Analysis of differentially expressed serum proteins during development of Obesity

By

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RESEARCH DISSERTATION

Submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

in

BIOCHEMISTRY

in the

FACULTY OF SCIENCE & AGRICULTURE

(School of Molecular & Life Sciences)

at the

University of Limpopo

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2009

Diabetes Research Group of the

South African MEDICAL RESEARCH COUNCIL

BUILDING A HEALTHY NATION THROUGH RESEARCH

Declaration

I declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Master of Science in Biochemistry has not been previously submitted by me for a degree at this or any other University; that it is my work in design and in execution, and all material contained therein has been duly acknowledged.

NRS SIBUYI (MISS) 29/06/2009

Initials & Surname (Title) **Date**

Dedication

I dedicate this work to my parents Thompson and Milinah, my brothers: Mack, Sydney, Reineck, Edwell and Quirin; my sisters: Julia, Agnes and Pinky and my beautiful flower Lee for believing in me, your support and mostly your love. You guys are the greatest and I love you very much.

"Life is a gift and every breath we take is a gift from God. We all have the power to make the decision to either live life a 100% or just to float along, allowing influences to control our lives.

There is no right or wrong way of living this wondrous journey, but if we can live it the best possible way we can, enjoying everything around us. Being in "this moment" constantly growing and learning, appreciating and loving, we would be much richer for it. "Anonymous"

Acknowledgements

From the bottom of my heart I sincerely thank the following people.

1. My heavenly Father for always looking out for me and knowing my needs, and giving me strength to finish up the project.

2. National Research Foundation and MRC Diabetes Research Group for funding this research.

3. Prof Mampuru for giving me this wonderful opportunity and believing in me.

4. Dr Madiehe for your patience, encouragement and allowing me to work with you and in your laboratories.

5. Dr Ndimba for helping out with the proteomics work and allowing us to work in your laboratory.

6. Prof J Rees for your advice and encouragement.

7. Mr Phineas Mokgotho for helping out with the registration and other academic stuff I needed help with. It really means a lot to me.

8. My DRG family: Kwazi Gabuza for being there for me from the beginning without complaining about me crowding your space but mostly for being my body guard and co-co supervisor. Don't know what I would have done without u…probably "ngi yeka yonk' into ngi yo funi umsebenzi eGoli"

Peggy Mobo: you taught me how to run my first gel and for that I'll forever be grateful and for helping out with the animal studies.

Andrew Tomboer: without you my project would have definitely fallen apart. Thank you very much for everything

Dr Amanda Skepu: for all the assistance and guidance that you gave me throughout this study.

Nelusha Gounden, Cleo Dodgen, Refiloe Mofokeng Siphelele Zulu, Carli Dimitrich, Siyamcela Genu, Nomgcobo Dodo, Euphodia Makondo, and Ntheveleni Thovhogi.

You guys really helped me to understand the true meaning of family, most of all you were there for me throughout my research journey. It wasn't easy and it could have been worse without you holding my hand, and believing me: the cakes worked wonders (thought of it made me rush to work every morning). You guys rock.

9. University of the Western Cape - Proteomics Research Group:

Dr Ludivine Thomas for the mass spectrometry analysis.

Nonkululeko Ndabambi, Xolisa Melamane, Rudo Ngara, Simphiwe Mahuzi and James Sehata for all your help with the proteomics work.

10. Vusi Mbazima and Koena Sekhula for keeping in touch, with you I know coming home won't be so lonely.

11. My parents and siblings, I feel really honoured to be part of you guys and there's nothing I can do without your love and support, thank you so much. I love you.

12. My dearling daughter Lee for her patience and letting me do this. Love you sweet thing.

13. Last but not least: everyone who has been part of this journey. Not mentioning your names doesn't mean you're least important to me, on the contrary I couldn't have done it without your love and support. You probably won't see this but I am grateful to have you in my life, my own angels sent to keep me sane.

THANK YOU SO MUCH

Abstract

Obesity is a complex and chronic disease that is associated with many complications including type 2 diabetes (T2D). However, the mechanism leading to these events is unclear. To determine the role played by obesity in the development of T2D, it was purposed to examine serum protein profiles of diet-induced obese Wistar rats. These protein(s) will be used as potential biomarkers for early detection, diagnosis, as target for drug discovery and hopefully treatment of obesity and obesity-induced disorders. An animal model of obesity-induced T2D was established by feeding male Wistar rats a diet with a high fat content for 44 weeks. Weight changes and food intake were monitored weekly during the diet phase. Fasting blood glucose levels were measured while blood was collected for serum preparation every second week for the first eight weeks; using these parameters, a model for the identification of diet-induced obese rats was established. Serum protein profiles were then compared between the two groups using 2D-PAGE analysis coupled to MALDI-MS at termination of the study. Several proteins showed differences in their expression when compared between the low fat (LF) and high fat (HF) groups. However, only proteins that showed expression that was either two fold low or high between the two groups were considered to be differentially expressed.

High fat fed rats weighed significantly heavier starting at the fourth week on diet and throughout the study, their glucose homeostasis and serum protein expression was altered. Three protein spots were identified as dysregulated in the HF group, where Apolipoprotein-AIV was found to have been up-regulated whereas C-reactive protein and Fetuin-A were down-regulated. These proteins might help in the understanding of the mechanism(s) that underlie the pathogenesis of obesity and its related disorders. However, further studies are required to determine the relationship between these proteins, obesity development and its comorbidities.

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LIST OF ABBREVIATIONS

2-D	Two-dimensional
2-DE	Two-dimensional electrophoresis
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
ACN	Acetonitrile
Apo A-IV	Apolipoprotein A-IV
APS	Ammonium persulfate
AR	Adiposity rebound
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
BW	Body weight
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1 propanesulfonate
CRP	C-reactive protein
DIO	Diet-induced obesity
DM	Diabetes mellitus
DTT	1,4-dithiothreitol
EQ	Equilibration buffer
ESI	Electrospray ionization
FFA	Free fatty acid
FIRKO	Fat-specific insulin receptor knockout
FIZZ	Found in inflammatory zone
GAD65	Glutamic acid decarboxylase
HAPs	High abundant proteins
HbAlc	Glycosylated haemoglobin
HCl	Hydrochloric acid
HDL	High density lipoprotein
HF	High fat
HRP	Horseradish peroxidase
IAA	Iodoacetamide
IAP	Islet amyloid polypeptide
IEF	Isoelectric focusing
IGF-1	Insulin-like growth factor 1
IL	Interleukin
IPG	Immobilized pH gradient
KCl	Calcium chloride
LAPs	Low abundant proteins

LB	Lysis buffer
LF	Low fat
m/z	Mass-to-charge
MALDI	Matrix-assisted laser desorption ionization
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MetS	Metabolic syndrome
mRNA	Messenger RNA
MS	Mass spectrometry
MW	Molecular weight
NCBI	National Centre for Biotechnology Information
NF-κB	Nuclear factor-kappa B
NH_4HCO_3	Ammonium bicarbonate
NIDDM	Non-insulin dependent diabetes mellitus
PBS	Phosphate buffered saline
pI	Isoelectric point
PMF	Peptide mass fingerprint
PPARγ	Peroxisome proliferator-activated receptor-gamma
RELM	Resistin-like molecule
SA	South Africa
SDS	Sodium dodecyl sulphate
SELDI-TOF	Surface-enhanced laser desorption/ionization time-of-flight
SES	Socioeconomic status
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TBST	Tris-buffered saline Tween-20
TCA	Trichloroacetic acid
TEMED	*N*,*N*,*N'*,*N'*-tetramethylethane-1,2-diamine
TNF-α	Tumour necrosis factor-alpha
TZDs	Thiazolidinediones
V	Volt
V.hr	Volt hour
WAT	White adipose tissue
α-cells	Alpha cells
β-cells	Beta cells

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CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Obesity-induced type 2 diabetes is a health problem that existed for a very long period; hence the term diabesity was coined to describe the link between the two conditions. Diabesity is triggered by interactions between genetic and environmental factors. Obesity is considered to be a strong risk factor and maybe a cause for the later development of T2D (Felber and Golay, 2002; Golay and Ybarra, 2005). The statistics indicate that 60–90% of all type 2 diabetics are or have been obese (Stumvoll *et al*., 2005), and the relative risk for an obese patient to develop T2D was found to be 10-fold for women and 11.2-fold for men (Field *et al*., 2001). Statistically the rise in obesity proved to be in direct proportion with the increase in T2D, with every kilogramme of weight gained increasing the risk of diabetes between 4.5 and 9% (Golay and Ybarra, 2005).

Diabesity is the most challenging condition that affects millions of people worldwide and has serious health problems that increase morbidity and mortality (Permana *et al*., 2004). The prevalence of diabesity has reached epidemic proportions globally (Gustafson, 2006), making the medical care costs of its single components a considerable economic burden for patients, families, and society. Productivity losses have been estimated to be $40 billion, which was almost half of the medical costs ($92 billion) associated with diabetes in 2002 in the US alone (Tunceli *et al*., 2005), and the indirect costs attributable to obesity to be $47.6 billion a year in 2003. These costs represent the value of lost output caused by morbidity and mortality, and may have a greater impact than direct costs at the personal and societal levels (Montague, 2003). Although obesity is an observable trait, T2D is mainly asymptomatic in its early stages and may remain undiagnosed for years. About a third of all people with diabetes worldwide do not know they have it. Early detection of these conditions and treatment is of the utmost importance to prevent the complications associated with T2D, or intervention strategies that can delay progression of the disease (Hirsch, 2002).

Inspite of the established pathophysiological basis of T1D, considerable uncertainty surrounds that of T2D which results from relative insulin deficiency. In T2D the pancreatic beta-cells become progressively less able to secrete sufficient insulin to maintain normal carbohydrate and lipid homeostasis (Bell and Polonsky, 2001). Available medical therapies for T2D are usually able to better the condition, but rarely restore the normal metabolism, leaving many patients exposed to debilitating and life-threatening complications (McCarthy, 2004).

The use of appropriate animal models comprises an important part of the overall strategy for elucidating the mechanisms that underlie the development of human T2D and obesity (Boustany-Kari *et al*., 2006). These include understanding the natural history of the disease - the genetic and evolutionary basis of the disease, identifying targets for therapy, and evaluating interventions and treatments. Several animal models have been established, and the ideal animal model for such studies is one that recreates the complete natural history of these disorders as they occur in human populations (Kaplan and Wagner, 2006). The success of these models can be measured based on the specificity and sensitivity of the approaches used to unravel the underlying mechanisms of these diseases (Thongboonkerd, 2004).

Proteins play an important role in most biological functions. They partake in cell signaling and signal transduction. These are the most important biological functions in which the cell react and respond to its environment, and defects in signal transduction pathways result in various diseases (Berg *et al*., 2002b). Therefore, identification and quantification of dysregulated proteins through proteomics can assist in understanding functional changes during the onset of obesity and progression to diabetes. Although proteomics is a relatively new field, proteomic techniques have been extensively applied to several fields of medicine to better understand the normal physiology, to define the pathophysiology of diseases, and to identify novel biomarkers and new therapeutic targets in the past few years (Thongboonkerd, 2004). These proteomics studies entail the use of two-dimensional gel electrophoresis for profiling complex protein mixtures based on their charge and molecular mass. This is coupled to mass spectrometry or other

methods for protein identification. Proteomics has been used in recent years for studying protein-protein interactions; characterization of macromolecular complexes using antibodies, nucleic acids, or other molecules as bait (tag) followed by identification using mass spectrometry and computational methods for structure or function prediction. Therefore the study of a subset of the proteome is feasible, and could be applied to study biological systems and diseases (Dunn and Pennington, 2004).

1.1 METABOLIC SYNDROME

Metabolic syndrome (MetS) describes a cluster of metabolic abnormalities as outlined in Figure 1.1, which are diagnosed concomitantly and are considered to be an important cause of mortality and morbidity for cardiovascular diseases (Lakka *et al*., 2002). Visceral obesity, as described later, is the major risk factor for MetS (Fülop *et al*., 2006). Insulin resistance is thought to be the underlying metabolic defect explaining the syndrome (Marks, 1996; Scholz-Ahrens and Schrezenmeir, 2006; Trevisan *et al*., 1998).

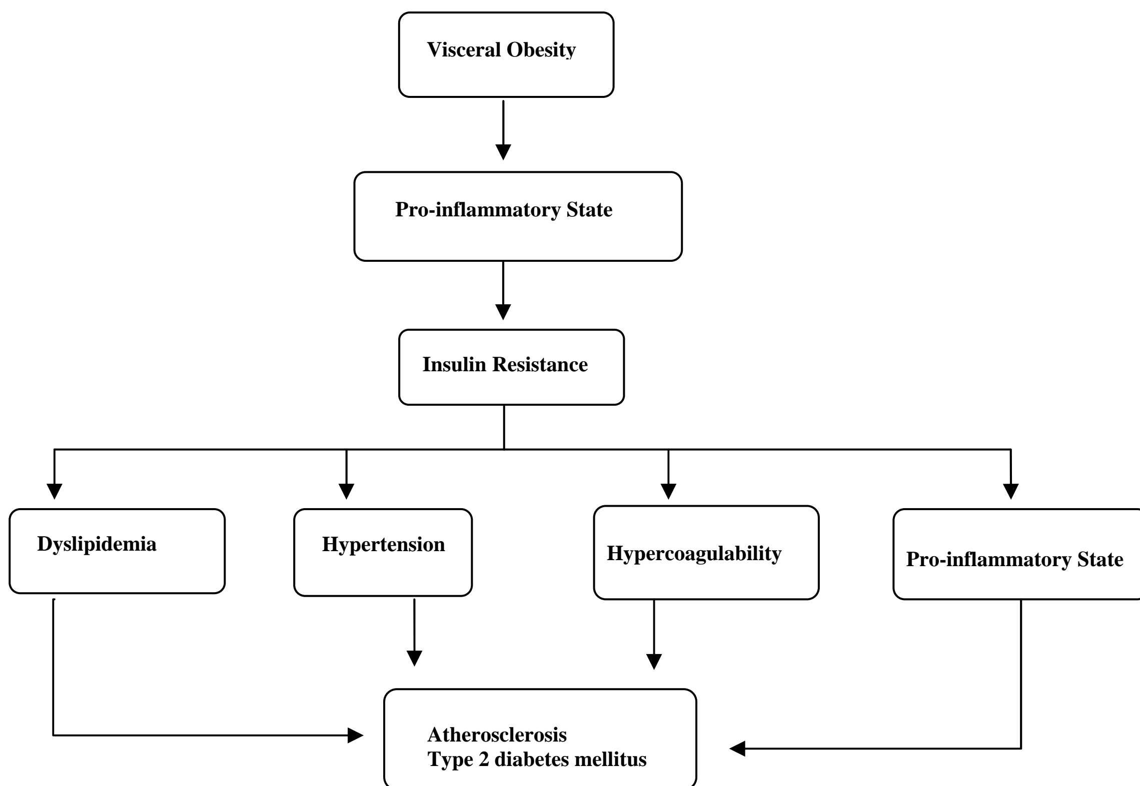

Figure 1.1: The current concept of the metabolic syndrome (Fülop *et al*., 2006).

Since the first description of the syndrome, four other metabolic abnormalities have been added to that originally described. These are: dyslipidaemia, hypertension, low HDL cholesterol and disturbances in glucose metabolism (Trevisan *et al*., 1998).

The global prevalence of the metabolic syndrome or its single components has dramatically increased in the recent past, and shows a tendency to increase in the future. The increase of these metabolic disorders is accompanied by dramatic changes in lifestyles, mainly reduced physical activity and a change in dietary habits, which includes increased consumption of fast foods and soft drinks (Lakka *et al*., 2002). The syndrome has become one of the nation's costliest health concerns, with the current cost of its single components estimated to be at $132 billion per year (about R924 billion in SA currency) in the United States for diabetes alone. In 2002, at least half of the fifteen member states of the European Union had obesity levels of greater than 20% which costs €32.8 billion per annum. The reduction of the socioeconomic status and individual burden derived from the high costs that are attributable to the metabolic syndrome should have high priority. The genetic predisposition, environmental and lifestyle factors plays a major role in the development of the MetS. Based on public health recommendations, environmental factors, like physical activity and nutrition, can be modulated cost-effectively by individuals themselves (Scholz-Ahrens and Schrezenmeir, 2006).

1.2 OBESITY

Obesity is a chronic metabolic disorder that results when energy intake exceed energy expenditure for a prolonged period (Mutch and Clement, 2006). Obesity is assessed by a BMI of 30 $kg.m^{-2}$ or greater in adults (Kopelman, 2000). Childhood obesity (2 to 20 years of age) is measured differently due to variations that occur throughout childhood (Proietto and Baur, 2004). It is based on BMI-for-age, and is assessed in relation to the weight of other children of a similar age and gender. Children and adolescents with a BMI between the 85th to 95th percentiles (percentile refers to the relative position of the child's BMI among children of the same gender and age) are considered to be overweight while those with a BMI greater than 95th percentile are obese (Collins, 2005; Proietto and Baur, 2004).

The global epidemic of obesity results from a combination of genetic susceptibility, increased availability of high-energy foods and sedentary lifestyle (Kopelman, 2000; Lean, 2000). The prevalence of obesity has been increasing at an alarming rate for several

decades in both adults and children reaching epidemic proportions world wide (Artiss *et al*., 2006). In SA, combined figures for all ethnic groups in 1998 estimated that 57% women and 27% men were obese for the adult population. The prevalence for women was especially higher for blacks at 34% followed by coloureds at 28% when compared to Indians and whites (Van der Merwe and Pepper, 2006).

Obesity poses a major threat to health and longevity, and is rapidly replacing malnutrition and infectious diseases as the most significant contributor to ill health in the United States (Olshansky *et al*., 2005). Excess body fat is strongly associated with increased risk of developing a number of potentially serious health problems including hypertension, T2D, stroke, cancer, sleep apnoea, gout and cardiovascular diseases (NIDDK, 1990; National Institutes of Health, 2002; Kopelman, 2002). Together with its comorbidities, obesity markedly increases aging process thereby reducing life expectancy (Artiss *et al*., 2006; Haslam and James, 2005).

1.2.1 Body Mass Index Information

BMI is the gold standard in medicine for assessment of healthy weight, overweight and obesity. BMI is calculated by dividing body weight in kilograms by the square of height in meters (Collins, 2005; Kopelman, 2000).

Table 1.1: Standards used for assessing BMI in adults

BMI (kg/m^2)	**Classification**
<18.5	Underweight
18.5 – 24.9	Normal weight
25 – 29.9	Over weight
30 or more	Obese
30 – 34.9	Grade I
35- 39.9	Grade II
40 or more	Grade III

BMI results are interpreted according to Table 1.1 as proposed by the World Health Organization. BMI of 18.5 to 24.9 kg/m^2 is considered normal and healthy. A BMI of 25.0 to 29.9 kg/m^2 is considered overweight which might lead to moderate health risks. Obesity is defined as a BMI of 30 kg/m^2 or greater whereas a BMI of 30.0 to 34.9, 35.0 to 39.9, and 40.0 kg/m^2 or greater, are classified as grade I, II, and III obesity respectively. Severe clinical obesity includes morbid obesity with a BMI greater than 40 kg/m^2 and malignant obesity at greater than 50 kg/m^2. However, BMI has some deficiencies and does not account for individual frame size, muscularity, fat distribution and the changes of ageing (Collins, 2005; Merrick *et al*., 2002; Welborn *et al*., 2000).

In a study done by Kaluski *et al*., (2007) obesity was significantly associated with socioeconomic status (SES) especially among women. Women with lower SES as a result of lower height and higher weight were classified as obese. However the opposite was observed in men, the risk of overestimating obesity prevalence by using the BMI was lower in taller men and men with higher SES were found to be heavier. The results showed non-significant differences in BMI between men of the higher and the lower SES. Calculating BMI is sometimes compromised due to the effects of increased weight and the decrease in height. For instance, a change in weight by a certain percentage results in a similar percentage change in BMI, whereas a change in height leads to twice the percentage change in BMI (Kaluski *et al*., 2007).

1.2.2 Aetiology of Obesity

Although the development of obesity has a potential genetic component, the mechanism thereof is still not known. Genetic influences are difficult to elucidate and identification of the genes is not easily achieved in familial studies. Moreover, the influence that genotype might have on the aetiology of obesity is generally attenuated by non-genetic factors (Afridi and Khan, 2004). The tendency to gain weight is hereditary; however, family members share not only genes but also diet and lifestyle habits that may contribute to obesity. Separating these lifestyle factors from genetic factors is often difficult, especially with evidence pointing to heredity as a strong determinant of obesity (Afridi and Khan, 2004).

Genetic studies on monozygotic and dizygotic twins proved beyond doubt that obesity development is influenced by genetic factors, which account for as much as 50 to 80% of variance in monozygotic twins (Mitchell *et al*., 2007; Sorensen and Echwald, 2001). Moreover, studies of adopted twins showed no relationship between the body weight of those children and their adoptive parents, but a close correlation with their biological parents (Sorensen and Echwald, 2001).

Although genetic factors certainly play an important role in the susceptibility for obesity, the real culprit is the environment that promotes a sedentary lifestyle and long-term energy imbalance due to excessive caloric intake relative to energy expenditure (Fruhbeck *et al*., 2001). Thus, the development of obesity is dependent on the disequilibrium between energy intake and energy expenditure during an extended period of time (Brandt *et al*., 2006; Racette *et al.,* 2003).

Weight gain during adulthood is characterized by adipocyte hypertrophy, a process by which fat cells or adipocytes increase in size to accommodate the excess lipid. In an evolutionary context, the ability to store excess energy in adipose tissue was essential for survival, because energy could be drawn from this storage depot in times of famine. Paradoxically, this survival characteristic is disadvantageous when food is continuously abundant and palatable (Racette *et al.,* 2003).

1.2.3 Critical Periods for Obesity Development

There are critical periods in life when exposure to certain environmental factors may increase the risk for development of obesity. And the weight gained during these critical periods of life commonly lead to an increased number (as opposed to increased size) of fat cells and make obesity more difficult to treat (Pocai *et al*., 2006). These critical periods include: prenatal period, adiposity rebound, puberty and adolescence and pregnancy.

Prenatal period:

Several epidemiological and animal studies support the hypothesis that prenatal malnutrition can have deleterious effects and give rise to obesity in adulthood especially when challenged postnatally with a hypercalorioc diet (Sardinha *et al*., 2006).

In humans, data from the Dutch famine studies have been used to understand the effects of nutritional deficiency in foetal life to subsequent development of obesity. Military inductees at age 19 years who were born to mothers exposed to famine during pregnancy had a higher frequency of obesity as compared to controls (Ravelli *et al*., 1976). Exposure to famine during prenatal period results in a two-fold increase in frequency of obesity in adulthood as compared with a control group (Law *et al*., 1992). The Dutch famine experience suggested that the first trimester of pregnancy represents a critical period of vulnerability to the subsequent development of obesity. Nutrient limitation during development is considered to modify the hypothalamus and the set point for energy balance (Raman, 2002). This proves that the events occurring during embryonic life can result in permanent changes in structure and function in the developing foetus even after withdrawal of the adverse stimulus (Levitt and Lambert, 2002; Sardinha *et al*., 2006).

Adiposity rebound:

Adiposity rebound (AR) refers to the time at which BMI increases after its nadir (the point of maximal leanness or minimal BMI) in childhood. Children have a rapid increase in BMI during the first year of life. After 9 to 12 months of age, BMI declines and reaches a minimum before beginning a gradual increase through adolescence and most of adulthood. Adiposity rebound may be a critical period in childhood for the development of obesity. An early AR is associated with higher BMI in adolescence and in early adulthood (Dorosty *et al*., 2000; Whitaker *et al*., 1998).

Puberty and adolescence:

Puberty is a time of rapid change in size and shape for both boys and girls. In girls earlier age at menarche is associated with obesity, independent of childhood BMI and other potential confounding factors (Pierce and Leon, 2005). Biochemically, puberty is

associated with a physiological increase in insulin resistance (Smith *et al.*, 1988). In relation to these changes in insulin metabolism, post-pubertal fat deposition in both females and males tends to be more central rather than general. Moreover, dietary patterns and levels of physical activity are largely formed in adolescence and persist into adulthood (Lawlor and Chaturvedi, 2006).

***Pregnancy*:**

The third trimester during pregnancy presents a critical period for the onset of obesity on the unborn children. For instance, infants born to diabetic mothers demonstrate both increase in body weight and body fat. Subsequently, the prevalence of obesity in these children is increased when they reach 15 to 19 years of age (Raman, 2002).

The maternal weight gain during pregnancy and post-partum weight retention is an important predictor of the mother's risk of subsequent obesity and diabetes (Sattar and Greer, 2002). It has been proposed that the antenatal period offers a unique period in the life course during which women at risk of future obesity and its comorbidities such as diabetes and cardiovascular disease might be identified, at a time when they might be particularly receptive to health promotion or disease prevention interventions (Lawlor and Chaturvedi, 2006).

1.2.4 Factors That Influence Body Weight Gain

There are numerous factors involved in weight management, some of which an individual has either no control over or has the potential to control. What causes one person to gain weight while another person maintains their weight is the subject of intense investigation. Several factors that contribute to differences in weight gain have been described: age, gender, physical activity level, diet or food preferences and also genetic or hereditary factors.

Age:

As a person grows older, the metabolic rate slows down and a person does not require many calories to maintain the weight. Obesity reaches maximal rates among middle aged (40-60 year) adults; with rates faster in women than men and fastest in postmenopausal

women than their premenopausal counterparts. With advancing age body weight becomes progressively more difficult to maintain. Therefore, individuals gain weight regardless of similar habits such as eating the same diet and doing the same activities as they did when they were younger (Myers, 2004).

Energy requirements and expenditure decrease with advancing age. Physical activity which account for a portion of the energy expenditure is reduced in the elderly. This is due to decrease in all metabolically active tissues such as skeletal muscle, brain and visceral organs (National Academies Press, 2004).

Gender:

Males have a higher resting metabolic rate than females, so males require more calories to maintain their body weight. This higher resting metabolic rate is primarily due to the increased lean body mass that men have compared to women. Additionally, when women enter menopause, their metabolic rates decrease significantly. That is part of the reason why many women start gaining weight after menopause (Myers, 2004).

Physical activity level:

Physical activity is a very important component for weight control at an individual level, and an effective adjuvant to dietary management for weight loss and maintenance (Centers for Disease Control and Prevention, 1999). Physical inactivity appears to be both a cause and a consequence of obesity, whereas active lifestyle is protective against development of obesity and chronic disease (Stubbs and Lee, 2004). Physical activity in obese individuals reduces appetite and increases the body's ability to metabolize fat as an energy source. The evidence in the last 25 years shows that the increase in obesity prevalence is due the decreased level of physical activity in everyday life, such as availability of modern transportation (Myers, 2004).

Diet or food preferences:

Diet and food preference plays a significant role in both development and control of obesity. For instance, increased dietary fat intake from high fat diets has been implicated in the development of obesity in animals and humans (Afridi and Khan, 2004) due to

energy density of fat, their palatability and the weak satiety effect. Dietary choices are influenced by sensory properties such as taste, smell and texture of food; and fats or high energy food are usually preferred over other foods because of its flavour that greatly contribute to eating pleasure (Schrauwen and Westerterp, 2000). In the last 25 years, the availability of high fat foods, such as fast foods, combined with decreased physical activity was found to be the major factor in the increase in the prevalence of obesity (Afridi and Khan, 2004; Drewnowski, 2004).

Genetic or hereditary factors:

Heredity is associated not only with obesity, but also with thinness. It most closely correlates with maternal weight. If the mother is over weight or obese as an adult especially during pregnancy, there is approximately a 75% chance that the children will be heavy. If the mother is thin, there is also a 75% chance that the children will be thin. It is related to metabolic processes inherited primarily from the biological mother (Myers, 2004).

1.2.5 Treatment of Obesity

Since obesity is a major risk factor for the development of serious chronic diseases, its treatment and management may lead to an early intervention for prevention of these maladies and their consequences (Guri *et al*., 2006).

The clinical approach to obesity treatment includes life style modification, pharmacotherapy which is limited and surgery which is only recommended for morbidly obese patients with at least one of obesity-induced disorder. The common lifestyle modification for treatment of obesity is cognitive-behavioural therapy (standard behaviour treatment), which includes behavioural modification, dietary intervention and physical activity (Cooper and Fairburn, 2001). The standard dietary intervention approach is a low-fat diet with less than 25% of calories from fat with a calorie deficit of 500 to 1000 kcal per day, which will result in 0.5 to 1 kg weight loss per week. Calorie prescription is usually based on the person's initial weight. In addition to restricting energy intake, physical activity to increase energy expenditure is encouraged or

prescribed. A report on the study of the behavioural treatment of obesity done by Jeffery *et al* (2000) for over 20 years had shown a dramatically improved short-term treatment efficacy but has been less successful in improving long-term success in adults. Long-term maintenance of weight loss is problematic. This could be because compensatory metabolic processes resist the maintenance of the altered body weight set point. Individuals regain approximately 35% of their lost weight in a year after stopping treatment. Therefore, the greatest challenge in the treatment of obesity is identifying strategies that can improve long-term maintenance of weight loss (Burke *et al*., 2006; Leibel *et al*., 1995).

Pharmacotherapy coupled with the lifestyle modification is recommended for patients who have difficulty achieving their weight-loss goals through lifestyle modification after six months of effort (Lee and Aronne, 2007). Few anti-obesity drugs are available for use for a period no longer than two years. These drugs fall into two categories: appetite suppressants and fat absorption inhibitors. Appetite suppressants act on neurotransmitters (i.e., norepinephrine, serotonin, and dopamine) within the central nervous system, whereas drugs that inhibit nutrient absorption act on pancreatic enzymes to prevent the conversion of dietary fat into absorbable free fatty acids. At the moment there are only two drugs approved for long-term use in the treatment of obese type 2 diabetic patients: sibutramine and orlistat (Lee and Aronne, 2007).

Sibutramine:

Sibutramine is a serotonin and norepinephrine reuptake inhibitor that reduces body weight by suppressing appetite. Phentermine is the most widely prescribed appetite suppressant weight loss agent for the treatment of obesity; however its use is restricted for only up to two years due to lack of information from long-term clinical trials. Their use in some obese patients is contraindicated because it inhibits the reuptake of norepinephrine and may increase their blood pressure. Other side effects of sibutramine include increased heart rate, insomnia, constipation, headache and abdominal pain. For normotensive obese patients, sibutramine, in combination with diet and behavioural

modifications, has been demonstrated to have beneficial effects (Artiss *et al*., 2006; Lee and Aronne, 2007).

Orlistat:

Orlistat is a pancreatic lipase inhibitor that reduces fat intake by inhibiting fat absorption from the diet through inhibition of pancreatic lipase activity in the small intestine. When taken in conjunction with a hypoenergetic diet it can induce modest weight loss and better weight maintenance than diet alone (Hollander *et al*., 1998). However, in the absence of major dietary changes; the adverse effects of gastrointestinal discomfort, flatulence, and diarrhoea have limited its use. In addition, its long-term efficacy and safety have not been established (Artiss *et al*., 2006).

1.3 DIABETES

Diabetes mellitus (DM) commonly known as diabetes or sugar diabetes is a chronic medical condition which occurs when the human body is unable to produce or use insulin properly. This could be as a consequence of variable interaction of genetic and environmental factors. Diabetes mellitus is characterized by varying or persistent hyperglycaemia (elevated glucose levels) accompanied by abnormal insulin secretion after a meal (Singh *et al*., 2005).

Diabetes afflicts approximately 6% of the entire population globally. Due to increased prevalence and propensity of diabetes to cause end-organ damage and its complications, about 100 billion dollars in the United States is spent on health care costs annually (Korc, 2003; Shetty *et al*., 2004). Even though it was regarded as a disease of minor consequence to world health, it now occupies one of the main causes of serious maladies in the 21st century. The number of people with diabetes is expected to increase from current estimate of 150 million in 2010 to 300 million in 2025 (Sang *et al*., 2005).

1.3.1 Classification of Diabetes

Several types of diabetes exist, including maturity-onset diabetes of the young which is characterized by onset of hyperglycaemia at an early age (before age of 25 years) and

gestational diabetes which is defined as a degree of glucose intolerance recognized during pregnancy (American Diabetes Association, 2004). However only the two main forms of diabetes are going to be discussed, namely, type 1 and type 2 diabetes. They are classified based on their aetiology and not their insulin dependence and usually characterized by hyperglycaemia due to defects in insulin secretion, insulin action, or both (Wareham and O'Rahilly, 1998).

1.3.1.1 Type 1 Diabetes

T1D is also known as childhood or juvenile onset diabetes because it is most commonly diagnosed in children and adolescents, but can also occur in adults. It is an insulin dependent form of diabetes (Couri *et al*., 2006). T1D results from an autoimmune mediated destruction of the insulin-producing ß-cells in the pancreatic islets of Langerhans by T-lymphocytes, which leads to an absolute deficiency of insulin. The global increase in its incidence strongly suggests that the action of putative environmental risk factors such as viruses or nutritional factors play a major role early in life, possibly in utero (Leslie and Elliott, 1994). Therefore, T1D results from a complex interplay between the ß-cell, the immune system, and the environment factors in genetically susceptible individuals (Sparre *et al*., 2005; Stene *et al*., 2001).

T1D accounts for about 5 to 10% of all cases of diabetes. Although most T1D cases are immune-mediated, there are other forms of T1D that are characterized by the loss of β-cells without evidence of autoimmunity (Bekris *et al*., 2006; Couri *et al*., 2006). These include atypical or idiopathic T1D, which is characterized by diabetic ketoacidosis as the initial clinical presentation, lack of autoimmune markers at diagnosis and has physical characteristics that are more typical of patients with T2D. This type is common in young obese African-Americans but also occur in other ethnic groups. Usually patients start treatment with insulin therapy to maintain glycaemic control followed by either diet or oral hypoglycaemic agents as in T2D (Bennett *et al*., 2001; Piñero-Piloña *et al*., 2001).

Typical T1D disease unfolds through the following two main stages: firstly the occult phase - insulitis, occurs when a mixed population of leukocytes invade the islets

promoting β-cell death; and secondly the overt phase - diabetes, occurs when most of β-cells have been destroyed and the pancreas can no longer produce sufficient insulin to control blood glucose levels. The factors initiating the destructive process are largely unknown and no genes or proteins are specific for most T1D cases (Denis *et al*., 2004; Sparre *et al*., 2005). Multiple autoantibodies such as those recognising glutamic acid decarboxylase, insulin, islet cell antigen 512, phogrin and islet amyloid polypeptide and human leukocyte antigen gene products (DR4-DQ8 and DR3-DQ2) have been implicated in the pathogenesis of T1D (Danke *et al*., 2005; Gillespie, 2006). Although these antibodies and genes are common in 90% of newly diagnosed diabetic patients and often appear years before the clinical onset of the disease, their functional role in the pathogenesis of T1D is still not clear. These proteins and genes are also implicated in other autoimmune diseases such as autoimmune thyroid disease and stiff-man syndrome and polyendocrine autoimmune disorders (Reijonen *et al*., 2000).

(i) Causes of T1D

In T1D, the body has little or no insulin due to attack and destruction of the β-cells in the pancreas by the immune system. What causes this autoimmune response is not clear; however the condition can either be triggered by environmental and genetic factors in susceptible individuals. Although viral infection is considered to be one of the potent environmental factors that play a role in the pathogenesis of T1D (Hyoty *et al*., 1988), several proinflammatory cytokines, in particular IL-18, have also been reported to be associated with the pathogenesis of this autoimmune disease (Hennige and Hans-Ulrich, 2004; Masuda *et al*., 2007).

(ii) Signs and Symptoms of T1D

The signs and symptoms of T1D often appear after a flu-like illness and gradually intensify over the course of a few weeks. Typical features include: Increased thirst and frequent urination, extreme hunger and weight loss.

Increased thirst and frequent urination:

Excess glucose builds up in the bloodstream, and then the glucose pulls water from the body's tissues, resulting in dehydration and eventually thirst. This leads to drinking more

fluids and frequent urination. The excess glucose in the bloodstream passes through the kidneys and leaves the body through urine (Barrett and Porter, 2006; NIDDK, 2006; Torpy *et al*., 2003).

Extreme hunger:

The basic defect in type 1 diabetes is an inability to produce insulin, the hormone necessary for glucose uptake by cells to fuel their functions. This leaves the muscles and organs energy depleted. This triggers intense need for energy and thus induces hunger. Even when the stomach is filled the hunger persists because without insulin, the glucose produced from dietary carbohydrates never reaches the energy-starved tissues (NIDDK, 2006).

Weight Loss:

Despite eating a lot to relieve the constant hunger, people with T1D lose weight, sometimes rapidly because the cells are deprived of glucose which is lost into the urine. Without the energy supplied by glucose, cells die at an increased rate before they can divide and replace themselves. Muscle tissues and fat stores shrink, and consequently body weight decreases (Barrett and Porter, 2006; Torpy *et al*., 2003).

(iii) Treatment of T1D

Traditional treatments of T1D involve numerous daily insulin injections, lifestyle adjustments and rigorous glucose control. The treatment must be continued indefinitely. Insulin treatment is the only treatment option since numerous trials with agents that suppress or modulate immune function have failed to preserve β-cell function long term (Bekris *et al*., 2006). It is speculated that drug targets of factors important to disease pathogenesis may provide safe and effective adjuvant treatment to preserve β-cell in autoantibody positive subjects who are at maximum risk for disease (Couri *et al*., 2006). T-cells are the main targets for the preventive strategies for T1D. Other preventive strategies that can be applied to Type 1 diabetics include preventing initiation of autoimmunity against the β-cells and reversal of ongoing autoimmunity through β-cell regeneration as outlined in Figure 1.2. T1D can also be prevented by identifying and

eliminating environmental insults followed by exposing the immune system to β-cell antigen (Gillespie, 2006).

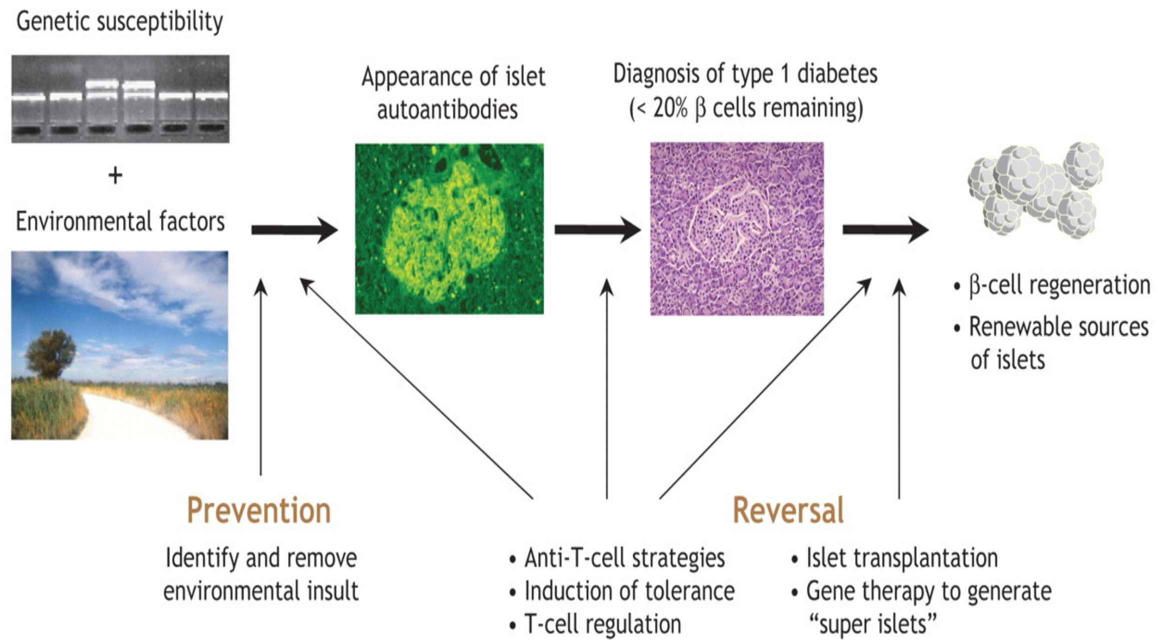

Figure 1.2: Potential targets for therapeutic intervention of T1D (Gillespie, 2006).

Transplantation of either the whole pancreas, pancreatic islets or of stem cells to replace the destroyed or inactive cells is a strategy that can result in an independence from exogenous insulin, persistent normoglycaemia, normal HbA1c level, without the risk of hypoglycaemia. However, pancreas and islets transplantations require immunosuppressive therapy for life, which is associated with the toxicity of the drugs, incidence of frequent infections and malignancy (Shapiro *et al*., 2000). Pancreas transplantation is a serious surgical intervention, and carries numerous risks and complications. Since 2000 islet cell transplantations have been performed, and intensive research is currently under way for alternative sources of β-cells (Wsikowa *et al*., 2004).

Although pancreatic transplantation has offered a successful therapeutic approach for many years, there are insufficient donor organs or cells available. With respect to islet transplantation a further difficulty is that many of the current agents damage β-cells or induce peripheral insulin resistance and in some patients results in inadequate glucose

control despite insulin therapy. The hope that such an approach would result in long-term freedom from the need for exogenous insulin, with stabilization of the secondary complications of diabetes, has failed to materialize in practice (Bekris *et al*., 2006; Shapiro *et al*., 2000).

1.3.1.2 Type 2 Diabetes

T2D occurs much later than T1D, usually after the age of 30, hence the name adult onset diabetes. It is the most common form of diabetes and independent of insulin. About 90 to 95 percent of people with diabetes have T2D (Los Angeles Chinese Learning Centre, 2003). Unlike T1D, T2D is a heterogeneous disorder characterized by a progressive decline in insulin action followed by the inability of β-cells to compensate for insulin resistance due to pancreatic β-cell dysfunction. Insulin resistance is a characteristic metabolic defect that precedes overt β-cell dysfunction and is primarily associated with resistance to insulin-mediated glucose disposal at the periphery and compensatory hyperinsulinaemia. Insulin resistance can be caused by a defect in glucose uptake in skeletal muscle and adipose tissues, and impaired glucose production in the liver (Musselman *et al*., 2003).

The β-cells compensate for insulin resistance by secreting more insulin to maintain the glucose homeostasis (Weir and Bonner-Weir, 2007). In time, however, this β-cell function gets impaired leading to deterioration in glucose homeostasis and subsequent development of impaired glucose tolerance and frank diabetes. Relative insulin deficiency occurs as the day-long circulating insulin concentrations in patients with T2D are almost comparable or slightly elevated in absolute terms to the values in normal individuals despite the hyperglycaemia (Sasaoka *et al*., 2006).

(i) Factors Associated with T2D

Genetic predisposition, age, obesity, diet, physical inactivity, ethnicity and sedentary life style are the major risk factors involved in the development of T2D as outlined in Figure 1.3., with about 80% of the individuals diagnosed with T2D being obese (Srinivasan *et*

al., 2005). The genetic contribution to T2D is even stronger than in T1D, with a concordance rate over 90% in identical twins (Musselman *et al*., 2003).

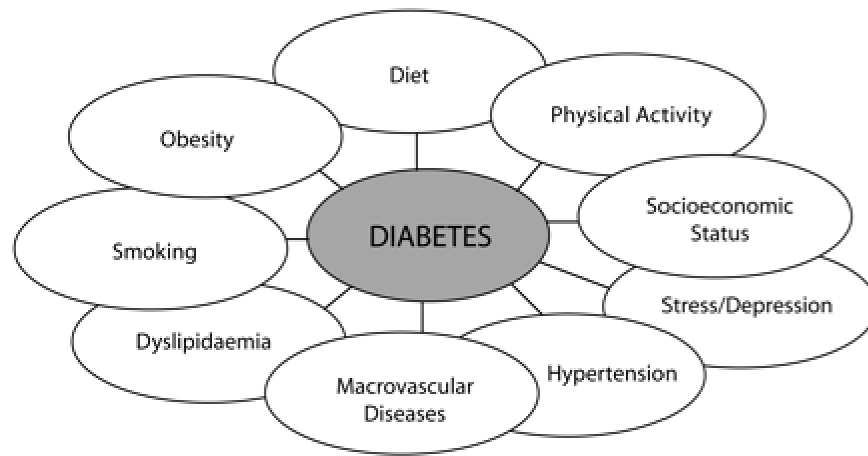

Figure 1.3: Factors associated with type 2 diabetes as a composite chronic disease (Colagiuri *et al*., 2006).

Though it used to affect adults, T2D is increasingly being diagnosed in children and adolescents in recent years. When it is diagnosed, the pancreas is still able to produce sufficient insulin, however, the body fails to use the insulin effectively. After several years, the pancreatic β-cells becomes exhausted, insulin production decreases and glucose builds up in the blood and the body is unable to use its main source of fuel efficiently (Docherty, 2001; Sang *et al*., 2005; Winzell *et al*., 2003).

There is evidence that both insulin resistance and progressive pancreatic β-cell failure are key pathomechanisms in the natural history of T2D. However, it is unclear how these abnormalities arise and how they are related to typical T2D features (Zozulinska and Wierusz-Wysocka, 2006). This condition is more complex than T1D, but is often easier to treat since insulin is still produced, especially in the initial years. It may go unnoticed for 4-7 years in a patient before diagnosis, since the symptoms are typically milder and can be sporadic. However, severe complications can result from undiagnosed T2D, including renal failure and coronary artery disease (Dhatariya, 2003; Smith, 2005).

(ii) Treatment of T2D

Although early events in the pathogenesis of T2D are driven mostly by β-cell dysfunction and insulin resistance, obesity is an important contributor for its development (Chan *et al*., 2007). Therefore, weight control and long-term management of obesity are central to prevention and the optimal management of people with T2D. However, T2D is a chronic and relapsing disease which can be rarely cured but can only be managed through lifestyle intervention or palliation to lower blood glucose concentrations. Lifestyle modification is an important aspect in the management of diabetes, with dietary intervention, weight management, physical activity and smoking cessation being critical parts of diabetes health care as they are important for good glycaemic control and the prevention of microvascular and maybe macrovascular complications (The Diabetes Control and Complications Trial Research Group, 1993). Lifestyle modifications lower blood glucose concentrations and also decrease risk factors for coexisting diseases. Lifestyle modification is recommended for the majority of overweight patients with T2D to help control their glucose metabolism. Failure to achieve lower glucose levels through lifestyle interventions alone, a controlled-energy diet and regular aerobic exercise with pharmacotherapy is recommended. (Chan *et al*., 2007; Inzucchi, 2002). Several approved hypoglycaemic agents are available for improving glycaemic control in type 2 diabetic patients. These include: sulfonylureas, meglitinides, biguanides and thiazolidinediones (Cho *et al*., 2006; Hong *et al*., 2004; Shetty *et al*., 2004).

Sulfonylureas:

Sulfonylureas are the first oral agents to be available for treatment of type 2 diabetic patients. They lower blood glucose by stimulating the release of insulin from the pancreas by binding to the sulfonylurea receptor on the pancreatic beta cells. All the sulfonylureas are equally efficacious and reduce the HbAlc by 1% to 2% (Inzucchi, 2002). Selection of sulfonylureas is based on their propensity to cause hypoglycaemic episodes, their pharmacokinetic and pharmacodynamic profiles and cost (Levy and Cohen, 2003).

The first-generation sulfonylureas are acetohexamide, chlorpropamide, tolazamide, and tolbutamide, and the second-generation agents are glyburide, glipizide and glimepiride.

Second-generation agents are the most commonly prescribed sulfonylureas, and with the exception of glyburide, they are associated with the least risk of hypoglycaemic episodes (Cheng and Fantus, 2005). The use of both the first and second generation sulfonylureas in patients allergic to sulfonamides is not recommended as they might cause anaphylaxis, rash, or life-threatening reactions such as Stevens-Johnson syndrome (Levy and Cohen, 2003).

Meglitinides:

Meglitinides are insulin secretagogues that stimulate the release of insulin from the pancreatic β cells to reduce postprandial hyperglycaemia. The release of insulin is glucose dependent and diminishes at lower glucose concentrations. The available meglitinides include nateglinide and repaglinide. Although the meglitinides are not considered first-line agents for T2D and are chemically unrelated to sulfonylureas, they may offer an alternative to the sulfonylureas in patients who either have a history of sulfonamide allergy, or have postprandial hyperglycaemia, or who are at high risk for hypoglycaemia because of erratic meals (Levy and Cohen, 2003).

Biguanides:

Initially three antidiabetic biguanides were used in the 1950s, namely: metformin, phenformin and buformin. Due to high incidence of lactic acidosis phenformin was withdrawn in the 1970s and buformin received limited use in a few countries, leaving metformin as the main biguanide on a global basis. Since then, metformin has been the most favourable and the least expensive of the oral antidiabetic agents used (Krentz and Bailey, 2005).

Metformin serves as an alternative antidiabetic first-line agent. It has been the mainstay of oral hypoglycaemic agent treatment since the 1950s and the only biguanide used in routine clinical practice. It inhibits glucose absorption from the gastrointestinal tract, inhibits hepatic gluconeogenesis, and improves insulin sensitivity by increasing peripheral glucose uptake and utilization. Metformin can also be used in combination with other classes of oral anti-diabetic agents, or with insulin. It is recommended as first-

line pharmacotherapy for overweight and obese patients with T2D but can be as effective in normal weight T2D patients. Metformin is contraindicated in patients who are treated pharmacologically for chronic heart failure and those with renal insufficiency as a precaution against drug accumulation (Levy and Cohen, 2003; Oiknine and Mooradian, 2003).

Thiazolidinediones:

Thiazolidinediones (TZDs) are a class of antidiabetic agents that improves insulin sensitivity and reduce plasma glucose and blood pressure in subjects with T2D. Thiazolidinediones bind and activate PPARγ, but there is no direct evidence to conclusively implicate this receptor in the regulation of mammalian glucose homeostasis (Barroso *et al*., 1999). In clinical studies, TZDs consistently lower fasting and postprandial glucose concentrations as well as free fatty acid concentrations (Nolan *et al*., 1994). In most studies TZDs also decreased insulin concentrations, indicating its role as insulin sensitizer, which has been confirmed by direct measurements in *in vivo* studies in humans. Treatment of nondiabetic subjects or those with T2D for three to six months with TZDs, e.g. troglitazone, rosiglitazone, or pioglitazone increased insulin-stimulated glucose uptake in peripheral tissues (Nolan *et al*., 1994). In similar studies, TZDs increased hepatic and adipose tissue insulin sensitivity. Moreover, insulin secretory responses, even after adjustment for an improvement in insulin sensitivity, have increased in subjects with impaired glucose tolerance and T2D. Paradoxically, these improvements are accompanied by weight gain and an increase in the subcutaneous adipose-tissue mass. Rosiglitazone and pioglitazone are currently approved in most countries for the treatment of hyperglycaemia in patients with T2D, either as monotherapy or in combination with sulfonylureas or metformin (Mayerson *et al*., 2002; Yki-Järvinen, 2004).

All these therapies however, have limited efficacy, tolerability and significant mechanism-based side effects. Sulfonylureas for instance enhance weight gain and about 20–25% of patients with T2D are unresponsive to the sulfonylureas initially, and subsequent failure of these agents occurs in 5–10% of patients per year. Other oral hypoglycaemic agents are associated with episodes of hypoglycaemia, whereas some

hardly address the underlying defects such as obesity and insulin resistance. Metformin increases the risk of lactic acidosis especially in patients with renal insufficiency (Davies and Srinivasan, 2006; Harrigan, *et al*., 2001; Moller, 2001). Often oral therapy regimens are insufficient to achieve glycaemic control in many patients with T2D, due to the progressive nature of the disease that goes from insulin resistance to β-cell failure and ultimately insulin deficiency. Furthermore, the stepwise approach of adding additional oral agents in current treatment algorithms often only prolongs the failure to achieve glycaemic control and increases the risk of diabetes related complications and the likelihood of β-cell failure (Vinik, 2006).

1.3.2 Long-Term Complications of Diabetes

How does diabetes cause problems in so many parts of the body? Diabetes is characterized by persistent hyperglycaemia which causes damage to nerves and blood vessels in different parts of the body. Over a period of months or years, diabetics stand a greater risk of suffering from any of the following three general categories of diabetes complications, as summarized in Figure 1.4:

Neuropathy (Nerve Damage):

Neuropathy refers to nerve disorders that lead to numbness and sometimes pain and weakness in the hands, arms, feet and legs. Neuropathy can also cause problems in the digestive system, heart, and sex organs. About 25% of patients with 10 years of history of diabetes (both type 1 and type 2) have some degree of nerve damage, but not everyone experiences physical symptoms. Neuropathies are more common in people who have had diabetes for at least 25 years, who are overweight, have poor blood glucose control, and have high blood pressure (NIDDK, 2002). The incidence of neuropathy increases with duration of diabetes and is accelerated by poor management of the disease. The most common type is peripheral neuropathy, which affects the arms and legs and increases the chance of foot injuries, which, if left untreated, can lead to amputation (Rao and Aruin, 2006; Vincent *et al*., 2004).

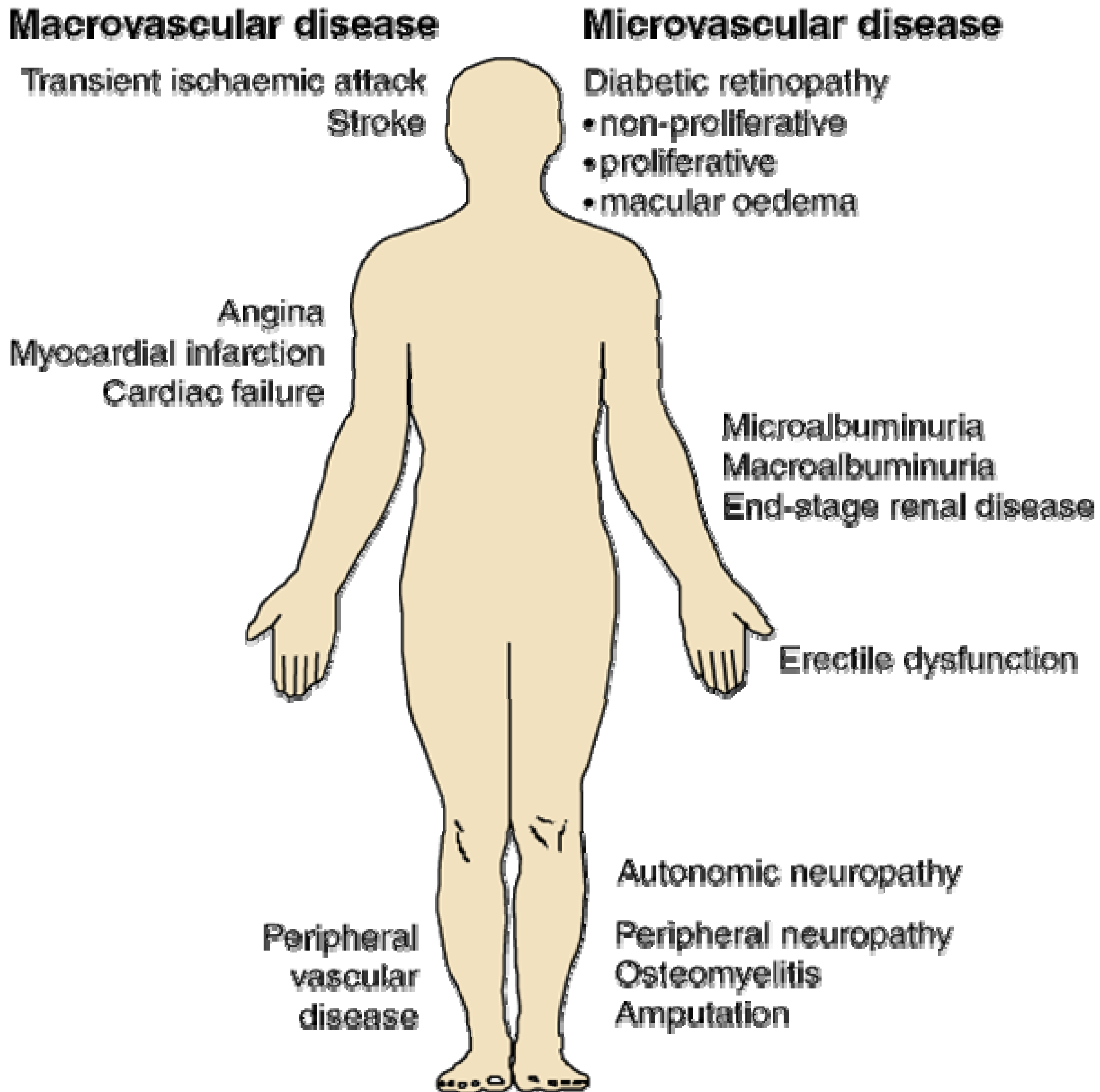

Figure 1.4: Complications of diabetes (Bate and Jerums, 2003)**.**

Macrovascular disease (Damage to large blood vessels):

Macrovascular diseases (myocardial infarction, congestive cardiac failure and stroke) accounts for more than 70% of deaths in patients with diabetes (Malmberg *et al*., 2005). Overall mortality has more than doubled in both type 1 and type 2 diabetic patients compared with age-matched and sex-matched non-diabetic people. Treating young to middle-aged female patients suffering from macrovascular disease with insulin worsened the condition, the role played by insulin in this situation remains unclear (Fisher, 2006).

High blood glucose is a risk factor for development of atherosclerosis, which can lead to a heart attack, stroke or poor circulation in the feet. Cardiovascular disease is the leading cause of diabetes-related death. Adults with diabetes have heart disease death rates about 2 to 4 times higher than adults without diabetes. The risk of stroke is also 2 to 4 times greater for people with diabetes (Bate and Jerums, 2003).

Microvascular disease (Damage to small blood vessels such as capillaries): Microvascular complications are highly prevalent especially in type 1 diabetics and occur early during the course of disease. It affects the small vessels, such as those supplying the retina, nerves, and kidneys. Consequentially, this leads to retinopathy and blindness, neuropathy, which may result in lower limb amputation, and nephropathy often leading to end-stage renal disease requiring dialysis or transplantation. The exact mechanism is not clear, but hyperglycaemia results in thickening of capillary walls and reduces blood circulation to the skin, arms, legs, feet, eyes and kidneys (Bate and Jerums, 2003).

1.4 UNDERSTANDING BLOOD SUGAR AND INSULIN

1.4.1 Regulation of Glucose Metabolism

Glucose is a simple sugar found in food, an essential nutrient that provides energy for cellular functions. After meals, carbohydrates are digested in the stomach and the intestines into glucose. The glucose is absorbed by the intestinal cells into the bloodstream, and is carried by blood to all the cells in the body. However, glucose cannot enter the cells by itself; it needs assistance from insulin in order to penetrate the cell walls. Insulin therefore acts as a regulator of glucose metabolism in the body (Nolan, 2006).

A continuous supply of glucose is necessary to ensure proper function and survival of all organs within the body. While low glucose levels produce cellular death, chronic high levels of glucose can also result in organ damage (Yeo and Sawdon, 2007). Therefore, the blood glucose level is maintained at a physiological set point of 5 mM. Glucose homeostasis within the body is primarily regulated by the liver and skeletal muscle

through insulin and glucagon. Following a meal (Figure 1.5), the blood glucose levels increases and triggers insulin secretion by the pancreatic β-cells. Insulin stimulates glucose uptake by the skeletal muscle and adipose tissue. This action enhances glucose usage in the skeletal muscle and adipose tissues. Glucose is rapidly phosphorylated as it enters the muscle and either subsequently stored as glycogen in the liver or oxidized to generate energy in the form of ATP. In adipocytes, glucose is stored as lipid, due to increased uptake of glucose and activation of lipid synthetic enzymes. At this stage glycogen breakdown is suppressed by inhibiting enzymes of the gluconeogenetic pathway (Norman, 2002; Saltiel, 2001).

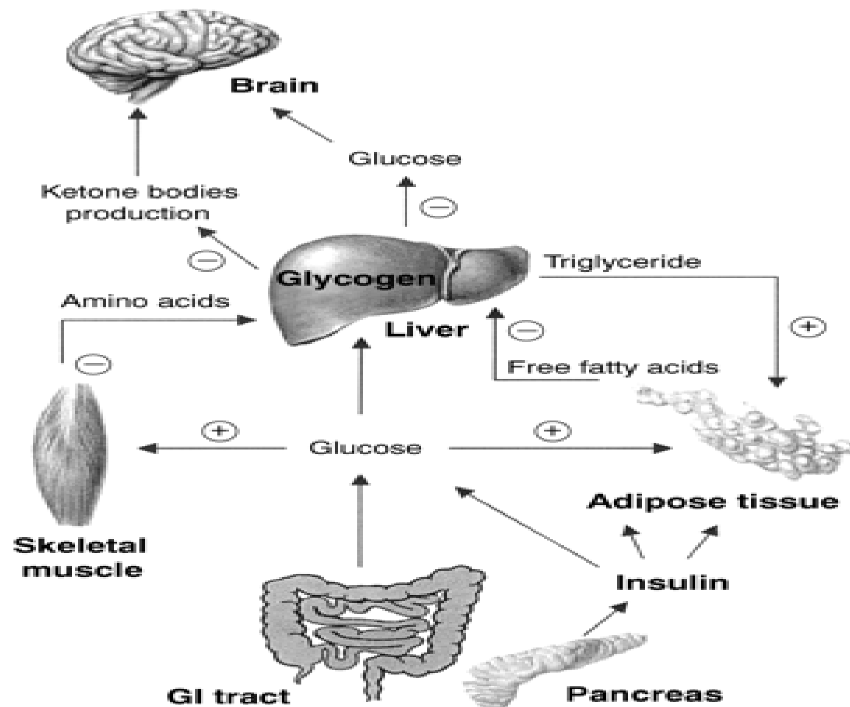

Figure 1.5: Insulin action after a meal. Selected actions of insulin are indicated with (+) up regulation or (-) down regulation. Insulin activates transport of glucose to muscle and adipose tissue, and also promotes synthesis of glycogen and triglycerides by the liver (Van Tilburg *et al.,* 2001).

Glucose uptake is the rate-limiting step in glucose utilization and storage and should be maintained at its physiological set point to avoid complications associated with high blood sugar. The balance between the utilization and production of glucose is maintained at equilibrium by two opposing hormones, insulin (glucose-lowering hormone) and glucagon (glucose-elevating hormone). Insulin is secreted from the β-cells in the pancreas

in response to an increased plasma glucose following a meal (Saltiel, 2001). When plasma glucose falls during fasting or exercise, glucagon is secreted by α-cells to enhance glucose output from the liver by gluconeogenesis (synthesis of glucose from pyruvate) and glycogenolysis (synthesis of glucose from glycogen). Both α- and β-cells are extremely sensitive to glucose concentrations and can regulate hormone synthesis and release in response to small changes in plasma glucose levels. Insulin stimulates glucose uptake, utilization and storage, while suppressing hepatic glucose production, and thus reducing plasma glucose levels. Glucagon on the other hand, promotes the release of stored and newly synthesized glucose into the bloodstream. These two hormones act in concert to ensure that glucose homeostasis is maintained throughout a wide variety of physiological conditions (Norman, 2002).

1.4.2 Insulin

Insulin is a polypeptide hormone containing 51 amino acids which is synthesized, stored and secreted by the β-cells in the islets of Langerhans. Insulin has a molecular weight of 5.8 kDa and its structure varies slightly between species. The β-cells possess a sophisticated glucose-sensing system, and insulin secretion by these cells is a highly regulated process. Insulin regulates blood glucose levels by two main mechanisms: inhibiting glucose production by the liver and stimulating glucose uptake (Yeo and Sawdon, 2007).

The three major target tissues for insulin action are muscle, liver and adipose tissue. Besides stimulating the uptake, utilization and storage of glucose as glycogen, insulin action affects several other processes at the cellular level such as amino acid uptake, lipolysis in adipocytes, Na^+-K^+ pumps, protein synthesis, gene expression, DNA synthesis and apoptosis. Insulin also inhibits the production and release of glucose by the liver, due to the blockade of gluconeogenesis and glycogenolysis (Charbonnel, 2005; Rajiv *et al*., 1997).

Insulin is used medically in patients with T1D because of an absolute deficiency of the hormone.Patients with T2D however, have either relatively low insulin production or insulin resistance or both, and in the long run will require insulin administration when

other medications become insufficient in controlling blood glucose levels (Charbonnel, 2005).

Insufficient production or failure of insulin to stimulate target sites in liver, muscle and adipose tissues causes insulin resistance due to decreased insulin levels or activity. This results in high levels of glucose in the blood and eventually to type 2 diabetes (NIDDM, non-insulin dependent diabetes mellitus) as outlined in Figure 1.6. Insulin resistance results in a reduced ability of insulin to promote glucose uptake and usage by skeletal muscle and fat cells (Nolan, 2006; Matsumoto *et al*., 2006).

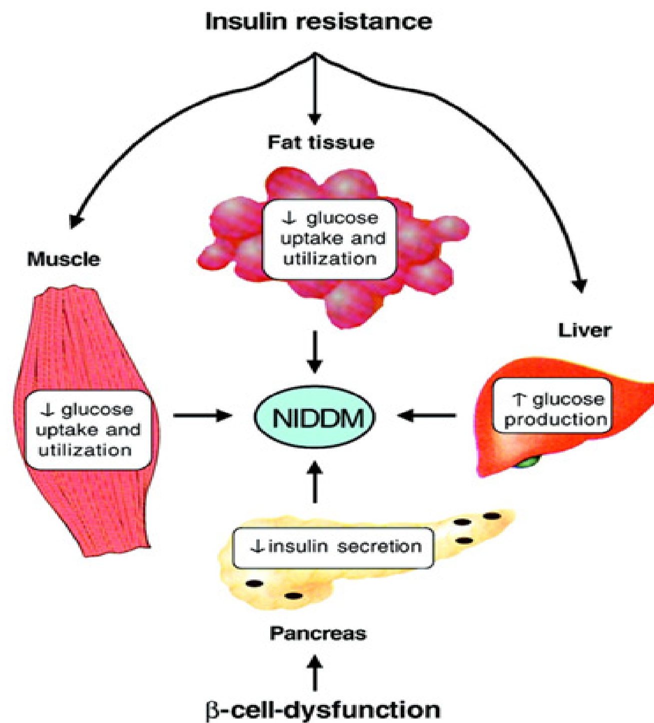

Figure 1.6: Insulin resistance in target tissues, along with β-cell failure, leads to NIDDM /T2D (Baudry *et al*., 2002).

Suppression of hepatic glucose production by insulin resistance, leads to hyperinsulinemia (increased secretion of insulin) to overcome insulin resistance. Failure of the β-cells to compensate for the insulin resistance despite the increased insulin secretion results in T2D development (Baudry *et al*., 2002). Insulin resistance and β-cell dysfunction are the major characteristics of T2D (Kahn, 1994).

1.5 “DIABESITY" - THE OBESITY-DIABETES LINK

The connection between obesity and diabetes is so visible, because 97 percent of all cases of T2D are caused by excessive weight that some health experts have coined a new term, Diabesity (Zimmet *et al*., 2001). The word encapsulates the diabetic/weight connection and is a step towards the wider cluster of symptoms of metabolic syndrome, a frequent precursor of coronary heart disease. Obesity is a major risk factor in the development of diabetes because overweight people have a slower metabolic rate compared to people of the same weight (Marchand, 2002). This slower metabolic rate, combined with a high fat diet and a genetic tendency to retain fat may exacerbate the epidemic of overweight (Shishkov, 1999).

The transition from obesity to diabetes is due to progressive defect in insulin secretion that accompanies the progressive rise in insulin resistance (Warram *et al,* 1990). The defects in insulin secretion and resistance appear during the early stages of obesity development, and both become worse as the patient becomes diabetic. Thus, the deviation in the classic hyperbolic relationship (change in insulin action compensated by insulin secretion) caused by insulin resistance and insulin secretion, and the glucose allostasis concept (Stumvoll *et al*., 2003) remain prevailing concepts in the development of diabesity. Fortunately, the transition to obesity-induced T2D is fully reversible in its early stages (Golay and Ybarra, 2005).

1.6 ADIPOKINES: HORMONES ASSOCIATED WITH DIABESITY

Adipokines are proteins produced mainly by adipocytes (Fantuzzi, 2005). These hormones are believed to influence body weight homeostasis, affect insulin action, glucose, fat metabolism and consequently insulin resistance, which ultimately lead to T2D (Lau *et al*., 2005).

Circulating adipokine levels appear to correlate closely with adiposity in animals and humans, with increasing levels in obese and insulin resistant states (Wajchenberg, 2000).

Impaired insulin action in adipose tissue increases the rate of lipolysis and FFA secretion. The increased flux of FFAs impairs insulin secretion and induces insulin resistance in muscle and the liver by interfering with glucose transport and insulin-mediated glucose uptake exerting negative influences on cardiovascular health (Lau *et al*., 2005). The hormones that have been implicated include among others: resistin, leptin, adiponectin, adipsin and visfatin.

1.6.1 Resistin

This hormone, discovered in 2001, was found to be released by adipocytes and to interfere with the activity of insulin implicated in pathogenesis of diabesity in rodent models (Berger, 2001; Kusminski *et al*., 2005). This serendipitous observation was made during a search for targets of the TZDs, a class of insulin sensitizing drugs (Steppan *et al*., 2001). The hormone was named resistin from the original observation that it induced insulin resistance in mice (Fantuzzi, 2005). It occurs at higher levels in obese individuals than in those with normal weight. Resistin was identified in the blood of normal mice, which rose after feeding them a HF diet. Injecting mice with recombinant resistin caused impaired glucose tolerance, increased blood glucose levels and the mice eventually developed the symptoms of diabetes. Obese mice given an anti-resistin antibody had an improved blood sugar levels and insulin action whereas an anti-diabetic drug decreased their resistin levels (Steppan *et al*., 2001).

Resistin is a 12.56 kDa peptide hormone containing 108 amino acids as a propeptide. In humans, serum resistin occurs in several different high molecular weight isoforms with resistin-immunoreactivity at molecular weights of 10 to 20 kDa, as well as from 45 and 55 kDa (Gerber *et al*., 2005). The biological action of resistin is not yet fully understood; studies suggest a physiological role in obesity-associated insulin resistance (Risch *et al*., 2006).

Resistin belongs to a novel class of cysteine-rich secreted proteins named FIZZ or RELM, which have unique patterns of tissue expression (Holcomb *et al*., 2000). Although resistin has been generally known as a mediator of obesity-associated insulin

resistance, there are some contradictory data showing that the level of resistin is not related to obesity or insulin resistance (Chung *et al*., 2006; Lubos *et al*., 2006).

Resistin expression *in vivo* is specific to white adipose tissue. Resistin levels are increased in diet-induced obesity as well as in genetic models of obesity and insulin resistance (Steppan *et al*., 2001). Although resistin has been implicated as a link between obesity and insulin resistance in rodents; the role of resistin in humans is unclear, because unlike the situation in rodents, it is expressed at higher levels in monocytes and macrophages than in adipocytes in humans (Jung *et al*., 2006). The abundant expression of resistin in monocytes/macrophages in humans suggests that it might have another pathophysiologic role (Jung *et al*., 2006; Steppan *et al*., 2001).

1.6.2 Leptin

The hypothesis for the existence of a peripheral factor that informs the brain of energy status was first proposed in the 1950s (Kennedy, 1953; Eiden *et al*., 2001), and formed the basis for further investigations that led eventually to characterization of the obese (o*b*/*ob*) mouse. Ob/ob mouse is a homozygous mutant that lacks a critical factor for regulation of body weight. Coleman characterized the phenotype of the obese mouse which included massive obesity, hyperphagia, insulin resistance, and cold intolerance (Coleman, 1978). However, it was not until 1994 that Zhang *et al* (1994) characterized, through positional cloning and sequencing, the genotype responsible for the syndrome. The gene was termed *ob* and the product leptin. The term leptin came from the Greek word "leptos" meaning "thin", to describe the body weight-reducing effects of leptin on mice (Zieba *et al*., 2005).

Leptin is a peptide hormone product containing 167 amino acids with a molecular mass of 16 kDa. Human leptin is 84% identical to mouse and 83% identical to rat leptin (Zhang *et al*., 1994). Mouse and human leptin both share many structural similarities to other members of the helical cytokine family, including IL-6, IL-12, IL-15, growth hormone, prolactin, granulocyte colony-stimulating factor and oncostatin M (Prolo *et al*., 1998). The leptin gene is highly conserved across species, and is located on chromosome 7q31.3 in humans and on chromosome 4q22 and 6 in rats and mice, respectively. It is mainly

produced by adipocytes (Zhang *et al.* 1994); however, more recent studies have confirmed that other tissues including placenta, ovaries, skeletal muscle, stomach, foetal cartilage, pituitary, mammary tissue and liver also express leptin (Zieba *et al*., 2005).

Leptin acts as a satiety signal, regulating appetite and body weight in both humans and rodents (Koerner *et al*., 2005). Leptin acts on the central circuits in the hypothalamus in order to reduce food intake and increase energy expenditure. Thus, leptin plays a crucial role in the control of body fat stores, appetite and maintenance of body weight through coordinated regulation of metabolism, the autonomic nervous system, and body energy balance (Figure 1.7). Recent studies have revealed direct effects of leptin in the periphery, partly through interactions with other peripherally acting hormones such as insulin (Otero *et al*., 2005; Zieba *et al*., 2005).

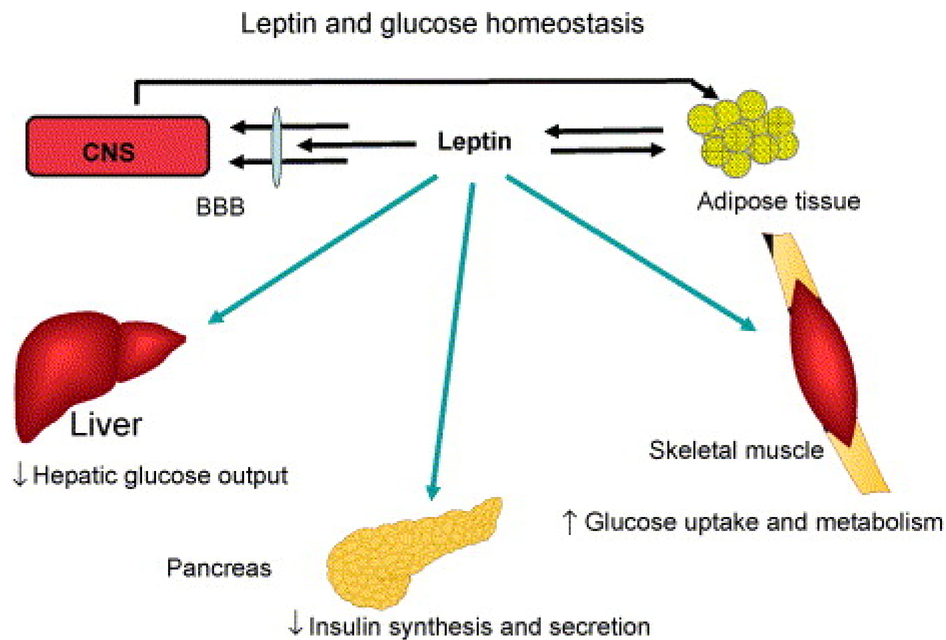

Figure 1.7: Effects of leptin on glucose homeostasis. Leptin is secreted from the adipocytes. It crosses the blood–brain barrier by a saturable transport system, and reaches to the arcuate nucleus of hypothalamus to control food intake, energy expenditure and neuroendocrine function. Circulating leptin also has direct peripheral effects: (1) it decreases hepatic glucose output in the liver, (2) increases glucose uptake and metabolism in skeletal muscle and (3) decreases insulin synthesis and secretion from the pancreatic beta cells. CNS: central nervous system, BBB: blood–brain barrier (Bulent and Ibrahim, 2006).

Leptin may be found circulating in the free form or complexed with leptin-binding proteins, and this characteristic appears to be species-specific and dependent upon physiological status. In humans, the half-life of free leptin is about 30 minutes, with the kidneys being responsible for approximately 80% of leptin clearance from the peripheral circulation (Zieba *et al*., 2005).

Leptin regulates body weight by informing the hypothalamus about the adipose tissue mass through its receptor (Ob-R). Ob-R is encoded by the diabetes (*db*) gene cloned from the mouse choroids plexus (Campfield *et al*., 1995; Hegyi *et al*., 2004). The Ob-R has several isoforms Ob-R (a-f) generated by alternative mRNA splicing which differ in the length of the intracellular domain (Fei *et al*., 1997; Tartaglia *et al*., 1995). The short form (Ob-Ra) was detected in almost all tissues whereas the longest form, Ob-Rb, was found to be highly expressed in the hypothalamus and also in some peripheral tissues (Kieffer *et al*., 1996). Leptin effects are mediated by binding to the Ob-R in the hypothalamus through the Jak/STAT pathway (Hegyi *et al*., 2004). Administration of leptin in *ob/ob* mice inhibits food intake and reduces body weight *via* activation of specific brain receptors (Tartaglia *et al*., 1995; Lee *et al*., 1996). Leptin receptor mutations in mice resulted in severe obesity and T2D (Fei *et al*., 1997). Conversely, leptin deficiency in mice and humans causes obesity and may also cause diabetes syndromes, including hyperinsulinaemia (Montague *et al*., 2003).

1.6.3 Adiponectin

Adiponectin also known as adipoQ or Acrp30 is an adipocyte-secreted hormone and has a molecular weight of 30 kDa. It has insulin-sensitizing effects and increases fat oxidation in humans (Lau *et al*., 2005; Saely *et al*., 2007). Adiponectin is composed of a collagenous domain and a globular that is structurally similar to complement factor C1q (Goldfine and Kahn, 2003; Scherer *et al*., 1995). The globular domain is structurally similar to TNF-α (Fantuzzi, 2005). The expression and secretion of adiponectin in human visceral adipose tissue is regulated negatively by glucocorticoids and TNF-α, and positively by insulin and Insulin-like growth factor 1. Adiponectin levels are decreased in

obese state and are inversely correlated to insulin-resistant states and high-sensitivity C-reactive protein levels in humans and animals (Lau *et al*., 2005).

Although adipocytes are the most important source of adiponectin, serum adiponectin concentrations are inversely associated with obesity, insulin resistance and T2D in rodents and humans, whereas increased serum adiponectin concentrations are associated with improved insulin sensitivity (Berg *et al*., 2002a). Obese subjects were found to have reduced adiponectin levels while patients with anorexia nervosa had increased levels. Adiponectin levels are significantly reduced in patients with T2D, and the mechanism by which the insulin-resistant state is associated with low levels of adiponectin remains elusive. However, TNF-α, which is increased in the WAT of obese subjects, might suppress adiponectin production. On the other hand, adiponectin was to reduce the production and activity of TNF-α. Adiponectin also possess anti-inflammatory activities that inhibit IL-6 production and induces production of anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist (Wolf *et al*., 2004). Inhibition of NF-κB by adiponectin might explain at least part of these effects (Fantuzzi, 2005).

In accordance with its insulin-sensitizing role, transgenic mice lacking adiponectin show impaired insulin sensitivity (Berg *et al*., 2001). Moreover, heterozygous PPARγ knockout mice and mice with a fat-specific insulin receptor knockout have reduced body fat mass and have increased longevity despite normal or increased food intake. These mice are protected from age and diet-induced obesity, adipocyte hypertrophy and insulin resistance. Protein and gene expression analysis of these mice revealed that adiponectin was over expressed; suggesting that increased adiponectin secretion could help to compensate for the adipocyte-specific insulin resistance in FIRKO mice, and could further explain the phenotype of these mice with normal insulin sensitivity and normal glucose homeostasis (Blüher, 2005).

1.6.4 Adipsin

Adipsin is a serine protease which was first isolated from adipocytes in 1986 (Min and Spiegelman, 1986; Fain *et al*., 2007). It was later discovered that adipsin corresponds to

complement factor D in human subjects (Rosen *et al.*, 1989), and the rate-limiting enzyme in the alternative pathway of complement activation (White *et al*., 1992).

Adipsin and several other components of both the classical and alternative complement cascade, are primarily expressed by adipocytes in mice, and by both adipocytes and monocytes-macrophages in human subjects (Fantuzzi, 2005; White *et al*., 1992). Adipsin levels are reduced in murine models of obesity but either increased or unchanged in obese human subjects (Fantuzzi, 2005). Subsequently, it was reported that adipsin expression is severely impaired in both genetic and acquired obesity in rodents (Flier *et al*., 1987).

1.6.5 Visfatin

Visfatin is a 52 kDa cytokine expressed in lymphocytes and is predominantly secreted from visceral adipose tissue (Koerner *et al*., 2005). However, previous studies revealed that visfatin was highly enriched in subcutaneous adipose tissue in lean and more insulin-sensitive subjects, and concluded its expression in visceral and subcutaneous adipose tissue was regulated oppositely with BMI (Varma *et al*., 2007). It has a glucose-lowering effect and plays a role in glucose homeostasis (Zahorska-Markiewicz *et al*., 2007).

1.7 ANIMAL MODELS FOR STUDYING PATHOPHYSIOLOGY OF DIABESITY

Knowledge of the aetiology and regulation of physiological basis of human chronic diseases is owed largely to studies that were conducted on animals. Animal models have been a cornerstone of many studies on environmental effects, such as epigenetics, responses to high-fat diets and the identification and development of pharmaceuticals for treatments. Compared with human studies, use of animal models is easier to manage and also faster due to availability of enhanced techniques and some destructive methods that allow more accurate description of the phenotype (Carroll *et al*., 2004; Speakman *et al*., 2007).

Animal models for human diseases are necessary and important because they provide valuable insights and lead the way to potential novel therapeutic approaches in human

subjects. They ideally resemble, in as close a manner as possible a particular behavioural or physiological trait of man. Rodents, especially rats and mice, have been the predominant models and have comparable physiology and metabolism, digestive systems and susceptibility to metabolic traits which are very important to man. Moreover, rats and mice like man are omnivores, they share similar complex taste and are similar neuroanatomically, especially with regard to hypothalamic, limbic and brainstem systems (Thibault *et al*., 2004).

1.7.1 Animal Models for Human Obesity

Historically, the use of animal models for obesity dates as far back as 1905 after the discovery of obese yellow coat (*agouti*) mice (Cuenot, 1905). In 1942 scientists were able to produce obese rats by electrolytically lesioning the ventromedial hypothalamus of the rat leading to establishing the role played by the hypothalamus in regulating body weight (Giridharan, 1998).

Several animal models of human obesity exist to date and have been classified as either genetic or nongenetic:

Genetic Models of Obesity:
These are heritable forms of obesity showing the importance of genetic factors in the development of obesity. Genetic models of obesity especially mice and rats, have been used for the identification, isolation and establishment of the importance of genetic predisposition in the development of obesity (Giridharan, 1998).

Non-Genetic Models of Obesity:
This type can be produced in mice and rats, surgically, chemically or by dietary means. Destruction of the ventromedial part of the hypothalamus by microsurgery or by chemical agents such as gold thioglucose results in hyperphagia causing obesity in rodents. These hypothalamic rodent models of obesity have contributed immensely to the understanding of the central role of the hypothalamus and the role of afferent and efferent signals in maintaining energy homeostasis. In addition to hypothalamic destruction and dietary

manipulations, endocrine manipulations are also used for the induction of obesity. An endocrine manipulation involves the use of hormones such as insulin and corticosteroids which plays a major role in the metabolic rate and lipogenesis and increases the rate of fat deposition. These hormones manipulate energy balance and are useful in understanding the aetiology of obesity. Diet-induced obesity can be achieved by overfeeding an energy dense diet with a high percentage of fat and that is highly palatable (Giridharan, 1998).

Several animal models for obesity exist. The monogenic obesity models include the *db/db* and *ob/ob* mice, *Agouti* mice, Zucker *fa/fa* and JCR:LA-*cp* rats. Sprague–Dawley and Wistar rats serve as polygenic model of obesity. Obesity in these rats is induced by feeding a diet high in fat and sugar (Boustany-Kari *et al*., 2006; Carroll *et al*, 2004). These prolific and variable rodent models have advanced our understanding of the genetics and physiology of human obesity. Rodents are extremely valuable experimental models that have human-like primary metabolic pathways and can be genetically manipulated in many ways. The vast spectrum of existing genetic variation, coupled with a short generation time and low husbandry costs, make rodents well suited to studying obesity. Indeed, rodent strains undergoing long-term directed selection for obesity-related traits have been particularly useful as a model to characterize the complex genetic basis of obesity in humans, establishing a strong heritable component for body-fat proportion and quantifying the genetic and phenotypic correlations between obesity and traits involved in energy balance (Pomp, 1999).

1.7.2 Animal Models for T2D

Pathogenesis of T2D is very complex involving interaction of many genes with environmental factors, making its analysis in humans very difficult. Diabetes research in humans is hindered by ethical concerns because instigation of the disease in man is not allowed. Animal models of T2D show characteristics of hyperglycaemia, defects in glucose metabolism and sometimes diabetic complications such as nephropathy (Srinivasan *et al*., 2005; Srinivasan and Ramarao, 2007).

Many studies have been carried out to determine the genetic factors involved in T2D (Van Tilburg *et al.,* 2001), using animal models with similar phenotypic characteristics to

those of human T2D patients (Nojima *et al*., 2006). Several established diabetic animal models with obesity such as the Wistar fatty rat, Otsuka Long Evans Tokushima Fatty rat and spontaneously diabetic Torii rat have been used to understand pathogenesis of T2D and to test the pharmacological effects of new drugs. Animal models having features of the disease of interest have been used to investigate and also to understand the underlying mechanisms during the onset, progression and diagnosis of diseases (Masuyama *et al.*, 2003; Yamada *et al*., 2006). In spite of that, there are other non obese diabetic rodent models which are used for the investigation of T2D in non obese individuals. Examples include the Goto-Kakizaki rat and AKITA mouse (Srinivasan and Ramarao, 2007).

1.8 PROTEOMICS

Proteomics is a new area of science that began when Marc Wilkins used the term *'Proteome'* describing the PROTEin complement expressed by a genOME, in public for the first time in 1994, but its first tool (two dimensional gel electrophoresis) has been used since 1975 (O'Farrell, 1975). Proteomics is the scientific approach to study the complete protein complement, or the proteome within a cell or tissue from their identity, quantity, and function. Proteomics is one among various 'OMICS' fields such as transcriptomics, metabolomics, interactomics, lipomics, and glycomics that have been growing rapidly in the postgenomic era and helped in understanding gene function. During the past few years, proteomics has been extensively applied to several fields of medicine to better understand normal physiology, to define the pathophysiology of diseases, and to identify novel biomarkers and new therapeutic targets. The size and complexity of the human proteome is part of what makes proteomics a very complex science. Proteomics is still a relatively new field and offers the opportunity to discover untold information about the human proteome. In contrast to the genome, the proteome is dynamic and is in constant flux because of a combination of factors such as differential splicing of the respective mRNAs, posttranslational modifications, and temporal and functional regulation of gene expression and identifying and understanding these changes is the underlying theme in proteomics (Gygi *et al*., 1999; Huber, 2003; Thongboonkerd, 2004).

The cause of most human disease lies in the functional dysregulation of protein interactions, leading to over- or under-expression of the particular proteins within the body. Proteomics has evolved from advances in scientific knowledge and technology and understanding the role that protein networks play in disease aetiology will create enormous clinical opportunities, because these pathways represent the drug targets that can be used for therapeutic interventions. Proteomics is a particularly rich source of biological information because proteins are involved in almost all biological activities and they also have diverse properties, which collectively contribute greatly to our understanding of biological systems. Many different technologies have been and are still being developed to collect the information contained in the properties of proteins. In the future, it will be possible to analyze the state of protein signal pathways in the disease-altered cells before, during, and after therapy, heralding the advent of true patient-tailored therapy (Liotta *et al*., 2001; Patterson and Aebersold, 2003).

1.8.1 Serum Proteomics

Most diseases manifest themselves by more or less severe changes in human physiology, and this serves as the basis for therapeutic interventions and diagnosis of diseases. Many biochemical and cellular parameters are routinely measured in biological fluids (Bischoff and Luider, 2004). For diagnostic purposes easily accessible body fluids are preferred, hence blood and its components (especially plasma and sera) have always been used. Blood provides a major link between all cells, tissues and organs and therefore contains all representative proteins produced in the entire body following cell or tissue damage. These proteins that are synthesized, secreted, shed, or lost from cells and tissues throughout the body and can be correlated to pathological conditions and present opportunities for potential clinical utility for therapeutic intervention or as diagnostic or prognostic biomarkers (Schrader and Schulz-Knappe, 2001).

Serum proteome analysis has the potential to facilitate disease diagnosis and therapeutic monitoring as it contains thousands of proteins that has the potential to elucidate the pathophysiological mechanism of diseases and serve as indicators of diseases (Matsumura *et al*., 2006). However, serum presents a major technical challenge as it

contains proteins that vary in concentrations, with 22 dominant proteins such as albumin, immunoglobulins, transferrin, haptoglobin, transthyretin, hemopexin, α_1-antitrypsin, α_1-acid glycoprotein α_2-macroglobulin and lipoproteins that make up 99% of total human serum (Veenstra *et al*., 2005). The remaining 1% are low molecular weight proteins (Mr<10,000) that includes, hormones, cytokines, growth factors and proteolytic fragments of high molecular weight proteins which are of interest in proteomics studies. These low molecular weight proteins play a critical role in biological processes and some of them can be of potential use diagnostically or therapeutically, for instance, insulin is used for blood glucose regulation and Angiotensin II for blood pressure regulation (Adkins *et al*., 2002; Zheng *et al*., 2006). The presence of high abundant proteins makes detection of low abundant proteins very difficult (Merell *et al*., 2004).

Despite these challenges, there are various sample preparations such as fractionation and precipitation that can be applied to remove most abundant proteins (albumin and immunoglobulins) thereby reducing the complexity of serum. These standardized procedures for serum profiling are very crucial for obtaining reliable biomarkers, since slight changes in a given sample preparation procedure can lead to very different protein profiles (Luque-Garcia and Neubert, 2007). The potential of serum proteomics to investigate the underlying mechanisms of the metabolic diseases has been poorly addressed and there are not many reports on the disorders especially in South Africa (Matsumura *et al*., 2006).

1.8.2 Biological Marker

Biological marker or biomarker is a substance that accurately indicates a biological state as well as the physiologic state of an organism. In disease state it can be found in increased or low amount in the blood, urine, sweat, stool, or tissues compared to normal circumstances. Recently, the definition has been broadened to include biological characteristics that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (Mayeux, 2004; CCFA, 2006). The utility of a biomarker lies in its ability to

provide an early indication of the disease, to monitor disease progression, to provide ease of detection, and to provide a factor measurable across populations (Srinivas *et al*., 2002).

Biomarkers would enhance the positive predictive value of a diagnostic test and minimize false positives or false negatives; however, many issues such as the specificity of the protein biomarkers, their efficacy between gender and among races and ethnic groups, and their ability to identify interindividual differences in susceptibility for monitoring high-risk groups have to be addressed. To be of public health value, the assay should be adaptable to a high-throughput format with minimal misclassification. For a biomarker to be useful, its variation must be greater than the inherent error in the measurement. The variance of the biomarker is the target source of variation that defines the hypothesis to be tested by the experiment. In scientific measurements, there are many other sources of variation that may confound the detection of biomarkers. In mass spectrometry (MS) experiments for instance, reagents used for sample preparation may sometimes have a pronounced effect on the spectra. In addition, small changes in protein concentrations have to be differentiated from a diverse biological background that encompasses variations of age, diet, genetics, time, physical activity and gender (Harrington *et al*., 2006; Srinivas *et al*., 2002).

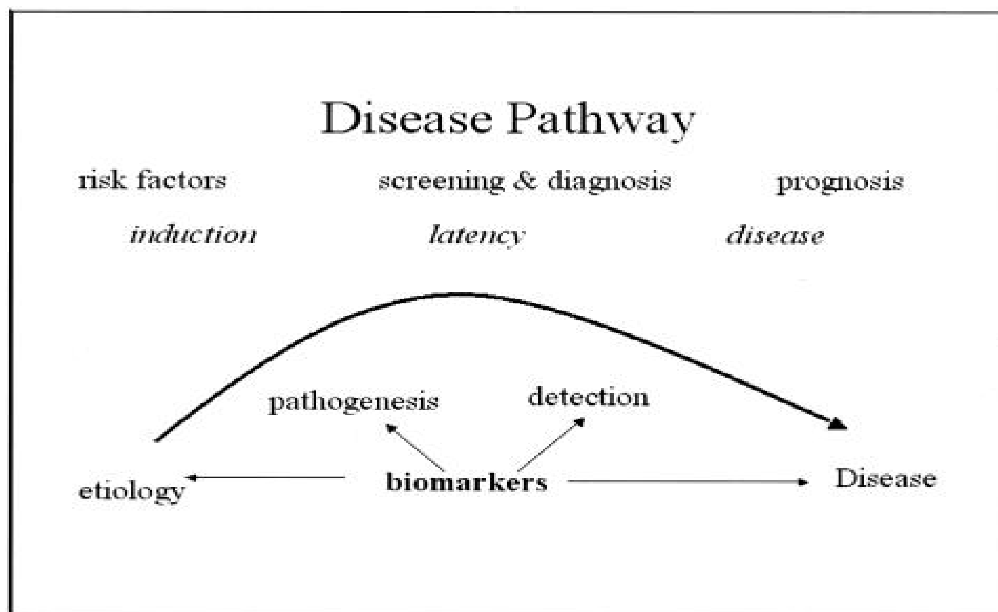

Figure 1.8: Disease pathway and potential impact of biomarkers (Mayeux, 2004).

Biomarkers can be used in early detection and diagnosis of diseases, monitoring of therapy, and eventually in prevention and risk assessment of diseases. As illustrated in Figure 1.8, biomarkers can be used for development of treatment depending on the stage of the disease which can be used for early detection, diagnosis and as a therapy. The ability of biomarkers to identify interindividual variations in susceptibility for monitoring high-risk groups should be assessed. The biomarkers should be reflective of the disease process and help identify harmful exposures. The specificity of the discovered biomarker has to be measured on model systems to infer whether a marker is a part of disease pathogenesis and not part of an adaptive response to the stimuli. The effect of other comorbid conditions on the specificity of a particular biomarker should also be assessed (Srinivas *et al*., 2002).

1.8.3 Proteomics Technologies

The term proteomics was brought to the limelight about a decade ago, and even though this new term was used to introduce the concept of exploring changes in all proteins expressed by a genome, the tools used in proteomic analysis are borrowed from traditional biochemistry. Although this approach is commonly used to characterize all protein species within a given cell, many researchers are also taking advantage of proteomic technology to elucidate protein changes between healthy and diseased states (Nair *et al*., 2004).

Proteomic technologies make it possible for the identification of differentially expressed proteins caused by the disease process in an accurate manner. The inherent advantage afforded to proteomics is that the identified protein is itself the biological endpoint. Several changes occur to the cells as they transform from its normal morphology to a pathologic state. These changes at the protein level as a result of altered expression, differential protein modification, changes in specific activity or aberrant localization affect cellular functioning (Srinivas *et al*., 2002).

A large number of tools are now available to the proteomics researcher for determining protein identity, relative protein expression or protein interactions in purified protein

complexes. Many diseases can be described as diseases of the regulation of protein function, and as such should be amenable to study by proteomic techniques (Bernhard *et al*., 2004; Katz *et al*., 2005).

1.8.3.1 Two-Dimensional Polyacrylamide Gel Electrophoresis

The two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) technique is one of the most powerful proteomics tools for the separation and quantification of proteins in an electric field. 2D-PAGE was initially described by O'Farrell in 1975 and has since evolved markedly as one of the core technologies for the analysis of complex protein mixtures extracted from biologic samples due to its resolution power and sensitivity. The proteins are separated in two steps, firstly according to their isoelectric point (pI) using isoelectric focusing and secondly their molecular weight carried out on polyacrylamide gels as a matrix. Since the proteins are separated based on their pI (pH at which the protein has no net charge) and mass by these two parameters, it is possible to obtain a uniform distribution of protein spots across a two-dimensional gel. This technique has resolved 1100 different components from *Escherichia coli* and should be capable of resolving a maximum of 5000 proteins (O'Farrell, 1975).

The reproducibility provided by the 2D-PAGE separation is sufficient to allow comparisons between healthy and diseased samples. The 2D-PAGE system can resolve proteins differing in a single charge and can be used in the analysis of *in vivo* modifications resulting in a change in charge. Proteins whose charge is changed by missense mutations can also be identified. To increase the reproducibility and resolution of the 2D-PAGE, Görg and co-workers initiated the use of immobilized pH gradient (IPG) strips for the first dimension in 1988 (Cristea *et al*., 2004; Huber, 2003; Natarajan *et al*., 2005).

1.8.3.2 Mass Spectrometry

Mass spectrometry is an analytical technique that measures the molecular mass to charge (m/z) ratio of molecules that have been ionized. Mass spectrometry provides essential tools that can be used for the identification of unknown compounds, to quantify known

compounds and to determine the structure and chemical properties of molecules. Detection of compounds can be accomplished with very minute quantities for a compound of mass 1000 Daltons, that is, compounds can be identified at very low concentration in chemically complex mixtures. It also allows detection of minor mass changes, such as the substitution of one amino acid for another, or a post-translational modification (Ashcroft, 2006; ASMS, 2001).

Various MS techniques are used for determining the m/z of gas-phase ions, the methods varies in producing gas-phase ions from analytes and their subsequent detection. However, there are two commonly used techniques for protein/peptide profiling: electrospray ionization and matrix-assisted laser desorption ionization (MALDI). Electrospray ionization is used to convert charged analytes within a liquid sample to gas-phase ions through high voltage, heat and drying gases; and MALDI requires that the analytes be mixed with photoreactive small molecules, dried on a metal surface and subsequently exposed to laser irradiation (Katz *et al*., 2005).

Mass spectral techniques have the strong advantage in that they can be used in the analysis of components of a mixture without prior identification. The dramatic progress in mass spectrometry-based methods of protein identification has triggered a new quest for disease-associated biomarkers. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and surface-enhanced laser desorption/ionization mass spectrometry, provide effective means to explore the less studied information of the human serum proteome: the low abundant proteins (LAPs). These LAPs are promising for the detection of important biomarkers. Due to the significant experimental problems imposed by high abundant proteins (HAPs), it is important to effectively remove these species prior to mass spectrometry analysis of the low-molecular-weight serum and plasma proteomes (Chertov *et al*., 2005; Katz *et al*., 2005).

1.8.4 Application of Proteomics to Obesity and T2D

The complexity of diabesity and its complications requires an unbiased scientific method such as proteomics to study the disease. At present many proteomic studies focused on

changes of the serum proteome (Zhao *et al*., 2008), urine (Rao *et al*., 2007), vitreous body (Ouchi *et al*., 2005), and affected organs such as pancreas (Jägerbrink *et al*., 2007), brain and kidneys under the diabetic state in rats and mice. Potential protein markers or related proteins that may offer a new theoretical foundation for the pathogenesis, diagnosis and treatment of the disease have been identified (Liu *et al*., 2007). In humans, eight potential markers were found in skeletal muscle biopsies of obese type 2 diabetic patients (Hojlund *et al*., 2003), with three proteins dysregulated in red blood cell membranes of patients with Chinese T2D. However further studies were required to evaluate these proteins as disease specific markers before they can be used in diagnosis or drug targets (Jiang *et al*., 2003).

In rodent models of obesity, several proteins with altered expression were identified in the liver, white and brown adipose tissue, and muscle (Kussmann *et al*., 2006). Expression levels of GDF-3/Vgr-2 were also found to be reduced by obesity in diabetic mice but increased in obese non-diabetic mice. Further studies are required to determine the role of GDF-3/Vgr-2 in diabesity (Witthuhn and Bernlohr, 2001).
Relatively few proteomic studies to investigate the T2D pathogenesis have been published to date despite the defined target organs; pancreas, skeletal muscle and liver (Scott *et al*., 2005). Although previous studies have revealed complex and detailed altered protein expression profiles in different tissues of diabetic subjects, many functional implications remain to be answered. And no single protein seems to be responsible for the development of diabetes (Sparre *et al*., 2005).

1.8.5 Challenges and Limitations of Proteomic Analysis

As opposed to genomics; there are a number of challenges faced in proteomics studies. For starters the genome is static and the same in every cell whereas the proteome is dynamic and constantly changing in response to external stimuli. As a result, the proteome has many proteins contrary to the genes in the genome as a result of different post translational modifications (Huber, 2003).

An ideal reproducible sample preparation protocol that will isolate the full complement of proteins in a given biological sample eliminating post-extraction modifications and non-specific contaminants, like keratins, nucleic acids, and artefacts is of vital importance. Sample preparation for a proteomic analysis is delicate and an intricate work due to protein molecular sizes, charge state, protein conformational states, post-translational modifications, and other physicochemical properties. These properties have a strong impact on whether the proteins will migrate in the 2D gels (Garbis *et al*., 2005; Huber, 2003).

Another major challenge, especially when serum is used, comes from detection of LAP that may become important drug targets and disease markers such as transcription factors, kinases and regulatory proteins. Serum contains HAP which makes up 99% of the total protein content with the prime targets making up to 1%. For the low copy number proteins there are no amplification techniques analogous to polymerase chain reaction for nucleic acids, hence approaches for removal of HAPs and enrichment of LAPs are needed. Therefore success in the search of markers in serum depends on depletion of at least albumin and IgG either by chromatography or precipitation which is very expensive and time consuming (Corthesy-Theulaz *et al*., 2005; Graves and Haystead, 2002).

A technical key problem stems from the reproducibility and stability of 2-DE. Although impressive improvements on the technique have been made, 2D electrophoresis remains a labour-intensive, low-throughput approach that requires a large amount of sample. It has limited range for protein identification, difficulty in detecting proteins with extreme MW and pI. Despite the challenges, when coupled to other complementary technologies proteomics offer a potential to provide new insights into biology and the ability to study complex biological systems (Adkins *et al*., 2007; Graves and Haystead, 2002).

1.9 AIMS AND OBJECTIVES OF THE STUDY

1.9.1 Aims

The aim of the study was to:

- ❖ To identify proteins markers of obesity in rat serum during its development and progression to full blown diabetes using the proteomics approach.

1.9.2 Objectives

The specific objectives of the study were:

- ❖ To induce obesity in Wistar rats by feeding high fat diet
- ❖ To collect blood samples and establish a method for enrichment of low abundant proteins.
- ❖ To analyze the protein profile of the serum through 2 dimensional gel analysis coupled to mass spectrometry.
- ❖ To validate the identified proteins using western blot

1.9.3 Hypothesis

Serum protein profiles are altered by high fat feeding during development of obesity and progression to type 2 diabetes in Wistar rats.

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Equipment

- X-ray processor (Agfa, RSA)
- Filter paper, paper bridges, Hybond-P nitrocellulose membrane, X-ray film (Amersham Biosciences, RSA)
- Immobiline reswelling tray, Ettan IPGphor II IEF apparatus (Amersham Biosciences, UK)
- Proteomics Analyzer (Voyager DE PRO MALDI MS,) (Applied Biosystems, UK)
- Mini-Protean III system, Mini-Protean III Dodeca system, Molecular Imager Pharos FX system, Quantity One Software, PDQuest version 8.0 2-D gel analysis software, ExQuest spot cutter (Bio-Rad, UK)
- PROC GENMOD software (MRC Biostatics Unit, RSA)
- ImageJ Software (National Institute of Health, USA)
- Digital camera (Olympus Optical, US)
- Ultraspec 1000 spectrophotometer (Pharmacia Biotech, USA)
- Glucometer, Accu-Chek Sensor (Roche, RSA)
- Eppendorf refrigerated microcentrifuge Model 5417R (Sigma-Aldrich, RSA)
- Block heater (Stuart Scientifica, USA)
- Vortex (VELP Scientifica, EU)
- Microcentrifuge tubes (Whitehead Scientific, RSA)

2.1.2 Chemicals and reagents

- Mineral oil (Plus one drystrip cover fluid) (Amersham Biosciences, RSA)
- Bradford dye reagent concentrate, Tween-20, BSA, ethanol , NH_4HCO_3, ACN, Trifluoroacetic acid, DTT, IAA, Bio-Lyte 3-10 buffer , Flamingo fluorescent stain (Bio-Rad, RSA)
- Chow (rat food) (Epol, RSA)

- Page ruler unstained protein ladder (Fermantas, RSA)
- Acetic acid, CHAPS, glycerol, HCl, KCl, TCA, acetone (Merck, RSA)
- Ammonium persulfate (APS), Trypsin (Promega, UK)
- Methanol (Merck, RSA)
- Halothane (Safe Life Pharmaceuticals, RSA)
- 2-Mercaptoethanol, bovine serum albumin, bromophenol blue, glycine,
- Coomassie brilliant blue R-250, TEMED, 40% Acrylamide/bis-acrylamide, SDS thiourea, urea, low melting agarose, glycine, alpha-cyano-4-hydroxycinnamic acid matrix, Ponceau S stain (Sigma-Aldrich, RSA)
- Primary antibodies (rabbit polyclonal anti-Apo A-IV, mouse monoclonal anti-CRP, goat polyclonal anti-Fetuin-A), secondary antibodies (goat anti-rabbit IgG-HRP, donkey anti-goat IgG-HRP, rabbit anti-mouse IgG-HRP), LumiGLO Chemiluminescent substrate system (Whitehead Scientific, RSA)

All the equipment, chemicals and reagents used for this study were of the highest analytical grade obtained from reputable suppliers.

2.2 METHODS

2.2.1 ANIMAL STUDIES

2.2.1.1 Animals and diets

The animals were maintained and treated in accordance with the MRC Ethics Committee on research animals. The animals were kept in the MRC primate unit resource facilities. For this study: ten week old male Wistar rats from the breeding colony at the MRC primate unit were used. The rats were given standard rodent chow upon weaning at three weeks of age. After two weeks of adaptation to the housing and diet conditions, the animals were matched by weight (165.4 ± 3.2 g, mean body weight ± S.E.) and divided into two groups of *7* rats each. One group served as the control (LF) group and fed chow as a low fat diet and the other group was used as an experimental group (HF) and fed a diet with high fat content to induce obesity. The diet composition was prepared as in

Table 2.1 (information on dietary composition proprietary to the Diabetes Research Group). Rats were fed *ad libitum* on their specific diets with free access to fresh water for 44 weeks. The rats were singly caged in a temperature controlled environment at 23°C, and subjected on a 12 hours light/dark cycle.

Table 2.1: Low Fat and High Fat diet composition

g/100g DRY DIET	LF (%)	HF (%)
Carbohydrates	51	37
Fat	2.5	39
Proteins	8	10
Fibre	6	3
Indigestible Components	22.5	11

2.2.1.2 Rat weight measurements

Rats were weighed at the beginning of the study before they started on their respective diets and weekly after they started eating their respective diets until the end of the experiment.

2.2.1.3 Collection of blood samples

Blood was obtained by tail bleeding from baseline to the 8th week and also at termination of the animal experiment (44 weeks). On the day of collecting blood, the rats were deprived food for 4 hours; this was done early in the morning. The rats were slightly anaesthetized with halothane. Blood was collected in 1.5 ml microcentrifuge tubes from rats by nicking the tip of the tail and blood was stored on ice. The blood was separated by centrifugation at 7 000 xg for 10 minutes at 4°C; the serum was collected, aliquoted in 1.5 ml microcentrifuge tubes and stored at -80°C until further processing for proteomic analysis.

2.2.1.4 Fasting glucose measurements

While collecting the blood sample, fasting blood glucose levels were measured with a Glucometer. The drop of blood was put on the strip and values recorded. The data, along with weight data, were used as phenotypic parameters of the animals.

Rats from the HF diet displaying the following symptoms: overweight and high fasting glucose levels were considered diabetic, and the rats on LF displaying normal profiles were considered as age-matched controls.

2.2.1.5 Food intake assessment

Food intake was measured twice weekly throughout the study. Left overs and spillages were weighed and food intake calculated using the following equation:

Food intake = Food provided – (spillage + left over)

The mean food intake per rat was estimated by subtracting the weight of food spillage and left over from the initial food provided and dividing by the number of rats in the cage.

2.2.2 SERUM PROTEOMICS

2.2.2.1 Removal of high abundant proteins

For reproducible proteomic analysis, serum albumin was removed due to its interference with the detection of potential biomarkers which could be present in low abundance by using 10% TCA in ice cold acetone, volume per volume (v/v). The serum was precipitated by addition of four volumes of 10% TCA/acetone according to Chen *et al.*, (2005) with some modifications. The sample was briefly vortexed and incubated at -20°C for 90 minutes. The sample was then centrifuged at 20 800 xg for 15 minutes at 4°C. The supernatant was transferred to a new tube and left on ice. The precipitate or pellet was washed with 500 µl cold acetone and incubated for 15 minutes at -20°C, then centrifuged

as before for 15 minutes and the supernatant transferred to the other supernatant. Five hundred microliters of fresh acetone was added to the supernatant, vortexed briefly and incubated at -20°C for 90 minutes and centrifuged as before and the supernatant discarded. Both the precipitate and its supernatant were air dried for 15-30 seconds and resuspended in 200 µl of solubilization or lysis buffer and stored at -20°C until use.

2.2.2.2 Protein quantification

Protein concentration of the serum was determined with the Bradford assay using bovine serum albumin as a standard (Bradford, 1976). Different standard concentrations (0, 50, 100, 200, 400 and 500 µg/ml) were prepared from a 5mg/ml bovine serum albumin (BSA) stock solution. The ingredients were mixed together with the Bradford dye reagent added last as illustrated in Table 2.2, and incubated at room temperature for 5 minutes and the absorbance read at 595 nm using a spectrophotometer. The absorbencies of both the standards and serum samples were recorded. Duplicate samples were prepared.

Table 2.2: Preparations of the standards and sample for protein estimation.

STANDARD PREPARATION							SERUM SAMPLE
	Amount required in µl						
[BSA] (µg/ml)	0	50	100	200	400	500	-
dH_2O	80	80	80	80	80	80	80
BSA (5 mg/ml stock)	0	1	2	4	8	10	0
LB	10	9	8	6	2	0	5
0.1 M HCl	10	10	10	10	10	10	10
Serum sample	0	0	0	0	0	0	5
Bradford reagent	900	900	900	900	900	900	900

The absorbance values of the standards were used to plot a standard curve: absorbance vs [BSA]. The equation generated by the linear regression was used to calculate the concentration of the protein sample using the measured absorbance.

2.2.2.3 One-Dimensional gel electrophoresis

Samples were retrieved from -20°C and thawed on ice. Both the albumin-depleted (precipitates) and albumin-containing (supernatants) samples obtained from the TCA/acetone precipitation were analysed by SDS-PAGE on a 12% acrylamide gel using the Mini Protean III system (Bio-Rad, UK) with 1 mm thickness gels. Sample containing 20 µg of protein was mixed with an equal amount of 2X Laemmli sample buffer and boiled at 99°C for 5 minutes in a block heater (Stuart Scientifica, USA). The samples were left to cool at room temperature then pulse spun for 8 seconds in a microcentrifuge. After loading the samples on the gel, the gel was run at 110 V through the stacking gel and at 120 V through the resolving gel. After electrophoresis the gels were stained with Coomassie staining solution for at least 30 minutes to overnight while shaking on an orbital shaker. The gels were then destained and images captured with a digital camera.

2.2.2.4 Two-Dimensional gel electrophoresis

(i) Rehydration of strips (sample preparation)

Samples were applied to 7 cm IPG strips (Bio-Rad, RSA) with a pH range 4-7 by in-gel rehydration. Sample containing 100 µg of total protein were solubilised in a solution with 1-1.5% 1,4-dithiothreitol (DTT) and Bio-Lyte 3-10 buffer (ampholyte) with a trace of bromophenol blue and adjusted to a total of 125 µl with lysis buffer, the sample was pipetted into the grooves of the immobiline reswelling tray. The outer sheets were peeled from the strips, the strips immersed (gel side down) into the grooves of the reswelling tray without trapping air bubbles. The strips were overlaid with 2-3 ml of mineral oil (Plus one drystrip cover fluid) each, the tray was covered with its lid and passively rehydrated overnight (12-16 hours) at room temperature.

(ii) First Dimension: Isoelectric Focusing

The rehydrated strips were removed from the reswelling tray using forceps and rinsed with distilled water for a second, then blotted on the moist filter paper to remove excess reswelling solution and the mineral oil to avoid urea crystallization on the gel surface. This is normally responsible for prolonged IEF and empty vertical lanes in the stained 2-

D pattern (Gorg *et al*., 1988). The strips were then placed in the grooves of the focusing tray of the Ettan IPGphor II IEF apparatus (Amersham Biosciences, UK); gel side up and according to charge orientation (i.e., the anode or "+" mark on the strip should correspond with the same mark on the focusing tray). Paper bridges soaked with distilled water and blotted with filter paper, were placed at the edges of each strip and the electrodes placed on top of the paper bridges. The strips were overlaid with mineral oil and the IEF ran following the three step protocol: the initial voltage was limited to 250 V for 10 minutes, then increased to 3500 V for 2800 V.hr and continued at 3500 V for 3700 V.hr. The current was limited to 50 µA per strip at the optimum temperature of 20°C.

(iii) Equilibration of IPG strips

After focusing, the strips were removed from the focusing tray using forceps, rinsed with distilled water and blotted with a moist filter paper to remove excess oil and then transferred to a clean rehydration tray (gel side up). The strips were first equilibrated in equilibration buffer (EQ) buffer containing 2% (w/v) DTT for 15 minutes, followed by another 15 minutes equilibration in the above buffer containing 2.5% (w/v) iodoacetamide (IAA) per ml buffer in a shaker. A volume of 2.5 ml per strip was used for both buffers when using the rehydration tray. Equilibration buffer was discarded by decanting the tray.

(iv) Second Dimension: SDS-PAGE

Traditionally 12% SDS gels were cast using the Mini-Protean III Dodeca system (Bio-Rad, UK) with gel thickness of 1 mm. The strips were rinsed in the 1x SDS running buffer using forceps and gently placed on the top of the gel with the gel side towards the outer or short plate. Strips were gently brought to close contact with filter paper. Three microliters of the molecular weight marker (Page ruler unstained protein ladder) was spotted on a piece of a filter paper and put beside the strips on the gel. The strips were overlaid with warm 0.5% low melting agarose solution prepared in 1x SDS running buffer with added bromophenol blue, the agarose was allowed to set for 5 minutes. The gels were immersed in the electrophoresis tank, filled with 1x SDS running buffer and ran

at 110 V for 20 minutes and continued at 150 V until the bromophenol blue marker reached 0.3 cm from the bottom of the gel.

(v) Flamingo staining

The gels were fixed in ethanol/acetic acid/water solution (4/1/5) for at least 2 hours to over night on an orbital shaker. The fix solution was removed and the gels were stained with 1X Flamingo fluorescent stain solution prepared in dH_2O. The gels were covered with aluminium foil and stained for at least 3 hours on a shaker.

2.2.2.5 Quantitative analysis of gel images

Gel images from the diabetic and control serum samples were scanned on a Molecular Imager Pharos FX system (Bio-Rad, UK). Image analysis including image editing, spot finding, quantitation and matching was carried out using Quantity One Software (Bio-Rad, UK) and PDQuest version 8.0 2-D gel analysis software (Bio-Rad, UK). Protein spots were detected by following the PDQuest software instructions using the following parameters: horizontal and vertical streaking removal with radius of 33, smoothing by Power Mean filter with kernel size 3×3, speckle removal at sensitivity of 50. Each image was checked manually to remove false spots and to add the faint spots that were missed by the PDQuest software. A reference or master gel image was created that included all of the spot information found in all the gels. Spot numbers (SSP) were assigned by PDQuest software after being matched in all the gels, i.e., the same spot on all the gels was given the same SSP for comparison.

The densities of protein spots were normalized using the Local Regression Model to allow accurate comparison of the spot quantity between the gels from the two different samples. The spots were then quantitatively compared between HF and LF groups. Protein spots were considered to be differentially expressed if the difference between the averages of spot densities between the two groups was 2-fold greater or less.

2.2.2.6 In gel proteolysis

The protein spots that were differentially expressed between the sample from the diabetic rats and their controls were excised from the gels using the ExQuest spot cutter, two spots each from the albumin-depleted and albumin-containing samples. The protein spots were tryptic digested following the protocol adapted from Shevchenko *et al*., (1996). The obtained gel pieces were transferred to a 96-well plate and rinsed with dH_2O and washed twice for 5 minutes with 50 mM NH_4HCO_3 to remove SDS buffers. The washing solution was discarded after each wash. The gel piece was again washed once with 50 mM NH_4HCO_3 for 30 minutes with occasional vortexing. The wash solution was discarded, and the gel pieces washed twice with the 50% 50 mM NH_4HCO_3/ 50% acetonitrile (ACN) for 30 minutes with occasional vortexing to distain the gel. The solution was discarded, followed by addition of 100% ACN to each tube and incubated for 5 minutes. The ACN solution was discarded and the gel pieces left to dry completely in a speed vacuum.

The gel pieces were rehydrated in 0.05 μg trypsin solution prepared in 25 mM NH_4HCO_3 pH 8.0 for 15-30 minutes at 4^oC to avoid auto digestion of trypsin. The gel pieces were then covered by 25 mM NH_4HCO_3 solution and incubated overnight at 37^oC. After digestion, the reaction was stopped by adding 50 μl of 1% trifluoroacetic acid and incubated for 2 to 4 hours. A fraction of the resulting solution, together with alpha-cyano-4-hydroxycinnamic acid matrix, was spotted onto a MALDI target plate and allowed to air-dry for mass spectrometric analysis over a mass range of m/z 550 to 1,500. The Proteomics Analyzer (Voyager DE PRO MALDI MS) (Applied Biosystems, UK) was used to identify the obtained mass spectra that was searched through Mascot search engine (refer to table 2.3 for search parameters) (http://www.matrixscience.com) and queried against the NCBI protein database for identification.

Table 2.3: Search parameters used for Mascot search engine

Mascot search Parameters	
Type of search	Peptide Mass Fingerprint
Enzyme	Trypsin
Variable modifications	Oxidation (M)
Mass values	Monoisotopic
Protein Mass	Unrestricted
Peptide Mass Tolerance	± 100 parts per minute
Peptide Charge State	1+
Max Missed Cleavages	1

2.2.3 WESTERN BLOT ANALYSIS

2.2.3.1 Western blot validation

Conventional western blot was used to confirm the identities of the differentially expressed proteins. Five time points (0, 4, 6, 8 and 44 weeks) were compared between the two groups to determine at what time point the identified proteins expression levels started to change. Serum protein concentration was estimated using the Bradford assay. Twenty (20) and ten (10) micrograms (µg) of serum proteins for albumin-depleted (pellets) and albumin-containing (supernatant) sample, respectively were resolved by 12% SDS-PAGE following the same conditions as described in section 2.2.2.3 (one-dimensional gel electrophoresis). The proteins were electroblotted onto Hybond-P nitrocellulose membrane. Briefly, filter paper, the membrane and fibre pads (the size of the gel) were first soaked in 1x blotting buffer for 30 minutes prior to use. The membrane sandwich was assembled according to the manufacturer's instructions using forceps to handle the membranes and filter papers. The sandwich was placed into the transfer assembly and the transfer tank filled with transfer buffer, and the cooling block placed into the transfer apparatus. The tank was then placed in ice and the protein bands transferred at 150 V for 1 hour. The membranes were stained with 0.5% Ponceau S

staining solution to visualize and ascertain efficient transfer of protein from the gel to the membrane. The membranes were marked with a pencil and rinsed with tris-buffered saline Tween-20 (TBST) to remove the stain and incubated with the blocking buffer (5% Carnation non-fat dry milk in TBST) overnight at 4°C while shaking on an orbital shaker. The blocking solution was discarded and the membranes washed with TBST for a few seconds.

The membranes were probed with 1:5000 primary antibody prepared in antibody binding buffer for 1 hour at room temperature. The antibodies used were rabbit polyclonal anti-Apo A-IV antibodies: 200 µg/ml; mouse monoclonal anti-CRP antibodies: 100 µg/ml and goat polyclonal anti-Fetuin-A antibodies: 200 µg/ml. The antibodies were removed and the blots were washed three times in TBST for 10 minutes each. The blots were incubated with 1: 10 000 secondary antibody conjugated to HRP in antibody binding buffer for 1 hour at room temperature. Apo A-IV: 200 µg/0.5 ml concentrated goat anti-rabbit IgG-HRP; CRP: 200 µg/0.5 ml concentrated rabbit anti-mouse IgG-HRP conjugated; Fetuin A: 200 µg/0.5 ml concentrated donkey anti-goat IgG-HRP conjugated. The membranes were washed three times in TBST for 10 minutes each. The blots were developed with LumiGLO chemiluminescent substrate system then exposed to X-ray film for 15 minutes in a dark room and the reactive bands detected in an X-ray processor and the image recorded with a Digital Camera.

One membrane per time point was used for the proteins (CRP and Fetuin-A) from the albumin-depleted sample. After exposing the film to the membrane probed with anti-CRP antibodies for 15 minutes, the same membrane was used for incubation with anti-Fetuin-A antibodies following the steps for western blot stripping (section 2.2.3.2).

2.2.3.2 Western blot stripping

The blots were washed thrice in TBST for 10 minutes with agitation to remove the chemiluminescent substrates. The blots were then incubated at 60°C for 30 minutes in the stripping buffer. The stripping buffer (100 mM 2-mercaptoethanol, 2% SDS and 62. 5 mM Tris pH 6.8) was removed and the blots washed 3 times in TBST for 10 minutes. The

blots were then re-blocked and re-probed with primary and secondary antibodies as described in section 2.2.3.1.

2.2.4 Densitometry analysis

Protein levels were evaluated using densitometry. The X-ray films obtained from western blotting were scanned with a Molecular Imager Pharos FX scanner (Bio-Rad, UK). Image analysis including image editing and quantitation were carried out using ImageJ Software.

2.2.5 Statistical analysis

Statistical analysis was performed by the MRC Biostatistics Unit. Results were presented as means ± SEM. The data for body weights were analyzed by PROC GENMOD procedure and differences were considered statistically significant if $P < 0.05$. Statistical analysis for 2D gel analysis, comparison between LF and HF groups was assessed using the quantitative, student's t-test and Boolean analysis sets by the PDQuest and the relationships were considered statistically significant when $p < 0.05$.

CHAPTER 3: RESULTS

3.1 ANIMAL STUDIES

3.1.1 Body weights

Weekly measured body mass of rats during the eleven month period is shown in Figure 3.1. At the beginning of the study both groups had similar average body weights. Exposure to high fat diet led to a significant increase in body weight gain for the HF group. The significant difference was evident by the beginning of the fourth week and throughout the duration of the study. Towards the end of the study the body weights for HF group declined due to deaths of rats in the group.

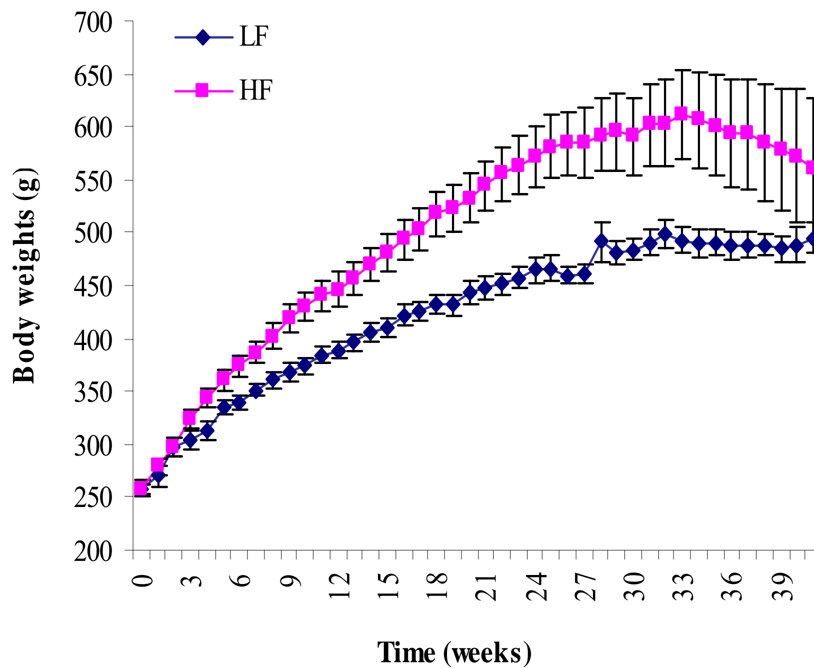

Figure 3.1: Time course of changes in body weight of control rats (LF) and rats with diet induced obesity (HF) for 44 weeks period. The difference between the two groups was significant ($P < 0.05$) starting from the fourth week in the diet as determined by PROC GENMOD statistical analysis.

At the end of the study the HF group gained more weight (302.7 ± 67.9 g) compared to LF group (236.1 ± 11.9g) as illustrated in Figure 3.2. The weight gained by the HF group at 44 weeks was 66.6g on average more than the average weight of the LF group.

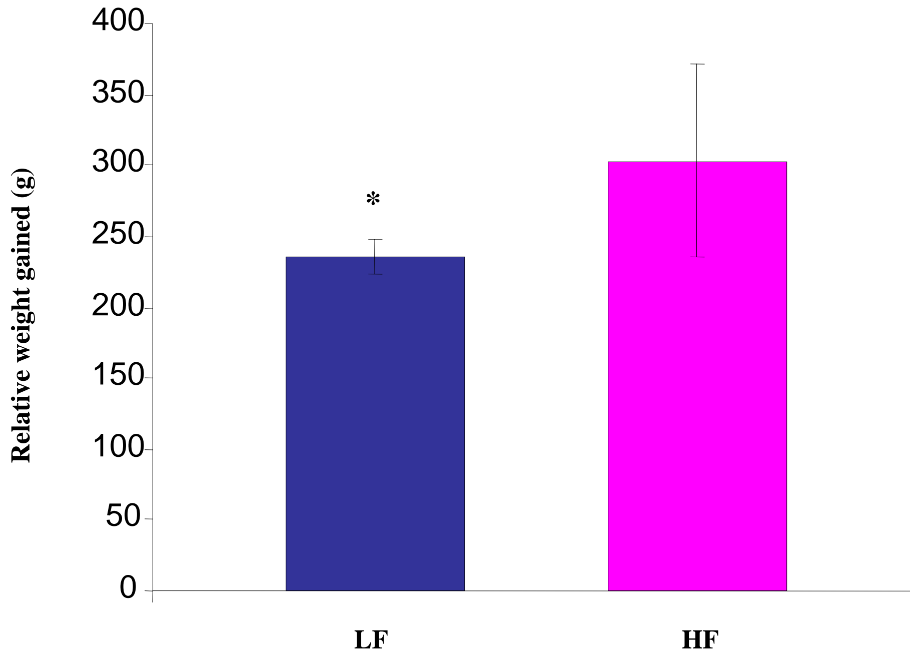

Figure 3.2: Body weight gained at the end of the study for both the LF and HF groups, with the HF heavier than the LF group. * The difference of the weight gained is statistically significant with $p < 0.05$.

3.1.2 Food intake

Cumulatively both HF and LF groups ate the same amount of food in the first four weeks. The food intake for the LF group became slightly higher from the fifth week and continued to increase until the 34th week compared to the HF group (Figure 3.3). The difference was statistically significant from the ninth week until the 33rd week. Towards the end of the study i.e., week 35-37 the intake for the LF group did not show a significant difference to that of HF group.

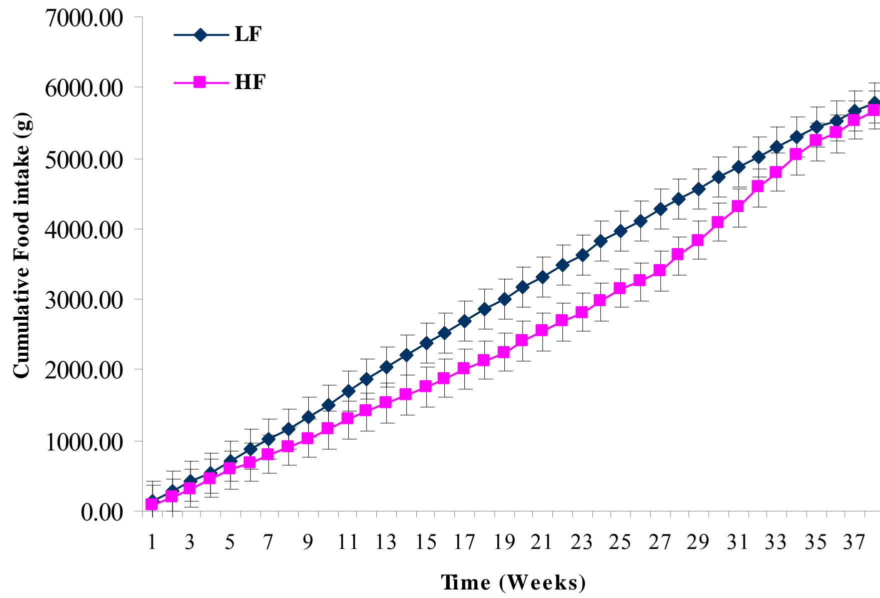

Figure 3.3: Mean cumulative food intake in obese and control rats.

Although the LF group had higher food intake (g) compared to the HF group, the HF had higher energy intake from the beginning throughout the entire study (Figure 3.4). This is due to increased calories from the HF diet: 1 g of the HF diet contains 5.54 calories whereas 1 g of the low fat diet has 2.67 calories. The calories were calculated based on the nutritional information of the diet provided the supplier.

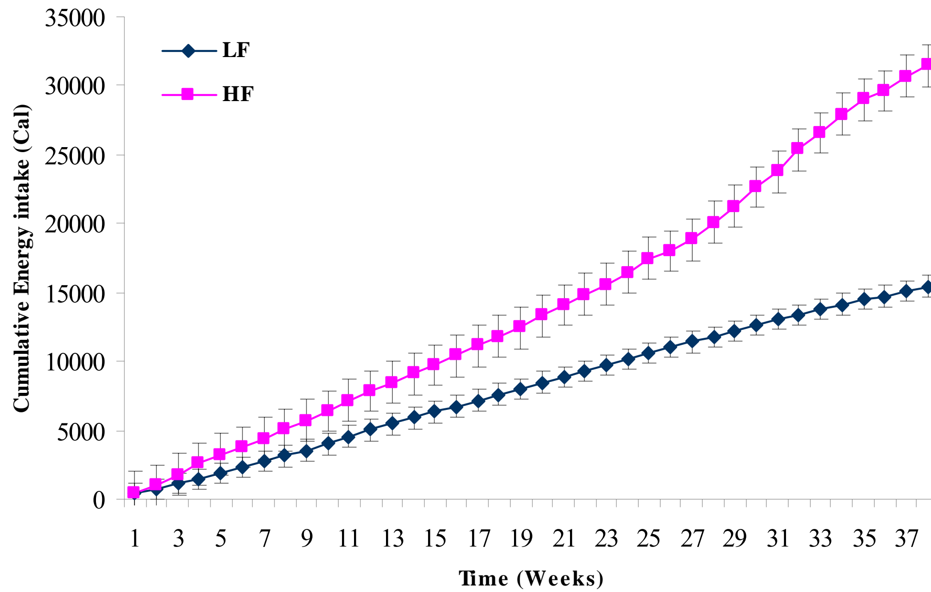

Figure 3.4: Cumulative energy intake (EI) for the LF and HF groups. EI was high for HF group compared to LF group, and the difference was statistically significant $p < 0.05$.

3.1.3 Fasting blood glucose levels

The fasting blood glucose levels were measured at baseline, every second week for the first 8 weeks and at termination. The glucose levels showed a similar trend for both the LF and HF groups (Figure 3.5). The HF group had higher glucose levels starting from the fourth week on the high fat diet and persisted throughout the study period (Figures 3.5 and 3.6). The difference was not statistically significant.

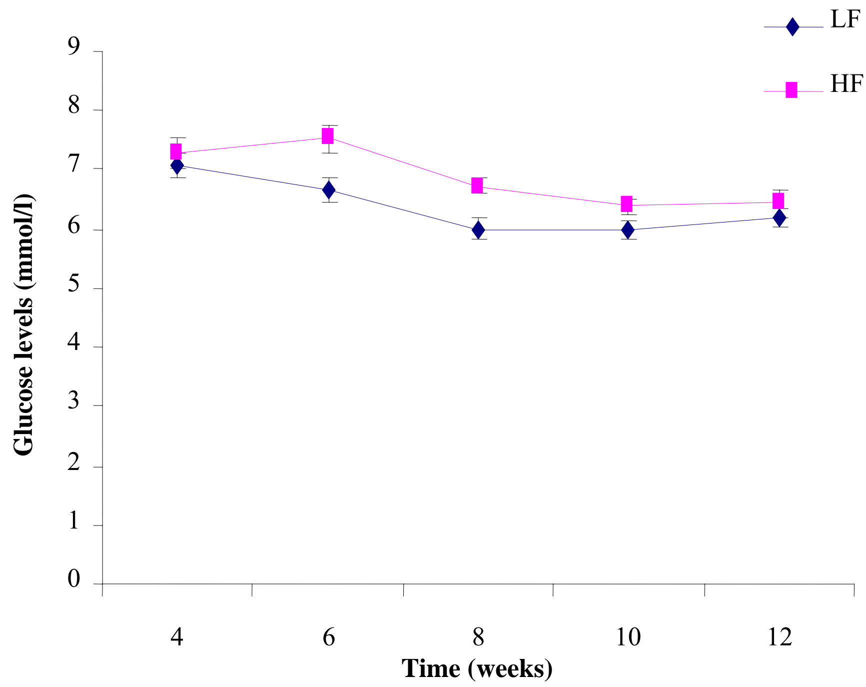

Figure 3.5: Fasting blood glucose levels for the first twelve weeks in the LF and HF group.

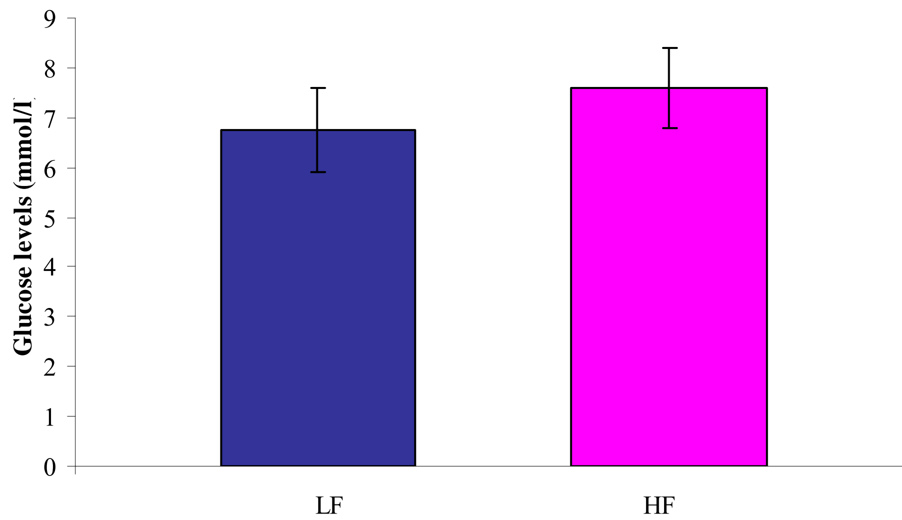

Figure 3.6: Glucose levels for the LF and HF groups at the end of the study.

3.1.4 Tissue weights

To determine which body tissues were responsible for the increased body weight, tissues were dissected and weighed at the end of the experiment. As shown in Table 3.1 the weights of kidneys, heart, testes, pancreas and spleen were heavier in obese rats than their control group, however the differences in these tissue weights between the LF and HF groups were not statistically significant. The adrenal glands weighed the same in both groups. The adipose tissue weights: Epididymal, inguinal and retroperitoneal fat pads were significantly reduced in the LF group compared to those in the HF group.

Table 3. 1: Various tissue weights for both the LF and HF groups.

Tissues	**LF**	**HF**
Liver	14.18 ± 1.59	13.72 ± 0.80
Kidneys	2.9 ± 0.20	3.2 ± 0.24
Heart	1.38 ± 0.09	1.56 ± 0.10
Testes	2.86 ± 0.09	3.12± 0.07
Epididymal	12.56 ± 4.64	12.88 ± 3.07[a]
Inguinal	27.56 ± 4.93	33.12 ± 12.98[a]
Retroperitoneal	19.08 ± 11.59	21.04 ± 9.62[a]
Perirenal	3.02 ± 1.56	2.92 ± 0.67[a]
Adrenal	0.1 ± 0.00	0.1 ± 0.00
Pancreas	0.70 ± 0.05	0.72 ± 0.05
Spleen	0.64 ± 0.05	0.72 ± 0.06

With "a" indicating that the difference between the two groups is statistically significant for the HF group with $p < 0.05$.

Adiposity Index

The adiposity index was calculated as the percentage of the sum of all white adipose tissue pads in relation to the final body weight. The adiposity index was higher for the HF group (40.0 ± 9.6%) when compared to the LF group (9.5 ± 1.3%) as shown in Figure 3.7. The difference between the two groups was highly significant.

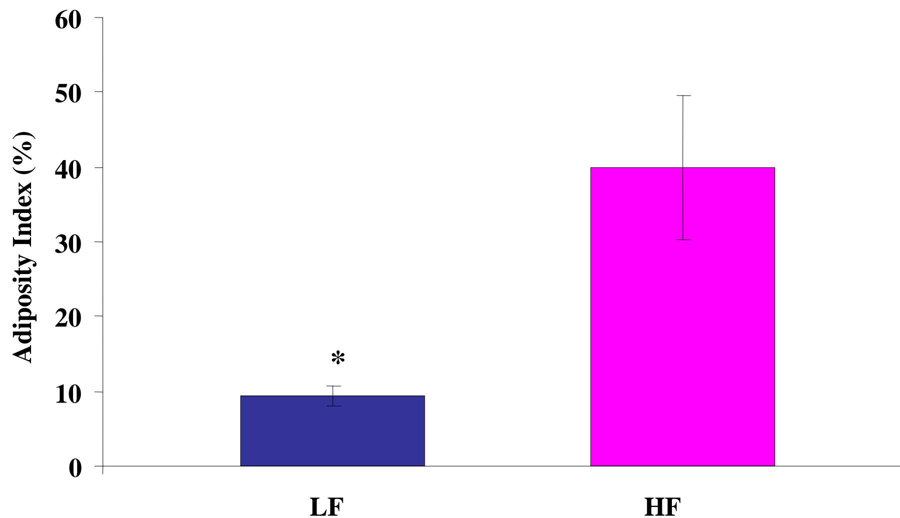

Figure 3.7: Adiposity index for the LF and HF groups at the end of the study. * $p < 0.05$.

3.1.5 Tissue weights relative to body weights

To determine what percentage weight each tissue contributed to the final body weight, the weight of each tissue was divided by body weight at termination and multiplied by 100 percent. As shown in Figure 3.8 the weight of kidneys and the liver contributed significantly more to the final body weight (BW at termination) of the rats on the low fat diet compared to those on HF. The adrenal, heart, pancreas, testes, spleen and the perirenal fat pad did not have any significant effect on the final weights of the rats in both groups. The weight contributed by the white adipose tissues shown in Figure 3.9 was highly significant for the HF group ($p < 0.004$) compared to LF group.

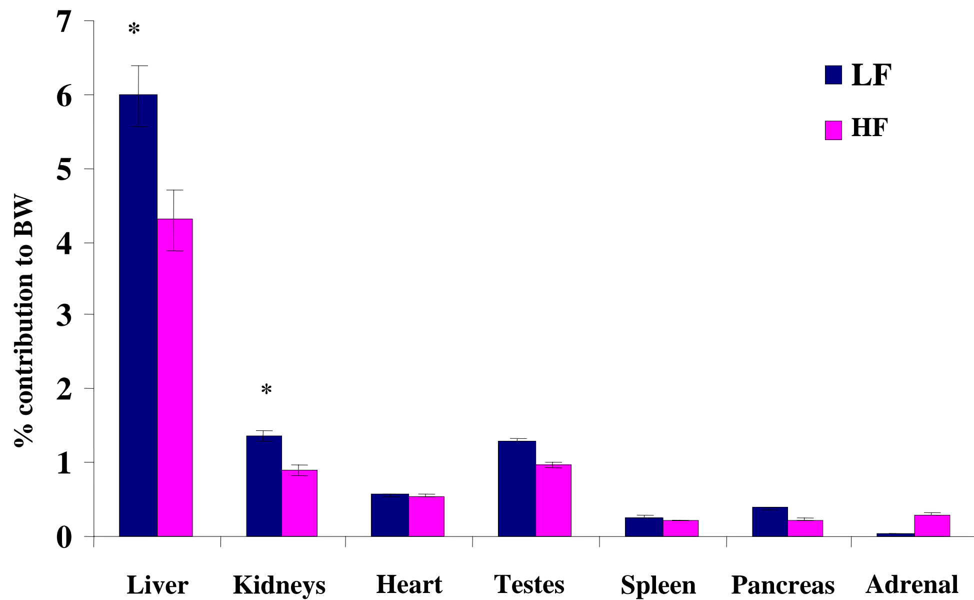

Figure 3.8: Percentage contribution of various tissues to the total body weight gained by rats for LF and HF groups. * Difference between the two groups is highly significant with $p < 0.004$.

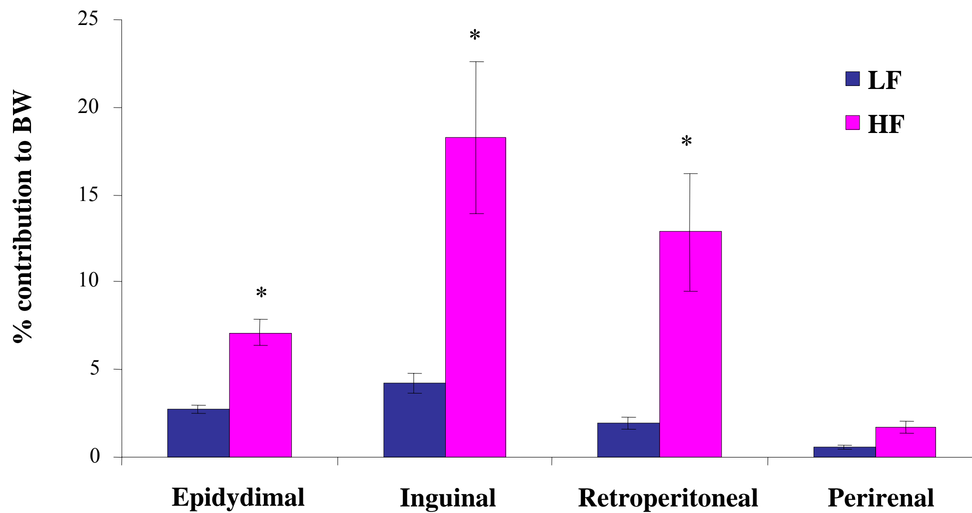

Figure 3.9: Percentage contribution of fat pads to the total body weight gained by rats for LF and HF groups. * Difference between LF and HF group is significant with the $p < 0.05$.

3.2 PROTEOMICS

3.2.1 SDS-PAGE

Serum proteome analysis requires removal or depletion of high abundant proteins as they interfere or mask resolution of low abundant proteins as shown in Figure 3.10A. Twenty micrograms of crude serum proteins was poorly resolved in a 10% SDS-PAGE, and diluting the serum with PBS showed some improvement (Figure 3.10B). However, not all proteins were clearly resolved and some proteins which are present at low concentrations were diluted to below detection levels.

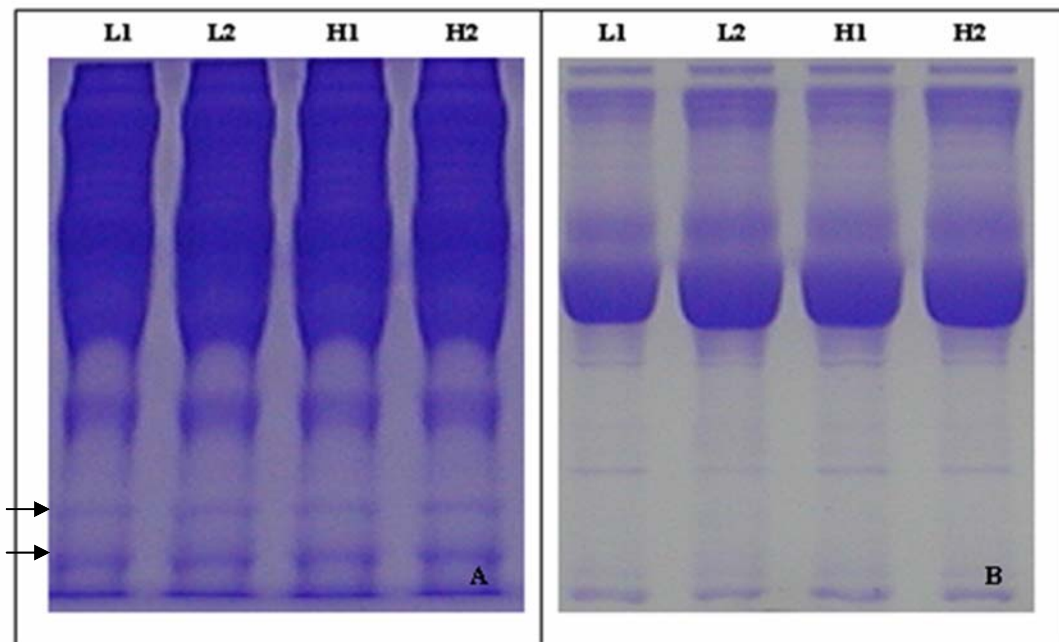

Figure 3.10: SDS-PAGE analysis of crude serum samples (A) and PBS diluted serum samples (B), two samples were from the low fat group (L1 and L2) and the other two from the high fat group (H1 and H2). Arrows shows proteins that disappeared following dilution of the serum with PBS.

Various chemical precipitation methods were carried out to address the problem: starting with 10% 1.5 M Tris-HCl and urea. Tris-HCl and urea precipitation was able to remove some high abundant proteins, however, the resolution was still poor (Figure 3.11). Ten percent TCA/acetone precipitation (weight/volume) was able to deplete most of albumin when compared to Tris-HCl and urea but removed most other proteins with the albumin.

The 10% TCA/acetone (v/v) was more effective and hence the method of choice for albumin depletion in the serum.

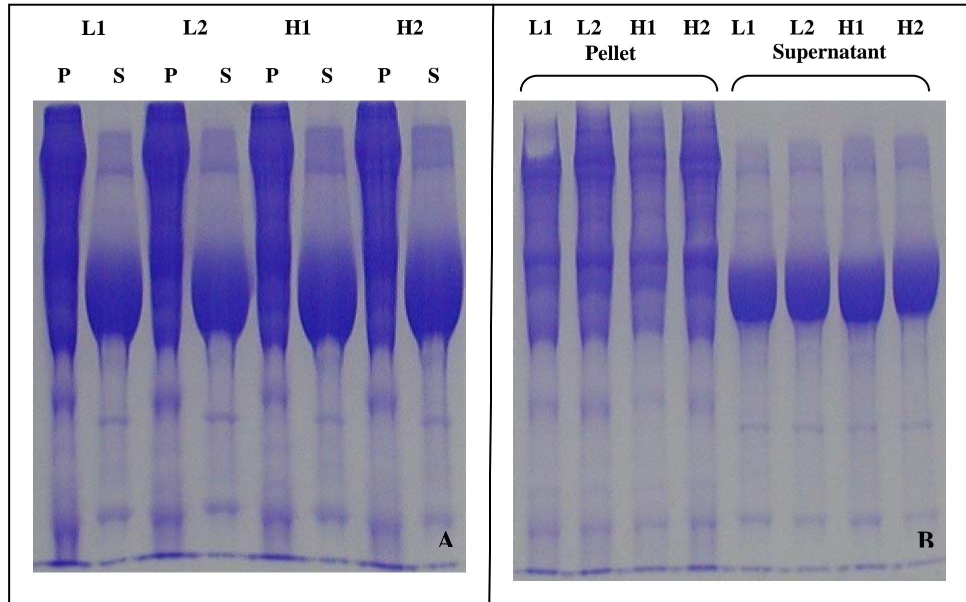

Figure 3.11: SDS-PAGE analysis of albumin depleted serum samples (A) 1.5M Tris-HCl and (B) urea precipitation for the LF (L1-L2) and HF (H1-H2) groups. The proteins were resolved on a 12% gel. : P: pellet/ albumin-depleted sample and S: supernatant/ albumin-containing sample.

Using the 10% TCA/acetone (v/v) precipitation method, four bands were visually identified as dysregulated in the albumin-depleted or pellet gel (Figure 3.12A), two bands above 170 kDa and the one above 26 kDa appeared to be down regulated in the HF group, while the one around 55 kDa was up regulated in the HF group when compared to the LF group. For the supernatants (Figure 3.12B) only two bands at 43 and 34 kDa were up regulated in the HF group, however the results still have to be confirmed using 2D gel analysis.

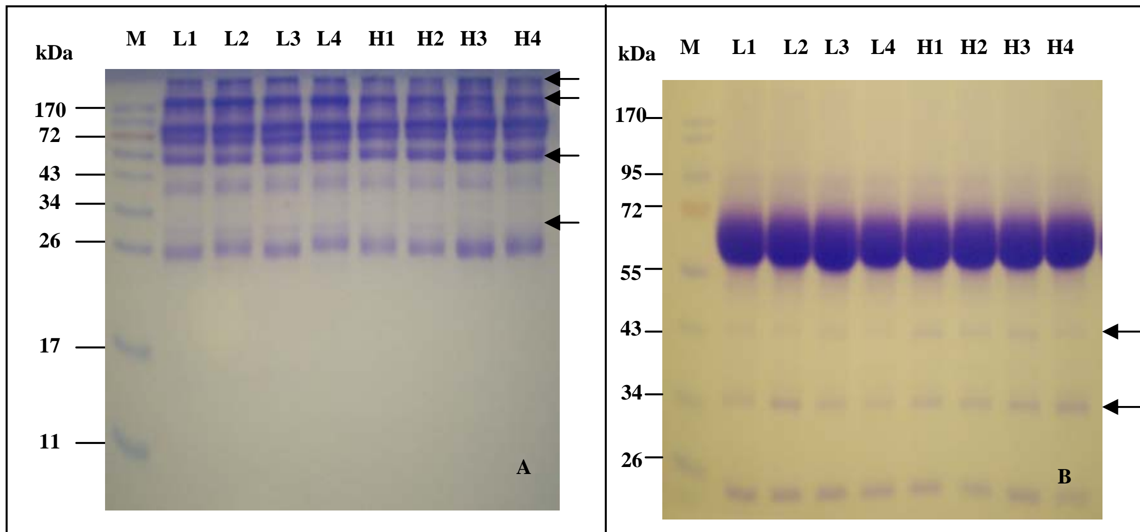

Figure 3.12: SDS-PAGE analysis of albumin-depleted serum samples (A) and supernatants containing albumin serum samples (B) after TCA precipitation for the LF (L1-L4) and HF (H1-H4) groups. Twenty (20) µg of protein was resolved on a 12% gel. The arrows indicate proteins that appear to be dysregulated between the two groups.

3.2.2 Two-dimensional gel electrophoresis

Hundred micrograms of serum proteins from four different rats per group were resolved by 2D-PAGE. The samples were first resolved based on their isoelectric points in the first dimension followed by their molecular mass in the second dimension. Over eighty spots were resolved in both the precipitate and supernatant as shown in Figures 3.13 and 3.14. Three gels in each group were used for comparison and only the spots that were present in all the gels per group were considered.

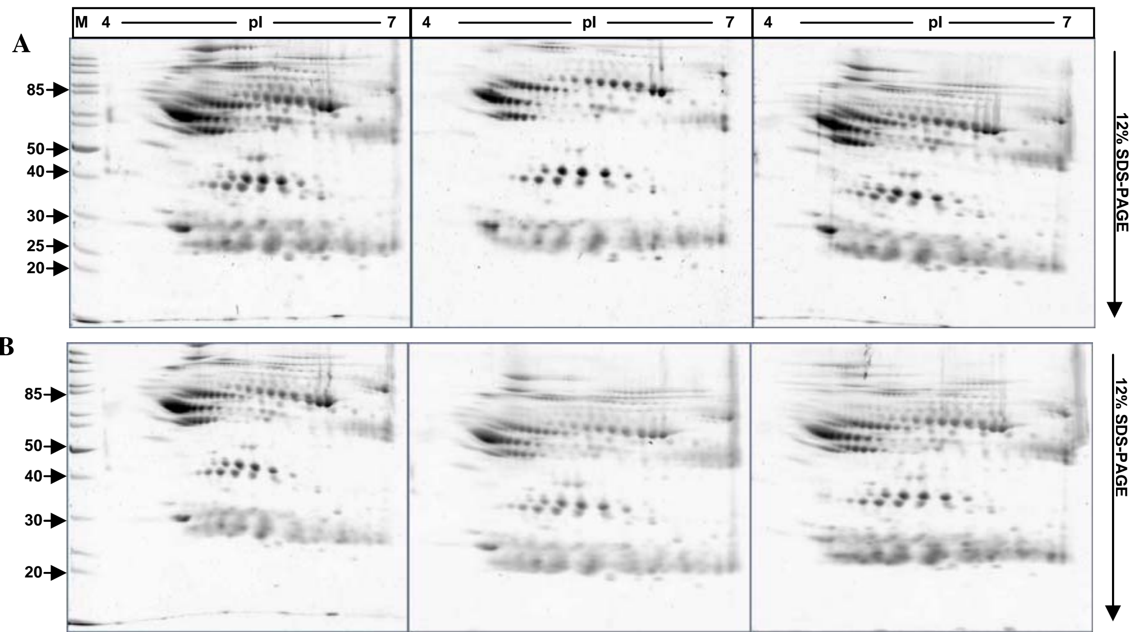

Figure 3.13: Representative Flamingo stained 2D gels from rat serum at 44 weeks. Hundred (100) µg protein of albumin-depleted samples was resolved by 2D-PAGE in a 7 cm IPG strip with 4-7 pH range; A are gels from LF group and B from HF group. M = molecular weight markers in kDa.

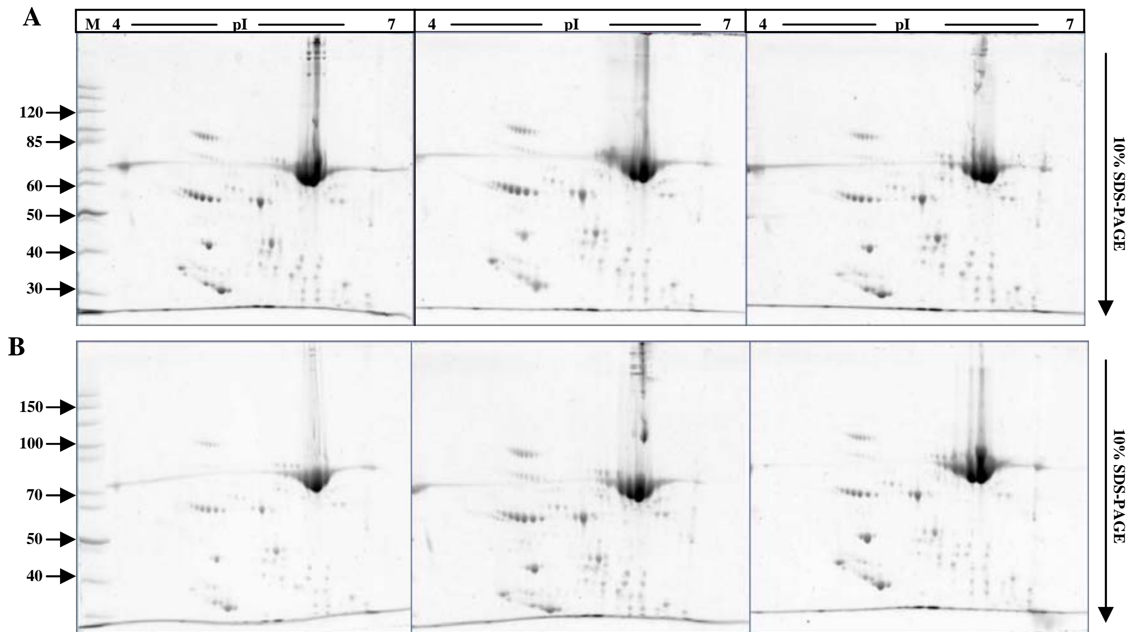

Figure 3.14: Representative Flamingo stained 2D gels from rat serum at 44 weeks. Hundred (100) µg protein of albumin-containing samples was resolved by 2D-PAGE in a 7 cm IPG strip with 4-7 pH range; A are gels from LF group and B from HF group. M = molecular weight markers in kDa.

3.2.3 IMAGE ANALYSIS

Three gels per group were chosen for PDQuest analysis. The gels were compared quantitatively, qualitatively and statistically, and a number of spots showed significant variations between the two groups that were either induced or repressed by the HF diet. From the 84 matched spots in the pellet or albumin-depleted sample (Figure 3.15), 8 spots showed differences that were statistically significant.

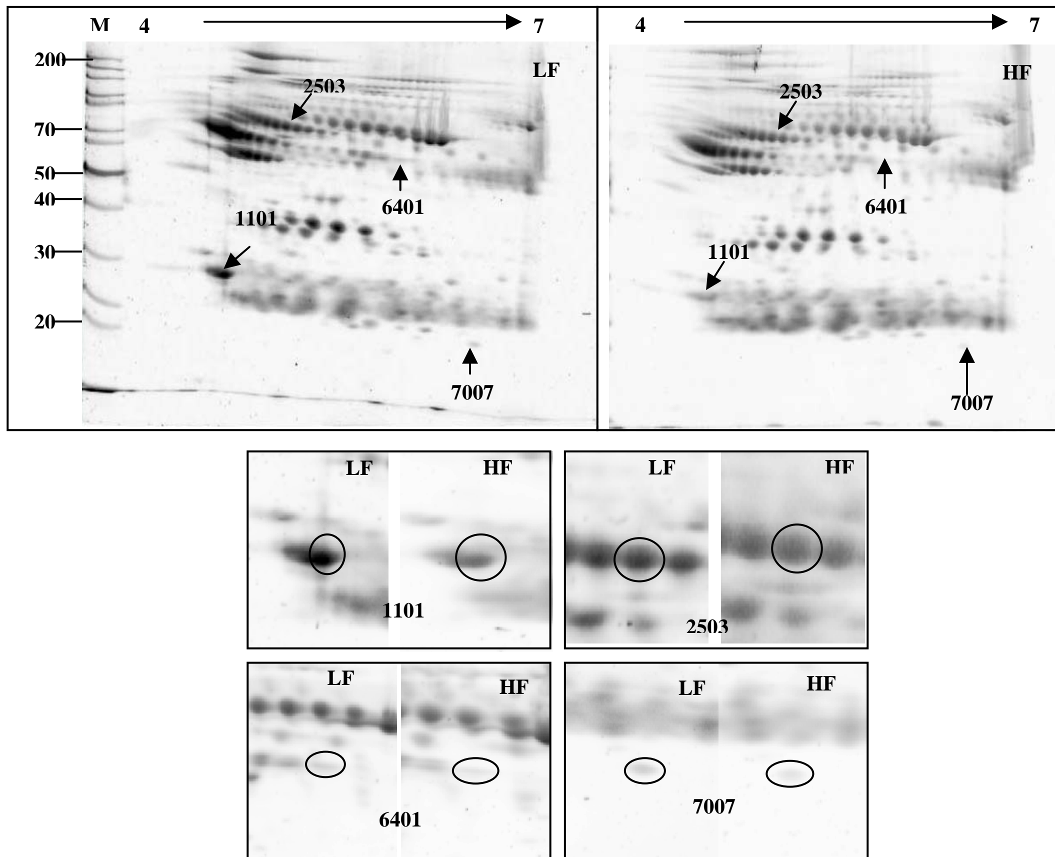

Figure 3.15: 2D Maps with magnified images of spots that were found to be dysregulated in HF from albumin-depleted sample when compared to LF group. The arrow is pointing at the spot that was found to be significantly dysregulated in HF group when compared to the LF group.

The spots were in the molecular mass range of 10- 200 kDa in the two groups. Five spots (1101, 2311, 2503, 6401 and 7007) showed a difference of 2-fold less or greater spot intensity in the HF group as compared to the control group. Two spots 2311 and 6401 were too faint to be analysed or seen in the gels. The two spots were found to be up-

regulated and the other three spots (1101, 2503 and 7007) were down-regulated in the HF group.

Eighty spots in the supernatant or albumin-containing sample (Figure 3.16) were compared between the two groups, ten spots showed differential expression that were statistically significant. Only one spot (2301) showed a 2-fold expression difference. The spot was up-regulated in the HF group compared to the LF group. To ascertain that variations between the two groups were due to obesity development, spots that showed 2-fold or more expression differences were considered to be differentially expressed.

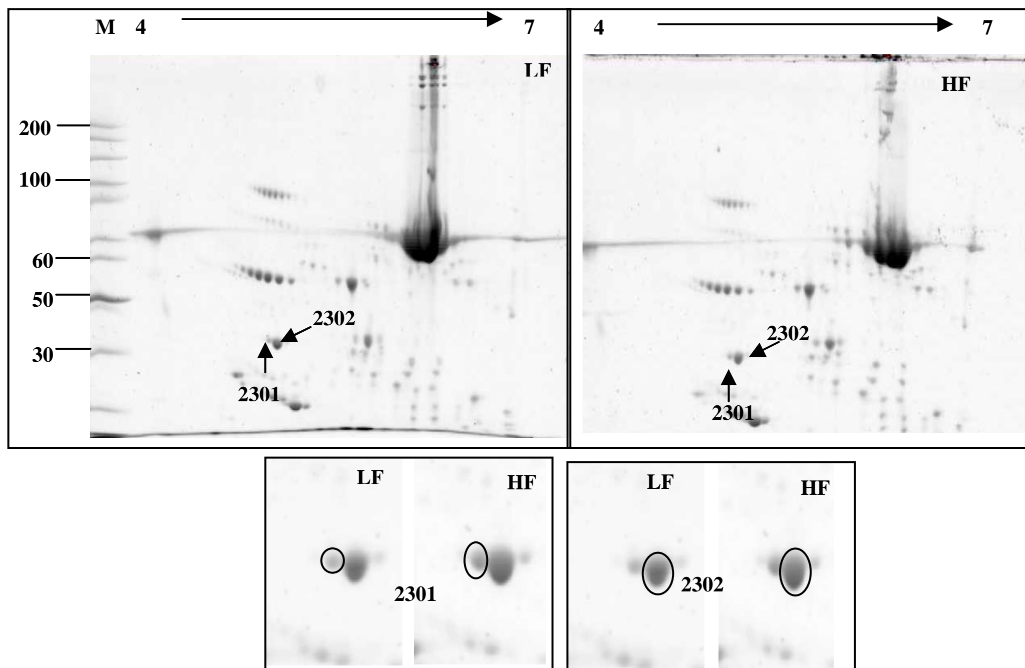

Figure 3.16: 2D maps and magnified images of spots that were found to be quantitatively and stastically dysregulated in HF when compared to LF group in the albumin-containing sample.

Scatter plot for the albumin-depleted sample

The scatter plot was used to identify relationships in the protein expression levels between the LF and the HF group. The spots were either up- or down-regulated in the HF group when compared to the LF group. Five protein spots were dysregulated (Figure 3.17): two spots were up-regulated (2311 and 6401) and three spots were down-regulated (1101, 2503 and 7007) in the HF group compared to the LF group.

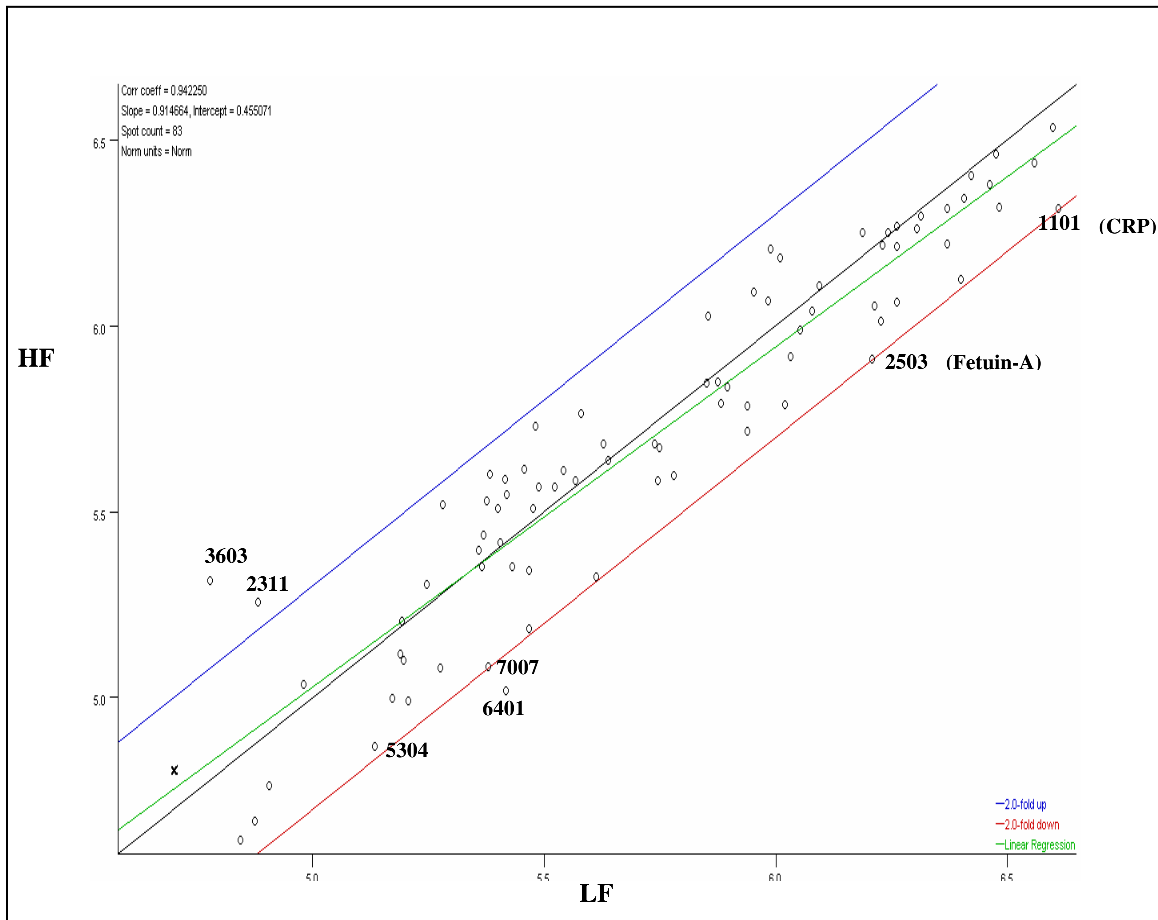

Figure 3.17: Scatter plot showing the correlation between replicate gels for both the LF and HF groups in pellets or albumin-depleted sample.

Scatter plot for the albumin-containing sample

From the scatter plot only one spot was found with the expression level of 2-fold or greater (Figure 3.18).

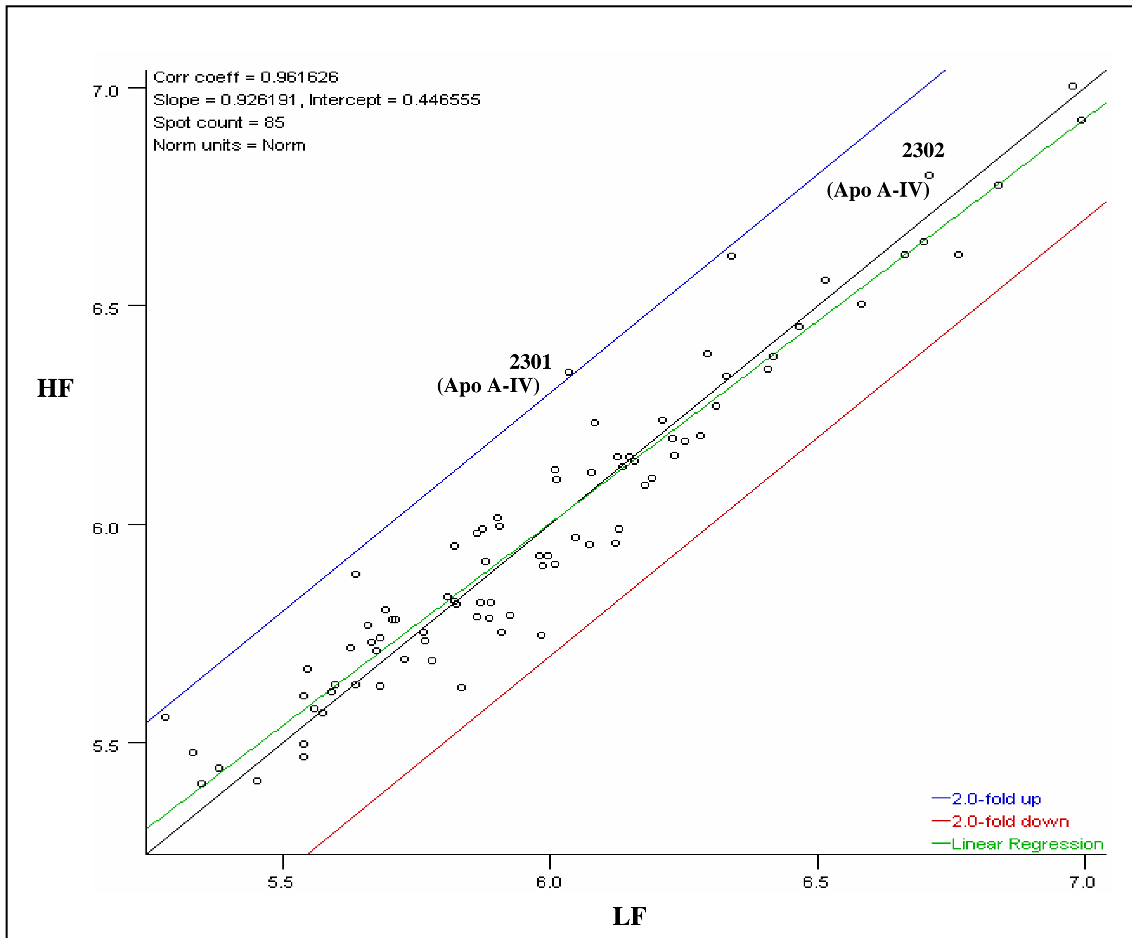

Figure 3.18: Scatter plot showing the correlation between replicate gels for both the LF and HF groups in supernatants or albumin-containing sample.

3.2.4 Mass Spectrometry Analysis

Two spots with altered protein expression from each sample were chosen for identification by MALDI-TOF mass spectrometry, these were spots 2503 and 1101 from the albumin-depleted sample and 2301 and 2302 from the albumin-containing sample.

Mass spectra of the protein spots were obtained by digesting the proteins with trypsin into smaller peptides and measure their masses with MALDI-TOF MS. The obtained masses, called peptide mass fingerprints, were compared *in silico* using Mascot to known protein sequences in the NCBI database to identify the proteins. The results were statistically analyzed to find the best match.

3.2.4.1 Mass spectrum of albumin-depleted sample

Only one spot (1101) was digested successfully in the albumin-depleted sample. The results obtained for spot 2503 was inconclusive. A mass spectrum acquired from the tryptic digested spot is shown in Figure 3.19.

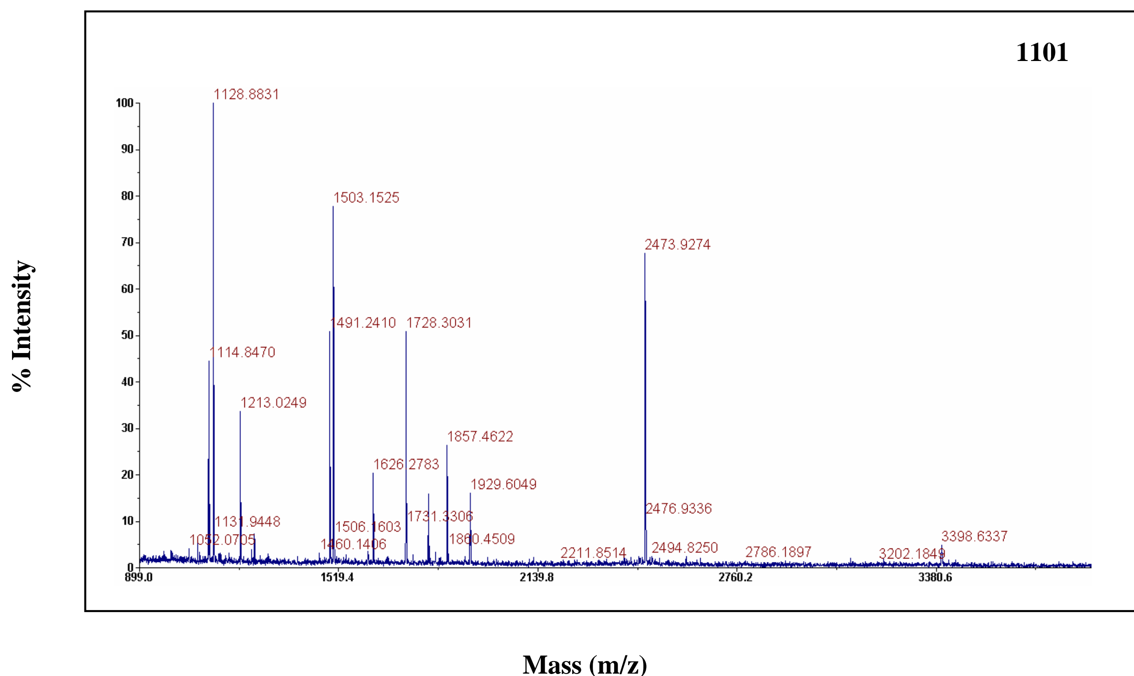

Figure 3.19: Mass spectrum from the Voyager DE STR MALDI TOF MS measurement of tryptic digested proteins. MALDI-TOF MS analysis showed a peptide mass fingerprint spectrum of the spot cut from the albumin-depleted sample.

The mass spectrum shows the relative abundance of each ion and the increasing mass to charge (m/z) ratio of the resulting peaks of the fragmented spot. Since MALDI-TOF uses a positive ionization method that generates singly charged ions with a charge of 1+ regardless of the molecular mass, then the m/z gives the mass of the isotope directly.

3.2.4.2 Mass spectrum of albumin-containing sample

Mass spectra obtained from the digested spots in the supernatant are shown in Figure 3.20. Seven similar peaks were identified from the spectra: i.e., 1018.5, 12.61.6, 1287.6, 1417.7, 1656.8, 2379.0 and 2812.3 m/z. The peaks were found to be of the same mass but differed in their abundance, and this suggested that the spots might be the same protein.

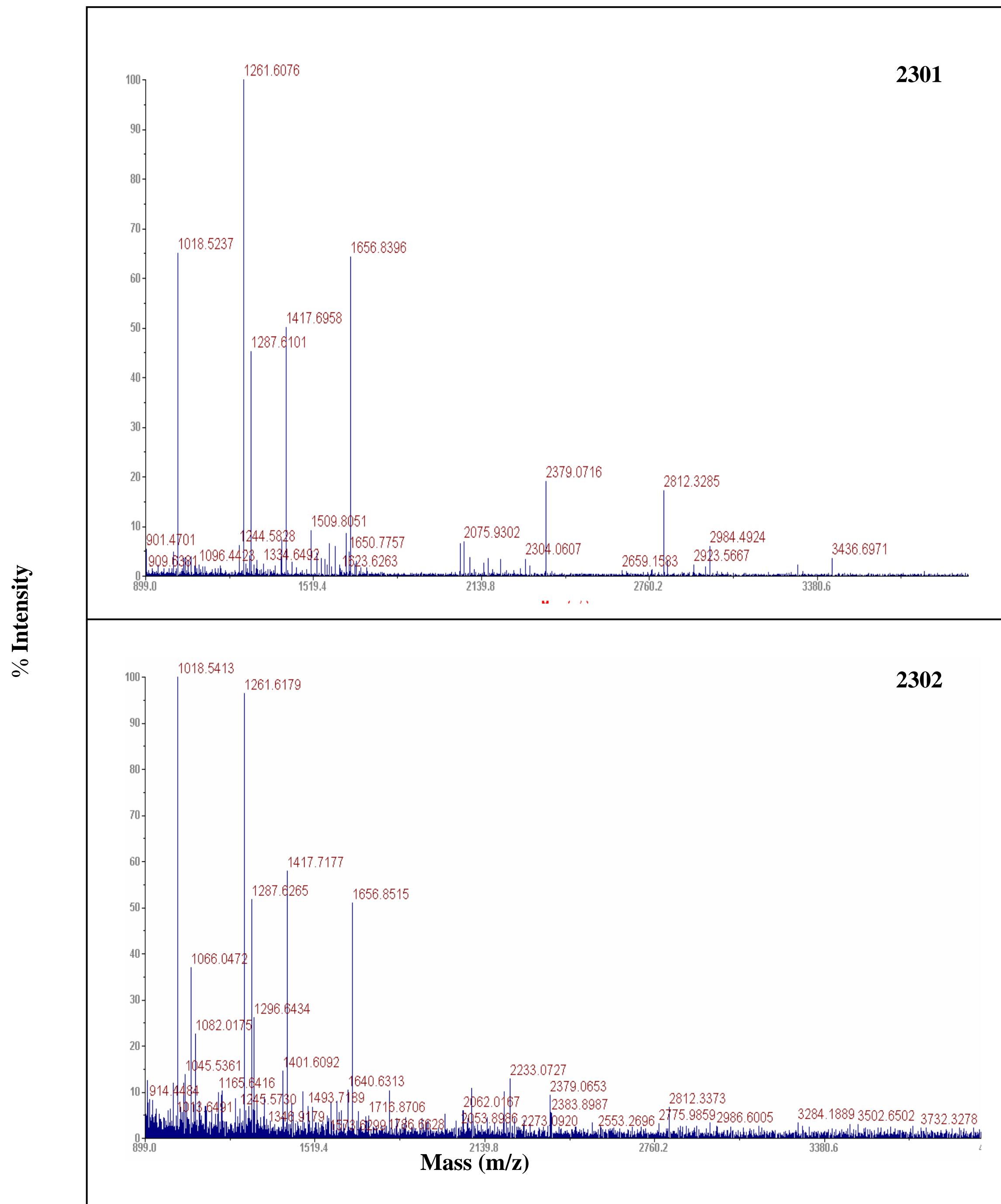

Figure 3.20: Mass spectrum from the Voyager DE STR MALDI TOF MS measurement of tryptic digested proteins. MALDI-TOF MS analysis showed a monoisotopic peptide mass fingerprint spectrum of the spots cut from albumin-containing sample.

3.2.4.3 Protein sequences of matched proteins

Protein sequences obtained from the trypsin digested spots were blasted in the NCBI database to determine which protein best matched the sequence. The matched sequences in the known proteins are highlighted in red. For spot 1101, seven peptides were matched to the C-reactive protein, as shown in Figure 3.21.

Spot 1101: CRP

```
   1 MYRIATKTWH VQVLFVYSVP GSSALFAGVP AALLHALVLW LLSSVEAIAT
  51 LLSLVLYSFH DASSVHSCQV IPSEADSGFV NYIKAMIWEK ELLLKGDLNH
 101 SEADELGAKQ ALALGAYRLL ISPRWGVYRK EGGTQDPNNI KYLGVTLTKQ
 151 VKDLYNKNFK TLKKEIEEDL RRWKDLPCSW IGRINIVKMP ILPKAIYRFN
 201 AIPIKIPIQF FKELDRTICK FIWNNKKPRI AKAILNNKRT SGGITIPELK
 251 QYYRAIAIKT AWYWYRDRQI DQWNRIEDPE MNPHTYGHLI FDKGAKTIQW
 301 KKDSIFSKWC WFNWRSTCRR MQIDPCLSPC TKLKSKWIKD LHIKADTLKL
 351 IEEKLGKHLE HMGTGKNFLN KTPMAYALRS RIDKWDLIKL QSFCKAKDTV
 401 VRTNRQPTDW EKIFTNPTTD RGLISKIYKE LKKLDRRETN NPIKKWGSEL
 451 NKEFTAEECR MAEKHLKKCS TSLVIREMQI KTTLRFHLTP VRMAKIKNSD
 501 DSRCWRGCGE RGTLLHCWWG CKLVQPFWKS VWRILRKLDI ELPEDPAIPL
 551 LGIYPKDAPT YKKDTCSTMF IAALFIIARS WKEPRCPSTE EWIQKMWYIY
 601 TMEYYSAIKN NDFMKFVGKW LELENIILSE LTQSQKDIHD MSKQAFVFPG
 651 VSATAYVSLE AESKKPLEAF TVCLYAHADV SRSFSIFSYA TKTSFNEILL
 701 FWTRGQGFSI AVGGPEILFS ASEIPEVPTH ICATWESATG IVELWLDGKP
 751 RVRKSLQKGY IVGTNASIIL GQEQDSYGGG FDANQSLVGD IGDVNMWDFV
 801 LSPEQINAVY VGRVFSPNVL NWRALKYETH GDVFIKPQLW PLTDCSQVQV
 851 GQRPPHKTDT LKLIEEKVGK NLGHMGTGPH EAQEGKTKVW MLQSFLEGGT
 901 KILTGGNMER KCGIEERPSR DCPTWGSSPH SATKPRQHCG YQEVHADRNL
 951 IQLSPERLCQ SLTNTEVDAH SQPLTEKEVP NGGVREKTEG VEGVCNPIRR
1001 TVSSNQPYPV RDPRD
```

Figure 3.21: Mascot search showing the matched peptides (in red) of the spot cut from the albumin-depleted sample in the known protein sequences of C-reactive protein.

From the albumin-containing sample or the supernatant: a pair of spots with the same molecular masses and different pI was shown to have some similar sequences (the peptides are underlined in Figure 3.22) that matched in the same protein. By comparing the sequences covered by the PMFs these spots appeared to be the same protein, Apolipoprotein A-IV. In spot 2301, 12 out of 49 peptides were matched to the sequence of Apo A-IV and for 2302, 14 out of 20 matched.

Spot 2301: Apolipoprotein A-IV

```
  1 MFLKAVVLTV ALVAITGTQA EVTSDQVANV MWDYFTQLSN NAKEAVEQLQ
 51 KTDVTQQLNT LFQDKLGNIN TYADDLQNKL VPFAVQLSGH LTKETERVRE
101 EIQKELEDLR ANMMPHANKV SQMFGDNVQK LQEHLRPYAT DLQAQINAQT
151 QDMKRQLTPY IQRMQTTIQD NVENLQSSMV PFANELKEKF NQNMEGLKGQ
201 LTPRANELKA TIDQNLEDLR SRLAPLAEGV QEKLNHQMEG LAFQMKKNAE
251 ELHTKVSTNI DQLQKNLAPL VEDVQSKLKG NTEGLQKSLE DLNKQLDQQV
301 EVFRRAVEPL GDKFNMALVQ QMEKFRQQLG SDSGDVESHL SFLEKNLREK
351 VSSFMSTLQK KGSPDQPLAL PLPEQVQEQV QEQVQPKPLE S
```

Spot 2302: Apolipoprotein A-IV

```
  1 MFLKAVVLTV ALVAITGTQA EVTSDQVANV MWDYFTQLSN NAKEAVEQLQ
 51 KTDVTQQLNT LFQDKLGNIN TYADDLQNKL VPFAVQLSGH LTKETERVRE
101 EIQKELEDLR ANMMPHANKV SQMFGDNVQK LQEHLRPYAT DLQAQINAQT
151 QDMKRQLTPY IQRMQTTIQD NVENLQSSMV PFANELKEKF NQNMEGLKGQ
201 LTPRANELKA TIDQNLEDLR SRLAPLAEGV QEKLNHQMEG LAFQMKKNAE
251 ELHTKVSTNI DQLQKNLAPL VEDVQSKLKG NTEGLQKSLE DLNKQLDQQV
301 EVFRRAVEPL GDKFNMALVQ QMEKFRQQLG SDSGDVESHL SFLEKNLREK
351 VSSFMSTLQK KGSPDQPLAL PLPEQVQEQV QEQVQPKPLE S
```

Figure 3.22: Mascot search showing the matched peptides (in red) of the cut spots in the known protein sequences of Apolipoprotein A-IV and sequences that matched in both the two spots (underlined) from the albumin-containing sample.

3.2.4.4 Probability based Mowse score

The Mowse score is derived from -10*Log (P), where P is the probability that the observed match is a random event. A probability based Mowse score of 60 (1101) in Figure 3.23 indicated that the identified peptides match the CRP sequence with a high degree of certainty; and a score of 84 (2301) and 193 (2302) for Apo A-IV in Figure 3.24. The shaded area represents scores with the greatest statistical uncertainty, the unshaded area indicates scores that are statistically significant. Protein scores greater than 68 for the three spots were considered to be a significant match of the proteins ($p < 0.05$).

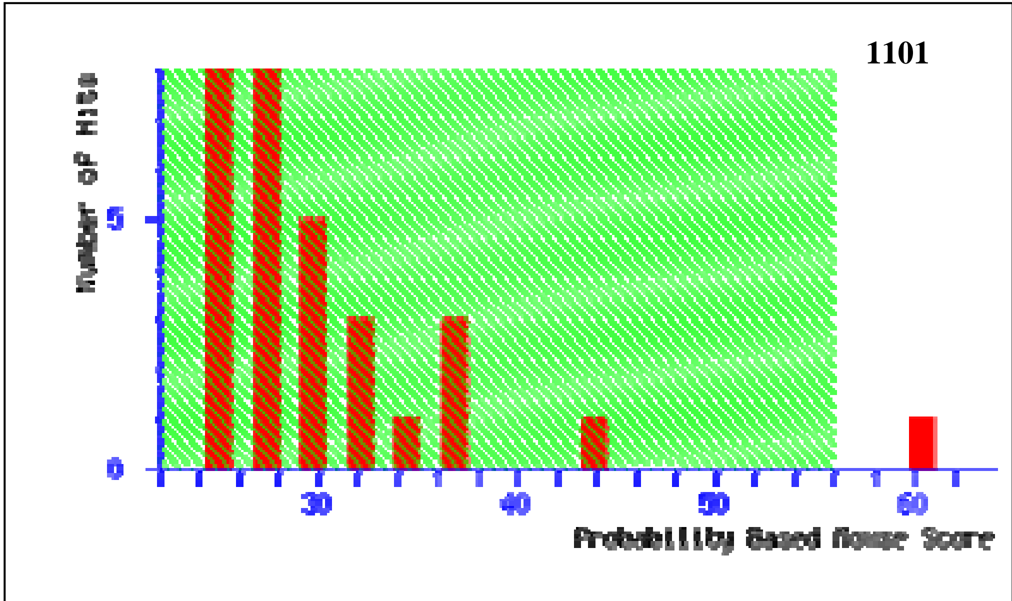

Figure 3.23: Probability based MOWSE scores of the albumin-depleted samples. Data were internally calibrated. Peptide mass tolerances were set to ± 100 ppm for database searches.

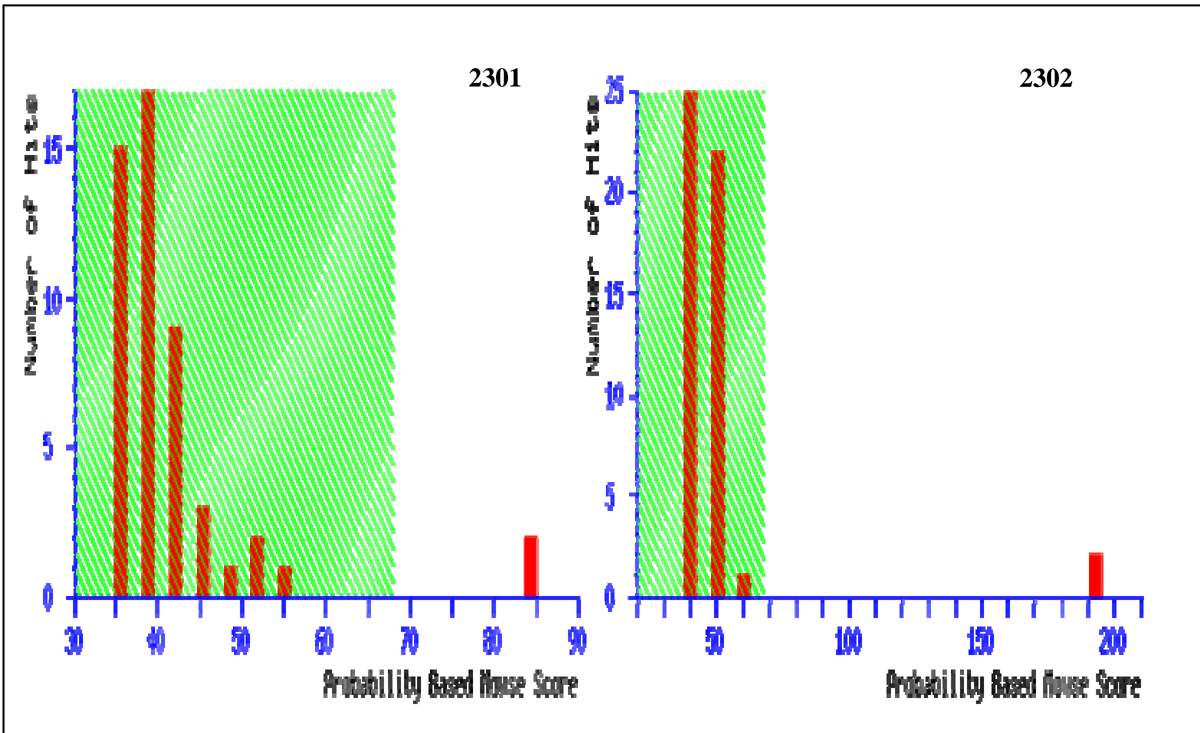

Figure 3.24: Probability based MOWSE scores of the albumin-containing samples. Data were internally calibrated. Peptide mass tolerances were set to ± 100 ppm for database searches.

3.2.4.5 Protein identification

The PMF analysis was used for the identification of the proteins based on the mass measurement of enzymatically digested proteins by MALDI-TOF MS and comparison of the generated spectra with the theoretical fingerprints from NCBI. Three out of four protein spots were identified by the Mascot search engine through the NCBI database as shown in Table 3.2. The other spot was identified as Fetuin-A through serum 2D-PAGE reference maps published by the rat serum protein study at http://linux.farma.unimi.it. The protein identities were confirmed by western blot analysis.

Table 3. 2: Differentially expressed serum proteins in obese rats as compared to the lean rats.

SSP No.	Protein Name	NCBI Accession No.	pI		MW (kDa)		Peptides matched/ Unmatched	PMF Probability Score	% Sequence Coverage
			Gel	Theoretical	Gel	Theoretical			
1101	CRP	Q7TMA9_RAT	4.9	9.21[a]	25-30	11.6[a]	7/?	60 *	8
2301	Apo A-IV	Q5BK92_RAT	5.1	5.18	30-40	44.5[a]	12/49	84 *	33
2302	Apo A-IV	Q5BK92_RAT	5.2	5.18	30-40	44.5[a]	14/20	193 *	35
2503	Fetuin-A	?	5.4	?	70-85	?	?	?	?

The proteins were identified by MALDI-TOF MS, PMF obtained were used to search the protein identities through NCBI database by the Mascot search engine. SSP No.: Standard spot number; pI: Isoelectric point; MW: Molecular weight; PMF: Peptide mass fingerprint; ?: results were not obtained from the MALDI-TOF MS; *: Scores are statistically significant ($p < 0.05$). [a] The discrepancy between the experimental and theoretical pI and MW of the identifies proteins might be due to post translational modifications on development of obesity.

3.3 WESTERN BLOT ANALYSIS

3.3.1 Western blot and densitometry analyses

CRP, Fetuin-A and Apo A-IV were subjected to one-dimensional western blot analysis to confirm their identity, and to further ensure that proteins were indeed over- (Apo A-IV) or under-expressed (CRP and Fetuin-A) in obese rats. The expression levels of the proteins were compared between the LF and HF group for all the time points (baseline, 4, 6, 8 and 44 weeks) using the ImageJ software.

Protein expression in the albumin-depleted sample

Two distinct bands were detected at 25 kDa by anti-CRP antibodies as shown in Figure 3.25A. The densitometric quantitation in Figure 3.25B showed that CRP's expression was not consistent for the first 6 weeks. Baseline expression was down for the LF group when compared to the HF group, increased at the fourth week and decreased again at the sixth week, reaching maximal levels at 8 weeks.

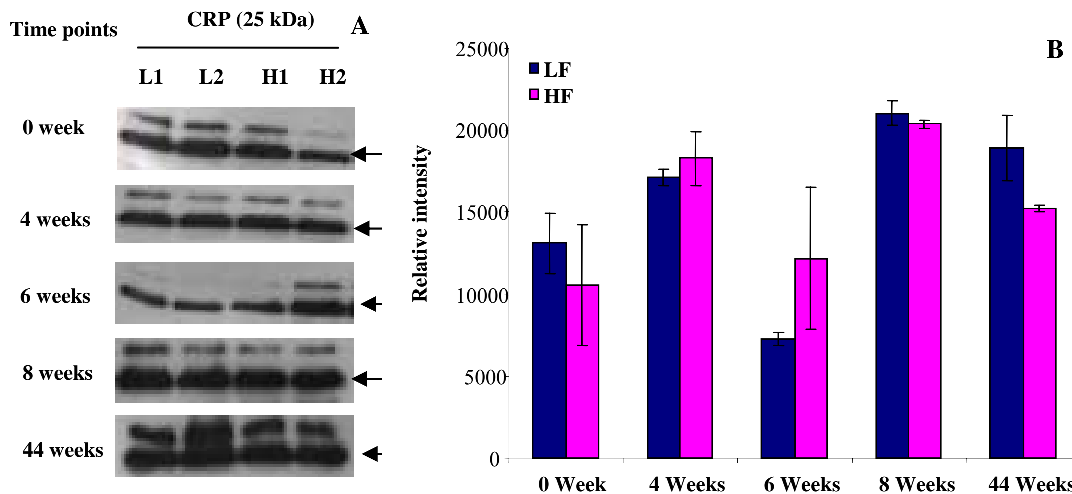

Figure 3.25: Western blot and densitometric analyses confirming the expression of CRP at different time points (0, 4, 6, 8 and 44 weeks). Twenty micrograms albumin-depleted sample was subjected to 12% SDS-PAGE, blotted onto nitrocellulose membrane and probed with anti-CRP antibodies (A). The arrow signifies the protein of interest. Relative band intensity of CRP (B) at different time points was compared. Values, as determined by image J software, are mean values of two rats per group.

A single band at 58 kDa was detected by anti-Fetuin-A antibodies as illustrated in Figure 3.26A. Through densitometric analysis, the protein expression was compared between the two groups at different time points. The expression remained low for the first six weeks for the HF group and increased at the 8th and 44th weeks (Figure 3.28B).

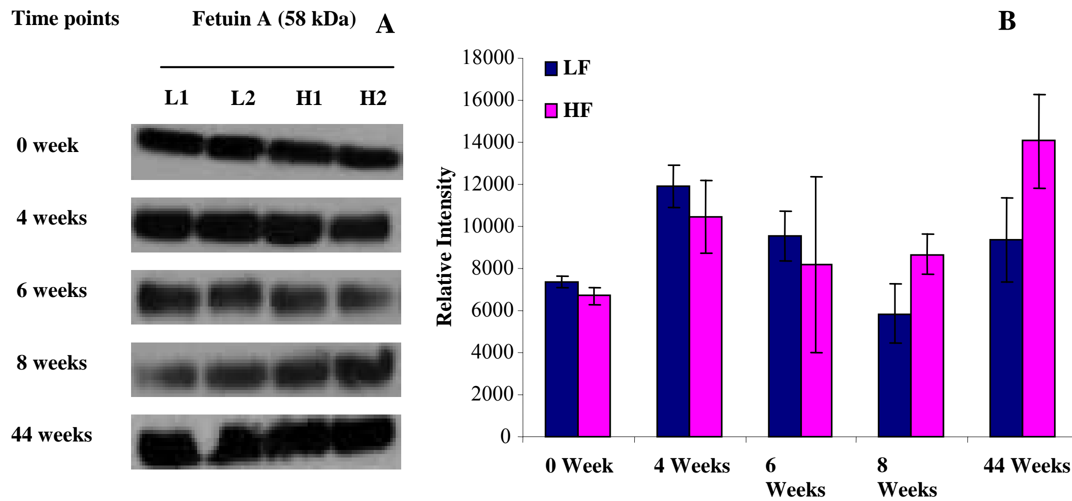

Figure 3.26: Western blot and densitometric analyses confirming the expression of Fetuin-A at different time points (0, 4, 6, 8 and 44 weeks). Twenty micrograms albumin-depleted sample was subjected to 12% SDS-PAGE, blotted onto nitrocellulose membrane and probed with anti-Fetuin-A antibodies (A). Relative band intensity of Fetuin-A (B) at different time points on either the LF or HF group was compared. Values, as determined by image J software, are mean values of two rats per group.

Protein expression in the albumin-containing sample

Apo-AIV showed some differences in its expression between the two groups that can be visually seen in Figure 3.27A from the densitometric analysis. The two groups started with similar levels of Apo-AIV at baseline that increased on the fourth week of HF feeding as illustrated in Figure 3.27B. The HF group had increased levels of Apo A-IV from the fourth and remained high for in the last three time points when compared to the LF group.

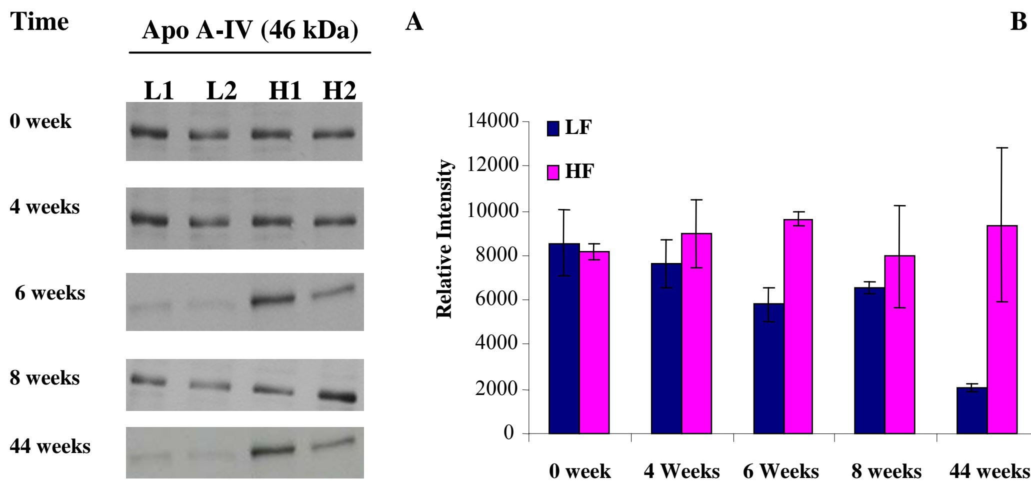

Figure 3.27: Western blot and densitometric analyses confirming the expression of Apo-AIV at different time points (0, 4, 6, 8 and 44 weeks). Ten micrograms of albumin-containing sample was subjected to 12% SDS-PAGE, blotted onto nitrocellulose membrane and probed with anti-Apo-AIV antibodies (A). Relative band intensity of Apo-AIV (B) at different time points was compared. Values, as determined by image J software, are mean values of two rats per group.

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1 DISCUSSION

The increasing prevalence of human obesity poses a serious health threat in the developed and developing countries. Obesity is the most challenging condition and a matter of great concern due to its association with conditions such as cardiovascular disease and T2D. This study focussed on obesity as a major contributor to development of T2D. Obesity and T2D are two commonly occurring and highly interrelated conditions that have approached epidemic proportions in recent years. The onset of T2D is highly correlated with obesity with as many as 90% of type 2 diabetics being overweight or obese (List *et al*., 2007). Despite the major progress made in regulating energy balance and understanding the molecular mechanisms leading to obesity in both human and animal studies, no safe or effective treatment has been found (Kolonin *et al*., 2004; Woods *et al*., 2003). Obesity is a chronic condition that is mostly managed through palliation; unfortunately, the usefulness of commercially available drugs for the treatment of obesity has proven disappointing due, in part, to poor efficacy and off-target side effects that raise compliance issues (Ma *et al*., 2006).

Since, both obesity and T2D are multifactorial disorders this complicates genetic studies of these diseases and what make matters worse is that T2D does not show any clinical symptoms until the disease is in its advanced stages and difficult to treat successfully (Storlien *et al*., 1997; Woods *et al*., 2003). Therefore, novel approaches are needed that can unravel the biochemical and physiological processes that occur during the development of obesity and progression to T2D. This will give insights into obesity and obesity-associated diseases, and ways to prevent it in susceptible individuals. Hence the purpose of this study was to determine changes in serum protein profiles that occurred during the transition from the lean to obese state in Wistar rats using a proteomics approach. The approach is attractive in that the altered protein profile might be used for diagnosis. The identified biomarkers for early detection may thus be used and may serve as potential targets for drug discovery.

To accomplish this aim, an animal model of diet induced obesity was established using male Wistar rats. The rats in the high fat (HF) group were fed an in-house prepared high fat diet for an extended period of time that resulted in increased body weight gain of the rats. These rats started with similar body weights as the base line control rats and by the end of the fourth week the HF group had become overweight as illustrated in Figure 3.1. At the end of the study the HF fed rats were heavier than the rats in the low fat (LF) group, taking into account that some rats in the HF group lost weight towards the end of the study. Other animal studies (especially in rodents) proved that eight weeks of high fat diet feeding was sufficient for the induction of obesity (Harrold *et al*., 2000). This study lasted forty four weeks to allow enough time for T2D to manifest in the HF rats as it occurs in human state. Obesity-induced T2D through HF diet requires more time to develop unlike chemical-induced T2D (it can be induced by partial destruction of the β-cells in the pancreas using streptozotocin/ STZ) that can develop within a week in rodent models of diabetes. However the doses of STZ used have to be carefully measured to create stable T2D that cannot revert or deteriorate to T1D (Arulmozhi *et al*., 2004).

There was a highly significant correlation between energy intake and weight gained by the HF group, which proved that positive energy balance is an important determinant factor in the development of obesity. This confirmed that disequilibrium between energy intake and expenditure together with diet composition plays a key role in weight gain not only in humans (Thomas *et al*., 1992), but in animals as well. Despite the increased food intake in the LF group relative to that of the HF group, the HF group had higher energy intake due to high calories from fat. This increased energy intake consequently resulted in positive energy balance and weight gain. Failure to increase energy expenditure or decrease the food intake results in increased fat mass (Smith *et al*., 2000). This has been demonstrated in many studies where normal rats fed high fat diet *ad libitum* become obese due to excess dietary fat (West and York, 1998); therefore, failure to compensate the energy intake results in increased fat mass (Lovejoy *et al*., 2002). Accordingly, the excess fat is then stored in white adipose tissues which explains the increased fat pads observed in the HF group when compared to the LF group (Table 3.1). HF diets produce

a consistent and significant increase in body fat content that is dependent on the amount of fat in the diet and the duration of feeding (Hill *et al*., 2000).

The rats gained weight rapidly and became quite obese after consumption of a high proportion of energy from fat for 44 weeks. It is evident from animal experiments that the percentage of energy derived from fat in the diet is positively correlated with body fat content. This proves that obesity can be induced by HF diets in animals such as monkeys, dogs, pigs, hamsters, squirrels and rodents (West and York, 1998) just like in humans (Artiss, 2006). In the study done by Héliès *et al* (2005) the hypercaloric diets were used to trigger obesity development in obesity-resistant Lou/C rats and obesity-prone Fischer 344 rats, diet-induced obesity was observed in Fischer 344 rats but not in LOU/C rats. This study showed that the interaction between the genes and environmental factors plays a very crucial role for obesity development in genetically susceptible individuals as well as animals (Héliès *et al*., 2005). Diets rich in energy and high in fat are a major factor within the current obesogenic environment, which is involved in alteration of homeostatic and regulatory systems in the body further complicating the disease state. The possibility that these kinds of diets and the energy contained therein, fuelled the current obesity epidemic. This has increased interest in rodent models of diet-induced obesity which mimic the human condition (Mercer and Archer, 2005).

Although the increase in the body weight was used as an indicator of obesity, the study did not only focus on obesity but also determined whether this model exhibited other characteristics similar to those in obese humans. The study demonstrated that the glucose homeostasis was altered in animals fed a HF diet. The HF fed rats exhibited high glucose levels starting in the fourth week on the diet and throughout the entire study (Figures 3.5 and 3.6). The glucose levels between the two groups were statistically insignificant, and the oral glucose tolerance test (OGTT) would have determined if the rats were diabetic or not. However, studies done in our laboratories using the same animal model proved that the rats become glucose intolerant after eight weeks of high fat feeding through the OGTT.

Previous studies have shown that persistent hyperglycaemia is strongly associated with insulin resistance which is an important risk factor in development of obesity-induced T2D (Roche *et al*., 2006a). Other studies have shown that Wistar rats can develop glucose intolerance with long term feeding (32 weeks) of HF diet and that high fat diet and genetic predisposition is essential for development of T2D (Chalkley *et al*., 2002). The rat model used in this study has been reproducible over several experiments and displayed similar features to human obesity. These rats gained more weight after eating the HF diet, had increased glucose levels and adipose tissues when compared with the rats on the low fat diet (Figures 3.1, 3.5, 3.6 and Table 3.1). Therefore, this model proved to be appropriate for studies of factors responsible for transition from obesity to T2D that resembles the human disease condition and could be useful in attempts to decipher issues central to obesity development and progression to diabetes.

The hypothesis for this study was that the serum protein profile would be altered by high fat diet during induction of obesity. In order to test this hypothesis, serum was obtained from blood samples collected from lean and HF diet induced obese rats. Serum proteins were analyzed by 2D-PAGE coupled with MALDI-TOF MS. Since proteins are involved in most cellular processes, their cumulative expression profile is expected to reflect the specific activity of cells. Therefore, the use of a proteomics approach in this study is justified. Proteomics has the potential of describing the protein expression profile associated with a specific disease phenotype. Moreover, relatively few proteomics studies in human or animal models that investigated obesity and T2D individually have been published to date despite the defined target organs, such as the pancreas, liver and skeletal muscles in the case of diabetes (Sparre *et al*., 2005) and white adipose tissues for obesity (Coppack, 2005).

In recent years, serum proteomics has become increasingly important. This was driven by the hope of finding new molecular diagnostic biomarkers and potential therapeutic targets for specific diseases. Indeed, serum analysis measures homeostatic levels of metabolites throughout the organism that are reflective of the physiologic state of an organism (Schrader and Schulz-Knappe, 2001). However, few proteomics studies were conducted

using serum due to the presence of high abundant proteins (HAPs) that often obscure the detection of low abundant proteins (LAPs), and because of this challenge, many researchers opted to study tissues and cells because their protein content is evenly distributed (Kussmann *et al*., 2006). Particular tissues express specific proteins exclusively as compared to serum which contains proteins from all over the body, and this complicates serum proteome studies as illustrated in Figure 3.10A. Despite its complexity, serum has an important clinical value for the identification and detection of biomarkers. Blood perfuses through different tissues and therefore contains proteins whose levels are reflective of ongoing changes occurring within tissues in response to physiologic and pathological states (Schrader and Schulz-Knappe, 2001).

Therefore, the characterization of serum protein profiles will enable the discovery of i of reliable disease biomarkers. Serum proteome presents many beneficial attributes for proteomic investigation since it has high protein content (60-80 mg/ml), with albumin contributing 60% of the overall protein content (Tirumalai *et al*., 2003). Albumin together with eleven other highly concentrated proteins, *viz*: immunoglobulins (A, M and G), fibrinogen, transferrin, haptoglobin, alpha 2-macroglobulin, alpha 1-acid glycoprotein, alpha 1-antitrypsin and high density lipoproteins (Apo A-I and Apo A-II) constitute 95% mass of serum proteins. These HAPs interfere with the detection of the other LAPs and also proteins with low molecular weights (Roche *et al*., 2006b). These LAPs are made up of several classes of physiologically important proteins such as cytokines, chemokines, peptide hormones, as well as proteolytic fragments of larger proteins (Tirumalai *et al*., 2003). If the LAPs in a sample are of interests, it is essential to remove the HAPs first to facilitate visualization of LAPs for downstream proteomics analysis.

The present study established an optimized approach (adapted from Chen *et al.*, 2005) for depletion of HAPs through precipitation by TCA/acetone solution. The method removes at least 80% of albumin, allowing high sample load and concentrating proteins present at low concentrations and low molecular weight (Figures 3.12-3.14). Removal of albumin enhanced protein resolution for both 1- and 2-D gel electrophoresis and made it easier for detection of proteins that are around the same molecular weight as albumin. The number

of protein bands and spots identified in both the 1- and 2-D gels demonstrated the efficiency of the method for the removal of HAPs, particularly albumin, thereby enriching the detection of the other serum proteins present in small amounts as illustrated in Figures 3.12- 3.14. Both the precipitate (albumin-depleted) and supernatant (albumin-containing) samples were analysed to make sure that important proteins were not discarded because albumin is a carrier protein that can bind and transport small molecules and peptides within the circulatory system (Tirumalai *et al*., 2003).

Further, proteomic analysis revealed altered protein expression that was either induced or repressed in the HF group as compared to the LF group (Figures 3.17 and 3.18). Eleven spots were significantly dysregulated in the precipitate or albumin depleted sample and ten spots in the supernatant or albumin containing sample. However, only spots that showed expression difference of 2-fold lower or greater between the two groups were considered for further analysis. Two spots per sample were chosen for identification in the albumin-depleted and albumin-containing samples (Figures 3.15 and 3.16). The spots were chosen based on their resolution and quantities on the gels. The proteins were identified as C-reactive protein (CRP) and Fetuin-A in the pellet or albumin-depleted sample, while both the spots in the supernatant or albumin-containing sample were identified as Apolipoprotein A-IV. Fetuin-A, is also known as α_2-Heremans-Schmid glycoprotein (AHSG) or phosphoprotein of 63 kDa in rats (Olivier *et al*., 2000). Fetuin A and CRP were down-regulated whereas Apo A-IV was up-regulated in the HF group as illustrated in Figures 3.17 and 3.18.

The identities of these proteins were further confirmed by western blot analysis; and through western blot the different time points were compared to determine as to when the changes in protein expression started (Figures 3.25A, 3.26A and 3.27A). Densitometric analysis revealed that the alteration began at 8 weeks for both CRP and Fetuin-A as shown in Figures 3.25B and 3.26B, respectively; whereas Apo-AIV's expression increased from the 4th week on the diet (Figure 3.27B). Apo-AIV, as discussed below, is involved in lipid transportation and therefore is expected to increase as fat increases in the body. Therefore, there is a need to investigate whether the change in protein

expression is due to the development of the disease instead of the diet. The western blot results for Fetuin-A did not correlate with the 2-D gel analysis. The spot which was later identified as Fetuin-A was down-regulated in this study; this is because one dimensional electrophoresis was used for western blots. So instead of getting distinct spots as in two-dimensional electrophoresis, a single band with all the spots with different pI is obtained at the same molecular weight.

Fetuin-A is an abundant circulating blood protein that is produced mainly in the liver. It has diverse biological functions including regulation of calcium homeostasis and inhibition of insulin-induced autophosphorylation of the insulin receptor (IR) and IR-tyrosine kinase (TK) activity *in vitro*, *in vivo* and also in intact cells (Inoue *et al*., 2008; Mathews *et al*., 2002). Insulin stimulates tyrosine phosphorylation of the insulin receptor and insulin binding to the receptor; this activates a tyrosine kinase enzymatic activity inherent to the receptor (Ren and Davidoff, 2002). The phosphorylation action of this protein sets into motion a cascade of signaling events leading to the uptake of glucose into muscle and adipocytes. Failure to dispose of glucose leads to insulin resistance, which is central to the pathophysiology of T2D. At the cellular level, insulin resistance is characterized by IR down-regulation, reduced IR kinase activity and/or defects in the intracellular signaling responses to insulin. The Fetuin-A or *ASHG* gene in humans is located on chromosome 3q27 and has been identified as a susceptibility locus for T2D and metabolic syndrome. Genetic ablation of the gene in mice enhanced insulin signal transduction and increased whole-body insulin sensitivity and caused resistance to weight gain when fed a HF diet (Hennige *et al*., 2008; Olivier *et al*., 2000).

Apo A-IV is a 46 kDa glycoprotein in humans (Tso and Liu, 2004). It is exclusively secreted by the small intestine in humans and by both the small intestine and the liver in rodents (Liu *et al*., 2001). Apo A-IV is composed of three fractions: lipid-free Apo A-IV representing about 4% of total Apo A-IV, a fraction associated with Apo A-I (LpA-I:A-IV, 12%), an Apo A-I free, lipoprotein fraction (LpA-IV, 84%) when separated by a non-disruptive separation technique (Wong *et al*., 2007). Its synthesis in the small intestines is

stimulated by fat absorption, which might be mediated by chylomicron formation (Tso and Liu, 2004).

Apo A-IV is regarded as a satiety signal that controls the food intake and bodyweight. Apo A-IV plays a role in the catabolism of triglyceride-rich lipoproteins and in reverse cholesterol transport (Kronenberg *et al*., 2000; Strobl *et al*., 1993). *In vitro*, Apo A-IV has been shown to activate the enzymes lecithin: cholesterol acyl transferase thereby facilitating the action of cholesterol ester transfer protein, and modulates lipoprotein lipase activity. In addition to these classical roles in lipid metabolism, Apo A-IV possesses anti-atherogenic and anti-oxidant properties (Wong *et al*., 2007).

C-reactive protein is an acute-phase sensitive marker for systemic inflammation (Visser *et al*., 1999), infection and tissue damage (Pepys and Hirschfield, 2003). Accumulating evidence suggests that inflammation may play a crucial intermediary role in the pathogenesis of insulin resistance. CRP is used as a physiological marker for inflammation that is associated with, and overt T2D. Therefore, inflammation might play a role in development of T2D through its association with hyperglycaemia, insulin resistance, thereby linking diabetes with other inflammatory-induced diseases. CRP mediates the acute phase response derived *via* IL-6 dependent hepatic biosynthesis. Administration of human recombinant IL-6 in rodent models of glucose metabolism induces gluconeogenesis, increased glucose levels that is compensated by increased insulin secretion. Infusion of subcutaneous recombinant IL-6 in humans induces a similar metabolic responses observed in rodents (Pradhan *et al*., 2001). The physiological mechanisms linking elevated CRP to these disorders are still not clear, but their association is believed to be mediated by adipose tissue since it is an important source of circulating inflammatory cytokines (Greenfield *et al*., 2004). Unfortunately, CRP production is part of the nonspecific acute-phase response to many different forms of pathologies. Therefore, it can never be diagnostic on its own (Pepys and Hirschfield, 2003).

CRP, Fetuin-A and Apo A-IV have been implicated in the obese and diabetes state before, but all three proteins have not been linked in a single study before. Of interest, Fetuin-A and CRP expression was found to be increased in a number of studies and down-regulated in this study. This might be due to the fact that 2-D analysis is able to clearly resolve proteins with the same molecular weight but different pI, and it might be important to find out what kind of modifications occurred to these proteins. In a study done by Matsumura *et al* (2006), Fetuin-A's expression was increased in obese T2D model rats (OLETF rats) at 34 weeks of age when compared to their control (LETO rats). Serum was diluted 100X and then separated using a 2-D liquid chromatographic fractionation system; the system separates proteins based on their pI and hydrophobicity. Despite these observations, to our knowledge, there are no published data evaluating the relationship between Fetuin A, CRP and Apo A-IV, and the development of obesity and obesity-induced T2D using 2-D analysis coupled to MS at different time points. The closest to this study was the research done by Zhao and colleagues (2008), however it only determined differentially expressed serum proteins at the termination of their study; i.e., at eight weeks where the rats were already obese. In contrast, the present study evaluated expression starting at the baseline serum levels through the first eight weeks including the termination point to determine the exact time where expression started changing. This might be the time that calls for intervention and by using these proteins independently or together it might be possible to predict future risks for this disease among apparently healthy individuals or those susceptible to obesity development. These proteins, together with their genes, might be useful targets that can help in the prevention or inhibition of the development of obesity or progression to T2D or any disorder associated with obesity.

4.2 CONCLUSION

An animal model of HF diet induced obesity that developed T2D features common to human obesity when fed HF diet *ad libitum* was established. The study proved that environmental and dietary factors do influence experimental results such as glucose metabolism, tissue and body weights. In this regard, Wistar rats present an ideal

polygenic model for obesity studies. In this way, it might be possible to target most, if not all the complications associated with obesity at once rather than dealing with single components (T2D, insulin resistance) at a time. Serum proteomic analysis revealed proteins that had altered expression in rats fed a HF diet. Ultimately, these altered proteins may uncover dysregulated pathways that lead to the onset and progression of obesity and its associated diseases, and may also provide a starting point for the development of potential therapeutic and preventive measures and improved management of the T2D and obesity.

Further studies are required to determine the identities of the other 17 proteins whose expression were found to be statistically meaningful and to determine if these proteins are obesity specific proteins. Future work should also attempt to evaluate whether the change in protein concentration is not due to other factors such as fat and protein content in the diet; further fractionation of the pellet might be helpful in enhancing the low copy proteins that were not analyzed in this study.

The study was successful in developing a rat model of diet-induced obesity by feeding male Wistar rats a HF diet, the rat model closely simulated the development and abnormalities of obesity as it occurs in humans. Through proteomic analysis serum proteins that were differentially expressed were identified. These proteins might shed some light in the pathophysiology, development and disease mechanisms that result in obesity-induced T2D.

CHAPTER 5: REFERENCES

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Appendix

A1: Abstracts for Poster Presentations

1. **Dietary supplementation with *Aloe ferox* extracts reverses obesity in rats**

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Aloe ferox Miller, commonly known as the Cape Aloe is an endemic succulent plant native to South Africa and Lesotho. It has been used for both medicinal and cosmetic purposes e.g. wound healing, as a laxative and for the treatment of STIs. More recent pharmacological studies have shown aloe extracts to possess anti-tumour, anti-microbial, anti-inflammatory and anti-diabetic activity. Because of its reputed anti-diabetic activity, we decided to investigate its effect on obesity, the most important risk factor for Type 2 diabetes. Obesity is a multifactorial disease caused by gene and environmental interaction and imbalance in energy intake and expenditure. It is a risk factor for debilitating chronic diseases such as diabetes and cardivascular diseases. This study investigated the antiobesity effect of diet-supplemented *Aloe ferox* extracts in rats. Male Wistar rats were made obese by feeding a high fat diet, then assigned to three groups (n = 6 each): the HF-C, the HF-NS1 and HF-NS2 groups fed the HF diet, HF plus NS1 or NS2 aloe extracts, respectively. The NS2 extract significantly reduced body weight, fat pads and liver weight, and blood glucose levels compared to the control and HF-NS1. Although NS1 significantly lost body weight, paradoxically tissue weights and glucose levels were not significantly different from control rats. This data suggests that the NS2 extract has both antiobesity and antidiabetic properties. Further studies are needed to understand the mechanism by which NS2 induces weight loss in HF diet-induced obese rats.

- Presented at the 33rd Congress of the South African Association of Botanists, held at University of Cape Town from 14-18 January 2007.

2. **Serum proteome analysis of diet-induced obese rats**

Nicole Sibuyi[1,3], Abram Madiehe[1], Mmabatho Mobo[1], Bongani Ndimba[2], Xolisa Melamane[2], Amanda Skepu[1], Kwazi Gabuza[1], Leseilane Mampuru[3]

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Obesity is a chronic condition that arises when an individual's genetic make up is susceptible to an environment that promotes energy consumption over expenditure. However the mechanism that underlies the diet-induced obesity (DIO) remains elusive, hence this study was designed to identify serum proteins that are differentially expressed during obesity development. To accomplish this goal, an animal model of dietary-induced obesity was established to better understand the normal physiology and pathophysiology of DIO. Wistar rats were divided into two groups (n=7/group): one group was fed chow as a control and the other group a high fat diet to induce obesity. Body weight and food intakes were measured weekly. Blood samples were collected and glucose levels were measured in the first eight weeks and at termination. Serum was prepared by centrifugation and prepared for 2D-PAGE analysis coupled with MALDI-TOF mass spectrometry. Feeding a HF diet resulted in increased body weight gain, which was associated with altered glucose homeostasis. Several proteins showed significant differential expressions between the two groups, which was either suppressed or induced by the HF diet. The resulting serum proteomics data may constitute potential biomarkers for the diagnosis of predisposition to obesity due to environment.

❖ Presented at the 2nd SA Proteomics and genomics conference held at University of the Western Cape from 03-05 March 2008.

A2: PREPARATION OF SOLUTIONS AND REAGENTS REQUIRED

Protein Precipitation

Lysis (LB) buffer:

7 M Urea

2 M Thiourea

4% CHAPS

Protein quantification:

5 mg/ml stock bovine serum albumin (BSA) in lysis buffer

0.1 M HCl

Bradford dye reagent concentrate

SDS-PAGE:

2X Laemmli sample buffer:

4% SDS

10% 2-Mercaptoethanol

0.004% Bromophenol blue

20% Glycerol

0.125 M Tris-HCl, pH 6.8

SDS-PAGE Gels:

1.5 M Tris pH 8.8: Dissolve 90.86 g Tris in 400 ml distilled water (dH_2O), used HCl/NaOH to adjust pH to 8.8 and filled up to 500 ml. Stored at 4^oC.

0.5 M Tris pH 6.8: For 200 ml, mixed 66.6 ml of 1.5 M Tris pH 8.8 to 150 ml with dH_2O, adjusted pH to 6.8 with HCl/ NaOH then filled up to 200ml.

10% SDS: Dissolved 10 g in 100 ml dH_2O and stored at room temperature.

10% APS: Dissolved 1 g APS in 10 ml dH_2O and stored at 4^oC.

Table: Preparation of resolving and stacking gels.

COMPONENTS	AMOUNT REQUIRED (ml) RESOLVING GELS		STACKING GEL
	10% Acrylamide	12% Acrylamide	(5% Acrylamide)
dH_2O	7.15	6.4	3.6
40% Acrylamide/bis-acrylamide	3.75	4.5	0.625
0.5 M Tris pH 6.8	0	0	0.630
1.5 M Tris pH 8.8	3.8	3.8	0
10% SDS	0.15	0.15	0.05
10% APS	0.15	0.15	0.05
TEMED	0.006	0.006	0.005

10X SDS running buffer:

10 g SDS

30.3 g Tris

144.1 g Glycine (make to 1L with dH_2O)

Coomassie stain:

0.1% Coomassie brilliant blue R-250

40% Methanol

10% Acetic acid in dH_2O

Destaining solution:

40% Methanol

10% Acetic acid in dH_2O

2-D Equilibration buffer:

6 M Urea = 36 g

20% SDS = 10 ml (2%)

1.5 M Tris-HCl pH 8.8 = 3.3 ml (0.05 M)

50% Glycerol = 40 ml (20%), with a tinge of Bromophenol blue adjusted to 100 ml with dH_2O.

Western Blot Analysis

10X TBST/ Wash buffer:

250 mM Tris

1400 mM NaCl

30 mM KCl

0.5% Tween-20, adjusted pH to 7.4 then filled up to required volume with dH_2O.

10X Blotting / Transfer buffer:

0.25 M Tris – 30.28 g

1.92 M Glycine – 144 g

0.1% SDS – 1 g (optional). Make up to a litre with dH_2O, made in advance and refrigerated.

1X Blotting:

100 ml 10X blotting buffer

200 ml Methanol – make up to a litre with dH_2O.

Blocking buffer:

5% Carnation nonfat dry milk in TBST. The same buffer was used for preparation of the primary and the secondary antibodies as the **Antibody binding buffer**. (Caution: The buffer must be at 4°C for electrophoretic transfer).

Stripping Buffer:

100 mM 2-mercaptoethanol - 0.35 ml

2% SDS - 10 ml of 10% SDS

62. 5 mM Tris, pH 6.8 - 12.5 ml of 0.25 M Tris, pH 6.8 make up to 50 ml with dH_2O