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Evaluation of Acylation Stimulating Protein (ASP) and adipokines in relationship with determinants of obesity and its consequences

Thèse

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Résumé

L'obésité est associée avec plusieurs désordres métaboliques d'envergure dont le diabète, les maladies cardiovasculaires et la stéatose hépatique. De nouvelles études visant le développement des traitements efficaces contre l'obésité et ses complications ont été entreprises afin d'élucider les mécanismes pathophysiologiques par lesquels l'obésité induit ou amplifie ses conséquences négatives. Le tissu adipeux sécrète plusieurs hormones ou adipokines qui sont impliquées dans la régulation du poids corporel ainsi que l'homéostasie métabolique. La protéine stimulant l'acylation (ASP) est une adipokine stimulant la synthèse des triglycérides et leur stockage au niveau du tissu adipeux (et ceci, en agissant à travers son récepteur : le C5L2).

Cette thèse se penche sur diverses populations humaines et évalue les changements au niveau de différentes adipokines, plus particulièrement l'ASP, en lien avec les facteurs déterminants de l'obésité. Cet objectif global s'est concrétisé à travers quatre études : I) L'évaluation des niveaux d'adipokines chez des patients consommant des breuvages édulcorés au glucose ou au fructose afin de déterminer les effets de la composition de la diète sur la fonction du tissu adipeux, II) Une étude transversale évaluant le lien entre l'ASP et les facteurs de risque cardiométabolique dans une population à risque, III) une étude chez des femmes souffrant d'obésité sévère qui ont subi une chirurgie bariatrique, afin de déterminer les associations entre l'expression hépatique des récepteurs liés au facteur du complément C3 avec les niveaux d'hormones sexuelles et d'adipokines post-chirurgie, et IV) l'évaluation des niveaux sanguins d'adipokines ainsi que de l'expression du C3 et des récepteurs qui y sont reliés dans les tissus adipeux viscéral vs sous-cutané en lien avec le syndrome métabolique, les hormones sexuelles et le profil métabolique.

Nous avons démontré que l'ASP et son récepteur offraient différentes réponses en fonction du sexe, de la présence d'un désordre métabolique, des niveaux d'hormones sexuelles, de l'organe impliqué ainsi que de la composition de la diète : tous des facteurs déterminants pour l'obésité. En conclusion, ces résultats suggèrent que l'ASP agit comme

médiateur entre les facteurs exogènes et les évènements biologiques menant à l'obésité et ses conséquences métaboliques.

Abstract

Obesity is associated with many major metabolic disorders, especially diabetes, cardiovascular disorders and fatty liver disease. Aimed at developing effective therapies for obesity and its complications, new research has intensified to elucidate the pathophysiological mechanisms by which obesity induces or amplifies its major adverse consequences. Adipose tissue, as an endocrine organ, secretes several hormones termed "adipokines" that are involved in energy homeostasis and weight regulation. Dysfunction of adipokine pathways has been recognized as a key etiological factor of obesity-induced disorders. Acylation stimulating protein (ASP) is an adipokine that stimulates triglyceride synthesis and storage in adipose tissue by enhancing glucose and fatty acid uptake. ASP acts via its receptor C5L2.

This thesis investigates several human populations under varying external and internal conditions and evaluates changes in adipokines, in particular ASP and its related proteins, in association with obesity determinants. This overall aim is achieved through four studies including the following: I) evaluation of adipokines in healthy overweight/obese adults consuming glucose- or fructose-sweetened beverages to determine the effects of diet composition on adipose tissue function II) a cross-sectional population-based study to determine fasting serum ASP and its relationships with cardiometabolic risk factors in a relatively high risk adult population III) a study on severely-obese pre/post-menopausal women, who underwent bariatric surgery, to determine associations of hepatic gene expression of complement C3 related receptors, sex hormones, adipokines and metabolic profiles as well as evaluating obesity improvement after surgery IV) a study on women with a wide age and BMI range to determine plasma adipokine levels and adipose tissue depot gene expression of C3 and related receptors in association with metabolic syndrome criteria, ovarian hormones and metabolic profile.

I found different responses of ASP and its receptor according to gender, metabolic disorder, sex hormone levels, organ involvement and diet composition: all factors critical as

obesity determinants. The results presented here demonstrate that ASP may mediate the link between obesity-related exogenous factors and biologic events that lead to obesity consequences. In conclusion, these findings validate that obesity is a low-grade inflammatory status with multi-organ involvement, evidencing sex differences and dynamic interactions between immune and metabolic response determinants.

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Abbreviations

AO	Abdominal obesity
ALT	Alanine transaminase
ALP	Alkaline phosphatase
ApoA1/B	Apolipoprotein A1/B
AKt	Protein kinase B
ASP	Acylation stimulating protein
AST	Aspartate transaminase
AT	Adipose tissue
BMI	Body weight index
BPD	Biliopancreatic Diversion
BW	Body weight
CHD	Coronary heart disease
C5L2	C5a-like receptor 2
CVD	Cardiovascular diseases
DM	Diabetes Mellitus
DNL	De novo lipogenesis
E1	Estrone
E2	Estradiol
FSH	Follicular stimulating hormone
FCH	Familial combine hyperlipidemia
GGT	Gamma glutamyl transpeptidase
HDL	High-density lipoprotein
HFCS	High-fructose corn syrup
GLU	Glucose
IL	Interleukin
IR	Insulin resistance
KO	Knockout
LDL	Low-density lipoprotein
MAb	Monoclonal antibody
MAC	Membrane attack complex
MetS	Metabolic syndrome Met S
MHO	Metabolically healthy obese
NAFLD	Non alcoholic fatty liver disease
NEFA	Non-Estrified Fatty Acids
PAb	Polyclonal antibody
PCOS	Polycystic ovary syndrome
RYGB	Roux-en-Y gastric bypass
SAT	Subcutaneous Adipose Tissue
SBP	Systolic blood pressure
SC	Subcutaneous
SSBs	Sucrose-sweetened beverages
TARF	The Turkish Adult Risk Factor
T2D	Type 2 diabetes

TC	Total cholesterol
TEF	Thermal effect of food
TG	Triglyceride
VAT	Visceral adipose tissue
WAT	White Adipose Tissue
WC	Waist Circumference
WHO	World health organization
WHR	Waist-to-hip ratio
WT	Wild Type

Dedication

Dedicated to my parents, my wife and my son; Artin.

Acknowledgment

I would not have been able to complete this journey without the aid and support of countless people over the past four years. My first and sincere appreciation goes to my supervisor Dr. Katherine Cianflone for taking me on as a graduate student and believing in me. I would also like to thank her for being open to ideas and for her excellent guidance, caring, patience, and providing me with an excellent atmosphere for conducting research. Besides my supervisor, I would like to express my deep gratitude and respect to my co-director Dr. André Tchernof, for his invaluable support, guidance and knowledge over the years.

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Avant-Propos

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Chapter Two is composed of the manuscript entitled "Effects of Sugar-sweetened Beverages on circulating Acylation Stimulating Protein, Leptin & Adiponectin: Associations with Metabolic Parameters" co-authored by Reza Rezvani, Katherine Cianflone, John P. McGahan, Lars Berglun, Nancy L. Keim, Steven C. Griffen, Peter J. Havel, Kimber Stanhope, and has been published in the journal *Obesity (Silver Spring)* in December 2013. The study was conceived and executed by Dr. Kimber L. Stanhope and her team. My contribution to this study includes measuring the adipokine hormones in the plasma samples, performing statistical analysis of the data, contributing to the development of the hypothesis, and writing the manuscript.

Chapter Three contains the manuscript "Apparent sex-specific divergence of Acylation Stimulating Protein (ASP) levels with respect to metabolic parameters of pathogenic and clinical relevance" co-authored by Reza Rezvani, Altan Onat, Gunay Can and Katherine Cianflone, which has been published in the *Journal of Endocrinology and Metabolism* in February 2012. Dr. Altan Onat and his team performed the recruitment, the study protocol, and collection of samples. My contribution to the study includes measuring ASP in the blood samples, performing statistical analysis, contributing to the development of the hypothesis and writing the manuscript.

Chapter Four contains the manuscript entitled "Cross sectional associations of Acylation Stimulating Protein (ASP) and adipose tissue gene expression with estradiol and progesterone in pre and postmenopausal women" co-authored by Reza Rezvani, Abhishek

Gupta, Jessica Smith, Pegah Poursharifi, Picard Marceau, Louis Pérusse, Claude Bouchard, André Tchernof and Katherine Cianflone, which was accepted for publication in the Journal of *Clinical Endocrinology* in June 2014. I had a principal role in implantation, study design, statistical analysis, and writing the manuscript. Dr Abhishek Gupta and Dr Pegah Poursharifi helped me in plasma measurements and gene expression experiments. Samples were provided by Dr. Picard Marceau, Dr. Louis Pérusse and Dr. André Tchernof. In addition, I would also like to thank the collaboration of the members of the department of bariatric surgery, IUCPQ and tissue bank of Laval hospital.

Chapter Five contains the article "Complement receptors C5aR and C5L2 are associated with metabolic profile, sex hormones and liver enzymes in obese women pre and post bariatric surgery" co authored by Reza Rezvani, Jessica Smith, Marc Lapointe, Picard Marceau, Andre Tchernof, Katherine Cianflone and was accepted for publication in the *Journal of Obesity* in February 2014. Dr Picard Marceau supervised recruitment and collection of samples. My contribution to this manuscript includes assisting in the development and design of the project, performing of the laboratory assays for adipokines in blood and sex hormones and gene expression experiments, statistical analysis, and writing the manuscript.

Chapter 1

Introduction

1.1 General Introduction

Over last three decades, obesity rates have more than doubled globally and are now considered as a major health hazard. In some countries the prevalence rates of overweight and obesity have increased so dramatically that healthy-weight adults are now in the minority (1) Because of the numerous harmful health consequences associated with obesity, there has been a parallel increase in obesity-related investigations aimed at preventing obesity.

Most people in most countries are no longer at risk of a negative energy balance, which was common in our “hunter-gatherer days” (2). Instead, humans face a new problem that coincided with the start of industrialization: positive energy balance. Although we live in the 21st century, humans carry the history of thousands of centuries of ancestral genes that have been naturally selected to store energy for long periods of limited food availability and even starvation (3). This genetic background helps protect against weight loss and not weight gain. However, the obesity epidemic has only existed for a few decades. Our sedentary life style, diet composition and individual genetic susceptibility collectively affect energy balance and body weight over time. Thus, various combinations of factors can cause a positive energy balance leading to overabundant fat storage: excess food intake, decreased energy expenditure, or a combination of both, the latter often being observed in obese patients.

1.1.1 Energy balance

Human physiology is consistent with the first law of thermodynamics "energy can be neither created nor destroyed " or, put another way, "Energy intake = Energy expenditure" (4). This simple concept of energy balance is a statement of the principle of energy conservation in human and helps us to understand the development of an overweight state and obesity, which, by definition, is an excessive accumulation of energy stored as body fat. Thus, for most cases, this equation can be changed to (5):

Energy intake = Energy expenditure + change in body macronutrient stores

For humans, energy intake is the amount of chemical energy entering the body from the food and fluids we consume that can be liberated via metabolism and thus is measured as metabolizable energy. Energy expenditure is the heat released by the body through resting metabolism, the thermic effects of meals, and physical activity. Prospective studies (6)(7) have illustrated that research on weight maintenance and weight loss should consider the effects of the intervention on energy balance overall and not just energy intake or energy expenditure alone (5).

1.1.2 Components of Energy Intake

Energy intake includes 3 major macronutrient groups—carbohydrate (CH), protein (Pr), and fat (Figure 1.1) and a smaller component from alcohol. The net absorption of dietary energy components (～2–10%) varies among individuals and is dependent on diet composition, food preparation, and intestinal factors (8). Energy yielding nutrients for carbohydrate (4 kcal/g), fat (9 kcal/g), protein (4 kcal/g) and alcohol (7 kcal/g) represent population averages for metabolizable energy, which is the amount of fuel actually available to cells for biological processes.

1.1.3 Components of Energy Expenditure

Absorbed carbohydrates, proteins, and fats are transformed *in vivo* to substrates that can ultimately either be oxidized to produce metabolically useful energy that drives biological processes or they may be stored. Expended energy reflects fuels metabolized for

growth, body maintenance needs, physical activity, pregnancy and lactation, and many other processes. Total energy expenditure is composed primarily of I) the resting energy Expenditure (REE) or basal metabolic rate (BMR), II) the thermic effect of food (TEF) and III) energy expenditure derived from physical activity (PA) (Figure 1.1) (8).

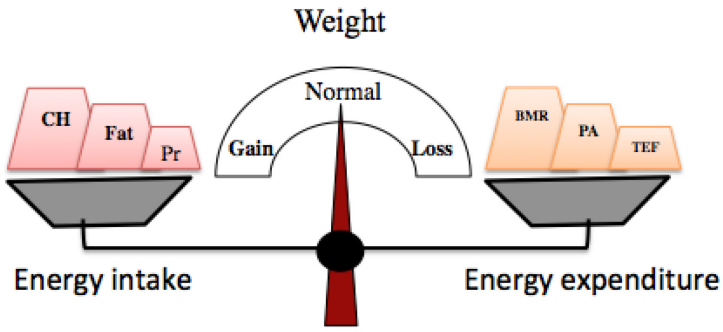


Figure 1.1 Energy balance; Components of energy intake and energy expenditure

1.1.4 Components of storage

Carbohydrate is stored mainly in the form of intracellular glycogen in skeletal muscles and liver. The total mass of glycogen (relative to total body energy stores) is small and turnover is rapid; maximal amounts are observed in the postprandial state. *Protein* in body takes many specific forms and, as with glycogen, is associated with water but at a lower value per gram. *Lipid* in the form of triglycerides, which are present within different tissues such as adipose tissue and liver, is the largest source of stored energy in most adults and has no water associated with it (9). A lean adult has in average 35 billion adipocytes, each containing ~ 0.4–0.6 μ g triglyceride and a severely obese can have four times as many adipocytes, each containing twice as much lipid (8).

1.1.5 Body composition

Any imbalance between utilization of these components relative to diet composition, will lead to an alteration in body composition. The energy stored per unit body weight of carbohydrate, protein and lipid is different, especially when accounting for the associated intracellular water (9). Therefore, changes in body weight are expected when

the macronutrient composition of the diet is altered, even when the energy content of the diet is held constant, particularly over the short term (8).

At a molecular level, body composition consists of six major components; water, lipids, protein, carbohydrates, bone minerals and soft tissue minerals. Among several models describing body composition, the two-component model is most commonly used, and divides the body into I) fat mass (FM), which includes fat from all body sources including brain, bone marrow (skeleton) and adipose tissue and II) fat-free mass (FFM), which include water, protein, and mineral components (9). Given this definition, to achieve long-term weight loss, the loss of fat mass while maintaining FFM is desirable (10). It should be noted that "lean body mass" is different from "fat free mass" and represents primarily muscle, a major determinant of the resting metabolic rate, which varies with changes in hydration and dehydration (9).

1.1.6 Fat storage

Fat mass is the most variable component in body composition, ranging from 5% to 60% (11) of body weight, and is affected by age, gender and ethnicity as well as environmental factors. Fat distribution refers to the relative amount of fat in compartments where fat in adipose tissue and non-adipose tissue are stored. The main depots are in adipose tissue including the subcutaneous adipose tissue (SAT) (stores > 80% of total body fat) and visceral adipose tissue (VAT) (10-20% in men and 5-10% in women of total body fat) compartments (11). In non adipose tissue locations, there are small but important amounts in intra-muscular, perivascular, and liver locations.

1.1.7 Regulation of body weight and energy balance

For a traditional Inuit hunter or fisherman in northern Québec, a higher percentage of fat mass could likely be beneficial, preventing excessive loss of body heat during winter. A pregnant woman or a breast-feeding mother needs sufficient body fat to support conception, fetal growth and post-natal feeding. At the most simple level (Figure 1.1), regulation of energy balance is simply a case of balancing food intake against the energy used by the body, but this is not so simple. Many factors are involved in regulating body

weight and composition such as neurochemicals, body fat stores, protein mass, hormones and postprandial factors. Energy intake and expenditure are both regulated by the central nervous system, with the hypothalamus playing a central role (12), although clearly some aspects are also subject to voluntary control. It is important to remember that in the period of human evolution, the most of these systems have arisen based on pressure to conserve energy in the face of shortage, rather than limiting it at times of excess (3).

The hypothalamus receives signals from the periphery, both in the short term, indicating the end of a meal, and also in the long term, signaling body energy stores (13) These signals are integrated and help determine future food intake, and also, to some extent, energy expenditure. Short-term controls are concerned primarily with factors governing hunger, appetite, and satiety (14). Satiety is associated with the postprandial state when excess food is being stored and hunger is associated with the post-absorptive state when those stores are being mobilized (14). The hunger signals are much stronger than those for satiety and it is easier to supersede the signals for satiety (12). Long-term regulation seems to involve a feedback mechanism in which a signal from the adipose mass is released when normal body composition is disturbed, as when weight loss occurs (13). Adipocytes secrete hormones also termed Adipokines, which act as signaling molecules involving both short and long-term controls (15).

1.2 Obesity

Obesity is a complex, multifactorial state of excessive fat involving environmental (social and cultural), genetic, physiologic, metabolic, behavioral, and psychological components (16). Obesity can be defined as a chronic "disease" as it increases the risk of medical illness and premature death. A simple and convenient way of defining obesity and overweight promulgated by the World Health Organization (WHO) and the Canadian National Institute of Health (CNIH) is based on body mass index (BMI).

Table 1.1 WHO classification of BMI

Classification (WHO)	Principal cut-off points Kg/m^2	Risk of BMI Co-morbidities
Underweight	<18.50	Low*
Normal range	18.50-24.99	Average
Overweight	25.00-29.99	Increase
Obese class I	30.00-34.99	Moderate-severe
Obese class II	35.00-39.99	Moderate-severe
Obese class III	≥40.00	Severe

* increases in risk of other medical problems, http://www.who.int/nmh/publications

Classification of overweight and obesity is based on data gathered from population-based epidemiology studies that evaluated the relationship between obesity and rates of mortality and morbidity that are adiposity related (Table 1.1). BMI (calculated as weight/height^2 in units of kg/m^2) between 25 and 29.9 is considered to be overweight. Obesity is characterized by an excess of body fat and is usually diagnosed based on a BMI greater than 30 kg/m^2 and is further subdivided into Class I–III (Table1.1).

There is some evidence to suggest that Asian populations have different associations between BMI, percentage of body fat, and health risks than do European populations (17). A WHO expert consultation identified further potential public health action points (23.0, 27.5, 32.5, and 37.5 kg/m2) along the continuum of BMI, and proposed methods by which countries could make decisions about the definitions of increased risk

for their population (17). Therefore, China (18) uses a BMI of 28 for obesity and Japan (19) uses a BMI cut-off of 25 kg/m^2 as a cut-off.

1.2.1 Epidemiology of obesity

The global epidemic of obesity is now recognized as one of the most important public health problems facing the world today. The prevalence of obesity has increased dramatically worldwide over the last decades. For example the prevalence of obesity has nearly doubled between 1980 and 2008 (20). According to the World Health Organization (WHO) report, 35% of adults aged 20 yrs and older were overweight (34% men and 35% women) in 2008 including 10% men and 14% women being considered as obese (WHO 2010) (21) .

The 2010 International Obesity Task Force (IOTF) analysis (22) estimated that approximately 1.0 billion adults are overweight (BMI: 25-29.9) and a further 475 million are obese. When Asian-specific cut-of points for the definition of obesity (BMI>28) are taken into account, the number of adults considered obese globally is over 600 million (22). The IOTF estimates that up to 200 million school-age children are either overweight or obese, 40-50 million of which are classified as obese (23). Up to one-third of adults in some Westernized countries are obese and very few countries remain unaffected by obesity (24). By 2015, the WHO predicts that these numbers will increase to 2.3 billion overweight and 700 million obese (24) Predictions suggest that if this secular trend continues with undiminished strength, by 2030 up to 57.8% of the world's adult population (3.3 billion people) could be either overweight or obese (25).

In the United States, the 2010 National Health and Nutrition Examination Survey (NHANES) estimated that the age-adjusted obesity prevalence was 35.7% with no sex differences (26). The age-adjusted prevalence of overweight and obesity combined (BMI $\geq$ 25 kg/m^2) was 68.8% in 2010 with a mean BMI of 28.7 kg/m^2 in the US population. However, data from the last decade (1999–2010) suggest that the prevalence of obesity may have plateaued in the USA (27).

In Canada, the prevalence of obesity is lower than in the United States. Based on nationally representative surveys the prevalence of obesity in Canadian adults increased from 10% in 1970-72 to 26% (27% men, 25% women) in 2009-11 (28). Furthermore in Canada, 29% of men and 41% of women reach cut-off values for waist circumference (WC; above 102 cm in men and 88 cm in women) suggesting the presence of abdominal obesity, with mean WC values of 95.1 cm for men and 87.3 cm for women (28). The prevalence of obesity in children has tripled since 1981, and based on the World Health Organization BMI growth standards, 12% of Canadian school-age children were obese in 2009/11(28).

According to the IOTF report in 2010, in the European Union, approximately 60% of adults and over 12 million children are overweight or obese. A recent European study concluded that in a worst-case scenario almost every third European adult might be obese by the year 2015 (29). While the distribution in America demonstrates a high proportion of overweight and obese people (62% and 26% respectively, in both sexes), South East Asia shows the lowest prevalence (14% overweight in both sexes and 3% for obesity) (21). Growth in population size, population aging, urbanization and changes in lifestyle, including increases in total calorie intake and reductions in physical activity, all contribute to an epidemic of overweight and obesity in developing regions (25).

1.2.2 Obesity Determinants

Two key issues have been highlighted in obesity review articles. First, obesity results from an imbalance in energy intake and energy expenditure. And second, obesity develops from a complex interaction between a variety of factors including genetic, physiologic, environmental, psychological, social, economic, and even political.

On the other hand, maintenance of a healthy body weight and body composition, which is important for the achievement of lifelong health, occurs through successful energy balance maintenance within a normal range (30). Energy balance systems (31) are themselves governed by the interaction with both internal and external environmental factors. Therefore, to understand the causes of obesity it is necessary to know what factors are involved in regulating energy balance.

1.2.2.1 Internal environment factors

Genetic factors*:* It is well known that genetic factors influence the body's tendency to store energy either as fat or as lean body tissue. The heritable factors are likely to be responsible for 45–75% of the inter-individual variation in BMI (32) . Obesity clusters within families and recent data show that this is not just due to traditional genetic transmission or similar familial environments but may also be due to epigenetic factors (33). The presence of obesity and accompanying metabolic derangements, especially insulin resistance, during pregnancy have been shown to transmit or imprint characteristics in offspring in animal models (34). In one study of (35) children aged 2.5–25 yrs born either before or after maternal bariatric surgery, improvements in cardiometabolic markers sustained into adolescence were attributable to an improved intrauterine environment particularly in children born after maternal bariatric surgery (36).

However, although the genetics of obesity is a highly researched area, just a small number of rare single genetic abnormalities have been discovered. Leptin deficiency is a monogenic defect that has been found in a very small number of humans with severe, early onset obesity (37). A pronounced feature in such people is increased appetite, with hyperphagia as the predominant problem; the metabolic rate is normal. These subjects may

also have problems with fertility due to hypogonadotropic hypogonadism (38). The molecular bases of the classical obesity syndromes, such as Prader-Willi syndrome and Bardet-Beidl syndrome have been definitely associated with defects in imprinted genes on chromosomes (39).

Neuroendocrine factors: The neuroendocrine causes of obesity include hypothyroidism, Cushing's syndrome, growth hormone deficiency, hypogonadism, and polycystic ovary syndrome (40). Eating disorders, notably binge eating disorders and night eating syndrome, also give rise to obesity. The industrially produced substances that can affect endocrine function may also be a contributing factor to the etiology of obesity. They include dichlorodiphenyltrichloroethane, some polychlorinated biphenols and some alkylphenols that may act by disturbing endogenous hormonal regulation (41).

Psychiatric factors: Obesity is not regarded as a psychiatric disorder, but the risk of obesity is increased in patients with psychiatric disorders such as depression and anxiety. On the other hand, obese people suffer from stigmatization and discrimination in many life domains.

1.2.2.2 External environment factors

The food and diet composition have shifted in ways that promote overeating. The Westernization of diets, with an increase in availability of high caloric foods, especially of fat, and also over-consumption of refined carbohydrates and fructose, certainly contribute to the epidemic of obesity (42). This type of foods tends to be energy dense, is usually inexpensive and easy to prepare with heavy marketing for both adults and children: these factors contribute to increased daily caloric intake (43). Experimental studies show that consumption of such food leads to diminished satiety vs. consumption of less energy dense foods, and encourages overconsumption, suggesting that the peripheral and hypothalamic regulatory systems are less sensitive to a high fat diet (44)(45). These high calorie products are frequently consumed by millions of peoples who are struggling to meet the economic and scheduling demands of today’s fast-paced lifestyle.

Physical activity levels have also dramatically decreased in the past several decades (46). It has been estimated that less than half of US adults engaged in recommended levels of physical activity in 2005 (47). There is less access to physical activity, less physical education in schools (48) and more time is spent on sedentary behaviors such as television watching, using the Internet, smartphones and playing video games (49). The innumerable advances in technology developed over the past few decades have made many tasks more efficient, but in the process have ultimately decreased the number of calories expended (i.e., TV remote controls, automatic garage door opener, sit-down lawnmowers etc.).

Medications that can cause weight gain include antidepressants, antidiabetic drugs, anticonvulsants, antipsychotic medication, beta-blockers, and steroid hormones. Weight gain is associated with several commonly used medications including psychotropic medications, diabetic treatments, antihypertensives, steroid hormones and contraceptives, antihistamines, and protease inhibitors. The deleterious effects of drug-induced weight gain include increased risks for developing type 2 diabetes, hypertension, hyperlipidemia, as well as poor medication compliance (50).

1.3 Dietary macronutrients

Dietary macronutrient effects on body weight depend not only on the amount consumed, but also on their type. Thus, a diet high in carbohydrates and/or fat will encourage weight gain (51). However, the development of complicated obesity will depend on whether carbohydrates are simple or complex, and whether fats are saturated or polyunsaturated (52)(53). The current obesity epidemic is thought to be largely attributable to excessive consumption of palatable foods, high in refined (simple) sugars and saturated fats (51) resulting in higher energy intake. It should be kept in mind that the weight loss achieved is associated with the duration of the diet and restriction of energy intake, but not with restriction of carbohydrates

1.3.1 Fat

Fat refers to the class of nutrients known as lipids. The lipid family includes triglycerides, phospholipids and sterols. Fat is a more concentrated energy source than other energy nutrients (9 kcal/g fat vs 4 kcal/g sugar and protein). Studies on humans and animals have demonstrated that diets high in saturated fats induce weight gain, insulin resistance, and hyperlipidemia (54).

Numerous cross-sectional studies have clearly shown positive associations between the proportion of total energy intake covered by fat and body fatness (55). Increasing low-fat and fat-free dairy products have been recommended in the Dietary Guidelines for Americans (DGA) for achieving nutrient adequacy, disease prevention and overall good health (56). A recent systemic review and meta-analysis of 33 randomized controlled trials in adults has shown that lowering the proportion of energy intake from total fat was associated with lower body weight (by 1.6 kg), BMI, WC as well as small but statistically significant improvements in lipid profile and blood pressure, suggesting a lack of harm on other major cardiovascular risk factors (57).

By contrast, a number of longitudinal studies have been unable to establish any association between self-reported dietary fat and carbohydrate intakes, and subsequent weight change (55). Especially in the case of fat intake vs. weight change, the results on

intake of saturated (SFA), or polyunsaturated (PUFA) fatty acids against development of obesity indicate both a positive (58) or no significant association (59). Furthermore, results of a longitudinal association study between dairy consumption and changes of body weight and waist circumference in the Framingham Heart Study (60) showed no beneficial relation of total low-fat dairy or skim/low-fat milk with long-term change in weight or WC. Therefore, the strength of the data providing evidence for a causal link between dietary fat and obesity has been challenged by new questions on dietary fat effects on obesity.

Consequently, it has been suggested that saturated fat should be replaced by monounsaturated fat rather than by carbohydrate. Field et al. (58) showed associations of mono-unsaturated fatty acid (MUFA) with protection against weight gain, however this finding was not confirmed in another study (59). Studies on the role of trans-fatty acids (TFA) suggested that TFA, when substituted for carbohydrates or PUFA, were associated with increased waist circumference (60). Field et al. also (58) found a positive association between TFA intake and weight gain. Hence, all theses studies showed that high intake of TFA predict weight gain. The lack of multiple data on specific combinations prevents us from making a stronger conclusion.

Therefore, the focus on dietary fat may likely be a distraction from the more significant causes of obesity and metabolic disorders. Increasing evidence now suggests that the rise in consumption of carbohydrates, particularly refined sugars high in fructose, appears to be at least one potentially important contributing factor (61).

1.3.2 Carbohydrate

Dietary carbohydrates include simple (sugars) and complex (starches and fibers) carbohydrates. Disaccharides and starches are a major source of energy. In the course of digestion (62), they are hydrolyzed by specific glycosidase to their monosaccharide components, which are absorbed into the circulation from the intestine. The monosaccharaides are then transported to cells in various tissues, traversing the cell membrane through facilitative transporters. Glucose is transported into cells of many different tissues through the GLUT family of transporters. Inside cells, glucose is phosphorylated, and can be converted to glycogen primarily in liver and skeletal muscle, or

it can be routed through the energy releasing pathways of glycolysis and the tricarboxylic acid cycle in all cell for ATP production (9).

Howard et al. reported that higher intake of total carbohydrates protected against weight gain in women (63), but Halkjaer et al. (64) did not find such an association between carbohydrate intake and change in body weight or waist circumference. The source of carbohydrates may be relevant, however, since Halkjaer et al. reported a positive association between change in waist circumference and carbohydrates from foods with simple sugars, potatoes and from refined grains in women. By contrast, they also found that high carbohydrate intake from vegetables (women only) and fruit protected against an increase in waist circumference.

1.3.3 Glucose vs. Fructose

Glucose (62)(9), commonly known as blood sugar, serves as an essential energy source for all the body's activities. Glucose is one of the two sugars in every disaccharide and the unit from which the polysaccharides are made almost exclusively. One of these polysaccharides, starch, is the chief food source of energy for all the world's people; another, glycogen, is an important storage form of energy in the body.

Fructose (62)(9) is the sweetest of the natural sugars. Surprisingly, fructose has exactly the same chemical formula as glucose (C6H12O6) but its structure differs. Fructose occurs naturally in fruits and honey; other sources include products such as soft drinks, ready-to eat cereals, and desserts that have been sweetened with high-fructose corn syrup.

Fructose has some differences from glucose (62)(65)(66)(9); I) Fructose differs from glucose by the presence of a keto group attached to carbon 2 of the molecule, whereas glucose presents an aldehyde group at carbon 1. II) Its metabolism differs markedly from that of glucose due to its almost complete hepatic extraction and rapid hepatic conversion into glucose, glycogen, lactate, and fat. III) Fructose is absorbed from the gastrointestinal tract by a mechanism different from glucose absorption. Fructose is transported into the enterocyte through a specific fructose transporter, GLUT5, located at the apical pole of the enterocyte. Contrary to glucose, this process does not require ATP hydrolysis and is

independent of sodium absorption. IV) Glucose directly stimulates insulin release from the pancreatic β-cell, but fructose does not. V) Fructose also enters muscle and other cells without depending on insulin, whereas most glucose enters cells in an insulin-dependent manner and finally, VI) Once inside the cell, fructose can enter the pathways that provide the triglyceride backbone (glycerol) more efficiently than glucose.

Fructose was initially thought to be advisable for patients with diabetes due to its low Glycemic Index (67) (68), which is indicative of plasma glucose and insulin response to foods. However, a growing body of evidence in animal studies clearly indicates that long-term consumption of fructose is associated with increased risk of diabetes through effects on adiposity and independently through other metabolic effects (66). Although the evidence is controversial in humans (69), high fructose intake has indeed been shown to cause dyslipidemia and to impair hepatic insulin sensitivity (70) (71) (72).

1.3.4 High Fructose consumption

In 2009, the American Heart Association released a scientific statement calling for reductions in added sugar intake to 100 to 150 kcal/day for most Americans as a means of reducing obesity and cardiovascular disease risk (73). Over the past several decades, daily caloric intake has increased by 150 to 300 kcal (differing by age and sex), and about 50% of the increased calories have come from the consumption of caloric-sweetened beverages (74). Overall, the world average sugar consumption per capita has increased by 16% over the past 20 years, from 56 g/day in 1986 to 65 g/day in 2007 (65). South America and Oceania are the highest sugar consumers, followed by Europe, while low sugar consumption is recorded for Asia and Africa (65). Over the last few decades, fructose has become a major constituent of our modern diet (74). Dietary fructose comes from two principal sources, food such as fruits and vegetables, and from refined products that contain sucrose as high-fructose corn syrup (62).

Interestingly, the patterns of development of the obesity epidemic and overconsumption of sugar-sweetened beverages have overlapped over the last few decades (75). This is shown by the changing intake of fructose and high-fructose corn syrup (HFCS)

intake plotted against the increasing prevalence of obesity (Figure 1.2) (76). Although this pattern cannot prove the role of HFCS as a culprit, it provides testable evidence for a potential role of fructose in development of obesity.

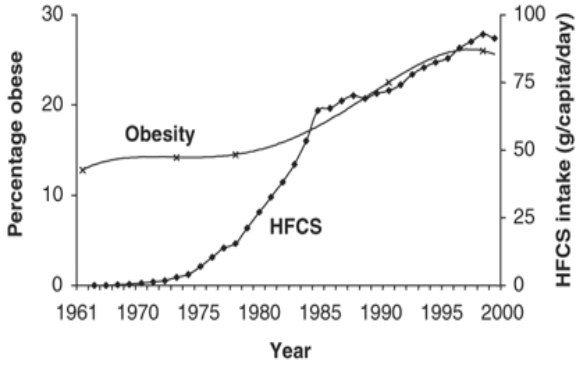


Figure 1.2 Changing intake of fructose and high-fructose corn syrup intake plotted against the increasing prevalence of obesity.

Adapted from Bray et al. 2004

It is commonly assumed that high-fructose corn syrup contains 42–55 % fructose and in some brands, up to 65% (77). The consumption of HFCS has increased largely because of the increased consumption of beverages and foods sweetened with HFCS, including carbonated and noncarbonated beverages and juices, breakfast cereals, bread and other baked goods, canned fruits, jams and jellies, condiments, and prepared desserts (74). In the Longitudinal Study of Child Development in Quebec (1998–2002)(78), 6.9% of children who were non-consumers of sugar-sweetened beverages between meals, from the age of 2.5–4.5 years, were overweight, compared with 15.4% of children who consumed sugar-sweetened soft drinks 4–6 times or more per week between meals.

Thus, high consumption of fructose, as occurs with the increasing consumption of soft drinks and the use of high-fructose corn sweeteners, may be a ‘fat equivalent’ (76). In contrast to a high-fructose diet, a diet that is high in glucose does not lead to hypertension, elevated plasma triglycerides, insulin resistance or hyperinsulinemia. It therefore seems that fructose is more damaging sugar component in terms of cardiovascular risk (79).

Several mechanisms have been suggested for the effects of fructose that come from drinking either HFCS or sucrose-sweetened beverages (71)(79)(80). 1) An increase in overall energy intake due to consumption of soft drinks supplementing a regular diet is the most obvious mechanism. In general, energy obtained from soft drinks is not completely compensated for by a reduction in energy intake from other foods. 2) Fructose is metabolized primarily in the liver where it is converted to fructose-1-phosphate from which it can readily become a substrate for the backbone of the triglyceride molecule. 3) The metabolism of fructose in the liver generates adenosine 5'phosphate that is a substrate for conversion to uric acid through a process that alters nitric oxide generation. 4) The fructose consumption reduced post-meal insulin exposure may lead to reduced activation of lipo protein lipase (LPL) in SAT and less triglyceride (TG) uptake in SAT, thus increased TG uptake/accumulation in VAT (53). 5) In the liver, fructose-containing sugar will up-regulate de novo lipogenesis (DNL), which will increase liver lipid levels, and also concurrently inhibit fatty acid oxidation (53).

Furthermore, there is now growing evidence to challenge the view that fructose metabolism occurs primarily in the liver and kidney, suggesting that its role in the brain may have important implications for neuronal function (81). Recently, van der Borght's group raised the possibility that there is at least some neurobiological evidence for the detrimental effect of high fructose consumption on cognition (82). These finding reviewed by Yon MA et al. (77) show that in rats, appetite-related and inflammatory signals appear to mediate impaired hippocampal neurogenesis resulting from the chronic intake of fructose, at concentrations much lower than those consumed by humans, and these effects are independent of obesity. Whether or not this phenomenon extends to brainstem regions of feeding regulation may depend on fructose concentration and the duration of consumption interacting with the site of the neurogenic niche (77). About 10 % (w/v) of fructose appears sufficient to impair hippocampal neurogenesis in rodents (83)(84).

1.4 Sex difference in obesity

The prevalence of obesity is higher in women than in men in most countries around the world. It has long been recognized that men and premenopausal women differ in their fat distribution, which is called ‘gynoid’ and ‘android’ (for women and men respectively) (85). Despite the generally lower population prevalence of obesity in men, obese men are at substantial risk of obesity-related chronic diseases because of fat accumulation in abdominal, visceral depots (86). Because of the significantly increased cardiometabolic risk associated with abdominal fat in men (and postmenopausal women), it is important to understand mechanisms that determine where fat accumulates.

1.4.1 Sex differences in energy metabolism

It is well recognized that body size and fat-free mass are strong determinants of energy expenditure (EE) (87). In one cross-sectional study including white adults, lean mass contributed to 63%, fat mass to 6%, and age to 2% of the variability of basal metabolic rate between subjects (88). Because women tend to have less weight, less height, less fat-free mass but more fat mass than men at any given BMI, it is not surprising that in absolute terms women have lower EE. Furthermore, the decline in resting EE with age has been found to be greater in women than in men, suggesting that women may be at greater risk for obesity with aging (89).

However, lower total and physical activity energy expenditure, as well as resting energy expenditure, in women than in men have been reported (90). These differences may be at least partly explained by body composition differences (91). Physical activity energy expenditure is related to percent body fat in men but not in women (92)(90) . In addition, it has been reported that physical training programs are not associated with significant fat loss in women suggesting that women may compensate for higher energy expenditure by increasing energy intake to a greater extent than do men (93). A stronger relationship between body composition and type of food intake also has been demonstrated in women than men (90). Women with higher fat intake and lower carbohydrate intake were found to have a significantly higher percentage of body fat but this was not true for men (90).

1.4.2 Sex differences in postprandial state

Higher postprandial lipemia (circulating triglycerides and non-esterified fatty acids) in men compared with women in response to food intake has been previously reported (94). However, visceral adipose tissue accumulation was a significantly important contributing factor to the exaggerated postprandial triglyceride response in men (95). Previously, it has been shown that postprandial lipemia can be predicted by fasting triglyceridemia, and men have been reported to have substantially higher fasting triglyceride levels than do women (96). Liver fat content, which is significantly higher in men than in women, is also associated with postprandial lipemia (97)(98). The sex hormone estrogen has been suggested to contribute to postprandial triglyceride levels (99).

1.4.3 Sex differences in body fat distribution

Body fat distribution is significantly different between men and women. Multiple studies have shown the importance of adipose tissue distribution (86) (100). Men tend to have central fat distribution by storing fat in their trunk and abdomen, whereas women tend to store fat in their hips and thighs (peripheral fat distribution) (100).

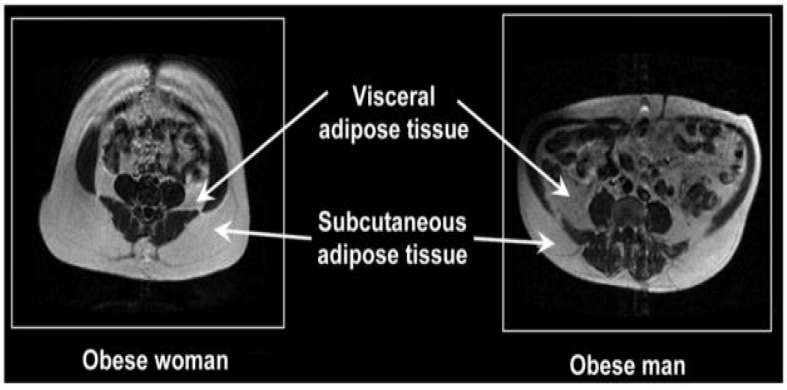


Figure 1.3 Cross-sectional abdominal magnetic resonance grey-level images of an obese woman and an obese man.

Adapted from Geer EB, Shen W. Gend Med. 2009;6 Suppl 1:60-75.

Therefore, compared with women, men generally have more VAT and less SAT, as measured by magnetic resonance imaging (MRI) or computerized tomography (CT) scans

(Figure 1.3). It is well recognized that estrogen-deficient postmenopausal women tend to accumulate more abdominal and visceral fat while premenopausal women accumulate more lower body (gluteo-femoral) fat. Interestingly, the sex difference in abdominal vs. gluteo-femoral fat cell size persists even in extreme obesity; the increases in thigh fat mass in women are related to increased fat cell hyperplasia rather than increased fat cell size (101). The tendency towards a peripheral fat distribution in women is associated with improved insulin sensitivity (102), compared with the central fat distribution observed in men which is associated with elevated postprandial insulin, non-esterified fatty acids, and triglyceride levels (94). The association of fatty liver with visceral adiposity, dyslipidemia (103), and insulin resistance may explain the increased insulin resistance observed in men compared with women.

Explanations for the associations between increased visceral adiposity, hepatic fat accumulation and hepatic insulin resistance have been proposed. Some hypotheses propose increased free fatty acids (FFAs) supply to the liver from inflamed, hypertrophied adipose tissue (104) as well as the combination of decreased adiponectin production coupled with increased proinflammatory cytokines TNF-α and IL-6 production from adipose tissue (105)(106). Sex dimorphism in regional adiposity has been clearly demonstrated across age groups such as in the study by Kotani et al. (107). Cross sectional studies have shown that visceral adipose tissue deposition was found to increase with age mostly in men and in menopausal and postmenopausal women (100).

Sex differences in body fat distribution appear to be largely a result of differences in sex hormones between men and women. Previously, Krotkiewski et al. (108)(109) suggested that sex hormones might be involved in regulating the typical gender differences in regional body fat distribution. It has been well recognized that the sex hormone estrogen is key in maintaining the sex-specific fat distribution patterns in humans (85). In female rats, ovariectomy increases (110) visceral fat and decreases subcutaneous fat and estrogen treatment reverses this effect (111)(112). The same result after administration of exogenous estradiol to male rats has been illustrated when compared to male rats not given estrogen (112) . Previously, research has shown that male and female aromatase-knockout (ArKO) mice, which are estrogen-deficient and have elevated testosterone, accumulate more intra-

abdominal adipose tissue with increased adipocyte size in the gonadal and intra-renal depots (113). In addition, Male ArKO mice also develop fatty liver, as do aromatase-deficient men, and this is reversible with estradiol treatment (114).

Sex differences have also been investigated on effects of sex hormones on lipoprotein lipase activity and lipolysis. Higher adipose tissue LPL activity (promoting fat storage) has been shown specifically during lactation in femoral adipocytes (subcutaneous adipocytes) compared with abdominal adipocytes in premenopausal women but not in postmenopausal women (115). Furthermore, estrogen treatment in postmenopausal women restores LPL activity of the femoral adipocytes and decreases lipolytic response in subcutaneous adipocytes but not in abdominal adipocytes (116). It has been shown that ovariectomized female rats are significantly less sensitive to leptin's anorexic action in the brain and that estradiol treatment reverses this leptin resistance. Furthermore, responsiveness to central leptin in male mice is enhanced by peripheral estradiol administration (112).

1.4.4 Sex differences in cardiometabolic risk

The presence of central adiposity, regardless of gender, is a risk factor for development of insulin resistance and diabetes (117), thus premenopausal women typically have decreased cardiometabolic risk relative to men and posmenopausal women. In the Diabetes Prevention Program's population of 3234 patients, baseline waist circumference was the strongest predictor of diabetes in both sexes (118). Several epidemiological studies suggest that a high waist-to-hip ratio is a better predictor of all-cause and cardiovascular disease mortality than a large waist circumference alone (119). Furthermore, numerous evidences suggest that a larger hip circumference (larger gluteo-femoral fat stores) are protective against both CVD and metabolic risk in multiple ethnic groups, independent of waist circumference or abdominal fat (120). The cutoff points for increased risk in men are larger than those for women in the diagnostic criteria of the metabolic syndrome (eg, waist circumference of 102 vs 88 cm, respectively) (117).

More interestingly, transplantation of subcutaneous fat (inguinal region) into the intra-abdominal compartment of male mice on a high-fat diet resulted in significant

protective effects on adiposity, insulin sensitivity and glucose tolerance (121). It would be interesting to see if similar or even greater protective effects could be conferred from transplanting female subcutaneous fat, given that in humans, the protective effect of a large hip circumference with regards to CVD morbidity and mortality was significant only in women, with just borderline significance on total mortality in men (122).

Divergent roles of visceral and subcutaneous fat in terms of cardiometabolic risk factors can be explained by differences in histology and metabolic activity of these two fat tissues. Specifically, visceral adipocytes are more sensitive to catecholamine-induced lipolysis and less sensitive to insulin's antilipolytic effect than are subcutaneous adipocytes (123)(124). This higher sensitivity to catecholamines and lipolytic activity may lead to enhance delivery of free faty acids (FFA) directly via the portal system to the liver, resulting in elevated FFA, glucose and very-low-density lipoproteins in circulation, and decreased hepatic insulin clearance (125). Therefore, increases in visceral adipose tissue contribute to dyslipidemia, enhanced gluconeogenesis, and insulin resistance (125).

Many studies (reviewed in (86)) have shown that women have larger low density lipoprotein (LDL) particles than men. High levels of small, dense LDL particles are associated with an increased coronary hearth disease (CHD) risk. Carr et al. (126) suggested that higher hepatic lipase activity in men, which affects LDL and high density lipoprotein (HDL) heterogeneity and is responsive to testosterone stimulation, might also explain the gender difference in the cardiovascular risk profile. In addition, women have larger HDL particles than men, a phenotype generally associated with an overall low-risk lipoprotein-lipid profile (100). It has been demonstrated that although premenopausal women had more total body fat than men, they also had lower visceral adipose tissue accumulation and a better metabolic risk profile (100).

1.4.5 Depot-Specific Adipose Tissue Sex Steroids

Adipose tissue distribution per se may contribute to gender differences in insulin resistance via metabolism and release of sex hormones. Although the adrenal glands and gonads are the primary source of sex hormones in circulation, adipose tissue can contribute up to 50% of circulating testosterone in premenopausal women and 100% of circulating

estrogen in postmenopausal women (127). Adipose tissue aromatase in adipose tissue converts androgens to estrogens: androstenedione to estrone and testosterone to estradiol. Furthermore, adipose tissue 17β-hydroxysteroid dehydrogenase (17βHSD) mediates the conversion of weak androgens or estrogens to their more potent counterparts: androstenedione to testosterone and estrone to estradiol (125). Expression of 17βHSD in subcutaneous adipose tissue is relatively less than aromatase, whereas visceral adipose tissue expresses more 17βHSD than aromatase. Thus, more visceral adipose tissue may express relatively more 17βHSD and as a consequence more local androgen production. Furthermore, mice with targeted ablation of aromatase have increased visceral adiposity, insulin resistance, dyslipidemia, and hepatic steatosis (128)(113).

1.5 Sex hormones and obesity in women

The average obese female encounters many obstacles to achieving a healthy life because of a complex mix of social, cultural, and physiologic factors. Obese women often have disturbances in sex hormone metabolism, and as a result, menstrual irregularities and infertility more than non-obese women.

1.5.1 Estrogen

At least in part, gender differences in body composition; fat distribution and energy homeostasis may be due to the effect of sex hormones. The decrease in insulin sensitivity with menopause, and subsequent improvement with estrogen replacement, suggests that estrogen has a favorable effect on insulin sensitivity and may play a role in glucose homeostasis in women (reviewed in (125)). Even in men, complete lack of estrogen synthesis or activity is associated with insulin resistance (129). It has been shown that in males, insulin resistance increases during adolescence, despite significant decreases in adiposity whereas in females, body fat significantly increases but insulin resistance does not significantly change (110). Thus, insulin resistance occurs independently of increasing lean and decreasing fat mass, possibly due to the relative decrease in estrogen in males compared with females.

1.5.1.1 Estrogen effects on insulin function and glucose homeostasis

Estrogen may be protective against the development of insulin resistance and diabetes. Human and animal studies have shown that estrogen plays a role in the maintenance of glucose homeostasis and substrate metabolism. In humans, it has been demonstrated that women have a higher 17β-estradiol concentration than men during exercise, which was associated with significantly more lipid and less carbohydrate metabolism (130). In animals, estrogen is protective against hyperglycemia in diabetes, by reducing hepatic glucose production and enhancing glucose transport in the muscle (125)(131). Estrogen also has antioxidant properties; a protection against oxidative stress has been shown in mice, mediated via increase in gene expression, including those encoding antioxidant enzymes superoxide dismutase and glutathione peroxidase (132). Furthermore, in conditions of oxidative stress, estrogen has been found to protect pancreatic β-cell function and survival (131). Finally, pregnancy as a high estrogen state is characterized by reducing insulin sensitivity (insulin resistance), but other hormonal changes during pregnancy also affect insulin sensitivity (110).

1.5.1.2 Estrogen effects on obesity and fat distribution

Estrogen may have protective effects against obesity, in particular visceral obesity, in humans and in animals (125). Studies in animals suggest that the sex hormone estrogen may have a role in the prevention of obesity (131). Tara M. D'Eon et al have demonstrated that ovariectomized animals have increased adiposity compared with animals with intact ovaries (133) (133). Estrogen treatment significantly reduced adipose mass and adipocyte size in these animals, possibly via decreased expression of lipogenic genes in adipose tissue, liver, and skeletal muscle. They suggested that in muscle, estrogen appears to promote the use of lipid as fuel by partitioning FFAs toward oxidation and away from TG storage by upregulating the expression of peroxisome proliferation activator receptor-δ.

Estrogen may also have beneficial effects on adipose tissue distribution. Previously, Kotani et al in 1994 (107) evaluated the role of aging on adipose tissue distribution and demonstrated that, compared with premenopausal women, visceral adipose tissue in postmenopausal women was significantly larger. In another study, women receiving

hormone replacement therapy (HRT) had lower waist circumferences than did the never-users, again suggesting that estrogen may reduce central adiposity in humans (134). The preferential deposition of adipose tissue in the subcutaneous in women versus deposition in the viscera in men may be related to the higher level of estrogen in women compared with men (125).

Of the two types of estrogen receptors (ERα and ERβ), ERα appears to be the one involved in the regulation of fat distribution (85). Silencing of ERα in the ventromedial nucleus of hypothalamus in female mice results in increased body weight, decreased energy expenditure, increased visceral adiposity and decreased leptin sensitivity (135). In male ER knockout mice, the obesity-promoting effect and disturbed lipoprotein profile appear to be mediated specifically through ERα, as ERα knockouts exhibit increased fat mass while ERβ knockout mice do not (136). Association of polymorphisms in the estrogen receptor alpha gene with body fat distribution has been shown in human studies. Okura et al.(137) have demonstrated that ERα gene polymorphisms predict abdominal obesity in women, but not in men, suggesting a possible sex dimorphism in the effects of ERα (137).

1.5.1.3 Estrogen has anti-inflammatory properties

Human studies have shown that increased proinflammatory cytokine levels typically produced by immune cells, including tumor necrosis factor α (TNF-α), interlukin-1 and 6 (IL-1, IL-6), are associated with menopause and theses levels are substantially lower in women receiving hormone replacement therapy (138). Animal studies also have shown that circulating levels of TNF-α were significantly higher in ovariectomized rats compared with estrogen-replaced ovariectomized rats or those with endogenous estrogen production (139). These elevations in TNF-α were associated with impaired vascular function due to decreased nitric oxide levels. Given the association between increased proinflammatory markers and obesity-related complications such as insulin resistance (140), the association between estrogen and decreased cytokines may play a role in gender differences in insulin resistance.

1.5.2 Progesterone

Some studies have shown that progesterone levels are changed in obese women. Women who were obese had 20% lower progesterone levels compared with more lean women (BMI<20) averaging 43 years in age (141). Another study among older premenopausal (mean age 47 years) women also found lower (−35%) daily levels of progesterone among obese compared to normal weight women (142). A very recent study showed that compared with normal weight women, obese women averaged lower levels of progesterone (-15%,), luteal hormone (LH: -17%), follicle-stimulating hormone (FSH: -23%) and higher free estradiol (+22%) across the menstrual cycle (143). The decrease in progesterone has been explained as a consequence of the decreased mean levels of LH throughout the cycle including the luteal phase.

The effects of progesterone on adipose tissue function are less investigated; however, a series of *in vivo* and *in vitro* studies has suggested that progesterone may act as a lipogenic hormone (144). Studies identifying progesterone receptors in mature human adipocytes are limited (145). Gray and Wade (146) were able to identify progesterone binding sites in adipose tissue from ovariectomized–adrenalectomized rats that were primed with estrogen . Progesterone was reported to stimulate fat storage by enhancing lipoprotein lipase activity (147) and increasing the expression of transcription factors that control synthesis of fatty acid synthase (148). In 1997, Björntrop initially suggested that progesterone could be involved in female fat distribution pattern through its anti-glucocorticoid effects (149). This notion was partially supported by studies showing progesterone binding with high affinity to glucocorticoid receptors inhibiting abdominal fat accumulation stimulated by cortisol (147). Zhang Y. et al (2008) demonstrated that preadipocytes and mature adipocytes efficiently generate progesterone metabolites (such as 20-α hydroxyl-progesterone), which is more consistent with relative modest effects of progesterone on abdominal fat cell differentiation (150). Progesterone is also involved in the complex regulation of appetite, yet it does not significantly influence feeding behavior in ovariectomized rats, except when administered at non-physiological, pharmacological doses (151). However, in the presence of estrogen, progesterone does stimulate appetite and promote weight gain (152).

1.6 Endocrine function of adipose tissue and adipokines

Obesity is the result of hypertrophy and hyperplasia of adipocytes and consequently the expansion of white adipose tissue (WAT). The main roles of WAT are the storage of triglycerides during positive energy balance and fatty acid release when the energy balance is negative. This regulation is achieved through endocrine, paracrine and autocrine pathways that allow adipocytes to regulate the metabolism in other fat cells located in liver, muscle, brain and pancreas (153).

Adipose tissue function is categorized into three parts (reviewed in (154)): 1) lipid metabolism (lipogenesis and lipolysis) including fat storage and free fatty acid release 2) glucose metabolism, adipose tissue catabolizes triglycerides providing glycerol and fatty acids that participate in glucose metabolism in liver and other tissues 3) production and secretion of adipokines, which include hormones, cytokines and other proteins with specific biological functions.

In obese subjects, white adipocytes have increased release of free fatty acids via lipolysis leading to an overflow of lipids, which has been considered a key reason for obesity-associated insulin resistance and hepatosteatosis for several decades (155). Adipokines mediate the crosstalk between adipose tissue and other key metabolic organs, especially the liver, muscle, and pancreas, as well as the CNS (156). Given this notion, dysfunctions in adipokine pathways can result in impaired organ communications and metabolic abnormalities in multiple tissues, thereby constituting a critical pathological component in the development of metabolic disease (157).

As a secretory organ, adipose tissue displays several unusual characteristics (158). First, adipose tissue is composed of distinct cell types: mature adipocytes and stromal-vascular cells as well as various immune cells (macrophages, T cells, neutrophils, lymphocytes) all of which may contribute to a variable extent to adipose tissue secretory function. Second, adipose tissue is heterogeneous. The different fat depots (visceral and subcutaneous) are different not only in terms of metabolic capacities, but also in adipokine secretion pattern, which may have local indirect consequences on AT by autocrine or

paracrine mechanisms and/or may directly impact the liver for substances produced by intra-peritoneal fat. When the contribution of each depot to systemic levels of adipokines is considered, it should be kept in mind that intra-abdominal fat only represents 15% of total fat in lean and obese individuals (159). Moreover, organs other than AT may contribute to systemic levels of some adipokines.

1.6.1 Adipocyte function and metabolic inflammation

Hotamisligil et al. (1993) (reviewed in (160)) postulated the first functional link between obesity and inflammation by showing that adipose tissue in obese mice secretes TNFα, and they also demonstrated that adipocyte-derived TNFα plays a direct role in obesity-induced insulin resistance. Over the years, this has evolved into a concept of "metabolic inflammation", which has been widely accepted as an important mechanistic connection between obesity and its complications (Hotamisligil 2006) (161). It has been demonstrated that adipose tissue produces an array of cytokines and chemokines such as IL6 and MCP1, which either positively or negatively regulate systemic glucose and lipid metabolism. In 2003, two studies reported that obesity induces macrophage infiltration of adipose tissue in both mice and humans (162)(163), providing an explanation for the source of adipose-derived cytokines but more importantly, demonstrating for the first time the close juxtaposition between immune and metabolic cells in a metabolic organ.

Obesity induces a shift to activated macrophages in adipose tissue leading to increased levels of proinflammatory cytokines and reactive oxygen species (ROS), which induce insulin resistance (164). In recent years, nearly all major immune cells have been identified in adipose tissue and are actively involved in the endocrine function of adipose tissue in systemic metabolic regulation (160). Furthermore, the close physical and signaling interactions between immune and metabolic cells also exist in all major metabolic organs of obese subjects especially the liver, muscle, and pancreas, indicating that metabolic inflammation is a universal feature and a pathological basis for obesity-induced metabolic dysfunction (160).

Several potential underlying causes for obesity-induced adipose inflammation have been proposed in recent years (160). Adipose tissue expansion in the development of

obesity can cause hypoxia, which induces compensatory angiogenesis. Infiltrated macrophages in adipose tissue have also been proposed to be a mechanism to remove apoptotic cells (165). In addition, endotoxemia associated with altered gut permeability and obesity might potentiate adipose inflammation (166).

On the other hand, "Inflammation during obesity, is not all bad" (167). Macrophages and proinflammatory cytokines are essential for adipose remodeling and adipocyte differentiation and might be necessary for the body to adapt to the excess energy and maintain metabolic homeostasis (160). Expression of proinflammatory cytokines (leptin, IL-1, TNF-α, IL-6, etc.) is enhanced in adipocytes, macrophages, and lymphocytes (167). On the other hand, to control chronic inflammation, the anti-inflammation molecules (adiponectin, IL-10, etc.) are activated to balance the inflammatory impact (167).

The inflammation may act in the peripheral organs/tissues as well as in the central nervous system to regulate energy balance (168)(169). In the periphery, inflammation may induce fat mobilization and oxidation to promote energy expenditure and may induce energy disposal through glucose excretion in urine as a result of insulin resistance and hyperglycemia. In the central nervous system (CNS), inflammation may inhibit food intake and activate neurons for energy expenditure. If this feedback system is deficient (called "inflammation resistance"), energy expenditure will be interrupted and fat will accumulate in the body, thereby increasing adiposity (168). The anti-inflammatory molecules tend to promote energy (fat and glucose) storage and improve insulin action, whereas the proinflammatory activities facilitate weight loss and yet impair insulin action (167). However, more studies are needed to fully understand the mechanism of beneficial inflammatory responses associated with various stages of obesity.

1.7 Key adipokines in obesity-induced metabolic disorders

1.7.1 Leptin

Leptin is one of the most potent adipocytokines in metabolic regulation and energy hemostasis. Leptin plays a central role in regulating food intake, energy expenditure and neuroendocrine function by signaling nutritional status to other organs especially the hypothalamus (170). Leptin stimulates oxidation of fatty acids in muscles by activating AMP-activated protein kinase (171) and regulates lipogenesis in liver by inhibiting the fatty acid synthesis pathway (172). Therefore, leptin prevents the accumulation of lipids in non-adipose tissues, which can lead to functional impairments known as "lipotoxicity"". Anti-diabetic beneficial effects of leptin are independent of its regulation of body weight and energy intake (173).

All these effects are mediated mainly via activation of two multiple pathways downstream of leptin receptors (174). The energy balance regulation of leptin is mediated by activation of the JAK–STAT pathway, which is essential for regulation of energy balance and controls expression of anorexic neuropeptides (175). The anti-diabetic effect of leptin is mediated by centrally activating the phosphatidylinositol-3-kinase (PI3K)/AKT pathway that stimulates insulin sensitivity in the peripheral tissues (176).

Leptin also has proinflammatoty properties that contribute to its overall effects in body weight regulation through suppressing energy intake and enhancing energy expenditure (168). In addition, leptin has been implicated in a number of immune dysfunctions (177). Interestingly, inflammation induced by metabolic stress also negatively regulates leptin signaling in a manner similar to insulin receptor signaling (178).

Many studies have shown that leptin concentrations are significantly higher in women than men, a finding associated with the higher body fat and intramyocellular content observed in women (125). This difference has been explained by sex hormone effects and different adipose tissue distribution. In men, androgens have a negative association with leptin concentrations (179), and androgen treatment decreases leptin levels in hypogonadal men (180). Estrogen increases leptin concentration (181) and higher leptin

concentrations in women are associated with larger adipocytes and significantly higher expressions in SAT versus VAT (182). Such evidence highlights the role of leptin as a metabolic signal of energy sufficiency (183) as well as leptin resistance observed in states of increased adiposity (184).

Long-term leptin treatment improves hepatic and peripheral glucose metabolism in insulin resistant lipodystrophic patients and improves liver histology (185) (185). Leptin receptors are expressed on hepatic stellate cells and leptin is believed to be involved in liver fibrosis (186). Leptin-deficient mice develop obesity; however, they fail to develop liver fibrosis during steatohepatitis or in response to chronic toxic liver injury (187). Restoration of physiological levels of circulating leptin, but not correction of the obese phenotype by dietary manipulation, restored fibrosis indicating an essential role in developing liver fibrosis (188).

1.7.2 Adiponectin

Adiponectin, a hormone secreted almost exclusively by adipose tissue, is identified as an abundantly-secreted adipokine (189). Adiponectin is an insulin-sensitizing hormone. Adiponectin lowers glucose production in the liver (190) and improves insulin sensitivity in the muscle and liver by increasing FFA oxidation (191). Recombinant adiponectin can enhance insulin action and partially reverse insulin resistance in obese mice (191). Most animal studies (192)(191) but not all (193) have reported that adiponectin deficiency develops into insulin resistance and is associated with inflammatory markers in adipose tissue and reduced responsiveness to PPARγ. Adiponectin has also antiatherogenic effects as reported previously (reviewed in (160)). In addition, a protective effect in ischemic heart disease through AMPK and cyclooxygenase 2 pathways has been demonstrated (194) Adiponectin signaling is mediated by two adiponectin receptors, adipoR1 and R2 (195). Adiponectin receptors knockout causes lipid accumulation, inflammation, and insulin resistance (196). Clinical studies demonstrated that plasma adiponectin levels decrease in obesity-induced disorders and type 2 diabetic patients (197). Adiponectin levels are also negatively associated with adiposity and fasting glucose (198). Recently, a multi-ethnic

meta-analysis indicated that numerous genetic loci associated with adiponectin levels influence the risk of insulin resistance and type 2 diabetes (199).

Adiponectin also has anti-inflammatory effects that contribute to its protective role against metabolic stress in obesity. Adiponectin suppresses TNFα production in obese mice (200), and adiponectin-deficient mice have high levels of TNFα in adipose tissue (201). Low levels of plasma adiponectin are associated with increased C-reactive protein in humans (202). Adiponectin enhances the clearance of apoptotic cells by facilitating their opsonization and uptake by macrophages (203). Some of the anti-atherogenic effects of adiponectin are also mediated by its role in the suppression of inflammatory responses. Adiponectin inhibits nuclear factor-κB (NFκB) activity and its downstream adhesion molecules leading to reduced monocyte adhesion to endothelial cells (204). In addition, adiponectin confers vascular-protective activities by suppressing the apoptosis of endothelial cells (205)

It has been reported that adiponectin levels are significantly higher in women than in men (206), even after adjusting for differences in BMI (207). This study also found that lower adiponectin levels were more closely associated with hyperglycemia and diabetes in women than in men. Potential reasons for lower adiponectin levels in men than in women may be due to the inhibitory effect of androgens on adiponectin levels (208), higher visceral adiposity, or lower insulin sensitivity in men.

In the liver, adiponectin increases insulin sensitivity and regulates FFA metabolism by suppressing lipogenesis and activation of FFA oxidation (209). Adiponectin levels correlate negatively with liver fat and hepatic insulin resistance in non alcoholic fatty liver disease (NAFLD) (210) suggesting that hypoadiponectinemia is part of a metabolic disturbance characterized by central fat accumulation. Moreover, low adiponectin levels are associated with more extensive inflammation in NAFLD patients (211), suggesting an anti-inflammatory role of adiponectin in controlling hepatic inflammation. This is highlighted by the fact that adiponectin administered in ob/ob mice alleviates liver steatosis and attenuates inflammation in NAFLD mouse models (212).

1.7.3 Acylation stimulating protein (ASP)

ASP is a 76-amino acid protein, which was recognized early on as an adipokine hormones produced by adipose tissue. In 1988, Cianflone and Sniderman conducted the first studies demonstrating that a protein component detected in circulating blood is responsible for the increased intracellular triglyceride (TG) esterification rates in human skin fibroblasts (213). This protein that was named based on its function—acylation-stimulating protein (ASP)—and was recognized to be identical to C3adesArg (214). Three proteins of the alternative complement system are involved in ASP production: C3, factor B and adipsin, all of which are synthesised and secreted by adipocytes (reviewed in (215)). The concentration and the structure of any one of these precursors may influence immune reaction as well as production of ASP. Following activation of the alternative complement pathway, C3 is proteolytically cleaved by C3 convertase to the chemotactic proinflammatory protein C3a and then immediately desarginated by carboxypeptidase N to a derivative C3a-desArg/ASP.

ASP in circulation increases substantially with overweight and obesity (216), and decreases with weight loss (217). ASP and its component proteins (C3, B and adipsin) have also been shown to be significantly increased in Type 2 diabetes and also in some studies of Type I diabetes (215). Even in non-obese Type 2 diabetics, plasma ASP and C3 levels are increased. ASP also increases in cardiovascular diseases, dyslipidemia as well as metabolic disorders such as familial combined hyperlipidemia (FCHL) and polycystic ovarian syndrome (PCOS) (215)(218)(219).

1.7.1.1 ASP and lipid metabolism

ASP increases triglyceride synthesis in fat-storing cells (reviewed in (215)). The effect is achieved through different ways: 1) ASP stimulates fatty acid incorporation into adipose triglycerides (FIAT). These effects are believed to involve activation of diacylglycerol acyltransferase, the final enzyme of the TG pathway. 2) ASP stimulates glucose transporters (GLUT4, GLUT1) to increase glucose intake in adipose tissue. 3) ASP inhibits intracellular lipolysis via inhibition of hormone-sensitive lipase (HSL)-mediated lipolysis.

1.7.1.2 ASP and Insulin

Previously, insulin was believed to be the major hormone that up-regulated triglyceride synthesis in adipocytes. These effects involve increased lipoprotein lipase activity, increased glucose transport and inhibition of lipolysis by hormone sensitive lipase (HSL) (reviewed in (220)). ASP, however, has been proven to markedly stimulate TG synthesis in a number of cell lines from mice and humans (215). For both FIAT and glucose transport, the effects of ASP are additive and independent to those of insulin (215)(221) a well-recognised lipogenic hormone. In the studies by Van Harmelen et al. (222), in the presence of both insulin and ASP, the combined effect was a reduction in net fatty acid output from the adipocytes, with almost complete (97%) re-esterification and storage of the fatty acid available. Thus, in combination with insulin, ASP can provide a powerful drive for fat storage, at least in isolated cells.

1.7.1.3 ASP-C5L2 pathway, extension of ASP functions

ASP manifests its metabolic and insulin-like effects on human adipocytes via its only known receptor C5L2. C5L2 was discovered in 2000 as a putative orphan receptor (GPR77) (223). C5L2 is expressed in various tissues of myeloid and non-myeloid origin and transcripts were detected in brain, placenta, ovary, testis, spleen and colon (reviewed in (223)). Surface expression of C5L2 was detected in lung, liver, heart, kidney, in adipose tissue and in skin fibroblasts as well as in neutrophils (223). C5L2, initially proposed as a nonfunctional receptor, has been shown to be actively involved in inflammatory conditions such as insulin resistance, asthma, and coronary artery disease (224)(225).

Animal models of the ASP–C5L2 pathway deficiency have been investigated (reviewed in (226)). *ASP*-knockout (KO) mice lacking ASP precursor protein C3 (C3KO), and the *C5L2*-KO mice (C5L2KO), lacking the ASP receptor have been examined. C3KO mice showed a delay in TG clearance, an effect that can be reversed with ASP injection (227)(228)(229) and also show reduced triglyceride synthesis in adipose tissue and increased food consumption. These mice were obesity resistant as they also exhibit increased energy, despite increased food intake. In addition, on a high fat diet, excess fuel in ASP-deficient mice is directed towards increased free fatty oxidation as an energetic fuel

in the skeletal muscle instead of being stored in adipocytes. Consequently, C3KO prevents lipotoxicity in the muscle through augmented lipid transport and mitochondrial usage (227)(228). Interestingly, C5L2KO mice showed some metabolic consequences that are quite similar to C3KO mice supporting the role of C5L2 as a functional ASP receptor, but still remaining controversial. These studies showed beneficial effects of C5L2 disruption. By contrast, in the presence of a high-fat-high-sucrose diet, the absence of C5L2 has been shown to increase insulin resistance (229). Other studies with C5L2KO mice have demonstrated a pathophysiological role for C5L2 in insulin resistance, and lipid metabolism (230)(231)(232)(233).

The significant changes in adipose tissue mass in ASP-deficient mice studies suggest that ASP action on adipose tissue may be mediated in several ways. I) ASP has acute effects on postprandial clearance of TG (lipemia) via influence on adipose tissue storage (234). II) ASP may participate in the recruitment of preadipocytes to become adipocytes (220). In addition, changes in feeding behavior, overall energy expenditure, and physical activity profile suggest effects that are centrally mediated (228). All these studies raise the possibility that ASP actions may not be limited to adipose tissue itself.

1.8 Obesity and Contribution of inflammation

Inflammation, a crucial component of the immune system is a routine response of innate immunity. Chronic inflammation – also called low-grade inflammation – is been associated with the development of several metabolic diseases. During the past decade, low-grade inflammation has come to be regarded as a key feature of obesity, atherosclerosis and type 2 diabetes (T2D) (235).

Two major metabolic organs can help to clarify this concept: liver and adipose tissue. These two organs have a special architectural organization, which is the contiguity of metabolic (adipocyte and hepatocyte) and immune cells (macrophages and Kupfer cells) as well as access to the blood network (Figure 1.4).

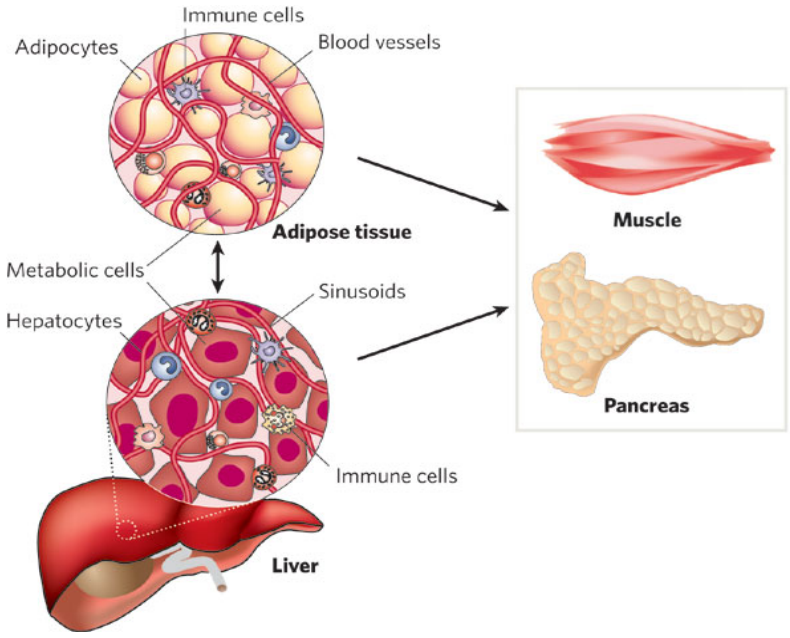


Figure 1.4 Architectural organization and proximity of principal metabolic and immune cells in adipose tissue and liver.

Adapted from G. S. Hotamisligil, Nature, vol. 444, no. 7121, pp. 860–7, Dec. 2006.

This arrangement of elements allows an interaction of immune and metabolic cells while both sets of cells have their own distinct roles. In addition, these organs can also signal to other organs, including the pancreas, brain and muscles. The interrelated functions in immune and metabolic cells in adipose and liver can therefore affect whole-body energy homeostasis and the inflammatory state (161). In mice studies, it has been found that obesity induces a proinflammatory profile of classically- activated macrophages (M1) in adipose tissue resulting in immune cell recruitment and low-grade inflammation (236).

1.8.1 Complement system; links between metabolism and inflammation

Complement system: The complement system is an ancient danger-sensing system that recognizes exogenous threats such as conserved microbial motifs as well as endogenous threats including altered-self molecules (e.g. following injury or hypoxia, virus-infection or tumor-related) and apoptotic cells (237). However, the complement system has been implicated in a multitude of processes in the course of development, degeneration, and regeneration (reviewed in (238)). Liver and adipose tissue can generate most of the complement factors. The complement cascade can be activated through three distinctive pathways: the classical, alternative and lectin pathways. The alternative pathway acts as an amplification loop for the classical and lectin pathways and can account for more than 80% of the total activation of the complement system (239) (Figure 1.5).

Anaphylatoxines: The cleavage of C5 and C3 has a central role in complement system activation. Breakdown products - C5a and C3a – are part of the anaphylatoxin family, together with C4a, and induce several biological responses (240). The C3a and C5a are anaphylatoxins with highly potent chemoattractant activity. They regulate vasodilation, increase the permeability of small blood vessels, and induce contraction of smooth muscles (241). In addition to their pro-inflammatory properties they regulate tissue regeneration and tissue fibrosis as well as CNS development (241)(238). In spite of the numerous proinflammatory effects, the main role of C3a appears to be in Th2-type inflammatory reactions. The protection of host from complement activation is through expression of complement regulatory proteins and factors (242), whereas carboxypeptidases (CpN serve

to degrade anaphylatoxins into their less immune active, desarginated (desArg) forms (238) (Figure 1.5).

Complement receptors: The anaphylatoxins bind to a family of three receptors, which belong to the superfamily of G-protein-coupled receptors (GPCR); the C3a receptor (C3aR), C5a receptor (C5aR) and C5a receptor-like 2 (C5L2). C5L2 also been postulated as a receptor for C5a, C5adesArg, C3a and C3adesArg. C5L2 may contribute to signaling induced by C5a and C3a through dimerization with other GPCRs such as C5aR or others (241). It has been suggested that C5L2 is a signaling receptor for ASP (C3adesArg) (243), but may also act as a decoy receptor for C5a, although the exact molecular signaling pathway remains controversial.

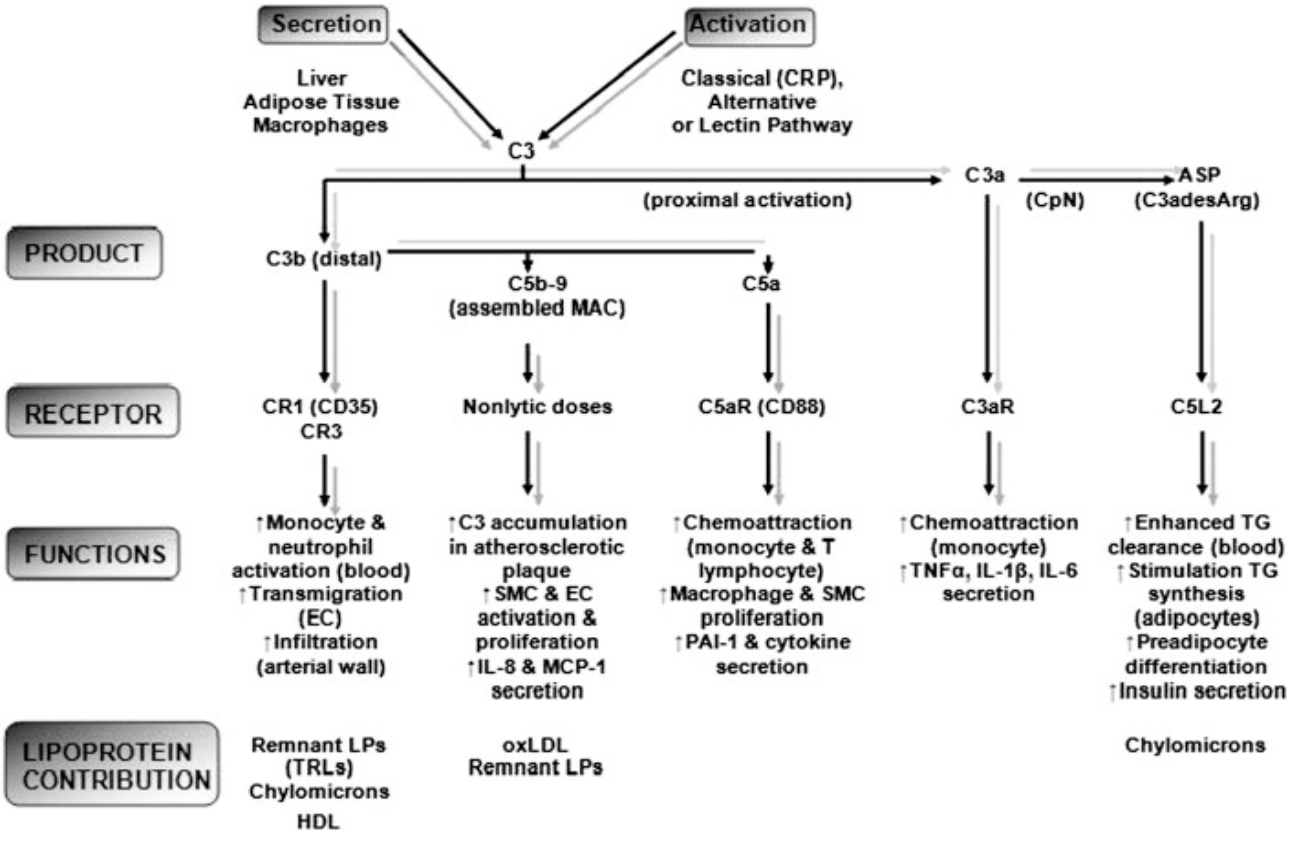


Figure 1.5 Generation of complement products and functions.

Adapted from Altan Onat et al. Complement C3 and cleavage products in cardiometabolic risk Clinica Chimica Acta, 2011, 1171 – 1179

1.8.2 C5L2-C5aR interaction

Both C5L2 and C5aR are expressed on myeloid and non-myeloid cells such as adipocytes and preadipocytes, although C5aR mRNA levels are typically higher than C5L2 (244)(245). The two receptors, C5aR and C5L2, have been proposed to have closely linked physical and functional interactions (244)(246) and this has been implicated in inflammatory conditions such as sepsis (247)(248). Likewise, it has been demonstrated that C5aR and C5L2 are both capable of forming homo- and heterodimers (246). Interestingly, both ASP and C5a have been found to stimulate internalization/colocalization of C5aR and C5L2 in J774 macrophages and 3T3-L1 adipocytes (246). The consequences of homo- or heterodimerization are not yet clear; however, this could be linked to alternative signaling or regulatory cell- and ligand-dependent responses to severe inflammatory conditions or metabolic modulations. Although there are only a few studies using C5aR and/or C5L2 antibodies/antagonists or knockout models, the results are consistent with coupling of immune and metabolic aspects in adipocytes (249)(250). However, the distinctive roles of C5aR and C5L2 in immunity and adipocyte function and signaling are not yet clear.

Roy et al. suggested an immune-metabolic role for C5aR in energy expenditure and fat storage in *in vivo* studies using C5aRKO mice (251), while Lim et al. demonstrated C5a effects on lipid and glucose metabolism in adipocytes by using a C5aR-selective antagonist which interfered with C5a-mediated fatty acid uptake (250). Recently, the well-defined proinflammatory C5a-C5aR pathway has been targeted for pharmacological therapy via inhibition of C5 cleavage, C5a blocking antibodies or C5aR antagonists for treatment of sepsis, cardiovascular diseases, autoimmune disorders, asthma, and psoriasis (251). However, the consequences of interfering with the C5a-C5aR pathway could also have a metabolic impact on C5L2 signaling and this requires clear knowledge and consideration of C5L2 and its ligand- and tissue-specific effects (225).

1.9 Obesity consequences

1.9. 1 Health consequences of obesity and morbidity

The health consequences of obesity range from a number of complications that impact nearly all organ systems and results in an increase risk of deadly complications (Figure 1.6). WHO has estimated that worldwide 2.8 million people worldwide die each year as a result of being overweight or obese, and an estimated 35.8 million (2.3%) of global disability-adjusted life-years are caused by overweight or obesity (252).

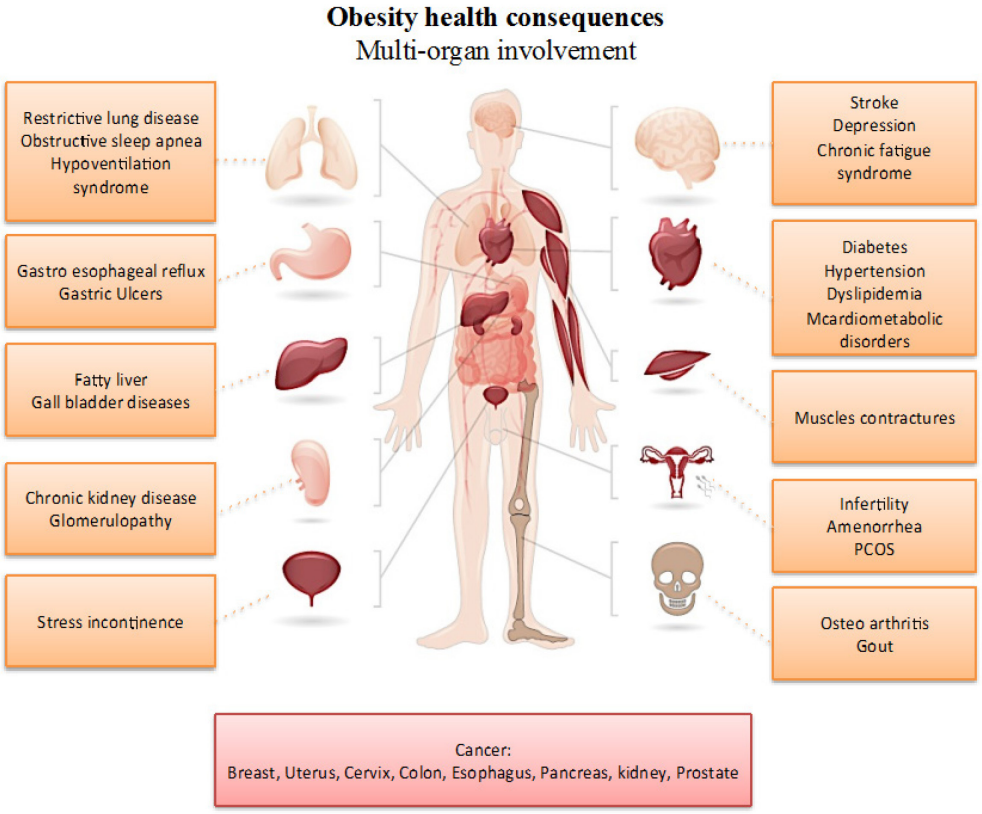


Figure 1.6 Obesity- related complications

Garrow, J.S. "Obesity and related diseases", 1988, ISBN: 0-443-03798-1

In one large, pooled analysis of prospective studies (253), both overweight and obesity (and possibly underweight) were associated with increased all-cause mortality in

analyses restricted to participants who never smoked and did not have diagnosed cancer or heart disease. The lowest all-cause mortality was generally observed in the BMI range of 20.0 to 24.9. Longer follow-up attenuated the associations with lower BMI levels (253). On average, a BMI of 30–35 reduces life expectancy by 2–4 years while severe obesity (BMI>40) reduces life expectancy by 10 years (254).

Obesity is a major risk factor for several common and important diseases (Figure 1.6) T2D, CVD and certain cancers and is thought to contribute to many more (42). Diabetes and CVD are, together with cancer and chronic respiratory disease, the world's biggest killers, causing an estimated 35 million deaths each year (255). However, although obesity is a risk factor for insulin resistance (IR) and T2D, and a significant risk factor for CVD, not every obese patient is insulin resistant or at high risk of T2D and CVD (256). In Canada, obesity is a major contributor to morbidity and mortality. For instance, 61% to 74% of type 2 diabetes cases, 17% to 32% of osteoarthritis cases, 14% to 21% of colorectal cancers, 8% to 14% of depression cases, and 20% of premature deaths that occur in Canadian adults are estimated to be directly attributable to obesity (28).

1.9. 2 Cardiovascular risk factors and obesity

The global cardiovascular risk is the probability of suffering from a coronary event or stroke in a given period of time (257). There are a number of well-defined classical risk factors for CVD, including smoking, high levels of low-density lipoprotein cholesterol (LDL-C), low levels of high density lipoprotein cholesterol (HDL-C), T2D, and hypertension (117)(258). However, a substantial number of high-risk individuals do not manifest these risk factors. For this reason, it is important to identify tools that can be used together to provide a broader assessment of a patient's overall risk. The most widely used instruments for assessing of cardiovascular disease are the Framingham Risk Score, based on a long-term observational study of a population, and validated on divergent populations, and PROCAM (Prospective Cardiovascular Munster Study) calculator (reviewed in (258)). As mentioned, although obesity is a risk factor for insulin resistance and type 2 diabetes, and a significant risk factor for cardiovascular disease, not every obese patient is insulin resistant or at high risk of T2D and cardiovascular disease (256). This explains why obesity

has been an ill-defined modifiable CVD risk factor compared with others such as hypertension, smoking and cholesterol (high LDL)/low HDL) (258).

1.9. 3 Metabolic Syndrome

The metabolic syndrome (MetS) refers to a clustering of cardiovascular risk factors that include insulin resistance, obesity, atherogenic dyslipidemia and hypertention (259). The modern concept of MetS began when Reaven proposed that insulin resistance provided a common mechanism underlying the associated abnormalities of blood pressure, HDL-C, triacylglycerol and glucose tolerance (255). Reaven did not include abdominal obesity, which has also been hypothesized to be an underlying factor as part of the condition (260). This pathophysiological concept was not desined for clinical or epidemiological use (255).

There are currently several separate definitions to identify individuals with the metabolic syndrome (Table 1.2). The initial WHO and EGIR classifications required the measurement of insulin resistance and are primarily used in the research environment (261). ATP III classified components of MetS that related to cardiovascular disease. According to ATP III, underlying risk factors for CVD are obesity (especially abdominal obesity), physical inactivity, and atherogenic diet. The major risk factors are cigarette smoking, hypertension, elevated LDL-C, low HDL-C, family history of premature CHD, and aging, and emerging risk factors included elevated TG, small LDL particles, insulin resistance, glucose intolerance, proinflammatory state, and prothrombotic state (262).

Definitions of MetS that were published by the AHA/NHLBI and the IDF enabled clinicians to identify patients at risk for this disorder; but the criteria of these definitions differed slightly (263) (Table 1.2). The five screening variables used to identify those with MetS are waist circumference, circulating levels of triglycerides and of HDL-C, fasting glycemia and blood pressure. In October 2009, these two panels (AHA/NHLBI & IDF) met again and generated in a combined definition of MetS (263). They included the waist circumference cutoff points that are specific to country and population, considering the ethnic heterogeneity of patients.

NO	CRITERIA FOR METABOLIC SYNDROME	OBESITY		DYSLIPIDEMIA		BLOOD PRESSURE	GLUCOSE	INSULIN RESISTANCE (IR)	OTHER
		MALE	FEMALE	MALE	FEMALE				
1	WHO (5th or 6th+ ≥2 criteria) 1999	WHR>0.90 and/ or BMI >30 kg/m2	WHR>0.85 and/or BMI >30 kg/m2	TG ≥150 mg/dL (≥1.7 mM); HDL-C < 35mg/dL (0.9mM)	TG≥150 mg/dL (≥1.7 mM); HDL-C <39 mg/dL (<1mM)	≥140/90 mmHg	T2D, impaired glucose tolerance, impaired fasting glucose	IR measured under hyperinsulinemi ceau glycaemic conditions	urinary albumin excretion rate ≥ 20 ug/min or albumin:creatine ≥30mg/g
2	EGIR	WC>94 cm	WC>80 cm	TG≥177 mg/dL(≥2 mM); HDL-C < 39mg/dL (<1mM)		≥140/90 mmHg or on medication	fasting glucose >110 mg/dL (≥6.1mM)	IR	–
3	NCEP-ATP III (≥3criteria)	Abdominal obesity WC ≥ 88 cm	Abdominal obesity WC ≥ 102 cm	TG≥150 mg/dL(≥1.7 mM); HDL-C <40mg/dL or on therapy	TG≥150 mg/dL; HDL-C <50mg/dL or on therapy	≥130/85 mmHg or on therapy	fasting glucose >110 mg/ dL(≥6.1mM)	–	–
4	AHA/NHLBI or updated NCEP criteria 2005	WC>88 cm (Asian≥80)	WC >102 cm (Asian ≥ 90)	TG≥150 mg/dL (≥1.7 mM); HDL-C <40mg/dL (1.0 mM) or on therapy	TG≥150 mg/dL (≥1.7 mM); HDL-C <50mg/dL or on therapy	≥130/85 mmHg or on therapy	fasting glucose >110 mg/ dL(≥6.1mM)	–	–
5	IDF (1st+ ≥2 other criteria) 2005	Ethnicity-specific WC (≥90 cm for men and ≥80 for women)		TG≥150 mg/dL(≥1.7 mM); HDL-C <40mg/dL(1.03) or on therapy	TG≥150 mg/dL (≥1.7 mM); HDL-C < 50mg/dL(1.03) or on therapy	≥130/85 mmHg or on thrapy	fasting glucose >110 mg/dL(≥5.6 mM)	–	–

Table 1.2 Metabolic syndrome diagnostic criteria

Adapted from Gupta A, Gupta V. Metabolic syndrome: what are the risks for humans? BioScience Trends. 2010; 4(5): 204-212.

1.9. 3.1 Prevalence of metabolic syndrome and cardiovascular diseases

The prevalence of metabolic syndrome has increased dramatically in recent years. The increasing prevalence of metabolic syndrome contributes to an increase in the prevalence of CVD and diabetes. Various studies based on the results of the National Health and Nutrition Examination Survey (NHANES) indicate that over a 10- to 15-year period, the prevalence of obesity, MetS and diabetes has increased by 35%, 48%, and 19%, respectively (reviewed in (264)). Prevalence of MetS will clearly vary depending on the definition applied, the ethnicity and age of the study population (261). The prevalence of MetS is approximately 34.6% in the United States, 17.8–34.0% in Europe and 12.8–41.1% in Asia (265). The prevalence increases linearly from the age of 20 until age 50, when it reaches a plateau (260). Hispanics and African-Americans have the greatest risk for developing MetS, followed by Caucasians. Asians have the lowest risk, at least in the United States (260). One report suggested that MetS could be responsible for approx 7% of total mortality, regardless of the cause, and up to 17% of CVD (266). Similarly, a report from the Framingham Heart Offspring Study showed that the contribution of MetS to the risk of CVD was 34% in men and 16% in women (267). In that analysis, the components of the syndrome that contributed most to the CVD outcomes were high blood pressure (33%) and low HDL-cholesterol (25%). A meta-analysis of 37 longitudinal studies found a 78% increased risk for CVD events and death in people with MetS (268).

1.9. 3.2 Pathogenesis of metabolic syndrome

There is no accepted central underlying mechanism (255), but based on current definitions of metabolic syndrome, it may be divided into four central features:

1- Insulin resistance: In insulin resistance, three predominant tissues that use glucose; adipose, muscle and liver, do not respond appropriately to insulin, and circulating glucose levels remain high, which leads to pathological states. Insulin increases local blood flow in tissues through the activation of endothelial nitric oxide (NO), leading to vasodilation effects in capillary (few minutes) and in larger-resistance vessels (by 2 hours) (reviewed in (259)). Both of these effects contribute to increased delivery of glucose and

insulin into tissues. The vascular effects of insulin couple glucose homeostasis with blood flow and contribute to glucose metabolism at physiological concentrations of insulin (269). Thus, insulin signaling coordinately affects peripheral glucose use, vascular tone and blood flow. In insulin resistance, inhibition of PI3K pathway, which mediates the metabolic effects of insulin (glucose, lipid and protein metabolism), leads to a reduction in endothelial nitric oxide production, resulting in endothelial dysfunction (259). This abnormality, in addition to a reduction in GLUT4 translocation, leads to decreased skeletal muscle glucose uptake (270). Hence, the mechanisms that contribute to insulin resistance can also affect vascular function (reviewed in (259)).

2- Visceral adiposity: Visceral fat in comparison with the subcutaneous tissue, represent a metabolically active organ, strongly related to insulin resistance (271). Adipocytes from visceral fat have a very different histology and biology from subcutaneous fat (100). Subcutaneous fat tissue is characterized by small, insulin-sensitive adipocytes, without vascular stroma and cellular infiltration. Fat taken from visceral compartments and composed of large, insulin resistance adipocytes, has a well-developed vasculature with infiltration of inflammatory cell (272).

In a review article by J.P. Déspres et al. (117), 3 scenarios have been proposed to explain the relation of visceral adiposity to the metabolic syndrome (Figure 1.7): (1) The "portal/visceral hypothesis" proposes that the hyperlipolytic state of the omental adipose tissue contributes to expose the liver high concentrations of free fatty acids into portal vein of obese subjects leading to hyperinsulinemia, hyperglycemia (increased hepatic glucose production), and hypertriglyceridemia (increased VLDL-apolipoprotein B secretion). (2) The “endocrine paradigm hypothesis” postulates that adipose tissue, as an endocrine organ, is a source of adipokines and inflammatory cytokines which contribute to the metabolic consequences of the presence of visceral obesity. (3) Ectopic adipose tissue depots, which surround organs and blood vessels, are a result of the relative inability of subcutaneous adipose tissue to act as a protective metabolic sink because of its inability to expand (lipodystrophy) or because it has become hypertrophied, dysfunctional and insulin resistant.

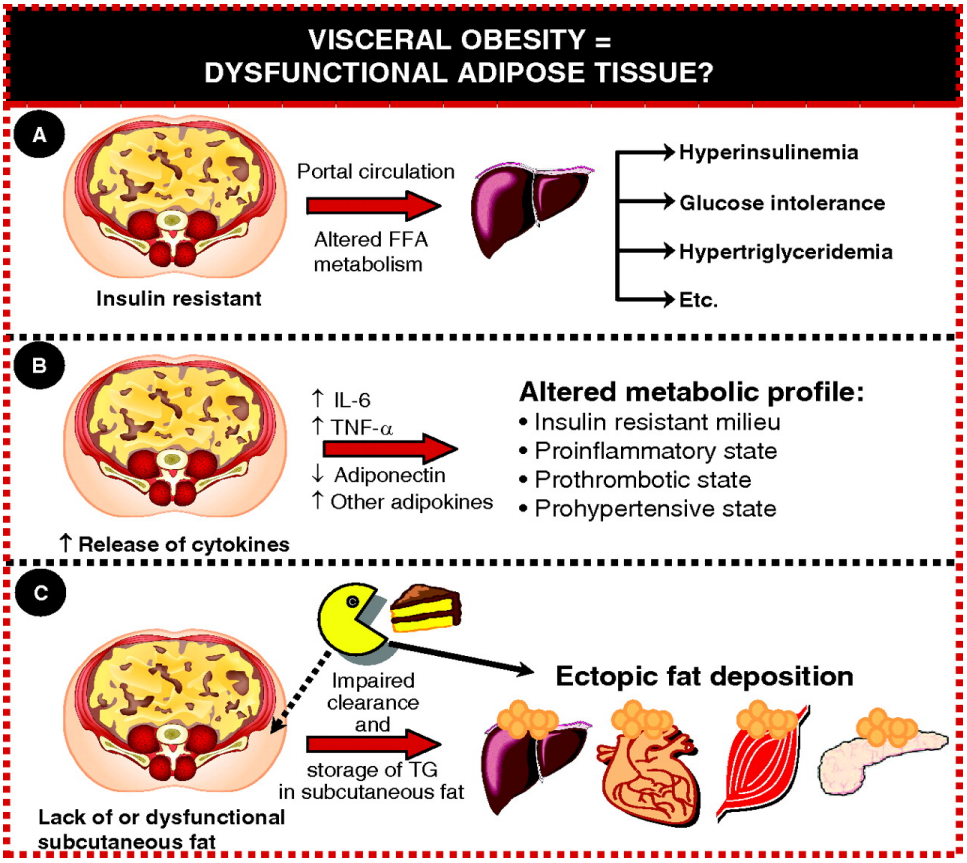


Figure 1.7 Proposed mechanisms by which visceral obesity could be linked to the atherothrombotic-inflammatory abnormalities of insulin resistance

Adapted from Després J et al. Arterioscler Thromb Vasc Biol 2008; 28:1039-1049

3- Atherogenic dyslipidemia: Insulin resistance and visceral obesity are associated with atherogenic dyslipidemia. The key features of atherogenic dyslipidemia are high plasma triglyceride levels, low HDL cholesterol levels and increases in small, dense LDL (272) . Insulin resistance leads to atherogenic dyslipidemia in three proposed mechanisms (reviewed in (269)): First, insulin normally suppresses lipolysis in adipocytes; so impaired insulin signaling increases lipolysis, resulting in increased FFA levels. In the liver, FFAs serve as a substrate for the synthesis of triglycerides. FFAs also stabilize the production of apoB, the major lipoprotein of VLDL particles, resulting in more VLDL production. Second, insulin normally degrades hepatic apoB through PI3K-dependent pathways, so insulin resistance directly increases VLDL production. Third, insulin regulates the activity of lipoprotein lipase, the rate-limiting and major mediator of VLDL clearance.

4- Endothelial dysfunction: All the components of MetS can individually impair endothelial function (265). Endothelial dysfunction is the final common pathway between many cardiovascular risk factors and the development of atherosclerosis (259). Several mechanisms are implicated in the pathogenesis of endothelial dysfunction. Decreased NO availability appears to play a major role and may result from reduced NO production and/or increased inactivation by reactive oxygen species (273). In addition, reduced availability of other vasodilating agents (including prostacyclin and endothelium-derived hyperpolarizing factors) and/or increased production or activity of vasoconstrictive substances (including endothelin-1 and angiotensin II) are also implicated (265).

1.9. 3.3 Diagnosis of metabolic syndrome: waist circumference challenge

Regarding the association between visceral adiposity and the presence of the features of metabolic syndrome, the measurement of waist circumference (WC) has been proposed as a crude anthropometric correlate of abdominal and visceral adiposity (271). In IDEA study (International Day for the Evaluation of Abdominal Obesity), which evaluated 168,000 patients on five continents, it was clearly found that, independent of BMI, waist circumference was consistently associated with the prevalence of T2D (274). In addition, at any given BMI value, patients with diabetes had a higher WC, showing the clear discriminating value of the WC (275). Imaging studies have revealed that this association between risk of diabetes and enlarged waist circumference is attributable mainly to visceral adipose tissue accumulation, which has been shown to be a major risk factor for the development of T2D (276). The NCEP-ATP III made an index of abdominal adiposity (assessed by WC) and not obesity (assessed by BMI) as 1 of 5 criteria on which clinicians could diagnose the syndrome. However, the relationship of WC to abdominal adiposity, especially visceral or intra-abdominal obesity, is age- and gender- as well as ethnicity-dependent (100).

Some studies have indicated that African-American women have lower amounts of VAT for a given waist circumference, BMI, or WHR compared to white women (277). Thus, a given anthropometric measurement may represent different amounts of VAT and/or SAT in different racial/ethnic groups. Furthermore, if VAT is the underlying culprit

contributing to metabolic risk, current BMI and waist circumference cutoff points to define risk may overestimate or underestimate risk in different racial/ethnic groups (278). Regarding ethnicity, the IDF has recognized this problem and proposed to lower the WC cut-offs (which is a mandatory criterion in IDF evaluation of metabolic syndrome) for some ethnic groups (117). However, although WC is a fairly good correlate of amount of total abdominal fat, it cannot distinguish visceral adiposity from amount of subcutaneous abdominal fat (117).

As a simple initial screening approach distinguishing visceral obesity from subcutaneously obese patients, Després and his colleagues have previously proposed that the simultaneous presence of fasting hypertriglyceridemia and an increased WC (hypertriglyceridemia waist) could represent a simple clinical phenotype to identify patients with an excess of visceral adipose tissue (117). Recently, some studies have indicated that neck circumference may also be an independent correlate of metabolic risk factors above and beyond BMI and waist circumference (279).

1.9. 4 Fatty liver

Alcoholic and nonalcoholic fatty liver diseases represent the two common metabolic disorders and important causes of liver disease. They both consist of a spectrum ranging from simple hepatic steatosis, to steatohepatitis, liver fibrosis and cirrhosis (280)(281). With the availability of magnetic resonance spectroscopy, very strong associations have been reported between liver fat content and features of the cardiometabolic risk profile predicting risk of type 2 diabetes and CVD (282)(283).

Considering that liver is a central organ for the control of carbohydrate and lipid metabolism (284) and a major site of insulin uptake and degradation as well as some study results (282)(285), it has been suggested that the associations between visceral adiposity and metabolic complications could be entirely explained by the concomitant increases in liver fat content, although reported correlations between coronary atherosclerotic calcification and visceral adipose tissue are stronger than with liver fat (286).

It has been suggested that a high liver fat content, associated with abdominal obesity, may result in a reduced hepatic extraction of insulin, leading to increased intrahepatic insulin exposure (286). In addition, hepatic glucose output is increased among subjects with a high liver fat content (287). This phenomenon contributes to insulin resistance and can explain the hyperglycemic state of type 2 diabetes.

The fatty liver releases more triglyceride-rich lipoproteins through an overproduction of large VLDL1 particles (103). Increased lipid availability also protects apolipoprotein B against its local degradation in the hepatocyte, explaining the elevated plasma apolipoprotein B concentrations observed among individuals with a high liver fat content (288). Thus, a high liver fat content can, by itself, largely explain the hyperinsulinemic, hyperglycemic, hypertriglyceridemic, and elevated apolipoprotein B dysmetabolic state of visceral obesity without involving a specific contribution of visceral adipose tissue (288). Steatosis and steatohepatitis could be derivable to the combined effects of insulin resistance and relative failure of adipokine mediators. Visceral fat has been postulated to play an important role via the release of free fatty acids and adipokines directly into the portal circulation. Indeed, the surgical removal of visceral, but not subcutaneous abdominal fat, improves peripheral and hepatic insulin sensitivity in some studies (289)(290).

Studies suggest that the contribution of FFAs derived from visceral fat lipolysis to hepatic triglyceride content is relatively small. Only 5%–20% of the fatty acids entering the portal circulation originate from visceral adipose tissue (291), the remainder is derived from upper and lower body subcutaneous fat. Furthermore, only ~60 % (277) of triglycerides within steatotic livers originate from adipose tissue–derived fatty acids (i.e., lipolysis) (292). However, the metabolic connection of visceral fat and hepatic fat is unlikely to be mediated via fatty acid delivery and uptake alone.

1.9. 4.1 Fatty liver and the complement system; confluence of metabolic and inflammation

Inflammatory response to steatosis and fat accumulation in hepatocytes can be linked via complement components. In alcoholic fatty liver disease, excessive ethanol consumption results in disturbances in lipid metabolism including increased lipogenesis,

reduced lipolysis, reduced AMP-activated protein kinase (AMPK) activity, production of reactive oxygen species (ROS) and pro-inflammatory cytokines, and activation of natural killer cells (reviewed in (293)).

In this context, C3-deficient mice have been found to be protected from alcohol-induced steatosis and from microvesicular and macrovesicular hepatic triglyceride accumulation (294)(295). In addition, C3-deficient mice on an ethanol diet have a decreased expression of lipogenic enzymes, elevated serum and liver adiponectin levels, and a reduced ethanol-mediated induction of serum alanine aminotransferase (ALT) activity (294)(295)(296). Whereas, mice deficient in C5 are not protected from steatosis but instead display decreased serum ALT and hepatic inflammation (296). Thus, C3 and C5 may contribute through different mechanisms to the pathogenesis of alcohol-induced liver disease. A role for the classical complement pathway in alcoholic liver disease has also been demonstrated (297).

Patients with NAFLD showed increased C3 deposition and plasma C3 and ASP levels that correlate with insulin resistance (298)(299). The accumulation of C3 in biopsies of NAFLD patients is associated with higher hepatic lectin and classic complement pathway, as well as higher hepatocyte apoptosis, neutrophil infiltration, and IL-8 and IL-6 expression. All these factors are positively correlated with the degree of steatosis (300). Furthermore, patients with progressive Nonalcoholic Steatohepatitis (NASH) have elevated expression of hepatic C3 (301). However, because liver function is decreased in cirrhosis, the serum concentrations of C3 and C4 are reduced in severe cirrhosis (302). Some, but not all (295)(303) rodent studies have reported that mice given a high-fat diet showed enhanced hepatic expression of Factor D/adipsin, a key component of the alternative pathway, suggesting a possible role for this complement component in the development of NAFLD (302). Interestingly, liver highly expresses C5L2 (243), which is associated *in vitro* (243) and *in viv*o (230) with triglyceride synthesis. C3−/− mice and C5L2-deficient mice on a high-fat diet are prone to develop enhanced hepatic steatosis as a result of increased hepatic triglyceride content, increased lipogenesis-related gene expression, hepatic glucose uptake, and reduced fatty acid oxidation, as determined by hydroxyacyl-

Coenzyme A dehydrogenase activity (230)(294)(296). Together, these findings, suggest a protective role for C3 and C5L2 in the development of hepatic steatosis.

The role of complement factor C3 in partial hepatectomy-induced liver regeneration has been investigated. Hepatectomy is associated with transient fat accumulation in the liver as a result of the induction of lipogenic enzymes (304). However, C3-deficient mice develop enhanced steatosis after partial hepatectomy. This effect is likely a result of the absence of ASP, as shown by ASP reconstitution experiments (305). Interestingly, complement is also linked to the regulation of the subsequent proliferative response. Mice deficient in either C3 or C5 show increased lethality and decreased regenerative potential after partial hepatectomy (306)(307)(308). Double deficiency in C3 and C5 results in an aggravated phenotype, which can be reversed by concomitant administration of C3a and C5a (306)(307).

Finally, a role for C5L2 and ASP in liver regeneration has also been suggested, since administration of ASP in C3−/− mice restores adequate liver regeneration (305). Given the detrimental actions of complement, and especially C3, as pro-inflammatory molecules in hepatic ischemia-reperfusion injury (309), a delicate balance must exist between complement-mediated injury and regeneration (305). In particular, C3 deficiency or C3 inhibition protects mice from hepatic ischemia-reperfusion injury, even in obesity, which normally exacerbates ischemia/reperfusion injury (303). However, when ischemia–reperfusion injury is combined with partial hepatectomy, C3 deficiency results in more severe hepatic injury (305).

1.10 Metabolically Healthy Obese, a controversial issue?

Since 1980, a range of metabolically healthy and unhealthy obese and non-obese phenotypes has been recognized (310). The obese phenotype may exist in the absence of metabolic abnormalities such as dyslipidaemia, insulin resistance, hypertension and an unfavorable inflammatory profile. These individuals are referred to as "metabolically healthy obese (MHO)". Similarly, not all non-obese individuals present a healthy metabolic profile.

1.10.1 Defining "metabolically healthy obesity"

Several definitions are currently used to describe metabolic health, resulting in a wide variation in prevalence estimates from 6–40 % of the obese population (reviewed in (311)). There are several limitations to defining "metabolically healthy obesity": Firstly, there is no standard metabolic health definition, different inclusion criteria have been used to distinguish metabolically healthy from unhealthy subjects thus making comparisons between studies difficult. For example, not all metabolic health definitions include insulin resistance (Aguilar-Salinas (312), Meigs (313)), blood pressure or fasting plasma glucose concentrations (Karelis (314)), while others consider inflammatory markers (Wildman (315)).

Secondly, the limitations in different methods to define obesity, in addition to BMI, ethnic and sex specific waist circumference cut-points and body fat percentage (BF%), derive from a range of body composition methods, and are also used to define abdominal and overall obesity, respectively (311). While waist circumference correlates with visceral adipose tissue, it does not take into account depot-specific body fat. On the other hand, BMI cannot recognize differences between lean and fat body mass, therefore persons of short stature or muscular build may be misclassified. The comparison of the association between BMI, WC and BF% with cardiovascular risk factors has shown that waist circumference and BF% were more strongly associated with MetS and CVD risk, respectively (316). In addition, data from a large cross-sectional study suggests that using BMI may underestimate obesity prevalence (317). Furthermore, recent examination of

markers of glucose metabolism according to obesity classification revealed that BF% might be a better determinant for pre-diabetes and T2D development (318).

1.10.2 Mortality and the metabolically healthy obese

A recent large systematic review and meta-analysis of 2.88 million individuals confirmed significantly higher all-cause mortality with obesity when all grades are combined (319). Interestingly, however, individuals obese with BMI 30 to <35 kg/ (grade 1) were not associated with higher mortality. These conflicting findings may be, at least in part, accounted for by different obesity-associated metabolic health phenotypes.

Conflicting results have been reported from a few prospective studies tracking the development of cardiometabolic disease and mortality in MHO (reviewed in (311)). These finding have reported that, for most metabolic health definitions, obese subjects, whether metabolically healthy or not, carry an elevated risk of CVD and mortality (reviewed in (311)). These findings suggest that the MHO phenotype is not as benign and apparently healthy as initially considered (311). In contrast a number of studies have reported MHO individuals not to be at increased risk of CVD and all-cause mortality (320).

It has been suggested that the MHO phenotype starts in childhood and persists into adulthood (321). Whether the metabolic health status of obese individuals transitions between healthy and unhealthy states over time is unclear, but it may account for the observed decreasing MHO prevalence with age (321). However it is possible that transition between MHO and metabolically unhealthy obese over the follow-up period, as well as a lack of a unique MHO definition and different follow-up times, may account for conflicting findings regards CVD and mortality outcomes (311). Previous prospective studies have considered the MHO phenotype as a static condition. A recent longitudinal study found that persistent MHO status was associated with favorable cardiometabolic outcomes, but that MHO status was transient for one third of subjects (322) suggesting that metabolically unhealthy obese is a progressive phenotype along which MHO represents a dynamic intermediate stage.

Future studies examining MHO would benefit from including longitudinal follow-up to allow differences in disease incidences, examination of transition between MHO and metabolically unhealthy obese, and all-cause mortality risk according to different MHO criteria to be ascertained (311).

1.10.3 Determinants of metabolically healthy obese

Diet and life style factors: Although it is clear that both diet composition and lifestyle factors such as physical activity are significant contributors to the obesity epidemic, evidence supporting the role of diet and physical activity in MHO has been inconsistent to date (323), which may reflect the range of metabolic health criteria used to define MHO as well as limited data available.. Despite some conflicting findings, which may have arisen due to inconsistencies in how metabolic health was defined, collectively, these data suggest that the beneficial effects of physical activity on cardiometabolic risk factors are evident even among obese subjects (311).

Inflammatory markers: Some studies have shown more favorable inflammatory status among MHO subjects (reviewed in (311)). A small study of obese subjects undergoing bariatric surgery recently demonstrated that adipose tissue in MHO individuals is characterized by smaller fat cells, reduced macrophage infiltration and a more favorable inflammatory profile (324). In contrast Wildman et al., reported that despite not finding increased 10-year risk of CVD among metabolically healthy overweight/obese women these subjects still displayed abnormal levels of inflammatory markers (325). These conflicting findings may be accounted for by differences in ethnicity and age-group, small subject numbers in some studies and limited inflammatory profiling together with different metabolic health criteria being used to define MHO (reviewed in (326)). Indeed a recent comparative study investigating inflammatory markers according to different definitions of MHO concluded that the associations between pro-inflammatory cytokines and MHO are definition dependent (327).

Adipocytokines: Previously, one study examined a range of adipocytokines and did not report any differences between obese subjects with or without the MetS (328). On the other hand, higher adiponectin levels have been reported among MHO subjects (312)(329).

Data from mice studies suggest that increased adiponectin levels promote metabolic flexibility of adipose tissue (330). Thus the raised levels observed in MHO may partly account for their ability to maintain proper metabolic function under metabolically challenging circumstances (311). Another recent study has reported that metabolically healthy obese and non-obese subjects presented with lower complement component C3 concentrations and, depending on metabolic health definition, also lower levels of CRP, IL-6, TNF-α, plasminogen activator inhibitor-1 (PAI-1) and WBCs and higher adiponectin concentrations (331).

These data suggest that significant differences in inflammatory profiles between MHO and metabolically unhealthy obese individuals (311)(326). Therefore, reduced inflammatory status increases the likelihood of metabolic health, particularly among obese subjects (320). These findings support the hypothesis that MHO adipose tissue has enhanced adipogenic capacity resulting in increased lipid storage and reduced metabolic dysfunction such as insulin resistance and lipotoxicity (311).

1.11 Obesity treatment

Obesity must be considered as a chronic disease associated with multiple comorbidities and, thus, any treatment approach must also be multifaceted and reflect the complex etiology of obesity. The two main non-surgical approaches for the treatment of obesity and related complications are lifestyle interventions and pharmacotherapy.

The guidelines published by the National Heart, Lung and Blood Institute in 1998, suggest "weight reduction of 5%–10% from baseline is associated with improvements in cardiometabolic risk", with consequent reductions in obesity-related morbidity and mortality rates (332) ((333)Pi-Sunyer, Becker DM, Bouchard, et al 1998). Weight should be lost at a rate of 1 to 2 pounds per week based on a calorie deficit of 500–1000 kcal/day" (333). The two main non-surgical approaches for the treatment of obesity and related complications are lifestyle interventions with adjunctive pharmacotherapy. Although lifestyle modification remains the cornerstone of obesity interventions, its effectiveness is frequently limited by significant weight regain in the long-term. Bariatric surgery has become a standard of care in managing patients with a BMI > 40 or BMI > 35 with serious obesity-related co-morbidities (Table 1.3).

Table 1.3 Bariatric surgery indications

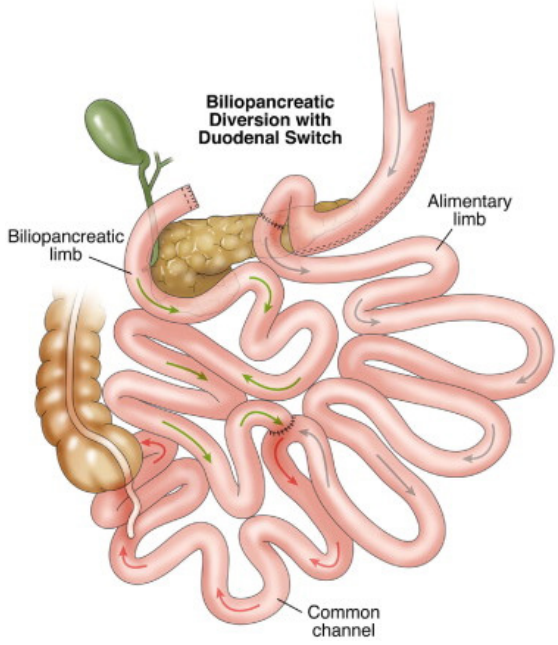
INDICATIONS OF BARIATRIC SURGERY
BMI ≥ 40
BMI ≥35 with obesity-related comorbid conditions, such as • **Hypertension** • **Impaired glucose tolerance** • **Diabetes mellitus** • **Hyperlipidemia** • **Obstructive sleep apnea**
Documented failure of medical management (diet + exercise; medication)
Psychological ability to undergo surgery
Absence of other chronic disease

Adapted from Abeles, Deborah, Shikora, Scott A 2004

Available procedures include laparoscopic and open Roux-en-Y gastric bypass (RYGB), sleeve gastrectomy, adjustable gastric band, vertical banded gastroplasty, duodenal switch and biliopancreatic diversion (334). RYGB is currently the most widely performed bariatric surgical procedure. In a meta-analysis of 136 studies including 22,094 patients, RYGB resulted in an average excess body weight loss of 62%, with resolution of diabetes in 84%, of hypertension in 68%, of obstructive sleep apnea in 81%, and improved hyperlipidaemia in 97% (335).

Biliopancreatic diversion (BPD) is one of the most effective operations. This technic has gained wide acceptance, especially in European and Canadian obesity clinics owing to an approximately 70% long term excess weight loss, ameliorating type 2 diabetes, and correcting many features of the metabolic syndrome (336). This operation is both restrictive and malabsorptive. It involves a partial gastrectomy, with the gastric remnant anastomosed to the distal ileum (Figure1.8).

Figure 1.8 Biliopancreatic diversion with Duodenal Switch



Elder KA, Wolfe BM. Gastroenterology 2007; 132:2253-2271

The proximal ileum is also anastomosed to the terminal ileum, creating a common channel approximately 50 to 100 cm from the ileocecal valve (337)(334). This approach leads to diversion of bile and pancreatic secretions to the distal small bowel, thereby creating a mild form of malabsorption and major changes in the secretion of gastrointestinal

peptides (338)(337). Biliopancreatic diversion (BPD) is one of the most effective operations: it significantly reduces the morbidity and mortality associated with severe obesity by inducing significant, long-term weight loss, ameliorating type 2 diabetes, and correcting many features of the metabolic syndrome (336). The diverting bile, and pancreatic secretions to the distal small bowel resulting in decreased fat absorption (339)(340) and major changes in the secretion of gastrointestinal peptides (341)(338)(342). Superior outcomes of diversionary procedures such as gastric bypass (343)(344) and BPD (343)(344) are related to altered hormonal signals associated with obesity and food intake.

Unlike non-surgery intervention, the effects of bariatric surgery seem to be sustained in the long term. The recently updated Swedish Obese Subjects Study demonstrated mean changes in body weight after bariatric surgery −23% (at 2 years), −17% (at 10 years), −16% (at 15 years) and −18% (at 20 years) (345). Despite proven efficacy and the fact that mortality from bariatric surgery is comparable to that of cholecystectomy or appendectomy in bariatric centers with high surgical volumes, it is estimated that less than 1% of obese subjects who qualify for bariatric surgery will undergo such intervention (346). High costs and the complications of bariatric surgery are the main hurdles for widespread use. Early complications include anastomotic leaks, internal hernias, thromboembolic events, bowel obstruction, GI hemorrhage and wound complications. Late complications include gallstone formation, marginal ulceration, anastomotic stricture, incisional hernia, gastro-gastric fistula, dumping syndrome, micronutrient deficiencies and weight regain.

Current obesity treatment guidelines do not distinguish between MHO and metabolically unhealthy obese subjects, and recommend weight loss for all obese individuals, starting with lifestyle intervention (333). There is no suggestion that MHO individuals should not be treated, but stratification of obese individuals based on their metabolic health phenotype may be important in the early identification of those who should be prioritized for pharmacological and lifestyle intervention and in determining the most appropriate therapeutic strategy (311).

1.11.1 Do we need a gender-specific approach for obesity?

In spide of lacking strong evidence to support major gender differences in weight loss response to dietary restriction, some studies do suggest that females lose less weight with a comparable degree of energy restriction than do males even after matching for initial body weight (347). Women have been shown to lose less weight than men after bariatric surgery (348). As mentioned before, physical exercise as an approach to weight loss may be less effective in women than in men (86). Furthermore, studies consistently find that men lose more visceral fat during weight loss than do women (349), an effect also seen in mice subjected to caloric restriction or lipectomy (350). Postmenopausal women lose less visceral fat during weight reduction than premenopausal women (351), a finding likely related to the important role of estrogen in regulating abdominal fat stores in women as discussed above.

With regard to long-term regulation of body weight and energy balance, some studies have suggested that consuming more frequent, smaller meals is associated with lower body weight (352). It appears, however, that the effect of meal frequency on the regulation of energy balance differs between men and women; while the relationship between increased meal frequency and reduced appetite/body weight is strong in men, it is absent in women (353)(354). This difference has been explained by sex differences in fat-free mass, such that meal frequency is a function of energy expenditure only in those individuals with high fat-free mass (i.e. men) (354). These data raise this question that if consuming more frequent small meals throughout the day is more effective for weight regulation in men than women. However, a recent weight loss intervention has shown no difference in weight loss, reduction in waist circumference, and fat or lean mass when the same amount of energy is consumed as frequent meals or less frequent meals (85). Further research is needed, however, in order to determine whether this finding from epidemiological observations can be translated into clinical interventions.

Sex differences have also been reported in the reduction of health risk factors with weight loss. In the Diabetes Prevention Program, a large randomized trial of lifestyle vs. metformin in adults at high risk for developing diabetes, weight loss of >3% of body

weight produced a greater reduction in serum glucose, insulin and lipids in men than in women (355). In the Stanislas Family Study conducted in France (356), weight gain over 5 years was related to cardiometabolic risk factors in a sex-specific way. While weight gain worsened blood pressure, serum lipids and uric acid in both men and women, apolipoprotein A1 and several liver enzymes were worsened only in men, and serum high sensitivity C-reactive protein and haptoglobin (inflammatory markers) were worsened only in women (356).

1.12 Objectives and hypotheses

The global epidemic of obesity is now recognized as one of the most important public health problems facing the world today. It has become increasingly evident that white adipose tissue-derived adipokines mediate the link between obesity-related exogenous factors (diet, lifestyle) and various biologic events (such as pre and post menopausal status) that lead to obesity consequences (cardiometabolic disorders). Here, in this thesis, I present data on recently-described adipokines, with particular attention to the roles of ASP in such conditions.

In the first study, I investigated effects of diet, sugar-sweetened beverages, on circulating Acylation Stimulating Protein, Leptin & Adiponectin and their associations with metabolic parameters. I hypothesized that long-term sugar consumption leads to changes in circulating levels of the adipocyte hormones ASP, adiponectin, and leptin, both in the fasting and postprandial states.

In the second study, I evaluated fasting serum ASP in a relatively high-risk Turkish adult population in relationship with cardio-metabolic risk factors. I hypothesized that cardiometabolic risk factors in a high-risk population may be associated with high ASP levels.

In the third study, I investigated the association of circulating ASP and C3 related receptors in adipose depots with plasma levels of ovarian hormones and adiponectin as well as metabolic syndrome parameters among women (pre-menopausal and post-menopausal). I hypothesized that both circulating ASP levels and gene expression of related proteins in subcutaneous and omental adipose tissue would be influenced by sex hormones in women.

In the fourth study, I investigated ovarian sex hormones, adipokines and metabolic factors and their association with gene expression (mRNA) of ASP-related proteins in liver tissue of pre and postmenopausal severely obese women. I hypothesized that hepatic gene expression of immune complement C3-related receptors (C3aR, C5aR and C5L2) would be associated with pre/post-menopausal status and metabolic profile in severely obese.

Chapter 2

Effects of Sugar-sweetened Beverages on circulating Acylation Stimulating Protein, Leptin & Adiponectin: Associations with Metabolic Parameters

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2.1 Résumé

Des sujets consommant des breuvages édulcorés au fructose ou au glucose pour une période de 10 semaines ont démontré des gains de poids et une masse grasse comparables. Les sujets ayant reçu du fructose ont développé un volume de gras viscéral plus grand, une dérégulation lipidique et une baisse de sensibilité à l'insuline vs les sujets recevant du glucose. L'objectif de cette étude était d'évaluer l'effet de la consommation de glucose et de fructose sur la production d'adipokines et les relations avec le métabolisme et la sensibilité à l'insuline. Des sujets obèses ont reçu 25% de leur apport calorique journalier à partir de boissons sucrées au glucose ou au fructose pour 8 semaines suivi de 2 semaines de retour à une diète balancée.

Les concentrations postprandiales d'ASP étaient significativement élevées aux semaines 2 et 8 et ce changement corrélait avec leurs niveaux de triglycérides sanguins. L'adiponectine était significativement réduite à la semaines 10 dans les deux groupes et corrélait de façon inverse avec le volume de tissu adipeux intra-abdominal. Les profils de leptine étaient plus élevés avec le groupe obtenant du glucose et ceux-ci corrélaient avec les changements des niveaux d'insuline sur 24 heures. Ces changements des niveaux d'adipokines pourraient contribuer à la dysfonction métabolique induite par la consommation de fructose.

2.2 Abstract

Background: Subjects consuming fructose- or glucose-sweetened beverages for 10 weeks gained comparable amounts of body weight and fat mass. However, fructose consumption increased intra-abdominal fat volume, promoted lipid dysregulation, and decreased insulin sensitivity, but glucose consumption did not. Objective: To evaluate fructose and glucose consumption effects on plasma ASP, adiponectin, and leptin concentrations in these subjects, and to determine the relationship with changes in energy intake, body weight, adiposity, circulating triglycerides, and insulin sensitivity. Study design: 32 overweight/obese adults consumed glucose- or fructose-sweetened beverages that provided 25% of their energy requirements along with their usual ad libitum diets for 8 weeks. They then consumed the sweetened beverages for 2 weeks, as part of a standardized, energy-balanced diet. Plasma hormone and lipid concentrations were measured at baseline and 2, 8 and 10 weeks intervention, and body adiposity and insulin sensitivity were measured at baseline and 10 weeks. Results: Fasting and postprandial ASP concentrations were significantly increased at 2 and/or 8 weeks when the sugar-sweetened beverages were consumed with ad libitum diets. Increases in ASP correlated with changes in late-evening triglycerides concentrations. The presence of metabolic syndrome risk factors influenced changes of postprandial ASP. At 10 weeks fasting adiponectin levels were significantly decreased in both groups of subjects, with the decreases being inversely associated with baseline intra-abdominal fat volume. Sugar consumption increased fasting leptin concentrations and these increases were associated with the changes in body weight. 24-h leptin profiles were increased during glucose compared with fructose consumption, and correlated with changes of 24-h insulin levels. Conclusion: Sugar consumption increased ASP, fasting and postprandial leptin concentrations and decreased adiponectin. These changes were, respectively, associated with changes of postprandial triglycerides, body weight, 24-h circulating insulin concentrations and baseline intra-abdominal fat volume. These changes in adipokines may further contribute to fructose-induced metabolic dysfunction.

2.3 Introduction

Consumption of fructose at 25% of energy requirement for 10 weeks increased de novo lipogenesis (DNL), increased visceral adipose deposition, promoted lipid dysregulation, and decreased insulin sensitivity in older, overweight/obese men and women [1]. None of these adverse effects were observed in a group of subjects consuming glucose, who gained the same amount of weight as the subjects consuming fructose (~1.4 kg) [1]. These results are important because they illustrate that the major dietary sugars, fructose and glucose, have markedly different metabolic effects in humans, and that the differences are independent of weight gain.

It is well known that the circulating concentrations of acylation stimulating hormone (ASP), leptin and adiponectin; adipocyte hormones which are regulators of lipid metabolism and/or insulin sensitivity; are affected by changes in body weight and body fat mass and distribution [2,3,4,5]. Less is known about their modulation in response to the macronutrient content of the diet, and sugars in particular. The objective of this study was to determine the effects of fructose and glucose consumption on circulating ASP, leptin and adiponectin levels in the same subjects who consumed 25% of energy requirement as fructose- or glucose-sweetened beverages for 10 weeks [1]. We also investigated how the effects of fructose and glucose on these hormones related to the previously reported [1] changes in energy balance, body adiposity, circulating lipids, and insulin sensitivity in these subjects.

ASP is identical to C3a-des-Arg, and is produced through interaction of three proteins of the alternate complement system: C3, factor B, and adipsin (factor D), all of which are synthesized and secreted by adipocytes [5]. ASP increases triglyceride (TG) synthesis in adipose tissue through (i) increasing glucose transport via translocation of glucose transporters Glut 1, 2, and 3 [6]; (ii) stimulating fatty acid uptake and esterification [7]; through (iii) increasing the activity of enzymes related to fat storage, such as diacylglycerol-acyltransferase [8], thereby (iv) enhancing the efficiency of lipoprotein lipase (LPL) [9,10] which hydrolyses chylomicron TG [11] by reducing fatty acid inhibition and (v) by inhibiting hormone sensitive lipase [12]. Adiposity is an important determinant of

circulating ASP levels, which are elevated in obese subjects [13] and decrease with weight loss [14], although this is not the only determinant, as patients with metabolic dysfunction, such as type 2 diabetes, cardiovascular disease or polycystic ovary disease have increased ASP even in the absence of obesity [15,16,17,18]. We hypothesize that, because subjects consuming fructose exhibited both increased weight gain and increased metabolic dysfunction, consumption of fructose would increase levels of ASP more than consumption of glucose.

Adiponectin has insulin-sensitizing effects that appear to be mediated in part by its effects to activate AMP kinase, increase fatty acid oxidation and decrease ectopic fat accumulation [3,19]. Adiponectin has also been described as having anti-atherogenic and anti-inflammatory properties [20]. It prevents obesity-induced endothelial dysfunction by promoting nitric oxide production, suppresses activation and promotes repair of endothelial cells, and inhibits reactive oxygen species and apoptosis [21]. Unlike ASP, circulating adiponectin concentrations are lowered in obese animals and humans, and are inversely correlated with visceral adipose [19]. We hypothesize that, because consumption of fructose increased accumulation of visceral adipose, subjects consuming fructose will have greater decreases of adiponectin than subjects consuming glucose.

Leptin is a key regulator of energy homeostasis [22,23], which, along with insulin, acts in the hypothalamus and other areas of the brain to reduce energy intake and increase energy expenditure. Leptin can affect insulin sensitivity and this may be mediated by its effects to stimulate fatty acid oxidation, thus attenuating lipid accumulation in peripheral tissue [2]. Like ASP, circulating leptin concentrations are positively associated with increased body adiposity [22]. However, in vitro studies in isolated adipocytes [24] and in vivo studies in humans [25,26] have demonstrated that production of the adipocyte hormone leptin is strongly regulated by insulin-mediated glucose metabolism. Consumption of fructose compared to glucose results in lowered postprandial glucose and insulin excursions, therefore in short term studies, the 24-hour leptin area under the curve (AUC) was reduced by 25-30% when normal weight women [27] and overweight men and women [28] consumed meals accompanied with fructose- compared with glucose-sweetened

beverages. Thus, we hypothesize that sustained fructose consumption will lower 24-h circulating leptin profiles compared with sustained glucose consumption

2.4 Methods

2.4.1 Study design

The study design and methodology have been previously described [1] and are detailed on the online supplement. Briefly, during the baseline phase of the study, subjects resided in the UCD Clinical and Translational Science Center's Clinical Research Center (CCRC) for 2 weeks and consumed an energy-balanced, high complex carbohydrate (55%) diet. Procedures conducted during the baseline CCRC visit included DXA scans for quantification of total body fat, abdominal computerized tomography scans for quantification of total, extra- and intra- abdominal fat area, oral glucose tolerance tests with deuterated glucose disposal for assessment of insulin sensitivity and 24-h serial blood collections. A detailed description of the 24-h serial blood collections is available on the online supplement. Subjects then began an 8-week outpatient intervention and consumed either fructose- (n=17) or glucose-sweetened (n=15) beverages at 25% of energy requirements with self-selected ad libitum diets. The subjects returned to the CCRC after 2 outpatient weeks for 2 days, and then again for the final 2 weeks of the intervention for inpatient metabolic studies, during which the glucose- or fructose-sweetened beverages were consumed as part of an energy-balanced diet. 24-h blood collections were conducted during baseline and after 2, 8 and 10 weeks of dietary intervention.

2.4.2 Subjects

The subjects were 40 to 72 years of age with BMIs of 25–35 kg/m^2. Details concerning recruitment and inclusion/exclusion criteria have been previously reported [1] and are available on the online supplement. The subjects provided informed written consent and the study protocol was approved the Institutional Review Board of the University of California, Davis.

2.4.3 Plasma Assays

Plasma concentrations of fasting ASP, adiponectin, and leptin were measured in plasma samples collected or pooled from the 8:00, 8:30, 9:00-h timepoints. Postprandial concentrations of ASP were measured in samples pooled from the 22:00, 23:00, 23:30-h timepoints. Leptin concentrations were measured in samples collected from all 33 postprandial timepoints and the incremental 24-h AUC over the morning nadir was calculated by the trapezoidal method [27]. Plasma ASP was measured as previously described [7]. Leptin, adiponectin, and insulin were measured by radioimmunoassay (Millipore Inc., St. Charles, MO). Triglyceride (TG) concentrations were determined with a Polychem Chemistry Analyzer (PolyMedCo, Inc., Cortdland, NY).

2.4.4 Statistical analysis

The absolute change (Δ from 2 wk, 8wk, 10wk when 25%E fructose or glucose/30%E complex carbohydrate was consumed compared with 0 wk when 55%E complex carbohydrate was consumed) for each outcome was analyzed with SAS 9.3 (SAS, Cary, NC) in a mixed procedures (PROC MIXED) model with time, sugar, gender and number of metabolic syndrome risk factors (MSRF) as factors. All models included adjustment for outcome concentration at 0wk (baseline). MSRF were those defined by the American Heart Association/National Heart Lung and Blood Institute (83, 84), with subjects grouped into 2 levels of MSRF (0 and 1 MSRF vs 2 and 3 MSRF). Insignificant 3-way and 4-way interactions were removed if they decreased the precision of the model. Outcomes that were significantly affected by glucose or fructose consumption were identified as least squares means (LS means) of the change significantly different than zero. All outcomes were tested with the following continuous covariables: baseline value and the change (2 wk – 0 wk) in %body fat, total, extra-, and intra-abdominal fat, and insulin sensitivity index. The following time-level covariables were also tested: absolute change (Δ from 2 wk, 8wk, and 10wk compared with 0 wk) of body weight (ΔBW), previous day's energy intake, late-evening TG concentrations, and 24-h insulin AUC. Outcomes with significant interactions were further analyzed in sugar- or gender-specific RM models.

Values are reported as mean ± standard error (SEM). A $P<0.05$ was considered statistically significant for all analyses.

2.5 Results

Baseline characteristics of the subjects have been previously reported and are available on the online supplement (Table S1). The absolute values of outcomes and covariables at all measured timepoints are provided in Table 2.1. There were no significant differences between the 2 experimental groups in any of the measured anthropomorphic characteristics or metabolic parameters at baseline. The P-values of the associations of anthropometric and metabolic parameter with sugar-induced changes in adipocyte hormone concentrations are shown on the online supplement (Table S2).

Consumption of fructose increased both fasting (Figure 2.1A) and postprandial (Figure 2.1B) ASP concentrations at 2wk (Fasting: P=0.0006; Postprandial: P=0.0015) and 8wk (Fasting: P=0.0007; Postprandial: P=0.0007), but not at 10wk. Consumption of glucose increased both fasting (P=0.031) and postprandial (P=0.020) ASP concentrations at 8wk only. The trend for ASP levels to be higher at 2wk and 8wk was significant in the postprandial state (P=0.0029, effect of time), and nearly so in the fasting state (P=0.054). The changes of fasting and postprandial ASP were not significantly affected by sugar (Fasting: P=0.16; Postprandial: P=0.29).

Three time-level covariates were tested, and the change of fasting ASP was significantly associated with the changes in late-evening TG concentrations (P=0.0051), but not changes in body weight or previous day's energy intake (Table S2). Simple regression analysis shows (Figure 2.1C) that the subjects with larger increases of the late evening TG peaks had greater increases of fasting ASP concentrations ($r=0.510$, $P<0.0001$). The change in the late-evening TG concentrations (P=0.046) was also a significant contributor to the variations in postprandial ASP. None of the continuous covariates tested in the model (baseline levels and the change (10wk – 0 wk) of %body fat, total, extra-, intra-abdominal fat, insulin sensitivity index) were associated with the changes of fasting or postprandial ASP (Table S2).

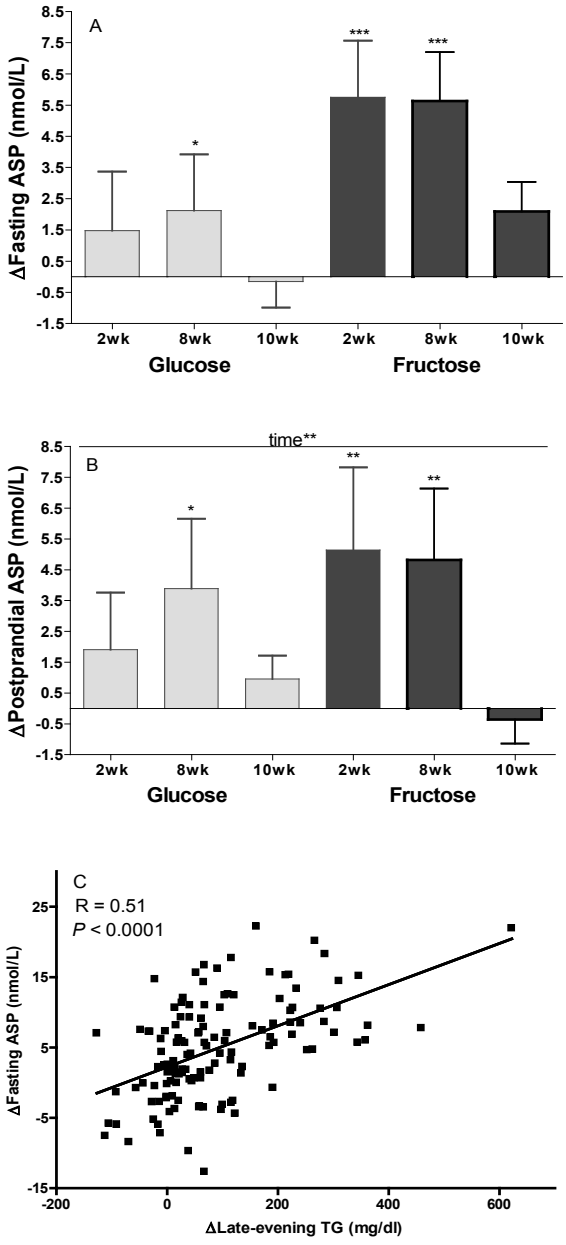

Figure 2.1 Effects of glucose or fructose consumption on fasting and postprandial plasma ASP concentrations.

Changes (Δ) at 2wk, 8wk and 10wk compared to baseline (0wk) in the fasting (A) and postprandial (B) ASP levels of subjects who consumed 25% of energy requirement as either glucose (n=15) or fructose-sweetened beverages (n=17). Effect of: Time** $p<0.01$ in PROC MIXED 4-factor (sugar, time, gender, MSRF) repeated measures (RM) model with outcome at 0wk (OutcomeB) as continuous covariable. *P<0.05, **P<0.01, ***P<0.001, Least squares mean of the change different from zero. Results are presented as mean ± SEM. (C) Correlation between change (Δ) in fasting ASP and change (Δ) in late-evening (postprandial) triglyceride (TG) at 2wk, 8wk and 10wk.

The effects of gender (P=0.47) and MSRF (P=0.12) on the changes of fasting ASP were not significant, however the effect of plasma ASP concentrations at baseline was highly significant (P<0.0001). When subjects were divided into 2 equal groups based on baseline ASP concentrations, subjects with low ASP concentrations (<7.0 nmol/mL) had markedly higher changes in fasting ASP at 2wk (+9.1±1.3 vs -1.6±1.5 nmol/mL, P<0.0001) and 8wk (+8.5±1.1 vs -0.5±1.5 nmol/mL, P<0.0001) than subjects with high baseline ASP concentrations.

Subjects with 0-1 MSRF had greater increases of postprandial ASP levels at all three intervention time-points than subjects with 2-3 MSRF (P=0.019). The effects of MSRF were independent of the effects of baseline postprandial ASP concentrations, which, as with fasting ASP, were inversely associated with the changes in postprandial ASP (P=0.0007). In the postprandial state, the effects of gender (P=0.058) were nearly significant, and there was a significant sugar x gender x time effect (P=0.045), due to the women consuming fructose having larger increases of ASP than the men consuming fructose.

The sugar-specific models demonstrate that the significant effects of time and MSRF on postprandial ASP in the 2-sugar model were mainly mediated by consumption of fructose. In the glucose-specific model the effects of time (P=0.24), MSRF (P=0.98), and MSRF x time (P=0.88) were not significant (Figure 2.2A), nor were the effects of gender (P=0.83) and gender x time (P=0.79) (Figure 2.2C). In the fructose-specific model the effects of time (P=0.0003), MSRF (P=0.0087), and MSRF x time (P=0.0005) were all statistically significant (Figure 2.2B), as were the effects of gender (P=0.045) and gender x time (P=0.0004) (Figure 2.2D).

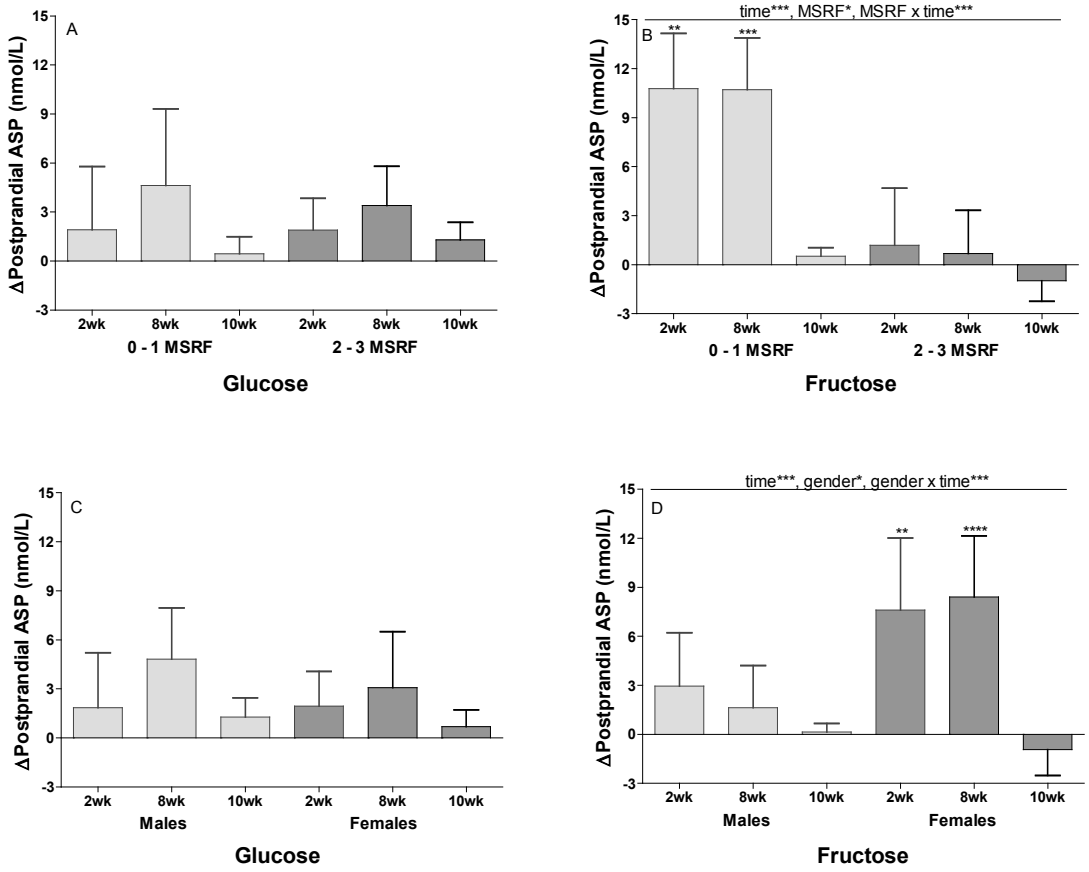

Figure 2.2 Effects of metabolic syndrome risk factors (MSRF) and gender on the changes in postprandial plasma ASP concentrations in subjects consuming glucose or fructose.

Changes at 2wk, 8wk and 10wk compared to baseline (0wk) in postprandial ASP levels of subjects grouped by number of MSRF during consumption of glucose (A) (n=15) or fructose-sweetened beverages (B) (n=17). Changes at 2wk, 8wk and 10wk compared to baseline (0wk) in postprandial ASP levels of subjects grouped by gender during consumption of glucose (C) (n=15) or fructose-sweetened beverages (D) (n=17). Effect of: Time*** $p<0.001$, MSRF* $p<0.05$, MSRF x time*** $p<0.001$, gender* $p<0.05$, gender x time*** $p<0.001$ in sugar-specific PROC MIXED 3-factor (time, gender, MSRF) RM model with outcome at 0wk as continuous covariable. **P<0.01, ***P<0.001, ****P<0.0001, Least squares mean of the change different from zero.

The changes of fasting plasma adiponectin concentrations (Figure 2.3A) were not significantly affected by sugar (P=0.10), MSRF (P=0.37) or gender (P=0.087), but were affected by time (P<0.001). Compared to baseline (0wk) levels, adiponectin concentrations in subjects consuming glucose were increased at 2wk (P=0.023), and decreased at 10wk (P=0.028). Circulating adiponectin concentrations were unchanged at 2wk (P=0.77) and

decreased at 10wk (P=0.0011) in subjects consuming fructose. There was a significant effect of sugar x gender x time (P=0.039). Gender-specific models showed a significant effect of sugar x time in women (Figure 3B; P=0.0011), but not in men (Figure 2.3C; P=0.57), with women consuming glucose having larger increases of plasma adiponectin concentrations at wk2 and wk8 than at wk10.

The changes of fasting adiponectin concentrations were influenced by total (P=0.024) and intra-abdominal (P=0.036) fat volume at baseline (Table S2). The subjects who had lower baseline levels of total and intra-abdominal fat exhibited the greater decreases in adiponectin. Simple regression showed that the relationship between baseline intra-abdominal fat and the changes in adiponectin was especially strong at 10wk (P=0.0004, r=0.59, Figure 2.3D). The adiposity associations with adiponectin were independent of baseline adiponectin concentration, which was also a significant covariate in the model (P=0.0014). Subjects with high baseline levels of adiponectin (>7 ug/ml) exhibited greater decreases (-1.5±0.4 ug/ml) of circulating adiponectin at 10wk than subjects with low baseline levels (-0.5±0.2 ug/ml).

Both subjects consuming fructose (8wk: P=0.0081; 10wk: P=0.027) and glucose (8wk: P=0.023; 10wk: P=0.032) had significantly increased fasting leptin concentrations at 8wk and 10wk (Figure 4), but these changes were not significantly affected by sugar (P=0.84), time (P=0.36), MSRF (P=0.84), or gender (P=0.44). The changes of fasting leptin were significantly affected by the changes in body weight (P=0.010), with those subjects gaining the most weight having the largest increases of leptin. The change in %body fat from 0wk to10wk was a marginally significant contributor to the changes in fasting leptin (P=0.055), while the changes and baseline levels of total, extra-, and intra-abdominal fat were not (Table S2).

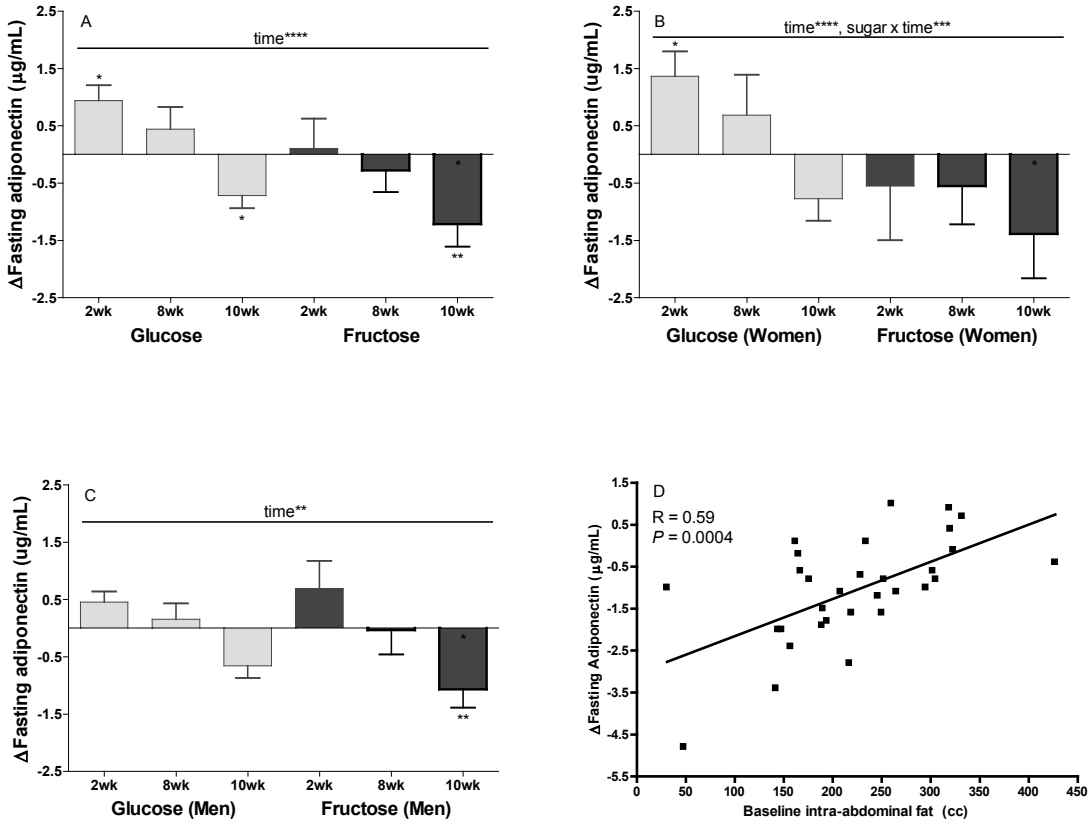

Figure 2.3 Effects of glucose or fructose consumption on fasting plasma adiponectin concentrations in overweight and obese subjects.

Changes (Δ) at 2wk, 8wk and 10wk compared to baseline (0wk) in the fasting (A) adiponectin levels of subjects who consumed 25% of energy requirement as either glucose (n=15) or fructose-sweetened beverages (n=17). Effect of: Time**** P<0.0001 in PROC MIXED 4-factor (sugar, time, gender, MSRF) RM model with outcome at 0wk as continuous covariable. *P<0.05, **P<0.01, Least squares mean of the change different from zero. Changes at 2wk, 8wk and 10wk compared to baseline (0wk) in fasting adiponectin levels of women (B) and men (C) during consumption of glucose (n=15) or fructose-sweetened beverages (B). Effect of: Time** P<0.05, Time**** P<0.0001, Sugar x time*** P<0.001 in gender-specific PROC MIXED 3-factor (time, sugar, MSRF) RM model with outcome at 0wk as continuous covariable. *P<0.05, Least squares mean of the change different from zero. Results are presented as mean ± SEM. (D) Correlation between th 10wk changes (Δ) in fasting adiponectin and baseline intra-abdominal fat volume.

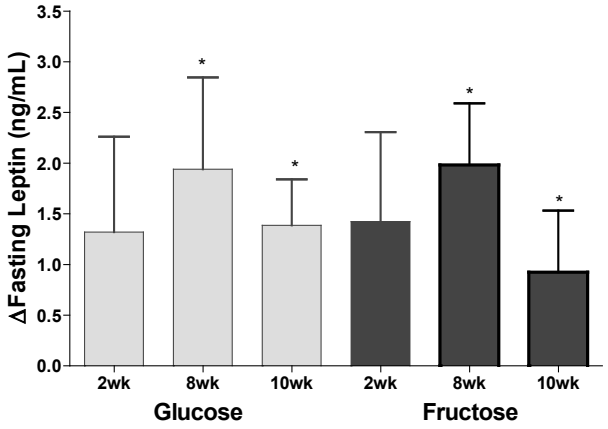

Figure 2.4 Effects of glucose or fructose consumption on fasting leptin concentrations.

Changes (Δ) at 2wk, 8wk and 10wk compared to baseline (0wk) in the fasting leptin levels of subjects who consumed 25% of energy requirement as either glucose (n=15) or fructose-sweetened beverages (n=17). PROC MIXED 4-factor (sugar, time, gender, MSRF) RM model with outcome at 0wk as continuous covariable. *P<0.05, Least squares mean of the change different from zero.

The changes in postprandial leptin were significantly affected by sugar (P=0.026) with subjects consuming glucose tending to have increased and subjects consuming fructose tending to have decreased 24-h leptin AUCs compared to when they consumed the baseline complex carbohydrate diet (Figure 2.5). These changes were not affected by time (P=0.51), MSRF (P=0.56), or gender (P=0.24) (Table 2.1). The change (10wk – 0wk) of %body fat (P=0.031) contributed significantly to the changes of the leptin AUC, and simple regression showed there was a strong positive relationship at 10wk (r=0.39; P=0.0015). The changes of the 24-h insulin AUC significantly affected the changes of 24-h leptin AUC (P=0.0035), while completely negating the significant effect of sugar (P=0.70) and lessening the effect of change of %body fat (P=0.093) on circulating leptin concentrations. Simple regression including all 3 intervention timepoints (Figure 2.5D) showed the expected positive relationship between the changes of the leptin and insulin AUCs (P<0.0001, r=0.40).

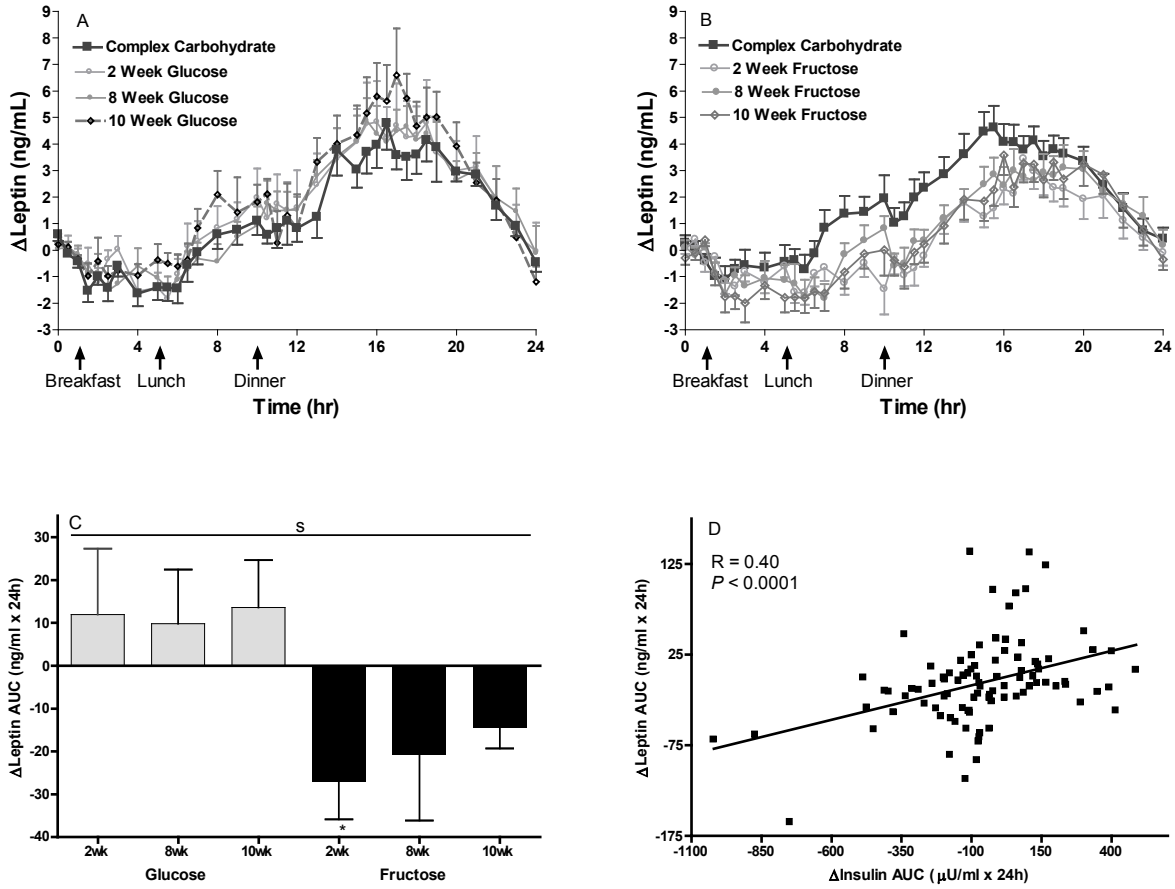

Figure 2.5 Effects of glucose or fructose consumption on 24-h leptin profiles.

24-h circulating TG concentrations in subjects before and after 2, 8, and 10 weeks of consuming glucose-sweetened beverages (A) (n=14) or fructose-sweetened beverages (B) (n=17). (C) Changes (Δ) at 2wk, 8wk and 10wk compared to baseline (0wk) in the 24-h leptin AUCs of the subjects who consumed glucose or fructose-sweetened beverages. Effect of: Sugar* $P < 0.05$ in PROC MIXED 4-factor (sugar, time, gender, MSRF) RM model. Mean ± SEM. (D) Correlation between changes (Δ) in 24-h leptin AUC and 24-h insulin AUC at 2wk, 8wk and 10wk.

Table 1: Outcome and covariable concentrations before and after consumption of glucose- or fructose-sweetened beverages

Variable	Sugar (n)	Complex Carb 0 wk	Sugar 2 wk	Sugar 8 wk	Sugar 10 wk	Factors/ interactions/ covariable	*P* value
Preceding diet		Energy balance	*Ad libitum*	*Ad libitum*	Energy balance		
Fasting ASP[A]	Glucose (n=15)	9.8 ± 1.4	11.3 ± 1.3	12.0 ± 1.3[B]	9.7 ± 1.3	Sugar	0.16
(nmol/l)	Fructose (n=17)	7.0 ± 0.8	12.7 ± 1.3[D]	12.6 ± 1.1[D]	9.0 ± 0.9	Gender	0.47
						MSRF	0.12
						$Outcome_B$	<0.0001
Postprandial ASP[A]	Glucose (n=14)[G]	9.7 ± 1.2	11.6 ± 1.4	13.6 ± 1.7[B]	10.7 ± 1.4	Sugar	0.29
(nmol/l)	Fructose (n=17)	9.2 ± 1.3	14.4 ± 1.8[D]	14.0 ± 1.6[D]	8.9 ± 1.1	Gender	0.058
						MSRF	0.019
						Sugar×Gender×*t*	0.045
						$Outcome_B$	0.0007
Fasting Adiponectin[A]	Glucose (n=14)[G]	7.7 ± 1.1	8.6 ± 1.2[B]	8.1 ± 1.2	7.0 ± 1.1[B]	Sugar	0.1000
(ug/ml)	Fructose (n=17)	8.1 ± 1.2	8.2 ± 1.0	7.9 ± 1.1	6.9 ± 1.0[C]	Gender	0.087
						MSRF	0.37
						Sugar×Gender×*t*	0.039
						$Outcome_B$	0.0014
Fasting Leptin[A]	Glucose (n=15)	18.3 ± 3.5	19.6 ± 3.2	20.2 ± 3.4[B]	19.7 ± 3.4[B]	Sugar	0.84
(ng/ml)	Fructose (n=17)	17.3 ± 3.5	18.7 ± 3.1	19.3 ± 3.7[B]	18.2 ± 3.5[B]	Gender	0.46
						MSRF	0.84
						$Outcome_B$	0.072
24-h Leptin AUC[A]	Glucose (n=14)	71.2 ± 10.6	83.1 ± 21.3	81.0 ± 13.6	84.8 ± 15.9	Sugar	0.026
(ng/ml x 24-h)	Fructose (n=17)	78.9 ± 11.3	51.8 ± 11.3[B]	58.1 ± 15.6	64.3 ± 11.6	Gender	0.24
						MSRF	0.56
						$Outcome_B$	0.24
Body Weight[A]	Glucose (n=15)	86.0 ± 2.8	86.8 ± 2.8[D]	87.5 ± 2.9[D]	87.5 ± 3.0[D]	Sugar	0.39
(kg)	Fructose (n=17)	85.5 ± 2.6	86.1 ± 2.7[C]	86.9 ± 2.6[E]	86.8 ± 2.6[D]	Gender	0.11
						MSRF	0.32
						$Outcome_B$	0.1
Prior day's energy intake[A]	Glucose (n=15)	2,376 ± 72	2,895 ± 197[C]	2,803 ± 147[D]	2,376 ± 72	Sugar	0.61
(kcal)	Fructose (n=17)	2,469 ± 92	3,232 ± 155[E]	2,845 ± 170[C]	2,469 ± 92	Gender	0.0072
						MSRF	0.25
Late-evening TG peaks[A]	Glucose (n=15)	202.4 ± 24.6	234.7 ± 21.7	227.2 ± 20.5	214.2 ± 20.3	Sugar	0.0069
(mg/dl)	Fructose (n=17)	211.1 ± 28.3	295.5 ± 36.7[C]	282.7 ± 35.4[C]	274.9 ± 31.1[D]	Gender	0.071
						MSRF	0.49
						$Outcome_B$	0.018
24-h Insulin AUC[A]	Glucose (n=14)	596.1 ± 118.7	699.6 ± 112.0	693.0 ± 112.9	757.5 ± 129.6[C]	Sugar	<0.0001
(ng/ml x 24-h)	Fructose (n=17)	681.4 ± 112.6	426.1 ± 66.6[E]	489.1 ± 85.8[D]	480.4 ± 72.9[D]	Gender	0.56
						MSRF	0.64
						$Outcome_B$	<0.0001
Body Fat[B]	Glucose (n=15)	35.6 ± 2.1			36.1 ± 2.0[G]	Sugar	0.84
(%)	Fructose (n=17)	33.6 ± 2.3			33.9 ± 2.2	Gender	0.33
						MSRF	0.86
Total Abdominal Fat[B]	Glucose (n=14)	765 ± 57			794 ± 53	Sugar	0.36
(cc)	Fructose (n=17)	683 ± 55			731 ± 51[G]	Gender	0.11
						MSRF	0.61
Extra-Abdominal Fat[B]	Glucose (n=14)	522 ± 59			544 ± 61[G]	Sugar	0.64
(cc)	Fructose (n=17)	476 ± 43			495 ± 38	Gender	0.82
						MSRF	0.40
Intra-Abdominal Fat[B]	Glucose (n=14)[G]	243 ± 21			250 ± 23	Sugar	0.059
(cc)	Fructose (n=17)	207 ± 21			235 ± 25[H]	Gender	0.049
						MSRF	0.21
Insulin Sensitivity[B]	Glucose (n=14)	0.236 ± 0.036			0.210 ± 0.021	Sugar	0.030[E]
(mmoles 2H20/ 4-	Fructose (n=17)	0.254 ± 0.049			0.208 ± 0.040[H]	Gender	0.033[E]
h Insulin AUC)						MSRF	0.19[E]

[A]PROC MIXED 4-factor (sugar, time, gender, MSRF) RM model on change from 0wk with outcome at 0wk (OutcomeB) as continuous covariable.

[B]$P < 0.05$, [C]$P < 0.01$, [D]$P < 0.001$, [E]$P < 0.0001$ Least squares mean of the change from 0 wk different from zero.

[F]General Linear Model (GLM) 3-factor ANOVA on 10 wk vs 0 wk difference as previously reported (Stanhope, JCI, 2009).

[G]$P < 0.05$, [H]$P < 0.01$, Paired t test, 10wk vs 0wk

t: time

Mean ± SEM

Table 2.1 Outcome and covariable concentrations before and after consumption of glucose or fructose- sweetenrd beverages

2.6 Discussion

In this study we examined circulating concentrations of the adipocyte hormones, ASP, leptin and adiponectin in relationship with dietary effects and metabolic consequences of long-term fructose and glucose consumption in overweight and obese men and women. Consumption of fructose markedly increased plasma ASP levels at 2 and 8wk, but not at 10wk. Consumption of glucose also resulted in significant, although less marked, increases in ASP at 8wk. The changes in both fasting and postprandial ASP were associated with the changes of late-evening triglyceride concentrations.

Along with LPL and insulin, ASP has an important role in TG clearance from the blood for storage in adipose tissue. Principally, ASP stimulates reesterification of fatty acids for synthesis of TG within the adipocyte. The positive association between the changes in ASP and postprandial TG levels suggest that postprandial TG may be involved in the regulation of ASP production. We have suggested several mechanisms for the increased levels of postprandial TG in subjects consuming fructose including[25]: 1. Increased DNL from fructose which increases hepatic lipid and VLDL production and secretion. 2. Decreased LPL activity due to lowered post-meal insulin responses, 3. Competition between VLDL and chylomicron for LPL binding sites. We also suggested that positive energy balance contributed to the increases in postprandial TG at 2 and 8wk[25]. It is not known which, if any, of these mechanisms explain the association between the changes of ASP and postprandial TG. While there is evidence from in vitro studies which demonstrates that chylomicrons increase ASP production and VLDLs have minimal effect [(29)], little is known about the regulation of ASP by circulating lipids/lipoproteins in vivo. The significant increases of postprandial ASP at 2 and 8wk in subjects consuming fructose, and the lack of increase at 10wk when they were consuming the energy balanced diet, suggest that positive energy balance may augment the effects of postprandial TG to increase ASP.

The very few studies that have investigated acute or chronic effects of macronutrients on human ASP concentration suggest ASP-mediated non-esterified fatty acids (NEFA) trapping (and re-esterification) could be an important determinant of a

healthy lipoprotein phenotype [30]. A diet high in trans fatty acids decreased fasting ASP levels compared with diets that were high in polyunsaturated fatty acids in hypercholesterolemic women [31]. The decrease was associated with reduced clearance of fatty acids and higher levels of apoB. In 141 healthy men and women the acute ASP response to a high fat/high-energy meal significantly predicted postprandial TG and NEFA clearance, and both ASP response and lipid clearance were inversely related to fasting plasma ASP levels [32]. Specifically this study shows that subjects with high plasma ASP had a decreased ASP response to an oral fat challenge compared with subjects with low plasma ASP. The present data are in accord with these acute effects, and demonstrate that subjects with low fasting ASP had significantly higher ASP responses after 2 and 8 weeks of exposure to fructose-induced postprandial hypertriglyceridemia than the subjects with high fasting ASP concentrations. Interestingly, we also show that subjects consuming fructose with 0-1 MSRF had higher postprandial ASP responses than those with 2-3 MSRF, and the effect of MSRF was independent of the effect of baseline postprandial ASP concentrations. Our results support the suggestion that ASP-mediated NEFA trapping could be associated with a healthy lipoprotein phenotype. Also supportive is a recent report that the adipose gene expression of factors related with lipid uptake and processing, such as ASP, LPL, low density lipoprotein receptor protein 1, and fatty acid binding protein 4 were lower in morbidly obese patients than in lean healthy persons [33]. Thus, although obesity results in high levels of circulating ASP, high ASP responses to diets that induce increased levels of TG may be an adaptive response to maintain metabolic equilibrium.

Fasting adiponectin concentrations were significantly decreased at 10wk in both groups of subjects, although there was a small, but significant increase of adiponectin at 2wk in subjects consuming glucose. As reviewed [19], there is evidence to suggest that adiponectin production by adipocytes may be regulated by insulin-stimulated glucose utilization. In 3T3-L1 cells, adiponectin production increased following insulin and high glucose treatment [34], while adiponectin mRNA increased with augmentedinsulin-stimulated glucose uptake[35]. Thus, the regulation of adiponectin production by insulin-stimulated glucose utilization may possibly explain the significant increase of adiponectin levels during consumption of glucose-, but not fructose-sweetened beverages at 2 weeks. The reversal of this effect, and the significant decrease in adiponectin measured in both

groups of subjects at 10 weeks could be attributed to their comparable weight gain as it is well documented that adiponectin levels are inversely related to adiposity. However, weight gain does not always result in reduced adiponectin levels as recently reported in young, healthy subjects who gained approximately 6 kg in 4 weeks by increasing fast food consumption and decreasing exercise; while fasting insulin, leptin and CRP increased, plasma adiponectin levels were unchanged [36].

It is well-documented that visceral adiposity is an important determinant of plasma adiponectin concentrations in human [19], however in the present study the changes of visceral fat did not correlate with the changes of adiponectin. Instead, baseline levels of abdominal and visceral adiposity were significant contributors, with higher levels of abdominal and visceral adiposity being more protective against decreases in adiponectin than lower levels. Similarly, low baseline levels of adiponectin were associated with smaller decreases of adiponectin. A potential explanation for these surprising, independent relationships is visceral adipocyte size. As reviewed by Swarbrick and Havel [19], there is evidence to suggest that visceral adipocytes, which have been shown to produce more adiponectin than subcutaneous adipocytes, become less insulin-sensitive and secrete less adiponectin as they enlarge. Healthier subjects with higher baseline concentrations of adiponectin and lower amounts of visceral adipose are expected to have smaller, insulin-sensitive visceral adipocytes that have a greater capacity for further enlargement. With enlargement these visceral adipocytes become less insulin-sensitive and produce less adiponectin. The subjects with lower baseline adiponectin concentrations and higher levels of visceral adipose are likely to have started with larger visceral adipocytes in which adiponectin production was already impaired. Therefore further enlargement of these cells had little effect on adiponectin concentrations.

Recently it was reported that higher adioponectin levels predicted weight gain in the participants in the Nurses' Health Study [37]. The authors suggested that high adiponectin production by adipocytes might be a sign of "healthy" adipose tissue with further capacity to store fat. However, in two other studies, higher baseline adiponectin concentrations were predictive of greater loss of weight and body fat in morbidly obese subjects after Roux-en-

Y gastric bypass surgery [38,39]. Clearly, more research is needed to understand the association and role of adiponectin concentrations in metabolic disease.

Fasting leptin concentrations increased in both groups of subjects with the changes from baseline being significantly and positively associated with the changes of body weight. Of the 2-timepoint covariates, the change in total body fat (10wk – 0wk) showed a marginally significant and much stronger relationship to the changes of fasting leptin than the changes in total, intra- and extra-abdominal adipose. This is expected as it has been previously reported that leptin expression [40] and secretion [41] is higher in subcutaneous adipose samples compared to visceral samples from the same subjects.

In contrast to fasting leptin, the 24-h circulating leptin profiles, as assessed by the 24-h AUCs, were differentially affected by the 2 sugars. In subjects consuming glucose, the leptin AUC tended to increase, and in subjects consuming fructose it tended to decrease. We have previously shown this differential effect of glucose and fructose consumption on 24-hour circulating leptin profiles in normal-weight women [27] and in overweight men and women [28] who consumed glucose- or fructose-sweetened beverages with meals for one day. To our knowledge this is the first study to document that the differential effects of fructose and glucose on 24-hour circulating leptin concentrations are sustained during long-term consumption of the 2 sugars.

Because we have reported that insulin-stimulated glucose utilization mediates leptin production and secretion by adipocytes [24], we also tested the changes in 24-h glucose and insulin AUC, and the amplitude of the post-meal glucose and insulin peaks [42] as time-level covariates in the statistical model for leptin AUC. These results showed that the 24-h insulin AUC was positively associated with the 24-h leptin AUC, and that the differential effects of fructose and glucose on circulating leptin profiles were dependent on their differential effects on 24-h insulin AUC.

When designing this study we had originally hypothesized that consumption of fructose compared to glucose would lead to a reduction of circulating leptin concentrations which would lead to increased energy intake and/or decreased energy expenditure and weight gain. To test this hypothesis, we included in the study protocol an 8-week outpatient

period during which subjects consumed their usual diets ad libitum along with the glucose- or fructose-sweetened beverages. It is interesting that 24-h circulating leptin levels were lowered in subjects consuming fructose compared with those consuming glucose, yet there were no differences in reported energy intake or weight gain between the 2 groups

2.7 Conclusion

In this study, long-term sugar consumption with ad libitum feeding leads to changes in circulating levels of the adipocyte hormones ASP, adiponectin, fasting and postprandial leptin. The changes of each of these hormones were associated with specific and distinct metabolic changes. Fasting and postprandial ASP were associated with postprandial triglycerides, adiponectin with baseline abdominal/visceral fat, fasting leptin with body weight, and 24-h leptin profiles with 24-h circulating insulin concentrations. More research is needed to determine if the changes observed in the adipocyte hormones are involved in the multiple metabolic derangements induced by consumption of fructose.

2.8 References

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Chapter 3

Apparent sex-specific divergence of Acylation Stimulating Protein (ASP) levels with respect to metabolic parameters of pathogenetic and clinical relevance

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3.1 Résumé

La protéine stimulant l'acylation (ASP) est une hormone provenant du tissu adipeux qui stimule la synthèse des triglycérides et le transport du glucose. Des liens entre l'ASP ainsi que son précurseur, la protéine C3 du complément, ont été observés avec l'obésité, la résistance à l'insuline, le diabète ainsi que les maladies cardiovasculaires. L'objectif de cette étude était d'évaluer les niveaux d'ASP et de C3 et leurs liens avec des facteurs de risques cardiométaboliques dans une population d'adultes Turques. Les niveaux d'ASP chez les femmes étaient plus faibles que chez les hommes ($p=0.059$) et ceci a été observé lorsqu'il y avait présence ou non de désordres cardiométaboliques. De façon intéressante, l'ASP corrélait de façon différente entre les sexes avec des paramètres tels que, les triglycérides, le glucose, la taille, l'âge etc. Ce dimorphisme sexuel est un aspect propre à cette population Turque qui a aussi été observé pour divers paramètre lipidiques (HDL, LP(a)) ainsi que son association avec les risques cardiométaboliques liés au tabagisme et à la prise d'alcool et l'état pro-inflammatoire lié à l'adiposité. Les données présentées ici sur l'ASP corrobore avec ce qui a déjà été observé en terme de différences sexuelles mais aussi de corrélation entre l'ASP et l'état métabolique des sujets.

3.2 Abstract

Background: Acylation stimulating protein (ASP) is an adipose tissue-derived hormone that regulates triglyceride (TG) synthesis and glucose transport. Associations of ASP and/or its precursor complement C3 have been demonstrated with obesity, insulin resistance, diabetes, and cardiovascular diseases. *Objective*: To determine fasting serum ASP in a relatively high-risk Turkish adult population sample and assess relationships with cardiometabolic risk factors. *Study design*: Cross-sectional population-based study recruiting 224 men and women from the Turkish Adult Risk Factor (TARF) study. *Results*: Mean ASP levels in women (271 nmol/l) tended to be lower than men (290.2 nmol/l) (p=0.059), and this was also true in most subgroups with *vs*. without cardiometabolic disorders. Interestingly, correlations with ASP diverged in direction across genders with TG, glucose, height, age and other risk variables. *Conclusion*: Gender divergence is an aspect of this Turkish population that has also been noted for various lipid parameters (HDL, Lp(a)), association of cardiometabolic risk with smoking and alcohol intake and the response of the pro-inflammatory state to adiposity. This is consistent in the present study, where metabolic states correlate with ASP, but are divergent between genders.

3.3 Introduction

Cardiometabolic risk is a constellation of metabolic and underlying risk factors that significantly increase an individual's risk of having a cardiovascular event or developing metabolic abnormalities such as type 2 diabetes (T2D). Metabolic syndrome (MetS) has been considered as a specific subset of cardiometabolic risks that, when clustered together, impart a relative increase in risk of cardiovascular disease (CVD)(1). Approximately 9%, 21%, and 35% of normal weight, overweight, and obese adolescents (2) and 23.5%, 48.7% and 68.3% of normal weight, overweight, and obese adults respectively (3) have cardiometabolic risk factor clustering in U.S. The prevalence of metabolic syndrome (MetS) is approximately 34.6% in the United States, 17.8–34.0% in Europe and 12.8–41.1% in Asia (4). Various studies based on the results of National Health and Nutrition Examination (NHANES) (2,3) indicate that over a 10- to 15- year period, the prevalence of obesity, metabolic syndrome (MetS), and diabetes has increased by 34%, 48%, and 19% respectively. Obese individuals are characterized by a state of chronic low-grade inflammation (5) that may be casual in the development of insulin resistance and other disorders associated with obesity, such as hyperlipidemia, metabolic syndrome, or atherosclerosis (5).

Recently, adipose tissue has been recognized as a rich source of hormones (adipokines), some of which are pro-inflammatory and anti-inflammatory mediators (6). Alteration of adipose tissue function, including modified adipokine secretion, plays a key role in the pathogenesis of obesity and metabolic disorders (7). Acylation Stimulating Protein (ASP, aka C3adesArg) is one candidate, potentially contributing to the etiology of metabolic syndrome and cardiovascular disorders (8). ASP is an adipokine generated by the ordered interaction of complement C3, factor B, and adipsin through activation of the alternative complement pathway, all produced by adipocytes (ASP review (9)). However, ASP/C3adesArg is also generated systemically following pro-inflammatory immune activation (8).

The main known roles of ASP are stimulation of free fatty acid incorporation into adipose tissue by increasing triglyceride synthesis and storage (9), increase in glucose

uptake through enhanced translocation of glucose transporters (9) and reduction of triglyceride lipolysis in adipocytes through inhibition of hormone sensitive lipase (9). The autocrine effect of ASP in human adipose tissue was shown to be mediated through binding to its receptor, C5L2, mediated through activation of protein kinase C, PI3kinase and Akt (10).

Several studies have been shown that fasting ASP levels are increased in subjects with obesity (11,12), insulin resistance (13), and type 2 diabetes (T2D) (14,15). These studies strongly support the idea that obesity and/or insulin resistance are associated with increased ASP levels (16). However, higher ASP values were also observed in several metabolic disorders including the cardiovascular disease (17), polycystic ovary syndrome (18), renal disease (19) and nonalcoholic steatotic hepatitis (13) and dyslipidemia (9), without necessarily being associated with obesity and/or insulin resistance.

The prevalence of obesity and its consequences are rapidly increasing in developing countries (20). Obesity and abdominal obesity are major and growing problems for Turkish adults, especially for Turkish women (20). The Turkish population is known for the following characteristics: 1) The prevalence of obesity in the adult Turkish population (20.6% in men and 39.9% in women) is higher than most Western European countries (10-25% in Europe) and comparable with the United States (29% in with men and 50% in black women) (20, 21). 2) The prevalence of obesity is similar in rural and urban areas in Turkey (20). 3) In the Turkish population there is a high prevalence of cardiovascular risk factors including metabolic syndrome components (Onat A, review,(22)). Consequently, there is an increased rate of cardiovascular disease and type 2 diabetes (22). 4) Studies suggest that prevalence of abdominal obesity, MetS, and T2D are significantly as great among Turkish women as men (23,24). 5) Adult Turks are recognized to generally have lower levels of plasma cholesterol and higher levels of triglyceride (TG) than Westerners (22). The Turkish Adult Risk Factor (TARF) study, an established prospective population-based study, has contributed novel information on characterization of this population, including disorders with systemic inflammation (22).

The aims of this study were: to evaluate the adipokine ASP, in a large sample from a homogenous Turkish population and to evaluate the relationship of this adipokine with cardiometabolic risk factors in men as compared to women.

3.4 Methods

3.4.1 Sample population

224 adult men and women were recruited randomly from participants of the 2005-2008 follow-up surveys of the TARF Study, a prospective study on cardiac disease and its risk factors in a representative sample of adults in Turkey, carried out biennially since 1990 in 59 communities throughout 7 geographical regions of the country (22). Samples from available deep-frozen sera were randomly selected for study, without knowledge of any clinical or biochemical data. The study was approved by the Ethics Committee of the Medical Faculty, Istanbul University. Written informed consent for participation was obtained from all individuals. Data were obtained on medical and familial history via a questionnaire, physical examination, and blood samples.

3.4.2 Measurement of risk factors

Blood pressure (BP) was measured with an aneroid sphygmomanometer (Erka, Germany) in the sitting position on the right arm, and the mean of two recordings 3 min apart was recorded. Waist circumference was measured with the subject standing and wearing only underwear, at the level midway between the lower rib margin and the iliac crest. Blood samples were collected, after an overnight fast >11 hours, spun at 1000g and shipped on cooled gel packs to Istanbul to be stored at -75^{o}C, until analyzed in a central laboratory. Serum concentrations of apolipoprotein apoB, apoA-I and C-reactive protein (CRP) were measured by nephelometry (BN Prospec, Behring Diagnostics, Westwood, MA). Plasma fibrinogen levels were assayed by modified Clauss method using Behring Fibrinometer II coagulometer and multifibren U kit. Serum concentrations of triglycerides and glucose, measured in the fasting state, as well as of total, low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol (LDL-C, HDL-C plus 2nd generation,

direct quantification) were determined using enzymatic kits from Roche Diagnostics with a Hitachi 902 autoanalyzer.

Fasting concentrations of sex hormone-binding globulin (SHBG) and insulin were assayed by electrochemiluminescence immunoassay (ECLIA) on Roche Elecsys 2010 immunautoanalyzer (Roche Diagnostics, Mannheim, Germany). ASP concentration was measured using an in-house sandwich ELISA, following previously published methodology (26).

3.4.3 Definitions

Atherogenic dyslipidemia was defined as TG>1.7 mmol/L and HDL-C≤1.03 mmol/L (28). MetS was positive when 3 of 5 criteria of the National Cholesterol Education Program ATP-III were met, modified for prediabetes (fasting glucose 5.56-6.95 mmol/L) (27) with male central obesity defined as a waist circumference >95 cm, as assessed in the Turkish Adult Risk Factor study (28). In 2% of individuals exhibiting two MetS components and missing data on fasting triglycerides, the MetS status of a previous survey was adopted. Diabetes was diagnosed with the criteria of the American Diabetes Association (29), namely by self-report, plasma fasting glucose>7mmol/L or with 2-h postprandial glucose >11.1 mmol/L. HOMA was calculated as [insulin (mIU/L) x glucose (mmol/L)]/ 22.5. Nonfatal coronary heart disease (CHD) was identified by presence of angina pectoris, a history of myocardial infarction with/without accompanying Minnesota codes of the ECG (30) or a history of myocardial revascularization. Typical angina and, in women, age>45 years, were prerequisites for a diagnosis when angina was isolated. ECG changes of “ischemic type” of greater than minor degree (Codes 1.1-2, 4.1-2, 5.1-2, 7.1) were considered as myocardial infarct sequelae or myocardial ischemia, respectively.

3.4.4 Data statistical analysis

This study is a cross-sectional population-based study. The study population characteristics according to gender are reported as mean values ± standard deviations for continuous variables and proportions for categorical variables. Graphical and statistical analyses were performed using GraphPad Prism and SPSS. Two sided t-tests and Fisher's

exact test were used to analyze the differences between means and proportions of two groups, respectively. Spearman's correlation coefficients served to analyze univariate correlations. A value of $p<0.05$ was considered statistically significant. Stepwise multiple forward regression analysis was applied for the study of association of ASP with cardiometabolic risk factors.

3.5 Results

The study sample consisted of 224 adult Turkish men and women. The average age of the population was 51.5 yrs. This group was middle-aged to older, overweight (mean BMI=28.1 men and 29.9 women), prone to abdominal obesity and similar to the overall cohort of the Turkish Adult Risk Factor study. In the group as a whole, presence of cardiometabolic disorders (at least one of these disorders; CHD, MetS, hypertension (HT), T2D) was 60.7% (60.8% in men, 60.6% in women). The study group characteristics and metabolic profiles, separated based on gender are shown in Table 3.1.

There was no significant difference in age, waist and blood pressure values between men and women, but there were significant differences in height (men>women, $p<0.001$) and BMI (women>men, $p<0.01$). In general there was no significant difference between men and women in distribution of cardiovascular and metabolic disorders (Table 3.1). Total cholesterol, HDL-C, TG, glucose, creatinine, apolipoprotein A1, sex hormone binding globulin (SHBG), and testosterone were significantly different between men and women, but apolipoprotein B and C-reactive protein, were not significantly different. The ASP values ranged from 51 to 1277 with a geometric mean value of 290.2 nmol/l (SD±1.86-fold) in men and 271 nmol/l (SD±1.73-fold) in women ($p=0.059$)(Table 3.1).

In Figure 3.1, geometric mean ASP concentrations based on presence/absence of specific cardiometabolic disorders (CHD, metabolic syndrome, type-2 diabetes and hypertension) is shown in men and women. Levels were non-significantly higher in men with MetS and diabetes than without. Otherwise, including overall in women, ASP concentrations were non-significantly lower in the presence of cardiometabolic disorders.

Parameters	Unit	Women n=94 Mean	SD	Men n=130 Mean	SD	p-value
Age	Year	52.2	12.9	51	11.1	Ns
Waist	Cm	95.3	15.5	97.8	10.4	Ns
Height	Cm	156.2	6.60	168.5	6.70	<0.001
BMI	kg/m^2	29.9	5.20	28.1	3.90	<0.01
Systolic BP	mmHg	127	22.5	125.4	21.5	Ns
Diastolic BP	mmHg	76.4	10.5	76.5	10.6	Ns
Total C	mg/dl	200.6	42.6	188.8	43	<0.05
HDL C	mg/dl	51.1	11.8	41.4	10.8	<0.001
LDL C	mg/dl	120.7	35.3	114.8	36.8	Ns
TG	mg/dl	148	76.6	181.9	119.4	<0.01
Glucose	mg/dl	97.2	22	106.9	41.5	<0.05
Creatinine	mg/l	0.75	0.15	0.93	0.16	<0.001
ApoAI	mg/dl	145	23.2	128.8	22.6	<0.001
ApoB	mg/dl	103	34	100.1	31.9	Ns
Insulin¶	µIU/mL	8.97	2.09	7.76	1.89	Ns
CRP	mg/l	3.10	3.30	3.90	5.40	Ns
Fibrinogen	mg/dl	3.20	0.84	3.13	1.03	Ns
SHBG	nmol/l	61.1	37.7	42.5	24.5	<0.001
Testosterone	nmol/l	0.56	7.39	10	9.08	<0.05
ASP*	nmol/l	271	242-305	305	274-339	0.059

Metabolic disorder	Women N/%	Men N/%	Fisher test
CHD	7 / 7.4%	16 / 12.3%	Ns
MetS	44 / 47%	61 / 47%	Ns
T2D	7 / 7.4%	22 / 17%	0.068
HT	45 / 48%	49 / 38 %	Ns

Table 3.1 Characteristics of the study population according to sex.

log-transformed values. Mean and SD are provided for men and women (*for ASP, geometric mean and 95th confidence limits are provided), where Apo apolipoprotein, BP blood pressure, C cholesterol, CHD coronary heart disease, CRP C-reactive protein, HT hypertension, MetS metabolic syndrome, T2D type 2 diabetes, TG triglyceride, SHBG sex hormone binding globulin.

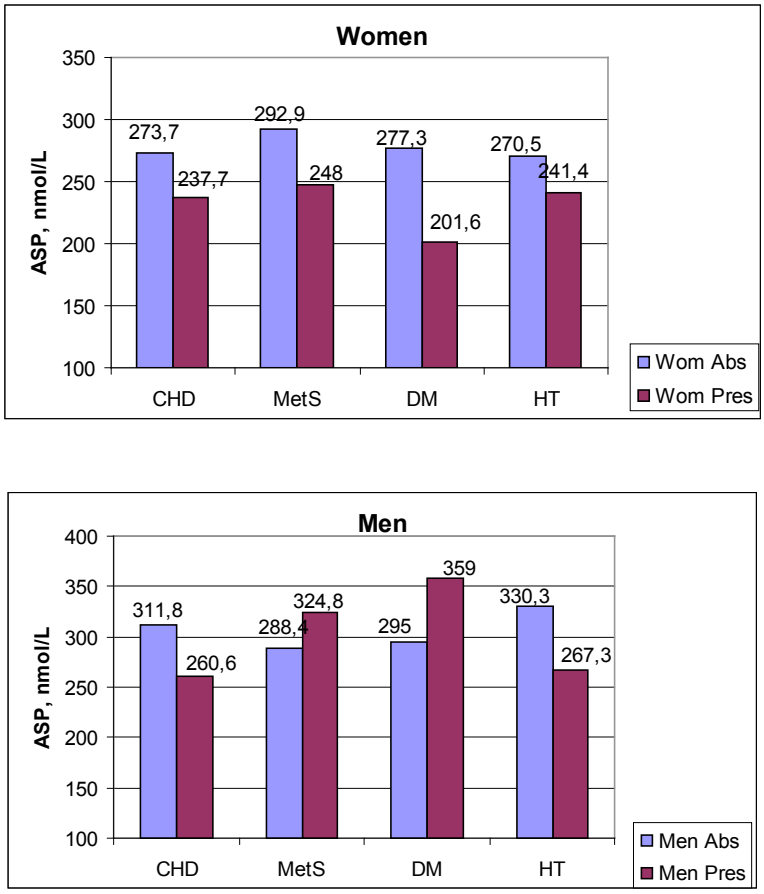

Figure 3.1 Geometric mean serum ASP concentrations in women (upper panel) and men (lower panel) without and with CHD, metabolic syndrome, type-2 diabetes and hypertension.

Levels were non-significantly higher in men with MetS and diabetes than without. Otherwise, including overall in women, ASP concentrations were non-significantly lower in the presence of cardiometabolic disorders. Log-transformed ASP values.

Correlation of ASP with various parameters in these subgroups is shown in Table 3.2. Overall, subjects with hypertension (HT) had several factors that correlated with ASP including SHBG, C-reactive protein (CRP), fibrinogen and creatinine. In subjects without hypertension there were positive correlations with height and negative with LDL-C. Similarly, ASP correlated with height, HDLC and SHBG in subjects with metabolic syndrome, but not in those without.

	All subjects (224)			
	r	*p value*	*r*	*p value*
Height	**0.17**	**0.01**		
SHBG	**-0.1**	**0.08**		
Parameters	*Healthy (88)*		*Non-Healthy (136)*	
Height	0.17	0.12	**0.16**	**0.07**
SHBG	0.04	0.77	**-0.30**	**<0.01**
Parameters	*Non-MetS (119)*		*With MetS (105)*	
Glucose	**-0.17**	**0.08**	0.13	0.22
Height	0.1	0.29	**0.15**	**0.03**
HDL-C	-0.09	0.31	**-0.20**	**0.04**
SHBG	-0.02	0.86	**-0.31**	**<0.01**
Parameters	*Without HT (130)*		*With HT (94)*	
Height	**0.19**	**0.03**	0.07	0.47
HDL-C	**-0.16**	**0.07**	-0.06	0.56
LDL-C	**-0.18**	**0.04**	0.13	0.21
SHBG	-0.03	0.77	**-0.33**	**<0.01**
CRP	0.05	0.52	**0.21**	**<0.05**
FIBRINOGEN	-0.04	0.68	**0.25**	**<0.05**
CREATININE	0.13	0.17	**-0.21**	**0.05**
AGE	0.06	0.47	**-0.19**	**0.06**

Table 3.2 Spearman correlation coefficients of ASP with certain variables according to metabolic disorders.

Spearman Correlation (r values) and significance level (p values) are provided according to metabolic disorder where CRP C reactive protein, HT hypertension, MetS metabolic syndromeSHBG sex hormone binding globulin. Values with p<0.1 are indicated in bold.

Correlation analysis of ASP was then performed stratified by sex, presence of MetS or other metabolic disorders (Table 3.3). Overall, correlations with ASP were stronger in men than in women in whom certain variables exhibited the opposite direction to those in men. Correlations of ASP overall in men were significant and positive with height, triglyceride, and glucose and tended to be so with CRP, with an inverse correlation with age and SHBG. In men with cardiometabolic disorders or with MetS, these correlations persisted and even tended to be accentuated as compared to men without MetS. Overall these correlations with ASP were in the opposite direction in women, even in healthy women. Significant inverse correlations were displayed with fasting glucose, LDL

cholesterol, and testosterone and tended to be so with total cholesterol and fasting TG. Significant positive correlations with waist girth, (diastolic) BP, and age have been shown. These correlations were strongly attenuated in the presence of MetS or otherwise "non-healthy" women. Correlation of ASP to height tended to be inversely, although very weakly, correlated in "non-healthy" women.

	r	p value	r	p value
All (224)	**Men (130)**		**Women (94)**	
Age	**-0.19**	0.030	0.08	0.47
Height	**0.26**	0.003	-0.03	0.79
Glucose	**0.19**	0.031	**-0.23**	0.03
Triglyceride	**0.19**	0.031	**-0.18**	0.09
CRP	**0.16**	0.069	0.04	0.73
Testosterone	0.05	0.630	**-0.26**	0.04
SHBG	**-0.19**	0.064	**-0.01**	0.95
Healthy (88)	Men (51)		Women (37)	
LDL-C	0	0.964	**-0.36**	0.03
Creatinine	**0.26**	0.079	**-0.12**	0.49
Non-healthy (138)	Men (79)		Women (59)	
Age	**-0.29**	0.010	0.13	0.35
Height	**0.27**	0.016	-0.17	0.20
BMI	**0.22**	0.049	0.13	0.33
Triglyceride	**0.24**	0.037	-0.15	0.26
HDL-C	**-0.30**	0.025	0.16	0.22
Glucose	**0.21**	0.046	**-0.27**	0.05
SHBG	**-0.32**	0.014	-0.19	0.23
Without MetS (119)	Men (69)		Women (50)	
Age	**-0.21**	0.083	0.16	0.28
Height	**0.27**	0.024	-0.06	0.70
WC	-0.10	0.393	**0.31**	0.03
Diastolic BP	-0.12	0.316	**0.33**	0.02
Systolic BP	-0.09	0.485	**0.26**	0.07
TC	0.07	0.558	**-0.24**	0.09
With MetS (105)	Men (61)		Women (44)	
Height	**0.23**	0.075	-0.21	0.18
Glucose	**0.31**	0.020	-0.21	0.17
Fibrinogen	**0.28**	0.061	0.04	0.83
SHGB	**-0.28**	0.064	-0.20	0.26
CRP	**0.23**	0.049	0.03	0.86

Table 3.3 Spearman correlation coefficients of ASP with certain variables according to gender

Spearman Correlation (r values) and significance level (p values) are provided according to metabolic disorder where BMI body mass index, BP blood pressure, C cholesterol, CRP C reactive protein, HT hypertension, MetS metabolic syndrome, SHBG sex hormone binding globulin, WC waist circumference. Values with $p<0.1$ are indicated in bold.

3.6 Discussion

The salient findings in this population-based cross-sectional study among middle-aged Turkish adults were: 1) Higher average ASP concentrations in comparison with those reported in other ethnicities. 2) Diverging correlations across genders between ASP levels on the one hand and TG, glucose and height among other risk variables.

The average concentration of ASP in all Turkish subjects in this study was generally higher (mean= 344.7±214.2, median=292.1 nmol/l) than in other studies (9). Factors such as obesity, metabolic disorders, ethnic and nutritional differences may all contribute to this (9). The ASP values measured here were higher than other ethnic groups such as Caucasian (12,31,32), African American (31), Pima Indian (12), Inuit from Nunavik (32) and Chinese (33) that have been measured so far, although the present group also contained a much higher percentage of known non-healthy subjects. ASP values were more similar to severely obese subjects (11). Further, there was a borderline sex difference (p=0.059) between men and women, which was not seen in a normal-weight North American population, although there were differences between severely obese men and women (11). As mentioned above, the present group has a high percentage of overweight and obese subjects as well as metabolic disorders (hypertension, metabolic syndrome, cardiovascular disease, type 2 diabetes). As mentioned above, previous studies show that obese subjects, T2D subjects and subjects with cardiovascular diseases have higher ASP levels (58-400%) vs. normal weight controls (9). A study by Yang et al. showed an increased ASP level in diabetic subjects even in the absence of obesity (15), and this was also true of women with polycystic ovary disease (18). All of these factors likely contributed to the higher ASP levels.

The level of ASP may also be influenced by the concentration of its precursors (9). ASP precursor components adipsin, C3, and factor B increase in obesity by approximately 30% for C3, 45% for factor B, and 37% for adipsin, however, small changes in substrate (C3) and enzyme (adipsin) may produce much larger changes in product (ASP) (34). In several studies, C3 mRNA expression has been reported to be increased in older vs. younger subjects and in omental vs. subcutaneous tissue (9).

A number of studies have demonstrated that plasma ASP correlates positively with various indices of body size (9). This includes BMI and percentage ideal body weight (17,35), waist to thigh or waist to hip ratio (33), total fat mass or percentage body fat (17,36). In this study, BMI in non-healthy men had a significant positive correlation with ASP concentration and waist circumference, in women (without metabolic syndrome), this also correlated. Height was a body size marker that consistently correlated significantly with ASP in men, regardless of the group tested. Stepwise multiple forward analysis in this study indicated that height was an independent covariate of ASP only in men (results are not shown). In Caucasian population studies, it has been shown that height has an inverse association with CVD (37). However, in rapidly developing populations, the protective effect of height on cardiovascular mortality or its risk factors is less obvious (38). In an Asian (Hong Kong) population, height was inversely associated with increased blood pressure and raised fasting plasma glucose but only after adjustment for central obesity (38).

In both the present study and other studies, ASP consistently correlated with plasma lipid profile, factors commonly related to metabolic syndrome, diabetes and cardiovascular disease. Whether the increase in plasma ASP is a cause or a consequence of abnormal lipid metabolism cannot be determined simply by correlation studies, but examination of ASP function is supportive of linkages. It has been proposed that a decrease in the ASP cell-surface receptor concentration or ASP response are related to hyperapoB, and that an elevated plasma ASP in patients with hyperapoB identifies individuals likely to have reduced cellular response to ASP stimulated peripheral TG synthesis and glucose transport (39). Further, there are strong supports that ASP binding and subsequent response may be a significant factor in determining regional differences in fat distribution (40).

Enhanced ASP action in subcutaneous tissue can lead to an increase in triglyceride storage. Impaired ASP action in visceral adipose tissue, however, can contribute to decreased efficiency of triglyceride storage and increase circulating fatty acid fluxes, leading to metabolic imbalance and disorders that are commonly associated with visceral obesity, such as diabetes and cardiovascular disease (40). Reduced adipose tissue response to ASP could contribute to an increased fatty acid flux, which leads to stimulation of

hepatic apolipoprotein B lipoprotein production, explaining the associations between plasma ASP and lipid parameters (9). With the identification of an ASP receptor, C5L2, in adipose tissue and other insulin-sensitive tissues (41) the presence of chronically elevated levels of ASP, in conjunction with insulin resistance and plasma lipid abnormalities, would be consistent with the hypothesis of 'ASP resistance' (14,42).

Correlation between plasma ASP and other factors was different between men and women, and sex hormones could play a partial role in this. Overall, plasma ASP levels in normal weight girls were significantly higher than normal weight boys (33). An in vitro study by Wen et al. (43) indicated that physiological concentrations of progesterone as well as high concentrations of testosterone induce ASP resistance in both adipocytes and preadipocytes, although other explanations are possible. While ASP may be increased as a response to the insulin resistance, some cellular studies support the interpretation that the alteration in ASP pathway may be a direct response to the effect of the sex steroid hormones on downregulation of C5L2. For instance, physiological increases in sex steroid hormones in PCOS (polycystic ovary syndrome) and late pregnancy likely induce decreased C5L2 expression and signaling, leading to ASP resistance (18,44).

A study by Xia and Cianflone (34) demonstrated marked changes in expression of factors involved in ASP generation associated with increased obesity that were very different in men and women. Specifically, with increased BMI, in women there was a decreased expression of C3 and adipsin, while in men there was increased expression of C3, factor B, and adipsin. In women, subcutaneous tissue was the primary target, while in men visceral tissue was more often affected. These results also demonstrated a greater relative expression in visceral than subcutaneous tissue with development of obesity in both men and women. Koistinen et al. demonstrated an increase in C3 mRNA in adipose tissue from obese men compared to lean men (42). While these obese men only demonstrated a slight but not significant increase in plasma C3, they did have a substantial increase in plasma ASP. Interestingly, the level of C3 mRNA correlated inversely with glucose disposal rate but positively with BMI and postprandial triglyceride clearance (42).

Other divergent gender related associations have also been noted in this Turkish population. Gender influences the pro-inflammatory response to overall adiposity, central obesity and associated impaired function of HDL, with women affected to a greater extent (45). Association of inflammatory mediators is largely independent of MetS components in men, but tends to act in conjunction in women (45). Gender modulates response of cardiometabolic risk variables to moderate alcohol consumption and smoking status (45). Aggregation of Lp(a) to apoA1 to form an immune complex contributes as a CHD risk factor (45) and gender divergence in Lp(a) and LP-PLA2 has also been noted in this population (25). This inverse correlation, particularly in women, is in line with an enhanced role of pro-inflammatory status/oxidative stress in the pathogenesis of cardiovascular risk in Turkish women compared with men, and might relate to various HDL parameters (HDL, apoA1, apo CIII) (25). The present findings pertaining to divergent associations in genders of fasting glucose, triglycerides and height (and circulating total testosterone) could be consistent with a notion that ASP is related to variations in Lp (a) and LP-PLA2 (data not shown) and possibly HDL and related parameters, reflecting a pro-inflammatory state.

This population-based study has potential limitations in being cross-sectional in design wherein the causal role of ASP in cardiometabolic risk cannot be assessed. Secondly, the sample size for some cardiometabolic disorders (CHD, T2D) was less than adequate and precluded correlation analyses in subgroups. On the other hand, the availability of diverse lipid and non-lipid parameters, and identification of various cardiometabolic disorders in a population-based study comprising adults prone to enhanced low-grade inflammation, form its strength.

We conclude that ASP levels not only reflect dysglycemia and atherogenic dyslipidemia in men but also other inflammation and anti-inflammatory biomarkers as well as height. The addition of MetS marginally accentuates these associations. In contrast, in women, correlations with ASP (notably triglycerides, glucose, LDL-C and height) are in the opposite direction, as are those with serum Lp (a) (data not shown), already in the absence of current definitions of MetS.

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Chapter 4

Cross sectional associations of Acylation Stimulating Protein (ASP) and adipose tissue gene expression with estradiol and progesterone in pre and postmenopausal women

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4.1 Résumé

Les hormones stéroïdiennes jouent un rôle important dans le métabolisme du gras et l'obésité. Nous avons donc proposé une implication entre la protéine stimulant l'acylation (ASP) et les hormones ovariennes. On a donc mesuré, chez 392 femmes âgées de 18 à 69 ans (avec un IMC entre 17 et 90 kg/m^2), les niveaux plasmatiques d'ASP, hormones sexuelles, glucose, adiponectine ainsi que les lipides et apolipoprotéines, en plus des facteurs déterminants du syndrome métabolique. Dans un sous-groupe, on a aussi mesuré l'expression génique du précurseur de l'ASP (C3) et des récepteurs qui y sont reliés; C5L2, C3aR et C5aR dans les tissus adipeux viscéral et sous-cutanés. L'ASP corrélait de façon négative avec l'estrogène ($p<0.0001$), le progestérone ($p<0.05$), l'adiponectine ($p<0.001$) ainsi que l'ApoA1 ($p<0.001$) alors qu'elle corrélait positivement avec les niveaux d'ApoB ($p<0.001$), la tension artérielle systolique ($p<0.001$), le tour de taille ($p<0.001$) et les triglycérides ($p<0.05$). Chez des sous-groupes de personnes non-obèses (maigre et surpoids), obèses mais métaboliquement saines (MHO) et obèses avec syndrome métabolique (MSO), on a observé une augmentation séquentielle des niveaux d'ASP ($p<0.001$) alors que ceux d'adiponectine ($P<0.0001$) et d'estradiol ($p<0.001$) diminuaient et que la progestérone restait similaire. D'un autre côté, la progestérone corrélait positivement avec l'expression génique de C3 dans le tissu viscéral ($p<0.05$) et négativement avec l'expression de C5L2 au niveau viscéral ($p<0.01$) et sous-cutané ($p<0.05$). Ces résultats soulignent le concept que les hormones sexuelles affectent de façon différente les niveaux d'ASP et l'expression génique dans le tissu adipeux et ceci, en fonction du dépôt adipeux.

4.2 Abstract

Sex steroid hormones play an important regulatory role in fat metabolism and obesity. Objective: We hypothesized involvement of interactions between ovarian hormones with Acylation Stimulating Protein (ASP). Design, Patients and measurements: In 392 women with wide age (18 to 69 years) and body size (BMI: 17 to 90 kg/m2) ranges, fasting plasma levels of ASP, ovarian hormones, glucose, adiponectin and lipids/apolipoproteins were assessed, along with determination of metabolic syndrome (MS) features. Gene expression of C3 (ASP precursor) and related receptors C5L2, C3aR and C5aR in subcutaneous and omental adipose tissues were measured in a subset. Results: ASP correlated negatively with concentrations of estradiol ($p<0.0001$), adiponectin ($p<0.001$) and apolipoprotein A1 ($p<0.001$) and positively with apolipoprotein B levels ($P<0.001$), systolic blood pressure ($p< 0.001$), waist circumference ($p<0.001$), and triglyceride concentrations ($p<0.01$). In aged-matched groups of lean, overweight, metabolically healthy obese (MHO) and obese with metabolic syndrome (MSO), there was a stepwise increase in ASP levels ($p<0.001$) while concentrations of adiponectin ($p<0.0001$) and estradiol ($p<0.001$) but not those of progesterone decreased. Progesterone but not estradiol levels correlated positively with C3 gene expression in omental adipose tissue ($p<0.05$) and negatively with C5L2 expression in both omental ($p<0.01$) and subcutaneous ($p<0.05$) adipose tissues. Conclusion: Our results are consistent with the concept that sex hormones differentially influence circulating ASP and adipose tissue gene expression of its related proteins in a depot-specific manner. ASP may play a role in the regulation of regional fat metabolism through interactions with sex hormones in women.

4.3 Introduction

Obesity is recognized as an important risk factor for metabolic syndrome, insulin resistance, dyslipidemia, Type 2 diabetes and cardiovascular disease. However, there can also be a clustering of cardiometabolic abnormalities among normal-weight individuals and, conversely, some overweight and obese individuals are considered metabolically healthy. Adipose tissue plays a central role in glucose and lipid metabolism [1]. The adipocyte secretes a variety of bioactive molecules, collectively termed adipokines, which mediate many autocrine and paracrine functions [1]. Adipose tissue accumulates in numerous depots, primarily grouped as subcutaneous and visceral depots with distinctive characteristics [2]. The differential expansion of these depots is of importance because the association with metabolic disorders is stronger for visceral than for subcutaneous [2], possibly because of proximity to the liver, or possibly because different adipose tissue depots express adipokines at different levels (reviewed by Tchernof A and Després JP, 2013) [2]. Moreover, differences in gene expression profiles or in biochemical and metabolic properties may also contribute to excess visceral adipose tissue being preferentially associated with metabolic dysfunction [2].

Acylation Stimulating Protein (ASP) is an adipokine that is identical to C3adesArg, a byproduct of innate immunity activation. It is derived from the cleavage product of complement C3 [3]. Cleavage of C3 generates C3a, which binds the C3aR receptor. C3a is then desarginated by carboxypeptidase to generate C3adesArg (ASP), which interacts with its receptor C5L2 [4]. ASP enhances lipid storage in adipocytes through stimulation of glucose uptake, fatty acid esterification, and lipoprotein lipase activity, effects that are independent and additive to insulin [3]. In addition to ASP, the C5L2 receptor also binds C5a and C3a. These proteins, which are structurally similar to ASP, also bind their classical receptors C3aR and C5aR, while C5L2 also hetero-dimerizes with C5aR. All three receptors have roles in immunity, as well as recently identified roles in energy homeostasis and lipid metabolism [5].

In addition to adipokines, sex hormones are widely involved in the regulation of adipose tissue metabolism and have been reported to affect insulin sensitivity, glucose homeostasis and body weight [6]. Sex hormones have also been shown to influence adipokine

secretion and adipose tissue function [6-8]. Studies have demonstrated that with decreased production of sex hormones there is an increase in the mass of abdominal, visceral adipose tissue [9], associated with increased risk factors, which have an important impact on women's health [2]. Ovarian hormones appear to be protective against the metabolic syndrome; prior to menopause, women have much fewer obesity-related metabolic disorders, and the prevalence of these metabolic disorders increases in women after menopause.

Estradiol, the predominant natural form of estrogen during reproductive age, is involved in many physiological functions, including development, growth, energy homeostasis, and reproductive physiology [10]. Estrogen-action is mediated by two specific estrogen receptors (ER) with possible effects on lipogenic enzymes such as lipoprotein lipase and hormone sensitive lipase [11]. An anti-lipolytic function for ER-α, specifically in subcutaneous adipose tissue, has been reported [12]. Progesterone plays an important role in ovulation, reproduction and in mammary gland growth.

The progesterone receptor has been identified in many tissues/organs including uterus, ovaries, mammary glands, bones, and central nervous system as well as adipose tissue [13]. The role of progesterone in energy homeostasis, lipid metabolism and fat distribution has been investigated in animal and human studies. Progesterone administration in female rats increased subcutaneous adipose tissue and altered gene expression of adipokines involved in energy homeostasis regulation [14]. Castrogiovanni et al. demonstrated that although the lipogenic effect of an obesogenic diet remained or was enhanced after previous progesterone administration, the metabolic, endocrine and adipose tissue dysfunction induced by diet could be prevented with high-dose progesterone treatment [15]. Furthermore, a specific stimulatory effect of progesterone on lipogenic enzyme gene expression in subcutaneous adipose tissue in female rats has been shown in a recent study [16]. Adipose tissue also has the enzymatic machinery to inactivate progesterone [17]. Although not completely consistent, *in vivo* and *in vitro* data have suggested that adipose tissue may represent a significant target organ for progesterone action affecting adipose tissue lipid metabolism and fat distribution, much like androgens and estrogens [1]. In human studies, a higher energy intake during luteal vs follicular phase was demonstrated [18]. Very recently, a randomized control trial by Prior et al. demonstrated that administration of oral micronized progesterone for three months to healthy early postmenopausal women was

neutral towards cardiovascular and inflammatory markers (such as BMI, glucose, blood pressure), but tended to improve endothelial function [19].

Previous human studies have shown gender and regional differences in ASP levels and function (both binding and activity) in adipose tissue in mice and humans (reviewed by Saleh J. 2011 [20]). As progesterone levels were found to be closely and significantly linked to plasma ASP levels *in vivo* [20], cell studies were conducted to evaluate sex hormone effects on ASP response *in vitro*. Adipocytes incubated with high level progesterone and estradiol rapidly demonstrate a dose-dependent inhibition of ASP function [7;8] and decreases in C5L2 expression [7]. However the effect of menopausal status coupled to obesity on circulating ASP levels and gene expression of its related receptors in omental and subcutaneous adipose tissues with a wide range of BMI has not been evaluated. Given the published data, we hypothesized that both circulating ASP levels and related gene expressions would vary as a function of sex hormone levels in women.

4.4 Methods

4.4.1 Study subjects

Blood samples were obtained from three sources: I) CRIUCPQ (Centre de Recherche Institut Universitaire de Cardiologie et de Pneumologie de Québec) Tissue Bank (http://www.criucpq.ulaval.ca/index.php/en/tissue-bank): 253 women (all severely obese) who were undergoing bariatric surgery with biliopancreatic diversion with pre-operative fasting blood samples and adipose tissue samples taken at the time of bariatric surgery (sub-group only) II) the Gynecology Unit, Laval University Medical Center: 41 women who were candidates for elective surgery (total abdominal hysterectomy or right/left and bilateral salpingo-oophorectomy) with pre-operative fasting blood samples and adipose tissue samples taken at the time of surgery (sub-group only) and III), Quebec Family Study (QFS): 98 women with fasting blood samples available. For the QFS study, families of French descent (Caucasians) were recruited with three cycles of data collection between 1979 and 2002 in Quebec City, to participate in a longitudinal prospective cohort study to examine genetic determinants of fitness, obesity, and cardiovascular and diabetes risk

factors. The design of QFS and methods are described elsewhere [21] and the results have been recently reviewed by Chaput et al [22].

All subjects (from all three sources) met the following eligibility criteria for inclusion in the present evaluation: adult women, no ovariectomy, no lipid lowering medication, no diabetes, and fasting blood sample availability. All blood samples in first and second groups were obtained before surgery. Altogether, the analysis group included 392 women, aged between 18-69 years old, and all data were pooled prior to analysis. Among these subjects, there were 139 individuals from the first and second group with frozen subcutaneous and omental adipose tissue samples available for gene expression analysis. The ethics committee of the CRIUCPQ approved the experimental protocol. Laval University Medical Center approved the study of subgroup II. The QFS study was previously approved by the Medical Ethics Committee of Laval University. All participants provided written informed consent for participation in medical research.

4.4.2 Study design and criteria for metabolic syndrome

Individuals were classified based on BMI as lean (BMI<25kg/m^2), overweight (25kg/m^2 <BMI<30kg/m^2) and obese (BMI≥30kg/m^2). Obese subjects were further divided according to presence (metabolic syndrome obese, MSO) or absence (metabolically healthy obese, MHO) of metabolic syndrome or according to the number of metabolic syndrome features from 0=having no feature to 5= having all 5 features. Metabolic syndrome was defined according to the US National Cholesterol Education Program Adult Treatment Panel III guidelines and modified as recommended in the latest American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement [23] with a lower cutoff for fasting glucose (5.6 mmol/L). Metabolic syndrome (MSO) was defined as having 3 of the following metabolic risk factors: (1) central obesity (waist circumference >88 cm in women), (2) hypertriglyceridemia (fasting triglycerides >1.69 mmol/L, (3) low HDL cholesterol (fasting HDL<1.29 mmol/L in women), (4) glucose intolerance (fasting glucose >5.6 mmol/L, and (5) hypertension (sitting blood pressure 130/85 mm Hg obtained as a mean of two readings taken after resting for at least 10 minutes or on regular antihypertensive medications). MHO was defined as having ≤ 2 metabolic risk factors.

4.4.3 Clinical and Plasma Measurements

Clinical Assessment: Anthropometric measurements (height, weight, waist circumference, hip circumference) and blood pressure were measured on the day before or on the morning of surgery/blood sampling. Premenopausal and postmenopausal status was determined based on self-reporting by the subject.

Plasma analysis: Blood samples were obtained from participants after overnight fasting and collected into EDTA-containing tubes. The hospital clinical biochemistry laboratory measured fasting plasma glucose, triglyceride, total cholesterol, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), apolipoprotein B (Apo B), and apolipoprotein A1 (Apo A1) according to standardized clinical methodology. The following assays were measured in the research laboratory for all samples: estrone and estradiol (RIA, Beckman Coulter Canada LP, Mississauga, ON), adiponectin (RIA, Millipore, Billerica. MA, USA), progesterone (ELISA, ALPCO, Salem, NH, US) and C-reactive protein (CRP), ELISA, Ray Biotech, Burlington, ON) following instructions of the manufacturers. ASP concentration was measured using an in-house sandwich ELISA, following previously-published methodology [24].

4.4.4 Adipose tissue Sampling and Analysis:

Biopsies: Adipose tissue biopsy samples were obtained from subcutaneous and omental depots during bariatric or gynecological surgery. Adipose tissue samples were washed with sterile Krebs-Ringer-HEPES buffer, placed in liquid nitrogen, and then immediately transported and stored at -80^{o}C.

RNA Extraction and Real Time qPCR Analysis: All samples (maximum 100 mg adipose tissue) were homogenized in Qiazol (Qiagen Inc, Mississauga, ON, Canada). Total RNA was extracted from homogenates using the RNeasy Plus Universal Mini Kit (Qiagen Inc.) according to the manufacturer's instructions. From the total amount, 0.1 μg of purified RNA was retrotranscribed to cDNA using a QuantiTec® Reverse Transcription Kit (Qiagen Inc.) with a final volume of 20 uL. Genomic DNA contamination was eliminated by DNase treatment included in QuantiTec® Reverse Transcription Kit. For real-time PCR

evaluation of gene expression, 1 ul of cDNA was used for each reaction. RT2 SYBR® Green qPCR Master Mix (Qiagen Inc.) was used and a 3-step PCR was performed using CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, ON, Canada), using the following protocol: an initial denaturation step at 95°C for 10 minutes, 39 cycles of 95°C for 15 s, 55° C for 10 40 s, 72°C for 30 s was followed by a final extension step of 95°C for 10 s and melt curve 65°C to 95° C. Real time RT-PCR was performed to quantify human C3, C3aR, C5aR, and C5L2 relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). C5L2 (GPR77: QT00243971) and C3 (Hs_C3_2_SG, NM_000064) primers were purchased from Qiagen (QuantiTect Primer Assay, Qiagen, Mississauga, ON, Canada). The sequences for the GAPDH, C3aR and C5aR primers obtained from Alpha-DNA (Montreal, Canada) were: C3aR1-Right; 5′-AGCAGAGAAAGACGCCATTG-3′, C3aR1-Left; 5′-ACTGTGGCTAAGTGTGGGGA-3′, C5R1-Right; 5′- TATCCACAGGGGTGTTGAGG-3′, C5R1- Left; 5'-GCCCAGGAGACCAGAACAT–3′, and GAPD-Right; 5′-AATGAAGGGGTCATTGATGG-3′, GAPD-Left; 5′-AAGGTGAAGGTCGGAGTCAA-3′. For data analysis the ΔΔCt method was used, as performed with Bio-Rad CFX manager software (version 1.5)(Bio-Rad Laboratories). All procedures followed Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines including specificity, appropriate controls and assay performance [25].

4.5 Statistics

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA, USA) or Sigmastat 3.5 (Systat Software Inc, San Jose, CA, USA). Descriptive parameters are provided as mean ± SEM or in percentages. For non-normally distributed parameters, values were log-transformed for statistical analysis. Normality was assessed by Shapiro-Wilk test. Two sided t-tests were used to analyze the differences between means and proportions of two groups. The 95% confidence interval for the difference between two means was used to specify a range of values within which the difference between the means of the two populations may lie. One-way ANOVA analyses were used to compare more than two groups (as indicated). Spearman rank correlation coefficient was used for evaluating the degree of linear association or correlation between

two independent variables, to be conservative given the use of multiple statistical tests. A $p<0.05$ was considered statistically significant for all analyses

4.6 Results

4.6.1 Relationship of ASP with sex hormones, anthropometric values and metabolic profile

Characteristics of the participants are given in Table (4.1). The subjects covered a wide age range (18-69 years, average age 41 years old) and BMI (16.3 to 89.9 kg/m^2), with 30% postmenopausal. Plasma ASP ranged from 2.8 to 125 nmol/L and correlated with estradiol ($r=-0.24$, $p<0.001$), a correlation that was maintained when the postmenopausal women were excluded (Figure 4.1A, premenopausal $n=274$, $r=-0.23$, $p<0.001$). While there was a weak correlation of ASP with progesterone ($r=-0.14$, $p<0.05$) this was lost when postmenopausal women were excluded. ASP also correlated with adiponectin ($p<0.001$) and CRP ($p<0.0001$) as well as with most of the metabolic syndrome criteria including waist circumference ($p<0.001$), BMI ($p<0.0001$), blood pressure (systolic and diastolic; both $p<0.01$) and triglyceride levels ($p<0.01$) and other circulating lipid parameters Apo B, Apo A1, and Apo A1/B ratio ($p<0.0001$, $p<0.001$ and $p<0.0001$, respectively) (Table 4.1) correlations which remained significant with exclusion of postmenopausal women (data not shown).

The subjects were grouped by plasma ASP quartiles to evaluate the associations with metabolic and lipid parameters and adiponectin. There was a decrease in sex hormones across the four groups (estradiol $p<0.001$; progesterone $p<0.05$, ANOVA with trend) although there was no difference in age across these groups. As well, Apo A1 and adiponectin decreased (Figure 4.1B, C), while CRP and ApoB increased (ANOVA $p<0.001$, Figure 1B, C). Metabolic syndrome parameters including systolic blood pressure ($p<0.001$), diastolic blood pressure ($p<0.05$), BMI ($p<0.05$), waist circumference ($p<0.001$), and triglyceride concentrations ($p<0.05$) increased significantly across ASP groups (Figure 4.1D). Exclusion of postmenopausal women did not change the results (data not shown).

Table 4.1 Baseline characteristics, anthropometric measurements, and metabolic analysis of participants.

							ASP correlations	
	Parameters	Mean	SEM	MIN	MAX	95% CI of Mean	r	p value
	Age (yrs)	41.2	0.5	18.3	69	40.0 - 42.2	0	ns
Metabolic syndrome criteria	**Waist Circumference (cm)**	123	2	58	195	120 - 127	0.22	P<0.001
	BMI (kg/m^2)	41.9	0.70	16.8	89.9	40.7 - 43.6	0.26	P<0.0001
	Systolic BP (mmHg)	130	1	85	188	128 - 132	0.21	P<0.01
	Diastolic BP (mmHg)	79.6	0.70	29	185	78.1 - 80.6	0.17	P<0.01
	Glucose (mmol/L)	5.5	0.10	3.6	15.4	5.35 - 5.62	0.10	ns
	Triglyceride (mmol/L)	1.53	0.05	0.45	4.48	1.46 - 1.61	0.16	P<0.01
	HDL-C (mmol/L)	1.34	0.02	0.36	2.45	1.31 - 1.37	-0.06	ns
Circulating parameters	**Total Cholesterol (mmol/L)**	4.99	0.05	1.52	15.1	4.88 - 5.09	0.09	ns
	LDL-C (mmol/L)	2.94	0.04	0.75	6.29	2.86 - 3.02	0.08	ns
	Apo B (g/L)	0.89	0.02	0.23	2.35	0.84 - 0.92	0.22	P<0.0001
	Apo A1 (g/L)	1.10	0.02	0.06	1.95	1.02 - 1.10	-0.20	P<0.001
	Apo A1/B	1.43	0.04	0.05	4.58	1.36 - 1.51	-0.26	P<0.0001
	ASP (nmol/L)	26.6	0.90	2.8	125	24.4 - 27.7		
	Adiponectin (µg/ml)	15.7	1	1	112	13.7 - 17.7	-0.23	P<0.001
	CRP (mg/L)	8.64	0.49	0.15	34.8	7.67 - 9.61	0.30	P<0.0001
Ovarian hormones	**Estradiol (pmol/L)**	184	9.97	18.4	963	164 - 203	-0.24	P<0.0001
	Estrone (pg/ml)	71.2	2.70	9.10	322	65.9 - 76.6	0.10	ns
	Progesterone (nmol/L)	6.70	0.38	0.25	48.9	5.95 - 7.44	-0.14	P<0.05

Spearman rank correlation coefficients of ASP with variables. APO; Apolipoprotein, BMI; Body Mass Index, BP; blood pressure, CRP; C-reactive protein, HDL-C; high density lipoprotein-cholesterol, LDL-C; low density lipoprotein-cholesterol. Ranges represent minimum to maximum values. Results are expressed as mean ± SEM for n=392 women, $p<0.05$ is considered significant, where p ns indicates not significant.

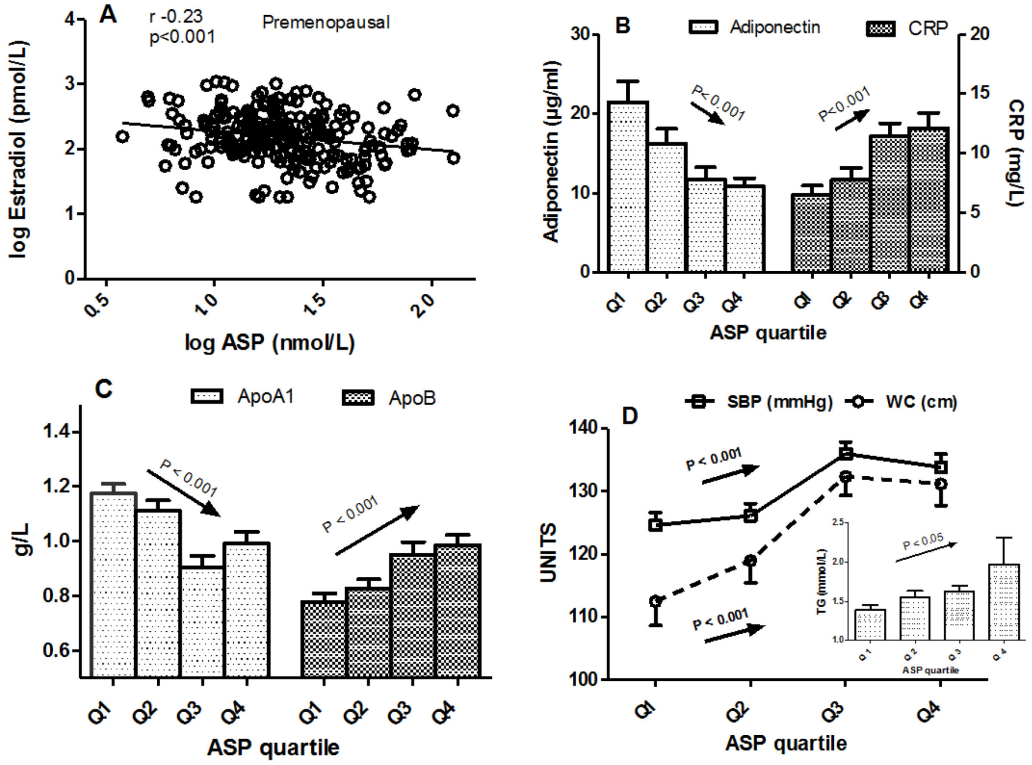

Figure 4.1 Association of acylation stimulating protein (ASP) with ovarian hormones and metabolic profile.

A) Linear plot correlations of estradiol with ASP plasma levels in premenopausal women. (B) adiponectin (right *y*-axis), C-reactive protein (CRP) (left *y*-axis) and (C) Apolipoprotein A1 (ApoA1), Apolipoprotein B (ApoB) plasma levels in subjects divided based on ASP quartile (pre- and postmenopausal women). (D) Metabolic syndrome parameters: systolic blood pressure (SBP), waist circumference (WC) and triglyceride (TG, inset) in ASP quartile groups (pre-and postmenopausal women). Statistical differences were analysed with one-way anova test for parametric data and Kruskal–Wallis for nonparametric values where $P < 0{\cdot}05$ is considered as significant. Results are expressed as mean ± SEM.

4.6.2 Comparison of adipokines and sex hormones with metabolic profiles between Lean, Overweight, and Obese MHO and MSO groups:

Subjects were then separated into four groups: (i) Lean subjects (BMI<25 kg/m^2 (ii) Overweight subjects (25kg/m^2 <BMI<30kg/m^2) (iii) Metabolically healthy obese subjects (MHO: BMI>30, MS score ≤ 2) and (iv) Obese subjects with metabolic syndrome (MSO: BMI>30, MS score ≥3). The group characteristics are given in Table 4.2. As shown in Figure 4.2 (A and B) the trend from Lean to Overweight to MHO to MSO for adipokines and sex hormones indicated that ASP increased overall (Figure 4.2A, $p<0.001$) while adiponectin decreased ($p<0.0001$). This trend was also seen with estradiol, which decreased (Figure 4.2B, $p<0.001$), but not with progesterone (Figure 4.2B) or estrone (data not shown). One-way ANOVA P values for waist circumference, BMI, systolic BP, diastolic BP, glucose, TG, HDL-C, Apo A1 and Apo A1/B ratio were all significant (Table 4.2). Individual comparisons (ANOVA post-hoc test and 95% confidence intervals (95%CI) indicated that MHO, in comparison with the Lean group, had significantly higher levels of ASP ($p<0.05$) but lower concentrations of estradiol ($p<0.05$) and adiponectin ($p<0.001$), although there was no difference in age (Figure 4.2B). There was also no difference in estrone (data not shown) and progesterone (Figure 4.2B). In the MSO group, ASP was higher, while adiponectin and estradiol were lower as compared to Lean, although there was no difference in age (Figure 4.2A and B). As expected, several differences were found in metabolic parameters in the MHO and MSO groups, which were significantly different from the Lean group (Table 4.2).

Spearman correlations among study parameters in the three individual groups showed that the associations of ASP with estradiol in Lean vs Overweight vs MHO vs MSO was progressively stronger (Lean $r=-0.19$, $p=0.2$, Overweight $r=0.16$, $p=0.5$, MHO $r=-0.27$, $P<0.05$ and MSO $r=-0.35$, $P<0.001$). In the MSO group, ASP correlated with Apo B levels ($r=0.26$, $p<0.0001$), the Apo A1/B ratio ($r=-0.24$, $p<0.01$) and progesterone concentrations ($r=-0.19$, $p<0.05$).

Table 4.2 Comparison of Non-Obese, Metabolically Healthy Obese (MHO) and Obese with Metabolic Syndrome (MSO) groups.

	Lean (N=71)		Overweight (N=45)		MHO (N=60)		MSO (N=210)				
Pre/Post-Menopausal	**63/3**		**40/5**		**46/14**		**144/66**			**MSO vs Lean**	**MSO vs MHO**
Parameters	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**ANOVA**	**95% CI**	**95% CI**
Age (yrs)	41	1.29	46	1.38	40	1.32	41	0.76	ns	_	_
Waist Circumference (cm)	74.1	0.89	86.9	1.27	134**	3.43	144**††††	1.25	p<0.0001	-74.1 to -65.4	-15.9 to -4.32
BMI (kg/m^2)	22.2	0.21	27.3	0.23	45.5***	1.4	50.4***††††	0.63	p<0.0001	-30.4 to -26.1	-7.68 to -2.19
Systolic BP (mmHg)	112	1.94	117	1.7	123***	1.46	141***††††	1.06	p<0.0001	-33.7 to -25.3	-22.6 to -14.2
Diastolic BP (mmHg)	68.3	1.28	71.1	1.44	74.7***	0.92	86.5***††††	0.88	p<0.0001	-21.5 to -14.9	-15.1 to -8.40
Glucose (mmol/L)	5.08	0.07	5.02	0.09	5.3*	0.11	5.79**	0.12	p<0.01	-1.11 to -0.31	_
Triglyceride (mmol/L)	1.19	0.06	1.45	0.09	1.28*	0.08	1.73**†	0.05	p<0.0001	-0.74 to -0.35	-0.52 to -0.04
LDL-C (mmol/L)	2.75	0.08	3.32	0.11	2.81	0.13	2.95*	0.02	ns	-0.40 to -0.00	_
HDL-C (mmol/L)	1.46	0.04	1.41	0.04	1.41	0.03	1.26**††††	0.05	p<0.0001	0.12 to 0.27	0.07 to 0.22
Total Cholesterol (mmol/L)	4.73	0.10	5.48	0.11	4.82	0.26	4.96	0.06	ns	_	_
Apo B (g/L)	0.89	0.02	1.04	0.03	0.77*	0.04	0.89†	0.03	ns	_	-0.24 to -0.00
Apo A1 (g/L)	1.35	0.02	1.40	0.03	1.06**	0.05	0.9**††	0.03	p<0.0001	0.35 to 0.54	0.05 to 0.26
Apo (A1/B)	1.59	0.05	1.41	0.05	1.67	0.11	1.32*††	0.06	p<0.01	0.06 to 0.47	0.11 to 0.59
CRP (mg/L)	1.92	0.36	2.10	0.30	10.3***	1.33	14.39***††	0.64	p<0.0001	-14.2 to -10.7	-6.73 to -1.43

Statistical differences were determined by one-way ANOVA. APO; Apolipoprotein, BMI; Body Mass Index, BP; blood pressure, CRP; C-reactive protein, HDL-C; high density lipoprotein- cholesterol, LDL-C; low density lipoprotein-cholesterol, Results are expressed as mean ± SEM, where *p<0.05, **p<0.001, ***p<0.0001, vs Lean group, and †p<0.05, †† p<0.01, †††p<0.001, ††††p<0.0001 vs MHO group and p ns indicates non-significan

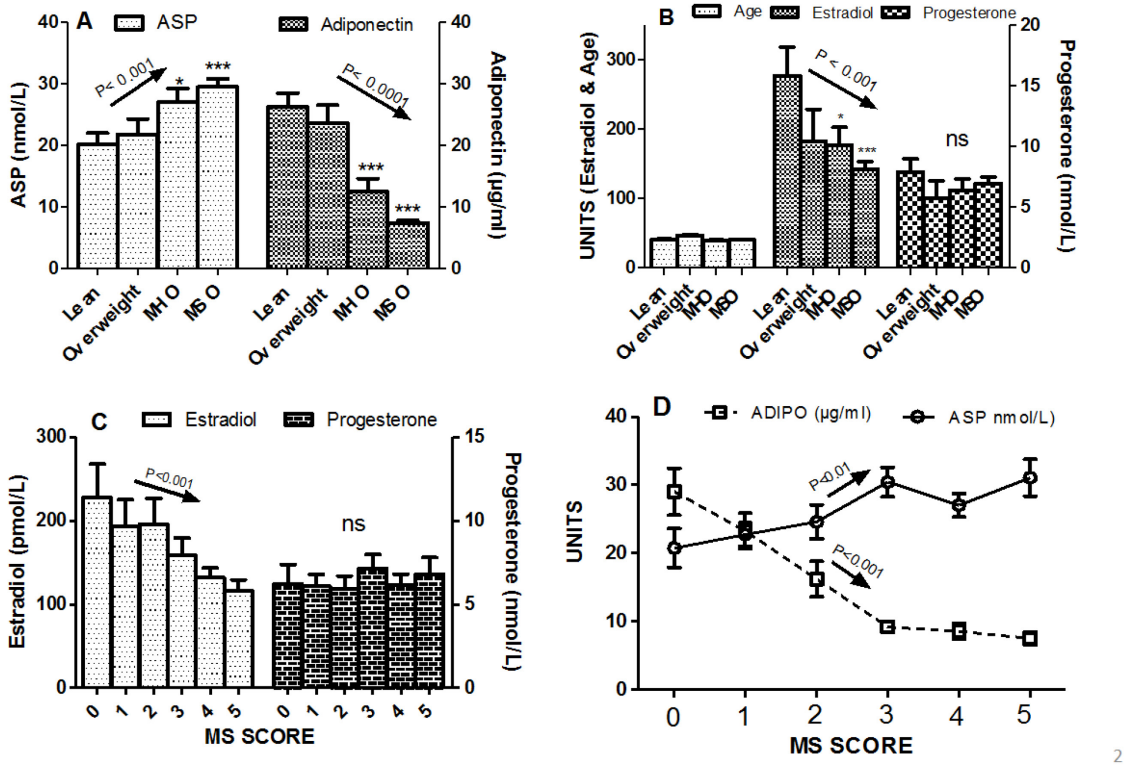

Figure 4.2 Acylation stimulating protein (ASP), adiponectin and ovarian hormone levels in subjects based on obesity and metabolic syndrome

(A, B) Circulating levels of ASP, ovarian hormones, adiponectin and age in lean, overweight, metabolically healthy obese (MHO) and obese with metabolic syndrome (MSO) groups assessed by one-way anova. (C,D Circulating levels of ASP, adiponectin (ADIPO) and ovarian hormones (estradiol and progesterone) in groups separated based on the number of diagnostic metabolic syndrome (MS) criteria met by individuals where no metabolic syndrome is present indicated as a score ≤2 (MS−) and Metabolic Syndrome (MS+) is a score ≥3. Statistical differences were determined by one-way anova where $P < 0{\cdot}05$ is considered significant. Results are expressed as mean ± SEM where $*P < 0{\cdot}05$, $***P < 0{\cdot}001$ and P ns indicates not significant.

4.6.3 Association of metabolic syndrome score with adipokines and sex hormones

The relationship with metabolic syndrome was further examined evaluating the individual MS score (number of MS features) with sex hormones and adipokines (Figure 4.2C and D). Overall, ASP levels increased ($p<0.01$) with increasing MS score, while adiponectin ($p<0.001$) and estradiol ($p<0.001$) decreased with no change in progesterone and estrone (data not shown). These relationships remained with exclusion of the postmenopausal women (data not shown). Spearman rank correlation analysis showed a significant correlation of MS score with total cholesterol ($r=0.11$, $p<0.05$), Apo A1 ($r=-0.47$, $p<0.00001$) and Apo (A1/B) ($r=-0.33$, $p<0.00001$).

4.6.4 mRNA expression of C3 and receptors C5L2, C5aR and C3aR in adipose tissue

In a subset of subjects (n=139) with subcutaneous and omental adipose tissue samples, expression of ASP-related genes (ASP precursor protein complement C3, ASP receptor C5L2 and related receptors C3aR and C5aR) were evaluated for associations with sex hormones: estradiol, progesterone and estrone. The characteristics of this subgroup were comparable to the overall group (Table 4.1). Although no association was found between estradiol and mRNA abundance of these genes, progesterone correlated negatively with C5L2 in both omental and subcutaneous adipose tissue (Figure 4.3A and B). C3aR also correlated inversely with progesterone in subcutaneous adipose tissue, while C3 had a positive correlation with progesterone in omental adipose tissue (Figure 4.3C and D).

As the subjects ranged in BMI from 19.5 to 78.9 kg/m^2, the association with progesterone was further evaluated in Non-Obese (Lean and Overweight, n=35) vs Obese (n=104) subjects, which were then further divided into 2 equal groups based on progesterone (top 50%, bottom 50%) as shown in Figure 4.4. For C3aR and C5aR, expression in omental tissue was comparable for all 4 groups. However in subcutaneous tissue, there was a progressive decrease in expression levels with increasing progesterone and increasing obesity (Figure 4.4A and B). By contrast, for C3 there was no difference in the 4 groups in subcutaneous tissue, but increasing expression in both Non-Obese and Obese with higher progesterone (Figure 4.4D) while C5L2 mRNA expressions in both

Non-Obese and Obese subjects decreased significantly ($p<0.05$) in omental adipose tissue of the high progesterone groups (Figure 4.4C).

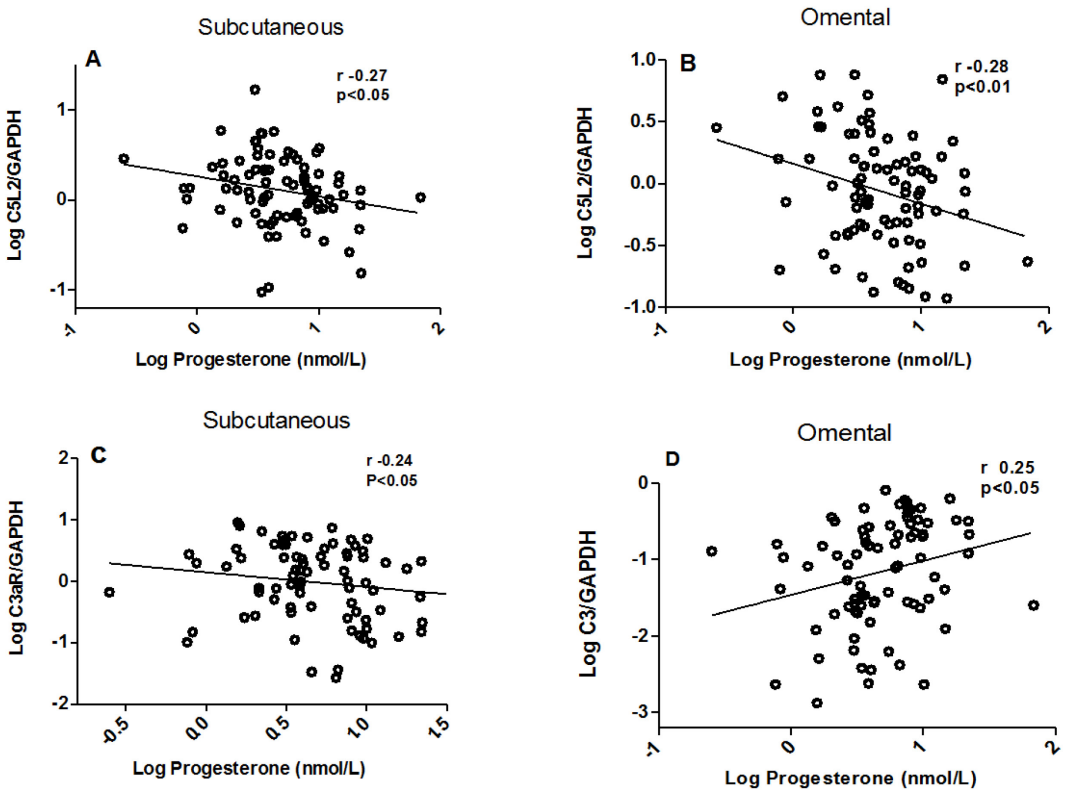

Figure 4.3 Correlation of circulating progesterone levels with gene expression of C3 and related receptors in subcutaneous and omental adipose tissue.

Linear plot correlation of progesterone levels with (A) subcutaneous C5L2 mRNA expression, (B) omental C5L2 gene expression (C) subcutaneous C3aR gene expression and (D) omental C3 expression in adipose tissue. Coefficients are given for Pearson correlation (r) where $p<0.05$ is considered significant.

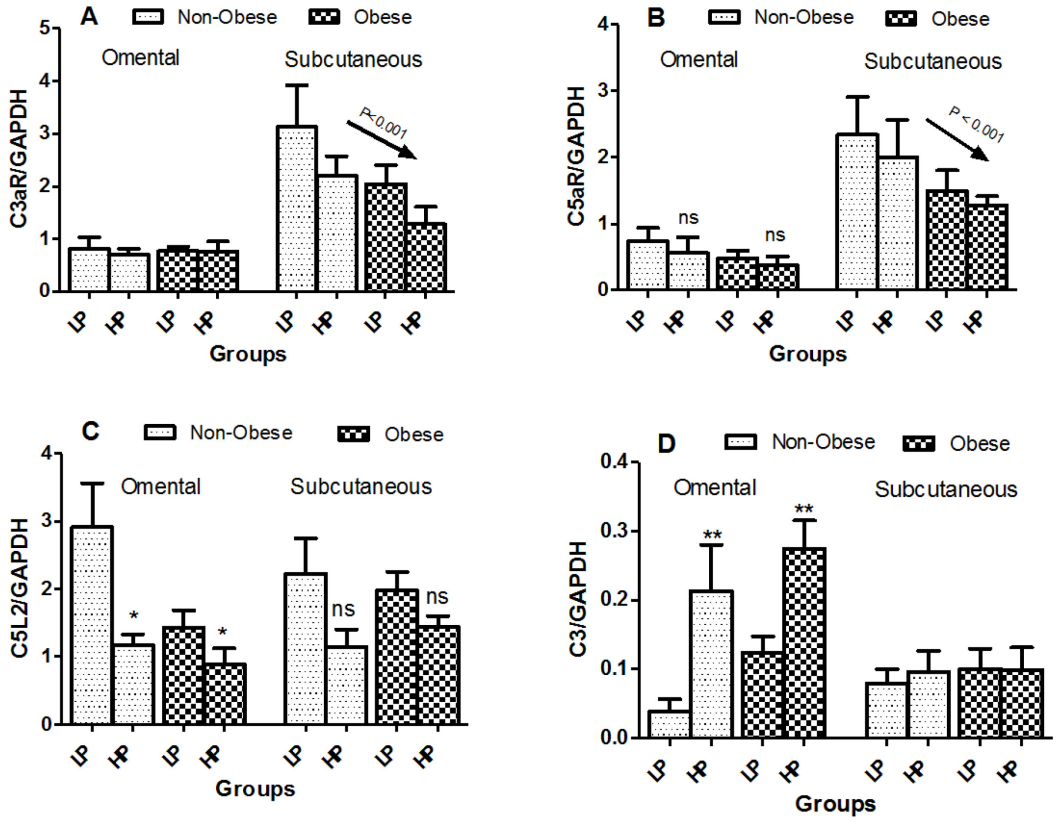

Figure 4.4 Gene expressions relative to progesterone in Non-obese and Obese subjects.

Groups were classified based on BMI into Non-Obese and Obese and further separated based on progesterone levels (high progesterone =HP and low progesterone =LP). Comparison of mRNA expression of A) C3aR B) C5aR C) C5L2 and D) C3 in both subcutaneous and omental adipose tissues. Statistical differences were determined by one-way ANOVA for more than 2 groups and Mann Whitney test for two groups (for non-parametric values) where $p<0.05$ is considered significant and p ns indicates not significant. Results are expressed as mean ± SEM.

4.7 Discussion

In this study, we investigated circulating ASP and its association with plasma levels of ovarian hormones and adiponectin as well as metabolic syndrome parameters among women with a wide range in age, body size, and ovarian hormone status (pre-menopausal and post-menopausal). Expression of relevant genes in omental and subcutaneous adipose tissues was evaluated in a subsample. Collectively, the results indicate: 1) an association of circulating ASP with the ovarian hormones estradiol and progesterone; 2) an association of progesterone but not estradiol with C3 and receptors C5L2, C5aR and C3aR gene expressions in adipose tissue; 3) associations of ASP and estradiol (but not progesterone) with components of the metabolic syndrome; 4) under conditions of high progesterone there is down-regulation of C5L2 (ASP receptor) and up-regulation of C3 expression (ASP precursor) in omental but not subcutaneous adipose tissue.

Earlier findings in humans point to a significant association between circulating concentrations of reproductive hormones and ASP variations in women. ASP is increased in late gestation, luteal phase of the menstrual cycle and in polycystic ovary syndrome [20]. Plasma ASP levels in late pre-pubertal girls are significantly higher than in boys of comparable developmental stage [26], while in women of reproductive age, ASP levels are significantly lower than in males of comparable age and BMI [27], indirectly suggesting direct ovarian hormone influences on ASP production. It should be noted that ASP levels are rapidly responsive to changes in ovarian hormones. ASP levels did not change across the follicular phase of the cycle but changed significantly starting in the ovulatory phase and sharply increased in the mid-luteal phase coinciding with increasing progesterone levels [20].

Such rapid and direct effects of ovarian hormones on ASP production have also been demonstrated *in vitro*. In cultured adipocytes, physiological levels of estradiol, and, to a greater extent progesterone, decreased ASP production without affecting C3 production, suggesting an effect on conversion of C3 to ASP [8]. These results are consistent with our findings of a negative correlation between circulating ASP and progesterone or estradiol levels as well as with a positive correlation between omental adipose tissue C3 expression

and progesterone. The impact of estradiol and progesterone in other tissues or cell types may also be informative: estradiol is a powerful stimulus for C3 production in uterine epithelial cells [28], and estradiol increases C3 expression in ovarian as well as parametrial adipose tissue [29]. *In vivo* vaginal administration of the progesterone antagonist RU486 increased cervical C3 deposition, macrophage infiltration and increased serum C3adesArg (ASP) and C5adesArg levels in rats [30]. Although blood samples in this study were not obtained at a defined menstrual cycle stage such as the luteal phase, given the rapid effects of sex hormones in cell studies, we speculated that ASP levels and related gene expressions would change as a function of concurrent sex hormone levels in women, allowing us to generate further testable hypotheses.

On the other hand, decreased estradiol and progesterone in menopause or a defect in aromatase action are associated with insulin resistance and increased fat deposition [6]. As increased plasma ASP and complement C3 are also associated with obesity and insulin resistance [3], the ASP association with ovarian hormones may be indirect. Late pregnancy, characterized by increased estradiol and progesterone, is associated with both insulin resistance and increased plasma ASP [20]. Some studies have shown that estradiol can interfere with specific components of the insulin-signaling pathway involved in glucose transport stimulation in adipocytes and muscle [31]. As these pathways are also targeted by ASP, we speculate that there may be a mechanism of ovarian hormone-induced ASP resistance characterized by increased ASP. These published results are consistent with our present findings to the effect that plasma ASP levels are higher in severely obese women with the metabolic syndrome (vs other groups of comparable age) and are correlated inversely with estradiol and, to a lesser extent, progesterone levels.

The capacity of adipose tissue to produce ASP and the increased adipose tissue conversion of androgens into estrogens within adipose tissue in obese individuals, which is associated with hyperinsulinemia, hyperglycemia, and chronic inflammation [32], potentially supports the hypothesis of close interrelationships between ASP, ovarian hormones and insulin resistance, especially in adipose tissue. Yet, estrone, one of the products of aromatization known to be increased in obese women [33], was not related to ASP levels and expression of ASP-related transcripts in the present study. Other steroid hormone

conversions taking place locally in adipose tissue may be of relevance. For example, increased conversion of cortisone to cortisol by 11β-hydroxysteroid dehydrogenase type 1 in hypertrophied, abdominal adipocytes [34] may contribute to alter the dynamics of ovarian hormone action and ASP production and/or signaling in a depot-specific manner.

Similarly, adipose tissue gene expression of C3 and the related receptors C5L2, C5aR and C3aR was related to progesterone levels. In support of these findings, *in vitro* progesterone treatment of adipocytes leads to down-regulation of C5L2, as well as interference in the ASP–C5L2 signaling pathway [7], while progesterone diminished cell surface C5aR in macrophages [30]. Altogether, estradiol associations with plasma ASP, coupled to the progesterone associations with adipose tissue expression of ASP-related genes suggests a strong sex hormone influence on the ASP pathway.

As a consequence, physiological ovarian hormonal states wherein plasma ASP is high and the related receptors, particularly C5L2, are down-regulated, may be reflective of an ASP-resistant state. ASP resistance has been previously proposed in several studies [7;35] and may co-exist with an insulin resistant state. A recent study on diet-induced obesity in mice demonstrated increased plasma ASP, decreased adipose tissue C5L2 expression and signaling, and decreased *in vivo* ASP response, providing direct evidence for ASP resistance [35].

However, the present study was undertaken only in women and is based on a cross-sectional study design with only a single time-point evaluation. As samples were not taken within a defined menstrual cycle phase, direct progesterone associations are difficult to show. Further, to be conservative, due to multiple statistical testing, Spearman correlational analysis was used. These limitations prevent conclusions on cause-and-effect relationships.

In summary, our results, while primarily hypothesis generating, are consistent with the concept that ovarian hormones (estradiol and progesterone) may differentially influence circulating ASP and adipose tissue gene expression of its related proteins (C3, C5L2, C5aR and C3aR) in a depot-specific manner. Further, we speculate that changes in these hormones may favor the development of an "ASP-resistant" state. Taken together, this

cross-sectional study of the metabolic profile based on circulating parameters and adipose tissue gene expression suggests that the ASP pathway may play a role in the regulation of regional fat metabolism through interaction with ovarian hormones in women.

4.8 References

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Chapter 5

Complement receptors C5aR and C5L2 are associated with metabolic profile, sex hormones and liver enzymes in obese women pre- and post-bariatric surgery

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5.1 Résumé

L'obésité est associée à une dysfonction métabolique qui présente des différences entre les sexes ainsi qu'un état inflammatoire de faible niveau. Nous avons proposé que l'expression hépatique des récepteurs immunitaires reliés au facteur du complément C3 (C3aR, C5aR, and C5L2) serait associée au statut ménopausal ainsi qu'au profil métabolique de femmes souffrant d'obésité sévère. Notre hypothèse étant que le ratio C5L2/C5aR prédirait le profil métabolique suite à une chirurgie bariatrique, potentiellement en agissant à travers les voies ASP/C5L2 (métabolique) et C5a/C5aR (immunitaire). Les résultats pour 91 patientes ayant subi une chirurgie bariatrique démontrent que l'expression hépatique d'ARNm de C5L2 était plus élevée chez les femmes pré-ménopausées ($p<0.01$) et que ceci corrélait positivement avec : les niveaux d'estradiol, d'estrone, d'ApoB, d'ApoA1, du ratio ApoA1/B, le tour de taille, l'âge et les niveaux de LDL-C ($p<0.05$). Malgré le fait que les niveaux d'ASP étaient plus faibles chez les femmes pré-ménopausées ($p<0.01$), les niveaux hépatiques du ratio d'ARNm C5L2/C5aR étaient augmentées ($p<0.001$) et corrélaient positivement avec les niveaux d'estrone ($p<0.01$) et d'estradiol ($p<0.001$) mais négativement avec l'ApoB en circulation ainsi que les enzymes hépatiques : ALT, AST, GGT ($p<0.05$). Au cours des 12 mois suivants la chirurgie, dans le groupe avec un ratio d'expression d'ARNm C5L2/C5aR plus faible, les niveaux d'enzymes hépatiques étaient plus élevés (ALP et ALT, $p<0.05$, AST et GGT, $p<0.001$ 2-way-ANOVA). L'association C5L2-C5aR avec d'autres médiateurs tels les estrogènes, pourrait contribuer au métabolisme hépatique ainsi qu'à l'inflammation.

5.2 Abstract

Objective: Obesity is associated with metabolic dysfunction with sex differences and chronic, low-grade inflammation. We proposed that hepatic expression of immune complement C3 related receptors (C3aR, C5aR, C5L2) would be associated with pre/post-menopausal status and metabolic profile in severely obese women. We hypothesized that C5L2/C5aR ratio, potentially influencing the ASP/C5L2 metabolic vs. C5a/C5aR immune response, would predict metabolic profiles after weight loss surgery. Materials/Methods: Fasting plasma (hormone, lipid and enzyme analysis) and liver biopsies (RT-PCR gene expression) were obtained from 91 women during surgery. Results: Hepatic C5L2 mRNA expression was elevated in pre- vs. postmenopausal women ($p<0.01$), and correlated positively with circulating estradiol, estrone, ApoB, ApoA1, ApoA1/B, waist circumference, age and LDL-C (all $p<0.05$). While plasma ASP was lower in pre- vs postmenopausal women ($p<0.01$), the hepatic C5L2/C5aR mRNA ratio was increased ($p<0.001$), correlated positively with estrone ($p<0.01$) and estradiol ($p<0.001$) and negatively with circulating ApoB and liver enzymes ALT, AST, GGT (all $p<0.05$). Over 12-months postoperatively (4 time points), all liver enzyme levels in the low C5L2/C5aR mRNA ratio group remained higher (ALP and ALT, both $p<0.05$, AST and GGT, both $p<0.001$ 2-way-ANOVA). Conclusion: C5L2-C5aR association with other mediators including estrogens may contribute to hepatic metabolic and inflammatory function.

5.3 Introduction

Obesity is associated with increased morbidity and mortality from cardiovascular disease, type 2 diabetes and fatty liver disease, all of which have been clearly linked to a chronic, low-grade inflammatory status. Multi-organ involvement of obesity-induced inflammation (liver and adipose tissue), sex differences in obesity and obesity-related conditions (including: pre vs. postmenopausal status) and dynamic interactions between immune and metabolic responses (termed metaflammation) [1] are all considered important determinants of metabolic disease in obesity.

The complement system is recognized as a key immune regulatory system for cell and tissue homeostasis [2]. Complement component C3 plays a central role in the activation of the complement system [3]. One major source of circulating complement proteins, such as C3, is the liver [4], although adipose tissue and macrophages also secrete C3 [5]. The liver is constantly exposed to complement-activating pathogens via the portal venous system [6]. Proximal and distal activation of complement C3 leads to production of anaphylatoxins C3a and C5a, respectively, which have multiple immune functions including stimulation of histamine secretion and oxidative burst, chemotactic activity as well as secretion of various cytokines (reviewed in [7]). In recent years, C3a and C5a have also been found to have multiple metabolic functions in tissue homeostasis [8, 9, 10], tissue regeneration [11] as well as brain development [12].

In circulation, C5a and C3a are rapidly cleaved by carboxypeptidases to generate C5adesArg and C3adesArg, respectively. C3adesArg, also known as acylation stimulating protein (ASP), is a lipogenic hormone, involved in lipid storage and energy homeostasis (reviewed in [13]). ASP stimulates free fatty acid incorporation into adipose tissue by

increasing triglyceride synthesis and glucose uptake and reducing triglyceride lipolysis in adipocytes [13]. These complement components bind to a family of three receptors, which belong to the superfamily of G-protein-coupled receptors: the C3a receptor (C3aR), C5a receptor (C5aR) and C5a receptor-like 2 (C5L2). All three receptors (C3aR, C5aR, C5L2) have demonstrated roles in the immune process with more recent data in knockout (KO) mice demonstrating emerging roles in energy metabolism [8, 10, 14, 13].

C3aR, which binds C3a, has demonstrated roles in asthma, sepsis, liver regeneration as well as neuron maturation [12]. Further, based on studies in C3aR knockout mice, C3aR plays a role in insulin resistance and adipose tissue macrophage infiltration [9].

C5aR, which binds C5a and, to a lesser extent, C5adesArg, is involved in many inflammatory diseases including asthma, sepsis, rheumatoid arthritis and inflammatory bowel diseases as well as cancer and liver diseases [7]. One previous study showed that C5a stimulates food intake after central administration [15], while a very recent study in C5aR knockout mice demonstrated decreased body weight and fat storage regardless of diet (low fat chow or diet-induced obesity regimen) [8].

C5L2 binds C5a as well as C5adesArg, the latter with a higher affinity than to C5aR [16]. C5L2 has been postulated to be both a non-signaling decoy receptor for C5a [17, 18] as well as a signaling receptor [19]. In previous studies, we have demonstrated a role for C5L2 in ASP function [20, 21], with binding of ASP/C3adesArg and C3a to C5L2 [20, 16], downstream signaling activation [19, 22] and functional output (such as increased glucose uptake and TG synthesis) [23, 24], however the binding of ASP/C3adesArg to C5L2 remains controversial [25]. Recent studies have demonstrated formation of C5L2/C5aR homo- and heterodimers, with co-localization upon stimulation with either ASP/C3adesArg or C5a [26], as confirmed recently [27].

Sex steroids are an additional factor impacting body fat distribution patterns, circulating lipids and prevalence of metabolic diseases. Recent studies have shown that 17**β-**estradiol may play a role in reducing the inflammatory response in adipose tissue as well as the cardiovascular and neural systems [28, 29, 30]. Further, various cell studies have indicated that sex hormones may play a role in the regulation of C3 [31], C5aR [32, 33] and the C5L2/ASP response [34].

In relation to liver function specifically, a recent study in humans indicated that plasma C3a was associated with liver steatosis and hepatocellular injury in individuals consuming considerable amounts of alcohol daily as well as in severely obese people [35]. C3KO mice and C5L2KO mice on a high-fat diet are prone to develop enhanced hepatic steatosis as a result of increased hepatic triglyceride content, lipogenesis-related gene expression and hepatic glucose uptake, and reduced fatty acid oxidation [8, 24, 36]. A role for C5L2 and ASP in liver regeneration in mice has also been suggested, as administration of ASP in C3KO mice restores adequate liver regeneration, an effect absent in C5L2 knockout mice [37]. Together, these findings, suggest a protective role for C3/C3 peptides and C5L2 against the development of hepatic steatosis.

Based on this, we hypothesized that hepatic expression of C3, C3aR, C5aR, C5L2 in humans would be associated with hormonal status and specific metabolic profiles in severely obese pre- and post-menopausal women. Further, we hypothesized that specific liver expression patterns might predict improvement in post-operative metabolic profile and liver enzymes of subjects over one year following biliopancreatic diversion surgery to induce weight loss.

5.4 Materials and Method

5.4.1 Ethics Statement

The experimental protocol was approved by the university hospital ethics committee (CRIUCPQ): Centre de Recherche de l'Institut Universitaire de Cardiologie et Pneumologie du Québec) and all participants provided written informed consent for participation in medical research.

5.4.2 Study subjects

Samples were selected from the CRIUCPQ Tissue Bank (http://www.criucpq.ulaval.ca/index.php/en/tissue-bank) among severely obese women who had undergone bariatric surgery with biliopancreatic diversion (BPD) based on the following criteria: surgery within a 3 year period (2007-2010), no ovariectomy, no lipid lowering medication, no diabetes, liver biopsy and blood sample availability. Of the 252 pre- and postmenopausal severely obese women identified, estradiol was measured (see below) and 91 subjects were chosen based on their plasma estradiol levels; $<25^{th}$ percentile (n=46) and $> 75^{th}$ percentile (n=45) of the cohort tested. Fasting blood samples were collected prior to the surgery, and at 3, 6 and 12 months post operatively. Body composition was assessed prior to surgery and at 12 months. The experimental protocol was approved by the ethics committee of the CRIUCPQ and all participants provided written informed consent for participation in medical research. Patient selection criteria for bariatric surgery included body mass index (BMI), the presence of co-morbidities and a history of prior weight loss attempts.

5.4.3 Clinical and Plasma Measurements

Clinical Assessment: Anthropometric measurements (height, weight, waist circumference and hip circumference) were measured the day before surgery. Height, circumferences, and body weight of subjects were measured on a scale with 0.5 cm and 0.5 kg increments, respectively, and BMI (kg/m^2) was calculated. Blood pressure was recorded using an automatic blood pressure cuff.

Pre-operation Plasma analysis: Blood samples were obtained from participants after overnight fasting and collected into EDTA-containing tubes. The hospital clinical biochemistry laboratory measured fasting plasma glucose (GLU), triglyceride (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), apolipoprotein B (Apo B), apolipoprotein A1 (Apo A1), and liver enzymes aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT). The following assays were measured directly in the research laboratory: Estrone (E1) and estradiol (E2) (both RIA, Beckman Coulter Canada LP, Mississauga, ON), adiponectin (RIA, Millipore, Billerica. MA, USA) and sex hormone binding globulin (SHBG) (ELISA, ALPCO, Salem, NH, US) following instructions of the manufacturers. ASP concentration was measured using an in-house sandwich ELISA, following previously published methodology (38).

Post-operation analysis: Weight, fasting TG, HDL-C, LDL-C, GLU, and liver enzymes (ALT, AST, ALP and GGT) were measured at 3, 6 and 12 months after surgery as described above.

5.4.4 Liver Sampling and Analysis

Biopsies: Liver biopsies were performed according to standard CRIUCPQ Tissue Bank procedures, and approved by the ethics committee of the CRIUCPQ. Liver biopsy samples (250-500 mg tissue) were obtained during bariatric surgery. The liver biopsies were washed with sterile Kreb-Ringer-HEPES buffer, placed in liquid nitrogen, and then immediately transported and stored at -80^{o}C.

RNA Extraction and Real Time qPCR Analysis: All samples (maximum 40 mg liver tissue) were homogenized in Qiazole (Qiagen Inc, Mississauga, ON, Canada). Total RNA was extracted from homogenates using the RNeasy Plus Universal Mini Kit (Qiagen Inc.) according to the manufacturer's instructions. From the total amount, 0.1 μg of purified RNA was retrotranscribed to cDNA using a QuantiTec® Reverse Transcription Kit (Qiagen Inc.) with a final volume of 20 uL. Genomic DNA contamination was eliminated by DNase treatment included in QuantiTec® Reverse Transcription Kit. For real-time PCR evaluation of gene expression, 1 ul of cDNA was used for each reaction. RT2 SYBR® Green qPCR Master Mix (Qiagen Inc.) was used and a 3-step PCR was performed using CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, ON, Canada), using the following protocol: an initial denaturation step at 95°C for 10 minutes, 40 cycles of 95°C for 10 s, 55° C for 10 s, 72°C for 30 s was followed by a final extension step of 95°C for 10 s and melt curve 65°C to 95° C. Real time RT-PCR was performed to quantify human C3, C3aR, C5aR, and C5L2 relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene, with primers obtained from Alpha-DNA (Montreal, Canada).

The sequences for the primers used were: C5L2/GPR77- Right; 5′ TCCGAGAGGTCGCTGTAATC-3′, C5L2/GPR77-Left; 5′-TCAAGGACTCCCAAAACCAG-3′, C3aR1-Right; 5′-AGCAGAGAAAGACGCCATTG-3′, C3aR1-Left; 5′-ACTGTGGCTAAGTGTGGGGA-3′, C5R1-Right; 5′- TATCCACAGGGGTGTTGAGG-3′, C5R1- Left; 5'-GCCCAGGAGACCAGAACAT–3′, C3- Right; 5′-GCAATGATGTCCTCATCCAG-3′, C3-Left; 5′- CCTGGACTGCTGCAACTACA-3′, and GAPD-Right; 5′-AATGAAGGGGTCATTGATGG-3′, GAPD-Left; 5′-AAGGTGAAGGTCGGAGTCAA-3′. For data analysis the ΔΔCt method was used, as performed with Bio-Rad CFX manager software (version 1.5)(Bio-Rad Laboratories).

5.5 Statistics

Statistical analysis was performed using GraphPad Prism 5 (GraphPad software, CA, USA) or Sigmastat 3.5 (Systat software, San Jose, CA, USA). Descriptive parameters are provided as mean ± SEM or in percentages. For non-normally distributed parameters, values were log-transformed for statistical analysis. Two-tailed t-tests were used to analyze the differences between means and proportions of two groups. Two-way ANOVA analyses were used to compare among groups (as indicated). Pearson correlation was used to analyze bivariate correlation. Fisher exact test was used in the analysis of 2X2 contingency tables. Stepwise forward multiple regression analysis was used to assess how gene expression predicts variables of the metabolic profile. A $p<0.05$ was considered statistically significant for all analyses.

5.6 Results

5.6.1 Relationship of Hormone Status with Gene Expression

Baseline patient characteristics of the 91 severely obese women ranging in BMI ($37.5 \geq BMI \geq 78.9$ kg/m^2) and age (21-69 years) are given in Table 5.1. The subjects were grouped based on fasting estradiol (E2), into pre- and postmenopausal status groups as shown in Table 5.1. Biochemical analysis indicated that subjects in the high E2 subgroup ($p<0.0001$) also had higher levels of estrone (E1) ($p<0.0001$), progesterone ($p<0.05$), and were younger ($p<0.0001$) reflecting their premenopausal status. The high E2 group also had lower Apo B levels ($p<0.0001$), higher Apo A1 ($p=0.025$), and higher ApoA1/B ratio ($p<0.0001$), despite having significantly higher waist circumference ($p<0.05$) and lower HDL-C ($p <0.05$).

Hepatic gene expression of C3 and the three related receptors (C5L2, C3aR and C5aR) in pre- vs post-menopausal groups are shown in Figure 5.1A. While expression levels of C3, C3aR and C5aR were not different between the two groups, C5L2 gene expression was higher in premenopausal status women (higher E2)($p<0.01$). Further, C5L2 gene expression also correlated directly with plasma E2 and E1 levels (E2 $r=0.27$, $p<0.05$; E1 $r=0.42$, $p<0.0001$) as shown in Figure 1B and 1C, while C3 correlated inversely with SHBG ($r=-0.28$, $p<0.01$, data not shown).

	All subjects			Premenopausal status	Postmenopausal status	
Parameters	**Mean (n=91)**	**Min**	**Max**	**Mean (n=45)**	**Mean (n=46)**	**T TEST**
Estradiol (pg/ml)	**47.6 ± 5.3**	**6.1**	**294**	**81.4 ± 8.2**	**14.6 ± 0.7**	**N/A**
Age (yrs)	**40 ± 1**	**21**	**69**	**35 ± 1**	**45 ± 2**	**P<001**
BMI (kg/m^2)	**51.1 ± 0.9**	**37.5**	**78.9**	**51.9 ± 1.4**	**50.3 ± 1.1**	**NS**
Waist Circumference (cm)	**146 ± 2**	**119**	**184**	**150 ± 2**	**142 ± 2**	**P <0.05**
Hip Circumference (cm)	**137 ± 2**	**97**	**177**	**136 ± 3**	**138 ± 2**	**NS**
SBP (mmHg)	**136 ± 2**	**109**	**186**	**137 ± 2**	**136 ± 2**	**NS**
DBP (mmHg)	**84.4 ± 1.3**	**43.0**	**113**	**83.3 ± 2.2**	**85.5 ± 1.4**	**NS**
Glucose (mmol/L)	**5.6 ± 0.1**	**3.7**	**11.6**	**5.4 ± 0.2**	**5.7 ± 0.1**	**NS**
Cholesterol (mmol/L)	**4.9 ± 0.1**	**3.1**	**7.3**	**4.8 ± 0.1**	**5.0 ± 0.1**	**NS**
HDL-C (mmol/L)	**1.31 ± 0.03**	**0.61**	**2.26**	**1.24 ± 0.04**	**1.37 ± 0.05**	**P<0.05**
LDL-C (mmol/L)	**2.8 ± 0.1**	**1.0**	**4.9**	**2.8 ± 0.1**	**2.8 ± 0.1**	**NS**
Triglyceride (mmol/L)	**1.7 ± 0.1**	**0.6**	**4.5**	**1.7 ± 0.1**	**1.8 ± 0.1**	**NS**
ApoB (g/L)	**0.84 ± 0.05**	**0.27**	**2.27**	**0.64 ± 0.04**	**1.04 ± 0.07**	**P<0001**
ApoA1 (g/L)	**0.81 ± 0.04**	**0.06**	**1.95**	**0.98 ± 0.04**	**0.79 ± 0.07**	**P<0.05**
Apo A1/B	**1.4 ± 0.1**	**0.1**	**4.6**	**1.8 ± 0.1**	**1.1 ± 0.1**	**P<0001**
Adiponectin (µgl/ml)	**7.5 ± 0.9**	**2.5**	**41.1**	**6.3 ± 0.5**	**9.6 ± 1.9**	**NS**
Estrone (pg/ml)	**75 ± 5**	**9**	**267**	**106 ± 7**	**44 ± 4**	**P<0001**
SHBG (pg/ml)	**181 ± 12**	**15.2**	**573**	**162 ± 15**	**199 ± 18**	**NS**
Progesterone (ng/ml)	**2.4 ± 0.3**	**0.1**	**6.9**	**2.9 ± 0.4**	**1.6 ± 0.3**	**P<0.05**
AST (IU/L)	**23.4 ± 1.9**	**12.2**	**123**	**20.5 ± 6.6**	**25.1± 18.9**	**NS**
GGT (IU/L)	**40.3 ± 6.8**	**10.1**	**382**	**39.1 ± 75.2**	**40.9 ± 33.7**	**NS**
ALP (IU/L)	**95.5 ± 3.8**	**49.0**	**210**	**99.9 ± 28.8**	**92.7 ± 29.8**	**NS**
ALT (IU/L)	**25.5 ± 1.5**	**2.0**	**61.0**	**23.8 ± 11.3**	**26.6 ± 12.6**	**NS**

Table 5.1 Characteristics of pre- and postmenopausal Obese Women.

Estradial was measured in the 252 severely obese women evaluated, and subjects in the lowest quartile (<25th percentile, n=46 postmenopausal) and highest quartile (> 75th percentile, n=45 premenopausal) were selected for further evaluation of plasma and liver tissue, and were compared by t-test. Apo; Apolipoprotein, ALT; Alanine transaminase, ALP; Alkaline phosphatase, AST; Aspartate transaminase, BMI; Body Mass Index, DBP; diastolic blood pressure, GGT; Gamma-glutamyl transpeptidase, HDL-C; high density lipoprotein-cholesterol, LDL-C; low density lipoprotein-cholesterol, SHBG; sex hormone-binding globulin, SBP; systolic blood pressure. $P < 0.05$ is considered significant.

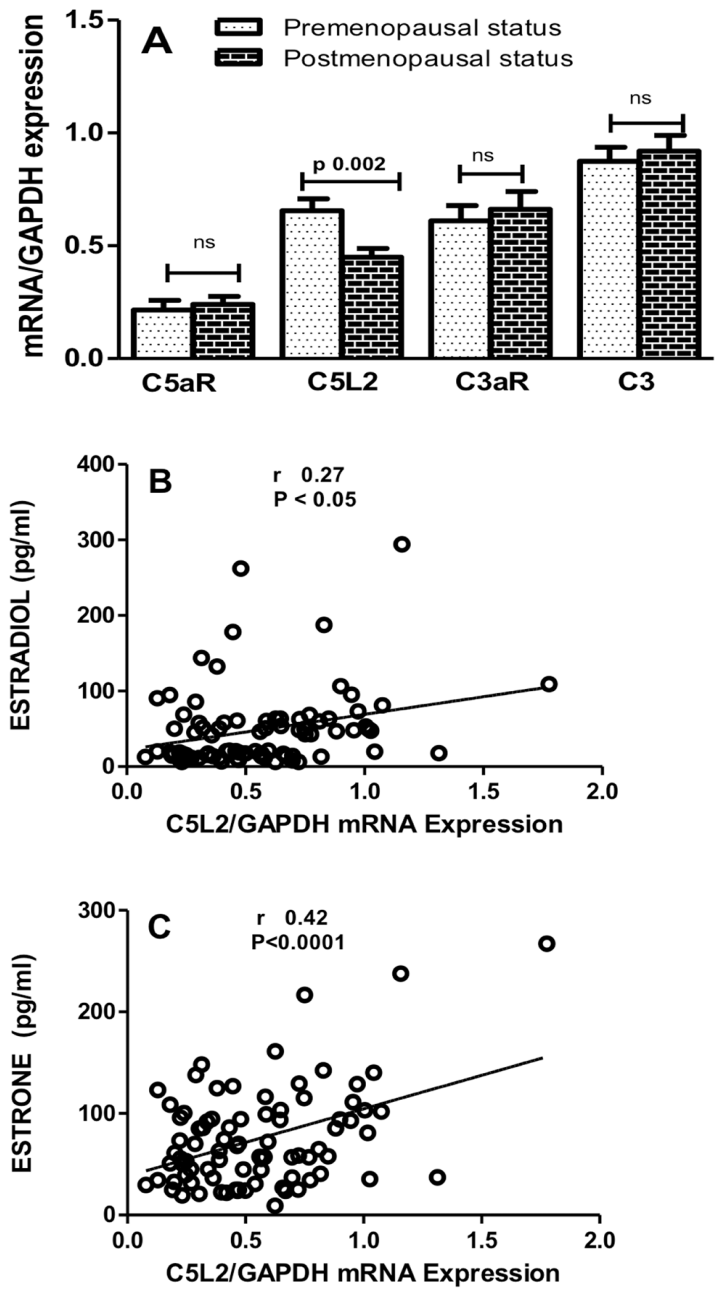

Figure 1

Figure 5.1 Association of liver C5L2 mRNA expression with circulating estrogen levels.

mRNA was extracted from liver biopsies, reverse-transcribed to cDNA and gene expression was evaluated by RT-PCR with the indicated primers with all results expressed relative to GAPDH using the $\Delta\Delta$Ct method as described in detail in Methods. A) Hepatic mRNA expression levels of C3 and the three related receptors (C5L2, C3aR and C5aR) in the pre- vs. postmenopausal groups. (B & C) Linear correlation plot of C5L2 mRNA expression with plasma estradiol (E2) and estrone (E1).

5.6.2 Hepatic Complement Gene Expression and Metabolic Profile

The relationships between hepatic gene expression and various anthropometric parameters and metabolic profile were then evaluated. Hepatic C3 expression correlated inversely with the liver enzyme GGT ($r=-0.28$, $p<0.05$, data not shown). C3aR was associated with adiponectin (Figure 5.2A, $r=0.36$, $p<0.05$) while C5aR correlated with BMI (Figure 5.2B, $r=0.34$, $p<0.01$) and liver enzyme AST ($r=0.32$, $p<0.05$, data not shown). Hepatic gene expression of C5L2 correlated with ApoA1 (Figure 5.2C, $r=0.25$, $p<0.05$), ApoA1/B ($r=0.25$, $p<0.05$, data not shown) and waist circumference ($r=0.25$, $p<0.05$, data not shown), with inverse correlations between C5L2 and ApoB (Figure 5.2D, $r=-0.25$, $p<0.05$), age ($r=-0.26$, $p<0.05$, data not shown) and LDL-C ($r=-0.23$, $p<0.05$, data not shown).

Forward stepwise regression analyses indicated that the dependent variable C5aR could be predicted from a linear combination of the independent variables age, BMI and glucose (pooled $r^2=0.20$, $p<0.001$) and the dependent variable C5L2 could be best predicted from a linear combination of independent variables LDL-C and E1 (pooled $r^2=0.24$ $p<0.001$).

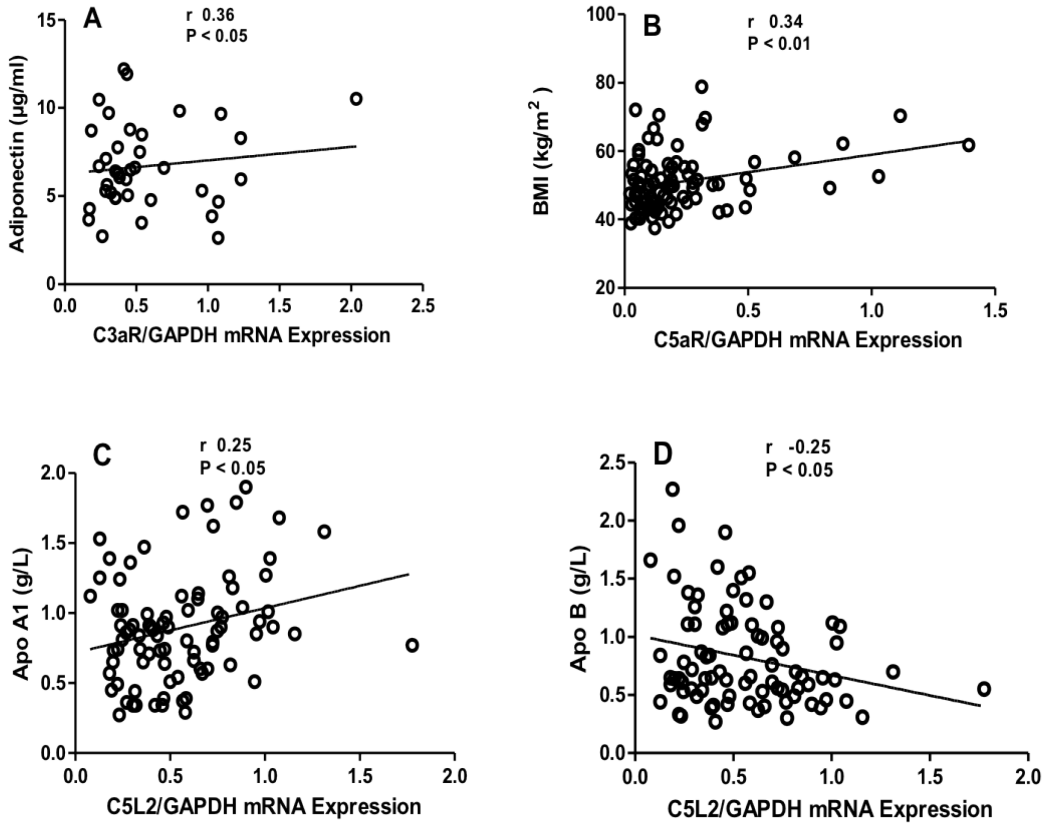

Figure 5.2 Hepatic mRNA Expression relative to Metabolic Parameters.

A-D: Linear plot correlations of hepatic mRNA expressions and metabolic parameters. mRNA was extracted from liver biopsies, reverse-transcribed to cDNA and gene expression was evaluated by RT-PCR with the indicated primers with all results expressed relative to GAPDH using the $\Delta\Delta$Ct method as described in detail in Methods.

5.6.3 C5L2/C5aR Ratio and Metabolic Parameters

As recent *in vitro* experimental evidence indicates a direct interaction (heterodimerization) between C5aR and C5L2 receptors [38, 26] which could affect subsequent cellular signaling and response, we tested associations of the C5L2/C5aR mRNA ratio with various metabolic indices in our subjects. While plasma ASP levels were significantly lower in premenopausal women vs postmenopausal women ($p<0.01$), the hepatic C5L2/C5aR mRNA ratio was higher (Figure 5.3A, $p<0.001$). Further, positive correlations between C5L2/C5aR mRNA ratio and sex hormones were identified (E1: $r=0.28$, $p<0.01$ and E2: $r=0.38$, $p<0.001$, data not shown).

Interestingly, the hepatic C5L2/C5aR mRNA ratio was inversely associated with circulating liver enzymes (ALT, AST and GGT) and ApoB (Figure 5.3B-E), as well as age ($r=-0.29$, $p<0.01$, data not shown). Because of significant correlations between the C5L2/C5aR mRNA ratio and pre-operative liver enzymes and metabolic parameters, subjects were partitioned based on high vs. low C5L2/C5aR mRNA ratio. Before bariatric surgery, ALT, AST and GGT but not ALP were significantly higher in the low-C5L2/C5aR group, with a lower ApoA1/B ratio, although there was no significant difference in BMI between these two groups (Figure 5.3F).

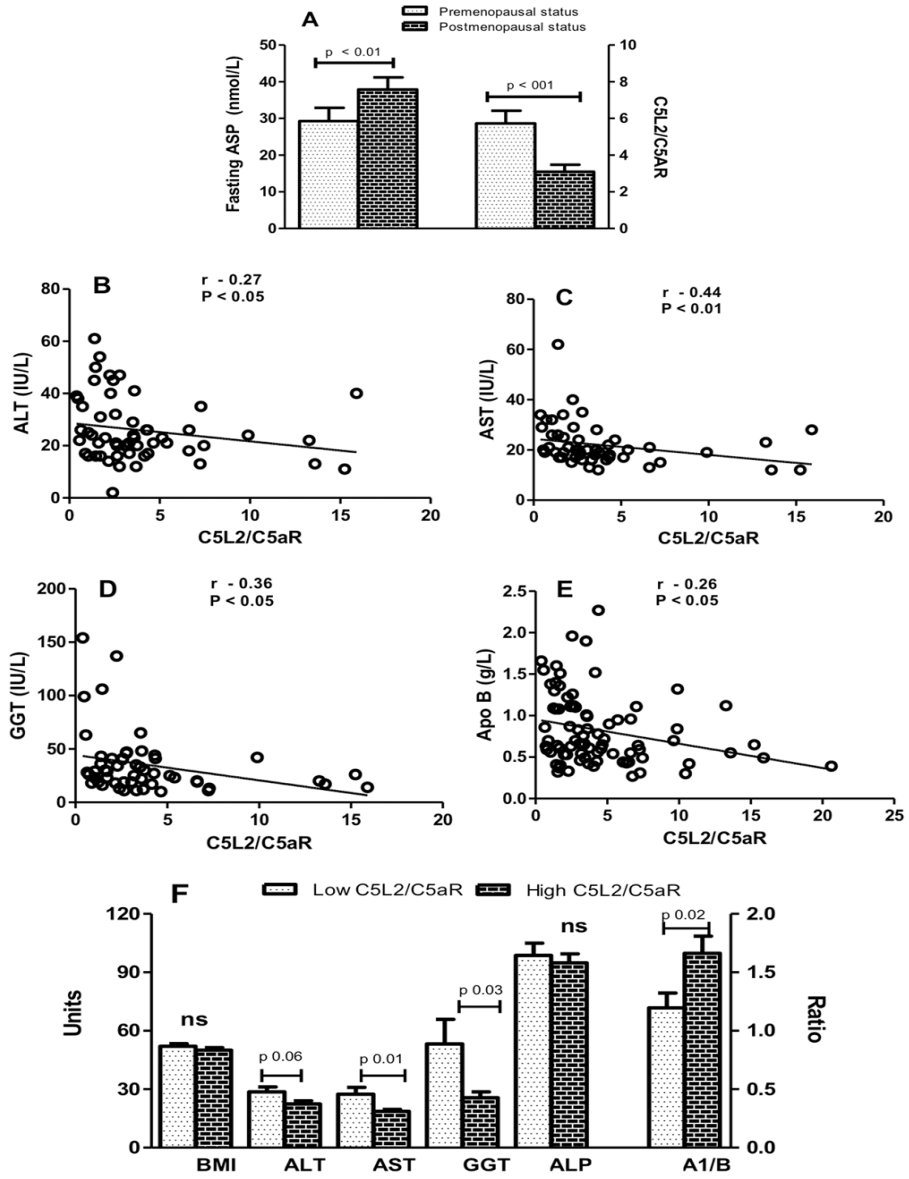

Figure 3

Figure 5.3 Association of hepatic C5L2/C5aR mRNA ratio with menopausal status and metabolic profile.

mRNA was extracted from liver biopsies, reverse-transcribed to cDNA and gene expression was evaluated by RT-PCR with the indicated primers with all results expressed relative to GAPDH using the $\Delta\Delta$Ct method as described in detail in Methods. (A): Comparison of hepatic C5L2/C5aR mRNA expression ratio and plasma ASP levels in premenopausal and postmenopausal groups. ASP values were log transformed for statistical analysis. (B-D): linear plot correlation of liver enzymes ALT, AST and GGT with C5L2/C5aR mRNA ratio, (E): linear plot correlation of ApoB and C5L2/C5aR mRNA ratio. (F): Comparison of preoperative BMI (kg/m^2) and fasting liver enzymes (IU/L) (left y-axis) and ApoA1/B ratio (right y-axis) before bariatric surgery in subjects separated based on low vs. high C5L2/C5aR mRNA ratio, compared by unpaired student T test.

5.6.4 Relationship of C5L2/C5aR ratio with bariatric surgery outcome

Metabolic profile and anthropometric results were evaluated postoperatively and compared to baseline data. As expected, the anthropometric and metabolic profile of subjects substantially improved over a 12-month follow-up after bariatric surgery. Hepatic C5L2 mRNA expression was positively associated with % weight loss (Figure 5.4A, $r=0.22$, $p<0.05$).

Following bariatric surgery, subjects were routinely followed in the surgical clinic at 3, 6 and 12 months. Globally, over the 12 month period, all liver enzyme levels in the low C5L2/C5aR mRNA ratio group remained higher than in the high C5L2/C5aR mRNA ratio group (Figures 5.5 A-D, ALP $p<0.05$, ALT $p<0.05$, AST $p<0.001$ and GGT $p=0.0002$ by 2-way ANOVA), although there was no significant different in BMI reduction curves (data not shown). One-year changes in BMI, LDL-C, HDL-C, total cholesterol, glucose and triglyceride are shown in Figure 5.4B. Although the expected improvements in all parameters were not significantly different between the two groups, the relative change in TG was significantly different between the low and high C5L2/C5aR mRNA groups (Fisher exact test, $p=0.02$).

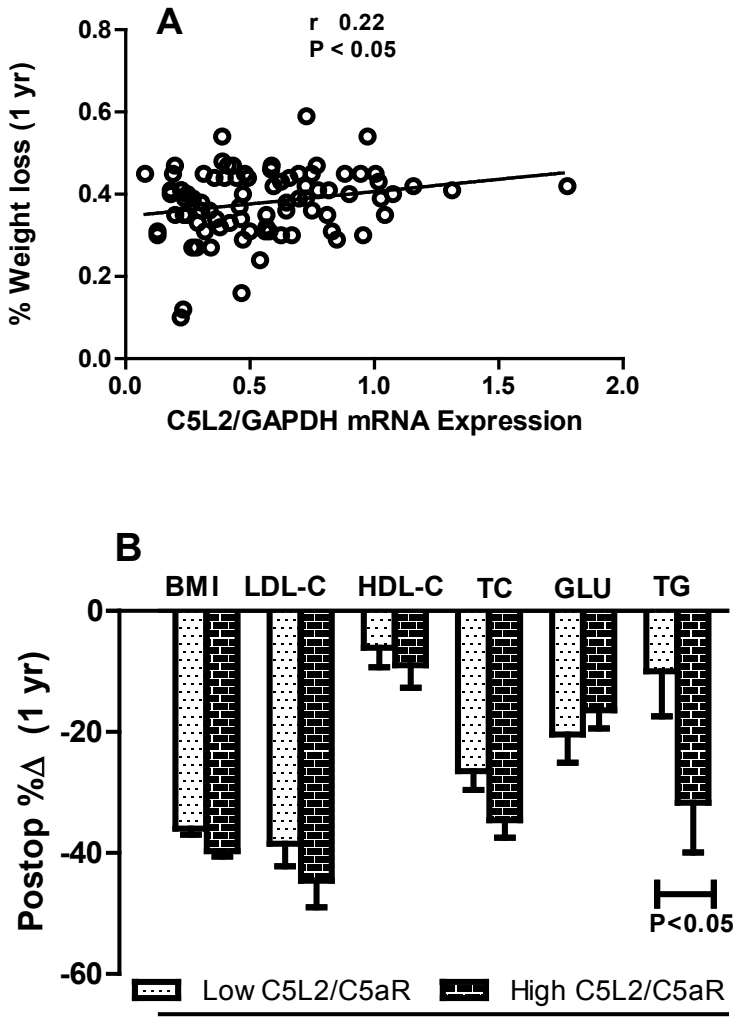

Figure 5.4 Metabolic profile after surgery.

mRNA was extracted from liver biopsies, reverse-transcribed to cDNA and gene expression was evaluated by RT-PCR with the indicated primers with all results expressed relative to GAPDH using the $\Delta\Delta$Ct method as described in detail in Methods. (A) Linear correlation plot of liver C5L2 mRNA expression at time of surgery vs % weight loss 12 months after surgery, analyzed by Pearson correlation test. (B) Percentage changes of metabolic profile before and after bariatric surgery and categorical comparisons between the high and low C5L2/C5aR mRNA ratio subgroups, analyzed by Fisher exact test.. BMI; Body Mass Index, GLU; fasting glucose, HDL-C; high density lipoprotein- cholesterol, LDL-C; low density lipoprotein-cholesterol, TG; triglyceride, TC; total cholesterol.

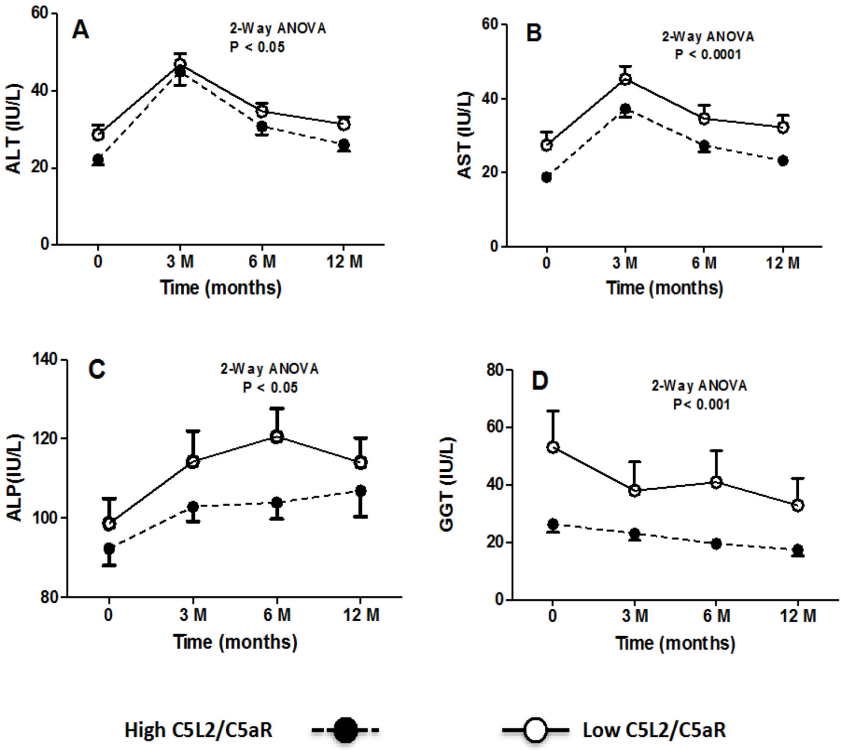

Figure 5.5 Plasma liver enzyme levels relative to C5L2/C5aR Expression.

(A-D) Plasma liver enzyme levels before surgery and during one year following surgery in high vs low C5L2/C5aR mRNA ratio subgroups, 2-way ANOVA comparison.

5.7 Discussion

In this study we investigated liver tissue mRNA expression of complement C3 and its cleavage product receptors, C3aR, C5aR and C5L2 and their relationship with hormonal status among severely obese women who underwent BPD. In addition, we assessed the subjects' metabolic profile before and up to one year after surgery in relation to partitioning between high and low liver C5L2/C5aR mRNA ratio. The major findings of this study are: 1) associations of hepatic complement-related gene expression in severely obese women with sex hormones and metabolic profile and 2) associations of hepatic C5L2/C5aR ratio with pre- and post-operative metabolic profile.

Although many studies have examined the role of complement C3, and to a lesser extent, the role of related receptors C5L2, C5aR and C3aR, overall the major focus of these studies has been in an immune context. More recently, however, the impact of C5L2, C5aR, and C3aR in the area of energy (lipid and glucose) metabolism has been recognized as a result of studies in gene knockout mice [8, 9, 10, 14].

However, there is little information available on gender and sex hormone influence on complement-related receptors, and their potential roles in liver function. In the present study, the positive correlation of hepatic C5L2 (and C5L2/C5aR ratio) with circulating estrogens (E1, E2), and the decrease in expression in post-menopausal women indirectly suggest an estrogenic influence. *In vitro* cellular and *in vivo* animal studies support the notion that estrogens increase adipose C3 expression [31] and neural C5aR expression [32] but have differential effects on C5L2 depending on estrogen receptor targeting (ERα and β) in different tissues [33]. It is now well recognized that most tissues in both men and women are influenced by estrogens [39], and the liver is particularly sensitive, including effects on

lipid metabolism [40]. Estrogen withdrawal results in increased hepatic lipogenesis, decreased VLDL lipoprotein production and secretion and decreased lipid oxidation with associated gene expression changes [40, 41]. In the present study, given the correlations between C5L2 and various lipid parameters, including apoA1 and apoB, estrogens may mediate the effects either directly, or indirectly via hepatic lipid changes.

The increased plasma ASP levels commonly seen in metabolic dysfunction (including type 2 diabetes and cardiovascular diseases) have been proposed as indicating an "ASP resistant state" [24, 14, 42]. A recent study demonstrated that diet-induced obesity in mice results in increased plasma ASP, decreased adipose C5L2 expression and decreased *in vivo* ASP response: providing proof-of-concept of ASP resistance [43]. The lower hepatic C5L2/C5aR mRNA ratio with yet higher ASP plasma level in postmenopausal obese women suggests the presence of ASP resistance in hepatic cells. Pathologically, if the ASP/C5L2 signaling is disturbed in adipose tissue, as in the case of decreased expression of the ASP receptor (a decrease in ASP function), this may enhance diversion of available glucose and fatty acids to other tissues (muscle, liver, arterial wall), leading to lipotoxicity, unless disposal mechanisms are up-regulated such as increased fatty acid oxidation [44]. This consequence was demonstrated in C5L2KO mice where it was shown that in response to reduced TG storage in white adipose tissue, C5L2KO mice developed a compensatory mechanism of increased muscle fat oxidation [23]. We speculate that the presence of ASP resistance in postmenopausal women may contribute to their metabolic dysfunction.

The C5L2/C5aR mRNA ratio was also informative in predicting post-operative outcomes: higher values of this ratio were associated with greater relative weight loss, greater decreases in fasting TG and lower liver enzymes both before surgery and over the follow-up. In the pre-operative state, increased C5L2/C5aR mRNA ratio was associated

with lower BMI, apoB and liver enzymes, and yet higher apoA1. Overall, these data suggest that a high hepatic C5L2/C5aR mRNA ratio is a beneficial feature, and raises the question of what the specific functional roles of C5L2 and C5aR are in relation to lipid metabolism in the liver.

While there is little data on the function of complement-related receptors directly in liver cells (either parenchymal hepatocytes or non-parenchymal cells), the complement system may contribute in either a beneficial or detrimental manner. In cells, C3 production in hepatocytes is stimulated by PPAR**α**, TNF**α**, IL-6 and LPS [45, 46] while C5a, via interaction with hepatic C5aR, is involved in proliferation, glycogen phosphorylase and glucose output [47, 48, 49]. *In vivo*, the complement system can both promote inflammation/injury and play a homeostatic role in repairing damaged tissue. While C3, C3aR, C5aR and C5L2 blockade/deficiency can protect from liver failure and improve sepsis survival in mice [50, 51], by contrast, the presence of these same proteins, and the C3 products ASP and C3a, play roles in promoting liver regeneration, liver transplant tolerance, and protection from steatosis [9, 37, 52, 53].

In relation to metabolic effects, an obesogenic diet induces increased liver expression of C3, C5aR, and C5L2 [54, 8, 14], while C3KO, C5aRKO, C5L2KO and C3aRKO mice are resistant to diet-induced obesity [55, 8, 23, 24, 9]. Finally, C3 and ASP/C3a have been implicated in human studies of fatty liver disease [56, 57].

How the balance between the various ligands (C3a, ASP, C5a/C5adesArg) and their receptors (C3aR, C5L2, C5aR) could influence overall hepatic function remains to be elucidated. Recent studies suggest that the ability of several ligands binding to the same receptor to evoke differential signaling and biological responses constitutes a phenomenon referred to as “biased agonism” [58]. Depending on the local environment and the

circumstances under which complement proteins are generated, they may contribute to cell and tissue homeostasis, to benefit or burden inflammation, or to tissue regeneration vs fibrosis [7]. For example, while in mid-grade sepsis, blockage (or gene absence) of either C5aR or C5L2 improved survival, in high-grade sepsis combined blockage of these two receptors was necessary [19]. Further, ASP demonstrated a biphasic role with a balance between inflammation/injury vs, regeneration [37].

While C3aR neither homodimerizes nor heterodimerizes, by contrast, C5aR and C5L2 both homo- and hetero-dimerize [26, 59]. The potential for cooperative interaction between C5L2 and C5aR has been evaluated in *in vivo* and *in vitro* studies. C5L2 acts as a positive modulator for both C5a- and C3a-induced responses in neutrophils, macrophages and fibroblasts and is critical for optimal signaling [60]. Conversely, another study on human neutrophils demonstrated that C5L2 functions as an intracellular receptor, becoming co-localized with C5aR after C5a binding, acting as a negative modulator through the beta-arrestin pathway [38]. Further, a recent study demonstrated formation of C5L2/C5aR heterodimers in adipocytes and macrophages, with co-internalization/co-localization upon stimulation with either ASP/C3adesArg or C5a [26]. Such dimerization may be one mechanism underlying the potential positive/negative modulatory effect of C5L2 on C5a or ASP/C3a effector functions.

Limitations of this study should be noted: all of the interpretations and conclusions are based on the evaluation of obese subjects, and all analyses were in women. In addition based on the limited availability of small quantities of frozen tissue from liver biopsies, the present study relied on C5aR and C5L2 mRNA expression without addressing the possible post-translational modifications, protein levels or functional assays of the examined

receptors. Further, as this is a cross-sectional study, the potential links between cause and effect can only be speculated upon.

5.8 Conclusion

In this study, evidence is presented to integrate data suggesting that complement-related proteins correlate with sex hormones and metabolic profile in liver tissue. C5L2-C5aR interactions and the association with other mediators, such as estrogens, may have a role in metabolic and inflammatory functions of complement-related proteins in human liver cells.

5.9 Refrences

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Chapter 6

Summary and discussion

6.1 Summary

This thesis investigated the role of adipokines, in particular ASP and their relationship with diet, sex hormones and cardio-metabolic risk factors in a series of experiments using human models. In general, these four studies provide additional evidence to show that:

I. Obesity is strongly linked to diet composition. Production and secretion of adipokines are dynamically regulated, including through nutritional influence.
II. Obesity is determined by interactions between exogenous and endogenous factors. The regulation of balance between energy intake and energy expenditure and the subsequent metabolic profile that evolves during positive energy imbalance are mediated by a complex network of signals originating from a number of endocrine tissues, such as adipose tissue, liver and gonads.
III. Obesity is a key player in the pathogenesis of multiple morbid disorders. There is a plethora of data indicating that changes in adipose tissue-secreted hormones respond not only to the mass of adipose tissue but also to presence of these abnormalities.

We have made the following novel observations:

I. From the first study (Chapter 2)
    - Long-term sugar consumption with ad libitum diet leads to changes in circulating levels of adipokines: ASP, adiponectin, and leptin.
    - Fasting and postprandial ASP were associated with postprandial triglycerides while in the fructose diet, women have greater increases in postprandial ASP than men.

- Fasting adiponectin was associated with baseline abdominal/visceral fat.
- The differential effects of fructose and glucose on 24-hour circulating leptin concentrations were sustained during long-term consumption of the 2 sugars.

II. From the second study (Chapter 3)

- There are higher average ASP concentrations in a Turkish population in comparison with those reported in other ethnicities.
- There are diverging correlations across genders between ASP levels and metabolic variables among cardiovascular risk factors.

III. From the third study (Chapter 4)

- There is a differential influence of sex hormones on circulating ASP and adipose tissue gene expression of related proteins in an adipose tissue depot-specific manner.

IV. From the fourth study (Chapter 5)

- There are correlations between sex hormones and hepatic complement-related proteins and metabolic profiles.
- There is an association of hepatic C5L2/C5aR ratio with pre- and post- operative profiles.

6.2 Metabolic syndrome and obesity, current concepts

Several pathophysiological explanations for the metabolic syndrome have been proposed involving insulin resistance, chronic inflammation and ectopic fat accumulation following adipose tissue saturation. For a long time, the metabolic syndrome was originally proposed as a cluster of metabolic abnormalities that could be potentially explained by a common pathophysiologic link, insulin resistance (357). More recently, the emphasis was directed to the functional failure of an organ, mainly adipose tissue that manifests through inflammatory response and adipokine dysregulation (42). Currently, the emphasis is on mechanistic molecular-level analyses of the link between inflammation and insulin resistance (358), a broad focus on the signaling pathways to account for the modulation of physiological changes and the commencement of pathological deviation .

This thesis effectively demonstrates that the mechanisms of obesity-related metabolic syndrome are intricately involved in multiple metabolically and structurally complex systems, that is, in tissue and cells which communicate with other cells within the same tissue, organ or at a systemic inter-organ level.

6.3 Obesity determinants

6.3.1 Adipose tissue dysfunction model

This model (reviewed in (359)) is proposed for explaining the metabolic syndrome and is based on the failure of the adipose tissue in buffering lipids during postprandial periods, which are an equilibrium between two states: draining free fatty acids that come mainly from triglyceride-rich lipoproteins during the postprandial period or releasing them during the fasting period. Switching between the two states is regulated by a multifactorial system including substrate and hormone levels as well as the functional state of the adipose tissue itself (359). A failure of the adipose tissue function in taking up dietary fat (being switched to releasing free fatty acids) might lead to excess lipid flux towards other tissues, during the postprandial period and even during the fasting period, and to a decreased clearance of triglyceride-rich lipoprotein particles (360). Concurrently, the increased availability of free fatty acids stimulates the liver, packaging them in triglycerides that are released in apoB-containing lipoproteins (VLDL). The interaction of these particles with HDL and LDL lead to the typical dyslipidemic profile (361). On the other hand, circulating fatty acids and those released by lipoprotein lipase modify the energy draining paths in peripheral tissues, impairing glucose use (359).

Given this profile, it is not surprising that in our studies (chapter 2, 3, 4, 5), ASP and its related proteins as well as leptin and adiponectin consistently correlated with plasma lipid profile, factors commonly related to metabolic syndrome, diabetes, cardiovascular disease and liver steatosis. Whether the increase in plasma ASP is a cause or a consequence of abnormal lipid metabolism cannot be determined simply by correlation studies, but examination of ASP function is supportive of such links.

In addition, adipose tissue is at the crossroads in the development of the metabolic syndrome, inflammation and atherosclerosis largely because the accumulation of excessive circulating energy in this tissue induces cell enlargement and stress (42). This accumulation leads to the release of alarm cytokines (362) which induces the infiltration of immune system cells (241)(238). The consequence of this process is the development of a nonclassical inflammatory state that becomes chronic because normal immune system mechanisms cannot successfully fight the agents (excess nutrients) ultimately responsible for the inflammatory response (241)(359). Therefore, adipose tissue affects not only metabolism but also many functions of organs and tissues, such as brain, muscle, liver, and blood vessels.

In chapter 4 and 5 we have demonstrated an association of complement C3-related immune system proteins in both liver and adipose tissue with metabolic profiles. Interestingly, association of C5L2/C5aR ratio, two anaphylatoxin receptors, with liver enzymes, sex hormones and metabolic profile could represent another example of the interaction of inflammatory and metabolic processes.

6.3.2 Adipose tissue dysfunction and diet

Nutrition represents a lifestyle element that can directly influence health; therefore preventive nutrition and weight control should become a main focus of health care systems. Despite wide publicity, and the popularity and promotion of low fat diets, nonetheless the putative decline in dietary fat consumption has not corresponded to a decrease in obesity, in fact, the opposite trend has emerged (54). Indeed, foods high in saturated fats and cholesterol have been shown to induce weight gain, insulin resistance, atherosclerosis and hyperlipidemia in humans and animals, but the emphasis on fat reduction has had no significant benefits relative to the obesity epidemic (61) . Increasing evidence now suggests that the emphasis must be put on the excess of energy derived from high-energy carbohydrate-rich diets, especially those containing excessive fructose, and the role in inducing obesity and metabolic syndrome (65)(84). However a very recent study by Chiu S. et al showed that an isocaloric exchange of fructose for other carbohydrates does not induce non-alcoholic fatty liver disease changes (363).

The processes involved in regulating energy homeostasis and intermediary lipid and carbohydrate metabolism are linked by common neuroendocrine mediators and adiposity-driven hormones such as leptin, ASP, and adiponectin. As we have shown in chapter 2, the production/function of all theses three adipocyte hormones appear to be regulated by diet and nutritional status (fasting/postprandial). We have shown dietary effects of long-term consumption of two marcronutrient on adipose tissue function.

6.3.2.1 Leptin

Long-term high fat diet induced obesity is characterized by hyperleptinemia and impaired leptin signaling (« leptin resistance ») to reduce food intake and increase energy expenditure in humans and mice (364)(365). Recently, Koch et al. have demonstrated that leptin deficient ob/ob mice treated by chronic intracerebroventricular infusion of leptin develop leptin resistance on a high fat diet independent of hyperleptinemia (366). They suggested hypothalamic inflammation might play a key role in the induction of diet-induced glucose intolerance. On the other hand, the 24-h diurnal leptin concentrations are reduced on a day when three high-fat meals are consumed when compared with high-carbohydrate/low-fat meals, which induced larger postprandial glucose excursions and greater insulin secretion (367). However, recently, it has been demonstrated that chronic central leptin treatment can decrease food intake, as well as body weight, in both high fat and low fat diet mice suggesting that diet-induced obese mice are not resistant to chronic central leptin treatment (368).

On a fructose diet, our results in chapter 2 showed sustained differential effects of fructose and glucose on 24-hour circulating leptin concentrations. This result is in agreement with data from in vitro experiments in isolated adipocytes (369) and from clinical studies in human subjects (370), supporting the idea that production of leptin is regulated by insulin-mediated glucose metabolism in adipocytes. The endocrine effects of dietary fat and fructose, resulting in decreased insulin secretion and leptin production, suggest a mechanism by which consumption of diets high in energy derived from fat and fructose, could lead to overconsumption of calories, weight gain, and obesity. However, fasting leptin concentrations increased in both groups with the changes from baseline being significantly and positively associated with the changes in body weight.

6.3.2.2 Adiponectin

Adiponectin knockout mice demonstrated aggravated high fat diet-induced obesity, metabolic derangements, and cardiac dysfunction (371). Recently, studies have shown an increase in adiponectin and adiponectin transgenic-overexpressing mice prevented diet-induced obesity and fatty liver, improved insulin sensitivity (372) and promoted adipose tissue vascularity (373) under conditions of long term high fat diet-induced obesity.

A recent study on diets high in monounsaturated and polyunsaturated fatty acids demonstrated that different types of dietary fatty acids regulate adiponectin production and proteins involved in fatty acid metabolism in adipose tissue. Yet the fatty acid composition of the diets had minimal effects on altering adipose tissue function and inflammation in the context of a long-term high fat diet (Enns et al 2014 (374)). A recent study in rhesus monkeys revealed that a high-fructose diet was related to insulin resistance and features of metabolic syndrome, with elevated leptin and decreased adiponectin concentrations (375).

In chapter 2 we showed a significant increase of adiponectin at 2 weeks in subjects consuming glucose and not fructose suggesting insulin-stimulated glucose utilization effect on adiponectin production. However, some results indicate that the total amount of caloric intake rather than the source of calories may play a more important role in controlling adiponectin levels (376). The importance of the visceral depot in determining circulating adiponectin concentrations is also supported by our results in chapter 2 showing association of fasting adiponectin with baseline abdominal/visceral fat.

6.3.2.3 ASP

In wild-type mice on a chow diet, ASP administration induces paradoxical glucose-sensitizing yet proinflammatory effects evidenced by plasma, adipose tissue, liver and skeletal muscle changes (377). On a high-fat diet, ASP exacerbates adipose tissue low-grade chronic inflammation, which is linked to other obesity-derived metabolic disorders such as insulin resistance. In C3KO mice, which are obligate ASP-deficient, a shift in the metabolic potential of skeletal muscle toward increased fatty acid utilization (227) has been reported during high fat feeding. In C5L2KO mice on a high fat diet, triglyceride storage in

white adipose tissue is reduced and postprandial triglyceride clearance is delayed (233) yet they develop more severe insulin resistance than wild type mice through altered substrate partitioning, ectopic fat deposition and a pro-inflammatory phenotype. Furthermore, a high fat/high fructose diet resulted in the development of ASP insensitivity in mice (229), which exhibited higher basal levels of plasma ASP and lower C5L2 expression in adipose tissue compared to chow-fed mice. Interestingly, injection of recombinant ASP failed to accelerate fat clearance (378). On a high fructose diet, the changes in fasting plasma ASP concentration observed in our study of extended sugar consumption are in line with the hypothesis that moderate or high sugar diet interventions promotes a state of positive energy intake and favors the development of dyslipidemia, and as a consequence increases adiposity biomarkers such as ASP.

Interestingly, we also have shown in chapter 2 that subjects consuming fructose with 0-1 metabolic risk factors had higher postprandial ASP responses than those with 2-3 risk factors. We suggested that although obesity results in high levels of circulating ASP, high ASP responses to diets that induce increased levels of TG may be an adaptive response to maintain metabolic equilibrium. This result is in agreement with the hypothesis that adaptive mechanisms, which helped man to survive in times of famine, infection and stress, may become maladaptive in the current obesogenic environment, favoring the manifestation of the metabolic syndrome (379), the same mechanisms as insulin resistance.

Fructose consumption led to increases in postprandial TG profiles and remnant lipoproteins (53). The differential effect of fructose versus glucose in post-heparin lipoprotein lipase activity demonstrated (380) may be one contributing factor leading to decreased peripheral postprandial TG clearance. On the other hand, in animal studies, a high fructose diet leads to an increase in plasma triglyceride concentrations (381). These TG, present as apoB48-containing chylomicrons, were shown to originate from fructose conversion into fatty acids within the enterocyte via intestinal *de novo* lipogenesis (382). Thus, fructose also stimulates chylomicron production. In consequence, food is one primary factor stimulating and regulating ASP production and secretion. This is supported by an *in vitro* study showing that chylomicrons stimulate adipocyte ASP production up to 150 fold (383).

However, fatty acids themselves do not affect ASP production in mature human adipocytes (220). *In vitro* data suggest that it is the interaction between chylomicrons specifically with the ASP precursor C3 which directly leads to increased ASP (384). The increased postprandial profile in *ad libitum* with fructose diets may be driving the increase in local ASP production. Although there was no difference in diurnal ASP levels in the general circulation, the persistent fructose-mediated chylomicron stimulation of local ASP production could, over time, explain a chronic increase in general circulating ASP levels (as evidenced in chapter 2).

However, the weight gain and positive energy balance cannot solely explain the metabolic consequences of fructose consumption, because these effects were absent in the glucose-consuming participants, despite a comparable weight gain. Stanhope and Havel suggested the possibility of an interaction of positive energy balance and/or weight gain with fructose consumption resulting in greater effects on lipid and carbohydrate metabolism than if the subjects had been studied only in a state of neutral energy balance (385). Increased consumption of fructose coupled to the *ad libitum* diet may be one factor that is contributing to increased ASP. Changes in ASP levels seem to be particularly associated with the combination of *ad libitum* and fructose feeding, suggesting a role of energy intake in ASP production. While ASP had no immediate diurnal or postprandial related changes, with fructose-*ad libitum* diets, circulating fasting and postprandial levels were consistently increased.

In addition, the increase in ASP correlated with changes in energy intake, postprandial lipemia, *de novo* lipogenesis (DNL), insulin sensitivity and visceral adipose tissue. However, whether the increased ASP levels result in a compensatory mechanism (related to insulin resistance) or lead in turn to an "ASP resistant" state remains to be determined. These associations are in agreement with previous studies (215), and raise the idea that ASP could be considered as a metabolic disorder biomarker. Further study on the contribution of these changes to metabolic outcomes will be needed. Therefore, our results in addition to previous animal studies show that ASP has a tissue-specific function, which is also influenced by diet composition and availability.

6.3.3 Adipose tissue dysfunction and sex hormones

6.3.3.1 Distribution of ASP in regional adipose tissue

The consequences of increased ASP production, especially in a site-specific manner, are relevant. A number of studies have examined the correlations of ASP, and its components with parameters that reflect body size or fat distribution. Many have demonstrated positive correlations of plasma ASP and C3 (ASP precursor) with BMI, total fat mass, percentage body fat and waist-to-hip ratio (215). These associations also have been shown in our studies. Because visceral adipose tissue as compared to subcutaneous adipose tissue has a limited capacity to prevent fatty acids from reaching the liver and stimulating hepatic lipoprotein synthesis (386), abdominal obesity may represent an example of impaired activity of the ASP pathway even if the dysfunction would be a secondary feature.

Growing evidence suggests that a tissue-specific function of ASP might be an important component of the hormonal interplay that enhances subcutaneous lipogenesis in females, and may therefore contribute to fat storage typical to females (387). The previous *in vitro* studies on regional and sex differences in adipose tissue response to ASP have shown higher triglyceride synthesis in subcutaneous adipose tissue compared with visceral adipose tissue (386) as well as higher binding affinity of ASP to plasma membrane extracts from subcutaneous adipose tissue compared with visceral adipose tissue particularly in women (388). As we have shown in chapter 4, under conditions of high progesterone, there is down-regulation of C5L2 (ASP receptor) and up-regulation of C3 expression (ASP precursor) in visceral but not subcutaneous adipose tissue.

In adipose tissue of women, mRNA expression of C3 and adipsin in subcutaneous adipose tissue is inversely correlated with BMI (215). In men, there was a pronounced increase in C3, adipsin, and Factor B mRNA expression in visceral adipose tissue with increasing BMI. Consequently, in both genders, the VAT-to-SAT ratio of all three factors involved in ASP production increased with increasing BMI. Dusserre et al. (389) suggested that ASP (in fact C3) gene expression was increased in visceral adipose tissue as a compensatory phenomenon to counteract the greater lipolytic capacity of the visceral cells.

In our fructose study (380), VAT area increased significantly with fructose supplementation, while SAT increased with glucose supplementation. In chapter 2 we suggest the fructose *ad libitum* effect on ASP may help to promote lipid deposition in VAT. This may be especially true under conditions of insulin resistance.

6.3.3.2 Influence of ovarian sex hormone on ASP production

Fasting plasma ASP levels have been shown to display sex differences. In chapter 3, we showed that in women, correlations with ASP (notably triglyceride, glucose, LDL-C, SHBG and height) are in the opposite direction than in men in the absence of MetS. In chapter 4, we demonstrated an association of ASP in circulation with sex hormones estradiol and progesterone. These findings may reflect increased responsiveness to ASP in women during reproductive age compared with men, and that as female hormone levels decrease in postmenopausal women, ASP resistance may develop. These findings are supported by studies showing that plasma C3 levels increased in older healthy women compared with younger women (390). The ASP resistance may also develop in obesity where obese females showed a higher level of ASP compared with obese males, which may also be a result of increased basal ASP production from subcutaneous fat mass in these women (216).

Our results, in addition to gender and regional differences in ASP levels and functions, as well as recent *in vivo* studies (391)(392)(393) are suggestive of hormonal influences on ASP production. We have shown (in chapter 5) an association of progesterone but not estradiol with C3 and related receptors C5L2, C5aR and C3aR gene expression in adipose tissue. In two longitudinal studies on normal weight young females, the ASP levels increased significantly starting in the ovulatory phase and sharply increased in the mid-luteal phase coinciding with increasing progesterone levels (391)(394). No significant correlation was seen with estrogen, luteinizing hormone or follicle-stimulating hormone. On the other hand, insulin did not show significant changes throughout the cycle and did not correlate with any of the female hormones measured (391). In agreement with these findings, it was shown in humans, that in the luteal phase, endometrium synthesizes

complement C3 *de novo*, whereas in the follicular phase, endometrium produces little or no C3 (395).

As progesterone levels were found to be tightly linked to plasma ASP levels *in vivo* and *in vitro* (396), Saleh et al. (387) hypothesized that progesterone may stimulate 'estrogen pretreated' adipocyte cells. The luteal phase in women is characterized by increased progesterone levels that are preceded or accompanied by estrogen elevations. Estrogen would therefore create significant changes in adipocyte function, which may include enhancing progesterone action by up-regulating specific binding sites (387).

Integrating our results and previously published results (391)(392)(393)(394)(395)(396), I speculate that there is an increased resistance to ASP-C5L2 function at high progesterone levels, and this may contribute to enhanced ASP production during high progesterone states such as the luteal menstrual phase and the mid-to-late pregnancy phase. This is comparable to the recognized variations in response to insulin, showing insulin responsiveness during early pregnancy and increased insulin resistance as pregnancy progresses where high progesterone levels, in spite of their lipogenic effects, exhibit anti-insulin effects (397).

6.3.3.3 Influence of sex hormones in ASP function

ASP metabolism may be altered in association with sex hormone disturbances probably contributing to accompanying lipid alterations (218)(398). High levels of ASP and C3 in PCOS women regardless of their BMI, and a decrease in ASP levels after treatment with metformin, are in agreement with this notion. An indirect evidence for the interplay between metabolic syndrome, ASP and sex hormone is the fact that, when treating components of one, parameters of the other can be improved as well.

Influence of sex hormones on circulation/function of ASP may be explained by the *ponderostat hypothesis*. It is proposed that the ponderostat system regulates lipid storage to an optimally adjusted mass, but that obesity-related metabolic disorders rapidly change the pre-established ponderostat settings in such a way that either the brain becomes less sensitive to the signal(s) from white adipose tissue or this tissue shows a decreased

capability to synthesize the signaling molecule(s) (399). It has been hypothesized that in obesity, the brain has lost its ability to control the size of adipose tissue (400) probably because of inadequate sensing of adipose tissue actual size due to either lower levels or insensitivity to ponderostat signals.

Estrone is synthesized in significant amounts by adipose tissue (401) which stores it largely as its oleoyl-ester (402), a precursor of a postulated ponderostat signal (403). The effects of oleoyl-ester on lipids can be summarized in the inhibition of lipogenesis in adipose tissue (404). These effects include decreased lipoprotein lipase expression and activity in adipose tissue (and hepatic lipase in liver), but increased activity in muscle (405), while intracellular lipolysis is unaffected (404). However, estrone levels were not related to ASP and its related proteins in our study. Therefore other, as yet unidentified, steroid hormone conversions may be of relevance.

I hypothesize that ASP levels increase in females, probably under sex hormone influence, and may enhance lipogenesis in favour of subcutaneous fat storage, as evidenced by functional and binding studies in vitro. On the other hand, reduced ASP responsiveness associated with decreased female hormone levels, may contribute to directing fat storage towards abdominal fat depots predisposing to adverse metabolic events. I conclude that although ASP levels and function are positively associated with increased fat mass. ASP levels may also be influenced by sex hormone changes that may enhance ASP action promoting subcutaneous fat storage characteristic in females.

6.4 Adipose tissue dysfunction, ASP and obesity related consequences

In chapter 3 and 4, we have shown evidence that points to a close crosstalk between metabolic organs (liver, adipose tissue) and innate immunity (C3 and its related proteins) in the course of metabolic disorders and liver steatosis. In particular, hormonal factors of innate immunity, C3, ASP and their related receptors are thought to contribute to metabolic dysregulation of adipose tissue or liver, and I propose that there are two major consequences of adipose dysfunction: 1) energy re-partitioning that provides the impetus for excess lipid accumulation in non-adipose tissues which is referred to as ectopic fat and

2) low-grade system/local inflammation. All these conditions are linked to the development of abdominal obesity and insulin resistance.

6.4.1 ASP and energy re-partitioning

With a lipogenic diet, surplus FFA flux to liver, skeletal muscle and pancreas provides the potential for excess lipid accumulation in non-adipose tissue, which is referred to as ectopic fat. This can lead to lipotoxicity of the cell, causing cellular dysfunction and even cell death. I speculate that, in an ASP resistance state, when fatty acid utilization / availability ratio is low, as in conditions of obesity and insulin resistance, there is a build-up of fatty acid intermediates which can induce insulin resistance. On the other hand, during fasting when fatty acid utilization relative to availability is high, as in high ASP sensitivity, these subjects will be more insulin sensitive. This is the case in endurance-trained athletes, where elevated skeletal muscle triglyceride are associated with greater energy demands and fatty acid oxidative capacity (406).

ASP-deficient mice have delayed postprandial TG clearance, are hyperphagic, and display increased energy expenditure with a shift in the metabolic potential of skeletal muscle toward increased fatty acid utilization (227)(228). By contrast, it has been demonstrated that C5L2KO mice fed a diabetogenic diet develop more severe insulin resistance than wild type mice through altered substrate partitioning, ectopic fat deposition and a pro-inflammatory phenotype (230). Lipid content of both liver and skeletal muscle is increased in C5L2KO mice in diet-induced obesity vs wild type (230). In chapter 5 we illustrated higher hepatic C5L2/C5aR ratio in pre-menopausal women while ASP levels were lower. Inversely, association of liver enzymes with this ratio is evident in subjects with high ASP levels (ASP resistant, postmenopausal women) where liver enzymes are higher, indicating liver steatosis status. Therefore, I suggest that ASP-C5L2 dysregulation has a role in energy re-partitioning, with the potential for excess lipid accumulation in non-adipose tissue. This can lead to lipotoxicity of the cell, causing cellular dysfunction and even cell death.

6.4.2 ASP and inflammation

In chapter 4 and 5, association of liver and adipose tissue complement cleavage proteins and receptors with metabolic profiles in women has been shown. Increasing evidence points to multiple functions of the complement system beyond immunity. Interestingly, the effects of complement seem to be context- and organ-dependent (238)(407). Complement components C3 and C5 and their derivatives C3a, C3adesArg (ASP), and C5a are central players influencing the physiology and pathology of liver and adipose tissue.

Basal levels of complement activation have rather beneficial metabolic effects, ranging from stimulation of insulin secretion in pancreatic β-cells (ASP, Factor H) to insulin-like actions with regards to adipocyte maturation and energy regulation (Factor D/adipsin, ASP, C3a, C5a) (238). In contrast, increased complement action can contribute to metabolic pathology. In the pancreas, complement activation can contribute to type 1 diabetes mellitus (T1DM) and T2DM. In obesity, complement components promote inflammatory cells and proteins to adipose tissue, thereby facilitating inflammation and the associated insulin resistance (222)(238). Interestingly, the complement system contributes to both liver homeostasis and disease (238).

Chronic recombinant ASP administration in C3 (-/-) mice enhances the high fat diet induced inflammatory response leading to an insulin-resistant state (378). C5L2 serves a dual function, binding the lipogenic hormone acylation stimulating protein (ASP), and C5a, involved in innate immunity. Disruption of C5L2 increases macrophage presence in white adipose tissue, contributing to obesity-associated pathologies, and further supports a dual role of complement in adipose tissue (230). Songqing et al have shown a dual role of ASP-C5L2 interaction in the balance between hepatic ischemia/reperfusion injury and liver regeneration in mice (305).

6.4.3 Fatty liver; an example for association of ectopic fat and inflammation

In chapter 5, we demonstrated that hepatic C3-related receptors were associated with metabolic profile in women. In fact, we hypothesized that the obesity-associated chronic low-grade inflammation seen in the liver is unequivocally linked to the development of metabolic syndrome and liver steatosis. C3 and C5 complement may contribute through different mechanisms to the pathogenesis of liver steatosis. Interestingly, the complement system contributes to both liver homeostasis and inflammatory diseases. Depending on the context, complement factors can act in either a beneficial or detrimental manner. For instance, the effects of complement activation during alcoholic and non-alcoholic liver disease are very distinct, while C3 (−/−) mice demonstrated a reduced steatosis in alcoholic liver disease (294), it did not change or rather enhanced the steatosis associated with high-fat diet (294)(296) and liver regeneration (305). Similar results have been reported by inhibition of C5 (293). C3-deficient mice have been found to be protected from alcohol-induced steatosis and from microvesicular and macrovesicular hepatic triglyceride accumulation (294)(295)(241). In addition, C3-deficient mice on an ethanol diet have a decreased expression of lipogenic enzymes, elevated serum and liver adiponectin levels, and a reduced ethanol-mediated induction of serum alanine aminotransferase activity (294)(296)(295) (241).

Higher C5L2/C5aR ratios in women with lower liver steatosis status (lower liver enzymes) demonstrates the potential of C5L2 to suppress C5a-C5aR-mediated responses, indicating two things: I) regulatory role of C5L2 in inflammatory function of C5aR, here as a liver protector protein II) evidence for interaction between C5L2-C5aR. In agreement with these results, a very recent publication on C5L2 (-/-) mice demonstrated dramatic increases in inflammation in allergic contact dermatitis as compared to wild-type mice. These increases were completely reversed following administration of mAb against C5aR (408). In another study, a protective role for C5L2 and ASP in liver regeneration in mice has also been suggested, as administration of ASP in C3KO mice restores adequate liver regeneration, an effect absent in C5L2KO mice (305).

6.4.4 ASP and cardiometabolic risk factors

Associations of ASP with cardiometabolic risk factors have been shown in chapters 3 and 4. Whether acute phase complement proteins such as C3, its cleavage product ASP and its related receptors are only biomarkers of inflammatory and cardiovascular diseases, or whether, they play crucial roles in cardiometabolic and athero-thrombosis (409) disorders remains to be proven. C3 has been recently identified as predicting coronary or cardiovascular events in several studies, whether independently (410) or dependently (411) of traditional established risk factors. In patients with coronary artery disease, complement proteins have been observed to be deposited and activated in the vessel wall (412). Plasma C3 levels may contribute independently through direct effects in atherosclerosis development or may simply reflect plasma ASP values.

We have shown in chapter 3 that there are diverging correlations across genders between ASP levels with some metabolic profiles among risk variables. Compared with women, C3 in men is independently and weakly related to the cluster of metabolic syndrome and exhibits an independent diabetogenic component (413), while in females C3 is tightly linked to a strong pro-inflammatory state, an additional component comprised in metabolic syndrome. Serum triglyceride levels were closely linked to C3 and ASP concentrations, mediating the risk for coronary heart disease (CHD). The role of gender is related to the question of whether elevated C3 and ASP may be part of the cluster of metabolic syndrome, or whether gender confers additional CHD risk (414).

Obesity itself may partly explain the increased C3 and ASP seen in metabolic syndrome and cardiovascular disease, as evidenced in chapter 4. On the other hand, C3 and cleavage products play a role in many processes that are altered in these cardiometabolic diseases. Activation of the complement C3 cascade leads to production and formation of anaphylotoxins, which likely mediate immune and inflammatory responses in ischemic myocardium (415).

Extensive activation of C3 and its activation products (C3a, C3b) are considered to be involved in the atherothrombotic process (416), and the association of complement C3b with lipid components in the vessel wall may induce an atherosclerotic process (417).

Elevated levels of activated complement byproducts including C3 have been found in blood and atherosclerotic plaques of patients with myocardial infarction (412). The complement dysregulation in the absence of CD55, a membrane inhibitor of the C3 convertase, provoked increased C3adesArg production that, in turn, caused altered lipid handling, resulting in atheroprotection and increased adiposity (418).

In the postprandial phase, lipoproteins such as chylomicrons and HDL contribute to C3 activation in the blood (419). The changing cytokine levels may contribute to the development of insulin resistance and metabolic syndrome (412). Current evidence suggests that elevated C3 and ASP concentrations constitute a prominent determinant of metabolic syndrome in both sexes and contribute in men independently and additively from the latter to the risks of diabetes and CHD. C3 appears to be part of the pro-inflammatory/oxidative state crucial in the mechanism of cardiometabolic disorders among women.

Results in chapter 3, and 4 evidence that elevated circulating C3 and ASP is not only a marker but may be a risk factor for the development of cardiometabolic disorders. Clinically, it may directly influence vulnerability of atherosclerotic plaques and enhance the development of acute coronary syndromes, or may trigger immune mechanisms and mediate impairment in function of apolipoproteins on HDL particles (420).

6.5 ASP resistance

Similar to insulin resistance, an ASP-resistant state has been proposed to contribute to the disturbed adipose tissue metabolism and dyslipidemia common in diabetes and cardiovascular disease. Hypothetically, ASP resistance would be evidenced by a reduced response to ASP, coupled with increased plasma ASP and associated lipids such as apoB (220). In other words, having increased levels of hormone (ASP; ligand) and increased levels of target substrates (triglyceride or glucose) yet decreased tissue receptor (C5L2) would be indicative of a decreased responsiveness. The underlying mechanism behind the C5L2 downregulation in adipose tissue in obesity remains to be elucidated. Chronically elevated ASP levels, as seen in obesity in both mice and humans (409), could be directly responsible for ASP resistance. Obesity is associated with increased fatty acids and inflammatory factors. Similarly, fatty acids (as dietary chylomicrons or non-esterified fatty acids) also increase ASP production and decrease C5L2 expression and protein (409)(226)(220) . In both instances this is associated with decreased ASP−C5L2 pathway functional responses.

In addition, a high ASP level in circulation could be explained as an adaptive mechanism which has also been proposed for insulin resistance (379). In addition to positive regulation of ASP function in energy homeostasis, nature has evolved a number of ways to change ASP action in response to certain conditions, including fasting, inflammation, stress and pregnancy in a way to allow mobilization of stored energy substrates. It has been shown in some physiological situations, such as late gestation in pregnancy or in the luteal phase of the menstrual cycle in women, that ASP is increased. In a cross sectional study (421) in the Inuit population in Northern Quebec, which has been characterized as having a lower risk for ischemic heart disease compared to Caucasian populations, ASP was still « sensitive » despite increased ASP levels suggesting that the higher ASP levels were in response to the traditionally higher dietary fat consumption as a compensatory mechanism.

In conditions of positive energy balance, in the current environment (Western lifestyle), limited “adipose tissue expandability” (422) and induced central obesity, may

lead to dysfunctional adipose tissue. This would then result in increased flux of FFAs into the circulation and uptake by the liver or muscle cells and other organs, as well as a low-grade inflammation, which together with chronic stress would activate the same mechanism as in insulin resistance, favoring the development of the metabolic syndrome. Thus, the manifestation of the metabolic syndrome in Western lifestyle could present a maladaptive phenotype in the current environment. In chapter 2 we have shown that consumption of both glucose and fructose markedly increased plasma ASP levels at 2 and 8 weeks. We believe this increase was a result of increasing postprandial triglyceride as well as a positive energy balance.

Furthermore, while ASP may be increased as a response to insulin resistance, some cellular studies support the interpretation that the alteration in ASP pathway may be a direct response to the effect of the sex steroid hormones on the downregulation of C5L2. For instance, physiological increases in sex steroid hormones in PCOS (polycystic ovary syndrome) likely induce decreased C5L2 expression and signaling, leading to ASP resistance (based on interpretations from cellular studies). In chapter 4 and 5 we have shown a higher ASP concentration in severely obese postmenopausal women vs premenopausal women, and associations of ASP and estradiol with components of the metabolic syndrome as well as a down-regulation of C5L2 (ASP receptor) and up-regulation of C3 expression (ASP precursor) in visceral but not subcutaneous adipose tissue in conditions of high progesterone. Sex hormones interfere with specific components of the insulin signaling pathway involved in glucose transport stimulation in adipocytes and muscle (423)(424)(425). As these signalling pathways are also targeted by ASP, I speculate that this may be a mechanism of ovarian hormone-induced ASP resistance characterized by increased ASP as “an adaptive stress response”.

6.6 Bariatric surgery outcome

By improving the components of the metabolic syndrome (dyslipidemia, hypertension, insulin resistance, and central obesity), bariatric surgery alters the cardiovascular risk profile of morbidly obese patients (426). While the contribution of gastrointestinal hormones to this improvement have been well documented, recent findings suggest that pancreatic hormones (glucagon and pancreatic polypeptides) as well as adipose tissue hormones (visfatin, leptin, ASP and adiponectin) may contribute to the resolution of insulin resistance in the long term (427). Bariatric surgery (specifically, BPD with duodenal switch) rapidly corrects ASP and C3 (within days) which is then associated with early improvement of insulin and glucose metabolism long before significant weight loss has occurred, it also improves or cures hypertension and corrects dyslipidemia (343)(428). Optimal results for the remission of obesity-related complications after bariatric surgery will occur especially if patients who are best suited to the surgery are identified. Therefore, to be able to make such decisions, we need preoperative information on the association between possible predictors and outcome. Duration of diabetes was the best predictor of success after gastric bypass surgery in Asian patients (429). Pre-surgery β-cell glucose sensitivity and meal-stimulated GLP-1 response were the only predictors of remission after Roux-en-Y gastric bypass and sleeve gastrectomy (430). C-peptide is also proposed as a predictor for remission of type 2 diabetes after bariatric surgery. Thus, bariatric surgery is recommended for obesity-related T2DM patients with elevated C-peptide (431). In addition, it has been shown that preoperative adiponectin concentrations may be predictive of the extent of weight loss, and both the changes in ASP and adiponectin are predictive of decreased apolipoprotein B and improved insulin action, respectively (343).

In chapter 5 we have shown that in the pre-operative state, increased C5L2/C5aR mRNA ratio was associated with lower BMI, apoB and liver enzymes, and yet higher apoA1. Further, higher values of hepatic C5L2/C5aR ratio were associated with greater relative weight loss, greater decreases in fasting triglyceride and lower liver enzymes both before surgery and over the follow-up. Overall, these data suggest that a high hepatic C5L2/C5aR mRNA ratio is a beneficial feature, but the specific functional roles of C5L2 and C5aR in relation to lipid metabolism in the liver remains to be elucidated.

6.7 Conclusion

ASP and its receptor C5L2 are important factors in the regulation of fat metabolism. ASP responds differently according to gender, ethnicity, metabolic disorder, sex hormone levels and diet composition. It acts as a key factor in the crossroad of adipo-hepatic axis, reproductive hormones and inflammation which are determinants of obesity and obesity consequences. This thesis favors a need for a conceptual shift from adipose tissue mass (defined as obesity) to adipose tissue function

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