Gene expression profile of the subcutaneous adipose tissue of WNIN /Obese and lean rat models: A comparative analysis

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By

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DECLARATION

I, Srivani Vinay, hereby declare that this thesis entitled "Gene expression profile of the subcutaneous adipose tissue of WNIN/ Obese and lean rat models: A comparative analysis" submitted by me under the guidance and supervision of Dr.Nasreen Z. Ehtesham, is original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University of Institution for the award of any degree or diploma.

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DECLARATION

I, Srivani Vinay, hereby declare that the work presented in my thesis is entirely original, plagiarism free and was carried out by me at molecular Biology Division at National Institute of Nutrition Hyderabad under the supervision of Dr. Nasreen Z. Ehtesham. I further declare that this work has not been submitted in part or in full, for the award of degree or diploma to any University or Institution. I hereby agree that my thesis can be deposited in Shodganga/INFLIBNET.
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Dedicated to
My Parents and Vinay

Thank you for your love
encouragement and trust

In Loving memory of
Dr. Madhuri. S

I am sure you are seeing this and very happy that it is finally completed
Thank you.

ABBREVATIONS

ABTS	2,2'-Azino- Bis(3- ethylbenzothiazoline-6- sulfoni acid
ACC	Acetyl Co A Carboxylase
ACTH	Adrenocorticotropic hormone
ADIPOR1	Adiponectin receptor1
ADIPOR2	Adiponectin receptor2
AI	Anthropometric indices
AMPK	AMP-activated protein kinase
Angplt4	Angiopoietin like -4
ASP	Acylation stimulating protein
ATF-6	Activating Transcription Factor 6
ATP	Adenosine Tri Phosphate
BiP	Binding immunoglobin protein
BCA	Bicinchoninic acid
BLASTN	nucleotide blast
BMP	Bone morphogenetic protein
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
CART	Cocaine- and amphetamine-regulated transcript
CCR2	C-C chemokine receptor type 2
cDNA	complimentary DNA
Ct	Threshold curve
CNS	Central nervous system
CRH	Corticotropin- relasing hormone
CVD	Cardiovascular Disorders
CHOP1	Cruxhalorhodopsin 1
CPT-1	Carnitine palmitoyltransferase I
C-REBP	cAMP-response element binding protein
DAG	Diacylglycerol
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl Sulfoxide
dNTP	deoxy nucleotide triphosphate
DNA	Deoxy ribonucleic acid
Ebf-2	Early B –cell factor 1
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ERAD	ER-associated proteiin degradation
ERK	Extracellular signal-regulated kinase
EtBr	Ethidium bromide
FAS	Fatty acid synthase

FASTA	FAST-All
FIZZ3	Found in inflammatory zone-3
FOXC2	Fork head box protein C2
GALP	Galanin-like peptide
GRP78	Glucose related protein
GSK-3β	Glycogen synthase kinase 3 beta
GI	gastrointestinal
GABA	Gamma-Aminobutyric acid
GLUT-4	Glucose transporter-4
H_20_2	Hydrogen Peroxide
HCl	Hydro Chloric Acid
HMW	High Molecular weight
HRP	Hore Radish peroxidase
KEGG	Kyoto Encyclopaedia of Genes and Genomes
IKK	IκB kinase
IP	Intra peritoneal
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4
IL-8	Interleukin-8
IL-12	Interleukin - 12
IP-10	C-X-C motif chemokine
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRS-1, 2	Insulin receptor substrate 1,2
IRE-1	inositol-requiring enzyme 1
LB	Luria-Bertani
LMW	Low Molecular Weight
MCS	Multiple Cloning Site
MCH	Melanin-concentrating hormone
MC3-R	Melanocortin receptor 3
MC4-R	Melanocortin receptor 4
MCD	Malonyl Co-A Dehydrogenase
$MgCl_2$	Magnesium chloride
MMP	Matrix metalloproteinase
MOPS	3-N-morpholinopropanesulfonic acid
Myf5	Myogenic factor 5
Ob	Obese
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NCBI	National Centre for Biotechnology Information
NFκB	Nuclear Factor Kappa Beta
NIRKO	neuron-specific insulin receptor knockout
NP40	Nonylphenoxypolyethoxylethanol
P38-MAPK	p38- Mitogen- activated protein kinases
PAGE	Polyacrylamide gel electrophoresis

PAI-1	Plasminogen activator inhibitor-1
PANTHER	Protein analysis through evolutionary relationships
Pax7 $^{+}$	Paired box protein 7+
PCR	polymerase chain reaction
PDH	Pyruvate dehydrogenase
PFK	Phosphofructo kinase
PGC-1α	PPAR gamma co-activator1- alpha
PERK	Protein kinase RNA-like Endoplasmic reticulum kinase
PI-3-kinase	Phosphoinositide 3-kinase
PIPES	Piperazine_ N, N'-bis(2-ethyllsulfonicacid)
PMSF	Phenylmethanesulfonyl fluoride
PPAR-γ	Peroxisome proliferator- activated receptor gamma
Prdm16	PR domain containing 16
PRb	Retinoblastoma protein
Q-PCR	Quantitative –Polymerase chain reaction
RANTES	Regulated on activation, normal T-cell expressed And Secreted
Ref-Seq	Reference Sequence
RNA	Ribonucleic Acid
RNI	Reactive Nitrogen Intermediates
ROS	Reactive oxygen species
RT-PCR	Reverse Transcriptase-Polymerase chain reaction
SDS	sodium dodecyl sulphate
Shox2	Short stature homeobox gene
SREBP-1c	Sterol regulatory element-binding protein 1
STAT3	Signal transducer and activator of transcription 3
TE	Tris-EDTA
T2DM	Type 2 Diabetes milletus
TGF-β	Transforming growth factor beta
TIMPS	Tissue inhibitor of metalloproteinase
TNFR1	Tumor necrosis factor receptor 1
Tris	Tris-hydroxyethyl amino methane
TZD	Thiazolidinedione
VEGF	Vascular endothelial growth factor
WNIN / Ob	Wistar NIN-Obese
XBP-1	X-Box Binding protein1
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galacto pyranoside
Zfp423	zinc finger protein 4
α-MSH	Alpha -melanocyte-stimulating hormone
β-MSH	Beta- melanocyte-stimulating hormone
B3-ADR	Beta 3 Adrenergic receptor
γ-MSH	Gamma -melanocyte-stimulating hormone
5-HT1B	5-hydroxytryptamine receptor 1B
5-HT2C	5-hydroxytryptamine receptor 2C

11β-HSD-1	11β-hydroxy steroid dehydrogenase-1
mg / ml	milligrams per millilitre
pg / ml	pictograms per millilitre
bp	base pair
kDa	kilodalton
kb	kilobase
rpm	rotations per minute
µg	Micrograms
%	Percent
µl	Microliter
µM	Micromolar
mM	Millimolar
$^{\circ}$C	Degrees Celsius
g	Acceleration due to gravity
M	Molar
U	Units
V	Volts

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Introduction

Chapter 1

1.1 Obesity

The term *'Obesity'* is derived from the Latin word *'obesitas'*, which means "stout, fat, or plump". It is a condition of excess fat accumulation in the body resulting from an imbalance in energy homeostasis.

1.1.1 Historical Perspective

In the past, obesity was less prevalent, as the energy was mainly spent to obtain a meal, and a fat body was associated with prosperity, fertility, and social status in the society (Bloomgarden, Z., T. 2003, Hayes et al., 2005, Singleton P, 2008). With the beginnings of industrialization, changes in lifestyle and food habits, views on a fat body have gradually changed and in a span of few years, obesity has emerged as a global health pandemic adversely affecting human morbidity and longevity.

1.1.2 Definition and Classification

The World Health Organization (WHO) defines overweight and obesity as excess accumulation of fat that may impair an individual's health (Yamada et al., 2007). Obesity being a risk factor for metabolic dysfunctions leads to increased prevalence of serious diseases like diabetes type II, hypertension, CVD, atherosclerosis, arthritis, dementia, Alzheimer's, cancer and so on (Figure 1.1) (Guh et al., 2009, Zammit et al., 2010, Flegal et al., 2013). The body weight classification chart released by WHO is considered as the international standard of reference for bodyweight. Anthropometric indices (AI) such as Body Mass Index (BMI), Waist Circumference (WC), Waist-to-Hip Ratio (WHR), and recently included Waist-to-Height Ratio (WHtR), are used to predict metabolic risk associated with obesity. The Body mass index (BMI) is the most commonly used index to classify body weight in adults. It is defined as a person's weight in kilograms divided by the square of height in meters (Kg / m^2). The WHO, defines a BMI greater than or

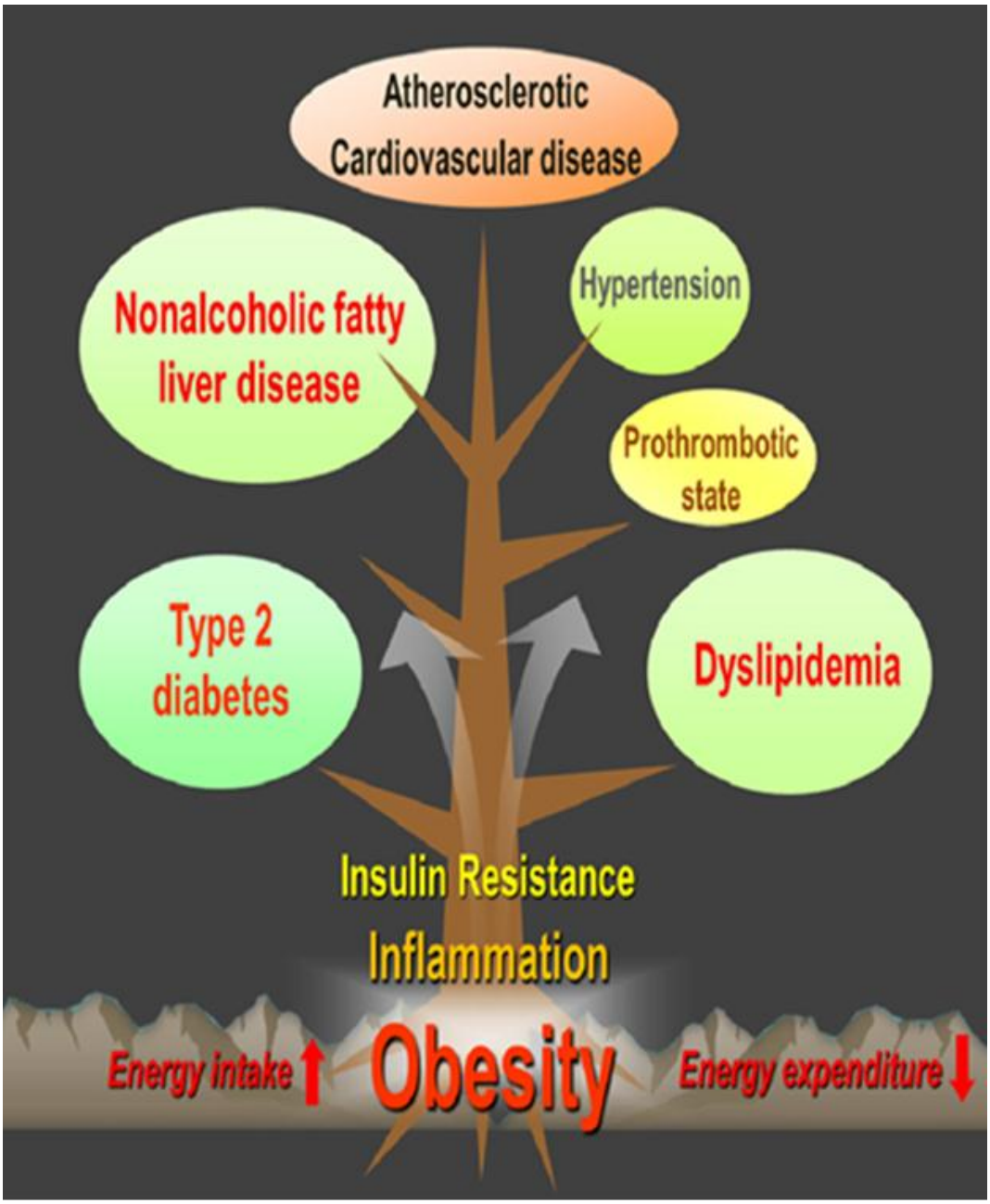

Figure 1.1: The concept of metabolic syndrome. The figure describes clinical complications branching out from obesity. An imbalance in the energy expenditure results in obesity and increases the risk for the development of variety of pathological conditions such as insulin resistance, type 2 diabetes, coronary diseases The development of inflammation in adipose tissue is linked to the obesity induced metabolic consequences like insulin resistance, type 2 diabetes, dyslipidemia, hypertension, coronary diseases.

equal to 25 as overweight and a BMI greater than or equal to 30 to be obese and also classify obesity further into three grades based on the severity. Other AI, like WC, WHR and WtHR were shown to accurately predict for visceral obesity and associated risk for CVD, hypertension, T2DM, and dyslipidemia in both sexes (Lee et al., 2008, Ashwell and Gibson, 2009). Recently**,** to account for the heterogeneity within obese individuals, a study suggested the obese with BMI above 30 should be further categorized into six types (Green et al., 2015).The BMI along with WC and WHR cut-off points chart released by the WHO is referred widely to classify and predict the risk factors associated with obesity (Table 1.1).

1.1.3 Prevalence

The prevalence of obesity worldwide (with BMI > 25 Kg /m^2) has continued to increase tremendously from the 1980s. This global incidence is of serious concern posing an additional burden on the developing countries such as India, Brazil, spreading even to Sub-Saharan African regions that are enormously impacting the global economy (Reddy, N., L. 2014). Obesity in children and adolescents in developed and developing countries is also accelerating (Marie et al., 2014). The WHO reports as per the year 2013 shows as much as 42 million children under the age of 5 were overweight or obese. As per the reports in 2014, more than 1.9 billion are overweight, of these over 600 million were obese. As per the WHO 2014 report of India, 60 % of total death is accounted by NCD diseases of which 25 % are CVD related deaths and 2 % from diabetes (Figure 1.2). The lower cutoff of value > 23 Kg /$m^2$for overweight and > 27 Kg /$m^2$for obesity has been proposed for Asian population.

Classification	BMI(kg/m2)		Disease risk(relative to normal weight waist circumference and waist to hip ratio		
	Principal cut-off points	Additional cut –off points	(M) >94cm (W) >80cm	(M) >102cm (W) >88cm	(M) ≥ 0.90 (W) ≥ 0.85
Underweight	< 18.50	<18.50			
Severe thinness	<16.00	< 16.00			
Moderate thinness	16.00-16.99	16.00-16.99			
Mild thinness	17.00-18.49	17.00-18.49			
Normal range	18.50-24.99	18.50-22.99 23.00-24.99			
Overweight	≥25.00	≥25.00	Increased	High	Increased
Pre-Obese	25.00-29.99	25.00-27.49 27.50-29.99			
Obese	≥30.00	≥30.00			
Obese class1	30.00-34.99	30.00-32.49 32.50-34.99	High	Very High	High
Obese class II	35.00-39.99	35.00-37.49 37.50-39.99	Very High	Very High	Very High
Obese class III	≥40.00	≥40.00	Extreme	Extreme	Extreme

Table 1.1: The international classification of body weight according to BMI. The table shows the international classification of body weight of an underweight, overweight and obese individual according to the BMI and their relative disease risk with respect to waist circumference and waist to hip ratio.

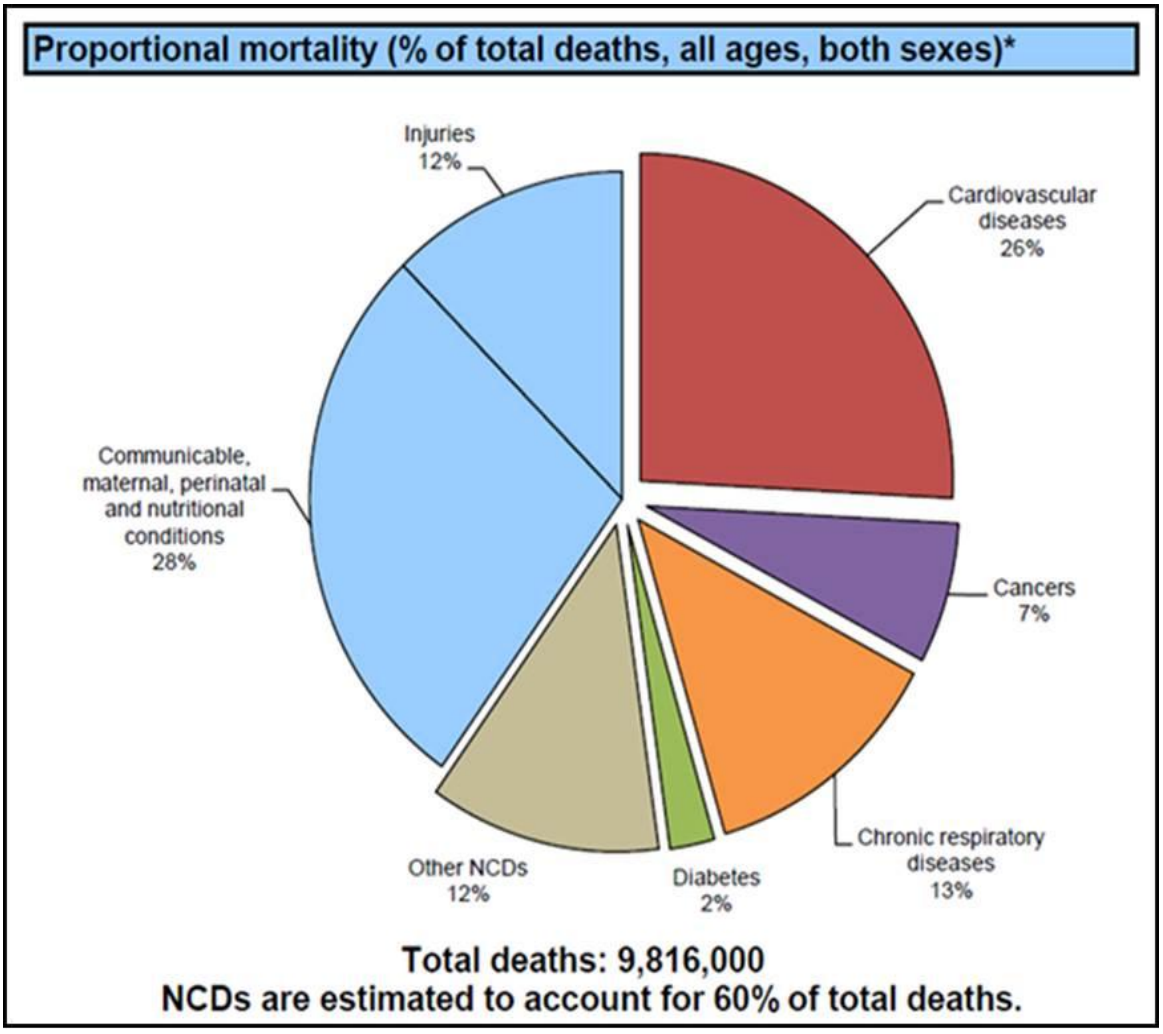

Figure 1.2: The percentage mortality profile of non-communicable diseases of India. The WHO reports on percentage of death occurring due to non-communicable disease in India as per year 2014 is almost 60 %.

1.2 Factors responsible for obesity

Obesity is a multifactorial disorder with genetic and environmental factors playing an important role in modulating energy balance. The complex interaction of susceptible genes in an obesogenic environment (food, sedentary lifestyle) reflects in the phenotype of the organism.

1.2.1 Genetic Factors

The percentage of genetic inheritance of fat mass varies from 30 -70 % (Bouchard C, 1991, Maes et al., 1997). Animal studies have identifying several single gene mutations (absence or dysfunctional) leading to obesity (monogenetic obesity). Subsequently, the homologous genes in humans have also been identified. Unlike the obesity seen in animals, three different categories of obesity has been identified in humans.

The 'monogenic obesity' that is usually rare and severe. Several groups have identified Ob (leptin), Ob-R (leptin receptor), and MC-4R (melanocortin receptor 4) mutations in humans which result in increased appetite (O'rahilly et al., 2003). Candidate gene and genome wide association studies have identified 9 loci involved in obesity (Choquet and Meyre, 2011). However, 'polygenic obesity' is the most common form of obesity which has increased prevalence worldwide. It is a result of a complex interaction between multiple factors, gene-gene and gene-environment (Figure 1.3). The polygenic animal models and the genome wide association studies have identified 58 loci contributing to polygenic obesity (Choquet and Meyre, 2011).

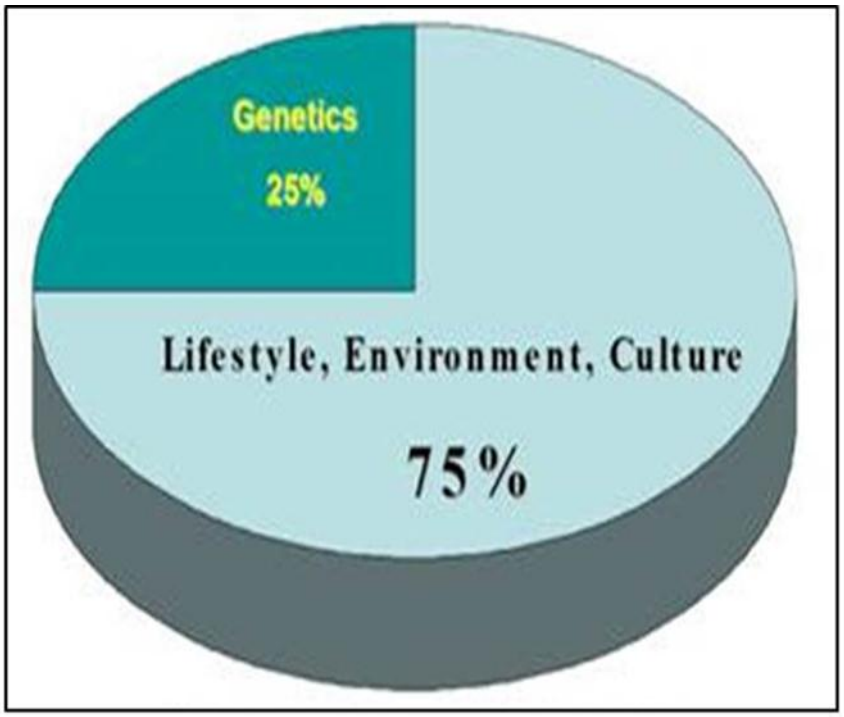

Figure 1.3: Factors contributing to obesity. The percentage of genetic and non-genetic factors that contribute to the development of obesity. The genetic factor range between 20 -70 % while the most predominant the non-genetic components that include lifestyle, social and regional factors.

1.2.2 Non-Genetic Factors

The obesity points to the crucial role of several environmental factors that are non-genetic in nature and are modifiable like physiological (physical activity), psychological diet, social status, ethnicity (McAllister et al., 2009). The increase intake of energy dense food along with decrease in physical activity are most common factors that are likely to have an impact on body weight (Figure 1.4).

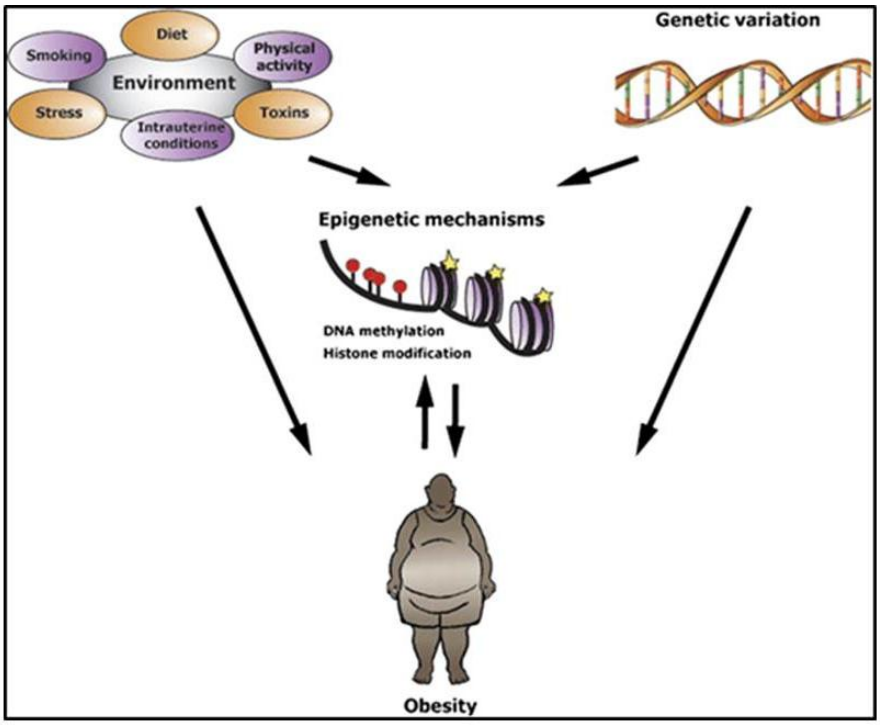

Figure 1.4: Genetic and environmental interactions determining obesity. A model describing the genetic and environmental interactions that leads to the development of obesity. The interplay of genetic interactions accompanied by epigenetic changes determines the gene-environment interactions.

1.3 The regulation of food intake and energy homeostasis

Food intake, one of the strong non-genetic factor contributing to obesity. It is one of the most natural instincts of an organism, very critical to derive energy for survival and an integral aspect of energy metabolism (Woods et al., 1998). Studying the regulation of food intake and its link to energy homeostasis has answered some of the key questions of energy homeostasis in the body. In simple terms, food intake is a process that initiates with hunger (urge to eat) followed by an appetite (choice of food) and terminates with satiety (no urge to eat). In the 19th century, several theories related to central regulation of food was proposed based on the strong experimental evidence and observations which,

revealed a complex, well-networked, self-regulatory central mechanism that can sense variation of energy requirements of an organism.

1.3.1 The hypothalamus

The classical experiments using electrical stimulation, lesions in the rat hypothalamus highlighted its role in energy homeostasis (Brobeck et al., 1946, Mayer et al., 1955). Other investigators confirmed this in a variety of species, including cats, dogs, and monkeys by replicating these observations and hypothesized "hypothalamic hyperphagia" results entirely from increased feeding behavior leading to obesity (Hetherington et al., 1940). The hypothalamus is the centre within the brain which receives and integrates diverse neurohormonal signals related to nutrition status of the body (Figure 1.5A). The hypothalamus consist of several nuclei involved in the regulation of food intake, the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the lateral hypothalamus area (LHA), the ventromedial hypothalamus (VMH), the dorsomedial (DMH) and the perifornical area (PFA) (Figure 1.5B). Experimental evidences have identified nutrient sensing neurons in the ARC, VMN and LH nuclei of the hypothalamus which are either stimulated or inhibited to modulate the food intake (Brobeck, J., R. 1946). Animal studies have shown that the bilateral lesions in the lateral area (LH) results in anorexia and weight loss, while the bilateral lesions in PVN caused hyperphagia and obesity. Thus, the lateral area (LH) is identified as the “feeding center" responsible for the urge to eat or the initiation of feeding, while the ventro-medial nucleus (VMN) is associated with "satiety center” capable of exerting inhibitory control over the lateral feeding centre when energy levels of the body are restored. (Anand and Brobeck, 1951, Bray et al., 1996).

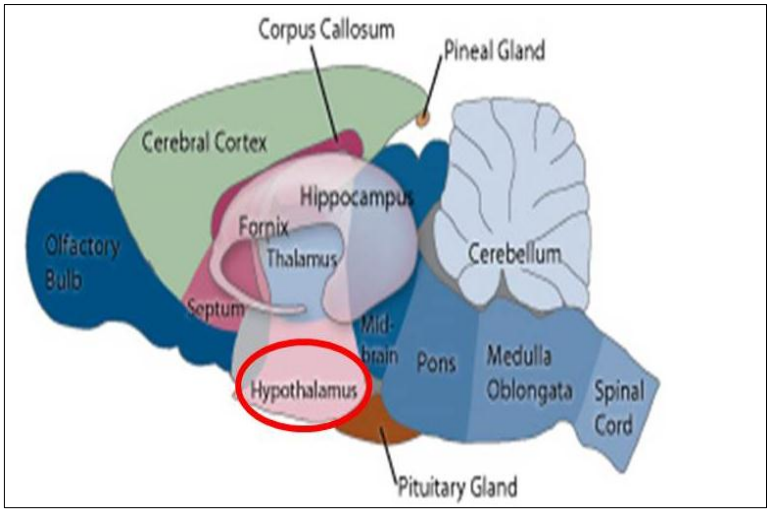

Figure 1.5A: The longitudinal view of rat Brain. The structure of rat brain showing the hypothalamus (circled in red). The hypothalamus is located ventrally below thalamus. It integrates central and metabolic signals from peripheral tissue like liver,adipose tissue to the higher regions of brain.

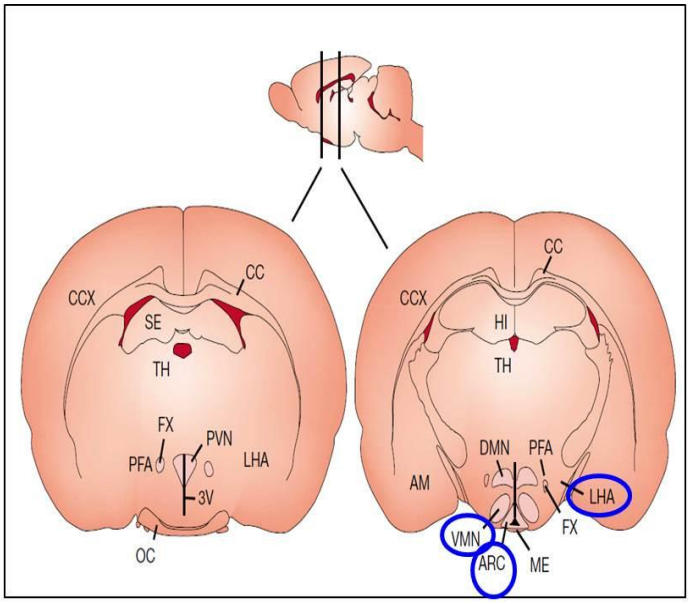

Figure 1.5B: The cross section of rat brain showing major hypothalamic regions involved in regulation of food intake and energy homeostasis. The cross section of brain at two levels indicated by two vertical lines shown in the left and right showing major hypothalamic regions implicated in regulation of food intake highlighted in circle. The Arcuate nucleus (ARC) is the center of peripheral nutrient integration. The first order neurons located in the Arcuate nucleus (ARC) respond to the adipose signals. Other regions include the ventromedial nucleus (VMN) - centre for satiety. The lateral hypothalamus area (LHA) - regulating the feeding behavior.

The arcuate nucleus (ARC) is an arc-like collection of neuronal cells located at the base of the median eminence around the third ventricle and has more access to blood supply than any region of brain. It is the centre of integration of various peripheral nutrient signals transmitted through efferent nerves. The ARC converts the nutrient signals to neuronal signals through projections into 'second order neurons' in the VMH, PVN, DMH, LHA and the PFA regions to further process the information.

The nucleus of the solitary tract (NTS) in the caudal brainstem receives various gastrointestinal (mechanical, chemical), peptide signals through the vagus nerves and conveys them to higher centres of the brain. These signals are assimilated with a reward value and received in the NTS as a neuronal response of CNS and transmitted for an appropriate physiological response (food intake or suppression, meal size **(**Travers and Norgren 1987).The NTS integrates peripheral (satiation and adipose) and neural signals that determine meal size (Broberger and Hokfelt, 2001).

1.3.2 The signals regulating food intake

Popular theories 'the lipostatic theory' and 'the set point theory' explained the mechanism of factors that control food intake that was centered around the amount of fat stores in the body (Kennedy G, 1953, Mu et. al., 1968). There are two different types of signals which regulate energy status of the body.

1.3.2.1 The Peripheral signals

1.3.2.2 The neuronal signals

The peripheral and neuronal signals are well networked to control food intake by regulating the meal size and satiation for short term or long term to maintain energy homeostasis (Figure 1.6).

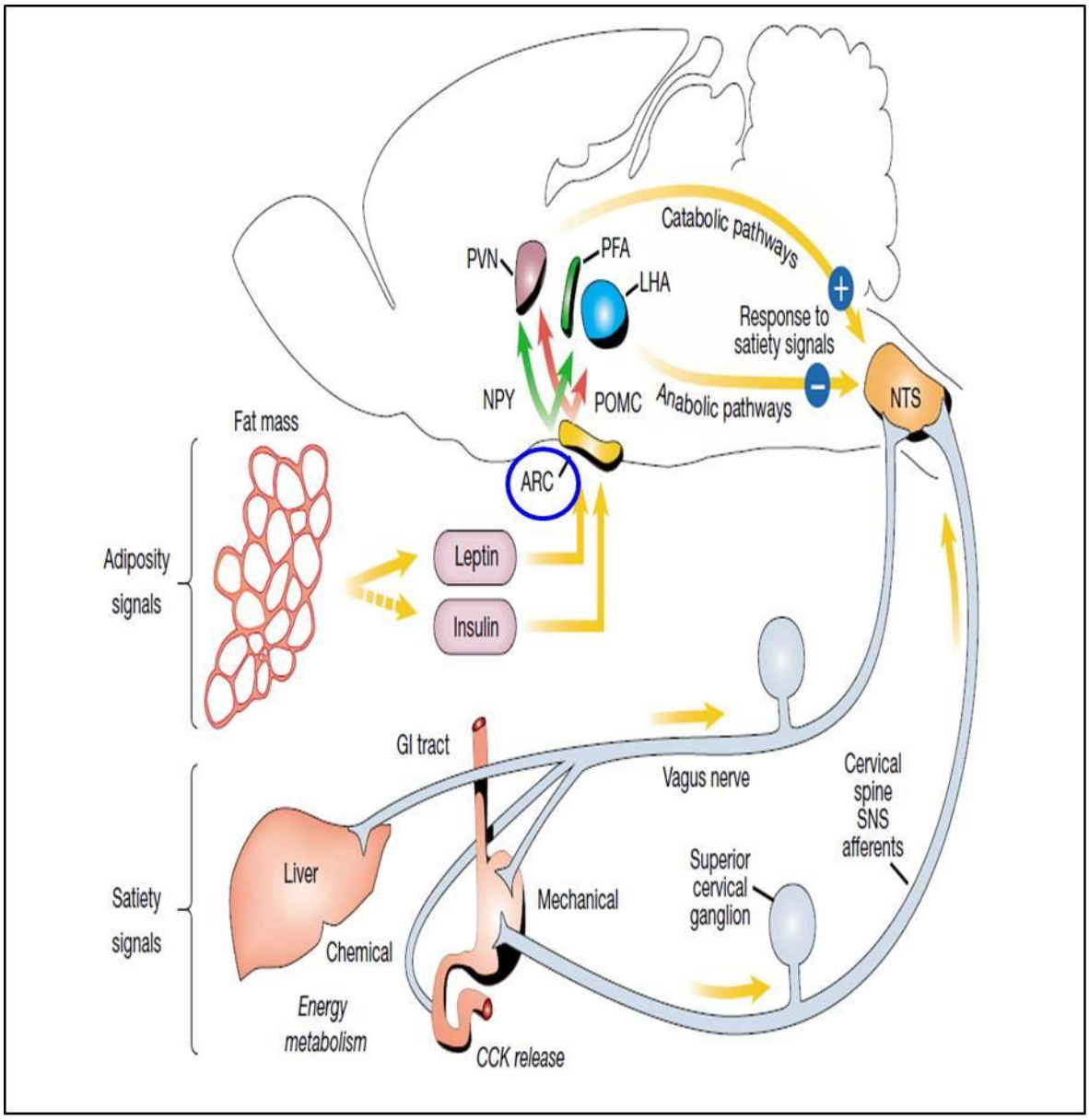

Figure1.6: Neuroendocrine model showing regulation of food intake. The model describes the integration of adiposity signals (leptin) from adipose tissue and (insulin) from pancreas and various satiety signals from gut and intestinal tract are integrated at the arcuate nucleus (ARC) circled in blue in the hypothalamus where the first order neurons respond and interact with the higher neuronal circuits in regulating appetite (meal size) and satiation.

1.3.2.1 The Peripheral signals

These signals are released by important peripheral organs like GI tract, pancreas, adipose, liver to the brain via the vagus nerves. They function in two ways (i) a short-term regulation which balances caloric intake and energy expenditure for short duration that may arise due to changes in activity with respect to the environment, and diet, and (ii) a long-term regulation which has a greater control for much longer period over body energy stores (Gasnier and Mayer, 1939). Later, these signals were termed as 'satiety factors' (Gibbs and Smith, 1973).The 'satiety factors' further can be divided into:

1. Satiation or Short term signal
2. Adipose or Long term signals

Satiation signals are short term satiety factors which suppress food intake however, do not contribute to reduction of overall body weight (Table 1.2). They are stimulated upon food ingestion and absorption of nutrients and impact within the duration of the meal by modulating meal size, increase the duration between meals by physiological processes such as reducing gut motility. Well studied short term signals are hormones that are secreted in the GI tract. The satiation roles of some peptides are described below:

1a. Cholecystokinin (CCK)

CCK was one of the first identified satiety hormone. It is released upon protein and fat digestion in gut and act via vasovagal reflexes. This peptide is produced by I-cells and mediates its action through CCK-1 receptors (Gibbs and Smith, 1973). The major forms identified in plasma are CCK-8, CCK-33 and CCK-39. Its main function is to induce satiety by stimulating pancreas to secrete enzymes, increase bile secretion, inhibits gastric secretion and gastric emptying (Konturek et al., 2004, Chandra and Liddle, 2007).

Hormones	Effect on Food Intake
CCK	Decreases
GLP-1	Decreases
PYY	Decreases
APO A-IV	Decreases
Enterostatin	Decreases
Bombesin	Decreases
Oxytomodulin	Decreases
Amylin	Decreases
Ghrelin	Increases

Table 1.2: List of GI Hormones. The list of various GI hormones that effect satiation by decreasing meal size or food intake.

1b. Peptide YY (PYY)

PYY is a member of a family of homologous peptides that includes pancreatic polypeptide and neuropeptide-Y (NPY). It is a 36 amino acid peptide initially identified from colon mucosa, rectum (Tatemoto et al., 1980) secretion is stimulated by lipids intake. The plasma levels of PYY were found to be elevated immediately after high fat diet (Pfluger et al., 2007).

1c. Bombesin like peptides

The family of bombesin like peptides includes bombesin and gastrin related peptide (GRP), neuromedin B (NMB). Bombesin peptides are exceptional as they act to increase the duration between subsequent meals thereby increasing satiety (Muurahainen et al., 1993, Stein and Woods, 1982). Mice deficient of GRP eat large meals and have reduced time between two meals subsequently developing obesity. Bombesin and GRP reduces food intake when administered systemically in humans and animals.

1d. Amylin

Amylin also called as islet amyloid polypeptide is secreted along with insulin from the β-cells of the pancreas. It reduces food intake by inhibiting gastric emptying, gastric acid secretion, and decrease glucagon secretion (Ludvik et al., 1997). They act through calcitonin receptors more as a hormone by directly stimulating neurons in the area of postrema in the hindbrain (Lutz, T., A. 2006).

1e. Glucagon like peptide (GLP -1, 2)

One of the cleavage products of pro-glucagon, glucagon like-peptide 1, 2 (GLP-1, 2) are mainly secreted from intestinal L-cells in ileum and colon (Holat, J., J. 1997, Giralt and Vergara, 1999). GLP-1 stimulates secretion of insulin and inhibits glucagon release, GI motility and is a major component of the "ileal brake" (Nauck et al., 1997).

1f. Ghrelin

Ghrelin, a 28 amino acid peptide stimulates sensation of hunger and feeding behaviour in conditions of fasting or starvation. It sharply rises before meal and falls in an hour of meal intake (Cumings et al., 2001). It has diverse functions that include protection of mucosal layer, stimulation of gastrin and HCl release and gastrointestinal motility control. Ghrelin initiates food intake by stimulating the NPY and AgRP expression in ARC of the hypothalamus (Trabaebert et al., 2002). Adipose signal's impact on the energy homeostasis is for a longer duration. They are secreted proportionally in response to body fat stores and reach the brain through circulation. Insulin and leptin are well studied hormone which act to regulate food intake and body weight in response to sensing the body fat stores (Stephans et al., 1998).The well-known hormones that function as long term signals or adipose signals are leptin and insulin.

2a. Leptin

Leptin is a 167-amino acid and a 16 kDa product of the Ob gene secreted mostly from adipocyte. It is the second adiposity signal identified in ob/ob obese mice (Zhang Y et al., 1994). Insulin stimulated glucose uptake stimulates leptin release from adipocyte. It acts via hypothalamic receptors to inhibit feeding and increase thermogenesis as a means to restore energy balance (Jéquier E, 2002, van Dijk G, 2001, Ahima, R., S. 2006). Leptin receptors are expressed widely in the hypothalamic nuclei including the ARC, PVN, VMN and LH perifornical areas as well as brain stem and caudal regions (Ahima et al., 2001). ICV injections of leptin have shown to decrease food intake and body weight (Wang et al., 1997).The ICV infusion in Ob mice showed decrease in food intake and glucose levels followed by reduction in weight and restored insulin sensitivity (Ahima et al., 1999). Leptin mediates its action by stimulating 'anorexigenic peptides' and suppressing the actions of NPY / AgRP and orexins (Ahima, R., S. 2005).

2b. Insulin

Insulin, a multifactorial hormone secreted from islet cells of pancreas that crosses blood brain barrier (BBB) is the first hormone implicated in the control of body weight by the CNS. It binds to insulin receptor expressed in several regions of the brain and reduces food intake by stimulating anorexigenic peptides and also signals brain about the fat stores (Woods et al 1974, 1979, Baskin et al., 1987). It cascades several other functions including growth, reproduction neuronal growth, and plasticity (Beilin et al., 2012). Studies in rodents have shown that direct administration of insulin into the brain inhibits food intake and reduces body weight, while mice lacking insulin receptors in the brain become obese (Bruning et al., 2000). Pharmacological drugs and hypothalamic lesion studies have shown the role of insulin by blocking the insulin signalling components (IRS-PI3K, IRS-2, insulin receptors) in the arcuate nucleus that resulted in increased

appetite and body weight (Niswender et al., 2003, Taguchi et al., 2007, Könner et al., 2011).

1.3.2.2 The Neuronal Signal

Neuronal signals that are stimulated after the peripheral signals are integrated and processed in the brain**.** They act to initiate (orexigenic) or suppress (anorexigenic) food intake through multiple pathways (Suzuki et al., 2012). The neuronal signals include the neuropeptides and neurotransmitters (orexigenic & anorexigenic) that act to induce ‘or inhibit food intake (Cone et al., 2001, Berthoud, H., R. 2002). Some of the neuropeptides regulated by adipose signals are listed below (Table 1.3A and B). Few functions of the orexigenic and anorexigenic neuropeptides and neurotransmitters that are important in food regulation are described below:

a. Orexigenic Peptides

The Neuropeptide Y (NPY) and (Agouti related protein (AgRP) are the most abundant and powerful orexigenic peptides expressed mainly in the primary neurons and also in several other regions of the hypothalamus (Shutter et al., 1971). NPY is a 36 amino acid peptide belonging to the pancreatic polypeptide family (Allen et al., 1983, William et al., 2000). The NPY and AgRP neurons, project into the other hypothalamic areas to exert their orexigenic effect such as the PVN, DMN and LHA. It stimulates feeding behavior upon starvation thus promoting positive energy balance (Clark et al., 1984, Stanley et al., 1986, Billington et al., 1991).

b. Anorexigenic peptides

The Pro-opiomelanocortin (POMC) /Cocaine and amphetamine-regulated transcript (CART) neurons are co-localized adjacent to NPY /AgRP. They function mainly to inhibit food intake. The melanocortins α-MSH, β-MSH, γ-MSH and ACTH are derived from POMC gene. The melanocortins mediate their effects through the

Anorexigenic Peptides	
Molecule	Action by neuronal Signal
α-MSH	Increases
CRH	Increases
TRH	Increases
CART	Increases
IL-1β	Increases
Urocortin	Increases
Seretonin	Increases

Table 1.3A: The anorexigenic peptides regulated by adipose signals to increase food intake.

Orexigenic Peptides	
Molecule	Action by neuronal signals
NPY	Decreases
AGRP	Decreases
MCH	Decreases
Hypocretins/Orexin	Decreases

Table 1.3B: The orexigenic peptides regulated by adipose signals to decrease food intake.

melanocortin receptors (MC1-5-R) show unique tissue expression (Voisey et al., 2003). Central activation inhibit feeding and leads to weight loss while, mutations of MC receptor (POMC) or blockade leads to increases feeding and weight gain (Lu, X., Y. 2001, Huszaer et al., 1997, Yaswen et al., 1999). Generation of MC4-R knockout mouse produced obesity very similar to Agouti (A^{vy}) mouse (Huszaer et al., 1997).

c. Neurotransmitters

The neurotransmitters relay the neuronal signal to the periphery organs. The principle neurotransmitters like GABA, acetylcholine, dopamine, and serotonin also have an important role modulating food intake. GABA is known to mediate food inhibition. GABA or its receptor antagonist when injected in LH region of hypothalamus suppresses food intake and thus decrease body weight (Turenius et al., 2009, Stanley et al., 2011). Dopamine is associated with brain motivation and reward system in inhibiting excessive food intake. In humans, low brain dopamine activity results in excessive food intake (Wang et al., 2002). Administration of dopamine agonists improves insulin sensitivity in animals and humans (Luo et al., 1999, Fulton et al., 2006). Likewise, serotonin exerts its activity on postsynaptic neuron receptors by increasing its retention thereby reducing food intake and body weight gain and increased satiation post food intake and absorption (Ribeiro et al., 2009, Petrissic et al., 1997). It acts via the two main receptors 5-HT1B and 5-HT2C.

1.4 Adipose Tissue - An Endocrine and Secretory organ

It is a loose connective tissue that occupies empty spaces in the viscera as fat pads protecting and cushioning the body. It stores surplus energy as triglyceride molecules (TGs) and releases in the form of free fatty acids (FFA) during energy deficit to meet the energy demands of the body. Initially understood as an inert tissue, adipocyte is redefined as a complex, metabolically dynamic endocrine organ (Siiteri, P., K. 1987) and subsequent identification and characterization of leptin in 1994 firmly established this (Zhang et al., 1991). The adipose tissue has certain characteristic features such as a) its capability for unlimited expansion to store excess energy as fat; b) its distribution as fat pads throughout the body, though not physically connected yet well-networked; c) its characteristic composition as a heterogeneous complex tissue consisting of adipose cells (adipocytes), stromo-vascular cells, immune cell, endothelial cells, blood vessels, connective tissue matrix, vascular and nerve innervations, d) its heterogeneity shown in regional fat depots with respect to its metabolic function and in their production of adipocytes (Proneca et al., 2014). It expresses several receptors and also secretes diverse hormones, cytokines and other bioactive peptides known as adipokines that allow it to respond and act locally (autocrine /paracrine) as well as centrally (Figure 1.7), facilitating cellular crosstalk with different organs system like muscle liver, immune system including brain (Paul Trayhurn and Stuart wood, 2004).

1.4.1 Types of Adipose tissue

In mammals, two types of adipose tissue, the brown adipose tissue (BAT) and the white adipose tissue (WAT) are identified that differ in colour, morphology, metabolic functions, physiology and gene expression (Cinti S, 2012). The relative amounts of white and brown AT are genetically determined and depend on several factors (age, sex, environmental, temperature and metabolic conditions). WAT is the main storage of

excess energy in the form of lipids, whereas BAT consumes energy to provide heat and restore body temperature. The balance between energy expenditure by BAT and storage in WAT critically regulates the volume and energy homeostasis (Rothwell and Stock 1979).

a. Brown adipose tissue (BAT)

The BAT was first identified by the Swiss naturalist Conrad Gessner in 1551 (Cannon and Nedergaard, 2008). The adipocytes are multilocular and brown in colour due to numerous mitochondria. They are mainly located in the interscapular, perirenal, and anterior subcutaneous sites in mammals. Evolutionarily, in humans, it was thought to be restricted to the neonatal and early childhood periods (Enerback S, 2010).The main function of BAT is to regulate body temperature and whole body fat through thermogenesis. Decrease in body temperature triggers the activity of uncoupling protein 1 (UCP1), exclusively expressed by brown adipocytes (Cannon and Nedergaard, 2004).

b. White adipose tissue (WAT)

White adipose tissues are the more predominant form of adipose tissue, represented mainly by superficial subcutaneous AT (SAT) and deep depots (visceral AT (VAT). The VAT is located inside the peritoneum as omental, mesenteric, retroperitoneal, perigonadal adipose tissue distributed around internal organs (e.g., stomach, liver, intestines, gonads and kidneys) to provide cushioning and protection. The fat accumulation differs in these anatomical locations with respect to metabolic condition and other factors such as age and gender. In rodents, subcutaneous fat is more in males than visceral fat, while reverse is true in humans (Bottner et al., 2004).The visceral adipose tissue (VAT) is more metabolically active than subcutaneous adipose tissue (SAT) and has a greater effect in their local and paracrine interaction with the surrounding blood vessels and lymph nodes (Robert et al., 2010).

1.4.2 Structure and function of White Adipose tissue

The importance of adiposity is not just on fat content but also its regional location. white adipose tissue is the main storage for excess energy on the form of lipids.

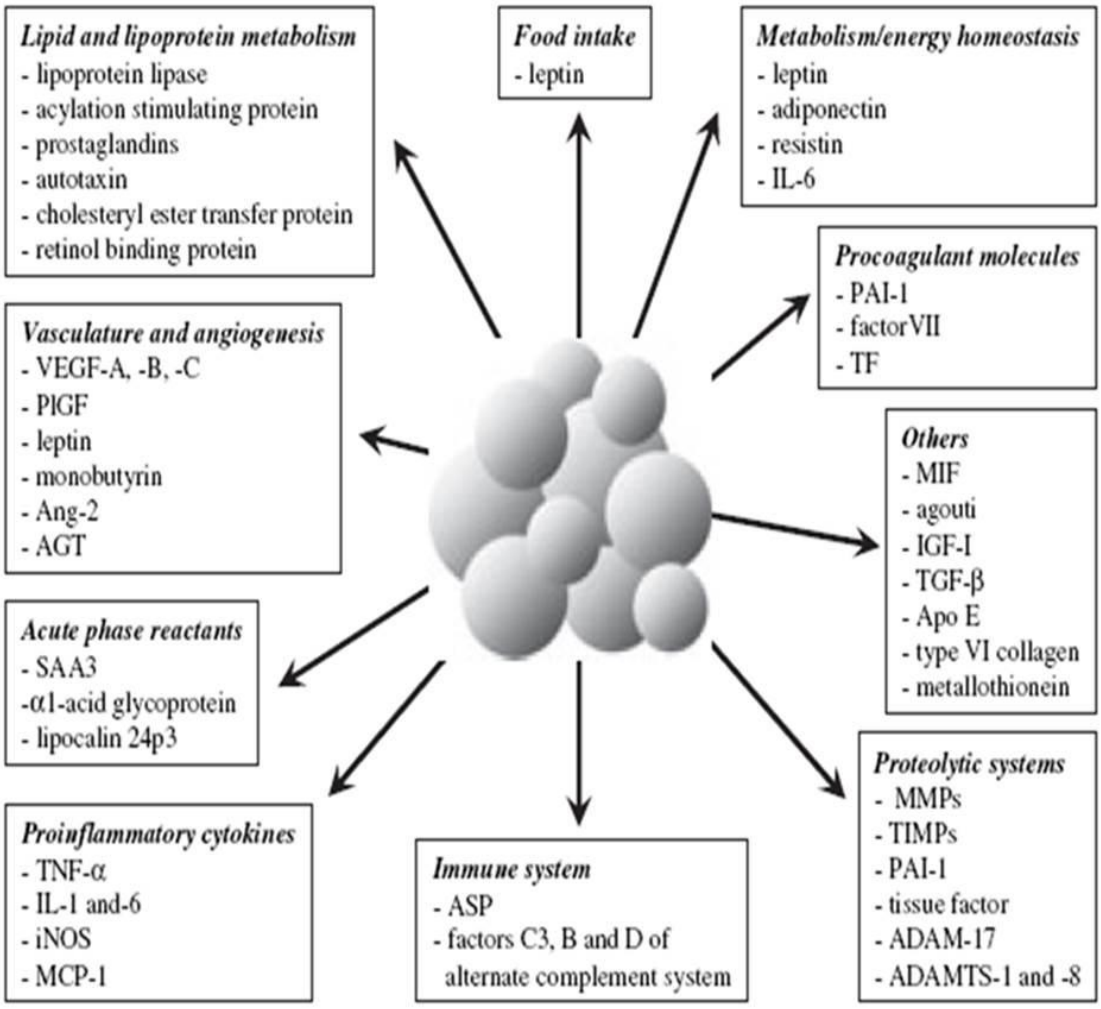

Figure 1.7: The diverse functions and adipokines secreted by adipose tissue. The adipose tissue is a major endocrine organ that secrets diverse adipokines that are involved in several physiological functions such as energy metabolism, immunity, inflammation. The diagram is an overview of the categorized endocrine role of adipose tissue and the secreted factors.

1.4.2.1 Structure and Functions

The WAT is a complex, heterogeneous mass containing adipocytes and the stromal vascular cells that includes mesenchymal cells, fibrocytes, pericytes, endothelial precursor cells, smooth muscle cells, immune cells, blood cells and pre-adipocytes (Fonesca-Alanis et al., 2007). In obesity, the adipose tissue is functionally dysregulated with altered secretions of diverse adipokines (hormone, peptides, cytokines) that disturb the energy metabolism and immune system leading to the development of insulin resistance, inflammation and further clinical complications.

1.4.2.2 Adipokines

Leptin

Leptin, mainly expressed in adipose tissue, and are present in lower levels in placenta, skeletal muscle, gastric and mammary glands and epithelium (Trayhum P, 1996, Fried et al., 2000). The effects of leptin on energy homeostasis are well documented. It mediates its actions through leptin receptor (Ob-Rb) expressed in peripheral organs and in the hypothalamus (brain) (Friedman and Halaas, 1998). Leptin enters through a saturable transport system and binds to the leptin receptor and signals via JAK-STAT-3 pathway (Tartagkia, L., A. 1997, Zabeau et al., 2003). Leptin acts as a 'starvation signal' and declines in conditions of fasting, weight loss and low fat stores. The leptin levels are proportional to body adiposity, food intake and body weight. Leptin suppresses food intake, stimulate fatty acid oxidation and thermogenesis.

Tumor necrosis factor (TNF-α)

The first adipose derived factor suggested representing a link between obesity, inflammation and diabetes that is secreted and expressed in white (Hotamisligil et al., 1993, Katsuki et al., 1998). Later, TNF-α was also identified to be released from the

infiltrated macrophages in WAT (Weisberg et al. 2003, Fain et al. 2004).TNF-α, a 17 kDa soluble molecule is synthesized and released into the circulation mediate signal through the receptors TNFR1 (Hotamisligil et al., 2003). The elevated TNF-α levels in obesity is strongly implicated in the pathogenesis of insulin resistance in the hepatocytes and adipose tissue and neutralization improved insulin resistance (Cai et al., 2005, Hotamisligil et al., 1993). TNF-α acts both in a paracrine and autocrine manner and regulates the expressions levels of several proteins like IL-6, IL-8, resistin, adiponectin, leptin, acute phase protein, haptoglobin (Prins et al. 1997, Coppack , 2001). It also regulates the function and development of WAT by stimulating lipolysis and inhibiting lipogenesis and adipogenesis (Hauner et al., 1995).

Adiponectin

A 30 kDa adipokines that is highly expressed in adipocytes with potent insulin sensitizing properties (Maeda et al., 1996, Pittas et al., 2004). The plasma negatively correlates to BMI and fat mass. Both protein and transcript levels are reduced in obesity (Hu et al., 1996, Ronti et al., 2006). They are found in serum as trimers (LMW) and multimers (HMW). The multimeric forms are active and low concentration in serum is related to impaired insulin sensitivity and T2DM (Waki et al., 2003). The interconversions of the two forms are regulated before secretion from the adipocytes (Schraw et al., 2008). There are two types of adiponectin receptors (ADIPOR1 &ADIPOR2), ADIPOR 1 has universal distribution and mediates the AMPK pathway while the other is specific to liver and leads to activation of PPARγ (Lee et al., 2008).

Interleukin-6 (IL-6)

IL-6 is a pro-inflammatory adipokine having pleiotropic effects (Papanicolau et al., 1998). IL-6 receptor (IL-6R) is homologous to the leptin receptor and exists as both

an approximately 80kDa membrane-bound form and approximately 50 kDa soluble forms (Mohamed-Ali et al., 1998). Epidemiological and genetic studies show that the IL-6 is associated with inflammation, insulin resistance, obesity, hyperinsulinemia, coronary heart disorder and diabetes type 2, suggesting a role in metabolic syndrome (Bulcao et al., 2006). In T2DM, the plasma levels are elevated and positively correlated to body fat mass and free fatty acids levels.

Monocyte chemoattractant protein-1 (MCP-1)

Monocyte chemoattractant protein-1 (MCP-1) is the most extensively studied CC chemokines produced predominantly by macrophages and endothelial cells and is a potent chemotactic factor for monocyte (Matshushima et al., 1989). The abundance of MCP-1 in white adipose tissue and plasma is increased in both genetically obese (ob/ob, db/db) and in Diet-Induced Obese (DIO) mice models (Sartipy and Loskutoff, 2003). The secretion is mostly from non-fat cells in adipose tissue and very less from adipocytes (Fain and Madan, 2005). The TNF-α enhances the expression of MCP-1 in humans as well as in murine adipocytes (Gerhardt et al., 2001). MCP-1 is upregulated by NFκB and p38-MAPK pathways (Fain and Madan, 2005). Moreover, MCP-1, contribute to the expansion and remodeling of adipose tissues and adipogenesis (Younce et al., 2009). MCP-1 links obesity to insulin resistance, hepatic steatosis, and macrophage infiltration into adipose tissue (Kanda et al., 2006, Panee et al., 2012).

Resistin

Resistin, is an adipokine that was a highlighted as molecular link between obesity and insulin resistance. It was discovered by three groups independently and identified as FIZZ3 protein in humans (Holocombb et al, 2000, Steppan et al., 2001, Kim et al., 2001, Rajala et al., 2002). Resistin contributes to the development of hyperglycemia and insulin

resistance (Flier, 2001, Steppan et al., 2001, Ehtesham, N., Z. 2001). Resistin is adipose secreted hormone that is induced during adipocyte differentiation and is downregulated by TZDs. Immunoneutralisation of resistin reduced hyperglycemia and improved insulin sensitivity in rodents (Steppan et al., 2001). Human and rodent resistin share sequence homology matches of 46.7 % at genomic and 64.4 % at mRNA level respectively. The mouse genomic sequence is approximately three times bigger than the human sequence which attributes to its functional differences (Ghosh et al., 2003**).** Resistin in rodents are secreted from adipocytes while in humans, they are highly expressed in monocytes, macrophage. There are differences in correlation of circulating levels of resistin to obesity in genetic models and diet induced models of obesity in rodents and humans (Way et al., 2001, Rajala et al., 2002). In rodents, the TNF-α seem to negatively regulate resistin expression (Shojima et al., 2002). The relationship of serum resistin and insulin resistance is still controversial in humans (Pereira et al., 2014). Resistin showed significant BMI-dependent associations with insulin resistance and factors linked with obesity and inflammation in patients with type 2 diabetes. Resistin in rodents link obesity to insulin resistance via pro-inflammatory pathways leading to hyperesistinemia which increases inflammation and insulin resistance (Mojiminiyi and Abdella, 2007, Lehrke et al., 2004) Although human and rodent resistin differ in their functions, circulating high levels of resistin is seen in altered metabolic conditions like obesity and diabetes in both rodents and humans (Lazar, M., A. 2007). Structurally, human resistin is secreted as intermolecular disulfide linked oligomer that is required to maintain its conformation and physiological function **(**Aruna et al., 2003, 2008). It has been identified as a surrogate marker in the onset and end point TB treatment (Ehtesham et al., 2011). The human resistin is also identified with chaperone like function (Suragani et al., 2013).

Although, resistin peripherally induces insulin resistance, it showed no effect on change in body weight or food intake (Rajala et al., 2003). Recently, centrally infused

resistin highlighted its role as a short term satiety molecule (Tovar et al., 2005), and as a hormone with dual effect showing decrease in food intake and regulated glucose level centrally but induced hepatic insulin resistance peripherally (Park et al., 2008). Overall, resistin detected in adipose tissue, is identified as a potential pathogenic factor that is increased in central adiposity (McTernan et al., 2000).

1.5 Animal models in obesity

The animal models are invaluable as they mimic characteristics of human obesity. They have been extensively used in a quest to understand the mechanisms of development obesity by pointing out some important molecules and device prevention with novel therapeutics. The advantages of easy handling, manipulation that can be extrapolated to human forms of obesity allow it to be extensively used experimental model.

1.5.1 Different models of animal obesity

Animal models studied in obesity can be broadly categorized into:

1. Models of mutations (monogenic or polygenic)
2. Models of manipulation (gene alterations by expression or ablation and exposing a genetically intact animal to obesogenic environment like the high fat diet).

All models show distinct pattern of obesity with hyperphagia, deviation in energy metabolism indicated by elevated metabolic parameters (hyperglycemia, cholesterol, and triglyceride) infertility (irreversible in females) and eventually develop co-morbidities like T2DM, insulin resistance, CVD. Different genetic models and non-genetic models of obesity are listed in table (1.4). The best worked out models are the monogenetic obese models arising from a single gene mutation arising from absence or dysfunctional gene. The well-studied models include the mutation of genes related to leptin pathway, (ob/ob

or the db/db mice, Zucker rat, ZDF, Koletsky rats) (Zhang et al., 1994), that provided a vital understanding of the adipose tissue secreting leptin and many other molecules and its receptors that function in energy homeostasis (Bray 1997,Chua et al.,1996). Animal models with single mutation identified with defective POMC, AgRP, MC4R genes that

Model	Mutation
Common monogenic models in mouse and rat	
Obese (ob) mouse	Leptin gene
Diabetes (db) mouse	Leptin receptor
Agouti yellow (Ay) mouse	Agouti gene
Tubby (tub)	Phosphodiesterase
Fat (fat)	Carboxy peptidase E
Zucker/fatty rat	Leptin receptor
Koletsky rat(kol)	Leptin receptor
Corpulent (cp)	Leptin receptor (null mutation)
ZDF	Leptin receptor
Wistar kyoto Faty rat	Zucker rat X Wistar kyoto
OLETF rat)	CCK 1 (spontaneous T@DM rats)
Knock out models	
AgRP	NPY
POMC	MC3/4
Polygenic models	
New zealand obese mouse	Japanese mouse (KK)
NH mouse M16 mouse	Spiny mouse
KK mouse Sand rat	Age related obesity onset (LOO mouse)
Osborne mendel rat	
Diet induce Obesity Models (high fat,cafeteria diets)	
C57BL/6 C57BL/6J C57BL/6N AKR/J	New Zealand obese Sprague-dawley rats (SD rats)
Non-rodent models	
Seasonal obesity models	
Syberian& syrian hamsters	Voles Lemmings

Table1.4: Different animal models available for obesity.

lie downstream of leptin and they are identified with defective POMC, AgRP, MC4R genes that lie downstream of leptin and the genetically engineered models of gene expression or ablation are extensively used to elucidate the molecular mechanisms.

1.5.2 WNIN / Ob rat model

The National Institute of Nutrition, Hyderabad, developed an in-house a genetic model of obesity in rat (WNIN / Ob) (Figure 1.8). The obese mutation is carried as an autosomal recessive trait affecting both the sexes and the obesity phenotype segregate in a typical Mendelian fashion (Giridharan et al., 1996).These animals are phenotypically characterized by hyperphagia, hyperinsulinemia and hypertriglyceridemia and hyperchelosterolemia and euglycemic showing visible phenotype of obesity as early as 35 days (Giridharan et al., 1996) and are known to be the world's fattiest rats (Jayaraman, K., S. 2005). The indigenous models were developed from a spontaneous mutation developed from a wistar line maintained through inbreeding over 90 years (since 1920). As a result, these animals are considered to be more genetically 'pure' in contrast to their western counterparts. These models develop almost all the major symptoms of metabolic degenerative conditions observed in human, making them ideal to study the metabolic syndromes at the molecular levels and being inbred can be compared better with their isogenic lean littermates. These animals gain weight rapidly and are too fat to move and prefer to lie close to food pellets. The mutants also lack grooming behavior and are infertile by 50 days. They show signs of rapid aging and tumors and other obesity related complications. Identification of the mutant loci in these animals is currently in progress, and so far no mutation was discovered in the known loci like leptin or its receptor (Kalashikam, et al., 2013).

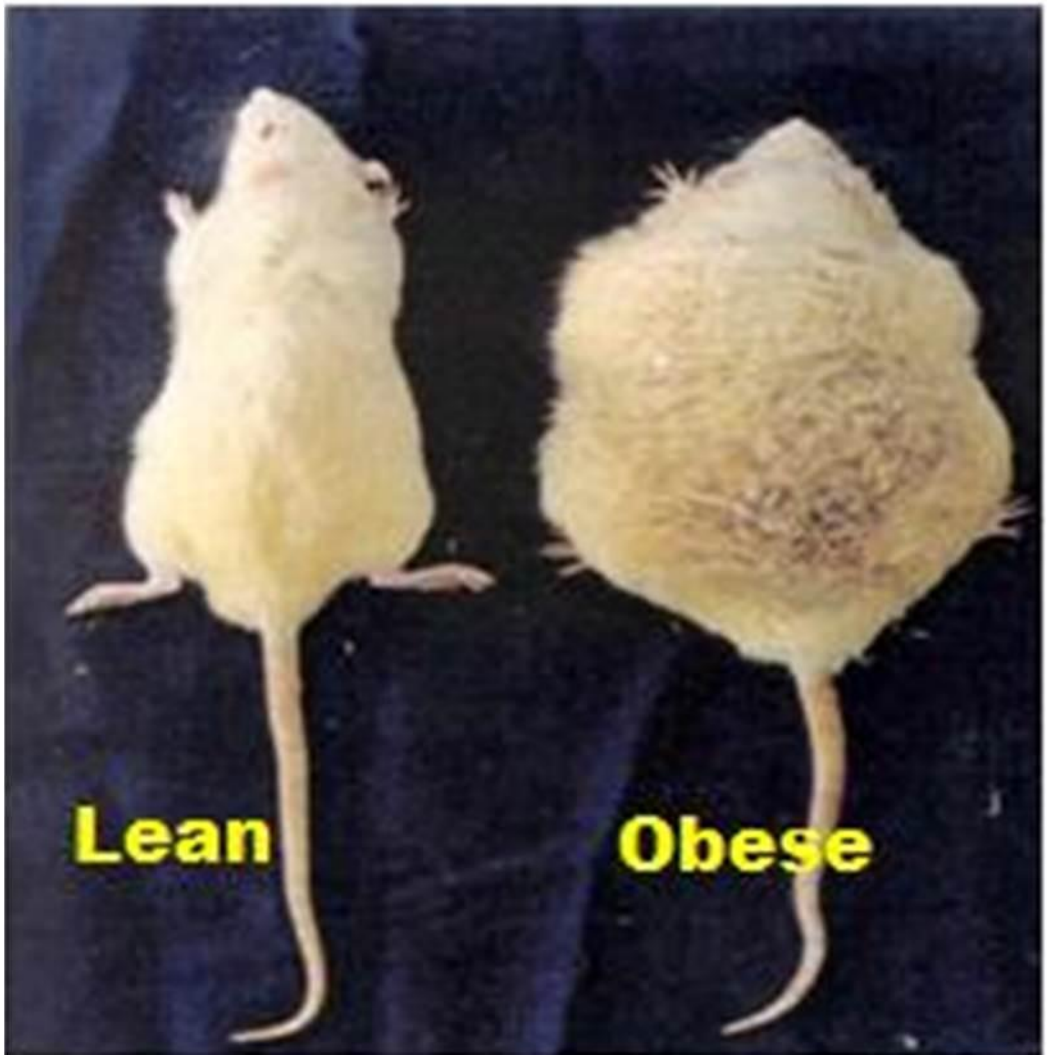

Figure 1.8: The WNIN / Ob and lean rat. The dorsal view of obese (WNIN / Ob) and lean rat. The WNIN / Ob Obese rat is a genetic obese rat model inherited in a typical Mendelian fashion. These animals gain weight rapidly and are twice the size of the lean counterpart.

1.6 Scope of the present study

The present study used three months male WNIN / Ob (obese and lean) rats for two different works. In the first part of the work, cDNA libraries of the subcutaneous adipose tissue of the obese and lean rats were generated to identify transcripts (cDNA) of lean and obese that represents a differential expression of genes in the tissue (i.e. overexpressed or repressed in obese rats). The bioinformatic analysis was done which classified the list of genes in to several functions and pathways. Some of the genes were validated using Q-PCR analysis. A comparative study of lean and obese rat of known pro-inflammatory cytokines were measured as a function of age (1, 3, 6, 12 months) in systemic as well as within the subcutaneous adipose tissue of male obese and lean rats. The work concluded with centrally infused human resistin in the hypothalamus to study the physiological effect on the body weight and food intake (energy metabolism) in these obese rats.

Chapter 2

Review of Literature

2.1 Adipose tissue (AT) - an active metabolic organ

The adipose tissue is a specialized connective tissue found in mammals, and functions as a fat storage site. There are two major types of inherently different adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT). Recently, specific surface markers have been identified to distinguish between these adipose tissues. The imbalances in the gene-gene and gene-environment interactions gradually lead to several dysfunctions within AT. Number of hormones released from AT such as, TNF α, leptin, adiponectin, angiotensin, IL-6, lipoprotein lipase and apolipoprotein E are responsible for different functions. The discovery of leptin and several other findings related to energy metabolism has changed our view on the functions of AT. The equilibrium between the WAT and BAT dictate the body fat levels by storing the triglycerides or utilizing excess for thermoregulation by BAT. Over the past two decades, studies on the plasticity and expandability of the WAT and BAT have been intensely investigated to understand the molecular mechanisms that determine its lineage, development and functions in order to find therapeutic solutions for obesity and related co-morbidities.

2.1.1 Tools to study gene expressions in AT

The progress made so far in understanding the molecular mechanisms of AT has been possible with the availability of advanced techniques for expression analysis such as serial analysis of gene expression (SAGE), differential display, subtractive hybridization, RT-PCR, high-throughput analysis (using gene chips, microarrays) for detection at transcript and protein levels. This has enabled comparative analysis of gene functions and their expression between different phenotypes (obese, diabetic, lean) and different anatomical locations of AT. Such studies in AT have shown differences in their order of fat storage, volume, function (signal response, secretion and expression) and gene expression profiles that vary in gender in both animals and humans (Gil et al.,2011). In

addition, animal studies using genetic (monogenetic, polygenic), diet induced obesity (DIO), pharmacological, knockout, transgenic models (specific gene studies by expression or ablation) as well as the use of *in-vitro* cell culture studies have immensely contributed to help understand the molecular aspects of functional, developmental lineages of adipocytes, simultaneously recognizing crucial role of a gene(s) or a specific pathway(s) in BAT and WAT that regulate energy homeostasis (Nadler et al., 2000).

2.1.1.1 Brown adipose tissue

The BAT functions to expend energy to restore body temperature. Unlike the white adipocytes, the molecular findings related to its functions and developments are very distinct (Billion and Dani, 2012). As reported by several lineage studies, brown adipocytes originate from the mesenchymal or the vascular endothelial (VE) progenitor cells expressing VE-cadherin^{+} along with Pax7^{+}/Myf5^{+} marker genes (myogenic marker) (Tran et al., 2012, Evan and Spiegelman, 2014). In addition, PPAR-γ, PGC-1α, PRDM16, Foxc2, are some of the transcriptional determinants identified to direct development processes of BAT, other molecules like irisin, natruiritic peptides, prostaglandins, Bmp-7,8, pRB, ZFP423, SMAD-3, TGF-β are required for its differentiation and functions (Seale et al., 2009, Hansen et al., 2004). Recently, beige adipocytes have been discovered in WAT which resemble white adipocytes in morphology but function similar to brown adipocytes (Wu et al., 2012, Park et al., 2014)**.** The current evidences demonstrate the presence of active BAT sites in adult rodents and humans (Nedergaard et al., 2007). In response to high fat diet, the role of BAT was indicated to protect from diet-induced obesity by robust decrease in adiposity with improved glucose tolerance and insulin sensitivity (Sale et al., 2011, Robidoux et al., 2014).

In humans, high degrees of obesity was associated with very little BAT reflecting the decrease in thermogenic capacity (Kozak et al., 2008, 2010, Wang et al., 2015). The brown adipocytes and the beige cells have beneficial metabolic effect and are a target of therapeutic solutions for combating human obesity and related metabolic disorders (Cinti S 2001, 2002, Ishibashi and Seale, 2010).

2.1.1.2 Subcutaneous and visceral adipose tissue

Several comparative studies (in animals and humans) of the major WAT depots, the VAT and the SAT using the current techniques in response to diet, drugs, gene expression or deletion identified several gene(s) and pathway(s) highly expressed or inactive. The VAT is identified to be metabolically and functionally active with the stromal vascular cells releasing prostaglandins, adipocytokines like TNF-α, IL-8, IL-1β, IL-6 and PAI (Fain et al., 2000, Fried et al., 1998, Alessi et al., 1997) that play a role in adipose dysfunction related to obesity. A comparative study in SAT and VAT of obese individuals has identified altered expression of networks of genes related to cardiovascular system development, inflammatory disease, endocrine and gastrointestinal disorders, cancer-reproductive disease, embryonic development, tumor morphology and cell cycle. Similarly, this has been shown in different animal models like cattle, pig and in humans (Vidal H, 2001, Hishikawa et al., 2005, Wu et al., 2008, Gerard et al., 2013).

Although the SAT is not a major contributor to major metabolic co-morbidities, but relative amounts is much more than other fat depots. It contributes to greater percent of free fatty acids (FFA), adiponectin, leptin (Soukas et al., 2000, Fraynet al., 2003). Studies related to extracellular matrix (ECM), identified some proteins playing a crucial role in cellular activity and tissue expansion that is expressed early in SAT and increases with tissue expansion (Poussin et al., 2008, Mori et al., 2014). The SAT also shows altered expression of genes related to adipogenesis consistent with an immature

adipocyte phenotype, which could reflect the expansion of the adipose tissue during obesity (Rodríguez-Acebes et al., 2010). It shows browning of adipocytes (trans-differentiation) and was identified to express some of the genes related to thermogenesis (SHOX2, PGC-1α, TLE-3, EBF-2, PRDM16) as seen in BAT (Cohen et al., 2014). The expression studies of PRDM16 in mice showed enriched expression in subcutaneous than visceral adipose depots. The adipose explants studies of animals and humans have shown that SAT responds better to insulin and secretes more adiponectin and less inflammatory cytokines (Altomonte et al., 2003, Kissebah and Krakower, 1994). Interestingly, the human biopsy studies have shown heterogeneity in gene expressions in different areas of subcutaneous adipose depots (upper abdomen, lower abdomen, flank and hip) with elevated levels related to the complement, coagulation cascades system and immune responses, basic biochemical metabolism including insulin signaling, the urea cycle, protein metabolism (glutamate, arginine and proline) in the lower abdomen (Kelley et al., 2000). Although, it is thought that SAT is relatively inert in contributing to metabolic complication, their functions lie beyond tissue expansion which are not fully understood and explored yet (Trayhum and Wood, 2004).

2.2 Metabolic disturbances in the adipose tissue redefining obesity

The AT is a nutrient sensitive tissue that responds rapidly with an ability to expand or reduce in order to maintain the energy homeostasis. Both deficit (lipodystrophy) and excess (hypertrophy) fat alters AT function (Hallberg et al., 2009).

In an altered state, such as obesity, considerable morphological, histological, functional and endocrinal changes occur within the WAT (Table 2.1). Healthy expansion of AT mass enhances recruitment of pre-adipocytes that differentiate into small adipocytes, along with minimal recruitment of other stromal cell types, and vascularization with minimal induction of ECM and inflammation (Ruderman et al.,

1981), (Figure 2.1). In an altered state particularly in obesity, the changes within AT is initiated with rapid growth of the fat pad through increase in adipocytes number and enlargement of existing adipocytes, limited vessel development as tissue expands, high degree of macrophage infiltration, secretions of pro-inflammatory cytokines, remodeling of the tissue and massive fibrosis is also seen (Ruderman et al., 1981). Consequently, deleterious physiological responses arises like hypoxia, adipocyte cell death, dysregulation in fatty acid fluxes enhanced pro-inflammatory chemokine and cytokine secretion, enhanced with macrophage infiltration that develops AT into a 'low-grade chronic inflammatory state' (Coppeck S.,W. 2005, Haugen and Drevon, 2007, Sun and Scherer, 2010). These sequential metabolic disturbances predispose obesity to multiple clinical conditions. Some insights in to these are described in the following sections.

2.2.1 Hypoxia

Hypoxia is a condition of inadequate oxygen below physiological levels. The WAT is not profusely innervated as the BAT; hence during expansion it quickly reaches to oxygen deprived state (Cinti S, 2005). Hypoxia is therefore an early determinant that limits healthy expansion of AT (Trayhurn and Wood, 2004). Evidences from animal and human studies in VAT and SAT report the development of hypoxia, reduced capillary density and vessels thickening as the adipose tissue expands (O'Rourke et al., 2011). The tissue oxygenation measure in different WAT depots of obese mice showed nearly 3 fold less levels than normal (< 40–50 mmHg) and is a direct evidence of hypoxia in rodent obesity (Ye et al., 2007). *In vitro* studies using cell lines 3T3-L1, primary adipocytes, and animal tissues shows overexpression of genes related to inflammation (leptin, IL-6, ANGPLT*4*, PAI-1, VEGF*)*, extracellular matrix proteins (macrophage migration inhibitory factor (MIF), the matrix metalloproteinase (MMP2 and MMP9) while adiponectin (ADIPOQ) and haptoglobin (HP) expression is decreased during hypoxia

Category of Change	Specific Change
Changes in non-adipose tissue	Increased fat content in liver Increased fat content in skeletal muscle Increased infiltration of organs with adipocytes
Macroscopic changes in adipose tissue	Increase in size of many or all adipose depots Changes in relative size of different adipose tissue depots
Histological changes	Changes in adipocyte number Changes in adipocyte size Changes in adipocyte differentiation and apoptosis Changes in non-adipocyte cell content Changes in neural network Changes in vasculature in adipose tissue
Functional changes in adipose tissue blood supply	blood flow Capillary permeability Changes in blood flow in response to food
Functional changes in adipose tissue intervention	Changes in sensory intervention of adipose tissue Changes in effector innervation of adipose tissue Changes in adrenoceptor number and type
Changes in adipose tissue in relation to energy storage	Uptake of glucose Uptake of fatty acids
Changes in adipose tissue in relation to energy release relation to food	Changes in responses of lipolysis in energy release relation to food Changes in response of lipolysis in relation to exercise
Changes in adipose tissue autocrine/paracrine function	Changes in secretion of paracrine factors
Changes in adipose tissue endocrine function	Changes in secretion of endocrine factors

Table 2.1: The changes in the structure and function(s) of adipose tissue in obesity. The list of specific morphological, histological, functional and endocrinal changes that occur within the adipose tissue seen in obesity

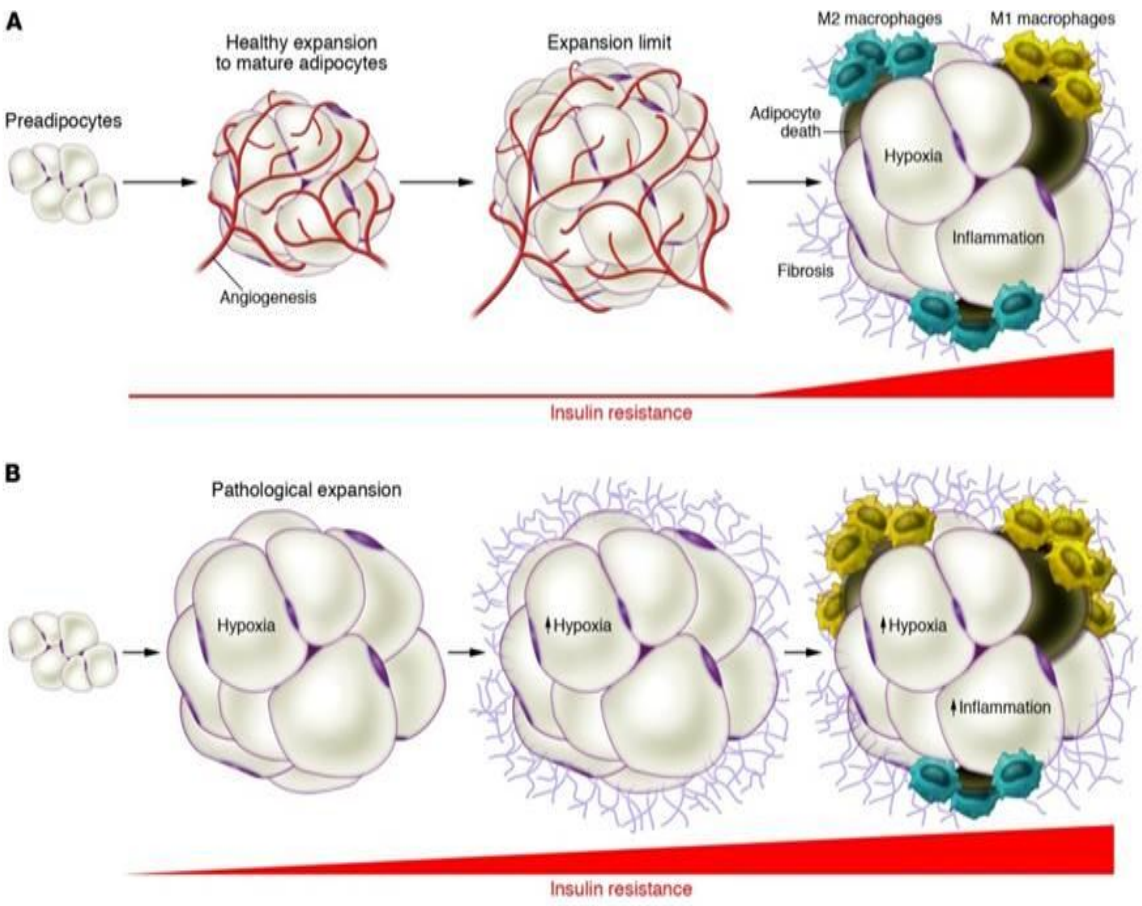

Figure 2.1: Healthy and unhealthy AT expansion. (A) Healthy AT expansion consists of an enlargement of AT through appropriate recruitment of pre-adipocytes along with an adequate angiogenic response and appropriate remodeling of the ECM. (B) In obesity, pathological AT expansion consists of massive enlargement of existing adipocytes, limited angiogenesis, and hypoxia with M1-stage macrophages prevail, leading to an inflammatory phenotype that is strongly associated with systemic insulin resistance.

(Lolmède et al., 2003, Wang et al., 2007). Bioinformatics analysis showed stimulation of several other genes/pathways and downregulation of PGC-1α and p38-MAPK signaling, and modulation of processes such as glucose utilization, lipid oxidation and cell death by hypoxia. The upregulation of ECM suggests that hypoxia-induced fibrosis in AT may be a key factor to stimulate the local inflammatory responses. In an interesting study, the hypoxia induced pre-adipocytes to secrete leptin, GLUT-1, IL-6, IL-4 and down regulates PPAR-γ and inhibits adipogenesis (Wang et al., 2008). Hypoxia induced complete loss of insulin-stimulated glucose uptake and immunostaining of AT demonstrates hypoxic areas accumulated with macrophages suggesting a link to inflammation and insulin resistance.

2.2.2 Endoplasmic Reticulum and Mitochondrial stress

The Endoplasmic Reticulum (ER) is an important site for cholesterol and steroid synthesis, triglyceride accumulation within adipocytes, post translational protein modification and trafficking that are assisted by chaperones (Kaufmann et al., 1999). The nutritional overload leading to increased tissue expansion, and triglyceride accumulation within adipocytes demands increased protein synthesis which leads to the functional inefficiencies of ER (ER stress). These results in accumulation of unfolded nascent proteins that are released into cytoplasm for proteosomal degradation responded by the unfolded protein response (UPR) to inhibit the abnormal protein synthesis through ATF-6, IRE-1, PERK signalling. The XBP-1 controls the expression of genes required for UPR signalling (Lee et al., 2008). The deletion of XBP-1 in mice showed reduction in fatty liver and cholesterol synthesis, hypolipidemia in liver and increased ER stress (Ron and Walter, 2007). Studies in Ob mice also showed elevated markers of ER stress with several other markers such as calnexin, and calreticulin protein chaperones such as BiP (GRP78), CHOP1, ERAD. It activates inflammation by activating JNK signalling

pathway (Horton, J., D. 2008, Gregor et al., 2007). The gradual elevation in ER stress leads to inflammation and insulin resistance eventually.

Mitochondrial dysfunction is defined as reduction in mitochondrial number, density, DNA, or function. Evidence on mitochondrial dysfunction leading to insulin resistance (IR) and vice versa has been well documented (Gianotti et al., 2008). In addition to ER stress, oxidative stress in mitochondria leading to generation of defective oxidation, increased ROS, peroxides, superoxides, free radicals also contribute to the etiology of adipocyte and muscle dysfunctions in obesity leading to insulin resistance (Wojtczak and Schonfeld, 1993). In state of adiposity, there is decrease in mitochondrial biogenesis, increased fatty acid oxidation and accumulation of lipid peroxides and ROS. ROS is shown to trigger inflammation by activating kinases (JNK, IκK pathways) that decrease insulin synthesis and signaling. The infusion of free fatty acids in mice showed increased peripheral insulin resistance and oxidative stress (Marfella et al., 1995). Recent studies consistently suggest that mitochondrial over activation actually induces insulin resistance, in which ATP blocks AMPK activity (Szendroedi et al., 2012).

2.2.3 Inflammation

Several factors like infection, elevated lipids, hypoxia, hyperplasia and hypertrophy within AT triggers inflammatory response. They are classified into interleukins, interferons, chemokines, hematopoietic factors and growth factors. Inflammation is the body's first response to a stimulus of infection, repair or tissue damage to clear necrotic cells. Inflammation also plays a role in many biological processes like growth, differentiation, cell division, and apoptosis. The increase in circulatory pro-inflammatory cytokines and the infiltration of macrophages secreting proteases, eicosanoids, cytokines and ROS, RNI intermediates are the two most striking discoveries within WAT that redefines obesity as a chronic 'low-grade' inflammatory

state (Cancello and Clement, 2006).The first molecular link to obesity and inflammatory state in the adipose tissue was demonstrated in rodent model of obesity in 1993. High expression of TNF-α was seen in adipocytes of obese rats and subsequent neutralization with TNF-α soluble antibody lead to an improvement in insulin sensitivity in these animals. Further, decrease in weight and circulating inflammatory marker improved insulin sensitivity. Similar reports from *in vitro* studies showed impaired insulin stimulated peripheral glucose uptake upon TNF-α treatment (Hotamisligil et al., 1993). These observations exhibited the existence of a strong role of inflammation in the development of insulin resistance. The inflamed adipose tissue secretes number of pro-inflammatory cytokines such as C-reactive protein (CRP), TNF-α, IL-6, and IL-8, chemokines like MCP-1 with receptor CCR2, IP-10 (Juge-Aubry et al., 2005) and anti-inflammatory mediators that have insulin sensitizing effect such as IL-10, IL-4, TGF-β and adiponectin (Friedman and Halaas, 1998, Welchhart et al., 2008, Mohammed Ali et al., 2001**)**. TNF-α also inhibits PPAR-γ function in the induction of insulin resistance (Ye J, 2008, Ghoshal et al., 2009, Shi et al., 2004) thus linking obesity induced inflammation to inslin resistance (Barbarroja et al.,2010). Furthermore, inflammation in adipose tissue is mediated by inflammatory adipokines produced by adipocytes and the infiltrated 'adipose tissue macrophages' (ATM).

The macrophages mediate immune responses to eliminate numerous molecules, ranging from small lipids to dead cells and colonies of pathogens. An increased level of macrophage markers in AT are reported in ob/ob mice, db/db mice and diet-induced obese (DIO) mice (Xu et al., 2003). Recently, T cells accumulation along with CCL5 / RANTES and its receptor CCr5 and CCr3 in WAT has been discovered (Wu et al., 2011). In humans, RANTES protein and transcript level are expressed more in the stromal vascular fraction of male visceral adipose tissue. Infiltration of macrophages and several immune cells like T cell, lymphocytes, NK cells, Mast cells in AT is a hallmark

inflammation (Weisberg et al.,2003, Rausch et al.,2008). The causes of increased macrophage infiltration in adipose tissue in obese individuals is not clear, however, the altered signalling by adipocytes, from lipotoxicity or reduced angiogenesis resulting local adipose hypoxia seem to trigger this response that eventually leads to several co-morbidities like insulin resistance, atherosclerosis, arthritis, CVD (Permana et al.,2006).

2.2.4 Insulin resistance

Insulin resistance (IR) is defined as failure of target tissues like pancreas β-cells, skeletal muscle, heart, vascular endothelium, neurons liver including adipose tissue to respond to insulin (Saltiel, A., R. 2001) (Reynisdottir et al., 1994, Kahn and Flier, 2000). The clinical studies demonstrated the presence of obese subjects who are euglycemic as well as a population with obese subjects which develop diabetes who showed a consistent presence of insulin resistance with T2DM and their non-diabetic offspring developing insulin resistance eventually. Similar correlation studies in animals and humans showed an IR state that eventually develop into diabetes type 2 and reversal upon treatment with insulin-sensitizing agents. This indicates that obesity is one of the prime causative factors leading to insulin resistance. IR is a physiological marker for the development of type 2 diabetes. Initially explained as 'Randle effect', the inhibition of glucose oxidation by excess fatty acid is a form of glucose intolerance that leads to, insulin resistance (Randle et al., 1963, Bergman and Ader, 2000). Several hypothesis proposed for IR include mitochondrial dysfunction, ER stress, hyperinsulinemia, lipotoxicity, inflammation, and aging. Insulin resistance is manifested by increased insulin secretion, increased hepatic glucose output with reduced glucose disposal in peripheral tissues (Kahn and Flier, 2000)

In insulin resistant state, there is decreased glycogen synthesis and increased glucose production. The skeletal muscle is accounted for maximal glucose intake for glucose oxidation and most of it to be stored as muscle glycogen. In insulin resistant

states, the glycogen stores are depleted and the glucose uptake is reduced. In adipose tissue, increased triglyceride synthesis, lipolysis and decreased glucose transport is seen. Several metabolites that are part of altered lipid and glucose metabolism facilitate and serve as markers of IR (like the expression of key enzymes PFK, PDH, ACC, MCD and AMPK). Key molecules like PPAR-γ, SREBP-1c, and C-REBP are well studied on their effect on glucose and fatty acid metabolism (Hue and Taegtmeyer, 2009). The defect in insulin stimulated glucose uptake is the rate limiting step for glycogen synthesis and glucose oxidation leads to IR and eventually proceeds to T2DM. Transgenic mice models generated to study insulin signaling components in different tissues highlight the failure of insulin stimulated glucose uptake and other physiological defects (Anindita Nandi et al., 2004, Kraegen et al., 2001). Hyperinsulinemia is seen in IR where plasma insulin levels are elevated as a result of over production of β-cells activity. Studies suggest that high insulin levels are induced by leptin resistance and fatty acids leading to insulin resistance. IR is primarily achieved through inhibition of insulin signaling by inactivation of IRS-1 and insulin receptor, the inhibition of PPAR-γ which directs lipid synthesis and storage in cells, through transcriptional activation of lipogenesis or by inducing expression of related enzymes. Population studies have linked systemic inflammation to insulin resistance (Grimble, R., F. 2002) and obesity induced adipose tissue inflammation leading to insulin resistance and predisposes to pathogenesis of T2D were reported in humans and rodent animal models (Hotamisligil, G., S. 2006, Shoelson et al., 2006, Schenk et al., 2008, Ouchi et al., 2011). AT as a result of fat accumulation turns hypoxic triggering inflammation with elevated expression of key adipcytokines like TNF-α, IL-1, IL-6, MCP-1 PAI-1, CRP leading to macrophage and immune cell infiltration. The accumulation of lipid metabolic intermediates like DAG, ceramide, ROS and H_2O_2 peroxides released as a result of ER and Mitochondrial stress are the physiological

changes in response to excess fat accumulation that gradually lead to IR and other serious clinical conditions (Ye et al., 2009).

2.3 Resistin: An adipokine with new roles

Identifying new functions of existing proteins and discovering many adipokines and hormones has immensely helped in the process of our understanding the pathophysiology of obesity. Resistin is one such discovered adipokine, a member of a secretory protein family known as resistin like molecules (RELMs). It was identified as an adipogenic hormone exclusively secreted from adipocytes that contributes to the development of insulin resistance. The human resistin is a functional ortholog of rodent form, and primarily secreted by monocytes and macrophages (Yang et al., 2003, Ghosh et al., 2003, Tomaru et al., 2009). It was focus of research interest as it was identified to link obesity to inflammation and insulin resistance (Hotamisligil, G., S. 2003). Rodent and human resistin showed differences in their genomic organizations, secretions and functions (Ghosh et al., 2003). Rodent resistin induced hepatic insulin resistance and increased glucose output while human resistin stimulated production of pro-inflammatory cytokines like IL-12, TNF-α (Silswal et al., 2005). The effects of different functions of resistin gradually lead to the effect of insulin resistance that predisposes to conditions such as diabetes, arthritis and CVD. The inflammatory pathways mediated by human resistin by elevated expression in non-adipose cells could be an alternate way to mediate insulin resistance. Accordingly, human resistin is identified with new roles to mediate insulin resistance through inflammatory mechanisms. The serum resistin was shown to be a useful biological marker in diabetic patients with CAD and restenosis (On et al., 2007), atherosclerosis (Reilly et al., 2005), cancer (Dalamaga M, 2014). Ehtesham et al. also reported on the utility of human resistin protein as a surrogate marker for monitoring tuberculosis disease onset and its treatment (Ehtesham et al., 2011). Recently, human

resistin was identified with novel role showing chaperone like activity *in vivo* (Suragani et al., 2013). However divergent in function, the high circulating levels of human resistin seen in altered metabolic conditions like obesity and diabetes possibly serves as marker for insulin resistance via inflammation. The peripheral effect of resistin on insulin resistance is well documented while the central effects are unknown.

The central action of resistin mediating anorectic effects is an addition to its emerging new roles. (Figure 2.2) In the recent years, resistin expression was reported in periventricular and arcuate nucleus regions of hypothalamus and pituitary regions of brain in ob/ob mice suggesting the presence of central mediated actions in line with leptin (Wilkinson et al., 2002, 2005). Several studies of acute and chronic resistin infusions primarily in rat models are reported. The central infusion of resistin in hypothalamus showed short - term satiety in rats but did not affect body weight (Tovar et al., 2005). Long term infusion of resistin showed decrease in food intake and increased peripheral insulin sensitivity but not as much in comparison with leptin and insulin. A report published explained the possibility of leptin and resistin independently improved energy homeostasis by modulating different hypothalamic signaling (Park et al., 2008, Brucelin S, 2008). Another report showed, central infusion of resistin in a wild type and in *Retn*-/- rats activated NPY in several regions of the hypothalamus and peripherally, induced hepatic insulin resistance by decreased expression of phosphorylated AMPK, GSK-3β and increased the activity of glycogenolytic enzymes, glucose 6-phosphotase, hepatic glucose output and expressions of pro-inflammatory cytokines associated with insulin resistance like SOCS-3, IL-6, TNF-α (Singhal et al., 2007, Muse et al., 2007). The activation of NPY expression are conflicting as some report the anorectic effects of resistin observed in rats model are associated with reduced expression of NPY, AgRP, while other reports maintain that the activation of NPY is needed to mediate peripheral effects in liver (Cifani etal., 2008). The central effects of resistin probably show

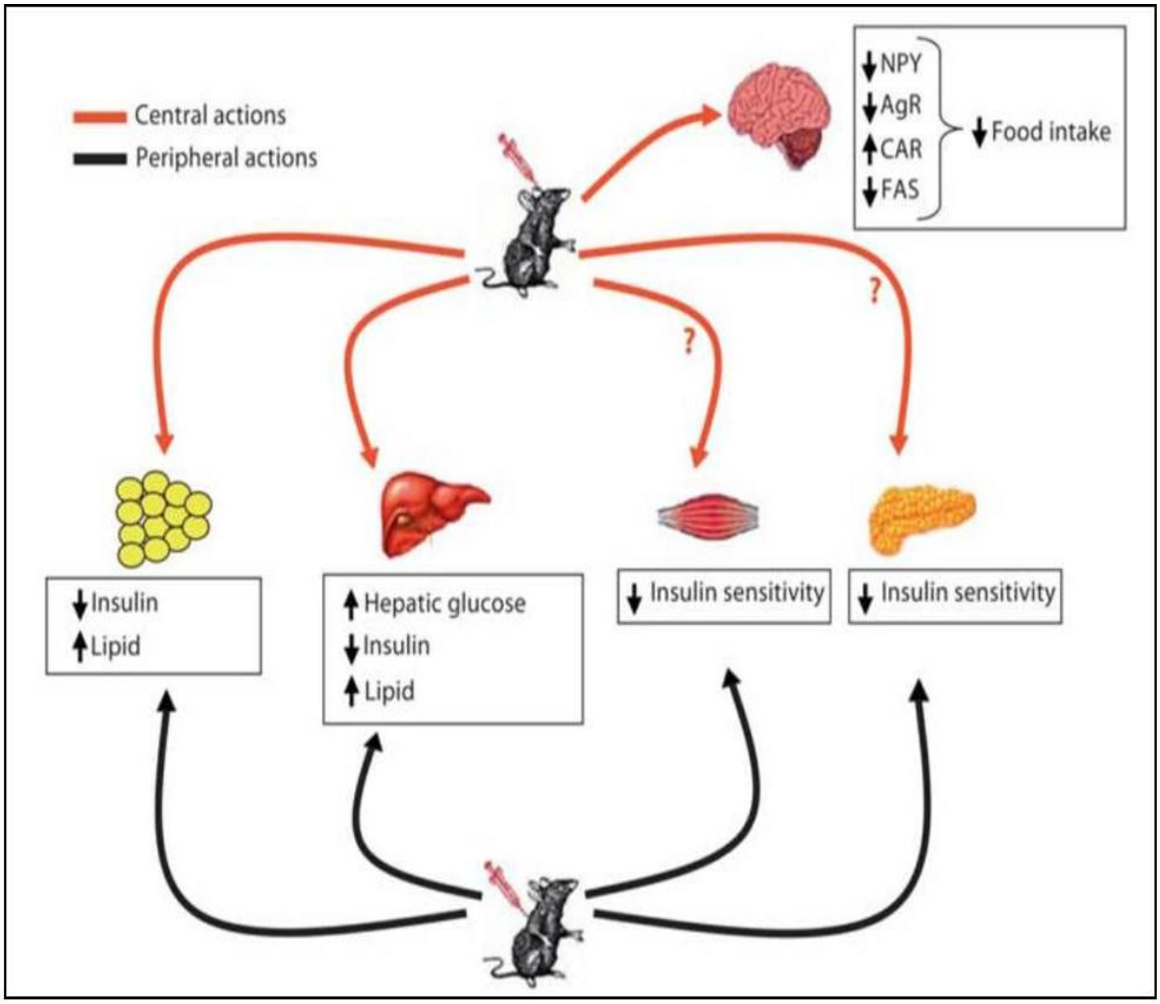

Figure 2.2: Central and peripheral effects of resistin. An illustrative showing different effects central and peripheral resistin on food intake, glucose homeostasis and lipid metabolism and insulin sensitivity seen in hypothalamus, adipose tissue, liver, muscle, pancreas.

differences depending upon the nutritional state, duration of infusion (chronic or acute) and regions within the hypothalamus. Supporting this, study on chronic and acute administration of resistin centrally in rats identified resistin to function as unique molecule exerting dual effects. It showed anorectic effects centrally and peripherally regulated fatty acid metabolism especially in liver and white adipose tissue (Vazquez et al., 2008). Another comparative study of chronic (for 6 days) and acute (for 90 minutes) administration of 10 µg resistin in the lateral ventricle and in the pituitary gland of both fast and fed rats also reflected in *in vitro* studies showed resistin strongly regulates lipid metabolism in the pituitary gland by increasing β-oxidation activity, decresing lipogenic enzymes like CPT-1, FAS, MCD and the expression of pro-inflammatory cytokines like IL-6, TNF-α opposite to its actions seen in liver (Rodriguz-Pacheco et al., 2013).

Central role of human resistin are being reported only recently. The peripheral effects of human resistin was studied in rodent model by expressing human resistin in macrophages but lacked any expression of murine resistin (Humanised mice resistin). These mice were subjected to high-fat diet and the effect on AT was studied. The WAT clearly showed accelerated inflammation and developed insulin resistance with time. This study pointed out that human and rodent resistin as functional orthologs contribute to insulin resistance through different mechanisms (Qatnani et al., 2009). Another report on the human resistin infusion for 14 days in the rat hypothalamus showed inhibition of insulin signaling components like (IR, AKT and ERK) associated with reduced IR expression and with upregulation of suppressor of cytokine signaling-3 (SOCS3) and phosphotyrosine phosphotae1B (PTP1B). Additionally, central resistin promoted the activation of the serine kinases Jun NH2 -terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) enhanced the serine phosphorylation of insulin receptor substrate 1(IRS1) and increased the expression of the pro-inflammatory cytokine IL-6, TNF-α in the hypothalamus and in key peripheral insulin sensitive tissues. Interestingly,

resistin was also shown to directly bind to Toll-like receptor - 4 (TLR - 4) receptors in the hypothalamus, leading to the activation of the associated pro-inflammatory pathways and development of insulin resistance (Benomar et al., 2013). Though there exists no uniformity in many of the reports on the role of resistin in suppressing food intake and insulin resistance, these studies highlight the existence of central mechanism of resistin in the regulation of lipid and glucose metabolism and its role in the development of insulin resistance through central mechanisms yet to be identified.

Chapter 3

Material & Methods

3.1 Generating subtracted cDNA libraries of subcutaneous adipose tissue of WNIN / Ob obese and lean rats

3.1.1 Tissue collections and Storage

The Obese rat (WNIN / Ob) and its lean counterpart were well maintained in the animal house facility of the National Centre for Laboratory Animal Science (NCLAS). The animals were kept in sterilized polypropylene cages with a layer of dry husk. The rat chow and water (autoclaved in the animal house) was given *ad libitum*. The animals were fasted a day before sacrifice. They were euthanized followed by cervical dislocation. The subcutaneous adipose tissues of three months old male lean and obese rats were taken out and wrapped and immediately flash frozen in liquid nitrogen. All the tissues were stored in -80 $^{\circ}$C until further use.

3.1.2 Total RNA isolation

3.1.2.1 Materials

The various reagents required for RNA isolation, Tri reagent, DEPC, deionized formamide, Sodium acetate, EDTA, Agarose and EtBr 10 mg / ml, 100 % ethanol were purchased from sigma Aldrich. chloroform, isopropanol, formaldehyde, glycerol, bromophenol blue dye was procured from Qualigens

Composition of buffers and solutions-

a) Loading Dye:

   50 % Glycerol, 1 mM EDTA, 0.4 % Bromophenol blue

b) Sample buffer:

   10 ml deionized formamide

   3.5 ml formaldehyde

1 ml 10X buffer

The solution was made up to 15 ml by adding nuclease free water.

c) 10X Formaldehyde denaturing running Buffer:

200 mM MOPS

50 mM sodium acetate

10 mM EDTA

pH to 7.0 adjusted with NaOH

The 10X buffer was filter sterilized using 0.2 μM filter and stored in 4 $^{\circ}$C. The running buffer was made to 1X as per the volume required for the gel electrophoresis by adjusting the volume with DEPC treated water.

3.1.2.2 Tissue homogenization

The subcutaneous adipose tissue was used from lean and obese rats for all the experiments. The adipose tissues of both lean and obese were weighed equally and not more than 300 mg was taken per 1 ml of trizol. The tissue was minced and put in the eppendorf tube containing zirconium beads (Unigentix) and homogenized in a regulated bead beater (Unigentix) for approximately 30 seconds. The homogenized tissue sample was centrifuged to remove tissues debris. The homogenate was transferred in a fresh 1.5 ml eppendorf and left at room temperature for 5-10 minutes.

3.1.2.3 RNA Isolation

The total RNA was isolated as per the manufacturer's instructions (Invitrogen). Briefly, the lean and obese tissue homogenate were prepared as explained above. 200 μl of chloroform was added and shaken vigorously and incubated at RT for 10 minutes. The samples were centrifuged at 12000g for 10 minutes and the aqueous phase was transferred to a fresh tube. This separates the aqueous layers containing RNA from an

inter phase containing genomic DNA and lower organic pink phase that has cellular protein lipids and glycoproteins. The aqueous phase is carefully pipetted out not touching the interphase and organic phase. The total RNA is precipitated by the addition of 500 µl of isopropanol. The tubes were gently mixed and left at room temperature for 10 minutes. RNA was pelleted by centrifugation at 12000g for 10 minutes at 4 °C. The pellet was washed two times with 70 % ethanol and centrifuged at 7000g for 5 minutes at 4 °C. The RNA pellet was air dried by inverting the tube and suspended in nuclease free water. The tubes were incubated at 60 °C for 20 minutes to make sure the entire solution is dissolved. The RNA samples (lean and obese) were aliquoted and stored in -80 °C. The concentration and quality of RNA was estimated by Nanodrop ND1000 spectrophotometer (Thermos Scientific).

3.1.2.4 Formaldehyde gel electrophoresis

The gel casting tray (BioRad) was set up in a laminar hood with 2 % agarose and formaldehyde (1.23 M), EtBr (0.5 ng / ml) was casted. The solidified gel with clear wells was then placed in the tank containing 1X formaldehyde running buffer. The RNA samples were prepared by adding 3 µl of RNA was added to 10 µl of sample buffer and 2 µl of nuclease free water. The solution was heated to 65-70 °C for 5 minutes and immediately placed in ice for 2 minutes. The samples were electrophoresed at 50 volts for 45 minutes. The gel is visualized and documented using the gel documentation system using the quantity one software (Bio-Rad). The RNA samples of both lean and obese were used for hybridization.

3.1.3 Ultra competent cell preparation

3.1.3.1 Materials

$CaCl_2.2H_20$, $MnCl_2$, PIPES, DMSO, Ampicillin were purchased from Sigma Aldrich and LB Media was purchased from Hychem.

3.1.3.2 Transformation

The *Escherichia coli* strain DH5α was used for ultra competent **c**ell preparation. Competent cells were prepared using the Inoue Method (Sambrook and Russell, 2006) to obtain high efficiency. The transformations of competent cells were done to calculate the transformation efficiency. Transformation efficiency $>10^8$, indicative of good competency were prepared and used for colony screening. The competent cell efficiency was calculated as below:

$$\frac{\text{Colony forming units (CFU)}}{\mu\text{g of DNA}} = \text{Efficiency of transformation}$$

3.1.4 Construction and screening of subtracted cDNA libraries

The cDNA library generated using subtractive hybridization enables a global view of expression patterns of various genes and their representative pathways from two comparative tissues, highlighting their potential role in an altered metabolic condition. The advantage of the technique has been well utilized to generate comparative cDNA libraries from different adipose tissues of well characterized genetic models like the fa/fa rat, ob/ob and db/db mice and other non-genetic diet induced models of obesity. The comparative gene expression analysis of healthy and obese adipose tissues has identified differential expression of several known as well as novel genes in different adipose depots. The WNIN / Ob rat is a recent addition to the available genetic obese models and

cDNA libraries of different adipose tissues and their gene expression analysis have not been attempted so far. The present study utilized the subcutaneous adipose tissues from 3 months old male, lean and obese rats to generate a comparative subtracted cDNA library.

3.1.4.1 Materials

Subtractive Hybridization was done using the cDNA Subtraction kit (BD PCR-Select^{TM}) purchased from BD Biosciences - Clontech laboratories. The cDNA cloning and ligation was done TA cloning kit (Promega), Ligation kit (Epicentre). LB plates (Tarsons) Agar, LB-medium (Hyxhem Ltd),10X PCR buffer with $MgCl_2$, 10 mM dNTPs, *Taq polymerase* 5 U / µl, IPTG, EDTA, Ampicillin, Ribonuclease, SDS were procured from Sigma Aldrich and X-Gal from USB.

3.1.4.2 Subtractive PCR Hybridization (SSH)

The hybridization was performed using (BD PCR-Select^{TM} cDNA Subtraction Kit) to selectively amplify differentially expressed genes in subcutaneous adipose tissue of lean and obese phenotype. It is similar to the traditional subtraction methods (Diatchenko et al., 1996, Gurskaya et al., 1996) with an advantage of starting with little quantities of RNA and easy amplification and purification of cDNA products of the two samples. This method amplifies and enriches the differentially expressed genes by 1000 fold and simultaneously normalizes and subtracts the abundant genes in the two samples. The Figure 3.1 describes the cDNA library construction of subcutaneous adipose tissue of WNIN / Ob rats using subtractive hybridization and subsequent cloning. The cDNA cloning resulted in subtracted gene library of subcutaneous adipose tissue. The cDNA transcripts obtained from the mRNA pools that are specifically expressed in the sample tissue is referred as tester and control cDNA is referred as

driver. The mRNA from both the tissues were converted to cDNAs and the tester and driver are hybridized, and the hybrid sequences are removed. The un-hybridized cDNAs represent the genes that are overexpressed in one tissue and suppressed in the other.

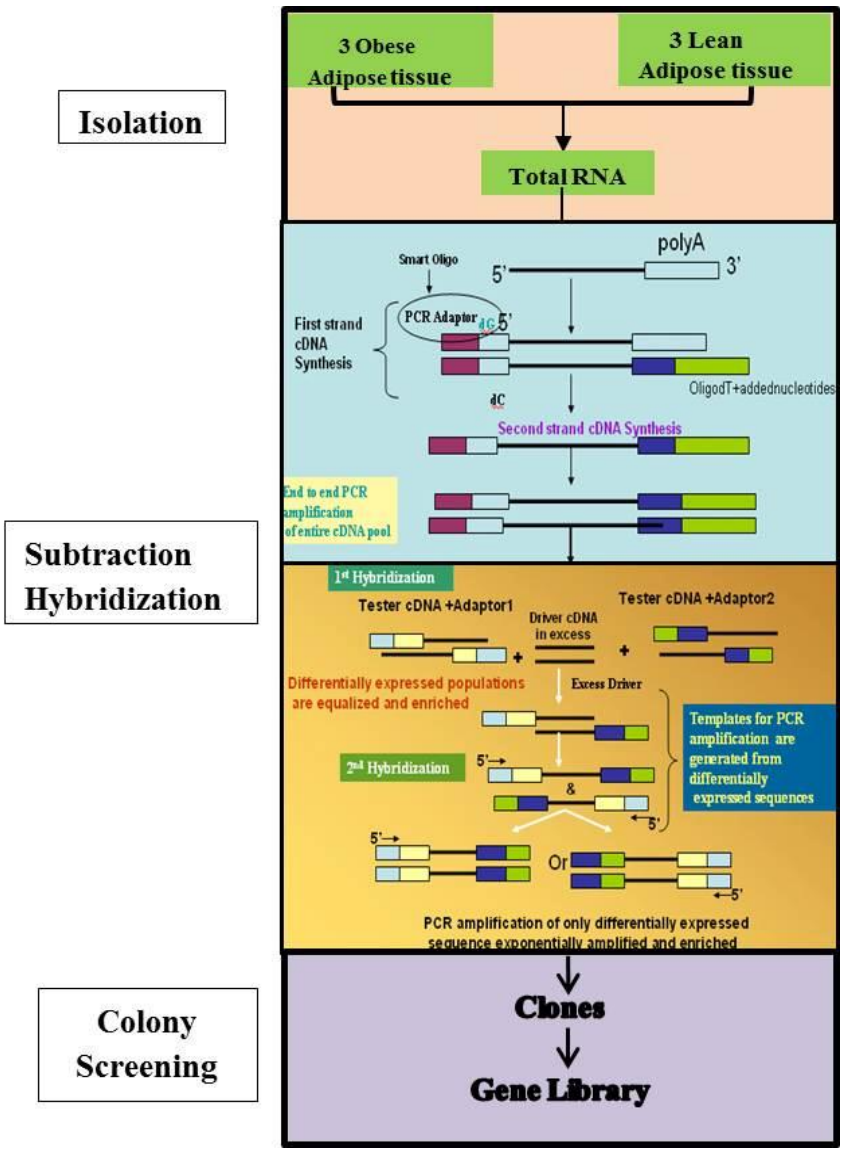

Figure 3.1: Schematic diagram of PCR -Select cDNA subtraction. The Total RNA pools from three obese and lean SAT were converted to cDNA pool. The forward and reverse subtraction resulted in enrichment of unhybrid sequence molecules that is upregulated only in the tester (samples), and identified with different adaptors at their ends. The obese was used as tester for forward hybridization and lean was used as tester for the reverse hybridization. The cDNA pools thus obtained were PCR amplified for and enriched for subsequent cloning. The cDNA cloning resulted in subtracted gene library of subcutaneous adipose tissue.

Thus, the forward and reverse subtractive hybridization was performed to identify the genes overexpressed (forward subtraction) and suppressed (reverse subtraction) in subcutaneous adipose tissue. The brief view on the hybridization procedure is given below:

1. First strand cDNA synthesis: Equal quantities of adipose tissue were taken from at least three obese rats (WNIN / Ob) and their lean littermates were homogenized and the total RNA was isolated as described in section 3.1.2. The RNA samples of both lean and obese with ratio 2:1 were used. The pooled totals RNA (1 μg) from three lean and obese rats were labelled as lean and obese respectively. The first strand cDNAs were synthesized from the total RNA pools of lean and obese.

2. Second-Strand cDNA Synthesis: The first strand synthesis of tester and driver were used for second strand synthesis as per the kit instruction to generate to double stranded cDNA for double digestion with *Rsa1* to generate shorter, blunt ends molecules for adaptor ligation

3. Adaptor ligation: The digested cDNA products of tester and driver were purified. The cDNA samples were paired for ligation and hybridization. The forward subtracted cDNA contains enriched sequences that are specific to the tester (obese) and reverse subtracted cDNA sequences are specific to lean which is the tester (lean cDNA was used as driver in forward subtraction). Each cDNA (tester and driver sample) was aliquoted into two separate tubes and ligated with Adaptor 1, Adaptor 2R followed by incubation at 16 $^{\circ}$C overnight in water bath (Hareus).

4. First hybridization: In the first hybridization, an excess driver cDNA (lean) was added to tester cDNA (obese) for forward subtraction, and an excess driver cDNA (obese) was added to each tester cDNA (lean) in the reverse subtraction.

5. Second hybridization: The second round of hybridization with fresh addition of excess driver (lean) cDNA for the forward subtraction and obese DNA in the case of

reverse subtraction samples to enrich the fraction of differentially expressed genes. The two sets of new hybrid molecules obtained from forward and reverse subtraction consist of differentially expressed cDNAs. The cDNA pools (obese and lean) were subjected to suppression PCR followed by another round of PCR amplification to exponentially amplify only the differentially expressed sequences.

3.1.4.3 TA cloning and Ligation

The subtracted PCR product obtained from obese and lean sample reflects a set of genes that are overexpressed or suppressed in subcutaneous adipose tissue of WNIN / Ob rats. The subtracted PCR products of obese and lean were cloned into pGEM-T vector by overnight ligation at 16 °C. The ligation was carried out as per manual instructions (Promega). 20 µl reactions was set up by adding 1 µl DNA ligase, 10 µl of 2X buffer to the ligation mixture of different ratio of insert to vector ration (1:1, 1:3, 3:1). The reaction was incubated at 16 °C overnight in water bath and later was transformed into DH5α competent cells.

3.1.4.4 Blue-white colony screening

It is a widely used technique in cloning and generating DNA libraries to identify recombinant bacteria that containing the cDNA inserts. Transformation was done using the bacterial mutant DH5α strain. The pGEM-T plasmid used for transformation has the alpha fragment that restores the β-galactosidase activity. The colonies with plasmid cDNA inserts that are ligated in the cloning site loose their alpha fragment activity (frame shift). The β-galactosidase activity is lost and the colonies appear white. While the colonies with no cDNA inserts have β-galactosidase activity restored and appears blue. The colony screening was done as described below:

The solidified agar plates containing ampicillin (100 µg / ml were supplemented with 0.5 mM IPTG and 80 µg / ml X-Gal. The transformations with DH5α competent cells were done using 2 µl of ligation mixture of forward and reverse subtraction samples**.** 100 µl of transformants (control and ligated plasmid) were spread plated on ampicillin / IPTG / X-Gal plates. The plates were incubated the plates overnight (16 –24 hours) in a 37 °C incubator (Hareus). The white colonies seen on the plate were the recombinant clones containing cDNA inserts of either obese or lean subcutaneous adipose tissue. The colonies were gently touched and stroked on two fresh agar plates linearly numbered making a working and a master plate.

3.1.5 Screening Transformants for Inserts

3.1.5.1 Materials

10X PCR buffer with $MgCl_2$, 10 mM dNTPs, *Taq polymerase* 5 U / µl, Ampicillin, Ribonuclease, SDS, ethanol were procured from Sigma Aldrich, NaOH, Tris, Potassium acetate, isopropanol from Qualigens. Restriction Enzymes *Sal1*, *Pst1*, *Sac1*, *Spe*, 10X restriction buffer, 10X BSA procured from New England Bio labs.

Buffers : Buffer 1: 25 mM Tris-Cl, 10mM EDTA (pH 8) with 10 ng / µl RNAse

Buffer 2: 0.2 N NaOH 1 % SDS

Buffer3: 3M Potassium acetate

TE buffer: 10 mM Tris-Cl, 1 mM EDTA (pH 7.4)

3.1.5.2 Plasmid isolation

The transformed white colonies containing the subtracted cDNA library were picked and inoculated into 2 ml LB broth containing ampicillin (100 µg / ml) and grown overnight at 37 °C. The plasmid isolation was done by miniprep method based on the

alkaline lysis method (Birnboim et al, 1979, Molecular Cloning: A Laboratory Manual Third Edition). Briefly, 2 ml bacterial culture was centrifuged (Sigma) for 5 minutes at 6000 rpm at room temperature. 300 µl of buffer 1 was added and stirred well to resuspend the pellet in the buffer. To this 300 µl of buffer 2 was added and gently mixed for few seconds and immediately 300 µl of buffer 3 was added. The eppendorf was gently mixed and kept in room temperature for 5 minutes followed by centrifugation at 10000 rpm for 5 minutes. The aqueous layer was carefully removed leaving the genomic DNA and the cytosolic components. The plasmid was precipitated by adding 600 µl of isopropanol to the supernatant. The solution was gently mixed and incubated at room temperature for few minutes followed by centrifugation at 10000 rpm for 10 minutes at room temperature. The pellet was washed twice by 1 ml of 75 % alcohol and centrifuged at 10000 rpm for 5 minutes at room temperature. The pellet was air dried and dissolved in TE buffer pH 7.4 or nuclease free water. The plasmid isolated was quantified by Nano drop ND 1000 spectrophotometer.

3.1.5.3 Restriction digestion of plasmids

The Restriction digestion of the plasmids (pGEM-T) isolated from colonies containing inserts confirmed the presence of inserts. The pGEM-T vector map shows the locations of several restriction enzymes flanking the insert site at the MCS (Figure 3.2). Accordingly, single and double digestions were done using one or a combination of enzymes *(Sal1, Pst1, Sac1, Spe1, Nco1*) with appropriate buffers showing 100 % activity (Molecular Cloning: A Laboratory Manual Third Edition). Briefly, the buffers were thawed and given a quick spin and a master mix containing appropriate buffer for 100 % enzyme activity, 10X BSA, restriction enzymes (5 units) were made for single or double digestion and the reaction volume was made up finally by adding the 1 µl plasmid. The tubes were incubated at 37 $^{\circ}$C incubator for 1 hour. The samples were visualized in a 1.5

% agarose gel. The linear plasmid is 3 kb in size. The DNA bands of single digestion more than 3 kb and double digestion with insert fallout were confirmed as positive clones.

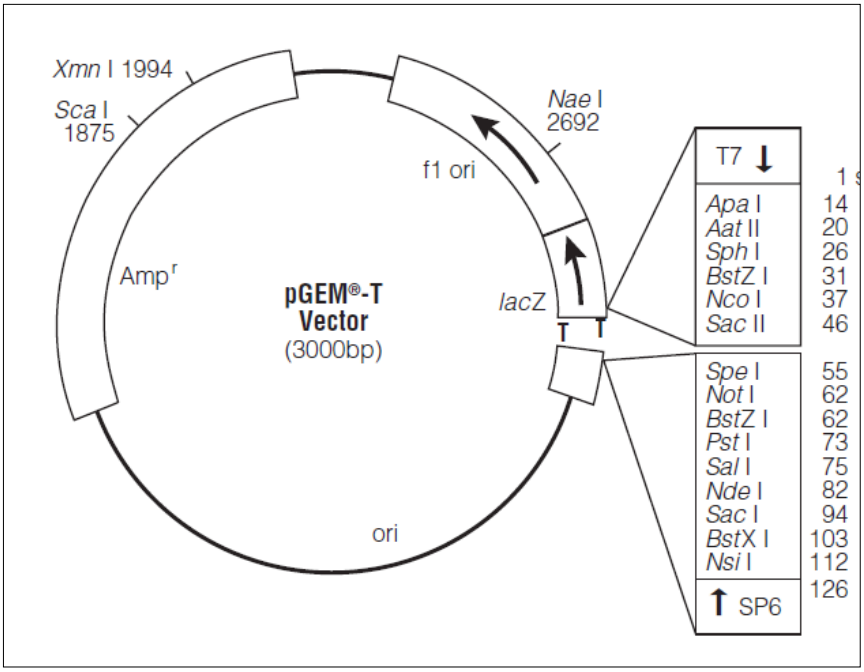

Figure 3.2: pGEM-T vector circle map**.** The pGEM-T vector map shows the cloning site with the list of restriction digestion enzymes. *Nco1*, *Spe1*, *Pst1*, *Sal1*, *Sac1* set of enzymes were used for single and double digestion. The universal primers T7 and SP6 used in colony PCR for confirming the presence of cDNA inserts.

3.1.5.4 Colony PCR

This is a PCR based protocol (Molecular Cloning: A Laboratory Manual Third Edition) to confirm the presence of DNA insert in the white colonies. The colony PCR was useful to quickly screen the colonies for the presence of cDNA inserts and size by using a combination of universal primers T7 and SP6 primer contained in the pGEM-T plasmid.

The Universal primer sequences mentioned below were used for colony PCR

T7 universal primer

5'TAATACGACTCACTATAGGG3'

SP6 universal primer

5'TAAGATATCACAGTGGATTTA3'

The colonies to be PCR amplified were gently touched with the tip and suspended in 200 µl nuclease free water. The tube containing bacterial suspension was heated for 95 °C for 10 minutes in water bath to lyse the cells. For multiple samples, master mix of PCR components was prepared and with the template added at last. The PCR was set up by adding following components and 5 µl of the cell suspension under following conditions 95 °C for 5 minutes, 30 cycles of 95 °C for 1 minute, 54 °C for 1.5 minutes, 72 °C for 1 minute with final extension at 72 °C for 7 minutes.

3.1.5.5 Automated DNA Sequencing

The DNA sequencing of the plasmids with confirmed cDNA inserts was performed on an Automated DNA sequencer ABI3100 (Applied Biosystems). Prior to sequencing the plasmids were purified by ethanol precipitation to remove unutilized primers, dNTPs, and salts. The sequencing was done using The Big Dye® Terminator kit (v3.1) (AB biosytems) which contained *Taq polymerase,* reaction buffers, and the four ddNTPS, labelled with different fluorescent dyes. The sequencing reaction of 10 µl containing Big Dye Terminator Mix, 100 ng plasmid (sample) and M13 universal primer (3.2 pm /well forward and reverse) was set up with denaturation at 96 °C for 1 minute, 25 cycles of 96 °C for 10 seconds, 50 °C for 10 seconds and extension at 60 °C for 4 minutes. The sequencing product was ethanol precipitated to remove the extra dyes, dNTPs and primers. The reaction volume was made up to 20 µl with water and 2 µl of 125 mM EDTA and 3M sodium acetate were added followed by 100 µl of absolute ethanol. All the samples in the 96 well plates were gently mixed and centrifuged at 13000

rpm for 30 minutes. The supernatant was discarded and 100 µl of 70 % ethanol was added. The samples were air dried and resuspended in 10 µl of injection buffer (HiDi formamide (Applied Biosystems).

Sequencing of the PCR products was performed to generate the amplicons that are terminated by the incorporation of fluorescently labeled dideoxynucleotides (ddNTPs) that results in a population of truncated products of varying lengths. These fragments are separated by high resolution capillary electrophoresis capable of separating fragments in a single nucleotide. The separated fragments are illuminated by LASER beams and the signals produced are captured by CCD camera.

3.2 Bioinformatic analysis of the Subtracted gene library

3.2.1 Basic Local Alignment Search Tool (BLAST)

The NCBI public domain is a free software program to search the protein or the nucleotide database available online **(http://blast.ncbi.nlm.nih.gov/Blast.cgi).** Sequenced cDNA inserts were identified using a free software program 'Basic Local Alignment Search Tool' (BLAST) (Altschul et al., 1990). By using various algorithms, sequence in query was identified or searched for sequence similarity with its nucleotide or protein default databases like protein, genomic and mRNA sequences (Ref-Seq) from NCBI Reference SequenceProject. These databases are frequently updated.

BLAST-N

The query (nucleotide sequence) was submitted in FASTA format, a widely used format to search nucleotide sequences (Pearson et al., 1988, Karlin et al., 1990). The BLASTN output displays sequence similarity of the query to the database using statistical theory. Each comparison produces a bit score and expect value (E-value). Bit score indicates how good the alignment of the query is to the database, higher the bit scores closer the

identity reflecting the degree of similarity and significance of matches. The expect value (E-value) indicates the statistical significance of the pairwise alignment and reflects the similarity to the database. The lower the E-value, more significant and similarities the query match to the database sequences. All the genes were identified based on lower E-value, and with maximum bit score, % homology and identity match to the rat mRNA sequences.

3.2.2 Gene Pathway analysis

The online NCBI public domain (http://blast.ncbi.nlm.nih.gov) has several databases like GO 'Ontology', KEGG and PANTHER pathway software are uniquely designed to provide complete information related to the gene. KEGG and PANTHER pathways are software tools that were used to classify the identified genes into their respective functional pathways in order to facilitate a global view of different biological and molecular functions of genes that are altered in the obese phenotype. The gene query searched is integrated into the NCBI database which enables the search typing using universal gene symbols and accession numbers. The cDNA inserts identified in BLASTN were analyzed through these softwares. The output provided gene information related to their functional pathways and also on family, biological process, cellular location and function.

3.2.3 Quantitative PCR studies

3.2.3.1 Materials

Superscript II Reverse Transcriptase, Oligo (dT), 4X RT buffer, 0.1 mM DTT, RNAse inhibitor, RNase H were purchased from Invitrogen. The 2X SYBR premix buffers (*Taq polymeras*e, SYBR green, dNTP mix) were procured from Takara. The following pair of primer sequences was used for Q-PCR:

SCD1: Forward: 5'GACAGGAGGGCAGGTTTC3'
Reverse: 5' GTAGTGGTGGTCGTGTAGG3'

LPL: Forward: 5' AGTTTGGCTCCAGAGTTTGAC3'
Reverse: 5' TCCAGGAATCAGATGACAAGTG 3'

ACS: Forward: 5' TCAGAGCAGTTCATCGGCATC 3'
Reverse: 5' TCGGTTCCAAGCGTGTCATAG 3'

MGLII: Forward: 5' GGATGGTGGTATCGGACTTC 3'
Reverse: 5' GCTGCTAGGATGGAGATGG 3'

CAPN1: Forward: 5' TGGAAGGAGCAGGAATGG 3'
Reverse: 5' TTGGAGTGGCAGTAAATGAG 3'

ALOX5-AP: Forward: 5' GCCAACCAGAACTGCGTAG 3'
Reverse: 5' TTGCCTCACGAACAGATACATC 3'

PPARG: Forward 5' AGCCCTTTGGTGACTTTATG 3'
Reverse 5' CTGTGACAATCTGCCTGAG 3'

β-ACTIN: Forward: 5' ACCAACTGGGACGACATGGA3'
Reverse: 5' TCTCAAACATGATCTGGGTCATCTT 3'

3.2.3.2 Q-PCR analysis

The Q-PCR was carried out as two steps PCR

1st step: The RNA samples of three months male, lean and obese were isolated from SAT as described in section 3.1.2. The first strand cDNA was synthesized for obese and lean RNA samples simultaneously using oligo (dT). The reactions were set up using the first strand cDNA synthesis kit (Invitrogen). Briefly, 5 µg of obese and lean RNA were used for cDNA synthesis. 1 µl of oligo (dT) 0.5 µg / µl, 0.5 mM dNTP mix were added to each tube and incubated at 65 °C for 5 minutes and quick chilled in ice for 2 minutes. 12 µl master mix containing 10X RT buffer, 25 mM $MgCl_2$, 0.1 M DTT were added to each tube and the tubes were incubated at 42 °C for 2 minutes. 1 µl of superscript II RT to each tube was added and incubated at 42 °C for 60 minutes. The reaction was terminated at 70 °C for 15 minutes. 1 µl of RNaseH was added to each tube and incubated for 20 minutes at 37 °C.

2nd step: Some of the genes identified to be overexpressed in the obese adipose tissue were validated by Q-PCR by the absolute quantification method. The $2^{-\Delta\Delta C_T}$ was used to calculate the relative fold expression of the gene in obese rat adipose samples. β-actin was used as an internal control. The cDNA dilution of 1:15 was used for standards from obese cDNA while 1:30 dilution were used for samples (obese and lean cDNA).The samples were run in duplicates and the reaction was repeated twice. 20 µl PCR reactions was set up in a 96 well plate (Bio-Rad) using 10 µl of cDNA dilutions (samples and standards) with 2X SYBR premix and 0.2 µM forward and reverse primer as per the kit instruction (Bio-Rad). The plate was sealed firmly and given gentle spin to avoid any air bubble. The PCR cycle was set up with 95 °C for 5 minutes, 40 cycles of 95 °C for 15 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds in iQcycler machine (BioRad). The inbuilt software (Bio-Rad) generated a standard curve, disassociation curve, R^2 value and the PCR efficiency. The disassociation curve showed a single peak at the melting temperature of the template. The single melt curves reflected the amplification of the PCR products, R^2 value of 0.99 and PCR efficiency of 95 % as per the software was maintained for all the PCR cycles.

3.3 Comparative analysis of Pro-inflammatory cytokines

A comparative analysis between, lean and obese rats of different age groups (1, 3, 6, 12 months respectively) was done to assess the relative levels of inflammatory cytokines. The concentration of some of the known pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, RANTES, MCP-1 were estimated in the serum and adipose tissue samples by ELISA.

3.3.1 Adipose tissue lysate preparation

The subcutaneous adipose tissues of lean and obese rats of different age groups were freshly isolated. The tissue lysates were prepared from 300 mg of tissue using lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 1 % NP40, 1 mM PMSF, 1 mM Aprotinin and Leupeptin 1 mM). The tissues were powdered using liquid nitrogen and further sonicated. The homogenate was centrifuged at 12000g for 10 minutes at 4 $^{\circ}$C. The supernatant was used for total protein estimation. All the samples were reconstituted to 1 mg / ml and 100 µl of 1:5 diluted samples were used to estimate the concentration of the cytokines. The concentration was expressed in pg / mg protein.

3.3.2 Serum preparation

The supra orbital vein in the eye was pierced using a capillary tube and nearly 2.5 ml of blood was collected in non-additive labelled vacutainers tubes, The capillary tube was carefully withdrawn and the eye was given a gentle wipe with spirit and released into cage, The serum was separated by leaving the blood samples for 1 hour at 37 $^{\circ}$C to clot or in 4 $^{\circ}$C overnight. The sample separates into a straw colored liquid distinct from the clot that settles in the bottom (which has platelets blood cells etc.). The samples were carefully centrifuged at 4000 rpm for 20 minutes at 4 $^{\circ}$C. The serum was pipetted out gently into a fresh eppendorf tube. The serum samples were clearly labeblled and stored in -20 $^{\circ}$C.

3.3.3 ELISA

The sandwich ELISA method was used to estimate the levels of pro-inflammatory cytokines. The antigen (cytokines) was immobilized between monoclonal rat capture antibody and biotinylated detection antibody. This complex was detected by the antibody conjugated HRP enzyme covalently linked to avidin. As avidin has strong binding affinity to biotin. The quantity of antigen is relatively expressed by visible colour

development by adding ABTS, as a substrate for the enzyme. The ELISA was done as per the manufacturer's protocol for the respective cytokine (Peprotech Inc.) in a 96 well microtiter plate (Costar) briefly explained below:

Capture Antibody: 100 µl / well of capture antibody was added and incubated at room temperature overnight later washed and blocked for 1 hour.

Standards and samples: 100 µl / well of standards and serum samples were added after wash and then incubated for two hours. For every cytokine analysis, the appropriate standards (provided in the kit) and their dilutions were prepared accordingly to estimate the concentration of the cytokine in the serum sample.

Detection Antibody: After washes, detection antibody was diluted to required concentration of respective cytokine in diluent and 100 µl was added to each well and incubated for 2 hours and washed. Avidin-HRP conjugate of 1:2000 dilution was used and 100 µl / well was added and incubated for 30 minutes and washed.

Colour development: ABTS liquid substrate was used for colour development. 100 µl / well of ABTS solution was added and kept in dark. The absorbance was monitored for 30 minutes for every 5 minutes at 405 nm wavelengths corrected to 650 nm and read by ELISA reader (Biotek power wave XS). KC junior software was used for analysis.

3.4 Intra-cerebroventricular infusion of recombinant human resisitin

3.4.1 Materials

TALON was purchased from Clontech laboratories. The Protein estimation kit was purchased from Pierce. Amicon concentrators with 3.5 kDa cut off were purchased from Millippore. The Alzet brain infusion kit II, Alzet mini osmotic pump 2001 was purchased from Durect Corporation. Stereotaxic surgical apparatus was purchased from Stoteling Corporation.

Animal house facility: Isolated room, cage rack, cages, cover, Water, Feed, disinfectant

Surgical tools: Spirit, Saline, Scalpel blades, Forceps blunt end, Mosquito, forceps, small, medium scissors, sieving needle and thread

Anesthetizing solution: Ketamine and xylazine

Painkiller and Antibiotics: Diclophenac (15 mg), Gentamycin

Accessories: 1 ml Syringe box (Dispovan), Gloves (Tarsons), Trimmer, Cotton, Autoclaved MilliQ water, blotting paper, Scale, Mask, Cap, Foot Cover, Bubble balance, Eye cover, Pencil, Tissue rolls, Balance, Marker

3.4.2 Purification of recombinant human resistin (rhRes)

Recombinant human resistin protein was purified by affinity chromatography as described by (Aruna *et al.*, 2003). Briefly, the recombinant resistin vector was sub cloned into PQE30 vector (pQEhRes) and transformed into *Escherichia coli* M15 cells. The cells were grown to log phase in LB broth containing kanamycin (25 μg / ml) and ampicillin (100 μg / ml) antibiotics at 37 $^{\circ}$C with constant stirring. The absorbance at wavelength $OD_{600\ nm}$ ranging between 0.4 - 0.6 after the cells reached log phase and induced with 1 mM IPTG for 16 hours at 18 $^{\circ}$C with constant shaking. The culture was harvested by centrifugation at 6000 rpm for 15 minutes at 4 $^{\circ}$C. The cell pellet was resuspended in lysis buffer (50 mM sodium phosphate pH 7.0, 300 mM NaCl, 0.1 mg / ml lysozyme and 8 M urea) and incubated for 1 hour at 37 $^{\circ}$C. Further lysis and cell disruption was done by sonication using a Branson ultrasound probe for three cycles each at 20 hertz for 3 times for 20 second each in ice. The total cell lysate was centrifuged at 13000 rpm for 30 minutes at 4 $^{\circ}$C. The supernatant was loaded onto a TALON column pre-equilibrated with lysis buffer to purify resistin under denaturing environment. Then column was washed with 10 bed volumes of wash buffer (lysis buffer with 5 mM imidazole) to remove any nonspecific unbound proteins. The bound protein (rhRes) was eluted from

the column with 150 mM imidazole in 1 ml frations. The fractions containing eluted protein were checked for purity on a 10 % Tris Tricine gel (BioRad). The eluted fractions were pooled and dialyzed against 20 mM Tris pH 8.0. The dialysis buffer was changed five times every three hours. The dialyzed protein reconstituted in 20 mM Tris was concentrated using centricon tubes with > 3kDa molecular weight membranes (Millipore) to 1 ml. The protein purified was estimated with BCA method (Pierce). The protein was stored in -20 $^{\circ}$C and used immediately for the human resistin infusion studies. 2 μg of rhRes protein was used for infusion in the rat brain.

3.4.3 Stereotaxic surgery for intra-cerebroventricular infusion of rhRes in lean and obese rat

The infusion studies were performed to assess the effect of human resistin, upon direct injection of protein (hRes) into the brain (hypothalamus) (Paxinos and Watson, 1985).

a. **Animal house facility**

The Animal room allotted was maintained sterile at 18 % humidity and a temperature of 22 ± 2 $^{\circ}$C. The cages and bottles were replaced every three days. Water, swabs and cotton was autoclaved. All the surgical items and apparatus were disinfected with spirit.

b. Stereotaxic surgery

The animals were anaesthetized by IP injection of ketamine 100 mg / kg body weight and xyalzine 10 mg / kg body weight. The anesthetized rats were checked by reflexes and placed on the stereotaxic surgery apparatus after shaving the hair on the head. The animal head was firmly secured with ears and mouth on the platform to get it

to a plane. Water was sprinkled on the rat's eye to lubricate and covered it so as to prevent from high intensity light during the surgery. The skin on the scalp was cut open exposing the bregma of the skull. The co-ordinates AP (- 2 mm), lateral (- 2 mm), DVN (- 4 mm) from bregma was set for placing the cannula which is the site of entry to the lateral ventricle. The site was marked with a sharp sterile pencil and 1 mm diameter deep hole was drilled with a motor fixed with a drill bead. The entire area was cleaned and the site of drilling was cleaned by small roll of paper or swab. A socket is made in the subcutaneous region at the back of the animal and the osmotic pump filled with saline (Alzet) was inserted. The cannula (Alzet) on the other end of the osmotic pump is placed at the drilled site (ICV) and sealed with locate acrylic adhesive (Alzet). The skin was sutured and cleaned with alcohol. 250 µl of diclophenac (painkiller) and 100 µl gentamycin (antibiotic) was injected to the animal. 3 ml of saline was injected to counter the dehydration during the surgical procedure. The animal was placed inside the cage with of water and rat chow *ad libitum* and left to recovery for a week.

c. **Osmotic Pump replacement**

The pump was activated with desired solution (20 mM Tris or 2 µg / µl hRes protein) for at least 12-14 hours. The pump was located externally and the area of the skin is shaved and an incision was made at a place close to the pump. The pump was replaced with the new pump into the tubing with no air bubbles. The next replacement of the pump was done at an area different from an earlier incision to avoid infection. The cut area was carefully sutured and cleaned with water and alcohol. The animal was carefully placed back into the cage and a surgery sutures on the head was carefully removed to reduce infection.

d. **Osmotic pump infusion**

Three months male lean and obese rats were used for this study and monitored for weekly changes in food intake and bodyweight after infusion with protein or vehicle. The animals in the study were divided into three groups each having three lean and obese rats. The pump infusion was divided into four stages (Figure 3.3). The figure 3.3 explains the experimental procedure of ICV infusion of human resistin.

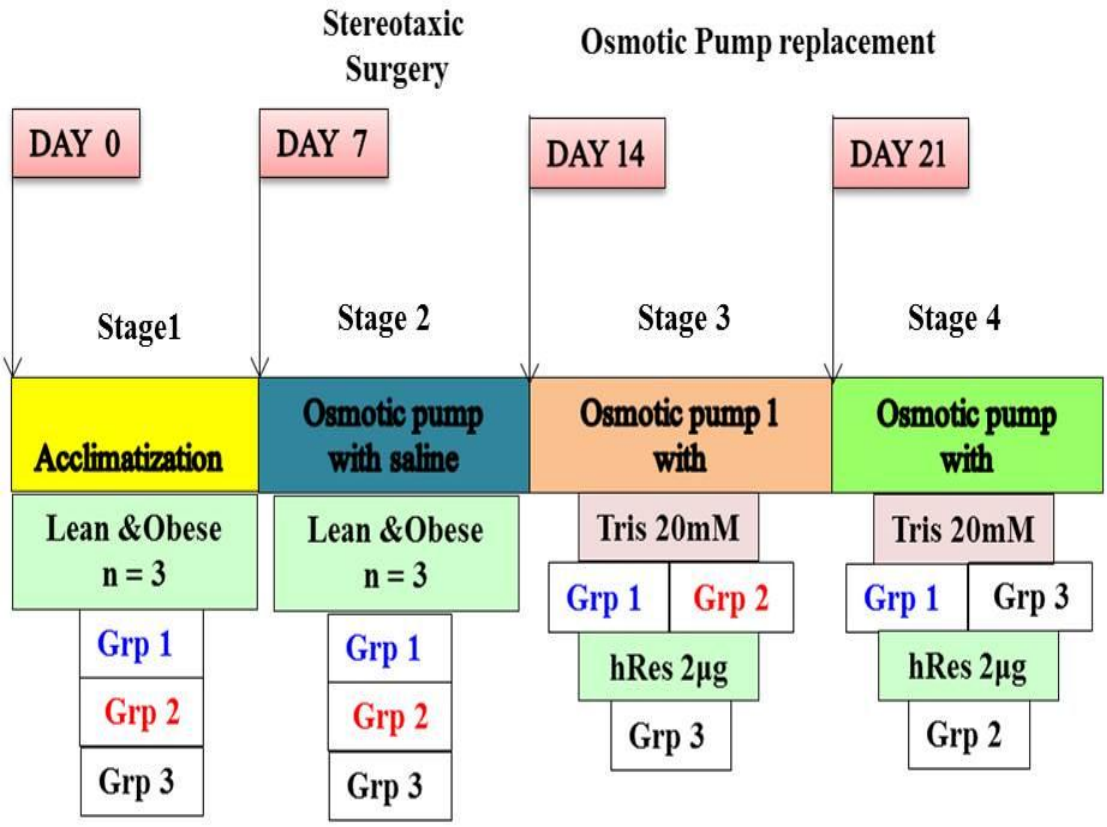

Figure 3.3: Schematic diagram of ICV infusion of human Resistin. The lean and obese animals were divided into three groups. The time line shows the stage of the experiment of each animal in the group. Week 1: Animals were acclimatized (yellow). Week 2: Recovery after ICV cannulation of animals in all the three groups (blue). Week 3 and 4 Change in osmotic pump in week 3(brown) and week 4 (green).

1st stage, Day 0- Animals taken from the colony were individually caged for acclimatization with water and food ad libitum

2nd stage, Day 7- ICV cannulation of animals in all the three groups by stereotaxic surgery with saline filled in the osmotic pump. The animals were left for recovery for a week with water and food pellets *ad libitum*.

3rd stage, Day 14- The old pump was replaced with a new osmotic pump containing vehicle (20 mM Tris) for Group 1 and 2 animals osmotic pump with protein (2 μg /μl rhRes) for group 3 animals received

4th stage, Day 21- In the second replacement, the osmotic pump of Group1 and 3 animals were replaced with vehicle (20 mM Tris) and in Group 2, the animals the pump was replaced with protein (2 μg/μl rhRes). The daily changes in the food intake and body weight were noted and monitored for a week. The osmotic infusion of rhRes followed by Tris in group 3 was used to check the effect of protein on food intake upon reversing the sequence. The food intake and bodyweight of animals in all the three groups were noted and calculated as weekly change.

3.5 Statistical significance

All results were expressed as mean ± SEM and they are representative of atleast three animals or two individual experiment. Significance was tested using student-test using Graphpad software. A probability of $P < 0.05$ was considered statistically significant.

Chapter 4

Results

4.1 Constructing subtracted cDNA library of the subcutaneous adipose of obese rat

The Subtractive hybridization is a powerful PCR based amplification technique that specifically enriches those cDNA fragments that differ between two tissues. In the present study, subtractive hybridization was done to obtain and identify the genes that are differentially expressed in the subcutaneous adipose tissue from the obese rat (WNIN / Ob) in comparison to its lean counterpart.

4.1.1 Suppressive Subtractive hybridization (SSH)

An equal amount of pooled adipose total RNA (1 μg) of three lean and obese tissues was used for first strand cDNA synthesis. At the second step, the double stranded cDNA was generated for both lean and obese cDNA samples. Both the cDNA pool after enriching with PCR was restriction digested and purified. The cDNA samples were paired for ligation and hybridization. Hybridization was carried out in forward (obese cDNA was used as tester and lean cDNA (driver) was used as reference) as well as reverse (lean cDNA was used as tester and obese cDNA was used as driver) direction to identify genes overexpressed or underexpressed respectively in the obese adipose tissue. The forward and reverse subtracted cDNA population was further enriched with PCR amplification. The highly enriched and amplified subtracted cDNAs of lean and obese adipose tissue were checked with its respective unsubtracted control resolved on 2 % agarose gel (Figure 4.1.1). The figure 4.1.1 shows the PCR amplified unsubtracted from lean and obese cDNA pool (lanes 1 and 2 respectively) in comparison to the subtracted cDNA pool generated from the lean and obese samples (Figure 4.1.1 lane 3 and 4 respectively).

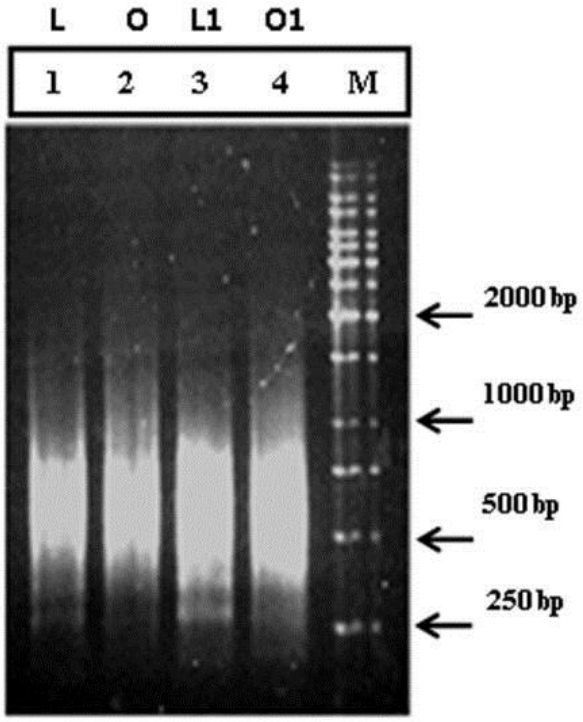

Figure 4.1.1: PCR amplification of unsubtracted and subtracted cDNA pools of lean and obese tissue. The subtractive hybridization using subcutaneous adipose tissue of lean and obese rats (n = 3) was performed and PCR amplified to enrich the subtracted cDNA pool of obese and lean. 5 µl of the PCR amplified cDNA subtractive pool from lean and obese (O1 and L1) obtained after forward and reverse subtraction was checked with unsubtracted (cDNA pool before hybridization) (L and O) resolved on a 2 % agarose gel. The PCR amplification showed enrichment of subtracted cDNA pools of lean and obese that ranged from 200 - 1000 bp. Lane 1and 2: PCR amplified unsubtracted from lean (L) and obese(O); Lane 3 and 4: PCR amplified cDNA subtractive pool of lean (L1) and obese(O1) M: 1 kb molecular weight marker (MBI fermentas).

4.1.2 Screening the transformants for the presence of cDNA inserts

The subtracted cDNA pools of lean and obese were ligated into pGEM-T vector (TA cloning vector) and processed as lean and obese colonies. The plasmids contain the cDNA inserts of lean and obese that are differentially expressed. These plasmids were further transformed into 'ultra competent' cells (DH5α cells with transformation efficiency of 10^8) for blue white colony screening. All the white (lean and obese) transformants were carefully numbered and a master plate was generated.

4.1.2.1 Plasmid isolation and restriction digestion

The presence of cDNA inserts in the colonies was confirmed with plasmid isolation followed by restriction digestion (single or double) (Figure 4.1.2A and B). The transformed white colonies containing the subtracted cDNA inserts (lean and obese) were inoculated into 2 ml LB broth containing ampicillin (100 µg /ml) and were grown overnight at 37 °C for plasmid isolation. The pGEM-T vector is a 3 kb plasmid. The cDNA inserts are ligated at multiple cloning site which also has restriction enzymes like *Nco1, Pst1, Sal1,* and *Sac.* Single digestion with 1 µl plasmid and 5 units of one or a combinations of two enzymes mentioned above for double digestion was performed at 37 °C for 1.5 hours and later resolved along with 1kb marker (MBI fermentas) on a 1.5 % agarose gel to confirm the presence of cDNA inserts. A linearized single or double digested plasmid without cDNA inserts is identified against 3 kb marker (Figure 4.1.2A, lane 1, 2, 3). The linearized plasmid with cDNA inserts upon single digestion resolved above 3 kb ladder position (Figure 4.1.2A, lanes 4-15), and the double digested plasmid showed the cDNA fall out (Figure 4.1.2B, lanes 1 to 5).

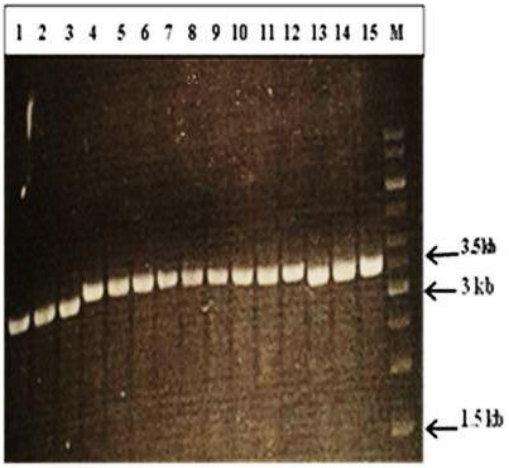

Figure 4.1.2A: Single Digestion of recombinant pGEM-T clones. The recombinant pGEM-T plasmids were ligated with cDNA inserts and transformed into DH5α cells. The presences of cDNA inserts in the positive colonies were confirmed by restriction digestion of plasmid (1 µl) using 5 units of restriction enzyme *(Nco1)* incubated for 37 °C for 1.5 hours. The linearized plasmids containing cDNA inserts showed bands above 3 kb as denoted by marker. Lane1-3: Single digested pGEM-T vector with no cDNA inserts. Lane 4 -15: Linearized plasmids with cDNA inserts after restriction digestion with a single restriction enzyme; M-1kb molecular weight marker (MBI fermentas).

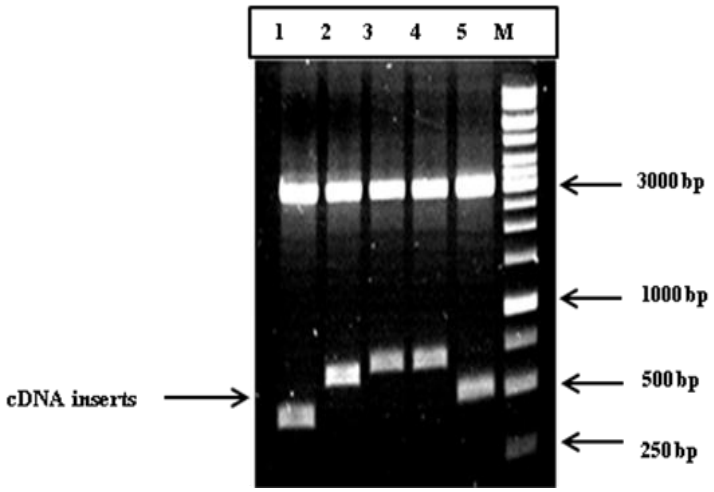

Figure 4.1.2B: Double Digestion of recombinant pGEM-T clones. The recombinant pGEM-T plasmids were double digested to confirm the presence of cDNA inserts. 1 µl of plasmid were digested with 5 units of restriction enzyme combination *(Nco1 and Sac1)* was used and incubated for 37 °C for 1.5 hours and resolved on 1.5 % gel. The positive plasmid clones show cDNA inserts upon double digestion. Lane 1-5: Positive recombinant plasmids double digested with cDNA inserts fall out; M: 1kb molecular weight marker (MBI fermentas).

4.1.2.2 Colony PCR

The colony PCR is a quick way to confirm the presence of cDNA inserts in the colonies. The combination of universal primers (M13 forward and M13 reverse or T7 and SP6) present in the pGEM-T vector was used for the PCR amplification. The white transformed colonies contain the lean and obese cDNA inserts. The PCR reaction was set up with 5 µl of the lysed cell suspension and the above mentioned primer combination. The forward and reverse primer sites flank on either side of the MCS. The PCR reaction will amplify cDNA insert if present in this region. The presence of cDNA inserts was visualized on a 1.5 % agarose gel (Figure 4.1.2C).

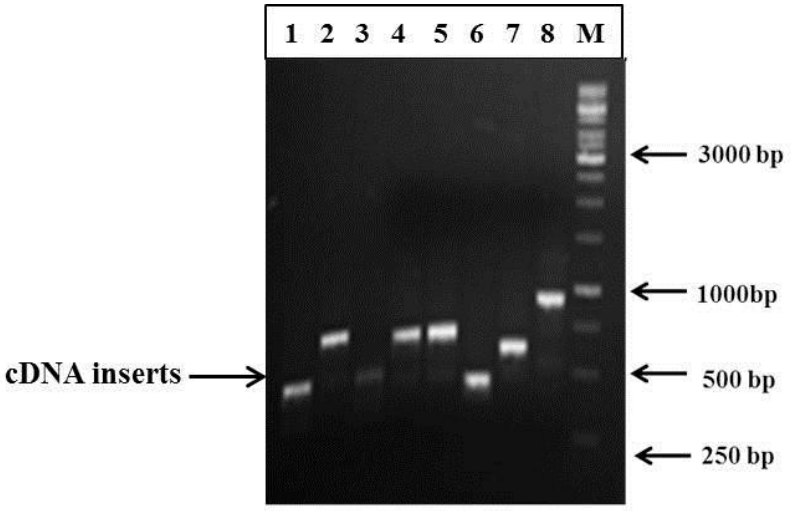

Figure 4.1.2C: Recombinant colonies PCR amplified cDNA inserts by Colony PCR. A small fraction of lysed cell suspension of the recombinant colonies were used to check for the presence of cDNA insert by PCR amplification using T7 and SP6 universal primers. The positive colonies showed PCR amplification of cDNA inserts that was visualized on a 2 % agarose gel. Lane 1-8: PCR amplified cDNA inserts from positive recombinant colonies; M: 1kb molecular weight marker (MBI fermentas).

4.1.3 Automated DNA sequencing

The subtracted cDNA pool of lean and obese were ligated into a pGEM-T vector and transformed into DH5-α cells. All the positive colonies represent a collection of various genes that are differentially expressed in lean and obese adipose tissue. The presence of cDNA inserts were confirmed by restriction digestion or colony PCR. All the recombinant plasmids of lean and obese library which confirmed the presence of inserts were numbered sequentially and sequenced. The plasmids were purified and sequencing was done as per the instruction protocol (Applied Biosystems). The plasmids with M13 primers (3.2 picomoles /well) were PCR amplified and ethanol purified and sequenced. The PCR products were terminated by incorporation of fluorescently labelled $ddNTP_S$. The PCR products were resolved on high resolution capillary electrophoresis capable of separating each nucleotide. The peaks are illuminated by LASER beams captured by inbuilt CCD camera resulting in a chromatogram. The DNA automated sequencer generated a chromatogram by series of uninterrupted sequence for all the amplicons sequenced. The purity of plasmid is proportional to clear peaks obtained for every cDNA sequenced. Figure 4.1.3A shows a good quality DNA sequence that generated clear peaks of nucleotides shown in different colours for each nucleotide. The FASTA sequence for each recombinant plasmid were labelled and used to identify the representative differentially expressed mRNA (Figure 4.1.3B).

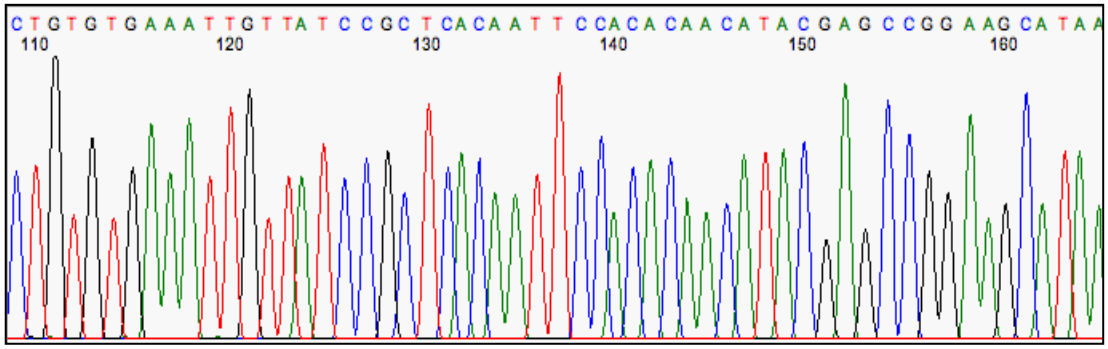

Figure 4.1.3A: Chromatogram generated for a recombinant plasmid after DNA sequencing. The recombinant plasmids were purified as per the kit instruction (Applied Biosystems) and processed with M13 primers for DNA sequencing. A typical chromatogram generated for one of the several cDNA contained plasmid that was sequenced to give a clear well separated peaks with different colours for each nucleotide G-black, T-red, C-blue, A –green.

CTCNGGAGGACACGCTGAAACTCTCACTCTGGGCGAGTCCTGATAAAACAGGCTCCTAAT
ACTGACCTACCTCAAGGGCAGTTCTGAGGTGATTAGAGCCTTTTCTTTTTTTAAGCAAGTG
TTTGGAGATGCATGGCAGAGGTTTTCCTGCAGACTTGTAAAGAGCTAGATGTGTTCTAAA
GACCATCTTTAGTCTACATTGCTCTTCCCAGAGATCTACAACTATGACCCAGCCAAAGTGC
AAGCCTACCTACTCACTGCCATAAAAACTATTCAGGAAACCTTTTCCTGGCCTGAATGAGA
TTTTCTTTTTTTTTTTATGTGGGGCGGTTATTTGTGACCCAAGTGTAATTTGGATGATTTTT
AATTAATATCAACTCTTGAAGCCTAATTGTACCTGCCCGGGCGGCCGCTCGAAATCCCGC
GGCCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTAT
TACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAA
CTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGC
ACCGATCGGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGACGCGCCCTGTAGC
GGCGCATTAGCGCGGCGGGTG

Figure 4.1.3B: Sequence of the cDNA inserts generated by DNA automated sequencer. The positive recombinant plasmids after confirming for the presence of cDNA inserts were processed in the automated DNA sequencer. A FASTA sequence obtained as a sequence output of nucleotide sequencing of one of the cDNA sequenced.

4.1.4 BASIC LOCAL ALIGNMENT TOOL (BLAST)

The BLAST is a software programme available in the NCBI public domain **(https://blast.ncbi.nlm.nih.gov)** was used to align the nucleotide query sequence with the selected database. The nucleotide blast (BLASTN) was used for shorter nucleotide query search. The software used the Ref-Seq RNA nucleotide of the database of rat (*Rattus Norvegicus)* to identify all the nucleotide sequences of all the sequenced cDNA clones. The nucleotide sequence of the cDNA inserts were entered in FASTA format. The highly similar sequences within the rat database with maximum % identity and sequence match was selected for the nucleotide query match.

NUCLEOTIDE BLAST- (BLASTN)

The BLAST output displayed graphical, tabular and alignment results of the nucleotide match with the mRNA in the rat database. The graphical representation of the BLAST output (Figure 4.1.4A) shows the nucleotide query with its length across the top in different colours. The subsequent horizontal lines below are the different sequence hits identified in the database by colours matched with the query length on the top. The lines in red show maximum identity to the query length and identity. The blast output table gives the significant sequence hits generated from the Ref-Seq RNA database. The table provides information for the probable mRNA identified for cDNA sequence and the information on the maximum similarity with the query, and percentage identity of RNA with the query and the E-value. The mRNA for the given nucleotide query is identified with low E-value and maximum identity and query coverage (Figure.4.1.4B). All the cDNA inserts were sequenced and BLAST searched to generate a gene library of RNA that is overexpressed in obese and lean subcutaneous adipose tissue.

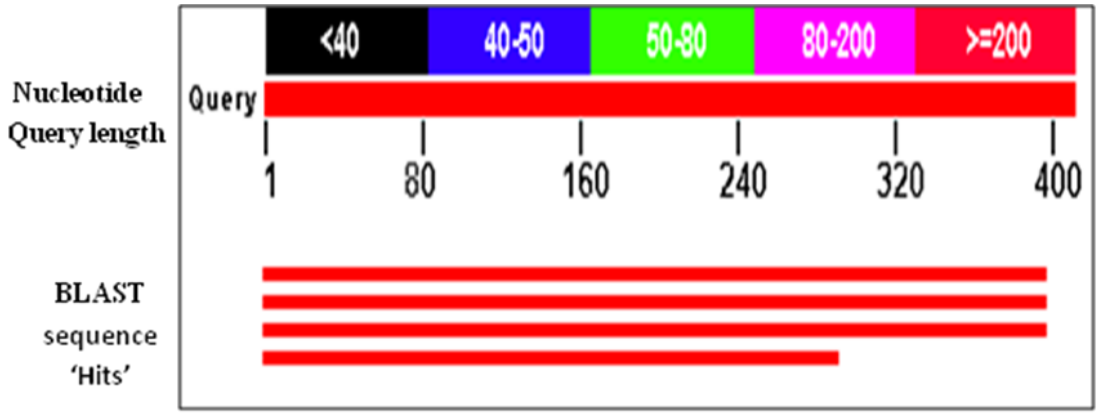

Figure 4.1.4A: The graphical representation of NCBI BLASTN output. The nucleotide query as FASTA was BLAST searched. The output contains various information about the nucleotide query. The length given in different colours was scaled on the top for query coverage. The sequence hits from the database after BLAST are represented by horizontal bars (in red) below. The BLAST 'hits' showing mRNA with red colored bars with maximum alignment was identified for the cDNA sequence.

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☑	PREDICTED: Rattus norvegicus stearoyl-Coenzyme A desaturase 1 (Scd1), transcript varian	721	721	96%	0.0	99%	XM_006231433.2
☐	Rattus norvegicus 1 BAC CH230-229J7 (Children's Hospital Oakland Research Institute) com	721	721	96%	0.0	99%	AC105487.6
☐	Rat liver stearyl-CoA desaturase mRNA, complete cds	699	699	96%	0.0	98%	J02585.1
☐	Rattus norvegicus stearoyl-CoA desaturase (Scd), mRNA	536	536	71%	6e-151	99%	NM_139192.2

Figure 4.1.4B: BLASTN 'hits' output table. The BLASTN output lists the significant sequence 'hits' in a table generated from the Ref-Seq RNA database. The BLAST output of one of the cDNA insert sequence shows information about the mRNA identity match and its description (highlighted in blue). The cDNA sequence matched with Scd1 gene with query coverage > 79 %, identity > 99 %, low E value of 6e-151 and accession number.

4.2 Bioinformatic analysis of the differentially expressed gene library

4.2.1 Subtracted gene library of subcutaneous adipose tissue of obese rat

The subtractive hybridization technique was used to compare and identify the genes overexpressed in obese and lean adipose tissue. The forward and reverse subtraction selectively amplified and enriched those genes that are specifically over-expressed in obese and lean subcutaneous adipose tissue. The overexpressed genes in the lean tissue obtained from reverse subtraction also reflect the set of genes suppressed in obese rat. The blue - white colony screening generated nearly 300 positive colonies from forward subtraction and nearly 500 white colonies from reverse subtraction that contained the overexpressed cDNA inserts of obese and lean respectively. The cDNA clones from corresponding colonies of obese or lean were numbered and identified.

4.2.1.1 Identification of mRNA genes obtained from the cDNA gene library

All the cDNA inserts representing the over expressed genes of lean and obese adipose tissue were sequenced and BLAST searched using BLASTN to identify the corresponding RNA (gene) using the Ref-Seq RNA database of rat (*Rattus Norvegicus*) available in the NCBI public domain **(https://blast.ncbi.nlm.nih.gov).** The BLAST output of both forward and reverse subtracted cDNA identified the RNA with alignment greater than 90 % identity. Table 4.1 lists some of the genes from the subtracted library overexpressed in obese adipose (O) and lean adipose tissue (L) with gene symbol, chromosome location and accession number.

Chromosome location	Accession No.	Symbol	Gene	Over-expressed tissue
1	XM_001053355	***Sorbs1***	similar to sorbin and SH3 domain containing 1 isoform 3	O
1p12	NM_001003404.1	***il22r2***	Interleukin 22 receptor, alpha 2	L
1q22	NM_019175.1	***Klk6***	Kallikrein 6	O
1q22	NM_173340.2	***Rpl13a***	Ribosomal protein L13A	O
1q22	NM_001106268.1	***Chsy1***	Carbohydrate (chondroitin) synthase 1 (predicted)	O
1q31	NM_012811.2	***Mfge8***	Milk fat globule-EGF factor 8 protein	L
1q35	NM_001024777.2		Similar to hypothetical protein MGC50721	L
1q35	NM_001006970.1	***Uqcrc2***	Ubiquinol cytochrome c reductase core protein 2	L
1q41	NM_030833.1	***Ifitm2***	Interferon induced transmembrane protein 2 (1-8D)	O
1q43	NM_012848.1	***Fth1***	Ferritin, heavy polypeptide 1	O
1q52	NM_012732.3	***Lip1***	Lysosomal acid lipase 1	L
1q51-q53	NM_031514.1	***Jak2***	Janus kinase 2	L
1q54	NM_139192.2	***Scd1***	Stearoyl-Coenzyme A desaturase 1	O
2q24	NM_001270961.1	***Cp***	Ceruloplasmin mRNA	L
2q26	NM_001106430.1	***Gst2***	Microsomal glutathione S-transferase 2	O
2q34	NM_001106444.1	***Krtcap2***	Keratinocyte associated protein 2 (predicted)	O
2q34	NM_001270961.1	***Hipk1***	Homeodomain interacting protein kinase 1	L
2q34	NM_017014.1	***Gst***	Glutathione s transferase	L
2q41	NM_012889.1	***Vcam1***	Vascular cell adhesion molecule 1	L
3q31-q32	NM_001044242		Hypothetical protein LOC311254 [Rattus norvegicus] similar to pyruvate dehydrogenase complex, component X	O
		Pdhx		O
3q32-q34	NM_012520.2	***Cat***	Catalase	O
3q41	NM_001014762.1	***Pdrg1***	p53 and DNA damage regulated 1	O
3q42	NM_001168543.1	***Pltp***	Phospholipid transfer protein (predicted)	O

Chromosome location	Accession No.	Symbol	Gene	Over Expressed tissue
4q42	NC_005103.3	***Ppar-γ***	Peroxisome proliferator-activated receptor gamma	
4q42	NM_133306	***Oldlr1***	Oxidized low density lipoprotein (lectin-like) receptor 1	O
4q44	NM_001107896.1	***Bcat1***	Branched chain aminotransferase 1, cytosolic	L
4q44	NM_017253.2	***Ppfibp1***	PTPRF interacting protein, binding protein 1 (liprin beta 1)	L
5q36	NM_022931.1	***Rims3***	Regulating synaptic membrane exocytosis 3	L
5q36	NM_022383.2	***Cap1***	CAP, adenylate cyclase-associated protein 1	L
5q36	NM_001025624.2	***Hmgn2***	High mobility group nucleosome binding domain	L
5q36		***Per3***	Period homolog 3(drosophila)	L
6	NM_026742.4	***Ndufa4***	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4	O
6q16	NM_001100540	***Iah1***	Isoamyl acetate-hydrolyzing esterase 1 homolog (S. cerevisiae)	O
6q22.33	NM_033515	***Arhgap18***	Rho GTPase activating protein 18	O
6q32	NC_005105.3		Immunoglobulin heavy chain (alpha polypeptide)	L
7q11	NM_001105939	***Naca***	Nascent-polypeptide-associated complex alpha polypeptide (predicted)	L
7q11	NM_134364	***Atp5b***	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	L
7q11	NM_030842.1	***Itga7***	Integrin alpha 7	L
7q12-q13	NM_001082477.2	***Igf1***	Insulin-like growth factor 1	O
7q13	NM_024129.1	***Dcn***	Decorin	L
7q22	NM_001011988.1	***Cnot2***	CCR4-NOT transcription complex, subunit 2	L
7q34	NM_001134880.1	***Chrac1***	Chromatin accessability complex1	L
7q34	NM_001008384.1	***Rac2***	RAS-related C3 botulinum substrate 2	L
7q36	NM_001008809	***Krt72***	Type II keratin Kb35	O
7q36	NM_199370.1	***Krt8***	Keratin complex 2, basic, gene 8	O
7q36	NM_012655.2,	***Sp1***	Sp1 transcription factor	L
8q21	NM_001025660.1	***Ei24***	Etoposide induced 2.4 mRNA [Rattus norvegicus]	O
8q24	NM_053638.1	***IDH3A***	Isocitrate dehydrogenase 3(NAD+)alfa IDH3A	L

Chromosome location	Accession No.	Symbol	Gene	Over-expressed tissue
			WDR45like mRNA	L
8q24	NM_001013110	***Tf***	Transferrin	L
8q32	BC166740.1	***Stt3b***	Similar to Oligosaccharyl transferase 3	L
9q31-32	NM_001034164	***Stat1***	Signal transducer and activator of transcription 1	L
9q36	NM_001109086	***D1Ertd622e***	Similar to D1Ertd622e protein (predicted)	O
10q24	NM_001108278.1	***Sat2***	Spermidine/spermine N1-acetyl transferase 2 (predicted)	O
10q24	NM_022690.2	***Ube2g1***	Ubiquitin-conjugating enzyme E2G 1 (UBC7 homolog, C. elegans	O
10q26	NM_080583	***Ap2b1***	Adapter related protein complex 2 beta 1 subunit	L
10q26	NM_031833	***Nme2***	Expressed in non-metastatic cells 2	O
10q31	NM_001105834	***Mrpl45***	Mitochondrial ribosomal protein L45 (predicted)	L
10q32.3	NM_001107076	***Rab40b***	Rab40b, member RAS oncogene family (predicted)	L
11q11	NM_013192.2	***Kcnj6***	Potassium inwardly-rectifying channel, subfamily J, member 6	O
11q22	NM_001024995.1	***(rrc33***	Leucine rich repeat containing 33	L
12p11	NM_017260.2	***Alox5ap***	Arachidonate 5-lipoxygenase activating protein	O
12	NM_001113570.1	***Gtf3a***	General transcription factor III A	O
12p11	NM_031144.2	***Actb***	Actin, beta	L
12q16	NM_001080147.1	***Anapc5***	Anaphase-promoting complex subunit 5 (predicted)	O
13q13	NM_001135868.1	***Slc45a3***	Solute carrier family 45, member 3 (predicted)	O
13q22	NM_017073.3	***Glul***	Glutamate-ammonia ligase	L
13q22	NM_019177.3	***Sell***	Selectin	L
13q24		***Ddr2***	Discoidin domain-containing receptor 2 precursor	L
13q26	NM_057139.2	***Hnrnpu***	Heterogeneous nuclear ribonucleoprotein U	O
14p21	NM_138874.2	***Csn1s1***	Casein alpha s1	O
14q21	NM_001012038.1	***Mtmr3***	Myotubularin related protein	O
			similar to LRRGT00152 (predicted) (RGD1560705_predicted), mRNA	O
16p14	NM_012598.2	***Lpl***	Lipoprotein lipase, mRNA	O
16p16	NM_207589.3	***Selk***	Selenoprotein K	O
16p16	NM_012570.2	***Glud1***	Glutamate dehydrogenase 1	O

Chromosome location	Accession No.	Symbol	Gene	Over-expressed tissue
16q11	NM_031819.1	***(Fat1***	Fat tumor suppressor homolog (Drosophila)	L
16q12.2	NM_001013188.1	***Leprotl1***	Leptin receptor overlapping transcript-like 1	O
17q12.1	NM_001012468.1	***RAB18***	RAB18, member RAS oncogene family	L
17q12.3	NM_031140.1	***Vim***	Vimentin	L
17q12.3	NM_024397.2	***Abi***	Abi-interactor 1	L
18p11	NM_021744.1	***Cd14***	CD14 antigen	L
18p12	NM_031693.2	***Syt4***	Synaptotagmin IV	L
18p13	NM_001114391	***Rpl7a***	Ribosomal protein L7a (predicted)	L
18q12.1	XM_001056272	***MetAP2***	Methionine aminopeptidase 2 (MetAP 2) (predicted)	O
19q12	NM_001025637	***Vps35***	Vacuolar protein sorting 35	O
19p11	NM_133295	***Psmb10***	Proteasome (prosome, macropain) subunit, beta type 10	O
20	NM_001044283.1	***Snx3***	Similar to sorting nexin 3 (LOC684097)	O
20p12	NM_212466	***Cfb***	Complement factor b	L

Table 4.1: List of genes identified to be differentially expressed in obese (O) and lean (L) adipose tissue. The genes that are over expressed in obese (O) and lean (L) adipose tissue are listed with their symbols and accession number as per the chromosome number. The list of genes overexpressed in lean adipose corresponds to repressed set of genes in the adipose tissue of obese rat.

4.2.1.2 Analysis of differentially expressed genes in the library

Subtractive hybridization (SSH) is very useful in generating a gene library enriched that is unique in collection and that is not predetermined in two comparable tissue samples. In this study, the genes differentially expressed in obese and lean rats are identified for the subcutaneous adipose tissue that offers an insight on the global expression of genes that are overexpressed in obese adipose tissue and also those genes that are suppressed in obese adipose (overexpressed in lean tissue) in metabolic conditions such as obesity. The global gene expression pattern of SAT of obese (WNIN / Ob) rat was analyzed with bioinformatics tools to classify them based on their chromosomal location (Figure 4.2.1), cellular location (Figure 4.2.2) and function (Figure 4.2.3), cellular process (Figure 4.2.4) and pathways analysis using GO 'Component', PANTHER CLASSIFICATION system and KEGG software (Figure 4.2.5). The chromosomal classification (Figure 4.2.1) shows that the overexpressed genes were identified mostly on chromosome 1 followed by chromosome 3, chromosome 7 and none on the chromosome 11. While the genes overexpressed in lean were mostly present on chromosome 2 followed by chromosome 8. There were no genes identified for the chromosome 14, 15, 19 in the library. The classification based on cellular location identified various locations of the cell such as cell membrane, cytosol, nucleus and several organelles (Figure 4.2.2). The genes overexpressed in obese adipose tissue were found more in the cytosolic locations followed by cell membrane and in many organelles such as ER, microsome, Golgi complex, while the repressed genes were abundant in the cell membrane followed by nucleus and cytosolic and cell organelles. The classification showed relatively low expression of membrane, nuclear and mitochondrial genes in obese rat indicating its impaired function in obesity. The classification based on the molecular functions showed various functions like catalytic, binding, transcription, enzyme regulator factors, structural and transport altered in the obese adipose tissue. The

catalytic and protein or nucleic acid binding functions were highest and the most effected and with decreased translational and antioxidant functions in the obese rat. (Figure 4.2.3). The classification based on the PANTHER GO 'Biological process' identified genes related to various cellular process like apoptotic, immunity, biogenesis, metabolic and developmental process The subtractive hybridization enriched several genes related to several cellular processes that are specifically altered in the obese adipose tissue (Figure 4.2.4). The figure 4.2.4 shows the number of overexpressed genes identified were related to metabolic processes such as carbohydrate, lipid and protein followed by developmental, biogenesis and regulatory processes and some of the other metabolic genes and the genes related to cellular localization (signaling, receptor binding) were also distinctly suppressed in the obese rat (overexpressed in lean). The KEGG and PANTHER pathway analysis software was used to identify the specific functional and biochemical pathways of the genes differentially expressed in subcutaneous adipose tissue of obese rat. KEGG pathways identified and generated visual pathway maps that represent the gene interaction(s) within a single or multiple pathways. In the forward subtracted library (over expressed in obese adipose tissue), the PPARγ pathway was identified with several other overexpressed genes such *as* Scd-1, Acsl1, Pltp, Olr1, Alox5-ap lying downstream to PPARγ pathway (Figure 4.2.5). Other pathways identified related to iron metabolism, integrin signaling, complement pathway were repressed in obese adipose tissue. The gene pathway analysis showed one or few components related to a pathway based on which gene functional pathways were listed to obtain an insight on the different altered pathways in subcutaneous adipose tissue. Based on the global view of different pathways identified, the obese adipose tissue showed metabolic (carbohydrate, lipid, amino acid) cellular binding, development and reproductive pathways to be highly altered.

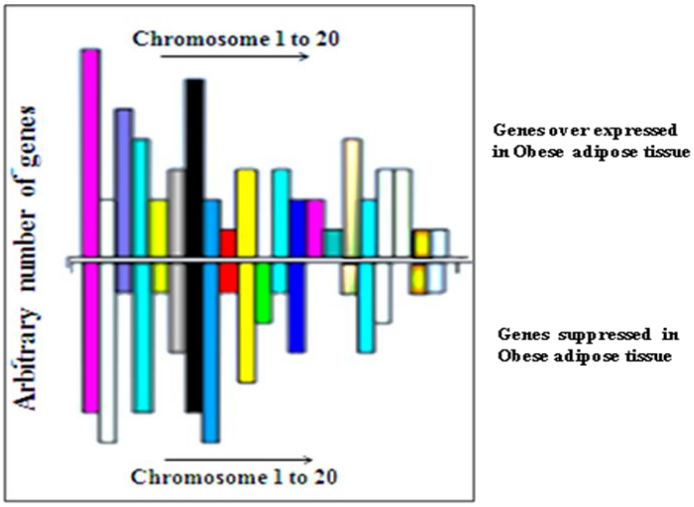

Figure 4.2.1: Chromosomal classification of the genes of the subtracted library of subcutaneous adipose tissue. The pictograph of the subtracted library shows classification of the overexpressed and repressed genes as per their chromosomal location. The graphs above the axis show the overexpressed set of genes and the suppressed (over expressed in lean) below the axis.

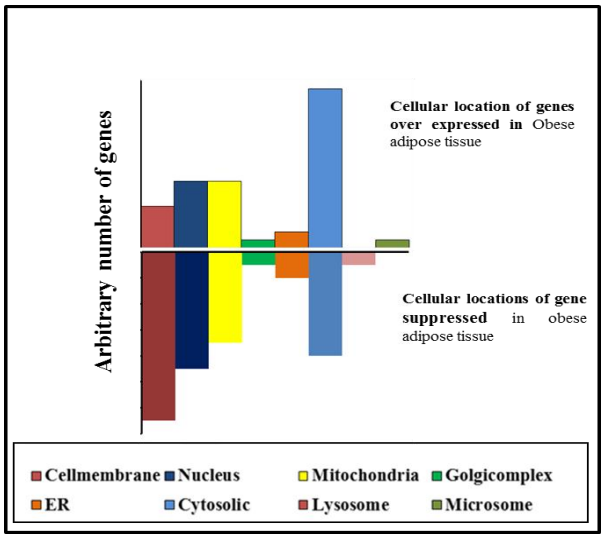

Figure 4.2.2: The differentially expressed genes of the subcutaneous adipose tissue classified as per cellular locations. The GO 'LOCATION' software component classified overexpressed and suppressed genes of obese subcutaneous adipose tissue as per their cellular site of function or expression. The pictograph above the axis represent the organelles which locate the over expressed genes and the bar graphs below the axis are those cellular location with repressed set of genes. The over expressed genes were located mostly in the mitochondria, while the genes identified to be repressed indicate decreased expression in the cellular membrane, nucleus.

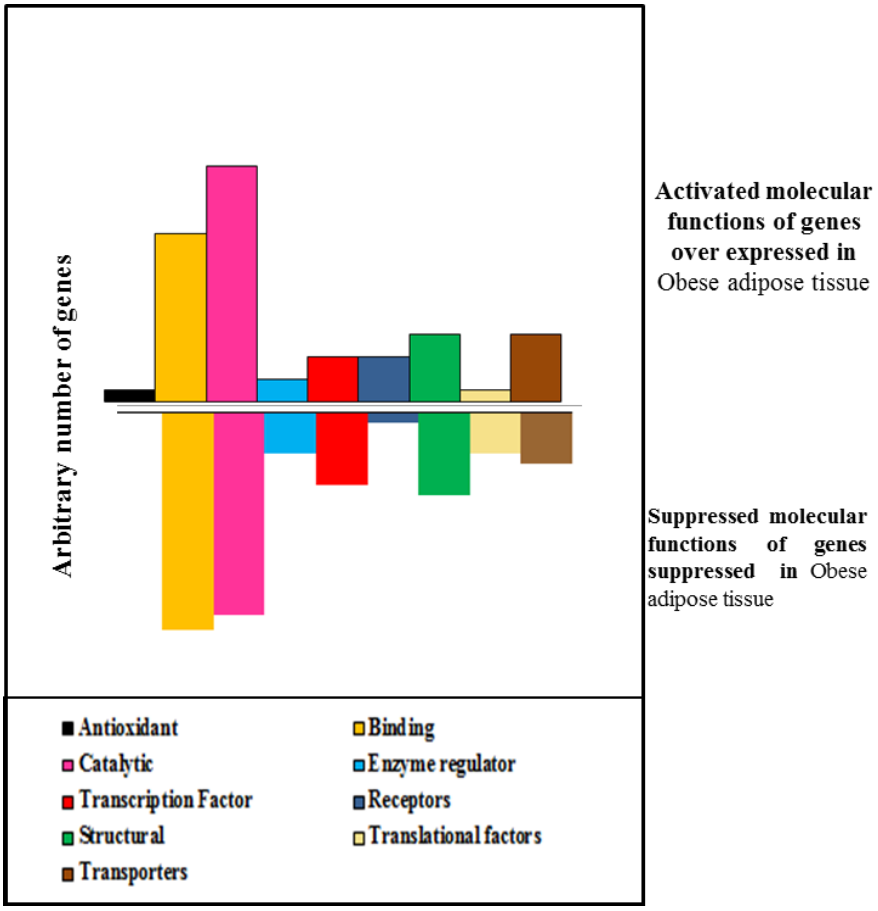

Figure 4.2.3: The differentially expressed genes of subcutaneous adipose tissue classified as per cellular functions. The GO 'FUNCTION' software component classified the overexpressed and suppressed genes of obese subcutaneous adipose tissue into various functions. The pictographs above the axis represent the molecular functions that are activated and the bar graphs below the axis below show reduced molecular functions. The catalytic (pink) and binding (yellow) (cell signaling, DNA binding) functions are more activated while transcriptional (red) and cytoskeletal (green) function are diminished in the obese tissue.

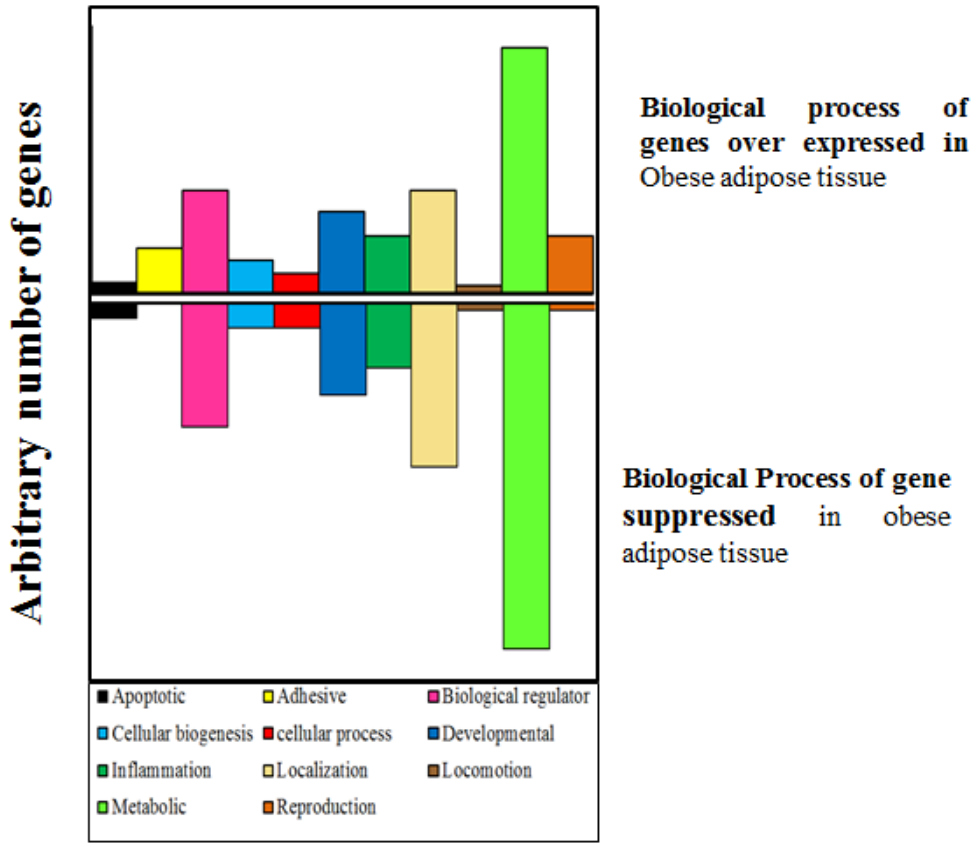

Figure 4.2.4: The differentially expressed genes of subcutaneous adipose tissue classified as per cellular processes. The GO 'PROCESS' component classified the overexpressed and suppressed genes of obese subcutaneous adipose tissue into various cellular processes. The pictographs above the axis represent the processes that are activated and the bar graphs below the axis below show reduced molecular functions. The metabolic processes (green) in the adipose tissue is highly altered (expressed and repressed) and the genes related to developmental (dark blue), regulatory (pink), functions are diminished in obese phenotype.

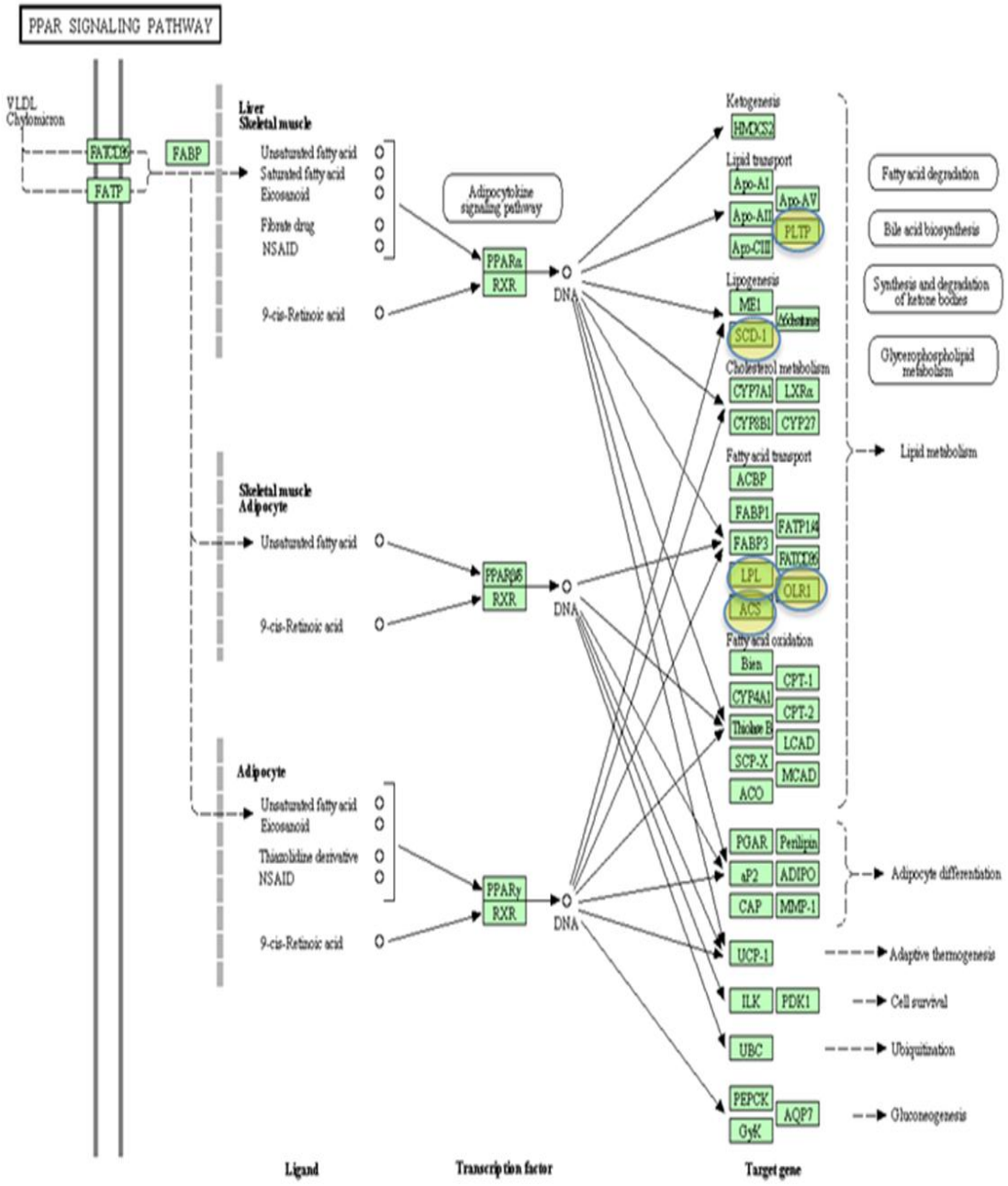

Figure 4.2.5: The PPARγ pathway. The pathway analysis using KEGG analysis identified the PPARγ pathway. The genes circled in yellow pathway are some of the differentially expressed (over expressed in obese) genes highlighted in yellow (Scd1, Lpl, Pltp, Olr1, Acs1) lying down stream to PPARγ pathway

4.2.1.3 Validation of gene expression using Q-PCR analysis

The Q-PCR analysis validated the bioinformatic analysis of the identified genes overexpressed in the obese adipose tissue. The relative fold of expression of some of the genes such as Scd1, AcsI, MglII, Alox5-ap, Lpl, and Capn1 was checked in the obese adipose in comparison to lean tissue. Q-PCR analyses of the genes were done using the absolute quantification method using β-Actin as internal control. The two step PCR was done to synthesize first strand cDNA synthesis from equal amounts of total RNA (5 μg) of obese and lean adipose tissue. 4 fold dilutions of standards from 1:30 diluted obese cDNA were prepared. The cDNA dilution of 1:15 obese and lean was used for samples. 20 μl PCR reaction was set up with standardized PCR conditions for every gene using 10 μl of cDNA dilutions and master mix containing primers (forward and reverse) and 2X SYBR green premix (buffers, nucleotides, *Taq polymerase*). The relative fold of expression of genes mentioned above were calculated using $2^{\wedge(-\Delta\Delta Ct)}$ method. The samples were run in triplicates and replicated twice for calculating the fold of expression. The obese adipose tissue showed significant increased expression of Scd-1 by 24 fold, Alox-5ap by 17 fold .MglII and Lpl genes were elevated by 9 and 7 fold respectively. Some of the other identified genes over expressed in the obese adipose like AcslI, Capn1, and Leprot-l1 showed a 2 fold increase in obese adipose tissue. There was no fold of expression of a hypothetical protein identified on the 15th chromosome from the forward subtracted library (Figure 4.2.6).

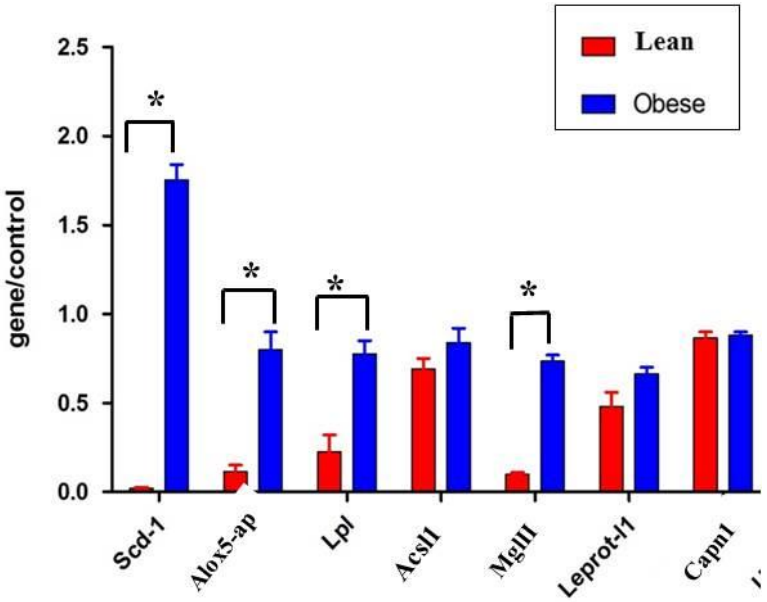

Figure 4.2.6: Q-PCR analysis of different genes in obese and lean adipose tissue. The Q-PCR studies validated the identification of some of the genes over expressed in obese adipose tissue. The total RNA (5 μg) isolated from 3 months lean and obese adipose tissue (n = 3) was used for cDNA synthesis for Q-PCR studies. The fold of expression of the genes Scd-1, Alox5-Ap, Lpl, Acsl1, MgIII, Leprotl1, and Capn1 was calculated. The fold of expression of Scd1 (24 fold), Alox5-Ap (17fold), Lpl (6 fold) and MglII (9fold) were significant. The values given are triplicates with experiments repeated twice. Error bars represent standard errors (SEM).

4.3 Pro-inflammatory cytokine analysis by ELISA

The immune and inflammatory pathways are linked to obesity induced pathogenesis leading to T2DM, CVD. Analyses of the subtracted cDNA library of the subcutaneous adipose tissue of obese rat revealed that genes related immune and inflammatory pathways were few. As a result, to obtain an overall effect of inflammation and its effect with time, the systemic as well as local tissue inflammation levels of some of the known pro-inflammatory cytokines were quantified by ELISA across the ages (1, 3, 6, 12 months) in male lean and obese rats.

4.3.1 Systemic and local (tissue) expression levels of pro-inflammatory cytokines

Lean and obese rats three in each age group (1, 3, 6, 12 months) were fasted and the blood samples were collected and serum was separated. Adipose tissues were collected and flash frozen. Total protein from 300 mg of adipose tissue of three lean and obese rats in each age group (1, 3, 6, 12 months) was estimated as described in material and methods. 1 mg / ml diluted five times in all the samples of tissue lysate were used for the experiment. 100 µl of serum and diluted tissue lysate was used for ELISA done as per the manufacturer's protocol (Peprotech). The concentrations were calculated based on the 4-parameter logistic fit by inbuilt software (Biotek). The concentration was expressed as pg / ml in serum and pg / mg protein. The expression analysis revealed a comparable yet an irregular increase in the expression levels of cytokines with age (months) in obese rats compared to lean counterpart. The levels of pro-inflammatory cytokines in obese were higher than lean (control), however it was not statistically significant.

4.3.2 Comparative analysis of serum and tissue levels of pro-inflammatory cytokines

The protein concentration of pro-inflammatory cytokines IL-1β, TNF-α, IL-6, RANTES, MCP-1 were determined in serum and tissue to compare the levels of cytokine expression and obtain a comparative systemic and local tissue inflammation.

IL-1β is a strong pro-inflammatory cytokine mediating systemic and organ specific influence. It is known to play important role in the accumulation of visceral fat by impairing adipogenesis, lipogenesis in the subcutaneous adipose tissue. The serum analysis of cytokine IL-1β (Figure 4.3.1A) showed increase in IL-1β expression in lean, but decreased in obese rats with age. The adipose tissue expression levels for both lean obese rats increased with age (Figure 4.3.1B) and were comparable to lean tissue.

TNF-α is an important biomarker that linked inflammation to obesity and diabetes. Studies have shown positive correlation to increasing subcutaneous fat mass. The systemic concentration of TNF-α in 3 months obese rat was relatively similar in lean (control) (Figure 4.3.2A). Although not significant, the overall pattern showed TNF-α levels to be more in obese than lean serum samples and the levels increased with age (Figure 4.3.2B). The adipose tissue expression of TNF-α also showed an increasing but not a significant trend across different ages across the age groups in lean and obese.

IL-6 cytokine affects various processes including the immune response, reproduction. The levels of cytokine estimated in serum of obese rat increased with age compared to lean (Figure 4.3.3A). The tissue concentration of IL-6 did not vary with control group and showed a steep increase at 12 months in obese (Figure 4.3.3B).

RANTES, a recently identified cytokine involved in T-cell infiltrate of adipose tissue during inflammation links immune and metabolic functions. The serum concentrations were constant and comparable to lean rats across the age groups (Figure 4.3.4A). The local (tissue) concentration of RANTES in the obese tissue increased from

1 month to 12 months compared to lean tissue but not significant (Figure 4.3.4B).

The MCP-1 cytokine plays an important role in the macrophage infiltration into the adipose tissue. Expression levels of MCP-1 were more in the stromal vascular fraction of the adipose tissue. The concentration of MCP-1 in the systemic circulation showed high levels in all the age groups of lean and obese rats (Figure 4.3.5A). The concentrations within the adipose tissue of lean remained constant while the obese tissue showed an increasing trend with a steep increase after 6 months (Figure 4.3.5B).

The concentration of the above mentioned biomarkers were estimated in serum and within the tissue to gain an idea on the levels of inflammation at different time points and a comparative analysis of the same with lean counterparts would imply the relative increase in inflammation in obesity in systemic levels and within the tissue. The analysis showed an increasing and irregular trend with values almost comparable to lean rats and not statistically significant. Concentrations of all the cytokines of 3 months obese in the serum and within the tissue were similar to control group and not significant. This indicates probably less inflammation at this time point.

It can be inferred that the WNIN / Ob obese phenotype with a healthy expansion of subcutaneous adipose tissue is able to resist inflammatory induced complications. Therefore, the cDNA library did not generate many inflammatory genes in the subcutaneous adipose tissue.

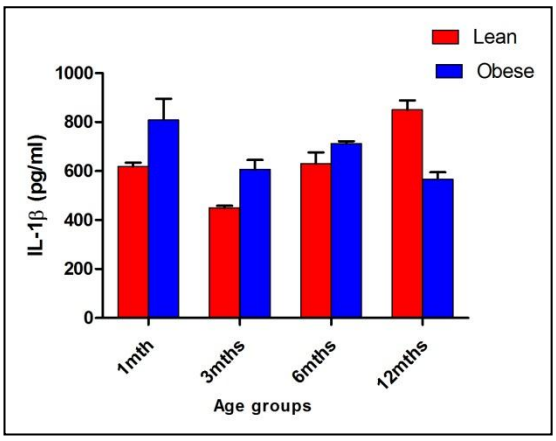

Figure 4.3.1A: Serum levels of 1L-1β in lean and obese rat across different ages. The blood collected from fasted lean and obese rats (n = 3). The serum was used for determining the concentration of 1L-1β expressed as (pg /ml) by ELISA. Error bars represent standard error (SEM). The values are cumulative of three animals. Student's t-test with value $P < 0.05$ was considered significant.

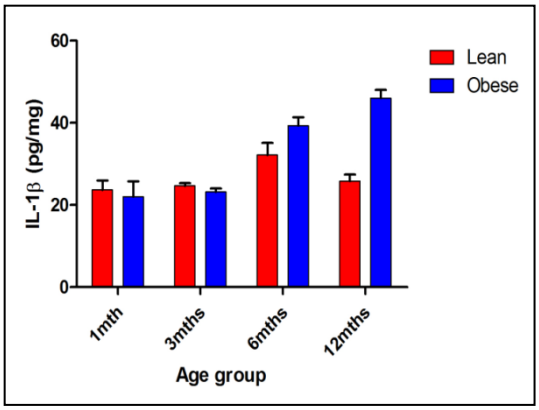

Figure 4.3.1B: The expression levels of 1L-1β in the subcutaneous adipose tissue of lean and obese in different ages. The concentration of 1L-1β measured in the adipose tissue. 300 mg of tissue of lean and obese (n = 3) from all age groups were homogenized and lysed with RIPA buffer and the concentration of 1L-1β expressed as (pg / mg protein) across ages. Error bars represent standard error (SEM). The values are cumulative of three animals. Student's t-test with value $P < 0.05$ was considered significant.

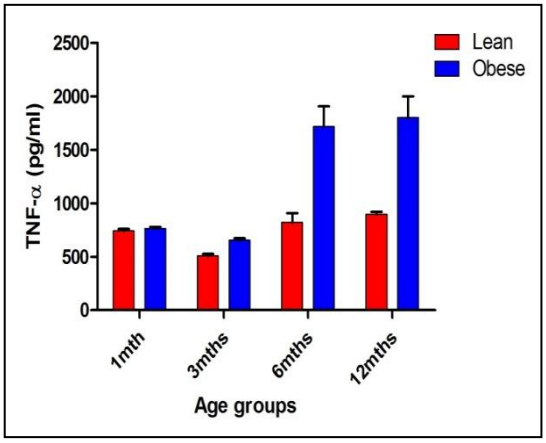

Figure 4.3.2A: Serum levels of TNF-α in lean and obese rat across different ages. Serum levels of TNF-α in lean and obese across different ages. The blood collected from fasted lean and obese (n = 3) of all age group and serum was separated. The concentration of TNF-α expressed (pg / ml) was estimated by ELISA. The values are cumulative of three animals. Error bars represent standard error (SEM). Student's t-test with value $P < 0.05$ was considered significant.

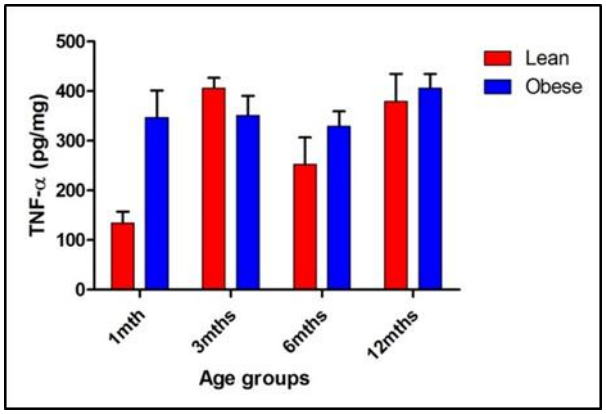

Figure 4.3.2B: The expression levels of TNF-α in the subcutaneous adipose tissue of lean and obese in different ages The concentration of TNF-α measured in the subcutaneous adipose tissue of lean and obese tissues in different age groups. 300 mg of tissue of lean and obese (n = 3) from all age groups were homogenized and lysed with RIPA buffer and the concentration of TNF-α expressed (pg /mg protein) across ages was determined by ELISA. The values are cumulative of three animals. Error bars represent standard error (SEM). Student's t-test with value $P < 0.05$ was considered significant.

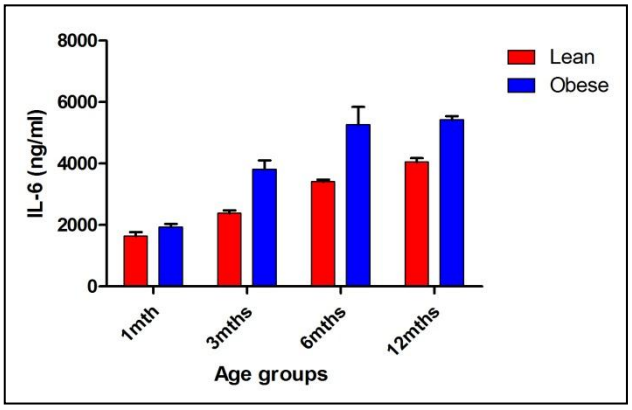

Figure 4.3.3A: Serum levels of IL-6 in lean and obese rat across different ages. Serum levels of IL-6 in lean and obese across different ages. The blood collected from fasted lean and obese (n = 3) of all age group and serum was separated. The concentration of IL-6 expressed (pg /ml) was estimated by ELISA. The values are cumulative of three animals. Error bars represent standard error (SEM). Student's t-test with value $P < 0.05$ was considered significant.

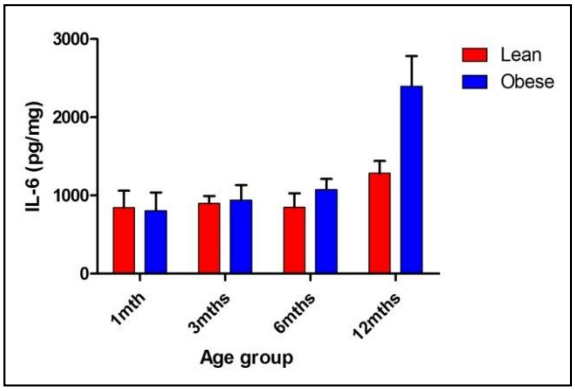

Figure 4.3.3B: The expression levels of IL-6 in the obese subcutaneous adipose tissue of lean and obese in different ages. The concentration of IL-6 measured in subcutaneous adipose tissue of lean and obese in different age groups. 300 mg of tissue of lean and obese (n = 3) from all age groups were homogenized and lysed with RIPA buffer and the concentration of IL-6 (expressed pg / mg protein) was determined by ELISA. The values are cumulative of three animals. Error bars represent standard error (SEM). Student's t-test with value $P < 0.05$ was considered significant.

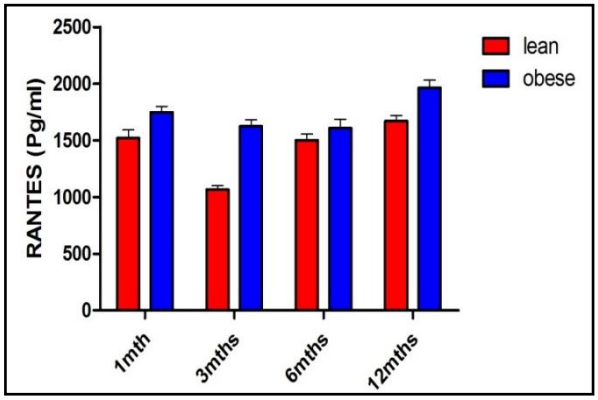

Figure 4.3.4A: Serum levels of RANTES in lean and obese rat across different ages. Serum levels of RANTES in lean and obese across different ages. The blood collected from fasted lean and obese rats (n = 3). The serum was used for determining the concentration of RANTES expressed as (pg /ml) by ELISA. Error bars represent standard error (SEM). The values are cumulative of three animals. Students t-test with value $P < 0.05$ was considered significant.

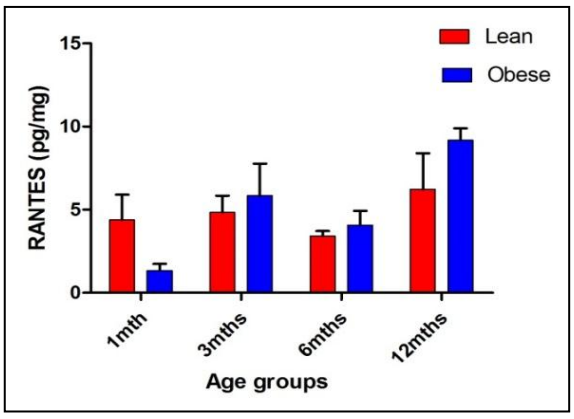

Figure 4.3.4B: The expression levels of RANTES in the subcutaneous adipose tissue of lean and obese in different ages. The concentration of RANTES measured in subcutaneous adipose tissue of lean and obese in different age groups. 300 mg of tissue of lean and obese (n = 3) from all age groups were homogenized and lysed with RIPA buffer and the concentration of RANTES expressed as (pg /mg protein) across ages. Error bars represent standard error (SEM). The values are cumulative of three animals. Student's t-test was done with values $P < 0.05$ was considered significant.

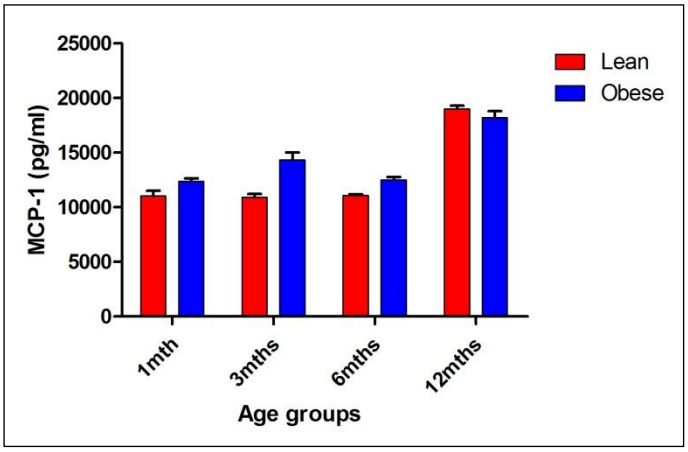

Figure 4.3.5A: Serum levels of MCP-1 in lean and obese rat across different ages. The blood collected from fasted lean and obese rats (n = 3). The serum was used for determining the concentration of MCP-1 expressed as (pg /ml) by ELISA. Error bars represent standard error (SEM). The values are cumulative of three animals. Student's t-test with value $P < 0.05$ was considered significant.

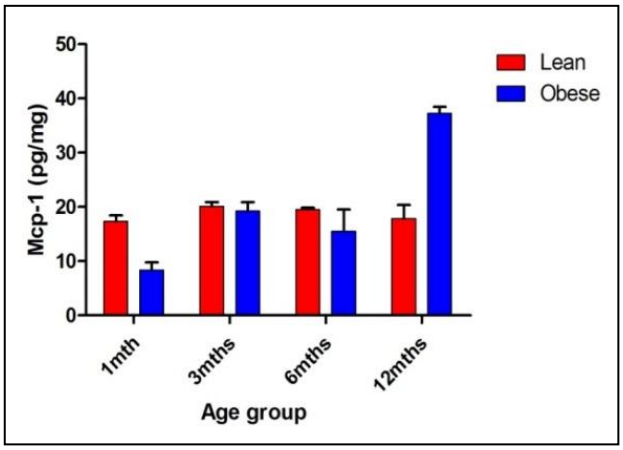

Figure 4.3.5B: The expression levels of MCP-1 in the subcutaneous adipose tissue of lean and obese in different ages. The MCP-1 cytokine concentration measured in subcutaneous adipose tissue of lean and obese in different age groups. 300 mg of tissue of lean and obese (n = 3) from all age groups were homogenized and lysed with RIPA buffer and the concentration of MCP-1 expressed (pg /mg protein) across ages was determined by ELISA. The values are cumulative of three animals. Error bars represent standard error (SEM). Student's t-test was done with p values < 0.05 was considered significant.

4.4 The Intracerebroventricular (ICV) infusion of recombinant human resistin (rhRes) protein

4.4.1 Purification of recombinant human resistin

The human resistin were cloned and expressed as His-tagged protein in *E.coli* and the recombinant protein was purified to homogeneity as described in material and methods. The pQE30hRes was transformed in M15 *E.coli* cells. The overexpressed recombinant was solubilized in urea (8M). The denatured protein was removed by affinity column chromatography and refolded subsequently by dialysis and gradual removal of urea. The protein was concentrated to 2 µg /µl in an amicon concentrator with a 3.5 kDa cut-off membrane. The fractions of purified recombinant protein were eluted and resolved on a 10 % Tris Tricne gel (Fig 4.4.1). The protein concentration was estimated by BCA method.

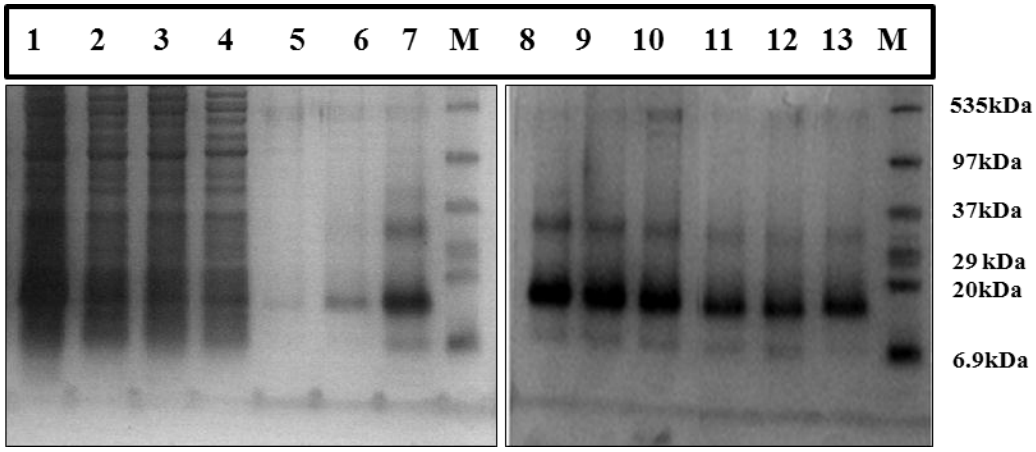

Figure 4.4.1: Purified rhRes by talon metal affinity chromatography observed in SDS-PAGE. The different fractions eluted during the purification of were analyzed on a 10 % Tris Tricine gel. Lane 1: IPTG induced rhRes, Lane 2: flow-through from Talon column, Lane 3 and 4: 5 mM imidazole wash, Lanes 5 - 13: elution fractions. The position of protein molecular size markers is indicated in extreme right of two gels.

4.4.2 ICV injection of rhRes in the obese and lean rats

The study aimed to find out the effect of recombinant human resistin (rhRes) injected in the brain (hypothalamus) on food intake and body weight of obese rat. Three months old male lean and obese rat, used for the experiment were first maintained individually in clean cages and isolated room (Figure 4.4.2A) and provided with rat chow and water *ad libitum* (Figure 4.4.2B) . The cages were changed every regularly. The animals were divided into three groups and three lean and obese rats were used for each group. (Figure 4.4.2 A and B)

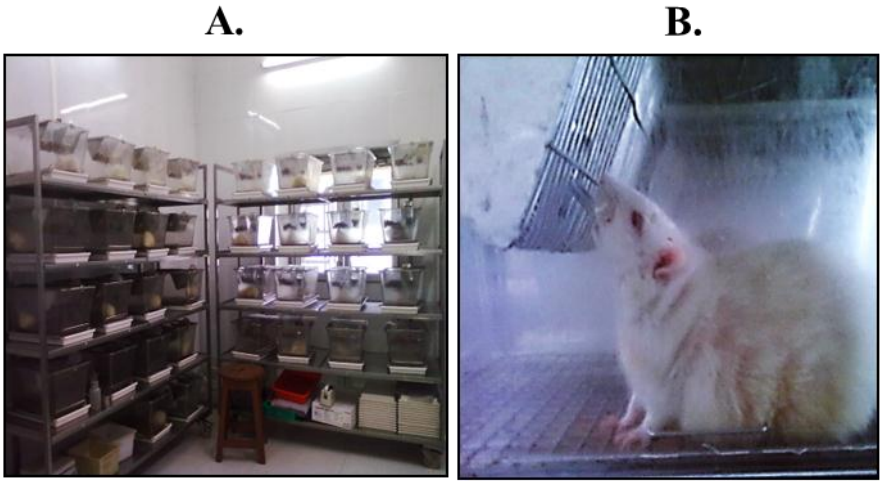

Figure 4.4.2A&B: Animal room facility for acclimatization of animals and stereotaxic surgery. (A) Animals maintained in individual clean cages in an isolated room with temperature maintained at 22± 2 ^{o}C (B) All the animals were provided with rat chow and autoclaved water *ad libitum*.

4.4.2.1 Stereotaxic surgery for the ICV cannulation

The stereotaxic surgery was done for all the animals to implant the cannula at the lateral ventricle (ICV) for infusion. The animal was anesthetized and the hair on the head was trimmed (Figure 4.4.3A). The animal was placed on the stereotaxic surgery platform inside the hood and the head was leveled firmly held with ear lobes and the eyes were

covered (Figure 4.4.3B). The incision on the scalp was cut open exposing the bregma of the skull. The coordinates from the bregma were noted and 1 mm diameter deep hole was drilled with a motor fixed with a driller bead (Figure 4.4.3C). The osmotic pump (Alzet osmotic pump 2001) was filled with saline was placed in the subcutaneous pouch at the back and the cannula (Alzet infusion kit II) on the other side was placed into the drilled site (ICV / lateral ventricle) (Figure 4.4.3D). The incision was sutured and cleaned (Figure 4.4.3E) and placed carefully into the cage for recovery with *ad libitum* rat chow and water (Figure 4.4.3F).

4.4.2.2 Infusion of recombinant human resistin

The pump infusion released protein or vehicle continuously for one week at the rate of 1 µl / hr. The osmotic pump infusion involved three stages for all the three groups (Figure 3.3, chapter 3). On day 7 (week 1), all the animals received saline pump for ICV cannulation. On day 14 (week 2), the lean and obese rat of group 1, 2 received a pump replacement of 20 mM Tris (vehicle), and the lean and obese rat of the group 3 received 2 µg rhRes that pumped protein 2 µg / µl / hr (protein) continuously for one week. On day 21 (week 3), similar procedure for the next change with tris or protein was done for all the groups.The lean and obese rat of group 1, 3 received a pump replacement of 20 mM Tris (vehicle), and the lean and obese rat of the group 2 received 2 µg rhRes. The incision made on the skin at the back during the surgery at the back to hold the osmotic pump facilitated easy replacement and the old pump (saline filled during ICV surgery) was removed and replaced with osmotic pump containing either 20 mM Tris or 2 µg rhRes protein after a week (Figure 4.4.4A-F). The skin was sutured and cleaned. The food intake and body weight was monitored for two weeks.

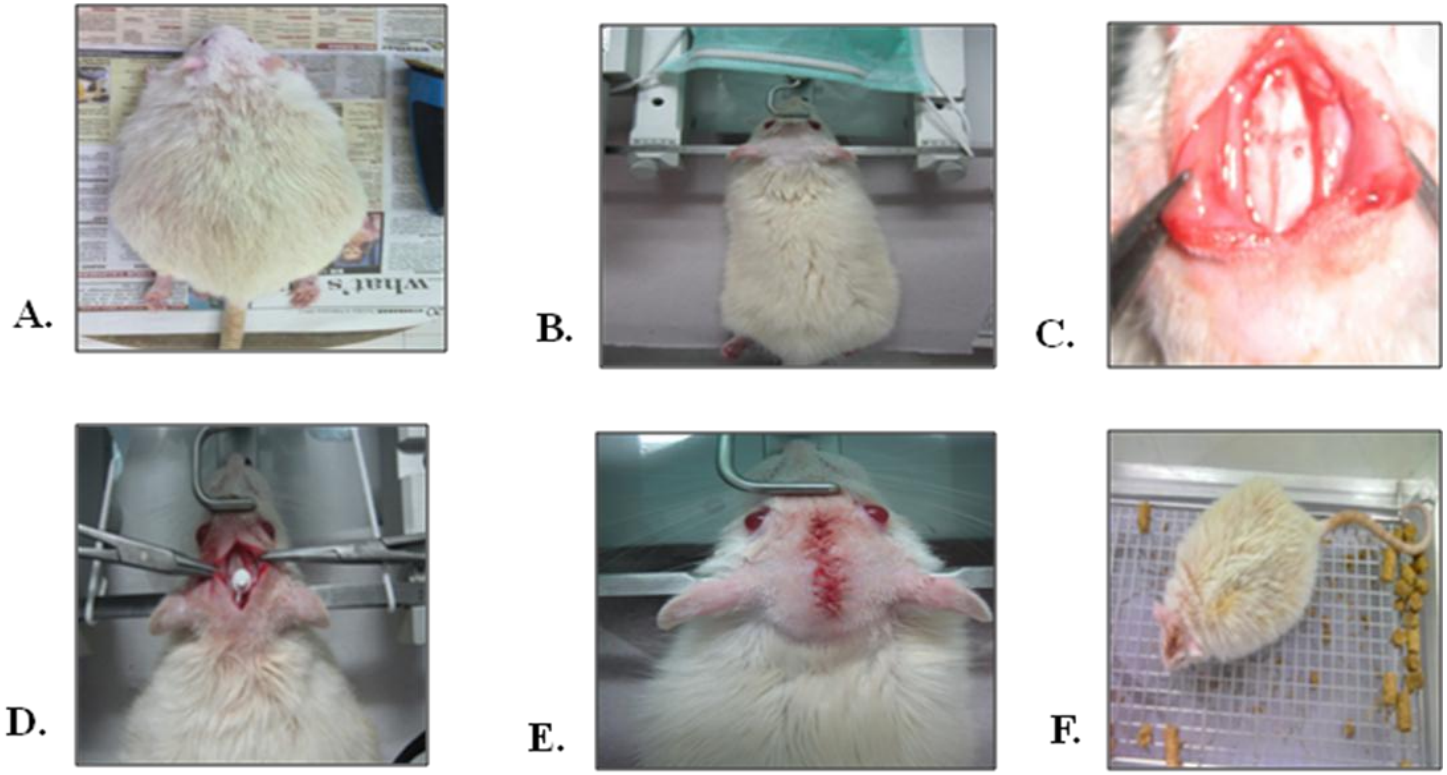

Figure 4.4.3A - F: Pictorial representation of the surgery procedure. The stereotaxic surgery performed for ICV infusion of rhRes protein. Clockwise: (A&B) Animals were anesthetized and the head was shaved and cleaned. The animal was assembled on the stereotaxic apparatus covering the eyes (C) The coordinates from bregma on the right side of the skull were noted and drilled in the right ventricle region (D) The osmotic pump pre-activated was put behind the subcutaneous pocket dorsally and the cannula was inserted into the hole and fixed firmly on the skull using an adhesive (E) after the cannula was firmly adhered the skin on the head was sutured and cleaned (F) The animal was carefully placed into the cage for recovery.

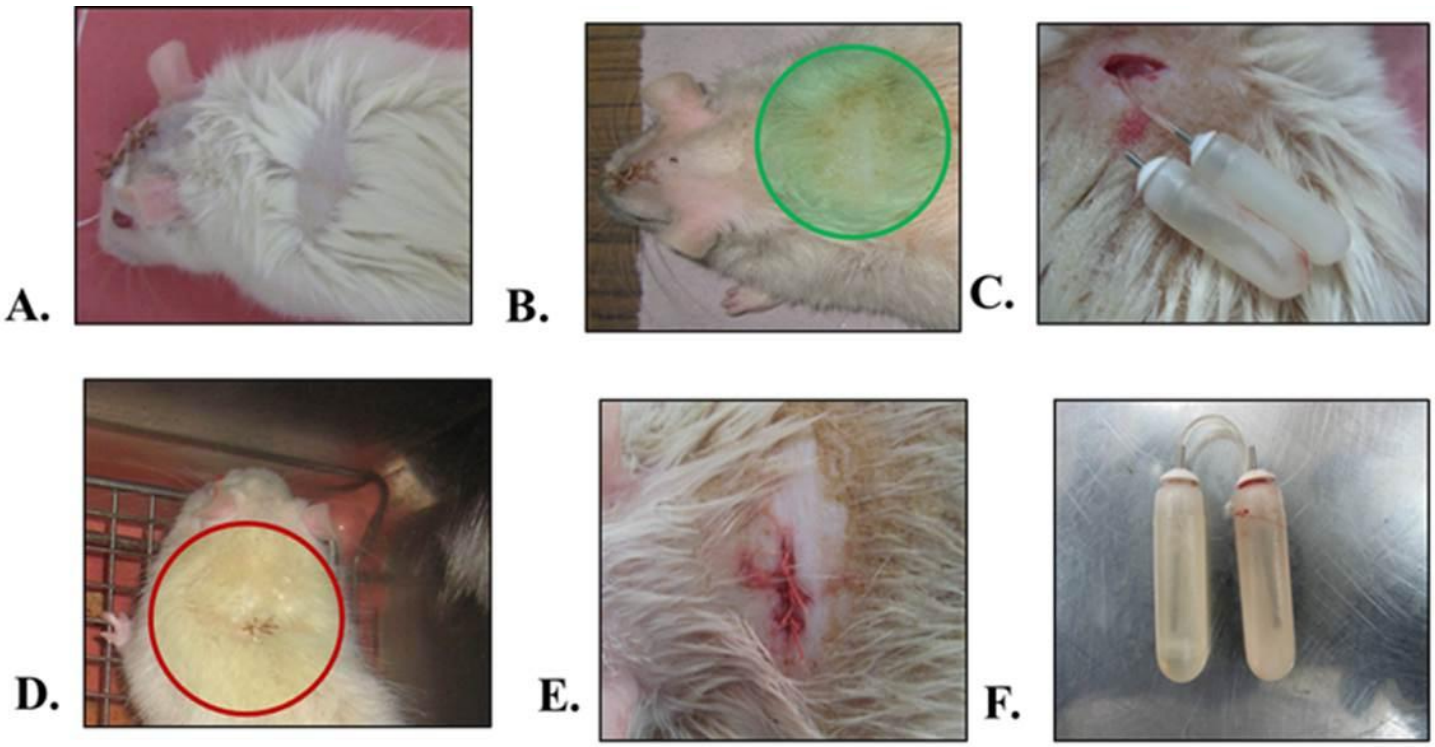

Figure 4.4.4A-F: Osmotic pump replacement with vehicle (20 mM Tris) or protein (2 µg rhRes). The lean and obese rats after surgery were implanted with pre-activated osmotic pump filled with vehicle (Tris) in group 1 and 2 or protein (rhRes) in group 3. Similar procedure for the next change after a week, osmotic pump with vehicle (Tris) in group1 and group 3 or protein (rhRes) in group 2 were implanted. Clockwise: A&B:Anesthetized rats on day 7 were replaced with pumps as per the group. An incision on the skin for the replacement of pump (an incision on the skin close to previous pump), C&D: The old pump on day 14 replaced with new pump containing vehicle or protein, After pump replacement the cut incision is sutures(circled in red) E: The dried suture of the previous incision was removed after which a new incision was made for the second replacement F: The empty osmotic pump collected on day 7 and day 14

4.4.2.3 Effect of infused rhRes on food intake and body weight in the obese and lean rat

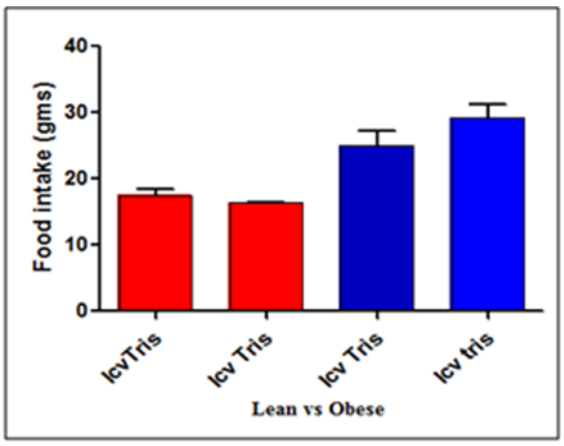

Figure 4.4.5A: Food intake comparisons after ICV infusion of Tris in group1 (control group). The lean and obese rats (n = 3) in group1 (control). The lean and obese rats in group 1 received infusion of vehicle (Tris 20 mM) for two weeks (week 1 and 2). The weekly changes in food intake were compared. The ICV Tris infusion showed no difference in food intake for both obese and lean. Student's paired t-test analysis with p value < 0.05 was considered significant. The values are cumulative of 3 animals taken in each group. Error bars represent standard error (SEM).

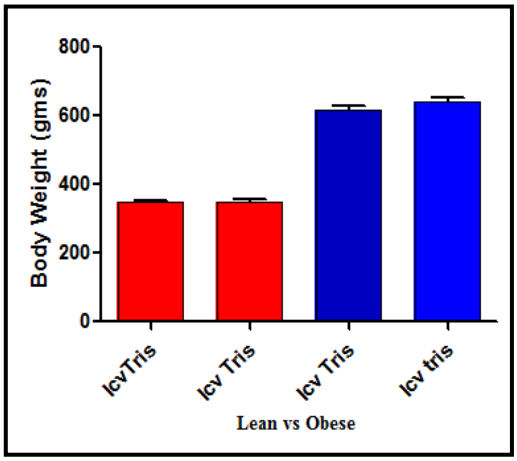

Figure 4.4.5B: Body weight comparisons after ICV infusion of Tris in group 1 (control group). The lean and obese rats in group 1 (n = 3) received only vehicle (Tris 20 mM) infusion for both the weeks (1 and 2). The weekly changes in body weight after pump infusion on week 1 and 2 were compared with lean rats. The changes in the body weight of lean and obese rats were not significant. Student's paired t-test analysis with p value < 0.05 was considered significant. The values are cumulative of 3 animals taken in each group. Error bars represent standard error (SEM).

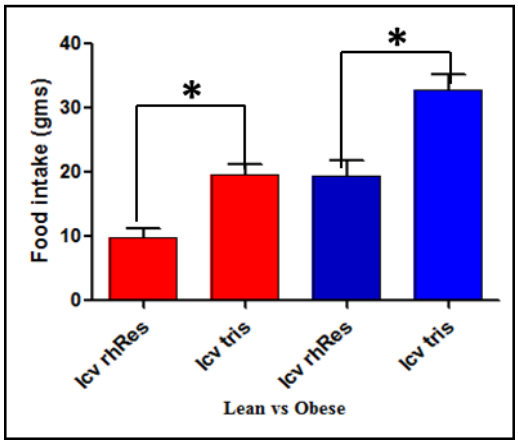

Figure 4.4.6A: Food intake comparisons after ICV infusion of rhRes in group 2. The lean and obese rats (n = 3) in group 2 received infusion of vehicle (Tris 20mM) in week 1 followed by rhRes (2 µg) in week 2. The weekly changes in food intake were compared. The ICV of rhRes decreased food intake in both lean and obese were significant. Student's paired t-test analysis with p value < 0.05 was considered significant. The values are cumulative of 3 animals taken in each group. Error bars represent standard error (SEM).

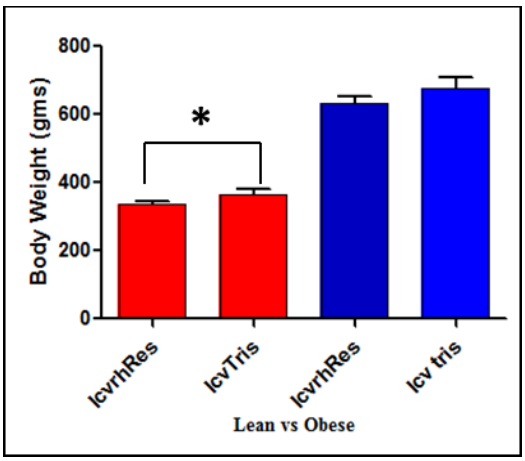

Figure 4.4.6B: Body weight comparisons after ICV infusion of rhRes in group 2. The lean and obese rats (n = 3) received vehicle (Tris 20 mM) in the week 1 followed by rhRes (2 µg) in the week 2. The weekly changes in the body weights were compared. The protein infusion reduced the body weight in both lean and obese rat. The decrease was barely significant for lean and not significant for obese. Student's paired t-test analysis with p value < 0.05 was considered significant. The values are cumulative of 3 animals taken in each group. Error bars represent standard error (SEM).

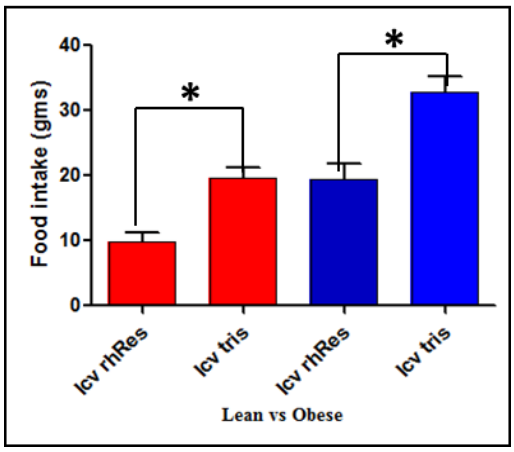

Figure 4.4.7A: Food intake comparisons after ICV infusion of rhRes in group 3 .The lean and obese rats received infusion of rhRes (2 µg) in the first week followed by vehicle (Tris 20 mM) in second week. The weekly changes in food intake were compared. The ICV infusion of rhRes reduced food intake in both lean and obese. The decrease in food intake seen in lean and obese was significant. Student's paired t-test analysis with p value < 0.05 was considered significant. The values are cumulative of 3 animals taken in each group. Error bars represent standard error (SEM).

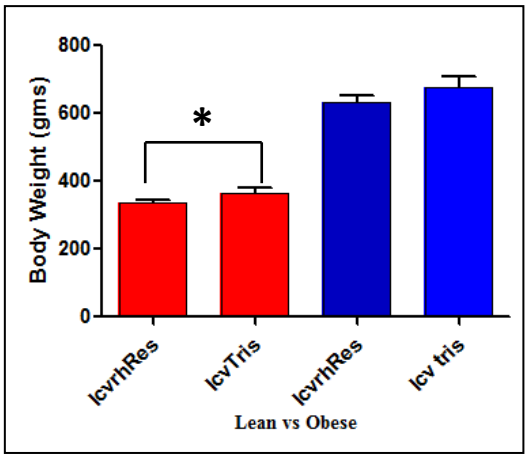

Figure 4.4.7B: Body weight comparisons after ICV infusion of rhRes in group 3.The lean and obese rats received rhRes (2 µg) in the first week followed by vehicle (Tris 20mM). The weekly changes in the bodyweights were compared. The protein infusion in week 1 reduced the body weight later increased after vehicle in week 2. The body weight of lean and obese rats decreased in the first week but was significant only in lean. Student's paired t-test analysis with p value < 0.05 was considered significant. The values are cumulative of 3 animals taken in each group. Error bars represent standard error (SEM).

The animals were divided into three groups, with each group having three lean and obese rats. After the recovery of animal from surgery, In the first week the group 1 and 2 rats (lean and obese) received 20 mM Tris (vehicle) osmotic pump replacement. While the third group (lean and obese received 2 μg / μl rhRes. The food intake and body weight measured for a week after which the osmotic pump was replaced with 20 mM Tris for group 1 and 3. The second group received 2 μg rhRes (protein) continuous infusion for a week. The daily food intake and body weight noted for another week. The change in food intake (grams) and in bodyweight per (grams) per week was calculated. Figure 4.4.5A and B shows the change in the food intake and body weight in group 1. The lean and obese in this group is the control group that received 20 mM Tris (vehicle for two weeks. The figure 4.4.6A and B of lean and obese rats of group 2 received Tris (vehicle) followed by rhRes (protein). There was a decrease in food intake and body weight in both lean and obese rats. The decrease in food intake was significant for both lean and obese. The decrease in body weight upon resistin infusion was barely significant for lean and the change was not significant in the obese rat. The group 3 lean and obese rats were infused with resistin in the first week (unlike group 2) followed by vehicle. The figure 4.4.7A and B shows the decrease in food intake and body weight of group 3 similar to the group 2. The change in the food intake and body weight was significant for lean and decrease in the food intake was significant for obese. The group 1 and group 3 were used as controls to understand the pattern of decrease in food intake or body weight in absence of resistin or resistin infused in reverse to group 2. The decrease in food intake as result of resistin infusion in both lean and obese rats indicates active central mechanisms of resistin in WNIN / Ob rats.

Chapter 5

Conclusions

5.1 WNIN / Ob obese rat – An addition to the genetic model of animal obesity

The rise in obesity shows an alarming trend globally. Although, obesity is not a sole factor for the increasing comorbidities, it is a major risk factor that accelerates several metabolic ailments like insulin resistance, T2DM, arthritis, CVD, cancers, neuronal disorders, depression, and infertility. Obesity is the result of imbalance in energy intake and expenditure. The complex network of gene-gene, gene-environmental factors regulates the development of obesity. The genetic factors influenced by an obesogenic environment could predispose an individual to increase body weight. The genetic factors vary from 30 – 70 % (Maes, H., H. 1997), while the non-genetic factors accompanied by epigenetic modifications seem to have more control in determining the development of the phenotype (Egger et al., 2004). Animal studies using both genetic and non-genetic models have significantly increased our understanding of the physiology of development of obesity. The genetic models with single gene defect of leptin, leptin receptor, pro-opiomelanocortin (POMC), Agouti related protein (AgRP), prohormone convertase 1, melanocortin 3 and 4 receptor lead to phenotype of abnormal eating behavior followed by a development of severe early-onset obesity. The widely used monogenic obese models Lep (ob/ob), Lepr (db/db), Agouti (A^y), fat (fat), Tubby, (tub) and Adipose (Ad) in mice and Fatty (fa/fa), Corpulent (cp) in rats have mutations as autosomal recessive traits and the gene mutations are related to leptin pathway (Lutz and Woods, 2012).

The WNIN / Ob obese rat is an in-house obese rat developed at the National Institute of Nutrition (NIN). It is a recent addition to the genetic models of obesity and genetically more pure than the other western animal models. The obese mutation is carried as an autosomal recessive trait affecting both the sexes and the obesity phenotype segregate in a typical Mendelian fashion (Giridharan et al., 1996). These obese rats are euglycemic and are characterized by a kinky tail and showed visible phenotype of

obesity from 35 days of age. They were reported to be the world's fattiest rats (Jayaraman, K., S. 2005). They also show hyperphagia, hyperinsulinemia, hypertriglyceridemia, hypercholesterolemia, infertility and reduced longevity very similar to other established models (Giridharan et al., 1996, Harishankar et al., 2011). They develop almost all the major metabolic degenerative conditions (such as cataract, tumours) observed in humans, making them ideal model to study the metabolic syndromes at the molecular levels. The genetic background is still unclear. It has been reported that there are no defects in leptin or leptin receptor (Rao et al., 2014). Recently, the genetic mutation of the WNIN / Ob was found to lie in the chromosome 5 in the region upstream to the promoter region of the leptin receptor (Kalashikam et al., 2013).

5.2 Construction of subtracted cDNA library by subtractive hybridization

The WNIN / Ob obese rat model have been well utilized to study histopathological changes (Harishankar et al., 2011), pancreatic studies (Venkateshan et al., 2013), adipose tissue gene expression levels (Madhira et al., 2011), neuronal studies (Reddy et al., 2014, Sinha et al., 2014) and vitamin A supplementation (Tiruvalluru et al., 2013). The studies on the differential gene expression analysis in the adipose depots were not done so far. The present work utilized subcutaneous adipose tissue to identify differentially regulated genes in the obese rat to understand a global regulation of various gene(s) of different pathways altered in response to obesity.

Similar to microarrays which are widely used that can show differential analysis of thousands of genes the subtractive technique also has limitations in not identifying many genes, but has an advantage to obtain a pattern of gene expressions that are not predetermined as in microarray. The cDNA library data matched with that of subcutaneous adipose of obese rat models and humans and showed most of the genes

similarly over expressed but probably varied in expression. The subtractive suppression hybridization was used to generate cDNA pool of differentially expressed gene in the subcutaneous tissue of three months old male lean and obese rat. The forward and reverse subtraction was done to generate cDNA pool that was overexpressed in lean and obese subcutaneous adipose tissue respectively which was further enriched by PCR (Figure 4.1.1). The cDNA pool obtained from forward and reverse subtracted pool were cloned in pGEM-T vector and transformed into *E.coli* cells for blue-white colony screening to prepare a library. Around 750 white colonies were selected through blue -white colony screening that contain the cDNA inserts of genes over expressed in lean and obese adipose tissue. The positive recombinant clones were restriction digested by single enzymes or combination of two enzymes to confirm the presence of inserts (Figure 4.1.2A and B). The plasmids with inserts upon single digestion resolved above 3 kb and some plasmids upon double digestion showed the cDNA insert. Colony PCR was done to PCR amplify the insert region in the plasmid to identify the presence of cDNA inserts in the recombinant colonies (Figure 4.1.2C). Over 500 confirmed positive plasmid clones containing the cDNA inserts were sequenced using DNA automated sequencer. The sequencing resulted in a chromatogram showing clear peaks of the nucleotide represented in different colours (Figure 4.1.3A) and the sequence of cDNA insert (Figure 4.1.3B).

5.3 Bioinformatic analysis of the differentially expressed genes in the subcutaneous tissue of the obese rat

The obese and lean cDNA forward and reverse subtracted cDNA pools were sequenced and the corresponding RNA was identified using bioinformatic tools. The sequences in the FASTA format were given as input and BLAST searched using NCBI BLASTN public database. The BLAST output identified several sequences against the Ref-Seq of rat database (Figure 4.1.4A). The mRNA identifiers with a maximum query

coverage and identity and low E-value were selected for the corresponding cDNA sequence given as query (Figure4.1.4B). BLAST search identified nearly 200 genes, 112 were identified to be repressed in obese (overexpressed in lean) and rest of the genes over expressed in obese from forward and reverse subtraction. The over expressed list of genes in obese (O) and lean (L) respectively represent the subtracted library of obese subcutaneous adipose tissue (Table 4.1). The table 4.1 lists the genes with their symbols and their differential expression in obese (O) or Lean (L) subcutaneous adipose tissue. The identified genes were further classified as per chromosome location, function, cellular location, biological process and the pathway analysis to understand the pattern of expression using GO 'annotations' in KEGG and PANTHER PATHWAY software tools. Several genes were overexpressed whose role have been already implicated in the development of obesity, in addition to some known and unknown genes were identified in the subtracted gene library of SAT. These classifications identified the genes belonging to different cellular locations as well as wide variety of metabolic pathways and molecular functions indicating a complex gene interaction in the development of obesity in these animals.

The chromosomal classification showed the over expressed genes being identified on chromosome 1 were Scd1, Klk6, Ifitm2, followed by 3 and 7 and none identified on chromosome 11. The repressed genes were located mostly on the chromosome 2 like Cp, Gst followed by 8. There were no genes identified on chromosome 14, 15, 19 in the library (Figure 4.2.1). Recent report on the gene mutation of this obese rat model was identified on the chromosome 5 in the promoter region upstream to leptin receptor. However, in this library, the genes identified to be over expressed on the chromosome 5 included few genes like nucleic acid binding protein, staufen RNA binding protein (Stau2), high mobility group nucleosome binding protein (Hmgn2) and repressed genes on the chromosome 5 were Cap2, Per3. There were no

genes identified related to leptin pathway in the library except for Jak 2 that was identified to be repressed in obese adipose tissue.

The classification of genes (overexpressed and suppressed in obese tissue) as per various cellular locations such as membrane, organelles (ER, lysosome, mitochondria, Golgi, Nucleus), cytoplasm revealed the over expressed genes to be highest in the cytosolic locations 28 % with genes like Vim, Dlg5, Vps35, Krt72, Snx3 belonging to signaling or cytoskeletal, transport functions followed by membrane and cellular organelles like mitochondria, Golgi complex, and microsome (Figure 4.2.2). The repressed genes were abundantly belonging to cell membrane followed by nuclear and cell organelle (mitochondria). The cytosolic proteins in adipocytes are major components of Golgi-ER transport system that mediate various intracellular activities like, signaling, protein and lipid transport, cell growth *etc*. Several cytosolic genes up regulated in the obese adipose tissue were components of Golgi-ER transport system like Sorbs1, Rims3, Syt4, Vps35, p28, Snx3, whereas other genes like Snx14, Rab18, Rab40, were identified to be repressed. The upregulated genes were found to encode the components that are involved in protein trafficking, Rab-GTPases are membrane proteins localized in the vesicles, lipid droplet, Golgi and ER compartments. While Rab18 is known to mediate lipolysis and lipogenesis in adipocytes and vesicular trafficking, Rab40 recruits the components of the ubiquitination machinery to regulate Wnt signaling. The genes related to ER stress like Naca, Pdrg1, Ces1d, and Ube2g1 were found to be up regulated in the obese adipose tissue whereas genes like Selk, Edem1 were down regulated. SelK is an endoplasmic reticulum (ER) resident protein and its biological function is not well understood. However, existing data identifies it to be an important player for preventing ER-stress induced apoptosis through unknown mechanisms. The altered gene expressions represented in the cellular locations like mitochondria, Golgi complex and cell membrane indicate their locations of impaired functions within the adipocyte in obesity

The classification based on GO ‘Ontology' of ‘molecular functions’ classified the genes in to various functions such as catalytic and binding functions, transcription, enzyme regulators, structural and transport (Figure 4.2.3). In the over representative genes, catalytic and nucleic acid or protein binding function constituted the highest percent (47 %) that contained genes like Stat1, Runx3, ribosomal proteins, Zcchc6, Uqcrc2, MetAP2, Eef1a1 and many more translational proteins. Genes related to antioxidant activity (1.4 %) identified few genes like Cat, Gst. The structural molecular activity constituted 8.3 % related to cytoskeletal and extracellular spaces indicative of tissue expansion. The genes repressed identified in obese (over expressed in lean) show gene related to catalytic and binding proteins close to 74 %. Several DNA binding proteins and transcription factors encoded by various genes like Sp1, Ap2b1, Gtf3a, Snapc5, Chac1 are up-regulated, however, genes like Rbbp-2, Dlg5, Lrrc33, Hipk, Ccr4. Chrac1, Dcn, Rac2, Per3, Naca, Jak2, Rims3, Cp, Pds5a, Atf5b, Tf, Cfb were down regulated.

The classification based on Go ‘Ontology’ of ‘biological processes’ divided the gene library into several functions that broadly fell into following categories such as a) metabolism (carbohydrate, lipid and amino acid), b) developmental, c) reproductive, d) biogenesis, e) apoptotic, f) signaling, g) structural, h) membrane bound, i) transporters, j) Nucleic acid binding proteins, k) immunity, and l) cellular process (Figure 4.2.4). The percentage of genes related to metabolism was close to 45.8 % like Lpl, Scd1, Glud1, Glul Acsl1, Eef1a1, Pdx, Bcat1, and Slc45a3. The percentage of gene related to cellular processes was close to 33.3 % that included genes like Vps35, Thoc1, Kcnj6, Snx14, sell, leprotl1, Igf-1, Syt4, Vim, Dlg5, Alox5ap, Snx14. About 13 % of over expressed genes were related to the developmental gene, 3 % of genes were related to reproduction functions. The gene repressed related to metabolism and cellular processes were close to 54 % and 33 % respectively.

This study also showed some of the inflammatory mediators like Ifitm2, C1qtnf1, Csn1s1, Steap4, Vim, Ppfibp1, Cpn, to be upregulated in obese rat whereas gene like Mgfe8, il22r2, Stat1 was found to be repressed. Studies on Csn1s1 have shown it to be pro-inflammatory and its immunomodulatory effects are mediated through its binding to the surface of monocytic cells and activate p38-MAPK pathway. Ppfibp1 was known to play an important role in immune response as well as in lipid absorption. Extracellular matrix serves as adipose tissue cytoskeleton which mediates cell communication related to cellular proliferation, differentiation, migration and secretion. The upregulated genes in obese related to extracellular matrix proteins include Vcam1, Krtcap2, Itga7, Krt72, kr8, Dlg5, Mtmr3, and Fat1. Vcam1 is important in inflammation, immune responses and is secreted by the endothelial cells. It is associated in the development of atherosclerosis and rheumatic arthritis. The pathway analysis showed, the genes required for lipid metabolism like Scd1, Mgl II , Lpl, ALOX5-AP, Oldlr, and Pltp were over expressed in the obese adipose tissue.

In silico pathway analysis shows that most of these genes act downstream to the PPARγ signaling pathway (Figure 4.2.5). Other signaling pathways identified for genes which were either over expressed or suppressed belonged to Integrin signaling pathways which showed Itga7 over-expressed while Rac2 in the same pathway was repressed. Psmb10 was over expressed in the adipose tissue that belonged to pathway related to Parkinson disease. Transferrin (Tf), ceruloplasmin (Cp) gene related to iron metabolism was down regulated

Epidemiological studies strongly link obesity to cancers. It is shown that obese individuals are at high risk of endometrial, ovarian, liver, oesophageal and colon cancers. The tumour formation in older aged WNIN / Ob obese rats were reported (Giridharan et al., 1996). Some of the genes like Arhgef12, Ei24, Slc45a3, eEf1α1, Metap2, Hipk1 primarily involved in transcriptional and translational regulation of cell cycle were up

regulated in the obese adipose tissue. In addition, some genes over expressed in the obese adipose tissue with apparently no known functions and were localized on the chromosomes 13^{th}, 15^{th} and X chromosome.

The bioinformatic analysis overall show disturbed regulation of gene expression related to metabolism (53.5 %), cellular process (35 %), developmental process (14.5 %). There was reduced antioxidant (1.2 %) and reproductive functions (2 %). Some of the overexpressed genes like Scd1, MglII, Lpl, Alox5-ap were validated using Q-PCR analysis. The Q-PCR analysis confirmed the bioinformatic analysis of genes identified to be overexpressed in the subcutaneous adipose tissue. Scd1 showed a maximum of 24 fold of expression in comparison to lean rat followed by Alox5-ap which was 17 fold higher in the obese rat. The MglII and Lpl genes showed 6 fold expression. The Q-PCR analysis of other genes like Acsl1, Capn1, Leprotl-1, hypothetical protein were not significant in expression (Figure 4.2.6).

5.4 Analysis of the systemic and tissue levels of pro-inflammatory cytokines

The subtracted library analyses identified few genes related to inflammation and immunity that was differentially expressed. The identified genes were not reflective of the inflammation milieu within the adipose tissue of the obese rat. The pro-inflammatory cytokines are implicated in the development of insulin resistance and other clinical complications. Therefore, systemic and local (adipose tissue) inflammation analysis of known pro-inflammatory markers IL-1β, IL-6, TNF-α, RANTES, MCP-1 was done across the ages using male obese and lean rats of 1, 3, 6, 12 months old to look at the inflammatory levels in the obese phenotype. The comparative analysis of the expression of pro-inflammatory levels showed an irregular but a gradual pattern of increase in the obese rats with age. The systemic and tissue inflammatory cytokine concentration gradually increased from the age of 6 months in obese rat. The comparison between lean

and obese was not found to be statistically significant.

IL-1β cytokine is involved in impairing adipogenesis, lipogenesis, insulin resistance in the subcutaneous adipose tissue (Speaker and Fleshner, 2012). The serum levels in the obese rat on an average was 673 ± 123 pg /ml in comparison to lean serum levels (634 ± 157 pg /ml). The serum levels showed a gradual decrease in obese rat groups while the expression levels of IL-1 β increase in lean serum (Figure 4.3.1A). The tissue concentration was estimated to be 5.45 ± 1.0 pg / mg in lean adipose tissue while the obese adipose tissue level on an average was 6.25 ± 1.6 pg / mg. The tissue inflammation showed gradual increase in lean and obese with age (Figure 4.3.1B).

The TNF-α cytokine levels are proportional to adipose tissue. It affects the LPL activity; modulate glucose homeostasis, leptin expression (Hotamisligil et al., 1999). The average concentration of serum levels of TNF-α in the obese rat 1209 ± 18 pg / ml compared to lean rats with an average concentration of 741 ± 167 pg / ml (Figure 4.3.2A). The tissue expression levels in the obese adipose tissue of TNF-α showed an average of 361 ± 64 pg / mg compared to the lean adipose tissue which showed 293 ± 129 pg / mg (Figure 4.3.2B). The concentration of TNF-α levels in the serum showed sharp rise in the obese rats after 6 months. However, within the tissue the concentration of TNF-α in obese remained almost comparable to control.

IL-6 is a multi-functional cytokine that correlates positively with obesity. It is implicated in the down regulation of LPL, stimulation of acute phase proteins, and activate hypothalamus-pituitary axis (Fried et al., 1997). The serum levels of IL-6 in the adipose tissue across the different age groups distinctly increased in obese compare to lean rats. The average concentration of IL-6 in obese serum was 3989 ± 155 pg / ml as against the lean serum showing concentration of 2866 ± 98.2 pg / ml (Figure 4.3.3A). The tissue expression levels in the obese rat were 260 ± 98.2 pg / mg compared to lean adipose tissue which had 193 ± 62 pg / mg (Figure 4.3.3B). The concentration of IL-6

within adipose tissue remained almost constant in lean and obese with a sharp rise in 12 months obese rat.

The regulated on activation, normal T cell expressed and secreted (RANTES) levels are proportional to adipocyte size, and levels are increased in impaired glucose tolerance. They are shown to mediate chronic immune activation (Madani et al., 2009). The serum level of lean was 1439.16 ± 248.87 pg / ml and in the obese rat showed an average concentration of 1735 ± 176 pg / ml. The serum levels of RANTES in the lean and obese rat were almost comparable across the four age groups (Figure 4.3.4A). The tissue concentration within the adipose tissue of obese rat showed 1.02 ± 0.6 pg / mg compared to lean adipose tissue where the concentration was 0.9 ± 0.4 pg / mg (Figure 4.3.4B). The concentration of RANTES within the adipose tissue increased with age and was comparable to lean adipose.

The MCP-1 cytokine play an important role in macrophage infiltration and are produced in the SV fraction, pre adipocytes and are elevated in obesity. It induces insulin resistance by impairing insulin stimulated glucose uptake and also adipogenesis (Gerhardt et al., 2001, Sartipy and Loskutoff, 2003). The serum MCP-1 level showed a very high expression similar to RANTES. The concentration of MCP-1 in the obese serum was 143349 ± 2570 pg / ml while the lean serum expressed a concentration of 12174 ± 500.3 pg / ml. The expression levels of the cytokine were higher in 6 months age group (Figure 4.3.5A). The expression level of MCP-1 in the lean adipose tissue on average was 18 ± 2.4 pg / mg while the obese adipose tissue showed an average concentration of 21 ± 10 pg / mg. The MCP-1 levels in the adipose tissue of the obese rat increased steeply form the 6 months age group (Figure 4.3.5B)

The cytokine concentrations estimated were used for comparative analysis with control (lean rat). The WNIN / Ob rat although phenotypically obese, is euglycemic.

They gradually develop glucose tolerance with age. The pro-inflammatory cytokine (systemic and tissue concentrations) analysis of obese rat were comparable to lean across the age groups. Moreover, the cDNA library was generated for subcutaneous adipose tissue of 3 months old obese rat, identified very few genes related to inflammatory pathway. It can be interpreted that the obese rats below 6 months are physiologically healthy with subcutaneous adipose tissue showing healthy expansion and are able to resist the deleterious effects of inflammation which deteriorates with age.

5.5 Intra-cerebroventricular infusion of human resistin

The list of novel and identified adipokines and hormones secreted from the adipose tissue are increasing. These secretory factors either facilitate or are directly involved in the obesity induced inflammation leading to insulin resistance and diabetes. Resistin is an adipose secreted hormone inducing insulin resistance in mice discovered a decade ago (Steppan and Lazar, 2002). Subsequently, the human resistin 12 kDa molecule was identified as an ortholog secreted mainly from monocytes and macrophages and mediating inflammation (Patel et al., 2003). Our laboratory works on biophysical and functions properties of resistin and identified several aspects of resistin (Ehtesham, N., Z. 2001). We have identified that mouse and human resistin differs in their genomic organization (Ghosh et al., 2003). Human resistin was reported to be secreted as a trimer forming intermolecular disulfide linkages. (Aruna et al., 2003, Raghu et al., 2004). Human resistin was also identified as a pro-inflammatory molecule, stimulating the secretion of IL-12 and TNF-α (Silswal et al., 2005). The role of human resistin identified as a surrogate biomarker in end-point determinant in the treatment of tuberculosis was reported (Ehtesham et al 2011). Recently, human resistin with chaperone like activity was also reported (Suragani et al., 2013). The obese rats are

hyperphagic inspite of the elevated levels of insulin and leptin peripherally. Similar to hyperinsulinemia and hyperleptinemia, resistin levels are also elevated in obesity. The level of resistin positively correlated with body weight in animal studies and humans (Nogueiras et al., 2010). The peripheral role of resistin is well known. The central mechanisms of resistin to act as a short term satiety molecule (Tovar et al., 2005, Cifani et al., 2008), and as dual nature protein mediating anorectic effects centrally and inducing hepatic insulin resistance peripherally (Muse et al., 2007) were reported. In the present work, the effect of central infusion of human resistin was studied. The human resistin with a concentration of 2 µg / µl was injected in the lateral ventricle (ICV). The inability of the systemic levels of resistin to cross the blood brain barrier is negated by the continuous infusion from a cannula and an osmotic pump implanted by stereotaxic surgery, permitting to mimic conditions within the brain and study the visible effect of resistin on energy metabolism (food intake and body weight).

The purification of recombinant human resistin (rhRes) was done as per the standardized laboratory procedure (Aruna et al., 2003). The purified rhRes was (Figure 4.4.1) was concentrated to 2 µg / µl for infusion through osmotic pump with a pump rate of (1 µl / hour). The three months old, male obese and lean rats used in the experiment were classified into three groups. Each group consisted of three obese and lean. All the animals were acclimatized into individual cages (Figure 4.4.2A-B) followed by intracerebral ventricular cannulation (ICV) with ad libitum food and water for recovery (Figure 4.4.3A-F)

After the recovery period, the osmotic pump implanted in the subcutaneous pouch during surgery was replaced with 20 mM Tris (vehicle) or rhRes (protein) (Figure 4.4.4A-F). Group 1 obese and lean rats were control group that received pump replacement with 20 mM Tris for two weeks. The group 2 received 20 mM Tris in the

first week followed by rhRes (2 μg) in the second week. The reverse was followed for the Group 3 which received protein in the first week followed by 20 mM Tris in second week. The change in body weight and food intake per week was noted for lean and obese rats. The change in food intake in the group 1 which received 20 mM Tris was 1.1 gms for lean and 4 grams in obese rat corresponding to increase by 6 % and 16 % (Figure 4.4.5A). The group 2 animals which received 20 mM Tris followed by rhRes decreased food intake by 4 grams in lean and 11 grams in obese corresponding to 24 % and 28 % decrease (Figure 4.4.6A). Similarly, the difference in the food intake in the group 3 animals that was infused with rhRes followed by 20 mM Tris showed decrease in food intake by 10 grams in lean and 11 grams in obese reflecting an initial decrease of 50 % and 35 % in the first week after the protein infusion. The increase in the percentage due to weight gain is reflective of no effect of 20 mM Tris given in the second week (Figure 4.4.7A). The average body weight before surgery of lean animals was 286 grams and obese rats weighed 446 grams. In group 1, infusion of 20 mM Tris in lean and obese rat showed difference of 5 grams in lean and 26 grams gain in obese (Figure 4.4.5B). The decrease in weight in group 2 after rhRes treatment in lean was 16 grams and 26 grams corresponding to a percent decrease of 4 % in obese and lean (Figure 4.4.6B). In the group 3, rhRes given in the first week followed by vehicle (20 mM Tris) showed decrease in body weight by 26 grams in lean and 44 grams in obese (Figure 4.4.7B). The difference in change in food intake and body weight when infused with Tris and protein were distinct and significant in group 2 and 3 lean and obese rat. The rise in difference of body weight in group 3 was due to the subsided effect of resistin and normal gain in body weight with time.

Resistin infusion centrally has shown decrease in food intake and body weight in both lean and obese. The decreases in bodyweight of obese were not significant and

barely significant for lean rats. It can be concluded that the resistin effects are functional in both lean and obese rat. However its effect on body weight was not significant in comparison with the known long term adipose signals like leptin, insulin. Resistin might act as a short term satiety molecule to inhibit meal size and intake with no drastic change in body weight.

Summary

Obesity is a global health concern that is rapidly escalating in the developed nations and is an additional health concern in the countries with emerging economies. Obesity, in simple terms is an excess accumulation of body weight. It can aptly be defined as a heterogeneous complex disorder of multiple etiologies characterized by excess body fat that threatens or affects socio-economic, mental or physical health (Sharma P, 2007). It is a risk factor associated with several other metabolic disorders like diabetes type 2, hypertension, cardiovascular diseases, atherosclerosis, cancer (Mokdad et al., 2003). The genetic and the non-genetic factors that include several environmental, social and psychological, regional, cultural factors have been identified. The genetic factors are non-modifiable and the percentage of genetic inheritance of fat mass varies from 40-70 % (Herrera et al., 2010). The drastic changes in lifestyle that are primarily inclined to sedentary lifestyle and intake of energy dense food is one of the environmental factor that plays a detrimental role in accumulation of excess fat in adipose tissue thereby altering its normal physiological functions. Over the past decade, animal models, genetic and non-genetic, have significantly advanced our understanding of the causative factors leading to the development of obesity. Single gene mutation resulting in obesity has been identified and well characterized in animal models (Liebel 1997, Tschöp et al., 2001). However, in humans, monogenetic forms of obesity are rare. Obesity is mostly polygenic in nature and is determined by the complex interactions of gene-gene and gene-environmental interactions (Mutch et al., 2006, Hinney et al., 2010).

Several studies in animal models have highlighted central regulation of food intake, energy metabolism and the co-ordination of brain with peripheral organs such as adipose tissue, muscle, liver in maintaining energy homeostasis (Schwartz et al., 2000). Several peripheral signals (short-term and long-term satiety factors) that are primarily released by important organs like GI tract, pancreas, adipose, liver to the brain via the vagus nerves regarding the energy stores for short and longer duration (Woods and

D'Alessio, 2008). Accordingly, the neuronal signals (orexigenic and anorexigenic, neurpopetides, neurotransmitters) respond to these peripheral signals by inducing feeling of 'hunger' or satiety and appropriately signal gluconeogenesis, glycogenolysis in liver or glucose uptake or lipolysis, or triglyceride accumulation in adipose tissue to maintain the energy balance (Arora and Anubhuti, 2006, Hellstorm et al., 2004). In obesity, such well networked mechanisms are affected as a result of either genetic mutation or altered expression of susceptible genes in response to obesogenic environment. The identification and characterization of leptin in 1994 firmly established the brain adipose 'cross talk' and redefined adipose tissue as an endocrine organ (Siiteri, P., K. 1987, Zhang et al. 1994). Over the years, the adipose tissue has become a focus of intense research to understand the aetiology and further explore the molecular functions of susceptible genes to find therapeutic solutions to combat obesity.

The adipose tissue is a loose connective tissue, heterogeneous in composition and distribution with different adipose depots throughout the body. As an endocrine organ, it secretes various adipokines and hormones related to diverse physiological functions such as, metabolism, vasculature and angiogenesis, insulin sensitivity, and inflammation in these depots (Kershaw and Flier, 2004). The different regional depots function as integrative unit to communicate signal from different organs system like muscle, liver as well as brain to balance energy homeostasis (Proneca et al., 2014**).** The brown and the white adipose tissue (BAT and WAT) are the two major types of adipose tissue. However, WAT is the major storage organ. The WAT depending on location is further divided into: 1. Visceral adipose tissue (VAT) 2. Subcutaneous adipose tissue (SAT). The subcutaneous fat is found beneath the skin, while the visceral fat includes the intra-abdominal fat such as omental, mesenteric and adipose tissue surrounding major organs such as liver, kidney. The VAT and SAT also differ in their physiological functions, cellular and molecular compositions and secretions and in their capacities to associate

with the risk factors of metabolic diseases **(**Palou et al., 2009, Wronska and Kmiec, 2012, Lee et al., 2013). With obesity, the adipose tissue is increasingly dysregulated, manifested by the infiltration of macrophages and immune cells and secretion of adipokines leading to a chronic 'low-grade chronic inflammation state' associated with obesity. Obesity induced Inflammation is linked to insulin resistance and diabetes type 2 (Pasarica et al., 2009). Considerable understanding has also emerged on the sequential developments triggered in response to AT expansion such as hypoxia, inflammation, ER and mitochondrial stress which eventually leads to insulin resistance. Hyperlipidemia and hyperglycemia caused by excess nutrients, lipolysis, and gluconeogenesis induce mitochondrial dysfunction, ER stress and oxidative stress to stimulate stress responsive signaling molecules such as JNK and IKKβ which is an important factor in the pathogenesis of T2D that is well established in humans and rodent animal models (Hotamisligil, 2006, Ouchi et al., 2011). These developments within adipose tissue reflect in their secretions, functions and gene expressions and interactions that differ greatly in different regional depots of adipose tissue. These variations probably are regulated by differences in cellular population, vascular innervations and anatomical location and a possible involvement of genetic factors and developmental genes **(**Trzeciak-Ryczek et al., 2011, Lee et al., 2013). The visceral fat is more metabolically active associated with the risk of metabolic diseases and relatively expresses more levels of TNF-a, IL-6, IL-1B, MCP-1 cytokines and macrophage infiltration than the subcutaneous fat (Lee and fried, 2010). Several studies report the association of visceral fat with the risk of metabolic diseases in comparison with subcutaneous fat and is widely accepted **(**Després, J., P. 2006, Shah et al., 2014). The molecular mechanisms of adipose tissue through altered gene expression is extensively studied in visceral adipose tissue and correlated to metabolic syndrome (Dusserre et al., 2000, Lemieux S 2001). Although depot differences in adipocyte metabolism and functions reflect in the pathogenesis of

obesity and related diseases, the relative contribution of VAT compared to subcutaneous fat is still controversial. Interestingly, 'the spill over' hypothesis suggests that when the SAT reaches its limit for expansion the VAT regions are signalled to accumulate fat (Danforth E, 2000) This suggests an implicative role of subcutaneous fat which is not fully explored. On the other hand, there are reports on the subcutaneous adipose tissue associated with peripheral insulin resistance (Pavan kumar Patel and Nicola Abate, 2013, Lê et al., 2011). The subcutaneous adipose tissue stores more than 70 % of total body fat and this relative abundance with an altered gene expression profile might significantly contribute to the changes occurring within AT and signal other depots that triggers obesity related inflammation leading to insulin resistance and other clinical complication gradually. Yet the different mechanism is still unclear. Moreover, the various genetic and developmental factors and its environmental interactions that determine the differences in fat distribution and functins and the relative clinical risk are not determined. Animal models in this regard offer a great help to study the gene expression analysis to identify key molecules that are differentially expressed and possibly involved in the pathogenesis. The studies of the thesis work have been carried out in an in-house obese rat model (WNIN / Ob).

These obese rats are the first and only genetic models of obesity and diabetes developed in India at the National Institute of Nutrition,(NIN) Hyderabad, India. An in-house obese rat model (WNIN / Ob) spontaneously arose from one of the inbred parental wistar colony (WNIN) maintained since 1920. They are genetically more 'pure' compared to other rat models which are outbred or randomly bred. The obesity is seen in both sexes, 47 % fat, sedentary, hyperphagic. The mutation is yet to be characterized. However, the preliminary work suspects it to be located on chromosome 5 (Kalashikam et al., 2013). There is no mutation in leptin or its receptor as found in other genetic models like zucker or corpulent rats. It segregates in a typical Mendelian fashion 1:2:1

(Lean: Carrier: Obese). The carriers and obese animals show 'kinky tail' phenotype due to fusion of caudal vertebre at the proximal and distal end of the tail at 50-60 days. The WNIN / Ob obese rat shows obese phenotype from the 35th day onwards and almost weighs twice in comparison to their lean littermates. The animals are hypoactive and similar to zucker rat model, the WNIN / Ob rats are euglycemic and show elevated levels of triglycerides, cholesterol, insulin, leptin. The female fertility is impaired while it is restored in males on pair feeding. The obese rat displays typical characteristics of human 'Metabolic Syndrome'. The differential expression studies in comparison with normal phenotype in various adipose tissues are one way to identify susceptible genes.

Hypothesis:

"**In an impaired state owing to energy excess, subcutaneous adipose tissue gradually alters in to a metabolically active site similar to the visceral fat."**

The study hypothesizes that under normal conditions, the subcutaneous fat has a protective and defensive mechanisms to counter the effects of accumulating fat within the adipose tissue. The deleterious effects of excess fat, gradually alters the subcutaneous fat into a 'metabolically active depot' like the visceral fat. The study aimed to explore how these differentially expressed genes in the subcutaneous adipose tissue especially the pro-inflammatory cytokines in the subcutaneous adipose tissue might play a role in the adipose tissue expansion or communication to the visceral fat in the accumulation of excess triglycerides in subcutaneous fat.

Objectives:

The thesis work attempted to look for differential gene expression profile of the subcutaneous adipose tissue of the obese rat by creating a gene library and primarily looked for pro-inflammatory molecules to dissect out its physiological function in

contributing to the obesity associated metabolic disorders in the obese rat model. The objectives of the study were:

1. To generate a gene expression profile of the subcutaneous adipose obese and lean rat for a comparative analysis
2. A comparative analysis on the expression level of known pro-inflammatory cytokines
3. The physiological role of human resistin on food intake and body weight (energy metabolism).

Results :

1. To generate a gene expression profile of the subcutaneous adipose obese and lean rat for a comparative analysis.

Subtractive hybridization

The PCR-Select cDNA Subtraction Kit (Clontech laboratories Inc.) was used to generate differential gene expression profile of the subcutaneous adipose tissue from three month old WNIN / Ob obese and lean rats. The forward (Obese vs lean) and reverse subtraction (lean vs obese) selectively amplified the genes that are over expressed in obese rat (suppressed in lean rat) and lean rat (suppressed in the obese rat).

Cloning and sequencing

The cDNAs obtained were cloned in the pGEM-T vector and transformed into DH5α competent cells. The blue - white colony screening identified nearly 800 colonies that were differentially expressed in the subcutaneous adipose tissue of the obese rat. The plasmids were isolated from all the positive colonies. The colony PCR and plasmid restriction digestion was done to confirm the presence of inserts which ranged between 200 bp to 1 kb pairs. All the positive colonies were subsequently sequenced.

Bioinformatic Analysis

The corresponding mRNAs were identified using NCBI blast (public database). The genes overexpressed in the obese and lean rat respectively were identified and further classified as per their

1. Chromosomal location
2. Cellular locations
3. Functions
4. Pathways.

Several genes were overexpressed whose roles have been already implicated in the development of obesity, in addition some unknown genes were differentially expressed. These genes belonged to a wide variety of metabolic pathways as well as were diverse in their cellular locations indicating a complex etiology of development of obesity in these animals.

Functionally, the genes broadly fell into the following categories:

1. Lipid and carbohydrate metabolism
2. Electrolyte Transporters
3. Components of signaling cascades
4. RNA-Binding proteins
5. Stress-associated proteins
6. Structural proteins
7. Inflammatory molecules
8. Transcription factors
9. Uncharacterized proteins

The subtractive technique has limitations in not identifying many genes as microarrays which are widely used that can show differential analysis of thousands of genes, but still has an advantage to find novel genes. The cDNA library data matched

with that of human subcutaneous adipose of obese rat models and humans showed most of the genes similarly over expressed but probably varied in expression. The genes related to lipid metabolism like Scd1, MglII, Lpl, Alox5-ap, Oldlr, and Pltp were over expressed in the obese adipose tissue. The KEGG pathway analysis shows that most of these genes act downstream to the PPARγ signalling pathway. Similarly, transferring pathway related to iron metabolism was repressed in obese rat. The cDNA library of the subcutaneous adipose tissue of the obese rat did not show any genes in the chromosome 5 or genes related leptin pathways. Several DNA binding proteins and transcription factors encoded by various genes like Sp1, Ap2b1, Gtf3a, Snapc5, Chac1 are up-regulated genes like Rbbp-2, Dlg5, Lrrc33, Hipk, Ccr4 are over expressed in lean rat (down regulated in obese rat). Currently, for most of these transcription factors we do not have any data regarding their roles in the pathophysiology of obesity. The cytosolic proteins in adipocytes are major components of Golgi-ER transport system that mediate various intra cellular activities like, signaling, protein, lipid transport, and recycle important for cell growth. Several cytosolic that were found to be differentially regulated in the obese and the lean adipose tissue in this study, were components of Golgi-ER transport system like Sorbs1, Rims3, Syt4, Vps35, p28, Snx3 were over expressed whereas other genes like Snx14, Glg, Rab18, Rab40, were repressed. The up regulated genes were found to encode the components that are involved in protein trafficking and the effect of altered expression is not investigated at the cellular level. The genes related to ER stress like Naca, Pdrg1, Ces1d, and Ube2g1 were found to be upregulated in the obese adipose tissue whereas expression levels for genes like Selk, Edem1 were more in the lean animals. This study also identified inflammatory mediators like Ifitm2, C1qtnf1, Csn1s1, Steap4, Vim, Ppfibp1, Capn1, were up regulated in obese rat whereas Mgfe8, Il22r2, Stat1 was found to be repressed. Extracellular matrix up regulated genes includes Vcam1, Krtcap2, Itga7, Krt72, kr8, Dlg5, Mtmr3, and Fat1. Epidemiological studies strongly link

obesity to cancers. It is shown that obese individuals are at high risk of some form of cancers like endometrial, ovarian, liver, esophageal and colon. In fact the WNIN / Ob obese rat model used in the study was found develop tumors especially at the older ages (Giridharan et al., 1996). Several genes like Arhgef12, Ei24, Slc45a3, eEf1a1, Metap2, Hipk1, primarily involved in cell cycle transcription and translation were upregulated in the obese adipose tissue. In addition, some genes overexpressed in the obese adipose tissue with apparently no known functions and were localized on the X, 13^{th} and 15^{th} chromosome. The Scd1 was the most identified gene in sequence which was over-expressed in the obese rat while transferrin was the gene most identified to be repressed in obese rat adipose tissue.

The library showed genes over-expressed in the obese adipose tissue belonging to the cytosolic locations followed by cell membrane and in many organelles such as ER, microsome, Golgi complex. Functionally, the genes over expressed in the obese rat, the catalytic and protein or nucleic acid binding functions were highest and the most effected in the obese rat. The bioinformatic analysis overall show disturbed regulation of gene expression related to metabolism (53.5 %), cellular process (35 %), developmental process (14.5 %). There was also reduced antioxidant (1.2 %) and reproductive functions (2 %).

Q-PCR Analysis

The bioinformatic analysis of the cDNA library that identified some of the over expressed genes related to metabolism, signalling components, inflammation, hypothetical proteins like Stearoyl Co-A desaturase (Scd1) Monoglyceride lipase(MglII) Lipoprotein lipase(Lpl), Arachidonic acid lipoxygenase -5 activating protein(Alox5-ap), hypothetical protein were validated using Q-PCR analysis. All the genes showed elevated expression in obese over lean rat that confirmed the cDNA library that is over expressed

in the obese rat. The Scd1 showed tremendous fold difference in obese rat. The hypothetical protein validated showed increased expression in obese but was not significant.

2. A comparative analysis on the expression level of known pro-inflammatory cytokines

The obesity induced changes within the AT increases levels of various pro-inflammatory cytokines. To check for the levels of systemic and tissue specific inflammation in WNIN / Ob rats, a comparative analysis of known pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, RANTES, MCP-1 across all ages (1, 3, 6, 12 months) was done using males obese and lean rat by ELISA (peprotch Inc.). The levels of protein expression increased sharply from 6 months of age in obese rats in systemic levels and adipose tissue. Overall, the expressions of the above mentioned pro-inflammatory cytokine levels increased with age in the obese rats but the expression levels were comparable to lean rats and not significant.

3. **The physiological role of the pro-inflammatory cytokine**

The library did not identify many pro-inflammatory cytokines and the systemic cytokine analysis showed no comparable trend. The thesis work completed with the study of centrally infused resistin. Our lab works on resistin protein and we have reported on several biophysical and functional aspects of human resistin (Aruna et al., 2003, 2008, Ghosh et al., 2003, Ehtesham et al., 2011, Suragani et al., 2013). The human and rodent resistin are functional orthologs. In rodents, it leads to insulin resistance while it is possible that the human resistin leads to similar effect through inflammation. The rodent and human resistin show 60 % and 50 % of homology at mRNA and protein level (Ghosh et al., 2003). We have also reported that human resistin as a pro-inflammatory

molecule that stimulates the synthesis and secretion of TNF-α and IL-12 from macrophages through NF-κB activated pathway (Silswal *et al* 2005).

After leptin, resistin is the second adipokine identified to be secreted from adipose tissue and also expressed in hypothalamus regions of the brain. Leptin and resistin in many ways are similar but functionally different. Like hyperinsulinemia and hyperleptinemia, resistin levels are also elevated in obesity. The continuous infusion permitted to mimic conditions of resitin levels within the brain to see its effect on food intake and body weight (energy metabolism). Leptin and insulin infusion centrally has shown to decrease food intake and body weight in many obese animal models (Perry and Wang, 2012). Several adipogenic hormones like leptin, adiponectin, IL-6 or insulin and satiation peptides like cholecystokinin (CCK), Protein peptide Y (PPY), glucagon like peptide 1 (GLP-1) have been injected peripherally and also centrally in different regions of the hypothalamus of the rats and mice that showed decrease in body weight and food intake (Mori et al., 1986, Wallenius et al., 2002, Park et al., 2011 Perry and Wang, 2012). Resistin levels are elevated peripherally in obesity but very few studies on the central mechanisms of resitin are reported but gathering attention (Tovar et al 2005, Muse et al., 2007). The human and rodent resistin are functional orthologs. In rodents, it leads to insulin resistance while it is possible that the human resistin leads to similar effect through inflammation. The rodent and human resistin show 60 % and 50 % of homology at mRNA and protein level. Therefore, it was interesting to see the effect of intra-cerebroventricular (ICV) infusion of resistin food intake and body weight. A pilot study on the effect of rat and human resistin was assessed using 2 µg / µl protein respectively purified by the standardized laboratory protocol. Interestingly, human resistin infused rats also showed decreased food intake and body weight. Further experiments were carried out using human resistin (hRes).

Sterotaxic surgery for cannula implantation

In this study, three pairs of obese and lean rats were divided into three groups. A mini osmotic pump was enclosed in the subscapular region in the dorsal side and the cannula in the intracerbebroventricle (ICV) was surgically implanted. All the animals were left to recovery with ad libitum rat chow and water for a week.

ICV infusion of human resistin

The ICV infusion was facilitated through implanting an osmotic pump in the subcutaneous pouch behind the back which pumps 1 μl/ hr. The first group received vehicle as Tris (20 mM), the second group received Tris (20 mM) and the third group received rhRes protein (2 μg / μl) in the first week. In the second week, the mini osmotic pump was replaced with Tris (20 mM) in the first group, while the second group and the third group received rhRes protein (2 μg / μl) and Tris (20 mM) respectively. The food intake and body weight was measured at a fixed time for both weeks to see the effect after protein treatment compared to vehicle. The effect of rhRes protein significantly decreased food intake but the decrease in body weight was barely significant in the lean rats. Interestingly, the obese also showed significant decrease in food intake but the decrease in body weight was not significant. The decrease in food intake and body weight was observed in both lean and obese which was very distinct from the group 1. This study shows that the resistin pathway is centrally functional in obese rat as in the lean rats. Acting as short-term satiety molecule to inhibit meal intake or meal size, it might not show overall effect on bodyweight in the obese rat which probably may be nullified in the presence of the genetic mutation that increases the body weight in the animal.

Salient findings:

1. The differential gene expression analysis of the subcutaneous lean and obese rat to identify and characterize the role of the pro-inflammatory cytokines in the subcutaneous adipose tissue was performed. The subtracted library showed more number of metabolic, transcription and cytosolic, developmental genes were up regulated and, the genes related to reproduction and antioxidant activity were down regulated over the pro-inflammatory genes.

2. The comparative analysis of known pro-inflammatory cytokines at systemic levels and within the subcutaneous adipose tissue was done to look at the levels of inflammation across the age group in lean and obese rats. The systemic and tissue concentrations of pro-inflammatory cytokines were comparable across the ages with the control (lean rat) and were not statistically significant. Although, the concentrations steeply increased after 6 months in male obese rats indicating basal levels of inflammation in the obese phenotype until 6 months of age.

3. The infusion studies of human resistin showed decreased food intake and body weight in obese and lean rat indicating resistin mechanism functional centrally in both lean and obese rat .

The obese (WNIN / Ob) rats are phenotypically obese and euglycemic. They gradually become glucose tolerant with age. The subtracted gene library was generated for 3 months old obese rat which did not identify many genes related to inflammation Moreover, The pro-inflammatory cytokines gradually increased with age but were comparable to lean rats. It can be inferred that the obese rats have a basal levels of inflammation until 6 months of age. It could also explain that the WNIN / Ob rats are ‘physiologically healthy’ obese rats with subcutaneous adipose tissue favouring a

physiologically healthy expansion and resisting the deleterious effects of obesity induced inflammation below 6 months of age after which the obesity induced inflammatory levels gradually increase. The physiological function of a pro-inflammatory molecule was studied using ICV infusion of human resistin (rhRes). Similar to previous reports on murine models, the ICV infusion using rhRes reduced food intake and body weight in lean and obese rats which indicated resistin being centrally active being centrally active also in the obese phenotype (WNIN / Ob) and may act as a short-term satiety molecule

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