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EFFECTS OF SEX STEROID HORMONES ON ACYLATION STIMULATING PROTEIN (ASP) AND RELATED GENE EXPRESSION

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Thesis submitted to the University of Nottingham for the degree of doctor of Philosophy, July 2017

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ABSTRACT

Metabolic complications of obesity are mainly linked to body fat distribution. Since body fat distribution is sex-linked, understanding the role of sex hormones in body fat distribution and storage is important. The acylation stimulating protein (ASP) is a novel lipogenic factor that is suggested to be influenced by hormonal changes. The aim of the present study was to investigate the effect of sex steroid hormones on ASP production and related gene expression, by using adipose tissue explants isolated from ovariectomized rats and the 3T3-L1 cell line generated from mouse embryonic fibroblast cells.

In *ex vivo* studies, 3-way ANOVA analysis showed an effect of hormones, fat depots and chylomicrons interactions on ASP production (p=0.005). Further analysis demonstrated that progesterone and estrogen (P&E) treatment stimulated ASP production (p=0.006) in comparison to estrogen group in subcutaneous adipose tissue explants. In the presence of chylomicrons, visceral adipose tissue explants incubated with P&E showed increased ASP secretion by 54% (p=0.003). The same treatment led to a significant reduction of ASP concentration in subcutaneous explant media (32% lower, P=0.03). C5L2 protein expression were more affected by hormone and chylomicrons interaction (p=0.025). The expression of C5L2 protein increased significantly by P&E treatment (p=0.04) in comparison to the control group in subcutaneous tissue explants. Fat depots and chylomicrons interaction showed a significant effects on effect on ASP's precursors C3 (p=0.003), factor B (p=0.009) and adipsin (p=0.001) mRNA expressions. DGAT1 have shown to be influenced by hormone and chylomicrons interactions (p=0.3) with no interaction effect on LPL mRNA expression.
Using 3T3-L1 cells, high doses of progesterone in the presence of estrogen significantly induced ASP production (p< 0.001). C5L2 protein expression increased in dose-dependent manner with progesterone or estrogen treatments. Treatment of 3T3-L1 adipocyte cells with P&E and chylomicrons had no effect on ASP production. Estrogen receptors (ERs) blockade increased the gene expression of C5L2 and PLC significantly by 5-fold.

In summary, female steroid hormones (P&E) may regulate fat distribution through their effects on ASP and C5L2 in subcutaneous adipose tissue. Chylomicrons modulated the ASP responses to P&E treatment in this tissue. P&E interaction with ASP was proven to be regulated through their receptors (PR and ERs) in 3T3-L1 adipocytes. These results demonstrated an important role of female sex steroid in fat distribution and adipocyte lipid metabolism by mediating ASP- C5L2 pathway. Therefore ASP is an important lipogenic factor that, under normal female hormonal states, may reduce the metabolic health risk in women.

ACKNOWLEDGEMENTs

First of all I would like to express my utmost appreciation to my supervisors, Prof Andrew Salter and Prof Simon Langley-Evans for their supervision and guidance through the time of research and writing. Their well-known expertise in lipid metabolism and research has helped tremendously in completing this thesis. I am also very grateful for all the opportunities and support was provided through my study.

I also would like to express my deepest gratitude to my role model in science Dr. Jumana Saleh from Sultan Qaboos University for non-ending guidance and encouragement. Thank you for all the discussions, ideas and feedback, it has been always a great help and absolutely invaluable.

I would like to express my sincere gratitude to all the technicians in the Division of Nutritional Sciences; Cathy Wells, Zoe Daniel, Kirsty Jewell, Sarah Jones and special thanks to Christine Essex for their technical support and advice.

My special thanks to all my friends in the Division for their kind support and great time.

Finally, I would like to thank and dedicate this thesis to my amazing family, Sultan, Saja and Faisal for the love and support. And to my parents, brothers and sisters for their endless prayers throughout the duration of research work.

PUBLISHED ARTICLES AND PAPERS PRESENTED AT CONFERENCES

Published articles:

1. **Al Riyami B**., El-Tahir M., Al Maskari S., Johnson E.H.& Saleh J. (2014). Acute effects of exogenous hormone administration on postprandial acylation stimulating protein levels in ovariectomized rats after a fat load. Journal of Nutrition and Metabolism, 2014: 7.

2. Saleh J., Farhan H., Al-Saqri I, **Al-Riyami B**., & Cianflone K. (2013).Plasma gamma-glutamyltransferase is strongly determined by acylation stimulating protein levels independent of insulin resistance in patients with acute coronary syndrome. Disease Markers 35 (13):155–161

Papers Presented at Conferences:

1. Saleh J., Farhan H., Al-Saqri I. , **Al-Riyami B**. & Cianflone K. (2013). Plasma Gamma-Glutamyltransferase is strongly determined by Acylation Stimulating Protein levels independent of Insulin Resistance in patients with Acute Coronary Syndrome. International Cardiology Symposium, Dubai, UAE, May 18-16 /2013.

2. **Al Riyami B**., Langly-Evan S., Salter A. &Saleh J. (2014). The Effect of sex hormones on acylation stimulating protein (ASP) production and its precursors, and related gene expressions in ovariectomized rat adipose tissue explant culture. 3rd Postgraduate conference, Society of Nutrition, Nottingham, UK, September 1-2/2014.

3. **Al Riyami B**., El-Tahir M, Al Maskari S., Johnson E.H.& Saleh J.(2014). Differential effects of reproductive hormone treatments on postprandial Acylation Stimulating Protein levels in ovariectomized rats after a fat load. The 2nd world congress of CLINICAL LIPIDOLOGY, Vienna, Austria,5-7 December 2014.

4. **B. Al Riyami**, S. Langley Evan and A. Salter (2015). The influence of sex hormones on ASP production and C5L2 and adipogenesis related gene expressions in ovariectomized rats ex vivo adipose tissue explant culture. Summer meeting, 6- 9 July 2015, The future of animal products in the human diet: Health and environmental concerns. Proceedings of the Nutrition Society 74(OCES), E281.

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LIST OF ABBREVIATION

AC	Adenylate Cyclase
ADRA2A	Adrenoceptor Alpha1 2A
Akt	Serine/Threonine Kinases
AMP Kinase	Adenosine Monophosphate-Activated Protein Kinase
ANG-II	Angiotensin II
ANP	Atrial Natriuretic Peptide
AR	Androgen Receptors
ASP	Acylation Stimulating Protein
AT	Adipose Tissue
ATGL	Adipose Triglyceride Lipase
BAT	Brown Adipose Tissue
BMI	Body Mass Index
BMR	Basal Metabolic Rate
BNP	Brain Natriuretic Peptide
BSA	Bovine Serum Albumin
C3adesArg	Complement 3a Desarginase
cAMP	Adenosine monophosphate
cDNA	Complementary DNA
CHD	Coronary Heart Disease
CHD	Coronary Heart Disease
CHO	Carbohydrates
CNS	Central Nervous System
CpB	Carboxypeptidase B
CRP	C-reactive Protein
CuSO4	Copper Sulfate
CYP11A1	Cholesterol Desmolase
CYP17A1	17α-hydroxylase/17,20 Lyase
CYP19A1	Aromatase
DAG	Diacylglycerol
DTT	Dithiothreitol
DGAT	Diacylglycerol Acyltransferase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immuno Sorbent Assay
ER	Estrogen Receptor
Factor D	Adipsin

FFA	Free Fatty Acid
FGF	Fibroblast Growth Factor
FIAF	Fasting-Induced Adipose Factor
GH	Growth Hormone
GLUT	Glucose Transporter
GP	G-Protein;
GPCRs	G-protein Coupled Receptor Receptors
GTT	Gamma-glutamyltransferase
HBSS	Hank's Balanced Salt Solution
HDL-C	High Density Lipoprotein Cholesterol
HEPES	4-(2-Hydroxyethyl)Piperazineethanesulfonic Acid
HSD17B1	17 β -Hydroxysteroid Dehydrogenase1
HSD3B	3β-Hydroxysteroid Dehydrogenase
HSL	Hormone Sensitive Lipoprotein Lipase
IDDM	Insulin Dependent Diabetes Mellitus
IGF-1	Insulin-like Growth Factor-1
IGFBP	Insulin-Like Growth Factor-Binding Protein
IL-6	Interleukin-6
IP3	Inositol 1,4,5-Trisphosphate
kDa	KiloDalton
KNa	Potassium Sodium
LDL-C	Low density Lipoprotein Cholesterol
LPL	Lipoprotein Lipase
MAPK	Mitogen Activated Protein Kinase
MC4R	Melanocortin-4-receptor
MCO-1	Chemoattractant Protein-1
mRNA	Messenger Ribonucleic Acid
NAFLD	Non-Alcoholic Fatty Liver Diseases
NaOH	Sodium Hydroxide
NCBI	National Center for Biotechnology Information
NEFA	Non Esterified Fatty Acid
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffered Saline
PCOS	Polycystic Ovarian Syndrome
PDK-1	3-Phosphoinositide-Dependent Protein Kinase-1
PI3K	Phosphoinositide-3-Kinase
PIP	Phosphatidylinositol 4,5-Bisphosphate
PIP2	Phosphatidylinositol 4,5-Biphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLA2	Phospholipase A2

PLC	Phospholipase C
PR	Progesterone Receptor
RA	Retinoic Acid
RAR	Retinoic Acid receptor
RMR	Resting Metabolic Rate
RXR	Retinoid X receptor
SRDFA2	Steroid -5-α-Reductase, αpolypeptide 2
SDS	Sodium Dodecyl Sulfate
SSH	Sex Steroid Hormones
SSHMR	Sex Steroid Hormone Membrane Receptor
SSHR	Sex Steroid Hormone Receptor
SSRE	Sex Steroid Response Element
TBS	Tris Buffered Saline
TG	Triglycerides
TGF β	Transforming Growth Factor Beta
TGFβ	Transforming Growth Factor β
TNF-α	Tumor Necrosis Factor-α
TTR	Transthyretin
Type 2 DM	Type 2 Diabetes Mellitus
UV	Ultraviolet
VLDL	Very Low-Density Lipoproteins
WAT	White Adipose Tissue
WHO	World Health Organization
20α-HSD	20α-Hydroxysteroid Dehydrogenase
3β -HSD	3β-Hydroxysteroid Hydrogenase

CHAPTER 1. INTRODUCTION

1.1 Obesity

Obesity is a growing global chronic condition that is present in both developed and developing countries. Obesity is a result of the interaction between genetic and environmental factors influencing a positive energy imbalance and excessive accumulation of fat in adipose tissue (Maes et al., 1997, Faith and Kral, 2006). Energy balance is a fundamental principle based on energy intake and energy expenditure. This definition helps to understand the development of obesity, which results from excess energy (when intake exceeds output) being stored as adipose tissue. In humans, the energy intake represents that consumed as food and drink and metabolized in the body. Energy expenditure is composed of the resting metabolism rate (RMR) and basal metabolic rate (BMR) which is the amount of energy required to fuel the body during rest, thermoregulation, and to sustain all vital functions and metabolic and degradative processes in the body. In addition, it includes the energy expended for digesting, absorbing and metabolizing food (diet-induced thermogenesis); and the energy needed during physical activity (Schoeller, 2009, Hill et al., 2012).

In 2014, The World Health Organization (WHO) estimated that there are more than 1.9 billion individuals whom are overweight and of these over 600 million classified as obese (WHO, 2016). Moreover, overweight and obesity are predicted to increase, with approximately 3.3 billion people being classified as such by 2030 with an expected economic burden of associated health care costs (Wei et

al., 2015). The international classification of weight by the World Health organization (WHO) is based on the body mass index (BMI) which is calculated by dividing the weight in kilograms per height meters squared (kg/m^2). It is proposed that BMI between 25 and 29.9kg/m^2 is considered to be overweight and a BMI greater than 30 kg/m^2 is defined as obesity (Keys et al., 1972), which is sub-classified into three levels as shown in **Table 1.1**.

Table 1.1 BMI weight classification proposed by WHO

Classification	BMI (kg/m^2)
Underweight	< 18.5
Normal weight	1805 -24.9
Overweight	≥ 25
Pre-obese	25-29.9
Obese I	30-34.9
Obese II	35-39.9
Obese III	≥ 40

1.1.1 Factors associated with obesity

Obesity is the result of a positive energy balance resulting from a complex interaction between various factors including genetic, environmental, physiological, social and economic. The overconsumption of high caloric food and refined carbohydrates has contributed to the increasing rate of the obesity and diabetes (Gross et al., 2004). Such energy-dense food is inexpensive and the

increased availability of this type of food increases the daily caloric intake. Eating disorders and other psychiatric disorders also increase the risk of obesity (Haines and Neumark-Sztainer, 2006). A low physical activity level is another important factor in developing obesity. In contemporary society, people are more engaged with technology and less likely to engage in physical activity and, as a consequence, less energy is expended by such individuals (Andersen et al., 1998).

Genetic factors have been suggested to contribute 45-75% of intra-individual variability in adiposity (Stunkard et al., 1986, Farooqi and O'Rahilly, 2007). These factors are likely to act through range of different mechanisms including regulation of energy balance, appetite regulation, nutrient absorption and distribution of fat and lean tissues (Farooqi and O'Rahilly, 2007). Attempts to identify specific 'obesity genes' have only recognized small number of monogenic defects. Mutations leading to leptin deficiency were some of the first to be discovered to increase obesity at an early age, by increasing appetite and therefore increasing body weight because of the excess energy balance (Farooqi et al., 2002). The central melanocortinergic pathways have also shown to play an important role in energy homeostasis (Yeo et al., 2003). One of the known receptors for melanocortin peptide; melanocortin-4-receptor (MC4R), has been shown to regulate appetite and body weight (Huszar et al., 1997). Yeo et al., reported that mutation in human MC4R triggers early onset of obesity representing the most common known cause of human obesity (Yeo et al., 1998). Hormonal dysregulation including hypothyroidism, growth hormone deficiency, Cushing's syndrome and polycystic ovary syndrome have also been shown to

increase body weight (Sjostrand and Eriksson, 2009). Some medications can induce weight gain including some diabetic treatments, steroid hormones, oral contraceptives, antihistamines and protease inhibitors (Rezvani, 2014).

1.1.2 Complications associated with obesity

The development of obesity has been associated with several serious chronic diseases including metabolic alterations affecting blood pressure, lipid profile, insulin resistance and other hormonal changes (Mitchell et al., 2011, Weisberg et al., 2003). A WHO report in 2002 indicated that 21% of ischemic heart disease, 58% of diabetes and 8-42% of cancers were attributed to a BMI above 25 kg/m^2 (WHO., 2002). Furthermore, a range of inflammatory mediators are produced in obesity, which increase the systemic inflammation that is associated with other diseases including osteoarthritis and rheumatoid arthritis (Balistreri et al., 2010).

Increasing evidence of the association between obesity and the metabolic and inflammatory complications has led to more investigations of the role of the adipose tissue as an active contributor to the development of pathological and physiological processes of these diseases. A summary of obesity factors and associated complications is presented in **Figure 1.1.**

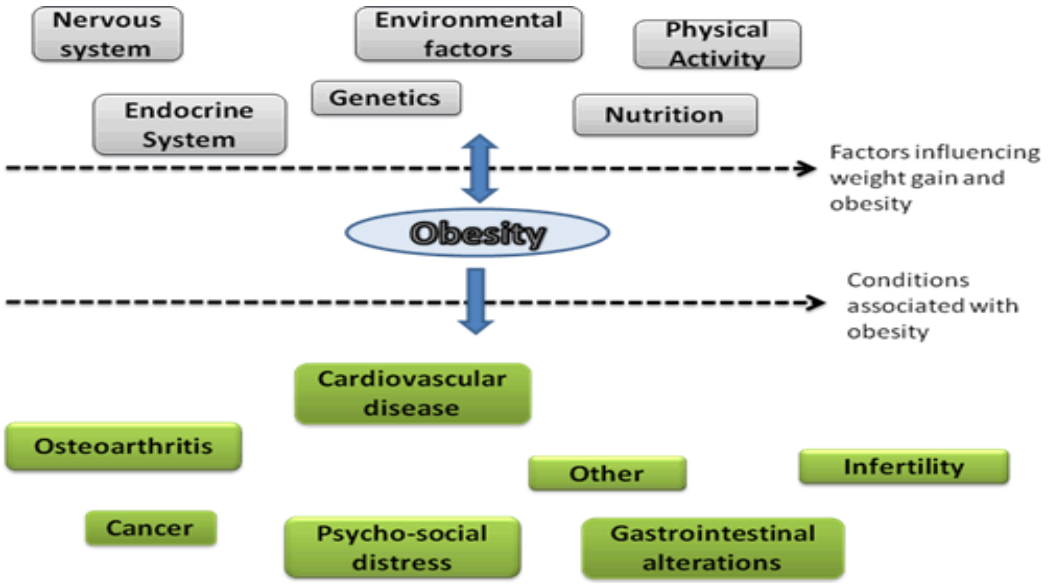

Figure 1.1 Factors influencing weight gain and obesity associated complications.

1.2 Adipose tissue

Obesity is characterized by the expansion of adipose tissue due to the accumulation of lipids in adipocytes (DiGirolamo et al., 1998). In an average, lean weight adult, adipose tissue constitutes between 10 to 20% of total body mass in males and 15-25% in females (Klaus, 2001). Adipose tissue is a specialized connective tissue that mainly consists of mature adipocytes and vascular stromal fractions of endothelial cells, fibroblasts, macrophages and preadipocytes which contribute to the structural integrity of the adipose tissue (Otto and Lane, 2005). Through the progression from the lean to obese state, adipose tissue starts to accumulate lipids in mature adipocytes which expand in size and leads to hypertrophy. In addition, adipocyte cell number increases through the differentiation of pre-adipocyte cells within adipose tissue, which enables hyperplastic expansion of adipose tissue in an attempt to cope with the increased

demand for triglyceride storage (Halberg et al., 2008). Adipose tissue is made of approximately 85% of lipid, and of that about 90-99% comprises triacylglycerols. The remaining weight of adipose tissue is water and protein (Albright and Stern, 1998). There are two types of adipose tissue present in mammals; white adipose tissue (WAT) and brown adipose tissue (BAT) that exhibit different functions, compositions and locations. BAT maintains body temperature and produces heat, whereas WAT is the most common and is mainly associated with energy storage and lipid mobilization (Gesta et al., 2007).

WAT is composed of large spherical adipocytes supported by connective tissue. White adipocytes are usually unilocular, containing a single large lipid droplet. The nucleus and mitochondria are pushed against the plasma membrane giving the cell a 'ring shape' (**Figure 1.2**). Adipocytes vary between 30 and 130 μm in diameter and the larger the cell size, the higher its metabolic activity and secretory functions (Wronska and Kmiec, 2012).

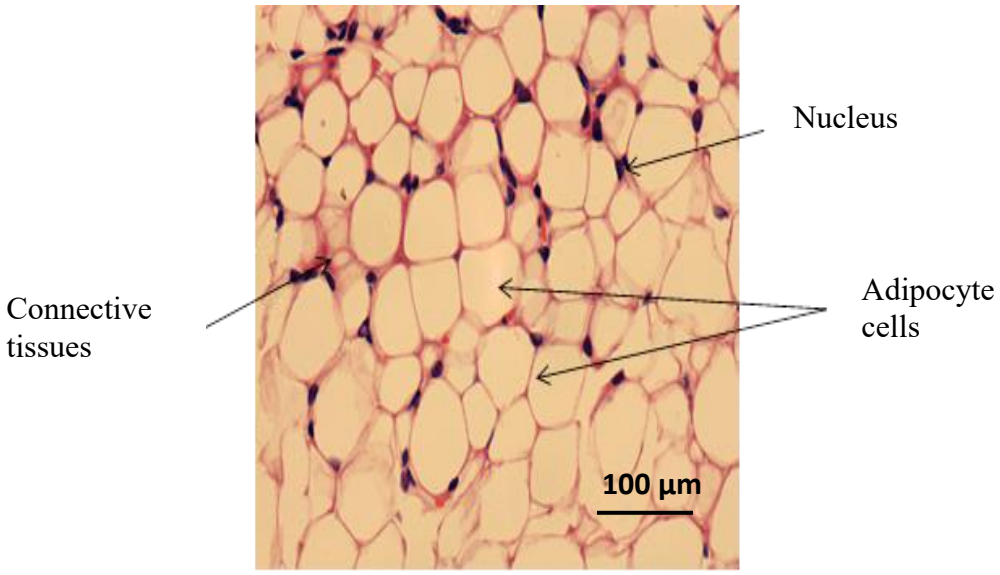

Figure 1.2 Histology slide of white adipose tissue (original magnification x100) showing mature adipocyte cells, nucleus and the connective tissues.

1.2.1 Regulation of fat storage

While the primary function of adipose tissue is as a fat storage reservoir, more recent research has also identified it as an active endocrine organ. Fat accumulation is determined by the balance between its synthesis (lipogenesis) and breakdown (lipolysis). In lipogenesis, free fatty acids (FFA) are esterified with glycerol to form triacylglycerol (TAG) within adipocytes. Plasma TG is the most significant source of such fatty acids which are circulating as TG-rich lipoproteins (Frayn et al., 2006). Dietary fats enter the blood circulation in the form chylomicrons through the intestine. TGs are incorporated into adipocytes under the action of lipoprotein lipase (LPL); an enzyme expressed in the adipose tissue and bound to the vascular endothelial cells. It hydrolyses the TG and releases fatty acids which combine with coenzyme A to form a thioester and are then re-

esterified into TG in the adipocyte (Albright and Stern, 1998, Wronska and Kmiec, 2012). Fatty acids are incorporated into TG and released from the liver in the form of very low density lipoprotein (VLDL) which can also be taken up by adipocytes in the same way.

In addition, adipocytes have the capacity for converting excess dietary carbohydrates (CHO) into Triacylglycerol (*de novo* lipogenesis), which has been estimated to contribute up to 10% of adipose tissue TG when carbohydrate intake exceeds energy requirement (Strawford et al., 2004). Glucose entering the adipocyte through insulin-dependent transporters (GLUT) can be used to form both the glycerol backbone of TG and new fatty acids. Glycolysis occurs to produce glycerol -3-phoshate, which is then used as the glycerol backbone of TG molecules. In addition acetyl coenzyme A produced from the hydrolysis of glucose can be converted to malonyl coenzyme A by acetyl coenzyme A carboxylase and then into fatty acids through the action of fatty acid synthase. As such, fatty acids carried by very low-density lipoproteins (VLDL) from the liver, by chylomicrons from the intestine and those produced by *de novo* lipogenesis can all be esterified with glycerol -3-phosphate to form lipid droplets of TGs (Strawford et al., 2004).

Under increased demand for energy, fatty acids are released by the hydrolysis (lipolysis) of stored TG, that is mobilized via the action of adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) to release free fatty acid (FFA) into the plasma (Karastergiou et al., 2012). FFAs released from VLDL-TG (liberated from liver) represent important metabolic fuels for tissues such as

muscle during fasting and starvation (Frayn et al., 2006, Frayn, 2010) . The glycerol backbone of the stored TG is also released into the circulation and represents an important substrate for hepatic gluconeogenesis. Various hormones are involved in the regulation of adipocyte fat metabolism with insulin playing a central role. Insulin inhibits hormone sensitive lipase (HSL) and activates LPL, thereby stimulating FFA uptake and TG synthesis in adipocytes. Fat storage is also regulated by other hormones that exhibit opposite functions to insulin such as glucagon, glucocorticoids and catecholamines in the fed and fasted state, or under stress (Coelho et al., 2013). The regulation of fat storage and mobilization in adipose tissue is illustrated in **Figure 1.3.**

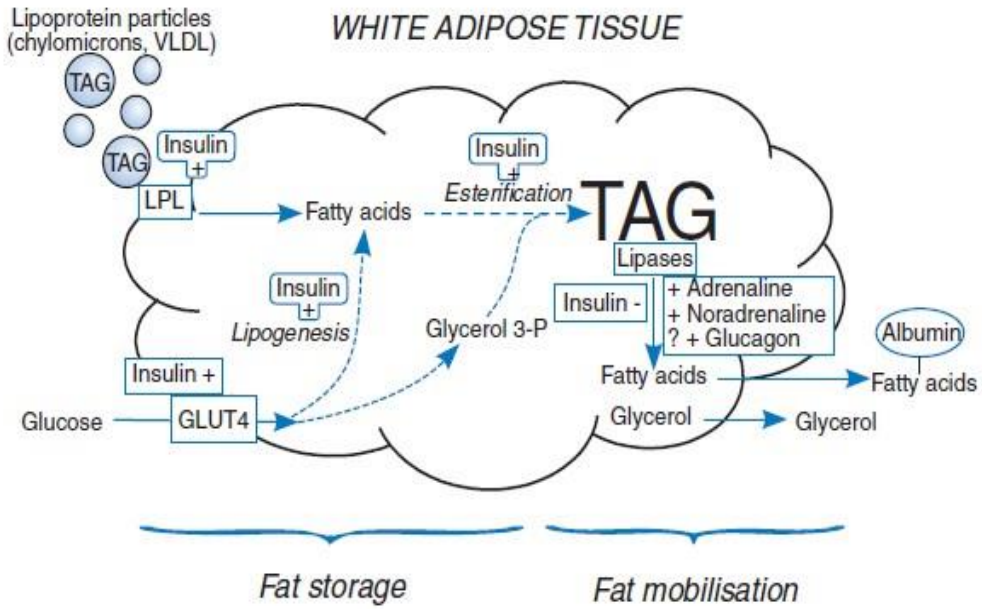

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Figure 1.3 Regulation of fat storage and mobilization in adipose tissue (Frayn, 2010).

1.2.2 Body fat distribution

In the 1950s, Vague was the first person to classify body fat distribution and to describe the upper body obesity (android–type) to be more associated with obesity complications than the lower body obesity (gynoid-type) (Vague, 1956).

The two major WAT depots are subcutaneous adipose tissue and visceral adipose tissue. Subcutaneous adipose tissue represents the most voluminous structure that stores approximately 80-90% of total body fat in the hypodermal layer of the skin (Karastergiou et al., 2012). Intra-abdominal (visceral) fat accounts for 6-20 % of total body fat and is localized within the abdominal cavity and mediastinum (Karastergiou et al., 2012). Visceral adipose accumulation generally increases the risk of adverse metabolic effects and cardiovascular diseases, compared to subcutaneous fat storage which is considered more protective (Jaffer et al., 2012). Visceral adipose tissue accumulation is associated with the intra-abdominal fat secretions, including inflammatory factors and fatty acids, that drain directly into the liver and systemic circulation in high concentrations (Kershaw and Flier, 2004). Visceral obesity (or as called abdominal obesity) can be measured by waist circumference and has been found to be a strong predictor of insulin resistance (Karter et al., 2005). This notion of a correlation between visceral fat and the alteration of glucose-insulin homeostasis has been shown to be more severe in overweight and obesity, independent subcutaneous adipose tissue (Ross et al., 2002). Cardiovascular disease metabolic risk profile (such as dyslipidemia, glucose tolerance, and blood pressure) also associates with increased deposition of visceral abdominal fat (Sironi et al., 2012). Many studies have shown that

obesity related to increased subcutaneous fat causes less adverse effect in patients metabolic profiles when compared to visceral adiposity (Despres et al., 1990, Pouliot et al., 1992, Ross et al., 2002). Earlier, it was reported by Després et al., that overweight and obese individuals showed different metabolic risk factor profiles such as insulin resistance and dyslipidemia (Despres et al., 1990). Abnormalities of the metabolic risk factor profile in obese patients were characterized by the excess of visceral adipose tissues, whereas patients with normal metabolic risk factor profile were characterized by subcutaneous obesity and low levels of visceral adipose tissue (Despres et al., 1990).

1.2.3 Visceral adiposity and metabolic syndrome

Metabolic syndrome refers to a cluster of several known risk factors for cardiovascular disease and type 2 diabetes which are associated with obesity and result from insulin resistance (Huang, 2009). Epidemiological studies have shown that body fat distribution is an independent contributor to metabolic syndrome and cardiovascular disease that is more associated to abdominal fat rather than to general adiposity (Kannel et al., 1991, Després, 2012). This concept was accepted in the early 1980s by various studies that produced solid evidence that ratio of waist to hip ratio and central obesity was correlated strongly with metabolic disorders and to cardiovascular diseases than total body fat (Kissebah et al., 1982, Lapidus et al., 1984, Ohlson et al., 1985, Krotkiewski et al., 1983).

Visceral adiposity is related to insulin resistance by decreasing insulin-mediated glucose uptake (Kershaw and Flier, 2004). This effect of visceral fat involves

increased production of pro-inflammatory adipokines such as, tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) that contribute to insulin resistance and vascular dysfunction. In addition, increased fluxes of FFAs and bioactive lipid intermediates have been shown to impair an important signaling pathway in triacylglycerol synthesis and glucose uptake known as PI3K-Akt (phosphoinositide-3-kinase/ serine/threonine kinases) pathway and thereby increase oxidative stress (Paul and John, 2007). PI3K-Akt signaling pathway has been shown to play an essential role in insulin regulation of glucose uptake by promoting membrane translocation of glucose transporter GLUT4 (Thong et al., 2005). Adipocyte energy homeostasis and FFA re-esterification processes have been shown to be partly regulated by insulin which inhibits lipolysis in a PI3K-dependent manner (Van Harmelen et al., 1999).

Another factor associated with increased visceral fat is the reduction of adiponectin production. Adiponectin is an important adipokine that enhances sensitization to insulin in various tissues in the body (Kadowaki et al., 2006). In a co-culture study of visceral and subcutaneous fat conducted by Halleux et al., the production of adiponectin from subcutaneous fat was inhibited, suggesting inhibitory factors secreted from visceral fat influenced its production (Halleux et al., 2001). These findings may explain the negative association between visceral adiposity and adiponectin levels observed with abdominal obesity. A reduced level of circulating adiponectin has been also correlated with reduced insulin sensitivity (Hotta et al., 2000).

Dyslipidemia has long been associated with obesity and, more specifically, abdominal obesity. This includes elevation of plasma TG levels, low density lipoprotein (LDL) and VLDL, accompanied by lowered levels of high density lipoprotein cholesterol (HDL-C) (Grundy, 2004).

The biological differences between male and female regional fat distribution are postulated to be associated with sex hormones, a notion that is supported by numerous epidemiological and clinical studies as reviewed by (Mayes and Watson, 2004). Men tend to store fat in the trunk and abdominal region, whereas women tend to store the fat at the hip and thigh regions (Tchernof and Despres, 2013). This will be an important focus for this thesis.

1.3 Sex steroid hormones

Steroids are lipophilic hormones that are synthesized by numerous organs including gonads (ovary and testis), the adrenal gland, placenta and to some extent by adipose tissue (Bjorntorp, 1997, Beato and Klug, 2000, Sanderson, 2006). Steroid hormones are synthesized solely from cholesterol, involving enzymatic reactions leading to the production of different sex hormones as illustrated in Figure 1.4 (Chen et al., 2011). Briefly, de novo synthesis of all steroid hormones starts with the action of desmolase enzyme to convert cholesterol to pregnenolone. Pregnenolone is converted progesterone by 3β-hydroxysteroid hydrogenase (3β -HSD). Pregnenolone and progesterone are the basic precursors for all other steroid hormones, that differ in the ring structure and the side chain attached to it (Sanderson, 2006).

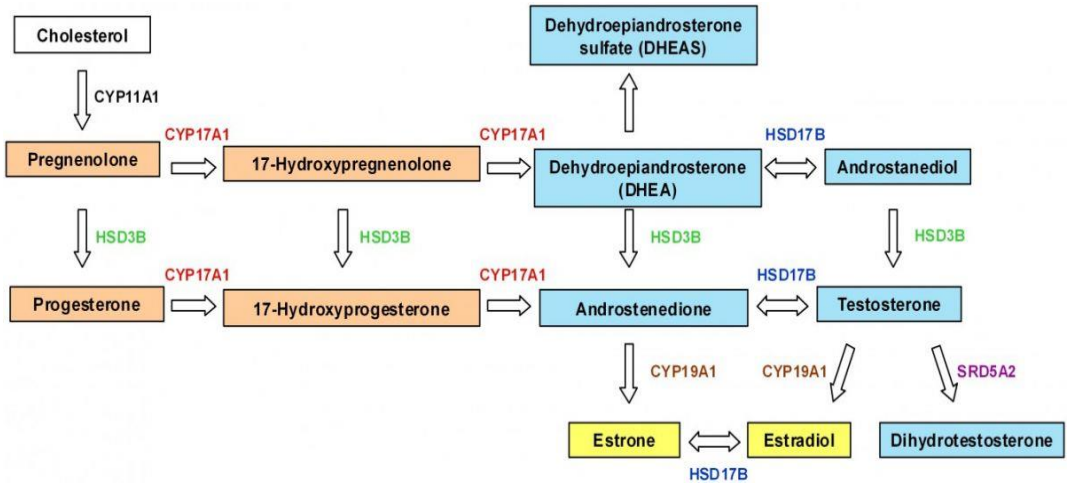


Figure 1.4 Sex steroid hormone biosynthesis pathways. CYP11A1: cholesterol desmolase, CYP17A1: 17α-hydroxylase/17, 20 lyase, HSD3B: 3β-hydroxysteroid dehydrogenase, HSD17B1: 17 β -hydroxysteroid dehydrogenase1, CYP19A1: aromatase, SRDFA2: steroid -5-α-reductase, αpolypeptide 2 (Chen et al., 2011).

Progesterone, estrogen and testosterone are the three major classes of sex steroids that play important roles in reproductive system. They have also been shown to influence carbohydrate and lipid metabolism, inflammatory responses, bone metabolism and brain function (Barengolts et al., 1990, Edwards, 2005). In addition to sexual development, estrogen and progesterone are associated with other weight regulatory hormones such as, insulin and cortisol. They have been found to influence the serum insulin levels by enhancing or reducing it respectively (Ahmadi and Oryan, 2009).

1.3.1 Sex steroids and body fat distribution

Visceral fat mass has been shown to increase with age in both males and females, though males and postmenopausal women tend to accumulate two-fold more visceral adipose than premenopausal women (Kotani et al., 1994). Hormonal reduction after the menopause has been linked with the increase of visceral fat accumulation and an increased risk of cardiovascular disease (Tchernof et al., 2000, Bjorntorp, 1996, Wajchenberg, 2000). This observation of increasing visceral fat in postmenopausal women is well recognized to be mainly related to estrogen deficiency. In animal studies, ovariectomized rats exhibit increased visceral fat and decreased subcutaneous fat accumulation, while administration of estrogen reverses this effect (Asarian and Geary, 2006, Clegg et al., 2006). In the aromatase-knockout mouse model (which are estrogen-deficient and have elevated testosterone), increased intra-abdominal adipose tissue accumulation is noted (Jones et al., 2000). It was initially proposed by Björntrop, in 1997, that progesterone influences body fat distribution in females through anti-glucocorticoid effects (Bjorntorp, 1997). This was supported by a study showing the affinity-binding of progesterone to the glucocorticoid receptor and the inhibition of cortisol–stimulated abdominal fat accumulation (Pedersen et al., 2003).

Moderate doses of testosterone have been reported to reduce abdominal obesity in men in a study carried by Marin et al., (Marin et al., 1993). The decrease of visceral fat improved glucose uptake, dyslipidemia and blood pressure. Women with polycystic ovary syndrome tend to develop abdominal obesity, where

androgenization is evident (Burghen et al., 1980, Evans et al., 1988). In postmenopausal women, the production of testosterone remains stable from the ovaries (Vermeulen, 1976). Cao et al. reported a positive association between free testosterone level and abdominal fat accumulation in postmenopausal women (Cao, 2007).

1.3.2 Molecular basis of sex steroid activity

While the specific mechanism of action of sex steroid hormones has not been completely established it appears to involve a combination of genomic and non-genomic actions through binding to membrane, intracellular and/or nuclear receptors (Mayes and Watson, 2004).

1.3.2.1 Genomic actions

Genomic mechanisms require the binding of the steroid hormone to a specific nuclear receptor in the adipocyte cell (**Figure 1.5**). Once the hormone binds to its receptor, it undergoes a conformational changes that allow the receptor to bind to specific DNA sequences or response elements that eventually modulate transcription of targeted genes (Gouva and Tsatsoulis, 2004, Mayes and Watson, 2004).

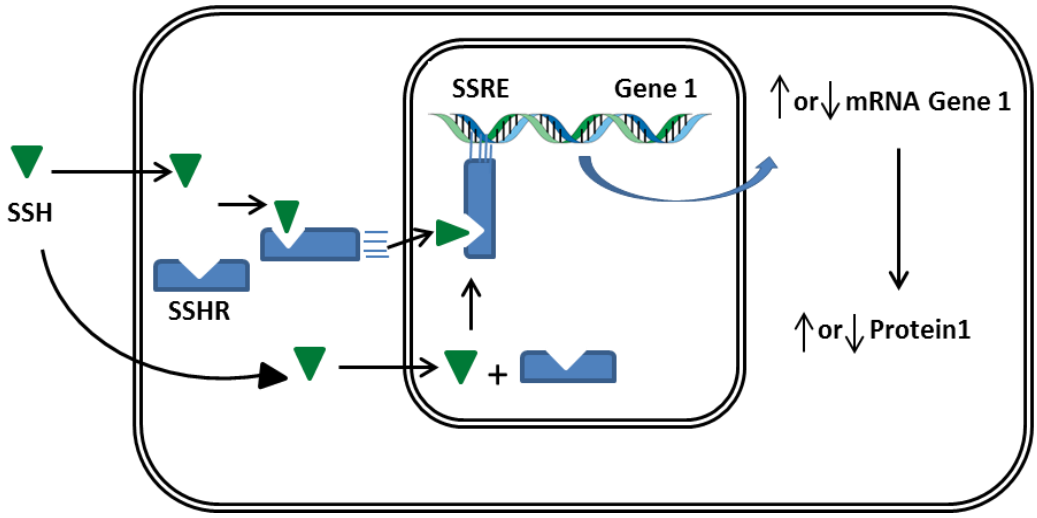


Figure 1.5 Transcriptional regulation mechanisms of sex steroid hormones. SSH, sex steroid hormones, SSHR, sex steroid hormone receptor, SSRE, sex steroid response element (adapted from (Mayes and Watson, 2004)).

1.3.2.2 Non-genomic actions

Non-genomic actions of sex steroid hormones are rapid responses to binding to cytoplasmic membrane receptors that regulate second messengers (Wajchenberg, 2000). This mechanism appears to regulate multiple membrane signaling pathways including the cyclic adenosine monophosphate (cAMP) and phosphoinositide cascades (**Figure 1.6**). These can mediate lipolysis and lipogenesis in adipose tissue (Marin et al., 1996). The activation of cAMP by steroid hormones would lead to lipolysis through the activation of HSL. The activation of the phosphoinositide cascade would lead to lipogenesis through protein kinase C (Marin et al., 1996).

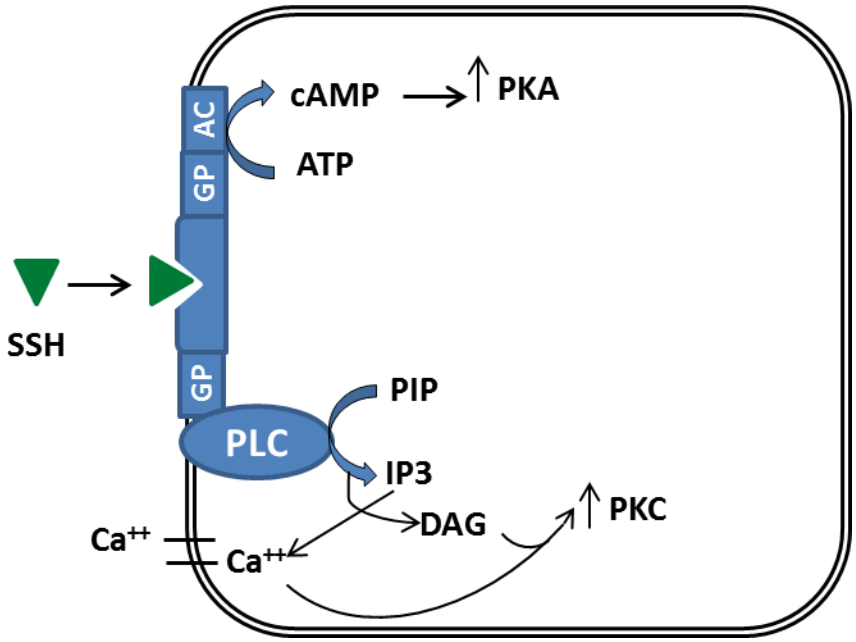


Figure 1.6 Non-genomic actions of sex steroid hormones on adipose tissue. SSH, sex steroid hormones, SSHMR, sex steroid hormone membrane receptor, GP, G-Protein; AC, adenylate cyclase, PKA, protein kinase A; PLC, phospholipase C; PIP, phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C. Adapted from (Mayes and Watson, 2004).

1.3.3 Sex steroid receptors in adipose tissue

In humans, estrogen receptor (ER) mRNA and protein have been identified in subcutaneous abdominal adipose tissue (Price and O'Brien, 1993, Mizutani et al., 1994). Pedersen et al. showed the presence of ER in mature adipocytes, but not in preadipocytes, and in the cytosols from visceral and subcutaneous adipose tissue in women (Pedersen et al., 1996). Later, he demonstrated the presence of two specific estrogen receptors (ER-α and ER-β) in different fat depots of both men and women (Pedersen et al., 2001). In human adipose tissue the distribution of ER-α was equal in subcutaneous and omental adipose tissue, while ER-β was found to be expressed more in abdominal compared to subcutaneous adipose

tissue (Pedersen et al., 2001). A study using cultured adipocytes, conducted by Anwar et al. showed that in response to estrogen treatment, the expression of ER-α was decreased and ER-β expression was increased in adipocytes from subcutaneous tissue. However, the inhibition of ER-α was not detected in omental adipocytes (Anwar et al., 2001). Independent of gender, knockout ER-α mice exhibited more obesity and lower energy expenditure (Heine et al., 2000). It was demonstrated by Naaz et al. that ER-α enhances the estrogen metabolic effect in regulating fat deposition and improving insulin sensitivity, whereas ER-β mediates the opposite effects, by increasing body weight and inducing insulin resistance (Naaz et al., 2002).

Studies investigating the presence of the progesterone receptor (PR) in mature human adipocytes are limited, with one suggesting they are not present (Rebuffe-Scrive et al., 1990). In contradiction, O'Brien et al. were able to detect PR using Northern blot analysis in subcutaneous adipose tissue of postmenopausal women, with higher expression in the stromal fraction when compared to adipocytes (O'Brien et al., 1998). In ovariectomized-adrenalectomized rats, Gray and Wade were able to identify a progesterone binding sites in adipose tissue that was primed with estrogen (Gray and Wade, 1979, Gray and Wade, 1980).

Androgen plays an important role in regulating central obesity and is considered to be a strong risk factor for cardiovascular disease and insulin resistance (Marin and Arver, 1998).The presence of androgen receptors (AR) in subcutaneous adipose tissue from men and women was reported by Pedersen et al. (Pedersen et al., 1996) and in both mature adipocyte and preadipocytes of men and women by

Dieudonne et al. (Dieudonne et al., 1998). The expression of AR and responsiveness to androgen was found to be higher in visceral than in subcutaneous human preadipocytes (Joyner et al., 2002).

1.3.4 Sex steroid hormones and lipid metabolism

The role of sex hormones in regulation of lipid metabolism has been extensively reviewed (Mittendorfer, 2005, Magkos and Mittendorfer, 2009, Pellegrini et al., 2014, Comitato et al., 2015). Females at reproductive age show lipid profiles that are consistent with a cardio-protective effects compared to men who tend to have more atherogenic lipid profiles (Wang et al., 2011). Estrogen treatment was shown to enhance, lipolytic responsiveness, whereas progesterone had no effect on lipolytic activity (Hansen et al., 1980). LPL has a key role in regulating adipose tissue lipid metabolism. As discussed below, LPL activity in adipose tissue has been shown to be influenced by different sex hormones that vary between men and women and in different regional fat depots.

1.3.4.1 Estrogen

Estrogen (mainly circulating as 17-estradiol (E2) form) is produced by the ovaries after the aromatization of androstenedione to estrone (E1) and is further converted to E2 (Mauvais-Jarvis et al., 2013). Estrogen mediates energy homeostasis through ER-α and ER-β. In human and rodent studies, females with ER-α gene mutation displayed increased visceral adiposity and decreased sensitivity to leptin (Okura et al., 2003, Heine et al., 2000, Musatov et al., 2007). Male ER-α knockout mice developed obesity associated with elevated lipoprotein profile

including cholesterol and LDL that was mediated by ER-α but not ER-β receptor (Ohlsson et al., 2000).

Estrogen directly affects LPL and HSL expression. The lack of estrogen in postmenopausal women has been linked to lipid metabolism disorders (Szafran and Smielak-Korombel, 1998). The regulation of LPL involves several hormones that may stimulate or inhibit LPL expression and activity. Animal studies have shown that ovariectomy of female rats' increases LPL expression in adipose tissue while estrogen replacement decreases LPL activity (Kim and Kalkhoff, 1975, Ramirez, 1981, Pedersen et al., 1992). Estrogen stimulates HSL enzyme and therefore increases lipolysis. The effect of estrogen on lipid metabolism is illustrated in **Figure 1.7** (Gao and Dahlman-Wright, 2013).

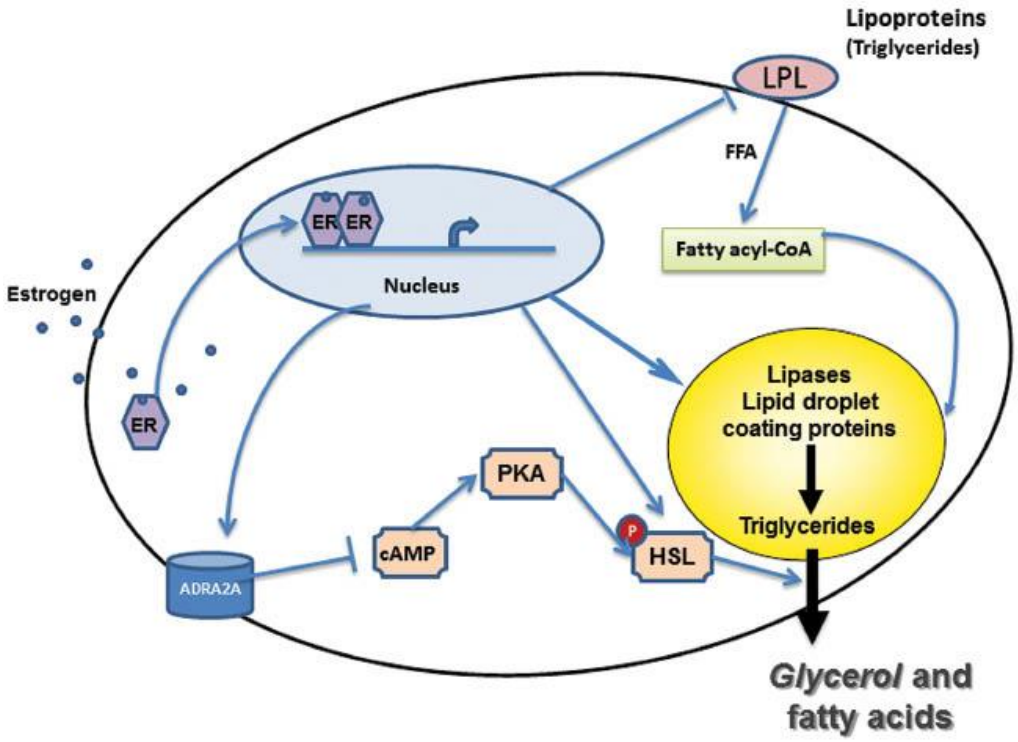


Figure 1.7 Estrogen effect in adipose tissue. ER, estrogen receptor; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; HSL; hormone sensitive lipase; LPL, lipoprotein lipase; ADRA2A, adrenoceptor alpha 2A (Gao and Dahlman-Wright, 2013).

1.3.4.2 Progesterone

Progesterone is a lipogenic hormone involved in adipose tissue metabolism (Lobo et al., 1993). In rats, progesterone increases subcutaneous adipose tissue and alters the expression of adipose secretions involved in energy regulation (Stelmanska et al., 2012). It can enhance glucose uptake, lipogenesis, lipolysis and lipoprotein lipase activity (Stelmanska et al., 2012). Some studies suggest progesterone opposes the effect of estrogen, stimulating lipid synthesis by increasing LPL activity (Kim and Kalkhoff, 1975, Shirling et al., 1981, Lacasa et al., 2001), however, others suggest that progesterone has no effect on LPL activity in

adipose tissue (Rebuffe-Scrive, 1987). In human studies, treatment of postmenopausal women with both progesterone and estrogen increased LPL activity in subcutaneous adipose tissue (Rebuffe-Scrive et al., 1987). The metabolic effect of progesterone is most profound during pregnancy. During early gestation, hormonal changes and increased progesterone favors body fat deposition along with hyperphagia and hyperinsulinemia (Kalkhoff, 1982). Progesterone also plays a role in adipose tissue metabolism through the action of 20α-hydroxysteroid dehydrogenase (20α-HSD) enzyme via the inactivation of progesterone (Blanchette et al., 2005). 20α-hydroxylation of progesterone was found to be high in women with omental obesity, whereas 20α-HSD activity correlated positively with omental adipocyte size and LPL activity (Blanchette et al., 2005, Blouin et al., 2005).

1.3.4.3 Testosterone

Low testosterone levels in men have been associated with increased body weight and disease pathology (Wang et al., 2011). Furthermore, obesity itself impairs testosterone production, promoting an increase in fat deposition (Cohen, 1999). In a longitudinal study, low testosterone levels were shown to increase the risk of hyperglycemia and cardiovascular disease in association with increased abdominal obesity (Shahani et al., 2008). Testosterone is another steroid hormone that has been shown to inhibit TAG uptake and LPL activity, resulting in lipid mobilization, specifically from visceral adipose tissue (Marin et al., 1996). A study by Blouin et al. showed that testosterone inhibited LPL activity in omental

fat in women, whereas in men it significantly inhibited LPL activity in omental and subcutaneous adipose tissues (Blouin et al., 2010).

Androgen receptor expression has been shown to be similar in both male and female adipose tissue (Joyner et al., 2002). However, the expression is higher in omental compared to subcutaneous adipose tissue in both sexes (Dieudonne et al., 1998, Rodriguez-Cuenca et al., 2005). It was also reported that AR expression is lower in mature adipocytes compared to preadipocytes, suggesting that mature cells may be less responsive to androgen stimulation when compared to preadipocytes (Dieudonne et al., 1998).

1.4 Adipose tissue secretions

Adipose tissue is currently viewed as an active secretory organ that is able to respond to signals that alter insulin sensitivity, lipid and energy metabolism, appetite, immunity, endocrine system and bone metabolism (Fantuzzi, 2005). The main role of WAT is to store TG during positive energy balance and to enhance the release of fatty acid when the energy balance is negative. In obesity, physiological secretions from adipose tissue are altered, with increased release of fatty acids, hormones and pro-inflammatory molecules, which have been considered key reasons for many of the metabolic disturbances in obesity (Kershaw and Flier, 2004, Balistreri et al., 2010).

Adipose tissue is composed of different cell types and different fat depots with different metabolic capacities and secretions. The discovery of leptin transformed the prespectives of an endocrine role of WAT in the regulation of many

physiological processes (Friedman and Halaas, 1998). Leptin affects energy expenditure, the immune and reproductive systems and has other metabolic effects such as the inhibition of insulin secretion from pancreatic beta-cells (Mohamed-Ali et al., 1998). Subsequently, a wide range of other protein factors have been found to be secreted from WAT (Trayhurn and Beattie, 2001)**,** as summarized in **Figure 1.8**.

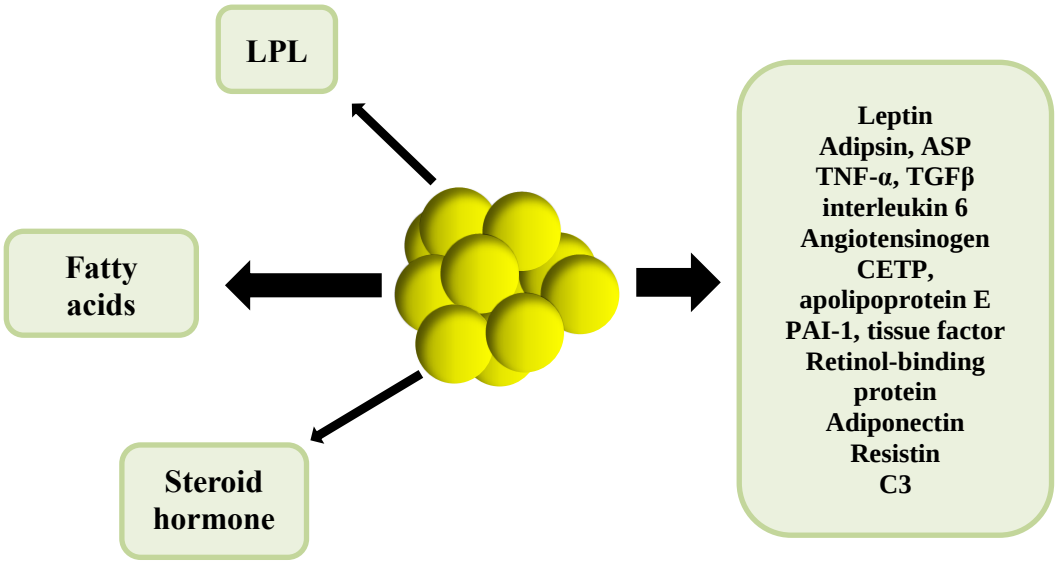


Figure 1.8 Proteins factors secreted from white adipose tissue. ASP, acylation-stimulating protein; CETP, cholesteryl ester transfer protein; FIAF, fasting-induced adipose factor; LPL, lipoprotein lipase; PAI-1, plasminogen activator inhibitor-1; TGFβ, transforming growth factor β; TNF-α, tumor necrosis factor α. Adapted from (Trayhurn and Beattie, 2001).

1.4.1 Lipoprotein lipase

LPL is the primary enzyme required to hydrolyze TG from circulating chylomicrons and VLDL to facilitate the uptake of FFA by various tissues (Merkel et al., 2002). It is synthesized and produced by adipocytes and transported to the capillary endothelial to act on the hydrolysis of the TG in circulating lipoproteins (Mayes and Watson, 2004). It is also expressed in other tissues such as muscles, liver, heart, kidney, pancreatic cells, nerves and lungs (Merkel et al., 2002).

Catalytically active form of LPL is a noncovalent homodimer that consist of two 55 kDa identical subunits (Iverius and Ostlund-Lindqvist, 1976, Osborne et al., 1985). LPL active site is anchored to heparin sulfate proteoglycans at the luminal surface of the vascular endothelium. In the circulation, LPL monomers are less bound to heparin sulfate leading to more circulation of inactive LPL (Lookene et al., 2004, Lookene et al., 1996). The dissociated of LPL dimers into monomers leads to loss of activity and it has been demonstrated *in vitro* studies to be effected by various physiological conditions such as pH, temperature and salt concentrations as reviewed in (Lookene et al., 2004, Lookene et al., 1996). The rate of LPL dimer dissociation showed to be dependent on LPL concentration and this process of inactivation includes irreversible unfolding of the dissociated monomers (Osborne et al., 1985).

LPL activity is regulated by various factors. For hydrolysis, LPL is activated by binding to apolipoprotein (apo) Cll, a component of the chylomicron and VLDL

particles (Havel et al., 1973), and inhibited by serum apoCIII and apoC1 (Brown and Baginsky, 1972). LPL is secreted by adipocytes into the interstitial space and transported across the capillary endothelial into the capillary lumen by glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein (GPIHBP1) (Davies et al., 2010). The absence of GPIHBP1 by mutation has shown to cause chylomicronemia and hyperlipidemia due to the blocked ability of GPIHBP1 binding to LPL in human (Beigneux et al., 2009). Angiopoietin-like 4 (ANGPTL4) proteins is another endogenous modulator that exhibited to inhibit LPL and regulate triglyceride by converting the catalytically active dimers of LPL to its inactive monomers (Sukonina et al., 2006, Yau et al., 2009). ANGPTL4 is highly expressed in adipose tissue and act mainly on LPL during fasting. This action would reduce triglyceride- driven fatty acid storage into adipose tissue and favor the intake of the fatty acids to tissues such as heart and muscles (Cushing et al., 2017). The deficiency of ANGPTL4 protein found to increases LPL-driven triglyceride clearance, reduces plasma triglycerides and lowers the risk of cardiovascular diseases (Dewey et al., 2016). Interestingly, it was found that ANGPTL4 can interrupt the binding affinity between LPL and GPIHBP1 providing an additional mechanism of the inhibitory role of ANGPTL4 on LPL (Chi et al., 2015).

Fat distribution is found to be regulated by LPL activity and it is regulated in a tissue-specific way that is dependent of the physiological needs of the targeted tissues (Preiss-Landl et al., 2002). In addition to its role in lipid storage, LPL has other specialized functions in different tissues, such mediating lipolysis in lung,

which is important in surfactant production (Hamosh et al., 1976). LPL overexpression is related to decreased insulin secretion and the increased LPL - delivered lipid uptake by the pancreatic islet cells that is associated with insulin resistance (Cruz et al., 2001). Dysfunction of LPL activity, due to mutation, leads to Familial Chylomicronemia Syndrome associated with increased TG levels, however patients with this condition mediate the uptake of TG pathway in the adipose tissue through the action of other lipases including hepatic and endothelial lipases (Merkel et al., 2002).

1.4.2 Cytokines

Cytokines are sometimes considered to be a pro-inflammatory molecules that are elevated in people with obesity and involved in the early stages of diseases such as atherosclerosis (Weyer et al., 2002). The number of macrophages within adipose tissue increases in obesity and contributes to the production of cytokines that induce inflammation (Weisberg et al., 2003). There are many types of cytokines that are classified as interleukins, interferons, chemokines and growth factors (Thalmann et al., 2007). Adipose tissue expresses a variety of cytokines such as interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCO-1) and complement C3, that are known to stimulate inflammation by the stimulation of acute-phase proteins such as C-reactive protein (CRP) or involoved in low-grade inflammation pathways (Weyer et al., 2000, Munkonda et al., 2012).

Elevated cytokine concentrations have been shown to directly affect cellular metabolism. For example, TNF-α directly decreases insulin sensitivity and increases lipolysis. Obese mice, deficient in TNF-α protein, have more sensitivity to insulin compared to wild type mice (Uysal et al., 1997). IL-6 increases TG levels, *in vivo,* by stimulating hepatic TG secretion and lipolysis (Nonogaki et al., 1995). Adipose tissue has also been shown to express receptors for various cytokines supporting a suggested model in which adipocytes are both the source, and target for inflammatory signals (Kershaw and Flier, 2004). Altogether, pro-inflammatory cytokines produced by adipose tissue and liver create a systemic inflammatory status that correlates to insulin resistance (Yudkin, 2003).

1.4.3 Adipokines

Adipokines are proteins secreted from adipose tissues and linked to food intake, energy expenditure, glucose and lipids metabolism, blood pressure, inflammation and immune function (Balistreri et al., 2010, Ravussin, 2002, Guerre-Millo, 2002, Procaccini et al., 2013). They act locally and systemically and respond to signals from other homonal networks and the central nervous system (CNS), thereby allowing adipose tissue to be at the centre of cross-communication between different body organs (Kershaw and Flier, 2004). The importance of adipokines is emphasized in obesity, when altered adipokine levels intiate a cascade of metabolic disturbances (Fantuzzi, 2005). The most notable adipokines involved in obesity and related disorders are reviewed briefly below.

1.4.3.1 Leptin

Leptin was discovered in 1994 by Zhang et al. and is predominantly expressed in WAT (Lago et al., 2007). Upon binding of leptin to its receptor, an activation of an intracellular signalling pathway occurs which stimulates energy expenditure and inhibits food intake (Cohen et al., 2001). Leptin receptors are found widely in adipose tissues, liver, muscles brain, and pancreatic cells indicating endocrine, paracrine and autocrine roles of leptin in energy metabolism (Feng et al., 2013). It is a satiety hormone functioning by signaling nutritional status to the hypothalamus, therefore playing a key role in regulating food intake and energy expenditure. In addition, leptin stimulates lipolysis, inhibits lipogensis and improves insulin sensitivity and glucose metabolism (Balistreri et al., 2010, Feng et al., 2013).

In ob/ob mice, the leptin gene is a mutated causing hyperphagia and weight gain (Tartaglia et al., 1995). Treatment of such mice with leptin reduces food intake and body weight (Levin et al., 1996). As reviewed by Havel, leptin increases insulin sensitivity by activating the AMP-protein kinase pathway and increasing muscle fatty acid oxidation (Havel, 2004). In obese subjects, circulating leptin concentrations increase and are directly related to the overall adiposity (Cooper et al., 2009). When fat stores are depleted, leptin concentrations reduce, stimulating hunger and decreasing energy expenditure, aimed at restoring fat stores to normal (Cooper et al., 2009). Women have higher leptin concentrations than men (Geer and Shen, 2009), a finding associated with sex hormones impacting on adipose tissue distribution.

1.4.3.2 Adiponectin

Adiponectin is another highly expressed hormone in adipose tissue and is also known as adipoQ or adipocyte complement-related protein. In contrast to leptin, adiponectin reduces with obesity (Arita et al., 2012) and in diabetes (Hotta et al., 2000). It affects metabolism by enhancing insulin sensitivity in the liver and muscles and increasing FFA oxidation is various tissues. It also decreases blood glucose, FFA and TG concentrations when given exogenously with a high fat and sugar diet in lean mice (Greenberg and Obin, 2006, Fruebis et al., 2001). In addition, it is considered as a protective factor against cardiovascular disease and inhibits inflammatory responses (Ouchi et al., 1999, Yokota et al., 2000). Adiponectin is negatively correlated with TG levels and positively correlated with plasma HDL-C levels (Shibata et al., 2012, Havel, 2004).

Adiponectin concentrations are inversely related to both total fat and intra-abdominal fat (Laughlin et al., 2007), however it was also reported to be produced more actively by visceral, rather than subcutanous fat cells (Motoshima et al., 2002). Plasma adiponectin concentration has been reported to be higher in women than in men independent of body size or body fat distribution (Cnop et al., 2003). This can be explained by the effect of androgens in men, decreasing plasma adiponectin concentration (Nishizawa et al., 2002).

1.4.3.3 Resistin

Resistin is a small polypeptide that is considered as an inflammatory factor produced from adipocytes, macrophages and other cell types in adipose tissue. In

human studies, a positive correlation between serum resistin levels and body fat content was reported (Kusminski et al., 2005). Resistin expression in adipose tissue is reduced on fasting with a parallel reduction in circulating blood levels (Trayhurn and Beattie, 2001). Resistin is expressed 15-fold more in visceral than subcutaneous adipose tissue. It has a significant effect on insulin resistance by impairing insulin mediated glucose uptake (Banerjee and Lazar, 2003, Steppan et al., 2001).

1.4.3.4 Adispin and Acylation Stimulating Protein

Adipsin was one of the first secreted proteins from adipose tissue to be identified that is expressed in a differentiation dependent manner in adipocyte cells (Cook et al., 1985). It was initially linked to obesity by an observation of reduced adipsin expression and circulating concentration in obese animal models (Flier et al., 1987). However, in human obesity adispin has been found to be increased and it has been shown to be involved in the synthesis of another protein of the alternative complement system named acylation stimulating protein (ASP) (Cianflone et al., 1999, Maslowska M et al., 1999). ASP is the main focus of this thesis.

1.5 Acylation Stiumlation Protein (ASP)

ASP is a hormone solely secreted by adipocytes and is derived from the C3 complex through the action of adipsin, factor B and carboxypeptidase (Cianflone et al., 1989a). ASP has an anabolic effect, by stimulating TG synthesis and glucose uptake. It also enhances postprandial fat clearance (Cianflone et al., 1999,

Cianflone et al., 2003, Saleh et al., 1998). ASP has been recognized to be a member of the "adipokines" family produced within the micro-environment of adipocytes in an paracrine manner. It was shown to be as potent as insulin in its fat storage effects (Cianflone et al., 1999, Cianflone et al., 1994). To date, insulin and ASP are the main fat storage hormones discovered.

ASP is a small peptide, consisting of 76-amino acids with a size of 8.9 KDa and has been shown to be identical to C3adesArg (Baldo et al., 1993). It was first reported and purified in 1989, by Cianflone and co-workers, and named based on its *in vitro* ability to stimulate TG storage in cultured human skin fibroblasts (Cianflone et al., 1989a). Since then, this small protein has attracted attention because of its important role in glucose and lipid metabolism.

ASP is a specific adipocyte hormone in that all three basic proteins, complement C3, factor B and adipsin required in the synthesis of ASP are produced by the adipocyte. Adipsin is a key enzyme involved in regulating the alternate complement pathway (White et al., 1992) . Complement C3 is cleaved to form ASP by a cascade of enzymes that is initiated by the action of adipsin on factor B and the protein C3 complex (Cianflone et al., 1999).

ASP is produced through the activation of complement alternative pathway. As reviewed by Cianflone et al., the process includes a cellular cleavage of C3 into two subunits known as C3 alpha (C3a) and C3 beta (C3b). Initially , factor B (B) binds to the activated C3b forming C3b-B complex in the presence of Mg^{2+} (Cianflone et al., 2003). Factor B then undergoes catalytic conformational

changes through the action of adipsin, generating a C3bBb complex, termed C3 convertase. Next, C3 convertase cleaves a single arginine-serine bond near the amino-terminal of the alpha chain of an intact C3 protein, producing C3a and new C3b proteins. At the final step, the amino acid arginine, at the carboxyl-terminal of C3a generated by the action of C3 convertase, is cleaved by carboxypeptidase B (CpB) enzyme to generate C3adesArg or ASP (Cianflone et al., 1999, Cianflone et al., 2003). Details of the cleaving process of C3 to ASP are illustrated in **Figure 1.9.**

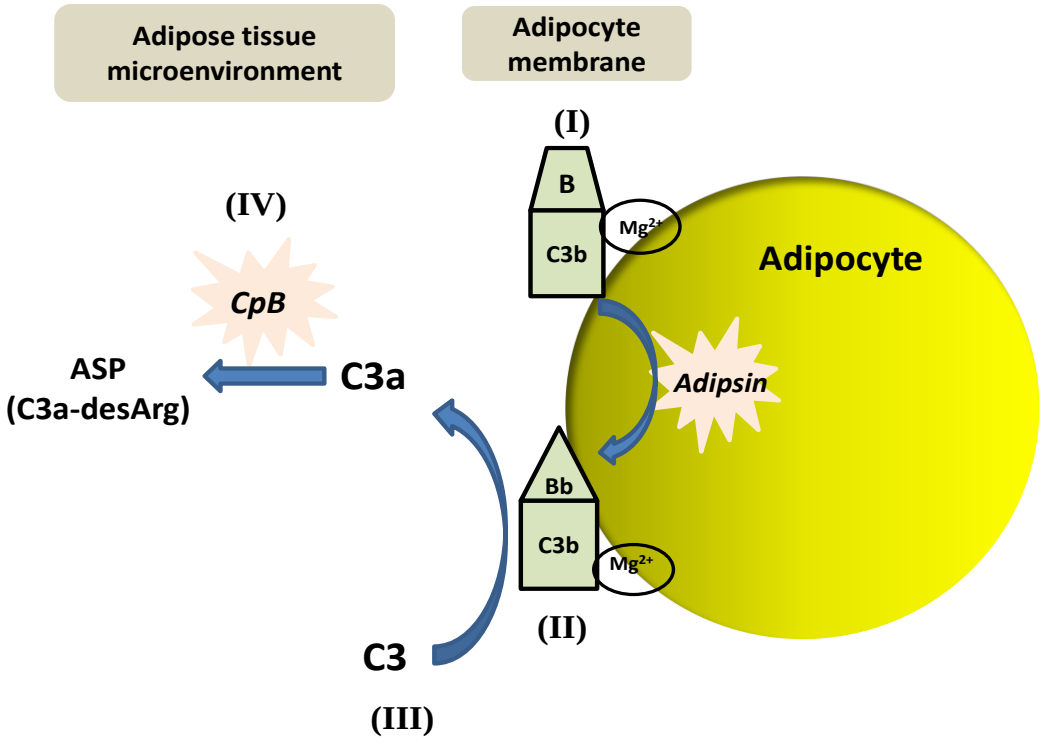


Figure 1.9 Enzymatic conversion of C3 to ASP: (I) formation of C3bB complex and membrane association (membrane); (II) cleavage by adipsin to generate C3bBb convertase (membrance); (III) cleavage of C3 by convertase to generate C3a and C3b; and (IV) cleavage of the c-terminal arginine of C3a by carboxypeptidase B (CpB)to produce C3adesArg (ASP) into the adipose tissue microenvironment. Adatpted from (Cianflone et al., 1999).

C3a is well documented as an immune modulator, whereas, ASP is considered to be a bio-inactive product, as it does not possess any immunocytologic activity (Baldo et al., 1993). C3a is a bioactive stimulator in proinflammatory responses mediated by its interaction with C3a receptor, whereas, the C3a-desArg form does not interact with the C3a receptors. (Fisette and Cianflone, 2010). Although both C3a and ASP differ in one amino acid, both exhibit very specific binding characteristics to their receptors which determines their distinctive functionality (Zwirner et al., 1999).

1.5.1 ASP precursors

The generation of C3a/C3adesArg is mediated through the interaction of adipsin, factor B, and complement C3 proteins. Adipose tissue displays an exclusive involvement of the components of the alternative pathway where ASP is likely to be synthesized in the microenvironment of adipocytes (Cianflone et al., 2003). While C3 and factor B are produced by adipose tissue and other tissues, such as liver, adipsin is primarily produced by adipose tissue and therefore plays a major role in regulating ASP synthesis (White et al., 1992).

Complement C3 is a major plasma protein that plays a key role in inflammation along with its mediator molecules (C3a, C5a, C4b and C3b). Complement C3a and C5a act through C3a and C5a receptors to promote infiltration of inflammatory cells in tissues like liver, lung and kidney (Mamane et al., 2009). C3 protein is also involved in the synthesis of ASP and therefore plays a role in fat storage (Cianflone et al., 1999). C3 has been shown to be elevated in obesity with high gene expression in omental adipose tissue in obese men and subjects

with diabetes and insulin resistance (Gabrielsson et al., 2003, Peake et al., 2004). It has been proposed that such elevation of C3 in diabetic patients is due to the reduced uptake of glucose and fatty acids in adipose tissue as a direct response to the activation of C3-ASP system (Engström et al., 2005). Fasting ASP levels are positively correlated to C3 concentrations in obesity suggesting that ASP production may be mediated by increased C3 in adiposity (Weyer et al., 2000).

Adipsin was first identified by Spiegelman et al., in 1983 in an adipocyte cell line (Spiegelman et al., 1983). It is a serine protease protein found to be homologous to complement factor D in human, which is a key enzyme in the regulation of the alternative complement pathway and therefore ASP production (White et al., 1992). It is the rate limiting enzyme in the alternative pathway of complement activation and plays a role in the regulation of systemic energy balance (Balistreri et al., 2010, Ukkola. O et al., 2003). In obese subject, adipsin has been shownto increases along with ASP levels (Maslowska M et al., 1999)

The elucidation of the Adipsin/ASP pathway in human adipocytes provided evidence of the involvement of adipsin in the ASP pathway triggering fat storage (Cianflone et al., 1994). The expression of the precursors of ASP in adipocytes was shown to be different in women and men consistent with ASP production. The expression of adipsin was shown to be downregulated in females and upregulated in males in order to compensate for the proposed ASP resistance (elevated ASP level) in omental tissue in male (Xia and Cianflone, 2003).

Factor B is also expressed in adipose tissue (Xia and Cianflone, 2003). *In vitro* studies using the 3T3-L1 cell line, showed ASP production increased 2-3 fold as factor B increased (Peake et al., 1997). Factor B was also shown to be associated significantly with insulin resistance, fasting glucose, TG and several metablic parameters (Moreno-Navarrete et al., 2010). The gene expression of factor B was higher in omental, compared to subcutaneous, adipose tissue in subjects with insulin resistance (Moreno-Navarrete et al., 2010).

1.5.2 Regulation of ASP production

ASP has been shown to markedly stimulate TG synthesis, additively and independently, of insulin (Faraj et al., 2004). In a study by (Van Harmelen et al., 1999); a combined effect of both insulin and ASP provided a powerful drive for fat storage with almost a complete re-esterification of available fatty acid and a reduction in net fatty acid output from adipocytes.

In addition to insulin, it has been reported that chylomicrons are potent stimulators of ASP production. In contrast, the addition of NEFA to cells *in vitro* did not affect ASP production and a minimal effect was observed of VLDL, LDL or HDL (Maslowska et al., 1997). Chylomicrons are intestinally derived postprandial lipoprotein particles that carry dietary fat. They are particles with an average diameter of 1000 nm composed of cholesterol, apolipoprotein, phospholipid and approximately 90% TG. The TG transported in the TG-rich lipoprotein particles. chylomicrons and VLDL, are hydrolyzed by LPL to FFA, which are taken by adipocytes (Clemente-Postigo et al., 2011).

ASP production in human adipocytes was examined in subcutaneous adipose tissue in response to dietary fat (Saleh et al., 1998), where ASP coordinates positively with TG deposition into adipose tissue. ASP efflux from adipose tissue increases postprandially in obese female subjects who display greater efficiency in clearing TG than lean female subjects, suggesting that ASP may contribute to obesity by the enhancing TG storage into subcutaneous adipose tissues (Kalant et al., 2000). The stimulatory effect of chylomicrons on ASP production was shown to be time-and concentration-dependent (Gao et al., 2011). Although the mechanism of the effect is not fully understood, it was suggested to be mediated through retinoic acid (RA) elements and transthyretin (TTR), a transport protein associated with chylomicrons (Scantlebury et al., 2001, Scantlebury, 1998). RA is recognized as an activator of retinoid X receptor (RXR), and retinoic acid receptor (RAR), responsive genes in adipocyte. In the same study, the effect of chylomicrons on ASP precursors showed that TTR stimulated an increase in C3 and potently increased the expression and secretion of both factor B and adipsin in human differentiated adipocytes (Scantlebury et al., 2001). In addition, the postprandial hyperchylomicronemia status surrounding the adipose tissue was shown to activate the complement pathway and accelerate C3 cleavage, resulting in over production of ASP (Fujita et al., 2007).

1.5.3 Role of ASP

ASP actively stimulates TG synthesis in fat-storing cells (Murray, 1999) through the esterification of fatty acids onto the glycerol backbone in adipocytes by increasing the activity of diacylglycerol acyltransferase (DGAT); the rate limiting

enzyme in TG synthesis. It also increases glucose uptake by enhancing translocation of glucose transporters (GLUT1, GLUT4) from intracellular sites to the plasma membrane surface (reviewed in (Cianflone et al., 2003)). Indirectly, ASP has been shown to enhance the activity of LPL in 3T3-L1 adipocyte by increasing the trapping of LPL-hydrolyzed NEFA within adipocytes (Faraj et al., 2004). ASP also mediates lipolysis by inhibiting HSL (Harmelen et al., 1999).

In animal studies, a C3 knockout (C3KO) mouse model, (ASP deficient mice), showed a delay in postprandial TG clearance after a fat load (Murray, 1999). This highlights the important role of ASP in the postprandial TG clearance. This effect was diminished by the administration of exogenous ASP, which accelerated the clearance of FFA and TG from the circulation after oral fat administration (Murray, 1999). A study by Saleh, reported that ASP administration enhanced postprandial TG clearance in obese rats supporting a role of ASP as a regulator of lipid metabolism (Saleh et al., 2001).

ASP-deficient mice have a reduced fat storage capacity, as illustrated by delayed postprandial TG clearance and decreased diacylglycerol acyltransferase (DGAT) activity (Paglialunga et al., 2008). The same study showed that by neutralizing the interaction between ASP and its receptor (C5L2), using antibodies, a delay in postprandial fat clearance was observed with a reduction in TG accumulation in adipose tissue and decreased LPL activity. Furthermore, treatment with anti-ASP showed increased body energy expenditure, oxygen consumption, muscle glucose oxidation and decreased glucose (glycogen) storage in the liver of mice (Paglialunga et al., 2010).

In healthy men and women, fasting ASP concentrations predict the rate of postprandial TG clearance; with higher fasting ASP levels linked to a delay in postprandial TG clearance (Cianflone et al., 2004). This delay, along with increased fasting ASP levels, has been suggested to represent a state of ASP resistance (Wen et al., 2008). Delayed fat clearance is also prominent in patients with genetic lipid metabolism disorders causing resistance in fatty acid uptake and storage (Meijssen, 2002). Marcil et al. showed dysfunction of ASP signaling related to the abnormal lipoprotein profile in familial combined hyperlipidemic subjects (Marcil et al., 2006).

1.5.4 ASP and metabolic disorders

In obesity and type 2 diabetes, ASP levels have been shown to be chronically elevated (Cianflone et al., 2004). In various studies including both humans and rodents, fasting blood ASP levels are shown to be elevated in obesity and associated metabolic disorders (Roy et al., 2008, Paglialunga et al., 2008). ASP concentrations decrease with weight loss and return to normal levels in obese women who lose weight (Faraj et al., 2004). Plasma C3, adipsin and factor B were also found to be decreased with weight loss (Hernandez-Mijares et al., 2012, Pomeroy et al., 1997, Lo et al., 2014). In obese subjects, higher ASP concentrations are clearly correlated with high BMI and body fat (Koistinen et al., 2001, Maslowska M et al., 1999, Weyer and Pratley, 1999) . In patients with acute coronary heart syndrome, ASP correlated with gamma-glutamyltransferase (GGT) that is identified as an independent cardiovascular risk factor (Saleh et al., 2013).

In a correlation study by Jiang et al., ASP and C3 were both elevated in the plasma of subjects with coronary heart disease (CHD). This correlation suggested a possible link of ASP and C3 to CHD, probably due to delayed fat clearance aggravating dyslipidemia (Jiang et al., 2014). In diabetic subjects, ASP levels increased in association with an increase in its component proteins (C3, adipsin and factor B) (Koistinen et al., 2001). ASP levels and C3 were documented to be elevated in diabetic subjects regardless of obesity (Yang et al., 2006).

ASP in the circulation increases substantially in women with polycystic ovary syndrome (POS) (Oktenli et al., 2007), in the nephrotic syndrome (Ozata et al., 2002) and other metabolic disorders associated with hyperlipidemia and cardiovascular disease (Murray et al., 2000, Jiang et al., 2014). Patients with hyperapolipoprotein B also showed elevated plasma ASP levels with a dysfunctional ASP pathway suggesting ASP resistance (Zhang et al., 1998, Cianflone et al., 1990). Non-alcoholic fatty liver diseases (NAFLD) patients have been found to have increased plasma C3 and ASP levels that correlated with insulin resistance (Yesilova et al., 2005).

ASP resistance (as shown both *in vivo* and *in vitro*) has been described to be similar to insulin resistance. A dysfunctional ASP action by cellular resistance to ASP binding is related to the alteration in fatty acid flux and the deposition in adipose tissue (Faraj et al., 2004). Interestingly, ASP resistance was recently observed in association with a chronic administration of ASP. In addition, an insulin resistant state was initiated with this chronic elevation in ASP

concentrations, which was suggested to reflect the increase in plasma ASP concentrations in obese subjects (Munkonda et al., 2012).

1.5.5 C5L2 receptor

ASP can only stimulate TG synthesis upon direct interaction with a receptor in the adipocytes (Murray, 1999). In 2003 the C5L2 receptor (also known as GPR77 and C5a-like receptor2) was confirmed to interact with ASP in adipocytes (Kalant et al., 2003). It is a 337-amino acid protein belonging to the G-protein family of receptors that share a common topology that consists of 7 transmembrane domains embedded intracellularly and extracellularly. G-protein coupled receptors (GPCRs) are important regulators of many physiological processes that control energy homeostasis (Tao Y.X. et al., 2013). C5L2 mRNA expression was seen in adipose tissue, muscle, brain, liver and immune cells (Kalant et al., 2005, Cui et al., 2009). In adipocytes, the presence of C5L2 is required for an ASP response, but its specific function is not yet fully explored in the other cell types (Fisette and Cianflone, 2010).

C5L2 showed high affinity binding to C5a protein and, in addition, it has a distinct binding site for C3a protein (Cain and Monk, 2002). It was first shown by Kalant et al. that C3adesArg (ASP) binds to C5L2 receptor, with high affinity, but not to the C3a receptor using HEK transfected cells with both receptors. It was interesting to note that despite the affinity of C5L2 for C5a/C5adesArg, only C3a and ASP proteins stimulated TG synthesis in adipocytes (Kalant et al., 2003). The high binding affinity between C5L2 and ASP increased TG synthesis and uptake of glucose (Kalant et al., 2005). In C5L2-/-knockout mice studies, adipose tissue

shows no response to ASP stimulation, confirming C5L2 to be the bioactive receptor for ASP (Paglialunga et al., 2007).

As a member of the subfamily of C5a and C3a receptors, it would be expected that C5L2 receptor has a functional role in the immune system. In sepsis, C5l2 binds with high affinity to C5a, however the binding does not stimulate G-protein signaling pathway activation (Rittirsch D et al., 2008). Li et al. showed that the binding of C5a protein to C5L2 does not initiate signaling, leading to the hypothesis that C5L2 functions as a decoy receptor (Li et al., 2012). C5L2 competes with C5aR for binding of C5a, preventing C5aR activation by removing the active complement fragments (C5a) from the extracellular environment and therefore mediating inflammatory responses of C5a-C5aR (Ward, 2009). In mice, blocking C5L2 receptor with an antibody results in increased plasma IL-6 concentrations when compared to mice in which C5L2 was not blocked (Gao et al., 2005). Collectively, C5L2 functions as a counter-balance to the pro-inflammatory effects of C5aR and studies have indicated that the presence of C5L2 may be of benefit in inflammatory states driven by C5a in various conditions such as sepsis, cystic fibrosis and chronic obstructive lung disease (Gerard et al., 2005).

1.6 ASP-C5L2 pathway

The ASP-C5L2 pathway plays an important role in lipid metabolism (Marcil et al., 2006). The binding of ASP to C5L2 was demonstrated to initiate intracellular signaling cascade events in the activation and translocation of protein kinase C

(PKC) (Baldo et al., 1995). Maslowska et al. (2006) proposed an intracellular signaling pathway scenario between ASP and C5L2. ASP-C5L2 receptor interaction causes conformational changes in the Gβ/γ and Gα subunit leading to phospholipase C (PLC) and phosphatidylinositol 3' –kinase (PI3K) activation (**Figure 1.10**). PLC and PI3K release intracellular signaling molecules (IP3, DAG, and Ca^{2+}) that furthermore activate intracellular enzymes, including PKC, PI3K and cPLA2.

The activation of both PLC and PI3K pathways is important to accomplish ASP-TG synthesis in adipocytes. This stimulation is independent and different from insulin-TG stimulation due to the fact that PLC activation is only involved in the ASP pathway and not in insulin (Maslowska et al., 2006).

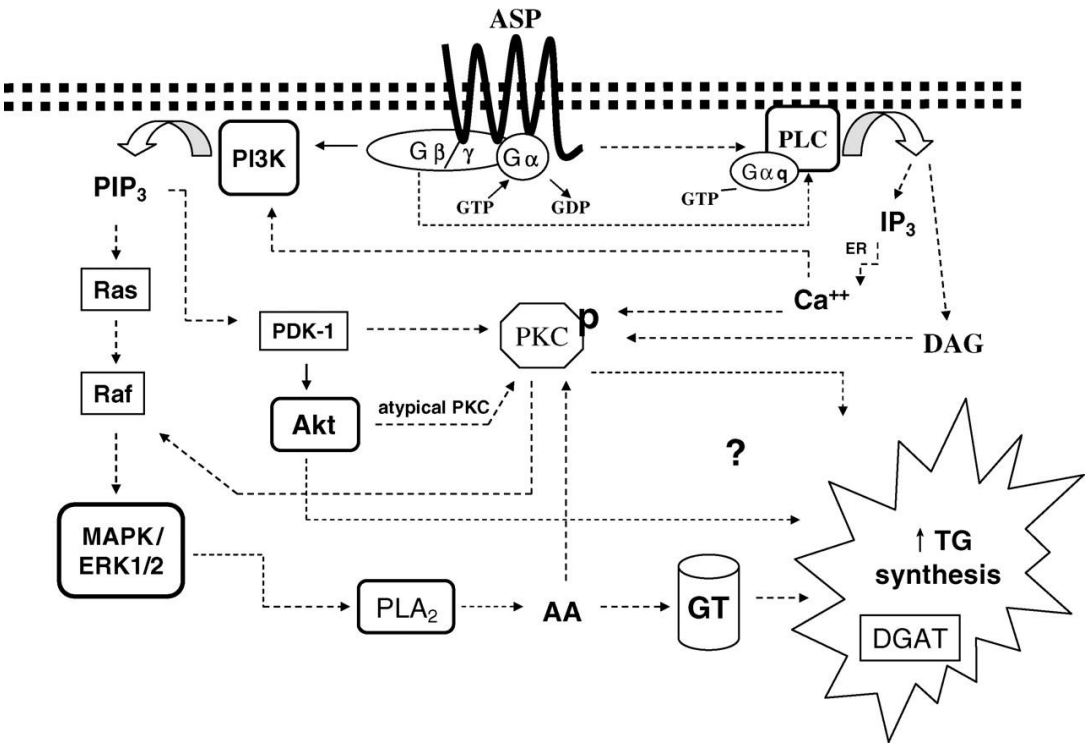


Figure 1.10 Proposed intracellular signaling pathway for ASP-stimulated TGs. AA; arachidonic acid, C5L2; ASP receptor, DAG; diacylglycerol, DGAT; diacylglycerol acyltransferase, ER; endoplasmic reticulum, GT; glucose transport, IP3; inositol 1,4,5-triphosphate, PDK-1; 3-phosphoinositide-dependent protein kinase-1, PIP2; phosphatidylinositol 4,5-biphosphate, PKC; protein kinase C (Maslowska et al., 2006).

1.6.1 Phospholipase C

Phospholipase C is a lipid metabolizing enzyme that regulates the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP_3). DAG activates protein kinase C, whilst IP_3 elicits the release of Ca^{2+}, followed by the activation of different Ca^{2+} -dependent pathways (Kayali et al., 1998, Putney and Tomita, 2012). In 3T3 adipocytes, the inhibition of PLC inhibited partially PI3K activity, and both components were shown to mediate glucose transporter activity (Kayali et al., 1998).

PLC-β is a member of the PLC enzyme family that has been linked to GPCRs (Litosch, 2002) . Upon GPCR activation, the G proteins subunits α, β and γ are dissociated and the Gα subunit binds to PLC-β to stimulate its activity (Litosch, 2002). G proteins-PLC-β activation promotes PIP2 hydrolysis and the mitogen activated protein kinase (MAPK) cascade (Santagata et al., 2001). PLC-β is regulated through a feedback mechanism mainly mediated by PKC phosphorylation (Yue et al., 2000).

1.6.2 Phosphoinositol 3 kinase

PI3Ks are part of a family of lipid kinases that phosphorylate the 3'-position hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns) (Dupont et al., 2012). PI3Ks can be divided into three different classes: class I, class II, and class III that differ in function and are regulated by several effector proteins (Jean and Kiger, 2014). Class 1 PI3K consists of one of catalytic subunit of p110α, p110β, or p110δ, a regulatory subunit referred to as p85 (Acosta-Martinez, 2011). P85-p110 complexes elicit signaling responses and recruit the kinase Akt, which controls multiple pathways (Vanhaesebroeck et al., 2012). Class I PI3K has different isoforms, with distinct functions mainly in cellular homeostasis and cross-talk with other signaling pathways (Jean and Kiger, 2014). P110α is an adipose tissue specific contributor in glucose and lipid homeostasis. The absence of p110α in mice (α-/-) results in a metabolic disturbance, including glucose intolerance and liver steatosis which influence body weight as a result of low energy expenditure (Knight et al., 2006). In addition to insulin, PI3K signaling

mediates the metabolic effect of hypothalamus-leptin action on glucose metabolism (Morton et al., 2005).

The PI3K and PKC pathways converge on MAPK/$ERK_{1/2}$ activation to stimulate calcium-dependent cytosolic phospholipase A2 (PLA2) phosphorylation (Sa et al., 1995). PLA2 stimulation is followed by increases in intracellular arachidonic acid (AA) that enhance PKC activity (Shinomura et al., 1991), and increase insulin-stimulated glucose uptake into adipocytes (Nugent et al., 2001). Maslowska et al. speculated that PLC, PI3K, PKC, MAPK/$ERK_{1/2}$ and PLA2 activation can lead to increased TG synthesis. Collectively, earlier studies showed that ASP increased glucose transporter (Yasruel et al., 1991) and DGAT activation (Cases et al., 1998) which, together, will maximize stimulation of TG synthesis in adipocytes.

1.7 Sex steroid hormones and ASP

Metabolic risk factors, including adipokines, have been shown to be different in men and women (Arrigo et al., 2011). As mentioned earlier, women have the tendency to accumulate fat subcutaneously with larger amount of fat mass compared to men who are more likely to accumulate fat in the abdominal region. Sex hormone and adipokine interactions are influenced by physiological changes such as pregnancy, aging and menopause (Stefańska and Odrowąż-Sypniewska, 2014). Different adipokines have different roles in appetite control and energy homeostasis. Similar to leptin, estrogen has been shown to reduce food intake, while testosterone stimulates appetite (Karim et al., 2014).

Studies with human adipose tissue have shown that the affinity for ASP binding was 3-fold higher in female subcutaneous fat compared with male subcutaneous fat (Saleh et al., 1999). Studies by Saleh et al., produced novel findings relating ASP to fat metabolism and hormonal changes in females (Saleh et al., 2009). ASP concentrations were found to strongly associate with fluctuating progesterone levels in females across the menstrual cycle whereas no association was found with insulin. In late pregnancy, ASP concentrations increased along with the natural increase of progesterone and estrogen hormones, and maternal TG levels strongly correlated with fetal birth weight (Saleh et al., 2007). Interestingly, cord blood ASP concentrations also correlated positively with fetal birth weight (Saleh et al., 2008). Elevations in ASP concentrations were seen in women with metabolic diseases that included hormonal disturbances such as polycystic ovary syndrome (Oktenli et al., 2007, Wu et al., 2009).

Based on these finding, Wen et al., (2008) investigated the potential effect of sex hormones on ASP function, using 3T3 cells. The findings showed that high doses of sex steroid hormones induced ASP production and reduced C5L2 expression, thereby causing an ASP resistance state, analogous to that found in mid and late pregnancy. Altogether, these recent findings suggest that ASP metabolism may be altered in association with sex hormones disorders.

1.8 Aims and Objectives

The prevalence of obesity has increased worldwide at an alarming rate over the last three decades. It is more pronounced in females than males (Badran and Laher, 2011, Lovejoy, 1998). As discussed in the previous sections, in mammals, adipose tissues are distributed as multi-fat depots across the body. Adipose tissue exerts a protective role, through its fat-storing capabilities, against lipotoxicity, that may result from increased free fatty acids and TG in the circulation. In addition, it produces a wide range of biologically active proteins, including adipokines, with varied functional roles (Greenberg and Obin, 2006, Balistreri et al., 2010). It is crucial to study and understand all newly emerging factors associated with obesity which might be part of therapeutic target in the future to prevent or reduce obesity.

A complex network of hormonal signals is involved in energy regulation and lipid metabolism (Mohamed-Ali et al., 1998). The relationship between sex hormones and adipose tissue distribution is not yet fully understood, despite the obvious association. ASP has attracted attention as it potently stimulates lipogenesis in adipocytes (Murray, 1999). Saleh et al., have reported novel findings relating ASP to fat metabolism and hormonal changes in females (Saleh et al., 2007, Saleh et al., 2009). Elevations in ASP levels were seen in women with metabolic diseases that included hormonal disturbances such as polycystic ovary syndrome (Oktenli et al., 2007, Wu et al., 2009). Recently, acute progesterone doses in ovariectomized rats were shown to enhance TG clearance after fat load (Al Riyami et al., 2014). The potential effect of sex hormones on ASP have been

investigated *in vitro* using 3T3 cells. High physiological levels of progesterone cause a resistance state similar to that of mid- and late pregnancy (Wen et al., 2008).

Based on the association between ASP and sex steroid hormones, the main hypothesis of this study was that female sex steroid hormones (progesterone and estrogen) affect the production of ASP and regulates related gene expression in different fat depots and therefore contributes to the fat distribution pattern fat load metabolism. It was also hypothesized that sex steroid hormones interact with ASP-C5L2 signaling pathway by estrogen and progesterone receptors. This hypothesis was addressed, firstly through an *ex vivo* study of adipose tissue explants from rodents, and secondly through *in vitro* studies using 3T3-L1 adipocyte cells.

1.8.1 Aims of *ex vivo* study

The release of metabolic factors, including adipokines, has been shown to be different in men and women (Arrigo et al., 2011). Affinity for ASP binding was three fold higher in female subcutaneous fat compared with male subcutaneous fat (Saleh et al., 1999). Sex hormones and adipokines interactions are influenced by physiological changes such as pregnancy, aging and menopause. As rodents exhibit a fat distribution that mimics humans, using explant fat pads will be an appropriate method to examine the effects of sex hormones on ASP production, function and receptor expression in regional adipose tissues.

The aim in this *ex vivo* study was to examine the direct effect of sex steroid hormones, at physiological concentration, on the ASP and related factors, released into the media of different fat depots (subcutaneous and visceral fat pads) obtained from ovariectomized rats. The expression of related genes in the tissue explants will also determine:

1. C5L2 mRNA (and protein) expression
1. Precursor's component: C3 mRNA, adipsin mRNA and factor B mRNA expression.
2. Lipogenic genes: DGAT1 mRNA and LPL mRNA expression.

This study also assessed the effect of the presence of chylomicrons. Chylomicrons are known to stimulate ASP production from adipose tissue *in vivo* and *in vitro* (Saleh et al., 1998, Gao et al., 2011). We investigated whether this stimulation is affected or modulated by the presence of sex hormones.

1.8.2 Aims of *in vitro* study

The 3T3-L1 adipocyte line has been shown to be a good model to investigate the impact of steroid hormones on the production of adipokines (Jung et al., 2013). Using cell lines like 3T3-L1 is an effective and less costly method to evaluate mechanisms involved in adipocyte metabolism (Poulos et al., 2010).

In three experiments; 3T3-L1 mature adipocytes will be used to examine the effect of female hormones (progesterone and estrogen) on ASP production and activity

Experiment 1: the direct dose-dependent effect of different female hormones (progesterone and estrogen) on ASP production, receptor expression and lipid accumulation in adipocyte.

Experiment 2: the time-dependent effect female hormones, in the presence of chylomicrons, on ASP production and lipid accumulation in cells at different time points.

Experiment 3: the interaction of progesterone and estrogen receptors on ASP-C5L2 signaling pathway using progesterone and estrogen antagonist treatment. The study aims to measure:

1. ASP production and C5L2 mRNA expression.
2. Intracellular TG accumulation.
3. PLC/PKCα mRNA and PI3K/AKT2 mRNA pathway components expression.
4. Lipogenic genes: DGAT1 mRNA, LPL mRNA and GULT4 mRNA expression.

CHAPTER 2. MATERIALS AND METHODS

2.1 Animal *ex vivo* tissue explants

2.1.1 Overview

The welfare of the animals and procedures carried out in this study were in accordance with the UK Home Office and the UK Animals (Scientific Procedures) Act (1986). The experimental procedures were completed under PROJECT LICENCE NO: 40/3598 and PERSONAL LICENCE NO 35774. The project protocol was approved by Animal Welfare and Ethical Review Body at BIO SUPPORT UNIT (BSU)-University of Nottingham. The animals were housed in a temperature controlled room (23°C), with a 12:12h light: dark cycle, and given free access to food and water.

2.1.2 Bilateral ovariectomy surgical procedure

8 week old female Wistar rats (n= 6) were ovariectomized (OVX) under general anaesthesia using isoflurane gas. All animals were pre-medicated with Metacam (2mg/kg) and Buprenorphine (0.05 mg/kg). The ovariectomy procedure was conducted as described by (Park et al., 2010). Anaesthesia was induced with 2.5% isoflurane and 1% oxygen supplied through a nose cone. Bilateral ovariectomy was performed by double dorsal lateral approach. After shaving the area, the locations of the ovaries were found by identifying the bulged area at the back of the abdomen when gentle pressure was applied to the middle of the spinal cord. A 1 cm skin incision was made and the skin was separated from the dorsolateral

abdominal muscle. Another incision was made to the muscles that allowed clear entrance into the peritoneal cavity. The layer of adipose tissue surrounding the ovary was gently displaced until the ovary and uterine tube were found. The ovarian tissue was carefully separated from the distal uterine horn by ligation and the ovary and surrounded tissue was completely removed. The remaining tissues were returned to the peritoneal cavity and the muscles and skin were sutured. Post-surgery hypothermia was prevented by controlling the cage temperature. Animals were medicated after the surgery with Metacam (2mg/kg) for 2 days. All rats were weighed throughout the recovery period and food intake, pain and stress were also monitored.

2.1.3 Tissue collection

The visceral fat pads refers to the combined omental and mesenteric fat depots which are found around the stomach and along the intestine respectively (Cinti, 2009). Mesenteric fat was dissected by stripping the intestine of fat starting from duodenum and ending by the colon. Subcutaneous adipose tissue was represented by the inguinal pads and the tissue was dissected from around the muscles. **Figure 2.1** shows the anatomic location of the extracted subcutaneous and visceral fat pads in rats.

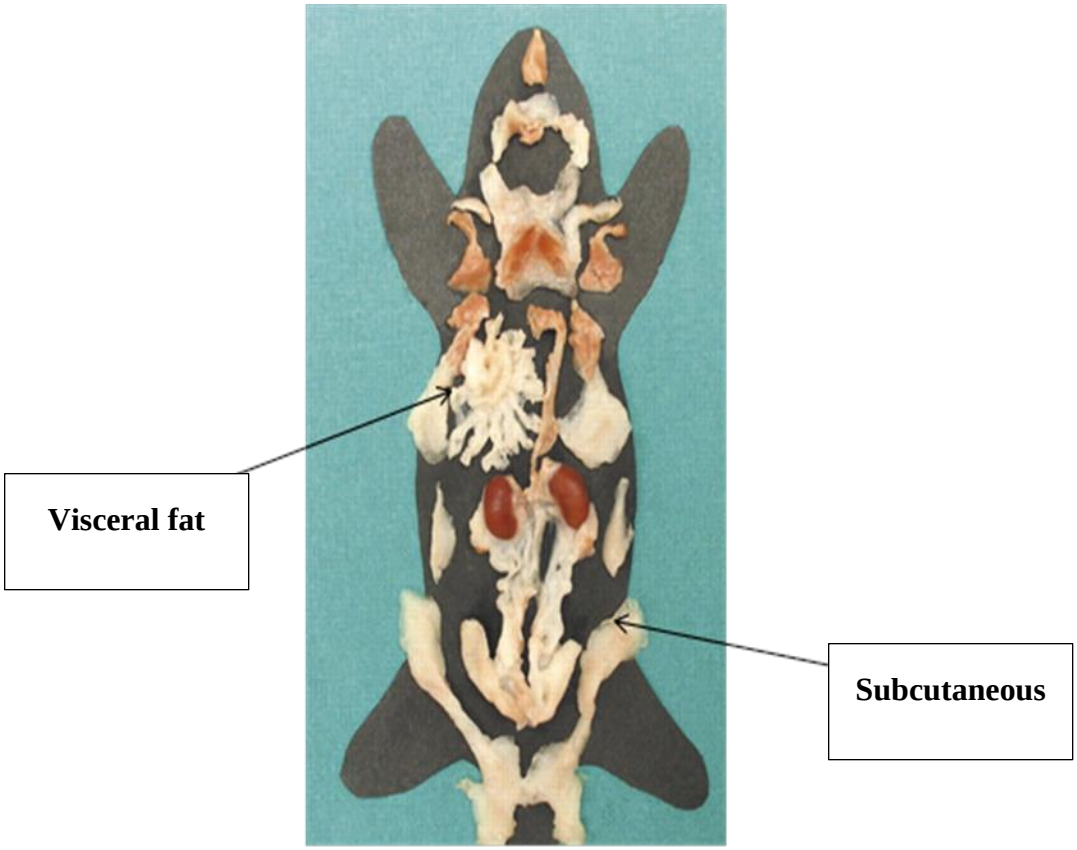


Figure 2.1 Subcutaneous and visceral fat pads anatomic locations in rat (Cinti, 2009).

Adipose tissue collected from these depots was cultured using the tissue explant protocol described in (Fried and Moustaid-Moussa, 2001). Two weeks after the OVX procedure animals were euthanized by raised $CO_{2,}$ followed by cervical dislocation. The animals were immediately dissected and subcutaneous and visceral adipose tissues were collected.

The tissue was transported from the Animal Unit in warm Hank's Balanced Salt Solution (HBSS, Gibco®). Under sterile conditions, in a tissue culture hood, the tissue was cleaned of connective tissues and visible blood vessels and finely minced into a size of ~1-2 mm^2. Tissue explants were then washed twice using

phosphate buffered saline (PBS) followed by final rinse with Dulbecco's Modified Eagle Medium (DMEM-F12) media (Life technologies). Tissue explants (weight ~ 100mg) were then cultured in 24- well plates with 300 µl of DMEM-F12 phenol free media. The media used did not contain phenol red as previous studies have shown this to have estrogen-like bioactivity (Xu Liu et al., 2013). The media contained 10% (v/v) Bovine serum albumin (BSA, Sigma Aldrich, UK), 1 % (v/v) penicillin- streptomycin 5000 U/ml (Gibco, UK) and hormone treatments as indicated. Explants were incubated at 37˚C in 5% CO_2 for 24 hours.

2.1.4 Hormone preparation

Sex hormones, progesterone, estrogen and testosterone, were purchased from Sigma. 1M stocks were prepared by dissolving the hormones in dimethyl sulfoxide (DMSO) according to manufacturer's protocol. Serial dilution of the stock was performed based on the requirement of each experiment using DMSO. In control groups, DMSO was added in equivalent amounts to the added hormone(s). Hormone stocks were stored at -20 ˚C in small aliquots. More details of the hormone concentrations used are provided in the materials and methods section of the *ex vivo* study (Chapter 3).

2.1.5 Media Analysis in tissue explant experiments

2.1.5.1 Rat C3a Enzyme-linked immunosorbent assay (ELISA)

ELISA is an antigen antibody reaction which is used to detect and quantify targeted substances in different samples. In this project, ASP was measured using rat C3a ELISA assay kit (Novateinbio©, USA). The technique used in this kit is a

sandwich ELISA and the general principle is described in Error! Reference source not found.. C3a monoclonal antibody was pre- coated on the provided microtiter plates. 50µL of the standard or media were added to allow C3a protein and the primary antibody to interact forming an antigen-antibody complex. To be able to quantify the amount of C3a protein in the sample, a second enzyme-linked antibody (horseradish peroxidase antibody (HRP-conjugated antibody)) was added and incubated for 1 hour at 37 ˚C. The primary and secondary antibodies form a "sandwich" and immobilize the targeted C3a protein on the plate. Then, the microtiter plate was thoroughly washed to remove all unbound components. Substrate solution (TMB) was added to each well and the enzyme (HRP) and substrate reacted together during short incubation period, of 15 minutes, at room temperature, avoiding light exposure. Only targeted protein and enzyme-conjugated antibody showed a color change. The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution (stop solution) and the color change was measured using a Bio-Rad microplate reader at a wave length of 450 nm.

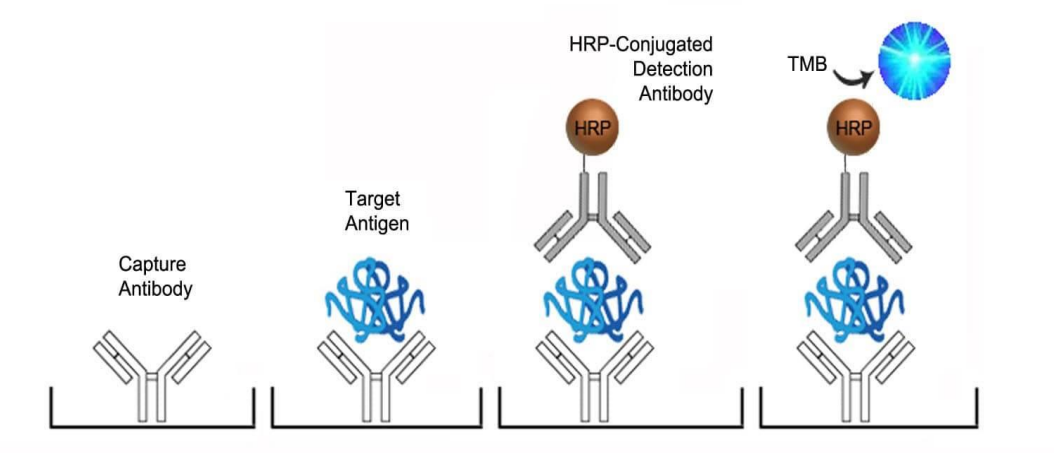


Figure 2.2 Sandwich ELISA principle (MyBiosource©).

A standard curve was used to determine the concentration of C3a in the media samples collected from tissue explants. Rat C3a ELISA standard curve raged from 25-800 µg/ml with a sensitivity of 5µg/ml was generated. **Figure 2.3** represents the standard curve with a regression co-efficient value of 0.95 and calculated average percentage of co-efficient variance value of 2.1%. C3a concentrations were determined directly from the standard curve and presented as µg /100 mg tissue weight.

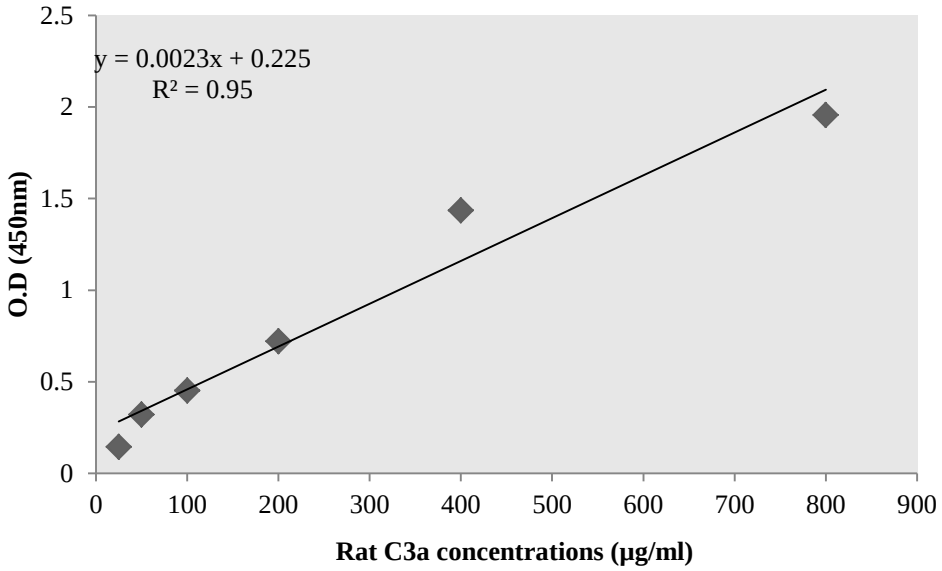


Figure 2.3 Rat C3a ELISA standard curve (R^2 =0.95, % CV= 2.1).

2.1.5.2 TG assay

The concentration of TG in samples of media was measured using TG assay kit. A range of standards, from 0 to 2 mg/ml, were prepared using SCal calibrator (Thermoscientific). 10 µl of standards/ samples were added to a 96 well flat bottom plate. 200 µl of Triglyceride reagent infinity (Thermoscientific) were added to each well. The plates were incubated for 15 minutes at 37 ˚C to and TG amount was determined using Bio-Rad plate reader at 550 nm. A standard curve was used to determine unknown concentration of different samples. Standard curve with concentrations of 0, 0.2, 0.4, 0.8, 1.2, 1.6, and 2 mg/ml was generated. **Figure 2.4** represent standard curve of TG assay (R^2 = 0.995) and with average coefficient of variance of 5.2%. In this assay Data Trol (Thermoscientific) were used with a reference value of TG measured between 0.8 and 0.91 mg/ml to

ensure quality and accuracy of the assay. Samples were diluted 1:5 and the obtained results were multiplied with the dilution factor.

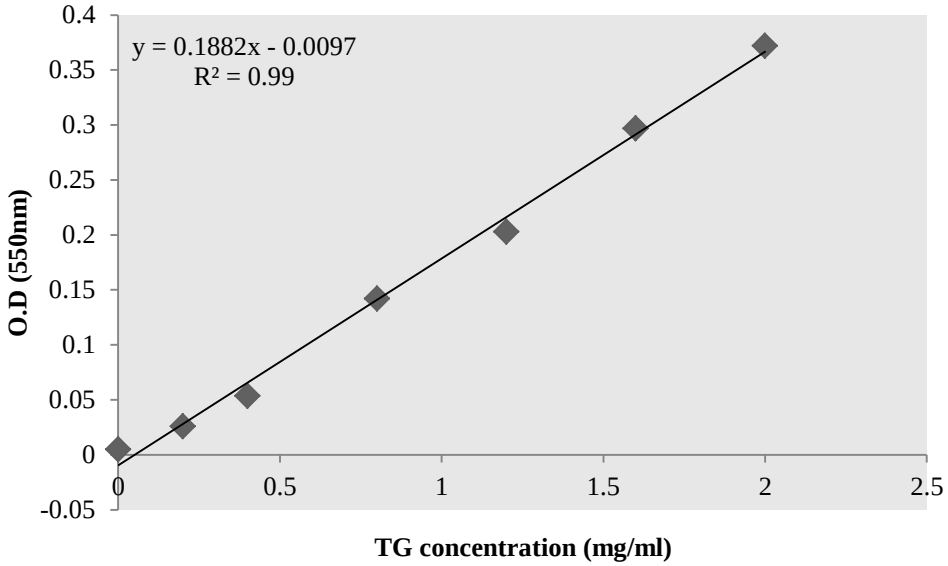


Figure 2.4 TG assay standard curve (R^2= 0.99, %CV 5.2).

2.2 Cell culture

2.2.1 Overview

For *in vitro* experiments 3T3-L1 cells were used. The 3T3 cell line was generated from mouse embryonic fibroblast cells and has been used extensively in biological research on adipocytes. Usually, preadipocyte 3T3 cells have a fibroblast-like morphology, however under certain conditions they differentiated into mature adipocytes that can accumulate TG (Green and Meuth, 1974). Although 3T3-L1 adipocytes provide no regional identity of the different fat depots, they are often used as a model to investigate secretory pathways and regulation (Millar et al., 2000, Melloni et al., 2013, Zeigerer et al., 2008).

2.2.2 3T3 cell culture, splitting and passage

3T3-L1 cells used in the study were originally provided by European Collection of Authenticated Cell Cultures and maintained within the Division of Nutritional Sciences, University of Nottingham with passage numbers between 8 and 12. 3T3 cells were cultured in T75 flasks using Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich, UK), 10% (v/v) Bovine serum albumin (BSA , Sigma Aldrich, UK), 1 % (v/v) penicillin- streptomycin 5000 U/ml (Gibco, UK) and 1% sodium pyruvate (Sigma, Aldrich) were added to the growth media. Preadipocytes cells were grown as fibroblast-like cells and maintained at a sub-confluent level to allow for serial passaging. The media were changed every 2 days through the growth of cells.

When cells reached confluence of ~80 %, the media was discarded from the flask and cells were washed with 10 ml of warmed PBS which was then discarded. To detach the cells from the flask, 2 ml of 0.25% Trypsin-EDTA (Invitrogen) solution (Invitrogen) was added and incubated at 37 ºC for 2 minutes. The presence of round floating cells, viewed under the microscope, was used to confirm detachment of all cells, and then 8 ml of growth media (DMEM, containing 10% (v/v) BSA, 1% (v/v) P/S and 1% (v/v) sodium pyruvate) was added to the flask to neutralize the trypsin-EDTA. The cell suspension was collected in universal tubes and centrifuged at 1000 rpm (centrifuge-Eppendorf 5702 R) for 5 minutes (4ºC). The supernatant was removed and cell pellet was then re-suspended in 10 ml of the growth media. 1 ml of the cell suspension was

transferred to a new T75 flask for further confluent growth or cells were counted in preparation for seeding.

2.2.3 Cell counting

While going through cell splitting, if cells were to be seeded onto a plate, preadipocytes in suspension were counted, in duplicate, using Luna-II™ fully automated cell counter (Biocompare, UK). 10 µl of the cell suspension were mixed with 10 µl (0.4%) trypan blue dye (Sigm, Aldrich, UK). 10 µl of the mixture were loaded into the counting slide and inserted into a cell counter. The results indicated the cell number and cell viability. Samples with at least 80% viability were used in seeding. For 12 well plates, 10,000 cells were seeded per well and maintained in 1-2 ml of growth media.

2.2.4 3T3 cell differentiation

After cells were seeded, the media were changed within 2 days of differentiation. Once the cells reached 80% confluence (Day 0), new media were added to the wells. On Day 2, preadipocytes were treated with differentiating media for 2 days. The differentiating media contained a hormone mix, consisting of 0.5 mM Isobutylmethylxanthine (IBMX), 250 nM dexamethasone and 170 nM insulin. Between days 4-12 the differentiating media, containing only insulin, was changed every 2 days. Throughout the cell differentiation period, pre-adipocytes start to change from fibroblast- like shape to rounded adipocytes as shown in **Figure 2.5**. The process of pre-adipocyte differentiation to mature adipocytes was monitored under the microscope from day 8 as cells started to accumulate lipid.

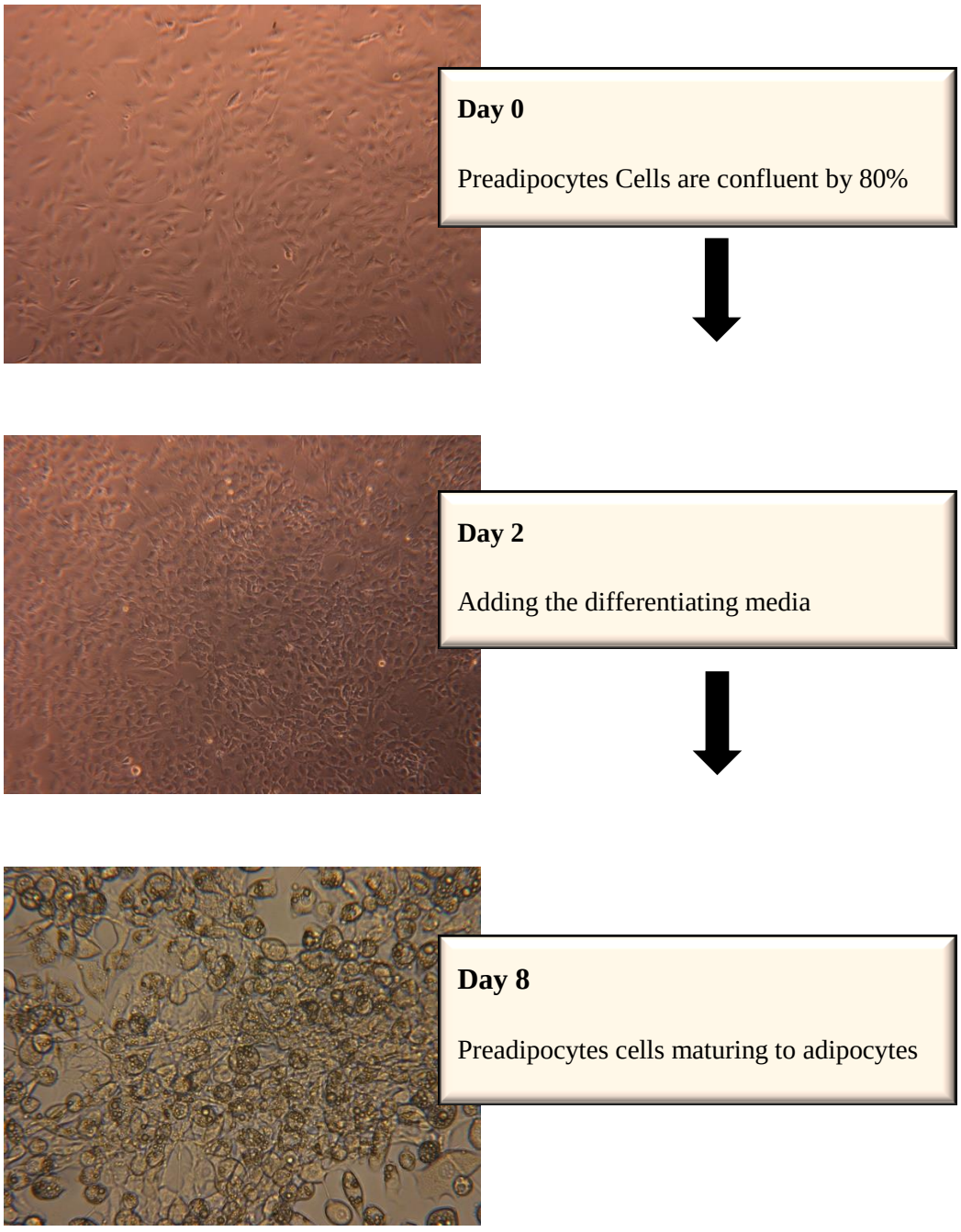


Figure 2.5 3T3-L1 cells preadipocytes' differentiating to adipocytes image was captured by light microscope at magnification of x100.

2.2.5 Cell freezing

For further use of 3T3-L1 cells, they were frozen in liquid nitrogen. During cell splitting (as in **section 2.2.2**), cells were re-suspended in 4 ml in fetal bovine serum (FBA), containing 10% (v/v) of DMSO (Sigma-Aldrich, UK). 1 ml of cell suspension was quickly transferred into cryovials and gradually frozen over night at -80 ºC at a rate of 1ºC decrease per hour using polystyrene insulation. The tubes were stored in liquid nitrogen for future use.

2.2.6 Hormone and antagonist preparation for cell culture study

Sex steroid hormones, progesterone and estrogen, used in cell culture were purchased from Sigma Aldrich and prepared as described previously in **section 2.1.4**. Hormones included in differentiation media were prepared according to manufacturer's protocol. Insulin (SAFC Biosciences ™, UK) was dissolved in cell culture grade water (Sigma Aldrich, UK). The insulin stock was initially adjusted pH to 2-3 with HCl and then re-adjusted with base buffer and the solution became clearer as it passed the isoelectric point (pH 5.3). The solution was sterile filtered using a filter of a pore size of 0.2 µm, before it was used in cell culture. Stocks of 50 mM of Isobutylmethylxanthine (IBMX) and 2.5 mM of dexamethasone were provided by the Division and required concentrations were prepared by the dilution in measured volumes of the treatment media.

The estrogen receptor α selective antagonist: MOO (1,3 Bis(4-hydroxyphenil)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride), and the estrogen receptor β antagonist: R,R-THC((R,R)-5,11-Diethyl-5,6,11,12-

tetrahyrro-2,8-chrysenediol) were purchased from Tocris Biosciences™. Mifepristone (Tocris Biosciences™) is a selective antagonist of the progesterone receptor with high affinity for progesterone. All antagonists were dissolved in DMSO and diluted in the media according to the required concentration.

2.2.7 Oil-Red O staining

This method was used to visualise lipid droplets in adipocytes by staining them with red colour. 0.5 % w/v of the Oil-Red O stock solution was prepared by dissolving 125 g of Oil Red O in 250 ml isopropanol and this was stored at – 20˚C. 0.3% v/v of Oil-Red O solution was prepared by diluting the 0.5% w/v stock solution with water (3:5) and the solution was filtered prior to the addition to cells. 3.7% v/v formaldehyde fixation solution was prepared by diluting 37% v/v formaldehyde, 10-fold in water. Both solutions were prepared immediately prior to staining the cells.

After the media was removed from the 6- wells plate, the cells were carefully washed twice with pre-warmed PBS avoiding any disruption of the cells. The cells then were fixed with formaldehyde solution for 30 minutes at room temperature. Cells were further washed with water and then 1 ml of the filtered Oil-Red O staining solution was added to the wells and incubated for 30 minutes at room temperature. The cells were washed twice with water. Lipid droplets appeared red under microscope. To quantify total lipid accumulation in cells, the Oil Red O stain was extracted by adding 500 µl of 100% isopropanol for 5 minutes into 12-well plate. 100% isopropanol was used as a background control to subtract background signal. Absorbance was measured at 492 nm.

2.2.8 Determination of Complement Fragment 3a and Complement Factor 3 in Cell Culture media

2.2.8.1 Mouse complement fragment 3a ELISA

C3a ELISA kit (Novateinbio, USA) was used to measure ASP level in the media of 3T3 cell cultures. A standard curve ranging from 15.6 -1000 pg /ml was prepared using a serial dilution of 10 ng of C3a stock provided with the kit. The assay sensitivity, as provided by the manufacturer, is down to 1 pg/ml. The regression coefficient value (R^2) was 0.95 and a calculated value of the percentage of co-efficient of variance (% CV) value was 3.6 as presented in **Figure 2.6.** The kit is based on standard sandwich enzyme-linked immunosorbent assay technology. Briefly, C3a specific antibody had been pre-coated onto the 96-well plate provided. The samples were added followed by the addition of biotinylated mouse C3a detection antibody. Streptavidin-HRP was added together with conjugated biotinylated antibody. Unbounded conjugates were washed away using the provided washing buffer. TMB substrate was used to visualize HRP enzymatic reaction. Finally, stop solution was added and the yellow colour was proportional to the amount of C3a in the media. The optical density of standards and samples was measured using a plate reader at a wavelength of 450 nm. From the generated standard curve, C3a concentration of samples was calculated. The results were expressed relative to total cell protein.

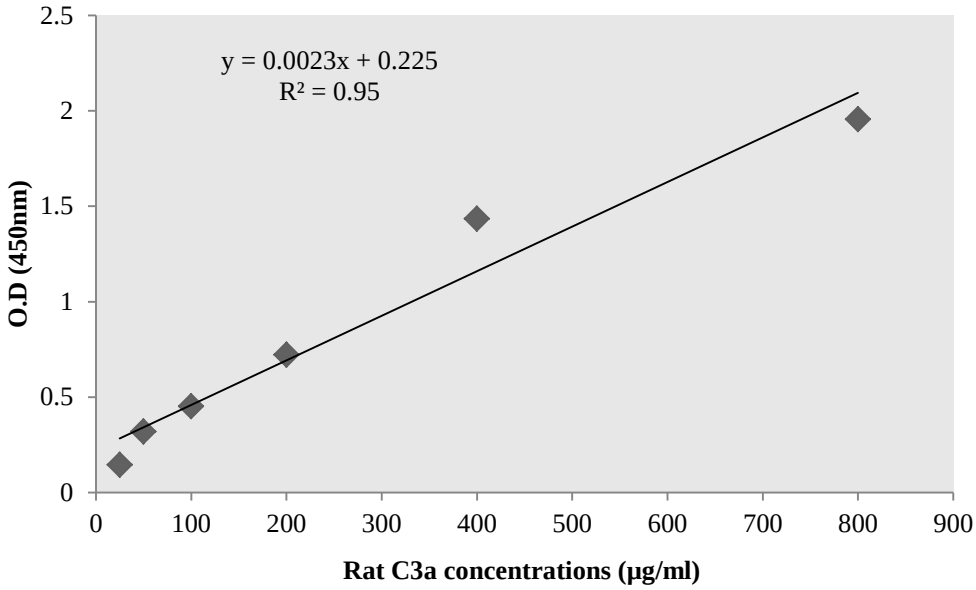


Figure 2.6 Mouse C3a ELISA standard curves (R^2= 0.95, %CV= 3.6).

2.2.8.2 Mouse complement factor 3 ELISA

Complement C3 plays a key role in the activation of alternative complement pathways. Mouse C3 ELISA (Novateinbio©, USA) was based on standard sandwich enzyme-linked immunosorbent assay technology. The standards ranged from 3.12-200 ng/ml and were prepared using a serial dilution of 200 ng/ml of C3 stock provided with the kit. The minimum detectable dose (sensitivity) of mouse C3 in the media was determined at 2.2 ng/ml and CV value of 5.8% as presented in **Figure 2.7.**

96-well plates were pre-coated with anti-mouse C3 specific antibody. Standards and samples were added to the wells, where C3 proteins were captured by the antibody. Subsequently, HRP conjugated anti-mouse C3 detection antibody was added and incubated at room temperature for 25 minutes leading to the formation of Ab-Ag-Ab sandwich. Unbounded conjugates were washed away, followed by

the addition of HRP substrate, TMB, resulting in the production of blue coloured product that changed to yellow after the addition of acidic stop solution. The intensity of the developed yellow colour was proportional to the amount of C3 in the media. The concentration of C3 in standards and samples was determined using a plate reader at 450 nm. The samples were diluted 1:2 and the concentration was multiplied by the dilution factor (x2). The results were expressed relatively to total cell protein.

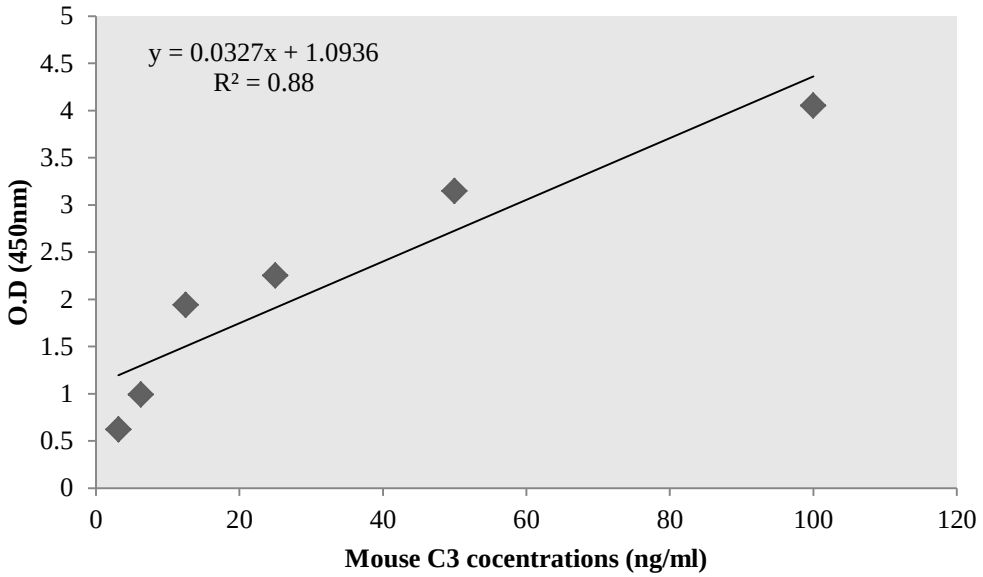


Figure 2.7 Mouse C3 ELISA standard curve (R^2=0.88, %CV= 5.8).

2.3 Isolation of Plasma Chylomicrons

The procedure was conducted in collaboration with the School of Life Sciences at the Queens Medical Centre (QMC) Nottingham with ethical approval (reference number: BT170320-16). In this study chylomicrons were isolated and added to

culture media to mimic postprandial changes in plasma TG following a meal. Dietary fats are digested in the lumen of small intestine, fatty acids absorbed and re-esterified into TG which is incorporated into chylomicrons (CM) before released into the circulation (Nauli et al., 2014).

50 ml of blood were obtained from a healthy subject, 3 hours following a fat-rich meal. Blood was collected on ice into vacutainer tubes containing EDTA anticoagulant. Plasma was isolated by low speed centrifugation (centrifuge-Eppendorf 5702 R) at 2800 rpm for 10 minutes at 4 ˚C. Chylomicrons were isolated from the plasma by preparative ultracentrifugation according to a modification of the method of (Havel RJ et al., 1955). 1.5ml plasma was pipetted into Beckman quick seal ultracentrifuge tubes. The tubes were then filled with 1.006g/ml KBr solution and sealed with Beckman Handheld Quick Seal tube topper. The tubes were balanced in 50.4 Rotor (Beckman Optima Ultracentrifuge XL-70) and centrifuged at 12000 rpm for 20 minutes at 12 ˚C under vacuum. The chylomicrons were recovered from the top 1 ml fraction by careful slicing of the ultracentrifuge tube using Kontron tube slicer. Triacylglycerol concentration was measured (in triplicate) using commercially available colorimetric enzyme assays as described in **section 2.1.5.2**

2.4 mRNA analysis

mRNA concentrations were determined by quantitative Real-time Polymerase Chain Reaction (qRT- PCR) method. The first step in this analysis was the extraction of RNA from tissue or cell samples, followed by reverse transcription

of RNA to synthesis cDNA. In qRT-PCR, cDNA for the gene of interest was amplified using specific primers and targeted mRNA was quantified by using standard curve method. Results were expressed relative to that of the house keeping gene; beta-actin.

2.4.1 Ribonucleic acid (RNA) extraction

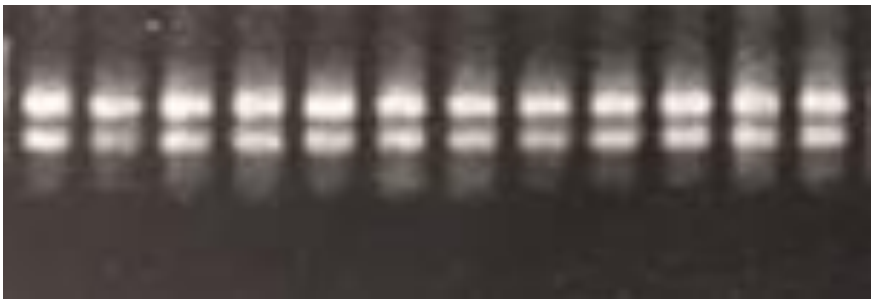
RNA is an unstable molecule that can degrade quickly after being extracted from the tissue (Siun and Beow, 2009). RNAlater stabilization reagent (Qiagen®) was used to store tissues and acts through quickly infusing into the tissues, resulting in immediate stabilization of RNA and preservation of RNA expression pattern. RNA in this study was extracted using RNeasy® Lipid Tissue Mini kit (Qiagen®). The procedure was conducted according to the instructions listed in the manufacturer's protocol.

Adipose tissue samples (~30 mg) were homogenized in 0.5 ml Qiazol lysis reagent using pestle pellet grinder. The homogenate was incubated at room temperature for 5 minutes. 100 µl of chloroform was added, mixed and centrifuged at 12,000 x g for 15 minutes at 4 ˚C. At this step, the homogenate solution was separated into aqueous and organic phase. The upper aqueous phase was transferred and mixed with 1 volume of 70% v/v ethanol. The mixture transferred to RNeasy mini spin column, centrifuged for 15 s at ≥ 8000 x g at 4 ˚C and the flow-through was discarded. At this step, the total RNA, bound to the column membrane, was treated with DNase I solution to remove any genomic DNA (gDNA) in the membrane. The treatment included a washing step by adding 350 µl of RW1 buffer followed by the addition of 80 µl DNase I solution

that was incubated for 15 minutes at room temperature. A further 350 µl of RW1 washing buffer was added and centrifuged at ≥ 8000 x g and this was followed by a final wash with 500 µl RPE washing buffer which was then centrifuged at ≥ 8000 x g for 2 minutes. Finally, the total RNA bound to the column membrane was eluted into a 1.5 ml tube by adding ~30 µl RNase- free water and centrifuged at ≥ 8000 x g for 1 minute.

2.4.2 RNA integrity and purity

RNA purity was tested using spectrophotometry (Nanodrop ND-1000, Thermoscientific). Nucleic acids absorb UV lights at 260nm and proteins absorb UV lights at a wavelength of 280 nm. Protein contamination in the samples could be measured through a ratio absorbance between 260/280nm (Fleige and Pfaffl, 2006). Sample ratios of 260/280 nm ranging from 1.8 - 2.0 suggested RNA was of sufficient purity. Total RNA integrity was examined by 1% w/v agarose gel non-denaturing electrophoresis where two clear bands of 28S and 18S could be observed in the RNA samples. 1% w/v of agarose gel was prepared by dissolving 1 g of agarose into 100 ml of 1x TAE buffer. The mixture was boiled and poured into gel apparatus and allowed to cool for 40 minutes. RNA samples (250ng/lane) were mixed with RNA loading buffer (50% glycerol, 1mM EDTA and 0.4% bromophenol blue) at dilution of 1:5 and loaded and the gel was placed in an electrophoresis tank and run at 100 V for 30 minutes. The gel was then placed in 1% w/v ethidium bromide solution for visualization. The bands (18S and 28S) in the gel for each sample were visualized under UV light and image was captured by Quantity-One software (Bio-Rad) (**Figure 2.8**).



F**igure 2.8** RNA integrity analysis by 1% agarose gel electrophoresis.

2.4.3 cDNA synthesis

A single strand complementary DNA (cDNA) of the RNA was synthesized via the action of reverse transcriptase enzyme prior to RT- PCR analysis (Freeman et al., 1999). RNA concentration was quantified using spectrophotometry (Nanodrop ND-1000, Thermoscientific). RNA was reverse transcribed to cDNA using first strand cDNA synthesis Kit (Roche®,USA). The template-primer reaction was prepared by adding 10 µl of total RNA (50ng/ µl), 2 µl of random hexamer primer (600 pmol/ µl) and 1 µl pf PCR-grade water. The process started with denaturation of the template-primer mixture by heating the tube at 65 ºC for 10 minutes using thermal block cycler. The next step involved the addition of 4 µl of 5 x reverse transcriptase reaction buffer, 0.5 µl of reverse transcriptase enzyme (20 U/ µl), 0.5 µl of RNase inhibitor (40 U/ µl) and 2 µl of deoxynucleotide mix (10 mM) to a final volume of 20 µl. All reagents were mixed carefully and heated through cycles of 25 ºC for 10 min and 55 ºC for 30 minutes. Finally, reverse transcriptase was inactivated by heating at 85 ºC for 5 minutes and then immediately placed on ice. cDNA samples used in q RT-PCR were all diluted 4 -

8 fold depending on optimization of the gene expression analysis. cDNA samples for each set of experiments were tested with validated primers to ensure the presence of cDNA and to prepare a pool of cDNA to be used as a standard curve in q RT-PCR.

2.4.4 Primers design and testing

Primers were designed using Primer Express v3.0 software (Applied Biosystems) using targeted species genome sequence from National Centre for Biotechnology Information (NCBI) online database. All primers were designed to avoid non-specific bindings by the span of exon/exon boundaries and to avoid any genomic DNA amplification. The primers were also design with a base length of 21 or less, optimum annealing temperatures no more than 5 ºC below the melting temperature and guanine-cytosine base pair content ranging from 40-60 %. (Mamedov et al., 2008) (Roux, 2009). Primers were acquired from Sigma Aldrich and stored at -20 ºC in at stock concentration of 100 µmol/l. All primers were diluted 1:10 before used in RT-PCR. Primers sequences used in each study are provided in the materials and methods section in each study. References will be provided for any primers used in previous publications.

A melting curve analysis was performed for all primers to ensure no primer-dimer products were produced in the reaction. The output of melting curve analysis preformed in qRT-PCR process is expected to show a single peak which indicates that no byproduct are produced due to non-specific binding or primer-dimer production as seen in **Figure 2.9**.

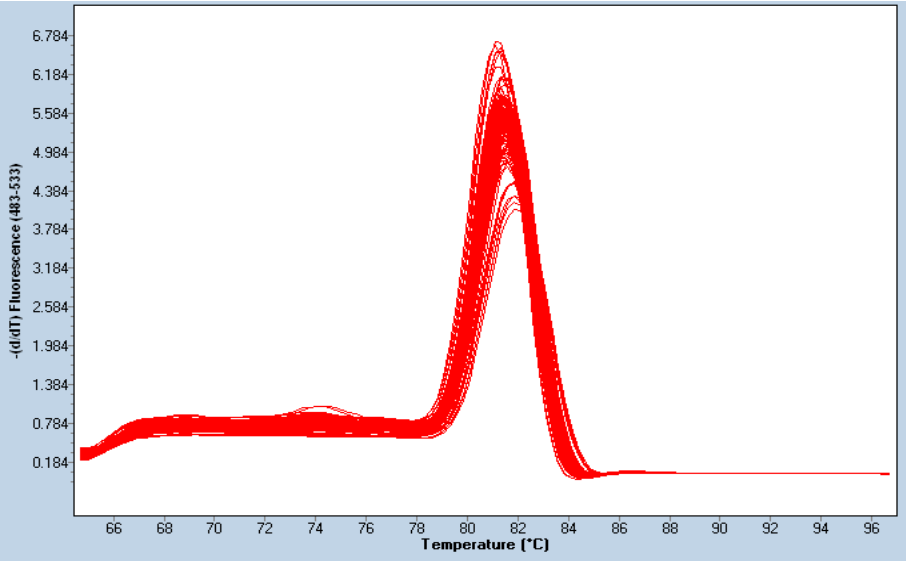


Figure 2.9 Representative melting curve analysis output of samples shows a single amplicon was generated in the PCR reaction.

2.4.5 qReal Time-PCR

qRT-PCR allows quantification of targeted mRNA in a sample using a pair of complimentary forward and reverse oligonucleotides primers in an enzymatic reaction. End-point product in RT-PCR was measured by the use of fluorescent intercalating dye (SYBR® green) (Freeman et al., 1999). PCR product was quantified by measuring a signal of fluorescence emitted by binding to double stranded DNA or cDNA. The threshold parameter was used to quantify and correlate the initial amount of targeted template. The amplification of the targeted sequence in the reaction in the repeated cycles is presented as a sigmoidal curve indicating the increasing fluorescent signal. The standard curve method was used to quantify the amount of amplified cDNA. The standard curve in this study was made up by serial dilutions of pooled cDNA samples. For relative gene

expression quantification, samples concentration was normalized for any variation, by relative expression of the ratio between the amounts of targeted gene to beta-actin (housekeeping gene).

RT-PCR was conducted using Lightcycler 480 (Roche, UK). In 384 well plate, the reaction included 5 µl cDNA, 7.5 µl SYBR®Green master mix (Roche, UK), 0.45 µl (0.3 uM) of forward primer, 0.45 µl reverse primer (0.3 uM) and 1.6 µl of RNase-free PCR water to a final volume of 15 µl per well. RT-PCR was programmed for each gene run by initial activation step of DNA polymerase enzymes including heating at 95ºC for 5 minutes. 45 cycles were performed, which involved denaturation for 10 minutes at 95ºC and combined annealing/ extension step for 30 seconds at 60ºC. The standard curve was produced by a serial dilution (1:2) of the cDNA pool with an efficiency ranged between 1.8 and 2.1 for every tested primer as seen in **Figure 2.10**.

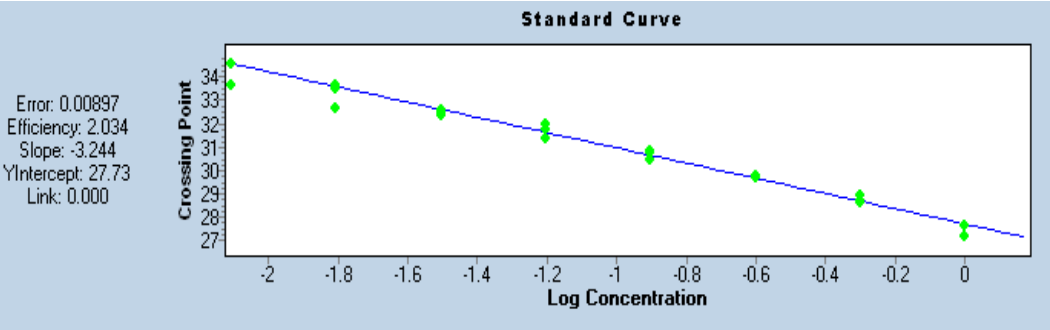


Figure 2.10 cDNA standard curve generated from pooled cDNA template by using serial dilutions (1:2). The representative standard curve shows an optimal efficiency of 2.03.

2.5 Protein quantification

Total protein in samples was quantified by 2-D Quant Kit (GE Healthcare Life Sciences) in *ex vivo* tissue explants and Lowry assay in cell culture studies. Protein concentration was determined by the change in colour for each sample that is proportional to protein concentration using this spectrophotometric technique.

2.5.1 Tissue protein extraction and quantification from adipose tissue

Protein was extracted from the adipose tissue samples using HNTG lysis buffer following protocol in (Syu and Saltiel, 1999). HNTG lysis buffer content is presented in **Table 2.1** and was made up to a final volume of 1 litre with distilled water. Prior to protein extraction, protease/phosphatase inhibitors (Protease Inhibitor Cocktail Set III, EDTA-free, Calbiochem®Diagnostics) were added to the lysis buffer to prevent any protease enzyme action on the samples.

Adipose tissue samples (from subcutaneous and visceral) were stored and frozen in RNAlater® solution. Approximately ~ 50 mg of adipose tissue was shredded by scalpel blade and homogenized in 300 µl of the extraction buffer using pestle pellet grinder devise. The homogenate was then centrifuged at 13000 rpm for 10 minutes at 4˚C. The supernatant was collected with the precaution to avoid the top layer of fat. In the case of disruption of the fat layer the sample was centrifuged again to collect the protein containing supernatant layer.

Table 2.1 HNTG protein extraction Lysis buffer.

Chemical	**Amount**
NaCl	150mM
HEPES	50mM
EDTA	2.5mM
% v/v glycerol	10
% v/v Triton	1

2.5.1.1 2-D Quant protein quantification

Protein concentration in extracted protein samples from adipose tissue was measured using the 2-D Quant Kit (GE Healthcare). The assay was performed according to the manufacturer protocol. A standard curve was prepared from a serial dilution using 2mg/ml bovine serum albumin (BSA) stock. In 1.5 ml tubes standards were added in concentrations of 0, 10, 20, 30, 40 and 50 µg/ml in duplicates. 30 µl of protein extracted samples were used in this assay. 500 µl of precipitant solution was added to each, mixed briefly by vortex and incubated at room temperature for 3 minutes. Next, a 500 µl of co-precipitant solution was

added, mixed briefly and centrifuged at 13 000 rpm for 10 minutes to sediment the protein from the mixture. The combination of precipitant and co-precipitant, precipitated proteins efficiently while avoiding interference from any contaminants. After centrifugation, the supernatant was discarded by careful pipetting so as not to disrupt the small protein pellets at the bottom of the tubes. The protein was re-suspended in alkaline solution of cupric ions by adding 100 µl of copper solution and 400 µl of distilled water to each tube. The protein pellets were mixed well with the solution by vortex. Finally, 1 ml of working color reagent was added to each tube and incubated at room temperature for 15-20 minutes. The color density is inversely related to the concentration of protein in the samples. Each sample was transferred to 1 ml cuvettes and the absorbance was measured using spectrophotometer with water as a reference at wavelength of 480 nm. From a generated standard curve the total protein concentrations were determined.

2.5.2 Determination of protein in cultured cells

Total cell protein from cultured cells was determined by Lowry assay. Protein was extracted from cells using HNTG lysis buffer (described in **section 2.5.1**). Cells were washed with warm PBS and scraped into 150 µl of lysis buffer. Cells in lysis buffer were homogenized using pestle pellet grinder for ~ 30 seconds and centrifuged at 13000 rpm for 10 minutes at 4 ˚C. The clarified supernatants were collected for further analysis.

2.5.2.1 Lowry assay

Before starting the assay, standard curve of BSA stock (5 mg/ml) ranged from 10 to 60 mg/ml of protein was prepared and used to determine unknown concentration of protein in cells. Prior to the assay, reagent 1 and reagent 2 were also prepared as in **Table 2.2**.

Table 2.2 Lowry assays reagent 1 and reagent 2 solutions content

Mixture	Content	Volume
Reagent 1	2% (w/v) Na_2CO_3 (in 0.1 M NaOH)	5 ml
	1% (w/v) $CuSO_4$	0.5 ml
	2% (w/v) KNa tartrate	0.5 ml
Reagent 2	Folin& Ciocalteu's phenol reagent	0.5 ml
	0.1 M NaOH	5 ml

In 96- flat bottom well plates, 50 µl of standard or sample were pipetted into the wells. 150 µl of 0.1M NaOH solution were added to make a total volume of 200 µl per well. 50 µl of reagent 1 was added and incubated for 5 minutes at room temperature. Subsequently, 50 µl of reagent 2 was added to all wells and left to stand for 20 minutes before the absorbance was measured by microplate reader (BioRad 680XR) at wavelength of 620 nm. Unknown concentration values were obtained from the constructed standard curve.

2.6 Western Blotting

Western blotting (or protein immunoblotting) is used to separate and quantify proteins based on molecular weight, through gel electrophoresis (Mahmood and Yang, 2012). In this study, the technique was used to measure C5L2 receptor protein expression in protein samples extracted from adipose tissue or cells. The process consisted of several steps of protein extraction and quantification (see **Section 5**): polyacrylamide gel electrophoresis, protein blot transfer, antibodies incubation and protein band detection and quantification. Western blotting was performed as described by (Flierl et al., 2009).

All samples were diluted with water to normalize to a concentration 15 µg of protein extracted from adipose tissues or cells. Due to the low protein content in the extracted samples, the samples were mixed with concentrated SDS-mix DTT (6 x; 5 volume of sample to 1 volume of SDS) with a final loading volume of 18 µl. 6x SDS was prepared and made up to 10 ml with water as in **Table 2.3**.

Table 2.3 SDS preparation (final volume: 10 ml).

Chemical	**Amount**
Glycerol	6 ml
1M Tris/HCl	3.75ml
SDS	1.2 g
DTT	0.462 g
Bromophenol Blue	Few grains

The samples and SDS mix were boiled to denature the protein and prevent any protein disulphide bond-cross-link. The samples were centrifuged at 13000 rpm for 3 minutes before loading.

2.6.1 Gel electrophoresis

The first step in Western blotting is to separate the proteins using gel electrophoresis. The gel running tank was filled with 1x Tris-Glycine electro-blotting buffer made from 10x buffer containing: 0.25mM Tris/ 1.92g glycine/ 1% (v/v) SDS (National Diagnostic, USA). The comb from a 4-10% mini Protean® TGX™ precast polyacrylamide gradient gel (Biorad) was removed carefully and clamped into the electrophoresis tank. Samples (~ 20 µl) and 10 µl of protein marker ladder (Precision Plus Protein Standard, Kaleidoscope colored, Biorad) were loaded into the wells of the gel and electrophoresis was run at 200V constant volts for approximately 30 minutes.

2.6.2 Western blotting

The next step involved the transfer of separated proteins in the gel to a Hybond ECL nitrocellulose membrane with 0.2 µm pore size (GE Healthcare). The nitrocellulose membrane and 4 sheets of 1mm filter paper were cut to approximate the same size as the gel. Prior to the blotting, the nitrocellulose membrane was activated by soaking in water for at least 10 minutes. After electrophoresis, the gel tank was dissembled and the gel was removed carefully and placed along with the nitrocellulose membrane, the filter papers and several sponges in Western blot buffer (or transfer buffer) for 5- 10 minutes. The transfer

buffer was prepared by mixing 60 g of glycine, 6 g of Tris base and 100 ml of isopropanol and the volume was made up to 2 liters with distilled water.

In holder cassette, a sandwich was prepared as showed in **Figure 2.11**. The gel was placed directly onto the nitrocellulose membrane and all air bubbles were rolled out. On either side of the gel-membrane, sponges and the filter paper were placed to firmly hold the membrane and gel. The clamp was placed in the Western blot transfer tank. Proteins migtated into the membrane when run at 200 mA (constant current) for 2 hours. Throughout the process the tank was kept cool by adding ice to the reservoir with constant stirring.

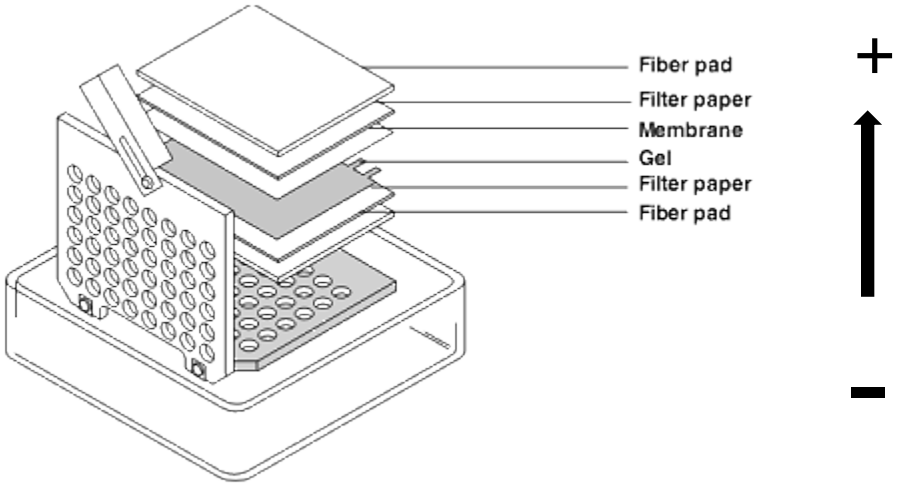


Figure 2.11 Schematic of transfer sandwich assembling for Western blotting.

After protein transfer, the membrane was washed using a washing buffer (T-TBS) that consisted of 200 ml of 1x TBS buffer (10x TBS: 90 g od NaCl, 200 ml 1 M Tris (PH 7.5) and the volume made up to 1 liter) mixed with 2 ml of Tween and made up to total volume of 2 liters. The membrane was then stained with diluted Ponceau S red stain to visualize and locate transferred protein bands. The stain was removed by washing with distilled water and T-TBS buffer.

The membrane was blocked using 2% (w/v) Amersham ™ ECL prime blocking reagent (GE Healthcare) that was mixed with TBS-T buffer for one hour at room temperature or overnight at 4 ºC. The membrane was then incubated for 1h at room temperature with primary rat polyclonal antibodies C5L2 (HP8018 Bycult Biotech, Germany) diluted 1:5000 with 2% (w/v) blocking reagent in T-TBS buffer. The membrane was washed with T-TBS buffer for at least 30 minutes which was replaced at least 5 times. It was then incubated with horseradish peroxidase (HRP)-labelled anti-rabbit IgG secondary antibody (GE Healthcare) at a dilution of 1:30,000 for 1h at room temperature. After washing the blot membrane for 30 minutes, the targeted protein was detected by incubation with enhanced chemiluminescence detection kit (ECL Selected™ Western Blotting detection Reagent, GE Healthcare) for 3-5 minutes. Beta actin protein was used as an internal control in Western blot. The blot (membrane) was treated with Restore Western Blot Stripping buffer (Thermo scientific) for 5 minutes and the membrane blocked again with 2% (w/v) of Amersham ™ ECL prime blocking reagent before using Beta actin primary antibody. Beta-actin monoclonal antibody (Sigma) was incubated for an hour at a dilution of 1: 10,000. The

membrane then washed with T-TBS for 30 minutes followed by the incubation with secondary horseradish peroxidase (HRP)-labelled anti-mouse secondary antibody at a dilution of 1:30,000. Proteins were detected using ECL Selected™ Western Blotting detection Reagent.

2.6.3 Detection

Based on the detection of light emission from secondary antibody that is linked to HRP, light emission was detected on x-ray films (Hyperfilm-ECL, GE Healthcare). Once the blot was incubated with the chemiluminescence detection reagents, the excess liquid was drained into filter paper and wrapped in plastic sheet protector. The wrapped membrane was placed in a film cassette and this process was carried in a dark room. In the dark room, a film was applied on top of the membrane and film was exposed for 1 to 3 minutes depending on the strength of the developed signals. The film then was developed by placing it in the developer solution for 90 secs, followed by rinsing it with water for 90 secs. Finally the film was placed in a fixing solution for 90 secs and allowed to dry. To obtain quantitative results Quantity-One analysis software was used to detect the targeted bands densitometry. The results were expressed relative to the internal β-actin control.

2.7 Statistics

All results are presented as mean ± standard error of the mean (SEM). Statistical analysis of data was performed using SPSS v22.0 statistical software. All data were subjected to the Shapiro-Wilk's normality test for parametric or non-

parametric distribution. Between groups statistical variation were analyzed by one way ANOVA, 3-way ANOVA and multivariate analysis (MANCOVA). When significant effects of the treatment were found; the analysis followed by mean comparison with Bonferroni post hoc, paired t test or Dunnett's Post hoc test. Pearson's test was used for correlation measurements of the effect of sex hormones on different biochemical parameters. Linear regression analysis was also performed to identify the main predictive factors. More details of statistical methods used are provided in the Chapters describing each of study. Statistical significance was set as $P < 0.05$.

CHAPTER 3. THE EFFECT OF SEX STEROID HORMONES ON ASP PRODUCTION AND RELATED GENE EXPRESSION IN *EX VIVO* OVARIECTOMIZED RAT'S ADIPOSE TISSUE

It was hypothesized that sex steroid hormones at physiological concentrations, will regulate ASP production and related gene expression in adipose tissue explants, in a depot-specific manner, and this may contribute to directing fat distribution. It was also hypothesized that the presence of chylomicrons, along with hormone treatments, may further modulate ASP production and lipogenic function in such adipose tissue explants.

3.1 Introduction

ASP has been demonstrated to have a great effect on lipogenesis by actively stimulating TG synthesis (Murray, 1999, Saleh et al., 1998). ASP can only stimulate TG synthesis through direct interaction with the C5L2 receptor in the adipocytes (Kalant et al., 2003). It increases the activity of diacylglycerol acyltransferase 1 (DGAT1) and glucose uptake, by enhancing translocation of glucose transporters (GLUT4) from intracellular sites to the plasma membrane surface (Cianflone et al., 2003). Indirectly, ASP increases the activity of lipoprotein lipase (LPL) by increasing the trapping of LPL-hydrolyzed non esterified fatty acid (NEFA) within adipocytes (Faraj et al., 2004, Kalant et al., 2000). It also reduces lipolysis by inhibiting hormone-sensitive lipase (HSL) (Harmelen et al., 1999). In a C3 knockout mouse study, delayed postprandial clearance was associated with the deficiency of circulating ASP (Murray, 1999). To date, the main known stimulators for ASP production are insulin and chylomicrons (Maslowska et al., 1997). Although the mechanism of the effect of chylomicrons on ASP production is not fully elucidated, it has been suggested to be mediated through retinoic acid (RA) and transthyretin (TTR) associated with chylomicrons (Scantlebury, 1998, Scantlebury et al., 2001).

Different hormones are involved in adipose tissue distribution including sex hormones, adrenal corticosteroids, growth hormone, insulin and adipokines (Bjorntorp, 1997). Sex hormone changes with age exert profound effects on adipose tissue metabolism and are associated with the differences in fat distribution between males and female (Bjorntorp, 1996). Sex hormones are

associated with the secretion of adipokines such as leptin and adiponectin from adipose tissue (Arrigo et al., 2011, Lizcano and Guzmán, 2014). A study using ovariectomized rats showed that progesterone and estrogen increased the level of leptin with a dose-dependent effect of progesterone (Rezai et al., 2011).

ASP has greater binding affinity for subcutaneous adipose tissues in females compared to males (Saleh et al., 1998). The studies of Saleh and coworkers have shown that ASP is regulated by fluctuation in female hormones, suggesting that ASP may work as a female lipogenic factor and therefore may be involved in fat distribution and metabolism in females (Saleh et al., 2009, Saleh et al., 2011). As rodents exhibit a similar fat distribution patterns to humans, explant tissue culture from OVX rats can be used to examine the cause and effect of sex hormones on ASP production -related gene expression in regional fat depots.

3.2 Materials and methods

In this section, the materials and methods are described briefly. Further details of each method and technique used are provided Chapter 2.

3.2.1 Animal experimental design

The concentration of ovarian steroids is expected to reach a nadir within 10 to 23 days after the OVX procedure (Murphy et al., 2013)**.** This procedure allowed to have more control of hormonal treatments and reduced the effect of *in vivo* circulating hormones produced by the ovaries. Two weeks after ovariectomy (OVX) rats were euthanized and adipose tissues were harvested. Subcutaneous and visceral adipose tissue explants were treated with different steroids hormones at physiological concentration, with or without chylomicrons (**Figure 3.1**).

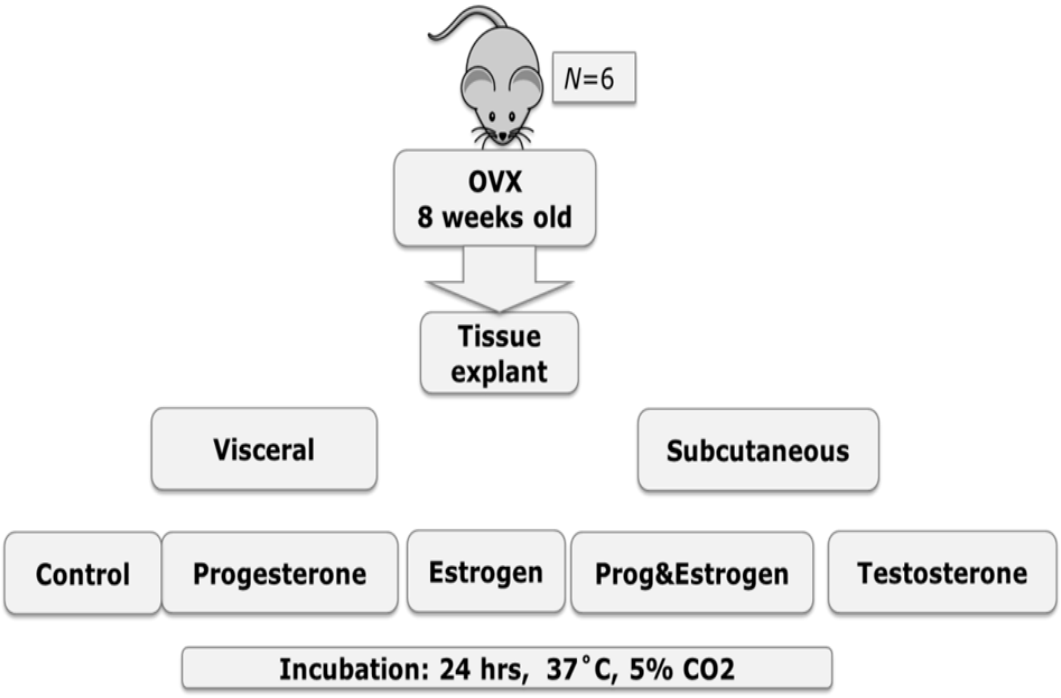


Figure 3.1 Experimental design for hormone treatments using tissue explants.

3.2.2 Tissue explants

The visceral (omental and mesenteric) fat pads are found around the stomach and along the intestine respectively (Cinti, 2009). Mesenteric fat was dissected by stripping the intestine of fat starting from duodenum and ending by the colon. Subcutaneous adipose tissue was taken from the inguinal pads and the tissue was dissected of the muscles.

Adipose tissue collected from depots was cultured using the tissue explant protocol as described in the literature (Fried and Moustaid-Moussa, 2001). Briefly, the tissue was transported from the animal unit in warm KBSS solution. The tissue was cleaned of connective tissues and visible blood vessels and finely minced (1-2 mm^2). Tissue explants were then washed twice using phosphate buffered saline (PBS) followed by final rinse with Dulbecco's Modified Eagle Medium (DMEM-F12) media (Life Technologies). Tissue explants (weight ~ 100mg) were then cultured in 24-well plates with 300 µl of DMEM-F12 phenol free media including 1% penicillin and streptomycin and 10% bovine serum albumin (BSA) with different hormone treatments and incubated at 37˚C and 5% CO_2 for 24 hours.

3.2.3 Hormone treatments

Tissue explants received treatments in two sets of experimental groups:

Group 1: Different hormone treatments (without chylomicrons).

Group 2: Different hormone treatments (with chylomicrons).

In triplicate, each tissue explant received one of the hormone treatments at the physiological levels as described in (Bendrik and Dabrosin, 2009, Wen et al., 2008) (**Table 3.1**).

Table 3.1 Hormone treatment concentrations in culture media.

Hormone treatments	Physiological level concentration
Progesterone	10^{-8} M
Estrogen	10^{-9} M
Progesterone & Estrogen	10^{-9} and 10^{-8} M
Testosterone	10^{-8} M

In 24 well culture plates, hormones and chylomicrons were added to the media according to the required concentrations in each experimental group. Chylomicrons were added at a concentration 100 µg/ml of TG, based on a previous study conducted by (Gao et al., 2011), where chylomicrons effects on ASP production were shown to be dose- and time-dependent. The aim of adding chylomicrons was to mimic lipaemia seen in the postprandial state. Each experiment included a control group that received a vehicle of DMSO. After 24 hours incubation, media was collected and stored immediately at – 80˚C for ASP production. Tissue explants were stored in RNAlater® at -80˚C for RNA and protein extraction.

3.2.4 Media analysis

3.2.4.1 Rat C3a ELISA

ASP was estimated using a C3a ELISA kit (Novateinbio, USA). C3a is rapidly converted to ASP (C3adesArg) due to the efficient action of carboxypeptidase

(Gupta et al., 2014), therefore, measuring C3a is representative of ASP level, taking in consideration the immediate and single use of thawed samples to avoid false generation of ASP in the samples. The technique applied in the ELISA was a quantitative sandwich immunoassay. The provided microtiter plate in the kit was pre-coated with rat's C3a monoclonal antibody; more details of the used protocol and generated standard curve are provided in the materials and methods (Chapter 2).

3.2.4.2 TG assay

This assay was used to determine the amount of TG in the collected media of tissue explants and in the chylomicrons fraction sample. All samples used were diluted 1:5 and 1:20 for media and chylomicrons respectively. The amounts of TG released from samples were measured using Triglycerides Infinity Reagent (Thermoscientific). More details of the generated standard curve found in chapter 2. For chylomicrons, TG concentration is presented as mg/ml, and in the media as mg /100 mg tissue weight.

3.2.5 Quantitative RT-PCR

Total RNA was extracted from ~ 30mg adipose tissue using an RNeasy lipid tissue mini kit (Qiagen®). RNA concentration was adjusted to 50 ng/µl. RNA was reverse transcribed to synthesize cDNA using Transcriptor First Strand cDNA synthesis Kit (Roche, UK). qRT-PCR was performed using Lightcycler 480® (Roche, UK). The reaction for each gene of interest was carried out in duplicate on 384 well plates. More details are described in materials and methods chapter

(Chapter 2). A standard curve (generated from pooled samples) was used to calculate gene expression determined by the ΔCT method and values were normalized to the house keeping gene beta-actin.

3.2.5.1 Primers design

Primers were designed using the primer analysis software Primer Express v3.0 (Applied Biosystems), based on available sequences on the NCBI Reference Sequence database and the Ensemble database. Melt-curve analysis for all primers sets were conducted and confirmed that no primer-dimers were produced in the absence of template cDNA.

The targeted genes in this study were all involved in ASP synthesis or function. The primer oligonucleotide sequences are presented in **Table 3.2**. C5L2 (also known as GPR77 and C5a-like receptor2) was identified as the ASP receptor (Kalant et al., 2003). The generation of ASP in the alternative complement pathway is mediated through the interaction of adipsin, factor B, and complement C3 in adipocytes (Cianflone et al., 2003).

Table 3.2 Oligonucleotides sequences for different genes in rats

Gene	Accession numbers	Forward sequence	Reverse sequence
C5L2	NM_001003710.2	5'GTCACCGCGGAATTACCA3'	5'TGGCCATGAACACCAATGG3'
C3	NM_016994.2	5' CCACTGTTTTCTATCGGATCTTCA 3'	5'TATTGGTTGTGGGAAGATAGAATGTC3'
Adipsin	NM_001077642.1	5' CTCTTTAAGCTCTCCCACAATGC 3	5' AGAGCGTGCCGGGTTTC 3'
Factor B	NM_212466.3	5' GGGATGCTCCGCCTGAA 3'	5' TGTTGTGCAAGCCATCAGTCA 3,
DGAT1	NM_053437.1	5' TCATCTTCTTCTATTGGCTTTTCCA 3'	5' AACTCGCGGTCTCCAAACTG 3'
LPL	NM_012598.2	5'GAGCCTGACCAAGAACTAAACTATGTACT3'	5' AAGCTCCCAGCACTCAGGAA 3'
β -actin	NM_031144.3	5' CGTGAAAAGATGACCCAGATCA 3'	5' CACAGCCTGGATGGCTACGT 3'

3.2.6 C5L2 protein expression

Western blotting was used to measure C5L2 protein concentration in adipose tissue samples as described by (Flierl et al., 2009). The process consisted of several steps including, protein extraction and quantification, polyacrylamide gel electrophoresis, protein blot transfer, incubation with antibody and protein band detection and quantification.

Adipose tissue samples (approximately 30 mg from visceral and subcutaneous) were homogenized in lysis buffer (protease/phosphatase inhibitors). Protein concentration was measured in each samples using 2-D Quant Kit (GE Healthcare). The assay was performed according to the manufacturer's protocol. Protein samples were prepared to a final concentration of 15µg and mixed with SDS solution. 20 µl of sample was loaded into 4-10% mini Protean® TGX precast polyacrylamide gradient gel (Biorad). The technique of Western blot is further described in more detail in Chapter 2. Protein bands were detected using chemiluminescence detection reagents. Film was developed and density of the C5L2 and beta-actin bands was quantified using Quantity-One analysis software. The results were expressed relative to the internal control (β-actin).

3.2.7 Statistical analysis

Parameters from adipose tissue taken from six OVX rats (with three biological triplicates, n=18) for each treatment were analyzed initially by 3-way ANOVA to determine any interaction effect of the independent variables; hormone treatments, chylomicrons and fat depots on the measured parameters. As tissue

explants were all obtained from 6 rats and used in triplicate for each treatment, the analysis was adjusted for different rats and therefore eliminating the possible effects of the biological differences of tissue explants between rats on the relationship between treatments, tissue depots and analyzed parameters.

Further analysis was carried out based on the output of the 3-way ANOVA results using multivariate analysis (MANCOVA) when hormonal treatment effect was found. In MANCOVA, different dependent variables (ASP and gene expressions) were analyzed based on the effect of the independent variable (the different hormone treatments) blocked for different rats as a covariant with advantage of reducing Type 1 errors. The main effect between treatment groups (pairwise comparison) was adjusted with Bonferroni post hoc test. 3-way ANOVA may highlight the general interaction effects, therefore more specific effect of hormonal treatments in relation to the different fat depots and the presence of absence of chylomicrons can be obtained through further analysis of MANCOVA.

When a significant interaction effect of depots or chylomicrons was found (no hormonal effect) as a result of the 3-way ANOVA analysis; paired t test was used to compare between the two fat depots (i.e. subcutaneous and visceral) or chylomicrons effect (i.e. with or without chylomicrons). Pearson correlation coefficient test was used to determine the association between all measured parameters. Linear regression analysis was also used to determine predictor

factors. All results in this study are presented as mean ±SEM and were analyzed using SPSS v23.0. Statistically significance was accepted at $p < 0.05$.

3.3 Results

3.3.1 Overview

In this *ex vivo* experimental study, results are presented for the effects of different hormone treatments on the production of ASP and related gene expression after 24 hours period of tissue culture. Fat tissues obtained from 6 rats and each treatment were applied in triplicate (total of n=18 for each treatment). The effects of hormones were examined in subcutaneous and visceral adipose tissue depots in the absence or presence of chylomicrons. ASP released into the media represents the production of ASP (µg/ mg) from 100 mg fat tissue. Gene expression for C5L2 receptor, precursors (C3, adipsin and factor B) and other lipogenic factors (DGAT1 and LPL) were measured by qRT-PCR and expressed as relative to the house keeping gene β-actin. C5L2 protein expression was measured by Western blot using C5L2 antibody and expressed relative to the house keeping (β-actin).

3.3.2 *Ex vivo* ASP production and C5L2 expression

3-way ANOVA indicated a highly significant interactive effect of depot site, chylomicrons and hormones on ASP ($F_{(4,263)} = 3.86$, $p < 0.005$) (**Table 3.3**). Subsequent analysis of the effects of hormones was therefore conducted independently for each depot (with and without chylomicrons) using MANCOVA analysis. It showed a significant effect of hormone treatments in subcutaneous adipose tissue explants without chylomicrons ($F_{(4,77)} = 3.37$, $p = 0.014$) and in the presence of chylomicrons ($F_{(4,78)} = 3.33$, $p = 0.015$). The primary effects were of the combination of progesterone and estrogen (P&E) treatment which increased

ASP levels in tissues without chylomicrons (p =0.006) when compared to estrogen group, but decreased it when chylomicrons were present (p =0.031) when compared to the control as shown in **Figure 3.2** (**A**) and (**B**). No effect of hormone treatments on ASP production in visceral tissue explants was seen in the absence of chylomicrons ($F_{(4,73)}$= 1.45, p=0.23) as shown in **Figure 3.2** (**C**). However, the effect of hormone treatments on ASP was augmented in the presence of chylomicrons ($F_{(4,67)}$= 7.67, p < 0.001). Significance was only apparent in the P&E treatment (p = 0.003) when compared to the control group (**Figure 3.2** (**D**)).

Table 3.3 Significance (*P*-values) of effects of depot, presence of chylomicrons and hormones and their interactions on ASP protein, C5L2 mRNA and C5L2 protein expression.

Effect	ASP	C5L2 mRNA	C5L2 protein
Hormone	0.008**	0.32	0.001**
Chylomicrons	0.12	0.96	0.001**
Depot	0.10	0.10	0.12
Chylomicrons*Hormone	0.09	0.03*	0.83
Depot*Hormone	0.02*	0.48	0.038*
Depot*chylomicrons	0.34	0.11	0.26
Depot*Chylomicrons*Hormone	0.005**	0.75	0.39

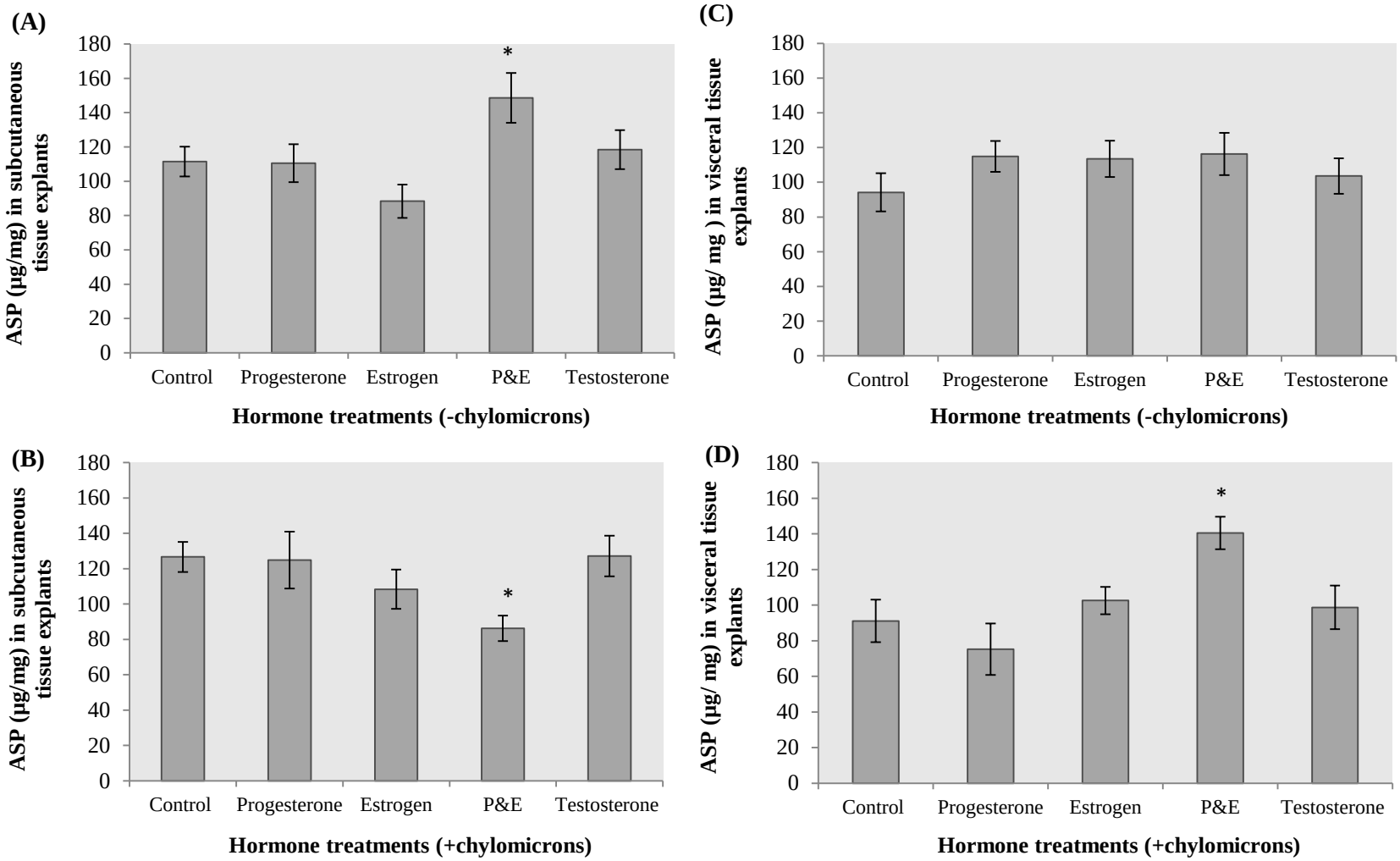


Figure 3.2 3-way ANOVA analysis showed a significant interaction of hormone treatments, fat depots and chylomicrons ($p<0.001$). Analysis was further carried by MANCOVA analysis. The effect of hormone treatments on ASP production in different adipose tissue explants cultured for 24 h in: (**A**) subcutaneous tissue explants (-chylomicrons) ($p =0.014$), P&E treatment increased ASP production in comparison to estrogen treatment group with $p=0.006$; (**B**) subcutaneous tissue explants (+chylomicrons) ($p =0.015$), P&E treatment decreased ASP production in comparison to the control group, $p=0.03$; (**C**) visceral tissue explants (-chylomicrons) ($p=0.23$); and (**D**) visceral tissue explants (+chylomicrons) ($p<0.001$), P&E treatment increased ASP production in comparison to the control group($p=0.003$). All data presented as mean± S.E.M (n=18).

C5L2 protein (n=4-5) was determined using Western blot technique for subcutaneous and visceral adipose tissue. A clear single band was visible for C5L2 receptor at ~60 KDa and for beta actin a band was seen at ~37 KDa. No significant variation was found between treatments, or tissues, in the beta-actin housekeeping gene expression as confirmed by ANOVA and beta actin normality and homogeneity were confirmed by Levene's test for all analysis.

3 way-ANOVA showed a significant interactive effect of hormones and depot on C5L2 protein expression ($F_{(4,67)} = 2.71$, $p=0.038$) (**Table 3.3**). Overall, subcutaneous adipose tissue expressed more C5L2 protein compared to visceral adipose tissue ($p= 0.005$, paired t test). Further analysis showed an increase expression in P&E treatment in subcutaneous (-chylomicrons) when compared to the control ($p=0.04$) as shown in **Figure 3.3** (**A**). Different hormone treatments showed no effect on C5L2 protein expression in subcutaneous tissue explants (+chylomicrons) or visceral tissue explants, in the absence of presence of chylomicrons (**Figure 3.3** (**B**), (**C**) and (**D**) respectively).

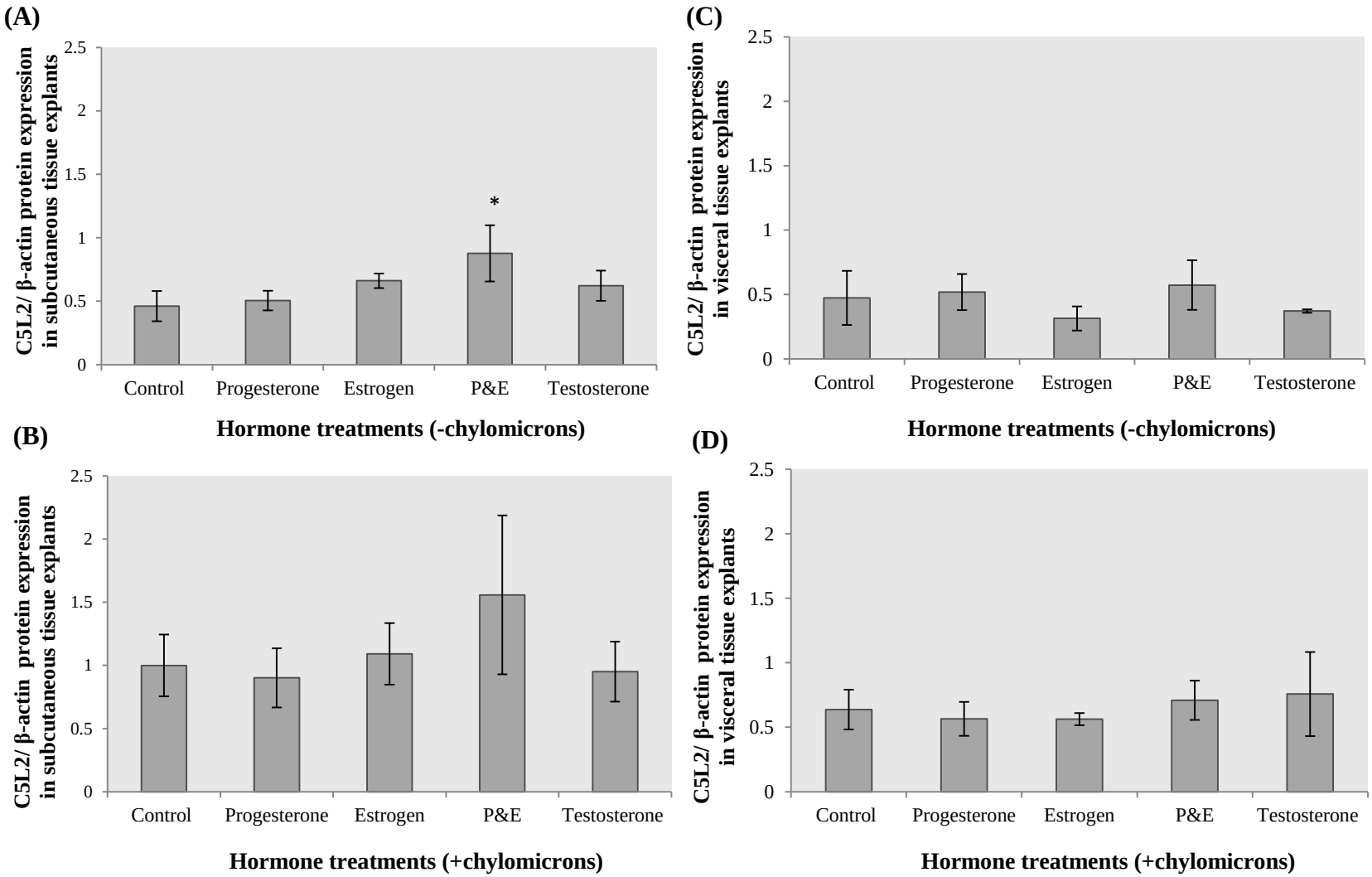


Figure 3.3 3-way ANOVA analysis showed a significant interaction of hormone treatments and fat depots (p=0.038). The Effect of hormone treatments (incubated for 24 h) on C5L2/β-actin protein expression was determined in: (**A**) subcutaneous tissue explants without chylomicrons, P&E treatment increased C5L2 protein expression in comparison to the control (p= 0.04); (**B**) subcutaneous tissue explants with chylomicron (p=0.7); (**C**) visceral tissue explants without chylomicrons (p=0.8); and (**D**) visceral tissue explants with chylomicrons (p= 0.9).All data presented as mean ±S.E.M (n=4-5).

C5L2 mRNA expression in subcutaneous tissue explants in the absence and presence of chylomicrons is shown in **Figure 3.4** (**A**) and (**B**) respectively, and in visceral tissue explants in the absence and presence of chylomicrons in **Figure 3.4** (**C**) and (**D**) respectively. C5L2 mRNA expression was mostly effected by the interaction of chylomicrons and hormones (p=0.025, 3-way ANOVA) (**Table 3.3**). As there was no depot effect on C5L2 mRNA, further analysis carried by MANCOVA for different hormonal treatments showed that treatment with chylomicrons in subcutaneous tissue explants was significant ($F_{(4,78)} = 2.1$, p=0.05). In the presence of chylomicrons, all hormonal treatments inhibited the gene expression of C5L2 in comparison with the control group; progesterone treatment (p= 0.02), estrogen treatment (p= 0.04), P&E treatment (p=0.02) and testosterone treatment (p=0.007), as shown in **Figure 3.4** (**B**).

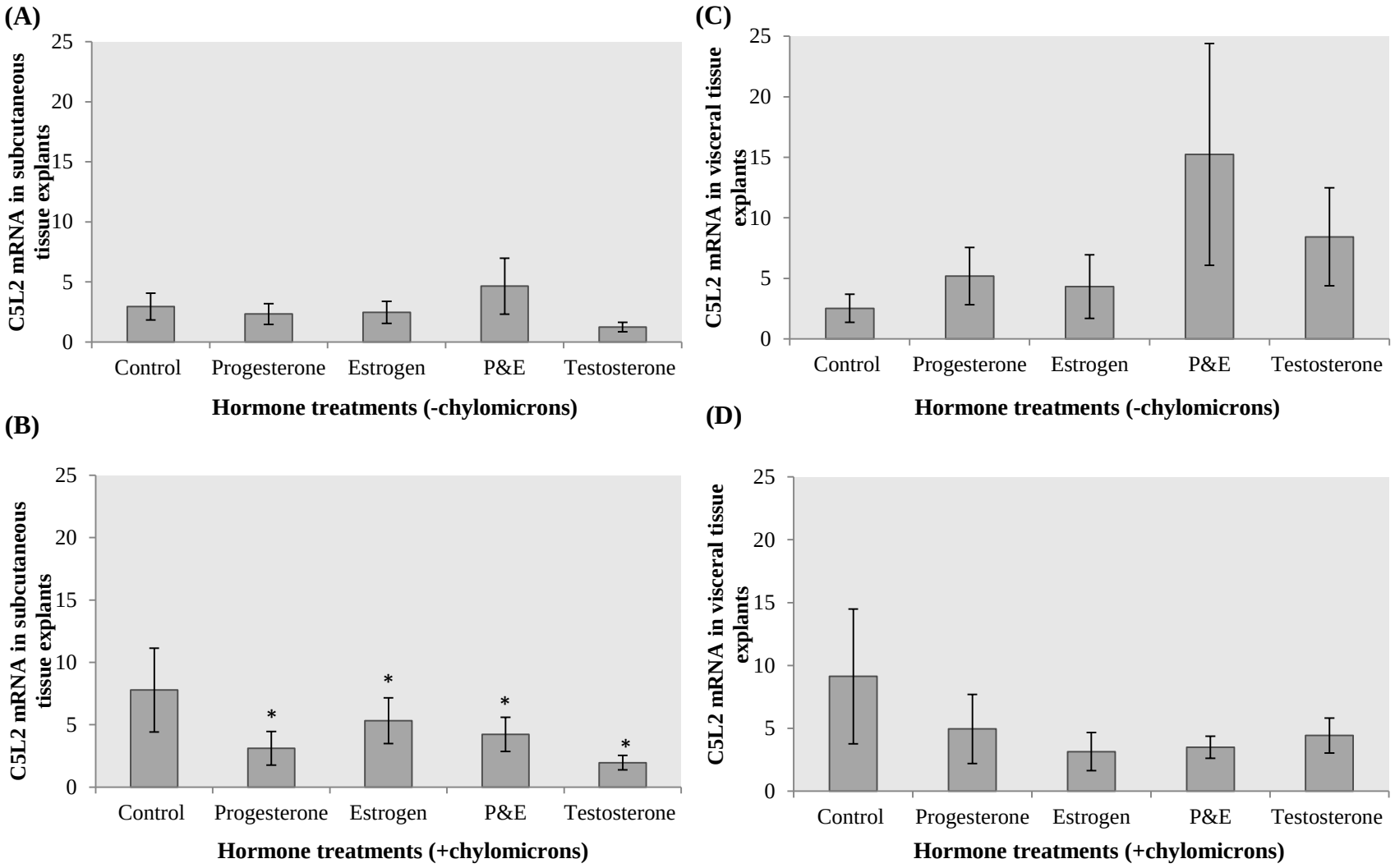


Figure 3.4 3-way ANOVA analysis showed a significant interaction of hormone treatments and chylomicrons (p=0.038). The Effect of hormone treatments incubation for 24 h on receptor C5L2 mRNA was determined in: (**A**) subcutaneous tissue explants without chylomicrons (p=0.5); (**B**) subcutaneous tissue explants with chylomicron (p=0.05); (**C**) visceral tissue explants without chylomicrons (p=0.4); and (**D**) visceral tissue explants with chylomicrons (p =0.7). All data presented as mean ±S.E.M (n=18).

3.3.3 Precursors (C3, factor B and adipsin) mRNA

Complement C3, adipsin and factor B are known precursors for ASP production. **Figure 3.5** shows the results of different fat depots with or without chylomicrons for (**A**) C3, (**B**) factorB and (**C**) adipsin. 3-way ANOVA analysis indicated an effect of depot and chylomicrons interaction on complement C3 ($p=0.005$), adipsin ($p <0.001$) and factor B ($p =0.01$) mRNA expression (**Table 3.4**).

Based on the findings, further analysis was carried using paired t test to compare fat depots and the presence or the absence of chylomicrons for each precursor. The presence of chylomicrons was found to suppress the expression of C3 in subcutaneous tissue when compared with subcutaneous (-chylomicrons) ($p <0.001$), and to visceral tissue (+chylomicrons) ($p <0.001$).

Similarly, overall expression of factor B decreased in subcutaneous (+chylomicrons) when compared to subcutaneous (-chylomicrons) ($p <0.001$) and to visceral (+chylomicrons) ($p =0.01$). The effect of chylomicrons was to increase the expression of factor B in visceral tissue explants ($p=0.003$), which is opposite to what we found in subcutaneous tissue explants.

Chylomicrons also suppressed the expression of adipsin in subcutaneous adipose tissue when compared to subcutaneous (-chylomicrons) ($p <0.001$) and to visceral (+chylomicrons) ($p <0.001$). In the absence of chylomicrons, subcutaneous tissue expressed more adipsin when compared to visceral tissue explants ($p <0.001$).

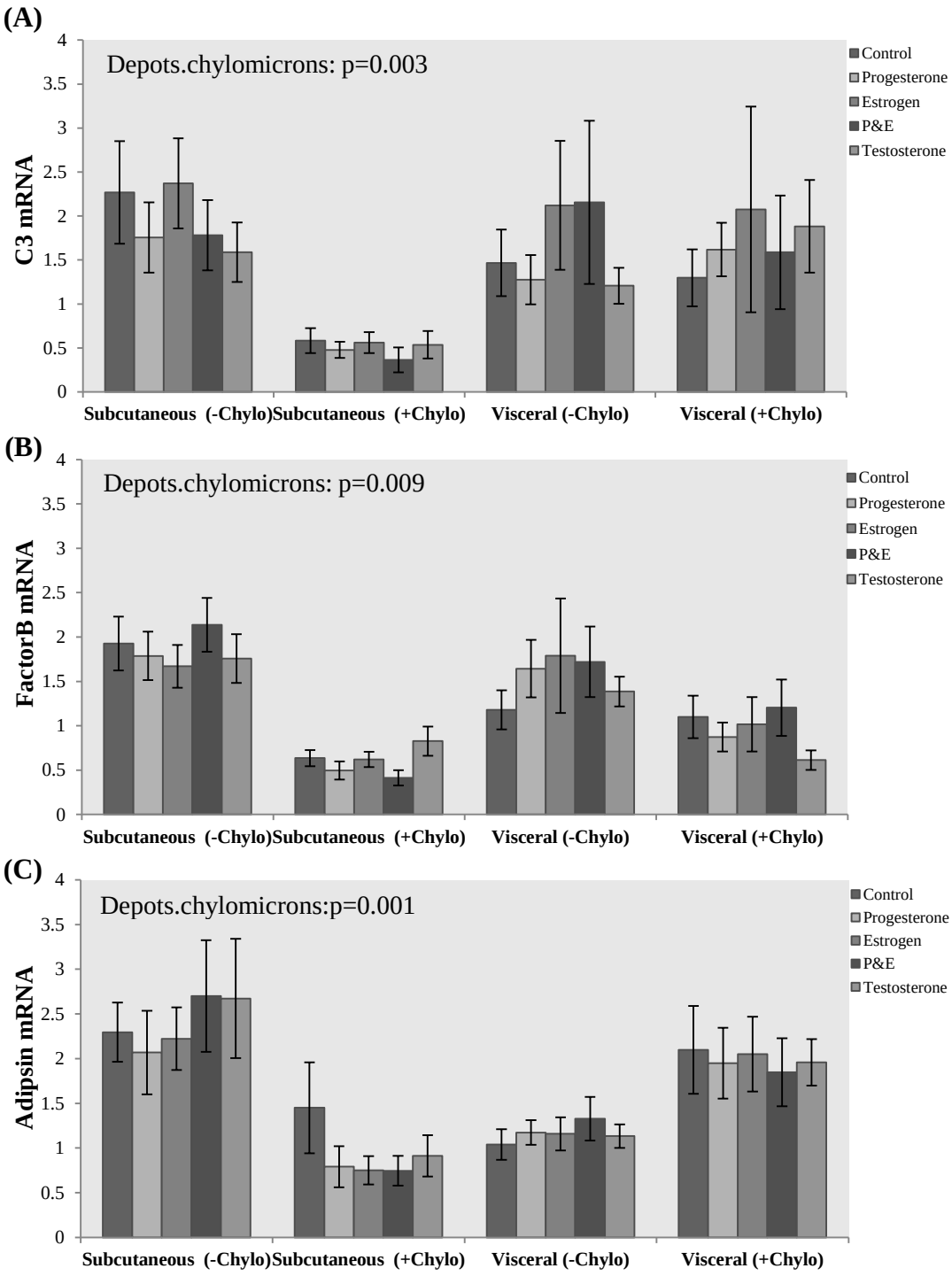


Figure 3.5 3-way ANOVA analysis showed a significant interaction effect of fat depots and chylomicrons on (**A**) C3 mRNA (p=0.003); (**B**) factorB mRNA (p=0.009); and (**C**) adipsin mRNA expression (p=0.001). Further details of 3-way ANOVA statistical differences and interactions are presented in **Table 3.4**. All data presented as mean ±S.E.M (n=18).

Table 3.4 Significance (*P*-values) of effects of depot, presence of chylomicrons and hormones and their interactions on C3 mRNA, factorB mRNA and adipsin mRNA.

Effect	C3 mRNA	FactorB mRNA	Adipsin mRNA
Hormone	0.79	0.89	0.84
Chylomicrons	0.01*	0.001**	0.23
Depot	0.02*	0.68	0.66
Chylomicrons*Hormone	0.81	0.96	0.56
Depot*Hormone	0.84	0.58	0.69
Depot*chylomicrons	0.003**	0.009**	0.001**
Depot*Chylomicrons*Hormone	0.92	0.67	0.84

3.3.4 DGAT 1 and LPL mRNA

In this section, the factors that influenced the expression of the lipogenic genes; DGAT1 and LPL were examined. DGAT1 mRNA and LPL mRNA concentration with different hormone treatments and tissue explants are presented in **Figure 3.6** (**A**) and (**B**) respectively**.** Overall, there was no effect of hormone treatments on gene expression in either depot. However, there was a significant depot and chylomicrons interaction on DGAT1 expression (p =0.05). Further analysis (paired t test) showed higher expression of DGAT1 in subcutaneous adipose tissue than visceral in the absence of chylomicrons of p value < 0.001. No interaction effect was found for the expression of LPL, therefore no further analysis was conducted.

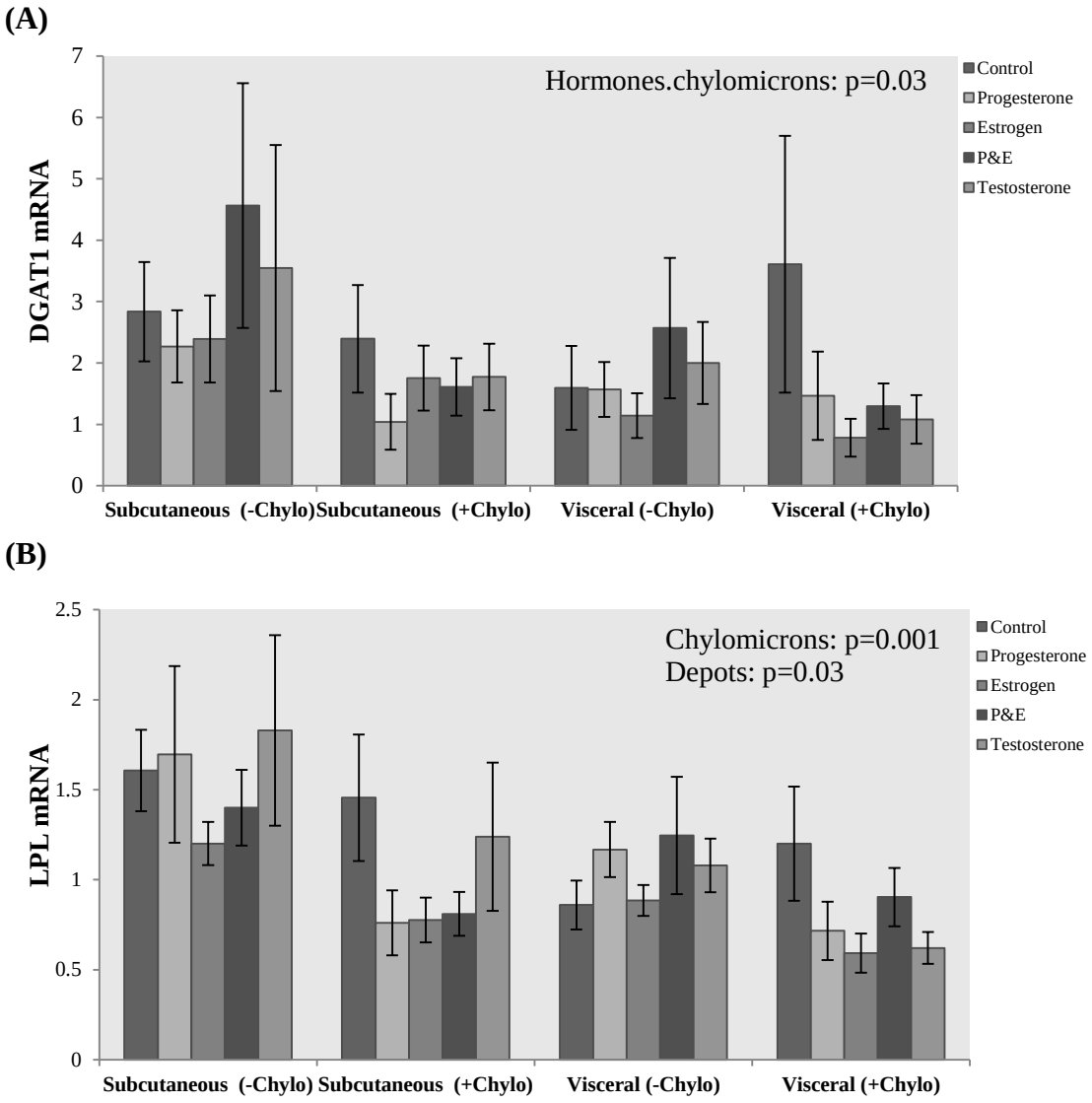


Figure 3.6 3-way ANOVA analysis showed a significant effect of: (**A**) chylomicrons and hormone treatments on DGAT1 mRNA (p=0.08) and (**B**) chylomicrons effect (p=0.001) and depot effect (p=0.03) on LPL mRNA. Further details of 3-way ANOVA statistical differences and interactions are presented in **Table 3.5.** All data presented as mean ±S.E.M (n=18).

Table 3.5 Significance (p-values) of effects of depot, presence of chylomicrons and hormones and their interactions on DGAT and LPL mRNA

Effect	DGAT1 mRNA	LPL mRNA
Hormone	0.36	0.25
Chylomicrons	0.08	0.001**
Depot	0.06	0.03*
Chylomicrons*Hormone	0.03*	0.22
Depot*Hormone	0.95	0.43
Depot*chylomicrons	0.09	0.11
Depot*Chylomicrons*Hormone	0.96	0.73

3.3.5 TG in tissue explant media

The effects of hormone treatments in the presence of chylomicrons upon TG levels in the media were measured in both depots. Fat depot affected TG concentration in the media ($F_{(4, 136)} = 60.03$, $p < 0.001$), as pairwise comparison showed more TG was found in visceral media (3.15 ± 0.093 mg/ml) compared to the subcutaneous TG level (2.21 ± 0.085mg/ml) with $p < 0.001$. MANCOVA analysis showed that different treatments had a significant effect in subcutaneous adipose tissue explants ($F_{(4, 78)} = 4.61$, $p = 0.002$). TG decreased significantly in the testosterone treated group when compared to the control group ($p = 0.002$) as presented in **Figure 3.7** (**A**). There was no significant effect of different hormone treatments on TG level in visceral adipose tissue ($F_{(4, 62)} = 0.56$, $p = 0.7$) (**Figure 3.7** (**B**)).

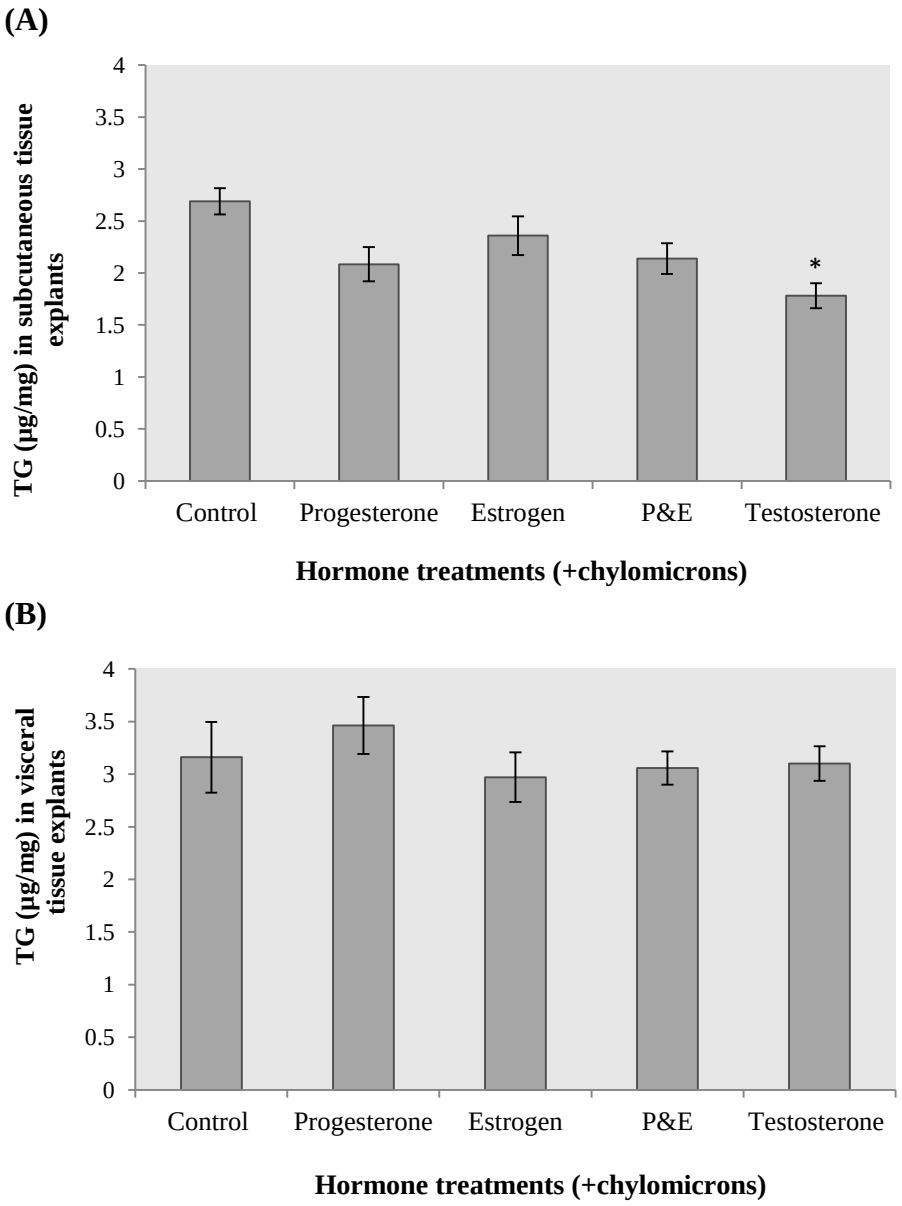


Figure 3.7 Hormone treatment effect on mean TG concentrations in tissues culture media (24 h) in the presence of chylomicrons (µg/mg) in: (**A**) subcutaneous tissue explants showed a significant effect on TG concentration ($p = 0.002$). TG testosterone treated group significantly lower than the control group (*$p = 0.002$); and (**B**) visceral tissue explants showed no significant effect of hormone treatments on TG concentration ($p = 0.7$). All data presented as mean ±S.E.M (n=18).

3.3.6 Relationship between expression of ASP, precursors and lipogenic genes

The relationship between different mRNA expression data sets was evaluated by Pearson correlation analysis, considering ASP, C5L2, precursors and lipogenic genes, with the control of hormone treatments, chylomicrons and different fat depots are presented in **Table 3.6**. Overall, there was no association between ASP and any of the other related genes. DGAT1 is an important gene in TG synthesis and it was positively correlated with C5L2 ($r=0.88$, $p<0.001$), adipsin ($r=0.46$, $p<0.001$) and LPL ($r=0.57$, $p<0.001$). In addition to its positive correlation with DGAT1, C5L2 gene expression correlated positively with Adipsin ($r=0.46$, $p<0.001$) and LPL ($r=0.57$, $p<0.001$).

Another important lipogenic factor involved with ASP function is LPL. LPL associated with ASP precursors positively, strongly with adipsin ($r=0.63$, $p<0.001$) and to a lesser extent with C3 ($r=0.27$, $p = 0.017$) and factor B ($r=0.23$, $p=0.04$)

Table 3.6 Pearson correlation matrix performed on ASP and related gene expressions.

	ASP	C5L2 mRNA	C3 mRNA	Adipsin mRNA	FactorB mRNA	DGAT1 mRNA	LPL mRNA
ASP	1.00						
C5L2	0.03	1.00					
C3	-0.17	-0.07	1.00				
Adipsin	-0.05	0.46**	0.19	1.00			
FactorB	-0.09	-0.002	0.79**	0.11	1.00		
DGAT1	0.04	0.88**	-0.06	0.45**	-0.02	1.00	
LPL	-0.10	0.57**	0.27*	0.63**	0.23*	0.69**	1.00

(*p< 0.01, ** p< 0.001)

A multiple regression analysis was performed with DGAT1 as dependent variable. DGAT1 mRNA expression was positively predicted by the expression of C5L2, adipsin and LPL. It also showed a degree of association with ASP with a P value of 0.06. DGAT1 showed a negative association with factor B (**Table 3.7**)

Table 3.7 Multiple regression analysis using DGAT1 as dependent variable

Variables	B	Std. Error	Beta	t	P-value
(Constant)	-2.17	0.47		-4.60	0.00
ASP	0.006	0.003	0.06	1.87	0.062
C5L2	0.09	0.01	0.27	7.15	0.00
C3	-0.04	0.10	-0.02	-0.39	0.69
Adipsin	0.38	0.14	0.12	2.76	0.006
FactorB	-0.52	0.18	-0.14	-2.91	0.004
LPL	3.29	0.24	0.62	13.74	0.00

3.3.6.1 ASP and C5L2 association analysis in P&E treatment

The main role of ASP is to clear fatty acids and stimulate TG uptake into adipocytes through its interaction with C5L2. The results showed that P&E treatment regulated the production of ASP from adipose tissue explants specifically when chylomicrons were added to the treatment media The relationship between ASP and C5L2 expression were further examined by correlation analysis due to the unexpected results of reduced ASP production in the P&E treated group in subcutaneous adipose tissue explants (in the presence of chylomicrons). ASP level was found to be negatively correlated with the level of C5L2 protein expression (r= - 0.89, p = 0.04) (**Figure 3.8**). Such negative association was not found in the control group (r= - 0.57, p = 0.32).

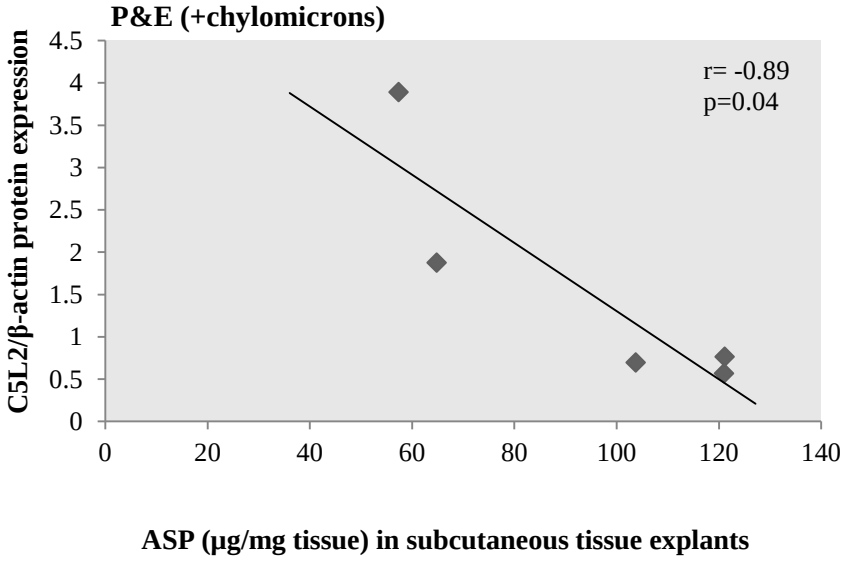


Figure 3.8 P&E (+chylomicrons) treated group in subcutaneous tissue explants correlation. ASP correlated negatively with C5L2 protein expression.

3.4 Discussion

Sex steroid hormones play an important role in the regulation of fat metabolism in adipose tissue. The effect of steroid hormone treatments on ASP production in the adipose tissue microenvironment is predicted to be more evident in adipose tissue cultures than in plasma. Several studies suggested that impact of ASP on lipogenesis is greater in women and in subcutaneous adipose tissue. Findings in this regard are collated in a review published by (Saleh et al., 2011) . In this *ex vivo* study, subcutaneous and visceral adipose tissue explants were cultured with different sex steroid hormones at physiological doses to investigate the direct effects of different conditional media treatments on ASP production and its association with expression of related genes.

The results demonstrated the significant effect of female steroid hormones in combination (P&E), interacting with fat depots and chylomicrons on ASP production. In this *ex vivo* study, subcutaneous and visceral tissue explants produced same amount of ASP in the controls. However, the results showed fundamental evidence of the stimulatory effect of the combination of progesterone and estrogen on ASP production in subcutaneous tissue explants, abut no effect of progesterone or estrogen alone. The addition of chylomicrons to P&E treated tissue reduced ASP production from subcutaneous tissue explants, but conversely increased the production from visceral adipose tissue. C5L2 protein expression and mRNA were found to be more responsive to hormone treatments in subcutaneous adipose tissue. C5L2 protein increased significantly in P&E (-chylomicrons) treatment in subcutaneous tissue explants. The gene expression of

the receptor was suppressed by the presence of chylomicrons. mRNA for ASP precursors C3, factor B and adipsin were all variable across the two fat depots and chylomicron treatment, with no effect of the hormonal treatment. The addition of chylomicrons inhibited the expression of the precursors only in subcutaneous tissue explants. mRNA for the lipogenic enzymes LPL and DGAT1 were not influenced by hormone treatment; however DGAT1 was expressed more in subcutaneous tissue explants and correlated strongly in this study with C5L2 and adipsin.

The results demonstrated a significant interactive effect of hormone treatments, fat depot and chylomicrons on ASP production. More specifically only the combination of female hormones (P&E treatment group) influenced ASP levels. P&E treatments increased ASP levels in subcutaneous tissue when only compared to estrogen group (**Figure 3.2** (**A**)). The effect of P&E in stimulating ASP production in subcutaneous adipose tissue is of an interest as the present of both hormones appears to stimulate ASP production into the media. Earlier findings suggested a relationship between progesterone and ASP in women during the menstrual cycle and a possible cooperation between ASP and progesterone in regards to a fat storage enhancement (Saleh et al., 2009). In pregnancy, ASP contributes to fetal lipogenesis and associates with pregnancy weight gain (Sodowski et al., 2008). Saleh et al. suggested that the hormonal changes during pregnancy may affect ASP's function as a lipogenic factor, reflected by the positive correlation between elevated ASP and TG in pregnant women (Saleh et al., 2007). This also supports our findings, as *in vivo* estrogen is always present in

the circulation and is synthesized by various tissues in addition to ovaries including adipose tissue (Nelson and Bulun, 2001). This may present a cross-talk effect between both hormones profoundly on ASP production. Such an effect was not seen in visceral tissue, reflecting the earlier finding that ASP stimulates TG synthesis to a higher extent in subcutaneous adipocytes and has a higher binding affinity and specificity to its receptor in subcutaneous adipose tissue, than in visceral adipose tissue (Walsh et al., 1989). The differences between males and females in body fat distribution may be related to differences in fat metabolism involving fatty acid mobilization, storage and oxidation (Comitato et al., 2015). As reviewed by Blaak (2001), females store postprandial fat in subcutaneous adipose more than men, and men tend to store more postprandial fat in visceral adipose tissues (Blaak, 2001). The fact that ASP production was increased in the presence of female hormones in subcutaneous tissue explants can explain part of the fat distribution mechanisms between males and females, and is consistent with the work of Rezvani et al., (2014).

Chylomicrons were added to the treatments media and control to mimic postprandial lipaemia. Takayuki and colleagues showed that the postprandial increase in chylomicrons activated the complement pathway resulting in ASP production (Fujita et al., 2007). Several studies (Saleh et al., 2011, Saleh et al., 1999) have suggested that ASP function is more influential in women, and specifically more in subcutaneous lipogenesis. Earlier findings supported the notion of increased ASP production in response to the postprandial fat (chylomicrons) (Saleh et al., 1998). Therefore, the physiological interaction of

chylomicrons with adipose tissue explants may not reflect the physiological interaction found *in vivo*. Fatty acids released from chylomicrons by the action of LPL, are taken up and converted into TG by adipocytes (Deckelbaum et al., 1990). Chylomicrons interact with ASP metabolism by either providing fatty acids for the uptake, or by direct interaction with adipocyte through retinoic acid (RA) and transthyretin (TTR) (Scantlebury et al., 2001, Scantlebury, 1998). Using tissue explants techniques in this study; chylomicrons interacted with only the outer surface area of the adipose tissue explants with minimal diffusion to capillaries and endothelium cells giving less interaction with LPL for hydrolysis and less stimulatory effect of RA or TTR. In addition time course factor is important to be investigating through shorter or longer incubational time as this was also shown to varies in different *in vitro* or *in vivo* studies (Saleh et al., 1998, Gao et al., 2011).

Unexpectedly, in the presence of chylomicrons with P&E treatment was found to decrease ASP concentrations significantly by 31.8% (P=0.03) in subcutaneous tissue explants compared to the controls (**Figure 3.2** (**B**)) while there was a significant increase by 54.23% in visceral adipose tissue explants (**Figure 3.2 (D)**). The presence of chylomicrons seems to modulate the effect of P&E treatment responses of stimulating ASP production in subcutaneous adipose tissue. The mechanism for this is unclear.

The effect of hormone treatments on C5L2 receptor expression was found to depend on chylomicrons or fat depot site at both the gene and protein expression level. C5L2 mRNA was suppressed by the presence of chylomicrons only in

subcutaneous adipose tissue in all treatment groups. This effect was not seen for C5L2 protein expression in the presence of chylomicrons. On the other hand, C5L2 protein expression was shown to be increased of in P&E treatment groups in subcutaneous tissue explants (**Figure 3.3**). Although there was no direct effect of progesterone or estrogen on the expression of C5L2, this work is the first to report a positive influence of the combination of both hormones (P&E) on C5L2 protein expression (without chylomicrons effect). *In vitro* studies using 3T3 cells, the addition of recombinant ASP and high doses of steroid hormones have suggested induction of an ASP resistance condition by suppressing C5L2 expression and this has been associated with altered adipose tissue function (Wen et al., 2008). Although we found no consistency in C5L2 gene and protein expression, there may be a negative feedback of high protein expression on the gene expression of the receptor regulated mainly in the P&E treatment group.

ASP synthesis is mediated by the interaction of C3, factor B and adipsin in the adipose tissues. Analysis showed that hormone treaments did not influence the expression of these precursors, and the precursors expression were more affected by the interaction of depot site and chylomicrons. Overall, the presence of chylomicrons suppressed the expression of C3, factor B and adipsin significantly in subcutanous adipose tissue. Without the influence of chylomicrons, adipsin protein was expressed more in subcutanous than visceral tissue explants. It was demonstrated earlier that the production of ASP was associated with increased mRNA expression of ASP precursors (Cianflone and Maslowska, 1995). In response to sex steroid hormonal effects on precursors expression, most reported

studies are based on association analysis. A study on obese females, reported a downregulation of the precursors adipsin and factor B expression in subcutaneous adipose tissues (Xia and Cianflone, 2003). Recently, in a cross-sectional study by Rezvani et al., the interaction between plasma ASP and ovarian hormones showed a regulatory effect on fat metabolism in depot specific manner (Rezvani et al., 2014). They found that progesterone was associated with C5L2 and C3 gene expression in adipose tissue, but not estrogen (Rezvani et al., 2014). Chylomicrons have been shown in a previous study to stimulate an increase in C3 and potently increase the expression of both factor B and adipsin in human differentiated adipocytes (Scantlebury et al., 2001). In contrast, the findings of the present work demonstrated that the stimulatory effect of chylomicrons was abolished by the presence of sex steroid hormones. The suppression of precursors in subcutaneous tissue explants (+chylomicrons) may partially explain the reduction of ASP concentration in P&E treatment groups, however this reduction was not shown to reduce the production of ASP in other treatment groups. In addition, correlation analysis controlled for various conditions in this experiment (**Table 3.6**) showed no relation between ASP and its precursors. However, further time course analysis needed to understand the participation of the precursors in the stimulation or the inhibition of ASP.

TG concentration in media was examined in subcutaneous and visceral adipose tissue in the presence of chylomicrons. The assay indicates TG concentrations indirectly by assessing glycerol concentration following a lipase mediated digestion of TG to release both glycerol and free fatty acids. It was anticipated

that the assessment of media TG concentrations would also reflect lipolysis of adipose tissue explants measured by the action of lipase to release glycerol (Du et al., 2011). The total glycerol concentration, therefore, considered to reflect total TG in the media. Overall TG (glycerol) concentration in subcutaneous adipose tissue media was significantly lower than visceral tissue explant media ($p=0.001$). The results of TG concentrations may also reflect the different physiological characteristics of subcutaneous and visceral tissue explants, with higher lipolytic activity in visceral adipose tissue (Wajchenberg, 2000).

Testosterone treatment led to a significant reduction in TG concentration in subcutaneous tissue ($p= 0.002$). In rodents, testosterone exhibits its lipolytic effect through the interaction with catecholamine and enhances the β_2-adrenoceptor signaling pathway (Arner, 2005, Xu et al., 1991). The difference between adipose tissues in response to testosterone has been more studied in humans, showing less lipolytic effects of testosterone in subcutaneous adipose tissue along with the less activity of β_2-adrenoceptor and HSL (Anderson et al., 2002, Large and Arner, 1998, Dicker et al., 2004). It was found that testosterone treatment in men redirected TG assimilation from visceral adipose tissue into subcutaneous adipose tissue after the intake of fat load (Marin et al., 1996). Studies of women with PCOS and elevated testosterone levels correlated with developed obesity. To date these studies were investigated in humans but not in rodents. By using rat adipose tissue explants in the present study, anti-lipolytic effect of testosterone hormone in adherent to subcutaneous fat tissue supported by earlier studies (Large and Arner, 1998). In the testosterone treated group, ASP levels correlated negatively

with media TG in subcutaneous adipose tissue explants (r=-0.62, p= 0.01) suggesting fat storage in females with elevated testosterone levels maybe linked with ASP action. Measuring TG concentration in the media is considered one of limitations in this experiment. The commercial kit used lipoprotein lipase enzyme to hydrolyze FAs from TG and releasing glycerol. With the fact that only FFA can be taken up by adipocytes, glycerol may remain constant in the media. Therefore only lipolytic activity is assumed depending glycerol concentrations into the media (Benz et al., 2012). In general this may not reflect the function of ASP in measuring FAs by adipocytes. It would be more important to assess the uptake of FAs and its ratio with glycerol concentration (Raclot and Oudart, 2000).

There was no significant effect of hormone treatments on LPL and DGAT1. LPL is a tissue specific enzyme that is responsive to fasting and feeding state. In adipose tissue, LPL activity increases after feeding and decreases during fasting, where insulin stimulates LPL expression and activity directly (Farese et al., 1991, Goldberg et al., 2009),. ASP indirectly stimulates LPL activity by enhancing the cellular uptake and estrification of hydrolyzed NEFA (Farese et al., 1991). Wu et al., investigated LPL activity and expression in adipose tissue explants. They found that using tissue explants may decrease LPL expression and activity to some extent due to the proteolysis of extracelluar LPL. The tissue explants were still able mediate the loss of LPL with in 6 hours of incubation (Wu et al., 2005). This may explain the unchanged level of LPL in different hormone treatments even with the presence of chylomicrons. Although LPL expression showed no

changes through this study, correlation analysis showed a positive correlation of LPL with all related gene expression; C5L2, C3, factor B, adipsin and DGAT1.

DGAT1 is an important enzyme involved directly in the ASP-C5L2 signaling pathway that triggers TG synthesis in adipocytes (Maslowska et al., 2006). In this study, the expression of DGAT1 was found to be higher in subcutaneous tissue explants than in visceral explants. The correlation analysis may explain further the effect of different hormone treatments on the measured parameters in this study in different fat depots. Partial correlation analysis controlled for all treatment groups, fat depots and chylomicrons showed a consistent relationship between C5L2 receptor and the DGAT1 in both tissue explants. ASP was found in an earlier study to stimulate adipose tissue DGAT1 activity by 90% suggesting a direct interaction of ASP with DGAT1 (Yasruel et al., 1991). DGAT1 expression was shown to be predicted by C5L2, factor B, adipsin and LPL (**Table 3.6**) in regression analysis, indicating that ASP may regulate DGAT1 indirectly through its receptor and precursors.

In summary, this study presents a comprehensive analysis of the effect of steroid hormones on ASP and most related genes in different fat depots. Although various studies have reported the association of the effect of female hormones on ASP, this is only study reporting the direct regulatory effect of progesterone and estrogen (in combination) in a fat depot dependent manner. In the natural female hormone cycle, a spike of estrogen is observed prior to the increase level of progesterone. In this experiment, only the presence of progesterone with estrogen has been found to effect ASP levels. In the fasting-like condition (absence of

chylomicrons), P&E hormone treatments significantly increased ASP production only in subcutaneous adipose tissue suggesting that sex hormones mediate ASP effects on fat distribution. The addition of chylomicrons augmented the effect of P&E treatments on ASP production in both subcutaneous and visceral adipose tissue explants. This may indicate an important role of sex steroid hormones in response to dietary fat, which is mediated by ASP. The effect of ASP was more pronounced in subcutaneous adipose tissue confirming the role of ASP in driving female fat distribution. P&E treatment contribute in fat distribution pattern and mediate the response to dietary fat by regulating ASP and C5L2.

Overall findings are important in identifying factors that regulate specific regional body fat distribution in perspective to the relationship with sex steroid hormones. The regional localization of stored fat may predict obesity-related health risk more than the total amount of body fat. In particular, increased visceral fat accumulation is associated with increased risk of metabolic disorders (Geer and Shen, 2009). Of interest with regards to sex differences, women exhibit adiposity and fat distribution associating with lower metabolic risk (Karastergiou et al., 2012). Although explanation of the direct effect of sex steroid hormones on fat storage localization is not fully known, this study provided evidence that ASP is mediating the effect of female steroid hormones directly by favoring subcutaneous over visceral fat storage. Therefore, ASP is an important driver of the protective effect of female sex hormones in many generated metabolic profile disorders that largely attributed to regional body fat distribution. As female hormone variations take place in response to biological changes or diseases (such

as polycystic ovary syndrome), it is interesting to explore the time and dose effect of female hormones on the production and function of ASP in adipocytes.

CHAPTER 4. DOSE- AND TIME-EFFECT OF FEMALE STEROID HORMONES TREATMENT ON ASP PRODUCTION

The *ex vivo* experiments reported in Chapter 3 indicated that the combination of female hormones, at physiological concentrations, influenced ASP production. However, *in vivo*, the female hormones fluctuate throughout the menstrual cycle and may also change in different physiological and pathological conditions. Chylomicrons modulated the effect of female hormones on ASP metabolism; however further investigation of the time course of this effect may help in elucidating potential mechanisms. To investigate these factors further it was decided to use the 3T3-L1 adipocyte cell line, thus reducing the use of animals and the biological variability associated with such experiments and allowing a greater range of doses and/or time points to be studied.

Therefore, it was hypothesized that treatment with progesterone, estrogen and a combined preparation would affect ASP, C3 production, C5L2 expression and lipid accumulation in adipocytes. A combination of both hormones in the presence of chylomicrons was hypothesized to affect ASP production and lipid accumulation in a time-dependent manner using 3T3-L1 cell line.

4.1 Dose effect of female steroids on ASP, C3, C5L2 and lipid accumulation

4.1.1 Introduction

Female sex steroids (i.e. progesterone and estrogen) play a key role in the distribution and metabolism of adipose tissue. They act through the activation of different signaling pathways influencing the expression of receptors or proteins in adipocytes, that will have an effect on preadipocyte differentiation, cell proliferation, lipid accumulation, adipocyte metabolism (lipolysis and lipogenesis) and on the secretion of different adipokines (Mayes and Watson, 2004) (Wen et al., 2008). In adipocytes, two factors are mainly responsible for regulation of lipid metabolism; insulin and ASP. Both have been shown to increase triglyceride synthesis, glucose uptake, lipoprotein activity and decrease hormone sensitive lipase, therefore, decreasing lipolysis (Cianflone et al., 2003).

ASP synthesis during cell differentiation increases with triglyceride synthesis in adipocytes (Cianflone et al., 2003). Differentiating preadipocytes showed an increased production of C3, adipsin and factor B resulting in the production of ASP (Choy et al., 1992). Insulin and chylomicrons are potent stimulators of ASP and C3 from human differentiated adipocytes (Maslowska et al., 1997, Scantlebury, 1998). Chylomicrons are intestinally driven postprandial lipoproteins that trigger the release of ASP from adipose tissue and coordinating in TG clearance (Saleh et al., 1998). The metabolism of chylomicrons involves the release of fatty acids from chylomicron particles by the action of lipoprotein lipase followed by the uptake of FFA and incorporated into TG by adipocyte

(Galton and Krone, 1991). Most of the studies in the literature have evaluated the relation between ASP and female sex steroids; however, there are very limited studies on the direct effect of progesterone and estrogen on ASP production and function. The secretion of other adipokines is also influenced by sex steroid hormones, for example estrogen effected positively the production of leptin (Shimizu et al., 1997, Hardie et al., 1997, Rezai et al., 2011), and this effect was weakened in the presence of progesterone (Rezai et al., 2011). Physiologically both hormones usually are circulated in the system together, and we confirmed earlier in this thesis that progesterone and estrogen in combination stimulated ASP production in adipose tissue explants. The aim of this study was, therefore, to evaluate the dose- effect of progesterone and estrogen (and combination) on ASP, C3 production, C5L2 expression and lipid accumulation using the 3T3-L1 cell line. The 3T3-L1 cell line is a well-established preadipocyte that was developed from murine Swiss 3T3 mouse (Green and Meuth, 1974). The advantage of using this cell line that it provides a homogenous response to treatments without confounding factors found in tissues driven from different donors (Ruiz-Ojeda et al., 2016).

4.1.2 Materials and methods

In this section a brief description is provided with more detailed explanation of the techniques used in the Materials and Methods chapter (Chapter 2).

4.1.2.1 Cell culture and treatments

3T3-L1 cells were grown in DMEM-phenol free media and low glucose (1000 mg/L) (Sigma) with 10% (v/v) FBS, and 1% (v/v) P/S cultured at 5% CO_2 at 37 °C. The preadipocyte cells were seeded at 40,000 cells per well in 6-well plates until they reached 80% confluent. Differentiating media was then used, included IBMX, dexamethasone and insulin for 2 days followed by media including only insulin up to day 8 or until the formation of mature adipocytes. Before cell treatment, the cells were maintained overnight without insulin in phenol-free media.

4.1.2.1.1 Cell treatment

Adipocytes were incubated for 24 hours with progesterone and estrogen at concentrations of 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M, which ranges from normal physiological to the pathological state. The control group had no hormonal treatment. The combination of both hormones (P&E) was also investigated with increasing dose of each in the presence of 10^{-7} of the other hormone. All treatments were incubated for 24 hours in 5% CO_2 at 37 °C.

4.1.2.2 Media and cell analysis

At the end of the treatment, cell culture media was collected and stored at -80 °C to determine ASP (C3a) and C3 using mouse ELISA (Novateinbio, USA). Cells were harvested in lysis buffer and homogenized to quantify total cell protein using Lowry assay method. C5L2 protein was determined using Western blot method. The cellular TG was measured in a homogenate of lysed cells using TG assay method as described in (Kang et al., 2013). Cells were extracted with 1 mL of lysis buffer (50 mM Tris, 0.15 M NaCl, 10 mM EDTA and 0.1 % (v/v) Tween-20) and pH of the buffer was adjusted at 7.5. The mixture was homogenized and the cellular lysate was used to measure for TG concentration using an enzymatic kit; Triglyceride Infinity Reagent (Thermoscientific), and reading absorbance at 550 nm (**section 1.1.5.2**).

4.1.2.3 Oil Red O staining

Cells were fixed with 10% (v/v) formalin for 30 minutes followed by washing with PBS. Then the cells were stained with Oil Red O staining and observed under a microscope. The total amount of lipid in each well was quantified by adding isopropanol and measuring the absorbance at 490 nm.

4.1.2.4 Statistical analysis

Data is reported as mean ±standard error of the mean (S.E.M.) of four biological replicates (n=4). Changes in ASP, C3, C5L2 protein expression and lipid accumulation were determined by one-way ANOVA. Dunnett's post hoc analysis

was used to determine the differences between different concentrations and the control group. Significance was set at $p < 0.05$.

4.1.3 Results

4.1.3.1 Effect of progesterone, estrogen and their combination on ASP, C3 production, ASP/C3 ratio and C5L2 protein expression

3T3-L1 adipocytes were incubated with increasing concentrations of progesterone, estrogen or in combination and then were evaluated for ASP and C3 production. As shown in **Figure 4.1** (**A**), increasing concentrations of progesterone had no significant effect on ASP production (p= 0.4). Estrogen treatment showed a trend to increase ASP production, however this did not reach statistical significance with p=0.06 (**Figure 4.1** (**B**)). Treatment with increasing concentrations of progesterone in the presence of 10^{-7} M estrogen increased ASP production significantly ($F_{(4,19)}$=9.52, $p < 0.001$). The highest concentrations of progesterone (+estrogen) increased ASP concentration significantly at 10^{-5} M ($p< 0.001$) and 10^{-6} M ($p<0.01$) relative to control **Figure 4.1** (**C**)). Increasing concentrations of estrogen in combination with progesterone (10^{-7} M) had no effect on ASP production (p=0.54) **Figure 4.1** (**D**)).

None of the hormone treatments had a significant effect on C3 production or on the conversion ratio of C3 to ASP, as shown in **Table 4.1**.

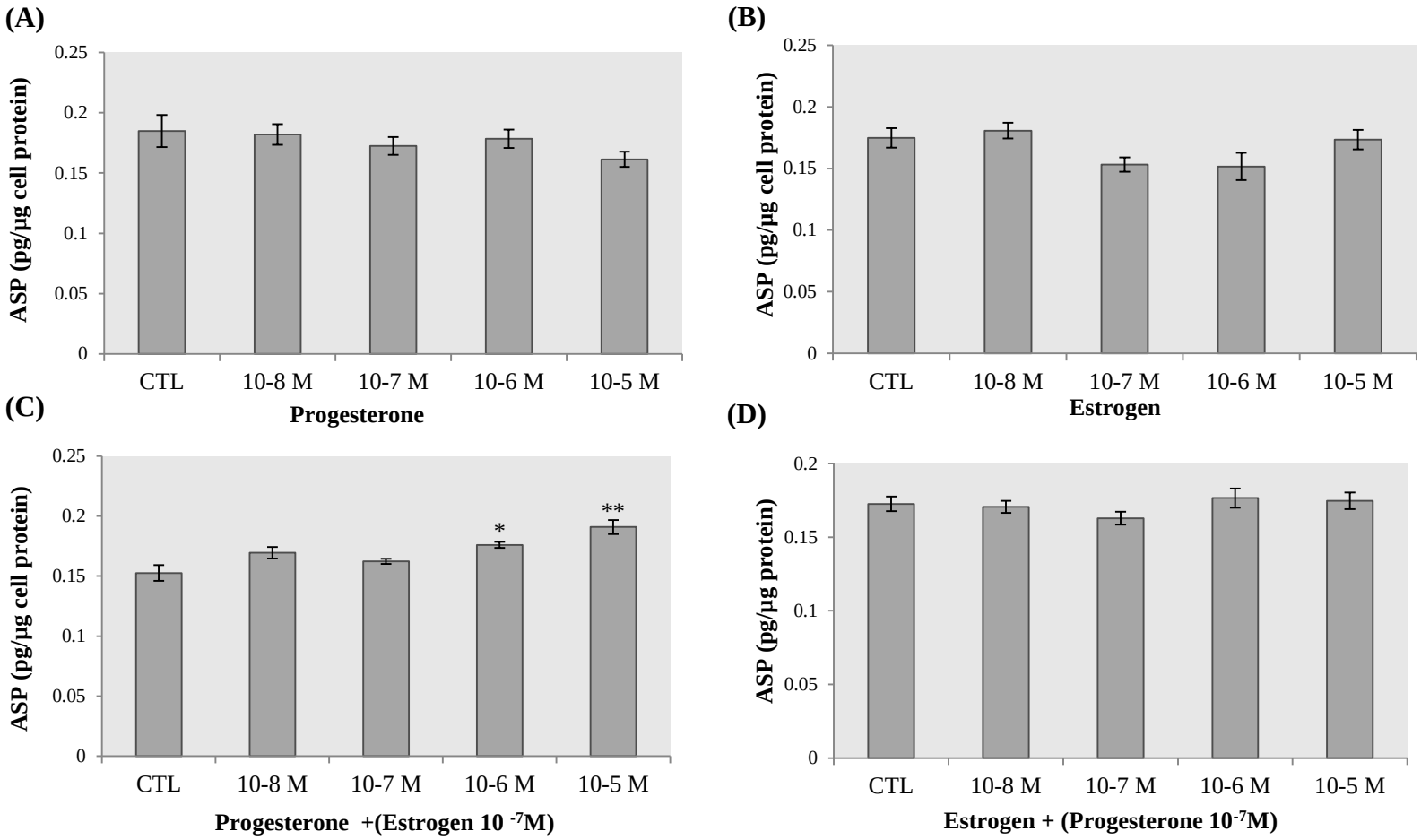


Figure 4.1 Dose-dependent effects of female steroid hormones on ASP production (pg/ µg cell protein) from 3T3-L1 adipocytes. (**A**) Progesterone treatment had no effect on ASP concentration (p=0.4), (**B**) Estrogen treatment had no effect on ASP concentration (p=0.06), (**C**) Progesterone treatment with estrogen (10^{-7}M) increased ASP production at 10^{-6} and 10^{-5} M relative to 0M control (*p<0.01, **p<0.001, respectively) and (**D**) Estrogen treatment with progesterone (10^{-7} M) had no effect on ASP concentration (p=0.54). All data presented as mean ±S.E.M (n=4) and analysis carried by ANOVA followed by Dunnett's' post hoc test.

Table 4.1 Dose-dependent effects of female steroid hormones on C3 production (ng/µg cell protein) and ASP/C3 ratio in 3T3-L1 adipocytes. All data presented as mean± S.E.M (n=4) and analysis carried by ANOVA test.

	C3 (ng/µg cell protein)				ASP/C3 ratio (ng/µg cell protein)			
	Progesterone	**Estrogen**	**Progesterone (+E 10^{-7}M)**	**Estrogen (+P 10^{-7}M)**	**Progesterone**	**Estrogen**	**Progesterone (+E 10^{-7}M)**	**Estrogen (+P 10^{-7}M)**
CTL	1.88± 0.1	1.76± 0.2	1.51±0.1	1.71±0.1	0.98 x10^{-3}±0.1	1.08 x10^{-3}±0.1	1.00 x10^{-3}±0.1	1.01 x10^{-3}±0.0
10^{-8} M	1.91± 0.1	1.86±0.1	1.82±0.1	1.97±0.1	1.03x10^{-3}±0.1	0.99 x10^{-3}±0.1	0.94 x10^{-3}±0.0	0.88 x10^{-3}±0.0
10^{-7} M	1.79± 0.1	1.66±0.1	1.64±0.1	1.84±0.1	1.02 x10^{-3}±0.0	1.02 x10^{-3}±0.1	0.93 x10^{-3}±0.1	1.02 x10^{-3}±0.1
10^{-6} M	1.92 ±0.1	1.66± 0.2	1.75±0.0	1.97±0.1	0.97 x10^{-3}±0.1	0.94 x10^{-3}±0.1	1.08 x10^{-3}± 0.1	0.98 x10^{-3}±0.1
10^{-5} M	1.59±0.2	1.98±0.2	1.87±0.1	1.94±0.1	1.21 x10^{-3}±0.1	1.11 x10^{-3}±0.1	1.01 x10^{-3}±0.2	1.09 x10^{-3}±0.1
P value	**0.32**	**0.27**	**0.12**	**0.38**	**0.07**	**0.06**	**0.71**	**0.28**

(**P**; progesterone, **E**; estrogen).

The effect of progesterone and estrogen was examined at increasing concentrations on C5L2 protein in 3T3–L1 adipocytes using Western blot method. The expression of C5L2 was analyzed relative to the β-actin protein and bands are presented in **Figure 4.2** in coordination with the treatment of different concentrations. Expression of β-actin showed no significant difference between different hormone treatments. It was interesting to find that progesterone and estrogen treatments individually had an effect on C5L2 protein expression ($p= 0.006$, $p=0.02$ respectively, ANOVA). Treatment with high dose of progesterone hormone (10^{-5} M) increased C5L2 expression ($p=0.004$, Dunnetts' test) as shown in **Figure 4.2** (**A**). Treatment with estrogen at concentrations of 10^{-8}, 10^{-7} and 10^{-6} M increased the expression of C5L2 with $p=0.03$, $p=0.02$ and $p=0.02$ respectively (**Figure 4.2** (**B**)).

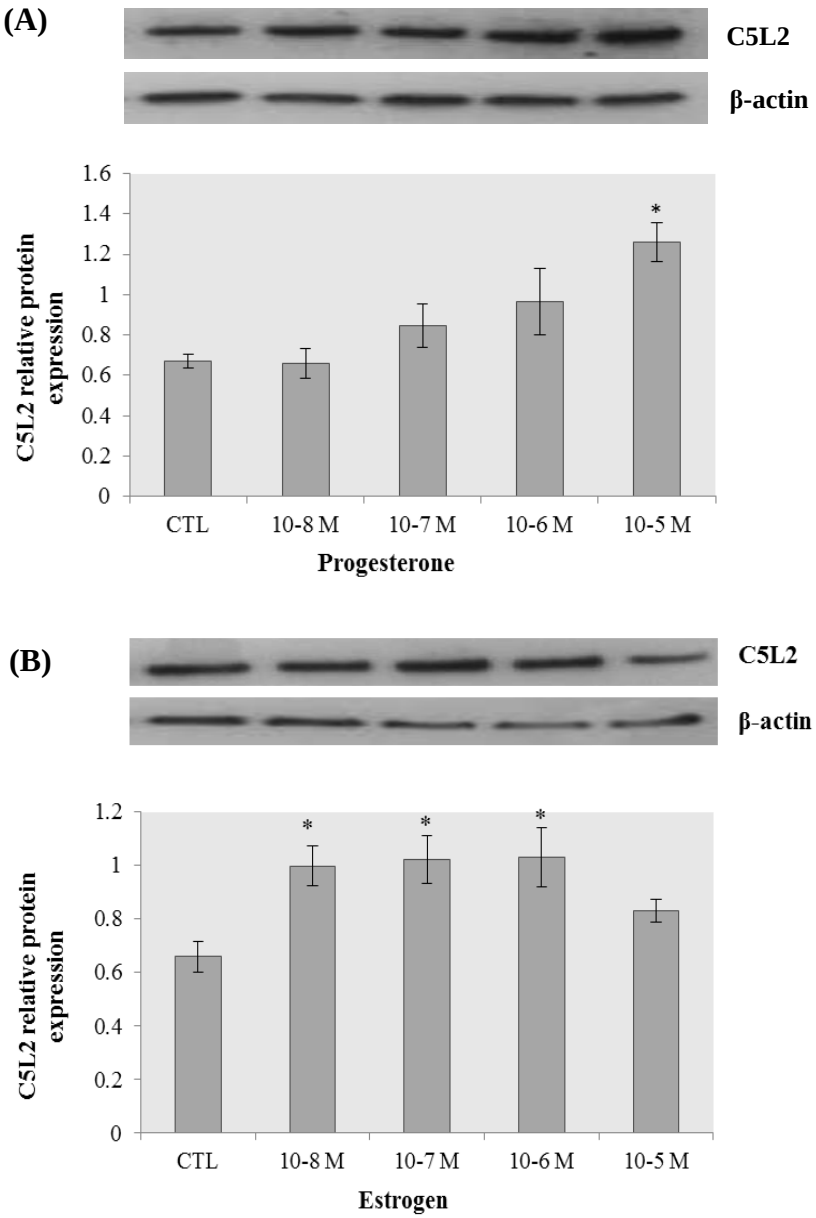


Figure 4.2 Dose-dependent effects of female steroid hormones on protein expression of C5L2 (relatively to β-actin) in 3T3-L1 adipocyte. Representative images are included for both C5L2 and β-actin arranged in accordance with treatment groups. (**A**) Progesterone treatment at concentration 10^{-5} M increased C5L2 expression significantly (*p=0.004), (**B**) Estrogen treatment at concentrations of 10^{-8} M (*p=0.03), 10^{-7} M (*p=0.02) and 10^{-6} M (*p=0.02) increased C5L2 protein expression. All data presented as mean± S.E.M (n=4). Analysis was determined by ANOVA followed by Dunnett's post hoc analysis.

There was no effect of varying progesterone with constant estrogen (10^{-7} M) on C5L2 expression (p= 0.36) as presented in **Figure 4.3** (**A**). Similarly, varying estrogen concentrations with progesterone (10^{-7}) did not affect C5L2 expression (p=0.32) (**Figure 4.3** (**B**)).

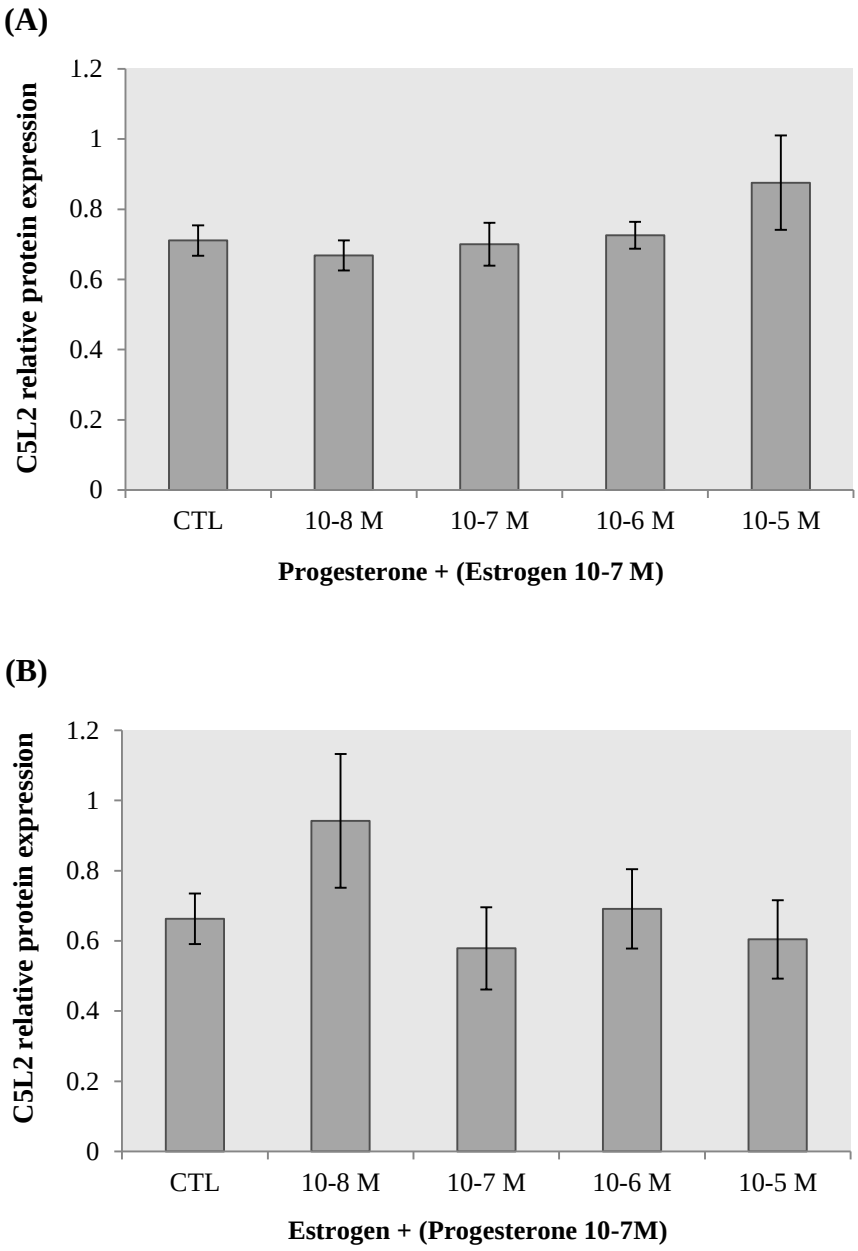


Figure 4.3 Effect of female sex hormones on C5L2/β-actin relative protein expression in 3T3-L1 adipocytes. (**A**) Progesterone treatment with estrogen (10^{-7}M) had no effect on C5L2 expression (p= 0.36), (**B**) Estrogen treatment with progesterone (10^{-7}M) had no effect C5L2 expression (p=0.32). All data presented as mean± S.E.M (n=4). Analysis was determined by ANOVA.

4.1.3.2 Effect of progesterone, estrogen and their combination on lipid accumulation

Figure 4.4 (**A**) shows a representative micrograph of mature 3T3-L1 adipocytes stained with Oil Red O. The staining represents total lipid accumulation that was quantified by eluting the stain with isopropanol and measured by microplate spectrophotometer. ANOVA analysis showed that different hormone treatments and different concentrations had no effect on total lipid accumulation in 3T3-L1 as presented in **Figure 4.4** (**B**), (**C**), (**D**) and (**E**).

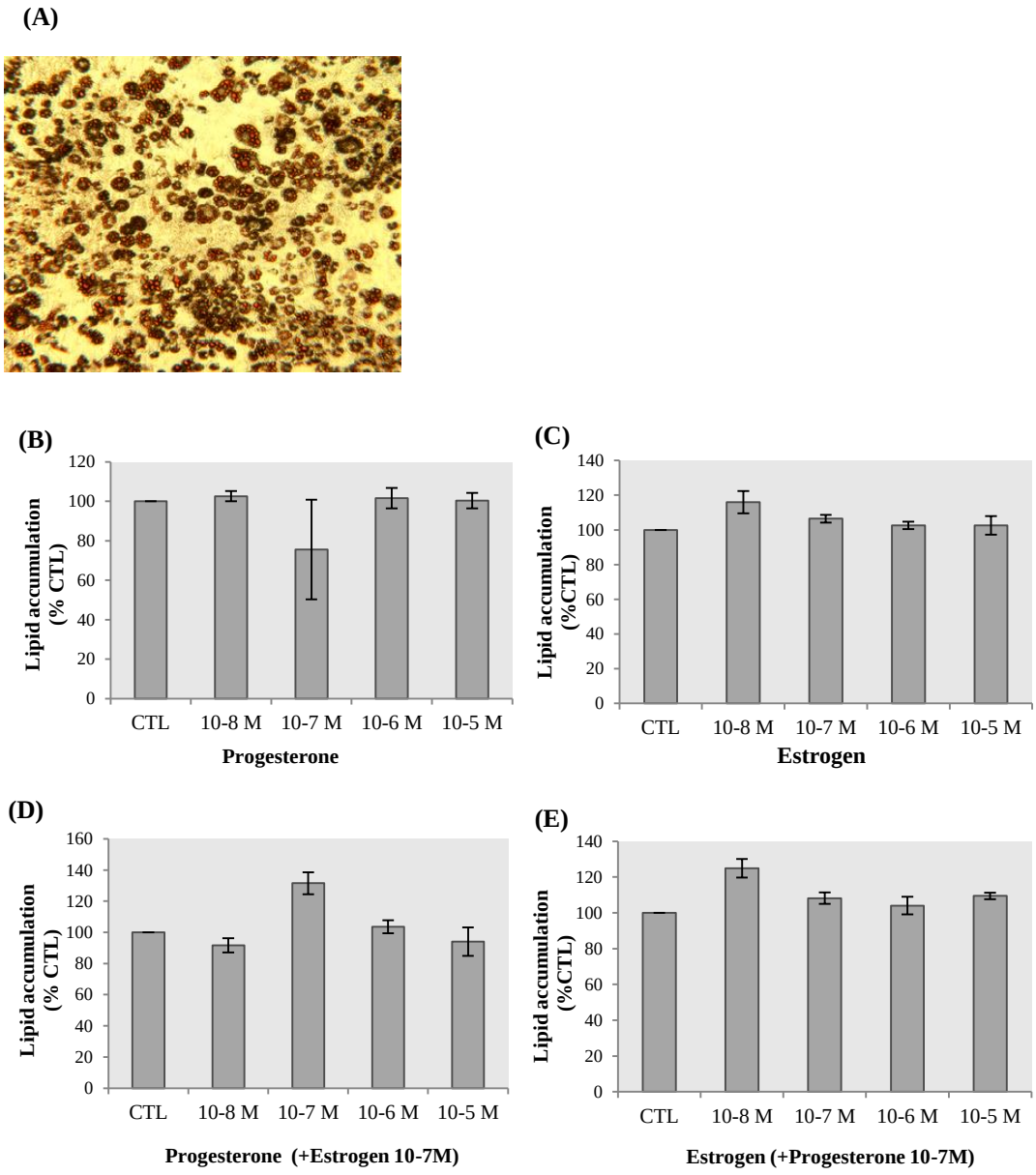


Figure 4.4 Lipid accumulation in 3T3-L1 adipocytes stained with Red Oil O. (**A**) representative image of stained adipocyte cells by light microscope (image captured at magnification of x100). Hormonal treatments at different concentrations had no effect on total lipid accumulation in (**B**) Progesterone treatment (p= 0.9), (**C**) Estrogen treatment (p=0.53), (**D**) Progesterone and constant estrogen treatment (p=0.23) and (**E**) Estrogen and constant progesterone treatment (p=0.43). All data presented as mean± S.E.M (n=4). Analysis was determined by ANOVA.

4.1.4 Discussion

Previous *in vivo* studies have shown a close link between ASP concentrations and female steroid hormones (Saleh et al., 1999, Saleh et al., 2007, Rezvani et al., 2014). The function of ASP in postprandial fat clearance was, for example, enhanced by acute dose of progesterone in ovariectomized rats (Al Riyami et al., 2014). Based on our earlier findings on the regulatory effects of progesterone and estrogen hormones on ASP in explant tissue, we aimed in this study to investigate the dose-effect of female steroid hormones on ASP production. The findings showed that increasing concentrations of progesterone and estrogen had no effect on releasing ASP and C3 from mature 3T3-L1 adipocytes. However, the combination of both hormones at high concentration stimulated ASP production but not C3 or C5L2 receptor expression. Furthermore, progesterone and estrogen increased C5L2 protein expression in a dose-dependent manner. ASP's main role is to increase TG uptake into adipocytes (Cianflone et al., 2003), though the stimulation of ASP production or the increased expression of C5L2 showed no effect on lipid accumulation in mature 3T3-L1 adipocytes.

In females, changes in fat distribution and lipid profile coincide with ovarian production during puberty and pregnancy (de Ridder et al., 1990, Ghio et al., 2011). The association between ovarian hormones and ASP was evident in the literature, where ASP correlated positively with the changes in progesterone concentrations in women menstrual cycle (Saleh et al., 2009). Increases in progesterone and estrogen during late pregnancy have also been shown to associate with an increase in ASP plasma concentrations (Saleh et al., 2007). In

our studies we were able to confirm a positive effect of progesterone and estrogen in combination on ASP produced from mature adipocytes in a concentration similar to late pregnancy (high physiological concentration of progesterone and estrogen (Xiu et al., 2016). High concentrations of progesterone and estrogen in pregnancy are characterized by insulin resistance and hyperlipidemia (Masuyama and Hiramatsu, 2011). Therefore, the increased concentration of circulating ASP was suggested to be as a result of ASP resistance. In a cross-sectional study, it was also speculated that ovarian hormones can induce ASP resistance in obese women with metabolic disorders (Rezvani et al., 2014). The increased hormonal ratio of progesterone/estrogen at late gestation is accompanied by increased lipid mobilization and elevated ASP concentrations (Saleh et al., 2007). The impaired function of ASP in pregnancy was associated with the increased circulating TG and this is possibly important to meet the increased fetal energy demands (Saleh et al., 2008).

The production of ASP has been related to the expression of C3, adipsin and factor B genes in differentiating adipocytes (Choy et al., 1992). The association between plasma ASP and C3 has been examined in various studies in humans with metabolic disorders and elevated lipid profiles (Cianflone et al., 2003). It was also examined in relation to conditions such as polycystic ovarian syndrome where ASP and C3 are elevated independently of obesity (Oktenli et al., 2007). The aim of this study was to evaluate C3 production and ASP/C3 conversion ratio with various concentrations of progesterone and estrogen. We found no effect of the hormonal treatments (at various concentrations) on C3 production, suggesting

that induced ASP production via progesterone and estrogen in combination, was independent of C3 concentration and not influenced by the conversion rate of C3 to ASP. This notion was is consistent with C3 mRNA expression in tissue explants (Chapter 3) and other *in vitro* studies (Gao et al., 2010). C3 is synthesized and produced from other organs than adipose tissue, such as uterus, that express more C3 in relation to hormonal fluctuation specifically estrogen (Sundstrom et al., 1989). However in adipocytes, a direct impact of estrogen or progesterone on C3 production was not determined previously.

The positive correlation between ASP concentrations and the expression of C5L2 in adipose tissue was demonstrated earlier (MacLaren et al., 2008). The C5L2 receptor is important in mediating the function of ASP and *in vitro* studies have shown that treatments with ASP and high doses of progesterone or chronic treatment with progesterone resulted in downregulation of C5L2 receptors in supporting the notion of ASP resistance (Wen et al., 2008). In the present study, C5L2 did not appear to be impacted by increased ASP concentrations; however this is the first time a direct positive effect of progesterone and estrogen (individually) on C5L2 protein expression, in a dose-dependent manner, has been reported. This contradicts previous *in vitro* studies that found an inhibitory effect of the steroid hormones on C5L2 mRNA expression (Wen et al., 2008, Gao et al., 2010). The opposing findings can be explained by the fact that the present experiment used a shorter incubation time than previous studies (24 vs 48 hours). This may result in an increased internalization and catabolism of ASP through the increased uptake by C5L2 receptor which is likely to lead to the reduction of ASP

and C5L2 (Gao et al., 2010). Considerate should also be noted that this is the only reported study of C5L2 protein expression, whilst previous studies reported the effect on C5L2 gene expression in adipocytes. The high expression of C5L2 may act as a negative feedback and in response inhibit C5L2 gene expression of C5L2. This may need further investigation to be confirmed in future studies.

The increased protein expression of C5L2 may be related to progesterone and estrogen function in adipocytes. Progesterone is known to maintain lipogenesis by the activation of LPL, increasing fatty acid synthase enzyme and enhance TG synthesis (Mayes and Watson, 2004). However this effect of progesterone on LPL were not found earlier in the *ex vivo* experiment, though, the activity of LPL and the time factor may play a key role in the lipogenesis effect of progesterone. A positive coordination between progesterone and ASP was demonstrated previously (Al Riyami et al., 2014) and by increasing C5L2 expression in response to progesterone in our study, this coordination between progesterone and ASP may also suggest C5L2 to be part of the lipogenic action of progesterone. Development of a resistant state by downregulating C5L2 receptor can found to be associated more with insulin resistance and conditions that are more related to metabolic syndromes. A study that included women with metabolic syndrome and high progesterone concentrations found downregulation of C5L2 expression in depot specific manner (Rezvani et al., 2014). Estrogen is known to favor an anti-lipogenic condition (Hod et al., 2016), and increasing circulated TG (MacLaren et al., 2008). These estrogenic effects may lead to the increase C5L2 expression as a compensatory effect of lipolysis in adipocytes. In a microarray

study, morbidly obese subjects with high concentrations of ASP and TGs showed an increase expression of C5L2 receptors in both subcutaneous and omental adipose tissue (MacLaren et al., 2010). Though we found a positive influence of progesterone or estrogen hormones on the expression of C5L2, the combination of both hormones did not show any effect on the expression of C5L2. Progesterone and estrogen in-combination have been demonstrated to increase ASP production, and this may lead to the uptake and internalization of C5L2 by ASP-C5L2 interaction.

Variation in ASP production or C5L2 expression would be expected to increase adipocyte lipid accumulation, which was analyzed in this study using Red Oil O staining of cells. We were not able to detect any changes in total lipid accumulation that corresponded with either ASP or C5L2 in response to hormonal treatment. As the media in this study didn't include any fat source (-chylomicrons); TG synthesis process would be dependent on the availability of glucose. Lipogenesis process in adipocyte is stimulated from the acetate and pyruvate produced by the breakdown of glucose in the adipocyte cells (Reshef et al., 2003). The synthesis of TG process is accomplished by the presence of insulin which has been controlled and removed from the treatment media in the present study. Therefore, it is not expected to detect any changes in lipid accumulation due to the lack of fat sources and insulin to facilitate the synthesis of TG from glucose.

The observation was also reported in 3T3-L1 adipocytes in response to a chronic treatment of sex steroid hormones that did not induce TG mass in adipocytes (Gao et al., 2010). ASP stimulates TG synthesis upon binding to its receptor C5L2 (Kalant et al., 2005). It can be concluded here that the coordination between increasing ASP production and increasing C5L2 expression is required for ASP-C5L2 interaction to stimulate lipid accumulation in adipocytes.

In summary, this is the only *in vitro* study using 3T3-L1 adipocytes to report a stimulatory effect of high doses of progesterone on ASP which is dependent on the presence of estrogen. The induction of ASP was found to be independent of C3 concentration and the conversion rate of C3 to ASP. Progesterone and estrogen increased expression of C5L2 suggesting a hormonal influence on both ASP production and C5L2 expression in adipocytes.

4.2 Time course of the effect of female sex steroids on ASP, C3, C5L2 and intracellular TG

4.2.1 Introduction

The production of ASP can be regulated by the expression or the concentration of the precursor C3 and by the conversion of C3 to ASP by the action of protein enzymes adipsin and factor B (Cianflone et al., 2003). So far, the regulation of the production of ASP and its precursor is not well understood. Various studies have highlighted the role of chylomicrons in regulating ASP production in adipose tissues. Earlier findings in human adipocyte culture studies demonstrated the stimulatory effect of chylomicrons on C3 and ASP production (Maslowska et al., 1997), an effect shown to be time- and dose-dependent (Scantlebury, 1998). *In vivo* and *in vitro* data have shown clearly that ASP is involved in the metabolism of chylomicrons by trapping the released FFA from chylomicrons (Sniderman et al., 1997).

The relationship between ASP and female steroid hormones is complex and there are conflicting reports suggest enhancing ASP function or directly inhibiting ASP production (Wen et al., 2008, Saleh et al., 2011). Based on the findings of the *ex vivo* study presented in chapter 3, female steroids (progesterone and estrogen) regulate the production of ASP in subcutaneous and visceral adipose tissue. Chylomicrons were shown to interact with different hormonal treatments affecting the production of ASP and C5L2. Therefore, we aimed in the present work to investigate the effect of progesterone and estrogen in combination with

chylomicrons on ASP, C3 production, C5L2 expression and lipid accumulation in 3T3-L1 adipocytes.

4.2.2 Material and methods

In this section a brief description are provided with more detailed explanation of the materials and method used in the Materials and Methods chapter (Chapter 2).

4.2.2.1 Cell culture and treatments

The preadipocyte cells were seeded into 6-well plates (n=4) until they reached 80% confluent in DMEM-free phenol media. Differentiating media was used up to day 8 or until the formation of mature adipocytes were confirmed by the microscope. Before cell treatment, the cells were washed with PBS and maintained overnight without insulin in phenol-free media.

4.2.2.1.1 Sex steroid treatments

Progesterone and estrogen in combination were shown in *ex vivo* experiment (Chapter 3) to regulate ASP production and this effect was augmented by chylomicrons at concentration of 100mg TG/ml. Similarly, insulin treatment with a similar concentration of chylomicrons induced ASP production in 3T3-L1 adipocytes (Gao et al., 2010), therefore we excluded the effect of insulin. In this experiment, there were two treatments; (**A**) the control group containing only chylomicrons (100mg TG/ml) and (**B**) the progesterone and estrogen (P&E) group at a concentration of 10^{-7} M with chylomicrons (100mg TG/ml). All treatments were incubated in 5% CO_2 at 37 °C for 0-h, 2-h, 8-h, 16- h and 24- h time course as this time was shown to be sufficient to induce ASP production.

4.2.2.2 Media and cell analysis

After the indicated treatment time, media was collected and stored at -80 °C to measure ASP and C3 using mouse ELISA (Novateinbio, USA). Cells were harvested in lysis buffer and homogenized to quantify total cell protein using the Lowry assay method. Protein expression of C5L2 was analysed using the western blot method. Intracellular TG was measured in cell lysed homogenates using the TG assay method as described in (Kang et al., 2013).

4.2.2.3 Statistical analysis

Data is reported as mean ± (S.E.M.) of the percentage of the control (% CTL) of four biological replicates (n=4). Changes in ASP, C3, C5L2 protein expression and intracellular TG mass overtime (incubational time points) were determined by repeated measure ANOVA (RMANOVA) with a Greenhouse-Geisser correction. This statistical approach is commonly used in repeated measures designs to monitor the effect of treatment over time. Bonferroni post hoc test was used to determine the differences between the mean of measured parameters at different time point of the treatment to the control group. Significance was set at $p < 0.05$.

4.2.3 Results

4.2.3.1 Effect of chylomicrons and P&E on ASP, and C3 production

ASP and C3 production were evaluated at different times after treatment with progesterone (10^{-7} M) and estrogen (10^{-7} M) (P&E) and chylomicrons (100 µg TG/ml), or with chylomicrons only using RMANOVA analysis for each treatment. ASP production did not change between time points with the chylomicron (p=0.19) and the P&E (+chylomicrons) treatments (p=0.54) (**Figure 4.5** (**A**) and (**B**)).

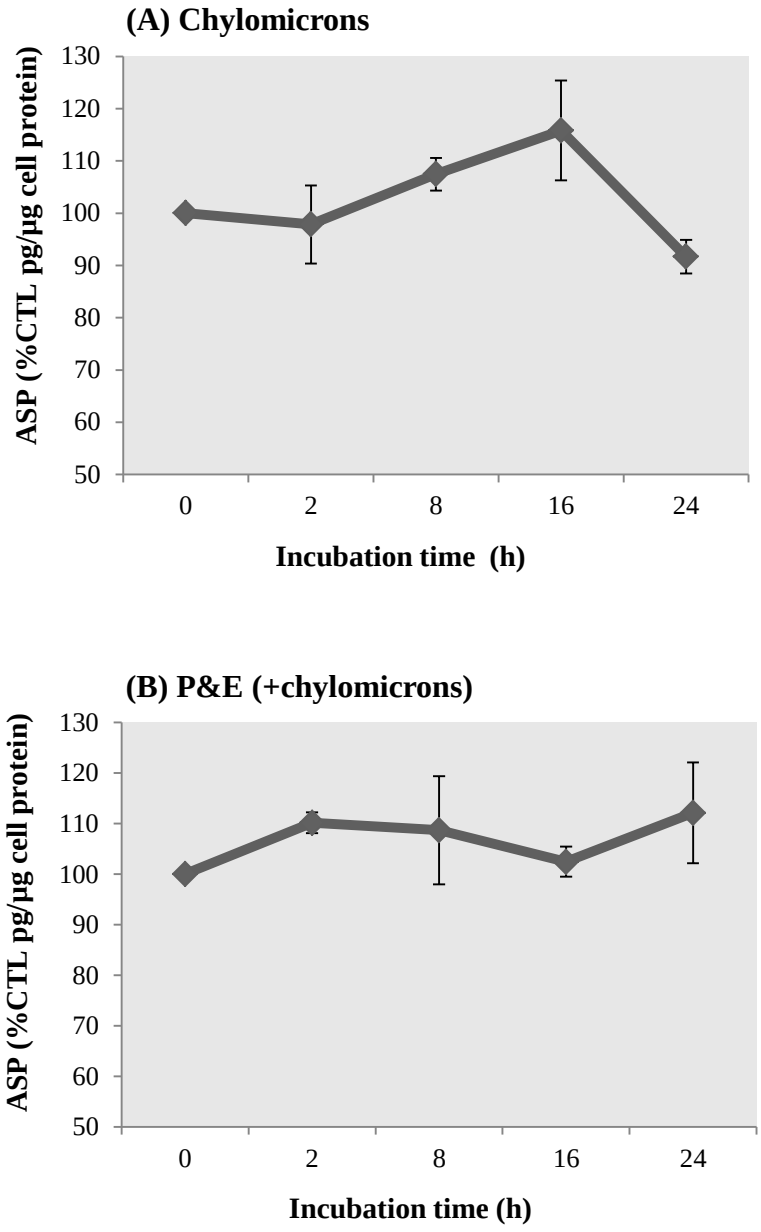


Figure 4.5 ASP production at different time points in 3T3-L1 adipocyte (n=4). (**A**) Chylomicrons had no effect at different incubation time (p=0.19), (**B**) P&E (+chylomicrons) treatment had no effect on the production of ASP at different incubation time (p=0.54). All data presented as mean ± (S.E.M.) of the percentage of the control (% CTL) (n=4). Analysis was carried using RMANOVA.

By contrast, C3 concentration at different treatment points was found to markedly increase (14-fold) in the chylomicrons treatment group ($F_{(1.94,5.82)}$=54.15, p< 0.001) and in the P&E (+chylomicrons) treatment group ($F_{(1.98,5.94)}$=10.08, *P*=0.01). In the chylomicron treatment group, post hoc test using Bonferroni correction showed an increase in C3 concentration at 8-h (p= 0.006), 16-h (p=0.007) and at 24-h (p=0.028) as shown in **Figure 4.6** (**A**). C3 concentration also increased to the same degree in the P&E (+chylomicron) treatment group at 2- h (p=0.03) and 16-h (p=0.001) (**Figure 4.6** (**B**)).

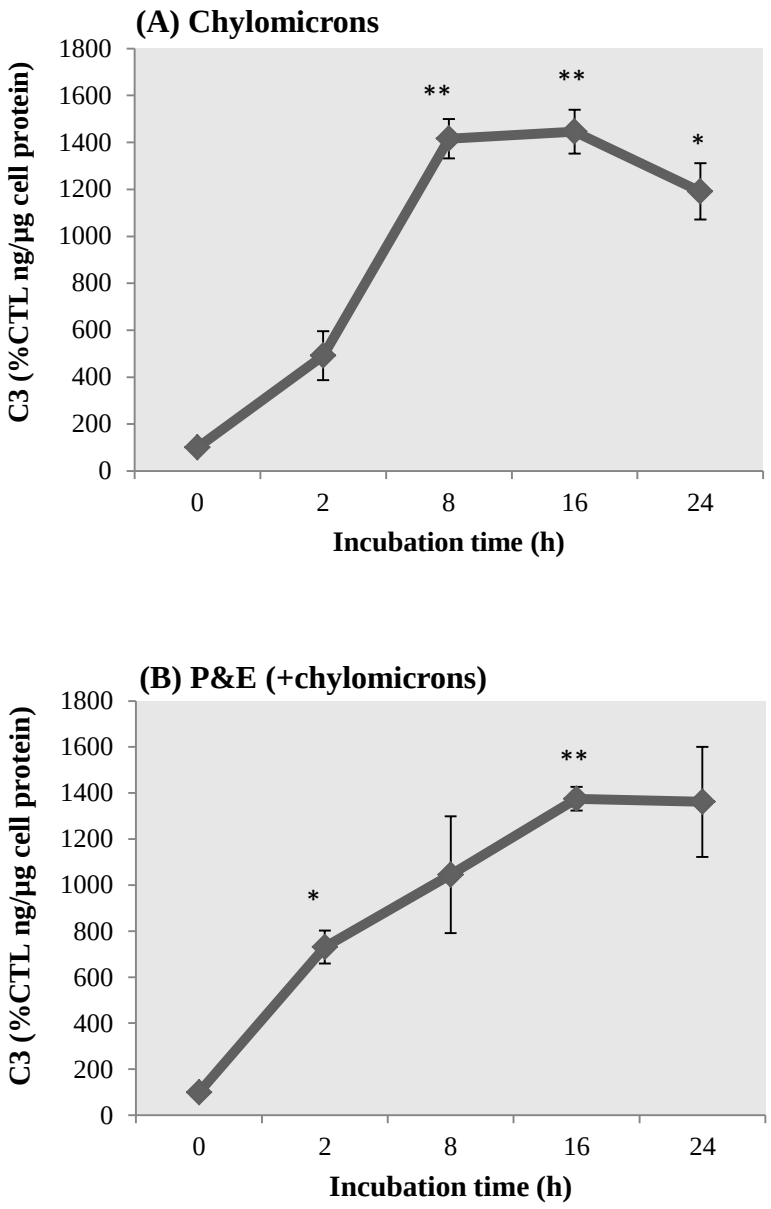


Figure 4.6 C3 production at different time points in 3T3-L1 adipocyte. (**A**) Chylomicrons increased C3 production significantly ($p<0.001$) at 8-h, 16-h and 24-h, (**B**) P&E (+chylomicrons) increased C3 production significantly ($p=0.01$) at 2-h and 16-h. **$p<0.001$, *$p< 0.01$. All data presented as mean ± (S.E.M.) of the percentage of the control (% CTL) (n=4). Analysis was carried using RMANOVA followed by Bonferroni post hoc test.

Consequently, the relative constant production of ASP compared with the increased precursor C3 meant that the ratio (ASP/C3) decreased dramatically when compared to the control (zero time; % CTL) in the chylomicron treated group ($F_{(1.09,3.27)}$ = 841.63, p< 0.001). The reduction in ASP/C3 ratio was determined at 2-, 8-, 16- and 24-h time points in comparison with the control (p<0.001) as presented in **Figure 4.7** (**A**). Similarly, the ASP/C3 ratio decreased in a time dependent manner with P&E (+chylomicrons) treatment ($F_{(1.12,3.35)}$ = 841.63, p< 0.001) at 2- h (p<0.001), 8-h (p=0.02), 16-hour (p<0.001) and 24-h (p<0.001) as shown in **Figure 4.7** (**B**).

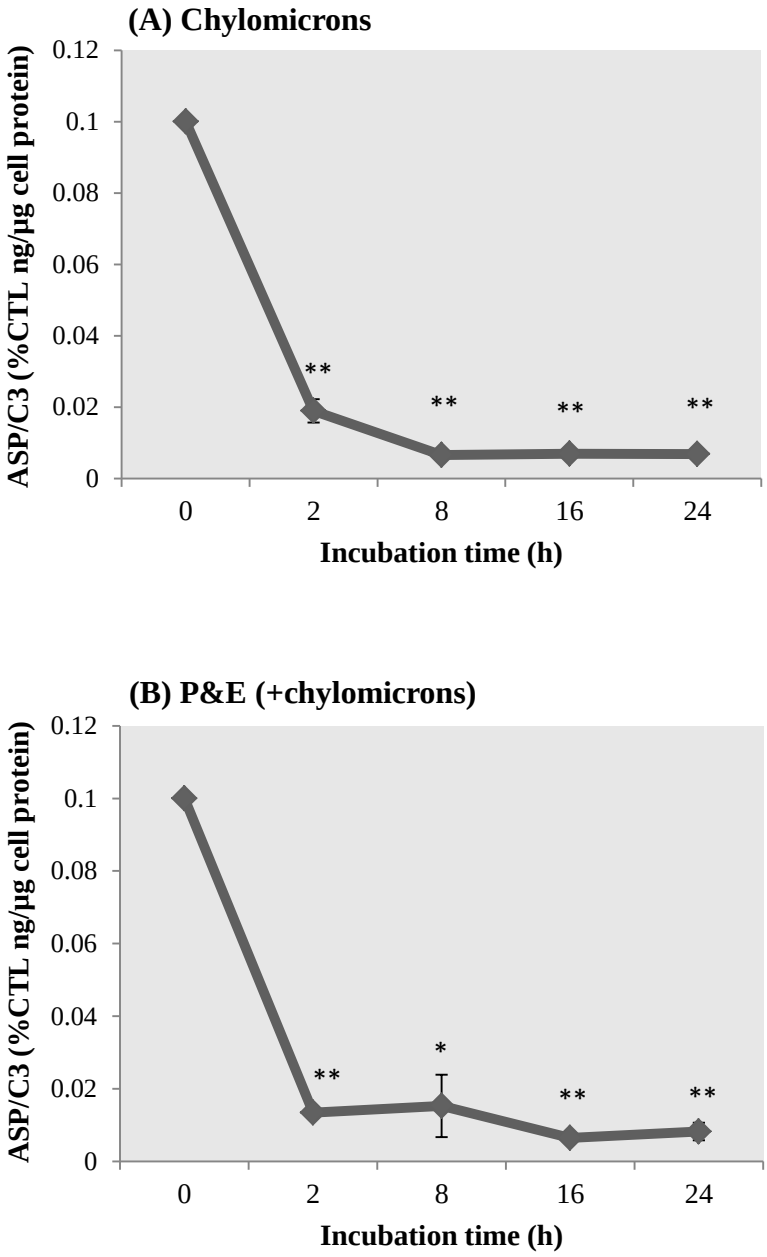


Figure 4.7 ASP/C3 productions at different time points in 3T3-L1 adipocyte. (**A**) Chylomicrons decreased ASP/C3 ratio significantly ($p<0.001$) at 2-, 8-, 16- and 24-h, (**B**) P&E (+chylomicrons) decreased ASP/C3 ratio significantly ($p<0.001$) at 2-, 8-, 16- and 24-h. **$p<0.001$, *$p< 0.01$. All data presented as mean ± (S.E.M.) of the percentage of the control (% CTL) (n=4). Analysis was carried using RMANOVA followed by post hoc using Bonferroni correction.

4.2.3.2 Effect of chylomicrons P&E (+chylomicrons) on C5L2 protein expression

There was no effect of chylomicron and P&E (+chylomicron) treatment on C5L2 receptor expression at different time points ($p=0.77$ and $p=0.37$, respectively by RMANOVA) (**Figure 4.8** (**A**) and (**B**)).

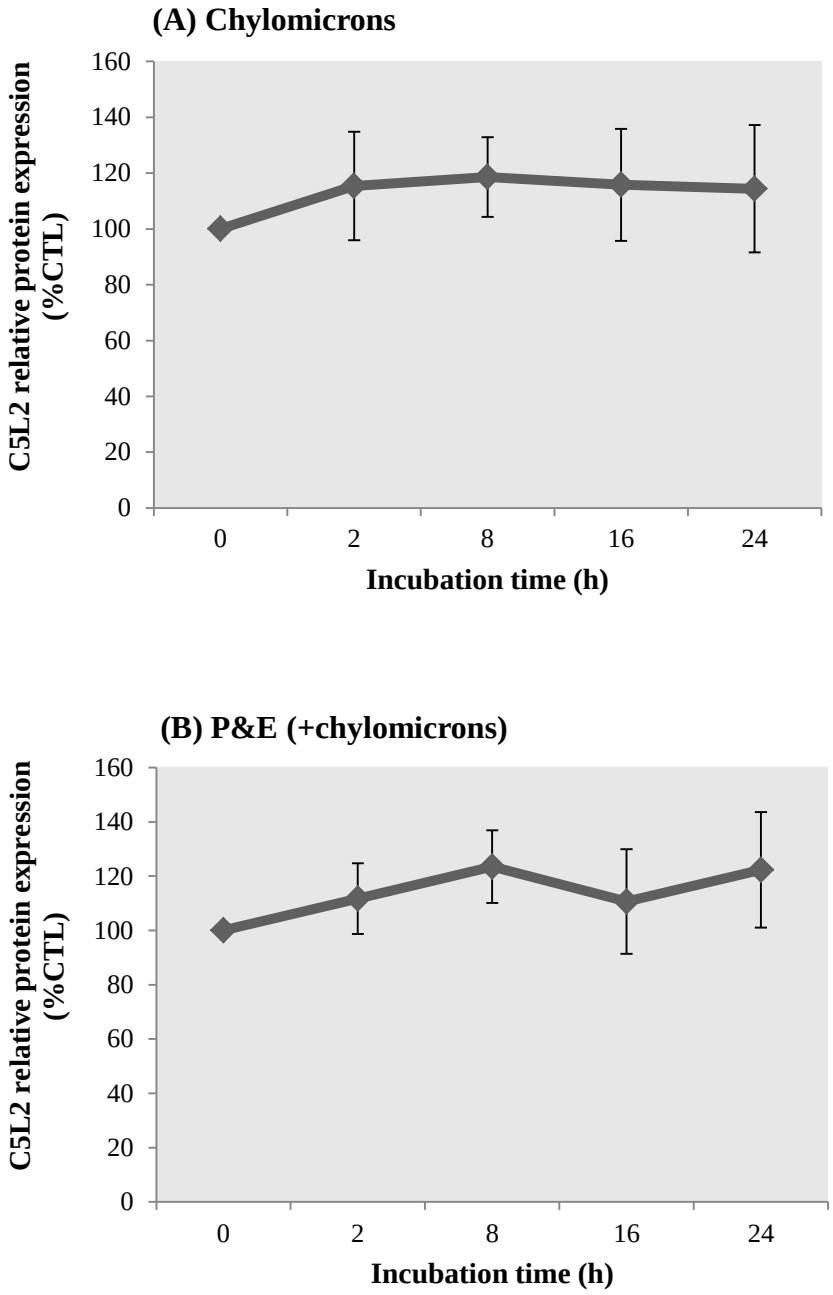


Figure 4.8 C5L2 relative protein expression to β-actin at different time points in 3T3-L1 adipocyte. (**A**) Chylomicrons treatment had no effect at different incubation time (p=0.77), (**B**) P&E (+chylomicrons) treatment had no effect on the expression of C5L2 at different incubation time (p=0.37). All data presented as mean ± (S.E.M.) of the percentage of the control (% CTL) (n=4). Analysis was carried using RMANOVA.

4.2.3.3 Effect of chylomicrons P&E (+chylomicrons) on intracellular TG mass

Lipid accumulation in adipocytes was determined by measuring intracellular TG in 3T3-L1 adipocytes for each treatment at different time points. RMANOVA showed no effect of chylomicrons (p=0.36) or P&E (+chylomicrons) (p= 0.19) treatments on total cell lipid at different time points as presented in **Figure 4.9** (**A**) and (**B**) respectively.

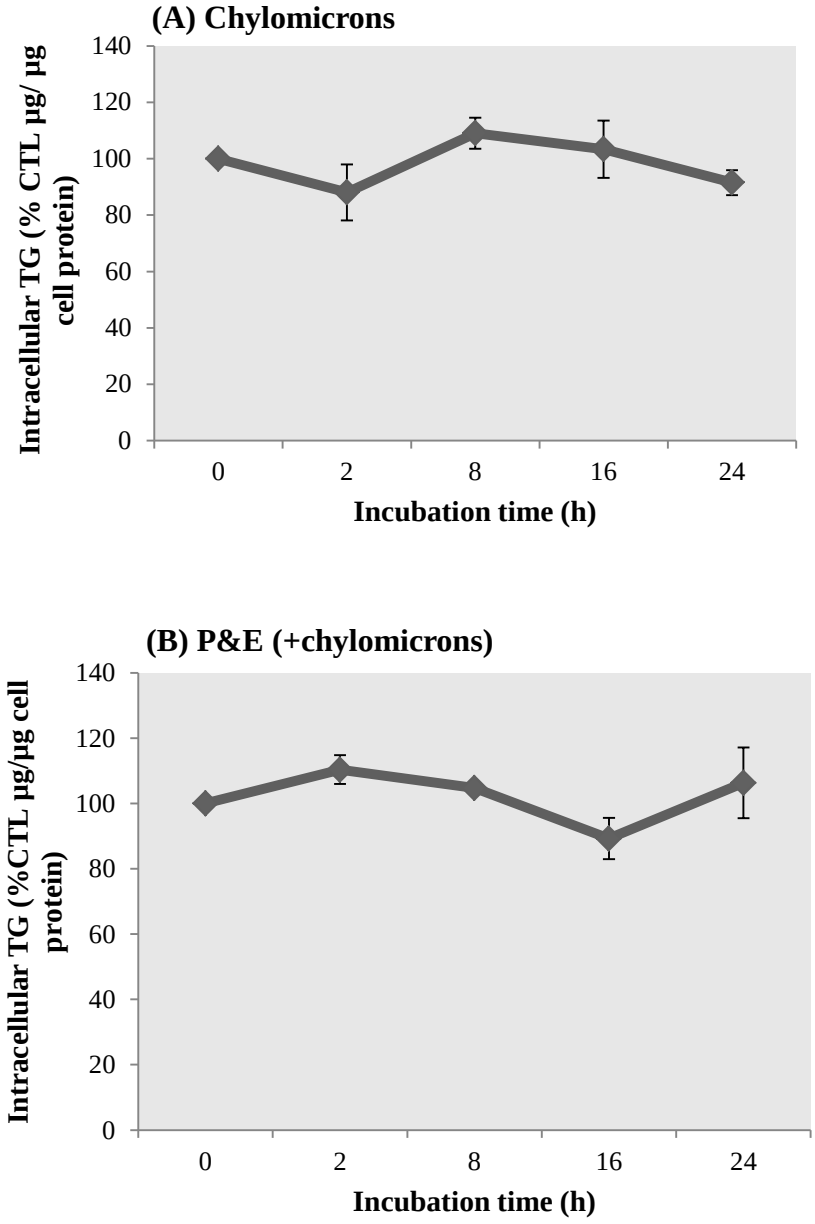


Figure 4.9 Intracellular TG measured at different time points in 3T3-L1 adipocyte. (**A**) Chylomicrons treatment had no effect at different time point (p=0.36), (**B**) P&E (+chylomicrons) treatment had no effect on the expression cell TG at different time point (p=0.19). All data presented as mean ± (S.E.M.) of the percentage of the control (% CTL) (n=4). Analysis was carried using RMANOVA.

Further analysis of total cellular mass of TG with other measured parameters using Pearson correlation, showed that intracellular TG correlated positively with ASP concentration (r=0.84, p<0.003) (**Figure 4.10 (A)**) and not significant with C5L2 (r= -0.05, p=0.85) (Figure 4.10 (**B**)) in the chylomicron treatment group

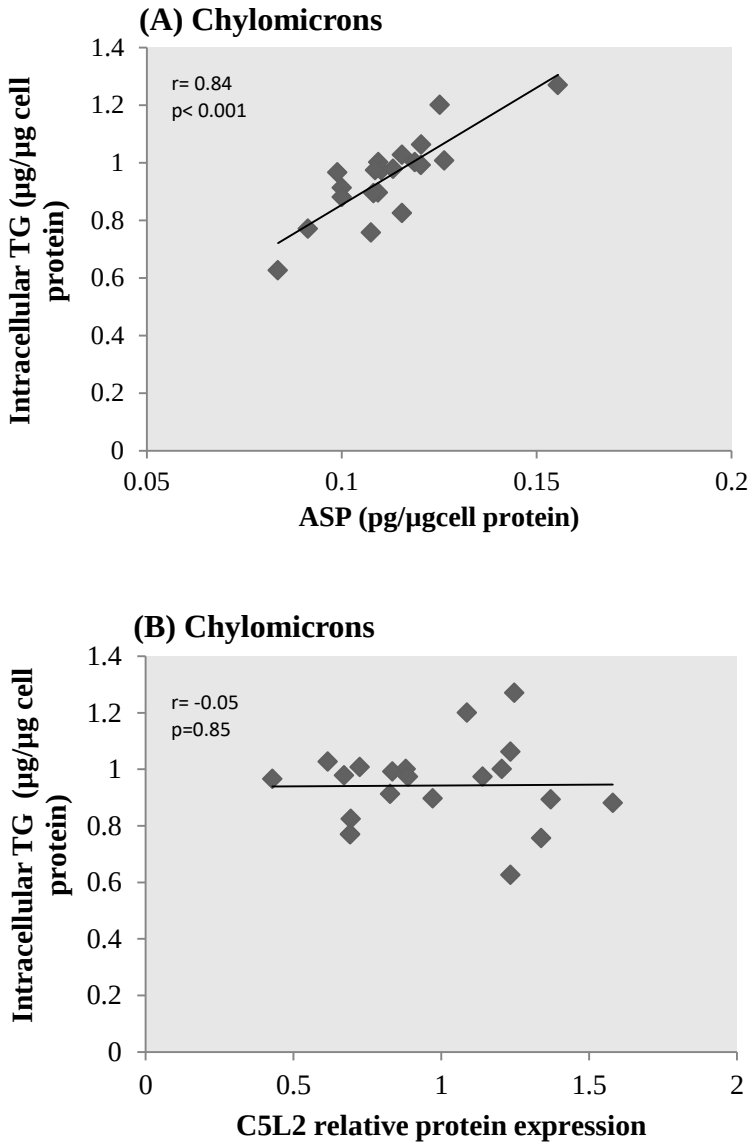


Figure 4.10 Correlation of intracellular TG with (**A**) ASP (p<0.001) (**B**) C5L2 (p=0.85) in chylomicron treatment group. Analysis carried by using Pearson correlation.

Interestingly, in the P&E (+chylomicron) treatment group, TG amount correlated positively with ASP (r=0.44, p=0.05) and C5L2 protein expression (r=0.56, p=0.01) as shown in **Figure 4.11** (**A**) and (**B**) respectively.

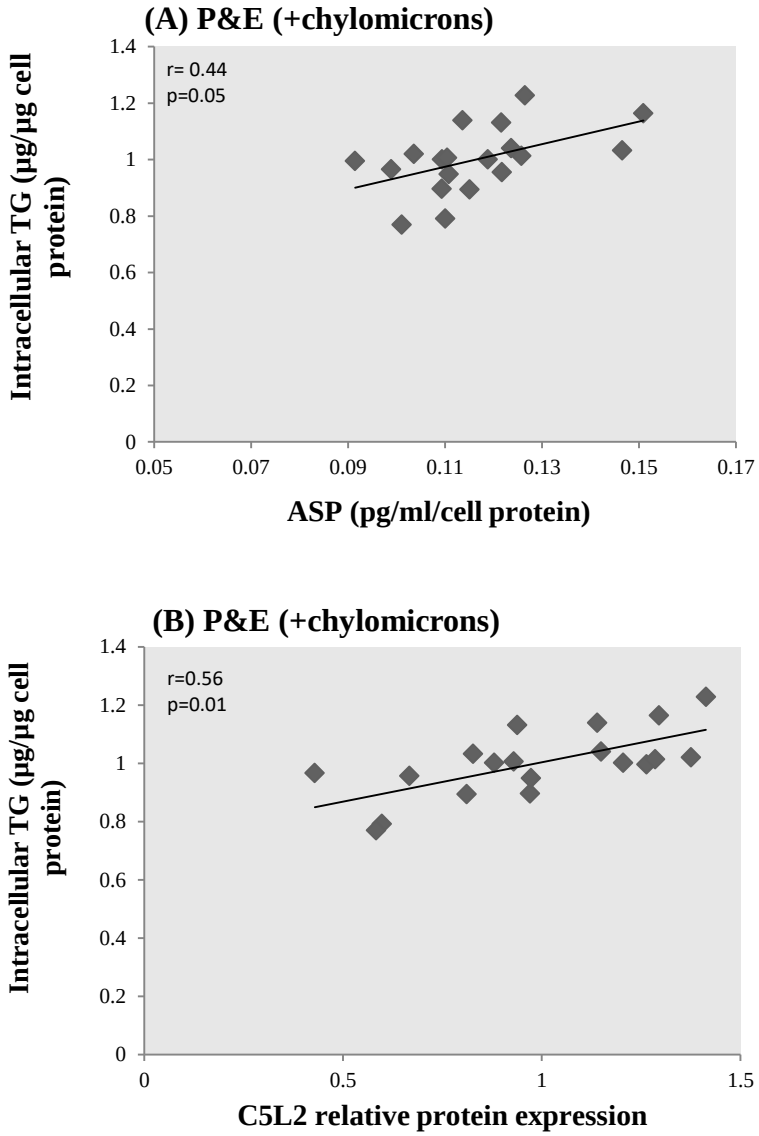


Figure 4.11 Correlation of intracellular TG with (A) ASP (p=0.05) and (B) C5L2 protein expression (p= 0.01) in P&E (+chylomicron) treatment group. Analysis carried by using Pearson correlation.

4.2.4 Discussion

ASP production from human differentiated adipocytes is associated with three protein factors (C3, adipsin and factor B), which promote triglyceride synthesis (Cianflone and Maslowska, 1995). Following the findings that plasma ASP increases after an oral fat load in human (Cianflone et al., 1989b), chylomicrons were defined as a key component in stimulating ASP and C3 production in cultured human adipocytes in a dose and time dependent manner (Maslowska et al., 1997). Female steroid hormones have been found to mediate ASP production as demonstrated earlier in this thesis. Progesterone also enhanced ASP function in clearing postprandial fat load in animals (Al Riyami et al., 2014). In the current experiment, we investigated the effect of female steroid hormone and chylomicrons on the production of ASP, C3 and C5L2 at different time points up to 24 hours using 3T3-L1 adipocytes in culture. Overall, we found no time-effect of P&E treatment (at the physiological concentration of 10^{-7} M) and low-dose chylomicrons (100 µg TG/ml) on ASP production, C5L2 expression and lipid accumulation. Chylomicrons were shown to have a dramatic stimulatory effect on C3 production in a time-dependent manner with no influence of P&E treatment. However, cell TG uptake in P&E treatment was found to associate with ASP and its receptor C5L2.

In the present work, chylomicrons (at low concentration) had no effect on ASP production after 24 h incubation, and we also found no enhancement of ASP production by P&E when added to the chylomicron treatment. On the other hand, C3 concentration increased significantly with both treatments with time

(**Figure 4.6** (**A**) and (**B**)). It was observed that P&E treatment accelerated the initiation of increased C3 production at 2 h time point when compared with only chylomicrons (C3 increased significantly at 8 h time point). C3 knockout, and therefore ASP deficient mice, studies had delayed postprandial TG clearance due to the lack of ASP synthesis in the alternative pathway (Murray, 1999, Persson et al., 2004, Paglialunga et al., 2008) . Postprandial TG-rich chylomicrons accelerated the generation of C3, therefore ASP production was increased in a time and dose-dependent manner (Fujita et al., 2007). Another factor shown to have an effect on C3 gene expression is estrogen which increased C3 expression and factor B in rats' uterus (Sundstrom et al., 1989, Kuivanen et al., 1989, Hasty et al., 1993) . Li S.H. and coworkers in 2002 carried interesting research on endometrium expression of C3 and factor B in mice. They found that during estrus cycle and pregnancy, C3 and factor B expression increased. Furthermore they injected OVX mice with estrogen hormone and same effect was obtained. However progesterone showed no effect on the expression of both genes, and by injecting both hormones, C3 expression increased and factor B expression modulated slightly (Li et al., 2002). Based on the above studies, in adipose tissue it appears that estrogen can enhance the production of C3 in response to chylomicrons in adipocytes.

The conversion rate of C3 to ASP is reflected by ASP/C3 ratio that was significantly decreased at all measured time points. Our findings were consistent with the findings of a study using differentiating human adipocytes where chylomicrons increased C3 significantly, but not ASP although it was produced in

parallel to C3 up to 24 h incubation (Scantlebury, 1998). In contrast, Gao et al., demonstrated that chylomicrons increased ASP concentration after 48 h incubational time, but not C3 and this was directly related to the significant effect of chylomicrons on the conversion of C3 to ASP (Gao et al., 2011). The discrepancy between the studies might be related to the shorter incubation period applied in this study under minimum dose of stimulation by chylomicrons and P&E. It would be of interest to investigate this further, as it may be that there is a lag between increased C3 and ASP production. The effect of chylomicrons seems to hinder the action of P&E on inducing ASP production as we demonstrated earlier in this chapter. This may occur through distinctive pathway largely by acting on high production of C3 to generate ASP to act on clearing postprandial fat load. However, we can't rule out the effect of P&E in the presence of chylomicrons based on the association analysis between ASP and TG that will be discussed further in this section. In the absence of chylomicrons, PE appears to exert long-term influences, which contribute to depot specific effects that we were able to see in the *ex vivo* experiment using subcutaneous and visceral tissue explants (Chapter 3).

Conversion rate is dependent on other factors that should be considered, such as the convertase activity of factor B and adipsin, that are further being affected by the action of known regulators such as factor H and I (Fujita et al., 2007, Gao et al., 2010), all of which could impact upon the relationship between C3 and ASP. Postprandial human studies have demonstrated no change in plasma ASP concentration (Koistinen et al., 2001, Ylitalo et al., 2002) or a slight tendency for

ASP to decrease in response to fat load over time (Weyer and Pratley, 1999, Faraj et al., 2001). It is only when a high fat load has been used that ASP has been found to increase (Faraj et al., 2001). Bioactive conversion of C3 to ASP is dependent on the enzyme adipsin and the presence of factor B. Insulin has been found to trigger adipsin activity, and in combination with chylomicrons, it was shown to enhance the chylomicron effect on C3 secretion and conversion (Maslowska et al., 1997). The factors involved in C3 conversion to ASP may play a more important role in the production of ASP, irrespective of overall C3 concentration. A number of studies of C3 and ASP suggested that only a very small proportion of C3 is converted to circulating ASP (Cianflone et al., 2003).

It has been suggested that high concentrations of steroid hormones interfere with ASP function by inhibiting glucose transporter stimulation and by the reduction of C5L2 expression (Wen et al., 2008). In the current experiment the expression of C5L2 did not change throughout the time course with either chylomicron or P&E treatments. ASP actions on TG synthesis and fatty acid uptake occur through interaction with C5L2 receptor, consequently, with no detectable change in ASP and C5L2, it might be expected that the intracellular TG concentration did not change over the 24 h period. Interestingly, correlation analysis showed that, in the chylomicron group, TG concentration in adipocytes was positively associated with ASP concentration. ASP directly regulates TG synthesis and clearance by stimulating DGAT and LPL activity (Yasruel et al., 1991, Sniderman et al., 1997). The production of ASP from adipose tissue has been shown to increase concurrently with TG clearance (Saleh et al., 1998) and the capacity for TG

synthesis in adipocytes is augmented with ASP efficiency in TG clearance. The notion of a positive correlation between ASP and efficiency in TG and NEFA clearance was studied in obese and ASP-deficient animal models (Murray, 1999, Saleh et al., 1999) . The administration of ASP in these models increased the clearance of TG and NEFA from the plasma. ASP has also been found to correlate positively with BMI in women and lowering TG concentration (Scantlebury-Manning et al., 2009). Clearance of postprandial TG has been demonstrated to be more efficient in women compared to men (Cianflone et al., 2004), indicating that women are more sensitive to ASP. In the present study, TG concentrations in adipocytes correlated positively with ASP and C5L2 receptor expression when female hormones were added with chylomicrons. The importance of C5L2 was highlighted in a C5L2 knockout mice by Paglialunga et al., who demonstrated that the lack of ASP-C5L2 interaction resulted in delays in TG clearance strengthening the role of C5L2 beside ASP in lipid metabolism (Paglialunga and Cianflone, 2007).

In summary, we can conclude that chylomicron stimulation of C3 did not increase ASP synthesis and P&E treatment, concurrent with chylomicrons did not enhance the conversion of C3 into ASP. However, P&E treatment was found to enhance the initiation of C3 production compared to treatment with chylomicrons alone. Various researchers have linked the induction of ASP to chronic exposure to elevated sex steroid hormones is associated more with metabolic syndrome. The fact that when cells were treated with P&E and chylomicrons, intracellular TG correlated positively with both ASP and C5L2 suggests the importance of female

sex steroid hormones in the regulating TG synthesis in response to postprandial fat. Collectively, without chylomicrons, P&E can stimulate ASP production and this might be more associated with long-term effect on specific fat depots regulation.

CHAPTER 5. REGULATION OF THE ASP-C5L2 SIGNALING PATHWAY BY ESTROGEN AND PROGESTERONE RECEPTORS'

Data presented earlier in this thesis, showed that treatment of 3T3-L1 cells with female steroid hormones impacted upon the production of ASP. For the experiments presented in the current chapter it was hypothesized that the actions of female steroid hormones on ASP production are mediated through their receptors in 3T3- L1 adipocytes. Furthermore, it was hypothesized that the blocking of estrogen or progesterone receptors would interfere with ASP signaling pathway components in adipocytes.

5.1 Introduction

G protein coupled receptor receptors (GPCRs) are important regulators of many physiological processes that control energy homeostasis (Tao Y.X. et al., 2013). C5L2 is part of the GPCR family and was identified as a second C5a receptor (C5a R2) that is highly expressed in adipose tissue with high binding affinity to ASP (Kalant et al., 2005). ASP-C5L2 binding stimulated TG synthesis via the initiation of intracellular signaling cascade, resulting from the activation of protein kinase C (PKC) and increasing DGAT1 activity and TG synthesis in adipocytes (Baldo et al., 1995).

The earliest investigation of ASP stimulation of the TG pathway, by Baldo et al., demonstrated the increases in intracellular DAG and protein kinase C (PKC) translocation, as well as the inhibition of PKC, affected TG synthesis (Baldo et al., 1995). Later, using a stimulated preadipocyte cell model, Maslowska et al, identified and proposed the ASP-C5L2 signaling pathway (Maslowska et al., 2006). The pathway starts with ASP activation of C5L2, which leads to Gα subunit dissociation, leading to the activation of both phospholipid lipase (PLC) and phosphoinositide-3-kinase (PI3K) pathways. The activation of both PLC and PI3K pathways is important for ASP-TG synthesis in adipocytes (Maslowska et al., 2006).

Female hormones play a role in fat distribution and lipid metabolism that is evident as hormonal levels change with age. Most sex steroid hormones are pooled in adipose tissue at a concentration 10-fold higher that the circulation and

so have impact on adipose tissue metabolism (Feher and Bodrogi, 1982). This regulatory effect of sex hormones is complex and dependent upon the activation of specific receptors inducing lipolytic and lipogenic signaling cascades in depot- and sex- dependent manner (Rodriguez-Cuenca et al., 2005). The relationship between steroid receptors and adipokines has been examined by a number of studies, particularly in relation to estrogen. The effect of estrogen and its receptors on vistatin and retinol-binding protein 4 (RBP4) adipokines using 3T3-L1 adipocytes has been investigated by Jung et al., (Jung et al., 2013). Both adipokines were shown to be elevated with obesity and related to insulin sensitivity (Fukuhara et al., 2005, Masaki et al., 2008). Estrogen receptors showed no effect on vistatin production or expression in 3T3 cells. However, estrogen showed a regulatory effect on the expression of retinol-binding protein 4 (RBP4) through ER α (Jung et al., 2013). In another study, leptin expression in 3T3 cells was induced by ER α and suppressed by ER β, with the ratio between both receptors regulating the expression of leptin (Yi et al., 2008).

Studies have demonstrated that ERs can mediate some signaling pathways which are related to the ASP-TG synthesis pathway, though there is little known yet about the cross-talk of female steroid receptors and ASP. As we demonstrated earlier, a combination of progesterone and estrogen (P&E) stimulated ASP production. The presence of chylomicrons with P&E treatment showed that intracellular TG has associated with both ASP and C5L2 (Chapter 4, section 4.2). In this chapter experiments are described aimed a investigating the effect of ERs and PR (by using ER and PR antagonists) on ASP-C5L2 signaling pathway in

3T3-L1 adipocytes, under the stimulatory effect of progesterone and estrogen treatments in the presence of chylomicrons. This is achieved by using steroid receptor antagonists which are selectively synthesized compounds that are used to study the biological functions of the targeted receptor. MPP and R, R-THC are both a highly selective antagonists for ER-α and ER-β respectively and mifepristone antagonist are PR antagonist.

5.2 Materials and methods

In this section a brief description of the methods used to conduct the experiments is provided, with more detailed explanation of the materials and methods used in Chapter 2.

5.2.1 Cell culture

Mouse 3T3-L1 cells are well characterized model for adipocytes during differentiation and mature adipocyte with fat droplets (Green and Kehinde, 1975, Fajas et al., 1998, Cornelius et al., 1994). 3T3 cells were seeded at 40,000 cell per well of 6-well plates (n= 5) until they reached 80% confluence using phenol free DMEM media (10%FBS, 1% P/S). 2 days after confluence the media was changed to the differentiating media that included IBMX, dexamethasone and insulin. The differentiation of the cells was maintained later by using media included only insulin for approximately 8 days until the formation of mature adipocytes, which was confirmed by light microscopy. Before cell treatment, the mature cells were washed with PBS and maintained for 16 hours (overnight) with phenol free DMEM media (10%FBS, 1% P/S) without insulin. Earlier findings by Gao et al., have shown that insulin and a low concentration of chylomicrons had a profound stimulatory effect on ASP production in 3T3-L1 cell culture (Gao et al., 2011). For this reason, Before cell treatment, the mature cells were washed with PBS and maintained for 16 hours (overnight) with phenol free DMEM media (10%FBS, 1% P/S) to exclude the effect of insulin.

5.2.2 Cell treatments

Cells were washed with PBS, and media containing treatments were added to the cells. The treatments included the antagonist drugs at concentration of 10^{-5} M of MPP (ERα receptor antagonist), (R-R)-THC (ER β receptor antagonist), MPP& (R,R)-THC and Mifepristone (PR antagonist) (Jung et al., 2013). Cells in each treatment group were cultured for 30 minutes with antagonists as described in (Yen et al., 2008), while the control group and P&E group were maintained with normal growth media. Then, the cells were washed again with PBS and incubated for 24 hours at 37 °C and 5 % CO2 with different treatments according to **Table 5.1**. All treatment groups (including the control) had a low dose of chylomicrons establishing a postprandial-like state for the cells.

Table 5.1 Treatment mixture in different groups

	Treatment	**Dose**
Control	Chylomicrons	TG (100 µg/ml)
P&E	Chylomicrons	TG (100 µg/ml)
	Progesterone	10^{-5} M
	Estrogen	10^{-7} M
MPP	Chylomicrons	TG (100 µg/ml)
	Progesterone	10^{-5} M
	Estrogen	10^{-7} M
	MPP	10^{-5} M
(R,R)-THC	Chylomicrons	TG (100 µg/ml)
	Progesterone	10^{-5} M
	Estrogen	10^{-7} M
	(R,R)-THC	10^{-5} M
MPP& (R,R)-THC	Chylomicrons	TG (100 µg/ml)
	Progesterone	10^{-5} M
	Estrogen	10^{-7} M
	MPP	10^{-5} M
	(R,R)-THC	10^{-5} M
Mifepristone	Chylomicrons	TG (100 µg/ml)
	Progesterone	10^{-5} M
	Estrogen	10^{-7} M
	Mifepristone	10^{-5} M

5.2.3 Media and cell harvest

At the end of treatment, media was collected and stored at -80 °C to measure ASP levels using mouse C3a ELISA (Novateinbio, USA) (more details found in chapter 4). The results were expressed relative to total protein. Cells were harvested for RNA by scraping wells in 150 µl of RNase-free PBS, which was then frozen at -80 °C until analysis. 50 µl of the cell-PBS were mixed with 50 µl of cell lysis buffer (2x). The cells were homogenized and total protein was quantified using Lowry assay method. Intracellular TG was measured in cell lysed homogenates using TG assay method as described in (Kang et al., 2013). Briefly cells were homogenate in lysis buffer and measured for TG concentration enzymatically (more details in **section 1.1.5.2** and **4.1.2.2.**)

5.2.4 Quantitative PCR

Total RNA was extracted from cells using the RNeasy ® Lipid Tissue mini kit (Qiagen). QIAzol lysis reagent was replaced by RTL lysis buffer (350 µl of RLT to 100 µl of cells in PBS). Following all the steps set out in the manufacturer's kit protocol (including on-column DNase digestion), 50 ng/ µl extracted RNA was used for cDNA synthesis using First Standard cDNA synthesis Kit (Roche). qRT-PCR was conducted on a Roche Lightcycler480 to determine the expression of different genes involved in the ASP-C5L2 signaling pathway. The results were normalized and presented as the gene expression relative to β-actin housekeeping gene. The primers used in this experiment section were designed using Primer Express v3.0 software or adapted from publications; PKC α from (Wen et al.,

2011) , PI3K from (Liu et al., 2014) and GLUT4 from (Im et al., 2006) (**Table 5.2).**

Table 5.2 Oligonucleotides sequences for different genes in mouse

Gene	Accession number	Forward sequence	Reverse sequence
DGAT1	NM_010046.2	5'CGACGGCTACTGGGATCTGA 3'	5'GATAATTGCTGAACCACTGTCTGA 3'
Akt2	NM_007434.4	5' CGCCCAGTCCATCACAATC 3	5' TCCGCTGGTCCAGTTCCA 3'
PLC-β	NM_001290790.1	5'CGGTGTGTAGAGTTAGACTGTTGGA3'	5' GGTGAAGCCGTGGGTGATAA 3'
PKC α	NM_011101.3	5' CCCATTCCAGAAGGAGATGA 3'	5' TTCCTGTCAGCAAGCATCAC 3'
PI3K	NM_001077495	5' CTCTCCTGTGCTGGCTACTGT 3'	5' GCTCTCGGTTGATTCCAAACT 3'
C5L2	NM_001146005.1	5' CCGACTTGTGTGTGGCATAGA 3'	5' AGCCAAAGAGGAATCGAACAGT 3'
LPL	NM_008509.2	5' GGCCGAGAGCGAGAACATT 3'	5'TCCACCTCCGTGTAAATCAAGA 3'
GLUT4	NM_009204.2	5' AGAGTCTAAAGCGCCT 3'	5' CCGAGACCAACGTGAA 3'
β -actin	NM_007393.5	5' GGCACCACACCTTCTACAATGAG 3'	5'ACATGATCTGGGTCATCTTTTCAC3'

5.2.5 Statistical analysis

In this experiment, Normality and homogeneity were assessed by Shapiro-Wilk normality test and Levene's test respectively. Data is reported as mean ±standard error of the mean (S.E.M.) for n=5 replicates per group. ASP and intracellular TG were measured relative to total cell protein.

Statistical difference between treatment groups was determined by one-way ANOVA. Where ANOVA was used, Dunnett's post hoc analysis was applied to compare specific groups to the control group. Pearson's correlation was preformed to assess relationships between ASP, TG and gene expression in different treatment groups. Significance was set at $p< 0.05$ for all statistical analysis.

5.3 Results

Progesterone and estrogen at concentrations of 10^{-5} M and 10^{-7} M respectively have been shown to stimulate ASP production in 3T3 cells earlier in this thesis (Chapter 4). Through the ASP-C5L2 signaling pathway, triglycerides are synthesized by a multistep pathway (Maslowska et al., 2006), where DGAT1 catalyzes the final reaction (Yen et al., 2008). In this study chylomicrons were added to all treatment groups along with P&E hormone treatments to stimulate the lipogenesis function of ASP.

5.3.1 ASP level in the media and intracellular TG and associations

ASP levels in the media were measured relative to cell total protein as data are presented in **Table 5.3**. Treatment of 3T3-L1 adipocytes with P&E and different antagonists showed no effect on ASP concentrations ($F_{(5,22)}= 0.75$, $p =0.6$). However, a significant effect of P&E was seen on intracellular TG level ($F_{(5,22)}= 12.9$, $p<0.001$, **Table 5.3**).

Table 5.3 ASP level in the media and intracellular TG level in 3T3-L1 adipocyte cells. Cells incubated for 24h with chylomicrons (control group) or with P&E (+chylomicrons) with different selective ERs or PR antagonist.

Treatments	ASP (pg/µg cell protein)	TG (µg/ µg cell protein)
Control (only chylomicrons)	0.73 ± 0.4	148.0 ± 7.5
P&E (+chylomicrons)	0.90 ± 0.6	174.6 ± 3.9 **
P&E (+chylomicrons) -MPP	0.27 ±0.04	135.8 ± 4.2
P&E (+chylomicrons) -(R,R)-THC	0.28 ±0.1	128.2 ± 4.2
P&E (+chylomicrons)-MPP- (R,R)-THC	0.47 ±0.1	130.6 ± 4.3
P&E (+chylomicrons)-Mifepristone	0.45 ±0.1	130.6 ± 4.3

All data presented as mean± S.E.M (n=5). Analysis was determined by ANOVA followed by Dunnett's test. ** p = 0.008, Dunnett's test

Partial correlation analysis, that was controlled for all treatments, between ASP and intracellular TG levels showed a positive association between the amount of TG in cells and the concentration of ASP in the media (r= 0.43, p =0.04) Furthermore, analysis confined to the P&E treatment group showed that ASP concentration was associated positively with expression of DGAT1 (r= 0.97, p = 0.034) and PKCα (r= 0.96, p = 0.042). DGAT1 expression correlated positively with both PKCα (r= 0.91, p = 0.034) and PI3K (r= 0.93, p = 0.024) gene expression.

5.3.2 Effects of ER-α , ER-β and PR antagonists [MPP, (R,R)-THC and Mifepristone) on the ASP-C5L2 signaling pathway

Based on earlier results showing an effect of P&E treatment on intracellular TG accumulation in 3T3-L1 cells, the effects of antagonist receptor treatments on ASP-C5l2 signaling pathway components were analyzed using the P&E treatment

group as the control (using Dunnett's post hoc test when significant was determined by ANOVA) in the following analysis. The cells were simultaneously treated with fixed concentration of progesterone (10^{-5} M) and estrogen (10^{-7} M) and low dose of chylomicrons (100 µg/ml).

5.3.2.1 C5L2 receptor mRNA expression

C5L2 receptor mRNA differed statistically between treatment groups ($F_{(5, 28)}$ =0.75, p =0.003). Post hoc tests showed that blocking both estrogen receptors (ER-α and ER-β) using MPP and (R,R)-THC antagonists combination, led to a significant increase in C5L2 gene expression (p = 0.002), the increase being almost 5-fold greater than expression in the P&E group (**Figure 5.1)**.

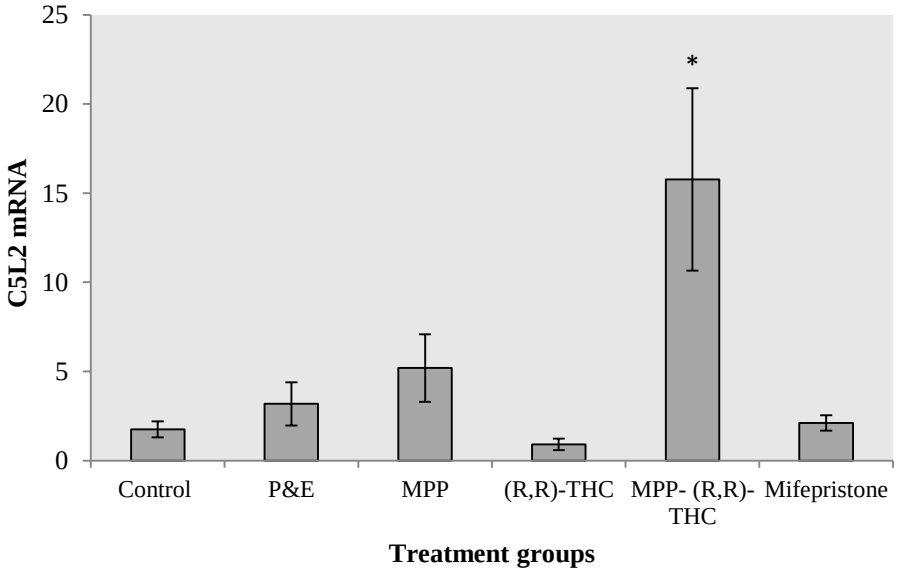


Figure 5.1 mRNA C5L2 relative gene expression differed statistically between treatment groups ($F_{(5, 28)}$ =0.75, p =0.003). C5L2 expression increased significantly in MPP and (R,R)-THC treated group (p = 0.002). All data presented as mean± S.E.M (n=5). Analysis was determined by ANOVA followed by Dunnett's test.

5.3.2.2 Effect of treatments on the PI3K/Akt pathway

ASP has been proposed to increase TG synthesis in adipocytes through the activation of both PI3K and PLC pathways (Maslowska et al., 2006). Using high doses of ERs and PR antagonists in this experiment along with P&E (+ chylomicrons) treatment did not show any effect on the gene expression levels of PI3K ($F_{(5,28)}$= 0.75, p = 0.59) or the downstream Akt ($F_{(5,27)}$= 0.68, p = 0.65) pathway as presented in **Figure 5.2** and **Figure 5.3** respectively.

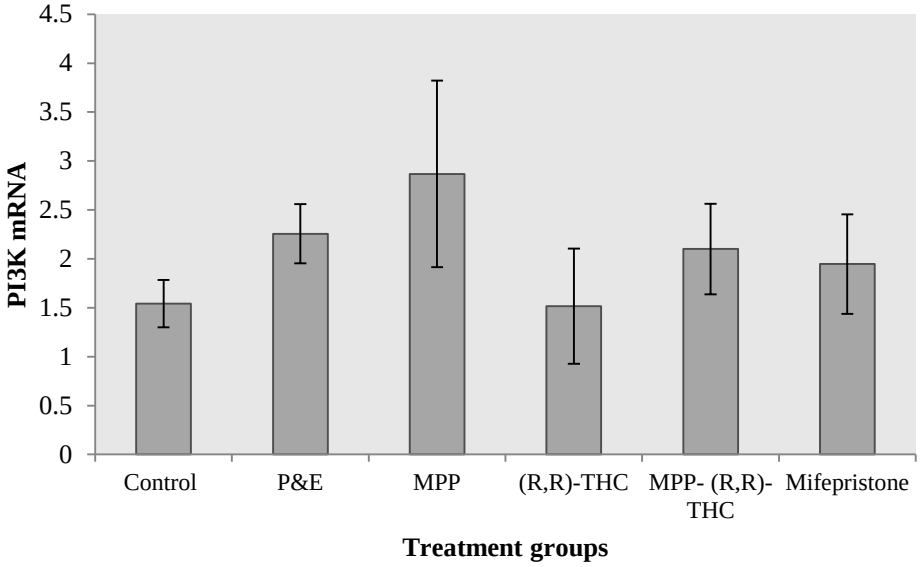


Figure 5.2 mRNA PI3K relative gene expression showed no significant differences between different treatment groups ($F_{(5,28)}$= 0.75, p = 0.59). All data presented as mean± S.E.M (n=5). Analysis was determined by ANOVA.

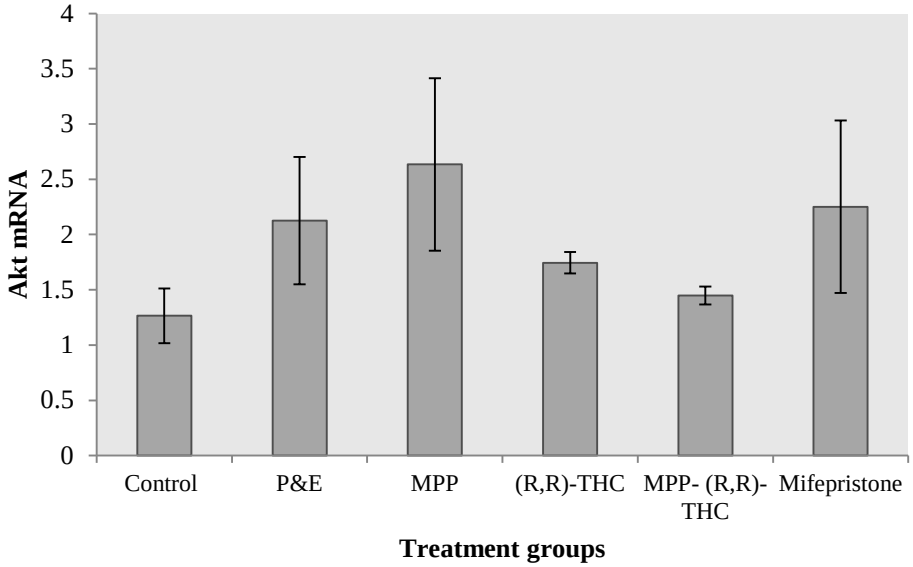


Figure 5.3 mRNA Akt relative gene expression showed no significant differences between different treatment groups ($F_{(5,27)}= 0.68$, $p = 0.65$). All data presented as mean± S.E.M (n=5). Analysis was determined by ANOVA.

5.3.2.3 Effect of treatments on PLC/PKCα and DGAT1

Antagonist treatments had an effect on PLC-β gene expression ($F_{(5.27)}= 7.63$, $p <0.001$) (**Figure 5.4**). A significant (5-fold) increase in PLC expression was noted when both ER-α and ER-β were inhibited by using MPP and (R,R)-THC antagonists in comparison to the P&E control group ($p = 0.001$).

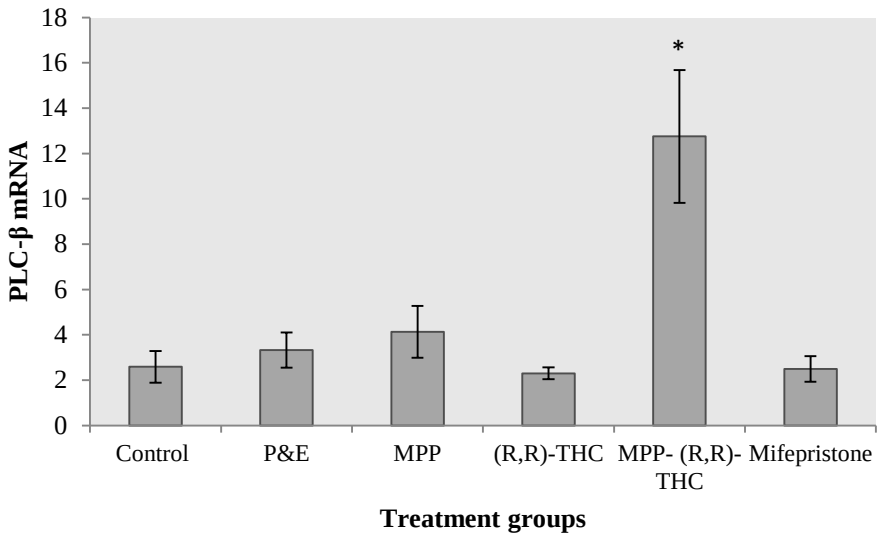


Figure 5.4 mRNA PLC-β relative gene expression differed statistically between treatment groups ($F_{(5,27)}$= 7.63, $p < 0.001$). PLC-β expression increased significantly in MPP and (R,R)-THC treated group ($p = 0.001$). All data presented as mean± S.E.M (n=5). Analysis was determined by ANOVA followed by Dunnett's test.

One way ANOVA showed no significant differences between different treatment groups on PKCα gene expression ($F_{(5,28)}$= 1.48, p = 0.23), as represented in **Figure 5.5**.

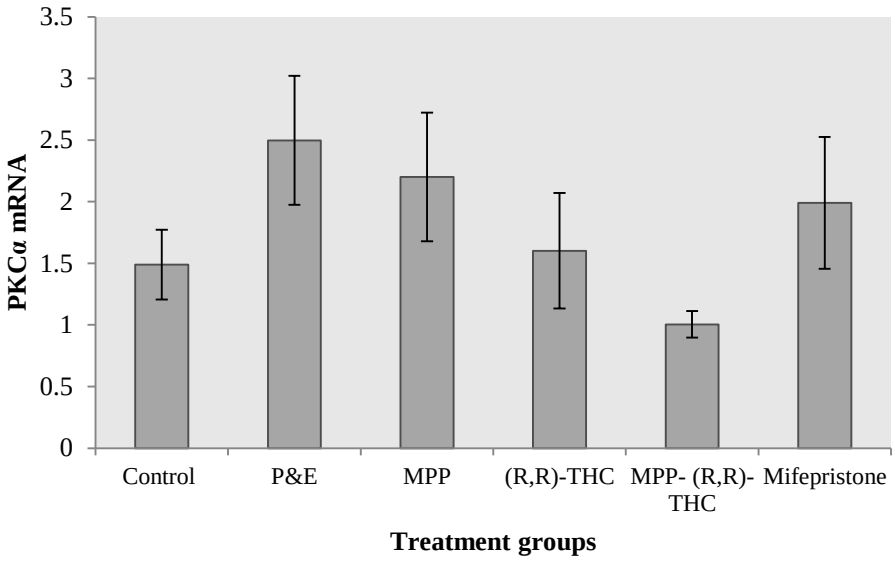


Figure 5.5 mRNA PKCα relative gene expression showed no significant differences between different treatment groups ($F_{(5,28)}$= 1.48, p = 0.23). All data presented as mean± S.E.M (n=5). Analysis was determined by ANOVA.

The treatments had no statistically significant effect on the expression of DGAT1 enzyme ($F_{(5,27)}$= 0.95, p = 0.97) (**Figure 5.6**).

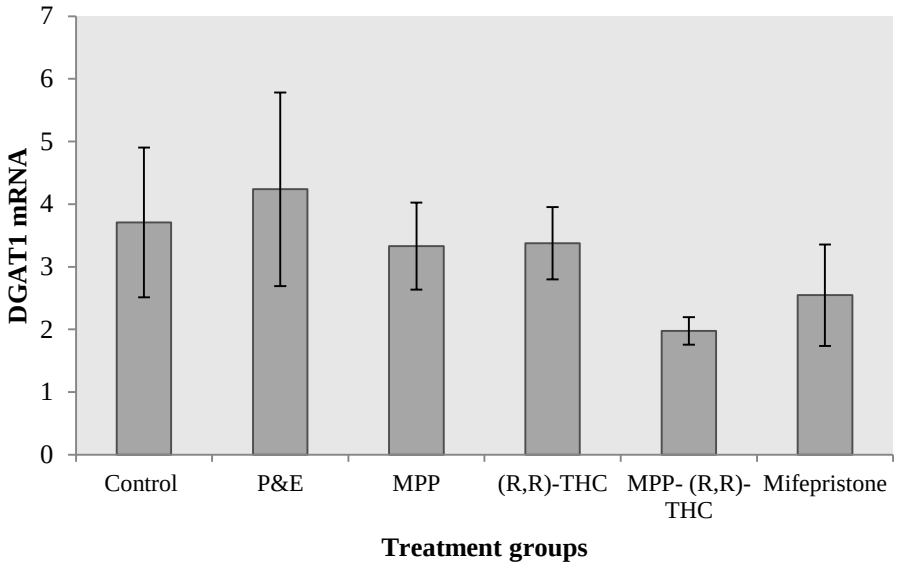


Figure 5.6 mRNA DGAT1 relative gene expression showed no significant differences between different treatment groups ($F_{(5,27)}$= 0.95, p = 0.97). All data presented as mean± S.E.M (n=5). Analysis was determined by ANOVA.

5.3.3 The effect of MPP, (R,R)-THC and Mifepristone antagonists on LPL and GLUT4

ASP is known to indirectly stimulate LPL by increasing the uptake of hydrolyzed TG (NEFA) into the adipocyte. ASP has also been shown to enhance glucose uptake by increasing the expression of glucose transporters (Cianflone et al., 2003). In this study there was no effect of different treatments on LPL gene expression ($F_{(5,27)}$= 1.85, p = 0.15) (**Figure 5.7**).

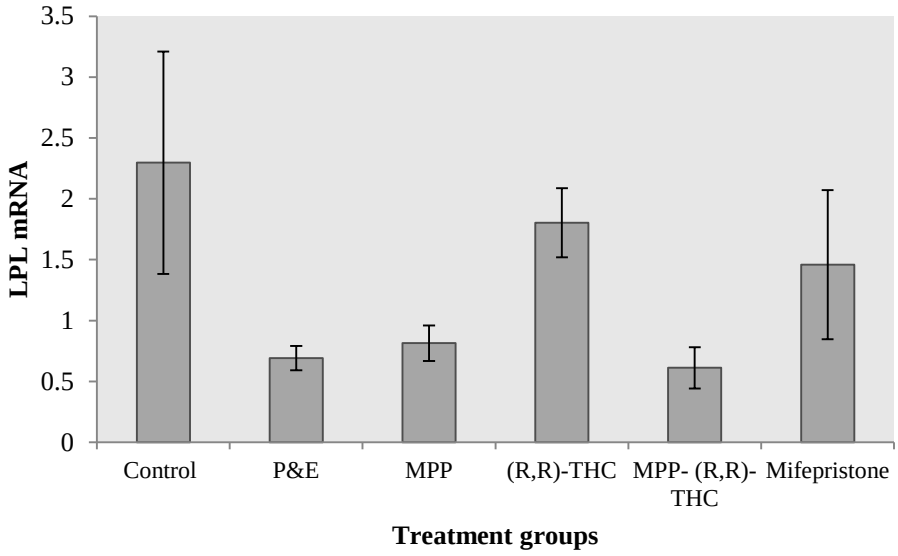


Figure 5.7 mRNA LPL relative gene expression showed no significant differences between different treatment ($F_{(5,27)}$= 1.85, p = 0.15). All data presented as mean± S.E.M (n=5). Analysis was determined by ANOVA.

ASP has been shown to increase glucose transporter and LPL activity in adipose tissue (Cianflone et al., 2003). We found that that the different antagonist treatments impacted on GLUT4 expression ($F_{(5,27)}$= 2.4, p = 0.07) (**Figure 5.8**).

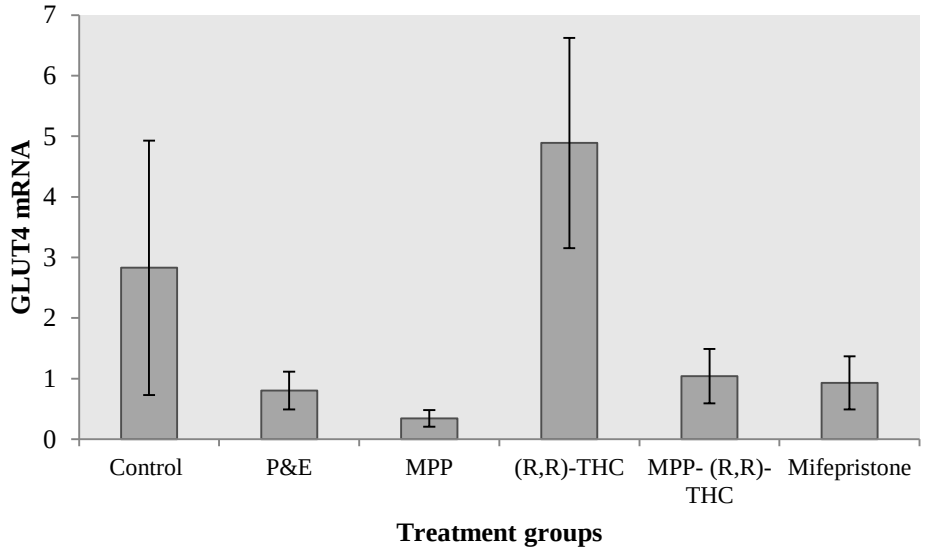


Figure 5.8 mRNA GLUT4 relative gene expression differed statistically between treatment groups ($F_{(5,27)}$= 2.4, p = 0.07). All data presented as mean± S.E.M (n=5). Analysis was determined by ANOVA followed by Dunnett's test.

5.3.4 Correlation analysis in ASP-C5L2 pathway

Partial correlation analysis, controlled for different treatments, was conducted to examine the relationship between different measured parameters (**Table 5.4**). The amount of intracellular TG associated strongly with ASP concentration in the media. In relation to signaling pathway components, TG level associated positively with PI3K and PKCα gene expression. It was demonstrated earlier that ASP-C5L2 pathway activation is dependent on the activation of PLC and PI3K pathways (Maslowska et al., 2006). Interestingly C5L2 correlated positively with

both PLC and PI3K gene expression. Correlation analysis also showed that LPL correlated positively with DGAT and GLUT4 gene expression, and all these factors are known to be enhanced by the action of ASP.

Table 5.4 Partial coefficient correlation of ASP, intracellular TG and related gene expressions controlled partially for different treatment group

	ASP	TG	DGAT1	Akt	PLC	PKCα	PI3K	C5L2	LPL
TG	**0.68								
DGAT1	-0.15	0.34							
Akt	-0.11	0.34	*0.55						
PLC	0.44	0.22	-0.24	-0.18					
PKCα	0.16	*0.62	0.44	**0.82	-0.19				
PI3K	0.49	*0.54	0.09	0.23	**0.67	0.42	1.00		
C5L2	0.46	0.23	-0.24	-0.06	**0.92	-0.07	**0.66		
LPL	-0.28	0.13	**0.85	**0.66	-0.46	0.46	-0.18	-0.39	
GLUT4	-0.19	-0.24	0.47	-0.02	-0.26	-0.20	-0.46	-0.31	*0.62

** $p < 0.001$, * $p < 0.05$

5.4 Discussion

Progesterone and estrogen in combination have been demonstrated earlier to stimulate ASP production in rat adipose tissue explants and in 3T3-L1 cells. To investigate the mechanism of P&E action on the ASP signaling pathway we specifically used ER antagonists and PR antagonists using 3T3-L1 cells. The treatments showed there were no significant effects on ASP production; however P&E (+chylomicrons) treatment increased intracellular TG which, in turn, was correlated with ASP concentrations. The combination of estrogen receptor antagonists (MPP-(R,R)-THC) markedly increased C5L2 and PLC expressions. GLUT4 expression was influenced mainly by ER-β as seen by using (R,R-THC) antagonist treatment. The blockade of progesterone or estrogen receptors was found to inhibit the effect of P&E hormones on increasing TG synthesis and may interact with the activity of ASP-C5L2 signaling pathway components.

In the past few years, there has been an increasing interest in the factors that regulate ASP production and mechanisms related to TG synthesis in adipocytes. Dietary fat, in form of chylomicrons, stimulates ASP and C3 and triggers the ASP pathway in human adipocytes (Scantlebury, 1998). In this study, ASP showed no significant increase in the chylomicrons and P&E treatment group. The presence of chylomicrons was found earlier to sharply increase C3 production and abolish, or delay, the stimulatory effect of P&E treatment on ASP production (chapter 4). Despite the unchanged ASP concentration in response to P&E (+chylomicrons) treatment, the lipid accumulation (determined by measuring intracellular TG level) was found to be significantly increased in the P&E treatment group (174.6

± 3.9 µg/ml/ cell protein) in comparison to the control group (148.0 ± 7.5 µg/ml/ cell protein) (**Table 5.3**). The amount of intracellular TG and media ASP levels were positively correlated, reflecting the functional role of ASP on the uptake of TG and fat storage. This strong link between ASP and TG has been demonstrated in many previous studies, especially during postprandial TG clearance in a number of situations including metabolic disturbance, weight variation and hormonal effects (Kalant et al., 2000, Cianflone et al., 2003, MacLaren et al., 2010, Al Riyami et al., 2014).

Correlation analysis confined to the P&E treatment group showed a positive association between ASP and DGAT1 gene expression ($r=0.95$, $p=0.047$). The effect of ASP on TG synthesis is mediated through increased DGAT activity and enhanced glucose uptake in adipose tissue (Cianflone et al., 2003). DGAT is the rate limiting enzyme involved in the TG synthesis pathway and its expression correlated positively with PI3K and PKCα gene expression. PI3K signaling cascade is known to mediate insulin effects on glucose homeostasis and lipolysis in response to nutritional changes (Duncan et al., 2007, Nelson et al., 2017). PI3K has also been reported to be activated by G-proteins and generates lipids through inducing a downstream specific signaling pathways such as Akt and PKC activation (Vanhaesebroeck et al., 2005). Based on the association findings, we postulated that the TG accumulation in response to P&E treatments in the adipocytes is stimulated through ASP signaling pathway components; PI3K and PKCα. We have also evaluated the effect of ERs subtypes and PRs on ASP production using selective antagonists. The results show that the blockade of

estrogen and progesterone receptors, using antagonist treatments, abolishes the increased intracellular TG caused by P&E treatment. This highlights the important role of estrogen and progesterone receptors in regulating TG synthesis in adipocytes in relation to ASP-C5L2 action.

Antagonist treatments for both ERs were found to increase the expression of C5L2 significantly (**Figure 5.1**). It was reported earlier by Wen et al., that high doses of progesterone or estrogen treatments, along with recombinant ASP protein, decreased C5L2 expression in 3T3 adipocytes (Wen et al., 2008). It seems that estrogen effects C5L2 expression in adipocytes, as indicated in the study of Wen et al., (Wen et al., 2008), with the effect mainly mediated by ER-α and -β. Selective antagonists for either ER-α or -β did not affect the expression of C5L2 and only by blocking both receptors did the expression of C5L2 increase significantly. Estrogen binding with ER-α/-β membrane receptors inhibits LPL (Homma et al., 2000) and activates cAMP and HSL, leading to lipolysis in adipose tissue (Shi et al., 2013). In addition, our findings suggests a lipolytic effect of estrogen through the inhibition of C5L2 via ER-α and -β and only by blocking the effect of both receptors can the inhibitory effect be abolished and C5L2 expression increased.

ER-α and -β antagonist treatments were also shown to increase the expression of PLC (**Figure 5.4**) in parallel with C5L2, suggesting that this gene must also be suppressed by estrogen, acting through either receptor subtype. PLC activation occurs through Gαq or Gβ/γ subunit (Lee et al., 1992), leading to the activation of intracellular enzymes including PKC, PI3K and Akt (Kayali et al., 1998, Yue et

al., 2000). PLC was reported to mediate TG synthesis stimulated by ASP in preadipocyte 3T3 cells (Maslowska et al., 2006). However, it was also demonstrated that the activation of the PLC and PI3K pathways are both required for the stimulation of ASP-TG. Even with the increased expression of C5L2 and PLC in this study produced by blocking the effect of estrogen receptor, we found no effect on the expression of PI3K, Akt, PKC and DGAT1. Interestingly, in association analysis, we found that C5L2 was associated positively with PLC and PI3K gene expression (**Table 5.4**) and this reemphasizes the findings of Maslowska et al., who suggested an important role of both PLC and PI3K in the ASP-C5L2 signaling pathway (Maslowska et al., 2006). Following the proposed signaling pathway for ASP by Maslowska et al., (Maslowska et al., 1997), PLC can only participate in TG synthesis through the activation of PKC, whereas PI3K can participate indirectly by the activation of PKC, through Akt, or directly by the activation of MAPK/$ERK_{1/2}$. Considering the fact that PLC and PI3K pathways are required for ASP-TG synthesis in preadipocytes (Maslowska et al., 1997), we suggest similar requirements in adipocytes in order activate the downstream PKCα and DGAT1 enzymes. Therefore, even with the association of C5L2 with PLC and PI3K, we found no change in intracellular TG concentration, confirming the importance of the activation of PI3K and downstream signaling components to maintain ASP-TG synthesis in adipocytes.

In the present study the comparison between different antagonist treatments and the P&E group showed no effect on LPL expression (**Figure 5.7**). Estrogen is known as a lipolysis hormone, preventing fat accumulation in adipose tissue.

Various studies have shown that ovariectomized rats increased LPL expression in adipose tissue leading to obesity (Ramirez, 1981, Pedersen et al., 1992), an effect that was reversed by estrogen administration. In contrast to estrogen action, progesterone is known to increase LPL activity. The opposing action between progesterone and estrogen seems to prevent any variation in LPL expression in adipocytes.

ER-β blocking increased GLUT4 gene expression. Estrogen is known to modulate insulin sensitivity and glucose homeostasis; however, the underlying mechanisms are not clearly understood. Estrogen was found to inhibit GLUT4 by the action of ER-β in muscles (Barros et al., 2006). Opposing the suppressive action of ERβ, ERα has been shown to be a positive regulator of GLUT4 expression and ERα (-/-) mice show a reduction of GLUT4 expression in muscles (Barros et al., 2006). Different treatment showed a trend of increased expression GLUT4 when ER-β was blocked; however this did not reach significance ($p=0.07$). This may supports the notion of the important role of ERβ in regulating glucose and lipid metabolism through the expression of GLUT4 in adipocytes. We were not able to detect a regulatory influence of ER-α, but the effect of ER-β was attenuated when we blocked ER-α in the combination treatment of MPP-(R,R)-THC, a finding that further confirm the opposite effect of both receptors in regulating glucose and energy homeostasis in adipocyte. GLUT4 and LPL have shown to associate positively (**Table 5.4**), suggesting a shared regulatory mechanism, mainly by the action of ER-β.

In summary, we confirmed the effect of P&E on increasing intracellular TG in association with ASP. The findings in this study demonstrated for the first time a direct link between ERs and ASP-C5L2 signaling pathway components. We found that ER–α and –β may play a role in suppressing C5L2 and PLC gene expression and, by ER -α and -β blockade, the expression of C5L2 and PLC increased significantly. However, we also found no effect of estrogen receptors on PKCα, PI3K and Akt. ERs may interfere with ASP-C5L2 signaling pathway, but the blockade of ERs or PR were found to impair ASP-TG synthesis. ER-β blockade in differentiated adipocytes appears to increase the expression of GLUT4 and this is may be independent of ASP action, as correlation analysis showed no link between GLUT4 and any of the ASP-C5L2 signaling components. With the consideration that we were not able to detect an ASP increase with P&E treatment, in the presence of chylomicrons, we recommend a further investigation of each hormone considering the addition of ASP (to confirm ASP stimulation) along with the antagonist treatments. As the mRNA concentrations may not reflect the expression and activity of the proteins produced, functional analysis and the activity of the signaling pathway components in ASP-TG synthesis pathway should be a focus for future work.

CHAPTER 6. CONCLUSIONS AND FUTURE WORK

This chapter presents a summary and the final conclusions obtained from all experimental work with recommendations of possible future work for each study.

6.1 Summary of novel findings from this thesis

The *ex vivo* and *in vitro* experimental work in this research project have yielded important findings that aid in understanding the possible mechanism of the action of female steroid hormones on adipose tissue distribution and metabolism.

The following are the novel key findings:

1. Progesterone and estrogen in combination (P&E) stimulated ASP production in subcutaneous (when compared to estrogen treatment group), but not in visceral adipose tissue. This may reflect on the importance of P&E hormones on fat distribution pattern related to female. Using the 3T3-L1 cell line showed a similar positive response to P&E treatment and that makes them a good model for any future analysis in relation to ASP and steroid hormones.
2. In explants from both depots, the effect of female steroid hormones (P&E) in mediating ASP production was augmented by the presence of chylomicrons. The effect was more profound in subcutaneous than visceral adipose tissue as reflected by more TG uptake into the adipocytes.

3. C5L2 protein expression was reported for the first time to have a positive response to female steroid hormones and to be potentially involved in ASP uptake and function in the presence of dietary fat.
4. Estrogen receptors (ER- α and -β) interact directly with ASP-C5L2 signaling pathway components and progesterone and estrogen receptors were found to be important in regulating ASP and TG synthesis in adipocytes.

6.2 Conclusions and future work direction

In the past few years, there has been an increasing interest in ASP as one of the anabolic controlling factors in adipose tissue. The identification of the ASP receptor, C5L2, (Kalant et al., 2005) has drawn even more interest in the role of ASP and C5L2 in the physiology of adipose tissue in relation to obesity and metabolic disorders (Cianflone et al., 2003). More lately a possible link between sex steroid hormones and ASP, particularly in women with hormonal changes such as in pregnancy or in conditions like polycystic ovarian syndrome, has been suggested (Saleh et al., 2007, Oktenli et al., 2007). The function of ASP in postprandial fat clearance was found to be more efficient in ovariectomized rats when given acute doses of progesterone (Al Riyami et al., 2014). The mechanism behind the effect of sex steroid hormones on ASP is not fully understood and few studies have investigated their direct effects in adipocytes.

Although several association studies were performed to investigate the link between sex hormone variations and ASP levels in humans and rodents (Saleh et

al., 2007, Saleh et al., 2009, Rezvani et al., 2014), the current work is the first study to investigate a cause-effect relationship between sex hormones and ASP in adipose tissue and an adipocyte cell line. The work presented in this thesis aimed to expand the understanding of the fundamental biological effects of, mainly female, sex steroids on the regulation of ASP in adipocytes. This was achieved first through *ex vivo* experimental work; using different fat depots from ovariectomized rats. Rodent models are appropriate as rats have been demonstrated to have similar fat distribution and lipid metabolism to humans (Bergen and Mersmann, 2005). The effect of sex steroid hormones at physiological concentrations on ASP production and related gene expression was examined in response to the absence or the presence of dietary fat (i.e chylomicrons). The study was carried further using the 3T3-L1 cell line as an adipocyte model to elicit the possible mechanisms in regards of the effect on female sex steroid hormones on ASP synthesis and signaling pathway.

Body fat distribution is significantly different between men and women (Kvist et al., 1988). The regional localization of stored fat may more strongly predict obesity-related health risk than the total amount of body fat. In particular, increased visceral fat accumulation is associated with increased risk of vascular diseases and other metabolic disorders (Despres et al., 1990). The divergent roles of subcutaneous and visceral adipose tissue from the perspective of cardiometabolic risk factors can be elucidated by the metabolic activities of these fat depots. Because of the significant increase of the metabolic risk associated with abdominal obesity in postmenopausal women, it is important to understand

factors and mechanisms that contribute to the localization of accumulated fat. The metabolic profile differences is largely attributed to gender-specific hormones (Tso et al., 2013). At reproductive age females typically store relatively less amount of visceral than subcutaneous fat storage in comparison to male subjects (Karastergiou et al., 2012). Interestingly, women at post-menopause develop an 'android' fat distribution that associates with metabolic abnormalities such as dyslipidemia and insulin resistance status (Geer and Shen, 2009). Therefore, female ovarian sex steroid hormones may have a protective role in lowering health risk in women by the regulation of fat distribution. ASP elevation in association with hormonal disturbance is seen in conditions like polycystic ovarian syndrome that may increase the risk of inflammation and cardiovascular diseases (Oktenli et al., 2007, Wu et al., 2009). An ASP resistance condition was proposed in relationship with elevated hormones during late pregnancy, where this may cause a hyperlipidemic state; providing more TGs that are required for fetal growth (Saleh et al., 2007). Although abnormalities in ASP profile were found with elevated female steroids and metabolic syndrome, there are no reports of variation in circulating ASP concentrations at physiological levels of female steroids. Therefore, the current findings may contribute in the establishment of understanding the mechanisms of long-term effects of female sex steroid hormones on fat distribution and the immediate response to postprandial fat in adipose tissue.

6.2.1 *Ex vivo* work limitation and concluding remarks

The focus of this study was to determine the direct effect of sex steroid hormones at physiological doses, on ASP production with the consideration of two factors; the presence or absence of chylomicrons and the differences in the regional fat depots (subcutaneous and visceral tissue explants). It was important as well to determine the expression of ASP receptor (C5L2), ASP precursors' and related lipogenic genes, in response to hormonal treatments and chylomicrons in different tissue explants to give a comprehensive understanding of ASP production. Overall, only the combination of female hormones, progesterone and estrogen (P&E), was found to stimulate the production of ASP and C5L2 protein receptor expression and this was confined to subcutaneous adipose tissue. There was no significant effect of hormone treatments on the expression of ASP precursors or the lipogenic genes (DGAT1 and LPL). However, precursors and DGAT1 genes were found to be more highly expressed in subcutaneous tissue than visceral tissue explants.

Studies have shown that women typically present higher body fat compared to men (Womersley, 1977, Jackson et al., 2002), but they exhibit better lipid profile and improved insulin sensitivity (Geer and Shen, 2009). The contradiction between higher body fat and enhanced metabolism in women compared to men is intuitively related to the differences in the biology and function of adipose tissue depots. It would be expected that women deposit fat subcutaneously more effectively than intra-abdominally. The findings supported a sex-specific depot effect of P&E at physiological levels, promoting storage of fat in subcutaneous

depots through the stimulation of ASP-C5L2 pathway. This may achieved by the mediation of LPL activity in adipocytes (Cianflone et al., 2003, Sniderman et al., 1997), and therefore attenuate lipolysis more in subcutaneous adipocytes than visceral, particularly in fasting state. Moreover, the earlier findings that visceral adipose tissue is more lipolytically active than subcutaneous (Jensen, 2008) could be explained by the action of ASP through the inhibition of lipolysis in subcutaneous adipose tissue. This provides a long-term effect of sex steroid hormones in women leading to the protective role of regional fat distribution.

The differences in fat distribution between men and women are abolished after menopause due to the loss of ovarian hormones with age, followed by redistribution of adipose tissue towards central adiposity (Ley et al., 1992). Considering that a substantial amount of estrogen is produced by adipose tissues (Simpson, 2003), the absence of progesterone seems to be important, along with the presence of estrogen, to regulate fat distribution in women. The positive coordination between P&E and ASP will be lost in menopause, potentially leading to changes in adipose tissue metabolism and, possibly, metabolic syndrome (Rezvani et al., 2014).

In the presence of chylomicrons, progesterone and estrogen (P&E) treatment was found to have an impact on ASP production in both subcutaneous and visceral tissue explants. In response to dietary fat and P&E treatment, ASP production increased from visceral tissue explants significantly, but no effect was found on C5L2 or any other related genes. In contrast, the production of ASP decreased significantly in subcutaneous tissue explants with P&E treatment. In response to

chylomicrons, C5L2 and precursor gene expression was found to be reduced significantly in subcutaneous tissue explants in all treatment groups. Furthermore, TG concentration was determined in the media for both tissue explants and it showed that the level of TG was significantly lower in subcutaneous when compared to visceral tissue explant media; reflecting the anti-lipolytic activity of subcutaneous tissue explants. Correlation analysis showed a negative association between ASP concentration in the media (in P&E treatment) and C5L2 protein expression, where C5L2 consistently associated positively with DGAT1. This suggests a higher uptake of ASP by C5L2 and the ASP-C5L2-DGAT1 activation pathway that facilitated TG synthesis in subcutaneous adipocytes. Insulin has been known for decades as the main regulator of TG storage in response to dietary fat (Frayn KN et al., 2003). Women have a tendency to store meal-derived TG-FAs preferentially in gluteo-femoral subcutaneous adipose tissue compared to men (Votruba and Jensen, 2006). The findings in this study suggest that the differences in fat deposition arise partially by directing post-absorptive FFA uptake into subcutaneous adipose tissue through the action of the ASP-C5L2 pathway regulated by progesterone and estrogen hormones at physiological levels. As research on the sex-specificity of adipose tissue function advances, the results of this study will add more detail on the influential effect of sex steroid hormones on the regulation of fatty acid homeostasis obtained from dietary fat in different fat depots.

The knowledge obtained from this chapter demonstrated an important interaction between sex steroid hormones and ASP. Earlier findings by various researchers

suggested an ASP resistance in conditions with elevated progesterone and estrogen hormones *in vivo* and *in vitro* (Saleh et al., 2007, Wen et al., 2008, Rezvani, 2014). Altered ASP metabolism is associated with increased plasma lipids in women with PCOS (Wu et al., 2009). The current study showed that female steroid hormones at physiological levels did not induce ASP resistance and may promote a positive health impact in women by effectively enhancing fat storage in subcutaneous adipose tissue. A simplified overview of the recent findings, collectively with earlier findings of the effects of sex steroid hormones on ASP, and possible health impact, is illustrated in **Figure 6.1.**

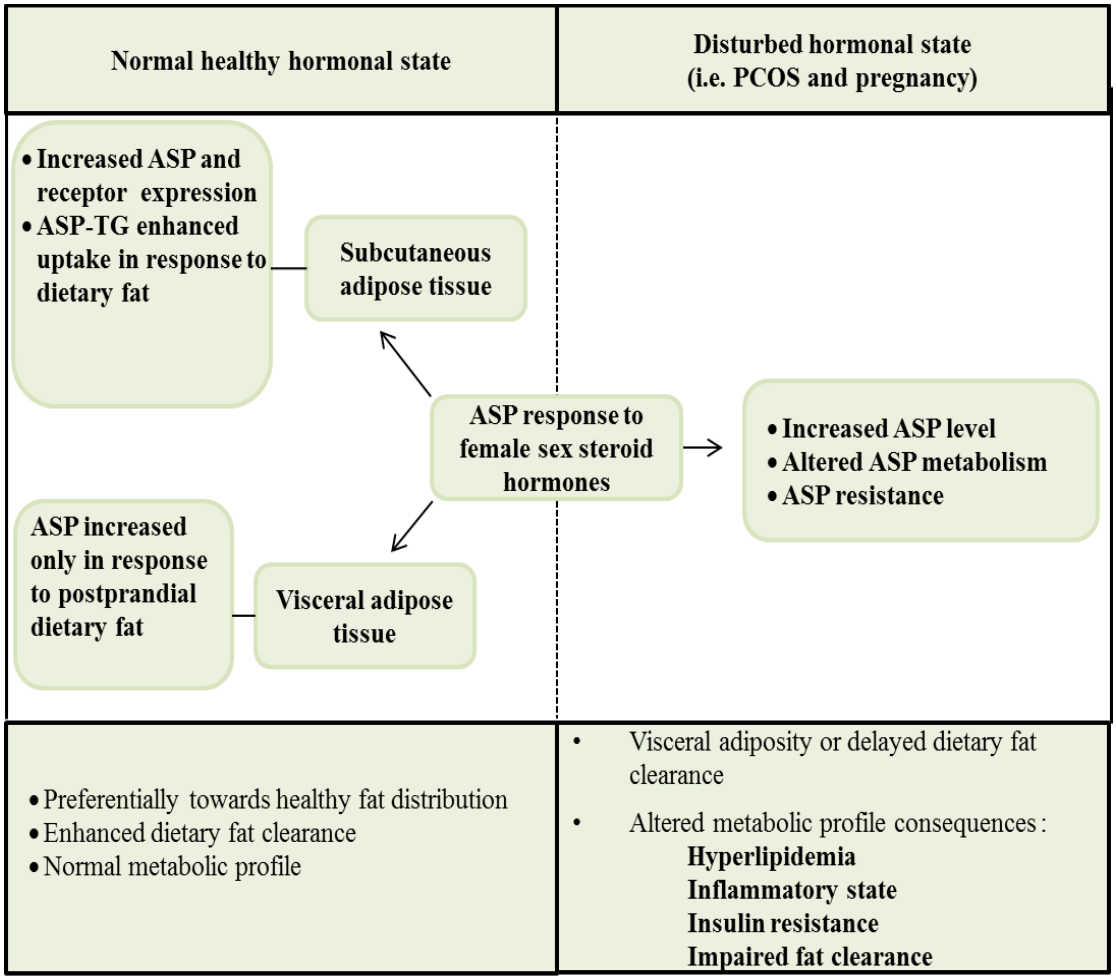


Figure 6.1 Overview of the effect of female sex steroid hormones on ASP and metabolic profile

The limitations of this study can be generalized in the lack of functional analysis that can measure ASP action on TG synthesis. In this study, glycerol concentration was used to estimate total TG in the media. This is not a reflective measure for FA uptake and synthesis into TG in adipocyte using dietary fat substrate (chylomicrons) supplied in the media. The future work can include measuring NEFA and calculate NEFA/glycerol ratio that may represent the

uptake of NEFA by adipocytes. As well, using labeled fatty acid is another method that can be used to measure TG synthesis in response to ASP production.

Future work should focus also on the expression of other related enzymes such as convertase and carboxypeptidase B that are involved in the synthesis of ASP. As there was no profound effect on the expression of the lipogenic enzymes, DGAT1 and LPL, further functional investigations can be carried on the activity of these enzymes specifically in response to dietary fat. ASP responded to hormones at physiological doses and the presence of chylomicrons was also found to play a key role in ASP metabolism in depot-specific regulation. However, increased hormonal doses were suggested to induce ASP resistance and impaired ASP metabolism by various studies. Therefore, time- and dose dependent investigation of female steroid hormones and chylomicrons is important to follow the pattern of ASP production that will reflect different physiological condition of hormones.

6.2.2 *In vitro* work concluding remark

Following the earlier findings of the *ex vivo* experiment on the effect of female hormone on ASP production (Chapter 3), effects of progesterone and estrogen treatments on ASP were studied using 3T3-L1 adipocytes. 3T3-L1 have been confirmed by earlier studies to produce ASP and express C5L2 (Wen et al., 2008, Gao et al., 2010). Although the use of 3T3-L1 cells give no information about regional identity, or how different fat depots may respond to treatments, responses of these cells are similar to primary adipocyte cell culture in respect to the

lipogenic pathway (Bergen and Mersmann, 2005), making it a good cell line model for the following investigations.

6.2.2.1 Dose-effect of female steroids on ASP

The findings clearly confirmed that ASP was stimulated only by the combination of both hormones (P&E), where the effect of a high dose of progesterone is dependent on the presence of estrogen. The production of ASP was found to be independent of C3 concentration in the media with no effect of hormonal treatments on C3/ASP conversion rate. The parallel increments of ASP and C3 were reported earlier in response of abnormal female sex hormone state (i.e. PCOS). Studies of postmenopausal women receiving hormone replacement therapy have shown an elevation of serum C3 and other complement proteins that might be involved in the development of cardiovascular diseases by inducing inflammation (Liu and Lv, 2008). Therefore, the biology of adipose tissue may be influenced by chronic exposure to abnormal hormonal status, due to age or disease, may alter the metabolism of ASP and C3 leading to increasing metabolic risks. However 'healthy' adipocytes may respond differently to hormones and the present study showed this response to be independent of C3 even in the presence of high doses of progesterone. To rule out ASP resistance in adipocytes, it was important to assess C5L2 expression, particularly by measuring the protein expression of the receptor, which we found earlier it is more responsive to hormonal treatments than gene expression. This is the only reported study that has investigated the protein expression of C5L2, where C5L2 expression increased in dose dependent manner with progesterone and estrogen hormones. This leads us

to postulate that the induction of expression of C5L2 by progesterone treatment can be a part of the lipogenic mechanism of progesterone in adipocytes. On the other hand, the increased expression of C5L2 in response to estrogen may be a compensatory response to the lipolysis effect of estrogen in adipocytes. The combination (P&E) of hormone treatments showed no effect on the receptor expression in response to the increased ASP production indicating no ASP resistance condition caused by increased female steroid hormones. The stimulatory effect of female hormones on ASP or C5L2 did not result in lipid accumulation in adipocytes in this study. Another limitations of this study is the lack of dietary substrate availability for ASP to function. The media only consisted of low glucose as source for lipogenesis in adipocyte. However with the control of insulin presence there was a low expectation for any lipogenesis process to occur in the present study. Insulin should also be considered to be in the media along with hormonal treatment to facilitate TG synthesis using glucose substrate and to be linked with ASP production and function. Another factor may influence TG synthesis is the incubation time factor of 24 h. Future work can investigate the effect of female hormones using longer incubation times to determine whether this increase may stimulate TG synthesis via ASP-C5L2 pathway or induce ASP resistance as proposed by previous research using 3T3 cells (Wen et al., 2008). To this point of this project, we could not determine any sign of ASP resistance because of the positive relationship between ASP and C5L2 expression. The determination of metabolic markers such as insulin sensitivity or inflammatory secretions may enable us to further assess the effect of P&E hormones of high doses on adipocytes.

6.2.2.2 Time- effect of female steroids on ASP

Following the marked effect of chylomicrons on ASP production in fat depots (Chapter 3), the focus of this study was to determine the effect of progesterone and estrogen (P&E) at high physiological concentrations, with chylomicrons, on ASP/C3 production. There was no difference in the production of ASP or the expression of C5L2 between chylomicrons treatment alone or in combination with P&E hormones. Chylomicrons were found to spike C3 production into the media in a time dependent manner with a significant increased initiated at the 8 h time point. C3 knockout, and therefore ASP deficient, mice have been reported to show a delay in postprandial TG clearance due to the lack of ASP synthesis in the alternative pathway (Murray, 1999, Persson et al., 2004, Paglialunga et al., 2008). Fujita et al. reported that postprandial TG-rich chylomicrons accelerated the generation of C3, and ASP was increased in a time and dose-dependent manner (Fujita et al., 2007). We have also noted the increasing amount of C3, however there was no detectable amount of ASP production in the cultured media. Again the time factor seems to be important factor in this study and varies from earlier studies. For instance Fujita et al., study, determined ASP generation within a short time course (120 min) (Fujita et al., 2007),whereas in Gao et al. study (Gao et al., 2011) ASP was increased after 48 h incubational period. Furthermore this may also suggest a further functional study of the effect of chylomicrons measuring factors such as factor H and I that were found to be depleted during C3 tick -over phenomenon (Fujita et al., 2007) .

With P&E treatment, C3 concentration increased significantly, a change that was initiated at the 2 h time point indicating that female hormones accelerated the production of C3 into the media. Estrogen was previously reported to increase C3 expression in uterus of rats (Sundstrom et al., 1989, Kuivanen et al., 1989), and effects of estrogen may also contribute to the increased production from adipocytes. Although we did not observe an increase in ASP production through the time course, intracellular TG concentrations in adipocytes correlated positively with ASP and C5L2 protein expression reflecting on the earlier findings of possible sex steroid hormones and ASP-receptor interaction, which enhances TG deposition into adipose tissue. Although women are typically observed to store more fat than men (Gallagher et al., 1996), sex steroid hormones have little impact on circulating lipid homeostasis (Wang et al., 2011, Clarkson and Mehaffey, 2009). There are a limited numbers of controlled studies regarding possible sex differences in the regulation of lipid metabolism by various adipokines (Ethun, 2016).

3T3-L1 adipocytes have been demonstrated to produce ASP in response to high doses of progesterone and estrogen. We found the use of physiological doses of P&E, with low doses of chylomicrons to be more influential in tissue explants, whereas in adipocyte cell culture it may not be efficient to produce detectable amounts of ASP. Cells may have produced smaller amounts of ASP that are hard to detect in the media. Having demonstrated a depot-specific response to hormones and dietary fat in the *ex vivo* experiment (Chapter 3), investigating hormone pathway interaction with ASP metabolism in different fat depots may be

a better approach to gaining further understanding of the effect of female hormones in determining the pattern of fat distribution. This can be achieved by using steroid hormones receptor blockade such as mifepristone that was clearly demonstrated by Langley and York (1990), to reverse obesity and reduce visceral fat in genetically obese animals (Langley and York, 1990).

There are some limitations in the present work that can be taken into consideration in planning extended future work. Firstly by using 3T3-L1 cells it was not possible to determine fat depot specificity of hormonal effects. Responses dependent on time are hard to assess between studies as this factor varies considerably between reported studies (Fujita et al., 2007, Wen et al., 2008, Gao et al., 2011). A functional study of TG incorporation into adipocytes may be studied with longer (or shorter) incubation times using labelled TG to improve the accuracy of measures of uptake. Using better methodology to measure intracellular TG can be applied to overcome the possible insensitivity that can associate with the present method. for example the presence of lipid droplet protein coat that may block the action of lipase leading to inaccurate measures of intracellular TG (Schwartz and Wolins, 2007). Finally, the production of ASP from 3T3-L1 was found to be very low and it might be hard to detect the significant variation between treatments. ASP might be measured using ELISA which gives higher sensitivity.

6.2.3 Female steroids receptor interactions with the ASP-C5L2 signaling pathway

One of the objectives set for the research work presented in this thesis was to examine the possible interaction of progesterone and estrogen with ASP-C5L2 signaling pathway components through their receptors. Blado et al., and Malowska et al., (Baldo et al., 1995, Maslowska et al., 2006), proposed a possible pathway for ASP-C5L2 action using the 3T3-L1 preadipocyte cell line. This involved the activation of PLC/PKC and PI3K/Akt pathways. The use of P&E treatment at higher concentrations (10^{-5} and 10^{-7} M, respectively) with chylomicrons was shown to significantly induce TG synthesis in adipocytes. The intracellular concentration of TG was shown to be associated positively with ASP, followed by an association between ASP, DGAT1, PKCα and PI3K reflecting an activation of the TG synthesis pathway. The increase of TG in adipocytes was abolished by the blockade of progesterone and estrogen receptors highlighting an initial effect of P&E by their receptors on ASP function.

Antagonism of the ERs (ER- α and –β) was shown to markedly increase the expression of C5L2 mRNA and PLC-β, suggesting that both receptors are important in the stimulation of TG synthesis in adipocytes and their inhibition is a result of the lipolytic effect of estrogen. GLUT4 showed to be correlated with LPL and ER-β had seems to have an effect on the expression of GLUT4, that can be suggested to be independent of the action on ASP signaling pathway; however, this may also interfere with the process of TG synthesis in adipocytes. C5L2 associated positively with PLC and PI3K; the main two signaling pathways found

to be important in the activation on ASP-C5L5 signaling pathway. Overall, the findings suggest that progesterone and estrogen seems to have a synergistic effect on ASP signaling pathway. Interrupting their function through their receptors may impair ASP-TG synthesis in adipocytes.

The novelty of this work was clearly demonstrated on reporting the clear interaction between female steroids receptors (mainly ERs) and the ASP-C5L2 signaling pathway. The presence of chylomicrons was found consistently to eliminate the increases of ASP production driven by P&E treatment; however the increase of intracellular TG in relation to ASP suggests the engagement of ASP in the process of TG synthesis. Future work should focus on each individual hormone and its receptor interaction with ASP-C5L2 signaling pathway. The response of ASP to P&E treatment was found to be altered in the presence of chylomicrons therefore we may suggest more control over the presence of chylomicrons in future experiments. The ASP signaling pathway may also be investigated in different fat depots in response to dietary fat and sex steroid hormones.

6.3 General conclusion

Fat storage process in adipose tissue is mediated by a host of different proteins, enzymes and hormones. ASP has been shown to be a powerful lipogenic factor that interacts with C5L2 and modulates TG synthesis. ASP therefore has a very important function and impairment of its interaction with C5L2 may generate a negative effect on lipid metabolism in adipose tissue. The work presented in this

thesis highlighted the role of female hormone-ASP interactions in relation to fat distribution and fat load (chylomicrons). Female steroid hormones (P&E) stimulated ASP directly and showed to be regulated in different fat tissue explants. This may reveal a regulatory mechanism of fat storage on female healthy form of fat distribution. The cause-effect association between P&E and ASP production was confirmed using 3T3-L1 adipocyte cells. Furthermore, female hormones exhibited a regulatory role on C5L2 protein expression, whereas ASP's precursors and related lipogenic factors were not influenced by hormonal treatment and were more associated with chylomicrons and different fats depots. In general future work may focus more on measuring protein expressions of precursors and conversion factors and the functional activities of the related lipogenic enzymes (DGAT1 and LPL). The cross-link between P&E and ASP-C5L2 signaling pathway were found to be mediated through ERs and PRs. Therefore, partial interference of ASP-C5L2 may provide a future target to study obesity. We speculate that abnormal ASP concentrations coupled with long-term hormonal disturbances can be considered in the future as a potential biomarker for the metabolic syndrome.

CHAPTER 7. REFERENCES

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