Identification of differentially expressed proteins in obese rats fed different high fat diets using proteomics and bioinformatics approaches

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Abstract

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Background

Obesity is a medical condition in which an energy imbalance leads to excessive accumulation of body fat. Obesity leads to a reduction in life expectancy through its association with chronic diseases of lifestyle. The prevalence of obesity is rapidly increasing throughout the world. It is now accepted that most cases of obesity result from an interaction between genetic and environmental factors. This rapid increase in obesity generally leads to an increase in morbidity and mortality from chronic diseases such as cardiovascular disease, type 2 diabetes, osteoarthritis and cancer of which obesity is a risk factor. There is a lack of information in molecular research to explain how obesity predisposes individuals to these diseases. Proteomics is a molecular tool and a set of techniques used to identify changes at protein level from a diseased state. This study aims to identify differentially expressed proteins in serum of obese rats fed different isocaloric diets using proteomics.

Methods

Twenty eight rats were used in this study. Twenty one male Wistar rats were fed three isocaloric high fat diets, which are lard diet (L) (n=7), lard fat free diet (LF) (n=7) and butterfat diet (BF) (n=7) to induce obesity. A control group was fed maintenance diet (C) (n=7). The dietary composition for the diets was as follows: C (1.3% fat, 67% CHO, 25% protein); L (35% fat, 50% CHO, 10% proteins); LF (19% fat, 57% CHO, 19% protein) and BF (15% fat, 59% CHO, 21% protein). The rats were body weight matched and assigned to four groups. Food intake and body weights were measured weekly. Blood glucose was measured using glucometer and blood collected at 0, 4 days, 4 weeks and 8 weeks of assigning rats to different groups. The serum was prepared. The serum was subjected to 2D gel electrophoresis and PD Quest analysis. Spots of interest were identified using MALDI TOF. Western blot was used to verify the PD Quest results. A bioinformatics review of identified proteins was performed.

Results

Feeding high fat diets to rats resulted in a significant weight gain for L and LF groups when compared to the C group ($p < 0.001$). The BF group weighed significantly more compared to C group ($p < 0.05$). Nine spots from LF, seven spots from BF and eleven spots from L were differentially expressed compared to those from C as determined by PD Quest analysis. Of the differentially expressed spots, the up-regulated spots were six, four and four for L, LF and BF, respectively. Down-regulated spots were five, four and three for L, LF and BF respectively. Three upregulated spots were identified using MALDI TOF MS to be Apolipoprotein AIV, C-reactive protein and Hemopexin-like.

The down regulated spot was identified to be Alpha 1 macroglobulin-like protein. Using a reference map, the other two proteins were identified to be Fetuin A and Fetuin B. Western blotting verification of the findings confirmed the increased levels of C-reactive protein for the L, LF and BF groups compared to the C group ($p < 0.01$). Increased levels of Fetuin A and Fetuin B were also confirmed for L, LF, and BF compared to C ($p < 0.05$). Western blotting also confirmed the results on Apolipoprotein AIV with groups on high fat diets having significantly high levels compared to the C group ($p < 0.05$). A bioinformatics analysis using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway showed the C-reactive protein to be involved in the nitrogen metabolism pathway. Apolipoprotein AIV seems to be associated with the activation of the peroxisome proliferation activated receptor (PPAR) signalling pathway.

Conclusion

This study managed to show the proteome variation that is brought about by obesity development. Further studies are required to study the role of these proteins in obesity pathogenesis and their usefulness as potential diagnostic/prognostic targets. What this study managed to reveal is the responsive proteins during obesity development and these proteins may be potential markers of obesity when studied in depth.

Keywords: High fat diet, Diet induced obesity, 2D Gel, MALDI TOF MS, Proteomics, Candidate biomarkers, Wistar rats, Serum, Western blot, differentially expressed proteins

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PLAGIARISM DECLARATION

I **Kwazikwakhe Gabuza**, student number **2660249** declare that **Identification of differentially expressed proteins in obese rats fed different high fat diets using proteomics and bioinformatics approaches** is my own work and that all the sources I have quoted have been indicated and acknowledged by means of complete references.

Signature: ---------- Date: 10 May 2013

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List of abbreviations

µg	microgram
µl	micro litre
1D-SDS PAGE	One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis
2D DIGE	Two dimensional difference gel electrophoresis
2D-PAGE	Two dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis
A1M	Alpha 1 macroglobulin
AHA	American Heart Association
ANOVA	Analysis of varience
Apo AIV	Apolipoprotein AIV
APS	Ammonium per sulphate
ATP	Adenosine tryphosphate
ATP-III	Adult Treatment Panel III
BAT	Brown adipose tissue
BDNF	Brain-derived neurotrophic factor
BF	Butterfat diet
BMI	Body mass index
BP	Blood pressure
BSA	Bovine serum albumin

CAD	Coronary artery disease
CBB	Coomassie brilliant blue
C	Maintenance diet
CCD	Charge-coupled device
CHAPS	(3-[(3-cholamidopropyl)dimethylamonio]-1-propane sulfonate)
CHD	Coronary heart disease
CHOs	Carbohydrates
CNS	Central nervous system
CPE	Carboxypeptidase E
CRP	C-reactive protein
CVD	Cardiovascular disease
ddH_2O	Double distilled water
DHAES	Dehydroepiandrosterone Sulfate
ECL	Electrochemiluminescence
EDTA	Ethylenediamine tetraacetic acid
EE	Energy expenditure
EFRMD	Excessive fat related metabolic disorders
EGIR	European Group for the Study of Insulin Resistance
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FDA	Food and Drug Administration
Fe	Iron
FFA	Free fatty acid

g	grams
GC	Gas chromatography
GenPept	GenBank Gene Products Data Bank
GLC	Gas liquid chromatography
HBT	Butylated hydroxytoluene
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol
HOMA	Homeostasis Model Assessment
HRP	Horseradish peroxidase
HFD	High fat diet
Hrs	hours
HTN	Hypertension
IAA	Iodoacetamide
IDF	International Diabetes Federation
IEF	Isoelectric focusing
IGT	Impaired glucose tolerance
IL-6	Interleukin 6
IPG	Immobilized pH gradient
IT	Ion trap
kDa	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
kVh	kilo volt hour
L	Lard diet

LB	Lysis buffer
LDL	Low density lipoprotein cholesterol
LEP	Leptin
LEPR	leptin receptor
LF	Lard fat free diet
m/z	mass to charge ratio
MALDI	Matrix Assisted Laser Desorption/Ionization
MC4R	Melanocortin 4 receptor
ml	millilitres
mM	Millimoles
mmol/l	millimoles per litre
MS	Mass spectrometry
MWCO	Molecular weight cut off
N_2	Nitrogen
n-3	Omega 3
n-6	Omega 6
nAChR	Nicotine acetylchotine receptors
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NCEP	National Cholesterol Education Program
no.	number
NTRK2	Neurotrophic tyrosine kinase receptor type 2
OGTT	Oral glucose tolerance test

PAGE	Polyacylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor-1
PCR	Polymerase chain reaction
PCSK1	Proprotein convertase substilisin/kexin type 1
pH	Potential of hydrogen
p*I*	Isoelectric point
PIR-PSD	Protein Information Resource Protein Sequence Database
PMF	Peptide mass finger printing
PMP	Protein model portal
POMC	Proopiomelarocotin
PPAR	Peroxisome proliferation receptor
PUFA	Polyunsaturated fatty acids
PVDF	Polyvinyledene fluoride
Q	Quadrupole
QTL	Quantitative trait loci
RefSeq	Reference Sequence
SDS	Sodium dodecyl sulphate
SIM 1	Single-minded 1
T2D	Type 2 diabetes
TAG	Triacylglycerol
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween20
TCA	Trichloroacetic acid

TEMED	N,N,N',N'-tetramethylethylenediamine
TNFα	Tumour necrosis factor
TOF	Time of flight
TrEMBL	Translation from EMBL
UV	Ultra violet
V	voltage
v/v	volume per volume
VLDL	Very low density lipoprotein cholesterol
w/v	weight per volume
WAT	White adipose tissue
WB	Western blot
WC	Waste circumference
WHO	World Health Organization
WHR	Waist hip ratio

Chapter 1: Literature Review

1.1 Obesity: Prevalence, Diagnosis, Causes and Treatment

Obesity is a complex chronic disease that results from the interaction of multiple genetic, cultural, socioeconomic, behavioural, physiologic, metabolic, cellular and molecular influences. It took time for the full spectrum of diseases linked to obesity to become apparent and for centuries obesity has been known to cause serious chronic diseases. Obesity is associated with a high incidence of co morbidities, including cardiovascular disease, type 2 (adult onset) diabetes, osteoarthritis and increased risk for many forms of cancer (Strader *et al.*, 1998). Coronary heart disease or heart failure, osteoarthritis (knee), hyperuricaemia, gout complications and complications of pregnancy e.g. eclampsia are amongst moderately increased risk health consequences. Increased risk consequences associated with obesity are cancer, impaired fertility/polycystic ovary syndrome, low back pain, increased risk during anaesthesia and fatal defects arising from maternal obesity.

Obesity occurs as a result an energy imbalance which happens when energy intake is greater than energy expenditure. The prevalence of obesity is rapidly increasing throughout the world; this is mostly attributed to environmental factors with high caloric diets on top of the list of these factors (Naderali and Williams, 2003). This is also demonstrated in this study. Numerous factors influence body weight gain and obesity and these therefore can be used to determine people at risk of developing obesity and obesity-induced disorders.

There are critical periods during development of obesity, the times during people's lives when exposure to certain factors may increase their risk for the onset of obesity. The prenatal period, the adiposity rebound and adolescence are proposed to be critical points in childhood. Pregnancy and the immediate postpartum period have been proposed are critical periods for women in adulthood. There are other factors that can influence body weight, factors that an individual has no control over. These include developmental determinants, genetic makeup, gender and age (Dietz, 2001).

Alcohol, smoking and drug therapy also has a huge impact in obesity development. Positive impact by moderate alcohol consumption has been seen through the reduction of coronary artery disease (CAD). Half of the coronary artery disease afforded by moderate alcohol consumption occurs through an increase in HDL cholesterol. On the other hand excessive alcohol consumption can exacerbate hypertension and lead to stroke, liver disease, alcoholic cardiomyopathy, cardiac dysrythmias and it also aggravates hypertriglyceridemia, which can be a problem in persons with baseline levels >500mg/dL (Langer *et al.*, 1992). Alcohol intake may have an impact on body weight (Dietz, 2001). Excessive consumption could result in excess energy being converted to fat (Dietz, 2001). Compared to non alcoholic beverages drinking alcohol has been shown to be associated with a greater energy intake which can be due to increased appetite. Despite alcohol's beneficial effects on CAD alcohol, should not be routinely prescribed since the benefits may not outweigh the risks (Dietz, 2001).

Smoking and obesity are not completely understood regarding their relationship. An acute increase of energy expenditure (EE) and reduction of appetite by nicotine is likely to be an explanation why smokers tend to have lower body weight than non-smokers. Furthermore are observations in previous studies that show frequent body weight gain that follows smoking cessation (Chiolero *et al.,* 2008). Cigarette smoking increases metabolic rate and may limit food intake. A study by Stamford et. al., (1986) reported that smoking cessation leads to significant weight gain of which 96% of the weight is fat, increased caloric consumption which could account for 69% of the weight gained over 48 days and a rise in HDL-C levels (Stamford *et al.,* 1986). In the same study it was found that the participants that return to smoking return to baseline weight in 1 year. This study is followed by various studies that focus on nicotine. A recent study revealed that nicotine temporarily reduces insulin sensitivity by stimulating the secretion of tumor necrosis factor alpha (TNFα) and free fatty acids (FFAs), whereas the long term stimulation of nicotinic acetylcholine receptors (nAChRs) by nicotine, in addition to autonomic nervous stimulation, contributes to better insulin sensitivity *in vivo* through the modulatory secretion of the adipocytokines (Liu *et al.*, 2004)

There are various drugs or pharmacological agents that produce weight gain. Glucocorticoids, hypoglycemic agents, certain antihypertensive agents, anti-allergens and numerous drugs that affect the central nervous system have been said to impact on weight gain. Glucocorticoids might function peripherally to produce the observed genotypic differences in the onset of fat accumulation and elevation of plasma leptin, not only by favouring leptin production but also by inducing

repartitioning of lipid storage in specific fat pads (subcutaneous fat) and the liver, where gluconeogenesis is stimulated by corticosterone (Smart *et al.*, 2006). Hypoglycaemic agents are known to have side effects and the drugs that are known to lead to weight gain are sulfonylureas and this is counteracted by the use of the drugs in this class in combination with metformin (Zinman *et al.*, 2010).

Racette et al., (2003) reported the prevalence of obesity based on age which shows an increase with an increase in age (Racette *et al.*, 2003). Projections by Odgen et. al. based on the body mass index showed 10.4 % of children 2 to 5 years of age, 15.3% of children 6 to 11 years of age and 15.5% of children 12 to 19 years of age to be overweight (Ogden *et al.*, 2002). That explained the increase of diabetes of the young back in the years and current. This shows an increase in obesity with an increase in age. This study above focused on the situation in America, in contrast to the study reported the same year by (Puoane *et al.*, 2002) showed the situation to decrease with age in South Africa. There were high obesity cases that were going down with increase in age. This might be because of the change to the situation that is now predominant amongst South Africans whereby children spend more time indoors living passive life of television and computer games.

There is data on different ethnic groups that has been collected over the years focusing on obesity. National Health and Nutrition Examination Survey (NHANES) indicated the ethnic differences in prevalence of overweight and obesity. The 1999-

2000 NHANES data reported men 20 years of age and older with BMI≥25 prevalence to 67.4 percent for non-Hispanic whites, 60.7 percent for non-Hispanic blacks and 74.7 percent for Mexican Americans. In women 20 years of age and older the prevalence of overweight was 57.3 percent in non-Hispanic whites, 77.3 percent for non-Hispanic blacks and 71.9 percent in Mexican Americans. Puoane et al (2002) reported on the race related prevalence of obesity in South Africa. Four major ethnic groups are recognized in South Africa these include Africans, Coloureds, Indians and Whites. According to the study by Puoane and colleagues they reported the prevalence of obesity (BMI>30 kg/m^2) in South African black women at 31-34% and in white women at 18-24% (Puoane *et al.*, 2002). In the Indian and coloured groups, the prevalence in women is 20-22% and 26-28% respectively. The prevalence in black, Indian and coloured men are 8%, 3-9% and 6-9% respectively, with the highest in men occurring in whites (15-20%).

The prevalence of obesity is increasing in most African countries, particularly in individuals living in urban areas (Van Der Merwe and Pepper, 2006). There are varying results depending on the parameters used to identify obesity. The use of the body mass index (BMI) has been found not to work in some ethnic groups but those ethnic groups may be diagnosed as obese using the waist to hip ratio (Amoah, 2003). Taking ethnicity to consideration in studying obesity is crucial and these studies have been done in most countries. There are differences in response to environment X gene interaction based on ethnicity. Some ethnic groups tend to respond more than others to the environmental changes with different energy distribution accompanied by the differences in expenditure.

Extensive studies have been conducted for obesity in different fields that include epidemiology, nutrition, genetics, and many more focus areas with the introduction of proteomics which is still new. The role of genes in obesity has been studied in animals as it is expanded in the following sections. Obesity studies in humans showed that genes are not oriented the same way in humans as in animals which shifted the focus to gene polymorphisms which answered quite a number of questions.

1.1.1 Prevalence and diagnosis of obesity

The global obesity prevalence has been reported to have reached epidemic proportion for over a decade now. The increase in people that move from rural to urban areas has risen dramatically as developing countries are advancing to technology based era of robotics and computers. Currently 300 million people worldwide can be considered as obese, and due to the rising trend in obesity prevalence, this figure could double by year 2025 if no action is taken against this threat (Flick *et al.*, 1977b, Formiguera and Canton, 2004). The prevalence of obesity has been shown to be underestimated especially in Sub-Saharan Africa. The studies by Puoane et. al., 2004 and Parker et. al., 1997 do not reflect the true situation of the impact of obesity in South Africa. The organizations that deal with statistics on obesity have not been active enough in terms of keeping the updates of the obesity standings in South Africa which is evidenced by the lack of most recent data on obesity epidemic.

The awareness has been spread on the press releases regarding the obesity state in South Africa. The figures below shows the published cases of obesity state in South Africa which is spreading in all age groups.

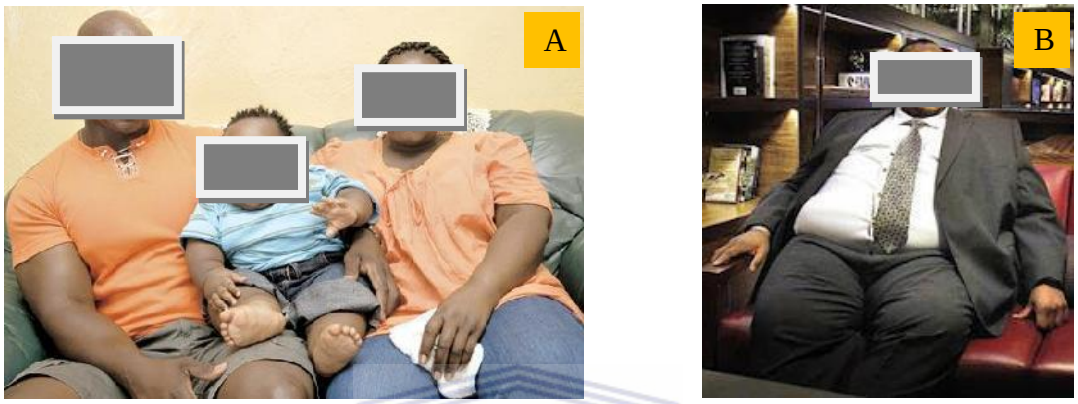


Figure 1.1: Images showing cases of obesity in South Africa as published by the press

The figure represents the cases as appeared on the newspaper where a toddler of 14 months of age was reported to weigh as much as 31kg (Mahlangu, 2012) (A). An obese adult (B) was shown in a different article (Laurence, 2012).

In the United States of America, 133.6 million (66%) adults are overweight or obese with 63.3 million (31.4%) that are considered being obese (Cannon and Kumar, 2009b). This means US as country is facing more than 90% cases of weight problems. Prevalence data in the United States of America is always available unlike in other countries but that can be due to the availability of the resources to run the studies towards the prevalence of obesity.

Body mass index (BMI) is commonly used for obesity diagnosis. BMI is the ratio of the weight (in kilograms) divided by height (in metres) squared: BMI = kg/m^2 (Rippe *et al.*, 1998). BMI is accepted as a better estimate of body fatness and health risk than body weight. There is a need to clarify that BMI is not a measure of body fatness but obese people tend to have excess body fat. BMI in overweight range is less healthy for most people, but in some cases may be acceptable for people who are muscular and have less fat. The BMI of >25 kg/m^2 is said to be for overweight individuals while the BMI of >30 kg/m^2 is considered to be obese (Leonhardt *et al.*, 1990). The World Health Organization classification of obesity is tabulated below to show more categories (James, 2004).

Table 1.1: WHO classification of obesity

Classification	BMI	Risk of comorbidities
Underweight	18.5	low*
Normal range	18.5-24.9	Average
Overweight	≥25	
Pre-obese	25.0-29.9	Increased
Obese class 1	30.0-34.9	Moderate
Obese class 2	35.0-39.9	Severe
Obese class3	40.0	Very severe

*increased risk of other clinical problems increased

Waist hip ratio (WHR) has been used as a predictor of obesity in some studies. The waist hip ratio is an index of body fat distribution which has been shown to be a predictor of coronary heart disease. It appears to be a measure of central obesity (Sosenko *et al.*, 1990). The recommendations of using waist circumference cut points of 40 inches (102cm) in men and 35 inches (88cm) in women to define central obesity has been used successfully to define the risk association (Beydoun and Wang, 2008). BMI is still crucial in epidemiological and clinical diagnosis of obesity and the WC is used to identify abdominal obesity.

1.1.2 Genetic and environmental causes of obesity

Obesity development has been associated with a number of factors. The obesity research has evolved to focus to factors leading to obesity. In most cases it is the combination of a lot which is now get presented as genetics x environment. This shows a bigger picture but the physiological changes go deeper to understanding what takes place during the change from a lean to an obese state. The excessive formation of adipose tissue that is due to excessive differentiation of adipocytes and storage of energy as fat is the most noted cause. The body homeostasis is the implicated process in the body weight gain because with proper energy balance obesity is least expected. Various organs contribute to energy homeostasis. A lot of processes take place in different organs as a way of controlling energy in the body. This takes place through energy storage or energy utilization. The organs that contribute to energy homeostasis are shown in (Figure 1.2).

There is an established communication of organs with the central nervous system especially the hypothalamus for the energy homeostasis. Satiety signals that are produced from gastrointestinal tract in response to the presence of food include gut peptide cholecystokinin, bombesin, gastrin-releasing peptide, neuromedin B and glucagon (Gale and Grant, 2004). Insulin and leptin modulate immediate peripheral satiety signals. These hormones directly target the central nervous system and inhibit food intake (Flick *et al.*, 1977a, Gale *et al.*, 2004). The figure shows that there are receptors in the central nervous system for the hormones and peptides produced peripherally which concludes the communication between peripheral organs with CNS. The breakdown in the communication may lead to a defect in the control of energy leading to the kick start of insulin resistance which leads to obesity and related conditions.

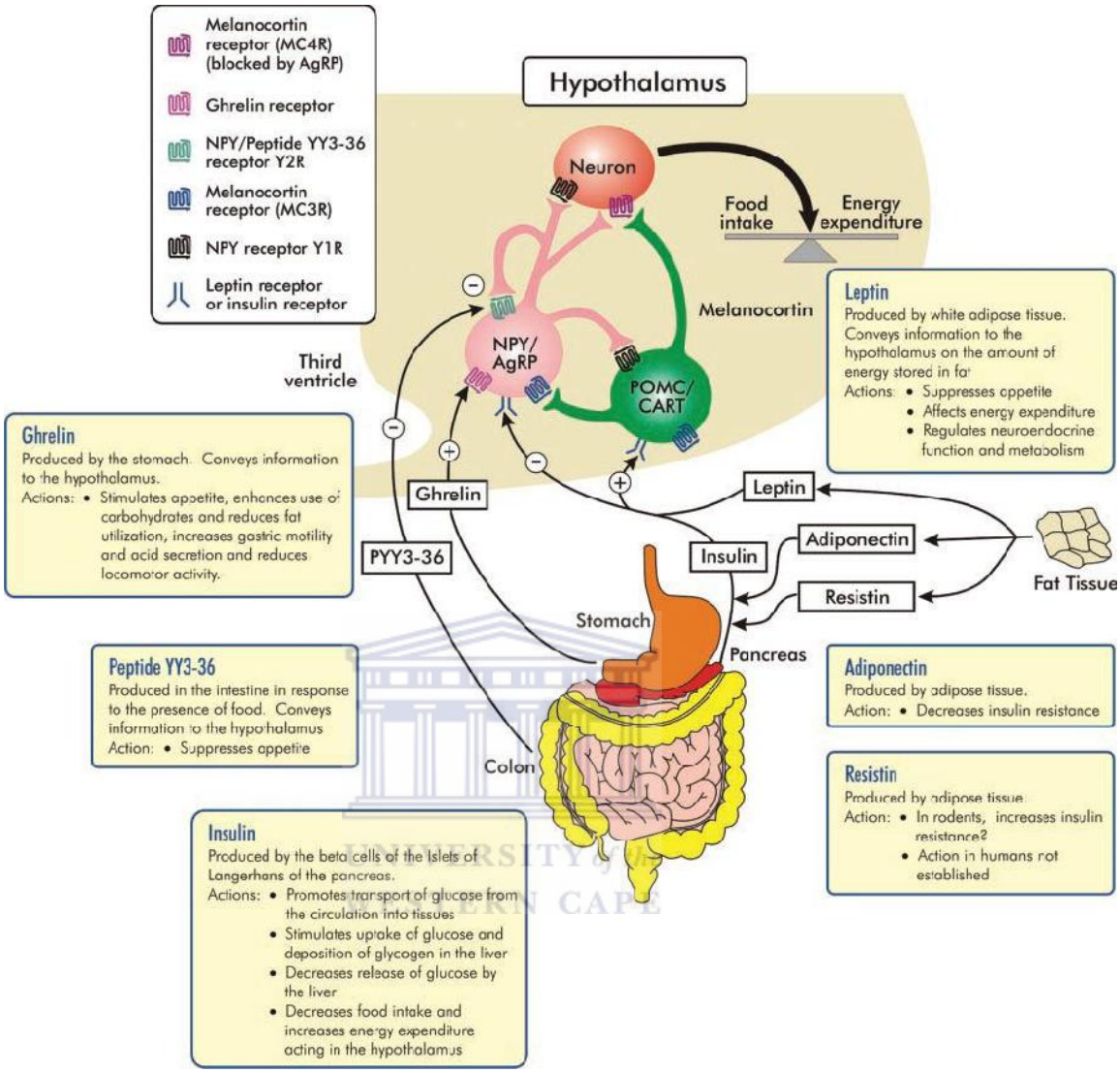


Figure 1.2: Various organs that play a major role in energy homeostasis (Gale et al., 2004)

The impact of diet to the development of obesity is very much noted. The definition of obesity as a condition that results from energy imbalance is based on dietary control. A challenge is to every individual to match the energy intake with the energy expenditure to overcome diet induced obesity. The abundance of calorie-dense and aggressive food marketing in mass media, supermarkets and restaurants, the large portions of food served outside the home, promoted high calorie consumption (Montague, 2003). This contributes to the increasing prevalence of obesity because besides exceeding the recommended limit of energy intake people do less physical activity to offset their consumption.

The content of food also contributes to the determination of energy that is consumed. Research in both humans and animals suggests that high fat diets that are low in complex carbohydrates promote obesity (Astrup and Finer, 2000). Fat contributes 9kcal/g compared to 4kcal/g for proteins and carbohydrates (CHO) leading to high energy intake from eating high fat foods than would be when eating a similar quantity of lower fat foods. Fat modifies the taste of food and in some people, it promotes excessive intake. This is because of the weaker satiation effect from dietary fat than CHOs, resulting in the over consumption of fat (Rolls and Rowe, 1979). Compared with dietary fat, CHOs require additional energy expenditure for digestion, assimilation and conversion to fat. Fat consumed need not be digested because of the state that they are at but only a little energy is needed for their storage. This means low energy in CHOs is also used for conversion and as a result the stored energy gets reduced than would have been for fat.

Carbohydrate intake contributes to the overall obesity development. There are postulates around the use of high protein and low carbohydrates in the diet. Increased calorie intake as CHOs has adverse physiological effects like increased insulin secretion, promotion of fat deposition and rise in serum triglycerides levels. Diet with low CHO can lead to a ketogenic state which has been hypothetically related to appetite suppression. Diet high in protein preserves lean body mass during weight loss. Protein compared to CHOs and fats is having highest thermogenic effect resulting in increased energy expenditure for a similar intake. A controversy has been raised by the role of CHO in soft drinks in producing obesity. French et al., (2000) suggest the increase in the consumption of soft drinks may contribute to the increased prevalence of obesity.

Dietary fats are the most water free source of energy for the body and are composed of 98% triglycerides which supply approximately two-thirds of the cells total energy requirement (Montague, 2011). Saturated fats have been proven to affect blood cholesterol levels. Saturated fats that raise cholesterol include lauric, myristic and palmitic acids which are found in butterfat, meat fat and vegetable oils that harden at room temperature e.g. coconut, and palm kernel oils (Stone, 1996). Monounsaturated fats also raise blood cholesterol, although not as potently as do saturated fats and they may lower HDL cholesterol (Mensink *et al.*, 1990). Polyunsaturated fatty acids (PUFA) lower serum cholesterol (Grundy, 1990). Two kinds of PUFA are n-6 PUFA and n-3 PUFA with sources of n-6 PUFA being seed oils such as corn, safflower, and cottonseed which are rich in linoleic acid. The n-3 PUFA include fish oil and linoleic acid, a plant source.

Food processing has seen more refining of food and the reduction of the required roughage. This is looked at as an improvement by the food industry. Food that is eaten by most people in developed world lacks fiber. Fiber is crucial portion of every diet because of the role that it plays in the process of digestion. Insoluble fiber contributes by promoting regular bowel movements and soluble fiber helps lower cholesterol (Kwiterovich, 1995). Rimm et al. (1996) reported that increased fiber is associated with reduction in coronary artery disease (Rimm *et al.*, 1996). That study further revealed an inverse association between total fiber intake (mainly vegetables, fruit, and cereal) and the risk of fatal and non-fatal myocardial infarction that was independent of fat intake (Rimm *et al.*, 1996).

Social aspects of life influences development of obesity. In South Africa there is an increased rate of social gatherings where people eat more food of animal origin compared to that of the plant origin. Restaurants also sell more meat than vegetables as their core business. Fast food outlets are found in most corners of South Africa as she continues to develop. Such a social behavior has long been reported in the US which is the leading country in obesity where Morgan and Goungetas, (1986) reported an increased snack consumption. The turnover in the work place setup has seen more physical work being done by robotics and personnel sit the whole day in front of computers. This alone gives a situation whereby the energy expenditure has been reduced as the intake of energy increases. The more urbanized African communities get the higher the rate of obesity and the less prudent their diets become (Kruger *et al.*, 2005, Puoane *et al.*, 2002).

Obesity has been studied based on biological, psychological and behavioural factors. Looking at the factors as mentioned above does not fully explain the rapid growth in obesity cases that has been witnessed in the past decades. There is growing agreement among researchers and policy makers that the social and physical environment may play an important role as well (Poortinga, 2006). Environments may be obesogenic in the sense that they promote caloric intake and/or discourage the expenditure of energy. The study in US showed that there was an increase in usage of vehicle through reports on an increase in miles of transportation done (Samimi *et al.*, 2009). In this study it was concluded that a 1% decrease in the use of automobiles can decrease obesity by 0.4%. Residents of neighbourhood with high population density, proximity to commercial destinations and good public transportation are more physically active than residents of less walkable neighbourhoods often deemed "surbuban" (Frank *et al.*, 2007).

To make up for the environment impact on obesity development changing an environment from obesogenic environment to an environment that promote energy expenditure is required. Studies have proven that positive energy balance results in accumulation of excess fat stored in the form of fat. The study that was conducted in Australia showed that to support the extensive survey that was conducted in USA, which found that the pervasive availability of vehicles, combined with heavy advertising and urban and suburban designs that support the vehicles as the most convenient transportation mode, is an important environmental influence on physical activity levels (Hinde and Dixon, 2005).

Obesity genetics has been studied for decades in both animals and humans (Fawcett & Barroso, 2010). Studies on obesity managed to reveal a number of genes associated with obesity to date (Harrera *et al.,* 2011). In animals the mouse has been widely studied genetically for obesity. There are five widely used monogenic mouse models of human obesity; those are diabetes, obese, yellow, tubby and fat (Naggert *et al.*, 1997). Mice homozygous for the obese mutation displayed features of hyperphagia leading to obesity and diabetes, inappropriately decreased energy expenditure, reproduction deficiency and stunted growth. The product of cloning obese gene was a protein leptin which itself has been studied extensively. Mice carrying mutation at diabetes locus exhibit a very similar phenotype to obese mice that studies suggested diabetes to be the receptor for leptin in the central nervous system (CNS). This has been found to be the lack of the long form of leptin receptor for the diabetes mouse leading to defects in the signalling across the blood brain barrier. Dominant alleles (yellow) at the agouti locus lead to a complex phenotype which includes maturity onset obesity, pigmentation defects, insulin resistance and an increased frequency of tumors. Fat mice carry an autosomal recessive mutation and display a range of abnormalities, including progressive adult onset obesity, hyperinsulinaemia and infertility. The mutant allele of fat showed to be a missense (serine→proline) mutation in carboxypeptidase E (CPE). The tubby mouse has an autosomal recessive mutation and displays a tripartite phenotype of blindness, deafness and maturity onset obesity. These mice increase their intake gradually in response in proportion. The mutations that were seen on mice do not reflect what is happening in humans.

The genetics of obesity has been studied in both humans and animals, which added to the body of knowledge the rare monogenic forms of obesity and polymorphisms. The uses of the information on genes showed to be dependent on the permissive environment for the genes to be triggered and kick start the weight gain process. Based on the previous studies available information on human genetics needs to be acknowledged.

1.1.2.1 Diet as an environmental factor for studying obesity in rats

Rodents are a major tool to study conditions affecting humans due to the great similarity and homology between the genomes of rodents and humans (Von Diemen, 2006). Outbred rat strains, which include Sprague Dowley and Wistar rat, are popular strains to study obesity as they readily gain weight on high fat diets (Gadja, 2008). Induction of obesity using diets depends on many factors including but not limited to susceptibility of the rats to obesity development (Choi, et. al., 2010). Susceptibility can be towards the sources of calories in the diet used e.g. high carbohydrate or high fat diet. The high fat diets that are mostly used for laboratory animal research contain more unsaturated fats such as lard, beef tallow or coconut oil and these diets are quite capable of inducing obesity in susceptible strains (Gadja, 2008).

1.1.3 Behavioural, pharmacological and surgical treatment of obesity

Treatment of obesity has been given attention in many ways. There are various approaches that are being used towards the treatment of obesity. The association of obesity with energy imbalance has been revealed more than two decades ago. Sliming

has been one of the approaches to the prevention of excess energy intake. The treatment of obesity can be grouped into categories; those are behavioural, pharmacotherapy and surgical interventions.

The successful treatment of obesity is marked by a preferential reduction of abdominal fat accompanied by amelioration of obesity related health risks, an improvement in quality of life and a reduction in mortality rate (Cannon and Kumar, 2009b). It is important to note that in most cases there is a higher failure to lose weight for the people that already gained excess weight. For a less ambitious approach goals can be set to reducing body weight and maintain lower body weight over a long time. To make things bearable for the people that cannot lose weight their goal can be to prevent further weight gain.

To date there is no medical treatment that promises to be successful in treatment of obesity. While the treatment of obesity aims to achieve best results with less harm to the patients none of the treatment strategies works without challenges. Behaviour based treatment fails because of the response of the patients that is dominated by a thought of obesity as a lifestyle than disease making them to have low self esteem and reduced eager to respond to the disease. The required change that is expected by patients when joining the behavioural change adds to their loss of faith in the therapy. The pharmacotherapy came with lots of negativity to practitioners because of the side effects involved. Most of the drugs used for obesity in the past were withdrawn because of life threatening side effects. The invasiveness of the surgical therapy makes a huge percentage of the obese community not to consider the treatment. Each

treatment will be discussed briefly in the following sections.

1.1.3.1 Behavioural therapy of obesity

Behavioural therapy is a method for systematically changing eating, exercise, or other behaviours that are thought to contribute to or maintains obesity (Foreyt and Poston, 1998). Behaviour therapy comprises self-monitoring (i.e. keeping food and activity logs), stimulus control (i.e. controlling cues associated with eating), stress management, nutrition education, slower eating habits, problem solving, the rewarding of changes in behaviour, cognitive restructuring, social support, physical activity and relapse prevention (Cannon and Kumar, 2009a). In the behavioural treatment what has been of note in most studies is that one must not assign high odds of success. Many researchers that did research on obesity treatment dating over two decades ago until the present are humbled by obesity in that they realised that it is difficult to maintain a new lower weight following a weight loss (Brownell, 2010).

The success of weight loss treatment depends on the structure, principles and techniques used with the patient (Corbalán *et al.*, 2007). Incorporation of psychological well being to interventions needs to be part of every patient's program not just a mere weight loss. Individuals with improved self esteem and body dissatisfaction have a higher likelihood of finishing among the successful weight loss group, to an extent independently of their initial weight loss (Palmeira *et al.*, 2010). A typical behavioural program, incorporating a 1200 kcal/day diet,

produces a weight loss of about 10% of initial body weight amongst the patients who complete the treatment (Cooper and Fairburn, 2001). The study by Corbalan et. al., (2009) raised more parameters that contribute to failure of behavioural therapy. In conclusion this study points out to the therapist ways to improve behavioural programs by considering that losing motivation to lose weight, being prone to stress related eating, being predisposed to eating when bored and thinking in black-and-white are some of the barriers for losing weight. Also emphasized is the encouragement of patients to record food intake and attending to group therapy.

1.1.3.2 Pharmacotherapy of obesity

Drugs that have been on the market prior to the 21st century include Dinitrophenol which is having thermogenic action resulting from uncoupling of oxidation phosphorylation from ATP formation. This drug was withdrawn from the market in the 1930s due to death cases from multi-system side effects (Finer, 2002). Synthesis of amphetamines followed the discovery of ephedrine from Chinese plant *Ephedra sinica*. These drugs were used as stimulants but it became apparent that they also suppress appetite and food intake. Related drugs were produced those are phentermine, diethylpropion and mazindol all three acting by blocking re-uptake of noradrenaline in hypothalamus neurones. Being stimulants some of the drugs with longer half life they interfere with sleep and cause unpleasant state of arousal. All three drugs were withdrawn in Europe but still available in other continents. Fenfluramine emerged in the 1960s with dexfenfluramine in the 1980s.

Pharmacotherapy in obesity led to discovery of a number of drugs that are partially successful but their challenge lies in the complexity of obesity itself. Many attempts have been made to correct metabolic disparity of the obesity condition, producing a number of reagents including Sibutramine (appetite suppressor), Orlistat (gastrointestinal lipid uptake inhibitor), and Fibrates (PPARα agonists) (Choi *et al.*, 2007). Orlistat and sibutramine were still on the market worldwide, until thesuspension of the sibutramine‟s marketing authorizations by the European Medicines Agency in January 2010 due to concerns that it could lead to increased risk of developing heart problems (Hsu *et al.*, 2010). The research in pharmacotherapy is perpetually producing drugs and many of the drugs are already in phase III of clinical trials. Remonabant, a drug that was developed to treat obesity with mechanisms of action involving cannabinoid receptors in the central nervous system and peripheral tissues (e.g. adipocytes), failed to win FDA approval and was recently withdrawn from the market in Europe because of its serious psychiatric adverse effects (Cannon and Kumar, 2009a).

1.1.3.3 Surgical therapy of obesity

Surgical treatment of obesity or bariatric surgery is a treatment option for morbidly obese patients with BMI ≥ 40kg/m^2 or a BMI ≥ 35 kg/m^2 and significant obesity-related comorbidities (Robertson, 2006). Procedures are categorised as restrictive or malabsorptive. Restrictive surgery aims to reduce the volume eaten at each meal, but does not alter the route ingested food takes through the gut e.g. gastric banding and vertical band gastroplasty. Malabsorptive surgery will usually include a restrictive

element but also includes surgical modification of the gastrointestinal tract to reduce nutrient absorption, normally bypassing part of the small intestine. Gastric bypass and duodenal switch are examples of malabsorptive surgery. Bariatric surgery is a highly effective treatment approach that results in a reduction in BMI of 10 to 15 kg/m^2 and a mean weight loss of 20 to 40 kg (Cannon and Kumar, 2009a).

Currently, surgery offers the only viable treatment option with long-term weight loss and maintenance for the morbidly obese (Miller, 2004). Surgical treatment of obesity is proving to be the best and most effective means of preventing the life-threatening complications and serious degenerative problems associated with morbid obesity. It is always advised that the patients that go for bariatric surgery must be ready for perioperation and postoperation. It is reported that bariatric surgery contributes to the loss of fat mass with a much lower loss of fat free mass (Frige *et al.*, 2009). In addition to that there is a reduction in visceral fat more than subcutaneous fat. This further improves glucose metabolism as it lowers blood glucose levels, insulin levels, insulin resistance (HOMA index) and reduces prevalence of type 2 diabetes mellitus and prevent its occurrence in obese subjects with impaired glucose tolerance (IGT) (Buchwald *et al.*, 2004, Pontiroli *et al.*, 2005). The need for the patient‟s preparedness contributes the most because that helps in standing the trauma of surgery and playing along in terms of behaviour change for the effectiveness of the procedure.

1.2 Obesity as a risk factor for chronic diseases of lifestyle

Many diseases have been found to be associated with obesity. The development of obesity has been associated with a variety of conditions including cancer, diabetes, metabolic syndrome, sleep apnoea and other conditions that continue to be associated with obesity. As obesity has long been defined as the accumulation of body fat the association of obesity with fatty liver has been increasing for researchers and the people in general. The accumulation of body fat has been associated with influx of fat in the body that leads to the development of adipose depots in all the organs of the body besides the adipose tissue. This is the increasing reason for the people to see the fat intake as the main cause of the abnormality and uncontrollable situation for those exposed to high fat diet and high caloric intake of any kind.

The prevalence of obesity-related health problems increases proportional as the BMI and the duration of obesity increase. It is always advisable for the people that suffer from co morbidities to reduce their body weight as a first step to resolve weight related problems. Obesity is thought to be the leading risk factor for osteoarthritis of the knee. As morbid obesity usually requires surgery many of the comorbidities interfere with the anaesthesia. What have been of concern to anaesthetists are diabetes and those diseases that are affecting the respiratory and cardiovascular systems.

Much co morbidities interrelate with each other as it is reported that the combination of gastric hyperacidity and an increased gastric volume with sleep apnoea, snoring and impaired upper airway reactivity, give rise to nocturnal micro aspiration. This combination precipitates lower airway reactivity and symptoms of asthma.

Comorbidities touch most of the medical issues orthopaedic, neurology, pulmonary, gastrointestinal, gynaecology, endocrine, cardiovascular, psychology, skin, metabolic, infectious, and oncology. Through this there is a chance of misdiagnosis and underestimation of obesity to date.

1.2.1 Obesity as a risk factor for impaired glucose regulation

Impaired glucose tolerance (IGT) and impaired fasting glycaemia (IFG) refer to the levels of blood glucose concentration above the normal range but below those which are diagnostic for diabetes (WHO, 1999). The two conditions are different in such a way that they refer to the inability to bring glucose levels to normal post-prandially and the other one refers to elevated fasting glucose levels in a fasting state. The values of IFG are fasting plasma glucose concentration of 6.1 mmol/l or greater; whole blood 5.6 mmol/l, but less than 7.0 mmol/l. If oral glucose tolerance test (OGTT) is performed, some individuals with IFG will have IGT or diabetes, but this cannot be determined without an OGTT. Previous studies, which have been carried out mainly in middle-aged people, have shown, although not quite consistently, that the deterioration in glucose tolerance is associated with at least positive family history of diabetes, overall obesity, central distribution of body fat, lack of physical exercise, and elevated systolic or diastolic pressure (Hiltunen *et al.*, 1997).

Studies also revealed the impaired regulation to be associated with brain atrophy and neuropsychological deficit which were found in preliminary studies comparing obese type 2 diabetic adolescents with obese non-diabetic adolescents (Messier *et al.*,

2011). The link between cognition and glucoregulation appears to occur in a continuum from impaired glucose tolerance to type 2 diabetes. Hyperinsulinaemia as well as high fasting glucose in non-diabetic older people are also associated with decreased cognitive function (Yau *et al.*, 2010, Rolandsson *et al.*, 2008). Impaired glucose regulation is central to most of the metabolic conditions because it starts with impaired regulation to conditions like diabetes and its complications that usually get to be controlled in very painful ways e.g. amputations.

1.2.2 Obesity as a risk factor for diabetes

Diabetes is one of the most costly and burdensome diseases in the world with increasing epidemic proportions worldwide. Diabetes mellitus represents a group of diseases of heterogeneous aetiology, characterized by chronic hyperglycaemia and other metabolic abnormalities, which are due to deficiency of insulin effect (Kuzuya *et al.*, 2002). Diabetes is the leading cause of renal failure in many populations in both developed and developing countries. Lower limb amputations are at least 10 times more common in people with diabetes than in non-diabetic individuals in developed countries; more than half of all non-traumatic lower limb amputations are due to diabetes (WHO, 2011).

The relationship between obesity and diabetes has long been raised in the research community. The challenge in the two states is that not all obese people are diabetic and not all diabetics are obese either. Obesity has been proven to lead to insulin resistance, insulin resistance leading to impaired glucose regulation that eventually

results to type 2 diabetes mellitus (T2DM).

1.2.3 Obesity as a risk factor for Coronary Heart Diseases and Hypertension

Heart attacks have been a concern due to the rise in number of people dying of heart attacks. Coronary heart disease (CHD) is a narrowing of the small blood vessels that supply blood and oxygen to the heart. Coronary artery disease is the other term for CHD. Atherosclerosis which occurs as the fatty material and other substances form a plaque build up on the walls of the arteries. This causes the narrowing of the coronary arteries leading to a slow down to a stop of the blood flow to the heart. People with CHD can experience chest pain (stable angina), shortness of breath, heart attack and other symptoms usually for active individuals.

Coronary heart diseases have been found to be influenced by various factors. These include dietary fat, smoking, alcohol consumption and lack of exercise (Talmud, 2007). Low density lipoprotein (LDL) particles promote atherosclerosis by entering the artery wall, becoming oxidized and subsequently being ingested by macrophages, to create rich foam cells, which develop to atherosclerotic plaques (Cromwell, 2007). Weight loss, even of 5-10% of total body weight has beneficial effects regarding coronary heart disease risk factors; total cholesterol, LDL cholesterol and triglycerides levels are decreased HDL cholesterol levels are increased, blood pressure levels are reduced and glucose metabolism is improved (Kastorini *et al.*, 2010). Mechanism by which cigarette smoking exerts its cardiovascular damaging effect is not clearly delineated (Chrysant, 2011). The most plausible mechanisms

include, lipid oxidation, inflammation and thrombosis, with lipid oxidation being the most dominant (Ambrose and Barua, 2004). Heavy alcohol consumption has been related to increased risk of cardiovascular disease, whereas moderate alcohol intake has been associated with a cardio-protective effect (Burazeri and Kark). Based on the above the American Heart Association (AHA) recommends that if alcoholic beverages are consumed, they should be limited to no more than 2 drinks per day for men and 1 drink per day for women, ideally with meals (Bhupathiraju and Tucker).

Hypertension (HTN) is a multi-factorial, polygenic disease that involves complex interactions between genetically determined homeostatic control mechanisms and environmental factors (Lerman *et al.*, 2005). The diagnosis of HTN is based on multiple conventional blood pressure (BP) measurements taken on separate occasions, in the sitting position by the use of mercury sphygmomanometer or another calibrated device and is universally defined as a systolic BP of 140 mmHg or higher, a systolic BP of 90 mmHg or higher, or both (Fagard). Approximately 95% of hypertensive patients have essential or primary HTN, which is a multi-factorial disease, resulting from an interaction between genetic and lifestyle/environment factors. These include being overweight, high salt intake, excessive alcohol consumption and physical inactivity.

Obesity and systemic arterial hypertension are two conditions that have been directly related. Thus, the incidence of hypertension in the obese population is greater than that in a non obese population (Ventura *et al.*, 1992). Visceral obesity promotes an increase in the availability of free fatty acids, potentially mediated through the

hydrolysis of stored adipocyte triglyceride by a range of lipases, including triglyceride lipase, lipoprotein lipase, hormone-sensitive lipase and endothelial lipase in adipose tissue (Chapman and Sposito, 2008). The accumulation of circulating free fatty acids lead to triglyceride accumulation in muscle and liver (in the form of hepatic steatosis) and to hyperglyceridaemia subsequent to enhanced hepatic production of VLDL. The rise in blood pressure may also be mediated by enhanced release of free fatty acids from the adipose tissue of obese patients in various mechanisms. These include the activation of α1-adrenoceptor-mediated vasoconstriction, attenuation of endothelial production of NO with induction of oxidative stress and inhibition of insulin-mediated vasodilation (Egan *et al.*, 2001, Sarafidis and Bakris, 2006).

1.2.4 Obesity as a risk factor for sleep apnea

Sleep apnea is a prevalent disorder characterized by loud snoring and daytime sleepiness. Fat infiltration to the upper airway has been shown to be related to the prevalence of obstructive sleep apnea, and impairment of the ventilatory drive, to central sleep apnea (Ramadan *et al.*, 2006). Repetitive cessation of airflow associated with arousal and hypoxemia has been seen in sleep apnea, further more there is an association of sleep apnea with morbidity and mortality from stroke and heart diseases (Li *et al.*, 2010). Studies have been done to establish the association of obesity and disorders like insulin resistance and impaired control of breathing (Ramadan *et al.*, 2006). Currently there is a difficulty in distinguishing the respective role of fat infiltration in the upper airway and impairment of ventilatory drive in

generation of sleep apnea because the disorder is diagnosed late in its course (Mehra and Redline, 2008).

Because of frequent co aggregation of obesity and sleep apnea it is often difficult to discern which health or pathophysiological effects are attributable to one or the other condition, and which effects may reflect additive or synergistic effects (Li et. al., 2010). Obesity has been stated to be a pro-inflammatory disorder for a long time which is something that has been reported for sleep apnea due to hypoxia/hyperoxia and sleep fragmentation which oftentimes cause or exacerbates pro-inflammatory states on sympathetic hyper reactivity or oxidative stress. Common pathways for both conditions include adipose tissue production of pro-inflammatory cytokines and chemokines such as TNF-α, IL-6, and leptin with reduction in adiponectin levels. Animal studies especially rodents demonstrated that background levels of obesity influence metabolic responses to intermittent hypoxemia. In humans pro-inflammatory responses to sleep apnea are also influenced by obesity and conversely obesity related effects on inflammation and cardiopulmonary disease are influenced by coexisting sleep apnea.

1.3 Metabolic syndrome

Obesity is a key component of metabolic syndrome, which is characterized by increased visceral adipose tissue, peripheral insulin resistance, hyperlipidemia and elevated blood pressure (Yan *et al.,* 2007). When high blood pressure, dyslipidaemia and insulin resistance present together, and are in turn associated with hyperglycemia

and visceral obesity, they are collectively identified as the metabolic syndrome (Chapman and Sposito, 2008). Indeed this syndrome is characterized by a risk factor constellation that predisposes patients to the accelerated development of atherosclerosis and CVD (Gami *et al.*, 2007). Table 4 shows the guidelines from the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) to define the metabolic syndrome on the basis of a combination of 3 or more key criteria among a series of 5 (Expert Panel on Detection and Adults, 2001).

Table1. 2: Features of commonly used definitions of metabolic syndrome (Expert Panel on Detection and Adults, 2001)

Defining level				
	WHO (WHO consultation, 1999). Impaired glucose tolerance glucose tolerance or diabetes and/ or insulin resistance and two other factors	EGIR (Balkau and Charles, 1999). Presence of fasting hyperinsulinemia (highest 25%) and two factors	ATP-III (NECP Expert Panel on Detection, 2001). Three or more of the following factors (TAGs and HDL counted separately)	IDF, Central obesity and two other factors
Central obesity	WHR ≥ 0.9 (men), 0.85 (women) and/ or BMI > 30 kg/m^2	Waist ≥ 94 cm (men), ≥ 80 cm (women)	Waist ≥ 102 cm (men), ≥ 88 cm (women)	Waist > 94 cm (European men), > 90 cm (Asian men), > 80 cm (women)
Blood pressure (mmHg)	≥ 140/90	≥ 140/90 or treated for hypertension	> 130/85 or treated for hypertension	≥ 130 SBP or ≥ 85 DBP or treated for hypertension
Dyslipidemia (mmol/l)	TAGs ≥ 1.7, HDL < 0.9 (men), < 1.0 (women)	TAG ≥ 2.0 or HDL-cholesterol < 1.0 or treated for dyslipidemia	TAGs ≥ 1.7, HDL-cholesterol: < 1.0 (men), < 1.3 (women)	TAGs ≥ 1.7, HDL-cholesterol, < 1.04 (men), < 1.29 (women)
Dysglycaemia (mmol/l)	Fasting glucose ≥ 1.6 and/ or 2h post-challenge glucose ≥ 7.8 on diabetes	Fasting plasma glucose > 1.6, but non-diabetes	Fasting plasma glucose ≥ 6.1	Fasting glucose ≥ 5.6 or previous diagnosis of impaired tolerance or diabetes
Insulin resistance	Glucose uptake during hyperinsulinemic-euglycaemic clamp in lowest quartile for population	Presence of fasting hyperinsulinaemia (i.e. among the highest 25% of the non-diabetic population)	Not applicable	Not applicable
Other factors	WHO (WHO consultation, 1999). Impaired glucose tolerance or diabetes and/ or insulin resistance and other factors	EGIR (Balkau and Charles, 1999). Presence of fasting hyperinsulinemia (highest 25%) and two other factors	Microalbuminuria (urinary albumin excretion > 20 µg/min or albumin/ creatinine ratio > 30 mg/g	None

1.3.1 Metabolic syndrome and controversy associated with the syndrome

There has been a differing of ideas regarding metabolic syndrome. The definitions of metabolic syndrome can differ as shown in table showing the definitions from ATPIII and WHO. Although including similar parameters but the cut off points are not the same. A review into metabolic syndrome highlighted a concern on fundamental, clinically important and critically missing information about the metabolic syndrome to warrant a more serious examination of whether medical science is doing any good by drawing attention to and labelling millions of people with a presumed disease that does not stand on firm ground (Kahn *et al.*, 2005). It is critical to mention that mostly the syndromes will never be put at the forefront because it is not the entire syndrome that gets identified as morbid but the individual risk factors and that causes a lot of debate around recognition of the syndromes and metabolic syndrome is not an exception.

Metabolic syndrome is a concept that has been slowly proving to be useful based on the studies that showed the likeliness of predicting the future development of cardiovascular disease/ diabetes (Aguilar-Salinas *et al.*, 2009). This comes from a series of definitions that has been used by various organizations to explain the syndrome. The initial definition of the syndrome was given by Dr Reaven in the Banting lecture that was published in 1998 where he postulated that insulin resistance and its compensatory insulinemia predisposed patients to hypertension, hyperlipidaemia and diabetes and thus was the underlying cause of much CVD referring to the condition as insulin resistance syndrome (Kahn *et al.*, 2005). The

improvement in a quest to utilize the concept towards prediction and prevention of morbid outcomes has graduated it to metabolic syndrome (MetS) (Bruce and Byrne, 2009). To date there is still a debate as to who is likely to develop the syndrome and there is still a lack of the tailor made diagnostic criteria for certain people since variations have been found to be dependent on parameters like race, age, ethnicity, etc.

1.3.2 Obesity as a component of the metabolic syndrome

The recognition of abdominal obesity as the most prevalent clinically measurable entity of the metabolic syndrome is one key advance of the concept (Després *et al.*, 2008). In the 21st century the research has been focusing on the lifestyle diseases and the concern that is brought by the clustering of diseases. The then known as the adult onset diabetes has been noticed in children of late. Obesity, which is the most common cause of insulin resistance in children is also associated with dyslipidemia, type 2 diabetes and long term vascular complications (Weiss *et al.*, 2004). This might explain the rise in obesity prevalence in children and the rise in type 2 diabetes in adolescents and children.

The association of obesity with metabolic syndrome is being shown diagrammatically in Figure 1.2 illustrating the possible correlates (A) of insulin resistance often found among individuals with excess visceral/ectopic fat. Panel B emphasizes the notion that the syndrome X/insulin resistance syndrome concept was based on pathophysiological considerations, whereas panel C highlights the fact that NCEP

ATP III and IDF metabolic syndrome is an entity identified by the presence of simple screening tools (Després *et al.*, 2008). Weight loss has been proven to improve the insulin resistance which is the component of the metabolic syndrome with diet and physical activity still at the forefront in achieving weight loss. The availability of food guides like the DASH diet and the US Department of Agriculture MyPyramid are designed to integrate sound dietary recommendations into a healthful way to eat for life (Hollander and Mechanick, 2008).

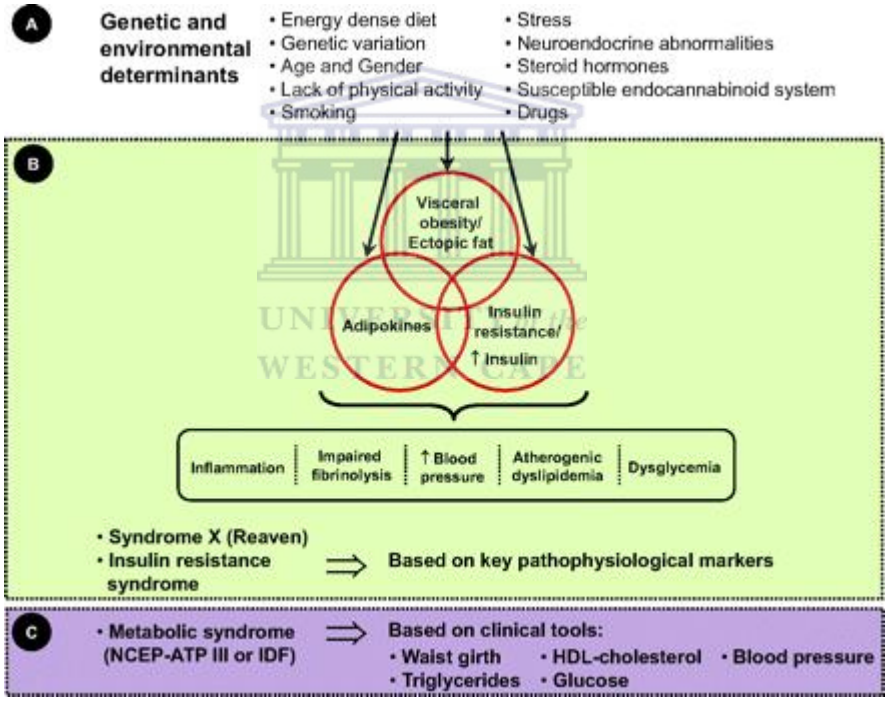


Figure 1.3: Simplified Metabolic Syndrome model taken from (Després et al., 2008)

1.3.3 Adipocytokines associated with metabolic syndrome

Identification of metabolic syndrome association with obesity has brought into light the increase in adipokines in the body system. Adipose tissue secretes a range of

peptide and protein hormones named adipokines implicated in the regulation of energy metabolism, and it is also a great source of inflammatory cytokines that participate in obesity related metabolism dysregulation (Roca-Rivada *et al.*, 2011). Such adipokines include adiponectin, leptin, resistin, tumour necrosis factor (TNF)-α, plasminogen activator inhibitor-1 (PAI-1) and interleukin (IL)-6 (Rolland *et al.*, 2011). Adiponectin increases fatty acid oxidation and reduces the synthesis of glucose in the liver. Ablation of the adiponectin gene has no dramatic effect on knock-out on a normal diet, but when places on a high-fat/sucrose diet they develop severe insulin resistance and exhibit lipid accumulation in muscles (Lago *et al.*, 2007b). This may explain the reduction in adiponectin levels for morbidly obese individuals (Oh *et al.*, 2007).

Leptin suppresses food intake and increases energy expenditure, decreasing in fasting state and increasing after eating (Hallikainen *et al.*, 2007). Adipokines, which are directly produced by adipocytes or adipose tissue macrophages, induce a low-grade chronic inflammatory state that could play a central role in obesity related cardiovascular complications and insulin resistance (Antuna-Puente *et al.*, 2008). It is already raised and agreed that the adipose tissue have a cross talk with other organs and this interaction is leading to energy regulation. The defect in the adipocytokine production can be dangerous to the body through failure in the communication of adipose tissue to the relevant organs for homeostasis resulting in states like insulin resistance.

1.4 Adiposopathy

Adiposopathy ("sick fat") is defined as pathogenic adipose tissue that is promoted by positive caloric balance, increased energy storage and sedentary life cycle in genetically and environmentally susceptible individuals that may directly promote CVD and may cause or worsen metabolic disease (Bays, 2011). The term adiposopathy arose from decades of adipose tissue scientific research and acknowledgement of the importance of central adiposity by major scientific organizations. The theory that white adipose tissue (WAT) could be an active contributor to the whole-body homeostasis rather than just a fat depot became tangible with the discovery of leptin in 1994 (Lago *et al.*, 2007a). Since then a lot of adipokines have been identified that are adipose tissue secreted adipokines as shown in table 1 with the risks associated with them.

Obesity alone does not always result in clinical metabolic disease, and not all patients with these metabolic diseases are overweight (Bays *et al.*, 2005). Since the identification of adipose tissue as an endocrine organ, the defects in the adipose tissue have been associated with a lot of disorders including metabolic disorders. During the positive caloric balance adiposopathy may be promoted by impaired adipogenesis, pathogenic fat distribution, impaired nutrient metabolism, abnormal adipose tissue factor activity, increased inflammation and dysfunctional „cross-talk" or pathological interactions with other body organ systems (Bays *et al.*, 2006). The changes at the adopokine level require the in depth analysis of molecules involved. Proteomics as used in our study maybe the approach that can identify not only the changes during the obesity development but it may also reveal the adiposopathy related changes

which will be useful in intervening on time before worse metabolic diseases kick start.

1.4.1 Adiposopathy diagnosis

As explained above adiposopathy is sick fat in simple terms. The fat pathogenesis is not dependent on the amount of fat. Currently there is no diagnosis for adiposopathy because there is currently no guarantee that the physical parameters that are used for diagnosing obesity for example can be used to predict the well being of the fat tissue. Just as there is a great variance in functionality and dysfunctionality of adipose tissue, further to that there is a variance in distribution of body fat among populations, and adipose tissue function and distribution are related (Bays *et al.*, 2005).

1.4.2 Major and minor criteria used to identify adiposopathy

The complications in the metabolic disorders research is due to the clustering of factors that contribute to deadly conditions in a long run. Adiposopathy has been identified to be exacerbated by fat accumulation (adiposity) and sedentary lifestyle in genetically susceptible individuals (Bays and Dujovne, 2006). The argument is raised by the increase of people that get excessive fat related metabolic disorders (EFRMD) beyond addressing adiposity and obesity. This gives an indication that the state of the adipose tissue itself needs to be taken into consideration as Bay, Abate, et al., 2005 mentioned that adipose tissue functioning needs to be looked at as well (Bays, 2005). Adiposopathy can be identified taking into consideration a lot of criteria because unlike fat accumulation and obesity but sick fat can be identified with more than just

physical parameters. Major criteria consist mostly of major parameter and the minor criteria are mostly the blood chemistry which is not usually looked at as shown in Table 1.3. The in depth details on criteria won't be touched in this thesis because the table is touching on the proposed idea. Adiposopathy concept is not fully accepted as yet but it will be able to shed light into why the people respond differently into diet as the time goes. Proteomics will be an enhancer also for the adiposopathy concept but this will be seen on the outcomes of the studies in diets and response like the current study.

Table 1.3: Proposed diagnostic criteria for the diagnosis of adiposopathy (Bays et al., 2005)

Major Criteria	Minor Criteria
1. Adiposity • Body mass index (BMI) ≥ 30kg/m^2 or • Waist circumference > 102cm (>40in) in men, or > 88cm (35in) in women or • Waist:hip ratio > 0.9 in men, or > 0.85 in women 2. Abnormalities in glucose metabolism • Fasting glucose blood levels ≥ 100mg/dl (6.0mmol/l) or • 1h post oral 75g glucose load blood glucose level ≥ 140mg/dl (7.8mmol/l) or	1. Microalbuminuria • Urinary albumin excretion rate > 20µg/min or • Albumin:creatinine ratio ≥ 30mg/g 2. Onset of androgenemia in women; especially with signs and symptoms of polycystic ovarian syndrome • Elevated total testosterone or DHAES in women, and possibly an increase in prolactin levels 3. Hepatosteatosis • Otherwise unexplained elevated hepatic

- Use of anti-diabetes drug treatment in Type 2 diabetes

3. Hypertension
    - Systolic blood pressure ≥ 130mm/Hg or diastolic blood pressure ≥ 140mm/Hg or
    - Use of antihypertensive drug treatment for high blood pressure
4. Dyslipidemia
    - Fasting triglycerides level > 150mg/dl (>1.7mmol/l) or
    - Fasting HDL-C level < 40mg/dl (<1.0mmol/l) in men or < 50mg/dl (< 1.3mmol/l) in women or
    - Use of lipid-altering drug treatment of high triglycerides or low HDL-C levels

transaminases, possibly with hepatic imaging revealing findings consistent with fatty liver

4. Hypercoagulable state
    - Otherwise unexplained thrombotic clinical event in patients with elevated BMI, with possibly an increase in plasminogen activator inhibitor (PAI-I)
5. Abnormalities of lipoprotein particle size and subclass distribution
    - Decreased LDL particle size with increased prevalence of “pattern B”
6. Hormone abnormalities of fat cell dysfunction
    - Elevated fasting insulin levels, and or decreased adiponectin levels or
    - Elevated insulin/leptin to adiponectin ratio

7. Metabolic markers of fat cell dysfunction
    - Elevated fasting or postprandial free fatty acid levels
8. Inflammatory markers of fat cell dysfunction
    - Elevated cytokine production (tumor necrosis factor and/or interleukin-6), with elevated C-reactive protein

1.4.3 Adiposopathy treatment

The use of therapies that treat individual pathologic components that collectively constitute adiposopathy can aid in improving the functioning of the adiposopathic fat cells and tissue thereof (Bays *et al.*, 2006). Weight loss through dietary interventions, regular physical exercise and some pharmacologic agents can improve fat function. PPAR gamma agonists have been said to increase the recruitment of new functional and healthy fat, causing apoptosis of "sick fat" or dysfunctional fat, and increasing the capacity of existing fat. Antidiabetes drugs may also improve glucose metabolism and thus improve the hyperglycaemia consequences of adiposopathy (Bays, et al., 2005). Current antiobesity agents such as orlistat and sibutramine have both been shown to reduce body weight and also improve many metabolic abnormalities of glucose and lipid metabolism that are otherwise associated with increased CHD risk (Bays., 2004).

Bariatric surgery is also said to improve the adipose tissue endocrine and immune response which is due to reduction in adipocyte size and a reduction in visceral adiposity (Bays, 2009). Weight loss seems to be showing positive results that are in line with adipocyte lifecycle hypothesis. The hypothesis states that the metabolic properties of an adipocyte vary predictably during its lifecycle. As an adipocyte matures, it accumulates triacylglycerol and becomes larger; that the rates of triacylglycerol synthesis and lipolysis are matched within adipocyte and that larger adipocyte, in general, have greater rates of triacylglycerol synthesis and concurrently, greater rates of lipolysis and therefore, larger adipocytes have greater rates of

transmembrane fatty acid flux, and that secretion of cytokines can also be related to adipocyte size with larger adipocytes having a more unfavourable profile of cytokine secretion than smaller adipocytes (Smith et al., 2006). The hypothesis points out the defects that takes place in the adipocytes and adipose tissue that need to be corrected and hence the focus of current therapy of obesity that aims at not just losing weight but also to rectify the adipose tissue functioning. Adiposopathy treatment is not officially accepted as the concept is still getting introduced.

1.4.4 Research on adiposopathy and the future possibility of considering it as a disease

The function of adipose tissue as an endocrine organ is proven by many studies. A crosstalk between the organs is now being studied to further understand the problem that leads to the increase in metabolic diseases. Adipose tissue is already spotted to be the target for novel therapies (Klein *et al.*, 2006). The adipose tissue performs three main functions which are the secretion of adipocyte-derived hormones (adipokines), energy dissipation/thermogenesis, and energy storage. The organs that have been given attention when it comes to metabolic disease have been organs either than adipose tissue. There is a mounting evidence that adiposopathy plays a role in metabolic disease. An emerging concept in treatment of obesity states that the development of antiobesity agents must not only reduce fat mass (adiposity) but also correct fat dysfunction (adiposopathy) (Bays, 2004, Scheen, 2008).

The adipose tissue produces adipokines that perform functions in the body and the

disturbance in the functionality of such adipokines may cause a problem to the body. It is known that the blood flow in gluteal adipose tissue is much lower than that in the abdominal adipose tissue (Tan *et al.*, 2004). Adiposopathy acknowledges that when positive caloric balance leads to adipocyte hypertrophy and visceral adiposity, then this may lead to pathogenic adipose tissue metabolic and immune response that promote metabolic disease (Bays *et al.*, 2008). This then try to breach the gap that is created by the lack of explanation of why some obese people are metabolically healthy? This can be related to the fat tissue being able to perform all the functions listed above to the necessary expectation by the body. Because research is on in various research avenues on adipose tissue the answers that arise from the research will enhance the chances of accepting adiposopathy as a disease. Facts are slowly piling up pointing at the contribution of sick fat in metabolic diseases which will see adiposopathy reducing late intervention because of the criteria that is currently used of using parameters like BMI to determine the need for treatment.

1.5 Advances in obesity research through genomics, proteomics and bioinformatics

Genomics is the study of all the genes and their functions. The pharmaceutical industry embraced genomics as a source of drug targets for decades now. There has been well established source of genomic information that came from long started studies of genes. The human genome project is the study that is influenced by the information that has been obtained from single gene studies. There has been a strong feeling about the break through that will come with full gene mapping leading to the completion of human genome project in 2003.

The success in genomics led to the birth of the other field that focused on studying all the proteins that are a result of the genome called proteomics. Proteomics promises to be the answer to the challenges that comes from the changes at protein level that shapes the phenotype of most of the organisms. The powerful and robust approach in proteomics has seen the evolution of proteomics from gel based to gel free proteomics and the discovery of the stains that are useful in identifying the post translational modifications which are role players in the changing of the phenotype. Both proteomics and genomics generate a lot of data in a short space of time and the storage of data and the utilization of such data will need proper and centralized method for easy accessibility of such data to anyone in need of such data.

Bioinformatics has proven to be a powerful tool in data storage, acquisition, and utilization towards addressing the problems encountered by the research community. The information that is available through search engines and databases currently covers most of the known information and the body of knowledge is not on a stand still. The use of bioinformatics in research saves time that would be required for an individual to search for the information that is available. When information is placed in a central location it becomes easier for research community to access. The obtained information remains to be utilized in a beneficial manner to address the problems encountered.

1.5.1 Application of genomics in obesity research

Genomics contributed a lot in the understanding of obesity. In 2004 the overall genes, markers and chromosomal regions that were associated or linked with human obesity phenotype were >600 (Perusse *et al.*, 2005). These were evidence from single gene mutation obesity cases, Mendelian disorders exhibiting obesity, quantitative trait loci (QTLs) from animal cross breeding experiments, association studies with candidate genes and linkages from genome scans. The focus in leptin-melanocortin pathway as a target for pharmacological intervention in patients with severe obesity turned out to be effective. The best illustration is certainly the case of a child with congenital leptin deficiency who was treated with subcutaneous injections of recombinant human leptin, leading to the correction of all the phenotypic abnormalities seen in this patient (Choquet and Meyre, 2010).

Single-gene defects causing obesity are rare in humans, with affected individuals suffering uncontrollable hunger and develop severe obesity at a young age (Wilding, 2003). The research in single genes managed to identify the situations associated with genes in order to be able to prioritise the gene that might be contributing to obesity (Figure 1.2). In the figure the condition that is associated with a defect in the gene which is usually due to mutation in that particular gene is listed. This knowledge of such conditions makes things easier for clinical applications of accumulated knowledge in genomics in prioritizing the gene that need to be sequenced first based on the properties seen in the patient. The monogenic obesity cases are rarely taking place with the majority of obesity cases being polygenic. Polygenic obesity is

commonly taking place in humans and this is challenging because it forms a cluster of conditions that are difficult to address. While acknowledging the contribution of genomics proteomics emerged to push the research a step further and the details on proteomics impact will be discussed in the next section.

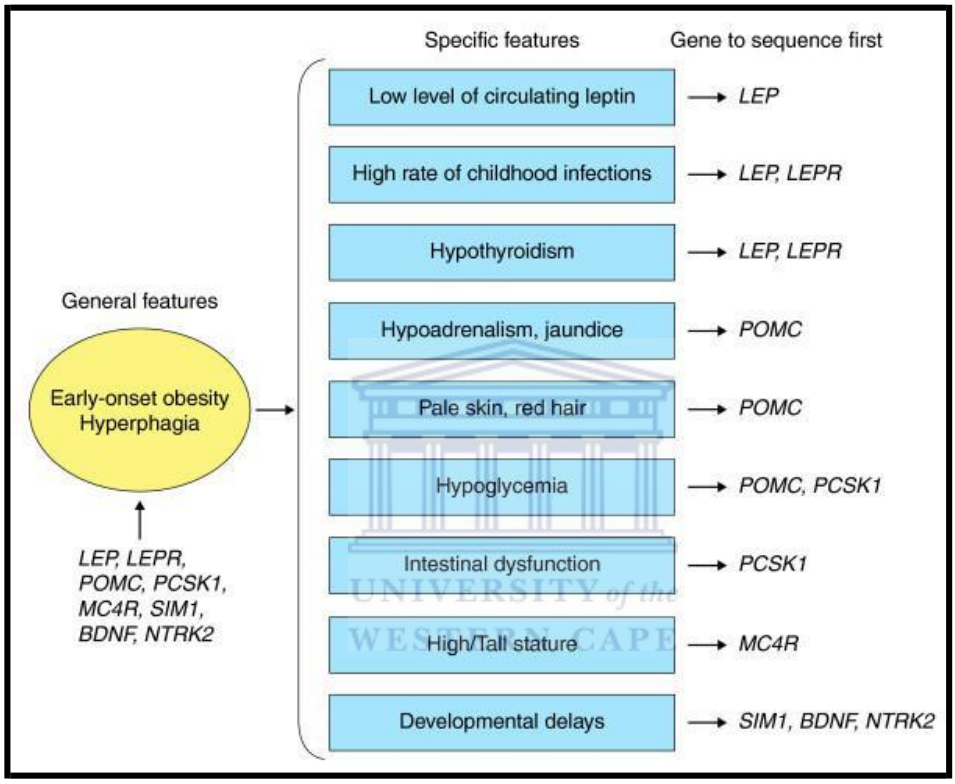


Table 1.4: Figure 1.4: Monogenic mutations associated with obesity (Wilding, 2003)

The figure shows the conditions and the single genes that are associated with their alterations. The defect in leptin and leptin receptor genes (*LEP* and *LEPR*) are associated with low leptin circulation, the defects may cause the reduction in circulating leptin or the reduction in effectiveness of leptin when the receptors are not fully functional. These effects can lead to development of obesity at childhood and hypothyroidism. *POMC gene* monogenic defect can lead to hypoadrenalism,

jaundice, pale skin, red hair and hypoglycaemia. On the other hand the hypoglycaemia and/or intestinal dysfunction can be the indication of monogenic effect of *PCSK1*. The development to high/tall stature indicates the defect in the MC4R gene in contrast to delays in development that is seen in most cases when there are monogenic defects associated with *SIM 1*, *BDNF* and/or *NTRK2*.

1.5.2 Application of proteomics in obesity research

1.5.2.1 Proteomics

Proteomics is defined as the analysis of the proteome, which is a complement of proteins in cells, tissue, organs, and physiological fluids as well as their interactions (Wang *et al.*, 2011). Proteomics research embraces two contrasting but complementary strategies, those are cell-mapping proteomics, which aims to define protein-protein interactions to build a picture of the complex networks that constitute intracellular signalling pathways and the second strategy, is protein expression which is based on the monitoring of global expression of large numbers proteins within a cell type or tissue, and quantitatively identifies how patterns of expression change in different circumstances (Simpson 2001). In addition to the above mentioned strategies there is also a need to know the structures of proteins in order to understand more and that is achieved using the so called structural proteomics which is the third proteomics strategy.

Proteomics employs 2D-PAGE and various chromatography techniques for separation of proteins (2) and soft ionization techniques, matrix-assisted laser

desorption ionization (MALDI) and electro-spray ionization to vaporize peptides and proteins enabling their analysis using mass spectrometry (MS) (1). Separation of ionized peptides or proteins in the high vacuum of the MS is based on the differences in their mass-to-charge (m/z) ratios, with time-of-flight (TOF) and electric ion trap as major mass-selective analyzers in MALDI and ESI instruments, respectively (Wang, 2006). The approach of the current study was based on expression proteomics using the gel protein separation and MALDI MS for analysis; therefore the following sections of this literature review will give details on the approaches as employed in this study.

1.5.2.2 The use of 2D PAGE for separation of proteins

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) separates thousands of proteins within a sample by their isoelectric points (pI) in the first dimension and their molecular weight in the second (Wu *et al.*, 2009). This method was introduced in 1975 by O Farrell to separate hundreds of cellular proteins under denaturing conditions (Issaq and Veenstra, 2008a). First dimension separation evolved from being performed in plastic or glass tubes where by the protein was run in the capillary tube and removed after running for second dimension as gel rods. An introduction of immobilized pH gradients (IPGs) (Bjellqvist *et al.*, 1982) improved the resolution and the reproducibility which was not there before the introduction of IPGs. Narrow IPG strips allowed a larger number of proteins to be separated than had been possible with standard 2D PAGE because a narrower pH range was spread out over a greater physical distance (Hoving *et al.*, 2000).

Advantage of using 2D PAGE is in the resolving many proteins at a go. What is a challenge is the between gel variation that makes it difficult to compare the protein from two different gels. The introduction of two dimensional difference gel electrophoresis (2D-DIGE) overcame the limitations of 2D PAGE by allowing up to three distinct protein mixtures to be separated within a single 2D PAGE (Unlu *et al.*, 1997). The 2D DIGE samples are tagged with dyes that bind to proteins prior to running them on a 2D PAGE. The staining and visualization of proteins are covered in the following section.

1.5.2.3 Detection of proteins in a 2D PAGE

Detection of proteins from the 2D PAGE is achieved using various staining methods with the most popular being Coomassie brilliant blue (CBB). CBB methods found widespread use for its low cost, ease of use and compatibility with most subsequent protein analysis and characterization methods such as MS (Gorg, 2007). Silver staining also gained popularity because of its sensitivity (in very low range), because it can be achieved with simple and cheap laboratory reagents, and because it does not require complicated and expensive hardware for the readout (Chevallet *et al.*, 2006). The uses of aldehyde-based fixatives/sensitizers are the most sensitive ones, but prevent subsequent protein analysis like MS due to protein cross-linkage (Westermeier and Marouga, 2005). Omission of aldehyde in fixatives and in the subsequent gel impregnating buffers (except in the developer), microchemical characterization by peptide mass fingerprinting (PMF) is possible, however at the

expense of sensitivity (Gorg, 2007).

Fluorescent-based protein detection methods have recently surpassed conventional technologies such as colloidal Coomassie blue and silver staining in terms of quantitative accuracy, detection sensitivity and compatibility with modern downstream protein identification and characterization procedures such as MS (Wayne F, 2002). Two major approaches for the fluorescent detections of proteins are used currently, there are i) covalent derivatization of proteins with fluorophores prior to IEF, and ii) post-electrophoretic protein staining by intercalation of fluorophores into the SDS micelles coating the proteins, or direct electrostatic interaction with the proteins (Gorg, 2007). Cyanine based dies are an example of pre-electrophoretic fluorescent labels that react with lysyl residues. The most prominent example of fluorescent dye that stains the proteins after electrophoretic separation has been completed is the ruthenium-based dye SYPRO Ruby (Berggren *et al.*, 2002). Commercially there are a lot of stains available for all the categories mentioned above but they won't be covered in this review.

1.5.2.3.1 Mass spectrometry

Mass spectrometry has improved from an invention that is almost a hundred years now by British Physicist JJ Thomson who invented the first mass spectrometer or "parabole spectrograph" as it was known at that time (Marchetti-Deschmann and Allmaier, 2011). Currently the technique is useful in various disciplines including chemistry, biochemistry, and structural biology. Mass spectrometer is consisting of three parts those are ion source, mass/charge (m/z) analyzer and detector. The sample

is getting analysed in the fragmented form. There are other mass spectrometers that further fragment the fragments like tandem MS or MS/MS. Fragments get ionized and the detected for the output of data. Ionization sources are discussed in the following sections.

1.5.2.3.2 Ion sources

The creation of intact molecular ions enables accurate measurements of molecular weight. The electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI) techniques revolutionized the analysis of biomolecules. Among desorption ionization techniques MALDI is the most appropriate for really high mass (≥ 60 kDa) analysis. The technique is inherently very sensitive, requiring only sample amounts in the low picomole range and exhibits a high tolerance towards contaminants (Allmaier *et al.*, 1995). MALDI ion source utilises energy from laser light that is converted into kinetic energy of the irradiated molecules or ions. The light gets directed towards a metal target plate of which the analyte has been crystallized in the presence of matrix molecules. This laser light is derived from a N_2 laser generating UV light with 337nm wavelength. The most commonly used matrix molecules are α-cyano-4-hydroxycinnamic acid for peptides less than 5000 Da and 3.5-dimethoxy-4-cinnamic acid, or sinapinic acid for proteins (Lin *et al.*, 2003). MALDI produces predominantly singly charged ions and is less sensitive to salts in the buffer than ESI, although salt and matrix adducts of analyte ions can form.

Electrospray ionization (ESI) is a soft method, allowing the formation of gas phase ions through a gentle process that makes possible the sensitive analysis of non-volatile and thermolabile compounds (Manisali *et al.*, 2006). ESI uses electrical energy to assist the transfer of ions from the solution into the gaseous phase. The transfer of ionic species from solution into the gaseous phase by ESI involves three steps: (1) dispersal of a fine spray of charged droplets, followed by (2) solvent evaporation and (3) ion ejection from the highly charged droplets (Ho *et al.*, 2003). The emitted ions are sampled by a sampling skimmer cone and then accelerated into the mass analyzer for subsequent analysis of molecular mass and measurements of ion intensity.

1.5.2.3.3 Analyzers

Mass analyzers separates the ions by their mass to charge (m/z) ratios utilizing electric and/ or magnetic fields to manipulate ions in a mass dependent manner. The mass analyzers that use electric field include time of flight (TOF), quadrupole (Q), and Ion trap (IT), while magnetic sector and ion cyclotron use magnetic field. TOF mass analyzer accelerates a packet of ions with a set of electric potentials and differentiates them by time they take to traverse a flight tube. An m/z value can be calculated from the time required to move from ion source to the detector. Quadrupole mass analyzer uses radiofrequency (RF) voltage applied to 4 metal rods with RF voltage of alternate polarity placed on opposite rods. The ratio of RF to DC selectively stabilizes the trajectory of ions of particular m/z value as they pass through the analyzer.

1.5.3 Verification of protein identifications

Proteomics studies and the information obtained from such studies can be a great step towards understanding conditions that get to be studied using proteomics. In order to have a stronger point from proteomics studies especially in the expression proteomics alternative approaches that verify the proteomics findings need to be used. The most utilized ways of verifying proteomics findings are Western blotting and enzyme-linked immunosorbent assay (ELISA). These two methods have been utilized in biomedical sciences for a long time because of their sensitivity. Because proteomics is involving a lot of approaches including the identification of highly expressed or least expressed proteins these two methods then get to be used to see if they also give the same result. This enables an enhanced confidence to the research or the study in question.

1.5.3.1 Western blot

Western blotting is a technique that is used to detect a protein from a sample with very high sensitivity. The commonly used approach involves the use of SDS PAGE to separate the proteins by their molecular weight, followed by transferring of the proteins to a nitrocellulose or polyvinylidine fluoride (PVDF) membrane. The primary antibody that recognises specifically the protein of interest on the membrane is added after blocking the membrane with BSA or fat free milk to avoid non-specific binding of the primary antibody to the membrane (Bergendahl *et al.*, 2003). The secondary antibody (for example, goat anti-mouse IgG) recognizes

the primary antibody according to its origin (for example, mouse), since it generally binds to all immunoglobulins from the particular organism that the primary antibody was isolated from. The second antibody is typically conjugated with an enzyme catalyzing a reaction that turns a colourless compound into a detectable dye and can be used universally with compatible primary antibodies. Older procedures used radioactively labelled secondary antibodies for sensitivity purposes, but recent developments make use of luminescent systems that reach similar performance without the disadvantages of working with radioactivity (MacPhee, 2010).

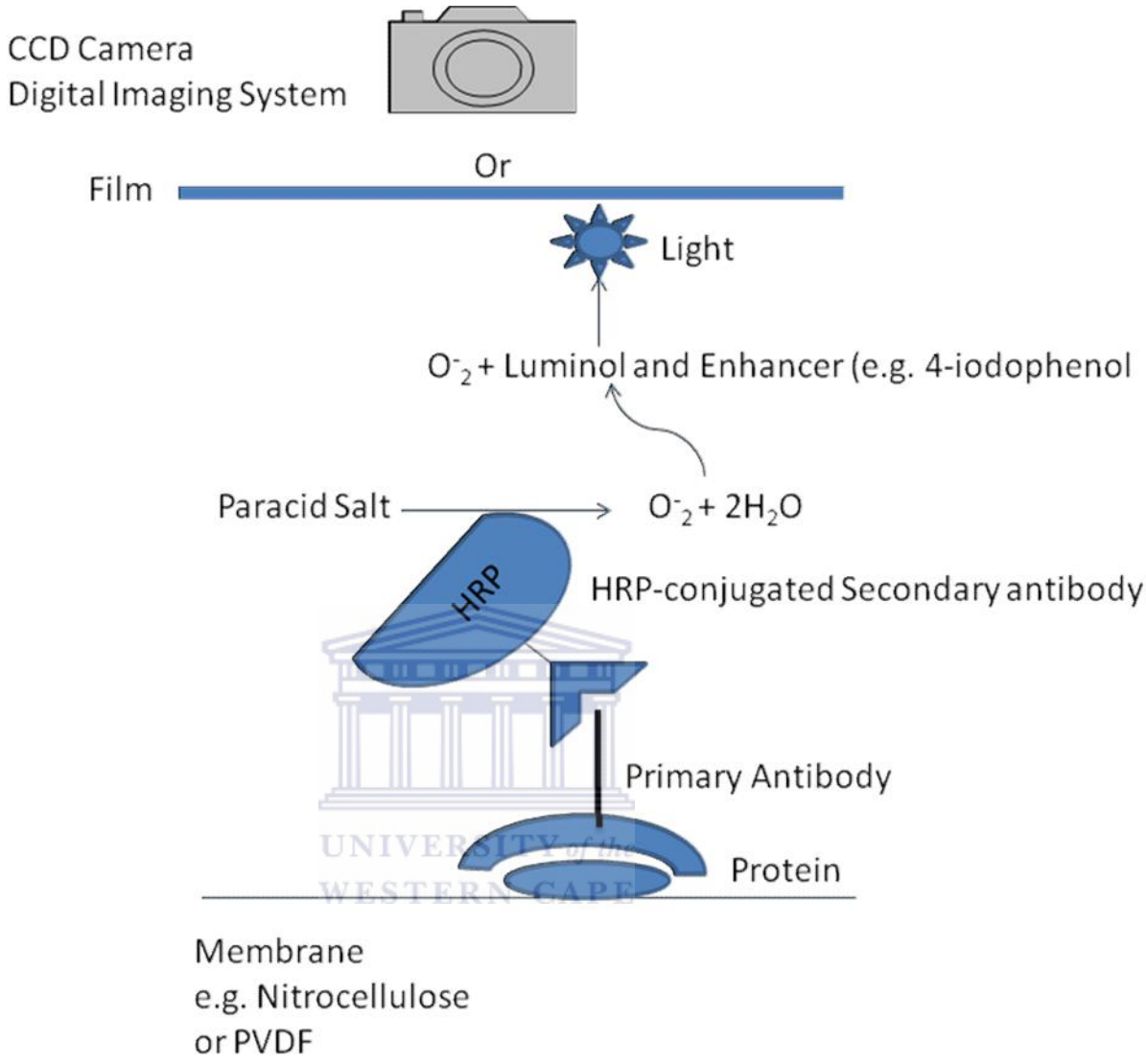


Figure 1.4: A schematic of ECL reaction and detection adapted from (MacPhee, 2010)

1.5.4 Blood, plasma and serum use for proteomics

1.5.4.1 Sample collection, handling and storage

Serum has been used extensively as a source of clues to various diseases and conditions. Serum is a potentially the most valuable specimen for biomarker elucidation because it constantly perfuse the tissues, it might be expected that the

onset or presence of disease may be determined by measuring the altered presence or abundance of the constituent molecular species in serum (Tirumalai *et al.*, 2003a). The standards in collection, handling and storage have been raised by too many studies. The manual by Gorg, et. al., (2000) is having a lot of procedures in dealing with serum for proteomics studies (Gorg *et al.*, 2000). Inappropriate handling of blood, serum or plasma collected for the determination of hormone concentration may lead to inaccurate endocrine data and diagnosis (Hegstad-Davies, 2006).

Studies on the effects of blood collection on many other types of laboratory suggest that optimization and standardization of collection tubes is an important element in reliable and standardization of collection tubes is an important element in reliable analysis of serum or plasma proteins (Luque-Garcia and Neubert, 2007). Various tubes have been studied by Drake et. al. (2004) and they found seven out of eleven tubes tested to add polymeric components to give multiple signals in the range of 1000-3000 m/z using saline instead of serum or plasma to eliminate confusion of thinking the peptides are from plasma or serum (Drake *et al.*, 2004). The use of anticoagulants has also been mentioned to have the changes to mass spectrometry protein profiles. The best temperature to store blood before separation is 4°C (Skogstrand *et al.*, 2008). Clotting time plays a role in the outcome of the serum study. Significant differences in MS protein/peptide peak intensities depending on clotting time. This is associated with the formation/accumulation of new peptides during and after the clotting process (Villanueva *et al.*, 2005). Many studies showed that the changes are not seen with the storage of serum for 24 hours at 4°C but changes start to show beyond that storage time (Marshall *et al.*, 2003). Furthermore

the storage was also found to be efficient at -20 to -80°C with some controversy regarding the freeze thaw cycles which can be avoided by aliquoting (Luque-Garcia and Neubert, 2007).

1.5.4.2 Depletion of highly abundant proteins

There is a huge challenge encountered when working with serum protein. This is attributed to by the availability of highly abundant proteins that contribute about 90% of proteins found in serum causing the difficulty in analysis of low abundant proteins. Strategies to accomplish success in studies that involve serum have been previously published. Amongst the listed approaches to depletion of high abundant proteins is the centrifugal ultrafiltraction, which utilizes the semi-permeable membrane to separate the proteins based on the molecular weight cut offs (MWCO) by centrifugal forcing of the liquid against the membrane. Special solvent conditions are required to disrupt protein-protein/ peptide interactions so that LMW components that may be bound to albumin or other large species are released and are free to pass through the membrane (Tirumalai *et al.*, 2003b).

One or more analytes can also be removed using solid phase extraction using columns or disk plates. Solid phase extraction columns are probably the most widely used for depletion of highly abundant proteins in plasma/serum samples as a first step in biomarker-related proteomics studies (Luque-Garcia and Neubert, 2007). The depletion columns have various types; they can be based on ion-exchange, metal-chelating, affinity ligands, bacterial proteins, antibodies, or combinations of these.

Solid phase extraction disks utilize the same principle as the columns. The main advantage associated with solid phase extraction disk plates is the increased ability for automation. The disk plates are generally used in a 96-well plate format allowing simultaneous processing of high number of samples by using robots (Koomen *et al.*, 2005). The other approach to depletion of high abundant proteins is organic solvent extraction. The precipitation with organic solvent in the presence of ion-paring agents dissociates peptides and smaller proteins from large abundant proteins, thereby facilitating their extraction.

1.5.4.3 TCA/Acetone precipitation of serum/plasma samples

Human plasma proteins originate from a variety of tissue and blood cells as a result of secretion or leakages. A plasma sample is obtained if the blood is withdrawn in the presence of an anticoagulant (EDTA, sodium citrate, or heparin) and centrifuged to remove blood cells (Hu *et al.*, 2006). In the absence of anticoagulant, a serum sample is obtained after the blood clots and cellular elements are centrifuged and removed. Lipids are widely present in biological fluids such as plasma complexed with proteins and this interaction reduces protein stability and might affect pI and MW (Ahmed, 2009). This could be the contributor to serum protein composition to be largely different from that of plasma. Precipitation in acetone or the combination of TCA/acetone remove lipids‟ elimination is more effective when proteins are in association with proteins in membranes (Ahmed, 2009).

Plasma and serum proteome analysis has been hampered by predominance of several

highly abundant proteins including albumins, immunoglobulins, alpha-1-antitrypsin, fibrinogen and haptoglobulin and their isoforms and fragments (Hu *et al.*, 2006). Protein precipitation with TCA/acetone or NaCl/ethanol is a useful approach for depletion of albumins (Huang *et al.*, 2005, Chen *et al.*, 2005). Removal of abundant has brought concern regarding whether less abundant serum proteins are removed along with albumin, hemoglobulins and other commonly depleted proteins, however the use of 5% acetonitrile in serum provide better enrichment of LMW proteins (Huang *et al.*, 2005). In this study after depletion of high abundant proteins both portions that is albumin containing and albumin depleted portion were used to account for the loss of loss protein with the albumin. Because of efficiency and the cost effectiveness the TCA/acetone use for depletion shows to be a useful approach over columns because of the cost involved with the use of columns.

1.5.5 Application of bioinformatics in obesity research

Bioinformatics is conceptualising biology in terms of molecules (in sense of Physical Chemistry) and applying “informatics techniques” (derived from discipline such as applied maths, computer science and statistics) to understand and organise the information associated with these molecules, on a larger scale (Luscombe and Gerstein, 2001). The term bioinformatics was coined by Hwa Lim in the late 1980s, and popularized in 1990s through its association with human genome project (Goodman, 2002). Bioinformatics is able to combine the wet lab and the readily available information to create new hypothesis towards the expansion of knowledge in biomedical research. Through bioinformatics it is now possible for an individual

skilled in bioinformatics to sort through a genome sequence, select for a gene(s), determine whether the gene has a known function, design PCR cloning primers and predict cellular location, molecular weight, solubility, p*I*, etc. of the protein, all *in silico*, i.e. in the computer, before performing any laboratory experiments (Chakravarti *et al.*, 2000).

The information generated through research is growing year in year out. It is necessary to have the findings to be organized and placed in a central location for efficient progression and generation of answers through research. The integration of biology and chemistry has been overlooked but currently there is an increase in collaborations in disciplines. Overall, the key challenge facing bioinformatics and indeed modern biology is to harness the mass of accumulating data and use it to create new understandings and new knowledge (Kahn, 2002). The long-term value of bioinformatics lies not in the tools developed and used, but in the conversion of data into the practical knowledge required for the delivery of better therapeutics (Persidis, 1999). Through bioinformatics the networks that are created by proteins in different locations in the body are accumulating to increase the understanding of obesity.

1.5.5.1 Bioinformatics improvements at gene and protein level

Investigation of each gene separately for its likely involvement in the disease process had to be done manually with the aid of available public database such as Online Mandelian Inheritance in Man (OMIM), Entrez and genome browsers, but now there are some promising bioimformatics tools. Six of these new tools are freely available online and those are Prioritizer, Geneseeker, PROSPECTR and SUSPECTS (P and

S), Disease Gene Prediction (DGP), Genes 2 Diseases (G2D) and Endeavour (Elbers *et al.,* 2007) (Elbers et.al., 2006). The information utilized by these tools is extracted from public online database, such as sequence data, medical literature, gene ontology and function annotation (GO) and information on biology, function and gene expression. These tools have been used to identify the common genes in obesity and T2D.

Bioinformatics is playing a crucial role in speeding up the protein research. Databases are available for 2D PAGE, mass spectra analysis e.g. peptide mass fingerprint, protein structure, protein interaction and protein role in metabolic pathways, etc. Mass spectrometry approaches are being used in protein identification and determining the nature of post-translational modifications (Sickmann *et al.,* 2003). Mass spectrometry and other methods make it possible to quickly identify large number of proteins, to map their interactions, to determine their location within the cell and to analyse their biological activities (Apweiler *et al.,* 2004). The databases that are being used differ in that some are sequence repositories that add little or no additional information to the sequence records they contain and generally make no effort to provide a non-redundant collection of sequences to users. Examples of sequence repositories are GenPept and NCBI‟s Entrez Proteins. Universal curated databases have additional information that is validated by expert biologists to enrich the sequence data. Such databases include PIR-PSD, Trembl and Swiss-Prot. Lately Swiss-Prot, TrEMBL and PIR-PSD have been merged to form the UniProt knowledgebase. This provides a cornerstone for scientists active in modern biological research (Apweiler *et al.,* 2004).

Another purpose of bioinformatics is to enable scientists in various researches to be able to draw biological conclusions. In recent years, most new generation of algorithms incorporate information from biological pathways into microarray data analysis (Goeman *et al.*, 2004, Subramanian *et al.*, 2005). Three popular pathways are referenced by such algorithms, those are KEGG, Ingenuity and Wikipathways (Soh *et al.*, 2010). Wikipathways is maintained by a community of professional users via the free and open wiki platform; KEGG is curated independently by a single lab from published literature while ingenuity is a commercial product (Soh *et al.*, 2010). The overall bioinformatics part of this study has been shaped by the above information in that the databases used are curated databases. For pathway analysis KEGG has been a database of choice since Ingenuity is a commercial product and was not available for use.

1.6 Problem statement

Obesity is a disease that is due to the accumulation of body energy stores due to increased adiposity that results mostly from high energy intake with reduced dissipation to create an imbalance in energy. The use of body mass index (BMI) and waist to hip ratio (WHR) has been successful in identifying individuals with obesity but these methods works to identify the individual at a late or compromised stage when reversing the condition is quite a challenge especially for the too ambitious individuals that require results immediately. It is known that adiposity is as a result of increase in fat storage which happens due to adipocyte differentiation or hypertrophy,

adipogenesis, and accumulation of free fatty acids in the blood. Blood, as a carrier of many components of the body and passing through the organs of the body, can be a useful material to use for tracking the changes that are due to adiposity, which is the contributor to obesity eventually. We hypothesized that using proteomics to study less invasively the serum proteins response to high fat diet feeding can reveal the trend that is taken by proteins in relation to the diets fed based on caloric content. This can lead to possibility of potential biomarkers being identified towards early detection of obesity and introduction of means to prevent the perpetuation of obesity.

1.6.1 Aims

- The aim of this study was to compare the changes in serum protein profiles of rats fed different diets
- To identify the proteins that are differentially expressed in high calorie diet fed groups compared to the control diet using peptide mass fingerprinting
- To validate the expression using Western blot analysis
- To determine whether the trend that the expression follows is dependent on diet or time
- To use bioinformatics to gather information on the proteins that were differentially expressed

1.6.2 Objectives

- To create a diet induced obese male Wistar rat model using a known lard diet and two other high fat diets that are new to our knowledge

- To use diets with different calorie content in terms of the energy source but with equivalent calorie content per gram of diet
- To monitor the intake of food over the duration of the study to make sure that there are no satiety differences
- To compare the proteome for the groups fed high fats diet compared to the control diet
- To identify the changes in relation to time by collecting blood at different time points
- To identify the proteins which are potential markers of response to high calorie intake
- To gather the information about the proteins found to be potential biomarkers regarding their role in metabolic pathways to complete the picture of the findings

Chapter 2: Materials and Methods

2.1 Materials and suppliers

Accucheck Precision Glucometer	(GlaxoSmithKline)
Acetonitrile	(Sigma Aldrich)
Acrylamide/ Bis acrylamide	(Sigma Aldrich)
Ammonium persulfate	(Promega)
Antibodies	(Santa Cruz Biotechnology)
Biolyte Ampholytes	(Bio Rad)
Bovine Serum Albumin	(Sigma Aldrich)
Bradford reagent	(Sigma Aldrich)
Bromophenol blue	(Merck)
Carbon dioxide	(Afrox)
CHAPS	(Sigma Aldrich)
Chemiluminescence X ray film	(GE Health Care)
Chow	(Epol)
Coomassie brilliant blue R 250	(Sigma Aldrich)
Dithiothreitol	(Sigma Aldrich)
ECL detection reagent	(Amersham)
Eppendorff microcentrifuge 5417R	(Sigma-Aldrich)
Fat free milk	(Nestle)
Filter papers	(Bio Rad)
Gel casting apparatus	(Bio Rad)
Glacial acetic acid	(Merck)

Glycerol	(Merck)
Glycine	(Sigma Aldrich)
Hydrochloric acid	(Merck)
Immobiline dry strip reswelling tray	(GE Health)
IPG strips (7cm, pH 4-7)	(Bio Rad)
Lauryl sulphate	(Sigma Aldrich)
Lumiglo	(KPL)
Mini-Protean III multi casting chamber	(Bio Rad)
Polyvinlidene Fluoride membrane	(PALL Life Sciences)
Sodium chloride	(Merck)
Thiourea	(Sigma Aldrich)
Trichloro-acetic acid (TCA)	(Sigma Aldrich)
Tris (Hydroxymethly) Methan	(Merck)
Trypsin	(Promega)
Tween 20	(BioRad)
Urea	(Sigma Aldrich)
Vortex	(Whitehead Scientific)
Voyager-DE PRO MALDI MS	(Applied Biosystems)
X-ray film processor	(Agfa)
Zip Tips	(Millipore)

2.2 Search engines and software used

MASCOT	(Matrix Science: http://www.matrixscience.com)
Image J	(National Institute of Health)
PD Quest	(Bio Rad)
Quantity One	(Bio Rad)
KEGG	Kyoto Encyclopedia of Genes and Genomes

2.3 Solutions

1.5 M Tris-HCl, pH 8.8	18.17 g Tris-base, set pH to 8.8 with HCl and make up to 100 ml with ddH_2O
10X TBS	25 mM Tris, 150 mM NaCl, 2 mM KCl,
10% APS	1 g ammonium per sulphate, 10 ml ddH_2O
10% TCA/acetone	10 ml Trichloroacetic acid, 90ml acetone
10X running buffer	250 mM Tris, 1.92 M Glycine, 1% SDS
1X Running buffer	25 mM Tris, 192 mM Glycine, 1% SDS

5% blocking solution	5 g fat free milk in 100 ml TBST add distilled H_2O to a litre
Agarose sealing mixture	0.5% (w/v) agarose, 0.002% (w/v) bromophenol blue in 1X running buffer
Coomassie de-staining solution	7% Ethanol, 2% acetic acid, fill up to 1L with ddH_2O
Coomassie staining solution	10% Ethanol, 7% Acetic acid, 0.05% Coomassie Blue R-250
Equilibration buffer 1	6 M Urea, 135 mM DTT, 30% Glycerol, 45 mM Tris base, 1.6% SDS, 0.002% bromophenol blue
Equilibration buffer 2	6 M Urea, 130 mM IAA, 30% Glycerol, 45 mM Tris base, 1.6% SDS, 0.002% bromophenol blue
Lysis buffer	9 M Urea, 2 M thiourea, 4% CHAPS in ddH_2O
Ponceau staining solution	0.1% Ponceau S (w/v) in 5% Acetic acid

Sample buffer	4.2 ml [0.5 M Tris-HCl pH, 6.8 (1 ml); 800 µl Glycerol; 1.6 ml 10% SDS (w/v); 400 µl 2-mercaptoethanol; 400 µl 0.05% (w/v) bromophenol blue]
Stripping buffer	20 ml SDS 10%; 12.5 ml of 0.25 M Tris, pH 6.8; 67.5 ml ultra pure water; 0.8 ml β-mercaptoethanol
TBST	100 ml of 10X TBS, make up to 800 ml with ddH_2O, set pH to 7.6 with HCL, fill up to 1 L with ddH2O, add 0.05% Tween 20
Transfer buffer	25 mM Tris, 192 mM Glycine, 10% (v/v) Methanol
Tris-HCl, pH 6.8	6.06 g Tris-base, set pH to 6.8 with HCl and make up to 100 ml with ddH_2O

2.4 Diets and feeding regimens

A diet-induced obesity study deserves careful planning of the high fat diets to avoid confounding factors like using cereal based diets as control diet like chow for purified diets (Gadja, 2008). In this study we used chow to formulate the diets. The lard diet was used before in the Medical Research Council's Research laboratory to study obesity (Sibuyi, 2009). The butterfat was used as a different source of energy and there is no work that has been published before that uses butterfat. Protein content was increased by the addition of fat free powdered milk to the lard fat free diet. The maintenance diet was also complemented with protein by adding fat free milk to the chow diet.

2.4.1 Preparation of the diets

Diets used in the present study were prepared in-house at the Medical Research Council's Diabetes Research laboratory. The ratios given in tables above are given for the preparation of a 100 grams diet. The dry material was weighed first for each diet. The mixing was performed after each addition. The fat and sunflower oil were mixed before adding to the mix. A litre of water was added for every four kilograms of diet. The diet was put on trays and flattened. The pizza cutter was used to cut it into small blocks for feeding. The diet was placed at 4°C walk in fridge for the solidification and storage. The diet was ready for feeding four hours after preparation. Obesity was induced by feeding the rats *ad libitum* diets are shown on tables.

Table 2.1 (a): Maintenance diet (C)

	Chow (g)	Fat free powd. milk (g)	Total (g)
Ratio	50	50	100
Protein	0.09	0.1605	0.2505
CHO	0.3975	0.2605	0.667
Fat	0.0125	0.0005	0.013
g/g of diet	0.5	0.5	1.0
kJ/g diet	5.5	7.225	12.725
Kcal/g diet	1.315	1.727	3.041

Table 2.1 (b): Lard high fat diet (L)

	Chow (g)	Lard (g)	Sumfl. Oil (g)	Sugar (g)	Vit. & min. (g)	Total (g)
Ratio	54.8	32	1.6	6.4	5.2	100
Protein	0.09864	0	0	0	0	0.09864
CHO	0.43566	0	0	0.064	0	0.49966
Fat	0.0137	0.32	0.016	0	0	0.3497
g/g of diet	0.548	0.32	0.016	0.064	0.052	1
kJ/g diet	6.028	4.84	0.6112	1.08672	0	18.4792
Kcal/g diet	1.441	2.83	0.116	0.26	0	4.41663

Table 2.1 (c): Lard Fat Free Powdered Milk Diet (LF)

	Chow (g)	Lard (g)	Fat free powd. milk (g)	Sunfl. Oil (g)	Total (g)
Ratio	50	17.5	32	0.5	100
Protein	0.09	0	0.10272	0	0.19272
CHO	0.3975	0	0.17248	0	0.56998
Fat	0.0125	0.175	0.00032	0.005	0.19282
g/g of diet	0.5	0.175	0.032	0.005	1
kJ/g diet	5.5	6.475	4.624	0.191	16.79
Kcal/g diet	1.315	1.548	1.105	0.064	4.013

Table 2.1 (d): Butterfat diet (BF)

	Chow (g)	Full cream powd. milk (g)	Total (g)
Ratio	50	50	100
Protein	0.09	0.1185	0.2085
CHO	0.3975	0.197	0.5945
Fat	0.0125	0.14	0.1525
g/g of diet	0.5	0.5	1
kJ/g diet	5.5	10.6	16.1
Kcal/g diet	1.315	2.533	3.848

2.4.2 Dietary fatty acid analysis by GC MS

Dietary fatty acids were quantified following the method similar to the one used by van Jaarsveld et. al. (2000), using heptadecanoic acid as an internal standard (Sigma Aldrich). Freeze dried food sample (2 g) was homogenized in 50 ml of chloroform/methanol (2:1) v/v containing 0.01% butylated hydroxytoluene (HBT) with a polytron. The homogenate was filtered with sintered glass funnels and the filtrate made up to 50 ml with chloroform/methanol (2:1) v/v. To 1 ml of the extract, 1ml of a 30 ug/ml heptane solution of heptadecanoic acid internal standard was added and solvents evaporated to dryness at 40°C under stream of nitrogen gas. To the dry residue, 2 ml of a 6% ethanolic-KOH solution was added, followed by heating at 70°C in an aluminium block for 2 hrs. After cooling, 1 ml of water and 2 ml of *n*-hexane were added and the samples were thoroughly mixed on a Vortex mixer. Of the top hexane layer, 1 ml aliquots were evaporated to dryness and re-dissolved in CS_2. Aliquots were then injected into a Varian Model 3700 GLC equipped with a flame ionization detector and a 1.2 m glass column (2 mm internal diameter) packed with 3% SP-2401 on 100/120 mesh Supelcoport (Supelco Inc., Bellefonte, PA, USA). Gas flow rates were: hydrogen, 20 ml/min; air, 200 ml/min; and nitrogen carrier gas, 25 ml/min. Temperatures were: injector, 280°C; column, 255°C; and detector, 290°C.

2.5 Induction of obesity using different high fat diets in a Wistar rat model

Twenty eight male Wistar rats were obtained from the Medical Research Council's Animal facility in Tygerberg (South Africa) at weaning. They were maintained on chow for four weeks. After four weeks the rats were body weight-matched and divided into four groups. They were single caged in wire meshed cages to allow food monitoring. The weights that were recorded during the grouping were used as baseline weights and the animals were assigned to different dietary groups. One group was continued on maintenance diet while three groups were switched to different high caloric diets. All animal protocols used in this study were approved by the University of Stellenbosch's Animal Ethics Committee.

2.5.1 Food intake and body weight measurements

To monitor the intake, food was weighed before giving it to the rats. Food spillage and food left was subtracted from the food given, to get the intake per rat. The average was used to determine intake per week. Body weights for the rats in different groups were recorded weekly. The body weight gain for each group was plotted in the graph as an average of the group's weight per week for the duration of the study that took eight weeks.

2.5.2 Blood glucose measurement and serum preparation

The animals were anaesthetized using 98% oxygen and 2% fluothane. The glucometer Precision ACCU check was calibrated using the calibration strip. The

strip was then substituted with a glucose strip for glucose measurement. The tip of the tail was cut using the scalpel. After cutting the tip of the tail a drop was placed on the glucose strip, blood glucose was measured following the manufacturer's instructions and the reading was recorded. A red light was used to warm the tail to enhance the blood flow to the tail. Blood was collected drop by drop into a 1.5 ml eppendorf tubes to a volume of approximately 100 µl. The tubes were kept on ice during collection until the blood was collected from all the groups. The tubes were removed from ice and centrifuged at 800 x g in a 4°C pre-cooled centrifuge for 15 minutes. The serum was transferred to new tubes in aliquots of 50 µl and stored at -80°C until use. This was performed at baseline, after 4 days and after 4 weeks during the obesity induction period.

2.5.3 Termination of animals and tissue collection

After eight weeks the animals were terminated for blood and tissue collection. The rats were anaesthetized using carbon dioxide and terminated by decapitation. A drop of blood was used to measure glucose as in 2.4.2. The blood was collected into a 10ml tube and the tube was placed on ice to clot. Serum was prepared as explained in 2.4.2. This was performed for each rat involved in the experiment. The rats were dissected using a sterile dissection kit. A pair of scissors was used to cut out fat pads which include inguinal, retroperitoneal, perirenal, epididymal and mesenteric fat pads. Other tissues that were collected include brain, liver, pancreas, spleen, kidneys, heart and adrenals. The tissues were removed from an animal, weighed and snap frozen in liquid nitrogen. Collected tissues were stored in foil at -80°C.

2.6 Protein expression analysis in serum using proteomics

2.6.1 Depletion of highly abundant proteins using TCA/acetone precipitation

Frozen samples were thawed and 50 µl of distilled water was added to an equal amount of the sample. To make a mixture homogenous a vortex was used. Four volumes of ice-cold 10% TCA-acetone (v/v) were added, and mixed by vortexing. The sample was incubated at -20°C for 90 minutes. Centrifugation at 16000 x g at 4°C for 15 minutes was performed and supernatant was carefully removed to a new tube. The pellet was washed once with 500 µl of ice-cold acetone. The pellet was incubated on ice for 15 minutes. The tube was centrifuged at 16000 x g at 4°C for 15 minutes; supernatant was added to the tube with the first supernatant and remnants of acetone were blotted using the paper towel. The tube with the supernatants was incubated at -20°C for 1hour after adding 500 µl of ice-cold acetone to make ~1500 µl. The tube was then centrifuged at 16000 x g at 4°C for 15 minutes and the supernatant was discarded and the paper towel was used to blot the remaining liquid. Pellets were resuspended in 100 µl lysis buffer (LB) (9M urea, 2M thiourea, 4% CHAPS). The first pellet was albumin depleted and the second pellet contained albumin.

2.6.2 Analysis of protein concentration using Bradford assay

The protein concentration was determined for each pellet as obtained in 2.5.1 using the Bradford assay. One µl of the sample was diluted with 49 µl of the solubilisation buffer. The standards were prepared using known amounts of bovine serum albumin (BSA). To 25 µl of diluted sample or BSA standard 1 ml of Bradford reagent was

added. The mixture of Bradford reagent and sample or BSA standard was incubated at 37°C for 30 minutes. The samples or BSA standards were then added to a microplate (125 µl/well) in triplicates. The plate was read on a spectrophotometer at wavelength 630 nm. The BSA absorbance values obtained were used to draw a standard curve. The concentrations of the samples were determined from the standard curve.

2.6.3 Analysis of TCA/acetone treated samples using SDS-PAGE

Quantified samples were mixed with an equal volume of 2X sample buffer (1:1). The samples were then boiled for 5 minutes. Fifty micrograms were loaded per well in a 12% SDS- PAGE with 5 µl Fermentas protein marker loaded in one well. The samples were electrophoresed at 120V (constant voltage) until the dye reached the bottom of the gel. The gel was removed from the glass plates, stacking gel was removed and the resolving gel was stained using Coomassie stain. Gel images were taken using the Bio-Rad gel doc system.

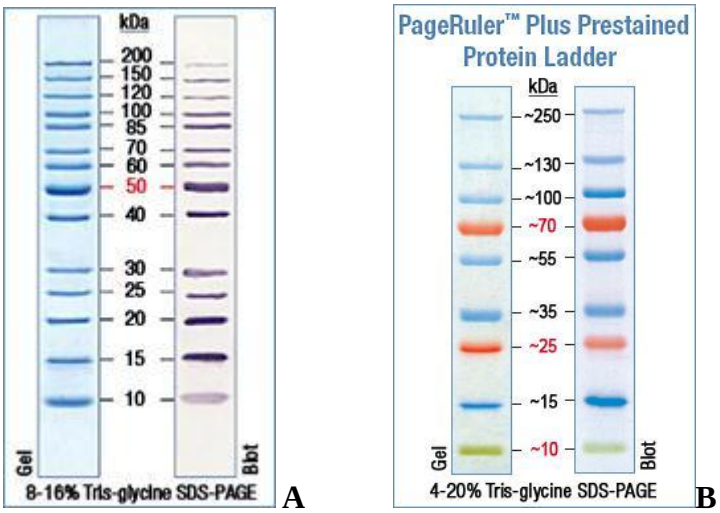


Figure 2.1: Fermentas markers that were used in gel electrophoresis (https://www.fermentas.com)

PageRuler Unstained Protein Ladder (A) and PageRuler Plus Prestained Protein Ladder (B)

2.6.4 Isoelectric focusing of proteins from TCA/acetone treated samples

Proteins samples as prepared in 2.5.1 were mixed with ampholytes, 50mM DTT, Lysis buffer to 200µl total volume recommended for the 7cm strips of 200µg total protein. The mixture was added in the re-swelling tray one sample each lane. The IPG strips were opened and placed gel towards the loaded sample on a reswelling tray. The tray was left for passive re- swelling overnight (at least up to 16 hours). The re-swollen strips were then placed on the isoelectric focusing machine and wet filter papers were put on edges prior to placing the electrodes. The machine was turned on and ran using the following protocol: first step at 7000V for 15 minutes, second step 8000V for 30 minutes and strips were run at 6475 Vhr running 12 strips.

2.6.5 Separation of protein on a second dimension following IEF

The strips were removed from the IEF machine and rinsed with distilled water. Excess water was blotted using a paper towel. The strips were then placed on the re-swelling tray gel-side up. The strips were equilibrated using equilibration buffers 1 and 2 respectively for 15 minutes each with shaking. During equilibration the protein ladder was added to ~4 mm^2 filter paper and left to dry. The 12% SDS PAGE was prepared without stacking gel. The equilibrated strips were rinsed with 1x running buffer and loaded on top of the SDS PAGE gel leaving a space for a filter paper space on the left side. The filter paper was loaded using a pair of forceps. To seal the strips and filter paper, cool 1% agarose gel with bromophenol blue was used. After polymerization of sealing agarose the SDS PAGE was run at 120V constant voltage until the dye reached the bottom of the gel.

2.6.6 Staining and imaging of SDS PAGE and 2D SDS PAGE

Gels were stained with Coomassie 1 overnight, Coomassie 2 for 30 minutes, and Coomassie 3 for 30 minutes. The gels were then destained using a destaining solution until a clear background was obtained and protein spots or bands were clearly visible. The gels were scanned using Bio Rad Pharox FX Imager and images were saved using Quantity One software. Saved images were cropped leaving the protein ladder off and processed to better visibility. The cropped images were loaded to PD Quest software for analysis.

2.6.7 PD Quest analysis of 2D SDS PAGEs for differentially expressed proteins

The loaded images 2.5.6 were analyzed using PD Quest software. The spots comparisons were performed after choosing the control group and experimental groups. The spots were either added or subtracted from gels to get the similar number of spots to compare across the groups. The spots were manually compared within and between groups to ensure the consistency in spots to be compared. The parameters that were to be used for analysis were chosen. The spots that were two fold high or less in intensity in experimental groups compared to control were considered enhanced or deregulated respectively. Those spots were considered for identification with MALDI MS.

2.6.8 Protein spots excision and in-gel digestion for MALDI TOF MS analysis

Spots that were considered to be differentially expressed from the experimental groups were excised using a pipette tip and placed in a sterile eppendorff tube. Water was added to a gel plug for rehydration and rinsing. Water was removed and 35 µl of acetonitrile was added to a gel plug. The gel plug was incubated with acetonitrile at room temperature (RT) for ten minutes to shrink and dehydrate. Acetonitrile was carefully removed using a sterile pipette and a gel plug was dried for ten minutes in a speed-vac. A re-swelling buffer [150 µl of 10 mM DTT in 100 mM NH_4HCO_3] was added and the tubes were incubated for 1 hr at 56°C. After 1 hr incubation the tube was cooled to RT and DTT solution was replaced with 150 µl (55 µl iodoacetamide in 100 mM NH_4HCO_3]. Incubation at RT for 45 minutes with occasional mixing by vortex was performed. The iodoacetamide solution was replaced with 150 µl of

acetonitrile and the tube was incubated at RT for 10 minutes. The washing steps were repeated as above. Acetonitrile was removed and a gel plug was dried by speed-vac for 10 minutes. The tube was placed in an ice cold water bath and a gel plug was swollen in 35 µl of digestion buffer [12.5 ng/µl trypsin (Promega sequence-grade modified porcine trypsin cat.# 511A) in 50 mM NH4HCO3]. The tube was left in ice water bath for 45 minutes after which the digestion buffer was removed. Ten microliters of 50 mM NH4HCO3 without trypsin was added and incubated at 37°C overnight. The gel plug was spun down for 1 minute at 14000 rpm. The supernatant was collected into a separate PCR tube. Twenty microliters of 20 mM NH4HCO3 enough to cover the gel plug was added. The tube was incubated for 10 minutes at RT. The supernatant was transferred to the PCR tube. Twenty five microliters of 5% formic acid, 50% acetonitrile were added to gel pieces and tubes were spun at 14000 rpm for 1 min, followed by transfer of formic/acetonitrile solution to the same PCR tube as above. The formic acid extraction was performed twice. The PCR tube was spun on the speed-vac to reduce the sample volume to approximately 10 µl. The digests were desalted with C18 Zip Tip and eluted with 70% acetonitrile and 0.1% Trifluoroacetic acid (TFA). The eluted peptides samples were mixed with saturated matrix of HCN spotted on a MALDI plate and analyzed using MALDI TOF TOF (Voyager-DE PRO). Generated mass sprectra from MS were loaded to MASCOT for identification. The high scoring identification from MASCOT was considered.

2.7 Verification of differentially expressed proteins by Western blot analysis

Proteins were obtained from the serum as explained in section 2.5.1 and an equal amount of sample and the sample buffer were mixed in a tube. The

mixture was boiled for 5 minutes using a heating block, removed and spun down. The sample was loaded on a 12% SDS PAGE. The gels were run until the dye front reached the bottom of the gel. The gels were removed and plated into container containing the transfer buffer. The PVDF membrane was cut according to the size of the gels, put in methanol for 30 seconds, rinsed in distilled water and set in the transfer buffer for 5 minutes with shaking. Filter paper and the foam were also prepared by immersing in the transfer buffer for about 15 minutes. The foam, filter paper, gel, membrane, filter paper, foam setup was done avoiding bubbles between membrane and the gel. The cassette was assembled and loaded in to the electrode and set in the tank. The transfer was performed at 150V constant voltage for 60 minutes. At the end of the run the membranes were removed and rinsed with TBST. The rinsed membranes were incubated in blocking solution [5% fat free powdered milk in TBST] for 1 hour with shaking. The blocking solution was removed and the membrane was washed 3 times with 1X TBST for 10 minutes each. The membrane was probed using a primary antibody 1:1000 overnight at 4°C with shaking. The membrane was washed with TBST as above and probed using the secondary antibody 1:4000 in blocking solution for 2 hours. The membrane was washed as above. The proteins were detected using the Lumiglo system (KPL). Equal amounts of solution A and B were mixed in the dark room with the safe light on. The mixture was applied in the membrane protein side up to cover the membrane. After a minute the mixture was removed and excess was blotted using a paper towel. The membrane was wrapped in a transparent plastic placed in the cassette and the hyper-film was placed on top of the wrapped membrane. The cassette was closed and the exposure was timed at 30 sec, 3 minutes, 5 and 10 minutes. The picture of a film was

shot using the Gel doc system and the pictures were saved for analysis using the Image J software (Schneider *et al.*, 2012). The densitometric volumes were used to compare the expressions.

2.8 Bioinformatics analysis and acquisition of information from databases

2.8.1 MASCOT identification of proteins

The proteins identified to be differentially expressed were further identified using mass spectrometry whereby the MASCOT database was used to compare the spectra as generated during mass spectrometry analysis. The identifications were taken based on the MOWSE scores. Highest MOWSE scores were considered to be the reliable identifications. Expasy was used to acquire the information that is readily available for the proteins that were identified to be differentially expressed. Using the orthology the links of the proteins identified with the pathways for metabolism were identified. The KEGG pathway analysis was used for this analysis.

2.8.2 Retrieval of protein information from Expasy

2.8.2.1 Uniprot information retrieval for proteins identified to be differentially expressed

The information about the proteins that were identified was obtained from the Expasy proteomics server. The protein name was used to get the information from the Uniprot database.

2.8.2.2 The PMP database use for available modelled structures of the protein

From the information acquired the accession number was used to get the structure as modelled in the Protein Model Portal database. The experimental structure of the protein was chosen when available. If the experimental structure is not available the modelled structure with highest sequence identity (ID) was used.

2.8.3 Orthology approach for identification of association of identified proteins to pathways using KEGG

The proteins were searched for their role in metabolic pathways. The information was not available. To get the association of the proteins with other pathway the orthologs of the proteins in questions were identified. The k values of the proteins were obtained and the search was performed on a KEGG pathway database. The highest e value was considered for protein's association with pathways. The pathways were considered for analysis and the literature was used to acquire more information on proteins.

2.9 Data analysis

The diet effects were tested by means of a linear mixed effects regression model. The fixed effects tested were diet, time and diet by time interaction. The random effects for each rat is a linear model on time with an intercept and slope as the parameters.

A procedure "lme" in R Program was used for a linear mixed effects regression

model. This is the model that we used: Weight = Diet + Diet*log (Time), 1+log (Time) /Rat……(1)

This is a model that takes into considers the change in the body weight cumulatively in relation to time. Diet is the initial body weight for each dietary group, whereas Diet* represents the weight in the successive weeks. It takes into consideration the time effect to weight gained.

Unless stated otherwise, all the other parameters were analyzed using the One way ANOVA (Graph Pad Prism 5). The values were considered to be statistically significant when $p \leq 0.05$.

Chapter 3: Induction of obesity using different diets

3.1 Introduction

There is no clear evidence to reveal the specific component of a diet (i.e. carbohydrate, fat, protein, vitamins, micronutrients) that influences the ways in which food energy is absorbed or used (Scheen, 2008). However it is an established fact that many strains of rats become obese when fed palatable diets containing large amounts of fat and most of the weight increase is carcass fat (Cobertt et. al. 1986). It is important to mention that the use of a high fat diet to create an obese animal model by researchers contributed to the gathering of information about obesity both in rats and mice. The type and amount of fatty acids used to prepare the diets has significant effects in various metabolic processes. The field of dietary research has looked more at fat consumption than the effect of glucose metabolism which is also found to contribute to the control of weight (Lichtenstein and Schwab, 2000). The rise in obesity and metabolic syndrome has been noticed and attributed to the global nutrition transition. A noticeable transition from traditional plant-based to more energy dense diet dominated by processed foods, sugar and high animal fat content together with sedentary lifestyle has been seen globally (Kimokoti and Millen, 2011).

An increased fatty acid content of the diet alters plasma lipoprotein profiles with an increase in the variety of fatty acids in terms of their chain lengths and the degree of saturation (Medei *et al.*, 2010). The current study utilizes gas chromatography to analyze fatty acid profiles for the four diets that were used during obesity induction. Other parameters that were analyzed include food intake, body weights,

blood glucose, adiposity and various tissue weights to get the physiological changes that are involved with dietary intake. The physiological response is widely used whereby the difference in tissue size tells the effect of diet. The following results show the change in weights, blood glucose, adiposity (fat depots) and the difference in fat contribution to the whole body weight (adiposity index).

3.2 Analysis of fatty acids in different diets using GC MS

The analysis of fatty acids for the diets was performed using the GC MS as detailed in Section 2.3.2. Results obtained were showed high percentages of saturated fatty acids for the three high fat diets compared to the control diet (Figure 3.1). Significantly high amounts of unsaturated long chain fatty acids [(linoleic acid (18:2)] content were found in the control diet than the three high fat diets. Significant in contrast the three high fat diets were high in the saturated long chain fatty acid [(stearic acid (18:0)] Table 3.1. The amounts of [palmitic acid (16:0)] were also high for the high fat diets with highest percentage contribution seen in the butterfat. The butterfat diet contained two folds or more of medium chain saturated fatty acids [myristic (14:0)] than the other two high fat diets and the control diet. The amount of monounsaturated fatty acids [oleic acid (18:1)] was close in percentage contribution across the diets Table 3.1.

Looking at the tabulated amounts for the diets (Table 2.1 a-d) it shows the distribution of fat contents between diets. It is noticeable that the control group has a very low fat content per gram of diet Table 2.1 (a). The dominant fatty acids

in the maintenance diet were polyunsaturated. More than 70% of the fat is unsaturated fat in the control diet. Overall the dominant fatty acids in the lard diet were saturated. There was no significant difference in the calories between the high caloric diets but the other two diets unlike the lard diet have a bit more calorie contribution from proteins and carbohydrates.

Table 3.1: GC MS results of fatty acid percentage contribution to the total fat in diets used

The diets which are maintenance diets (C), lard diet (L), lard fat free diet (LF) and butterfat diet (BF) were analyzed for their fatty acid profile. The fatty acids that are contained in each diet are given in the table under each diet in percentages. A scientific name of the fatty acid is given in the first column and the last column states the type of fatty acid

Names of fatty acids	**Systemic name**	C	**LF**	**L**	**BF**	**Type of FAs**
		% contribution to total fat				
α-linoleic acid (18:3 n-3)	9,12,15-octadecatrienoic	4	1	1	0	Unsaturated
γ-linoleic acid (18:3 n-6)	6,9,12-octadecatrienoic	0	0	0	1	Unsaturated
Arachidic acid (20:0)	Eicosanoic	1	1	0	1	Saturated
Palmitoleic acid (16:1 n-7)	Cis-9-hexadecanoic	1	1	2	2	Unsaturated
Myristic (14:0)	Tetradecanoic	1	8	3	3	Saturated
Palmitic acid (16:0)	Hexadecanoic	17	23	24	23	Saturated
Stearic acid (18:0)	Octadecanoic	7	15	22	22	Saturated
Oleic acid (18:1 n-9)	Cis-9-octadecanoic	27	33	39	37	Unsaturated
Linoleic acid (18:2 n-6)	9,12-octadecadienoic	42	9	9	11	Unsaturated

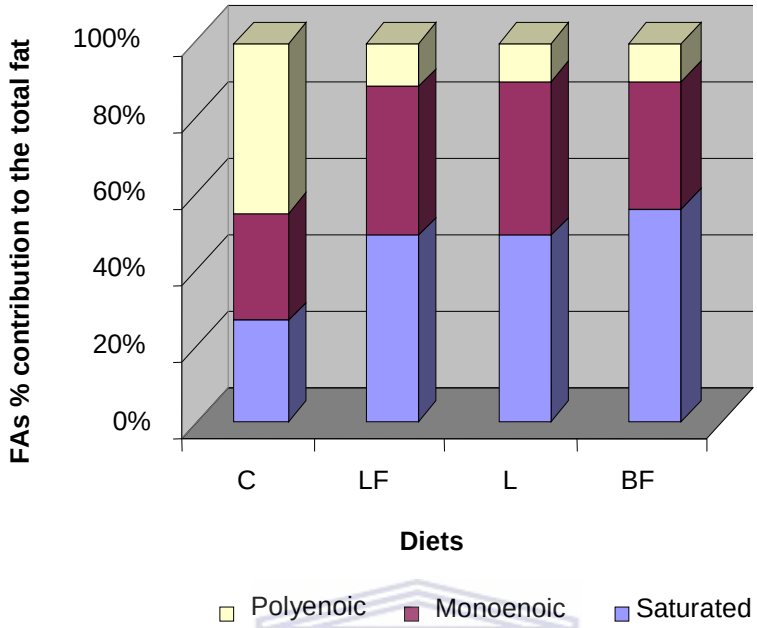


Figure 3.1: Percentage contribution of fatty acid to the total fats for the four diets used as analyzed by GC MS

Saturation of fats between diets is different with high percentage of un-saturation for the maintenance (C) diet. Three high fat diets i.e. lard fat free (LF), lard (L) and butterfat (BF) diets have the similar percentages of polyenoic fatty acids. The BF diet contained high amounts of saturated fats with least percentage of saturated fats in the C diet.

3.3 Induction of obesity by feeding different high fat diets in a Wistar rat model

The rats obtained at weaning were fed maintenance diet for four weeks for acclimatization followed by induction of obesity as explained in Section 2.4. Feeding high caloric diet to rats resulted in high weight gain for the rats on high caloric diets compared to the rats on maintenance diet. The effect of feeding high fat diet was recognised as early as the second week. Significant difference in weights for the animals that were fed lard high fat diet was recognised as early as week two whereas the animals on butterfat (BF) and lard fat free milk (LF) diet showed difference at week three. The differences in weights were seen throughout the experimental period for L and LF ($p<0.001$) when compared to the C diet fed group. The difference in body weight was also significant ($p<0.05$) for the BF fed group compared to the C fed group with group on BF being heavier. There was a response shown for the collection of blood at four weeks which was due to the starvation before blood collection. The control group showed a drop in body weight at week five, but it rose again at week six (Figure 3.2 A).

Food intake was monitored throughout the study as detailed in Section 2.4.1. The food intake by the group on the maintenance diet was high compared to the groups on high caloric diets (Figure 3.2 B). The cumulative food intake also showed a similar trend but with the group on maintenance diet having highest intake throughout the duration of the study though the rats started taking equal amount of food in the first week (Figure 3.3 A). The energy intake was the inverse of the food intake as seen in (Figure 3.3 B), which was due to the dietary content

that was found in different diets. The dietary content for C (1.3% fat, 67% CHO, 25% protein) contained the least fat amount than L (35% fat, 50% CHO, 10% protein); LF (19% fat, 57% CHO, 19% protein) and BF (15% fat, 59% CHO, 21% protein). More energy intake was from the L diet and this can be attributed to the high fat content with the least energy intake from the E diet but the difference was not significant. These results shows that the intake between the groups was not varying much based on the amount of food and energy intake needed per rat. These results suggest the differences in body weights to be due to the differences in energy storage between the groups.

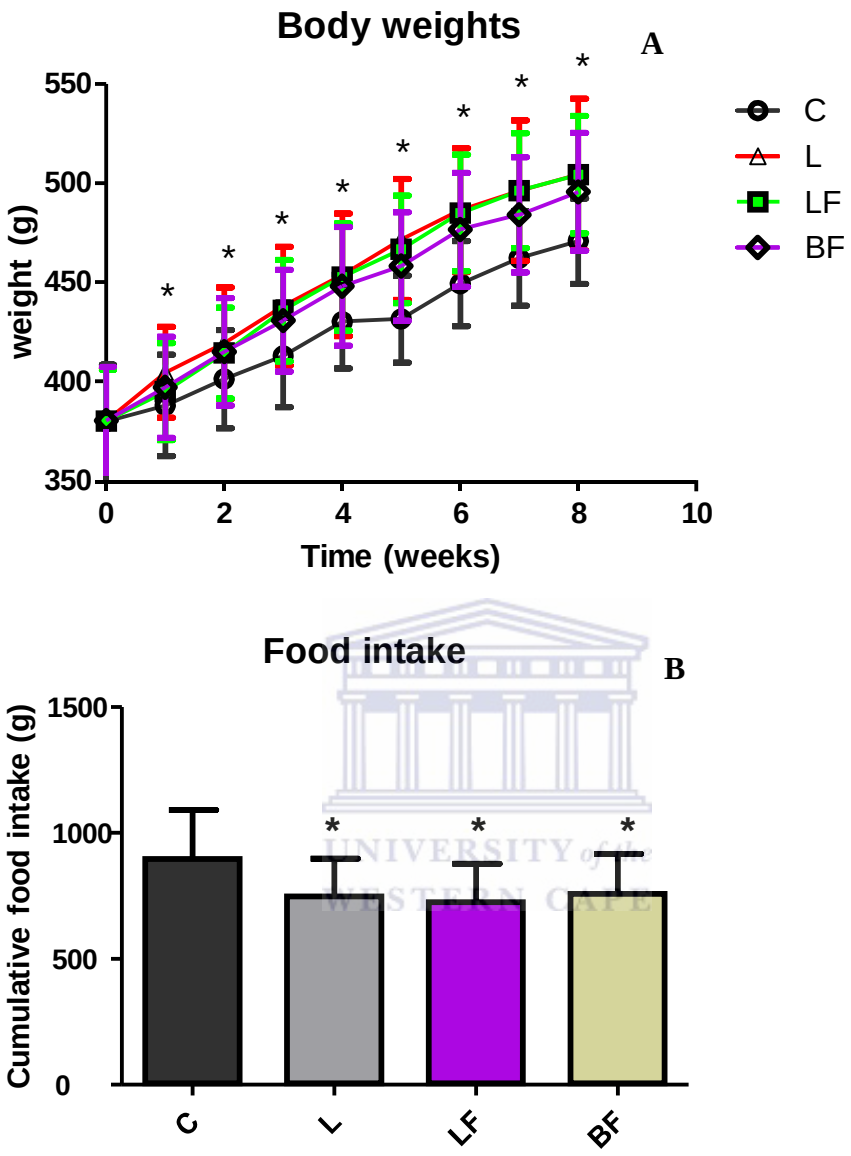


Figure 3.2: Rats body weights for eight weeks of obesity induction (A) and the amount of total intake over eight weeks per rat (B).

The figure (A) represents the body weight for the dietary groups which are maintenance diet fed group (C), lard fed group (L), lard fat free group (LF) and butterfat fed group (BF) for the duration of the experiment (8 weeks). The cumulative intake which is the average intake per rat throughout the period of the experiment is shown in B with low intake for the groups on high fat diet ($p < 0.05$).

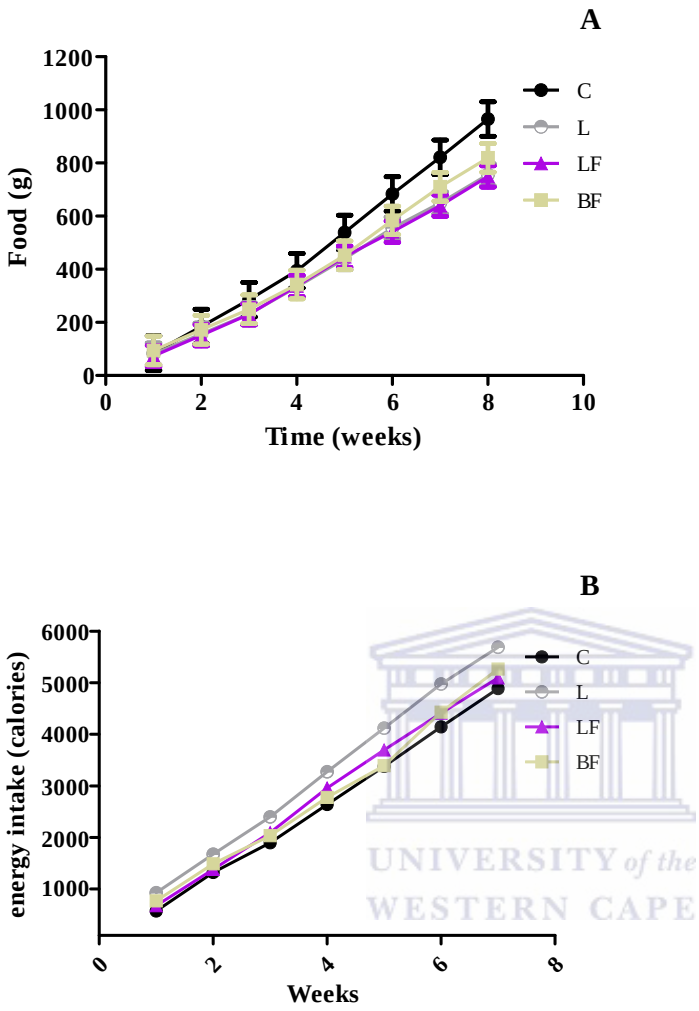


Figure 3.3: Cumulative energy (A) and food intake (B) over the period of eight weeks for all the dietary groups

The cumulative weekly food intake is shown in (A). Intake for the group on maintenance diet (C) being the highest followed by the butterfat fed groups. The lard and lard fat free groups show a similar intake. The cumulative caloric intake (B) shows to be the reverse of the food intake with C fed group having the lowest energy intake and the L fed being the highest.

3.4 Measurement of blood glucose and collection of blood for serum preparation

To monitor the chances in glucose levels, blood glucose was measured during the blood collection time points (Section 2.4.2). Blood glucose levels were not significantly different throughout the study. The group on BF diet showed high blood glucose levels at termination compared to the control group. The initial glucose levels were high but the levels eventually dropped. The other two groups on high caloric diets were having low blood glucose levels compared to the control group but the difference was not significant (Figure 3.4). The rise in glucose levels during the second collection time point could be attributed by blood collected under anaesthesia. Glucose measurements at termination were also performed after the carbon dioxide anaesthesia and decapitations. The glucose response can be at worst being associated with the pre-diabetes state but the rats were not diabetic at termination. At termination the postprandial glucose were 5.96±0.18, 8.52±0.39, 7.28±0.35 mmol/l for L, LF and C respectively. There was a rise in the BF group with postprandial glucose levels reaching 11.92±2.05 mmol/l.

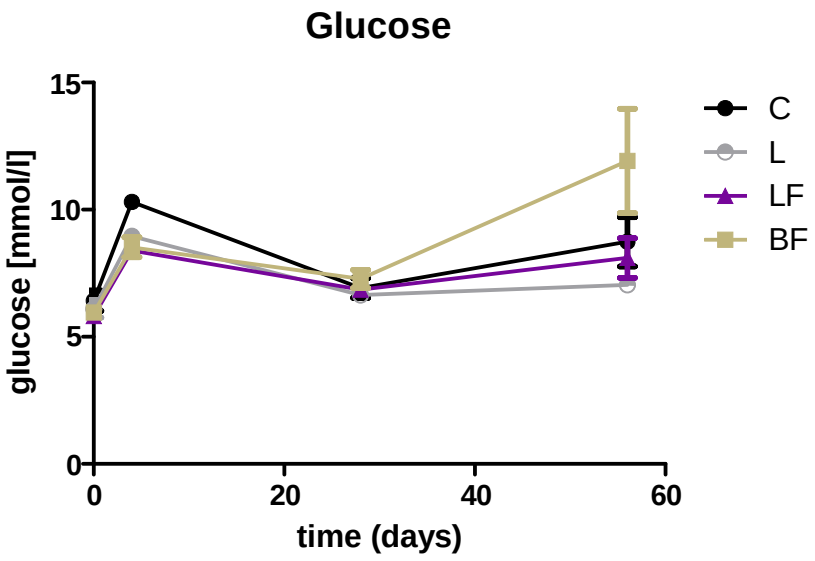


Figure 3.4: Glucose measurements from baseline to termination.

Baseline glucose measurements show similar levels for maintenance diet fed group (C), butterfat fed group (BF), lard fat free fed group (LF) and lard fed group (L). The levels rose for all the dietary groups during day four especially the C group. The levels were at normal levels when measure after four weeks and 8 weeks. Butterfat fed group showed high glucose levels at termination

3.5 Termination of animals and collection of tissues

At week eight the animals were terminated and the tissues were collected the details are in Section 2.4.3. Feeding high fat diets led to the accumulation of subcutaneous fat and abdominal fat for the rats on high fat diet. The images show the accumulation of white adipose tissue with just traces of brown adipose tissue (BAT) in the obese rats subcutaneously (Fig 3.5 A). The colour of the fat in lean rats that were in the control group showed a brownish colour suggestive of BAT. The abdominal area of the obese groups consisted of fat that seemed to be lining most of the abdominal organs compared to the abdominal area of the control group that showed very less obstruction from fat (Fig 3.5 B).

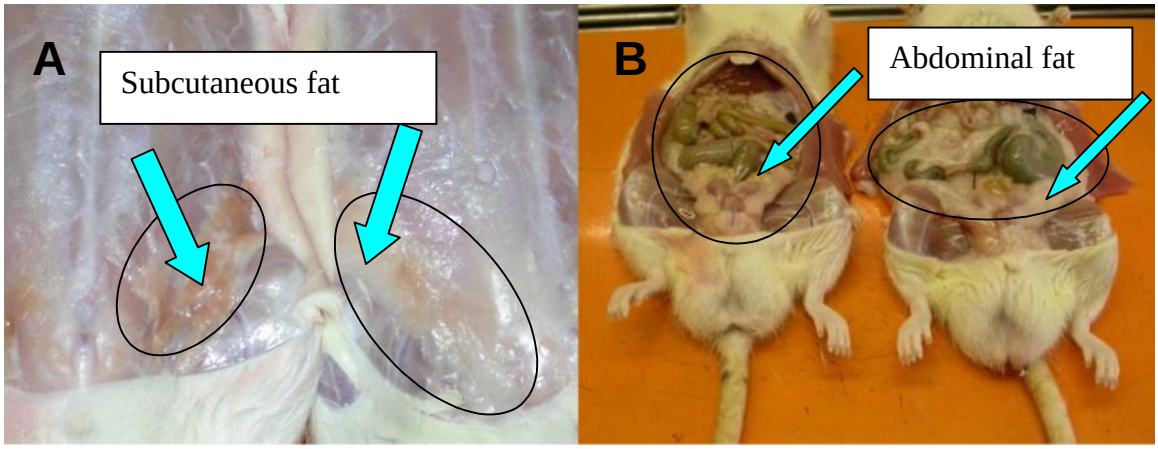


Figure 3.5: Accumulating fat tissue subcutaneously A and abdominally B

The accumulation of fat for the rats that were fed high fat diet was seen when rats were sacrificed. The subcutaneous fat (A) was different with representative from the group on maintenance diet (left) showing more of brown adipose tissue that the one from the group on high fat diet (right). Abdominal fat (B) was more for the representative from the high fat diet group (right) compared to the one for the maintenance diet fed group (left)

The weight contributed by fat in the total body weight was also seen in the adiposity index as calculated using an equation: Adiposity Index = sum (fat pad)/ total body weight X 100. The differences in adiposity index are shown in Figure 3.6. The group that was fed on lard showed more weight contribution from stored fat compared to the groups on chow ($p<0.01$). The other two groups on lard fat free and butterfat were also fatter than the control group but less than the lard group ($p<0.05$). This might be due to the differences in the storage of fat. On the other hand the fat was more on lard that could contribute to the high energy stored as fat.

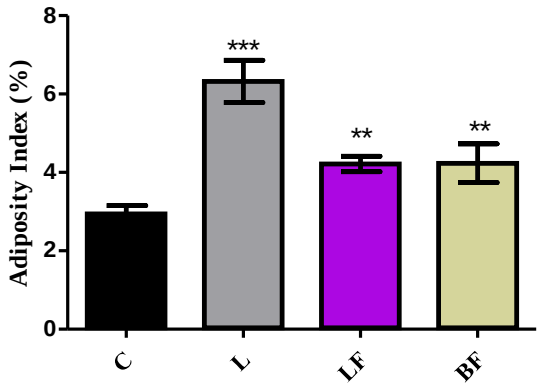


Figure 3.6: Percentage weight contribution of fat to the total body weight (adiposity index)

The percentage fat mass contribution to the body weight (body mass index) was significantly high $p < 0.001$(***) for the lard fed group (L) compared to the group on maintenance diet (C). The butterfat (BF) and the lard fat free (LF) fed groups had high adiposity index $p < 0.01$ (**) compared to the group on maintenance diet.

Weighing the organs for both the diet induced obese rats and the control group showed the significantly heavy fat tissues for the obese rats. The lard diet showed significantly heavy fat pads ($p<0.001$). The other two groups on high fat diet were also having heavy fat pads compared to the control but their fat pads were significantly weighing less compared to lard group ($p<0.05$). There was no significant difference in other organs those include liver, pancreas, adrenals, brain, heart and spleen. The epididymal, inguinal, retroperitoneal and perirenal fat were compared in this study.

Four fat pads that were weighed and compared were significantly heavier for the high caloric diets than that of the control group. The L diet fed group was having significantly heavy epididymal fat pad ($p<0.01$) when compared to the control group. The BF and LF diet fed groups also showed to have significantly heavy epididymal fat pads than the control group ($p<0.05$). A similar trend was seen for the

subcutaneous fat pads. The differences are shown in (Figure 3.7 A-D). The trend that was shown by the retroperitoneal and perirenal fat pads were also showing the significant difference for the L diet fed group ($p<0.01$) to that of the control diet, with significant difference between BF and LF fed groups when compared to the control group ($p<0.05$).

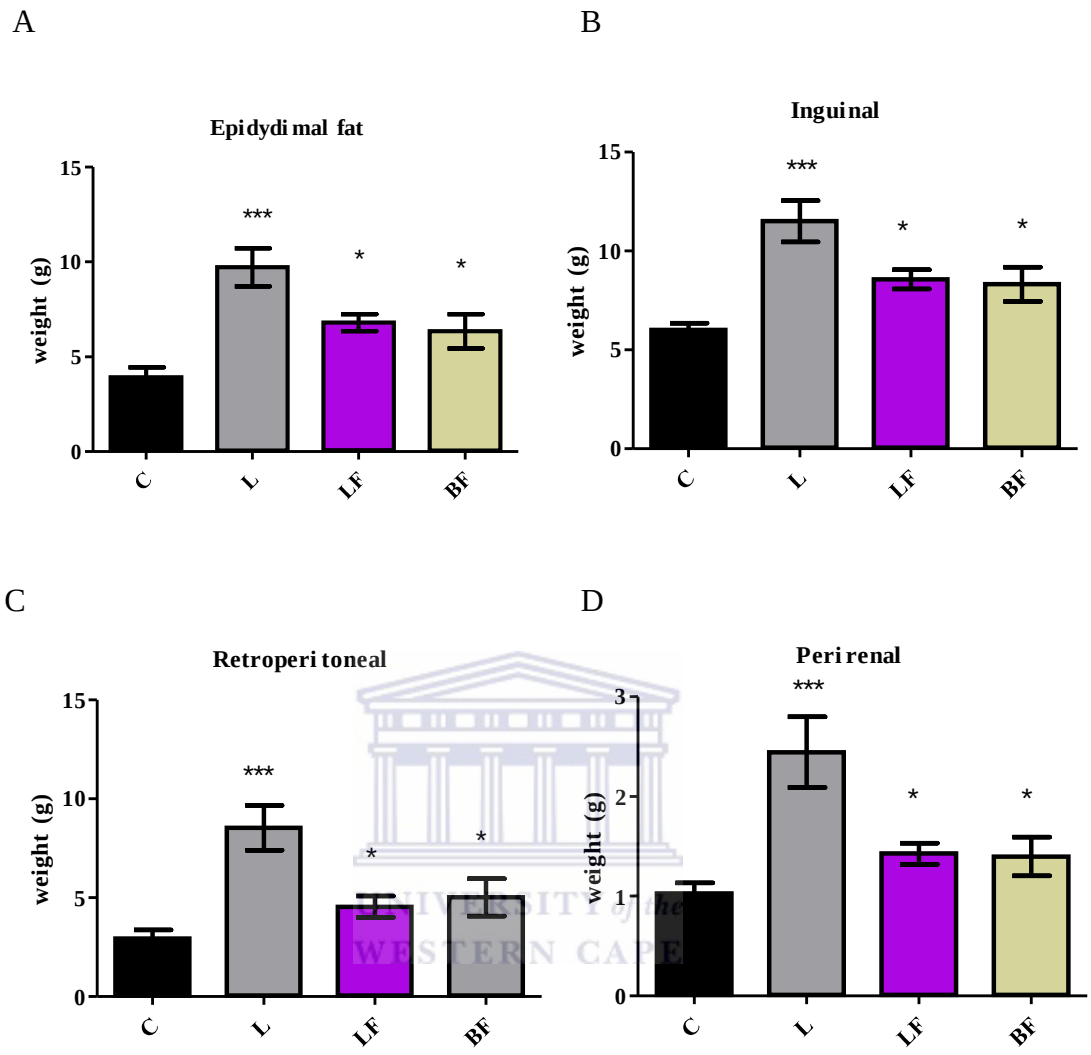


Figure 3.7: Comparison of fat pads for the groups on high fat diet to that of a control group

The figure shows the weights for epidydimal (A), inguinal (B), retroperitoneal (C) and perirenal (D). The weights for the rats on high fat diets were compared to the weight of the rats on maintenance diet. Fat tissue weights were significantly high for the lard fed group (L) $p < 0.001$ (***) compared to the maintenance diet fed group (C). The lard fat free fed group (LF) and the butterfat fed group (BF) showed significantly heavy fat tissue weights $p < 0.05$ compared to C group.

Adiposity index showed a similar pattern to the weight contribution of the fat pads. The tissue weights were not significantly different Table 3.2 shows the weights. The difference seen when analysing the fat tissue is not shown on tissue weight.

Table 3.2: The mean tissue weights for the rats in different dietary groups at termination

Tissue	C	L	LF	BF
Liver (g)	13.91 ± 0.50	14.74 ± 1.28	13.63 ± 0.54	13.19 ± 0.42
Pancreas (g)	1.81 ± 0.13	1.61 ± 0.13	1.97 ± 0.19	1.84 ± 0.07
Kidney (g)	3.26 ± 0.10	3.00 ± 0.07	2.97 ± 0.08	2.93 ± 0.08
Adrenals (g)	0.035 ± 0.00	0.040 ± 0.00	0.040 ± 0.00	0.040 ± 0.00
Brain (g)	2.00 ± 0.05	2.01 ± 0.05	1.94 ± 0.04	1.97 ± 0.05
Heart (g)	1.41 ± 0.06	1.63 ± 0.09	1.40 ± 0.06	1.34 ± 0.05
Spleen (g)	0.81 ± 0.03	0.69 ± 0.07	0.79 ± 0.04	0.80 ± 0.02

3.6 Discussion

The monitoring of parameters that can contribute towards positive energy balance entails measurement of a numerous aspects from food content to behaviour. As stated before the obesity epidemic is in part attributable to global nutrition transition, that sees the shift from the traditional plant-based foods to consumption of a more energy-dense diet (rich in animal fat, sugar and processed foods), reduced fiber, and a rise in sedentary lifestyles (Kimokoti and Millen, 2011) there is a need for scrutiny in studying obesity. The variation of dietary content is at the fore in the studies of obesity. Previous studies report that fats promote overeating and the development of obesity due to conversion of dietary fat to body fat at a low energy cost, high palatability of high fatty foods and low satiation from fat, calorie for calorie, than carbohydrates and proteins (Rogers, 1990).

In the current study the same batch of chow was milled and the diets were prepared for different groups to minimise variations. Because the fats that were used to prepare diets differed especially for the BF diet, in which butterfat was employed in its preparation, fatty acid analysis was performed. Compared to the control group the high fat diet fed groups contained high saturated fats. The high fat diet fed rats has been proven to increase body weight in many studies including the current study (Sibuyi, 2009). Our diets had a common factor which is the high fat compared to a similar study that focused on high fat low carbohydrate and a fat-sugar combination diet compared to the standard chow diet for 24 weeks (Pritchett and Hajnal, 2011). Both studies show high weight gain for rats on high caloric diet.

High protein diets have been found to reduce intake in days after starting feeding them to rats followed by gradual but not complete return to normal energy intake (Bensaïd *et al*., 2003). In most studies the high fat diet is the most studied diet, with high protein diet and high carbohydrate even less common, hence the interest in studying high fat diet because most of the complications have been identified using high fat diet. Fatty acid content and the types of fatty acids contained in diets have been of concern when studying obesity with studies proving the reduction of circulating plasma free fatty acids with reduction in dietary fat content (McCarty, 1995). The plasma FFAs were not measured in this study, of which knowing the fatty acid levels was going to be helpful especially when done together with insulin measurements in identifying the fatty acid effect to insulin.

The plasma glucose levels in rats that were fed high fat diets did not differ significantly to that of the control group. Glucose at baseline was the same for the groups followed by the rise at day four. It is not clear why there was a rise because this applied to the control group as well. At termination the glucose levels of the BF fed group were high which can be the high content of saturated fed contained in the diet. Previous studies have reported the high fat diets to induce obesity with glucose levels not differing significantly to the control groups for diet induced obese rats (Buettner *et al.*, 2006). Despite the fasting and post-prandial glucose that is misleading it has been proven that there is a glucose intolerance that kick starts as the intake of high fat diet progresses (Lichtenstein and Schwab, 2000). These results were seen in the studies where glucose tolerance and insulin tolerance tests were performed and those were not done for the current study (Buettner *et al.*, 2006).

Feeding high fat diet to rats results in the accumulation of fat which is reported by many studies (Choi, 2010). Furthermore there is a dependency on the proneness of the rat to develop obesity (Gao *et al.*, 2002). In our study we considered resistance to obesity by selecting high weight gainers to study them further. The studies similar to our study considered both low weight gainers as means of studying their source of resistance to obesity (Joo *et al.*, 2011; Choi *et al.*, 2010). The contribution from fat is high for the high fat diet fed studies due to the reason that high fat feeding promotes storage of fat.

Weighing the fat pads distinguished between the high fat diets fed groups from the control group to verifying that fat contributes a lot to the high body weights shown by high fat diet fed groups. This is also reported in other studies that the long term feeding of high fat diet, that is 8-14 weeks yield high adiposity indexes in rats (Nascimento *et al.*, 2011).

The diets used in this study showed different levels of obesity induction with high fat deposition showed by the lard diet. In an unpublished study in our lab lard diet showed to induce obesity for the female rats with protein malnutrition in their pups that showed low body size and poor hair growth. The use of the fat free powdered milk showed to have a difference in the pups' body size. This shows that the diets can contribute similar calories but dependent on the source the differences reflect in the body structure. The LF and BF fed groups showed significantly low adiposity indices compared to the L fed group. This could be possibly because of the calories in the diets that they were given that contributed to increment in other tissues either than fat tissue. Other organs that were weighed showed no significant difference. Although the body composition was not determined in this study the body weights were equal for the LF and L diet but different adiposity indices.

Chapter 4: Visualization and PD Quest quantification of differentially expressed proteins

4.1 Introduction

The emergence of proteomics as a discipline and a promising field is motivated by the need for the understanding of proteins as the functioning machinery within a biological system. Proteomics is has a potential of providing comprehensive qualitative and quantitative information on all the proteins, something that cannot be achieved using genomics or transcriptomics (Hebestreit, 2001). The closing of the gap in information at protein level that has been seen when using the genes or mRNA arrays is possible using proteomics since it is noticeable that the proteins are the ones performing the functions downstream and the ones that can address the phenotypic changes. An early diagnosis of obesity is required for timely interventions before the condition kick starts and get to extremities.

Serum potentially carries an archive of important histological and molecular information whose determination could serve to improve early disease detection (Tirumalai *et al.*, 2003b). The use of an animal model of diet induced obesity is at the forefront of solving the obesity epidemic since the previous studies showed the impact of obesity to be based on diet more than on genes as previously suggested (Holemans *et al.*, 2004). Caloric restriction still remains the most efficient way to promote weight loss (Viguerie *et al.*, 2005). Surely the profile of the proteins that runs in the blood can reveal information about the response post-translation through the studying of plasma or serum. Using the diet to study obesity is the

approach that will mimic closely the current situation in humans whereby people increase the intake of energy with reduced dissipation of energy, than focusing on the genes which are not likely to be causing such an increase in obesity pandemic. Genes can be expressed highly only to lack the enabling environment for the translation to take place. If the translation does takes place there are other forms that proteins undergo such as post-translation, these changes can only be seen when looking at the protein level. Proteomics is a discipline that enabled the creation of more knowledge about the changes that are brought about by the role the proteins play in the phenotype. With diet known to play a role in obesity development this study aims at looking for the changes that take place in the proteome in relation to the diet and time (changes that are brought by adiposity over time).

Serum proteins are a potential source of biomarkers. The challenge in the serum is the number of proteins that are highly abundant while the other proteins are present in low amounts. This calls for the depletion of high abundant proteins to make the visibility of low abundant proteins to be enhanced. Various ways were available to deplete abundant albumin those include methods that utilise high albumin affinity for certain dyes such as Cibacron-Blue and derivatives thereof (Chen *et al.,* 2005). Most of the methods that are used for the depletion lack specificity and hence the study by Chen, et. al. (2006) came with another perspective using trichloroacetic acid (TCA) a known method of protein precipitation which is forming an organic solvent soluble complex with albumin and acetone, which is the method that was adopted in the current study with some modifications. This modified protocol was used in our serum samples and keeping both the portions of samples to reduce the loss of information

that can arise from throwing one portion of the precipitated sample. Both the portions were analyzed using 1D SDS-PAGE and 2D SDS PAGE the same applied when analyzing the gel images.

4.1.1 Reduction of sample complexity by depletion of highly abundant proteins using TCA/acetone precipitation

Collected serum samples were processed using the TCA/acetone precipitation method, see Section 2.5.1. Two sets of protein profiles were obtained and separation of proteins using SDS PAGE showed a good resolution and distribution of the bands in the albumin containing pellet (Figure 4.1 A). As shown in Figure 4.1 B the separation was equally good in the albumin depleted pellet. The SDS PAGE for C, L, LF and BF showed to be visually similar. This shows that with careful preparation of samples the precipitation method yields comparable samples. The processing of serum samples using the TCA/acetone precipitation was applied because it is a method that can be used by any lab because the solvents are easily obtainable and are affordable.

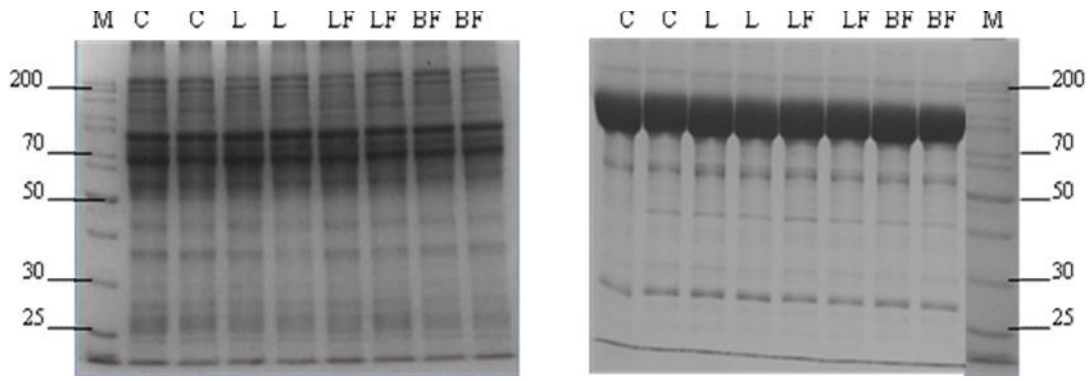


Figure 4.1: SDS PAGE albumin containing (A) and albumin depleted (B) serum sample from TCA/acetone precipitation

TCA/acetone precipitation of serum results in two portions, one with albumin depleted (A) and one that albumin rich (B). SDS-PAGE shows the profile for the groups on maintenance diet (C), lard diet (L), lard fat free diet (LF) and butterfat diet (BF). A pre-stained Fermentas protein ladder was loaded in each gel (M).

Splitting the proteome into two portions those are TCA precipitated and acetone precipitated is showing to be even beneficial when running the 2D PAGE electrophoresis. In Section 4.1.3 the separation of proteins in the 2D PAGE is discussed.

4.1.2 Analysis of protein concentration using Bradford method of protein quantification

After processing the serum samples the concentration of pellets obtained both albumin containing and albumin depleted pellets were quantified using the Bradford assay (Bradford, 1976), details are explained in Section 2.5.2. The concentrations for the samples were extrapolated from the bovine serum albumin (BSA) standard curve (Figure 4.2). The concentration of protein from albumin depleted sample was similar to that of albumin containing sample. This is showing that abundance that has been

reported for albumin and globulins in the serum and plasma samples is indeed huge enough to mask other proteins. Protein concentration was determined using the bovine serum albumin (BSA) standard curve. The albumin containing sample showed to have other proteins that precipitated with it with a possibility of some to be albumin binding protein.

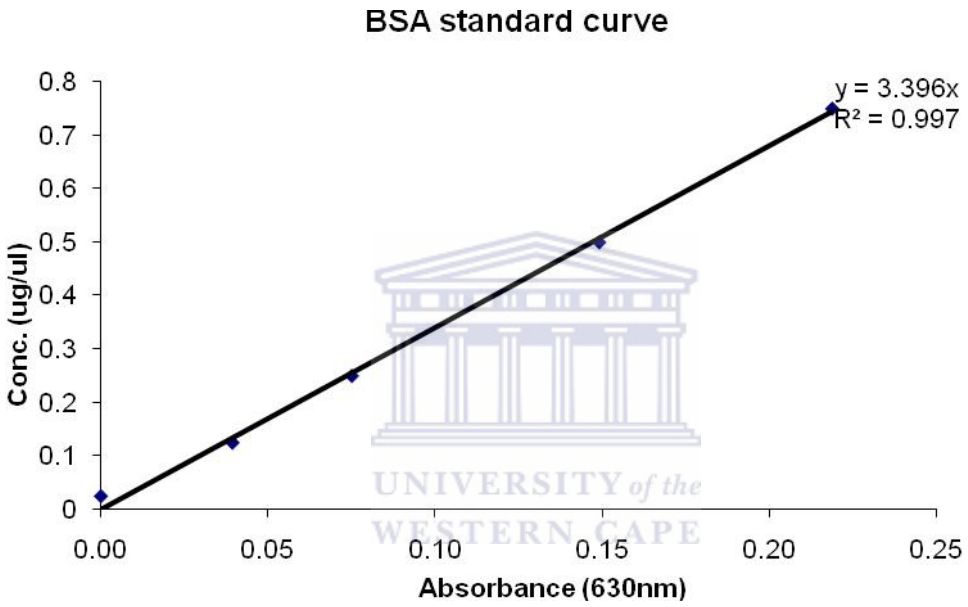


Figure 4.2: BSA standard curve representing the curves that were prepared for protein concentration throughout the project

4.1.3 Analysis of TCA/acetone treated samples using 2D SDS-PAGE

The serum samples were separated based on their pI for the first dimension and based on their molecular weight for the second dimension using the 2D PAGE (Section 2.5.4). Separating proteins using the 2D PAGE increased number of proteins that can be visualized as shown by a number of spots. The gel with the albumin depleted

sample contained many Coomassie visible spots (Fig 4.3. A), compared to the one with albumin (Fig 4.3. B). Albumin occupied a biggest portion of the gel showing albumin to contribute more to the concentration of the albumin containing pellet. Some spots showed to be on the same molecular weight with continuous shift to the side. This is mostly due to phosphorylation of proteins which affects the acidity of the protein shifting it to alkaline direction. Both gels were used for image analysis to ensure that the loss of information due to precipitation is minimized. If the 2D gels or SDS PAGE were to be superimposed it shows that some proteins that appear better in the albumin depleted sample would have been masked by albumin. The albumin depleted and albumin containing sample is shown in Figure 4.3 A and B respectively.

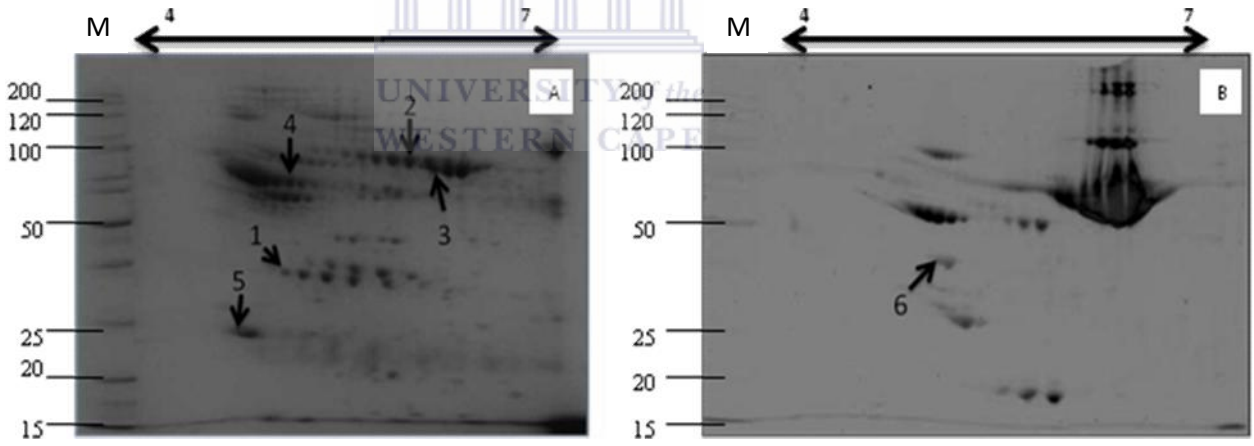


Figure 4.3: 2D gel image showing albumin depleted (A) and albumin containing sample (B).

The spots labelled 1-5 are the spots that were differentially expressed in the albumin depleted serum sample with obesity development (details in the following sections). The spot labelled 6 was differentially expressed in the albumin containing sample.

4.1.5 Analysis of differentially expressed proteins using PD Quest software

Two-dimensional gel electrophoresis was used to separate the protein. After 2D gel separation gels were stained with Coomassie brilliant blue and distained. Distained gels were scanned using Pharox FX imager system and images were stored as Quantity One images. The stored images were cropped and processed to prepare them for the PD Quest analysis. Cropped images were loaded in the PD Quest software. The scanner can be set to cover the scanning area based on the required information. The replicates that were used per group were three from four dietary groups giving us twelve gels in total Figure 4.4 page 113. Twelve gels (three replicates from each dietary group) when subjected to PD Quest the number of Coomassie visible spots were reported automatically by the software. A total of 154 spots were identified and matched manually for the differential expression between control group and high caloric diet fed groups. In the albumin depleted sample 64 spots were analyzed and 1 spot was differentially expressed.

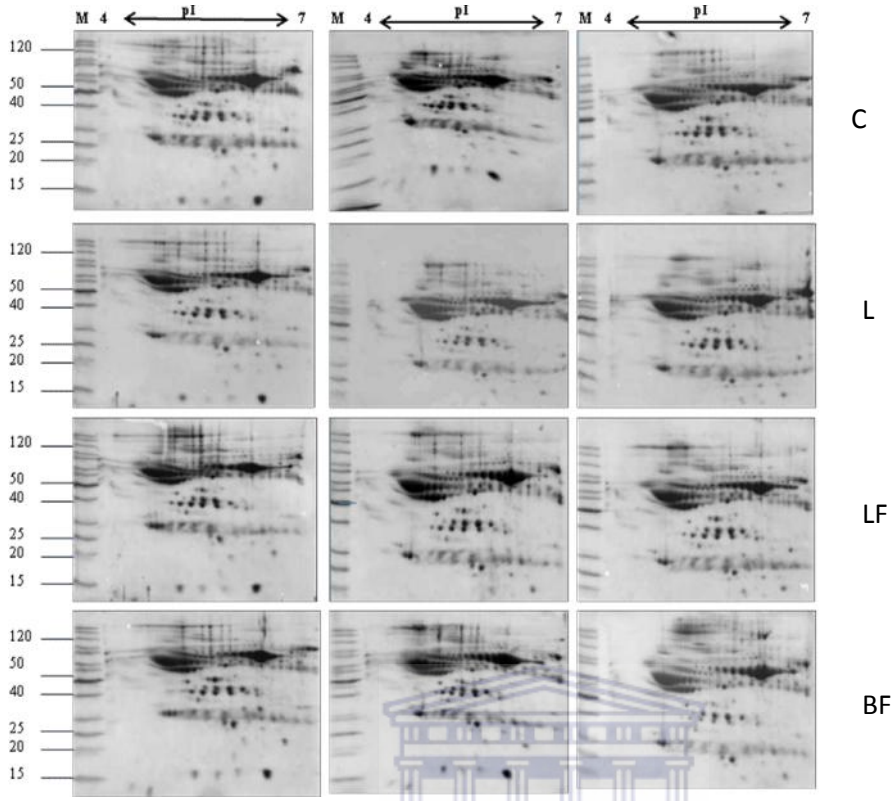


Figure 4.4: 2D Gel images scanned for PD Quest analysis with three replicates per group.

Images showing the replicates in four rows with C fed group (top row), followed by BF, LF and L fed groups respectively. Moving across are the best three representative gels from each group.

The same approach was used for the albumin containing pellet (Figure 4.5) where 12 gels that are consisting of three representative samples from each group were scanned at once using the same filter, cropped and PD Quest analyzed. As shown in the in Figure 4.4 the replicates that were used for analysis were scanned at once. Scanning gels at once reduces the variation that can be found between the gels. The same filter for PD Quest which is 2-fold was used for the identification of differentially expressed proteins. Two spots at the same molecular weight were identified to be up-regulated in the groups on high caloric diet from the albumin containing sample.

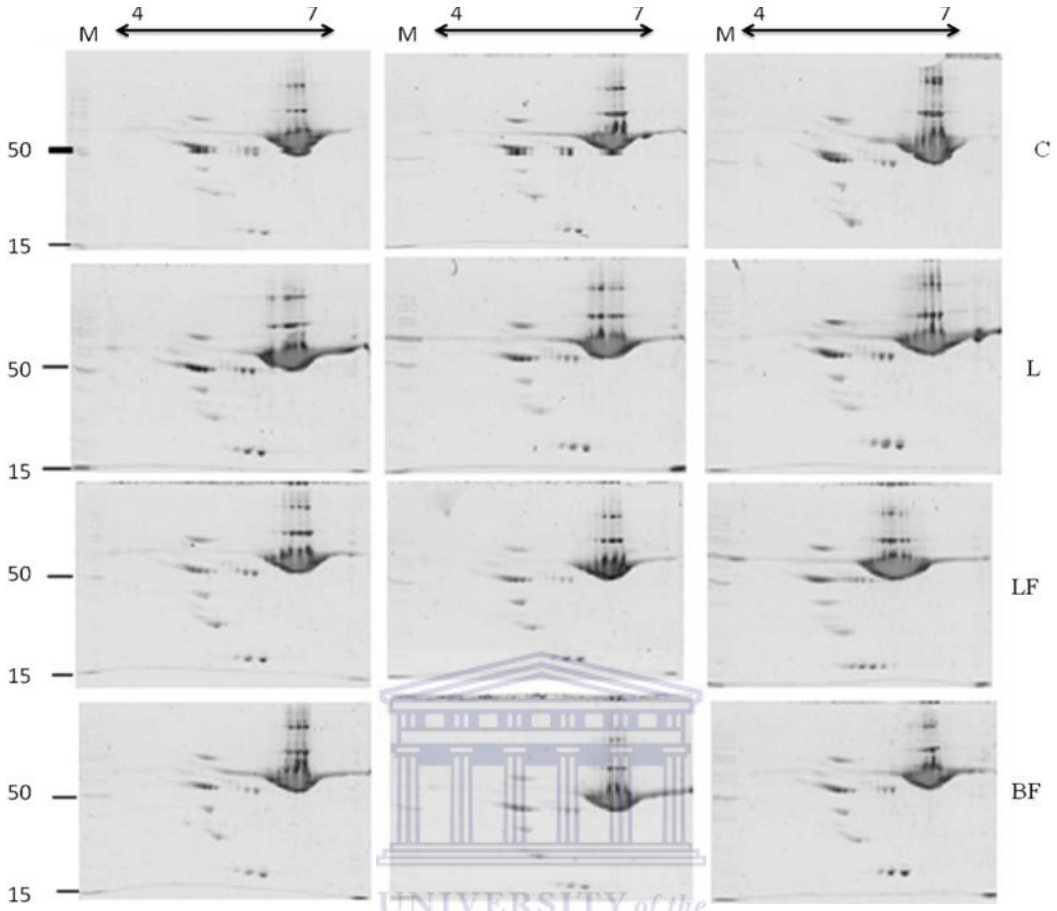


Figure 4.5: 2D gel images of albumin containing sample representative of four dietary groups.

Three replicates from each dietary group from left to right. The C diet replicates on the top followed by the BF, LF and L diet replicates respectively. Samples were run on a 4-7 pH IPG strip, M stands for the molecular weight marker Fermentas Page Ruler Unstained Protein Ladder.

Analyzing the spot by comparing the intensities using PD Quest gave the output of the spots that were two fold or more in intensity for high fat diet fed groups compared to the control group. The data could be obtained in a scatter-plot format for each high fat diet group compared to the control (Figures 4.6-4.8). The use of PD Quest is making things easier for the data presentation. The scatter plot is presenting the data visually with the spots that are outside the highest fold scale as the spots that are beyond 2 fold different compared to the control group. The regression line is the one that shows the similarity of the expression between the two groups.

For the easy tracing of the spot in question can also be presented as using the histogram representing each replicate. Upon clicking on the spot the spot number on the gel is labelled and that histogram gets presented. This is aiding in making sure that the spot and the expression is not mistaken for different spots. The results have been presented both on the scatter plot to show the number of spot that are beyond the set limit of 2 fold and the histogram to show the expression per replicate. The presentation on the histogram is also showing the number of replicates that were highly expressing a particular protein and the quantity and the statistics are used to determine if the protein is considered to be enhanced or suppressed in the test than in the control group. The Figures 4.12-4.16 are representing such figures that show the histogram presentation of the data.

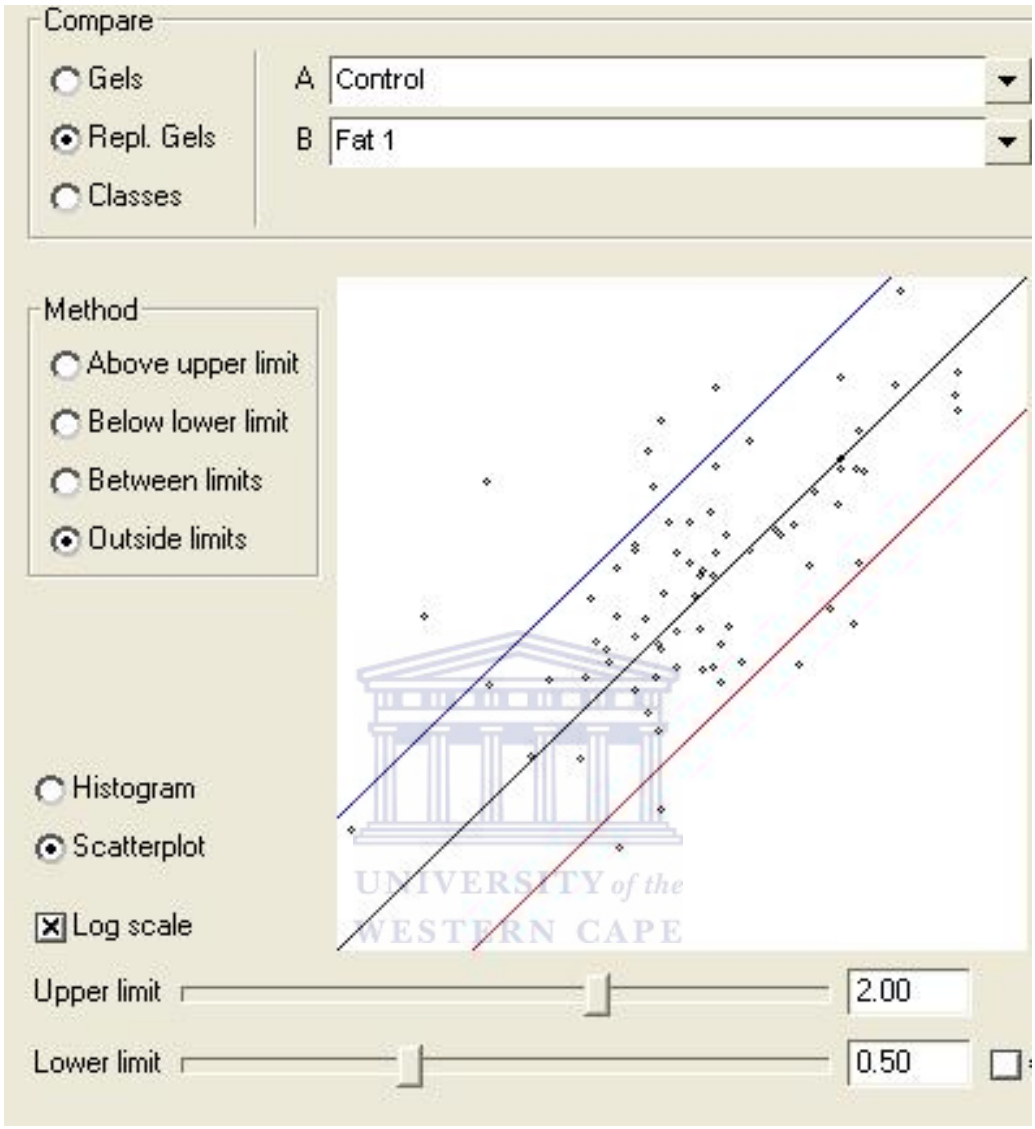


Figure 4.6: Scatter-plot showing the differences between spots for L fed group vs. control group.

Eleven spots that were identified to be quantitatively more than 2 fold different for the L fed group compared to the C fed group. Spots outside the blue line (above) that is six spots are enhanced while outside the red line (below) are five spots that were down regulated.

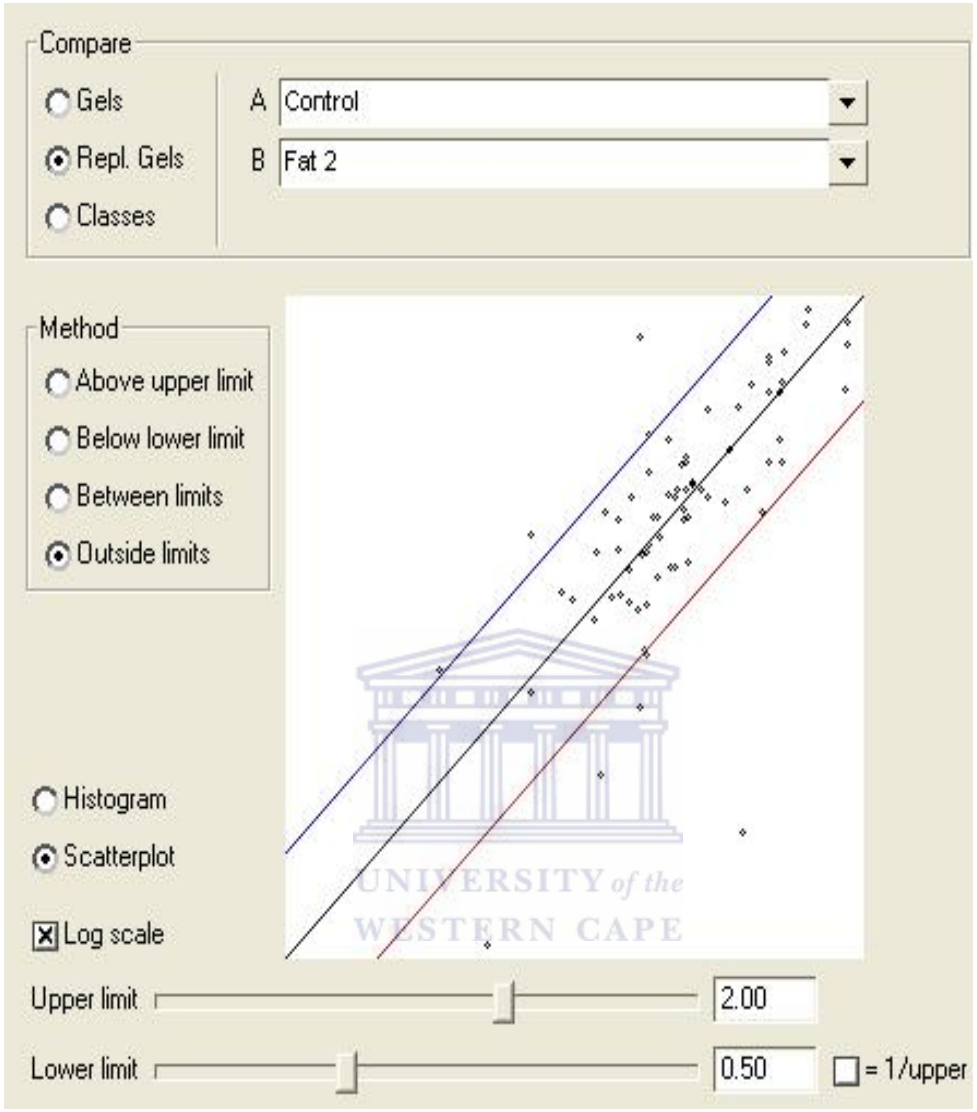


Figure 4.7: Scatter-plot showing the differences between spots for LF diet fed group vs. control group

The up-regulation of four spots seen for the lard fat free fed group when compared to the control group, with down-regulation of five spots (spots below the red line). The middle line is the regression line.

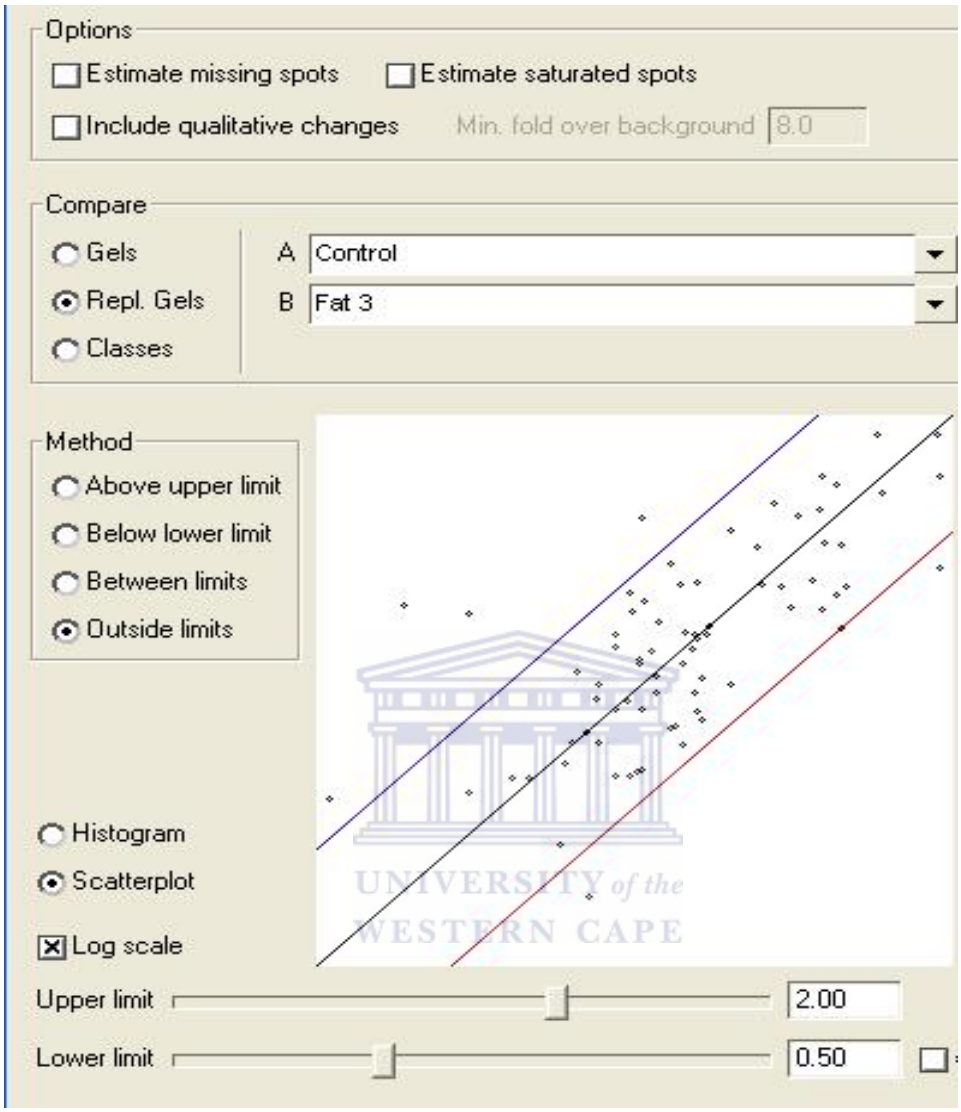


Figure 4.8: Scatter-plot showing the differences between spots for BF fed group vs. control group

Four proteins were up-regulated in the butterfat diet fed group (spots above the blue line), with two down-regulated proteins (spots below the red line) with the middle black line showing the regression line.

The comparison between the HFD fed groups showed that the spots that were differentially expressed for the L, LF and BF when compared to the C fed group are also expressed differently amongst groups. The L fed group according to the dietary

information is the group that contained the highest contribution of fat derived calories. The results in the previous chapter is showing the adiposity to be high for this group and the two other groups on high fat diet to be having less adiposity as revealed in the adiposity index. The L diet fed group compared to the LF fed diet group showed two spots that are highly expressed, those were spot SSP 0001 and SSP 7403. The expression of four spots was high in the L diet fed group compared to the BF diet fed group. One spot was less than two fold decreased in expression in the L diet fed group compared to both LF and BF diet fed groups.

The LF and BF diet fed groups also show difference in the expression of the proteins with four spots being highly expressed in the BF diet fed group. A single spot was suppressed in the LF diet fed group. The scatter plots shows how the HFDs fed groups compared are showed in the following figures. During the analysis Fat 1 is corresponding to the L fed diet, with Fat 2 being the LF fed diet group and the Fat 3 is the BF fed diet group. The Figures 4.9, 4.10 and 4.11 are the scatter plot representations of the groups‟ comparisons. The figures are showing the L and LF comparison, followed by the L and BF comparisons and the third scatter plot is the comparison of the LF and BF. The results are show that the expression is also different between the high fat diets fed groups. The results reveal that the difference in expression of a spot can be more than 2-fold for a particular HFD group compared to the control with a chance of a 2-fold more expression when compared to HFD group

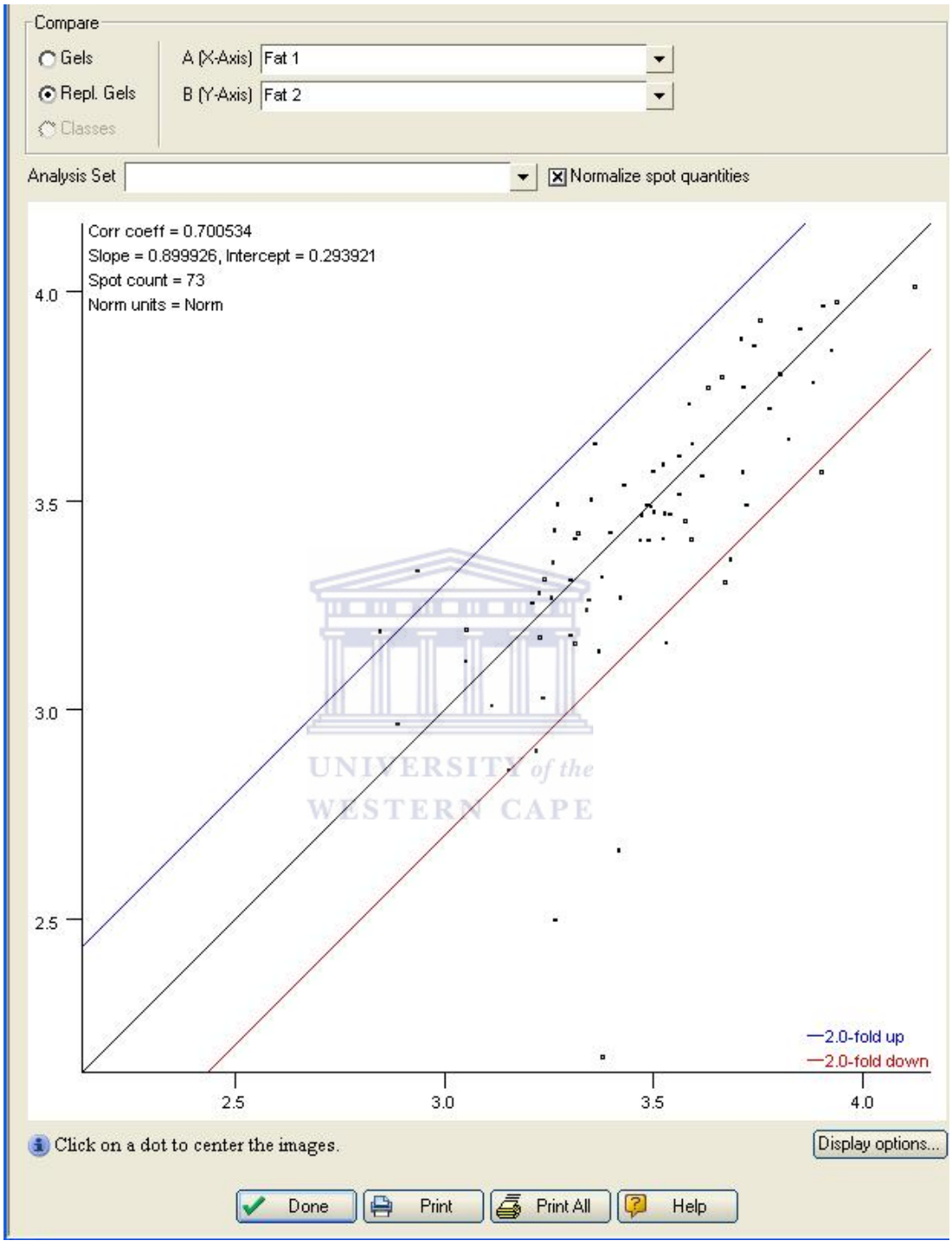


Figure 4.9: Scatter-plot showing the comparison of spots expression for L and LF fed groups

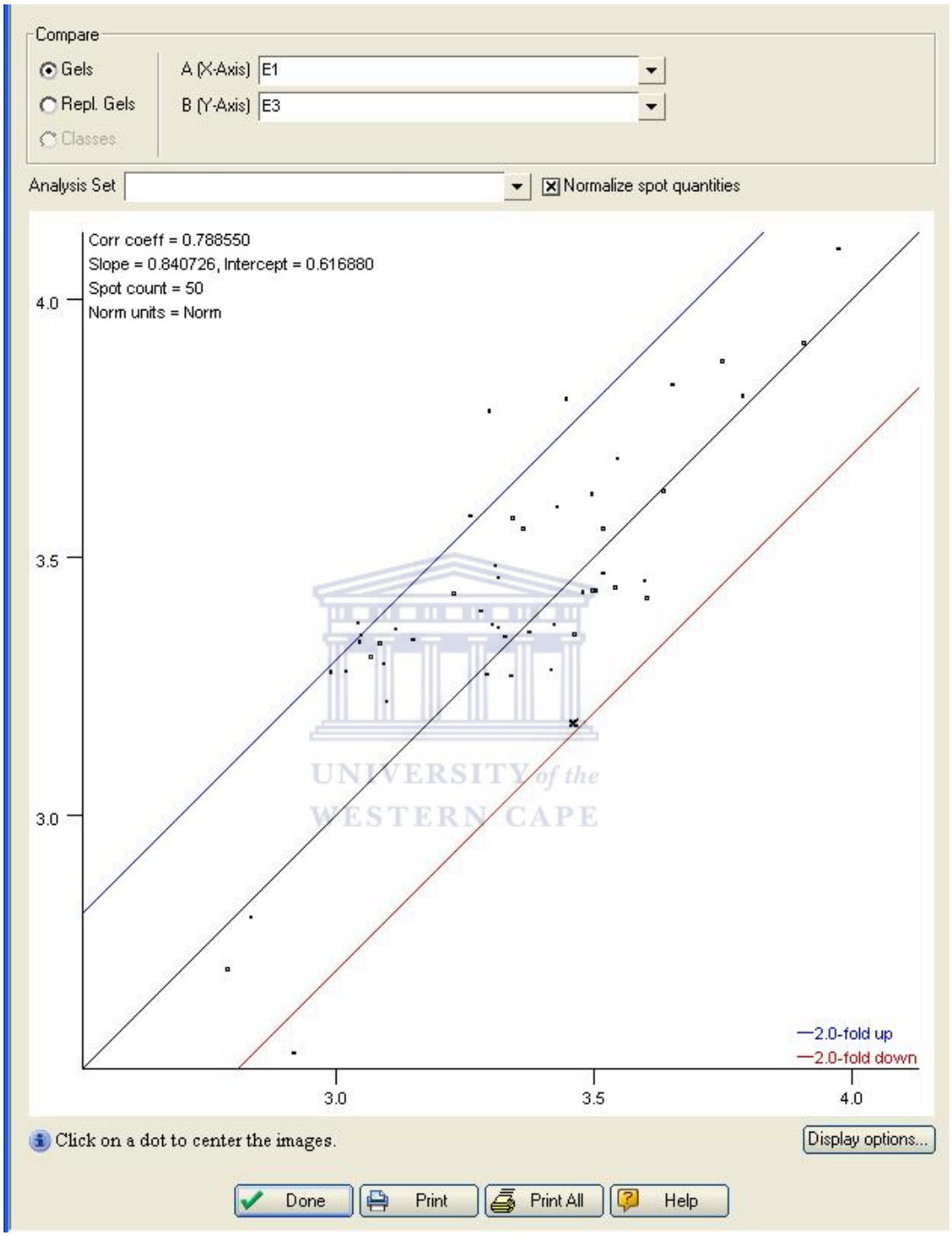


Figure 4.10: Scatter-plot of the L fed group compared to the BF fed group

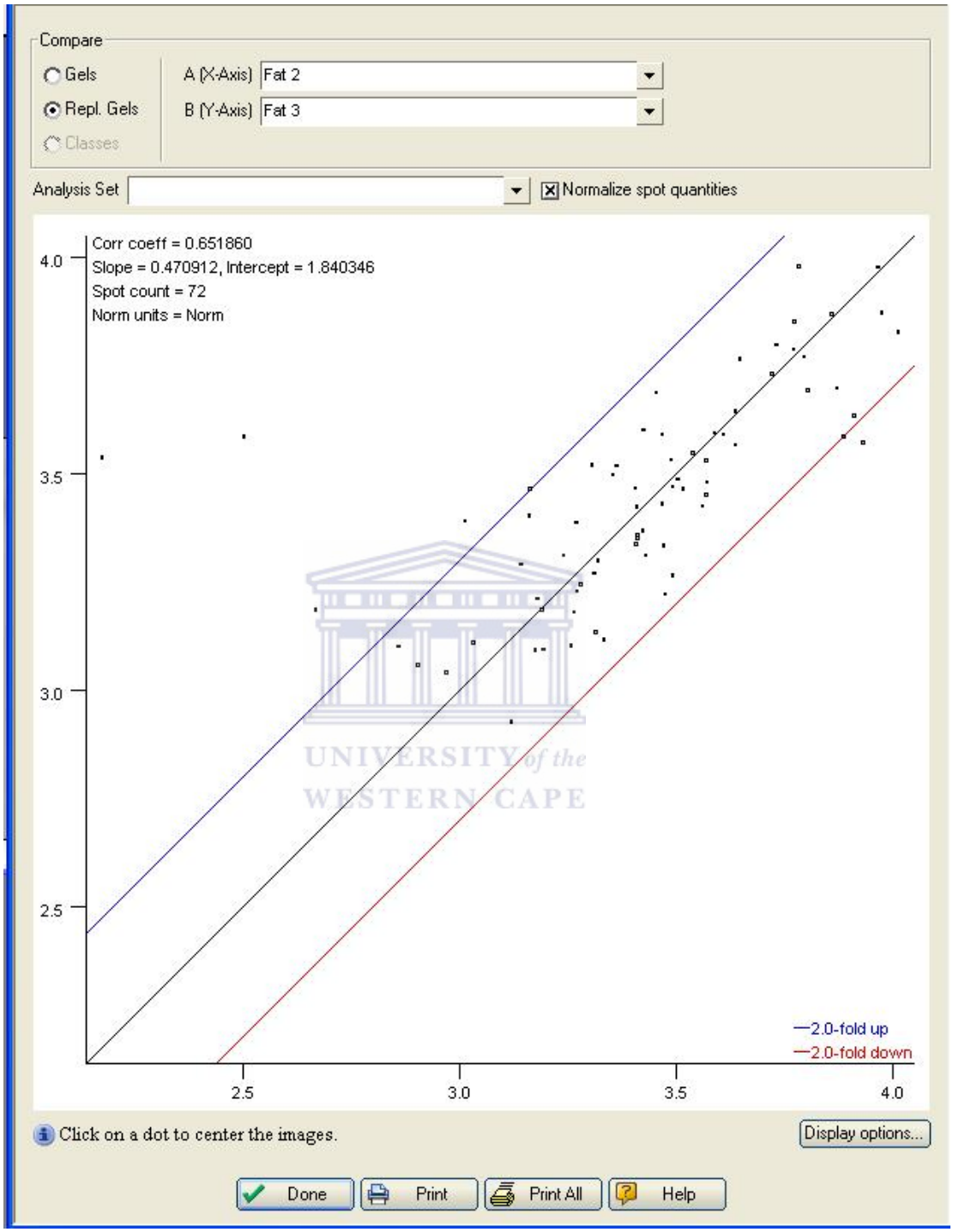


Figure 4.11: Scatter-plot presentation of the spots comparison between LF and BF fed groups

The spots that were differentially expressed between the groups were identified through clicking on the points that are beyond the limit (2-fold) on the scatter plot and get directed to the spot as it appears with a spot number showing on the gel. The following images show the spots that were found to be differentially expressed. To make it even easier to follow results PD Quest present tabulated and graphical results with markings on the gel images for an easy tracking of results. The protein identity was then obtained through mass spectrometry analysis or using the 2D map for the excision of spots for in-gel trypsin digests and mass spectrometry analysis. In the current chapter the spots will be given numbers (1-6) and the identification of the protein spots is discussed in the next chapter. PD Quest showed spot 1 (SSP 1101) to be highly expressed in the control group.

The spot was showing to be reduced with obesity development. The expression of SSP 1101 was very low to nearly fading on the 2D gel for the L group. The other two groups were also having a less intense SSP 1101. The adiposity index is following the same trend that was shown by the spot because as the adiposity index gets high the expression of the spot is getting reduced. This spot could be a potential marker for the fat deposition. Even the fat pads for the L group were heavier than that of all groups which tells the weight of the whole animal to be contributed by the fat than the lean body mass.

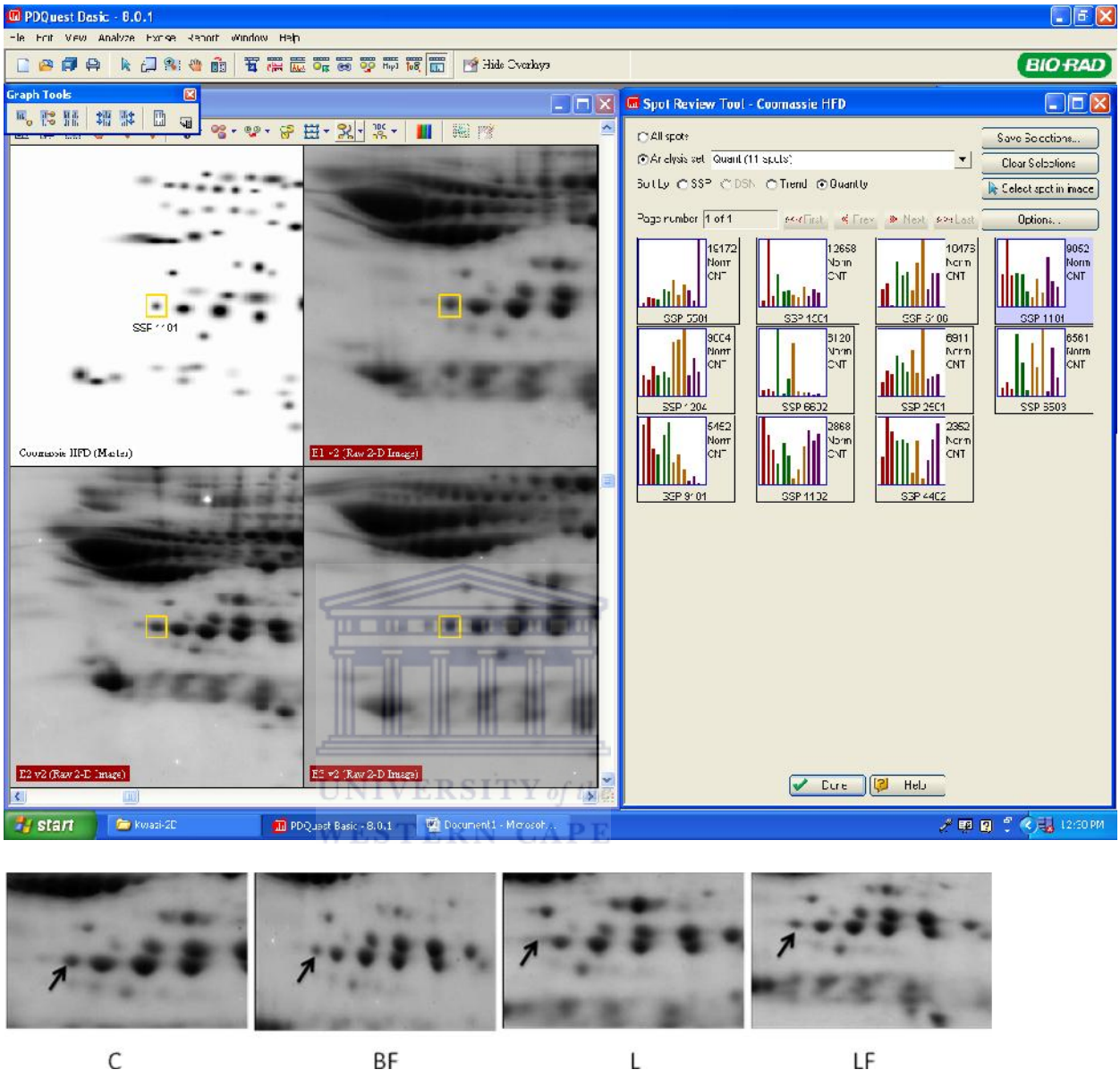


Figure 4.12: The PD Quest outcome of the analysis showing the differential expression of spot 1 corresponding to SSP 1101

The top figure shows the representative control replicates and the master gel with the spot that was analyzed marked with a yellow square. On the right hand side is the histogram corresponding to the spot number showing the replicates‟ expression of the protein from the four dietary groups. At the bottom are cropped images that show the

reduction of the spot intensity with the most fading spot being the spot from the L group and BF and LF also showing reduction of the spot 1 compared to the control group.

The PD Quest results were giving the output as statistical and quantitative results. The spots that were statistically significant and showing more than 2-fold difference were considered. The expression of spot 2 (SPSS 7403) showed to be high for the groups on high fat diet compared to the control group. The expression of spot 2 was low for all the animals in the control group and a representative scatter plot shows this (Figure 4.9).

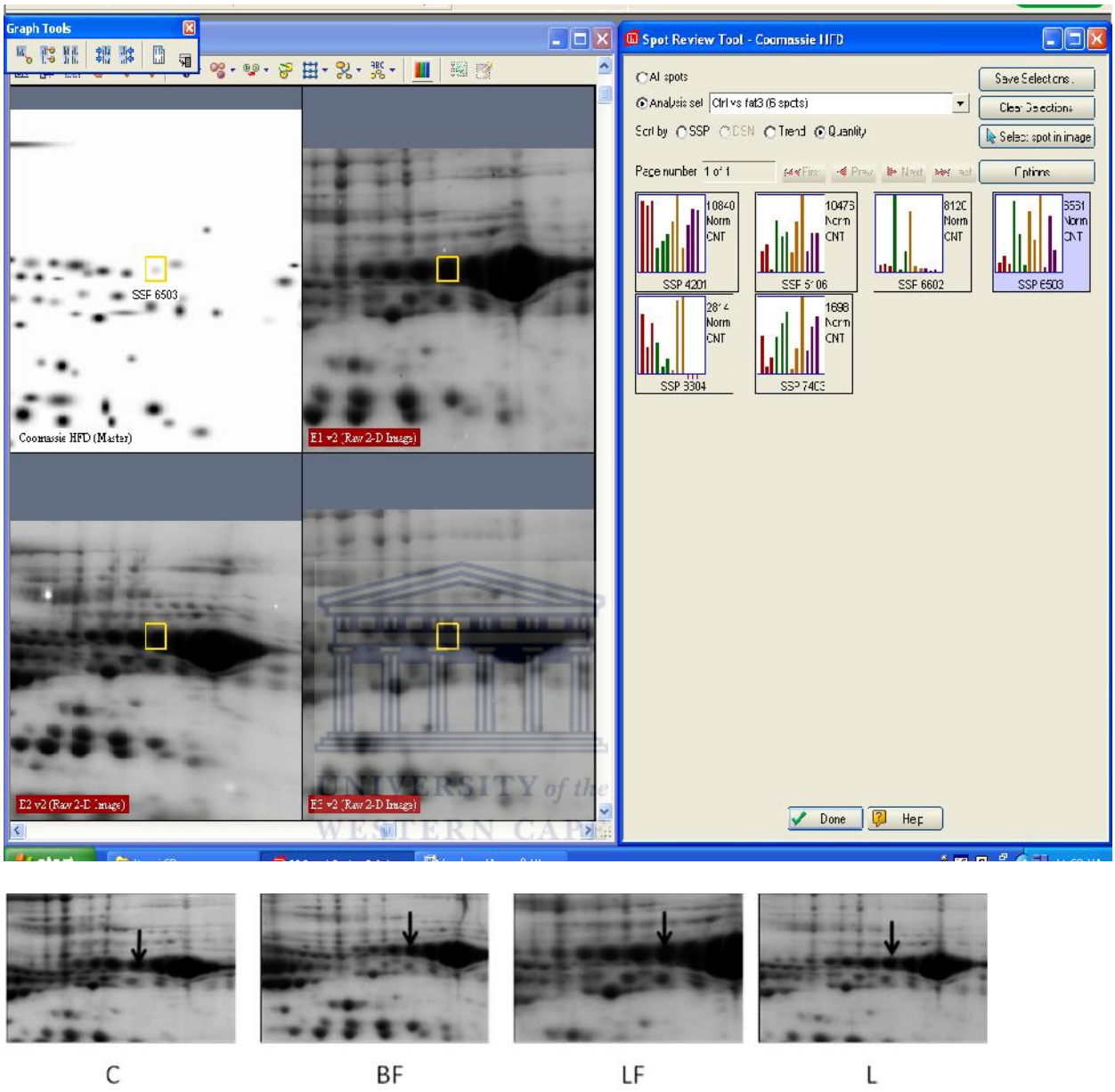
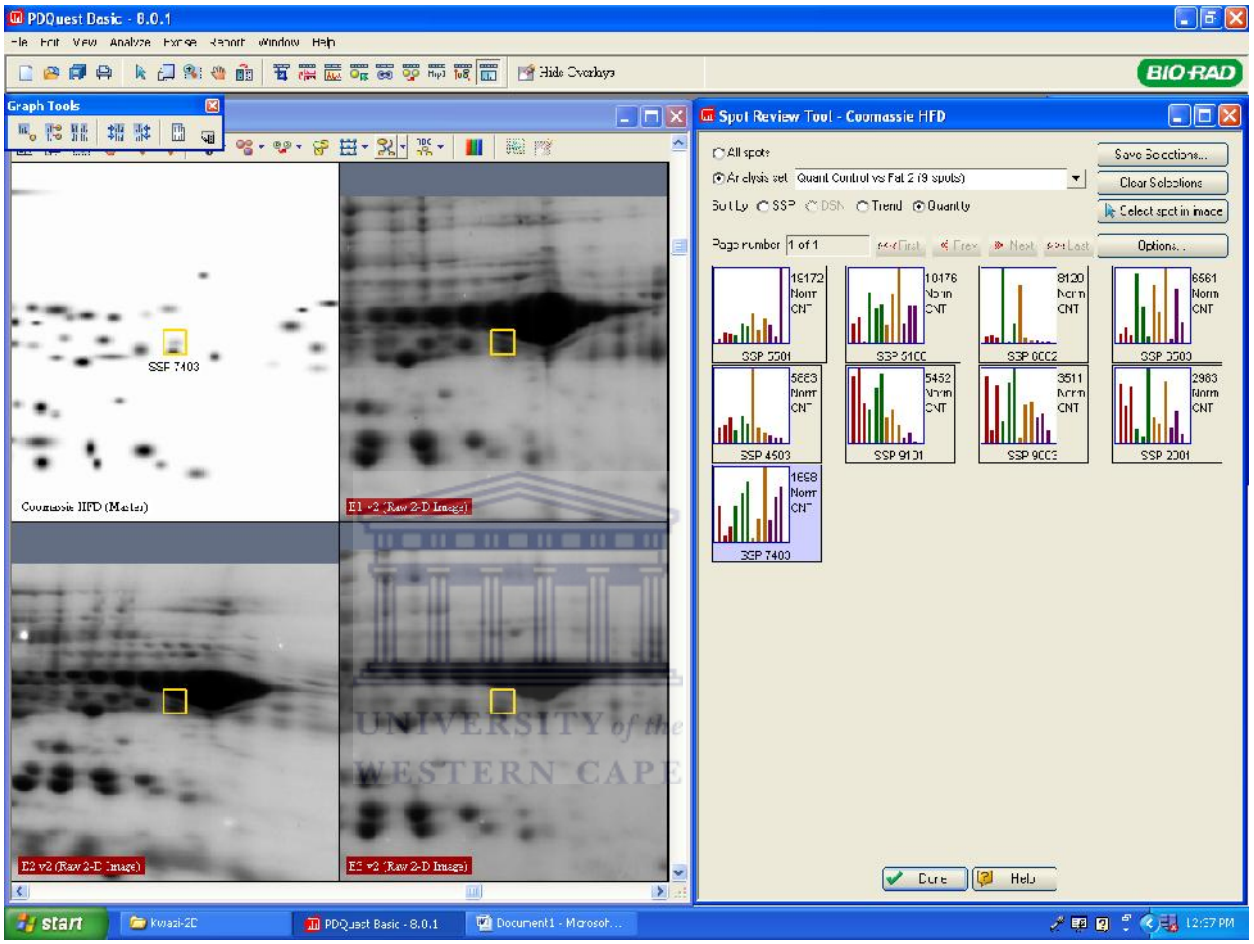


Figure 4.13: Histogram PD Quest output of the differences in spot 2 expression in different groups which was generated as SSP 6503

The spot that is analyzed is marked with a yellow square on the left pane of the window, with a histogram highlighted on the right showing the SSP 6503 (spot 2) expressions for C, L, LF and BF respectively. At the bottom are cropped images of representative gels that show the appearance of the spot in four groups.

The protein spots that were also up-regulated by feeding of high fat diet to the rats were spot 3 (SSP 7403) and spot 5 (SSP 0001) shown in (Figure 4.10 and 4.11).



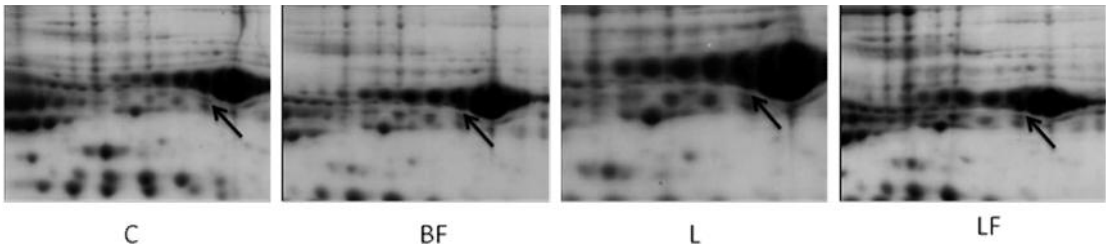


Figure 4.14: Figure 4.14: PD Quest results of the expression of spot 3 which had software generated number as SSP 7403

The spot number 7403 is shown on the master marked with a yellow square in all the gels marking the spot. The right pane is showing the histogram presentation of the spot expression. At the bottom are cropped representative images of the spots.

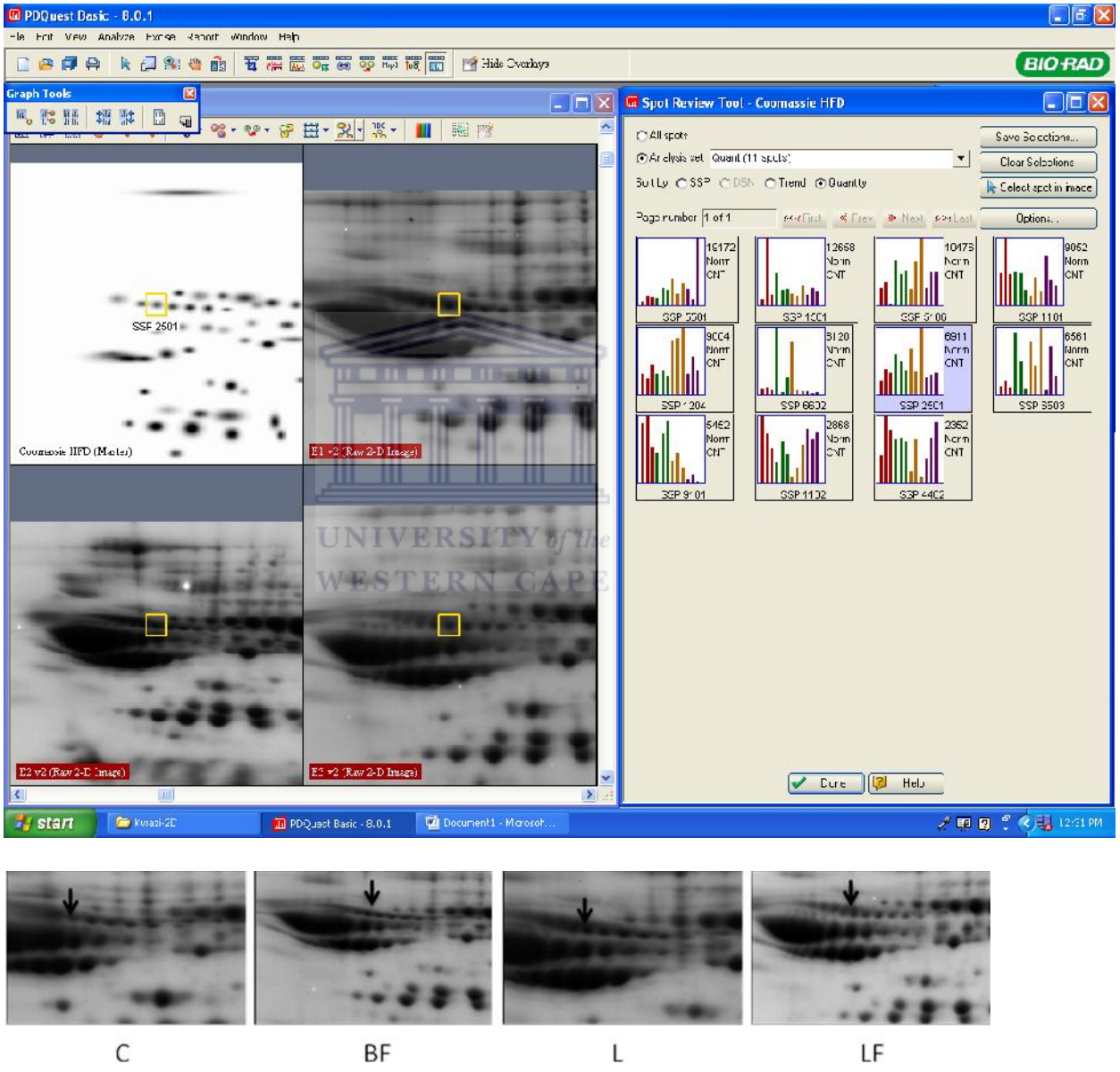


Figure 4.15: PD Quest results showing differential expression of spot 4 with software assigned number SSP 2501

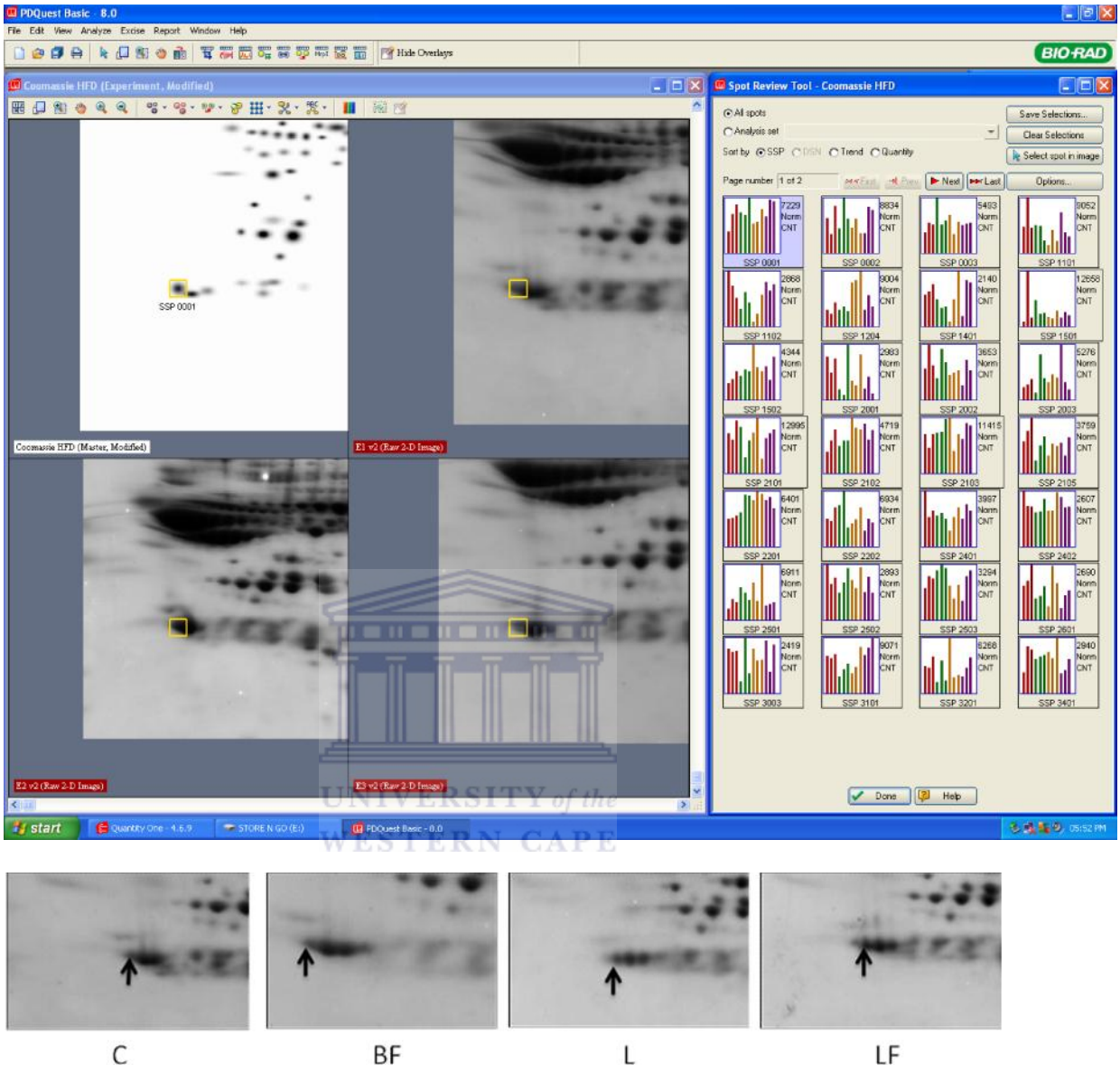


Figure 4.16: Figure 4.16: PD Quest analysis of the SSP 0001 (spot 5) with differential expression showed in the cropped images

The spot from the albumin containing were analyzed as well for the differential expression. One spot was more than 2-fold highly expressed for the groups that were fed high caloric diets. The spot also proved that the keeping of the albumin containing sample. The results are shown in Figure 4.16 for PD Quest

histogram result and the cropped images to show the representative spots per group for the spot 0001. This spot was differentially expressed in HFD groups compare to the control group with different intensities. The expression pattern followed the adiposity indexes. The L diet fed group expressed the spot more than the LF and BF fed groups. This spot could be the potential spot for the differentiation of the fat deposition which is the main identifier of obesity.

Discussion

Proteomics is promising great breakthroughs in the future based on the number of protein related information that can be revealed in a single experiment. The impaired protein synthesis in the muscle due to diet induced obesity shows the potential of the proteomics in understanding body processes (Anderson *et al.*, 2004). A study whereby the imaging software was used to get variation of the results, reports that there are variations even in two versions of the same analysis package after post hoc analysis (Stessl *et al.*, 2009). It is without a doubt that the plasma and other body fluids have played a major role in quick prognosis and diagnosis of diseases, but the majority of the findings were obtained while studying a single protein at a time.

The abundant proteins are the main concern in working with serum and this is the main focus in improving the number of candidate proteins that can be used as biomarkers. Albumin constitutes more than 50% of the total serum proteins, when removed it enables the increase in protein loads on immobilized pH gradient (IPG) gels with better visualization of low-abundance proteins, which may also result in the

loss of other albumin-bound low-abundance proteins (Sahab *et al.*, 2007). We used the organic solvent precipitation approach and kept all the samples that result from our precipitation to allow for the prevention of the loss of the albumin-bound low-abundant proteins as covered above which was seen to be the case in the samples where dyes are used for depletion of albumin. Pre-analytical variables have been seen to be key role players as well in the biomarker discovery in serum. The study by Ahmad et al. (2009) reported the variation of spectral intensities of the samples in relation to the storage time of the serum samples with the storage of samples for 10 months being the longest time for serum. We performed our proteomics work and identifications in less than 10 months which might be good for the results obtained and the identifications through peptide mass fingerprinting.

The use of 2D PAGE came with solutions in the analysis of many proteins at once. Iso-electric focusing (IEF) is having various pH gradients from narrow range to wide range. There is improvement in the resolution of proteins with the use of the narrow range strips because the narrow range is spreading over a greater physical distance (Issaq and Veenstra, 2008b). This allows the separation of the proteins with a similar pI to be separated with high resolution. Our study compared to the studies by Choi et. al (2012) mentioned above have fewer proteins that were identified to be differentially expressed which can be contributed largely by the strip size that was used in the studies. We used the mini strips, whereas they used the longer strips and stained with silver stain which is a sensitive stain. This study is an initial stage to finding the more responsive proteins in more than one diet. It also utilizes the widely used laboratory material and affordable techniques for laboratories. Our findings are

showing the proteins that are more obesity regulated than diet dependent proteins.

The spots that were revealed in the PD Quest analysis were found in the serum depleted and serum rich portion. Previous studies usually use other methods of depletion for abundant proteins. The proteins that were identified were below 200 kDa in the ladder. The identities of the spots that were differentially expressed are not known without the spots being identified.

Chapter 5: Identification of proteins by MALDI-TOF MS

Introduction

The utilization of image analysis for quantification of protein expressions in-gel is a powerful tool to show the proteins that are responsive during the diseased state or an unpleasant condition development. The number and the level of response of protein spots are not enough and they give limited information unless the spots are identified so as to get more details of the proteins identified in conjunction with the disease in question. The success of 2D-PAGE in separating the proteins came with challenges including identification. The identification of proteins over time was achieved by co migration of sample with purified protein or blotting as the main methods (Rabilloud *et al.*, 2010). The use of Edman's sequencing has been a gold standard for a long time but the protein to be identified need to be pure which is not accommodating 2D-PAGE proteins because of quantities required for analysis.

The numbers of proteins that are to be identified are usually high because 2D gel resolves even more proteins. This then require an increase in the identifications that can be made in a short space of time. The use of mass spectrometry is at the core of analysis and the accumulation of results is enhanced through mass spectrometry. MALDI TOF mass spectrometers are the most commonly used for their relative ease of use enabling even a non-specialist lab to readily access the technology for biomolecular analysis e.g. protein identification through peptide mass fingerprinting (PMF) (Zanders *et al.*, 2012). Proteins at their femtomole quantities can be identified following the excision of the protein from the gel, washing and destaining, reduction

and alkylation, in-gel trypsin digestion, MALDI TOF MS of the tryptic peptides and database searching of the PMF data. This method can manually process up to 96 proteins at once (Zanders *et al.*, 2012). This is showing an improvement that is of benefit to clinical studies as well that will bring high throughput. Versatility of MALDI TOF MS is recognised across different platforms that utilize the technology. In the chromosome analysis it is able to distinguish the difference in genes even if the difference is a single nucleotide (Hahn *et al.*, 2008).

In the current study the proteins that were found to be differentially expressed (Chapter 4) were subjected to the MALDI TOF analysis and four proteins were successfully identified. There were proteins were not identifiable but those proteins were identified from the serum proteome map (Gianazza *et al.*, 2002b). The identified proteins were searched for further information on databases. The results for the MALDI TOF analysis and the outcomes are discussed in the following sections. The identification of proteins was validated using the Western blot. The validation and the bioinformatics data mining are reported in chapter 6. The current chapter covers the PMF and map identified proteins.

5.1 Analysis of in-gel digested proteins using MALDI TOF MS and reference map

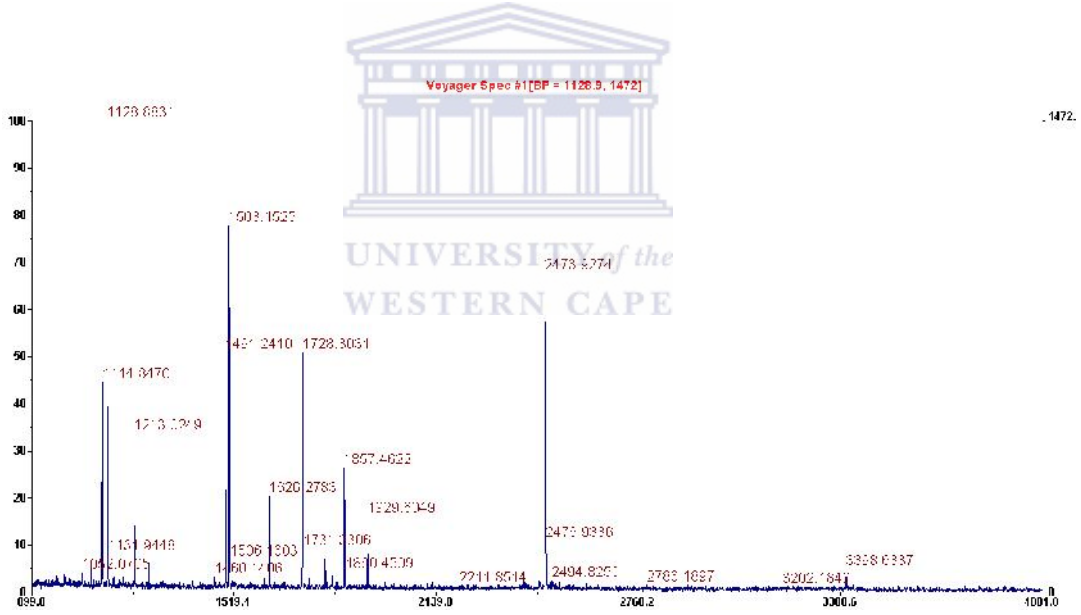
The spots that were identified to be differentially expressed (Chapter 4) were excised and in-gel digestion followed by identification (performed as detailed in Section 2.5.7). The identification of protein has improved since the use of mass spectrometry. In this study we used matrix assisted laser desorption/ionization time of flight (MALDI-TOF MS) for peptide mass fingerprinting. The proteins that were considered for identification were five in the albumin depleted sample and two spots in the albumin containing sample. Three spots in the albumin depleted sample were identified to be Hemopexin, alpha 1 macroglobulin and C-reactive protein. The other two proteins could not be identified. The mass spectra and amino acid sequences are shown in (figure 5.1-5.4). Apolipoprotein AIV was identified for the two spots that were excised from the albumin rich sample.

The identification of the proteins by MALDI-TOF was performed on digested peptides with an aid of the database searches to match the peptides. The MASCOT database was used with consideration of the identities that have the high MOWSE score. The identified peptides (matched peptides) are shown in red in the sequence as given with every spectrum in the results section. This section shows the outcome of the MALDI-TOF MS in identification of the in-gel digested proteins from the spots that were identified to be differentially expressed in the high caloric diet fed rats.

The identifications were corresponded with the 2D maps on serum that were published before. The study on inflammation (Giannaza et. al (2002) that used serum that contained most of the proteins labelled was considered for the identification of the

proteins that were not identifiable using MALDI-TOF MS. The confirmations of the proteins identified were performed using Western blot and the results are contained in the next chapter.

In the depleted sample the proteins that were identifiable using mass spectrometry were three in total. The other two proteins were identified from the 2D map as explained above seeing the matching of the mass spectrometry identified proteins with the identities on the map. Mass spectrometry identified proteins include the hemopexin (Figure 5.1).



1 MARTVVALNI LVLLGLCWSL AVANPLPAAH ETVAKGENGT KPDSDVIEHC

51 SDAWSFDATT MDHNGTMLFF KGEFVWRGHS GIRELISER**W KNPVTSVDAA**

101 FRGPDSVFLI KEDK**VWVYPP EKK**ENGYPKL FQEEFPGIPY PPDAAVECHR

151 GECQSEGVLF FQGNRKWFWD FATRTQK**ERS WPAVGNCTAA LR**WLERYYCF

201 QGNKFLR**FNP VTGEVPPRYP LDAR**DYFISC PGRGHGKLRN GTAHGNSTHP

251 MHSRCNADPG LSALLSDHR**G ATYAFSGSHY WR**LDSSRDGW HSWPIAHHWP

301 QGPSAVDAAF SWDEKVYLIQ GTQVYVFLTK GGNNLVSGYP KRLEKELGSP

351 PGISLDTIDA AFSCPGSSKL YVTSGR**RLWW LDLKSGAQAT WAELSWPHEK**

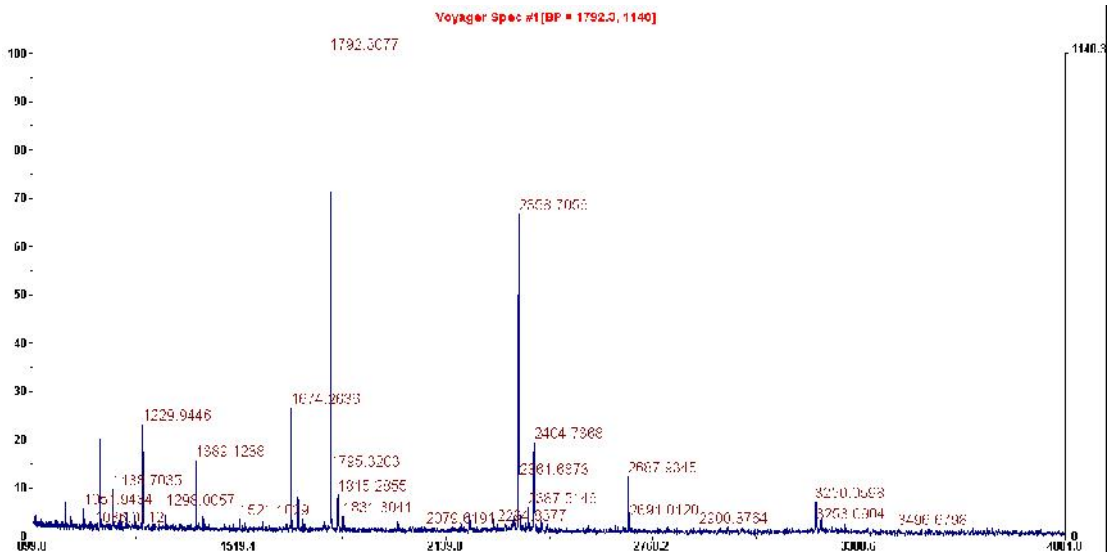
401 VDGALCLEKS LGPYSCSSNG PNLFFIHGPN LYCYSSIDKL NAAKSLPQPQ

451 KVNSILGCSQ

Figure 5.1: Mass spectrum and amino acid sequence for hemopexin

The coverage for hemopexin was good and the peak intensities shows to be good. The length of the matched peptides (red) shows the good coverage and the MOWSE score was high for hemopexin compared to other MASCOT returned identities

The other excised protein spot was identified to be alpha 1 macroglobulin-like protein because it was not validated using Western blotting with the spectrum and the sequence as shown in (Figure 5.2). The peptides that were matched were few but the coverage was good. The identified protein was the precursor which is found at a lower molecular weight because alpha 1 macroglobulin is having a high molecular weight but the excised spot was at a lower molecular weight, suggesting it was a low molecular weight fragment of alpha 1 macroglobulin.



1 MRRNQLPIPV FLLLLLLLPR DATAATGKPR YVVLVPSELY AGVPEKVCVH

51 LNHLNETVTL NVTLEYGVQY SNLLIDQAVD KDSSYCSSFT ISRPLSPSAL

101 IAVEIKGPTH HFIKKKSMWI TKAESPVFVQ TDKPIYKPGQ TVKFRVVSVD

151 ISFRPVNETF PVVYIENPKR NRIFQWQNVD LPGGLHQLSF PLSVEPALGI

201 YKVVVQKDSG KKIEHSFEVK EYVLPKFEVQ VKMPKTMAFL EEELVVTACG

251 LYTYGKPVPG LVTMKVCRKY TQSYSNCHGQ HSKSICEEFS KQADEKGCFR

301 QVVKTKVFQP RQKGYDMKIE VEAKIKEDGT GIELTGTGSC EIANTLSKLK

351 FTKANTFYRP GLPFFGQVLL VDEKGQPIPN KNLTVQVNSV RSQFTFTTDE

401 HGLANILIDT TNFTFSFMGI RVIYKQNNIC FDNWWVDEYH TQADHSAARI

451 FSPSRSYIQL ELVLGTLACG QTQEIRIHFL LNEDALKDAK DLTFYYLIKA

501 RGSIFNSGSH VLPLEQGKVK GVVSFPIRVE PGMAPVAKLI VYTILPNEEL

551 IADVQKFDIE KCFANTVNLS FPSAQSLPAS DTHLTVKATP LSLCALTAVD

601 QSVLLLKPEA KLSPQSIYNL LPQKAEQGAY LGPLPYKGGE NCIKAEDITH

651 NGIVYTPKQD LNDNDAYSVF QSIGLKIFTN TRVHKPRYCP MYQAYPPLPY

701 VGEPQALAMS AIPGAGYRSS NIRTSSMMMM GASEVAQEVE VRETVRKYFP

751 ETWIWDMVPL DLSGDGELPV KVPDTITEWK ASAFCLSGTT GLGLSSTISH

801 KVFQPFFLEL TLPYSVVRGE AFILKATVLN YMPHCIRIHV SLEMSPDFLA

851 VPVGSHEDSH CICGNERKTV SWAVTPKSLG EVNFTATAEA LQSPELCGNK

901 VAEVPALVQK DTVVKPVIVE PEGIEKEQTY NTLLCPQDAE LQENWTLDLP

951 ANVVEGSARA TQSVLGDILG SAMQNLQNLL QMPYGCGEQN MVLFVPNIYV

1001 LEYLNETQQL TEAIKSKAIS YLISGYQRQL NYQHSDGSYS TFGDRGMRHS

1051 QGNTWLTAFV LKAFAQAQSY IYIEKTHITN AFNWLSMKQR ENGCFQQSGS

1101 LLNNAMKGGV DDEVTLSAYI TIALLEMPLP VTHSVVRNAL FCLETAWASI

1151 SNSQESHVYT KALLAYAFAL AGNRAKRSEV LESLNKDAVN EEESVHWQRP

1201 KNVEENVREM RSFSYKPRAP SAEVEMTAYV LLAYLTSASS RPTRDLSSSD

1251 LTTASKIVKW ISK**QQNSHGG FSSTQDTVVA LQALSK**YGAA TFTK**SNKEVS**

1301 **VTIESSGTVS GTLHVNNGNR** LLLQEVRLAD LPGNYITKVS GSGCVYLQTS

1351 LK**YNILPEAE GEAPFTLKVN TLPLNFDKAE HHR**KFQIHIN VSYIGERPNS

1401 NMVIVDVK**MV SGFIPVKPSV KKLQDQSNIQ RTEVNTNHVL IYIEK**LTNQT

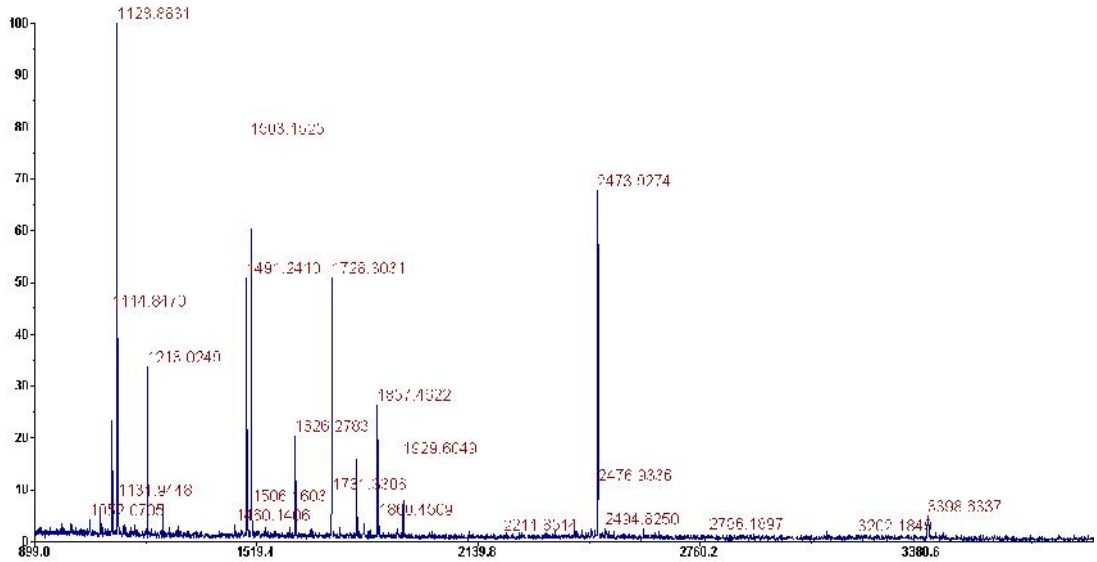
1451 MGFSFAVEQD IPVKNLKPAP VKVYDYYETD EFAIEEYSAP FSSDSEQGNA

1501

Figure 5.2: Mass spectrum and amino acid sequence for Alpha 1 macroglobulin

Few peptides matched for alpha 1 macroglobulin (red) but the coverage was enough to give an identity of the protein

The third identification in the albumin depleted sample was C-reactive protein. The identity was taken based on the MOWSE score and the peptide coverage. The matched peptides are showing to be found evenly distributed in the sequence as shown (Figure 5.3).



1 MYRIATKTWH VQVLFVYSVP GSSALFAGVP AALLHALVLW LLSSVEAIAT

51 LLSLVLYSFH DASSVHSCQV IPSEADSGFV NYIKAMIWEK ELLLKGDLNH

101 SEADELGAKQ ALALGAYRLL ISPRWGVYRK **EGGTQDPNNI K**YLGVTLTKQ

151 VKDLYNKNFK TLKKEIEEDL RRWKDLPCSW IGRINIVKMP ILPKAIYRFN

201 AIPIKIPIQF FKELDRTICK FIWNNKKPRI AKAILNNKRT SGGITIPELK

251 QYYRAIAIKT AWYWYR**DRQI DQWNR**IEDPE 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 MSK**QAFVFPG**

651 **VSATAYVSLE AESK**KPLEAF TVCLYAHADV SR**SFSIFSYA TKTSFNEILL**

701 **FWTR**GQGFSI AVGGPEILFS ASEIPEVPTH ICATWESATG IVELWLDGKP

751 RVRKSLQKGY IVGTNASIIL GQEQDSYGGG FDANQSLVGD IGDVNMWDFV

801 LSPEQINAVY VGR**VFSPNVL NWR**ALKYETH GDVFIKPQLW PLTDCSQVQV

851 GQRPPHKTDT LKLIEEKVGK NLGHMGTGPH EAQEGKTKVW MLQSFLEGGT

901 KILTGGNMER KCGIEERPSR DCPTWGSSPH SATKPRQHCG YQEVHADRNL

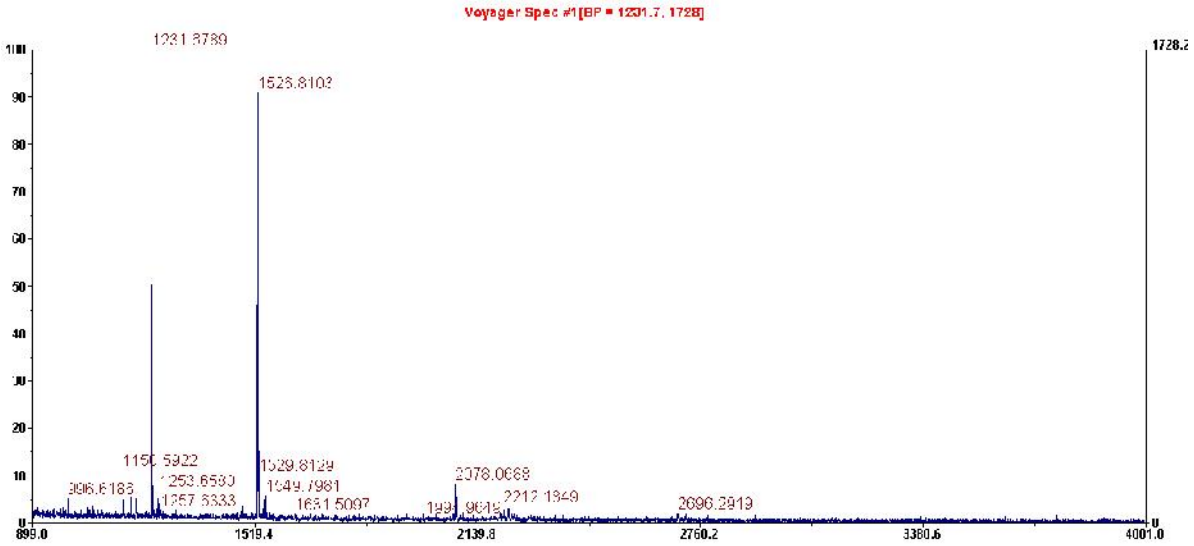
951 IQLSPERLCQ SLTNTEVDAH SQPLTEKEVP NGGVREKTEG VEGVCNPIRR

1001 **TVSSNQPYPV R**DPRD

Figure 5.3: Mass spectrum and amino acid sequence for C-reactive protein

The peaks that are found in the spectrum have high intensities. The distribution of matched peptides (red) from the sequence is even.

The protein that was identified to be differentially expressed in the albumin containing sample of the TCA/acetone precipitated serum was identified to be apolipoprotein AIV (Figure 5.4). The protein that was having a highest MOWSE score was chosen and it contained the highest peptide coverage. It was corresponding with the molecular weight of the spot that was excised for digestion at 46 kDa. The spots that were excised were side by side and both were identified to be Apo AIV.



1 MFLKAVVLTV ALVAITGTQA EVTSDQVANV MWDYFTQLSN NAKEAVEQLQ

51 KTDVTQQLNT LFQDKLGNIN TYADDLQNK**L VPFAVQLSGH LTK**ETERVR**E**

101 **EIQKELEDLR ANMMPHANK**V SQMFGDNVQK **LQEHLRPYAT DLQAQINAQT**

151 **QDMK**R**QLTPY IQR**MQTTIQD NVENLQSSMV PFANELKEK**F NQNMEGLK**GQ

201 LTPRANELK**A TIDQNLEDLR** SRLAPLAEGV QEK**LNHQMEG LAFQMK**KNAE

251 ELQTKVSTNI DQLQKNLAPL VEDVQSKLKG NTEGLQKSLE DLNK**QLDQQV**

301 **EVFRR**AVEPL GDKFNMALVQ QMEK**FRQQLG SDSGDVESHL SFLEK**NLREK

351 VSSFMSTLQK KGSPDQPLAL PLPEQVQEQV QEQVQPKPLE S

Figure 5.4: Mass spectrum and amino acid sequence for apolipoprotein AIV

The mass spectrum generated and the sequence that was retrieved from MASCOT database showing the matched peptides in red. The spectrum also shows the intensity of the peaks as plotted against the mass to charge ratio (m/z) for peptides.

The proteins that were identified are further tabulated (Table 5). The table is giving details of identified proteins. The accession number entry name and the gene that encode the protein are given in the table. The sequence coverage is also given on the table in percentages which shows the sensitivity of the MALDI TOF in identifying the peptides that belongs to a certain protein. There are even proteins that have a low coverage like the C-reactive protein that was identified to have eight percent coverage. There other two proteins marked with the RMI standing for the reference map identified proteins were taken from the reference map that was published in the inflammation study (Giannaza et. al (2002) and the 2D from a control was used for such identifications.

The use of the reference map in the current study was due to the MALDI TOF MS

not generating the spectra for the proteins. The map adopted from the inflammation study was used for the identification of the rest of the proteins (Gianazza *et al.*, 2002a). The map was similar to the gel image that was seen for this study (Chapter 4) but there were more proteins above pI 7 because a different IPG strip was used, which is a pI 3-10 compared to the pI 4-7 used in this thesis. The proteins were then mapped according the information showed in the map. The map shows the mixed sample with albumin available in the map. In this study we separated the abundant proteins from the less abundant proteins and improved the resolution of the proteins. The map aided in identification of Fetuin A and Fetuin B which are proteins that were also differentially expressed but were not identifiable with the mass spectrometry.

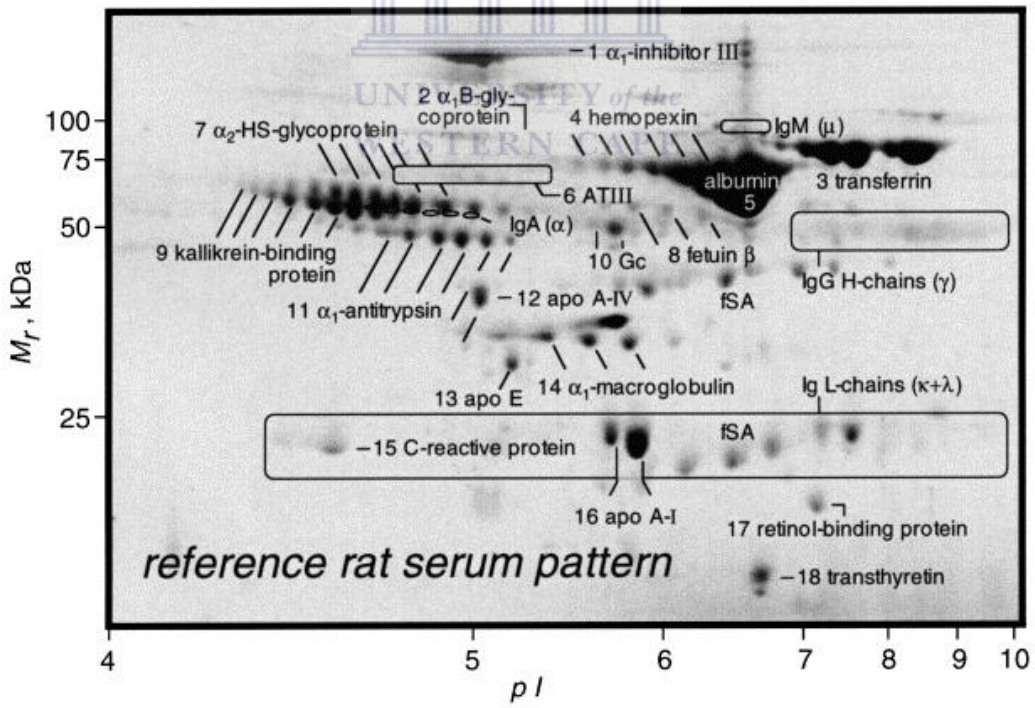


Figure 5.5: 2D map of a rat serum taken from Gianazza, et. al., 2002

Table 5: Peptide mass fingerprinting of differentially expressed spots

Spot no.	Protein	Accession no.	Score	Theoretical PI	In gel PI	Sequence Coverage	Regulation on HFD	Theoretical MW (kDa)	In gel MW(kDa)
1	Alpha 1 Macroglobulin-like	Q63041	67	6.46	4.9	8	↓	165	~40
2 (RMI)	Fetuin A	P24090	-	6.05	5.15	-	↑	59	~85
3 (RMI)	Fetuin B	Q9QX79	-	6.71	6.0	-	↑	41.5	~70
4	Hemopexin-like	Q5BKB4	77	7.58	5.85	25	↑	53	~100
5	C-reactive protein precursor	A42579	51	4.89	4.6	8	↑	25.4	~30
6	Apolipoprotein AIV	LPRTA4	84	5.12	5.1	33	↑	44	~45

RMI- reference map identified proteins with the information that is available obtained from uniprot search not NCBI

5.6 Discussion

The use of the proteomics approach to identify the changes at protein level is a promising to be a potential approach to giving the clues to the questions that arise from the disease development. Clinical breakthroughs brought about by proteomics have been seen. MALDI-TOF MS's clinical breakthrough that showed to be clinically successful is the use of MALDI-TOF in many routine clinical microbiology laboratories as a routine microbial profiling tool (Lescuyer *et al.*, 2010). Another study using LC-MS to study protein differences in tissues showed that there are differences in the tissues for the mice that are fed high fat diet to the proteins expressed by the mice fed low fat diet. The study focused on skeletal muscle and liver which made them report their observations that feeding leads to accumulation of skeletal muscle which was noticeable when they looked at fasted rats compared to the fed rats (Anderson *et al.*, 2004). Studying the tissues is an invasive way of obtaining the information but this study also reported that they observed the increase in plasma albumin levels. The use of the body fluids with less invasive approach like serum and plasma to non-invasive methods like the use of urine and saliva are of interest because they can enhance the delivery of biomarkers while encouraging the response from patients. The study on serum and plasma has been reported more in cancer research with some accumulation of the studies on obesity, but there is still a need to interrogate the proteome complexion in both serum and plasma in relation to obesity development because it can aid much in the identification of the role players and possible ways to intervene.

The numbers of spots that can be found to be differentially expressed are dependent on various parameters. The preparation of the sample to the stain that is used is also has an effect on the visibility of the spots. In the serum there are variations in the number of spots that are getting identified by different studies. The study by Zhao, et. al. (2008) identified seven proteins with one unnamed protein. The verification was performed for alpha 1 macroglobulin which showed to be down- regulated with obesity development. The studies by Choi, et. al. (2010 & 2012) showed differences in the serum proteome that is due to obesity and the differences that are gender based obesity proneness or obesity resistance.

The software used in studies to analyze the images is uses the similar principle of matching the spots with a master gel created as a reference gel. The studies above used UMAX Powerlook 1120 instead of Quantity One used in the current study. After the images were produced ImageMaster 2D software was used contrary to the use of PD Quest in our study. There have been reports of the variation between labs when it comes to the Proteomics finding which led to the Human Proteome Organization (HUPO), which tried to set the standards when it comes to procedures to use across the labs for human serum proteome. The identities show that there are common spots that have been found to be obesity associated in the current study and in the studies mentioned above. The spots that were identified in our study are the spots that responded to the increase in calories across the groups because those were the spots that were associated with obesity than a particular diet.

The serum map that has been produced in the study on inflammation by Gianazza., et. al. (2002b) is still having the similar pattern as other maps in the studies that are recently published including the current study. The map was similar to the gel image that was seen for our study but there were more proteins above pI 7 because a different IPG strip was used, which is a 3-10 compared to the 4-7 used in this thesis. The proteins were then mapped according the information showed in the map. The map shows the mixed sample with albumin available in the map. In this study we separated the abundant proteins from the less abundant proteins and improved the resolution of the proteins. The map aided in identification of Fetuin A and Fetuin B which are proteins that were also differentially expressed but were not identifiable with mass spectrometry.

Although the maps in the inflammation studies and the obesity study may have different proteins that are deregulated between the normal and acute phase the reference maps of the serum control are reliable for the identification of the spots that are unidentifiable with the mass spectrometer and hence the use of the reference map for identification of some unidentifiable proteins. The use of 2D for analysis of rat serum provides a reliable way to monitor the onset or progression of pathological state that is inflammatory component involving, furthermore it also aids in assessing the outcome of any therapeutic intervention (Gianazza *et al.*, 2012).

The identities that were found in the current study are show the proteins that are responsive to the development of obesity because the body mass indexes are high for all the rats that were fed on high caloric diets. The down-regulated protein was also

common and this gives the indication that these proteins may be potential markers for acute obesity development. Unlike other studies the current study is showing the use of the lard diet that is widely used together with other diets to show that the high caloric intake is not only referring to lard but to other diets. The regulation above 2-fold for the group on high caloric diets compared to the control shows that the identified proteins are highly responsive to accumulation of fat stores.

Chapter 6: Validation of proteins' differential expression through Western blot and bioinformatics of analyzed proteins

Introduction

The analysis of gel images and spots comparison through software is an important step towards identifying the spots that are differentially expressed between a normal and a diseased sample. The results obtained from such analysis always call for verification. Western blot is the widely used procedure for the verification of the expression of proteins and it is sensitive enough to pick up the proteins in picogram scale. This allows the use of Western blot to compare the intensity of bands as well as the confirmation of spots on a 2D PAGE. Studies where proteomics tools are used to verify protein expression in serum include studies in cancer (Villanueva *et al.*, 2005), nutrition (Kumar and Mann, 2009), diabetes (Sundsten and Ortsäter, 2009), obesity (Zhao *et al.*, 2008) and there are a lot other studies that utilize serum to identify differentially expressed proteins.

The use of Western blot as a method of verifying the proteomics results has been used by most of the researchers with ELISA being another method when Western blot is not used (Zhao *et al.*, 2008). ELISA is having a challenge of being costly compared to Western blot which allows repetitions and a room of troubleshooting. The use of antibodies in Western blot also poses a challenge because they are expensive but with the use of Coomassie stain for internal control (Welinder and Ekblad, 2010) this promises to keep Western blotting as a preferred method of verification.

When proteomics results have been verified there is a need of giving a biological meaning using bioinformatics. Bioinformatics is defined as a mean for functional analysis and data mining of data sets leading to biologically interpretable results and insights (Kumar and Mann, 2009). Bioinformatics was used in this study for protein identifications to mining the information that is readily available in order to find their association with obesity. This completes the understanding of the proteins and their functionality. Proteomics in conjunction with bioinformatics makes the success in high throughput data generation and answering of questions that arise regarding the differences between the diseased and normal state.

The combination of proteomics and bioinformatics is growing at a high speed. The ever improved proteomics approach is currently enabling the increase in the throughput while bioinformatics effort in completing the proteomic findings. The collection of data with obesity genomic loci from different species such as human, cattle, rat and mouse from a gene atlas, which includes a database and genomic view (Kunej T, 2012) shows the effort in trying to narrow the data and cut repetitions towards better understanding of obesity.

6.1 Western blot validation of the differential proteins identified using proteomics

The results obtained using the proteomics image analysis software can be confirmed using other protein quantification methods. The most sensitive methods which are ELISA and Western blot are usually methods of choice for their high sensitivity and

accuracy in determining the expression of protein. As a method to validate the results obtained in proteomics analysis we used Western blotting because it is worth considering the technical errors and other shortcomings in proteomics analysis. Western blot analysis was performed on the four proteins that were differentially expressed. Transferrin (TFR) was used as a loading control loading control. A study by Wilkins et. al. (2005) shows that transferrin levels do not change in the blood despite the disease state. There was a struggle in getting hold of commercial alpha 1 macroglobulin. Hemopexin was also not validated using the Western blotting even though the results could show the enhanced expression of the hemopexin spot on the gel.

When analyzing the Western blot for apolipoprotein AIV expression, there was a significant increase in the amount of apolipoprotein AIV in high caloric diet fed groups compared to the control group. The group on the L diet was having significantly high apolipoprotein AIV ($p<0.01$), but it was having less levels compared to LF and BF diet fed groups with highest expression seen in the BF fed group. The expression of apolipoprotein AIV has been found to be not too high with high caloric diets in some studies with high levels reported in other studies. The outcomes from the plasma and serum studies of apolipoprotein AIV are discussed further in section 7.1 where details are given with literature that has been looked at. There is no other lipoprotein that was identified to be commonly responsive to high caloric diet in this study.

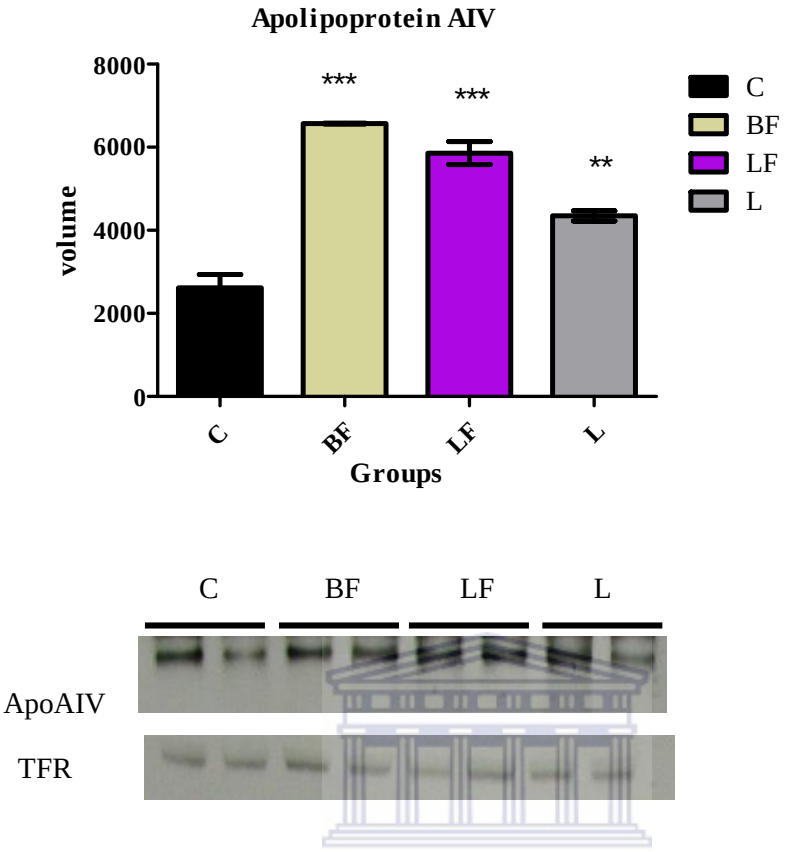


Figure 6.1 Figure 6.1 Western blot validation of apolipoprotein AIV showing a significantly low expression by the chow fed group (control)

The figure shows significantly high expression for the lard (L), lard fat free (LF) and butterfat (BF) fed groups compared to the maintenance (C) diet fed group. ** ($p < 0.01$); *** ($p < 0.001$)

The CRP has long been associated with inflammation. There is little information that link CRP to obesity. The association of CRP with obesity is rising from the results that have recently been reported and the discussion section contains details on this. In this study Western blotting confirmed the results that were obtained in proteomics study. Feeding high caloric diets increased the levels of CRP significantly ($p<0.01$). The highest expression was seen in the group that was fed L diet. There were similar expression levels for the BF and LF fed groups.

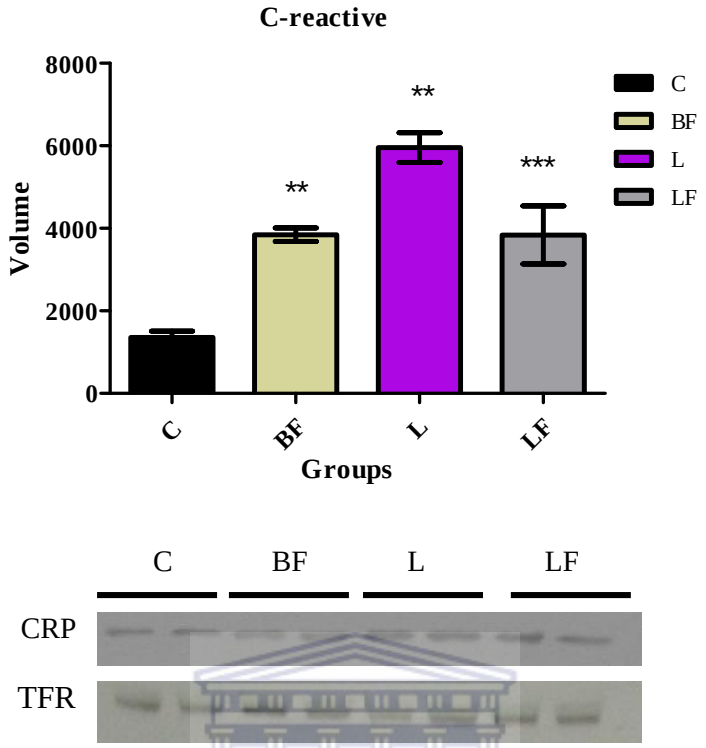


Figure 6.2: Western blot validation of PD Quest results on C-reactive protein

The expression of C-reactive protein (CRP) in serum for the rats that were fed high fat diets compared to the rats that were fed maintenance (C) diet. Lard fed group showed significantly high levels of CRP compared to C fed group $p < 0.001$ (***). The lard fat free (LF) and butterfat (BF) fed groups had high levels of CRP $p < 0.01$ (**) compared to C fed group. Transferrin (TFR) was used as a loading control.

Fetuin A increased with increase in caloric intake resulting in significantly high levels ($p<0.05$) for groups fed high caloric diets. The levels were high in L and LF fed groups. The results on fetuin A confirm the results that were obtained when using PD Quest to analyze the spot intensities for the high caloric diet fed groups compared to the C fed group (control group). The role of fetuin A in insulin resistance has been reported in studies that were focusing on the role the protein plays in the body. The effect of increase in fetuin A is not having established evidence in this study that it triggered insulin resistance or not as the glucose levels were shown not to be

significantly different in section 3.3.2 where glucose measurements were shown. The high levels of fetuin A could have been identified to be linked with insulin if insulin levels were included in the current study but insulin levels were not measured.

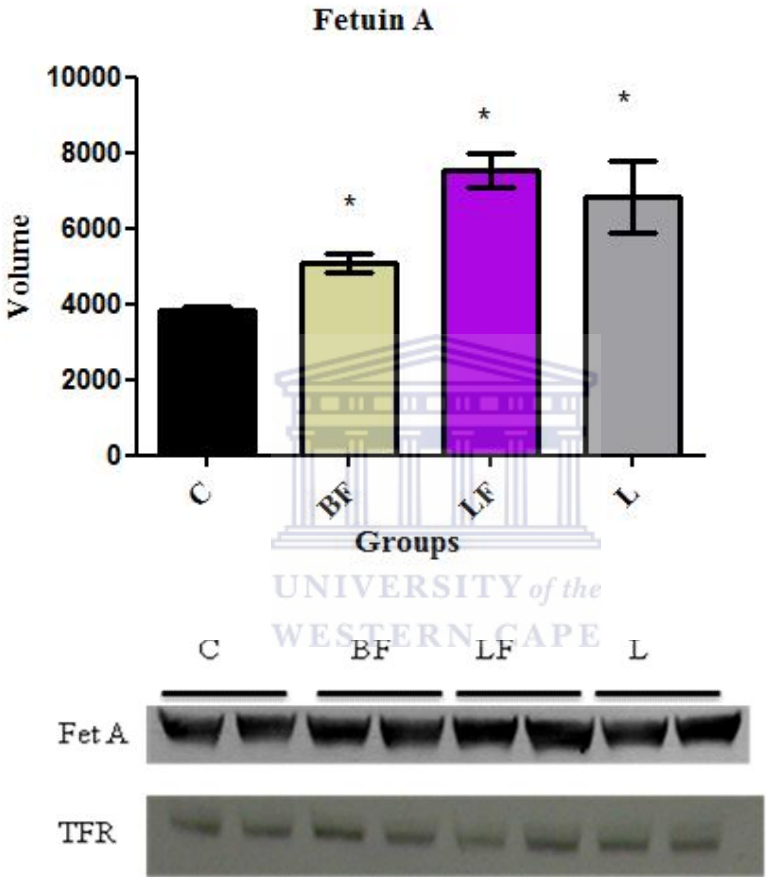


Figure 6.3: Validation of the Fetuin A by Western blot for the expression between the groups

Western blot validation shows the up-regulation of Fetuin A (Fet A) for the lard (L), lard fat free (LF) and butterfat (BF) compared to maintenance (C) fed group $p < 0.05$ (*). Transferrin (TFR) was used as a loading control.

Fetuin B which is another protein from the Fetuin family was identified to be highly expressed when high caloric diet was used to induce obesity in rats. The levels were

showing to be increasing with the increasing fat content of the diet. This was seen with high levels of Fetuin B seen when performing Western blotting in L, LF, and BF when compared to C. The levels were high for the three high caloric diet fed groups ($p<0.05$). When compared to C there were high levels for BF with more levels in LF and L of Fetuin B based on the Western blot analysis.

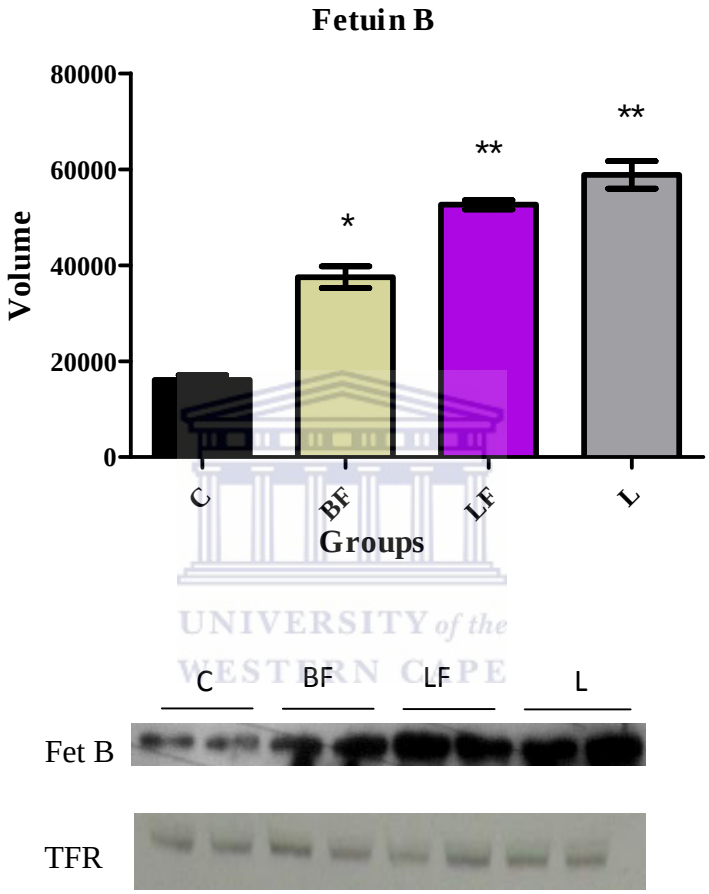


Figure 6.4: Fetuin B expression at termination

Significantly high expression of Fetuin B (Fet B) for the butterfat (BF) fed group $p < 0.05$ (*); lard fat free (LF) and lard (L) fed groups $p < 0.01$ (**) compared to maintenance (C) fed group. Transferrin (TFR) was used as a loading control.

6.2 Bioinformatics analysis of differentially expressed proteins

Bioinformatics is a discipline on its own. The utilization of bioinformatics in various studies is assisting with the utilization of the readily available information. This speeds up the generation of the information with an aid of centralizing the information that is known. Bioinformatics helped in finding the information about the proteins identified quicker. Expasy was used to get most of the information about the proteins from the UniProt database in this study. The proteins that were searched from UniProt are shown in table 6.1. The three dimensional structure of proteins were taken from the protein model portal (PMP) database. The structures shown are the cartoon model and the structure with the highest percentage identity. The KEGG helped in the orthologs study of the proteins in order to find the role that proteins might have in the metabolic pathways.

The information written about the proteins is the same information that is contained in the Uniprot database. Some of the information is not experimentally based information. It is a result of the various similarities that is identified from the protein in the same family or with the similar conformation. An approach to use the KEGG aids in creating the understanding of the role played by proteins in the pathways. It is worth mentioning that some of the proteins did not have any link to pathways even with the use of orthologs. Proteins have different functions and it is expected that while some are functional proteins others are structural proteins or just helping in other functioning either than pathways. The utilization of bioinformatics enhances the identification of proteins to diseases. In this study the orthologs were employed to

identify the pathways where proteins that were differentially expressed play a role based on the structural similarity of proteins and the possibility of the proteins acting on similar substrates as the proteins of similar structure with readily available information.

6.2.1 Bioinformatics review of alpha 1 macroglobulin

Alpha 1 macroglobulin (A1M) is a disulfide linked homotetramer existing as a 165 kDa subunit that is cleaved to a 45 kDa subunit. It is encoded by *A1m* gene also known as *Pzp* gene. It is made up of 1500 amino acids and it is completely sequenced. Alpha 1 macroglobulin is a serine protease inhibitor. The function by similarity (not known through experiments) as found in the Uniprot database is inhibition of all four classes of proteinases by a unique „trapping" mechanism. The protein has a peptide stretch called ' bait region' which contains specific cleavage sites for different proteinases. When a proteinase cleaves the bait region, a conformational change is induced in the protein which traps the proteinase. The entrapped enzyme remains active against low molecular weight substrates; activity against high molecular weight substrates is greatly reduced. Following cleavage in the bait region a thioester bond is hydrolized and mediates the covalent bonding to the proteinase.

Performing orthologs revealed that there are similarities between the structures of alpha 1 macroglobulin and alpha 2 macroglobulin. The structures as obtained using the protein model portal (PMP) showed to be similar as shown in figure 6.5. The competition experiments using rat receptor binding domain of alpha 1

macroglobulin and labelled human alpha 2 macroglobulin, revealed that recombinant version of rat alpha 1 macroglobulin have a high affinity for alpha 2 macroglobulin receptor as the 40 kDa light chain for alpha 1 macroglobulin (Nielsen *et al.*, 1995). In this study the conclusion was that macroglobulins interaction is determined by the C-terminal residue which is approximately 150 residues. In the complement and coagulation cascade alpha 1 macroglobulin is said to play a similar role as alpha 2 macroglobulin (red) in Figure 6.6 which is to inhibit the proteases. As one of the serine protease inhibitors the alpha 1 macroglobulin can inhibit the serine proteases from being triggered by either extrinsic or intrinsic pathways as they required for blood coagulation (Norris, 2003). The reduction in the expression levels of alpha 1 macroglobulin with development of obesity could be a contributor to atherosclerosis but that is not proven since currently there is limited information on alpha 1 macroglobulin.

A B

Figure 6.5: Alpha 1 macroglobulin (A) and alpha 2 macroglobulin (B)

The two proteins show structural similarities and they have been shown to have affinity for the same substrate. Based on the studies that have been done on alpha 2 macroglobulin it has been suggested that alpha 1 macroglobulin can have similarity in

function with alpha 2 macroglobulin in red (Figure 6.6). In the figure alpha 2 macroglobulin inhibits a number of proteases that are crucial to the coagulation pathway. The current study is not going to get into details in the pathways as this was not part of the study purpose but as a way of identifying the pathways and the roles of proteins in those pathways.

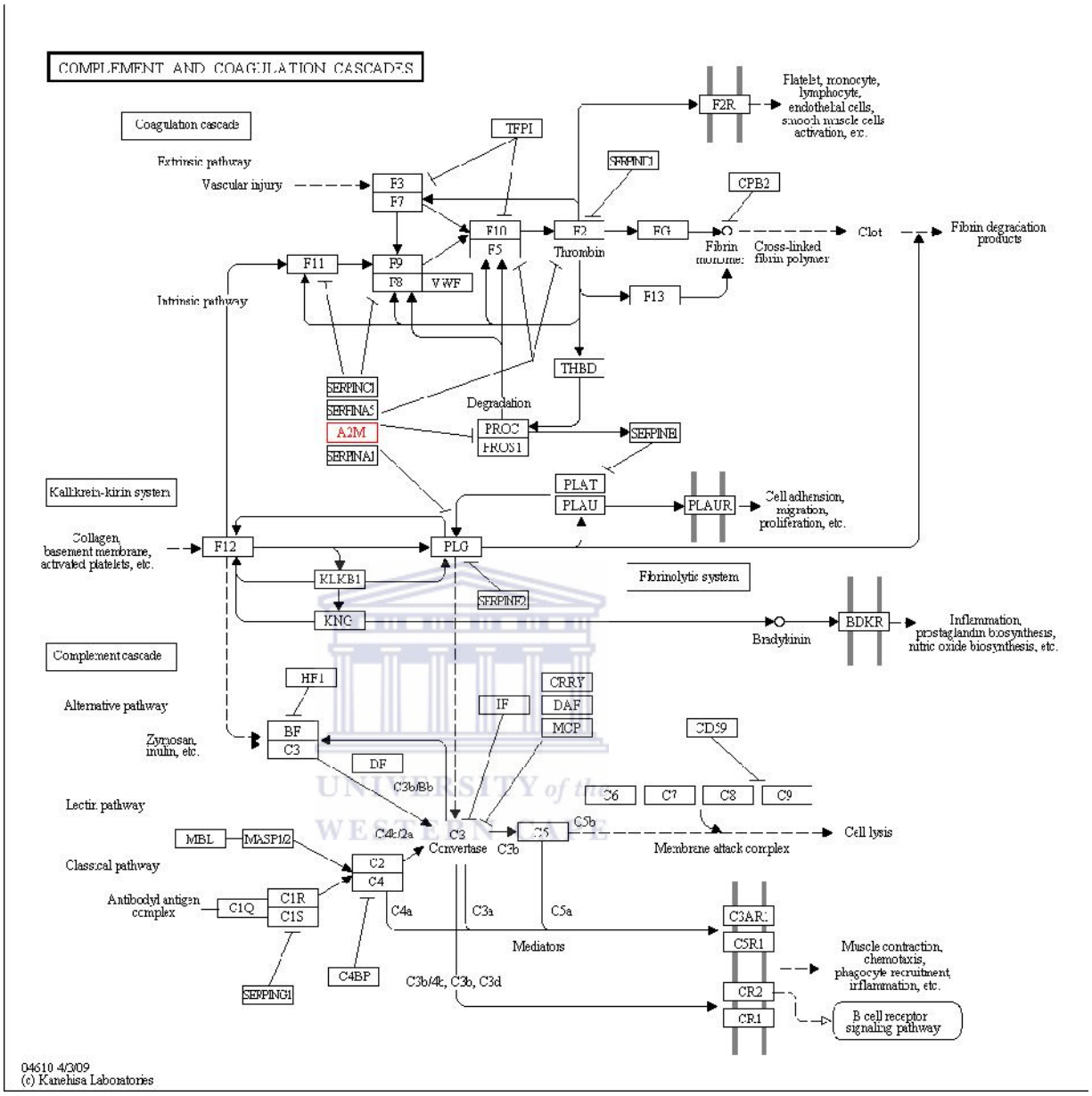


Figure 6.6: complement and coagulation cascade (KEGG pathways)

6.2.2 Bioinformatics review of apolipoprotein AIV

Apolipoprotein AIV (Apo A4) is a 391 amino acid long protein encoded by the *ApoA4* gene. It functions in chylomicrons and VLDL secretion and catabolism. It is also required for efficient activation lipoprotein lipase by Apolipoprotein C-II; potent

activation of LCAT. Apo A4 is a major component of HDL and chylomicrons secreted to the plasma. It contains nine of thirteen 22-amino acid repeats (each 22mer is actually a tandem array of two, A and B, related 11mers) occurring in this sequence that are predicted to be highly alpha-helical and many of these helices are amphipathic. They may therefore serve as lipid binding domains with lecithin cholesterol acyltransferase (LCAT) activating abilities.

Apolipoprotein is reported to be orthologous to apolipoprotein AI. Both proteins are lipid carrier proteins. The production of lipoproteins can be triggered by the accumulation of very low density lipoproteins (VLDL) and chylomicrons as shown in the pathway (figure 6.8) showing the chronology of processes taking place leading to more lipid transport proteins being produced.

A B

Figure 6.7: Apolipoprotein AIV and apolipoproteinAI

The accumulation of the apolipoprotein AI is shown in the pathway of which apolipoprotein AIV is also said to be produced through the peroxisome proliferated receptor activators (PPARs) signalling pathway. PPARs are transcription regulators which play a major role in metabolic and inflammatory regulation with far reaching medical implications (Feige *et al.*, 2006).

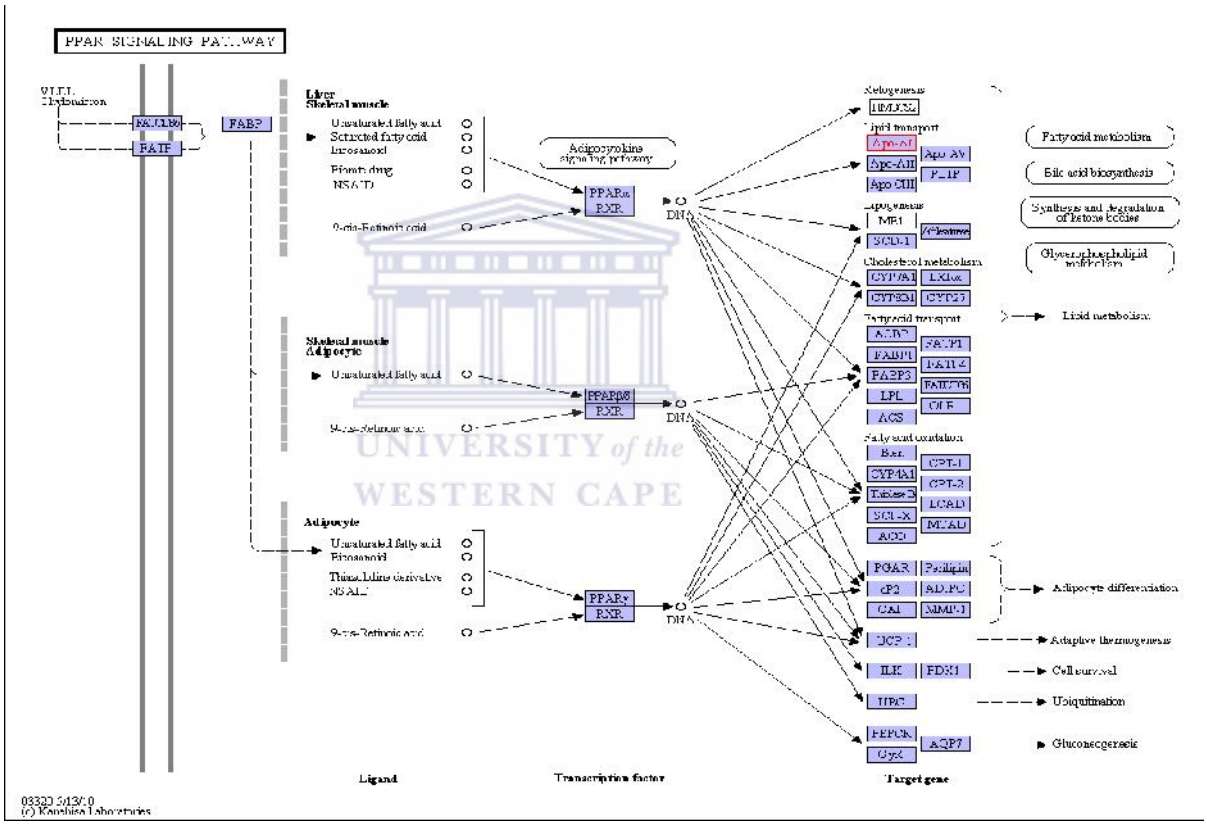


Figure 6.8: PPAR signalling pathway (KEGG pathways)

6.2.3 Bioinformatics review of C-reactive protein

C-reactive protein (CRP) encoded by the *Crp* gene is 230 amino acids long and a completely sequenced protein found in the plasma. It is homopentamer (pentaxin or pentraxin) having a discoid arrangement of 5 non-covalently bound subunits that are further processed into its mature form. Two of the five chains form a dimer linked by two interchain disulfide bonds located in the C-terminal heptapeptide and specific to rat CRP. Several functions have been associated with CRP. Most of the functions are associated with the host defence by promoting the agglutination, bacterial capsular swelling, phagocytosis and complement fixation through its calcium dependent binding to phosphorylcholine. It can interact with DNA and histones and may scavenge nuclear material released from damaged circulating cells. CRP is acting with an aid of the 2 calcium ions per subunit as cofactors.

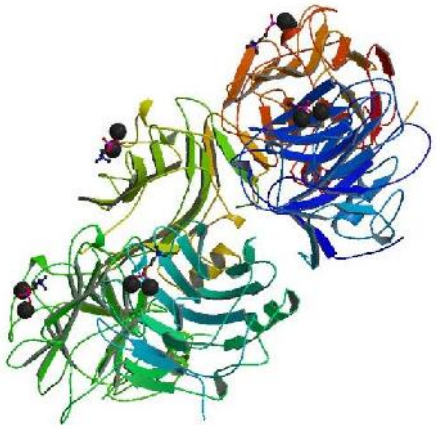


Figure 6 9: C-reactive protein structure as retrieved from the database

C-reactive protein is associated with inflammation in most studies. In the KEGG pathways (www.kegg.jp/kegg/pathway.html) CRP has been found to be orthologous to various proteins that play a role in nitrogen metabolism. The CRP levels were high in the high caloric diet fed groups and this elevation cannot be associated with inflammation in this study. However CRP is a sensitive marker for a systemic inflammation (Visser M, 1999). The high implication or association of CRP with inflammation has been argued and other possibilities have been pointed out of which ageing has also been one of the factors associated with rise in CRP levels (Kushner, 2001). There are a lot of studies that were done in inflammation that proved that the elevation of CRP is inflammation related. Furthermore, the accumulation of CRP has been associated with CHD because of various factors including the binding of CRP to LDL and its deposition to the most atherosclerotic plaques (Pepys and Hirschfield, 2003). Studies on CRP would be instrumental in obesity focused studies and CRP as a biomarker might be a challenge at the moment but it has been responsive for the groups that were fed high caloric diet in this study. Studies towards biomarker discovery can prove CRP to be of importance in obesity but that is not in the scope of the current study but CRP is a potential biomarker.

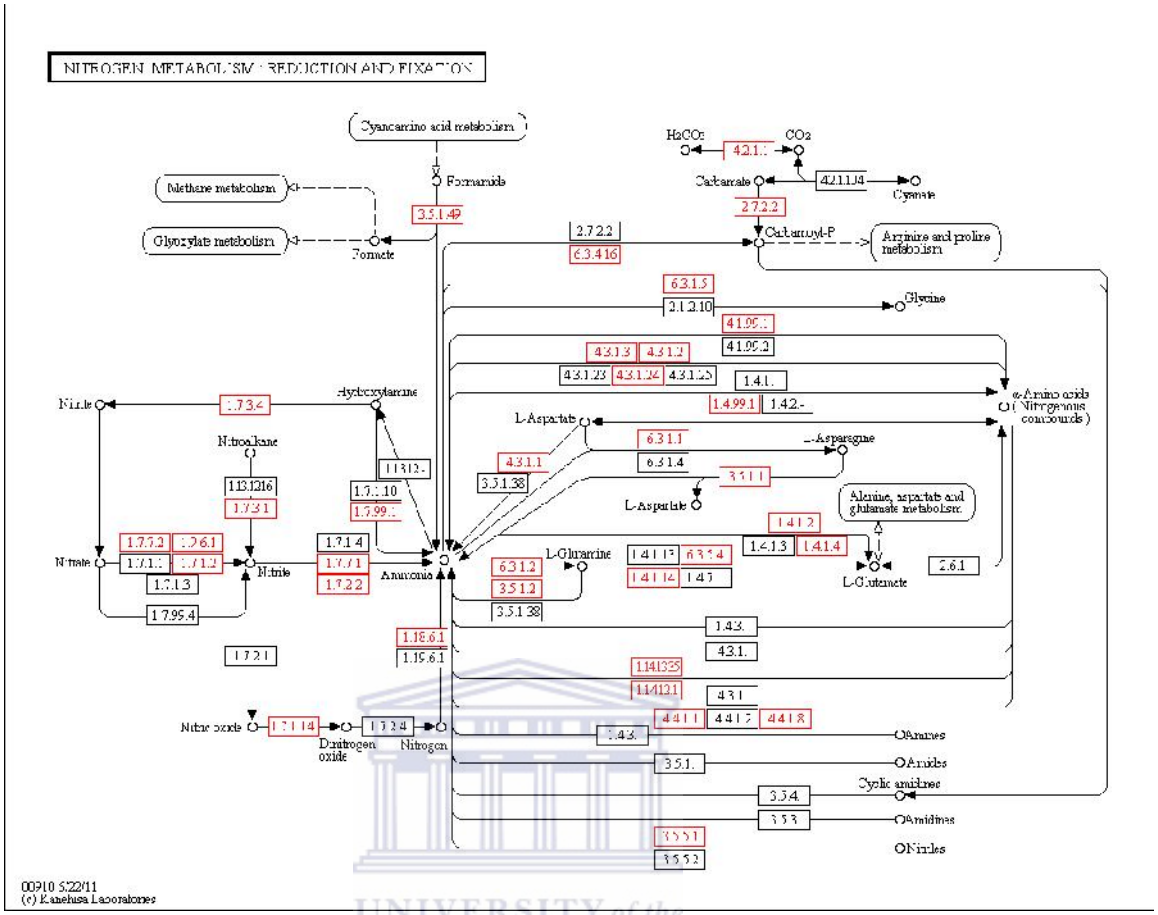


Figure 6.10: Nitrogen metabolism: Reduction and fixation (KEGG pathways)

6.2.4 Bioinformatics review of fetuin A

Fetuin A can be called alpha 2 HS glycoprotein or glycoprotein PP63. It is encoded by the *Ahsg* or *Fetua* gene that is synthesized in the liver and secreted by hepatocytes in the blood. The protein is 352 amino acids long with molecular weight of 63 kDa containing bone siliac acid. It is known to inhibit both insulin receptor tyrosine kinase activity and insulin stimulated receptor autophosphorylation and concomitantly antagonize the mitogenic effect of the hormone in cultured rat hepatoma cells.

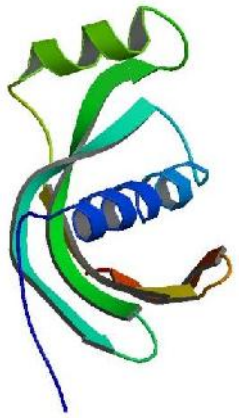


Figure 6.11: Fetuin A structure taken from Uniprot database (http://www.uniprot.org)

Fetuin A is also increased in expression with high caloric diet compared to the control group which can prove to be a bad thing to the metabolism as mentioned above that it inhibits insulin related function.

6.2.5 Bioinformatics review of fetuin B

Fetuin B also referred to as a Fetuin-like protein IRL 685 is a 378 amino acids protein. It is encoded by the *Fetub* gene. Fetuin B belongs to the Fetuin family which itself belongs to the cystatin superfamily and it is also proven that Fetuin A and Fetuin B are homologous (Olivier *et al.*, 2000b). In the study by Denecke et. al. (2003) fetuin B was shown to be expressed more in the liver. Functional analysis performed in the former study identified similarity between fetuin A and fetuin B in that both inhibit phosphate calcium precipitation with more activity shown by fetuin A. The information on fetuin B is not much compared to the information on fetuin A but the research on the functions of fetuin B in the body is still under investigation. The

structures for fetuins are similar as it can be seen in the previous and current section. Searching information on database revealed more information on fetuin A than on fetuin B with literature mentioning the identification of fetuin A to precede that of fetuin B.

A B

Figure 6.12: Fetuin B (A) and Kininogen 1 (KNG) (B)

The pathway search on KEGG pathways identified orthology for fetuin B and kininogen 1(KNG). Fetuin B had been associated with kallikrein-kinin system in the complement and coagulation cascades. Kinins are explained as the molecules involved in vascular tone regulation and inflammation (Costa-Neto *et al.*, 2008). Many studies have referred to obesity as an inflammatory associated disease. There was an increase in fetuin B with development of obesity in our study that might be the response that was counteracting the development of obesity. Recent study revealed the reduction of fetuin B in the obesity prone rats compared to

the obesity resistant rats (Choi *et al.*, 2012). The scope of the current study is not an in-depth study of pathways but the revelation of the possible functions of the proteins that were identified to be deregulated with obesity development. The results on fetuin B could be a step towards a potential candidate for biomarker or intervention target. The implication of fetuin B in the inflammatory related pathway could be an answer to the merging information when obesity and inflammation is studied.

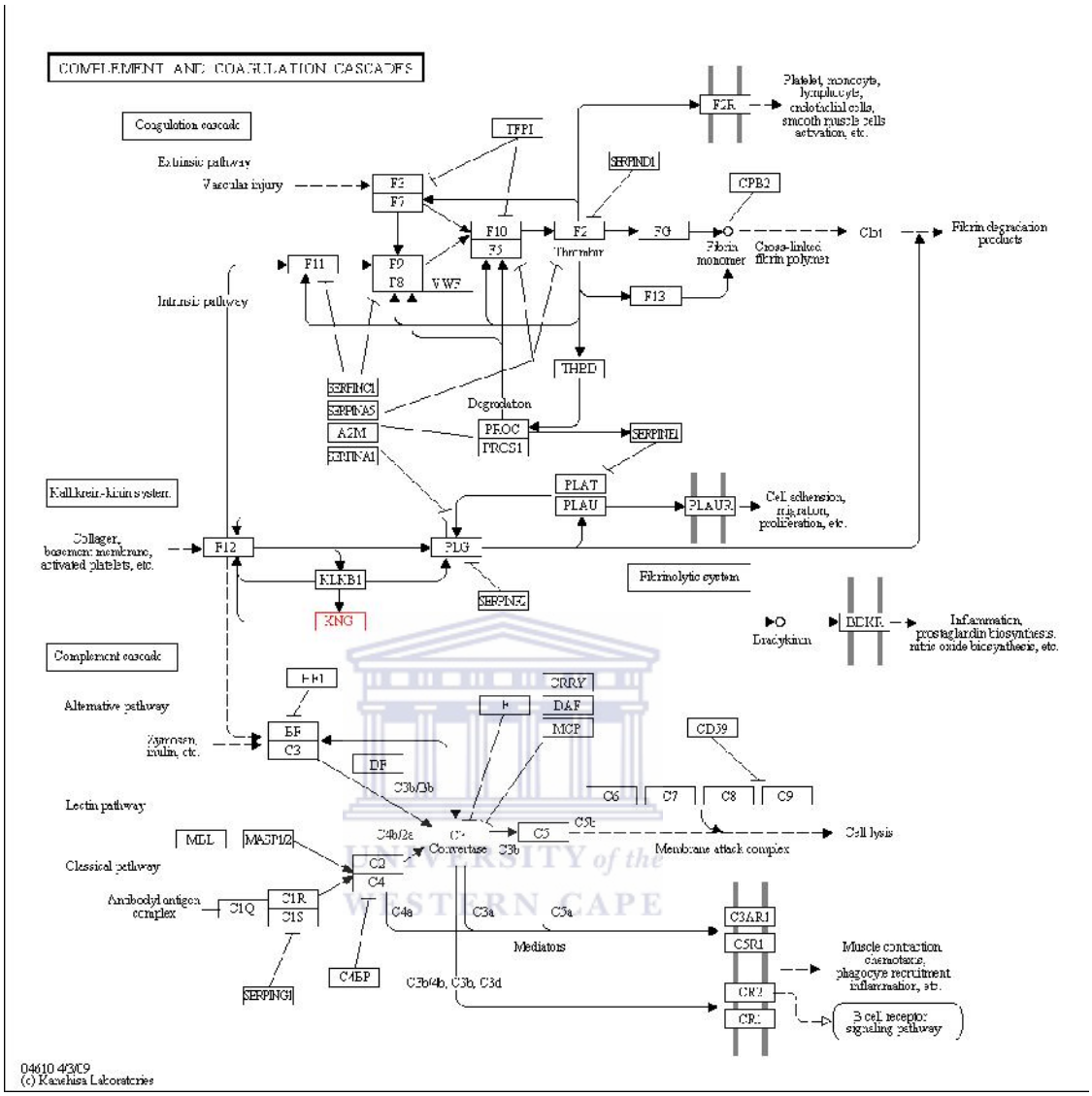


Figure 6.13: Complement and coagulation cascade (KEGG pathways)

6.2.6 Bioinformatics review of hemopexin

Hemopexin is a 460 amino acids protein encoded by *Hpx* gene. It is expressed in the liver and secreted into the plasma. It is completely sequenced and its function is binding the heme and transports it to the liver for breakdown and iron

recovery. The free hemopexin returns to the circulation.

Figure 6.14: Hemopexin structure taken from the Uniprot database

Western blot was used to determine the difference seen while analyzing the images by PD Quest. Transferrin has been shown not to change in the blood despite the disease states (Wilkins *et al.*, 2005). Transferrin was used as a loading control for the Western blots and showed no change in expression for all the time points. The changes were seen at week eight for all the proteins. There might be an earliest time than eight weeks where the difference in protein expression commences at blood level. Sampling was performed at baseline, four days, four week and eight weeks and all these time point did not show differences between the groups for three earlier time points but showed difference at week eight. There was an increased expression of C-reactive proteins for the rats on high fat diets after eight weeks.

The other validated protein was the apolipoprotein AIV as shown by the PD Quest analysis to be more than two-fold enhanced. Validation at termination showed the

groups fed on high fat diets to be enhanced compared to the control group. The difference in the apolipoprotein AIV was confirmed by using the albumin containing sample because the difference was picked on the same sample by PD Quest. The difference with the use of the Western blot confirmed the enhanced expression of the apolipoprotein AIV in high fat diet fed groups (Figure 5.7.2).

The fetuin A and fetuin B expression was also confirmed. The expression of Fetuin A and Fetuin B also got enhanced like the C-reactive protein and apolipoprotein AIV. The reduced expression showed by the alpha 1 macroglobulin-like in high caloric diet fed groups was not confirmed due to the unavailability of the antibodies against the protein. There was no supply for either Western blot or ELISA. The expression for the fetuin A is shown in Figure 5.7.3 and Fetuin B in Figure 5.7.4. There was no confirmation by western blot for hemopexin-like which was found to be up-regulated with development of obesity.

6.8 Discussion

The use of proteomics to reveal the changes in protein serum expression successfully revealed three proteins that were up-regulated and one down regulated protein in the albumin depleted sample while one up regulated protein was found in the serum containing sample. The proteins that were identified using the mass spectrometry analysis were alpha 1 macroglobulin-like and hemopexin-like in the albumin depleted sample. In the albumin containing sample apolipoprotein AIV was identified using the mass spectrometry. Other proteins were identified with an aid of the 2D map as taken from the inflammation study by (Gianazza *et al.*, 2002a). The overall number of proteins as identified using the map and mass spectrometry summed up to six proteins that were above two-fold up-regulated or down regulated with development of obesity.

Western blot validation of results also showed the up regulation of proteins at termination time point which took place at week eight of the experiment. Four proteins were validated using the Western blot and the antibodies against alpha 1 macroglobulin were not available. The study had only four blood collections for serum. The difference in protein expression was only seen at the termination time point (week eight) and Western blot verified this for proteins that were verified. The gap between week four and week eight seems to be big enough for a possibility that the differential expression could have been earlier than week eight. There should have been more information to clearly identify when the changes in the serum protein start to take place should have been collection in week five, six and seven. The results

confirm the PD Quest analysis that identified the differences in protein expression for the animals on high fat diet compared to the control group. The identifications by mass spectrometry also made firm the accuracy of the MALDI TOF in identifying the proteins that were identified as deregulated proteins. There is a confidence in the map as chosen to be used for other identifications because there was a similarity in the identifications with what was seen on the 2D map. The outcomes of the proteomics approach gives an indication of proteomics as a tool to study the changes at a protein level due to obesity revealing proteome change in serum. With the readily available information in the form of bioinformatics will be efficient in acquiring information of proteins found. Bioinformatics has been used successfully to acquire the information on identified proteins. The readily available information from Expasy is useful because the known information has been used to find the k-values used in the quest for ortholog information as obtained from the KEGG pathway database.

Chapter 7: General Discussion

7.1 Discovery of candidate biomarkers of obesity using Proteomics

Diet has been highly implicated in development of obesity and the obesity associated health risks with diabetes being the well pronounced because of the insulin resistance that kick start after obesity development and vice versa. The use of high caloric diet is in the forefront of obesity research as a way of finding out the effect that diet has in obesity development studies. The balance in lipid breakdown (lipolysis) and lipid synthesis (lipogenesis) is of crucial importance with mitochondria getting to be of high interest because of its crucial role in fatty acid oxidation. There is less literature on mitochondrial function in rodent models of high fat diet (HFD) induced obesity and they are inconclusive possibly due to different experimental conditions like the type and content of dietary fat, composition of other macronutrients, duration of the consumption and the amount of calories consumed (isocaloric vs hypercaloric) (Shiraev *et al.,* 2009). Obesity-related imbalance in the fatty acid supply and utilization results in excessive accumulation of intrahepatic TAG and the development of liver disorders such as non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (Ciapaite *et al.*, 2011).

In this study we looked at the effect of different high caloric diets in inducing obesity and the effect of such diets in the protein expression in the serum. Supplementation of the protein with the powdered milk instead of the casein which is not natural or condensed milk which increases the sweetness of the diet compared to the control diet is the new approach to our knowledge especially the use of BF diet. Obesity from

overconsumption of HFD is characterised by an abnormal increase in adipose tissue mass, mainly resulting from the excessive storage of TG within adipocytes (Ha *et al.*, 2011). This has been seen in our study as well with high adiposity index for the L diet fed group compared to the LF fed group which are diets of similar fat type with half the amount of fat for the LF and supplementation of the calories with protein from milk. The same was also the case with the adiposity index of the BF fed group that was lower than that of L fed group.

There is a rise in interest of knowing the fatty acid content of the diets that are used in nutritional studies in order to have a complete picture of the fats used than the amount of fat used based on weight. It is not only the fatty acids but also carbohydrates and proteins are being studied for their role in satiety which is one of the contributors to the control of body weight accumulation. An increased protein intake diet-related ketosis, and low glycemic index food consumption have been suggested to decrease appetite (Parra *et al.*, 2008). Mediterranean diets which include nuts and olive oil have salutary health effects. These foods have low content of saturated fatty acids (SFA) but a high content of unsaturated fatty acids, mainly polyunsaturated fatty acids (PUFA) in walnuts and monounsaturated fatty acids (MUFA) in olive oil (Casas-Agustench *et al.*, 2009).

Saturated fatty acids are associated with high intake compared to the unsaturated fatty acids which is due to the differences in satiety (Kien *et al.*, 2005). Other studies similar to this found no differences between saturated and unsaturated fat containing meals in terms of satiety (Poppitt *et al.*, 2005, Cooper *et al.*, 2011). Our

study also showed no difference in the intakes that could be associated with satiety because the food intakes were not differing significantly. Studies using labelled FAs have shown that oleic acid and other unsaturated FAs are more readily oxidized than are saturated FAs (Kien *et al.*, 2005). This might contribute to the difference that was seen in fat pad weights for the rats that were fed L diet when compared to other high caloric diets where significant difference was seen. Another parameter that should be noted is that the other high caloric diets also had fat content that was less than the L diet and protein that is high but as stated in the literature the energy utilised for conversion of protein to fat for storage is higher than the energy that is used for storage of fats. The variation in the diets used in this study covers the scenarios in different diets because diets have been studied individually as high fat diet or high protein diet with comparison to the maintenance. This study answers the variation of different energy sources in one experiment.

The use of weight and height for diagnosis of obesity is useful to date but with some shortfalls because the weight doesn't tell the weight from fat. Weight gain for the rats that were fed high caloric diets was significantly different from the control group as early as the second week for the animals fed L diet. All the animals were significantly different on the third week compared to the control. Considering a significant difference in the adiposity index but no significant difference in the body weight for the animals fed high caloric diets is indicative of the shortfall that can be brought about by the use of body weight for muscular subjects that are not high fat depots. It is not disputable that the use of parameters like BMI and WHR are useful and gives results in a short space of time especially for epidemiological studies. The

supplementation of this information by the newly identified approaches would be useful in improving the accuracy in the diagnosis of obesity. Proteomics as an emerging line of research and is promising to give the answers to the phenotype related changes in the body and the changes revealed by the proteomics downstream.

It is well known that the genetic makeup and the environmental exposure leads to obesity development. The improvement in the protein research with the discovery of 2D PAGE has enabled the studying of proteins with enhanced output. The invention of the soft ionization in the late 1980s of soft ionization techniques leading to MALDI-TOF MS has made possible the identification of proteins (Wang *et al.*, 2006). Cancer research utilised proteomics approach for years before the diabetes and obesity research took the proteomics route. The proteomics has been applied to study tissues including liver (Wang *et al.*, 2011), WAT (Joo *et al.*, 2011) and skeletal muscle (Kim *et al.*, 2010) to identify the protein response with obesity development. The outcomes on tissue samples were showing the expression of proteins to be different between rats fed high fat diet and the rats on maintenance diet.

A strong need to study the proteomics in the blood has been recommended for various reasons. Amongst the noted reasons are the less invasiveness of the use of blood and because the secreted protein migrate between tissues through blood (Choi *et al.*, 2010). The study of plasma proteome recently published showed seven protein that are differentially expressed when comparing rats fed HFD with rats on maintenance diet (Liu *et al.*, 2012). Unlike in our study the rats that were used in this study were further studied based on their ease of developing obesity (obesity prone)

and resistance to obesity (obesity resistance). Seventeen proteins were found to be differentially expressed between the groups of which seven of those proteins were significantly changed upon HFD feeding. In our study increasing calories altered six proteins, with five up-regulated proteins and one down-regulated protein in response to diet. Another difference between the two studies is the use of serum in our study and the depletion method that resulted into two portions with the down-regulated protein identified in the albumin containing portion. All the five up-regulated proteins were found in the albumin depleted portion.

MALDI MS identification of the differentially expressed proteins identified up-regulation of apolipoprotein AIV, C-reactive protein, Fetuin A, Fetuin B and hemopexin in the albumin depleted sample. The protein spot in the albumin containing sample (down-regulated protein) was also subjected to MALDI MS and it was identified to be alpha 1 macroglobulin. Down-regulation of alpha 1 macroglobulin was reported before in the study on serum where the protein was seen to fade with development of obesity (Zhao *et al.*, 2008). Similar results were reported in the plasma with down-regulation of alpha 1 macroglobulin with HFD diet feeding in both OP and OR rats (Choi *et al.*, 2010). Recently it was shown in the study of serum proteome in both male and female rats that alpha 1 macroglobulin is down-regulated by HFD feeding irrespective of gender. The studies together with the current study suggest alpha 1 macroglobulin to be a potential marker of increased caloric intake with less dependence of caloric source, gender and susceptibility of individual to obesity development.

These findings are corresponding with the studies done before. The study performed by Matsumura, et. al. (2006) using OLEFT rats, which presents metabolic disorder and type 2 diabetes comparing to the LETO which are resistant to metabolic disorders as a control revealed the difference at the serum proteome level. These results were obtained by the use of two-dimensional liquid chromatography system. There was an increase in apoliporotein AIV which is corresponding with the findings that were found in our study using PD Quest image analysis. The apolipoprotein AIV is the only lipoprotein that is directly influenced by the dietary lipids (Whited *et al.*, 2005). The study by Jung-Won Choi et. al. (2012) reported no significant difference in apolipoprotein AIV between the rats on LFD and OP rats whereas significantly reduced levels of apoAIV were seen on the OR rats. In a separate study by Liu et. al. (2012), unexpected results were seen whereby low levels of apoAIV were seen with high fat diet in both males and females. Study in prepurbetal children shows that the response to obesity development is marked by the rise in apolipoprotein AIV which is strengthening the possibility of apoAIV as obesity biomarker (Galata *et al.*, 2011). The varying reports on the apolipoprotein AIV response however makes it not to be readily accepted as a reliable protein to look at for the changes triggered by diet.

There are studies that reported the association of obesity with inflammation rendering CRP to be responsive to inflammatory. The proteomics outcome of CRP showed the increase in response to HFD in the current study. A different response of CRP to HFD was seen in the plasma proteomics analysis of HFD fed rats whereby obese rats show reduced CRP levels than lean rats (Choi *et al.*, 2010). A study by Hao Liu, 2012

reports the reduction of CRP with HFD feeding which was consistent for both male and female rats. In the current study CRP expression was high with the feeding of high caloric diet. A recent study showed that restricting calories in a high fat diet reduces the expression levels of the CRP in the adipose tissue (Park *et al.*, 2012). The results that are available regarding the expression of CRP in the blood are differing between studies in relation to various conditions. The study on human subject on caloric restriction for weight loss was conducted in obese women to find the link between CRP, obesity, insulin resistance and obesity (McLaughlin *et al.*, 2002). The results show that obese and insulin resistant individuals are having high CRP levels that fall parallel with weight loss-associated insulin resistance improvements. Using CRP as a biomarker is not possible because gender differences gives results that differ in relation to the race (strain), nutritional state and various environmental factors (Liu *et al.*, 2012).

The expression of the proteins in the fetuin family such as fetuin A and fetuin B also increased with HFD feeding. Similar results were found for the study in the plasma whereby fetuin A was increased in response to obesity regardless of the proneness to obesity in rats (Choi *et al.*, 2010), but gender dependent because in males there is a reduction in fetuin A with increase in females suggesting that fetuin A increase protects females from obesity (Liu *et al.*, 2012). Fetuin A has been studied by various researchers to find the role that the protein plays in the body. Fetuin A has been identified as a major protein during fetal life and is also involved in important function such as inhibition of the insulin receptor tyrosine kinase activity, protease inhibition activities and development-associated regulation of calcium metabolism

and osteogenesis (Olivier *et al.*, 2000a). The study on fetuin null mice showed that the knocking down of fetuin serves as a protection against the development of insulin resistance (Mathews *et al.*, 2002).

Hemopexin which is the protein produced in the liver has been shown to increase in the liver with obesity development in male and female obese rats (Wang *et al.*, 2011). In our study the expression of hemopexin was increased in the serum. This could be potential advantage for future studies should hemopexin be studied in relation to the metabolic processes in the liver. Having complete information will enable the use of less invasive method which is obtaining blood compared to the use of biopsies to obtain information about the obesity. Hemopexin studied in liver transplant studies showed that it is fucosylated in relation to the transplant rejection in genetically different models making it to be a potential indicator in studying the response (Pan *et al.*, 2010). The outcome of this study though it was focusing on different disease but liver is known for its role in metabolism and toxicity of which hemopexin shows to be a potential candidate in reporting the liver condition.

The use of proteomics is an approach that will speed the understanding of the body machinery which is proteins. The understanding of proteins and their role in diseases will enable the understanding of the proteins and the ways in which the proteins operate in relation to what is taking place in the body. This will be advantageous because the proteins and their state determine what is to happen in the body. Understanding the diet induced obesity will be a positive finding because there is a rise in the diet related diseases that prevails due to excess energy storage. The

availability of the information centralization through bioinformatics will be a big contributor to the continuation in the proteomics finding. Bioinformatics is covered in the next section. The validation of the expressions by Western blot is a reliable method and it is available to most laboratories over ELISA which is also a sensitive and quantitative but with quite high cost than Western blot.

7.2 Validation of candidate biomarkers of obesity using Western blot and bioinformatics

The main advantage of looking at the protein expression is the understanding of the functioning post translation. Various studies looked at the expression of protein in relation to the diet used but most of the time they use one high fat diet and the control diet which is limiting the results to one to one comparison of diets. In this study the protein expression was performed in more than one high caloric diet. At the present moment the outcome of protein expression obtained proteomics require the validation of the results using the Western blot or other quantitative methods. The use of the validation methods address the technical errors that can be encountered while identifying the proteins as differentially expressed. Various studies use Western blot as a method of choice for validation of proteomics results (Wang *et al.*, 2011). Some studies use ELISA and both the methods are preferred for their sensitivity.

The Western blot results for the proteins that were validated were corresponding with the expressions as analyzed using PD Quest. The corresponding results promise the potential of the use of proteomics in the future as a clinical procedure in screening for diseases and possibly intervention can be done following proteomics outcomes. The proteomics evolve at a high pace in recent times.

7.3 Concluding remarks, conclusion and future perspectives

The study was able to reveal that the rise in adiposity can be reflected in the serum proteome. This was shown by the biomarkers that followed the trend based on the storage of fat by animals in different groups. This can be used in future to tell the lifestyle of a person based on the profile looking at the abundance of the proteins that are insulin resistance perpetuators. Fetuin A is known to play a role in insulin resistance. Insulin resistance is one of the factors that contribute to a high prevalence of obese diabetic subjects. The other proteins that gets enhanced during high caloric intake which include CRP, apolipoprotein AIV, fetuin B and hemopexin rise interest in studying them further which can lead to identification of their potential as biomarkers in obesity. Alpha 1 macroglobulin reduction during obesity development is also showing this protein to be a potential biomarker for obesity in the future. This study managed to reveal the responsive proteins during obesity development of which some quantitative methods could have been employed to come with levels of proteins quantitatively to come up with quantitative amounts of proteins that can be associated with obesity state or possibility of obesity development. Studying the identified proteins was beyond the scope of this study. The study itself managed to show that the trend in protein response is having relation to calories which will be a powerful tool in the future in identification methods that are going to be used in personal medicine since the caloric intake in this study showed to be having a pattern for high caloric diets when compared to the control diet. Further studies for identification of proteins that are associated with obesity and possibly alterations with such proteins will be necessary to enable the combating of obesity.

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