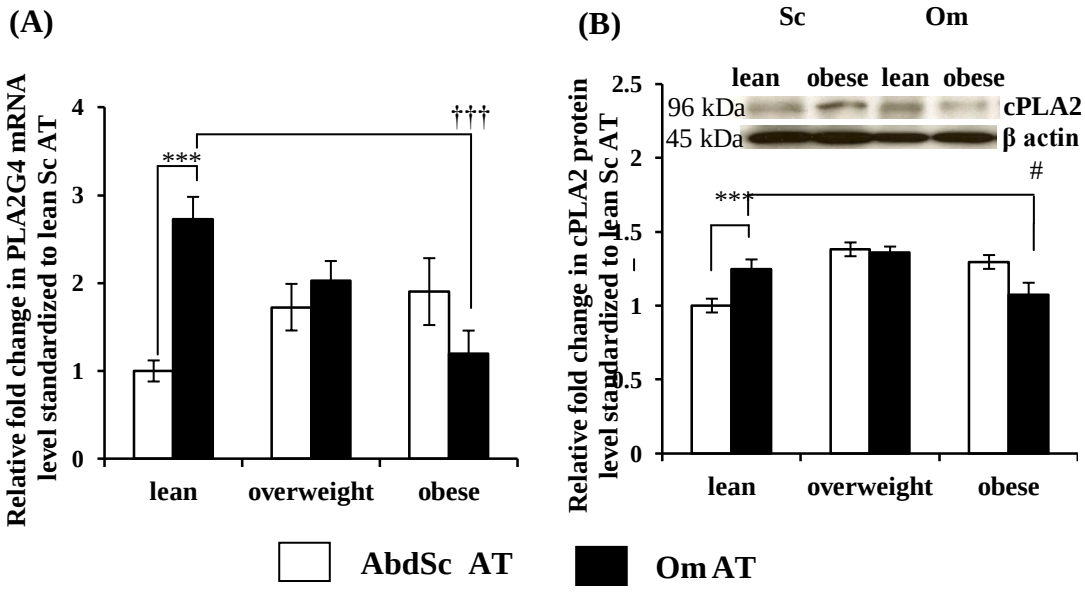

Figure. 3.3.2.2. mRNA and Protein expression of cPLA2 in AbdSc and Om AT from lean (n=9), overweight (n=10) and obese (n=5) non-diabetic subjects: (A) AbdSc and Om AT cPLA2 mRNA expression (PLA2G4). (B) AbdSc and Om AT cPLA2 protein expression. All RT-PCR and Western blot results were standardized to lean AbdSc AT. The difference in expression between AbdSc and Om AT was assessed by paired *t* test (*, *P<0.05*; ***, *P<0.001*). Unpaired *t* test with bonferroni adjustment (correction for significant *P<0.017*) was performed to compare mRNA levels across adiposity (#, *P<0.017*; ###, *P<0.001*, each group *vs.* lean Sc AT: *P<0.01*; †††, *P<0.001*, each group vs. lean Om AT). The mean of expression level of lean AbdSc AT was calculated and individual values measured in lean overweight and obese are normalized to this value.

There was no difference in iPLA2 (gene PLA2G6) gene expression between paired Abd Sc and Om AT and no noted effect on adiposity (Lean, Abd Sc: 11.85±0.12ΔCt; Om: 12.18±0.20ΔCt; Overweight, Abd Sc: 11.65±0.17ΔCt Om: 12.141±0.16ΔCt; Obese, Abd Sc: 12.02±0.26ΔCt Om: 12.62±0.15ΔCt; Fig 3.4.2.3 A). Protein analysis showed moderately significant increases in iPLA2 in Om AT compared with Abd Sc AT from all groups (*P<0.05*; Fig 3.4.2.3 B), although there were no significant differences in iPLA2 protein levels between lean, overweight and obese in both Abd Sc and Om AT (Fig 3.3.2.3 B).

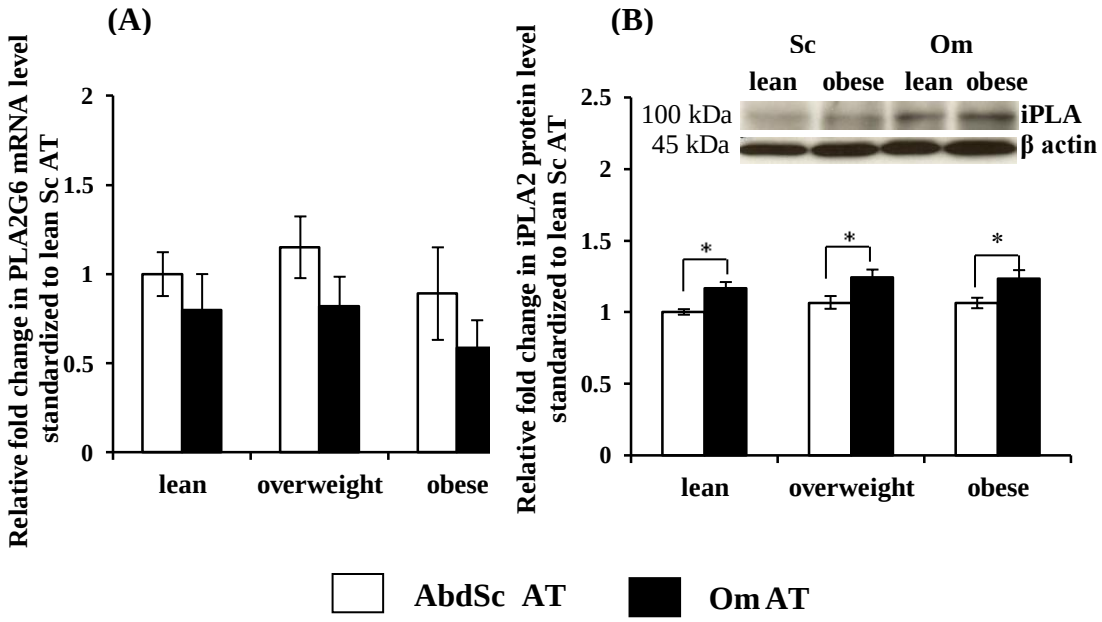

Figure. 3.3.2.3. **mRNA and Protein expression of iPLA2 in AbdSc and Om AT from lean (n=9), overweight (n=10) and obese (n=5) non-diabetic subjects**: (A) AbdSc and Om AT iPLA2 mRNA expression (PLA2G6); (B) AbdSc and Om AT iPLA2 protein expression. All RT-qPCR and Western blot results were standardized to lean AbdSc AT. The difference in expression between AbdSc and Om AT was assessed by paired *t* test (*, *P<0.05*; ***, *P<0.001*). Unpaired *t* test with bonferroni adjustment (correction for significant *P<0.017*) was performed to compare mRNA levels across adiposity. The mean of expression level of lean AbdSc AT was calculated and individual values measured in lean overweight and obese are normalized to this value

3.3.3 T2DM status influences the expression of PLA2 isoforms in Adipose tissue.

The LpPLA2 mRNA expression in T2DM AbdSc AT was approximately 5 fold higher than the non-T2DM AbdSc AT (*P<0.001*). Similarly, the Om AT LpPLA2 mRNA expression was higher in the T2DM group, at approximately 2 fold greater than the non-T2DM group (*P<0.01*; Fig 3.3.3.1 A). Further analysis of LpPLA2 protein expression confirmed the mRNA findings (Fig 3.3.3.1 B). These data suggest that LpPLA2 gene and protein expression appear to be up-regulated under T2DM conditions.

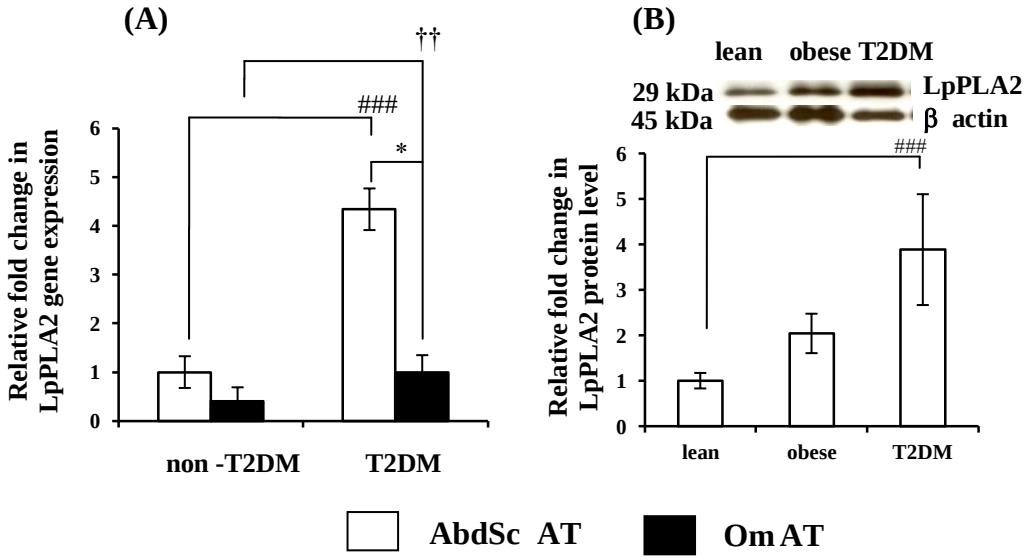

Figure 3.3.3.1 Comparison of mRNA and protein expression of tissue LpPLA2 between non-T2DM and T2DM: (A) LpPLA2 mRNA expression in AbdSc and Om AT compared between non-diabetic (n=24) and T2DM subjects (n=13). (B), LpPLA2 protein expression in AbdSc taken from lean, obese and obese T2DM. All RT-PCR and Western blot results were standardized to lean AbdSc AT. The difference in expression between AbdSc and Om AT was assessed by paired *t* test (*, *P<0.05*; ***, *P<0.001*). Unpaired *t* test with bonferroni adjustment (correction for significant *P<0.017*) was performed to compare mRNA levels across adiposity (#, *P<0.017*; ###, *P<0.001*, each group *vs.* lean Sc AT: †, *P<0.017*; ††, *P<0.01*; †††, *P<0.001*, each group vs. lean Om AT). The mean of expression level of lean AbdSc AT was calculated and individual values measured in lean overweight and obese are normalized to this value.

There were no significant differences in mRNA levels of cPLA2 and iPLA2 between the non-diabetic and T2DM groups, in either AbdSc AT or Om AT (Fig 3.3.3.2 A and B, respectively). This data indicated that among of PLA2 expression evaluated by microarray, RT-PCR and western blot, LpPLA2 was the only isoform that showed the higher expression in T2DM compared to non T2DM subjects.

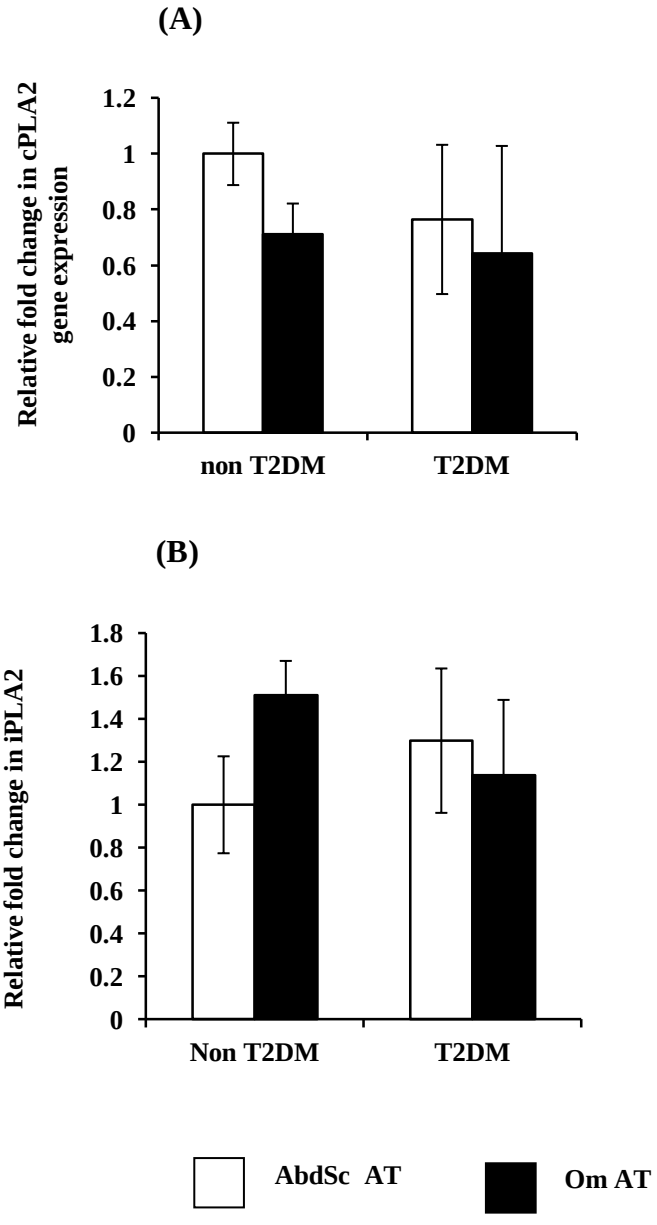

Figure 3.3.3.2 Comparison of mRNA of tissue cPLA2 and iPLA2 between non-T2DM and T2DM: (A) cPLA2 mRNA level in AbdSc and Om AT compared between non-diabetic (n=24) and T2DM subjects (n=13). (B), iPLA2 mRNA level in AbdSc and Om AT. Unpaired *t* test with bonferroni adjustment (correction for significant *P<0.017*) was performed to compare mRNA levels between non T2DM and T2DM.

3.3.4 Factors that influence mRNA and protein expression of LpPLA2 in AbdSc and Om AT

To investigate the importance of the macrophage as an active source of LpPLA2 in AT, the relationship between the macrophage specific marker, EMR1, and LpPLA2 mRNA expression was examined. The results highlighted that there was no significant correlation between EMR1 and LpPLA2. Taken together, these data support the concept that the increased LpPLA2 mRNA and protein expression observed in the AbdSc AT taken from T2DM subjects appears adipocyte derived (Figure 3.3.4.1). However, a significant, positive correlation between EMR1 and LpPLA2 was noted in Om AT (r=0.534, *P=0.049*), which has previously been reported as an active site of macrophage activity.

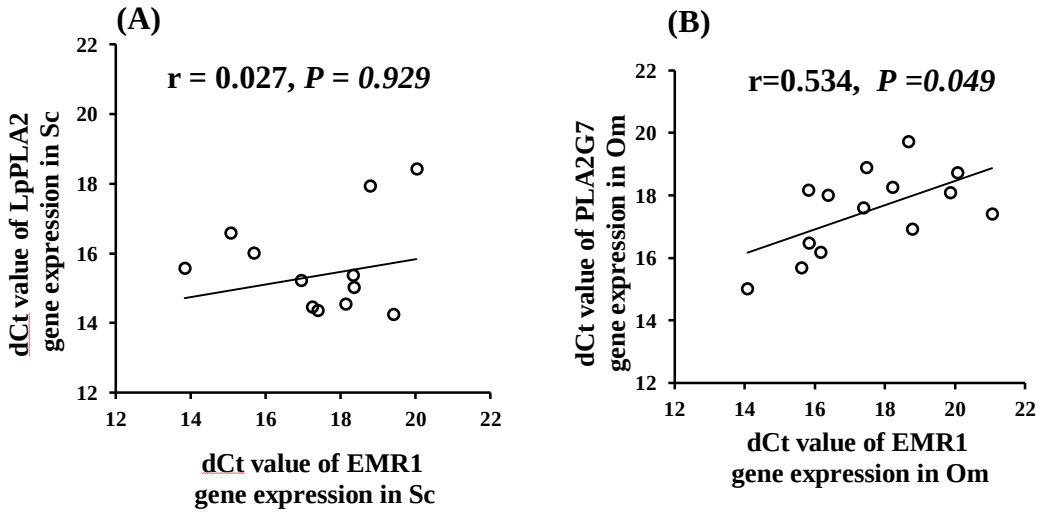

Figure 3.3.4.1 Factors that influence mRNA and protein expression of LpPLA2: Correlation analysis using Spearman's correlation coefficient with line of best fit between delta Ct values of LpPLA2 mRNA and macrophage specific marker, EMR1, in AbdSc AT (A) and Om AT (B) from T2DM subjects were tested. Significant correlation was set at *P<0.05*.

Immunohistochemistry showed the expression of LpPLA2 in the mature adipocytes taken from lean non-T2DM human AbdSc AT (Fig 3D (i)). LpPLA2 was observed to be concentrated in the adipocyte as denoted by the brown staining observed around each cell. Placenta tissue was used as a positive control for LpPLA2 expression with positive brown staining also shown (Fig 3..4.2 (iii)), with appropriate negative staining noted in both tissue sections (Fig 3.4.4.2 (ii) (iv)).

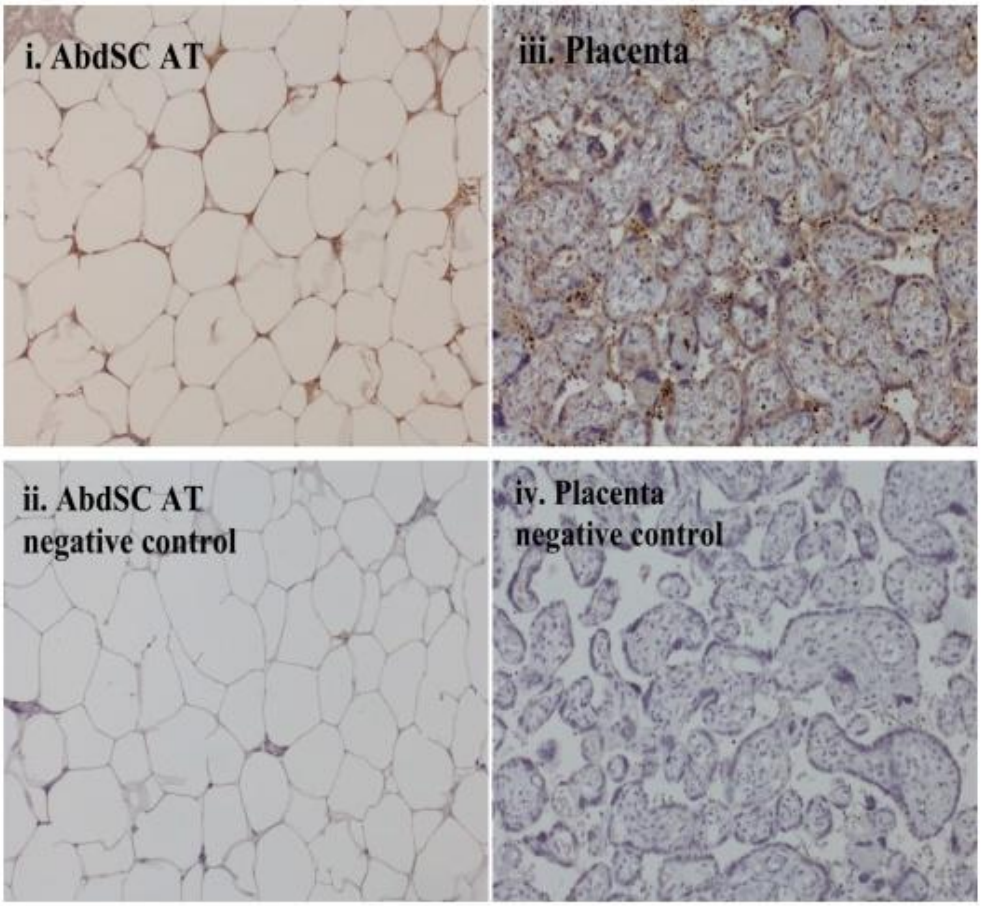

Figure 3.3.4.2 LpPLA2 expression in adipose tissue: Immunohistochemical expression of LpPLA2 detailed as follows: (i) AbdSc AT LpPLA2 positive staining (brown) (ii) negative control in AbdSc with omission of primary antibody (iii) positive control in placenta for LpPLA2 (iv) negative control in placenta with omission of primary antibody (i-iv magnification x200).

3.4.5 LpPLA2 expression in primary cell culture and human pre-adipocyte cell line.

The mRNA and protein expression of LpPLA2 was observed in both the human primary culture and the human adipocyte cell line, Chub S7, indicating adipocyte produced LpPLA2. The expression of LpPLA2 was not different between pre adipocyte and differentiated adipocyte at day 14 in both primary cells and cell line (Fig 3.3.4.3).

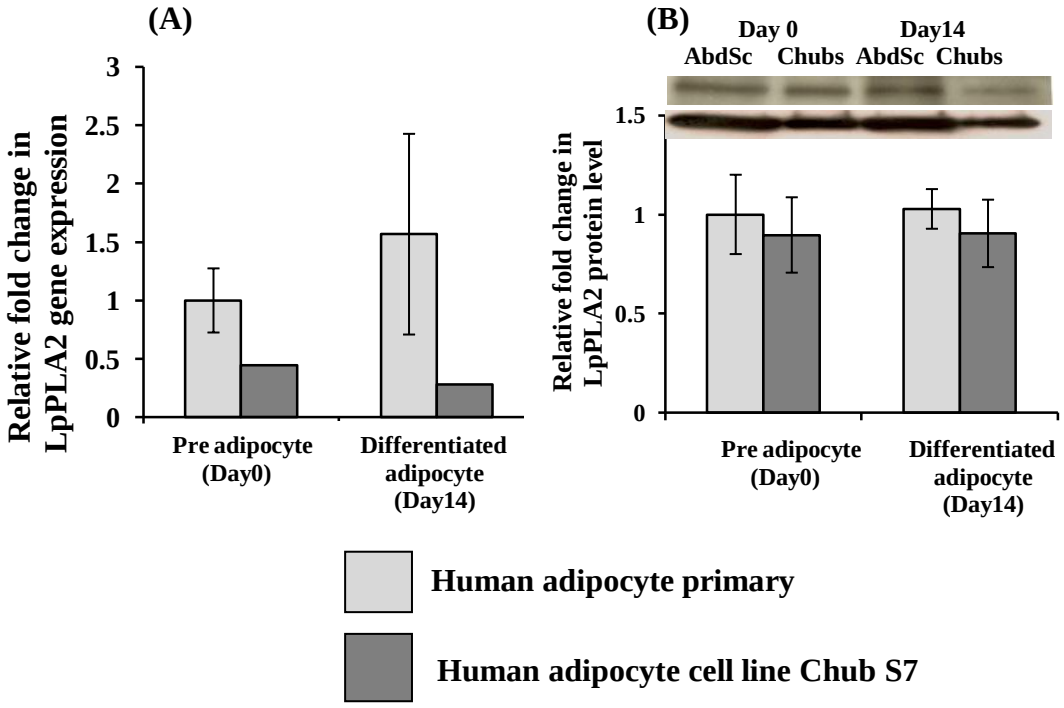

Figure 3.3.4.3 LpPLA2 expression in primary cell culture and cell line: (A), LpPLA2 mRNA expression. (B), protein expression in human primary culture (n=3) and human adipocyte cell line. Significant changes in expression between primary and cell culture, and pre adipocyte and differentiated adipocyte, were tested by student *t* test.

3.5 Discussion

Obesity is a condition that activates inflammation in adipose tissue, leading to high risk of T2DM and atherosclerosis (Gustafson, 2010; S. J. Kim et al., 2014; Olza et al., 2014). Our previous studies have shown that increasing adiposity and T2DM status contribute to an increased inflammatory response in human Epicardial AT (Baker et al., 2009) and AbdSc AT (Harte et al., 2013b). However, no study, to date, has examined LpPLA2 - a known mediator of inflammation in CVD - directly in human AT (Khakpour & Frishman, 2009; Kinney et al., 2011; McCullough, 2009; Toth et al., 2010). As weight gain heightens the inflammatory state, this may have specific relevance for cPLA2 which is known to be phosphorylated by p42/44 MAPKs, p38 MAPK and JNK, highlighting its potential inflammatory role (X. Wang et al., 2008). The iPLA2 isoform is also considered as a pathogenic inflammatory marker in vascular tissue, leading to increased oxidative stress and inflammation (Zhongwen Xie et al., 2010). Thus, this is the first study to examine the comparative and differential changes in expression of all PLA2 isoforms in AbdSc and Om AT across adiposity with or without T2DM.

This current study showed that upon examining all PLA2 isoforms in AbdSc and Om AT, in lean and obese subjects, two isoforms appeared to be significantly altered in AbdSc AT. Specifically, PLA2 G7 (LpPLA2) and PLA2 G6 (iPLA2) were significantly altered with increasing adiposity. However, both mRNA and protein data showed a slightly increased, but not significant, LpPLA2 AbdSc AT mRNA expression whilst iPLA2 mRNA expression in this depot remained unchanged. Between depots analysis indicated LpPLA2 mRNA expression appeared significantly higher in AbdSc AT than in Om AT, independent of adiposity.

Further studies examined gene expression changes comparing non-diabetic and T2DM AT samples. These studies highlighted dramatic changes in LpPLA2 gene expression in Abd Sc AT, with a 5 fold increase noted in LpPLA2 from non-diabetic AT compared with T2DM AT. In a similar fashion, Om AT LpPLA2 mRNA expression increased 2 fold. This data also showed that LpPLA2 expression was 5 fold higher in Abd Sc AT from T2DM subjects compared with Om AT from the same subjects. Due to such a significant change, and the knowledge that macrophages are a specific source of LpPLA2 which are known to be increased in obesity and T2DM, our studies utilised a specific macrophage marker, EMR1, to examine the relationship with LpPLA2 (Harford, Reynolds, McGillicuddy, & Roche, 2011; Khazen et al., 2005). This was specifically to identify how much contribution marcophages may make to the data observed in human AT.

From our studies, there was no correlation with EMR1 and LpPLA2 in AbdSc AT, in contrast to Om AT, suggesting that the changes observed in AbdSc AT LpPLA2 mRNA and protein expression appear to arise from the adipocyte itself. Such findings are in accord with previous studies examining other adipocyte inflammatory factors which also expressed in macrophages; for these studies other selective macrophage markers were utilised, such as CD45 and CD68, which again were unable to show a correlation in abdominal AT (Baker et al., 2009; McGee et al., 2011; McTernan et al., 2003) further supporting the concept that LpPLA2 changes observed were derived from the adipocyte and not the macrophages.

To further identify that the adipocyte itself was an important source of LpPLA2, immunohistochemical analysis was undertaken. Immunohistochemical staining highlighted that there was LpPLA2 in AT as denoted by brown staining accumulated around adipocyte cytoplasm attached to the adipocyte plasma

membrane, confirming that LpPLA2 protein is abundantly expressed in adipocyte. However it should still be noted thought that despite these data indicating LpPLA2 appears to be derived from AbdSc adipocytes, it should be stated that macrophages may still play a role in LpPLA2 activation within AT under different circumstances then in conditions or changing adiposity or T2DMs. Also it would be important to note that how circulating macrophages may be influenced in atherosclerotic disease may be very different.

As we are providing data as to the source of LpPLA2 within the adipocyte additional studies were undertaken to examine mRNA LpPLA2 was in both, primary human, AbdSc pre-adipocyte cell cultures and a secondary, human, adipocyte cell line, Chub S7 cells. Interestingly, there was no differences noted between LpPLA2 mRNA expression between pre-adipocyte and differentiated adipocyte at day 14 either primary or cell line cultures. This *in vitro* analysis suggested that LpPLA2 appears consistently present pre- and post- differentiation. The role LpPLA2 may have in adipocyte differentiation was not specifically investigated as the intervening days were not studied, which may have caused a change in LpPLA2 expression. However taken together, the *ex vivo* adipose tissue data and *in vitro* studies indicate LpPLA2 is expressed in human adipocytes.

Further analysis of cPLA2 indicated that there was a significant increase in mRNA expression with increasing adiposity in AbdSc AT, which was confirmed by protein analysis. In contrast, cPLA2 mRNA expression in Om AT was 2 fold higher in lean individuals compared with Abd Sc AT, which then reduced as adiposity increased, in an apparent step-wise opposing manner to cPLA2 expression in AbdSc AT. A similar but less pronounced change in cPLA2 protein expression was also noted across AbdSc and Om AT with increasing adiposity. Analysis of iPLA2

mRNA expression showed no significant change with adiposity in either Abd Sc or Om AT and only iPLA2 protein expression showed a modest increase in Om AT compared with Abd Sc AT with weight gain; this was lost in the obese group. However, there were no significant changes in cPLA2 and iPLA2 mRNA level in both AbdSc AT and Om AT between non T2DM and T2DM, indicating that diabetic status did not influent the expression of cPLA2 and iPLA2. In the case of cPLA2 and iPLA2, these isoforms are less well described and their functions in adipose tissue requires much more study compared with our understanding of LpPLA2's functions in tissues.

In summary, these studies highlight that human AT and the adipocyte itself appears to represent an active source of LpPLA2. LpPLA2 expression is raised in Abd Sc AT from obese non-diabetic subjects, which is further enhanced in the T2DM state. Furthermore as LpPLA2 can be secreted into blood circulation, it would be important to understand the both the direct association LpPLA2 may have with other metabolic makers, as the over-expression of oxidized fatty acid due to the increased LpPLA2 production in obesity and T2DM may promote further inflammation in T2DM and pathogenesis of complications such as atherosclerosis. As such studying the association circulating LpPLA2 in metabolic disease may help understand what factors influence how adiposity and T2DM increase the risk of cardiovascular disease.

CHAPTER 4 : The Systemic Relationships Between LpPLA2 and Metabolic Markers.

4.1 Introduction

Within the PLA2 family, LpPLA2 is the only secretory protein whilst cPLA2 and iPLA2 are both cytosolic proteins. The major source of LpPLA2 in the blood has been considered to arise from the monocyte derived macrophage, T lymphocyte and mast cells (Bao et al., 2008; Zhongwen Xie et al., 2010). LpPLA2 itself is known to bind to lipoproteins in the circulation, especially with LDL in normolipidemic subjects, whilst the minority of circulating LpPLA2 is also associated with HDL (Rosenbaum et al., 2012; Rubinstein & Izkhakov, 2011). Recently, studies have suggested that measuring circulating LpPLA2 could be viewed as a new marker for monitoring atherosclerosis (A. Cai et al., 2013; Mangili et al., 2014; Tousoulis, Papageorgiou, Androulakis, & Stefanadis, 2013). However, the cause and effect role of LpPLA2 in atherosclerosis remains unclear, part due to the fact that the anti-atherogenic and pro-atherogenic function of LpPLA2 has produced conflicting reports (S. B. Li et al., 2013; Rosenson & Hurt-Camejo, 2012; Rosenson & Stafforini, 2012; Tousoulis et al., 2013). LpPLA2 has the ability to remove oxidized phospho-lipids (PLs) and degrade platelet activating factor (PAF), stopping the oxidative process in LDL and HDL, resulting in anti-atherosclerosis and anti-inflammation.

However at the same time, LpPLA2 can produce lysophosphatidylcholine and oxidized free fatty acid, which both exhibit pro-atherogenic effects. Moreover, activation of LpPLA2 can produce arachidonic acid and consequently accumulate pro-inflammatory enzymes, such as active cyclooxygenase (COXs) and lipoxygenase (LOXs) which promote inflammation. Specifically COX2 is the enzyme responsible for synthesizing pro-inflammatory prostaglandins, and is the target of non-steroidal anti-inflammatory drugs (ibuprofen, aspirin) and COX2 inhibitors (Celebrex®)

(Aldrovandi & O'Donnell, 2013). Whilst lipoxygenases are also expressed in many tissues, including adipose tissue and 15-lipoxygenase appears to be able to generate both pro- and anti-inflammatory metabolites. 15-lipoxygenase in obesity promotes the onset of metabolic dysfunction and therapeutics against 15-lipoxygenase may be able to treat metabolic dysfunction (Chakrabarti et al., 2011; Cole, Kuhn, et al., 2012; Cole, Morris, Grzesik, Leone, & Nadler, 2012)**.**

The following studies highlight the proatherogenic function of LpPLA2. In a prospective cohort study, expression of LpPLA2 and lysophosphatidylcholine, LpPLA2 was identified to be higher in plaques taken from patients undergoing carotid endarterectomy, compared with patients without any cardiovascular event (Sun et al., 2001). Furthermore the treatment of darapladib, a PAF-acetylhydrolase (AH) inhibitor, in a diabetic/hypercholesterol swine model markedly reduced the lysophosphatidylcholine content in lesion and mitigated the development of atherosclerosis (Birbes et al., 2000). In contrast in other studies such as using the overexpression model by human LpPLA2 gene transfected apoliprotein E (apoE) $^{-/-}$ mice an anti-atherogenic effect was observed. The reduction of atherogenic lesions was more observed in male mice following gene transfer (Martinez & Moreno, 2001). The conflicting reports demonstrate that LpPLA2 might be involved in complementary or opposite pathways in atherosclerosis, the understanding of expression, function and regulation of LpPLA2 in specific tissue and diseases state in different species appears importance as well as the potential disease state where LpPLA2 function may alter.

However, despite expression evidence there is still no clear evidence of how LpPLA2 from the adipocyte plays a role on atherosclerosis. Previous analysis has indicated that there is higher expression of LpPLA2 in adipose tissue taken from

obese and T2DM subjects compared with lean, suggesting a potential pro-atherogenic role for LpPLA2 in subjects with T2DM. However to understand the influence of metabolic state further on circulating LpPLA2 (with an appreciable quantity perhaps being derived from adipose tissue in an increased metabolic dysfunctional state), the correlation between serum LpPLA2 and circulating metabolic markers were studied. Furthermore the association between LpPLA2 expressed in adipose tissue and inflammatory makers such as IL6, COX2 and 15-LOX were examined to understand how local inflammation derived from adipose tissue may impact and relate to circulating LpPLA2 in different metabolic states. Finally in vitro studies were undertaken to assess how ox-LDL a pro-inflammatory factor may influence LpPLA2 and inflammatory markers in a closed adipocyte culture system.

4.2 Materials and methods

4.2.1 Subjects: Serum and tissue collection

Fasting blood samples were taken from a female cohort, consisting of lean, (Age: 44.4±6.2yr; BMI: 22.1±0.2 kg/m^2, n=26) overweight (Age: 45.4±12.3yr; BMI: 26.9±0.2 kg/m^2, n=22), obese (Age: 49.0±9.1yr; BMI: 33.7±0.7 kg/m^2, n=24) and T2DM subjects (Age: 53.0±6.13yr; BMI: 44.2±1.8 kg/m^2, n=14). All subjects had their height, weight and BMI measurements taken using standard equipment. Detailed medical histories were taken and those non-diabetic subjects on medication considered to alter inflammatory status were excluded. In T2DM patients medical histories were taken. Ethical approval was obtained from the local research ethics committee and all patients gave written consent. Fasted blood samples were taken prior to surgery and serum levels were analysed as detailed below.

4.2.2. *In vivo* assessment of the biochemical profile

Fasting blood samples were collected from participating subjects and lipid profiles and fasting plasma glucose were determined using routine laboratory methods, undertaken in the biochemistry laboratory, at University Hospital Coventry and Warwickshire. In brief, the routine blood tests included glucose and a full cholesterol profile (TGs, HDL and LDL), as noted in Table 4.3.1.1. Ox-LDL and LpPLA2 were measured by ELISA (Mercodia Oxidized LDL ELISA, Sweden; intra-assay percentage coefficient of variation (CV) % = 6.4; inter assay % CV = 7.4) and R&D Systems Human PLA2G7/PAF-AH/LpPLA2 (Quantikine ELISA, UK; intra-assay % CV = 6.8, inter-assay % CV = 9.6). Insulin measurements were performed by a solid-phase enzyme amplified sensitivity multiplex immunoassay (Millipore,

U.K.), and glucose was measured by a glucose oxidase method (YSL 200 STAT plus).

4.2.3 Analysis of circulating endotoxin levels

Serum endotoxin was analysed using a commercially available QCL-1000 LAL End Point Assay (Lonza, NJ). The assay, and the CV values are noted, for the intra-assay CV = 3.9±0.46% and inter-assay CV = 9.6±0.75%, this assay has further been validated in our laboratory, as detailed previously (Creely et al., 2007; Harte et al., 2012).

4.2.4 RNA extraction and quantitative RT-PCR

RNA was extracted from samples using RNeasy lipid tissue kit (Qiagen, UK) according to the manufacturer's instructions. Extraction was followed by a DNase digestion step to remove any contaminating genomic DNA. RNA was quantitated using the Nanodrop ND-1000 Spectrophotometer (LabTech, UK) and 200ng of RNA from each sample was reverse transcribed using Bioscript Reverse Transcriptase (Bioline, UK) and random hexamers, according to the manufacturer's instructions. Quantitative real-time PCR was performed with pre-designed gene specific Taqman probes and primers (Applied Biosystems, UK, LpPLA2; Hs00173726_m1, cPLA2; Hs00233352_m1, iPLA2; Hs00185926_m1 and EMR1; Hs00173562_m1) in a reaction mix containing TaqMan universal PCR master mix (Applied Biosystems, UK). All reactions were multiplexed with the housekeeping gene 18S, provided as a pre-optimised control probe (Applied Biosystems, UK) enabling data to be expressed as delta threshold cycle (ΔCt) values (where ΔCt = Ct of 18S subtracted from Ct of gene of interest). Measurements were carried out in triplicate for each sample.

4.2.5 Cell cultures and human lipoprotein treatment

Human Chub S7 cells were maintained in Growth media, comprised of Dulbecco's modified Eagle's medium (DMEM/F12) with supplement and 10% FBS, 20U/ml penicillin/Streptomycin and transferrin at 37^0C and 5% CO_2 incubation. The cells were grown until confluent and subsequently differentiated (Promocell, UK). Differentiated adipocytes were incubated in DMEM, without FBS, overnight at 37^0C and 5% CO_2; the cells were treated with Ox-LDL (100 µg/mL; Kalen Biomedical, USA) for 3, 6, 24 and 48 hr, using 10% of PBS containing 154mM NaCl and 0.34mM EDTA as vehicle control. The treated cells were harvested for RNA isolation and protein extraction. Cytotoxicity based on the dye exclusion method was examined after 48 hr incubation. The percentages of cell viability of the cells treated with vehicle control and Ox-LDL was 93%and 92%, respectively.

4.2.6 Statistical Analysis

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 18.0 for Windows (SPSS UK Ltd, UK). Determination of correlations in gene expression analysis and variables of interest were performed using Pearson's correlation coefficient for normally distributed variables and Spearman's rank correlation coefficient for variables that were non-Gaussian (LpPLA2). Significance for these tests was set at $P<0.05$. For additional analysis where BMI was noted as a confounding factor for LpPLA2 levels, a partial correlation analysis controlling for BMI was performed. Multiple linear regression analysis was used to identify potential predictor of LpPLA2; whilst comparison of gene expression in *in vitro* experiments was tested for by one way ANOVA.

4.3 Results

4.3.1 Circulating metabolic markers and LpPLA2 levels.

T2DM subjects had significantly higher mean BMI (*P<0.001*), blood glucose (*P<0.001*), LDL (*P<0.01*), insulin (*P<0.001*), Ox-LDL (*P<0.05*) and endotoxin (*P<0.001*) levels and lower HDL (*P<0.001*), compared with lean non-diabetic subjects. Additionally, the significant difference in mean BMI (*P<0.001*), blood glucose (*P<0.001*), HDL (*P<0.001*), insulin (*P<0.001*) and endotoxin levels (*P<0.001*) were observed when compared with obese, non-diabetic subjects (Table 4.3.1.1).

Table 4.3.1.1 Baseline characteristics of non-diabetic and T2DM subjects.

	Non-T2DM (n=72)			
	Lean n=26	**Overweight n=22**	**Obese n=24**	**T2DM (n=14)**
BMI (Kg/m^2)	22.1±0.2	26.9±0.3*	33.7±0.7 ***	44.2±1.8*** †††
Glucose (mmol/L)	5.0±0.3	4.8±0.08	5.5±0.2	8.7±0.5***†††
Cholesterol (mmol/L)	4.8±0.2	5.3±0.2	5.7±0.3 *	5.0±0.3
Triglyceride (mmol/L)	0.9±0.06	1.04±0.12	1.7±0.2 **	1.4±0.2 **
LDL (mmol/L)	2.6±0.16	3.04 ±0.2	3.0±0.2	3.5±0.3 **
HDL (mmol/L)	1.8±0.08	1.8±0.1	1.6±0.09	1.2±0.1*** †††
Insulin (µIU/mL)	5.2±0.6	7.14±1.2	11.6±1.6 *	24.9±2.9 ***†††
Endotoxin (EU/mL)	2.1±0.2	2.8±0.2	2.5±0.3	7.8±0.9***
Ox-LDL (U/L)	39.67±1.4	43.01±3.2	49.76±4.7	63.37±6.7 *******
LpPLA2 (ng/mL)	125.04±8.3	140.62±9.2	138.17±12.4	135.35±10.1*****

Values were presented as mean ± SEM. Unpaired t test analysis was performed to identify differences between lean *vs* overweight, lean *vs* obese, lean *vs* T2DM *(*, P<0.05; **, P<0.01; ***, P<0.001*) and obese *vs* T2DM (†††, *P<0.001*).

4.3.2 Circulating LpPLA2 is associated with metabolic markers and endotoxin.

The relationships between LpPLA2 and metabolic markers were determined using linear regression analysis. There were significant positive correlations between serum LpPLA2 and cholesterol (r=0.618, *P<0.001*), TG (r=0.442, *P<0.001*), LDL (r=0.580, *P<0.001*), endotoxin (r=0.334, *P<0.001*) and Ox-LDL (r=0.365, *P<0.001*) in non-diabetic subjects (Fig 4.3.2.1).

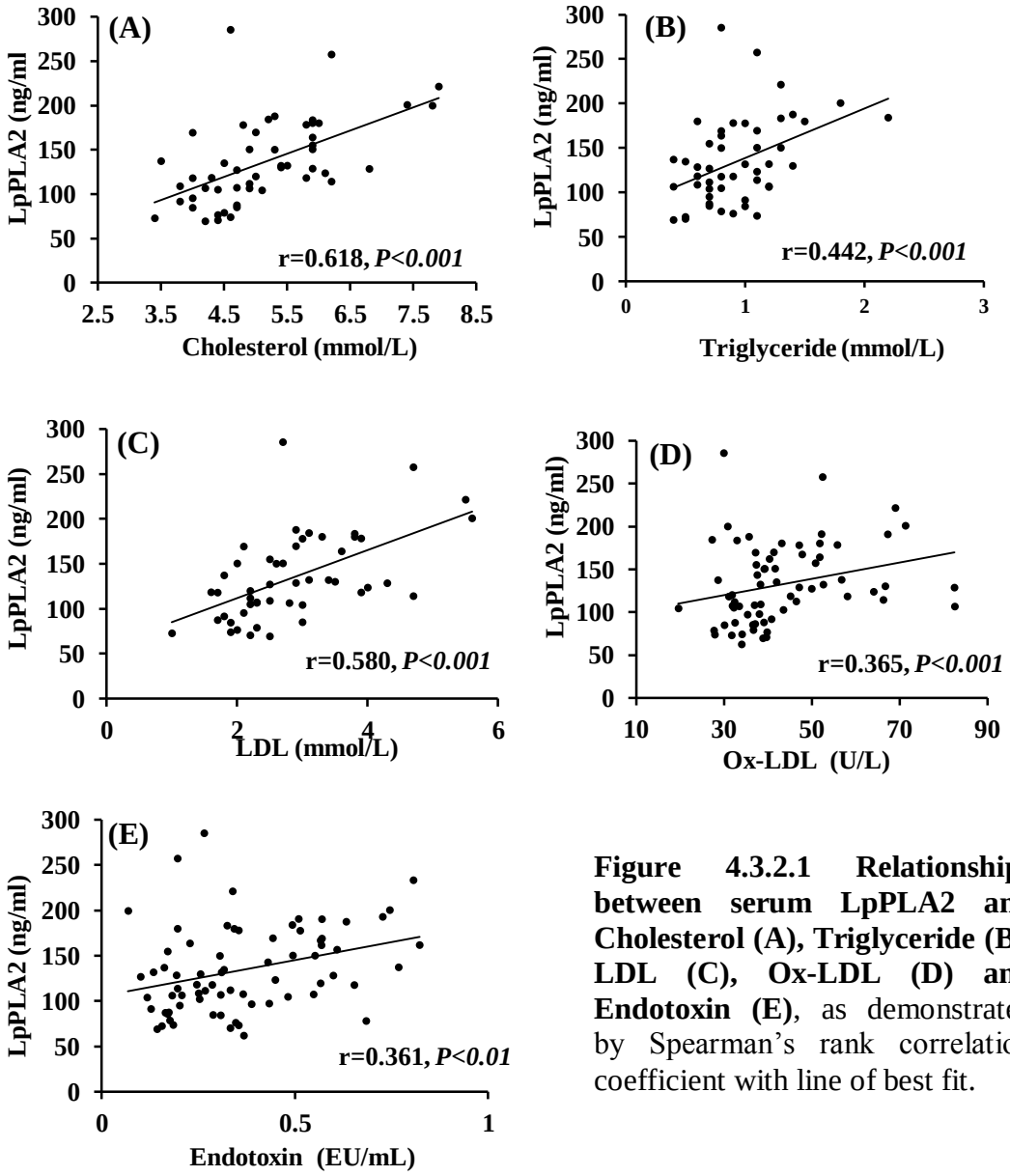

Figure 4.3.2.1 Relationships between serum LpPLA2 and Cholesterol (A), Triglyceride (B), LDL (C), Ox-LDL (D) and Endotoxin (E), as demonstrated by Spearman's rank correlation coefficient with line of best fit.

In addition, there were positive correlations between LpPLA2 and cholesterol (r=0.599, *P<0.05*), LDL (r=0.637, *P<0.01*) and Ox-LDL (r=0.791, *P<0.001*) in T2DM subjects (Table 4.3.2.1).

Table 4.3.2.1. Correlations between circulating LpPLA2 and metabolic markers

	non-T2DM				
	All	**lean**	**overweight**	**obese**	**T2DM**
	n=72	**n=26**	**n= 22**	**n=24**	**n=14**
BMI (Kg/m^2)	0.185	0.187	0.167	0.110	0.137
Glucose (mmol/L)	0.152	0.302	-0.160	0.316	0.484
Cholesterol (mmol/L)	0.618***	0.649**	0.489*	0.881***	0.599*
Triglyceride (mmol/L)	0.442***	0.479*	0.410	0.426	0.209
LDL (mmol/L)	0.580***	0.662**	0.349	0.784***	0.637**
HDL (mmol/L)	-0.264	-0.321	0.210	-0.651**	0.187
Insulin (μIU/mL)	0.182	0.214	0.417	0.007	0.088
Endotoxin (EU/mL)	0.334***	0.554**	0.156	0.395	0.379
Ox-LDL (U/L)	0.365***	0.529**	0.281	-0.100	0.791***

Values shown are correlation co-efficient *(*,P<0.05; **,P<0.01; *** P<0.001)*

Using partial correlation analysis, and controlling for BMI, positive associations between LPPLA2 and cholesterol (r=0.53, *P<0.001*), LDL (r=0.56, *P<0.001*), and Ox-LDL (r=0.34 *P*=0.027) remained significant across the combined cohort. Again using partial correlation analysis, and controlling for BMI, in subjects with T2DM alone showed a similar positive association between LpPLA2 and cholesterol (r=0.66, *P=0.019*), LDL (r=0.738, *P=0.006*), and Ox-LDL (r=0.75, *P=0.005*).

4.3.3 mRNA level of LpPLA2 and ALOX15 in human adipose tissue.

To study the roles of LpPLA2 in adipose tissue inflammation, mRNA level of ALOX15 was determined. The results from this study showed that there were no significant correlations between LpPLA2 and ALOX15, in either all subjects or those split by non T2DM or T2DM status (Fig 4.3.3.1).

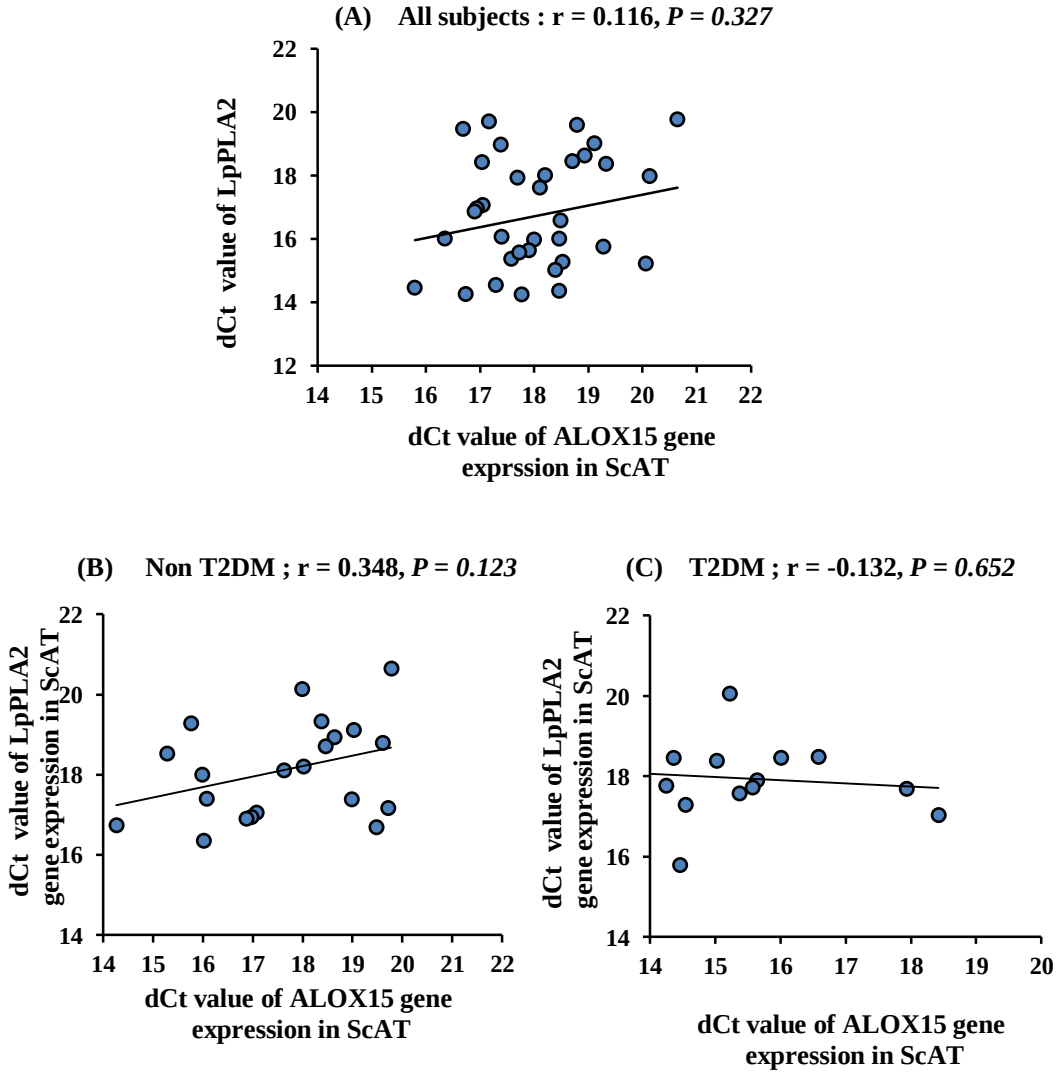

Figure 4.3.3.1. Correlation between LpPLA2 gene expression and ALOX15 gene expression for (A) All subjects, (B) Non T2DM subjects and (C) T2DM subjects. The line of best fit denoted as r and the corresponding P values are noted in the relevant graph.

4.3.4 mRNA level of LpPLA2 and COX2 in human adipose tissue.

This study showed that there were significant negative correlations between LpPLA2 and COX2 (*P<0.05*) in the T2DM subjects, whilst no significant correlations were observed in either the non-T2DM subjects or the whole cohort together.

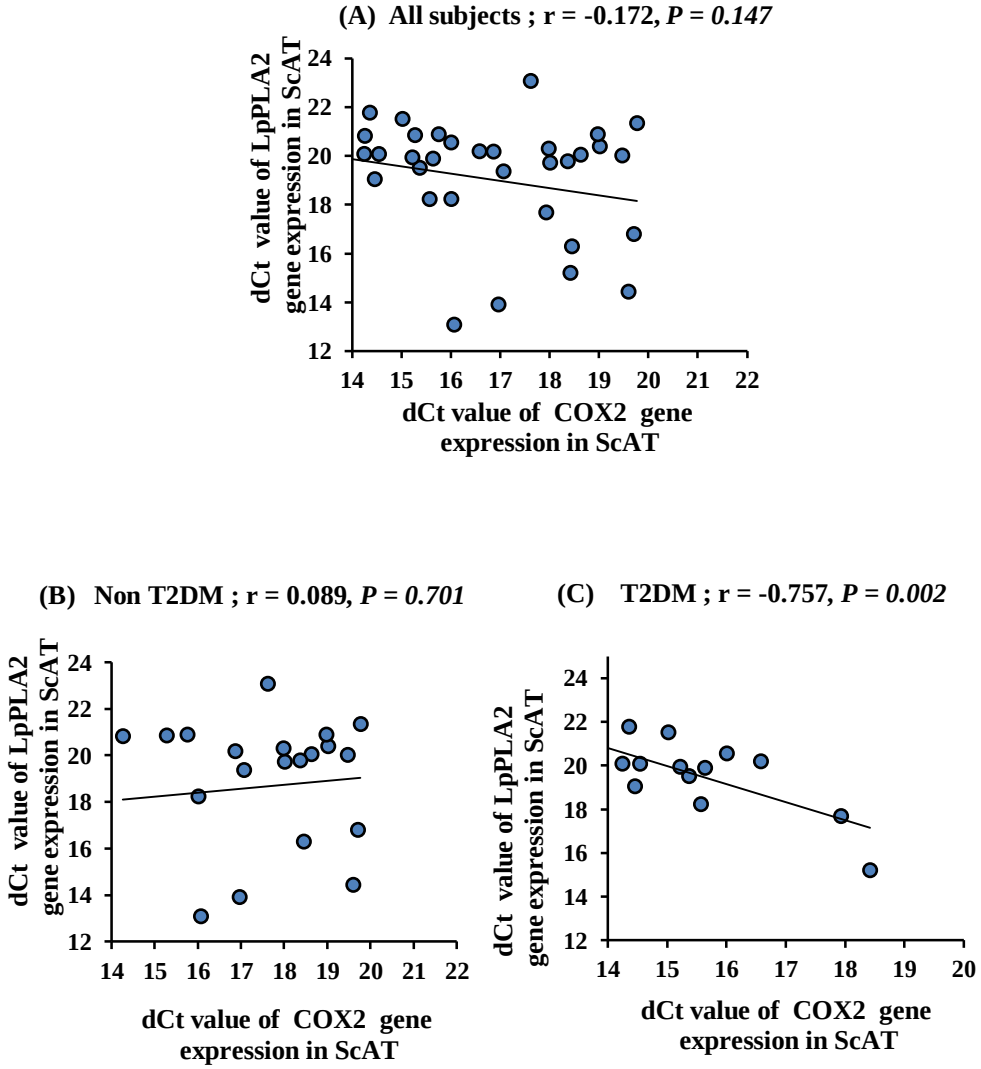

Figure 4.3.4.1. Correlation between LpPLA2 gene expression and COX2 gene expression for (A) All subjects, (B) Non T2DM subjects and (C) T2DM subjects. The line of best fit denoted as r and the corresponding P values are noted in the relevant graph.

4.3.5 mRNA level of LpPLA2 and IL-6 in human adipose tissue.

This study showed that there were significant negative correlations between LpPLA2 and IL-6 *(P<0.05)* in T2DM subjects whilst this correlation was noted observed either in the non-diabetic group or the group as a whole (Figure 4.3.5.1).

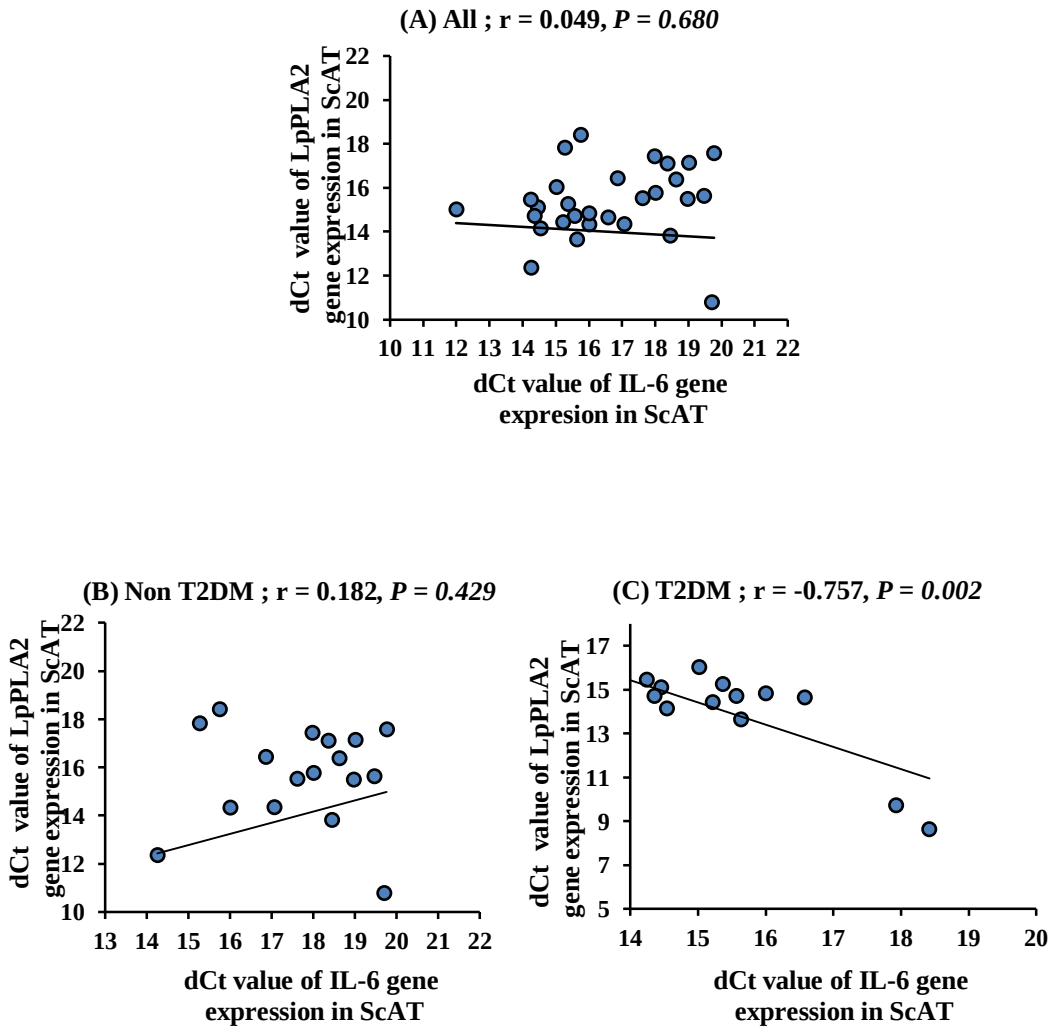

Figure 4.3.5.1. Correlation between LpPLA2 gene expression and IL-6 gene expression for (A) All subjects, (B) Non T2DM subjects and (C) T2DM subjects. The line of best fit denoted as r and the corresponding P values are noted in the relevant graph.

4.3.6 Effects of ox-LDL treatment on PLA2 expression in differentiated human adipocyte cell line, chub S7

Cells treated with Ox-LDL induced an acute rise in LpPLA2 expression, after 6 hr treatment and declined up until 24 hr (*P<0.001*; Fig 4.5.6.1 A). The gene expression of cPLA2 and iPLA2 was not modulated under the same stimulatory condition.

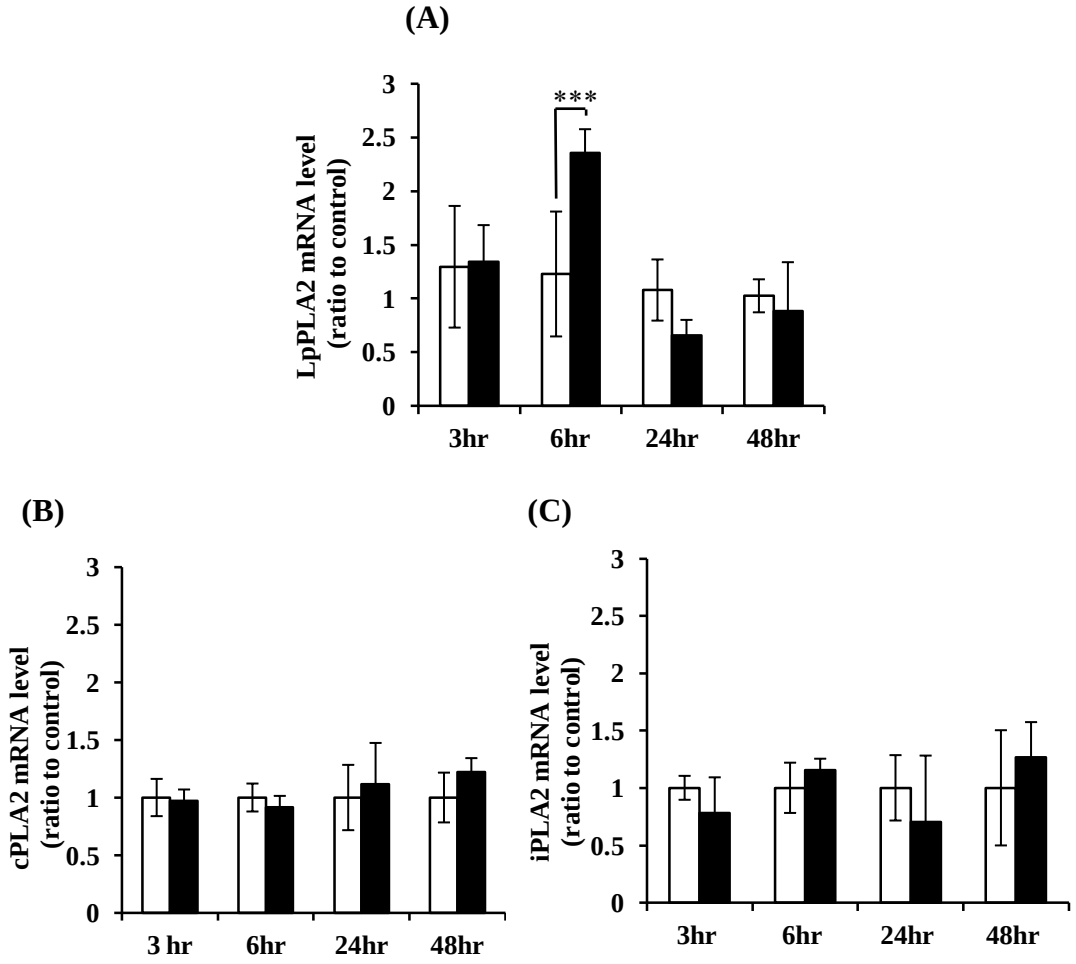

Figure. 4.5.6.1. Effects of ox-LDL on (A) LpPLA2 gene, (B) cPLA2 and (C) iPLA2 expression. Differentiated Chub-S7 adipocytes were treated with 100 µg/ml ox-LDL for 3, 6, 24 and 48 hr. Phosphate buffer saline containing 0.34 mM EDTA was used as control. mRNA level of LpPLA2 was measured by real-time RT-PCR. The data were normalized to 18S RNA. The fold change in expression was calculated relative to control of each time point. Protein level of LpPLA2 was measured by ELISA in cell homogenates. The difference of expression were assessed by one way ANOVA test (***, *P<0.001* vs control)

4.3.7 Effects of ox-LDL treatment on IL-6 and COX2 expression in differentiated human adipocyte cell line, chub S7

IL-6 gene expression was up-regulated within 3 h incubation when treated the cell with ox-LDL. However, ox-LDL treatment did not alter COX2 expression.

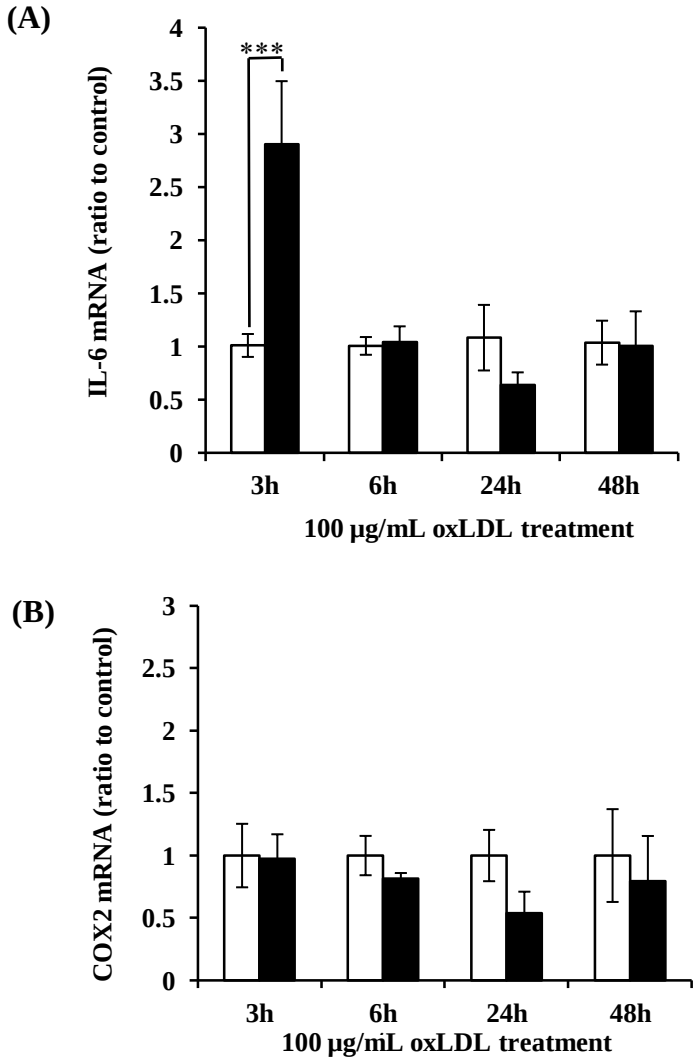

FIG. 4.5.7.1. Effects of ox-LDL on (A) IL-6 and (B) COX2 gene expression. Differentiated Chub-S7 adipocytes were treated with100 µg/ml ox-LDL for 3, 6, 24 and 48 hr. Phosphate buffer saline containing 0.34 mM EDTA was used as control. mRNA level of IL-6 and COX2 was measured by real-time RT-PCR. The data were normalized to 18S RNA. The fold change in expression was calculated relative to control of each time point. The difference of expression were assessed by one way ANOVA test (*P<0.001* vs control)

4.4 Discussion

In this study, it was observed that LpPLA2 correlated with specific cardiometabolic risk factors such as LDL-cholesterol, HDL-cholesterol, ox-LDL cholesterol, as well as a relative new biomarker of metabolic risk, endotoxin. Whilst such associations identified are not necessarily causal they do highlight the relevance of LpPLA2 as a potential new biomarker of cardiometabolic risk as other studies suggest (A. Cai et al., 2013; Gu et al., 2014; Hong et al., 2013; S. B. Li et al., 2013; Mangili et al., 2014). Furthermore examining subjects across adiposities and those with T2DM indicated that T2DM subjects develop a more pronounced metabolic dysfunction than obesity *per se*. This aspect appears relevant as a raised LpPLA2 level was noted in such T2DM subjects. Whilst the current *in vitro* adipocyte studies identified that oxidation of LDL was accompanied by an increase in LpPLA2 mRNA expression in a similar manner to prior studies in macrophages (Markakis et al., 2010; W.-Y. Wang et al., 2010).

Yet interestingly when examining the cohorts in more detail, considering the potential influence of adiposity, it was identified that controlling for BMI did not affect the positive correlation of circulating LpPLA2 with cholesterol, LDL or Ox-LDL across the combined cohort, or within the T2DM cohort. The correlation between lipids and LpPLA2 affirms previous studies and highlights how the relative distribution of LDL and HDL may impact on LpPLA2's function to either being atherogenic or atheroprotective, impacted by diabetic status and glycaemic control (Sanchez-Quesada et al., 2012).

Beyond the cardiometabolic parameters the positive correlation observed between endotoxin and LpPLA2 represents a novel insight into the effect of endotoxin on another inflammatory mechanism, in addition to the innate immune

cascade (Al-Attas et al., 2009; Baker et al., 2009; Creely et al., 2007). Previous studies have shown that endotoxin, as well as saturated fatty acids, may up-regulate the innate immune cascade, with activation of nuclear factor kappa B (NFκB) leading to down-stream activation of numerous pro-inflammatory factors (Creely et al., 2007; Youssef-Elabd et al., 2012). Interestingly, previous studies in endothelial cells and macrophages have also shown that the LpPLA2 signalling pathway is regulated, in part, by NFκB, which would also align with the mechanism through which endotoxin, as well as lipid mediators, influence circulating LpPLA2 (Baker et al., 2009; Creely et al., 2007; Senokuchi et al., 2005; Sonoki et al., 2008; Youssef-Elabd et al., 2012). This may therefore explain why endotoxin appears positively correlated with serum LpLA2.

The positive correlation between circulating LpPLA2 and ox-LDL were also observed in this study. Ox-LDL is widely considered as an important risk factor for cardiovascular diseases which recently, has been reported to augment the cardiovascular disease risk prediction within the Framingham model (Gómez et al., 2014). Similar to endotoxin, ox-LDL has also previously been reported as a regulator of LpPLA2 expression in monocyte and endothelial cells via p38 /NFκB pathway. In addition, LpPLA2 has been demonstrated to hydrolyze oxidized phospholipid in LDL particles, resulting in reduction of atherogenic effect of ox-LDL on foam cell formation. However during this process, lysophospholipid and oxidized free fatty acid are also gradually generated, leading to further increase oxidative progression and inflammation activation.

Since LpPLA2 plays this bi-functional enzyme role, the correlation between adipose tissue LpPLA2 mRNA expression and other inflammatory markers, such as ALOX15, COX2 and IL6 were studied to understand whether an *ex vivo* tissue

mRNA analysis of inflammatory markers may correlate with changes in LpPLA2 due to the known interactions through NFκB in adipose tissue (Baker et al., 2009; Harte et al., 2013b; McGee et al., 2011). However *ex vivo* mRNA analysis in adipose tissue taken from non T2DM subjects did not identify any correlations between LpPLA2 versus, IL-6, COX2 or ALOX15. Whilst sub-cohort analysis showed a negative correlation between LpPLA2 with COX2 and IL-6, in adipose tissue taken from T2DM subjects. This may indicate that in a pathological state such as T2DM, high expression of LpPLA2 in adipose tissue may not additionally promote inflammation but likely resolve inflammation in T2DM. However the reduction in mRNA of IL-6 and Cox2 may have arisen due to the influence of medications that the T2DM patients were on. Since previous research has shown that statins can reduce IL-6 and Cox2 expression directly from human adipose tissue, macrophages and endothelial cells (Baker et al., 2006; Q. Li et al., 2006; Massaro et al., 2010), whilst the effect of statins on LpPLA2 mRNA expression in adipose tissue has not been previously studied.

Due to the potential influencing effects of diabetes medication on inflammatory markers and LpPLA2 an *in vitro* study was undertaken to understand the impact of ox-LDL in an adipocyte culture system. Human differentiated adipocyte Chub S7 cells (Darimont et al., 2003) were treated with ox-LDL and gene expression changes measured over time.

From these studies it was identified for the first time that ox-LDL induced LpPLA2 expression in adipocyte after 6 h incubation and slowly returned into basal level within 24 h, whereas IL-6 mRNA levels were up-regulated within 3 h of incubation and declined within 6 h. Whereas ox-LDL treatment did not appear to alter either cPLA2 or iPLA2 expression. The expression of the two isoforms, cPLA2

and iPLA2 were determined as it has been previously reported that cPLA2 activity is activated by ox-LDL treatment, promoting inflammation in both mouse monocyte (W.-Y. Wang et al., 2010) and fibroblast (Lupo et al., 2007) cell lines. Thus, it was necessary to determine within in a human adipocyte system, if activation of cPLA2 and iPLA2 may account for the potential inflammation, which at least in these adipocyte cultures did not appear to be the case.

Also in this current study it appeared COX2 mRNA expression did not alter when treated with ox-LDL in adipocyte, suggesting that there were no direct association between activation of LpPLA2 by ox-LDL and COX2 in differentiated human adipocyte. COX2 was utilised as a candidate to increase in this culture system as prior studies with primary vascular smooth muscle cells taken from T2DM subjects had shown that COX2 protein expression increased in response to TG (Gordillo-Moscoso et al., 2013). However the change in culture system, metabolic state as well as differences in Cox2 in *ex vivo* adipose tissue from different metabolic states appear to impact on COX2 regulation and may therefore suggest its regulation is more complicated than first considered.

Taken together, the current *in vitro* data suggest that ox-LDL can up regulate both LpPLA2 and IL-6 in adipocytes whilst the *ex vivo* AT data was unable to confirm this in T2DM subjects probably due to interference through medicinal treatment. However LpPLA2 mRNA expression appears regulated by ox-LDL treatment in the Chub-S7 AbdSc cell line and these findings align with previous data which noted that LP-LA2 protein expression is higher in AbdSc AT compared to Om AT across all groups (noting that the differences in adipose tissue depot protein expression may influence cell responsiveness to ox-LDL).

In summary, these studies affirm and extend our current knowledge on circulating LpPLA2 and its association lipid profiles including ox-LDL and extend this through data on endotoxin. Furthermore linear regression analysis identified that ox-LDL was a significant predictor of circulating LpPLA2 which was confirmed by *in vitro* studies showing the regulatory effects of ox-LDL on Lp-PLA2 expression. However further studies are required to ascertain a direct cause and effect relationship between ox-LDL and LpPLA2. Furthermore as lysophospholipid and oxidized free fatty acid are products of LpPLA2 activity, high expression of LpPLA2 in adipocytes might be represent an important source of circulating ox-LDL in obese/T2DM subjects. As such further studies assessing the inhibition of LpPLA2 activity appear important to investigate its effect on LDL oxidation and LpPLA2 functional influence in increasing metabolic disease risk.

CHAPTER 5 : The *in vitro* Role of LpPLA2 in the Conversion of LDL into the Atherogenic ox-LDL in Human Adipocytes.

5.1 Introduction

Lipoprotein-associated phospholipase A2 (LpPLA2) is an enzyme that can hydrolyse polar phospholipids, such as those generated during the oxidation of low density lipoprotein (Ox-LDL), which results in the production of pro-inflammatory mediators, such as lysophosphatidylcholine and oxidised fatty acids(Rosenson & Stafforini, 2012). Recent interest in LpPLA2 has arisen due to a number of epidemiological studies that have shown circulating LpPLA2 to be up-regulated in conditions of inflammation and cardiovascular disease (CVD) (Khakpour & Frishman, 2009; Kinney et al., 2011; Oei et al., 2005; Winkler et al., 2007). Whilst limited studies, to date, have examined circulating LpPLA2 in patients with T2DM, studies have highlighted that LpPLA2 levels are positively correlated with body mass index (BMI), as well as triglycerides (TG), low density lipoprotein (LDL) and high density lipoprotein (HDL) and ox-LDL. Furthermore, elevated LpPLA2 levels have been associated with predicting T2DM (Maria Waegner et al., 2011; Nelson et al., 2012), although other reports have not been conclusive (Constantinides et al., 2011; Nelson et al., 2011). Much of the research, to date, has centred on CVD and the role of the macrophage as a major source of LpPLA2, although other cells types are also noted to express LpPLA2 including T lymphocytes, mast cells and hepatocytes (W.-Y. Wang et al., 2010; M. Yang et al., 2010; Zalewski et al., 2006).

In particular oxidative modification of LDL (oxLDL) is regularly associated with hypercholesterolemia and coronary artery disease. Furthermore despite the initial step of form cell formation in clearly understood which causes the atherosclerotic lesion to develop, the mechanisms underlying oxidative modification of LDL remains unclear (Vickers et al., 2009; W.-Y. Wang et al., 2010; M. Yang et al., 2010). However despite this, recent work has sought to understand the role of

LpPLA2 and its role in etiology on plaque formation through oxidative modification of LDL. These previous *in vitro* studies, using monocytes, have specifically examined the stimulatory effect of Ox-LDL on LpPLA2 activity, which indicates the potential influence of LpPLA2 on arterial inflammation (W.-Y. Wang et al., 2010); whilst in *vivo* studies have shown that Ox-LDL are significantly associated LpPLA2 in carotid endarterectomy (CEA) tissues (Vickers et al., 2009).

Other studies also highlight that elevated LpPLA2 activity promotes the development of vulnerable atherosclerotic plaques, and elevated plasma levels of this enzyme are associated with an increased risk of coronary events. Whilst a new selective oral inhibitor of lipoprotein-associated phospholipase A_2 known as Darapladib has been shown to reduce instability in plaques and reduce coronary events (Dave et al., 2014; Johnson et al., 2014; White et al., 2014). This inhibitor may also reduce the LpPLA2 enzymatic activity critical for eliciting macrophage responses associated with a variety of inflammatory processes and signaling pathways such as c-Jun-kinase (JNK) (Tzang et al., 2009).

However to date whilst it is understood that ox-LDL increases with obesity and that LpPLA2 can induce inflammation in macrophages no comparable study has been undertaken to examine LpPLA2 in human adipocytes; despite the knowledge that adipocytes possess many immune like cell characteristics. As such these current sought to determine whether the adipocyte can convert LDL to ox-LDL in cultured human adipocytes.

5.2 Methods and Materials

5.2.1 Cell cultures and human lipoprotein treatment

The AbdSc pre-adipocytes Chub S7 cells were grown in 6-well plates to confluence in phenol red-free Dulbecco's modified Eagle's medium (PRF-DMEM F-12) containing 10% bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and transferrin (5 µg/ml) at 37^0C, 5%CO_2 incubation until confluent. To undertake cell differentiation, AbdSc pre-adipocytes were maintained in promocell pre-adipocyte differentiation media (PromoCell, Germany) for 48 hr and, following this time period, the cells were maintained in the promocell adipocyte nutrition media (PromoCell, Germany) for fourteen days, with media changes every two days. Following the differentiation period the AbdSc differentiation adipocytes were given a 24 hour wash out period in PRF-DMEM/F12. To study the effect of LDL and Ox-LDL on LpPLA2 expression, a human AbdSc pre-adipocyte Chub S7 cell line was utilized, which originated from a female subject (Darimont et al., 2003). Chub S7 cells were maintained in growth media and differentiated under the same conditions as the human primary AbdSc pre-adipocyte cells, described above.

The differentiated AbdSc Chub S7 cells were treated with either LDL (200 µg/mL) or Ox-LDL (100 µg/mL; Kalen Biomedical, USA) for 3, 6, 24 and 48 hr, using 10% of PBS containing 154mM NaCl and 0.34mM EDTA as vehicle control. The treated differentiated AbdSc Chub S7cells were harvested for RNA isolation and protein extraction.

The differentiated AbdSc Chub S7 cells were also cultured with and without 20µM Darapladip, the LpPLA2 inhibitor in conjunction with Ox-LDL (100 µg/mL; Kalen Biomedical, USA) for 3, 6, 24 and 48 hr, using 10% of PBS containing

154mM NaCl and 0.34mM EDTA as vehicle control. The treated differentiated AbdSc Chub S7cells were harvested for protein extraction as detailed in Chapter 2.

5.2.3 Cellular Protein concentration

Human AbdSc Chub S7 cells following treatment were harvested and resuspended in Phosphosafe™ Extraction buffer (Novogen®, Merck, Germany). Protein concentrations were determined using the Bio-Rad Detergent Compatible protein assay kit (Bio-Rad Laboratories, CA) (Bradford, 1976) and quantified using the nanospectrophotometer (GeneFlow, UK).

5.2.3 Cell viability in the *in vitro* cultured Chub S7 cells

The viability of adipocytes was assessed using the trypan blue dye exclusion method and examined at 48hr following initial treatment. The percentages of cell viability of the cells treated with vehicle control, LDL and Ox-LDL was 93%, 97% and 92%, respectively.

5.2.4 Assessment of Ox-LDL and LpPLA2 in cultured cells

Ox-LDL and LpPLA2 were measured using commercially available solid phase enzyme-linked immunosorbant assay (ELISA; Mercodia Oxidized LDL ELISA, Sweden; intra-assay percentage coefficient of variation %CV = 6.4; inter assay % CV = 7.4) and R&D system Human PLA2G7/PAF-AH/LpPLA2 (Quantikine ELISA, UK; intra-assay %CV = 6.8, inter-assay %CV = 9.6).

5.2.5 Statistical Analysis

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 18.0 for Windows (SPSS UK Ltd, UK). Analysis of variance (ANOVA) was used to compare the effects of oxidized LDL on LDL and LpPLA2 expression across various time points. ANOVA with Bonferroni correction was undertaken in SPSS with automatic adjustment to the detailed significance dependent upon the number of comparisons.

5.3 Results

5.3.1 The *in vitro* effect of Ox-LDL on LpPLA2 expression in human adipocyte cell line, Chubs S7

Cells treated with Ox-LDL induced an acute rise in LpPLA2 mRNA, post 3 hr treatment, that slowly declined up until 24 hr as previously showed in Chapter 4 (*P<0.001*; Figure 5.3.1 A). LpPLA2 protein expression was significantly higher compared with control by 3 hr. The protein level gradually decreased after 6 h incubation (*P<0.001*; Figure 5.3.1 B).

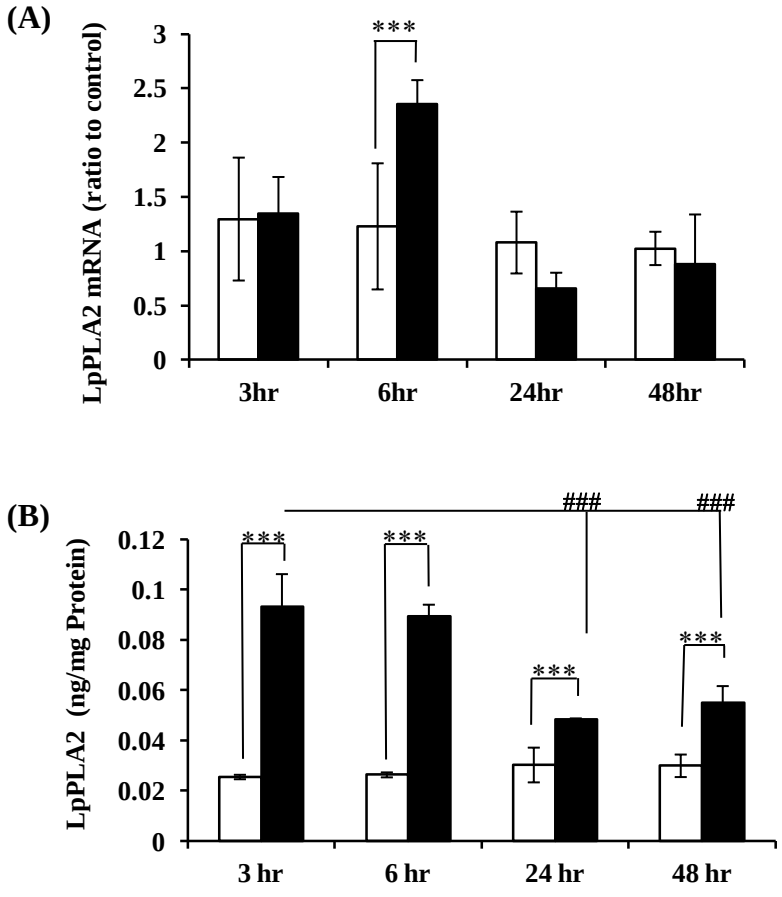

Figure 5.3.1 ox-LDL induced LpPLA2 expression. Differentiated Chub S7 adipocytes were treated with 100 µg/ml oxidized LDL for 3, 6, 24 and 48 hr. Phosphate buffer saline containing 0.34 mM EDTA was used as control. The intracellular protein level of LpPLA2 was measured by ELISA. The data were normalized to total protein. The difference in expression was assessed by two way ANOVA test (***, *P<0.001*, each time point vs. control; ###, *P<0.001*, each time vs. 3hr)

5.3.2 Activation of LpPLA2 by LDL in human adipocyte cell line, Chubs S7.

Reduction of LpPLA2 mRNA level was observed after treated the cells with LDL. After 48 hr incubation, the mRNA level significantly increased compared to control as previously showed in Chapter 4 (*P<0.001*; Figure 5.3.2 A). LpPLA2 protein level gradually decreased after 3 hr incubation (*P<0.001*) and then significantly increased at 48 hr incubation compared to 24 hr incubation (*P<0.001*; Figure 5.3.2 B)

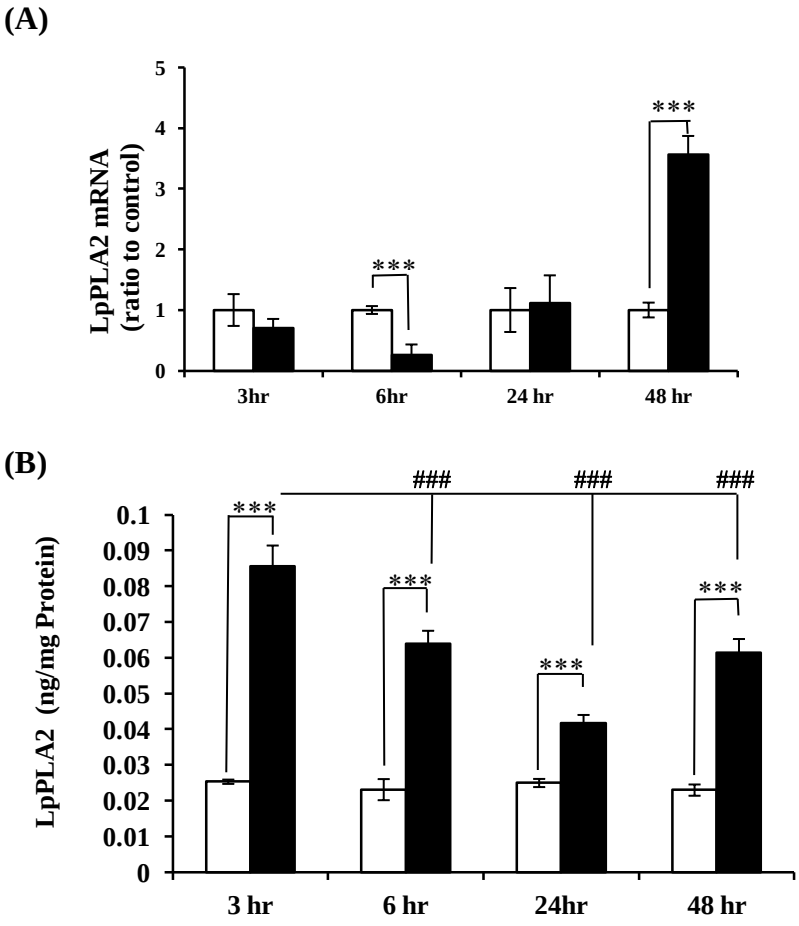

Figure 5.3.2 LDL induced LpPLA2 expression Differentiated Chub S7 adipocytes were treated with 200 µg/ml LDL for 3, 6, 24 and 48 hr. Phosphate buffer saline containing 0.34 mM EDTA was used as control. The intracellular protein level of LpPLA2 was measured by ELISA. The data were normalized to total protein The difference in expression was assessed by two way ANOVA test (*, *P<0.05*; ***, *P<0.001*, each time point vs. null; #, *P<0.001*, 48hr vs. 24 hr incubation

5.3.3 Oxidation of LDL by adipocytes was mediated by LpPLA2

Further analysis of LDL treatment determined whether the adipocyte cells were converting the LDL into Ox-LDL. The percentage of Ox-LDL in LDL treated media was determined, as shown in Figure 5.3.3. At 48 hr incubation, the Ox-LDL level had increased by 21.5% compared with the treatment media at 3 hr incubation (*P<0.05*)**.**

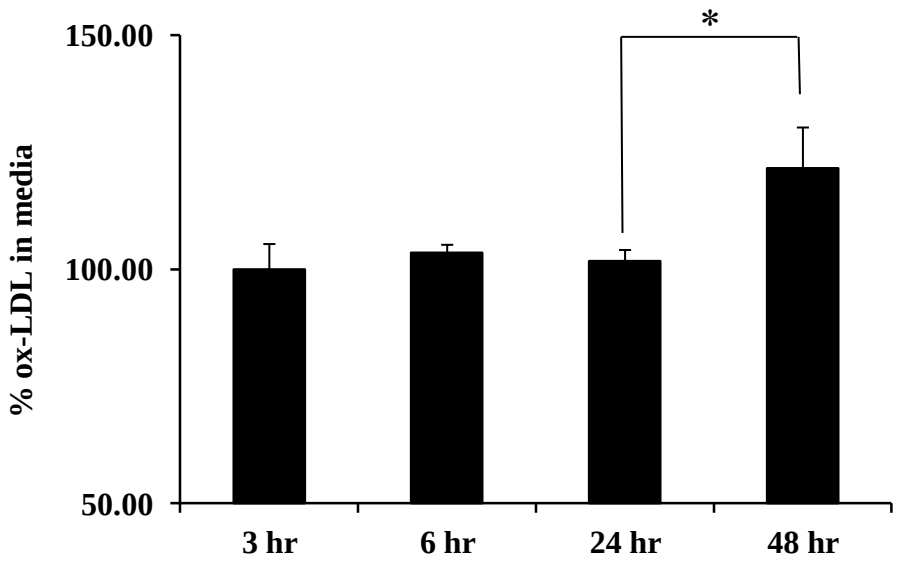

Figure 5.3.3 Increased ox-LDL in LDL treatment in human adipocyte cell line. Differentiated Chub S7 adipocytes were treated with 200 µg/ml LDL for 3, 6, 24 and 48 hr. Phosphate buffer saline containing 0.34 mM EDTA was used as control and Ox-LDL was measured by ELISA in conditioned media. Ox-LDL level at 3 hr was calculated as 100% ox-LDL in condition media. The change in % ox-LDL over time was assessed by two way ANOVA test with ∗, *P<0.05*, 48 hr vs. 3 hr incubation.

5.3.4 Changes in ox-LDL production by the use of an LpPLA2 inhibitor.

To confirm the ability of adipocyte in LDL modification via LpPLA2, the cells were treated with LDL plus a LpPLA2 inhibitor, darapladib. The ox-LDL level in conditioned media was approximately 19.8% lower in the cells treated with LDL plus darapladib after 72 incubation compared to the cells treated with LDL alone (*P<0.05*; Figure 5.3.4)

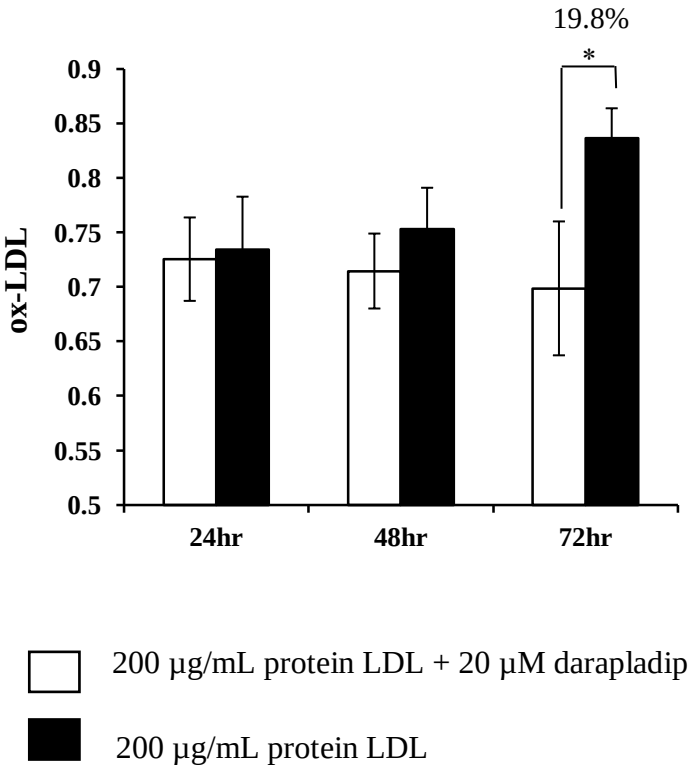

Figure 5.3.3 LpPLA2 inhibitor, darapladip, reduced ox-LDL production *in vitro*. Differentiated Chub S7 adipocytes were treated with 200 µg/ml LDL with or without 20 µM darapladib (SB) for 3, 6, 24 and 48 hr. Phosphate buffer saline containing 0.34 mM EDTA was used as control and Ox-LDL was measured by ELISA in conditioned media. The change in % ox-LDL over time was assessed by two way ANOVA test with ∗, *P<0.05*, 48 hr vs. 3 hr incubation.

5.4 Discussion

This present study is the first study to show that LpPLA2 within the adipocyte appears to regulate the production of ox-LDL suggesting that the adipocyte could act as another substantial source of ox-LDL production and may explain why increasing adiposity could contribute to rising ox-LDL levels. Furthermore the use of an LpPLA2 inhibitor provided further evidence that the production of Ox-LDL was regulated by LpPLA2 expression in the adipocyte. This work has important ramifications for highlighting that adipose tissue may act as separate and significant source of Ox-LDL production apart from foam cells within unstable atherogenic plaques noted in CAD (Dave et al., 2014; Johnson et al., 2014).

Due to the dynamic nature of LpPLA2 *in vitro* studies also observed that both Ox-LDL and LDL could induce LpPLA2 expression in human adipocyte. Ox-LDL treated cells acutely increased the new synthesis of LpPLA2 as observed by increasing mRNA expression noted as early as 6 hr post-incubation. Interestingly LpPLA2 protein level appeared to be induced at an even early time point, as early as 3 hr post-incubation. As such the raised LpPLA2 protein expression did not appear to be associated with mRNA production within the adipocyte. One rationale for this apparent inconsistency may arise due to the capability of the adipocyte to engulf Ox-LDL and LDL particles into the cell. This function has an important impact on LpPLA2, as following Ox-LDL uptake the adipocyte can digest the ox-LDL particle to release fatty acid, cholesterol as well as LpPLA2. Thus, the higher protein expression compared with the control observed at 3h post-incubation may appear to reflect the exogenous LpPLA2 available, whilst the higher mRNA expression observed at 6 hr post-incubation represents the newly synthesised LpPLA2 gene expression. The increased mRNA expression level resulted in LpPLA2 protein

expression to remain high inside the adipocyte at the 6 hr post-incubation time point. A similar phenomenon was also observed in the adipocytes treated with LDL where the LpPLA2 protein expression was higher than control 3 hr post-incubation and then gradually reduced over the 24 hr period. Unlike oxLDL treatment, the LpPLA2 mRNA level in the adipocyte treated with LDL showed a significant reduction in expression at 6 hr post-incubation, leading to a steady decline in LpPLA2 protein expression over time. Although, interestingly there was a subsequent up regulation of LpPLA2 mRNA and protein expression in LDL treated cells at 48 hr post-incubation. It appears that a couple of possible scenarios which occur *in vivo* could explain why such LDL modification may occur in *in vitro*.

It is clear that Ox-LDL is regularly associated with obesity induced hypercholesterolemia and CAD, with Ox-LDL known to promote foam cell formation, leading to causing the atherosclerotic lesion (Johnson et al., 2014). The mechanism underlying the *in vivo* oxidative modification of LDL remains unclear. However LDL is highly oxidized by metal ions such as Fe^{2+} and Cu^{2+} as well as superoxide ($O^{\cdot -}$) by Fenton reaction (Kappus, H. 1985)

$$O_2^{\cdot -} + Me^{(n+1)+} \longrightarrow O_2 + Me^{n+} \quad (1)$$

$$H_2O_2 + H^+ + Me^{n+} \longrightarrow HO^{\cdot} + H_2O + Me^{(n+1)+} \quad (2)$$

As such *in vitro* studies of smooth muscle cells (Heinecke, J 1984, Heinecke, J 1986), monocyte (Hiramatsu, K, 1986), macrophage (Wilkins, G, 1990) and endothelial cell (Steinbrecher, U.P., 1988) has established that there is a postulated release of superoxide which is considered to promote LDL modification. However, under physiological condition, oxidative stress induced by superoxide is controlled by the anti-oxidative system such as superoxide dismutase, NADH and glutathione

and superoxide itself has low reactivity to LDL (Bedwell, S., 1989). Additionally, *in vivo* studies have highlighted that the accumulation of cu^{2+} and Fe^{2+} has been observed in artery walls taken from atherosclerotic lesion, which may impact on LDL modification (Dubick, M., 1987, Smith, C. 1992); particularly as iron overloaded in thalassemia patients has been associated with a high risk of cardiovascular disease. Since, superoxide has less capacity in oxidation under physiological pH and iron and copper overloaded diseases are quite rare. However other mechanisms may also lead to LDL modification there through the dysregulation of pro- and anti-inflammatory mediators which can play role a role in the pathogenesis of cardiovascular disease as well. As mentioned in previous chapter, LpPLA2 is the bi-functional enzyme, containing both pro- and anti-inflammatory properties which depend on physiological and pathological state. In addition, previous studies have shown the up regulation of LpPLA2 in adipocyte taken from obese T2DM subjects and positive correlations between LpPLA2 and ox-LDL (Maria Waegner et al., 2011; Nelson et al., 2011).

As the early and late up-regulation of LpPLA2 up-regulation was observed in adipocyte treated with Ox-LDL and LDL, studies were undertaken to assess whether LpPLA2 may be interconverting LDL and Ox-LDL due to the relative changes over time as the adipocytes are held within a closed system. As such an LpPLA2 inhibitor, darapladib, was utilised to ascertain whether the relative changes in Ox-LDL production were directly related to LpPLA2 activation. The LpPLA2 inhibitor studies co-treating cells with LDL identified that that the production of resulting ox-LDL was significantly decreased with the inhibitor. Further due to LpPLA2s bi-directional nature of LpPLA2 in the adipocyte this may also help to explain why in such a closed system as LDL levels reduced and Ox-LDL increased, LpPLA2

expression may have altered. Such *in vitro* findings also indicate that LpPLA2 expression may have a significant impact on modifying *in vivo* systemic LDL. Therefore with increasing adiposity and rising LDL levels in patients with obesity and T2DM this leads to increased production of Ox-LDL without the necessarily further interconversion of Ox-LDL as noted *in vitro* as Ox-LDL can move away from the adipose tissue through the circulation. In conclusion these current *in vitro* studies highlight the functional capability of LpPLA2 within the human adipocyte and another key site of Ox-LDL production with implications for increased disease risk in patients with obesity.

CHAPTER 6 : Reduction of TLR4 Expression in Adipose Tissue after Bariatric Surgery in T2DM.

6.1 Introduction

Adipose tissue (AT) is an endocrine organ, releasing both pro-inflammatory cytokines (*e.g*. TNFα, IL-6, IL-1β and MCP-1) and anti-inflammatory cytokines (*e.g*. adiponectin, leptin, IL-1R and IL-10) (Giamila, 2005). There are much evidence supports that the imbalanced secretion of those adipokines contributes to low grade inflammatory diseases such as T2DM and cardiovascular diseases. In human adipose tissue, TNFα expression correlates with BMI, percentage of body fat and hyperinsulinemia, whereas weight loss decreases TNFα expression (G. Fisher et al., 2011). Similarly, increased circulating IL-6 level has been observed in obese and insulin resistant subjects (Roytblat et al., 2000; Vozarova et al., 2001). Moreover, TNFα and IL-6 have long-term effects in IRS-1, GLUT4 and PPAR gene suppression in 3T3 adipocytes, resulting in insulin resistance (Rotter, Nagaev, & Smith, 2003). In contrast adiponectin is widely known as the insulin-sensitizing adipokine with the capacity to suppress TNFα expression and induce IL-10 and IL-1Ra expression. Decreased circulating adiponectin concentration has been negatively associated with insulin resistance and T2DM. Whilst the molecular mechanisms of how the activation and changes in inflammation in AT remains unclear. It is apparent that increasing fat mass which expands fat droplets, reduced angiogenesis, increased cellular hypoxia, and innate immune activation may all contribute to inflammation in the obese AT state. In such cases the cellular response appears to mainly activate the IKK/NFκB and JNK pathway, consequently activating the production of chemokines and pro-inflammatory cytokines (J. Ye, 2009; Jianping Ye, 2011). Previous data in human adipose tissue affirms the up regulation of NFκB and JNK in obese state (Harte et al., 2013a). Studies have also sought to elucidate other systemic factors that

may mediate the inflammatory state in obesity. Studies have shown that free fatty acids are raised in obesity (Horowitz et al., 1999; Horowitz & Klein, 2000), with free fatty acids reported to induce NFκB activity via activated of the key innate immune receptors, the toll like receptors (TLRs) (Youssef-Elabd et al., 2012) and reduce glucose uptake in muscle cells (Hommelberg, Plat, Langen, Schols, & Mensink, 2009). Adiponectin also appears to regulate inflammation through TLRs and NFκB and peroxisome proliferator-activated receptor gamma 2 (PPARγ2) (Huang, Park, McMullen, & Nagy, 2008; Yamaguchi et al., 2005; Yessoufou et al., 2009).

Whilst obesity provides a state of chronic inflammation bariatric surgery provides an effective therapy for morbid obesity with T2DM to reduce the insulin resistant state and improve other co-morbidities associated with obesity such as hyperlipedemia, hypertension and obstructive sleep apnea (Bae, Lee, Yun, & Heo, 2014; Nienhuijs, de Zoete, Berende, de Hingh, & Smulders, 2010; Svane & Madsbad, 2014). A recent systemic bariatric surgery review which examined 136 studies with a total of 22094 patients (Buchwald et al., 2011) identified that 61.23% of patients reported weight loss, whilst 76.8% completely resolved their diabetes status. Furthermore the metabolic improvements such as resolving diabetic status were also coupled with reduction in serum triglyceride, VLDL and free fatty acids without significant weight loss post surgery (Curry et al., 2011; Magkos et al., 2010). However interestingly, no study to date has examined the potential impact of metabolic parameters on AT metabolism and the influence of weight loss to resolve the AT inflammation Therefore for this study, the inflammatory related gene expressions in AT were examined pre and 6 months post-bariatric surgical intervention and its relationship with metabolic changes due to surgery, particularly understanding the influence of *in vivo* and *in vitro* triglycerides on AT inflammation.

6.2 Materials and methods

6.2.1 Subjects: Serum and tissue collection

For this study, women were recruited with morbid obesity and T2DM and under-went bariatric surgery (n= 30). All subjects had their height, weight and BMI measurements taken using standard equipment. Detailed medical drug histories were taken and those subjects on medication considered to alter inflammatory status were excluded, including the thiazolidinediones. Ethical approval was obtained from the local research ethics committee and all patients gave written consent.

Subcutaneous abdominal adipose tissue samples and fasted blood samples before and 6 months post-surgery were obtained by needle biopsy. Serum levels were analysed as detailed below.

6.2.2 Surgical procedure

The bariatric surgery can be classified into two major techniques. One is related to restrictive techniques which reduce the stomach size in order to reduce food digestion *e.g.* adjustable gastric bands and sleeve gastractomy (SG). The another surgical techniques is related to a malabsorptive type surgery which reduces food intake and absorption by cutting off some part of stomach and pancreas and creating bypass between stomach and intestine *e.g.* biliopancreatic diversion (BPD) and the roux-en-Y gastric bypass. In this study, patients were operated by gastric banding (n=8), Sleeve gastrectomy (n=14) and Biliopancreatic diversion (n= 8). Clinical characteristic of participants are detailed in Table 6.3.1.1. Twenty five of 30 received metformin, six received sulfonylurea and one received insulin no effect of medication was noted in this study on the factors examined.

6.2.3 *In vivo* assessment of the biochemical profile

Fasting blood samples were collected from participating subjects and lipid profiles and fasting plasma glucose were determined using routine laboratory methods, as detailed in Chapter 2. In brief, the routine blood tests included glucose and a full cholesterol profile (TGs, HDL and LDL), as noted in Table 1. Insulin measurements were performed by a solid-phase enzyme amplified sensitivity multiplex immunoassay (Millipore, U.K.), and glucose was measured by a glucose oxidase method (YSL 200 STAT plus).

6.2.4 Protein determination and Western blot analysis

Human AT was homogenized and resuspended in Phosphosafe™ Extraction buffer (Novogen®, Merck, Germany). Protein concentrations were determined using the Bio-Rad Detergent Compatible protein assay kit (Bio-Rad Laboratories, CA) and quantified using the nanospectrophotometer (GeneFlow, UK). Western blot analysis was performed using a method previously described. For Western blotting, in brief, 40µg of protein samples were loaded onto a denaturing polyacrylamide gel (GeneFlow, UK). Protein expression of phosphorylated NFκB (1:1000, Cell Signaling, Denvers, MA) and TRAF6 (1:500, Sigma, UK) was assessed using rabbit monoclonal antibodies. Equal protein loading was confirmed by examining β actin protein expression. No statistical difference was observed in β actin expression for all samples analyzed. A chemiluminescent detection system, ECL/ECL^{+} (GE Healthcare, UK), enabled visualization of bands, whereas intensity was determined using densitometry (Genesnap, Syngene, UK).

6.2.5 RNA extraction and quantitative RT-PCR

RNA was extracted from samples using RNeasy lipid tissue kit (Qiagen, UK) according to the manufacturer's instructions. Extraction was followed by a DNase digestion step to remove any contaminating genomic DNA. RNA was quantitated using the Nanodrop ND-1000 Spectrophotometer (LabTech, UK) and 200ng of RNA from each sample was reverse transcribed using Bioscript Reverse Transcriptase (Bioline, UK) and random hexamers, according to the manufacturer's instructions. Quantitative real-time PCR was performed with pre-designed gene specific Taqman probes and primers (Applied Biosystems, UK, TLR4; Hs00152939_m1, TLR2; Hs00610100_m1, IL-6; Hs01075666_m1 and Adiponectin; Hs01004530_m1) in a reaction mix containing TaqMan universal PCR master mix (Applied Biosystems, UK). All reactions were multiplexed with the housekeeping gene 18S, provided as a pre-optimised control probe (Applied Biosystems, UK) enabling data to be expressed as delta threshold cycle (ΔCt) values (where ΔCt = Ct of 18S subtracted from Ct of gene of interest). Measurements were carried out in triplicate for each sample.

6.2.6 Statistical Analysis

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 18.0 for Windows (SPSS UK Ltd, UK). Student paired t-test was performed comparing mRNA and protein expression level pre- and post-surgery (6 months). Analysis of variance (ANOVA) was undertaken with bonferroni correction controlling for type 1 error which was used to compare mRNA and protein expression among groups with surgery type (adjusted *P*-value < *0.017*, considered as statistically significant). ANOVA with bonferroni correction was undertaken in SPSS with automatic adjustment to the detailed significance dependent upon the

number of comparisons. Determination of correlations in gene expression analysis and variables of interest were performed using Pearson's correlation coefficient for normally distributed variables and Spearman's rank correlation coefficient for variables that were non-Gaussian. Significance for these tests was set at $P<0.05$.

6.3 Results

6.3.1 Characteristics of T2DM subjects pre- and post-bariatric surgery.

It was observed that post surgery individual BMI (*P<0.001*), blood glucose (*P<0.001*), insulin (P<*0.001*), HOMA-IR (*P<0.001*), TG (*P<0.05*), Cholesterol (*P<0.001*) and LDL-cholesterol (*P<0.05*) were significantly improved (Table 6.3.1.1). According to individual surgery types, BMI reduction was 11.36% (4.63-18.55 %) for gastric banding, 10.76% (4.28-16.49%) for SG, 16.96% (14.41-19.09%) for BPD and 12.37% (4.68-19.09%) for overall. There was no significant difference in percent reduction of blood metabolic markers between gastric banding, SG and BPD as noted in Table 6.3.1.2. Bariatric surgery type had no selective effect on metabolic improvement following surgery.

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Table 6.3.1.1. Patients demographics and biochemical data pre- and post-surgery.

	ALL (n=30)		Gastric banding (n=8)		SG (n=14)		BPD (n=8)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Age	54.60±6.64	-	55.88±9.99	-	55.14±5.79	-	52.38±3.5	-
Days between pre- and post- surgery	170.0±23.8	-	163.75±21.34	-	183.57±17.84	-	152.50±23.15	-
BMI (Kg/m^2)	41.26±5.66	36.05±5.16***	42.26±7.54	36.83±8.20***	40.03±5.46	36.65±4.36***	43.30±2.56	35.29±2.60***
Fasting blood Glucose (mmol/L)	9.35±2.54	7.27±1.68***	9.88±2.77	7.28±1.72**	9.09±2.19	7.29±1.69*	9.28±3.09	7.23±1.83*
Insulin	28.52±19.15	16.29±9.25***	25.50±7.33	16.21±6.12*	26.40±20.47	16.29±11.34**	35.26±24.80	16.39±8.78 *
HOMA IR	11.93±8.92	5.13±3.01***	11.26±4.67	5.55±3.05**	11.12±9.64	5.06±3.23*	14.01±11.34	4.82±2.92*
Triglyceride (mmol/L)	1.90±1.11	1.46±0.68*	1.85±0.73	1.26±0.49*	2.10±0.51	1.45±0.73	1.60±0.77	1.67±0.75
Total cholesterol (mmol/L)	5.0±0.90	4.45±0.93***	4.89±0.93	4.41±0.70	4.98±0.75	4.83±0.84**	5.15±1.20	3.84±1.03***
LDL cholesterol (mmol/L)	3.04±0.90	2.74±0.78*	2.96±0.79	2.73±0.65	2.85±0.74	2.98±0.82	3.41±1.21	2.31±0.76**
HDL cholesterol (mmol/L)	1.09±0.31	1.04±0.32	1.07±0.30	1.10±0.29	1.14±0.35	1.17±0.32	1.00±0.24	0.76±0.13**
HDL/LDL ratio	0.39±0.16	0.41±0.16	0.38±0.13	0.43±0.16	0.43±0.19	0.43±0.18	0.33±0.15	0.36±0.12
TG/HDL ratio	2.01±1.15	1.60±1.00	1.91±1.00	1.30±0.74*	2.21±1.86	1.39±0.88	1.75±1.04	2.27±1.21

Table 6.3.1.2. Percent reduction in metabolic parameters Pre- and Post-surgery.

	% Reduction *(min, max)*			
	All	Gastric banding	SG	BPD
BMI (Kg/m^2)	12.37 *(3.68, 19.09)*	11.36 *(4.63, 18.55)*	10.76 *(4.28, 16.49)*	16.96 *(14.41, 19.09)*
Fasting blood Glucose (mmol/L)	18.32 *(-52.86, 50.00)*	25.24 *(14.29, 42.16)*	17.50 *(-17.31, 50.00)*	14.91 *(-52.86, 50.00)*
Insulin	36.69 *(-13.66, 100.00)*	32.98 *(-9.52, 59.09)*	30.31 *(-13.66, 68.75)*	44.79 *(5.17, 79.69)*
HOMA IR	49.88 *(-29.88, 100.00)*	50.44 *(8.91, 67.04)*	40.94 *(-29.88, 79.41)*	62.49 *(41.81, 83.72)*
Triglycerides	11.81 *(-72.93, 67.37)*	28.54 *(-3.88, 65.87)*	12.42 *(-25.93, 67.37)*	-7.64 *(-72.93, 36.70)*
Total cholesterol (mmol/L)	11.20 *(-26.84, 39.79)*	8.03 *(-26.84, 30.95)*	3.13 *(-23.91, 13.03)*	25.26 *(15.98, 39.79)*
LDL cholesterol (mmol/L)	5.91 *(-87.79, 51.20)*	0.28 *(-87.79, 47.34)*	-7.32 *(-65.35, 27.84)*	30.98 *(17.24, 51.20)*
HDL cholesterol (mmol/L)	3.19 *(-34.06, 37.96)*	-3.57 *(-26.58, 7.48)*	-4.30 *(-34.06, 17.68)*	22.33 *(7.04, 37.96)*
HDL/LDL ratio	-10.52 *(-121.86, 41.83)*	-15.85 *(-121.86, 32.59)*	-2.87 *(-85.78, 41.83)*	-16.23 *(-90.47, 9.06)*
TG/HDL ratio	3.16 *(-111.36, 72.09)*	29.59 *(-3.88, 67.11)*	12.25 *(-50.80, 72.09)*	-40.13 *(-111.36, 10.44)*

6.3.2 Bariatric surgery modulated inflammatory state in adipose tissue.

6.3.2.1 The change in TLRs expression following bariatric surgery

Comparison of pre- versus post-surgery highlighted that TLR4 mRNA (*P<0.01*) and TLR2 (*P<0.05*) were significantly reduced in AbdSc AT when analysed by student paired-*t* test. However the reduction of mRNA and protein expression did not appeared to be influenced by the different surgery type when controlled for BMI.

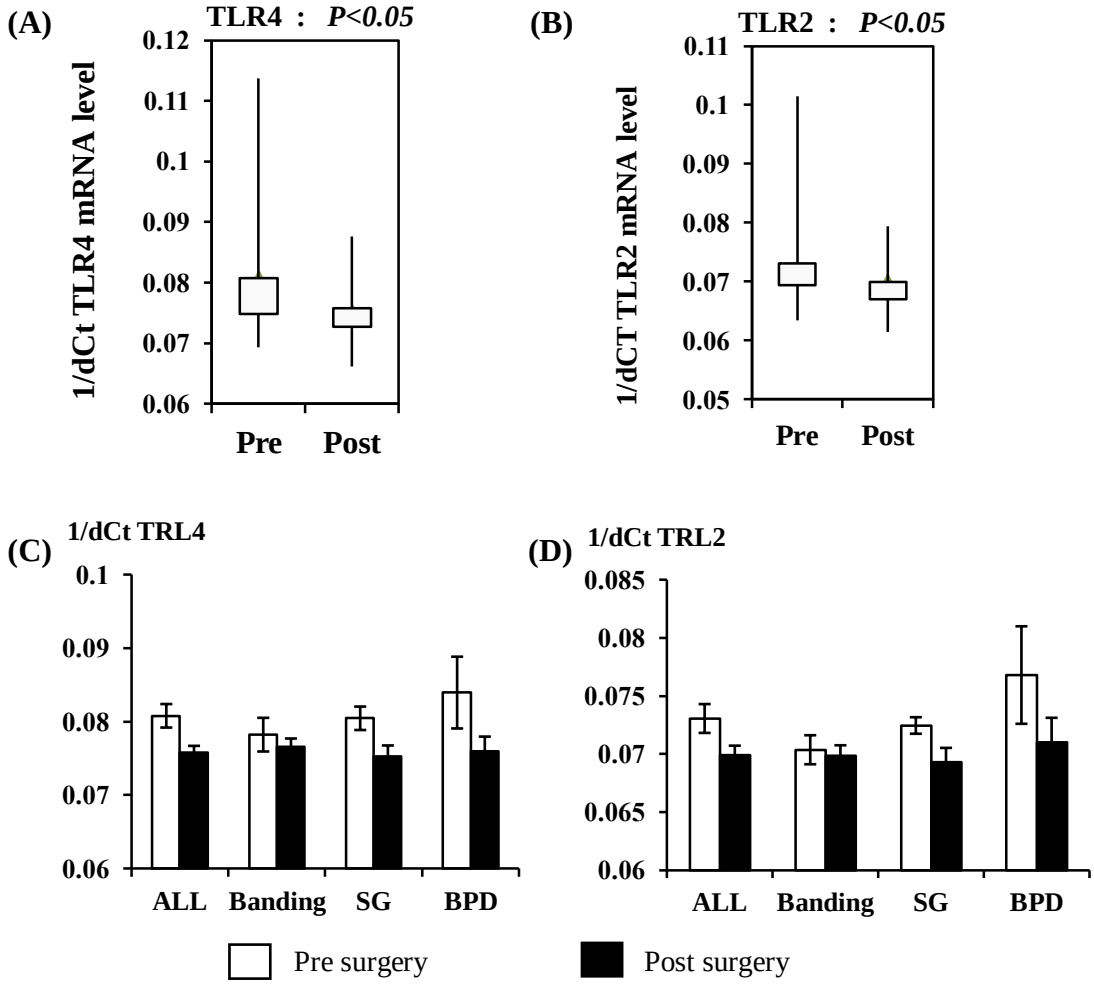

Fig 6.3.2.1. The comparison of (A) TLR4 expression and (B) TLR2 mRNA levels pre versus post surgery. Sub-division of (C) TLR4 mRNA and (D) TLR2 mRNA by surgery type. Data are represented as 1/normalized expression (target protein intensity normalized to β actin in the same sample target gene or Ct value normalized to 18s gene in the same sample).

6.3.2.2 Reduction of IL-6 and NFκB signaling molecule post-bariatric surgery.

Phosphorylated NFκB protein expression (*P<0.05*) in AbdSc AT taken from patients who underwent to bariatric surgery were observed. IL-6 mRNA levels were also shown to decrease post surgery (*P<0.05*). However again the mRNA reduction following surgery for NFκB and IL6 were not altered significantly by surgery type.

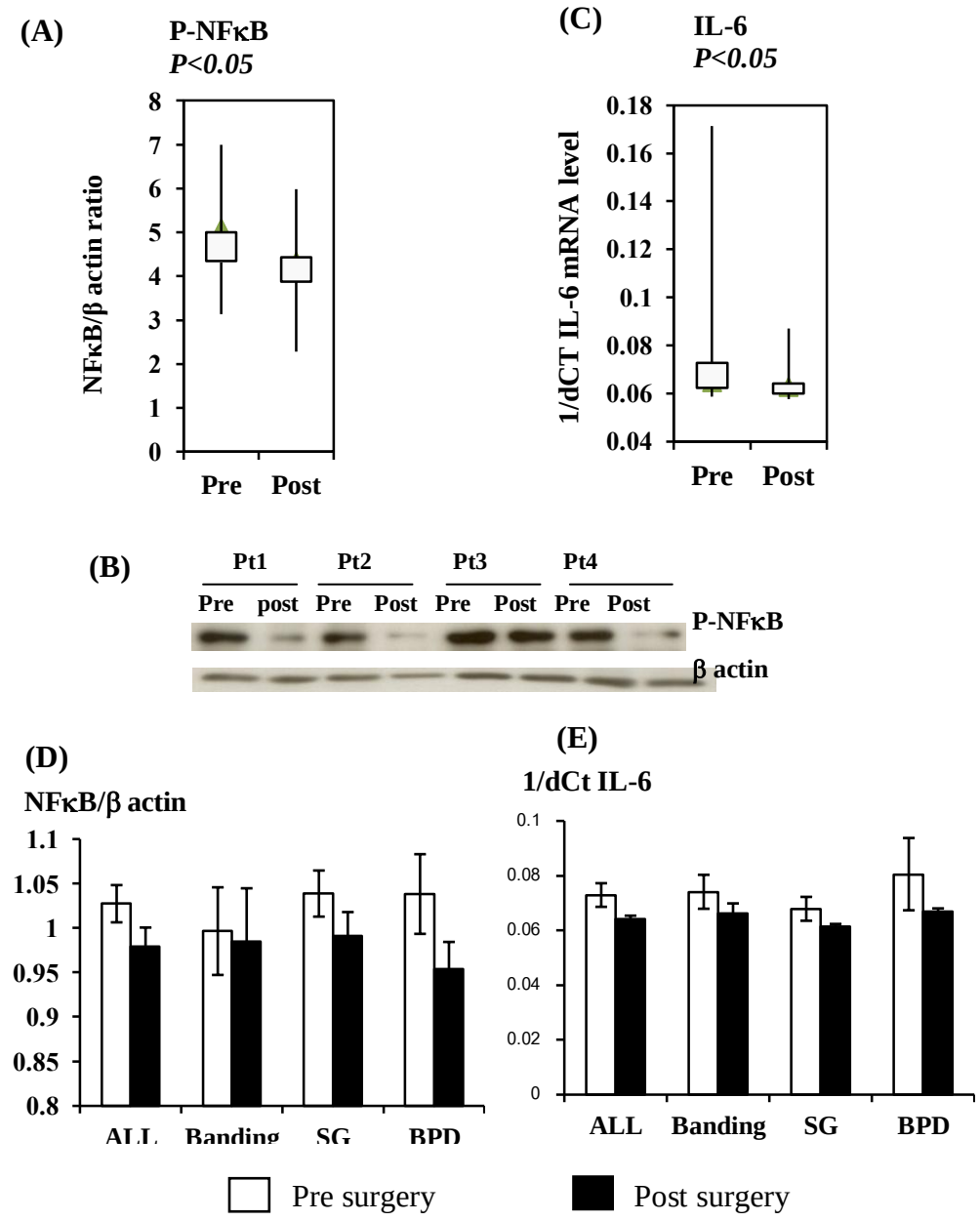

Fig 6.3.2.2. The comparison of (A) phosphorylated NFκB protein expression, (B) western blot of phosphorylated NFκB protein and (C) IL-6 mRNA levels pre versus post surgery. Sub-division of (D) NFκB protein expression and (E) IL-6 mRNA by surgery type. Data are represented as 1/normalized expression (target protein intensity normalized to β actin in the same sample target gene or Ct value normalized to 18s gene in the same sample).

6.3.2.3 Reduction of adiponectin and IκB signaling molecule post-bariatric surgery.

Adiponectin mRNA level (*P<0.001*) were reduced in AbdSc AT taken from patients who underwent bariatric surgery which was also observed for IκB (*P<0.05*). The reduction in the noted gene expressions was similar across bariatric surgery types (Figure 6.3.2.3).

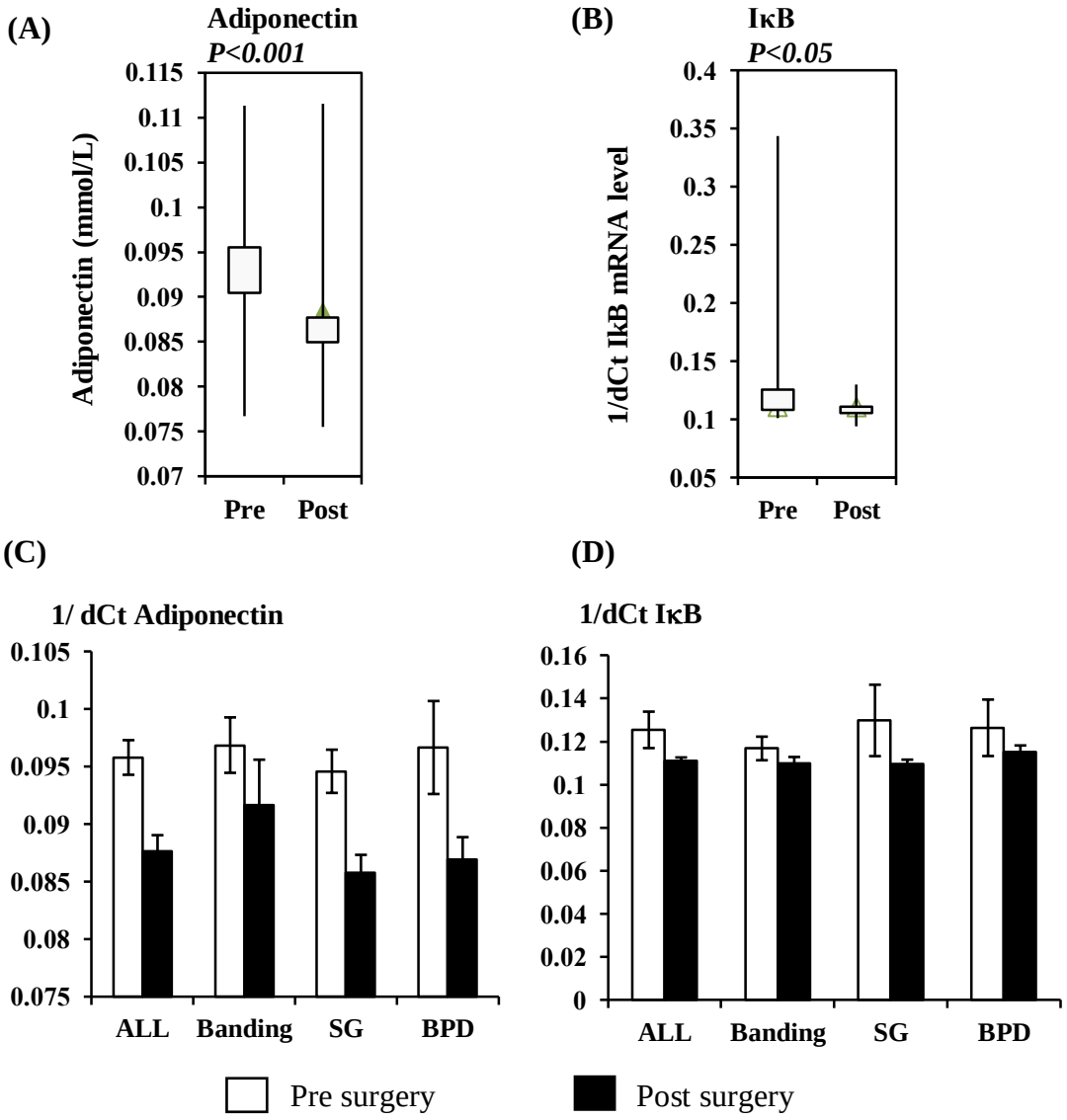

Fig 6.3.2.3. The comparison of (A) Adiponectin expression and (B) IκB mRNA levels pre versus post surgery. Sub-division of (C) Adiponectin mRNA and (D) IκB mRNA by surgery type. Data are represented as 1/normalized expression (target protein intensity normalized to β actin in the same sample target gene or Ct value normalized to 18s gene in the same sample).

6.3.3 Independent correlation of TLR4 expression and NFκB signalling molecules in AT post-surgery.

There were no observed correlations between the expression of TLR4, IL6, NFκB and TRAF6 in AT either pre- or post-surgery (Fig 6.3.3.1). Strong correlation between TLR4 and TLR2 were observed in both pre- and post-surgery (Pre-surgery r=0.890, *P<0.001*; Post surgery r=0.866, *P<0.001*). However there was a negative correlation observed between NFκB and adiponectin prior to surgery (r= -0.567, *P=0.002*). In addition post-surgery, there was a positive correlation noted between TLR4 and adiponectin (r=0.545, *P=0.003*), IκB (r=0.529, *P=0.003*), the NFκB inhibitory factor and TLR2.

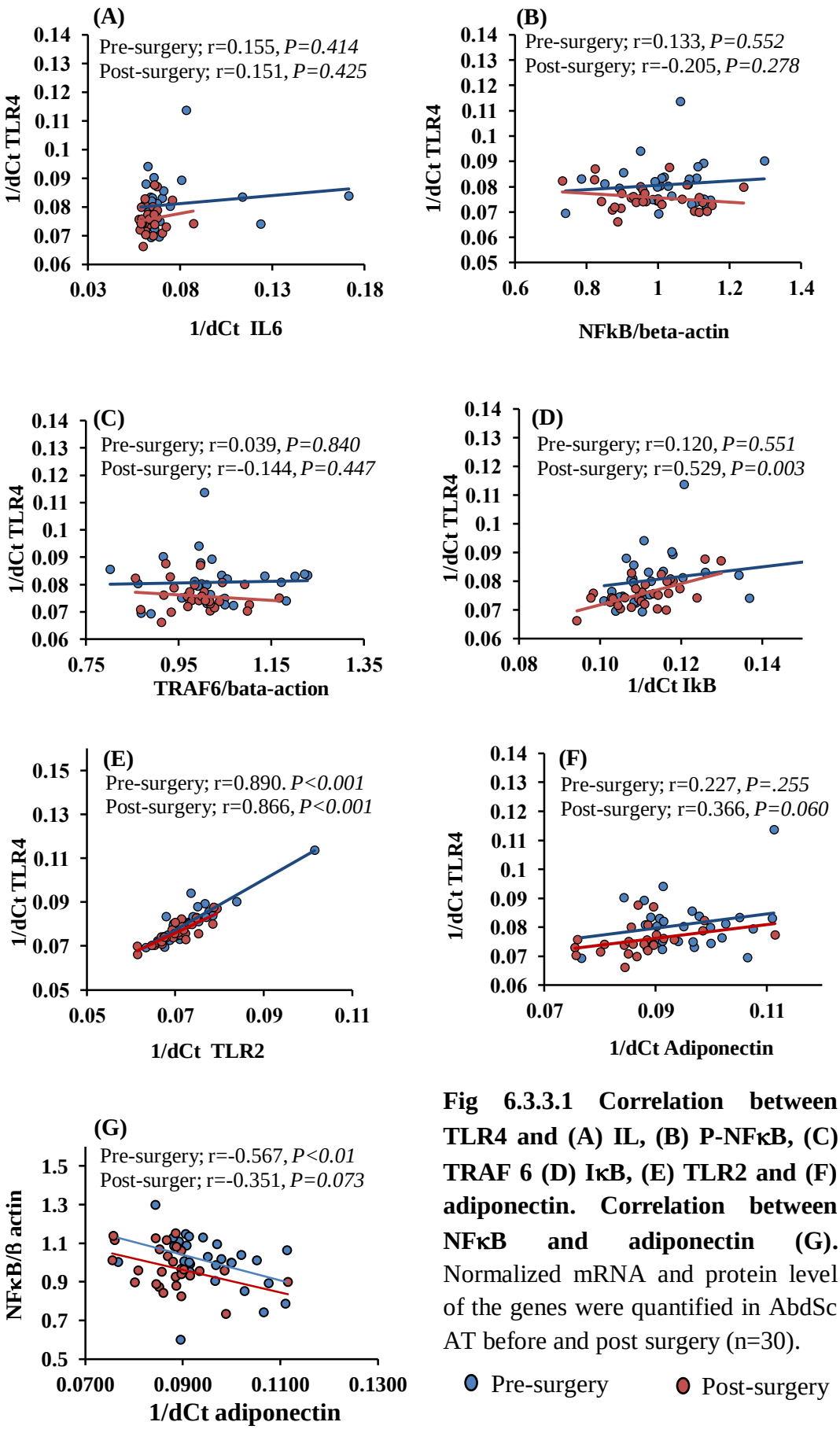

Fig 6.3.3.1 Correlation between TLR4 and (A) IL, (B) P-NFκB, (C) TRAF 6 (D) IκB, (E) TLR2 and (F) adiponectin. Correlation between NFκB and adiponectin (G). Normalized mRNA and protein level of the genes were quantified in AbdSc AT before and post surgery (n=30).

6.3.4 The associated changes in triglyceride levels and TLR4 mRNA reduction pre- versus post-surgery.

The triglyceride (TG) levels were split into quartiles, noting that when all subjects were grouped together, the third quartile (Q3) showed the greatest drop of serum TG (55.5% reduction post-surgery (*P<0.001*)) which was also associated with a significant reduction in TLR4 mRNA expression (*P<0.001*; Fig 6.3.4.1).

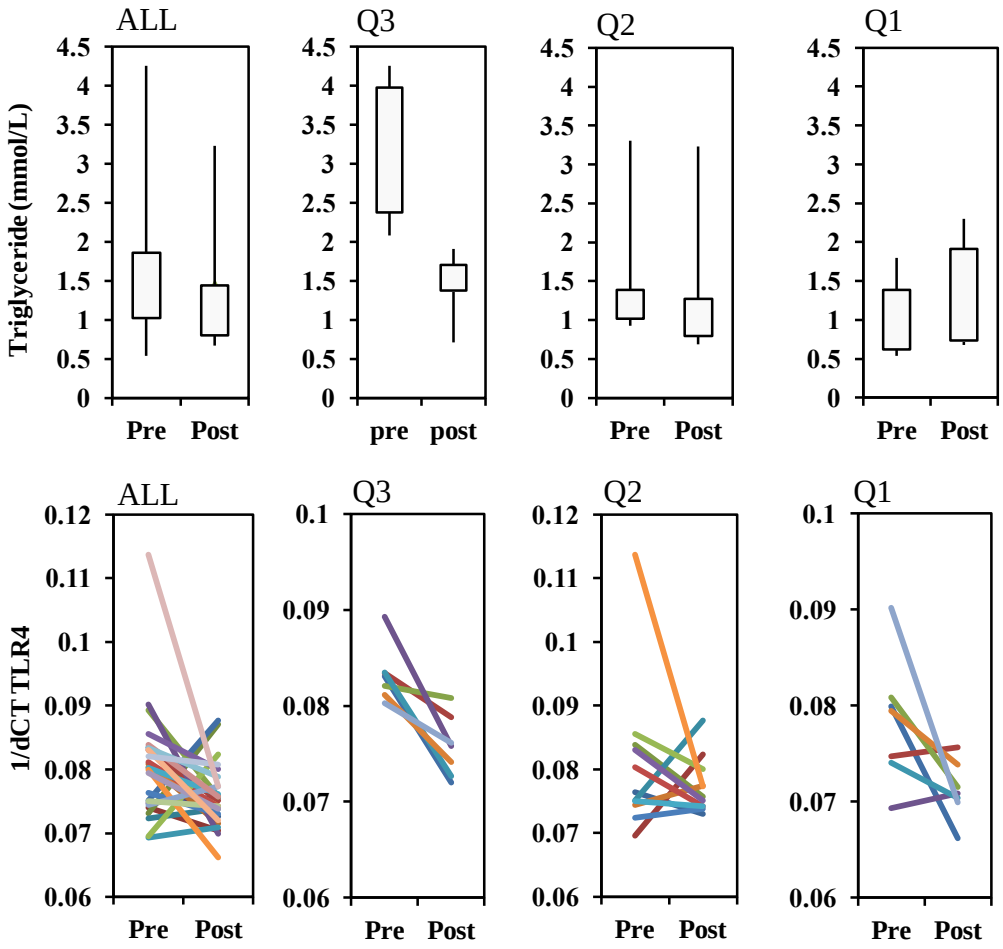

Fig 6.3.4.1 Changes in mRNA level of TLR4 in AbdSc AT and serum triglyceride. mRNA level of TLR4 data are represented as 1/ΔdCt. Delta change in triglyceride level (serum triglyceride before surgery – serum triglyceride after surgery) of each subject was calculated. Sub-groups were divided into quartiles according to delta changes in triglyceride level. First quartile (Q1) was the lowest 25% of serum triglyceride delta change while the third (Q3) was the highest 25% of triglyceride delta change. Second quartile was the delta changes that were in between Q3 and Q1.

6.3.5 Triglyceride rich lipoprotein, VLDL, induced TLR4 expression in differentiated human adipocytes.

The differentiated human adipocyte cell line, Chub S7, were treated with triglyceride rich lipoprotein or VLDL over a 24 hr period. The effect of these lipids on TLR4 mRNA expression highlighted that there was a dose dependent increase in TLR4 mRNA expression (Figure 6.3.5.1).

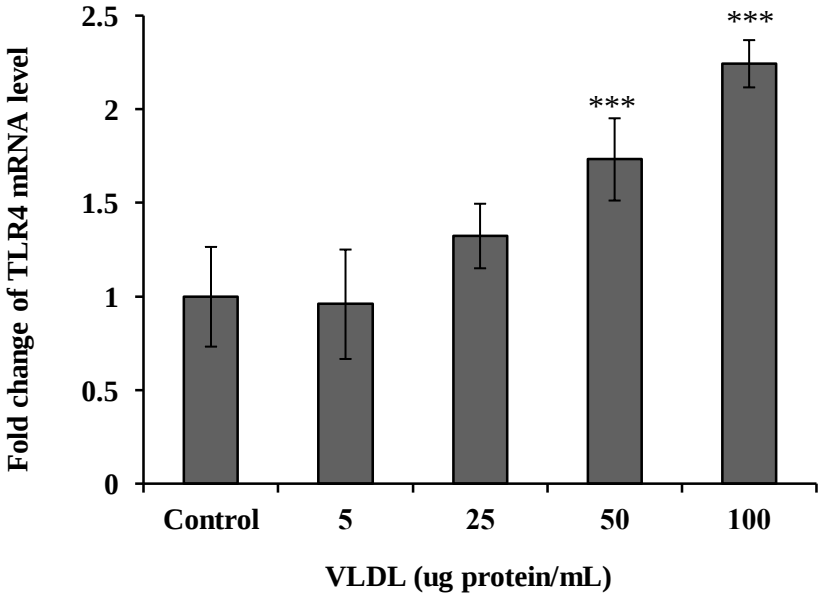

Figure 6.3.5.1. Effects of VLDL on TLR4 expression. Differentiated Chub-S7 adipocytes were treated with 0, 5, 25, 50 and 100 ug/mL for 24 hr. Phosphate buffer saline containing 0.34 mM EDTA was used as control. The TLR4 mRNA level was measured by RT-PCR. The data were normalized to 18S mRNA level. The difference in expression was assessed by two way ANOVA test; ***, *P<0.001*, each concentration vs. control cells)

6.4 Discussion

In this study, the improvement of fasting blood glucose, insulin, triglyceride, total cholesterol and LDL cholesterol post-bariatric surgery were observed. Although the degree of reduction did not appear to relate to the surgical intervention used. Whilst weight loss was clearly observed over time due to the bariatric surgery, all patients still remained classed as obese although there was metabolic improvements and significant changes noted in adipose tissue inflammation. As such this study cohort provided a unique model to study the molecules or pathways that might be involved in resolving inflammation in adipose tissue which may be coupled with systemic metabolic improvement independent of significant weight loss.

The similar findings also showed the improvement of metabolic and inflammatory state in patients was surgery type independence (Fenske et al., 2013). However, laparoscopic Roux-en-Y gastric bypass has been recently reported higher improvement of BMI, circulation insulin and total cholesterol compare to laparoscopic sleeve gastrectomy after one year surgery (Iannelli et al., 2013). After 2 years surgery, patients underwent Roux-en-Y gastric bypass showed more fat loss than patients who underwent gastric banding (Trakhtenbroit et al.). Differences in the findings of the effects of surgery type on BMI and metabolic markers could be contributed to weight loss, differences of baseline before surgery and duration after surgery. However, bariatric surgery clearly provides benefits to obese T2DM.

Expression of TLRs in adipocyte has been reported (Brenner et al., 2012; Poulain-Godefroy et al., 2010). The TLRs signaling cascade is triggered by binding complexes between TLRs and its ligands, such as lipopolysaccarides and fatty acid. The downstream of activation is mainly through the NFκB pathway which under

normal condition is inhibited by un-phosphorylated IκB. Pro inflammation cytokines such as TNFα can bind to its receptor and consequently phophorylate IκB into the active form which release NFκB. The active nuclear transcription factor, NFκB, then translocate into nuclease and stimulate pro-inflammatory cytokines expression (Fresno, Alvarez, & Cuesta, 2011). Expanding adipose tissue induced by high-fat diet induced the expression TLRs, resulting in activation of NFκB and MyD88 in mouse model (S. J. Kim et al., 2012). In this study, the expression of TLRs and NFκB signaling molecules in AbdSc AT taken from obese T2DM human subjects were evaluated. The reduction of pro-inflammatory makers, TLR4, TLR2, IL-6 and NFκB were observed after 6 months surgery, indicating the decline of inflammation in adipose tissue. However, there was no correlation between TLR4 expression and NFκB signalling molecules such as TRAF6 and NFκB itself, suggesting that NFκB was regulated by others pathway such as MAPK which mainly involved in insulin resistance signalling pathway.

Surprisingly, the reduction of anti-inflammatory markers such as adiponectin, IκB and IL-10, expression in adipose tissue were also observed. In contrast, previous studies showed the increase of circulating adiponectin after surgery. As mentioned above, there were some differences in weight loss, duration after surgery and patient characteristic baseline. The reduction of anti-inflammatory mediated molecules could be interrelated with the regulation of inflammatory response. In lean, adiponectin showed the positive correlation with inflammatory cytokines such as IL-6 and TNFα in order to protect the cells from uncontrollable inflammation, whilst, in obese T2DM, adiponectin was reduced and some studies showed the negative correlation with those inflammatory cytokines, indicating the uncontrollable inflammation in

obese T2DM. In this study, the negative correlation between adiponectin and NFκB was observed before surgery but after weight loss induced by bariatric surgery, there was no association between adiponectin and NFκB, demonstrating that adipose tissue become recover from uncontrollable inflammation. The further experiments studying the long term effects of bariatric surgery and kinetic changes of inflammation are required to verify the conclusion.

Because fatty acid can bind to TLR4, it is interesting to study how the reduction of lipid profiles after surgery associate with TLR4 in adipose tissue. In vitro study, primary human adipocyte treated with saturated fatty acid and high glucose increased the protein level of TLR4, indicating that TLR4 play at least in part of a long term inflammation in adipose tissue.

Thus, delta changes between pre- and post-surgery of serum triglyceride of each subject were calculated and put the data in order. Subjects were divided into three qualities according how well serum triglyceride was reduced. All subjects in the 3th quartile which were the first 25% of subjects whom circulating triglyride were reduced better than the others 75% of subjects showed the reduction of TLR4 expression, indicating that triglyceride was the factor that influenced TLR4 expression in adipose tissue.

Moreover, human differentiated adipocyte cells were treated with different concentration of triglyceride rich lipoprotein, VLDL. VLDL was used in this experiment because of limitation of triglyceride solubility and its mimic to the majority form of triglyceride in circulation. The expression of TLR4 was induced by VLDL in a dose dependent manner, confirming the in vivo finding.

Taken together, there is a reduction in AT inflammation as denoted by TLR and NFκB expression. The reduction in AT inflammation appears dependent on how successfully subjects reduce their serum triglyceride, which is supported by *in vitro* studies. These studies suggest that bariatric surgery lead to metabolic improvement with weight loss, whilst dietary intervention is still required to ensure triglyceride to reduce inflammation.

CHAPTER 7 : Discussion

7.1. Discussion

Whilst it is clear that increasing adiposity contributes to increased health risk our understanding of the multiple mechanisms where by adipose dysfunction can contribute to the increased risk of T2DM and CVD remains in its infancy. Part of the challenge to determine the mediators of this disease risk is the multiple factors that appear to be involved at any one time that may contribute to this risk, either from dietary sources, systemic patient profiles and how adipose tissue may function within this complex environment. Recent studies have highlighted that beyond glycaemic control, subjects with T2DM and CVD appear to be exposed to long-term sub-clinical inflammation which appears to exacerbate their metabolic condition over time. As such this thesis sought to evaluate the potential mediators of sub-clinical inflammation, principally focusing on the role of systemic lipids and the mechanisms of sub-clinical inflammation derived from adipose tissue and the adipocyte.

This thesis therefore examined the role of lipoprotein-associated phospholipase A2 (LpPLA2) a member of the phospholipase A2 super family of enzymes and its isoforms which has been shown to generate Ox-LDL, other pro-inflammatory mediators, such as lysophosphatidylcholine and oxidised fatty acids (Rosenson & Stafforini, 2012; Toth et al., 2010; Zalewski et al., 2006). The rationale behind this arose as LpPLA2 derived from macrophages and foam cells and has been shown to be up-regulated in conditions of inflammation such as CVD (Khakpour & Frishman, 2009; Kinney et al., 2011; Oei et al., 2005; Winkler et al., 2007), whilst studies on LpPLA2 and isoforms in adipocytes were absent, despite their clear similarities between the two cell types. The studies in this thesis principally highlighted that there was an association between circulating Ox-LDL and LpPLA2

which appeared to be related to adiposity and diabetic status. Further *in vitro* findings determined that LpPLA2 appeared to support the interconversion of LDL into Ox-LDL through the use of the adipocyte, since blockade of LpPLA2 signalling within the adipocyte partially blocked Ox-LDL production. Clinically this is relevant as previous studies have shown that association between Ox-LDL production and weight gain but the mechanisms of LDL modification in foam cells remain unclear (Maria Waegner et al., 2011; Nelson et al., 2012; Vickers et al., 2009; Wang et al., 2010; Yang et al., 2010). These studies however highlighted, in a controlled *in vitro* system, that the adipocyte could act as another source of Ox-LDL production, suggesting LpPLA2 could be targeted in adipose tissue to reduce systemic Ox-LDL production. Clearly, recent studies with the LpPLA2 inhibitor, Darapladib, have shown that this medication be an effective treatment for lowering CAD disease in unstable foam cell plaques (Johnson et al, 2014; Dave et al, 2014). As such the inhibitor may therefore be effective for targeting adipose tissue due to the similarities between cell types. Further it appears LpPLA2 enzymatic activity is critical for eliciting macrophage responses associated with a variety of inflammatory processes and signalling pathways such as c-Jun-kinase (JNK) so many have other downstream effects (Tzang et al, 2009).

Interestingly serum LpPLA2 had a number of positive correlation with cholesterol, triglycerides, LDL and Ox-LDL as might be anticipated but previous studies had apparently never examined the adipocyte as an active source of LpPLA2 signalling with the LpPLA2 protein expression raised in conditions of T2DM and may actively contribute to the Ox-LDL observed in *in vivo* studies, with recent studies confirming this and LpPLA2 acting as a potential predictive biomarker of T2DM risk (Maria Waegner et al., 2011; Nelson et al., 2012). In fact the current

thesis studies identified that despite controlling for BMI the positive correlation of circulating LpPLA2 with cholesterol, LDL or Ox-LDL across the combined cohort, or within the T2DM cohort was maintained suggesting the systemic lipid profile appears important (Sanchez-Quesada et al., 2012).

To examine the potential impact of lipids beyond the confines of LpPLA2 subsequent studies were undertaken to examine the relationship between systemic lipids and their association on the innate immune cascade in AT taken from patients before and following bariatric surgery. These studies highlighted that weight reduction had a significant impact on reducing the innate immune intracellular signalling cascade as well as systemic metabolic improvement which appeared to be irrespective of surgical intervention used which a previous *in vivo* study had noted as well (Fenske et al., 2013). However the success in reducing the subject's inflammation, as noted via assessing the key receptor of the innate immune pathway, the TLRs, appeared to be linked to how their systemic triglyceride levels dropped post-surgery as well. Furthermore additional *in vitro* studies identified the inflammatory nature of systemic lipids on the adipocyte, again highlighting the importance of the cell in sub-clinical inflammation.

Taken together these current *in vivo* and *in vitro* studies highlight the importance of systemic lipids to potentially influence local adipocyte function and regulation. Further that whilst human studies are critical to determine the mediators of increased health risk this needs to be coupled with both longitudinal studies and *in vitro* analysis to identify potential therapeutic targets as well as lifestyle intervention targets, particularly in reference to systemic lipids.

7.2 Limitations

Some of the details of the limitations have in part been discussed in the relevant chapters however it is important to note that all the subjects that took part in these studies were women. Whilst this could be viewed as strength and would have reduced the potential confounding factors that could arise through assessing two genders, within the same sample size obtained, it also means the analysis may only be applicable to women. Women were chosen for several reasons, including high attendance at clinical and follow up, willingness to engage more effectively with clinical research studies, willingness to have fat biopsies taken either during surgery or through needle biopsy post-surgery. Subsequent studies could repeat the current studies in men, which would potentially highlight similar findings but show men have a higher inflammatory risk which is coupled to their raised metabolic risk compared to women.

Human adipose tissue appears critical in the arising systemic inflammatory response and the inflammatory gene expression clearly changed over a 6 month period when assessing the subjects undergoing bariatric surgery. However a limitation in this study was that there were no adipose tissue biopsies taken within the first few days post-surgery, when the subjects appeared to reverse their T2DM status and improve their metabolic health. It would have been interesting to determine whether this lead to a significant reduction in the inflammatory response from adipose tissue and whether the type of surgery influenced that response. Also it would been interesting to increase the cohort size to try to evaluate the influence of medication pre and post-surgery on the subjects undergoing bariatric surgery but due to small sample size there was not attainable. Nevertheless, these current studies

have given a valuable insight into the role of lipids and the function of adipose tissue in understanding the inflammatory response in metabolic disease.

7.3. Future directions

This thesis shows the first evidence identifying that the mediators of sub-clinical inflammation such as systemic lipids can activate multiple concurrent mechanisms to increase metabolic disease risk. Further analysis of LpPLA2 and its isoforms in the peripheral metabolic and inflammatory pathways may also ascertain how these enzymes may change their functions to be more pro-anti-inflammatory and when and how this may occur. As such further studies would need to address how knock-down of LpPLA2 and its isoforms may affect adipocyte cell function and production of both pro- and anti-inflammatory cytokines. Also the current clinical use of an LpPLA2 inhibitor in CAD provides potential opportunities to understand the influence of such a drug on Ox-LDL production derived from adipose tissue which may also support to lower CAD and T2DM risk. The use of studies to examine the arteriovenous differences across human adipose from subjects in different metabolic states, with and without an LpPLA2 inhibitor may allow the assessment of how Ox-LDL and other pro-inflammatory cytokines may reduce with such an intervention. This may further highlight the potential *in vivo* importance of an LpPLA2 inhibitor on adipose tissue.

Further future studies could also explore other lipid factors on adipose tissue metabolism to identify how these factors may influence the innate immune cascade. Specifically studies undertaking low fat diet intervention studies coupled with AT biopsies measurement pre- and post-intervention to ascertain what impact this may make on systemic lipids as well as the inflammatory status of the adipocyte *ex vivo*

and *in vitro*. The *in vitro* studies could study how pre-and post-intervention the adipocyte handles various factors such as TG, FFAs, LDL, HDL and VLDL to see whether a suitable weight loss diet can change the potentially pathogenic nature of the adipocyte observed in obese conditions.

7.4. Conclusion

These studies have highlighted that the role and function of the adipocyte is complex and that there are many features and mechanisms of the adipocyte still to be explored. Despite the apparent lipid storage capacity of the adipocyte the adipocyte is a complex cell with multiple interactions vital to human physiology which makes adipocyte dysfunction in obese and diabetic conditions more problematic to resolve. However these current studies have shown the vital importance of systemic lipids control to reduce the pathogenic nature of the adipocyte. Furthermore unless substantial weight loss drugs can be devised it will be important to ensure that in metabolic disease systemic lipids profiles are actively managed to reduce further complications that may be derived due to the inflammatory response of the adipocyte.

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APPENDIX

Solution and Buffers

A.1 General cell culture solutions

A.1.1 Lysis buffer

Ammonium Chloride (NH_4Cl)	0.154	mol/L
Potassium Bicarbonate ($KHCO_3$)	10	mmol.L
Sterile water		

A.1.2 Collagenase

Collagenase powder	10	mg
Hank's Buffer Salt solution	50	mL
Pen/Strep	5	mL
Sterile water	445	mL

A1.3 Completed DMEM/F12 Phenol red free medium

DMFM/F12 Phenol red free medium	500	mL
Fetal Bovine Serum	50	mL
Pen/Strep	5	mL
Transferin	1	mL

A2. Western Blot solution

A2.1 4% Sodium dodecyl sulphate (4% SDS)

SDS	4	g
Water	100	mL

A2.2 Loading Buffer

125 mM Tris-HCl, pH 6.8	625	µL
4% SDS	200	µL
Glycerol	1	mL
Dithiothreitol	100	µL
Bromphenol Blue	125	µL
Distilled water	250	µL

A2.3 Phosphate Buffer Saline

Na_2HPO_4	1.44	g
KH_2PO_4	0.24	g
NaCl	8.0	g
KCl	0.2	9
Sterile water	1000	mL
Adjuste pH to 7.6		

A2.4 PBS-T

PBS	1000	mL
Tween 20	1	mL

A2.5 Blocking reagent

I-block powder	0.2	g
PBS-T	100	mL

A2.6 4% stacking gel (for 2 gels preparation)

Distilled water	6.1	mL
Acrylamide	1.3	mL
Stacking buffer	2.5	mL
10% APS	50	µL
TEMED	10	µL

A 2.7 10% separating gel (for 2 gels preparation)

Distilled water	7.8	mL
Acrylamide	6.6	mL
Resolving buffer	5.2	mL
10%APS	200	µL
TEMED	20	µL

A 2.8 7.5% separating gel (for 2 gels preparation)

Distilled water	9.4	mL
Acrylamide	5.0	mL
Resolving buffer	5.2	mL
10% APS	100	µL
TEMED	20	µL

ABSTRACT LIST:

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