

Genetics of Early Onset of Severe Obesity in a Consanguineous Population

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Abstract

Single gene mutations leading to obesity though rare have provided critical insights into the molecular and physiological mechanisms underlying control of energy homeostasis and body weight. No systematic studies have been carried out to assess the genetic spectrum of extremely obese cases (SDS>3) in a predominantly consanguineous population. In the present study we searched for obesity-associated mutations by a multi-layered combination of sequencing techniques in a cohort of 175 unrelated subjects with early onset severe obesity and their family members, from a Pakistani consanguineous population. All subjects were, initially, screened for mutations in of leptin (*LEP*) and melanocortin 4 receptor (*MC4R*) genes by Sanger sequencing. Subjects negative for mutations in these genes were screened for 27 obesity-associated genes using microdroplet PCR-enrichment followed by next generation sequencing (NGS). Genomic structural variations were assessed by genome-wide genotyping. Forty severely obese children and family members in whom mutations in known obesity-associated genes could not be identified using the aforementioned procedures were analysed by whole exome sequencing (WES). Hormone levels were analysed by immunoassays. Using Sanger and microdroplet-based sequencing, and genotyping, we identified 54 probands carrying 20 different homozygous loss-of-function mutations in 7 genes: *LEP* (n=35), leptin receptor (*LEPR*) (n=11), *MC4R* (n=4), Bardet–Biedl syndrome (*BBS*) (n=4) and Prader–Willi syndrome (*PWS*) (n=2). We also found a ~59 kb deletion in chromosome 1, encompassing *LEPR*. Lastly, WES revealed potentially pathogenic mutations in three susceptible genes, insulin induced gene 2 (*INSIG2*), Rho-associated protein kinase 1 (*ROCK1*), adenylyl cyclase 3 (*ADCY3*). Link of these genes to obesity is further supported by previous GWASs and/or animal studies. Notably, leptin values were significantly increased in *LEPR* deficient and cortisol levels raised in leptin deficient subjects as compared to those with *MC4R* mutations. Furthermore, gut hormones were shown to play a minimal role in inducing hyperphagia in subjects with congenital deficiency. A high prevalence (32%) of pathogenic mutation in this population, underscores the importance of comprehensive genetic screening of inbred populations to unravel new genes and signalling pathways modulating energy balance and providing leads to evidence-based patient management and wherever possible personalized treatment.

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Declaration of Originality

I hereby declare that this thesis for the submission of a Doctor of Philosophy is based on my own work. Proper recognition is given to the part of work performed in collaboration with other colleagues.

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Publications

The following is a list of publications arising from work carried out over the course of my PhD:

Original research articles

Saeed S, Bonnefond A, Manzoor J, Shabir F, Ayesha H, P, Philippe J, Durand E, Crouch H1, Sand O, Ali M, Butt T, Rathore AW, Falchi M, Arslan M, Froguel P (2015) Genetic variants in LEP, LEPR, and MC4R explain 30% of severe obesity in children from a consanguineous population. *Obesity* (Silver Spring, Md) 23: 1687-1695.

Saeed S, Bonnefond A, Manzoor J, Philippe J, Durand E, Arshad M, Sand O, Butt TA, Falchi M, Arslan M, Froguel P (2014) Novel LEPR mutations in obese Pakistani children identified by PCR-based enrichment combined with next generation sequencing. *Obesity* (Silver Spring) 22:1112-1117.

Bonnefond A, Philippe J, Durand E, Muller J, **Saeed S**, Arslan M, Martínez R, De Graeve F, Dhennin V, Rabearivelo I, Polak M, Cavé H, Castaño L, Vaxillaire M, Mandel JL, Sand O, Froguel P (2014) Highly sensitive diagnosis of 43 monogenic forms of diabetes or obesity through one-step PCR-based enrichment in combination with next-generation sequencing. *Diabetes Care* 37: 460-467.

Saeed S, Bech PR, Hafeez T, Alam R, Falchi M, Ghatei MA, Bloom SR, Arslan M, Froguel P (2014) Changes in levels of peripheral hormones controlling appetite are inconsistent with hyperphagia in leptin-deficient subjects. *Endocrine* 45: 401-408.

Saeed S, Butt TA, Anwer M, Arslan M, Froguel P (2012) High prevalence of leptin and melanocortin-4 receptor gene mutations in children with severe obesity from Pakistani consanguineous families. *Mol Genet Metab* 106: 121-126.

Book chapter

Al-Saud H, **Saeed S**, Dorajoo R, Froguel P (2014). Endocrinology: Obesity. *In*: Michael MF, Babyatsky MW, Giovanni MA (Eds) *Clinical Genomics*, McGraw Hill, New York. pp. 243-332.

Conference abstracts

Saeed S, Bonnefond A, Philippe J, Durand E, Manzoor J, Crouch H, Sand O, Arslan M and Froguel P. Sequential sequencing approach elucidates high proportion of monogenic obesity cases in a consanguineous population. The American Society of Human Genetics Conference 2015. October 6-10. Baltimore, Maryland, USA.

Saeed S, Bonnefond A, Philippe J, Durand E, Sand O, Manzoor J, Falchi M, Arslan M, Froguel P. Hotspot mutation regions in a consanguineous population - Leptin, Leptin receptor and Melanocortin 4 receptor genes. The European Society of Human Genetics Conference 2014 (# 2014-A-408-ESHG). 31 May -3 June 2014. Milan, Italy.

Saeed S, Bonnefond A, Butt TA, Philippe J, Durand E, Sand O, Manzoor J, Falchi M, Arslan M, Froguel P. Three percent of severely obese children from Pakistan carry mutations in LEPR: results of PCR-based enrichment combined with next generation sequencing. QBRI Symposium. Systems Biology of Diabetes: towards precision medicine. 26-27 January 2014. Doha, Qatar.

Saeed S, Falchi M, Ghatei M, Arslan M, and Froguel P. Ghrelin levels are low and unresponsive to food intake in homozygous but not in heterozygous carriers of LEP mutation. 2nd E.G.I.D. Symposium. Diabetes Research Breakthroughs: The Vintage. 10-11 Oct 2012. Lille, France.

Abbreviations

aCGH	Array comparative genomic hybridization
AHO	Albright hereditary osteodystrophy
ALS	Alstrom syndrome
ALS	Alstrom syndrome
ARC	Arcuate nucleus
BAF	B-allele Frequency
BBS	Bardet-biedl syndrome
BCE	Before the common era
BMI	Body mass index
CE	Capillary electrophoresis
CGH	Comparative genomic hybridization
CN	Copy number
CNS	Central nervous system
CNV	Copy number variation
Cs	Carpenter syndrome
CVO	Circumventricular organs
DAT	Description file
dbSNP	Single nucleotide polymorphism database
DNA	Deoxyribonucleic acid
FDA	Food and drug administration
FISH	Fluorescence in situ hybridization
FLI	Free leptin index
GSV	Genomic structural variant
GWAS	Genome wide association studies
GWAS	Genome-wide association study
HFD	High fat diet
HMM	Hidden markov model
HWE	Hardy-Weinberg equilibrium
IBD	Identity by descent
LD	Linkage disequilibrium
LH	Lateral hypothalamus
LOD	Logarithm of the odds
LRR	Log R ratio
MAD	Median absolute deviation
MAF	Minor allele frequency
MAP	Mapping file
ME	Mendelian error
MLPA	Multiplex ligation-dependent probe amplification
MPS	Massively parallel sequencing
mRNA	Messenger ribonucleic acid
NAHR	Non-allelic homologous recombination
NGS	Next generation sequencing
NTS	Nucleus tractus solitarius

PCR	Polymerase chain reaction
PED	Pedigree file
PHP	Pseudohypoparathyroidism
PPHP	Pseudopseudohypoparathyroidism
PVN	Paraventricular nucleus
PWS	Prader Willi syndrome
QC	Quality control
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROH	Runs of homozygosity
RT-PCR	Reverse transcription polymerase chain reaction
SNP	Single nucleotide polymorphism
T2D	Type 2 diabetes
VMH	Ventromedial hypothalamus
VMN	Ventromedian nucleus
VNTR	Variable number of tandem repeats
WES	Whole exome sequencing
WGS	Whole genome
WHO	World health organisation
WHR	Waist hip ratio

1. Chapter one

INTRODUCTION

Overweight and obesity are defined as abnormal or excessive fat accumulation that presents a risk to health [1]. The most commonly agreed measure of weight status in adults is the body mass index (BMI), which is based on the ratio of height and weight ($\text{BMI} = \text{kg}/\text{m}^2$). A BMI of 30 or higher is considered obese whereas a BMI of 35 or higher represents morbid obesity. For children, however, a BMI percentile for age is calculated by using a standard growth chart drawn from normal population where BMI greater than or equal to 97th and 99th percentile for gender and age are considered obese and morbidly obese, respectively [2].

1.1 A historical perspective

“We do not live in our own time alone; we carry our history within us.” [3]

Mankind has faced scarcity of food especially in times of recurrent droughts and famines for centuries in its early history. Procuring of food was a daunting task that involved strenuous efforts. Therefore, natural selection favoured development of integrated mechanisms of storing its own reserves of energy in the form of fat to be utilised during times of scarcity. In this inclement environment the individuals who could accumulate sufficient stores of fat from the amount of food available had an obvious advantage in survival and procreation. In ancient times, a woman who was bulky or overweight was thought to exhibit characteristics of fertility and of well-being. Such beliefs are probably manifested in the creation of ancient figurines found around the world such as the Venus of Willendorf, the lime stone statuette discovered in Austria (Figure 1.1) dating back to 25,000 BCE (late Palaeolithic period).

With the first agricultural revolution that started 10,000 year ago, the primitive method of procurement of food by hunting and gathering gave way to farming, herding and domestication of animals. Though production remained largely dependent on environmental or climatic conditions, the supply of food became more plentiful. Abundant food was available mostly to an elite group, that is members of the ruling and merchant classes, allowing them to grow large and put on weight. To be ‘stout’ or fat was considered a sign of affluence and power. Still, the common man, who toiled hard and long in the field for a pittance to grow crops for human and animal consumption remained lean and hungry. One of the earliest references to the ill effects of obesity on quality of life can be attributed to the famous Greek physician Hippocrates (c. 460-377 BC) who pronounced that ‘sudden deaths were more common in those who were naturally fat than

in the lean'. Some 500 years later, the leading Roman physician of his times , Galen (c. 129-199), categorised obesity into two types: 'moderate' and 'immoderate', perhaps the latter type referring to morbid form of obesity as we know it today. Avicenna (or Ibne-Sinä; 980-1037) the well-known thinker and philosopher who wrote more than 100 works on medicine, for the first time listed obesity as a disease. He also pointed out the association of obesity with diabetes.

From the Second Agricultural Revolution (1740-1850) triggering and coinciding with the First Industrial Revolution (1760-1840) in Europe and followed by the Green Revolution of the twentieth century in many parts of the globe (1945- late 1970's), food became progressively more plentiful, cheap and readily available. Research, development and technology transfer changed conventional farming and dairy methods to industrialised food production and brought about a significant change in the quality of life and human activity. In the apt words of Eknayan [4], the history of obesity relates to *"..... how what was (once) good became ugly and bad"*. Indeed the history of obesity cannot be separated from the history of food availability and an overt shift in quality and style of life.

In the first decades of the twentieth century, insurance companies were the first to express concern about the ill effects of obesity on health and were soon joined by the medical profession [5]. Obesity was medically documented as a morbidity and a forerunner of life-threatening complications such as cardiovascular disease, diabetes, some common types of cancer and respiratory afflictions [5, 6]. However, it has not been easy convincing the public that obesity is a disease.



Figure 1.1 The Venus of Willendorf. Created ~ c. 25,000 BCE in Vienna, Austria.

1.1.1 Human obesity in evolutionary context

Energy homeostasis depends on a balance of expenditure of energy with energy intake and whereas the former is continuous, the latter is an episodic process. The short term energy requirements during food intake intervals are met with glucose and glycogen metabolism (mobilization and storage). However, to ensure supply of energy during relatively long periods of food shortfall or deprivation (e.g. starvation, hibernation) or in anticipation of excessive energy expenditure (e.g. reproduction, migration) animals including humans have evolved mechanisms of storing energy as fat in periods of plentiful food supply for long-term sustenance in face of energy crises.

To explain the relatively recent emergence and phenomenal rise in incidence of obesity (metabolic syndrome) presumably in response to the developing scenario of food abundance and decline in physical activity, the ‘thrifty gene’ hypothesis was advanced by James Neel [7] almost more than half a century ago when present day knowledge about human genetics was still in its infancy. According to this concept genes favouring fat deposition were positively selected early in human evolution, to counteract frequent periods of starvation and famine experienced by ancestors of modern man [8]. Such a notion would imply that inheritance of ‘thrifty genes’ and thus of physiological mechanisms perfected over centuries favouring storage of body fat that once

served a useful purpose, is mainly responsible in the present day energy-permissive environment for the onset of obesity epidemic

The thrifty gene hypothesis to explain the emergence and prevalence of obesity in the present day obesogenic environment has been criticised mainly due to its failure to account for why some individuals grow fat while others do not while living under the same environmental conditions. To overcome this difficulty, an alternate proposition to explain the present day obesity epidemic in an evolutionary context, has been advanced by Speakman termed as the 'drifty gene' hypothesis [9, 10]. It argues that animals have evolved a 'dual intervention system' preventing them from storage of too little or too high amounts of fat, presumably regulated by two distinct albeit interacting, physiological mechanisms. Storing low amounts of fat will be under a strong negative selection pressure as paucity of food supply over any extended period will compromise survival and lead to starvation and death. Storage of fat will, therefore, be favoured by selection but up to a certain point. The resulting large size and consequent lack of agility due to excessive fat storage increases the risk of predation. The proposed dual intervention system will thus regulate the amount of fat so as to prevent its very low as well as very high build up. According to the drifty gene hypothesis, during the past 2 billion years of human evolution the risk of predation has been drastically reduced (due to bipedal mode of locomotion and use of fire and weapons) and the selection pressure on control of mechanisms responsible for the upper intervention point preventing excessive fat accumulation, has slackened. Consequently, random mutations in genes causing a variable drift in the upper intervention point would explain why some individuals are more disposed to obesity than others when exposed to the same scenario of food abundance and its uninterrupted availability [9]. However, notwithstanding the flaws inherent in the 'thrifty gene' hypothesis, the concept did bring into focus the obesity associated genes a study of which since then has led to our current understanding of the genetic and physiological basis of energy homeostasis.

1.2 Obesity epidemic

The previous four decades have seen a rapid change in human diet to high caloric food and adoption of a sedentary life style driven by a technologically advanced obesogenic environment, thus contributing to a phenomenal rise in the incidence and prevalence of obesity over a relatively

short time interval [11, 12]. Once considered a problem of technologically advanced and affluent countries, obesity together with its associated complications has now emerged as a major contributor to the global health burden and assumed the status of an epidemic as declared by the World Health Organisation (WHO) and considered a worldwide public-health crisis. According to WHO statistics, in 2014 more than 1.9 billion (39%) adults were overweight (BMI >25) and of these 600 million (13%) were obese (BMI >30) showing a 100% increase in worldwide prevalence of obesity since 1980 [1, 13]. At present, 50 per cent of the UK population is overweight and this percentage is set to increase. Among continents, Americas has the highest incidence of overweight and obesity with 62% overweight and 35% obese [14]. There has also been a phenomenal increase in prevalence of child obesity in recent years. In 2012, about 44 million children (<5 years of age) were overweight or obese showing almost an increase of 31 million since 1990 [15]. The staggering cost needed to deal with obesity epidemic is particularly detrimental to economies of developing countries that are already under a heavy burden of malnutrition and infectious diseases [16].

Obesity leading to serious health consequences like diabetes, cardiovascular disorders and many types of cancer, has emerged in recent times as one of the main causes of death globally. Statistics show that at least 2.8 million people die each year as a result of being overweight or obese [14]. This has led to an unprecedented explosion of research in all aspects of the disease - from molecular to sociological. However, it still remains an uphill task to find ways and means to contain the obesity epidemic.

1.3 Factors influencing obesity

A large body of evidence shows that both environmental and hereditary factors are important in determining the extent of adiposity and contribute to obesity epidemic [17, 18]. These two factors interact in an intricate manner hence in most cases it is difficult to clearly separate the genetic from the environmental contribution towards the obese phenotype.

1.3.1 Environmental contribution to obesity

Availability and abundance of cheaply marketed energy-dense food and decline of physical activity have been regarded as the two major components of the permeating obesogenic

environment over the last 2-3 decades resulting in the present day obesity epidemic (or pandemic) [19, 20]. In addition, there is growing evidence that other factors mostly accentuated by the changing environment and living conditions, may also significantly contribute to prevalence of obesity.

Infection by a number of microorganisms has been demonstrated to induce obesity in animal models and humans. Of various types of adenoviruses that cause obesity, the human adenovirus-36 (Ad-36) has been studied relatively well for its obesogenic effect in experimental animals as well as in humans [21]. However, not all types of adenoviruses cause obesity. Another well documented example of development of obesity due to infection in mice is provided by the canine distemper virus (CDV) and its effect has been attributed to a down regulation of hypothalamic leptin receptors [22, 23]. The relationship of infection with increased fat mass is not unexpected since immune cells like macrophages and adipocytes share a number of morphological homologies and functional characteristics [24].

In recent years there has been a growing interest in investigating the role of natural gut microbiome in health and disease. Various investigations demonstrate a significant role of gut microbiota in energy homeostasis and pathogenicity of obesity. The first concrete evidence of this effect came from an experiment where germ free mice were reported to carry 40% less body fat as compared to conventionally raised mice [25]. Furthermore, marked differences in the gut microbiota of genetically obese mice (ob/ob) and lean mice have been demonstrated [26]. Additionally obesity can be induced in germ free mice by introducing gut microbiota of genetically obese mice but not by colonising with gut microbiota from lean animals [27].

Epigenetic mechanisms enable differential expression of genes by switching on and off a particular set of DNA nucleotides mainly by methylation and demethylation or through other processes. Emerging evidence shows that epigenetic mechanisms are amenable to environmental influences especially during prenatal and early postnatal life and the changes could be permanent and heritable. Thus exposure to environmental factors during development can induce permanent alterations in epigenetic gene regulation. The increasing magnitude of some of the environmental factors can, therefore, potentially alter the epigenetic mechanisms of energy homeostasis and a change in epigenetic regulation of obesity genes could lead to obesity [17, 28].

Many chemicals used in industry as pesticides such as bisphenol A (BPA), polybrominated diphenyl ether (PBDE), organophosphates, phthalate, DDT, solvents and many others, are remarkably stable and through their release in the environment progressively accumulate and stored in fat and liver tissues of man and animals, concentrate in food chains and act as endocrine disruptors. There is evidence that these endocrine disrupting chemicals (EDC's) interfere mainly with androgen and estrogen signalling and bring about changes in metabolic homeostasis. Embryos are highly sensitive to the chemicals that are passed on through placenta or eggs. Since hormonal signals during development alter the timing and setting of gene expression during development through epigenetic mechanisms, these chemical agents bring about changes in the hormonal balance affecting not only reproductive processes but also a derangement of homeostatic mechanisms later in life that may lead to obesity [29].

Significant increase in the use of medications during the recent past, such as of psychotropic drugs, antihistamines, antihypertensives, antidiabetics, steroids, contraceptives and others, has been associated with increased weight gain and adiposity. According to the Third NAHNES report the use of psychotropic drugs alone in the United States has increased from 2.5% in 1988 to 8.1% in 2002 in adults [30]. Although the individual effects on weight gain due to these medications are small yet the increased prevalence of disorders for which these are prescribed, has appreciably contributed to the present day obesity epidemic.

In addition, the risk factors for development of obesity also include age, gender and ethnicity, amongst others [31]. A differential susceptibility to obesogenic environment has been demonstrated in different ethnic populations [32, 33]. Pima Indians, Pacific Islanders, and African- and Hispanic-Americans are more prone to obesity than their Caucasian counterparts when living under similar permissive environmental conditions [34, 35]. More recently, evidence though incomplete, has accumulated pointing out that sleep deficit, socio-economic and educational level, may also prove to be significant contributors to the obesity pandemic [31]. Environmental factors have also been shown to influence epigenetic mechanisms especially during early development and thus a change in epigenetic regulation of obesity genes could result in obesity [17, 28].

1.3.2 Genetic contribution to obesity

Although environmental factors contributing to the obesogenic environment, have undoubtedly driven the obesity epidemic in recent times, yet the wide inter-individual variation in BMI observed under more or less the same environmental conditions can only be attributed to a genetic (or heritable) predisposition to the condition [36]. The pioneering work of Stunkard and colleagues on twin studies in 1980's provided a heritability estimate of ~0.80 for body weight [37, 38]. Convincing evidence of a strong genetic influence on human obesity also came from an adoption study in Denmark that showed a high correlation of BMI of adopted children with that of their biological parents but no correlation with their adoptive parents [39]. Together, these and similar findings estimate heritability of obesity to be about 50-90%, implying that 50-90% of the variation in body weight in individuals sharing the same environment, is genetically attributable [40, 41]. However, a study on twins carried out in Finland, provides evidence that physical activity can modify to some extent the genetic risk for developing obesity [42].

Mainly based on genetic background and to some extent phenotypic characterization, obesity is conventionally categorized into 3 types - common polygenic, monogenic and syndromic.

1.4 Insights into molecular genetics of obesity

1.4.1 Common polygenic obesity

The genetic contribution to common obesity is complex and attributable to multiple obesity susceptibility loci. The individual effect size of these contributing variants is rather small that can so far explain only 2.7% of the BMI variation [43]. Initial efforts to uncover genetic basis of common form of obesity relied on two approaches – the genome-wide linkage studies and the candidate gene association approach. Later, the advent of array platforms for genome-wide SNP genotyping, a high-throughput methodology, enabled genome-wide association studies (GWAS), leading towards a better understanding of the genetic basis of complex disease.

1.4.1.1 Genome-wide linkage studies

Earlier, genome wide linkage approach has been used to search for previously unknown loci associated with the obesity phenotype. This approach relies on the relatedness of the individuals under investigation on the basis of co-segregation for certain regions in the genome with phenotypes in question [44]. Contrary to the candidate gene approach that relies on prior knowledge related to a disease gene, this technique identifies novel, previously unsuspected genetic loci associated with the disease. Although this method has been useful in mapping disease associated genes presenting monogenic form of obesity, it has demonstrated only a partial success in linking novel genomic loci with adiposity quantitative trait loci (QTLs) by analysing large pedigrees from populations. More than 30 genome-wide scans have resulted in identification of 250 susceptible loci with positive linkage results [45]. Some of these loci have attained significance levels and were identified in more than one study, such as 2p21–p23 [46-48], 6q22.31–q23.2 [49-51] and 20q11–q13 [52, 53]. However, no consistent replication for most of these loci could be reported [44] and even an effort to carry out a meta-analysis on more than 30,000 subjects, failed to identify a conclusive obesity related locus [54].

1.4.1.2 Candidate gene association studies

Candidate gene association study is a hypothesis driven approach that relies on the existing knowledge related to biology and pathophysiology of a particular disease. The candidacy of a gene is inferred from either positional or functional relevancy of the gene with susceptibility to the disease. The information could be derived from either animal models or from subjects with extreme phenotype [55]. Also, the candidacy of the gene may be inferred from position of the gene that lies near or within the genomic region that has been associated with the disease through linkage/association or due to any gene disruption event such as a chromosomal translocation or deletion [44]. These candidate genes are then tested at population level for their association with obesity-related traits.

A large number of candidate gene studies in relation to obesity have been carried out in previous years. These studies have brought about a steady increase in the number of associated loci. As per the latest update of Human Obesity Gene Map, 127 candidate genes have been reported [45]. Of the most widely replicated genes were melanocortin 4 receptor (*MC4R*) [56-58], prohormone

convertase 1/3 (*PCSK1*) [59, 60], brain-derived neurotrophic factor (*BDNF*) [61, 62], and b-adrenergic receptor 3 (*ADRB3*) [63, 64] genes. However, majority of the genes associated with obesity traits using this approach, failed to show consistent replication in successive studies [65].

1.4.1.3 Genome-wide association studies (GWAS)

Following the limited success achieved through genome-wide linkage and candidate gene studies [55, 66], development and use of GWAS enabled a hypothesis free association analysis of common variants at the genomic level. GWAS searches across the whole genome for single nucleotide polymorphisms (SNPs) that are more frequently associated with complex common disease and these studies have made it possible to identify a number of previously unknown disease associated loci.

***FTO* –the first GWAS identified obesity susceptibility locus**

In 2007, three independent genome-wide association studies led to the identification of *FTO* (renaming it the fat mass and obesity-associated gene), as the first success story of a GWAS-identified obesity susceptibility gene [67-69]. Since this discovery, SNPs mainly in the first intron of *FTO* have been shown to be consistently and robustly associated with body mass index (BMI) and other obesity related traits such as fat mass, waist-to-hip ratio and leptin levels across diverse populations and ethnicities and in adults as well as children [67, 70-72].

The unambiguous association of *FTO* intronic SNPs with BMI triggered a profusion of studies attempting to explain the causal relationship of *FTO* with body composition and energy homeostasis. Historically, *Fto* was one of the six deleted genes in chromosome 8 of the ‘Fuse toe’ (Ft) mouse (the other five genes included in the deletion were *Ir3*, *Ir5*, *Ir6*, *Fts* and *Rpgrip1l*) [73]. The *Ft* mutation was associated with fusion of the first to fourth toes and hyperplasia of thymus. *Fto* was later identified by cloning in 1999 [74].

Mice with global loss of *Fto* are characterized by growth retardation with a significant reduction in adipose tissue and lean body mass that has been attributed to an increase in energy expenditure [75]. Furthermore, *Fto* loss of function mutations in mice result in reduced body weight [76, 77]. Conversely, mice expressing extra copies of *Fto* are reported to have a dose dependent increase in body mass accompanied by an increase in food intake [78]. That *FTO* is

implicated in energy balance is also indirectly evidenced by its high expression in the hypothalamus [67] and reduced mRNA levels in the negative energy state [79]. These and similar studies strongly suggest that a disruption in *Fto* expression could lead to an imbalance in the control of energy homeostasis and body composition.

First direct observation of FTO deficiency in the human population came from study of a consanguineous family in which affected subjects carried a homozygous p.Arg316Gln mutation in a highly conserved region of FTO [80]. Affected individuals suffered growth retardation and early postnatal mortality like the *Fto* null mice but in addition, presented a number of malformations including developmental defects in the CNS and cardiovascular system. The parents of affected children were not reported obese. In vitro assays provided evidence of pathogenicity of the mutation that rendered the homozygous carriers of p.Arg316Gln mutation completely FTO protein deficient.

FTO protein has been shown to be a 2-oxyglutarate dependent oxygenase [81] and can demethylate single-stranded nucleic acids such as 6-methyladenosine (6meA), 3-methyluracil (3meU) and 3-methylthymine (3meT) [82]. Consequently it has been suggested that FTO might regulate the expression of genes involved in metabolism and energy balance. In line with its catalytic role *FTO*'s SNPs have been linked to the expression of gut hormones such as ghrelin [83]. It has been suggested that an over expression of FTO by high risk (AA) allele may enhance food intake through increased ghrelin production in response to reduced m6A methylation of ghrelin mRNA.

Another role of FTO, on similar lines, has been proposed in modulation of the reward system by its influence on dopaminergic circuitry within the mid-brain [84]. It was shown that in the *Fto* null-mouse there is less increase in extracellular dopamine when cocaine known to block dopamine transporter was administered as compared to the wild type littermate. *Fto* inactivation impairs cocaine induced increase in locomotor activity as compared to the control and have blunt increase in extracellular dopamine mirroring the phenotype observed when dopamine receptor type 2 (D2R) and type 3 (D3R) are impaired. Hess and co-workers further suggest a link of FTO's role as m6A demethylase (described above) and neuronal synapticity and demonstrate that transcripts that are selectively methylated in the *Fto* deficient mice are excessively linked to synaptic transmission and cell-cell signalling [84].

Recently influence of FTO on body composition and leptin sensitivity has been studied using *Fto* deficient mice on a high fat diet (HFD) [85]. It was observed that *Fto*^{+/-} and *Fto*^{-/-} mice on HFD in contrast to previous findings [75] increased their body fat but unlike similarly treated wild type mice, remained sensitive to leptin. Experimental evidence demonstrated that whereas normally NFκB signalling is up-regulated in response to HFD, *Fto* deficiency induces a down regulation of this signalling pathway. When NFκB signalling pathway was reactivated by TNFα, the *Fto* deficient animals became less sensitive to leptin. Furthermore, it was suggested that TRIP4, a transcriptional coactivator functionally links FTO to NFκB signalling. These investigations demonstrate that FTO can influence NFκB signalling and thereby leptin sensitivity under specific nutritional states.

There is emerging evidence that the association between *FTO* SNPs and BMI might be due to a role of intronic SNPs not only in the regulation of *FTO* itself but also of its neighbouring genes. Most of the focus has been drawn in this context to two of the neighbouring genes of *FTO*, *RPGRIP1L* and *IRX3*. *In vitro* and *in silico* studies suggest that *Rpgrip1l* might provide a mechanistic link connecting the *FTO* risk allele with obesity by way of its involvement in impairing leptin signalling [86]. *Rpgrip1l* encodes a protein localized in the primary cilium that regulates the correct positioning of the long form of leptin receptor (LEPR-b) [86]. The first intron of *FTO* contains a binding site for CUX1 that acts as transcriptional regulator of *Rpgrip1l* as well as *Fto* and the presence of a risk allele in the binding site of CUX1 weakens its affinity and interferes with its normal regulatory role [87]. Such an assumption is also supported by the observation that *in vitro* suppression of CUX1 expression results in a reduced expression of *FTO* and *RPGRIP1L*. The proposed model therefore suggests that *FTO* risk allele lowers the efficiency of the CUX1 leading to a reduced expression of *RPGRIP1L* which in turn interferes/inhibits localization of the long form of LEPRb in the vicinity of the primary cilium. The foregoing cascade of events would, therefore, result in reduced leptin signalling [86].

Intronic *FTO* SNPs have also been functionally linked to another neighbouring gene, iroquois-class homeodomain protein (*IRX3*). *IRX3* was one of the six deleted genes in the 'Fuse toe' (*Ft*) mouse model described earlier. In 2010 computational analysis suggested that *FTO* may contain highly conserved noncoding elements that could act as a potential regulatory element for *IRX3* [88]. Thus mapping of expression of quantitative trait loci in human brain samples demonstrated that BMI

associated SNPs (e.g. rs9930506) were linked to *IRX3* expression. Furthermore, mouse reporter assay provides convincing evidence that *IRX3* receives regulatory inputs from *FTO* [89]. Recently a study has attempted to explain the mechanistic basis for the association between the *FTO* locus and obesity by pointing to a pathway for adipocyte thermogenesis regulation that involves an upstream regulator - ARID5B, the *FTO* SNP (rs1421085), and downstream target genes (*IRX3* and *IRX5*). It was proposed that the risk allele contributes towards obesity by reducing mitochondrial thermogenesis in pre-adipocyte cells by way of disrupting conserved ARID5B repressor motif. This results in an increased expression of *IRX3* and *IRX5* implicating a developmental shift preferring lipid-storing white adipocytes over energy-dissipating beige adipocytes [90].

The influence of *FTO* SNP's on neighbouring genes and the possible underlying mechanisms have been reviewed in detail by Fawcett and Boroso [91] and Tung et al [92].

Meta-analyses of GWAS datasets

Since the discovery of *FTO*, large-scale genome-wide association studies have been carried out involving thousands of obese individuals in an attempt to uncover new loci associated with common and complex forms of obesity. The effort has made it possible to carry out meta-analyses of data derived from multiple populations and has led to the establishment of large international consortia such as the Genetic Investigation of Anthropometric Traits (GIANT).

The first meta-analysis of ~17,000 Caucasians combining 7 GWASs for BMI, confirmed a strong association of SNPs located 188 kb downstream from the *MC4R* gene with obesity related traits [93]. At the same time, in GWAS analysis of subjects of Indian origin the same locus was identified to be associated with waist circumference [94]. Since then several studies have successfully replicated a near-*MC4R* locus to be significantly associated with obesity related traits across diverse populations [95, 96] and in subjects of different age groups [97, 98].

In 2009 meta-analysis on more than 32,000 Caucasian samples from GIANT consortium [99] and almost a similar sample size (34,416) from the Diabetes Epidemiology: Collaborative Analysis Of Diagnostic criteria in Europe (DECOD) [100], while confirming association of with *MC4R* and *FTO*, found 6 and 7 new loci, respectively, 4 of which were common (*NEGR1*, *TMEM18*, *KCTD15* and *SH2B1*) from both sources. A study focused on subjects with early-onset extreme obesity reported another four putative loci including *NPC1*, *MAF*, *PTER* and *PRL*, associated with obesity [101].

In 2010, a GWAS was carried out by further expanding the sample number to 250,000 of individuals from 46 different populations of European origin. In this study Speliotes et al published 18 new loci and confirmed another 12 previously established BMI-associated loci [102]. By the end of 2010, 32 loci were associated with BMI through GWAS efforts. In 2012, two simultaneous investigations exclusively on Asian population that was the largest GWAS of BMI in individuals of non-European ancestry, jointly reported four new loci [103, 104].

In addition to GWASs for BMI, other obesity measures such as waist circumference and waist/hip ratio (WHR), fat percentage or fat mass have also been considered. These studies have resulted in establishment of several loci. For example in a study comprising ~77 000 individuals using WHR adjusted for BMI as the obesity measure, led to the identification of 13 loci [105]. By 2014, 77 loci were found to be associated with obesity related traits [66]. These studies have clearly indicated that differential fat distribution patterns could be attributed to distinct genetic components [106, 107].

Since for most of these identified loci we know very little about their pathophysiological role in relation to obese phenotype, therefore, these loci currently add very little to our understanding of their functional relationship to body composition and energy homeostasis. In an attempt to narrow down the gap between association and causation, a recent GWAS investigation aimed at not only to further expand the list of obesity associated loci but also to gain an insight into the physiological processes and molecular pathways that contribute to obesity. Recently a GWAS meta-analysis relating to BMI, by incorporating results from 300,000 subjects, was carried out to add to our understanding of the pathophysiology of common obesity [108]. This particular study, by using multiple complementary methods and expanding the number of BMI associated loci to 97, highlights the biology of potential causative genes at the associated loci. The aforementioned study provided evidence of contribution of the genes that were involved in synaptic plasticity, glutamate receptor activity and neuronal development, and thus could possibly modulate BMI. Furthermore, this study corroborates and compliments the well-founded evidence derived from monogenic form of obesity of the role of CNS and more specifically of genes expressed in hypothalamic part, in energy homeostasis and body weight regulation.

Another concurrent investigation aimed to enhance understanding of the genetic basis of body fat distribution, identified 49 loci associated with WHR adjusted for BMI, 33 of which were new

[109]. Majority of the variants were shown to be present in genes that are expressed in adipose tissue or in regions involved in regulatory control of adipocytes. Further pathway analyses provided insight into involvement of different processes affecting fat distribution such as adipogenesis, angiogenesis, transcriptional regulation and insulin resistance. To sum up, the aforementioned two studies have substantially increased our understanding of the molecular basis of obesity related traits and determined a direction for future research to uncover the complex biology of common obesity in terms of physiological and molecular mechanisms.

Interestingly, the GWASs demonstrated that most of the genes associated with Mendelian forms of human obesity (*LEPR*, *POMC*, *MC4R*, *BDNF*, *SH2B1* and *PCSK1*) are also enriched with more common variants associated with relatively small effects on BMI predisposing the individual to mild or moderate form of obesity in the general population [100, 101, 110-112]. This overlap with the monogenic obesity associated genes presumably indicates shared underlying mechanisms that could predispose an individual to a more severe monogenic as well as a more subtle polygenic form of obesity [113]. Conversely, the aforementioned findings also suggest the advisability to screen established loci identified through GWAS, in severely obese subjects with the objective to identify novel loci that could be associated with large effects on BMI to help pinpointing the causal genes [114].

1.4.2 Monogenic forms of obesity

Incidence of obesity due to a loss-of-function/pathogenic mutation in a single gene is a rare event. The first potential evidence of monogenic obesity was provided by the finding of two mutant mice, *ob/ob* and *db/db* belonging to different strains. The *ob/ob* mouse that was three times heavier than a normal mouse and also had reproductive dysfunction, was found in 1950 at Jackson Laboratories [115] whereas *db/db* mouse which was phenotypically very similar to *ob/ob* but also had diabetes, was discovered in the same laboratory in 1965 [116]. An elegant series of experiments carried out by Coleman through parabiosis of *ob/ob*, *db/db* and normal mice led to the suggestion of the presence of a circulating 'satiety factor' in normal mice which was absent in *ob/ob* mice and was produced in excess in *db/db* animals that were unresponsive or resistant to it [117, 118]. It was twenty years later that positional cloning of the *LEP* gene led to the discovery of its encoded adipocyte secreted hormone leptin [119] and thus confirmed the presence of the

blood-borne 'satiety factor' postulated earlier. Subsequent cloning of the leptin receptor (*LEPR*) [120] and the elucidation of the central leptin driven melanocortin 4 signalling [121], as the critical and principal mechanism for regulation of body weight and energy balance, spurred unprecedented research activity in the field of molecular and physiological basis of obesity.

Although the common form of obesity and its associated health and economic burden have been the major driving force for pursuit of unravelling the genetics of obesity yet the complexity of gene-gene and gene-environment interactions pose an obvious obstacle to such efforts in making a significant discovery. On the other hand, investigations on subjects with extreme obesity due to a single gene aberration provides an exceptionally strong association between the genetic make-up and the disease phenotype and has substantially contributed to our understanding of the pathophysiology of the disease revealing the underlying genetic basis of rare monogenic disorders [61, 71, 122-124].

First direct evidence in the human that obesity could be caused by mutations in a single gene came in 1997 through identification of a recessive homozygous mutation in the leptin gene in two severely obese cousins from an inbred family of Pakistani origin [125]. Extreme obesity presented by these two patients was shown to be due to a complete lack of leptin hormone (congenital leptin deficiency). This finding was followed by several investigations into possible contribution of other obesity related genes involved in the central leptin-melanocortin signalling and has led to extensive research on its mechanism of action fuelled by a quest to find novel therapeutic strategies for the treatment of human obesity [71, 126, 127]. Further impetus to these investigations was provided by an alarming rise in prevalence of obesity with its co-morbidities almost at the same period.

Monogenic forms of obesity have been identified in only 4-6% of severely obese subjects of European descent [71, 122], and are due to loss-of-function mutations in genes that have a key involvement in the hypothalamic control of energy balance via the leptin-melanocortin pathway and/or in regulation of neural development [128]. Mutations in these genes that disrupt these processes almost invariably result in severe obesity and hyperphagia in early childhood and are associated with various endocrine and immune disorders [128-130]. These include mutations in leptin (*LEP*) [125], leptin receptor (*LEPR*) [131, 132], preopiomelanocortin (*POMC*) [133], prohormone convertase 1 (*PCSK1*) [134, 135] or melanocortin 4 receptor (*MC4R*) gene [136-138].

Signals from other relatively recently proposed centrally located and presumably leptin-independent systems, implicated in regulation of body weight and energy balance such as those involving brain-derived neurotrophic factor (*BDNF*) [61] and its receptor, tropomyosin receptor kinase (*NTRK2*) [139, 140], and the transcription factor, single-minded 1 (*SIM1*) [141, 142], may also converge to influence the melanocortin pathway [143, 144]. Furthermore, there is growing evidence that some of the common variants associated with small increments in BMI identified by genome-wide association studies (GWAS) map within or in the vicinity of genes carrying rare variants with large effects [57, 58, 145].

Leptin

Leptin, the product of *LEP* gene, is an adipocyte derived hormone [119] and is also produced in smaller concentrations in other parts including brown adipocytes, stomach, placenta and skeletal muscle [146, 147]. Discovery of leptin gene in mice and subsequently in the human was undoubtedly a landmark in the field of obesity research. Indeed, well before its discovery, leptin's important role in regulation of body weight was foreseen through experiments carried out by Coleman described above [117, 118].

Studies both in animals and the human have demonstrated the close association between body fat, leptin mRNA and the plasma leptin levels [148, 149]. Leptin levels signal the state of energy stores to the brain and are elevated in situations of positive energy balance or obesity and decrease during negative energy balance such as in anorexia. Hence, leptin acts as a negative feedback regulator that helps the central nervous system in maintaining a set point for body fat stores and thus functions as a lipostat (control appetite and maintain constant body weight) [150]. Because of its crucial and vital role in control of body weight and energy balance, its biological and cellular characteristics have been extensively studied [150, 151].

Mutations in the *LEP* gene typically lead to complete absence of circulating hormone leptin – congenital leptin deficiency. Subjects with early onset obesity associated with congenital leptin deficiency lack satiety and consequently suffer from hyperphagia and severe obesity [61, 125]. Although leptin exerts its major effect on energy homeostasis by acting mainly through the arcuate melanocortin pathway (discussed below), recent evidence indicates other sites of its

action and pathways by which leptin may regulate a diverse range of physiological processes and functions [152].

Complete leptin deficiency besides leading to severe obesity at an early age, is also accompanied by hypogonadotropic hypogonadism and abnormal pubertal development [153]. *ob/ob* mice are infertile but the condition is reversible by recombinant leptin administration [154]. Likewise in the human, congenital leptin deficiency is associated with insufficient GnRH secretion establishing a hypogonadotrophic hypogonadism state and a failure to reach puberty. Lack of growth spurt, secondary sex characteristics, and menarche are also some of the characteristics that may be associated with congenital leptin deficiency in the human [153]. Exogenous leptin administration in these subjects, allows timely pubertal advancement signifying importance of leptin in pubertal development and reproductive health [155] indicating that the presence of circulating levels of leptin is required for normal activation of the hypothalamic-pituitary-gonadal axis at puberty [156].

Another significant role of leptin is its relationship to the immune system. Mice lacking leptin suffer from immunological abnormalities that are also normalized by administering recombinant leptin. Similarly, leptin deficiency in humans is accompanied by a down regulation of the immune response resulting in recurrent infection and mortality in childhood [156, 157]. In *ex vivo* studies, leptin has been shown to enhance macrophage activity thus promoting production of various cytokines like tumour necrosis factor- α (TNF- α), interleukin-6 (IL-6), and interleukin-12 (IL-12) [158].

In recent years leptin's involvement in mediating reward related activities has also been proposed. In rodents, leptin receptors are expressed in the ventral striatum (VS) - an area associated with pleasure and reward, through which leptin exerts its effect on the mesolimbic-dopamine system to decrease the incentive/reward associated with food intake [159]. Consistent with the idea of a role of leptin in the mesolimbic system, studies using functional magnetic resonance imaging (fMRI) have been used in subjects with leptin deficiency to elucidate leptin's role in both homeostatic and hedonic regulation of food intake. In the leptin-deficient state visualization of food images triggered a marked increase in neuronal activation of ventral striatum as compared to normal leptin sufficient subjects whereas, no differential activation of mesolimbic areas was noted following 7 days of leptin replacement [160].

With the exception of subjects with congenital leptin deficiency, obese individuals have higher leptin levels than their lean counterparts [150]. In common forms of obesity, the role of leptin as a negative feedback signal of adiposity appears to be attenuated and high levels of leptin in proportion to their fat mass fail to induce a loss of body weight [161]. The ineffectiveness of endogenous or exogenous leptin in obesity to regulate body weight is ascribed to leptin resistance; mechanisms that still remain largely controversial [126, 162]. One of the more plausible mechanisms proposed to explain leptin resistance in individuals with obesity involves the effect of over expression of the suppressor of cytokine signalling-3 (SOCS3) and/or protein-tyrosine phosphatase 1B (PTP1B) on downstream signalling of leptin [163]. Leptin resistant obese mice have increased hypothalamic SOCS3 and PTP1B mRNA expression [164-166] whereas both SOCS3 and PTP1B knockout mice have increased leptin sensitivity and reduction in body weight [126, 165]. A suppression of expression of these ligands could, therefore, provide a therapeutic strategy to overcome leptin resistance in cases of common obesity [161].

Some evidence in support of the blood brain barrier (BBB) as a crucial site mediating leptin resistance [167] is also available. Elevated level of triglycerides (hypertriglyceridemia) found associated with obesity are suggestive to some measure in inhibiting the transport of leptin across the BBB thus providing a mechanism for peripheral leptin resistance. Also, activity of the BBB transport system is shown to be reduced in diet-induced obesity (DIO) in mice [168].

Until 2011, in humans, only five distinct leptin mutations in 14 subjects had been described in the literature worldwide, which in the homozygous or compound heterozygous state led to congenital leptin deficiency and early onset severe obesity [125, 153, 157, 169-172]. During the course of this study another 3 mutations in 3 subjects have been identified [173-175]. The majority of the affected individuals belonged to consanguineous families.

The use of personalized medicine for obesity has so far been shown to be exceptionally successful only in relation to congenital leptin deficiency, a condition that can be completely reversed by treatment with recombinant leptin. Such an arrangement has been in place in the Western countries for more than a decade to treat leptin deficient subjects [156, 169, 176]. The recent approval of the recombinant leptin (Myalept) by US FDA, for treatment of congenital leptin deficiency and lipodystrophy, brings hope that treatment may soon become available to affected subjects in all other places irrespective of the geographical boundaries.

Leptin receptor (LEPR)

Leptin exerts its biological action through binding to the leptin receptor (LEPR), a member of class-1 cytokine receptor family. The expression of *LEPR* results in at least six isoforms of leptin receptors (ObRa-ObRf) due to alternative mRNA splicing. Whereas these isoforms have common extracellular domains, they differ in the length and sequence of their intracellular motifs. It is widely expressed in a variety of human tissues and is highly expressed in the hypothalamus, heart, liver, small intestine, prostate, and ovary [177-179]. Leptin signalling is effected only through the long isoform of receptor (ObRb) [177]. This receptor isoform is expressed mainly in the hypothalamus and mediates the downstream leptin signalling through activation of Janus kinase (JAK) and signal transducer and activator of transcription (STAT) proteins following the ligand binding and its dimerization [180]. The short isoforms ObRa and ObRc are believed to act as leptin transporters across the blood brain barrier (BBB) [177]. The ObRe is the soluble isoform that lacks an intracellular and transmembrane domain. ObRe has been shown to function as a leptin binding protein and its peripheral concentrations are used as a measure of free leptin index (FLI) [181].

In mice and humans both leptin and leptin receptor deficiency result almost in an identical phenotype of early-onset severe obesity accompanied by hyperphagia [131]. The absence of leptin action caused by functional pathogenic mutations in the *LEPR* like those of *LEP*, have been linked to hypogonadotropic hypogonadism and a failure of normal pubertal development in rodents and humans [131, 132, 154]. However, indication for a delayed but spontaneous onset of menstrual cycle in a few of the leptin receptor-deficient adults is on record [157]. In such cases it has been suggested that excessive accumulation of fat mass in these subjects may result in release of estrogen adequate for uterine development and onset of irregular menstrual cycles without development of secondary sexual characteristics [182]. One of these LEPR deficient patient has been reported to carry a natural pregnancy and given birth to a healthy child thus raising the question about the role of leptin signalling in human reproductive function [183].

Whereas *LEP* mutations result in a complete lack of its encoded protein, the pathogenic mutations in the *LEPR* render the carrier resistant to leptin action. The first reported pathogenic mutation in human *LEPR* was shown to be located in the splice donor site of exon 16 [131]. The resulting truncated LEPR protein, lacking both transmembrane and intracellular domains, was found to be secreted in the blood and to actively bind circulating leptin in high concentrations [132, 184].

Since then *LEPR* mutations have been identified in less than 25 subjects from diverse populations [63, 132, 185-188].

Proopiomelanocortin (POMC)

Proopiomelanocortin (POMC), a 241 amino acid polypeptide encoded by the *POMC* gene, is expressed mainly in the CNS (arcuate nucleus) and the anterior and intermediate (in rodents) lobes of the pituitary gland. Post-transcriptional tissue specific cleavage of POMC into smaller biologically active peptides (Figure 1.2) that also activate the melanocortin receptors (MCRs), influence diverse biological functions in the central nervous system, the adrenal gland, the skin and some other tissues. Amongst these cleavage products, α -melanocyte-stimulating hormone (α -MSH) serves as a ligand for MC4R in the CNS and thus mediates the downstream signalling of the melanocortin pathway [189].

In addition to its expression in CNS, POMC is predominantly expressed in the anterior pituitary (corticotrope cells) where its cleavage product adrenocorticotrophic hormone (ACTH) binds with the MC2R in the adrenal cortex to stimulate adrenal glucocorticoid synthesis and secretion. [190].

Homozygous or compound heterozygous pathogenic mutations that result in complete POMC deficiency are rare and have so far been reported in only eight cases [133, 191-194]. Patients with pathogenic *POMC* mutations present hyperphagia and obesity due to lack of α MSH and thereby an inactivation of its receptor, MC4R, in the hypothalamus and hypocortisolemia which is secondary to ACTH deficiency. In some of these investigations heterozygous carriers of *POMC* mutations also showed a high risk of obesity or being overweight [195, 196], suggesting that the defective allele in the heterozygous state can sufficiently interfere with the central melanocortin signalling to increase susceptibility to obesity. POMC deficiency, both in humans and rodents is also associated with pale skin and red hair consistent with the lack of MC1R receptor signalling in the absence of POMC derived α MSH. MC1R signalling is necessary to maintain phaeomelanin (yellow / red pigment) to eumelanin (brown / black pigment) ratio in the mammalian skin and hair colour [197, 198] and a disruption of which causes an increase of phaeomelanin.

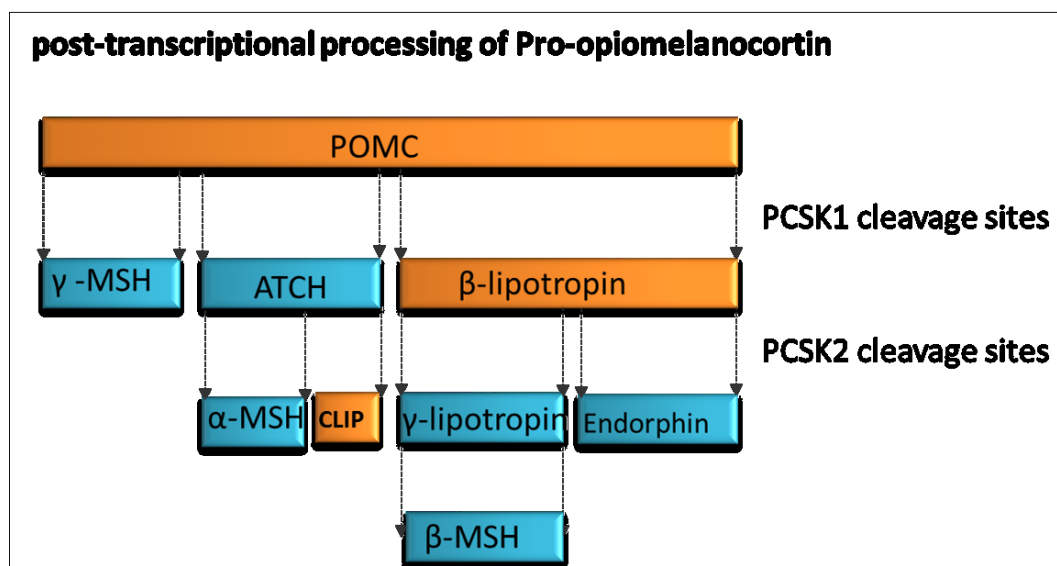


Figure 1.2 Processing of POMC precursor protein.

Proopiomelanocortin is post-translationally processed to produce several biologically active peptides, including adrenocorticotropin (ACTH) and α -, β - and γ -melanocyte-stimulating hormone (MSH). Arrows indicate the cleavage sites.

Melanocortin-4 receptor (MC4R)

MC4R, a G-protein coupled receptor activated by its ligand α -MSH, is highly expressed in the hypothalamic part of the brain and plays a significant role in melanocortin signalling and regulation of appetite and energy balance [121]. Compared to other rare single gene variants associated with obesity, mutations in the *MC4R* gene appear to be the more prevalent form of monogenic obesity. Studies regarding the frequency of *MC4R* mutations associated with human obesity estimate up to 6% of obese subjects and 1 in 1000 of UK population [199] although the prevalence may vary in different populations. The functional relevance of *MC4R* mutation to human obesity was first established in 1998 with a report of heterozygous frameshift mutation by two groups [138, 200]. Until 2000, 21 different mutations had been identified in this gene and associated with obesity. Of these 20 are reported in the heterozygous form while only one, p.Asn62Ser, was reported in the homozygous state in a subject from a consanguineous family of Pakistani origin [41]. By 2013, 166 autosomal dominant and recessive loss of function variations in the single coding exon (encodes for 322 AA) of *MC4R* gene have been described [201].

Most of the studies carried out on families indicate a co-dominant mode of inheritance with heterozygous subjects having a less severe obesity phenotype than homozygous carriers and with

a variable expression and penetrance [202-204]. In a French multigenerational familial study on *MC4R* mutation carriers an age and generation related effect on the penetrance of obesity has been demonstrated. This study shows that in older French generations (>52 years of age), the penetrance of heterozygous loss-of-function *MC4R* mutations was not more than 40%, and only a small minority of these individuals were self-reported to be obese (BMI>30) at the age of 20 years [202]. These observations suggest that the penetrance of *MC4R* mutations is variable and to a certain extent dependent on environmental and other factors.

The relatively high frequency of mutations in the *MC4R* gene urged scientists to carry out functional characterization to understand the underlying molecular mechanisms. Such studies have provided insights into different components of *MC4R* signalling including trafficking of receptor to the cell, ligand binding, activation and downstream signalling. A careful characterization of these defects has helped to identify mechanisms by which a specific receptor dysfunction could contribute to an increase in adiposity [203, 205, 206]. Most importantly such studies could also lead to development of therapeutic agents such as the use of small molecules that could act as pharmacological chaperones to rescue intracellular retained mutant *MC4R* receptor proteins [205].

Single-minded 1 (SIM1)

More recently, mutations in single-minded homolog 1 gene (*SIM1*) have been associated with severe obesity. *SIM1* encodes for its protein, SIM1, also known as Class E protein basic helix-loop-helix 14 (bHLHe14), a transcription factor, known to be involved in the development and function of the paraventricular nucleus (PVN) [207]. Mice with complete knockout of *sim1* (*Sim1*^{-/-}) die perinatally due to lack of PVN development [208]. Whereas haploinsufficiency of *SIM1* leads to obesity [208, 209], its over expression has been shown to protect against diet-induced obesity in rodents [210], indicating a possible involvement of SIM1 in energy balance. In spite of being hyperphagic and obese, *Sim1*^{+/-} mice undergo increased linear growth resembling *mc4r* mutants in appearance [144]. Also, *Sim1*^{+/-} mice are reported to have significantly reduced levels of *Mc4r* mRNA in the PVN [144]. *Sim1* overexpression observed in agouti yellow mice in which melanocortin signalling is impaired resulted in amelioration of hyperphagia and decrement of adiposity thus compensating for the defective *Mc4r* signalling [210]. The foregoing observations

support the notion of a physiological role of *SIM1* in regulation of food intake and body weight through a modulation of downstream pathway of leptin-melanocortin signalling.

Human *SIM1* deficiency was first reported by Holder and colleagues who described a de novo translocation between chromosomes 1p22.1 and 6q16.2 [211]. Subsequently, in patients with a Prader-Willi-like (PWL) phenotype, deletion of the 6q16.2-6q16.3 region that includes *SIM1* gene was also reported [212-214] suggesting its role in the development of PWL syndrome [215]. Further support to the involvement of *SIM1* in human obesity came with the identification of point mutations in this gene associated with severe obesity and PWL syndrome. In four recent investigations, screening of *SIM1* gene in obese and PWL syndrome cases, revealed 27 different mutations, 16 of which were accompanied by a significantly decreased expression of *SIM1* mRNA [141, 142, 216, 217] resulting in a spectrum of severity in obesity and of neurobehavioral anomalies. These findings also indicate a variable penetrance of *SIM1* mutations in a manner similar to those *MC4R* pathogenic variants and raise the possibility of environmental contribution in determining the severity of the condition in subjects carrying these mutations [141].

Brain-derived neurotrophic factor (BDNF) and tyrosine kinase B receptor (TrkB)

The brain-derived neurotrophic factor (BDNF) is a neural growth factor or neurotrophin which exerts its effect via the tyrosine kinase B receptor (TrkB). The BDNF by activating its receptor regulates neuronal proliferation, survival, and differentiation during development and affects synaptic plasticity in the adult brain [218, 219]. BDNF expression is also required for normal neuronal development and plasticity in the hypothalamic part of brain thus linking its role as a modulator of energy homeostasis [220].

The first direct evidence of BDNF involvement in energy homeostasis came when reduction in body weight, food intake and a reversion to normoglycemia were observed following intra-cerebroventricular injection of BDNF in genetically determined severely obese mice (*db/db*) [221]. Subsequent experiments on mice either heterozygous for the *BDNF* mutation or with conditional post-natal deletion of the gene in the brain region, resulted in the hyperphagic obese phenotype [222-224], suggesting that this neurotrophin may contribute in differentiation/regulation of central circuits of energy balance. It has also been indicated that BDNF/TrkB signalling exerts anorexigenic effect downstream of the *MC4R* [143, 223].

Indication that defective BDNF/TrkB signalling is associated with weight regulation and appetite control in the human was provided by a report of a *de novo* heterozygous mutation in *NTRK2* associated with severe obesity, impaired short-term memory and other developmental anomalies [140]. Further support to this observation was provided by identification of a BDNF-haploinsufficient child due to a paracentric inversion in the region encompassing the BDNF gene. In addition to severe obesity and hyperphagia, the patient also presented impaired cognitive function and hyperactive behaviour [61]. Altogether, these observations provide evidence of a significant involvement of BDNF/TrkB signalling in control of energy balance.

Proprotein convertase subtilisin/kexin type 1 (PCSK1/3)

PCSK1/3 formally known as PC1/3, is a member of serine endoproteases family involved in processing of inactive hormone and neuropeptide precursors into their biologically active form. PCSK1/3 expressed selectively in the neuroendocrine tissues, cleaves a number of precursor proteins including proinsulin, proglucagon, *POMC* and pro-neuropeptides, amongst others [225].

Pathogenic mutations of *PCSK1/3* that act in recessive form and cause complete lack of the encoded protein, are strongly linked to obesity [135, 226-228]. Congenital loss of PCSK1 protein in the human leads not only to obesity but to a number of other clinical phenotypes including intestinal dysfunction, hypocortisolemia, disruption of glucose homeostasis and endocrine dysfunctions due to defective processing of hormone precursors such as proglucagon, ACTH, proinsulin, pro-TRH and pro-GHRH. In another study partial PCSK1 deficiency due to heterozygous loss of function mutations have been associated with an 8.7-fold increased risk to develop obesity [60]. More recently, a unique dominant nonsense mutation in *PCSK1* gene (p.Arg80*) has been associated with obesity and glucose intolerance [229]. This mutation was found to co-segregate with obesity in a multi-generation family. Functional analysis revealed that the variant abolished PCSK1 enzyme activity.

Unexpectedly, *Pcsk1* null mice do not display an obesity phenotype but undergo a blunted growth accompanied by various neuroendocrine disorders [230]. However, the PC1/3^{N222D} mutant mouse represents a singular case leading to the obese phenotype [231]. This mutation resulted in lower levels of α -MSH and defective *POMC* processing.

Other putative monogenic obesity associated genes

To date, almost all genes that have been implicated in the development of obesity appear to modulate adiposity either by regulating appetite and energy homeostasis or influencing neurogenesis of brain areas that are crucial in energy balance. Recent efforts to identify new obesity associated genes influencing the leptin melanocortin domain have resulted in identification of melanocortin receptor accessory protein 2 (*MRAP2*) with a suggested role in modulation of MC4R signalling and consequent development of obesity in rodents and humans [232]. *Mrap2* knockout mice have been shown to develop obesity at an early age [232]. Moreover mutations related to *KSR2* – a scaffolding protein involved in Ras-Raf-MEK signalling pathway have been shown to have an effect on energy balance by way of a decrement of BMR [233], mimicking the phenotype observed in *Ksr2*^{-/-} mice [234]. These findings implicate *MRAP2* and *KSR2* encoded proteins as physiological regulators of energy homeostasis. Replication and further elucidation of these results are needed before mutations in these two genes could be associated as causal factors of monogenic obesity.

1.4.3 Syndromes associated with obesity

In addition to monogenic form of obesity in which excessive weight gain at early stages of life is the main presentation, a number of syndromes have been identified where obesity is one of the several other anomalous phenotypic expressions. These predominantly include developmental and cognitive delay, mental retardation, dysmorphic features and organ-specific developmental abnormalities [235]. Syndromic form of obesity may arise from structural abnormalities at the chromosome level involving several genes or as consequence of a mutation in a single gene with pleiotropic effects. Here we briefly describe some of the well-investigated forms of syndromic obesity from each of these two categories.

Chromosomal abnormalities - imprinting disorders:

Prader-Willi syndrome (PWS)

PWS is one of the more common forms of syndromic obesity. The syndrome presents infantile hypotonia, hypogonadism, growth hormone insufficiency, delayed development and feeding difficulties in early life. These symptoms are soon followed by hyperphagia leading to early onset

obesity. Other phenotypic characteristics include a distinctive facial appearance and short hands and feet [127, 236].

This complex obesity syndrome is caused by an absence of paternally inherited allele expression at 15q11–13 genomic region corresponding to where the maternally inherited allele is imprinted. Paternal deletion, maternal uniparental disomy, and imprinting defects are the three proposed mechanisms that could cause absence of paternal expression and result in development of the PWS. The deleted region is typically a ~6 Mb or ~5.3 Mb fragment depending on the position of the proximal breakpoints (BP1–BP3 and BP2–BP3, respectively) [237, 238]. However, unique deletion(s) with specified breakpoints have been reported in a small number of cases [239]. Some microdeletions ranging from 187 kb to 847 kb within *SNURF-SNRPN* and *snRNA* cluster region have also been observed indicating a major contribution of non-coding RNAs in this region in most if not all phenotypes related to PWS [240–242]. Further studies aimed at whether there is a functional involvement of other genes encompassing the deleted region may contribute to associating distinct genetic factors with specific clinical features accompanying this syndrome.

Albright hereditary osteodystrophy (AHO)

Phenotypic features associated with this syndrome in addition to obesity include short stature, round face, brachydactyly, and mental disability. All these features are collectively termed as the AHO. Various genetic and epigenetic events in the *GNAS1* gene have been shown to determine these phenotypes.

Inactivating mutations in the *GNAS1* cause a decrease in expression (~50%) and thus function of its encoded protein-Gs α , leading to development of AHO symptoms. Gs α is a critical component for signal transduction by activation of adenylyl cyclase and thereby causing an increase in intracellular adenosine monophosphate (cAMP). A tissue specific genomic imprinting is observed in the *GNAS1* region. Only the maternally inherited allele is expressed in tissues including renal tubules, thyroid and pituitary gland. The mutated maternal allele results in a resistance to parathyroid (PTH) whereas inactivating mutations in the paternal allele will not cause resistance to the hormone. Since elsewhere in the body, bi-allelic expression of *GNAS1* is present, mutation in either allele will reduce Gs α expression to half causing AHO. Mutations in the maternally inherited allele cause AHO and resistance to multiple hormones. The condition is termed as

pseudohypoparathyroidism (PHP) [243]. Paternal $Gs\alpha$ -inactivating mutations lead only to the AHO phenotype but without hormone resistance termed as pseudopseudohypoparathyroidism (PPHP) [244, 245].

Single gene defects - ciliary dysfunction

Cilia are the hair-like structures present on all types of cells in the body and play an important role in various cell functions including movement, perception of sensory input and signal transduction. Impaired ciliary function can lead to developmental abnormalities and deformities in several different organ systems. Abnormal formation and function of cilium caused by ablation of genes encoding for components involved in cilium integrity and function, have been linked to a constellation of phenotypically and genetically overlapping disorders collectively known as ciliopathies. Consequently there is a wide range of phenotypic outcomes of ciliary dysfunction in mammals. Of these obesity is one of the presentations in three syndromes namely Bardet-Biedl (BBS), Alstrom (ALS), and Carpenter (CA) syndromes [127]. Indeed, abnormal development of the primary cilia suggests a link between the ciliary function and Lep-LepR signalling in the hypothalamic satiety centres of the brain [246, 247].

Bardet-Biedl syndrome (BBS) is a pleiotropic and genetically heterogeneous autosomal recessive disorder. This syndrome is associated with several phenotypes including obesity, renal malformation, polydactyly, hypogenitalism and learning disabilities. To date, 18 genes (BBS1-18) have been associated with this syndrome by way of ciliary malfunctioning [248]. Proteins encoded by BBS genes are located at the base of the cilium and play their role in trafficking of particles in and out of the cilium [249, 250].

Alstrom syndrome (ALS) is rare autosomal recessive disorder typically characterized by a progressive neurosensory deficit, development of obesity at an early age, male hypogonadism and insulin resistance. Other clinical features associated with the disease include cardiomyopathy, hepatic triglyceridemia, short stature, fibrosis, and multiple organ failure [251, 252]. Unlike BBS, this disorder is reported to be genetically homogeneous since only ALMS1 gene has so far been reported to be associated with this syndrome. ALMS1 is widely expressed in centrosomes and basal bodies in ciliated cells suggesting the role of this protein in function and integrity of cilia [253]. The similarity in function of ALMS1 with BBS is suggested by a strong resemblance of

phenotypic features of the two syndromes. However, the absence of mental retardation and digital anomalies distinguish it from the BBS syndrome.

Carpenter syndrome (CS) is a very rare autosomal recessive disorder presenting truncal obesity, preaxial polydactyly of foot and craniosynostosis. Clinical features include hypogenitalism, congenital heart disease, mental retardation and dental anomalies [127, 254]. Mutations in *RAB23* have been implicated in the pathogenesis of CS. *RAB23* encodes a member of the RAB guanosine triphosphates (GTPase) family and is mainly involved in the intracellular membrane trafficking [255].

1.4.4 Beyond single gene defects - genomic structural variations

In addition to rare obesity associated Mendelian variants with a large effect and common variants with small effect, genomic copy number variations (CNV's) have strongly been implicated in some forms of extreme obesity. CNV's occur where a segment of DNA is duplicated or deleted and are estimated to account for nearly 18% of the heritable variance in gene expression. The proximal short arm of chromosome 16 is one such region that has strongly been associated with obesity. A smaller 220 kb and a larger 593 kb deletion found in 16p11.2 region were found to be associated with a predisposition to a highly penetrant form of obesity with a 43-fold increased risk of developing morbid obesity. The frequency of the 220-kb, SH2B1 inclusive, deletion in patients with severe obesity has been shown to be 0.51% (7/1362) [256] whereas the 593-kb deletion in the same chromosomal region had a frequency of 0.36% in obese individuals (15/4197) [257], presenting one of the more frequent genetic causes of obesity. This deletion has been consistently replicated in various independent investigations [258-260]. Whereas homozygosity of the 600 kb deletion in the short arm of chromosome 16 has been associated with severe obesity, corresponding duplication(s) in the same region result in extremely low BMI of affected patients. The reciprocity of deletions and duplications effects at the same locus present an interesting example of a 'mirror' effect on the phenotype [261].

The 220kb deleted region maps a strong candidate gene for obesity - Sarcoma (Src) homology 2 (SH2) B adaptor protein 1 (SH2B1). SH2B1 is an adaptor protein involved in signalling downstream of a various receptor tyrosine kinases and cytokine receptors including leptin receptor. Targeted disruption of *Sh2b1* in mice causes a striking leptin and insulin resistance, increased food intake

and severe obesity [262, 263]. SNPs within SH2B1 have also been linked to human obesity and BMI through several genome-wide association studies (GWAS) providing support for the involvement of SH2B1 in body weight regulation [99, 100, 264, 265]. Recently, several non-synonymous mutations have been identified and associated with obesity, insulin resistance and some of the behavioural abnormalities [266-269]. It has also been shown that variants present in N-terminal region exhibit a spectrum of maladaptive behaviour in addition to affecting food intake and body weight [269]. Interestingly SH2B1 α variant carriers were hyperinsulinemic but did not exhibit the behavioural phenotype observed in individuals with N-terminally located (1–631 region) mutation [268] suggesting genetic variants that disrupt isoforms other than SH2B1 β may be functionally significant. Taken together, these findings suggest a substantial role of copy number variants in susceptibility to obesity.

1.5 Physiological basis of energy homeostasis - role of CNS

Energy homeostasis implies a balance of energy intake with energy expenditure resulting in the maintenance of fat stores and body weight more or less at a set point, under normal conditions. Energy homeostasis like other homeostatic mechanisms is controlled and regulated by specific areas of the brain in relation to afferent peripheral and environmental signals interpreted and processed by a complex neurocircuitry, and translated into efferent signals affecting appropriate responses to maintain energy balance.

The key role of hypothalamic area of the brain in the control of energy homeostasis was indicated as early as 1940's by the demonstration that lesions of this part of the brain resulted in morbid obesity [270]. Since then a large body of morphological, physiological, genetic and pharmacological studies have established the importance of the hypothalamus in the regulation of food intake, body weight and energy homeostasis.

The hypothalamic neurons are concentrated in a number of nuclei including the arcuate (ARC), ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH), lateral hypothalamus and paraventricular (PVN) nuclei. These neurons are anatomically localized around the third ventricle and express receptors that are specific to metabolic signalling molecules originating peripherally as well as centrally [271]. Neurons comprising these nuclei receive multiple peripheral signals in the form of hormones, neuropeptides, neurotransmitters and metabolites and in turn produce

specific and coordinated physiological responses by inter- and intra-cellular signalling mechanisms to other brain regions [126, 272]. The circulating nutrient signals mostly converge to ARC situated ventrally and adjacent to the median eminence and the specific peptides expressed by its neurons play an important and well characterised role in regulating food intake and energy homeostasis.

The complex network of homeostatic circuitry also includes extra hypothalamic regions of the CNS such as the brainstem or nucleus of the solitary tract (NTS) a critical sensor of circulating signals for regulating food intake. This part of the brain contains various neuropeptides and receptors that have their role as appetite modulators and receives vagal inputs from the gastrointestinal tract on information about nutrient content, and projections of the hypothalamic nuclei to provide nutrient related information [273].

Of the peripheral hormonal signals that transmit the nutritional state of the body to the homeostatic system, leptin and insulin are considered as long term modulators of energy balance while peptides produced by the gut including ghrelin, peptide YY (PYY), glucagon-like peptide 1 (GLP-1) and cholecystokinin (CCK), along with vagal inputs are the main components of the short term food intake regulatory system. These hormones signal through their receptors and interacting by various feed-forward and feed-back mechanisms regulate appetite and energy balance [274].

In addition to homeostatic control of food intake and energy homeostasis, eating behavior is greatly influenced by cognition, emotional and reward related responses mediated by pre-cortex and areas of limbic system. Evidence is also available suggesting influence of peripheral metabolic factors on reward system [275, 276] in addition to acting as homeostatic mediators through the hypothalamus. Nutrition related information from these hedonic reward centers may also converge to central hypothalamic part to influence food intake by modulating the sense of pleasure associated with eating [126, 272].

Leptin and melanocortin pathway

Following the pioneering discovery of the obesity gene in 1994, and its encoded protein hormone leptin secreted by the adipose tissue [119], a great deal of attention has been given to search specific signalling molecules and pathways controlling our food intake and body weight leading

to the current understanding of mechanisms of action of leptin and its downstream mediators [71, 126, 127]. As a result of studies in rodents and the human and especially through insights derived from monogenic obesity, the central leptin melanocortin pathway has emerged as the principal signalling mechanism regulating energy balance.

The ARC is protected by the blood-brain barrier (BBB) and it is difficult for circulating molecules as large as leptin to access it by simple diffusion. It is generally held that leptin gets across this barrier that separates the ARC from the median eminence, by a 'saturable transporter system' [277]. The median eminence is provided with fenestrated capillaries, a part of the so-called circumventricular organs (CVO) that lie outside the BBB. It has been proposed that ARC LepRb bearing neurons in close vicinity to its border with median eminence may also be accessible to peripheral endocrine signals such as that of leptin. The border between ME and ARC is lined by highly modified glial cells, the tanycytes, that have been implicated in the process and mediate to transport leptin to the cerebrospinal fluid (tanycyte-mediated transport) [278, 279]. Moreover, there is evidence that some of the leptin receptor expressing neurons send projections across the barrier into the median eminence hence gaining direct access to the circulating hormone [280].

Intracellular leptin signalling - JAK2/STAT3 pathway

LepRb is the only form of leptin receptor that possesses all domains necessary for intracellular signalling [178] and initiates several signal transduction pathways [152, 281-283]. Of these the JAK2/STAT3 pathway is the best studied and is demonstrated to play a major role in energy homeostasis and body weight regulation [284]. Following leptin binding and receptor dimerization, JAK2 is autophosphorylated subsequently leading to phosphorylation of multiple tyrosines in LepRb (Tyr985/1107/1138) and enabling recruitment of downstream effectors. The phosphorylation of Y1138, provides a binding site for STAT3 protein which upon activation dissociates from the leptin receptor (Figure 1.3). Recently, Rho-kinase 1 (ROCK1) has also been reported to play a critical role in leptin signalling by phosphorylating JAK2 via a direct ROCK1-JAK2 interaction [285]. Following dimerization, the activated STAT3 translocates to the nucleus where it binds to the target gene to bring about a change in transcription. The promoter region of both *AgRP* and *POMC* contain a domain for STAT3 binding which then activates the transcription of *POMC* whereas inhibits the transcriptional activity of *AgRP* [286].

It was shown that mice with a targeted mutation at the Tyr¹¹³⁸ of the LepRb (s/s mice) were hyperphagic and obese but unlike mice with neural STAT3 deletions had normal body growth and fertility [287]. Mice with selective neural deletions of STAT3 like db/db mice, not only developed hyperphagia and obesity but were also defective in body growth and reproduction [152, 288]. These investigations indicate that STAT3-independent signals may be responsible for leptin mediated neuroendocrine regulation. JAK2/STAT3 pathway of leptin action has also been shown to suppress the orexigenic AgRP/NPY neurons [287, 289]. Disruption of JAK2/STAT3 in mice with mutation at Tyr¹¹³⁸ (s/s), results in an increased transcription of AgRP and NPY mRNA's and increased weight gain [162].

Additionally, LepRb signalling is shown to be affected by two adaptor molecules – SOCS3 and PTP-1B. These molecules when activated induce a negative feedback regulation resulting in an inhibition of tyrosine phosphorylation of LepR [290, 291]. Gene expression of SOCS3 is increased by STAT3 mediated leptin signalling which in turn binds to Tyr985 blocking leptin signalling. Initial findings demonstrated the involvement of Tyr985 for recruitment of SOCS3 [284, 291]. However, more recent studies suggest multiple binding sites within LepR [292]. Similar to SOCS3, leptin signalling results in an up-regulation of PTP-1B. Deletion of either *Socs3* or *Ptp1b* in POMC neurons leads to reduced adiposity, improved leptin sensitivity, and increased energy expenditure under HFD conditions [289, 293]. Moreover, changes in SOCS3 as well as PTP-1B expression in line with negative feedback mechanism of leptin signalling have been implicated in development of leptin resistance in relation to hyperleptinaemia observed in most cases of obesity [164, 165, 294].

PI3K signalling

In addition to JAK2/STAT3 pathway, activation of LepRb stimulates the phosphatidylinositol 3-kinase (PI3K) pathway in the hypothalamus [295]. Autophosphorylation at position Tyr813 by JAK2 causes binding of Src homology 2 B adapter protein 1 (SH2B1) that markedly increases JAK2 catalytic activity thus helping in recruitment and activation of downstream insulin receptor substrate proteins (IRS-1, IRS-2, IRS-4) to the LEPRb- JAK2 complex [296]. IRS's bind to the p85 regulatory subunit of PI3-kinase (PI3K) and activation of PI3K through a sequence of events leads to phosphorylation of FOXO1. Upon phosphorylation, FOXO1 leaves the nucleus to allow STAT3 binding to enhance the expression of AgRP/NPY and suppresses the expression of POMC (Figure 1.3) [297]. Hyperphagia accompanied with increased adiposity and hyperleptinaemia in IRS2-null

mice, provide evidence for a plausible participation of the IRS/PI3K cascade in leptin action [298]. The role of FOXO1 transcription factor in mediating IRS2/PI3K signalling to control energy balance may also explain the link between the downstream insulin and leptin signalling pathways [299, 300]. Furthermore, the overlapping PI3K pathways of leptin and insulin provide a possible explanation of the concomitant leptin and insulin resistance encountered so often in common obesity [126].

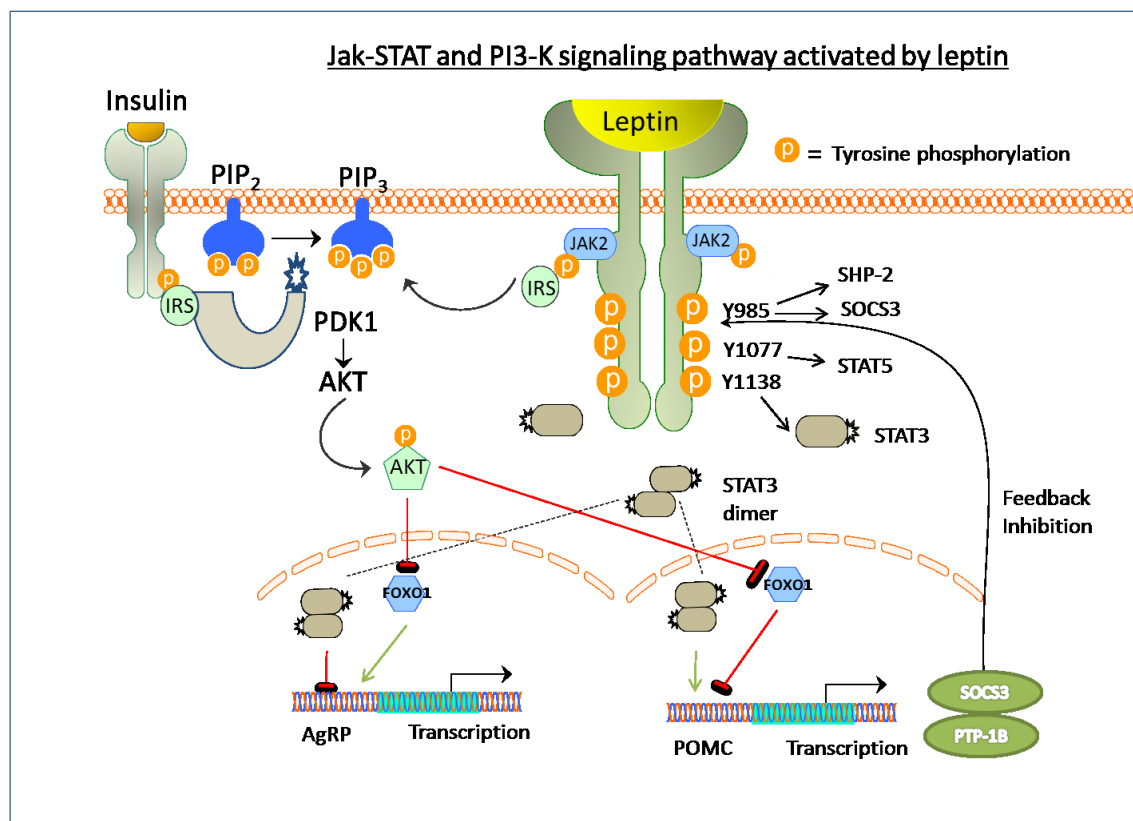


Figure 1.3 Leptin signalling pathway in the arcuate nucleus.

STAT3 activation and dimerization that take place after leptin's binding to LepRb allow p-STAT3 to enter in nucleus and bind to promotor regions of *Pomc* and *Agrp* to stimulate *Pomc* and inhibit *Agrp* expression. Activation of PI3K by leptin facilitates activation of phosphatidylinositol-3,4,5-triphosphate (PIP₃) which in turn leads to the activation of downstream targets such as Akt mediated by phosphoinositide-dependent kinase 1 (PDK1). Akt activation finally phosphorylates the transcription factor forkhead box protein O1 (FOXO1) and makes it depart from the nucleus and allows STAT3 to bind to *Pomc* and *Agrp* promoters.

Within the hypothalamic arcuate nucleus (ARC), leptin acts on two functionally opposing populations of neurons. One population of neurons synthesizes and releases three orexigenic neuropeptides: the agouti-related peptide (AgRP), the neuropeptide Y (NPY) and GABA [162, 287, 289]. The other set of neurons expresses genes that encode the POMC which is post-translationally cleaved to produce α - and β -melanocyte-stimulating hormone (α - and β -MSH). α -MSH acts as an anorexigenic signal and promotes energy expenditure by activation of melanocortin-4 receptor (MC4R) and to a lesser extent the melanocortin-3 receptor (MC3R) [301, 302]. POMC producing neurons also express cocaine and amphetamine-related transcript (CART). Both subsets of neurones in ARC are reciprocally regulated by leptin and make numerous connections with other hypothalamic nuclei, including the LH, PVN, VMN and DMN [189, 272]. Thus, the leptin-driven regulation of food intake results through a suppression of orexigenic and an induction of anorexigenic neuropeptides. Leptin exerts most of its effects on energy homeostasis through its long-form of receptor, LepRb, which is highly expressed in hypothalamic nuclei and other CNS regions involved in the control of energy balance [282]. In mice, ablation of LepRb in either POMC, AgRP, or both populations of neurons results in increased adiposity indicating the importance of leptin signalling in these neurones [303, 304]. However, the extent of adiposity observed is less than that observed in mice with global ablation of the receptor, demonstrating the involvement of other populations of LepR expressing neurons in the brain and indicating that leptin may act through different and independent neural systems to influence body weight and energy balance [287].

PVN has been shown to be a key site for LEP/LEPR signalling for regulation of food intake and energy balance. Electrolytic lesions in this part of the brain are accompanied by hyperphagic obesity. Release of α -MSH by POMC neurons, promotes satiety by binding to these melanocortin 4 receptors (MC4Rs) whereas inverse agonist of MC4R, AgRP, stimulates feeding. Expression of the transcription factor, SIM1, is shown to be necessary for normal PVN development and function. Partial deletion in the *SIM1* evokes decreased cellularity of PVN [305] and is associated with obesity due to increased food intake and neurobehavioral abnormalities.

Apart from well researched leptin driven melanocortin pathway in the hypothalamic area of the brain, evidence has been derived in the recent past of the existence of a leptin-independent melanocortin activation. Monogenic mutations and genetic manipulations influencing the brain-

derived neurotrophic factor (BDNF) and 5-hydroxytryptamine (5-HT) systems that result in obesity, indicate their involvement in energy homeostasis and body weight regulation [273]. Several lines of evidence are available linking BDNF signalling to energy balance in humans and rodents through its functional interactions with central as well as brainstem anorexigenic pathway. BDNF mainly expressed in VMH neurons acts as a downstream metabolic effector of MC4R signalling to mediate its role in the homeostatic control of food intake and body weight [223] [306]. Recent investigations also nominate 5-HT in regulation of energy homeostasis by activation of its specific receptors on POMC and AgRP neurons. BDNF-5HT signalling similar to that of leptin may, therefore, be regarded as a component of the central melanocortin system (Figure 1.4) [273]. Nonetheless, leptin undoubtedly appears to play the dominant role in driving the melanocortin signalling pathway, as its deficiency or that of its receptor, leads to one of the most severe types of obesity and hyperphagia, at a very early age.

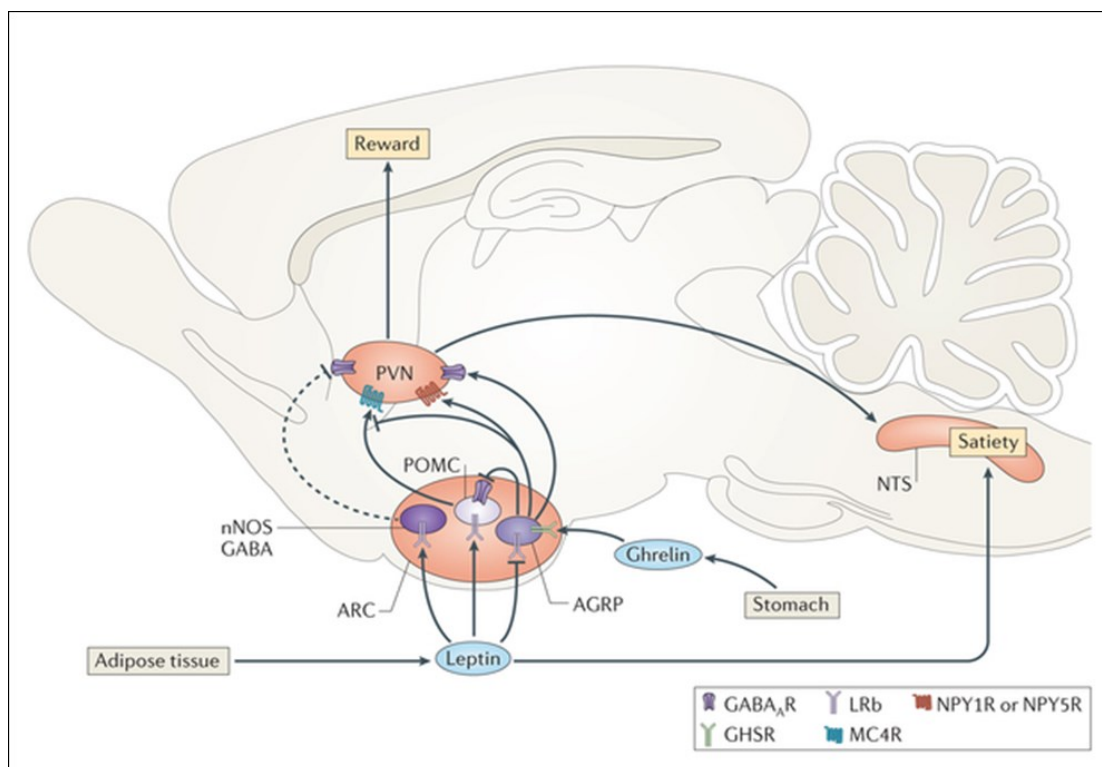


Figure 1.4 Neurocircuitry involved in energy homeostasis.

Neurons present in hypothalamic arcuate nucleus as well as in NTS are major respondents of peripheral signals such as leptin. In the state of positive energy balance, leptin activates anorexigenic (POMC) neurons and inhibits orexigenic (AGRP) neurons. Activated POMC neurons after cleavage and processing by PCSK1 give rise to MSH (not shown) that in turn serves as ligand for MC4R in PVN. Neurons in PVN such as oxytocin after activation inhibit feeding. Also VMH (not shown), which is a key site of BDNF expression, mediates downstream MC4R signalling to reduce food intake. Leptin responsive GABAergic neurons also mediate reduction in food intake through inhibiting downstream neural circuit that drive feeding. Collective effect from all these nuclei is conveyed to reward centre to modulate food intake (From Morton, Meek et al 2014 [307]).

1.6 Molecular approaches for diagnosis of obesity

1.6.1 Mutation screening from Sanger to next-generation sequencing

In 1976-77 Fredrick Sanger and his colleagues developed a nucleic acid sequencing technique that would be a watershed moment in the field of genetic analysis. The technique now generally known as Sanger sequencing was originally referred to as the ‘dideoxy sequencing’ or ‘chain termination sequencing’ [308]. The technique is based on chain termination chemistry—namely the dideoxynucleotides that contain hydrogen on the 3’ carbon instead of a hydroxyl group (OH) when integrated stop phosphodiester bonding between the dideoxynucleotide and the next nucleotide sequentially, and prevent further chain extension. Sanger sequencing undoubtedly proved a trailblazer for today’s innovative advancements in rapid and high-throughput sequencing techniques. Four decades after its discovery, Sanger sequencing is still considered the gold standard for nucleic acid sequencing and remains an important method for molecular genetic testing and validation for next generation sequencing results in most of the laboratories around the globe.

In 2003, the entire human genome was successfully sequenced unlocking the code of human life. The accomplishment of the ‘Human Genome Project’ marks it as one of the greatest feats in the history of human endeavour. Nucleotide analysis of the entire genome was carried out by using conventional (Sanger) sequencing and the first capillary-based automated sequencing instrument. Accurate and longer read lengths made possible by this procedure were of significant advantage in the absence of a template or reference sequence at that time. However, this first generation sequencing method used in the human genome sequencing, proved cost intensive and time consuming particularly for large scale genetic screenings. Since then efforts have been made to develop high throughput and cost effective sequencing technologies that offer technical and economic advantages over previous screening methods and could provide faster ways to identify genes and genetic variants of pathogenic significance. The advent of massively parallel sequencing (MPS) technologies developed in recent years have indeed made it possible to generate large amounts of sequence data rapidly and bring it down to a single-base resolution in a short time period at the scale of the whole human genome [309]. In principle, these newer technologies follow the same general concept as that of Sanger chemistry and CE sequencing. The main

difference lies in that newer technologies allow sequencing of millions of fragments in a massively parallel way as compared to sequencing of single DNA fragments as seen in CE. MPS has undoubtedly revolutionized the sequencing capabilities and has led to the development of next-generation sequencing (NGS) technologies [310].

Rapid advancements in terms of efficiency and cost effectiveness in NGS sequencing technologies is obvious from the fact that a single base sequencing run rate has climbed to one gigabase in 2005 and to 1.8 terabases in 2014 and has drastically reduced the cost of sequencing in terms of both money and time. NGS has enabled sequencing at population level and expedited discovery of genes and genetic variants associated with disease and to gain insights into the etiology of disease susceptibility [311].

Whole Genome Sequencing (WGS) is a powerful sequencing approach that enables characterisation of all protein coding and noncoding variations spanning across the genome. This technique has the capacity to identify genetic variations associated with a vast number of diseases, yet relatively high costs involved have restricted its wider use. As the cost of sequencing continues to drop with time it is hoped WGS will become a method of choice in the foreseeable future.

Whole Exome Sequencing (WES) technique is used to sequence all protein-coding regions in the whole genome (exome capture) and is now in fairly wide use. Since less than 2% of human genome contains majority (~85%) of the disease causing genes, WES, therefore, provides a cost-effective alternative to WGS, to identify candidate genes and mutations associated with various disease conditions [312] especially those that follow Mendelian inheritance. WES, has recently emerged as a useful diagnostic tool for disease gene identification [313-315]. In the recent past use of whole exome analysis in subjects carrying Mendelian mutations, has identified more than 100 causative genes [311, 316, 317] thus establishing the sensitivity and accuracy of the technique as well as its utility in clinical settings [318, 319].

Sequencing of targeted regions involves sequencing a subset of genes or genomic region of interest, enabling cost-effective and efficient detection of variants. This approach permits researchers to achieve excellent coverage (100x or higher) and thus allows identification of rare/novel variants that could be missed by WES and WGS. This method has already proven its

usefulness in clinical diagnostic testing by targeting selected genes to accommodate specific clinical needs.

A wide range of kits for targeted sequencing (including WES - since in principle it also comes under the category of targeted sequencing) are commercially available. These kits support one of the two main methods for DNA isolation and enrichment of targeted sequence from the human genome namely those that are hybridization based or those that are amplicon/multiplex PCR based.

Target enrichment through hybridization allows capturing a region of interest by hybridization to bionylated probes followed by magnetic pulldown. There are several commercially available enrichment systems that follow this principle. SureSelect and HaloPlex Target Enrichment provided by Agilent Technologies and Nextera Rapid Capture Custom Enrichment Kit offered by Illumina are highlighted amongst others.

Enrichment through amplicon generation allows enrichment of desired genes/regions by primer based PCR amplification. In most of the cases highly multiplexed PCR oligo sets are used to obtain amplicons of interest for subsequent MPS. There are several commercially available amplicon-based kits in the market, including Ion AmpliSeq (Life Technology, Carlsbad, CA, USA), TruSeq (Illumina, San Diego, CA, USA), Microdroplet PCR (RainDance Technology, Billerica, MA, USA) and Access Array (Fluidigm, San Francisco, CA, USA).

Microdroplet PCR (RainDance Technologies Inc., Billerica, MA, USA) implies microdroplet-based multiplex polymerase chain reaction (PCR) technology (RainDance). This technique provides for amplification of a large number of targeted regions in a cost/time effective manner and is considered ideally suited for identification of monogenic/rare mutations. Here primer library is combined with each sheared genomic DNA sample into droplets on a RDT1000 platform. Droplets are collected and amplified through PCR. Amplification products are recovered by breaking the emulsion (Figure 1.5). The **Access Array** system developed by Fluidigm, on the other hand, uses an integrated fluidic circuit (IFCs) and controllers to process samples. Before PCR is performed, the samples and primers are combined in the pre-PCR IFC Controller AX, which is then placed into the FC1 Cyclor for amplification. Ion **AmpliSeq** and **TruSeq** use a highly multiplex PCR method to amplify regions of interest.

Likewise there are several different next generation sequencing platforms available in the market. Each one employs a different sequencing chemistry such as pyrosequencing by Roche 454, reversible dye terminator chemistry by Illumina/Solexa and sequencing by ligation that has been introduced by Life Technologies. Demand of highly efficient and low cost technologies has resulted in development of several novel technologies leading to an era of third generation sequencing. As expected, the third generation sequencing platforms are characterised by new chemistry allowing less operation time and low cost. There are three noticeable third-generation sequencers in use: the Pacific Biosciences' realtime single molecule sequencing (PacBioRS) [320, 321], Complete Genomics' proanchor hybridization and ligation (cPAL) [322, 323], and Ion Torrent of Life Technologies Inc [324]. Recently, Oxford nanopore technology has employed a totally different sequencing approach where alterations in conductivity across the nanopore are measured as single DNA molecules pass through the membrane. In short, there has been an exponential improvement in the NGS technologies over the past few years in terms of efficiency and cost.

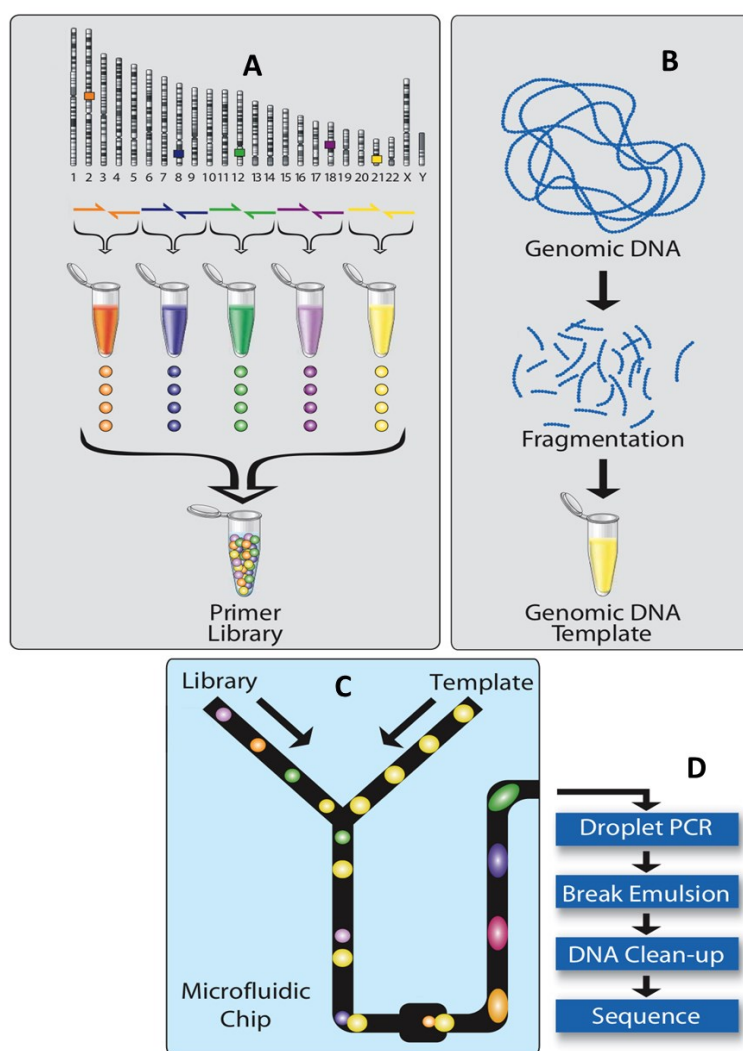


Figure 1.5 Microdroplet PCR-based target enrichment (RainDance technology) workflow.

(A) Primer library (forward and reverse primers) for each genes/regions of interest is designed using a proprietary pipeline. (B) Genomic DNA is fragmented, purified and end-repaired. (C) Purified genomic DNA droplets and primer pair droplets coated flow through the designated channels of the microfluidic chip of RDT1000 platform and reach the merge area where an induced electric field make two discrete droplets to combine to make a PCR droplet. (D) PCR droplets directly collected into a PCR tube are amplified on standard thermal cycler followed by emulsion breaking to release the PCR amplicons followed by purification and NGS sequencing. (From Tewhey, Warner et al. 2009 [325]).

1.6.2 CNV detection and validation

Copy number change at genome wide level can be inferred by employing array based techniques such as array competitive genome hybridization (aCGH) or genome wide SNP array. CNV's from targeted region of interest can also be detected by using multiplex ligation-dependent probe amplification (MLPA) or quantitative real-time PCR assays. Both techniques offer non-array based procedures for CNV detection.

1.6.2.1 Array based techniques

Array based methods such as array comparative genomic hybridization (aCGH) as well as SNP microarrays technologies infer copy number changes by comparing the test sample to an appropriate reference sample or population.

Array competitive genome hybridization (aCGH)

Platforms for this assay rely on the principle of comparative hybridization of test and reference samples. The signal ratio of both reference and test samples is normalised and is used to infer changes in copy number in the test sample. An increase in this ratio at any region will represent an increase in copy number in the test sample compared with the reference and similarly a decrease would signal a loss in copy number. Obviously use of well characterized reference sample is very crucial for accurate interpretation of data generated by array CGH [326].

SNP microarrays technologies

SNP arrays also follow hybridization method but unlike aCGH, these are performed on a single sample per array. In SNP genotyping arrays, highly specific probes are designed complementary to the test DNA with a difference in one nucleotide. The bases of interest are detected by single base extension method (Illumina) or by differential hybridization (Affymetrix). Since the genotyping data presented in this thesis have been generated on the Illumina platform a brief introduction to the Illumina workflow and data output is given as follows.

Illumina utilizes the proprietary beadchip technology. An individual Infinium bead assay genotypes a locus using two colors read system where one colour represents each allele. More than 5 million genotypes for a single individual can be assayed simultaneously on the surface of

each bead chip. These silica beads housed in with carefully framed micro wells are coated with oligonucleotides probes that target a particular locus in the genome. Sample DNA fragments are allowed to hybridize with the probes attached to bead chip and each probe would bind to a complimentary sequence in the sample on a location one base prior to the locus of interest. Allele specificity is conferred by a single base extension that incorporates one of the four labeled nucleotides followed by fluorescent staining. Once laser excites the nucleotide, the labels emit a signal that is detected by illumina scanner. Intensity value of each color conveys information of the allelic ratio. Data can then be analysed by GenomeStudio software to call genotypes and evaluate copy number variations across the genome (Figure 1.6).

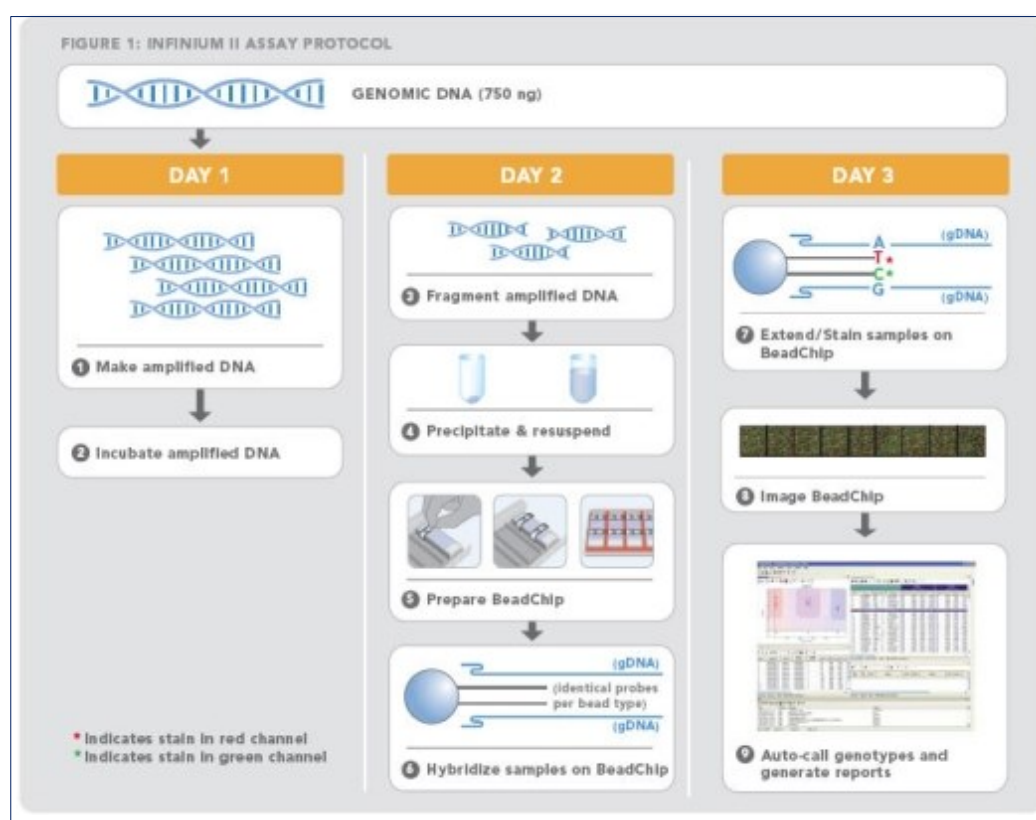


Figure 1.6 Schematic representation of the Illumina Infinium assay genome-wide SNP genotyping workflow. (From www.illumina.com).

For each SNP the two color-read out system gives an intensity value corresponding to each color. Polar transformation of these intensity values provides us the normalized intensity value (R), and allele intensity ratio (θ). Using these parameters on genoplot, LogR ratio and B Allele frequencies can be determined.

Log R ratio (logged ratio of the observed R) representing ratio of the sum of normalized signal intensity at each locus to the expected, is a matrix that normalises the signal intensities for CNV analysis. Expected allele frequency is computed by linear interpolation of the observed allele intensity ratio (θ) with respect to three canonical genotype clusters (genotypes AA, AB, and BB) from normal population data. A log base 2 transformation of this ratio yields the log R ratio:

$$\text{Log R Ratio} = \log_2(R \text{ observed}/R \text{ expected})$$

The BAF represents the frequency at which the B allele is called at a SNP, interpolated from the θ , by using the formula given below:

Eq no.	Sample θ is	B Allele Freq
1	<mean θ of AA cluster	0
2	between AA and AB	$0.5(\theta_{\text{SNP}} - \theta_{\text{AA}})/(\theta_{\text{AB}} - \theta_{\text{AA}})$
3	between AB and BB	$0.5 + 0.5(\theta_{\text{SNP}} - \theta_{\text{AB}})/(\theta_{\text{BB}} - \theta_{\text{AB}})$
4	>mean θ of BB cluster	1

Generally, both BAF and LRR are taken into account to determine CNV's. For BAF, deviation from 0, 0.5, or 1 (alleles AA, AB, and BB, respectively) may suggest the presence of an imbalance of copy number whereas, deviation of the LRR from an average of 0 suggests a higher or lower signal intensity than expected in the two chromosome copies (Figure 1.7).

Various statistical tools have been developed and used to analyse genotyping array data to infer copy number. These software packages/tools employ a range of statistical modelling approaches. An often used model for such analytical softwares is the Hidden Markov Model (HMM) that integrates data from multiple sources to infer CNV calls. Thus this model is designed to detect CNV by determining the hidden sequence of copy number state from observed LRRs and BAFs.

The software tools for CNV that implement HMM include QuantiSNP [327], PennCNV [328] and GenoCN [329]. Among these, PennCNV is more efficient in identifying large CNVs and generally has the lowest false discovery rate as compared to other similar tools [330].

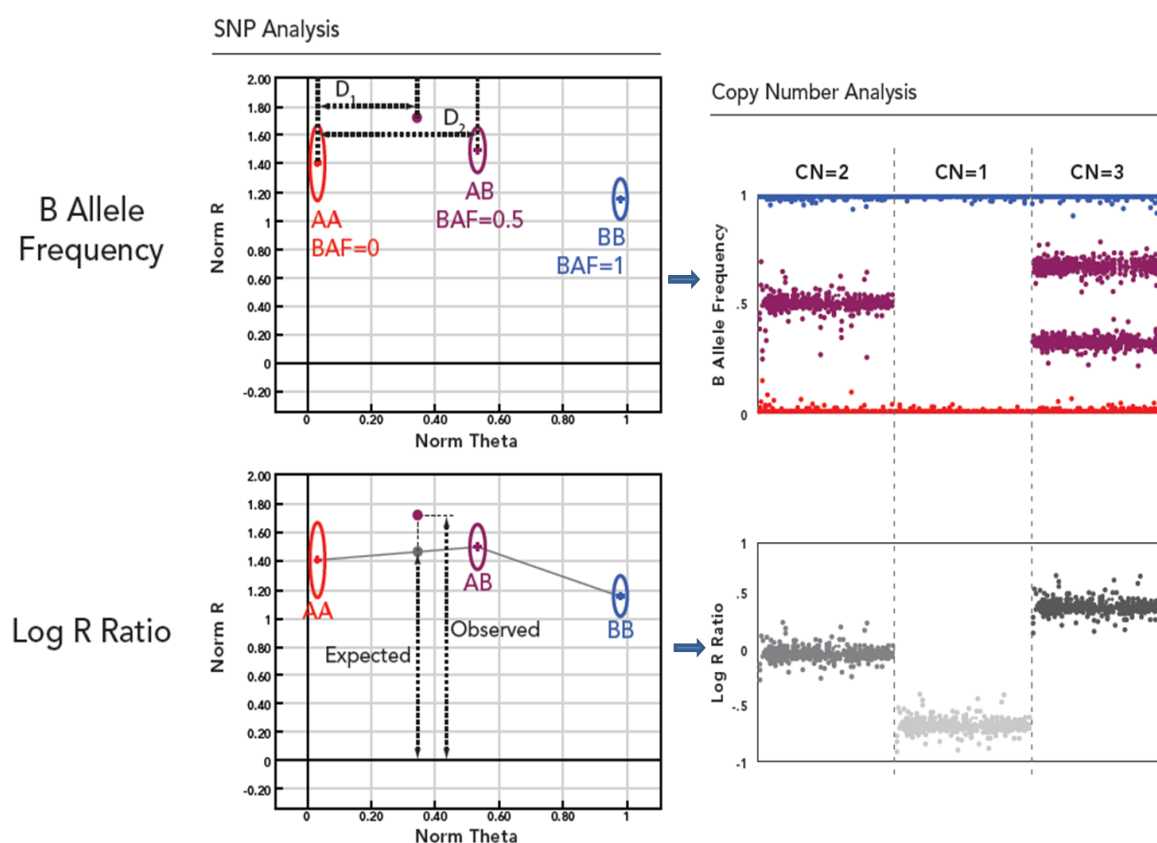


Figure 1.7 Derivation of the Log R Ratio (LRR) and B allele frequency (BAF) signal intensity measures by Illumina's GenomeStudio Algorithm.

The BAF is calculated using the subject theta value and a linear interpolation of the cluster defined B-allele frequencies assigned to each cluster (0.0, 0.5, 1.0). LogR ratio (log2 R ratio) is calculated as a ratio of observed normalised subject intensity to expected intensity. Where expected, intensity is calculated from observed allelic ratio with respect to linear interpolation of canonical genotype clusters AA, AB and BB. (Modified from Peiffer, Le et al. 2006 [331]).

1.6.2.2 Non-array based techniques

Multiplex Ligation-dependent Probe Amplification (MLPA)

Multiplex ligation-dependent probe amplification (MLPA) is a multiplex polymerase chain reaction-based method to infer copy number change in the desired regions. It is most frequently used non-array based method that provides a rapid and cost-effective procedure for copy number quantitation. Frequent use of this technique is also seen for validation of CNV detected by sequencing and genotyping data [256, 257]. Methylation-specific MLPA (MS-MLPA) has also been employed to detect changes in both DNA copy number and DNA methylation [332]. This technique has proved its usefulness for detecting disease related to imprinting disorders [333] .

A notable feature of MLPA that distinguishes it from other non-array based CNV detection methods is that in MLPA assay the probes attached to target sequence are amplified. The resulting products are fragments of different length (130 - 480 nt in length) that can be detected and analysed by capillary electrophoresis (Figure 1.8). Analysis of data includes normalizing peak values and comparisons with peak patterns obtained from reference samples to identify change in copy numbers. Analysis of MLPA can be performed by MLPA analysis software such as coffalyser.net (developed at MRC-Holland).

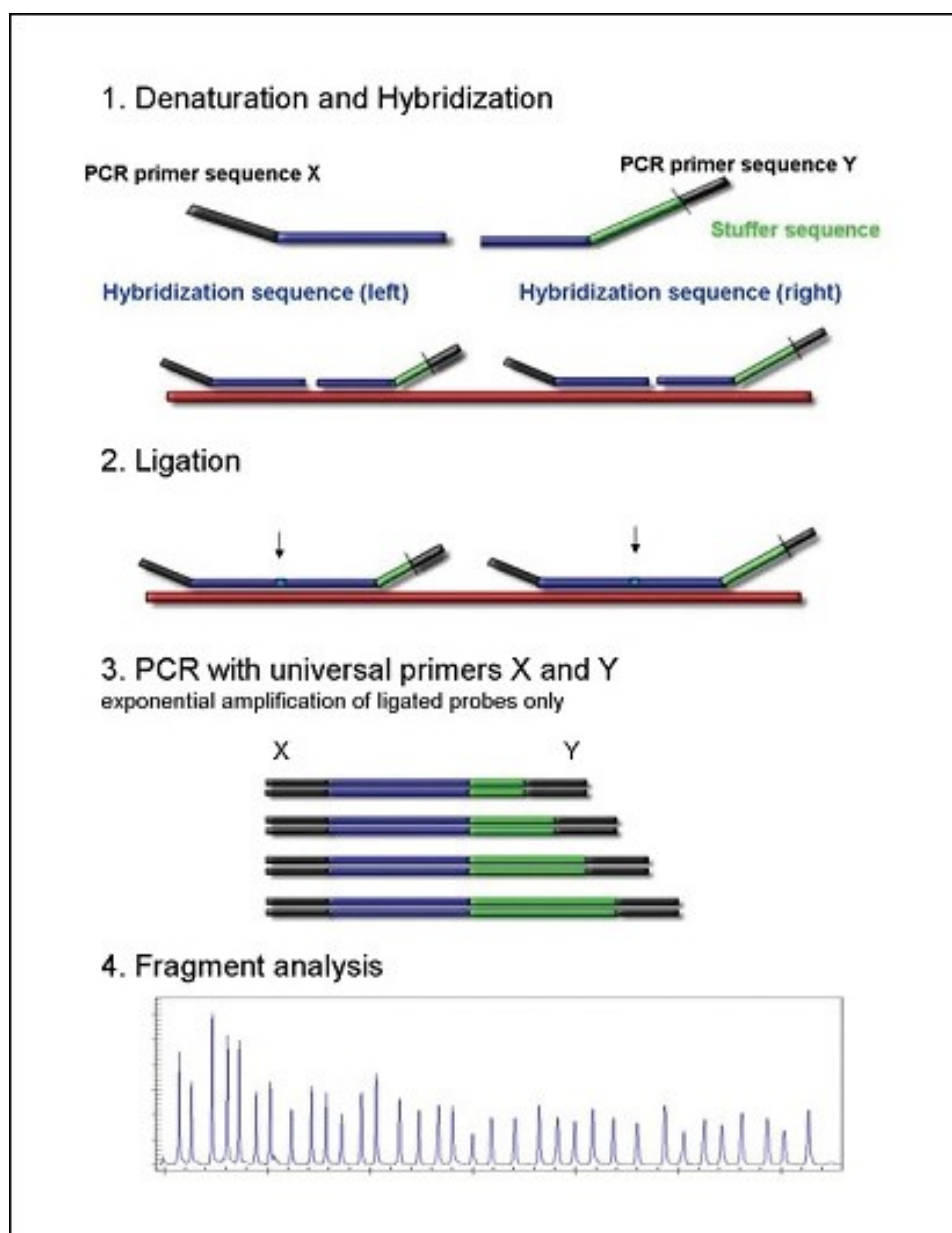


Figure 1.8 Multiplex Ligation-dependent Probe Amplification (MLPA).

Each probe set contains two specific oligonucleotides – short and long. The long probe nucleotide has stuffer sequence of variable length. In the first step the probes are hybridized with genomic DNA. Single base PCR reaction with one primer pair performs ligation reaction that is followed by PCR amplification with fluorescent labelled universal primer pair. High resolution capillary electrophoresis enables separation of PCR products in the form of electropherograms that are then normalised to infer copy number change in the test sample. (From www.mlpa.com).

Quantitative real-time PCR or real-time PCR (RT-PCR) is a refined version of the conventional PCR. It is used to amplify a targeted DNA segment and to simultaneously quantify the amplified product by monitoring the increase in fluorescence dye as the reaction progresses and the PCR product accumulates.

Conventionally qPCR is carried out by binding of a dye (such as SYBR Green) to the double stranded DNA [334], and each PCR cycle results in an increase in fluorescent intensity that is measured with a detector [335]. The method provides low cost and rapid detection of any change in DNA. Real time qPCR method was improved by employing a labelled probe complementary to the target DNA sequence such as in qPCR assays based on TaqMan chemistry (Applied Biosystems) [336]. These fluorogenic probes eliminate chances of any non-specific binding. qPCR can be used with advantage

for amplification of a region of interest with unknown copy number and a reference locus with known copy number using equal quantities of total starting DNA of each of the samples.

Other widely applied techniques for copy number detection include conventional karyotyping and fluorescent *in situ* hybridization (FISH) [337] and digital PCR (dPCR). The latter technique has greatly improved the limit of detection for copy number change and has been regarded to have a high level of accuracy for CNV analysis [338, 339].

1.6.2.3 Additional approaches for identification of highly penetrant or Mendelian mutations

Identification of a causative genetic variant for a disease that follows Mendelian inheritance seems to be less laborious compared to conditions that arise due to multigene effects. Nonetheless, investigating monogenic variants causing human disease still remains a major challenge.

Among the different approaches that have been adopted to identify causative mutations in monogenic disease, position mapping is a significant procedure that has resulted in determining a large number of genes associated with Mendelian diseases [340]. Position mapping identifies

physical locus of the gene in the human genome in a hypothesis free manner. There are two main methods through which position mapping can be achieved – linkage analysis and autozygosity mapping.

Positional mapping approach initially relied on genotyping of highly polymorphic microsatellites markers (STR) to pin down potentially causative intervals by a rather laborious process. The use of microsatellite markers has now been replaced by high-density genome-wide single nucleotide polymorphism (SNP) array offering a rapid and relatively accurate identification of chromosomal regions. The present era of NGS technologies has once again revived interest in genetic mapping to identify disease genes [341].

Several groups are taking advantage of the power of linkage analysis and homozygosity mapping and performing NGS experiments in parallel with these positional mapping techniques for the identification of disease genes. More recently attempts have been made to infer mapping from sequencing data [342].

Linkage analysis

Linkage analysis involves the mapping of those genomic regions that have a tendency to be transmitted together to the next generation without undergoing recombination during the meiosis event. The technique has been widely in use for many years as a first step in identifying the location of rare variants associated with a disease that follows Mendelian inheritance. It permits assigning numerical values to these regions in the form of LOD (logarithm of odds), which is a statistical presentation of a likelihood of that region being linked hence inherited together, and also reflects the probability of a certain region encompassing the disease gene. LOD score of 3 (or sometimes 3.3) is conventionally used as a cut-off that corresponds to a sample significance level of 0.0001 [343, 344]. However, large pedigrees with multiple affected individuals are needed to efficiently perform such an analysis.

Although this approach did not seem much useful for identifying recurrent genomic regions that accounts for milder form of obesity [345] as described above in the polygenic obesity section, it has been a highly effective strategy for identifying rare loci that carry variation exhibiting large effect. More recently cost effective availability of NGS has revived the interest in utilising this

approach to attain causative variant identification so that linkage analysis is once again in the forefront of studies on familial genetics [341].

Homozygosity mapping

In the context of autosomal recessive disorders, consanguinity offers a powerful approach/tool for disease gene mapping called homozygosity mapping [346]. Homozygosity mapping is a variation on linkage analysis that relies on the fact that the affected individuals from a consanguineous parentage are expected to be homozygous for causative alleles identical by descent (IBD). Homozygosity is not only confined to the specific disease locus but extends to the region spanning the disease locus. Searching for such blocks of homozygosity (regions of IBD) that are exclusively shared by the affected individual/s are expected to provide a powerful way to infer the candidate regions that include the associated allele/variant.

High-density single nucleotide polymorphism (SNP) array data can now be used to infer genome-wide homozygosity directly, by detecting contiguous homozygous segments in the genome referred to as genomic runs of homozygosity (ROH). Such ROH regions that are common in human population are assumed to be homozygous-by-descent [347] and can be inferred by publically available packages such as Plink [348].

In this era of next generation sequencing, this approach has been further revamped by pairing with WES or WGS experiment. This powerful approach of homozygosity mapping has provided us with additional evidence in our attempt to identify causative genetic variant in recessive disease aiming to narrow down the list of variants and to allow us to focus on only those genetic variants that lie in a critical locus homozygous in the affected subject. This provides a useful lead in the quest to identify the causal variant thus warranting their identification in inbred families as exemplified by Schrader et al [349], Puffenberger et al [350] and Wang et al [351] amongst several others.

Inbreeding and inbreeding depression

Inbreeding may be defined as 'production of offspring from the mating of individuals related by lineage'. Charles Darwin who published his theory of evolution, in 'Origin of Species' in 1859, was perhaps the first person to point out and document the harmful effects of inbreeding. In the same

era (late 19th century) the investigation of genetic transmission of characters or disease had also begun to emerge through the work performed by Gregory Mendel mainly through his breeding experiments on pea plants. Mendel, by taking into account the characteristics of the progeny, determined the inheritance of single traits as transmitted from parents to the offspring that are now referred to as the laws of Mendelian inheritance. The negative effects on fitness traits due to inbreeding can now be explained on the basis of increased chances of holding two mutated copies of an ancestral allele and an increase in homozygosity of such deleterious mutations leads to what is generally termed as inbreeding depression.

1.7 Global distribution of known consanguineous populations and major communities

The risk of carrying congenital abnormalities has been estimated to be ~2 times greater in offspring of consanguineous parents than those of unrelated parents [352, 353]. Moreover, consanguinity/inbreeding is considered to be the single most common cause of genetically related mortality due to increased chances of homozygosity of deleterious recessive mutations [354]. Regardless of the knowledge that inbreeding increases the risk of serious recessive disease, consanguineous marriages are the norm in 10 percent of the population and are widespread in many parts of the world [355]. The most predominant form of consanguinity is the first cousin union.

Presently, consanguineous unions are frequent in countries of Asia, Africa and the Middle East and in many smaller populations confined to certain geographical isolates [354, 356]. While these marriages are considered rather unusual in modern Western culture, consanguinity was not uncommon in the West until the latter half of the 19th century [357]. Although there is no religious restriction in most of the religions, consanguineous marriages are predominantly common in Muslim majority countries [358]. Whereas a decline in the practice has been observed in parts of Asia, Africa and the Indian subcontinent during the past years, there has only been a slight drop of consanguinity in the Muslim countries [6, 352, 359, 360]. Preference for consanguineous marriage amongst Muslim communities appears to be due to social and cultural traditions, rather than religious considerations.

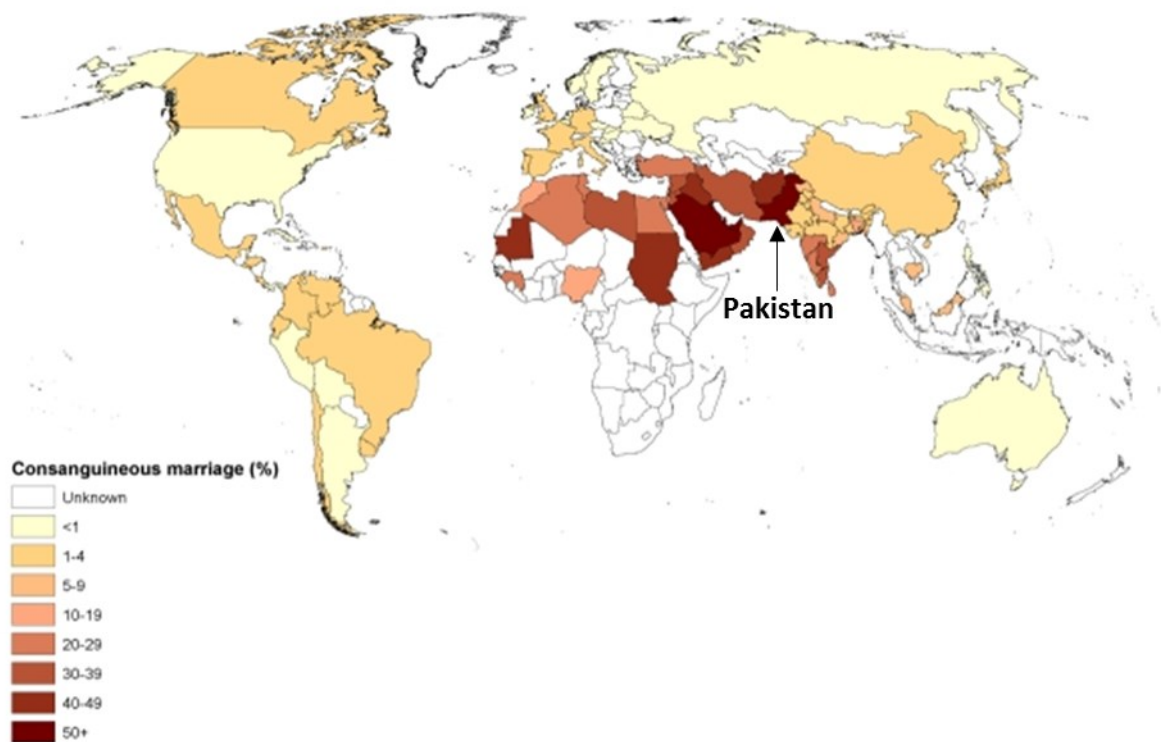


Figure 1.9 prevalence of consanguinity in countries around the world. Pakistan (by arrow) has >50% frequency of consanguineous marriages. (From Romeo and Bittles 2014 [361]).

Pakistan, a South Asian country, has one of the highest rates of inbreeding and prevalence of marriages between cousins or close relatives has been estimated to be between 60-70% cases [362, 363] (Figure 1.9). The practice is more predominant in rural areas and village isolates [354] and has been preferred over generations due to social and economic advantages such as protection of wealth, land and property and preservation of family norms and traditions [364].

Pakistan's population comprises many ethnic groups as a result of successive waves of migration from its neighbouring countries, mainly Iran, Afghanistan and India, during its history. The major ethnic groups include Punjabi, Sindhi, Pashtun, Baluchi and Mohajirs [354]. This ethnic composition also corresponds to the linguistic and to a large extent regional distribution. These major ethnic groups are composed of castes or sub-ethnic groups [356, 365].

Studies in UK showed that incidence of congenital anomalies and rates of infant deaths are markedly higher in children of Pakistani origin as compared to those of British and other ethnicities [353, 366]. A large study to estimate the incidence of congenital abnormalities in different ethnic groups was conducted in Bradford on pregnant mothers and babies born between 2007-2011 (the Born in Bradford Study) [353]. In this investigation, of 5127 babies born to mothers of Pakistani origin, 1922 (37%) were the outcome of first cousin unions. The study reported mortality in 9.7 per 1000 children of Pakistani origin as compared with a national rate of 5 in 1000. Majority of the deaths in Pakistani children were ascribed to chromosomal, genetic and congenital anomalies [367]. The study concluded that consanguinity doubles the risk of incidence of congenital anomalies. Consanguinity has also been accounted as the major risk factor of heritable anomalies irrespective of the social and economic status or life style [353].

In a separate investigation Morton et al [368] reported a significantly higher incidence of severe disabilities including hearing loss, learning disability and visual impairment in Pakistani children from Derbyshire. The incidence of these disabilities was shown to be 2% in children of Pakistani origin as compared to 0.56 to 0.87% in children of other nationalities [368].

1.8 Rationale and objectives of the study

Although recent technological advancements have evidently accelerated the pace of disease gene identification, yet we are still way off to narrow down the gap of missing or unexplained heritability of obesity [43, 102]. In recent years, a large part of the successes in unravelling the genetic basis of obesity has been derived from studies of the extreme phenotype caused by a disruption of the neuroendocrine pathways controlling appetite and energy homeostasis [71]. Inbred human populations offer a unique background for the discovery of genetic factors underlying many of the heritable anomalies including obesity. Indeed the first direct evidence of a homozygous recessive mutation in a gene (*LEP*) associated with severe obesity in human came from a consanguineous family of Pakistani origin [125]. Chances of occurrence of a deleterious recessive mutation in an individual in the homozygous state depend largely upon the level of inbreeding. Study of homozygosity in individuals with a long history of consanguinity, has shown that 11% of their genome may be homozygous resulting in a high risk of incidence of autosomal recessive disease [363]. Indeed, cousin marriages cause union of genomes, 1/8th of which is similar, leading to an increase in the coefficient of inbreeding (percentage of genome that is homozygous or identical by descent) and greater chances in incidence of recessive disorders in the offspring [369]. It is expected that genetic analysis of consanguineous families that has led to the identification of a number of rare variants with large effect could also be helpful in revealing recessive variants with a small or subtler effect size.

No systematic analysis to elucidate genetic causality of severe obesity has so far been carried out in specific predominantly consanguineous populations. Previously only 4-6% of randomly selected severely obese patients have been identified with monogenic obesity. Undertaking an intensive search to identify the genetic basis of severe obesity in a consanguineous population like that of Pakistan, therefore, appears in order to not only in furthering our knowledge of the genetic basis of obesity, but also to possibly bring in focus potential molecular targets in the perspective of an emerging era of personalised medicine.

In the context of aforementioned the main objectives of the study are summarised, as follows

- Estimate the proportion of monogenic forms of disease among cases of early onset severe obesity from a consanguineous population of Pakistan.
- Identify known and novel genetic variants in previously identified candidate genes for monogenic obesity.
- Elucidate the contribution of genomic structural variations in severe obesity.
- Identify novel genes that have not previously been associated with severe obesity in the human.
- Develop a rapid and cost-effective molecular diagnostics for severe obesity.

2. Chapter two

Materials and Methods

2.1 Study subjects

This investigation is based on 175 unrelated probands with early onset severe obesity with a BMI SDS for age ≥ 3 from consanguineous families. In addition, 380 family members were also included in the study. The subjects were mainly recruited from the Children's Hospital Lahore, Mayo Hospital, Lahore, and Allied Hospital, Faisalabad, Pakistan, following patient/parent written informed consent. The study protocol was approved by the institutional ethical committees. During the period under review, I visited Pakistan at four occasions to work with physicians and collaborators where I made regular visits to outdoor paediatrics clinics to recruit new subjects. Moreover, field visits were arranged to get samples from the patients and/or their family members. Probands and/or their parents were interviewed to obtain their family and medical history and to construct pedigrees spanning at least 3 generations. Anthropomorphic measurements and physical examination were carried out and blood samples were obtained in each case for subsequent DNA extraction and hormone estimations.

2.2 DNA extraction and quantification

Initially extraction of genomic DNA from peripheral blood leukocytes of 82 probands and 184 family members was carried out by myself using Gentra Puregene Blood Kit (Qiagen, Crawley, UK) as per instructions of the manufacturer. Subsequently I quantified the extracted DNA by NanoDrop (2000c, Thermo Scientific, Wilmington, DE, USA) and by qubit system. For DNA extraction of rest of the samples of 93 probands and 196 family members, DNA extraction facility from LGC genomic service (LGC Middlesex, UK) was used.

2.3 Genetic analysis

2.3.1 Screening of *LEP* and *MC4R* mutations

At the first step of genetic analysis, I performed genetic screening for *LEP* and *MC4R* genes. Coding exons of both genes were amplified followed by Sanger sequencing using BigDye terminator kit

(Applied Biosystems, Foster City, CA, USA) on an ABI 3730xl DNA sequencer. Exons 2 and 3 of *LEP* were amplified using 2 sets of primers. Exon 2 forward primer, 5'-GATGCATTTCAATACATATGTAG-3' and reverse primer, 5'-GTTTCTTGACTATCTGGGTCCAGTGC-3'; exon 3 forward primer, 5'-GCACTTGTTCTCCCTCTTCCT-3' and reverse primer, 5'-GTTCCCTCCCTTAACGTAGTCCT-3'. For exon 2, PCR was performed using AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), under the following conditions: 95° C 8 min, followed by 39 cycles of 95° C 30 s, 56° C 40 s, 72° C 1 min 10 s, followed by 72° C 10 min. Amplification of exon 3 of leptin gene was performed by using Clontech (Clontech Laboratories Inc, Mountain View, CA, USA) under the following conditions: 95° C 1 min, followed by 30 cycles of 95° C 30 s, 68° C 1 min, followed by 68° C 3 min. Coding region of the *MC4R* was amplified using the forward primer 5'-GTGAGCATGTGCGCACAGATTC -3' and the reverse primer 5'-GATATTCTCAACCAGTACCCTACA -3'. PCR was carried out using BioTaq (Bioline, London, UK) under the following conditions: 95° C 2 min, followed by 35 cycles of 95° C 40 s, 56° C 40 s and 72° C 1 min 20 s. PCR products were sequenced using BigDye Terminator kit (Applied Biosystems) and analysed on ABI 3730xl DNA sequencer. The sequencing reads were assembled and analysed by Mutation Surveyor DNA Variant Analysis software (SoftGenetics, State College, PA).

2.3.2 Microdroplet PCR-based enrichment and next generation sequencing (NGS).

To identify rare and novel variants in 27 genes associated with obesity, subjects that were found negative for *LEP* and *MC4R* mutations were further investigated by using PCR based microdroplet enrichment (RainDance Technologies) followed by NGS. The experiment was performed during my visit to CNRS UMR8199, Lille, France.

2.3.2.1 Primer Library Design

We selected 27 susceptible genes for monogenic and syndromic form of obesity from published material for this experiment. Table 2.1 provided the list of these genes. Subsequent to selection of genes, we designed the Primers for coding regions of all 27 genes included in the experiment using proprietary pipeline developed by RainDance Technologies (Lexington, MA). Moreover, 40 bp of flanking region of each exon and 1000 base upstream and downstream of first and last exon respectively were also included. For primer designing following criteria were used: primer length between 15 and 33 bp; namely guanine-cytosine content between 25 and 80%; melting

temperature between 56 and 60 C; amplicon length between 200 and 600 bp. The 355 targeted exons from 27 obesity associated genes were covered by 664 primer pairs that were encapsulated into microfluidic droplets via RainDance Technologies.

Laboratory work subsequent to primer designing that involved PCR enrichment and library preparation was performed during my visit to CNRS. Lille, France.

2.3.2.2 Microdroplet-Based PCR Enrichment

Three µg of DNA was fragmented to attain a length of 2–4 kb by sonication (Bioruptor NGS; Diagenode, Liege, Belgium) and purified using the MinElute system (Qiagen, Valencia, CA). The fragmented and purified DNA was assessed for quality and quantity by using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and ends were repaired. Subsequently, the primer library was combined with each sheared genomic DNA sample on the RDT1000 (RainDance Technologies), according to the manufacturer's protocol. A microdroplet-based PCR of 664 primer pairs encompassing 355 coding exons was performed. To release the PCR product, destabilizer reagent (RainDance Technologies) was added that resulted in the degradation of the emulsion. This was centrifuged briefly to separate oil from PCR product, which was then subjected to purification using MinElute columns (Qiagen). Final assessment of both quality and expected amplification profile was assessed on the 2100 Bioanalyzer (Agilent Technologies).

All samples enriched with targeted amplicons were sequenced on a HiSeq2000 (Illumina) in paired-ends. Reads were aligned to the Human Genome (hg19/GRC37) using the Burrows-Wheeler Aligner.

2.3.2.3 Library preparation and next generation sequencing

End repairment and concatenation of PCR product in order to get blunt ends was performed by NEB Quick blunting kit and the NEB Quick ligation kit (New England Biolabs, Ipswich, MA). The samples were purified using Agencourt AMPure XP beads (Beckman Coulter, Fullerton, CA) and sonicated to achieve 200 bp fragments (Bioruptor NGS; Diagenode). Adaptors were ligated to the fragments by using NEBNext Multiplex Oligos for Illumina (New England Biolabs). Finally, the

amplicon-enriched samples were sequenced on the HiSeq2000 (Illumina, San Diego, CA) in 76-bp paired-end reads by multiplexing 12 samples per lane.

2.3.2.4 Data Analysis

Bioinformatics expertise at Lille (CNRS UMR8199) was used for data analysis sequence. Briefly demultiplexing of sequence data were performed with CASAVA (version 1.8.2). Reads were aligned to the Human Genome (hg19/GRC37) using the Burrows-Wheeler Aligner (BWA-SW, version 0.6.1). SAMtools (version 0.1.18; algorithm "pileup" was employed for the variant calling.

2.3.2.5 Variant Prioritization

Following detection of all variants in the targeted regions, I carried out the filtration steps focusing on novel, nonsynonymous and homozygous variations. Also, novel variants present in splice donor and acceptor site were considered. Furthermore, I focused on heterozygous variants on those genes that are reported to show dominant models and show their effect in heterozygous state as well.

Any novel mutations identified by microdroplet-based PCR target enrichment and next generation sequencing were subsequently confirmed by me using conventional Sanger sequencing.

Table 2.1: List of genes for targeted sequencing using microdroplet PCR enrichment

No	Gene	Gene location	No of amplicons	Target size (bp)
1	<i>LEP</i>	7q31.3	13	5,577
2	<i>LEPR</i>	1p31	41	13,803
3	<i>MC4R</i>	18q22	7	3,488
4	<i>MKKS</i>	20p12	16	6,123
5	<i>MKS1</i>	17q22	23	5,440
6	<i>NTRK2</i>	9q22.1	59	22,705
7	<i>PCSK1</i>	5q15-q21	29	9,761
8	<i>POMC</i>	2p23.3	9	3,495
9	<i>SDCCAG8</i>	1q43	22	5,517
10	<i>SIM1</i>	6q16.3-q21	19	6,545
11	<i>TRIM32</i>	9q33.1	13	5,819
12	<i>TTC8</i>	14q31.3	20	4,953
13	<i>WDPCP</i>	2p15	25	6,209
14	<i>ALMS1</i>	2p13	45	16,072
15	<i>ARL6</i>	3q11.2	15	3,953
16	<i>BBS1</i>	11q13	24	6,123
17	<i>BBS2</i>	16q21	22	5,664
18	<i>BBS4</i>	15q22.3-q23	22	5,292
19	<i>BBS5</i>	2q31.1	22	5,583
20	<i>BBS7</i>	4q27	29	8,232
21	<i>BBS9</i>	7p14	29	7,169
22	<i>BBS10</i>	12q21.2	12	5,683
23	<i>BBS12</i>	4q27	13	5,582
24	<i>BDNF</i>	11p13	31	14,038
25	<i>CEP290</i>	12q21.32	59	12,517
26	<i>GNAS</i>	20q13.3	35	11,439
27	<i>SH2B1</i>	16p11.2	10	6,692

2.3.3 Whole Exome Sequencing (WES)

2.3.3.1 Target enrichment and library preparation

A subset of probands and their family members that has previously been screened for 27 obesity associated genes by Sanger sequencing and RainDance targeted sequencing techniques was selected for WES. For target enrichment and subsequent library preparation, instructions provided in user manual were followed. First experiment for target enrichment and library preparation on 4 samples for WES experiment was performed by me in our laboratory of Genomics of Common Disease. Briefly, the genomic DNA samples were fragmented to approximately 200 bp fragments by sonication. Ends were repaired and adenine bases were added to the 3' end of the DNA fragments. Following this, we ligated the indexing-specific paired-end adapter and amplified the adapter-ligated library. Hybridization: Following quality control, the prepared library was hybridized using SureSelect Human All Exon kit (Agilent Technologies Inc, Santa Clara, CA, USA). After hybridization in solution and capture on streptavidin-coated magnetic beads, the hybrid-selected DNA targets were purified by Dynal MyOne Streptavidin T1 (Invitrogen, Life Technologies Ltd, UK) and PCR-amplified to add index barcode tags for multiplexing. Samples were pooled for paired-end sequencing spread across 8 lanes of flow cell (multiplexed). For rest of the 36 probands and 59 family members, the target enrichment and library preparation through the same procedure, was performed in Lille using Bravo automated liquid handling platform (Agilent Technologies).

Bioinformatics expertise at Lille (CNRS UMR8199 & Institut de Biologie de Lille) was utilized for the analysis of the raw data. Briefly the reads were aligned to human genome build hg19 with CASAVA 1.8.2, variants were called using CASAVA 1.8.2 and GATK 2.5 and annotated with Ensembl v71 and dbNSFP 2.0.

2.3.3.2 Variant Prioritization

After variant calling and annotation, I implemented the subsequent filtering and bioinformatics variant analyses in order to identify causative mutations. Variants from each family were filtered

independently to identify specific homozygous mutation in that affected individual. For this the filtration steps mentioned in Figure 2.1 were performed (as described in chapter 6 section 6.3).

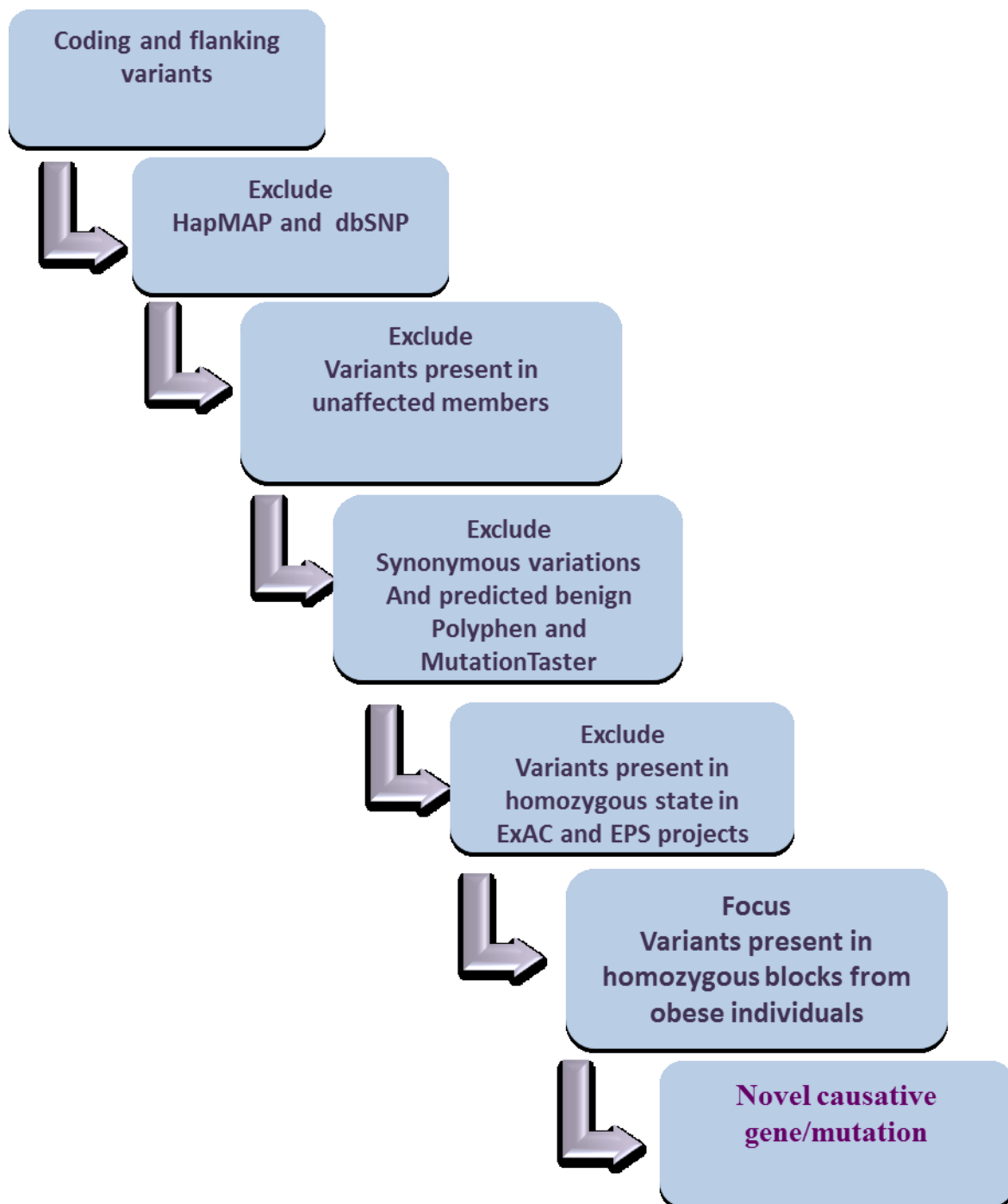


Figure 2.1 Step-wise filtration procedure carried out to identify causative variants.

2.3.4 SNP genotyping

Genome-wide high-density genotyping was performed by us in our lab facility, by using the HumanOmni 2.5S BeadChip KIT (Illumina Inc., San Diego, CA, USA) that contains over 2.5 million variants with capture capacity down to MAF 2.5%. The experiment was performed following instructions provided by the supplier. Briefly, the samples were denatured and whole genome amplified up to 1000-fold. Fragmentation step was carried out to cleave DNA into segments of 300-600 bp. DNA samples were purified and re-suspended prior to hybridization on bead chips followed by extension and staining (Xstaining). This procedure involves single base extension reaction using chain terminating dideoxynucleotides followed by the staining step. Subsequently, the beadchips were scanned and viewed on Illumina scanner to measure fluorescent intensity to enable genotype calls.

2.3.4.1 Creating genotyping project in genome studio and subsequent genotype calling

I created a genotyping project in the GenomeStudio using the standard cluster file and the genotypes were assigned to each SNP based on the florescent intensity data. One individual with less than 99% call rate was excluded from the study (Figure 2.2). Reproducibility and heritability report was also generated to make sure that parent to child heritability frequency (P-C Heritability Freq) is 0.99 or above. Finally the cluster file was exported and reports were generated for subsequent calling of runs of homozygosity as well as the CNV analysis.

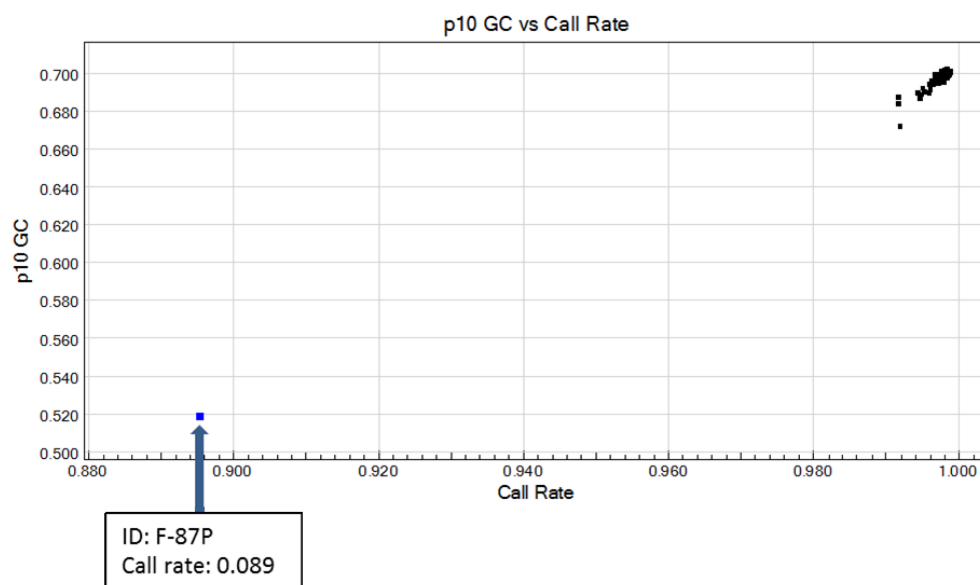


Figure 2.2 Scatter plot of call rate against 10% GenCall score

Cluster at the right top corner depicts pattern of samples behaviour. Better value of p10 GC depicts high quality of the reads.

2.3.4.1 Calling runs of homozygosity

As mentioned in introduction (section 1.6.2.3), linkage analysis or ROH are used to perform positional mapping. However, linkage analysis presents its usefulness when performed on a relatively large family with 2 or more affected individuals. Among all the families that have been included in WES analysis, only one family had more than one affected individuals. Therefore, homozygosity mapping was performed in the present case. This procedure consists of searching for a region of the genome that is autozygous in the inbred affected individuals. I performed the mapping of homozygosity using Plink open source whole genome association analysis toolset. The genotyping data were subjected to quality control so as to a) remove individuals missing more than 5% genotype data; b) exclude SNPs minor allele frequency (MAF=0.05); c) exclude markers that fail to comply with the Hardy-Weinberg test at a specified significance threshold (HWE=0.001) and d) exclude individuals and/or markers, on the basis of Mendel error rate (ME=0.05 0.1). Runs of homozygosity function (ROH) in Plink was utilized to identify regions where affected individuals were likely to have received twice the same ancestral ROH.

2.3.4.2 Copy number variation analysis by genotyping data

For the copy number analysis, I exported the called genotypes, B allele frequency (BAF) and log R ratio from GenomeStudio using default clustering files for each SNP. PFB (population frequency of B allele) was calculated based on the BAF of each marker. Identification of CNVs in this data set was carried out by me using PennCNV, an algorithm which employs a hidden Markov model for CNV prediction [328]. The Perl script Clean_cnv.pl, also developed by PennCNV, was used to merge adjacent CNVs automatically in order to get large CNVs. CNVs identified by PennCNV were also checked for the presence using CNV Partition plug-in provided by illumina.

2.4 Methods related to chapter 3

2.4.1 Sanger sequencing to confirm mutation in *LEPR*

Following identification of potential causative variants identified by targeted NGS sequencing, I confirmed the mutations for their presence by Sanger sequencing via the 3730 × I DNA Analyzer (Life Technologies, Carlsbad, CA, USA). Primers, encompassing region of interest were designed using primer 3 software (Table 2.2).

Table 2.2: primers used to amplify *LEPR* regions of interest

<i>LEPR</i> primers	
c.1675G>A	
Forward primer sequence - Exon 10	GATATTGCTTGATGAATACAGATG
Reverse primer sequence - Exon 10	TCTAATGCAATTAACTCTTACATATT
c.1810T>A	
Forward primer sequence - Exon 11	TGTACTTCAGGGCCCTTTAGA
Reverse primer sequence - Exon 11	GAAGCACACACTTATGCAC
c.2396-1G>T	
Forward primer sequence - Exon 15	CTATTGTCATGACTAGATAATTAG
Reverse primer sequence - Exon 15	CAATATTACTGCAAACAAATTAGGCAC
c.40G>A	
Forward primer sequence - Exon 1	GAGACTTATCTATAATCCCTTTCC
Reverse primer sequence - Exon 1	TTAGGAGGTTGGGAAGGGTTTT

Amplifications of *LEPR* exon 10 and exon 15 to confirm c.1675G>A and c.2396-1G>T mutations, respectively, were performed by using BioTaq (Bioline, London, UK). A 30 cycle PCR program was performed with denaturation at 95 °C for 30 sec, annealing at 56 °C for 40s and extension at 72 °C for 90 sec. Finally elongation was performed at 72 °C for 10 min. While for confirmation of c.1810T>A mutation in exon 11 of *LEPR* gene and c.40G>A in exon1, amplification was performed using Clontech (Clontech Laboratories Inc, Mountain View, CA, USA) under the following conditions: 95 °C 1 min, followed by 30 cycles of 95°C 30 sec, 68°C 1 min, followed by 68°C 3 min. Sequencing reads were assembled and analysed for the presence of the variant using Mutation Surveyor DNA Variant Analysis software (SoftGenetics, LLC, USA).

2.4.2 Characterization of 42 bp deletion mutation (c.1-2del42) in *LEP*

To further characterise a 42 bp deletion in *LEP*, identified in one of the family through Sanger sequencing, 2 ml of blood was drawn in PAXgene Blood RNA tube and was shipped to me, under dry ice. I extracted the RNA from blood cells of the proband, his parents and sibling using PAXgene RNA Blood kit (Qiagen, Germany) in accordance with the manufacturer instructions. Following RNA extraction, I characterized the deletion mutation. Reverse transcription polymerase reaction (RT-PCR) was carried out to acquire full length cDNA fragments containing the coding regions of the *LEP*, using random 6-mers primer (2.5U μ m) and dNTP mix (10mM each; provided PrimerScript RT-PCR kit (TaKaRa, Ohtsu, Shiga, Japan) added to 1 μ g of the template RNA and making up the volume to 10 μ l. Denaturation/annealing was carried out at 65°C for 5min. A reaction mixture containing 4 μ l of PrimerScript buffer (5x), 0.5 μ l of RNAase inhibitor, and 0.5 μ l of PrimerScript RTase and 5 μ l of RNAase free water was added to 10 μ l of denatured product. The condition of RT-PCR were set as follows: 30°C for 10 min, 42°C for 25 min followed by 95°C for 5 min. The synthesized cDNA was amplified using the following conditions: 94°C for 1 min; 45 cycles of 94°C for 30 sec, 65°C for 35 sec, and 72°C for 1 min.

Gel electrophoresis, with 1% agarose gel, of the amplified product from heterozygous parents resulted in two bands corresponding to the mutated whereas one band was found in the sample from unaffected control subject. The bands from 3% agarose gel were excised using sharp scalpel and were purified by QIAquick Gel Extraction Kit (Qiagen, Germany) according to manufacturer's protocol and followed by direct sequencing using primers as given in Table 2.3.

Table 2.3: PCR primers used to amplify cDNA of *LEP*.

Primers to amplify cDNA	
5' Primer Sequence - Exon 1	CGCAGCGCCAGCGGTTGCAA
3' Primer Sequence - Exon3	GCTGCCACAGCATGTCCTGC

2.4.3 Deletion mapping ~59 kb homozygous region in *LEPR*

In this experiment I performed mapping of deletion break points. Long range PCR amplicon was generated using SequalPrep Long range PCR Kit (Invitrogen, Carlsbad, CA). PCR amplification was performed with following PCR conditions: 4µl of SequalPrep10X Reaction Buffer (including dNTPs), 2 µl of SequalPrep PCR Enhancer A, 0.8 µl of dimethyl sulfoxide and 0.4 µl of SequalPrep Long Polymerase. All were mixed and added to 1 µl of a 2.5 µmol/l of primer and reverse primers and 100 ng of genomic DNA. Thermal cycling was performed under following conditions: 94°C for 2 minutes for activation followed by 10 cycles of 94°C for 10 sec, 66°C for 30 sec and extension at 68°C for 3 min. This was followed by 25 cycles of 94°C for 10 sec, 64°C for 30 sec and 68°C for 3 min with 20 sec increase in time every cycle, 72°C for 5 minutes and hold at 4°C. The amplicon was purified with ExoSAP-IT (USB) and sequenced using BigDye Terminator Cycle Sequencing kit (BigDye terminator kit Applied Biosystems, Foster City, CA). To identify deletion breakpoints, starting from last known undeleted region on the flanking side of the breakpoints, as identified by genotyping array (Figure 2.3), primers were designed using primer 3 (Table 2.4) and direct sequencing of amplified product was made. Breakpoints were detected by reading sequences using Mutation Surveyor DNA Variant Analysis software (SoftGenetics, LLC, USA).

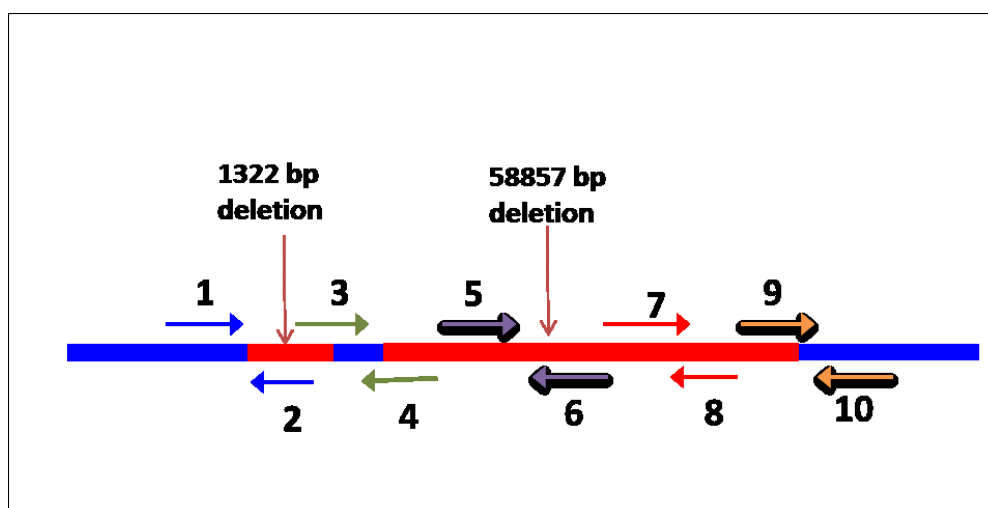


Figure 2.3 Primer spanning and within deleted region
Position of paired primers spanning and within the deleted region of the *LEPR* gene to identify deletions breakpoints.

Table 2.4: Primers used to check for deletion breakpoints of 1.3 Mb and 5.8 Mb homozygous deletions.

<i>LEPR</i> deletion primers	
Forward primer sequence 1	AGAGTTTGCTGATCCCAAGCTTAGAAC
Reverse primer sequence 2	CTAGCATATATATGAGCATCCAG
Forward primer sequence 3	CATTAAAGCCATGCAAATGTTGTTTC
Reverse primer sequence 4	AAGATGTTTAGGATCTGCACATTG
Forward primer sequence 5	GTCTTTACCACTTAAGGGGAGGGTAA
Reverse primer sequence 6	GTTTAACAGCAGTATATATTTACTTG
Forward primer sequence 7	TAAAATGCACAAGCCTTCGGTAT
Reverse primer sequence 8	GAAAATTCTCTATCCACAGATAG
Forward primer sequence 9	GCTTCCTCTTCACCTTCCATTG
Reverse primer sequence 10	AGAACATCAATTCGGTATAATAAG

2.5 Methods related to Chapter 4

2.5.1 Sanger sequencing

The potential causative variants in BBS genes that were initially identified by droplet based targeted sequencing, were confirmed for their presence by Sanger sequencing by myself. Primers, encompassing region of interest were designed using primer 3 software (Figure 2.5) and 3730 × l DNA Analyzer (Life Technologies, Carlsbad, CA, USA) was used to get sequence reads.

Table 2.5: Primers used to amplify *BBS* regions of interest.

<i>BBS2</i> primers	
c.406insG	
Forward primer sequence	GGGCCTGCATATTATCGTGTC
Reverse primer sequence	GAATTAACATTGTCTCCAGTAACC
<i>BBS10</i> primers	
c.271insT	
Forward primer sequence	GTGTCTGAAACGTTAGGAGAGC
Reverse primer sequence	TAAGATGTGGGAAGCCAGCC

Amplifications of *BBS2* to confirm c.406insG mutation was performed by using Clontech (Clontech Laboratories Inc, Mountain View, CA, USA) under the following conditions: 95°C 1 min, followed by 30 cycles of 95°C 30 sec, 68°C 1 min, followed by 68°C 3 min. Sequencing reads were assembled and analyzed for the presence of the variant using Mutation Surveyor DNA Variant Analysis software (SoftGenetics, LLC, USA). While for confirmation c.271insT in *BBS10* same enzyme was used but the PCR programme performed was by using BioTaq (Bioline, London, UK). A 30 cycle PCR program was performed with denaturation at 95°C for 30 sec, annealing at 56°C for 40s and extension at 72°C for 90 sec. Finally elongation was performed at 72°C for 10 min. While for confirmation of c.1810T>A mutation in exon 11 of *LEPR* gene and c.40G>A in exon1, amplification was performed 95 °C 3 min, followed by 35 cycles with denaturation at 95°C for 30 sec, annealing at 68°C for 45 sec and extension at 72 for 60 sec. Finally elongation was performed at 72°C for 10 min.

2.5.2 Multiplex Ligation-Dependent Probe Amplification

Subjects showing apparent heterozygous deletion 15q11-13 region were analysed using MLPA technique.

2.5.2.1 MLPA analysis using 343 probemix to confirm deletions/duplications in 15q11-13 region associated with Prader-Willi syndrome (PWS) and Angelman syndrome (AS)

I carried out the multiplex ligation-dependent probe amplification (MLPA) assay using P343 Autism-1 probemix (MRC Holland, Amsterdam, The Netherlands) to detect deletions/duplications in 15q11-13. These regions have previously been associated with autism and/or obesity. Principally MLPA is a relative technique and no conclusions regarding copy number change can be drawn from a single MLPA reaction of patient sample unless reference DNA samples are analysed simultaneously.

Purified DNA samples from two normal subjects were selected as reference samples as per manufacturer's instructions. Briefly, the procedure consisted of the following steps: 5ul of 150 ng genomic DNA rehydrated in DNA hydration solution (Qiagen, Crawley, UK), was denatured for 5 min at 98°C. After denaturation step, 1.5 µl of SALSA MLPA buffer and 1.5 µl of MLPA P343 probemix were added into each sample. The probes were allowed to hybridize to their targets for 20 h at 65°C. Following hybridization, 32µl of ligase-65 master mix (25 µl of dH₂O + 3 µl of ligase buffer A + 3 ul of ligase buffer B + 1ul of Ligase-65 enzyme) was added to each hybridized sample while samples were kept at 54°C and ligation reaction was performed (15 min at 54°C, 5 min at 98°C and paused at 20°C). Lastly, PCR mix (7.5 µl dH₂O + 2 µl SALSA PCR primer mix + 0.5 µl SALSA Polymerase) was added to ligation products that were amplified by PCR (35 cycles of 95°C for 30 sec, 60°C 30 sec, 72°C 60 sec followed by elongation step at 72°C for 20 min). PCR products were serially diluted (undiluted, 4x and 16x diluted) and taken forward for analysis. 0.7 µl PCR products from each tube were mixed with 0.3 µl of size standard (Rox-500 Genescan; Applied Biosystems, Foster City, CA) and 9 µl of deionized formamide. PCR products were analysed on a fluorescent capillary sequencer, Genescan (ABI 3730, Applied Biosystems, Darmstadt, Germany). Data generated by 26 probes in 15q region (Table 2.6) were normalised by Coffalyser (MLPA analysis tool developed at MRC-Holland).

Table 2.6: Probes in the MRC Holland P343-C1 AUTISM-1 kit within the 15q11-13 region.

No	gene	Map view	Chr. pos.
1	SNRPN-HB2-85	15-022,848294	15q11.2
2	SNRPN-HB2-85	15-022,858473	15q11.2
3	UBE3A	15-023,136395	15q11.2
4	UBE3A	15-023,150555	15q11.2
5	UBE3A	15-023,152935	15q11.2
6	UBE3A	15-023,167740	15q11.2
7	UBE3A	15-023,171919	15q11.2
8	ATP10A	15-023,484000	15q12
9	ATP10A	15-023,659036	15q12
10	GABRB3	15-024,344242	15q12
11	GABRB3	15-024,357380	15q12
12	GABRB3	15-024,363881	15q12
13	GABRB3	15-024,376510	15q12
14	GABRB3	15-024,379640	15q12
15	GABRB3	15-024,417665	15q12
16	GABRB3	15-024,568655	15q12
17	OCA2	15-025,770116	15q12
18	OCA2	15-026,017939	15q13.1
19	APBA2	15-027,196749	15q13.1
20	NDNL2	15-027,348739	15q13.1
21	TJP1	15-027,900944	15q13.1
22	TRPM1	15-029,081415	15q13.3
23	KLF13	15-029,452013	15q13.3
24	CHRNA7	15-030,191319	15q13.3
25	SCG5	15-030,759311	15q13.3
26	SCG5	15-030,776133	15q13.3

2.5.2.2 MS-MLPA to check for deletion breakpoints and methylation status in chromosome 15q11

In order to detect deletion breakpoints in PWS/AS region (initially identified by MLPA P343 Autism kit) and to analyse CpG island methylation of the 15q11 region, I performed the multiplex ligation-dependent probe amplification (MS-MLPA: ME028-B2 Prader Willi/Angelman probemix). The list of probes is provided in Table 2.7.

Denaturation and probe hybridization steps were similar to that of simple MLPA. Following hybridization, the product was diluted with 10 µl water and 3 µl of ligase buffer A, mixed well, and 10 µl of mixture was added in a second tube. Two ligase mixtures were prepared, a ligation mixture (8.25 µl dH₂O + 1.5 µl ligase buffer B + 0.25 µl ligase-65 enzyme) for copy number detection and a ligation-digestion mixture (7.75 µl dH₂O + 1.5 µl ligase buffer B + 0.25 µl Ligase-65 enzyme and 0.5 µl *Hha*I enzyme-Promega R6441, 10 units /µl) for methylation detection. At 49°C, 10 µl of each mixture were added in respective tubes and ligation and digestion step were carried through at 49° C for 30 min, followed by 5 min of heat inactivation of the enzymes at 98°C. Following ligation/digestion, 5µl of PCR reaction mix (3.75 µl dH₂O + 1µl SALSA PCR primer mix + 0.25 µl SALSA Polymerase) was prepared per sample and PCR was performed (35 cycles: 95°C 30 sec, 60°C 30 sec, 72°C 60 sec). PCR products were analysed in an identical manner as described in the previous section (2.5.2.1).

Table 2.7: Probes in the MRC Holland ME028-B2 Prader Willi/Angelman probemix within the 15q11-13 region.

No	Gene	Map view	Chr position
2	TUBGCP5	15-020.398303	15q11.2
31	NIPA1	15-020.612289	15q11.2
4	MKRN3	15-021.362818	15q11.2
29	MAGEL2	15-021.440355	15q11.2
32	NDN	15-021.482381	15q11.2
30	NDN	15-021.483412	15q11.2
18	SNRPN	15-022.619902	15q11.2
13	SNRPN	15-022.626072	15q11.2
17	SNRPN	15-022.690980	15q11.2
16	SNRPN	15-022.703328	15q11.2
15	SNRPN	15-022.716714	15q11.2
27	SNRPN	15-022.717321	15q11.2
14	SNRPN	15-022.751105	15q11.2
5	SNRPN	15-022.751214	15q11.2
7	SNRPN	15-022.751480	15q11.2
1	SNRPN	15-022.751773	15q11.2
19	SNRPN	15-022.764248	15q11.2
28	SNRPN	15-022.772555	15q11.2
10	SNRPN-HB2-85	15-022.848250	15q11.2
33	SNRPN-HB2-85	15-022.872658	15q11.2
21	SNRPN-HB2-85	15-022.888662	15q11.2
23	UBE3A	15-023.136395	15q11.2
20	UBE3A	15-023.156677	15q11.2
3	UBE3A	15-023.167740	15q11.2
8	UBE3A	15-023.171919	15q11.2
25	UBE3A	15-023.201674	15q11.2
6	UBE3A	15-023.235184	15q11.2
24	ATP10A	15-023.522207	15q12
12	ATP10A	15-023.659906	15q12
11	GABRB3	15-024.344242	15q12
26	GABRB3	15-024.363881	15q12
9	APBA2	15-027.196749	15q13.1
22	BLM	15-089.061432	15q26.1

2.5.2.3 MS-MLPA to confirm deletion and methylation status in chromosome 20q13.32

GNAS region

For the proband suspected to carry Albright hereditary osteodystrophy, I performed the MS-MLPA experiment using ME031 –GNAS probemix (MRC Holland, Amsterdam, The Netherlands; Table 2.8), in an identical manner as described in the previous sections 2.5.2.1 & 2.5.2.2.

Table 2.8: Probes in the MRC Holland ME031 –GNAS probemix within the 20q region.

No	Gene	Map view	Chr.pos.
1	NESPAS	20-056.850598	20q13.32
2	STX16	20-056.659882	20q13.32
3	GNASXL	20-056.862683	20q13.32
4	NESPAS	20-056.859205	20q13.32
5	STX16	20-056.675969	20q13.32
6	TH1L	20-056.989740	20q13.32
7	NESP55	20-056.848143	20q13.32
8	GNAS	20-056.917978	20q13.32
9	GNAS	20-056.900224	20q13.32
10	STX16	20-056.676511	20q13.32
11	GNAS	20-056.897495	20q13.32
12	STX16	20-056.679054	20q13.32
13	NESPAS	20-056.859408	20q13.32
14	NESP55	20-056.848321	20q13.32
15	GNAS	20-056.919132	20q13.32
16	GNASXL	20-056.862624	20q13.32
17	GNAS	20-056.911972	20q13.32
18	GNASXL	20-056.863596	20q13.32
19	NESPAS	20-056.859298	20q13.32
20	GNAS	20-056.918452	20q13.32
21	STX16	20-056.685865	20q13.32
22	GNAS	20-056.900164	20q13.32
23	NESPAS	20-056.850853	20q13.32
24	GNAS	20-056.913829	20q13.32
25	GNAS	20-056.897762	20q13.32
26	NESP55	20-056.848539	20q13.32
27	STX16	20-056.677727	20q13.32
28	GNAS	20-056.907400	20q13.32
29	GNAS	20-056.917576	20q13.32
30	GNASXL	20-056.863512	20q13.32
31	GNASXL	20-056.864358	20q13.32
32	GNAS	20-056.904036	20q13.32

2.6 Methods related to chapter 5

2.6.1 Hormone levels in subjects with monogenic obesity

The endocrine profile of subjects (described in detail in chapter 4) with pathogenic mutations in *LEP*, *LEPR* and *MC4R*, was assessed by us in the Department of Biological Sciences, Forman Christian College, Lahore, Pakistan, by measuring serum leptin, insulin, TSH and cortisol levels. Serum concentrations of these hormones were measured using commercially available ELISA kits (leptin: Organium Laboratories, Helsinki, Finland; insulin, TSH and cortisol: Monobind Inc, Lake Forest, CA, USA). Samples were assayed in duplicate. The intra- and inter-assay variations were < 11% for each assay.

2.6.2 Fasting and postprandial levels of gut hormones

The subjects for this study were drawn from two unrelated consanguineous Pakistani families with leptin deficient members that were previously screened for mutations in the coding region of *LEP*. Of the 13 family members included in this study, 5 individuals were homozygous (Group1: -/-) and 8 were heterozygous (Group 2: +/-) for the c.398delG mutation in the leptin gene. Additionally, 10 unrelated lean subjects with a wild type *LEP* sequence (Group 3: +/+), served as the control group. The study was approved by the institutional ethical committee and written informed consent was obtained in all cases from participants or their parents/guardians. The study was carried out in accordance with the principles of Declaration of Helsinki.

The subjects were presented in the morning after an overnight (10 h) fast. Anthropomorphic measurements and physical examination were made followed by measurement of blood glucose levels. None of the subjects were on regular medication. The subjects were presented with a mixed meal, *ad libitum*. Three to 5 ml venous blood was drawn 15 min before serving the meal and 60 min after its termination in each subject. The samples were collected in EDTA tubes kept on ice. The samples were centrifuged to separate plasma stored at -80°C until analysed.

Assessment of gut hormone levels was carried out by Paul R. Bech from the department of Medicine, Imperial College London and by myself. All plasma samples were analysed in duplicate. For PYY and GLP-1 measurements an established in-house RIA was used [370, 371]. The GLP-1 and

PYY assays had a sensitivity of 6 pmol/L and 18 pmol/L respectively. The coefficient of variation (CV) for both assay QCs were less than 10%. Total ghrelin concentrations were measured using a commercially available ELISA kit (Merck Millipore, Billerica, MA, USA). Samples were processed and analysed following the manufacture's recommendations. The intra- and inter-assay variations for these hormones were less than 10% for each assay.

2.6.3 Statistical analysis

Student's t test was used to assess differences between 2 groups. Comparisons between more than 2 groups were made by ANOVA followed by Tukey's multiple comparison test. Correlation was analysed by Pearson's correlation coefficient. Statistical analyses were carried out using GraphPad Prism version 5.00 (GraphPad Software Inc, San Diego, CA, USA).

2.7 Methods related to chapter 6

Novel mutations identified by whole exom sequencing analysis were confirmed by using conventional Sanger sequencing using following primers designed by primer3 software (Table 2.9).

Table 2.9: primers used to amplify regions of interest in *INSIG2*, *ADCY3* and *ROCK1* genes.

Primer	Sequence (5'-3')
<i>INSIG2</i> forward primer	TTCCCTTCTCAACAGAGGCA
<i>INSIG2</i> reverse primer	GAGTGACCACAGTTGCCAAG
<i>ADCY3</i> forward primer	AGAGCTCACACATCCACGAA
<i>ADCY3</i> reverse primer	CAGTCACTCCAGGGTTCTCC
<i>ROCK1</i> forward primer	ACATACCTTCCTCTGCCTTCTT
<i>ROCK1</i> reverse primer	TGTCTGACCTGTCTTTATCTAGCA

For confirmation of mutation in *INSIG2*, *ADCY3*, and *ROCK1*, I performed amplification of desired region using Clontech (Clontech Laboratories Inc, Mountain View, CA, USA) under the following conditions: 95°C 1 min, followed by 30 cycles of 95°C 30 sec, 68°C 1 min, followed by 68°C 3 min. This was followed by direct sequencing on ABI 3730xl DNA sequencer.

3. Chapter three

**leptin (*LEP*), leptin receptor (*LEPR*), and
melanocortin 4- receptor (*MC4-R*)
deficiency in our cohort of severely
obese children**

3.1 Introduction

As a result of genetic and molecular studies over the past two decades, the central melanocortin pathway triggered by the peripheral adipocyte hormone, leptin, has emerged as the critical signalling system regulating body weight and energy homeostasis [189]. Loss-of-function mutations in genes encoding proteins that make up the assembly of leptin-melanocortin signalling, almost invariably result in severe obesity and hyperphagia in early childhood and are associated with various endocrine and immune disorders [128-130].

Pathogenic mutations identified in leptin (*LEP*) and leptin receptor (*LEPR*) genes are rare and have so far been described in less than 50 obese individual worldwide and mainly from consanguineous families [125, 131, 132, 153, 157, 169-171, 173-175, 372]. Whereas *LEP* mutations result in a complete lack of its circulating protein, the loss of function mutations in the *LEPR* renders the carrier insensitive to leptin. In mice both leptin and leptin receptor deficiency results in almost identical early-onset severe obesity [373]. However, the human *LEPR* deficiency has been suggested to have a less severe effect than that caused by leptin deficiency [132].

Unlike *LEP* and *LEPR* mutations, the pathogenic mutations found in the *MC4R* gene cause the most prevalent form of monogenic obesity known so far. Indeed, prevalence of *MC4R* mutations associated with human obesity though variable among different studies has been reported to be 5-6% in obese subjects of diverse origin [203, 204]. However, the great majority of these mutations have been identified in the heterozygous state. Most of studies, both *in vivo* and *in vitro*, are suggestive of a co-dominant mode of inheritance with heterozygous subjects having a less severe phenotype than homozygous carriers [202, 203]. However the degree of penetrance of *MC4R* mutations has been demonstrated to be influenced by environmental factors [202].

To our understanding, no systematic assessment of prevalence of loss-of-function mutations in these three genes has been carried out in severely obese subjects in a single population with a relatively high degree of consanguinity. In the present study we have attempted to identify genetic variance in a cohort of randomly ascertained severely obese children from a single consanguineous population.

3.2 Mutations in *LEP*, *LEPR* and *MC4R* Genes

Causative variants described in these genes were identified through a multi-stage screening regimen involving Sanger sequencing, microdroplet PCR-enrichment (RainDance) followed by NGS, genotyping and WES, as detailed in chapter 2. Of a total of 175 subjects with severe obesity, 50 probands and 10 siblings were identified to carry mutations in *LEP*, *LEPR* and *MC4R* genes. In the description that follows the mutations are referred under the specific genes they impact regardless of the screening procedures used in their identification.

3.2.1 *LEP* gene mutations

Sanger sequencing analysis in our cohort of severely obese children revealed 35 unrelated subjects (20.5%) and 7 family members carrying 6 different loss-of-function mutations. Of these 4 were novel. All 42 affected subjects were also shown to be clinically leptin deficient. Parents of leptin deficient subjects were heterozygous carriers for the respective mutation. Physical characteristics of subjects with *LEP* mutations are given in Table 3.1 and Table 3.2.

3.2.1.1 c.398delG (p.Gly133_VfsX14)

This recessive frameshift mutation due to deletion of G from coding position 398 in exon 3 of the *LEP* gene in homozygous state, was identified in 29 unrelated probands. Twenty-seven of these were children <1-13 year (median: 1.2 year) and 2 adults (median age: 20) (Figure 3.1). The same mutation was also identified in 6 family members, 3 of which were adult females. This mutation disrupts the reading frame of the leptin gene resulting in 14 aberrant amino acids after glycine at 133 amino acid position, followed by a premature stop codon. All affected subjects had normal body weight at birth but started gaining excessive weight at the age of 1-4 months. Almost all subjects were reported to suffer from recurrent pulmonary infections and diarrhoea. Interestingly 28 out of 29 probands were also reported to belong to the same ethnic group (caste). Physical characteristics of subjects carrying this frameshift mutation are described in Table 3.1.

Table 3.1: Physical characteristics of subjects carrying frameshift c.398delG mutation in homozygous state.

Individuals No	Family No	ID	Gender	Age	SDS(BMI)
1	1	F-4P	F	0.7	4.9
2	2	F-13P	M	1.4	4.9
3	3	F-22P	M	7	4.2
4	4	F-16P	M	1	7.7
5	5	F-30P	M	3.3	7.5
6	6	F-53P	F	0.7	5.7
7	7	F-56P	F	0.7	5.5
8	8	F-60P	M	0.7	5.7
9	9	F-62P	F	2	7.4
10	9	F-62C	F	2.5	6.8
11	10	F-42P	M	1.7	9.0
12	10	F-42D	F	27.2	(63.28)
13	10	F-42E	F	16	(49.6)
14	11	F-71P	F	30	(57.7)
15	11	F-71D	F	8	4.2
16	11	F-71H	M	9	4.7
17	12	F-76P	M	0.7	6.8
18	12	F-76C	F	3	6.8
19	13	F-82P	M	9	4.9
20	14	F-111P	F	0.8	4.9
21	15	F-110P	F	1	5.7
22	16	F-122P	F	0.5	4.6
23	17	F-127P	M	21	(42.4)
24	18	F-130P	F	2	3.6
25	19	F-134P	F	0.8	7.6
26	20	F-138P	M	0.3	1.8
27	21	F-143P	F	0.7	6.6
28	22	F-153P	F	13	4.5
29	23	F-154P	M	18	(47.02)
30	24	F-157P	M	4	7.4
31	25	F-162P	F	1.2	4.9
32	26	F-172P	M	1.1	3.9
33	27	F-194P	M	0.7	4.2
34	28	F-195P	M	1.4	4.8
35	29	F-199P	M	1.1	5.8

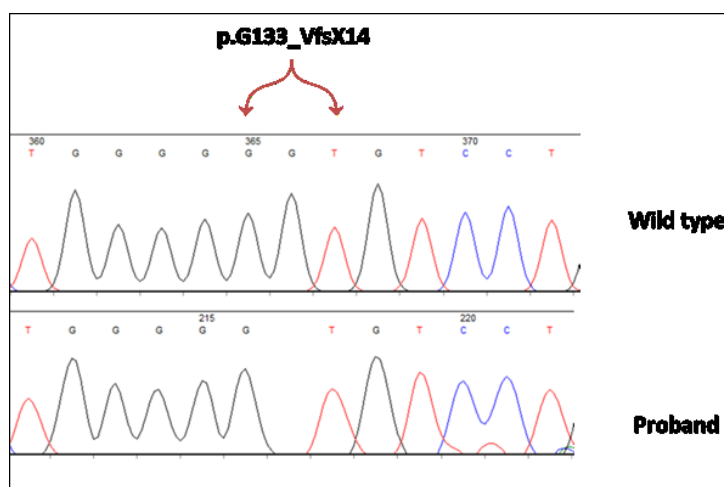


Figure 3.1 Sequencing scan of *LEP* at the site of mutation 398delG.

Scan demonstrating a homozygous 1 base deletion in exon 3 of *LEP* disrupting the reading frame. This mutation was identified in 29 unrelated probands and 6 family members with severe obesity.

3.2.1.2 c.1-44del42

This novel splice site mutation in the homozygous state identified in a 1.5 year old male child (F-75) with severe obesity, by direct sequencing of the gene, was due to a 42bp deletion of intron 1 of *LEP* (Figure 3.2A). This recessive deletion extending from position -44 to position -2 of the second exon (the first coding exon of *LEP*), involves an abnormal splicing resulting in a skipping of exon 2 as predicted by Automated Splice Site and Definition Analyses [374] (Figure 3.2B).

To further characterise this mutation, RNA was extracted from the blood leukocytes and the cDNA was prepared. Gel electrophoresis of the amplified product from heterozygous parents resulted in two bands corresponding to the mutated and wild type alleles, whereas a single band of respective size was found in the obese child and his wild type sibling (Figure 3.2C). Direct sequencing of cDNA from the proband and parents revealed absence of exon 2 transcripts in the mRNA of the affected proband whereas heterozygous parents carried both the wild-type and mutant transcripts (Figure 3.2D).

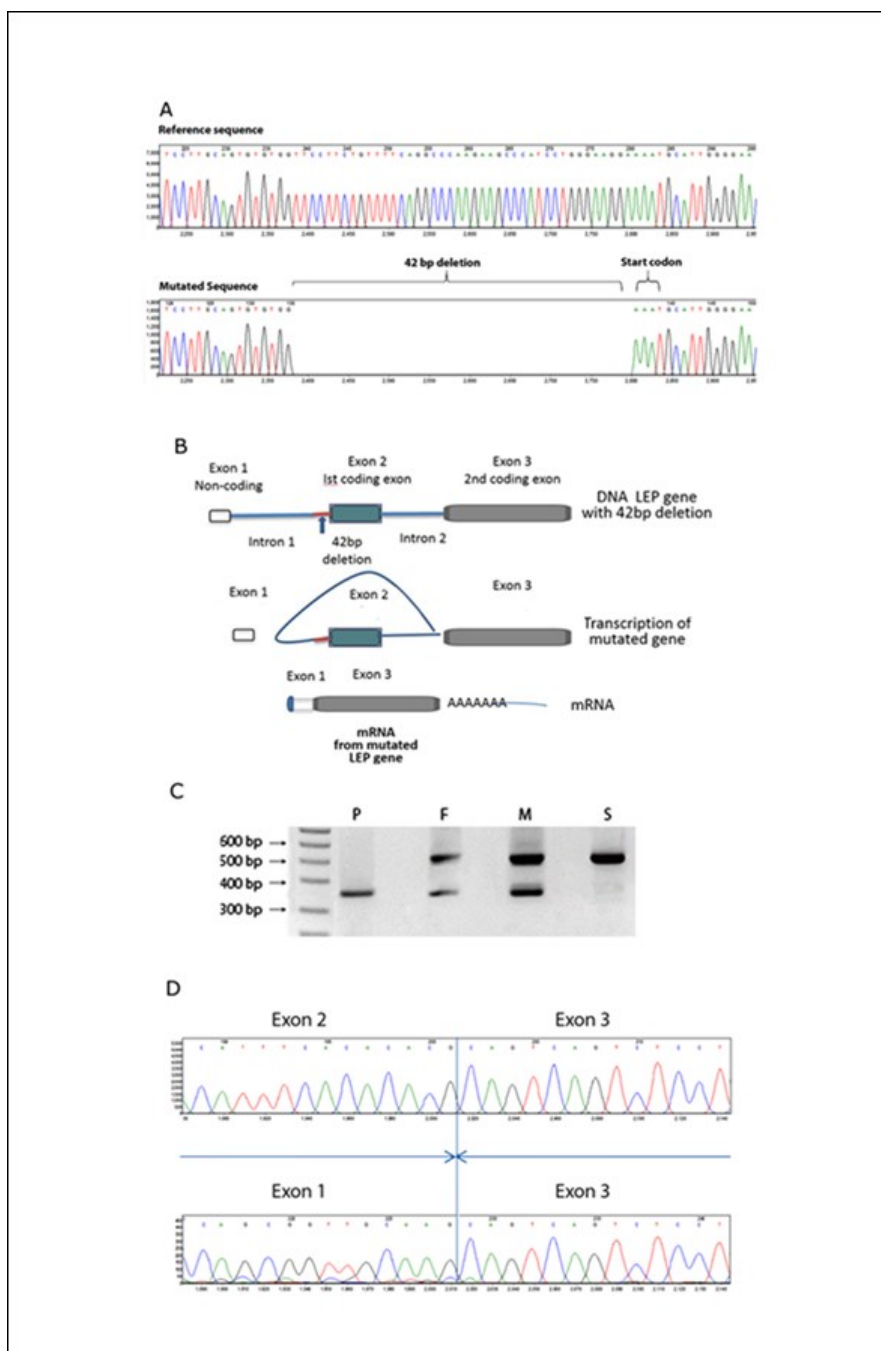


Figure 3.2 Sequence analysis and characterization of a novel homozygous 42 bp deletion in *LEP*.

(A) Sequence scans of reference and mutated *LEP* in the region of a 42 bp deletion in intron 1 that includes the splice acceptor site of exon 2. (B) Schematic presentation of the genomic organization of *LEP* indicating the position of the intronic deletion resulting in a skipping of exon 2 and the mRNA reconstruct transcribed by the mutated gene. (C) Gel electrophoresis of reverse transcriptase PCR amplified cDNA. The proband carrying homozygous deletion presents a single band of 360 bp, the heterozygous parents two bands of 360 and 530 bp, and the wild-type sibling a single band of 530 bp. P: proband; F: father; M: mother; S: wild-type sibling. (D) Results of sequencing of cDNA from wildtype (reference) and mutated subjects. The mutant sequence shows a lack of exon 2.

F-75: The proband was the youngest of three siblings of consanguineous parents from a village in the vicinity of Faisalabad city. He was presented at the hospital at the age of 1.5 years having difficulty in breathing. He was severely obese with a BMI SDS of 5.57 and was reported hyperphagic (Table 3.2). His lean brother was wild type for the mutation. Both parents were heterozygous for the said mutation. The eldest sister was reported to be obese and died at a young age.

3.2.1.3 c.350G>A (p.Cys117Tyr)

Sanger sequencing of the coding regions of *LEP* identified this novel missense recessive mutation in exon 3, in a 1.6 years old boy (Figure 3.3). The mutation results in a substitution of cysteine by tyrosine at residue 117, resulting in an impaired protein function as predicted by SIFT (score: 0), and MutationTaster (p-value: 1; P value in this case is the probability of the prediction. The value close to 1 indicates a high security of the prediction) softwares.

F-123: The proband born to consanguineous parents had a BMI SDS of 4.1 (Table 2). The proband presented severe obesity and was clinically leptin deficient. One of the paternal uncles was reported to be severely obese and died at the age of 10 years.

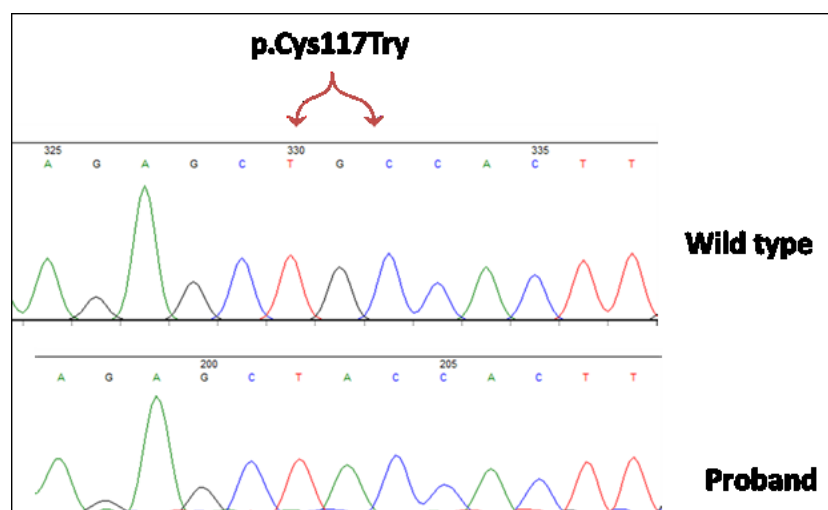


Figure 3.3 Sequencing scan of *LEP* at the site of mutation Cys117Tyr. Scan demonstrating a homozygous substitution (Cys117Tyr) in exon 3. This mutation was identified in F-123.

3.2.1.4 c.313C>T (p.Arg105Trp)

This mutation that involves a change of arginine to tryptophan at amino acid position 105 found in these two siblings has previously been described in a large Turkish family but is reported here for the first time in a Pakistani Christian obese family (Figure 3.4).

F-85: This missense mutation, in the homozygous state, was identified in two obese brothers 1.5 and 10 year old boys, with BMI SDS of 5.3 and 3.8, respectively (Table 3.2). Another two severely obese siblings in the same family were reported to have died at the age of 3 year and 2 month. One of the three sisters screened was found to be wild type.

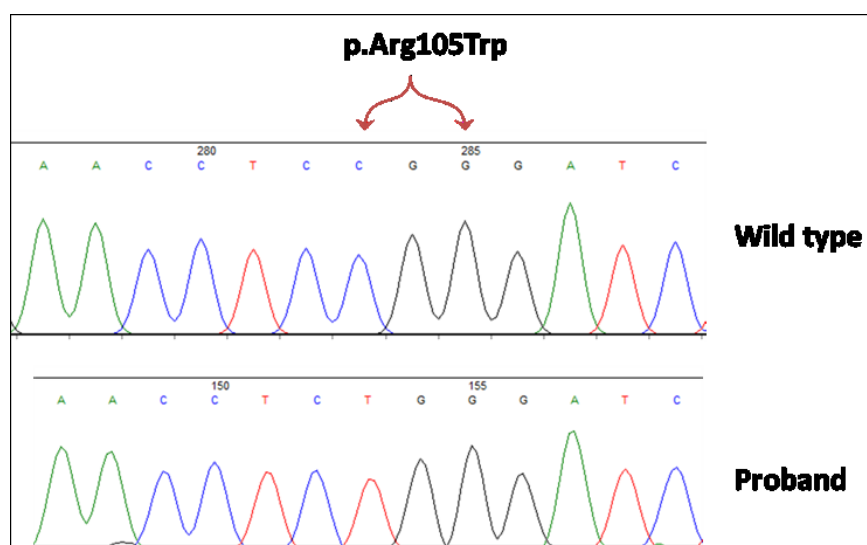


Figure 3.4 Sequencing scan of *LEP* at the site of mutation Arg105Trp
Scan demonstrating a homozygous substitution (Arg105Trp) in exon 3. This mutation was identified in *F-85P* and *F-85S*.

3.2.1.5 p.I35del (c.104_106delTCA)

This homozygous mutation involving deletion of three base pairs was identified in exon 2 of the leptin gene. The deletion involves amino acids 34 and 35 and results in deletion of isoleucine. This mutation is the only variant associated with extreme obesity that has so far been reported in exon 2 of *LEP* (Figure 3.5).

F-41: This three base deletion was identified in an 18 months old girl. She was normal weight at birth but started gaining weight after one month of age and by the age of 18 month weighed 15 kg with a BMI SDS of 4.7. At this age she was unable to crawl but suffered no other abnormalities or any other disease. The parents and a 4 years old male sibling were found to be heterozygous carriers. Whereas hyperphagia was reported in the proband, none of the family members who were heterozygous for this mutation had this condition and were of normal body weight.

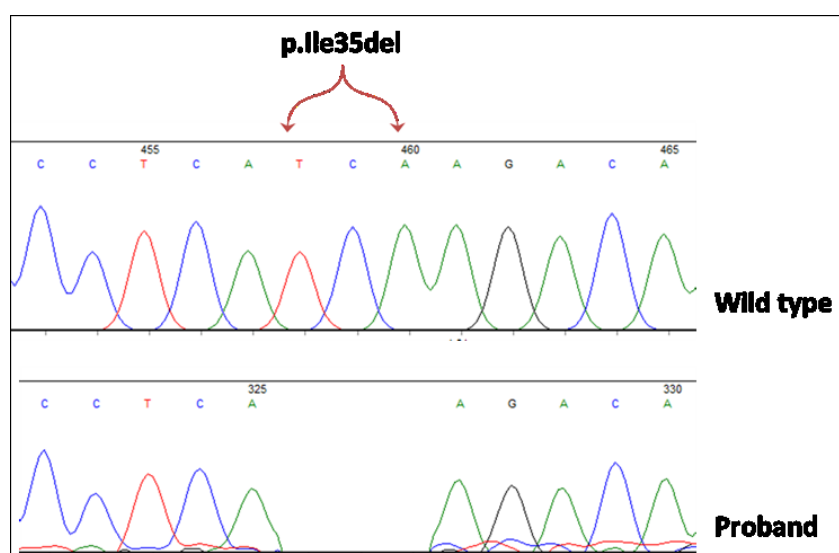


Figure 3.5 Sequencing scan of *LEP* at the site of a 3 base deletion.

Scan of *LEP* at the site of 3 base deletion (I35del) involving codons 35 and 36 in exon 2 resulting in deletion of isoleucine at position 34. This mutation was identified in *F-41*.

3.2.1.6 c.-29+1G>C

This substitution from G to C positioned at the splice donor site of exon 1 (non-coding) of *LEP* gene (Figure 3.6A) was found in two unrelated siblings. This substitution abolished the splice donor site as predicted by the Human Splicing Finder [374] (Figure. 3.6B).

F-132: This splice donor site mutation was identified in a 7 years old female from Okara district. She weighed normal at birth but developed hyperphagia and started gaining weight rapidly at 2 months of age. During her examination at the time of recruitment, she weighed 56 kg and had a BMI SDS of 3.2.

F-155: The same splice donor site mutation was identified in another 8 months old male child of consanguineous parents from Gujranwala. He was reported to belong to Ghorri caste. The proband started developing extreme obesity and hyperphagia at 4 months of his age and at the age of 8 months had a body weight of 13 kg with a BMI SDS of 3.8.

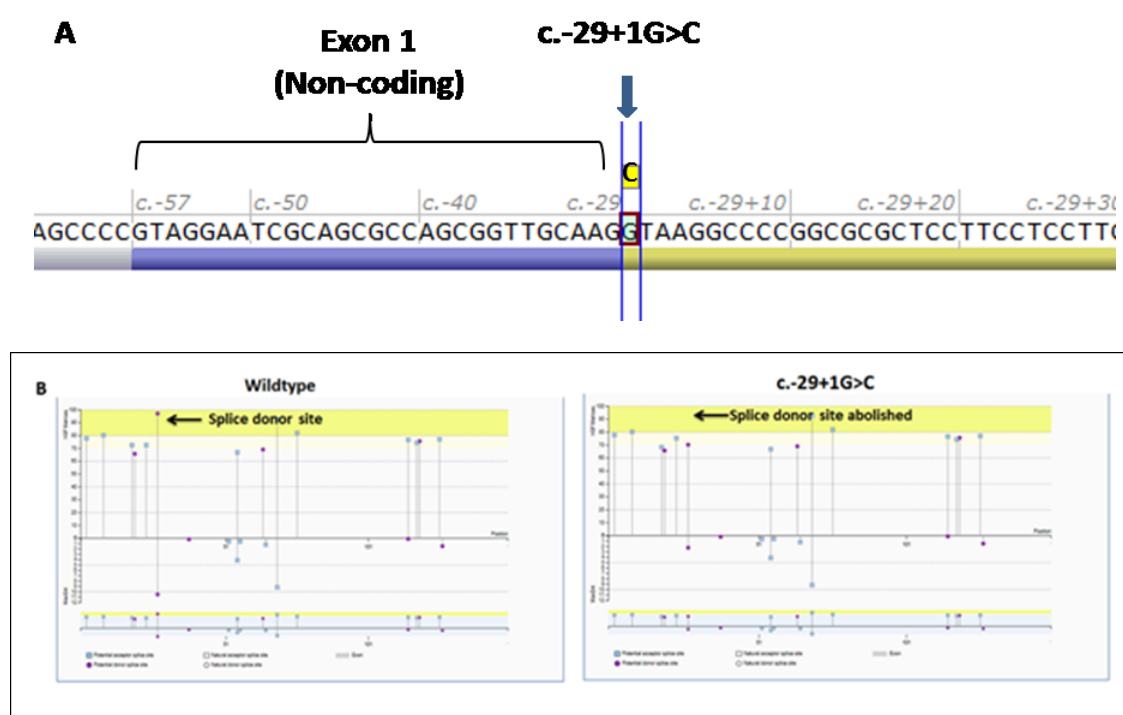


Figure 3.6 Splice donor site mutation (c.-29+1G>C) in intron 1 of leptin gene.

- (A) Diagrammatic presentation of non-coding exon 1; the mutation is indicated by arrow. (B) Schematic presentation of the splice donor site abolished in the mutant gene as predicted by the SpliceSiteFinder [374]. Splice donor site in yellow area (arrow) presents the strongest candidacy (above), abolishing of which can be seen in the mutant (below).

Table 3.2: Physical characteristics of subjects carrying mutations in *LEP* gene other than the frameshift variant.

No of individuals	No of families	ID	Mutation	Gender	Age	SDS
1	1	F-41P	c.104-106delTCA	F	1.5	4.7
2	2	F-75P	c.1-44del42	M	1.5	5.57
3	3	F-123P	c.350G>A	M	1.4	4.13
4	4	F-85P	c.313C>T	M	1.4	5.3
6	4	F-85S		M	10	3.8
7	5	F-132	c.-29+1G>C	F	0.7	3.2
8	6	F-155		F	0.2	3.8

P: proband; S: sibling

Mutations in bold are novel

3.3.2 *LEPR* gene mutation:

Microdroplet PCR-based enrichment and sequence analysis of the coding and flanking regions of 26 monogenic obesity associated genes, revealed 7, all novel, homozygous *LEPR* mutations in 11 probands (6.5%) and in one sibling, with early onset severe obesity. All mutations were found in regions of homozygosity (Figure 3.13). Parents of affected subjects were heterozygous for the respective mutations and had BMI within the normal range. Physical characteristics of the mutation carriers are described in Table 3.3.

3.3.2.1 **c.2396-1G>T**

This novel mutation in the homozygous state, which involved a G>T base substitution in the splice acceptor site of exon 15 (Figure 3.7), was found in four probands (*F-48*, *F-80*, *F-129* and *F-146*; Table 3.3), 3 females and one male. This mutation resulted in abnormal splicing of *LEPR* transcript by skipping of exon 15 as analysed by NetGene2 [375], Human Splicing Finder [374], and Automated Splice Site and Exon Definition Analyses [376].

***F-48*:** This proband, the first child of consanguineous parents from a remote village of the central Punjab province, was recruited at the age of 1.2 years when she weighed 22.5 kg and had a BMI

of 31.9 and BMI SDS of 5.9 (Table 3.3). She belonged to Tarrar caste. She weighed normal at birth but developed hyperphagia and started gaining weight rapidly at 2 months of age. During a follow up of the patient at the age of 2 years, she weighed 34 kg and had a BMI of 40.17. The child had no dysmorphic features but was reported to suffer from recurrent chest infections, dermatitis, reddening of skin, and breathing difficulty.

F-80: This proband was also a first female child of consanguineous parents. She was presented at hospital with excessively increasing body weight at 2 years of age. She belonged to Rajput caste. At the time of recruitment she had a BMI SDS of 3.3. Her parents were heterozygous for the mutation while a 0.6 year old male sibling was wild type.

F-129: She was the only child from a first cousin union and weighed 31 kg at the age of 1.4 year. Like members of *F-80* family, she also belonged to the Rajput caste. Interestingly, this proband also carried a homozygous missense mutation, D148N, in *BBS1* gene (described in section 4.2.1.4). This mutation was found in the largest homozygous region (37 Mb) identified in this proband. No polydactyly and/or other phenotypes associated with BBS were found in this proband. This mutation in *BBS1* has been reported in subjects from UK and US and shown to be pathogenic [377].

F-146: This male proband was recruited from the Faisalabad city at the age of 7 months. He also belonged to Rajput caste and weighed 11 kg.

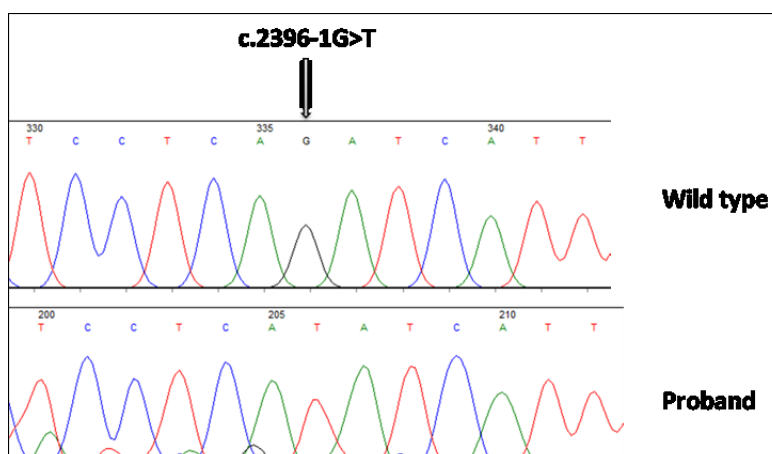


Figure 3.7 Sequencing scan of *LEPR* at the site of c.2396-1G>T mutation. Scan demonstrating a homozygous missense substitution from G to T at splice acceptor site of exon 15. This mutation was identified in 3 affected unrelated proband (*F-48*, *F-80*, *F-129*, *F-146*).

3.3.2.2 c.1674 G>A (p.Trp558Stop)

A second novel homozygous nonsense mutation of *LEPR* was identified in exon 10 (Figure 3.8), in two probands, a 0.7 year old girl (*F-59*) and a 1.2 year old boy (*F-81*) with early onset severe obesity and hyperphagia. A single base substitution of G to A created a premature stop codon resulting in a truncated protein comprising the first 558 of the 1165 amino acids of the *LEPR* protein. Both parents of the probands were verified to be heterozygous carriers for this mutation.

***F-59*:** This proband, the only child of first degree related parents belonging to Sheikh caste, was referred to the Children's Hospital Lahore at the age of 7 months. Since 3 months of age, she had an insatiable craving for food and underwent a rapid increase in body weight. At the age of 0.7 year she weighed 14.5 kg with a BMI of 36.5 and SDS for age 6.4. Two paternal uncles of the proband were reported to be severely obese at an early age and died during their childhood when they were 4-5 years old.

***F-81*:** The same homozygous nonsense mutation was also identified in another 1.2 year old boy with a BMI SDS of 5.5. The parents were heterozygous carriers for this mutation. The proband had developed extreme obesity and hyperphagia at an early age.

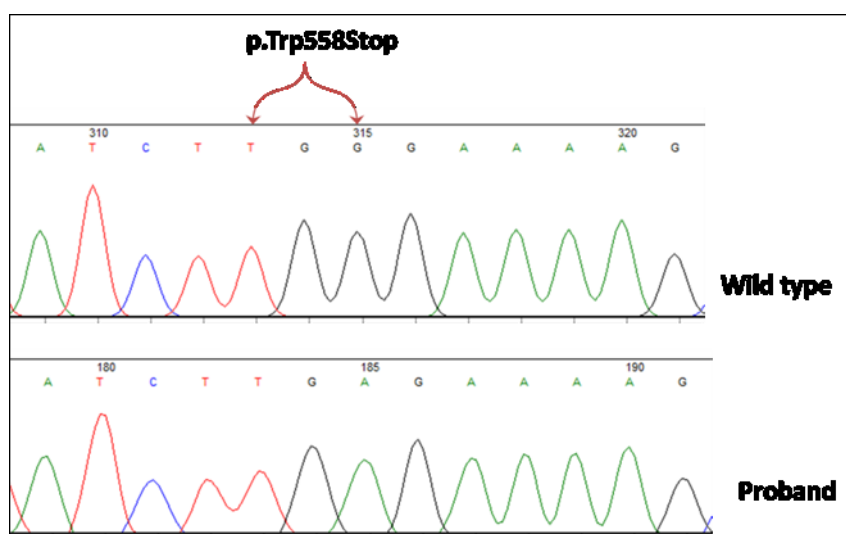


Figure 3.8 Sequencing scan of *LEPR* at the site of Trp558Stop mutation.

Scan of a mutation demonstrating a homozygous nonsense substitution at exon 10. This mutation was identified in 2 affected unrelated proband (*F-59, F-81*).

3.3.2.3 c.1810T>A (p.Cys604Ser)

The novel missense mutation was identified as due to substitution of T to A at position 1810 (Figure 3.9). The mutation results in a C604S residue exchange in exon 11 and is predicted to be deleterious (score: 0) by SIFT and damaging by PolyPhen softwares.

F-96: This mutation in homozygous state was found in two obese brothers 1 and 5 years old. The older brother was reported to be a slow learner. Other features included hypodontia, beak nose and retracted penis. The sibling was heterozygous carriers for the said mutation and was of normal body weight.

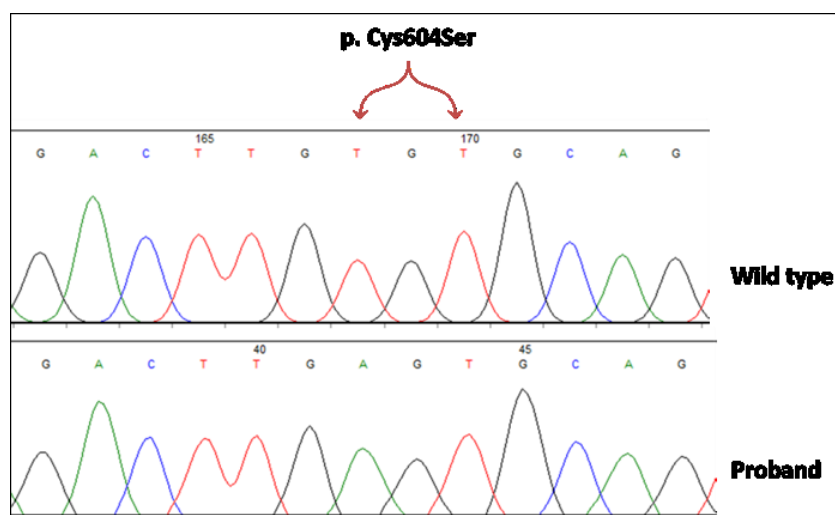


Figure 3.9 Sequencing scan of *LEPR* at the site of mutation p.C604S.

Scan demonstrating a homozygous missense substitution at exon 11. This mutation was identified in *F-96P* and *F-96S*.

3.3.2.4 c.40G>A (p.Glu14Lys)

This missense mutation was identified as a single base substitution at position 40 (last base in exon one) caused by a change of glutamic acid with lysine at codon4 to lysine (Figure 3.10) The mutation is reported to be disease causing (p-value: 1) by MutationTaster and tolerated by SIFT (score: 0.32). This mutation was initially identified by WES and subsequently validated by Sanger sequencing.

F-14: This male proband, older of the two children of consanguineous parents from Sahiwal, was recruited at the age of 2.3 years when he weighed 24.2 kg with a BMI SDS of 6.5. The family belonged to the Rehmani caste. His body weight was normal at birth but the child developed hyperphagia and started gaining weight rapidly as early as 1 month of age. The child had no dysmorphic features although achieving developmental milestones was somewhat delayed when the proband was 6 years old as reported by parents. The parents and sister were heterozygous for the mutation and had normal body weight.

3.3.2.5 c.2114G>A (p.Trp705Stop)

This novel nonsense mutation was identified in exon 13 of *LEPR* gene in a 9 months old boy. In this case a single base substitution (G>A) at position 2114 creates a premature stop codon resulting in a truncated protein comprising the first 705 of the 1165 amino acids of the LEPR protein (Fig 3.11).

F-150: This male proband, the only child of first degree related parents belonging to Dogar caste, was referred to the Children's Hospital at the age of 9 months when he weighed 14.2 kg with a BMI SDS of 3.8 His body weight was normal at birth but the child developed hyperphagia and started gaining weight rapidly as early as 2 months of age.

3.3.2.6 c.2153A>G (p.Asn718Ser)

The novel missense mutation, 12 codon apart from Trp705Stop, described above, was identified as due to substitution of A to G at position 2153 (Figure 3.11). The mutation results in an Asn718Ser residue exchange in exon 13 of *LEPR* gene and is predicted to be deleterious (score: 0) by SIFT and disease causing (p-value: 1) by MutationTaster.

F-142: This female proband, younger of the two children of consanguineous parents from Faisalabad, was referred to the Children's Hospital Lahore at the age of 8 months. She belonged to Jatt caste. Since 2 months of her age, she had an insatiable craving for food and underwent a rapid increase in body weight. At the age of 8 months she weighed 17.5 kg and had a BMI SDS for age of 5.8.

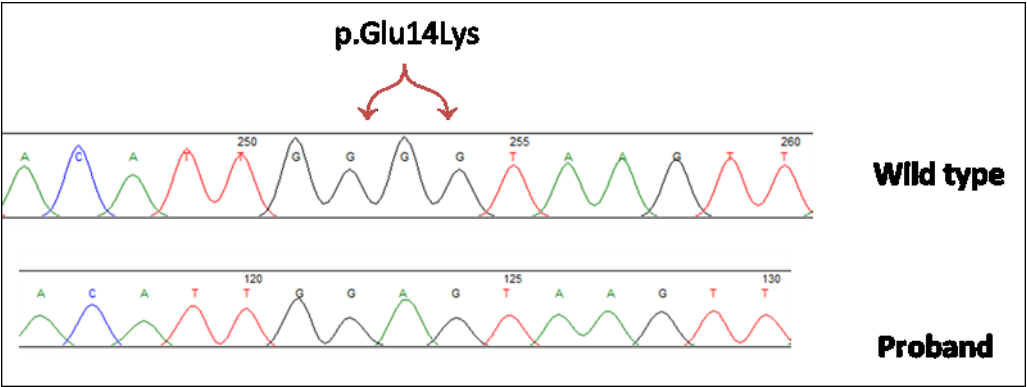


Figure 3.10 Sequencing scan of *LEPR* at the site of mutation Glu14Lys. Scan demonstrating a homozygous missense substitution at exon 1. This mutation was identified in *F-14*

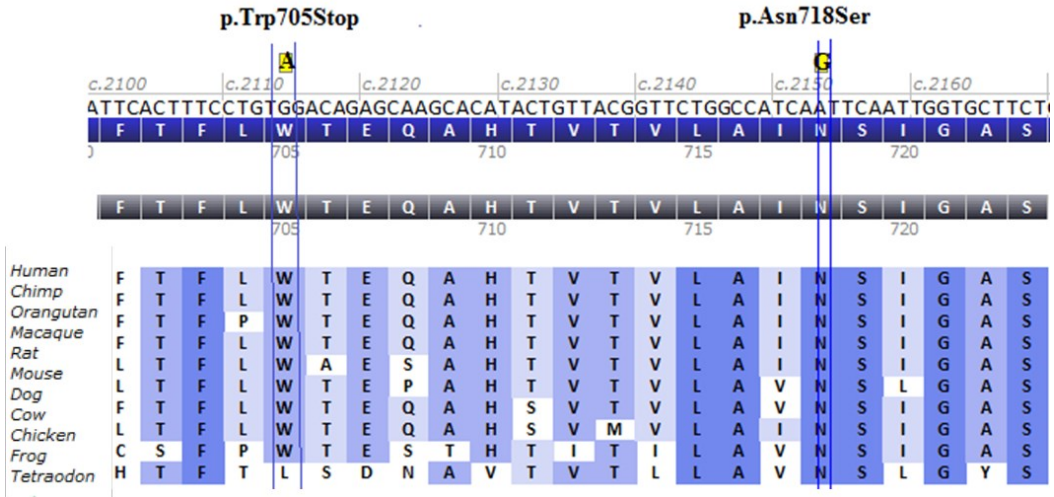


Figure 3.11 Diagrammatic presentation of p.Trp705Stop and p.Asn718Ser mutations. Scan of nonsense and missense mutations identified in exon 13 of *LEPR* gene. The mutations were identified in *F-150* and *F-142*, respectively.

Table 3.3: Physical characteristics of subjects carrying novel *LEPR* mutations in homozygous state.

No of individuals with mutation	No of families	ID	Mutation*	Gender	Age	SDS
1	1	F-48 P	c.2396-1G>T	F	1.2	5.9
2	2	F-80 P		F	2	3.3
3	3	F-129 P		F	1.6	8.6
4	4	F-146 P		M	0.6	3.0
5	5	F-59 P	c.1675G>A	F	0.7	6.4
6	6	F- 81 P		M	2.2	5.5
7	7	F-96 P	c.1810T>A	M	6	3.2
8	7	F-96 S		M	0.9	3.4
9	8	F-14 P	c.40G>A	M	2.3	6.5
10	9	F-142 P	c.2153A>G	F	0.7	5.8
11	10	F-150 P	c.2114G>A	M	0.8	3.8
12	11	F-95 P	1.3kb & 58.8kb homozygous deletion	M	0.6	4.5

P: proband; S: sibling

Mutations in bold are novel

*All mutations are novel

3.3.2.7 1.3 kb and 58.8 kb homozygous deletion

g.[66053156-66054477del1322];[66056233-66115089del58857insGTGTGTATATGGGGGT]

This type of homozygous deletion reported here for the first time in *LEPR* was identified in a 7 months old male child presenting early onset severe obesity. Conventional sequencing of *LEP* and *MC4R* and subsequent analysis by droplet PCR-based targeted enrichment followed by NGS against a panel of 27 obesity-associated genes failed to identify any pathogenic point mutation in this proband. Failure to get a signal in the latter assay prompted us to carry out CNV analysis of genotyping data that indicated deletions in a DNA segment spanning intron 3 of *LEPR*, extending to the end of the gene, and including the 3'-untranslated region and part of the intergenic region (Figure 3.12A). Deletion breakpoint mapping through primer walking revealed presence of two, 1322 and 58857bp, deletions (Figure 3.12B). The first deletion starts from position 66,053,156 of

chromosome 1 (1p31.3) and ends at position 66,054,477 whereas the second deletion extended from position 66,056,233 to position 66,115,089. The intervening segment of 1755bp, (chr1:66,054,478- 66,056,232), is preserved and is followed by a 16 bp (GTGTGTATATGGGGGT) insertion (Figure 3.12C). The parents of the proband were heterozygous for this copy number change.

F-95: The proband, a boy of 0.6 year of age had a body weight of 14 kg and a BMI SDS of 4.5. He was the only child of consanguineous parents who belonged to Rajput cast. Parents were heterozygous for the said deletion and were of normal body weight.

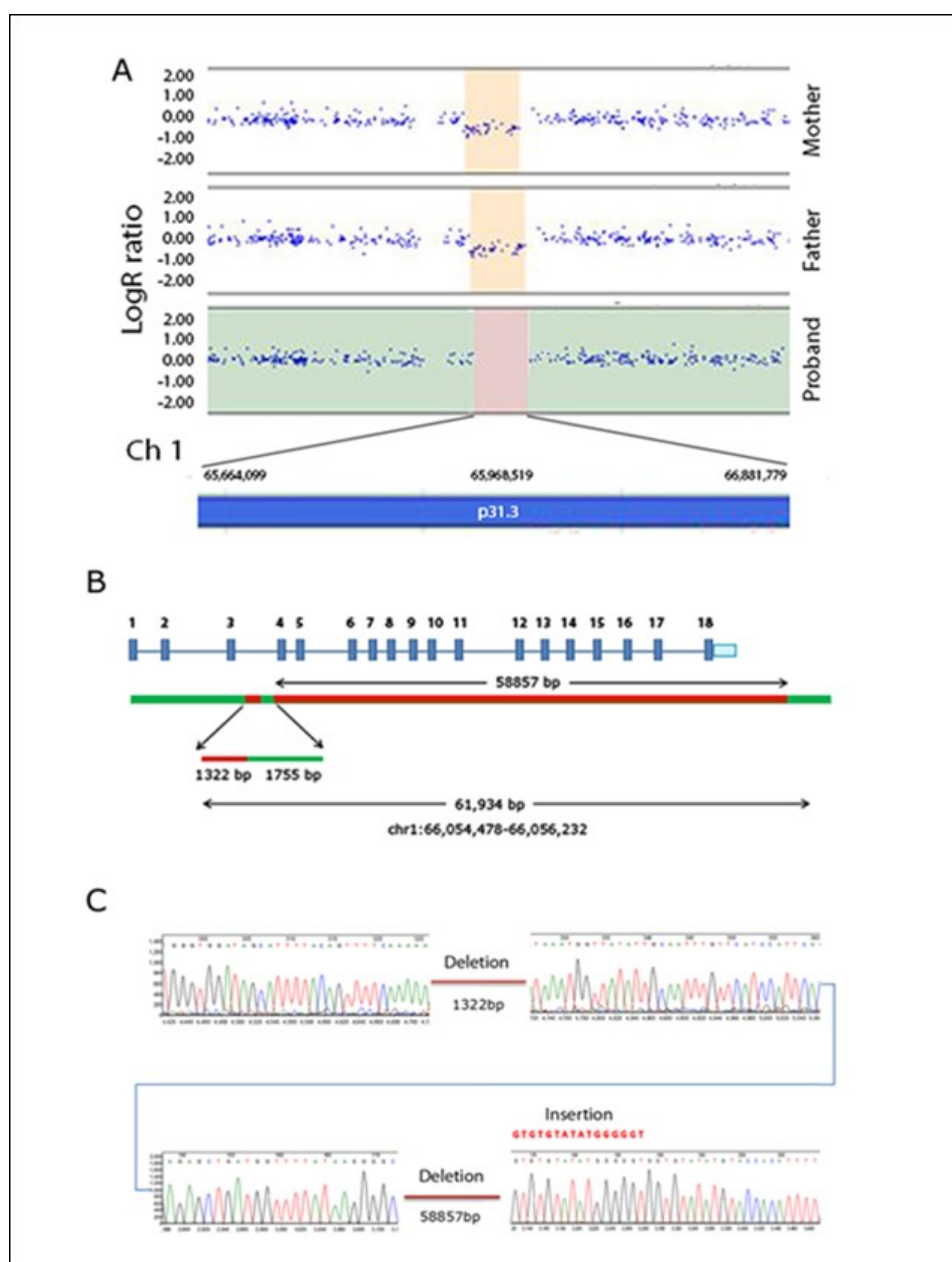


Figure 3.12 Two neighbouring novel homozygous deletions in *LEPR*.

Detection of two neighbouring novel homozygous deletion events (1322 and 58,857 bp) in *LEPR* and characterization of deletion breakpoint junctions (A) SNP array genotypes of the proband and parents representing copy number losses. LogR ratios were derived from SNP array genotyping by using CnvPartition and are shown on the y axis. Shift in the logR ratio in parents from 0 to 21 represents deletion of one copy (heterozygous) whereas in the proband both copies are deleted (homozygous). (B) Sequence analysis of deletion breakpoint junctions and flanking regions by long-range PCR and direct sequencing. The deleted parts are shown by red lines. An insertion of 16 bp with a sequence of GTGTGTATATGGGGGT was found at the 30 end of the 58,857 bp deletion. (C) Electropherogram of *LEPR* (1p31.3), indicates the position of breakpoint deletion junctions. The two deleted segments are represented by red lines.

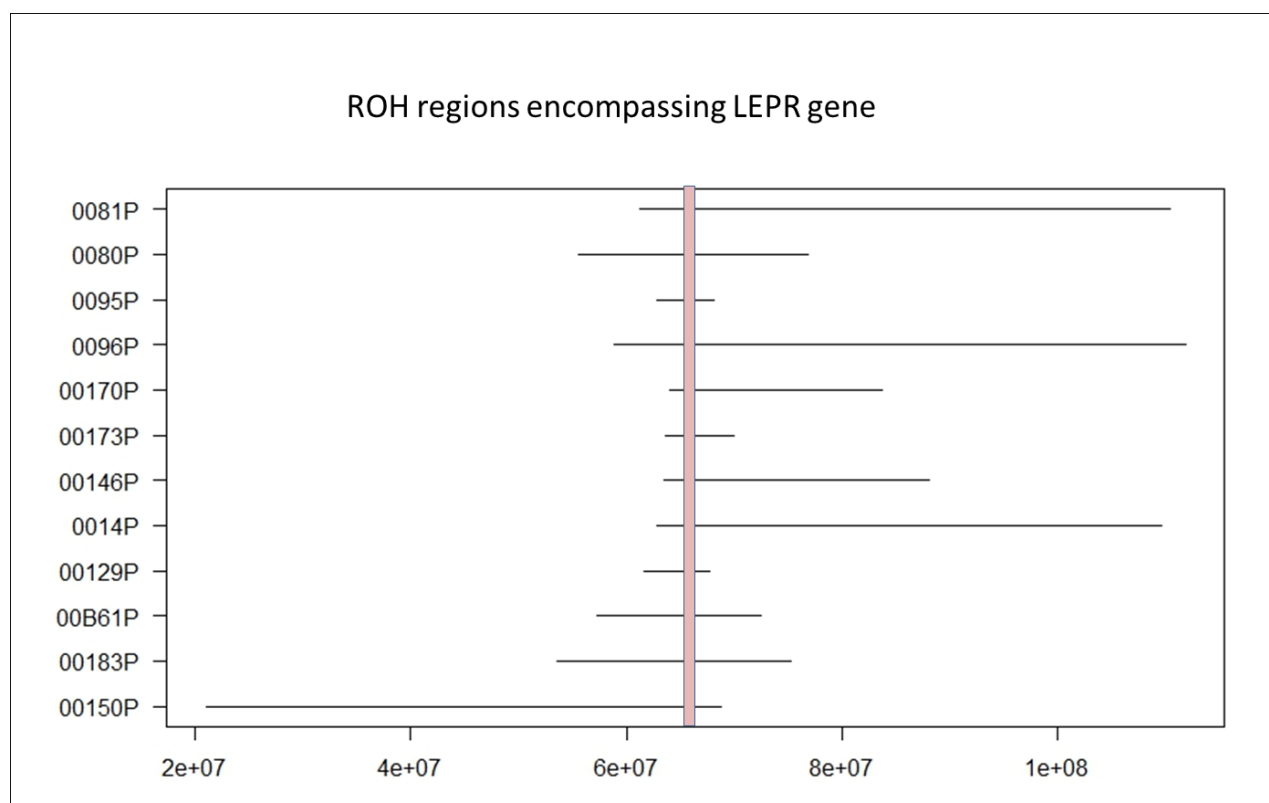


Figure 3.13 ROH encompassing *LEPR*.

Horizontal lines show regions of homozygosity as observed in probands with homozygous mutation in *LEPR* gene.

3.3.3 MC4R gene mutations

Through conventional sequencing technique 6 individuals from 4 unrelated families (2.3%) were found to carry 2 different mutations in homozygous state. One of these mutations previously described in the heterozygous state, is being reported here in the homozygous state for the first time. Physical characteristics of the mutant carriers are described in Table 3.4.

3.3.3.1 c.482 T>C (p.Met161Thr)

This missense mutation at coding position 161 of the only coding exon of melanocortin 4 receptor gene resulting in substitution of methionine to tyrosine, was identified in 2 probands and one of the siblings (Figure 3.14). Homozygosity of this mutation associated with extreme obesity is being

reported here for the first time. The mutation is predicted to be deleterious (score: 0.03) by SIFT and disease causing (p-value: 1) by MutationTaster.

F-32: This mutation in the homozygous state was identified in a 7 months old girl with early onset severe obesity and hyperphagia. She weighed normal at birth but started gaining weight rapidly in the following months attaining a body weight of 16 kg in the next 5 month. The heterozygous parents were overweight (BMI>25) but one of the siblings who was found heterozygous for the mutation had a normal body weight (comparable to the wild type sibling). The family belonged to Pathan caste.

F-77: This loss of function mutation in the homozygous state due to a single base substitution was also identified in two female obese cousins (F77 and F77S), 5 years and 10 month old with excessive body weight and hyperphagia. The BMI SDS of these girls was 3.7 and 4, respectively (Table 1). The normal weight male sibling of F-77 was wild type for the said mutation. In contrast to the subjects carrying the other *MC4R* mutation described herein, the age at onset of obesity was 2-3 months. The family belonged to Pathan caste.

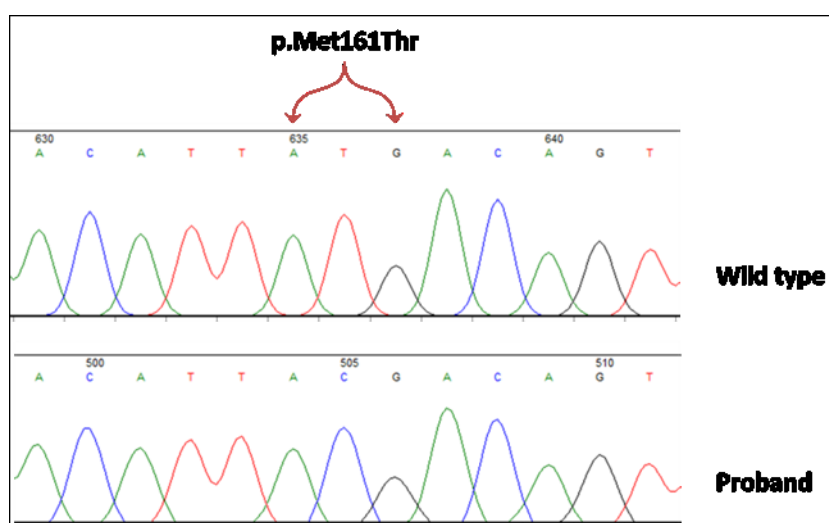


Figure 3.14 Sequencing scan of *MC4R* at the site of mutation p.Met161Thr. Scan demonstrating a homozygous missense substitution (Met161Thr) at the only coding exon of the gene. This mutation was identified in 2 affected probands and one sibling (*F-32*, *F-77P* and *F-77S*)

3.3.3.2 c.947T>C (p.Ile316Ser)

This missense mutation found in the coding region of *MC4R* resulted in a replacement of isoleucine by serine at amino acid position 316 (Fig 3.15). The mutation was predicted to be deleterious (score: 0.01) by SIFT and disease causing (p-value: 1) by MutationTaster.

F-58: This homozygous mutation in *MC4R* was found in a 15 years old male suffering from severe obesity. Hyperphagia and obesity in this boy were noticed after 10 years of age and by the age of 15 years, he had attained a body weight of 120 kg and a BMI of more than 49. The family had a history of diabetes and obesity and belonged to Sheikh caste.

F-74: This homozygous mutation in *MC4R* was found in two siblings, an 11 year old girl and a 13 year old boy, with severe obesity and hyperphagia. The BMI SDS score of the girl and the boy were

3.3 and 3, respectively and the age at onset of obesity was 4-6 years. The family belonged to Rajput caste.

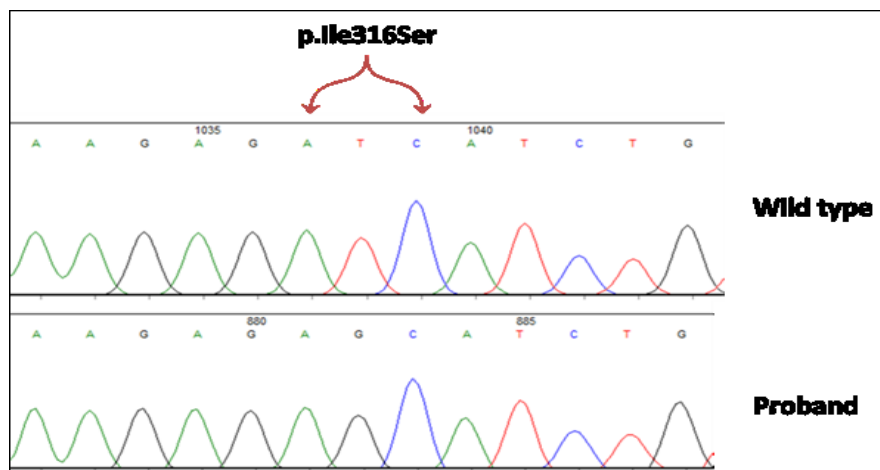


Figure 3.15 Sequencing scan of *MC4R* at the site of mutation p.Ile316Ser. Scan demonstrating a homozygous missense substitution at the only coding exon. This mutation was identified in 2 affected probands and one sibling (*F-58*, *F-74P* and *F-74S*).

Table 3.4: Physical characteristics of subjects carrying *MC4R* mutations in homozygous state.

No of individuals with mutation	No of families	IDs	Mutation	Gender	Age	SDS
1	1	F-32 P ^a	c.482T>C	F	0.6	5
2	2	F-77 P		F	5	3.7
3	2	F-77 S		F	0.9	4
4	3	F-58 P	c.947T>C	M	15	4
5	4	F-74 P		F	11	3.3
6	4	F-74 S		M	13	3

^a P: proband; S: sibling

The mutation in bold is reported here for the first time in homozygous state

3.4 Discussion

In this study we report the spectrum of monogenic obesity due to loss-of-function mutations in *LEP*, *LEPR* and *MC4R* genes, in 175 probands with early onset severe adiposity, from a consanguineous Pakistani population. Sequence analysis demonstrated that severe obesity in 30% of these subjects can be explained as due to incidence of rare or novel recessive mutations in the three genes. Amongst these 20% (n=35) had mutations in *LEP*, 6.5% in *LEPR* (n=11) whereas *MC4R* mutations were present in 2.5% (n=4), all in homozygous state.

By the end of 2011 and at the time the present study was initiated, only 5 different mutations in 14 children had been identified worldwide with congenital leptin deficiency [125, 153, 157, 169-172]. Eight of these affected children belonged to Pakistani ethnicity and were homozygous for an identical frameshift mutation (G133_VfsX14) in exon 3 of *LEP*. Later another 3 mutations in 3 subjects have been identified [173-175] (Figure 3.16 & Table 3.5).

In the present study we are reporting 42 children from 35 unrelated families carrying pathogenic mutations in the *LEP* gene. In the leptin deficient group we have identified 6 different mutations in *LEP* of which 4 were novel variants. Of note is the observation that a significant majority of the leptin deficient patients (29 of 35 unrelated probands) carried the frameshift mutation, G133_VfsX14, in the homozygous state unrelated for at least three generations. The same *LEP* frameshift mutation, was the first genetic variant discovered in association with severe obesity in

the human [125] and provided the initial impetus to genetic and molecular studies aimed at discovering the genetic and molecular basis of human obesity (regulation of body weight and energy balance) and has led to the foundation of obesity research. It is interesting to note that almost all the families with incidence of G133_VfsX14 mutation belonged to a closely-knit Pakistani caste, Arain, that constitutes one of the larger sub-ethnic populations of Punjab and that has preferred consanguineous marriages for many generations [356]. The foregoing leads us to hypothesise that G133_VfsX14 is a founder mutation in this caste.

One of the novel *LEP* mutations described here was due to a 42bp homozygous deletion at splice acceptor site. We further functionally characterized this mutation and showed that this deletion results in a truncated transcript lacking the first coding exon of the leptin gene that abolishes its normal function (Figure 3.2). Recently, we have found another novel missense mutation involving substitution of G to C that lies in the splice donor site of exon 1 (non-coding exon). As predicted by Splice Site Finder [374], the substitution eliminates this splice site and could result in encoding a non-functional protein (Figure 3.6B). Biochemical analysis of the two probands carrying this mutation showed undetectable circulating leptin levels (<1 ng/ul) as determined by ELISA. Two instances have also recently been reported by Wabitsch and co-workers [173, 378] where the aberrant protein encoded by the mutant *LEP* gene was secreted in circulation but lacked bioactivity in subjects carrying a homozygous *LEP* mutation, p.D100Y and p.N103K. In none of our *LEP* mutants we have so far found a similar condition. It may be surmised that in the majority of cases, the mutant leptin protein even when it is secreted in the blood, is not reactive to antibodies against the normal ligand, and thus escapes detection in routine immunoassays.

Another novel *LEP* mutation described by us was due to a missense substitution, c.350G>A (p.Cys117Tyr). With the exception of *LEP* mutation, p.Arg105Trp, identified here which has previously been described in a Turkish family, all pathogenic leptin variants so far identified in our cohort of severely obese children are population specific and have not been reported across various ethnic groups (Table 3.5).

Table 3.5: List of leptin gene mutation found by us and others

NO	Mutation found	Families	Affected subjects	Ethnicity	Inheritance	Reference
1	N103K	1	2	Egyptian	Homozygous	[170]
2	L72S	1	1	Australian	homozygous	[171]
3	R105W	1	5 or 4	Turkish	Homozygous	[153, 157, 176, 379, 380]
3	R105W	1	2	Pakistani	Homozygous	This study
4	398delG	5	8	Pakistani	Homozygous	[123, 125, 156, 169, 172, 174]
4	398delG	29	35	Pakistani	Homozygous	This study
5	c.481_482delCT	1	1	Pakistani	Homozygous	[174]
6	Q55X	1	1	Indian	Homozygous	[381]
7	p.D100Y	1	1	Turkish	Homozygous	[173]
8	c.104_106delTCA	1	1	Pakistani	Homozygous	This study
9	c.44-2del42	1	1	Pakistani	Homozygous	This study
10	C117Y	1	1	Pakistani	Homozygous	This study
11	c.-29+1G>C	2	2	Pakistani	Homozygous	This study

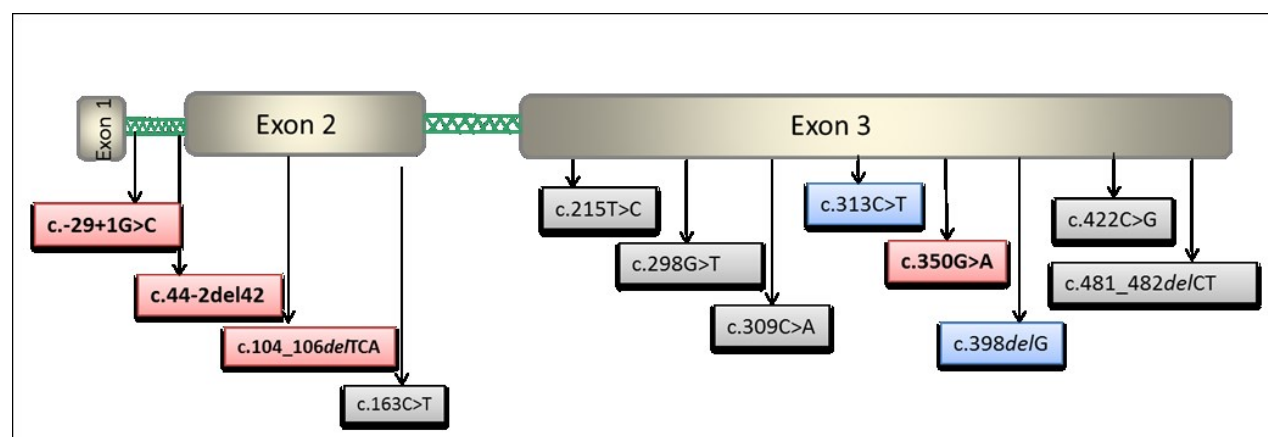


Figure 3.16: Position of homozygous *LEP* gene mutations identified in this study and by others. Grey: mutations identified by others; Blue: variants found by us and others; Red: novel mutations identified by us and described in this study.

Similar to those of *LEP*, *LEPR* mutations are also very rare and have so far found in less than 25 subjects [63, 132, 185-188] (Table 3.6). Our present study adds another 12 Individuals from 11 unrelated families with 7 novel homozygous variants (Table 3.3 and Table 3.6). This includes a unique copy number variation in *LEPR* gene found in a 0.6 year old boy, which involved two closely mapping homozygous deletions, 1.3 kb and 58.9 kb, starting from intron 3 and extending to the intergenic region. Two main mechanisms have been proposed for the formation of CNVs: non-allelic homologous recombination (NAHR) and non-homologous end joining (NHEJ) depending on 'microhomology' in place of long stretches of homologous sequences, at the CNV breakpoints [382]. A small insertion of 16 bp of unknown origin was observed at 3' of the deletion, which likely occurred as part of the NHEJ process. Previously, a deletion in *LEPR* was reported that includes the promoter, exons 1 and 2 [188] whereas deletion in our proband starts from intron 3.

In 4 of the unrelated probands, we identified a homozygous *LEPR* mutation involving a base substitution (G>T) at the intron/exon junction of the splice acceptor site of exon 15 resulting in an abnormal splicing of *LEPR* transcripts due to skipping of exon 15. This mutation renders the protein with 798 amino acids of the *LEPR* extracellular domain while it lacks both transmembrane and intracellular domains of the receptor.

The third loss-of-function *LEPR* mutation is a nonsense mutation (p.Trp558Stop) identified in the two unrelated probands. The said mutation results in a premature stop codon thus translating for a truncated *LEPR* protein comprising the first 558 amino acids of the extracellular domain, and lacking both trans-membrane and intracellular domains of the receptor. The fourth novel missense mutation, p.Cys604Ser, in exon 11 of *LEPR* gene was reported in two brothers from a consanguineous family. The older of the two (3 years old) was also reported suffering from developmental delay and mental disability. Two novel 12 amino acid apart mutations c.2114G>A (Trp705Stop) c.2153A>G (Lys718Ser) were also found in two unrelated severe obese probands.

As reported earlier by others [131, 132, 186, 187], all our *LEPR* deficient subjects presented with extreme adiposity and hyperphagia. However, we did not find any difference between the phenotypic features of the *LEPR* mutations and BMI and age matched leptin deficient children drawn from the same population. These observations are in contrast to a previous study in which phenotypic features of *LEPR* deficient subjects have been reported to be less severe and strikingly different from *LEP* mutant carriers [132, 204]. Our data match with animal studies where leptin

deficient mice, and mice lacking *LEPR* are phenotypically identical [152]. This phenotypic difference reported previously may be due to heterogeneity in patients' ethnic origin and age (as suggested by the investigators), which is not the case in our study.

Table 3.6: List of leptin receptor gene mutation found by us and others

No	Mutation	Affected subjects	Ethnicity	Reference
1	G>A in the donor splice site	3 (1 deceased)	Algerian	[63]
2	4-bp deletion in codon 22	3 (1 deceased)	Bangladeshi	[132]
3	11-bp deletion in codon 70	2	Turkish	[132]
4	66-bp deletion in codon 514	1 (deceased)	Iranian	[132]
5	W31X	3	Southern European	[132]
6	A409E	1	Turkish	[132]
7	W664R	1	Turkish	[132]
8	H684P	1	White (UK)	[132]
9	1-bp deletion in codon 15 and R612H	1	White (UK)	[132]
10	P316T	2	Egyptian	[187]
11	Uniparental Disomy	1	Not mentioned	[186]
12	p.C186AfsX27	1	Guinean	[185]
13	p.H160LfsX9	2	Sudanese	[185]
14	Deletion containing ex1 & 2 of LEPR	1	Not mentioned	[188]
15	p.799-1G>T	4	Pakistani	This study
16	p.Trp558*	2	Pakistani	This study
17	p.C604S	1	Pakistani	This study
18	1.3kb and 58.8kb deletion	1	Pakistani	This study
19	p.W705*	1	Pakistan	This study
20	p.N718S	1	Pakistan	This study
21	p.E14K	1	Pakistan	This study

Melanocortin deficiency has been regarded as the most common type of monogenic obesity in the West with 166 distinct variants of *MC4R* gene identified to date [201]. Previous studies demonstrate a co-dominant mode of inheritance and the majority of the variants described so far are documented in the heterozygous state. In our study we identified 6 subjects carrying *MC4R* homozygous mutation p.Met161Thr or p.Ile316Ser. The former mutation has previously been reported only in the heterozygous state in a 12 years old obese subject of Turkish origin [203].

The other homozygous *MC4R* mutation, I316S, previously reported in both homozygous and heterozygous carriers [383], was found by us in three 11-15 years old subjects. It is of note that the onset of obesity and hyperphagia, in these probands were reported to occur at a much advanced age (10–11 years) as compared to that of the child with the homozygous Met161Thr mutation.

Interestingly, we failed to identify any *MC4R* heterozygous mutants with obesity in our study. Heterozygous sibling of the probands with homozygous p.Met161Thr and p.Ile316Ser mutations were of normal weight and were not reported hyperphagic. In a previously reported multi-generational family study suggests that penetrance of heterozygous *MC4R* mutations is dependent of the obesogenic environment [202]. In the present investigation on severely obese Pakistani children, we also show that whereas onset of obesity in subjects with *MC4R* mutation p.I316S, was between 4-6 years, in those carrying M161T mutation, hyperphagia and increased adiposity were first noticed at a much earlier age of 3 months. The foregoing observation leads us to speculate that expression of obesity resulting from *MC4R* deficiency may not only be influenced by environmental factors but also in relation to age.

Hormone replacement therapy given to patients with congenital leptin deficiency brings about almost a complete reversal of clinical symptoms and results in normalisation of food-intake, body weight and energy homeostasis. Treatment with recombinant leptin of such rare cases has been in place in the West for more than a decade on a restricted or trial basis [169, 380] but has been lacking in countries like Pakistan until now, where there is a much higher incidence of the disease. During the last four years we have been in constant contact with drug companies that successively became responsible for the supply and compassionate use of recombinant leptin including Amylin, BMS, AstraZeneca and now Aegerion Pharmaceuticals, with the aim to deliver treatment to affected children in Pakistan but unfortunately with no significant success. The recent approval of the drug Myalept (recombinant leptin) by US Food and Drug Administration (FDA), for use in cases of congenital leptin deficiency and lipodystrophy, brings potential hope that treatment of affected children in other countries, may become available in the near future and to help provide these children a near normal life and prevent further child mortalities due to this disease.

Leptin replacement therapy for treatment of congenital leptin deficiency, as yet the only successful form of personalized medicine in the context of obesity, is obviously ineffective in LEPR

deficiency. It is imperative, therefore, to develop novel pharmacological strategies that could help in a direct activation of the downstream signalling of the melanocortin pathway thus circumventing its dependence on LEP-LEPR binding. Some of the potential candidates in this context include biomolecules such as the ciliary neurotrophic factor (CNTF) and its receptor agonist, axokine [384, 385]. Preliminary studies indicate that treatment with CNTF is accompanied by a decrease in adiposity both in humans and *db/db* mice [386].

Furthermore, novel small molecule compounds acting as pharmacological chaperones have been identified that can facilitate *in vitro* trafficking of the receptor protein to plasma membrane in cases of human *MC4R* mutants that result in intra-cytoplasmic retention of receptor protein and poor surface expression [387-389] but *in vivo* a positive impact in *MC4R*-deficient obese patients remains to be demonstrated. There is also the possibility that these pharmacological chaperones and highly potent *MC4R* agonists may find their use in the treatment of some types of common obesity for inducing a decrement in food intake and normalization of energy homeostasis [387, 388]. It is to be expected that personalized medicine based on molecular diagnosis shall in future play a key role in management of severe obesity with an unfolding of the genetic cause of different forms of this disease. The foregoing further emphasizes the importance of elucidating monogenic forms of obesity in consanguineous populations, in this perspective.

In summary, by using a multi-stage screening regimen we were able to identify genetic causality in 30% of unrelated obese children from this consanguineous population due to pathogenic mutations in *LEP*, *LEPR* or *MC4R* gene, compared to 3-5% in such cases reported from other populations. In our cohort of extremely obese subjects a lack of incidence of pathogenic variants in the remaining 25 known obesity associated genes is noticeable warranting a more thorough and exhaustive exome (or genome) analysis in the next phase. These findings also underscore the advisability of undertaking a comprehensive screening of obesity associated genes in other large consanguineous populations that offer a unique and valuable genetic material and which presumably could reveal new genes and pathways associated with body composition and energy balance.

4. Chapter four

Variants causing Prader-Willi-Syndrome (PWS) and Bardet-Biedl-Syndrome (BBS)

4.1 Introduction

Syndromic form of obesity presents besides excessive adiposity a wide range of associated clinical phenotypes such as organ specific developmental abnormalities and variable degrees of mental retardation. About 30 syndromic forms of obesity have so far been identified [235, 390]. In comparison with monogenic form of obesity, the genetic and molecular basis of syndromic disorders appears to be much more complex.

Bardet Beidl syndrome (BBS) is an example of genetically heterogeneous disorders, which is characterized by obesity, pigmentary retinopathy, polydactyly, hypogenitalism and mental retardation. The condition has been associated with 18 genes (BBS1-18) [248, 249]. This syndrome has been considered as a pleiotropic genetic disorder because of a significant variation in its expressivity and clinical variability [249, 391]. Although the causal factors of the BBS have remained obscure for a long time, more recent data and experimental evidence indicate that the condition is mainly attributable to a dysfunction of primary cilia. Inheritance of BBS is traditionally considered as autosomal recessive although notable exceptions exist suggesting its 'oligogenic' nature [392].

Whereas, BBS exemplifies pleiotropy and heterogeneity, Prader Willi syndrome (PWS) presents a complex interaction between epigenetic and genetic events during foetal development. PWS is phenotypically characterized by obesity, short stature, mental retardation, minimal pain perception and hyperphagia, and is caused by a deletion on the paternal chromosome 15 (15q11.2-q12). Three main events have been reported where loss of expression of paternal genes on the imprinted region is the most prevalent cause and explaining genetic basis in 75% of patients with PWS. However less than 3% of the affected subjects carry imprinting errors caused by microdeletions of the imprinting center at the *SNURF-SNRPN* gene locus and less than 1% due to paternal translocations. As described in Chapter 1, section 1.4.3 the vast majority of individuals with deletions in 15q11.2 region have one of two proximal breakpoints (BP1 or BP2) and a common distal breakpoint (BP3). These breakpoints arise due to presence of recurrent tandemly repeat sequences flanking the breakpoint regions that result in interstitial deletions of about 5-6 Mb in size. Some unique deletion(s) with specified breakpoints have been reported in a small

number of cases [239]. More recently, deletions between BP1 and BP2 and associated phenotypes have also been described [393, 394]. Until now, the definitive mechanistic link between defective gene products and the phenotype including dysregulation of energy balance remains to be elucidated.

It may be mentioned that categorization of the two forms of obesity, monogenic and syndromic, is arbitrary and mostly a matter of convenience. Syndromic obesity can also be caused by mutation in a single gene and monogenic obesity where the more prominent presenting feature is undoubtedly increased adiposity, is associated with more subtle and less emphasized phenotypic characteristics such as red hair, increased linear growth etc. and physiological abnormalities such as dysfunction of endocrine, reproductive and immune systems. However, various degrees of mental disability almost invariably an outcome of incidence of syndromic obesity have only rarely been reported in subjects with monogenic obesity.

To our understanding not much published information is available on the incidence of syndromic obesity in Pakistani population. In this chapter we are reporting cases of syndromic obesity identified to date in our cohort of Pakistani children with early onset severe obesity. We believe that continuation of such efforts will contribute to a better understanding of the phenotype-genotype relationship and lead to evidence-based diagnosis and management of patients afflicted with such a complex disease.

4.2 Results

In our cohort of severely obese children, 8 subjects from 6 unrelated families were identified with causal variations in BBS and PWS related genes by using microdroplet-based PCR followed by NGS, and CNV and MLPA analyses already described in detail in chapter 2. The variants identified in BBS genes included 2 frameshift insertion mutations of which one was novel and one compound heterozygous mutation. Deletions in PWS associated region were found in 2 probands and one of these subjects carried a novel intermediate size deletion with distinct breakpoints. In addition, a 1.3 Mb duplication in chr17 in a proband, was suggestive of Albright hereditary osteodystrophy (AHO). Altogether these mutations explain syndromic obesity in 4% of cases registered with us.

4.2.1 Mutations related to Bardet-Biedl syndrome (BBS)

4.2.1.1 Bardet-Biedl syndrome 2 (BBS2)

c.406insG (p.Ala136Glyfs*15)

Droplet PCR using RainDance technology and next generation sequencing revealed this novel insertion mutation at 406 position of exon 3 of the *BBS2* gene. The mutation results in a shifting of the frame starting from codon position 136 due to substitution of glycine in place of alanine followed by a premature stop codon after 13 aberrant amino acids. This results in a 450 amino acid long truncated protein compared to the normal 721 amino acid chain (Figure 4.1).

F-93: The two male siblings, 9 and 13 years old (*F-93* and *93C*, respectively), from a consanguineous family were found to carry this novel insertion mutation in the *BBS2* gene. Both siblings were severely obese with a BMI SDS of 3.9 and 3.0. They weighed normal at birth but rapidly started gaining weight at the age of 2-4 months accompanied by an insatiable craving for food. In both siblings, the vision became progressively impaired after 5 years of age and both were reported to have learning problems. A younger sister of affected brothers was of wild type while the parents were heterozygous carriers.

4.2.1.2 Bardet-Biedl syndrome 10 (BBS10)

c.271insT (p.Cys91Leufs*5)

This frameshift mutation in homozygous recessive state was identified in the last exon (exon 2) of *BBS10* gene due to the insertion of T between amino acid positions 271 and 272. This insertion resulted in a shifting of frame due to a substitution of cysteine by leucine at amino acid position 91 and appearance of the stop codon after three aberrant amino acids. Consequently, the mutation caused encoding of a 95 amino acid truncated protein compared to the normal 723 amino acid sequence (Figure 4.2).

F-73 and F-114: This recessive frameshift mutation in the BBS10 was identified in two unrelated 6.5 and 1 year old boys. The older boy (F-73) was born with normal body weight but was reported to experience intense appetite in early childhood and started to gain excessive body weight which was 43 kg with a BMI SDS of 4.2, at the time of recruitment. He also had defective vision and was slow in learning. The boy presented postaxial (ulnar) polydactyly of both hands. The other proband (F-114), identified with identical mutation, was reported to have low body weight at birth but rapidly gained weight from 2 months onwards and attained a BMI SDS of 4.93 at the age 1 year. In contrast to the other proband this child suffered postaxial polydactyly of feet.

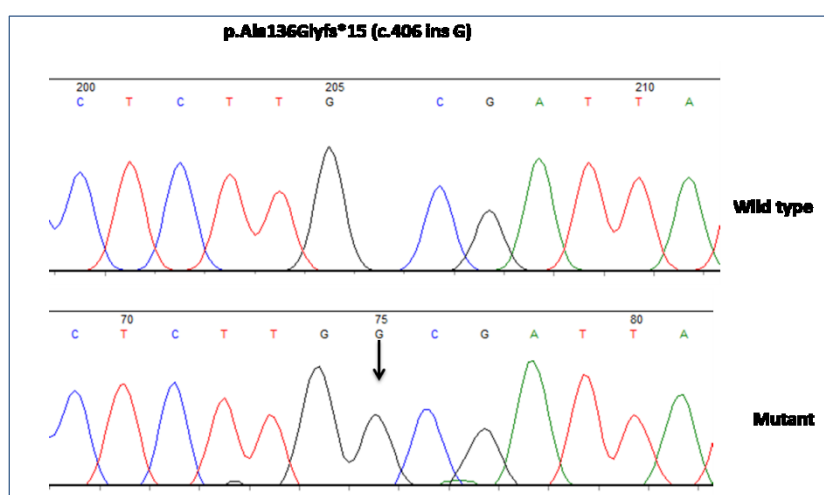


Figure 4.1 Sequencing scan of *BBS2* at the site of mutation 406insG. Scan of *BBS2* at the site of mutation demonstrating a homozygous 1 base deletion in exon 3 disrupting the reading frame. This mutation was identified in F-93P and F-93S.

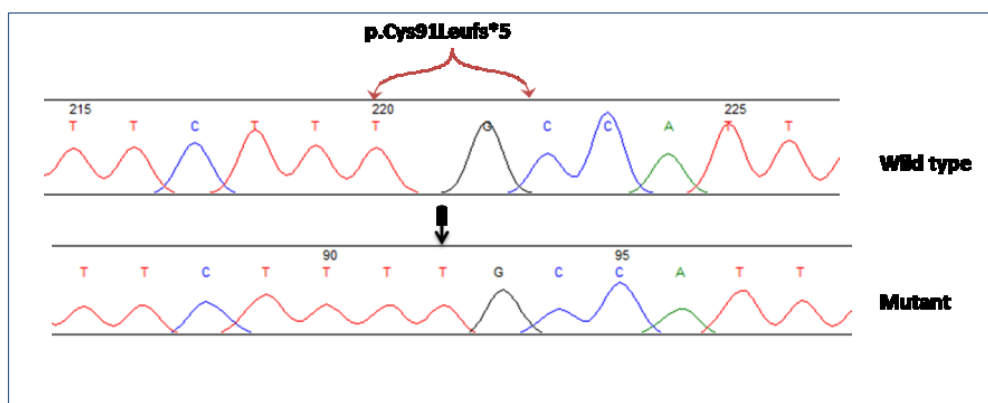


Figure 4.2 Sequencing scan of *BBS10* at the site of mutation 271insT.

The scan demonstrates a homozygous 1 base deletion in exon 2 disrupting the reading frame. This mutation was identified in *F-73* and *F-114*.

4.2.1.3 Bardet-Biedl syndrome 9 (*BBS9*)

c.635T>C (p.Leu212Ser) & c.662A>G (p.Glu221Gly) – compound heterozygous mutation

Another proband was found to carry 2 missense mutations in *BBS9*, c.635T>C and c.662A>G, both in heterozygous states. The two mutations were located 8 codons apart in exon 7 (Figure 4.3). One of the mutations was due to a substitution of leucine to serine at coding position 212 and was predicted to be disease causing (p-value: 0.791) by MutationTaster and deleterious (score: 0) by Sift. While the second substitution mutation was at position 221 resulting in a change of glutamate to glycine. The change is predicted to be disease causing (p-value: 1) by MutationTaster and deleterious (score: 0) by SIFT. This is the first report of the presence of a compound heterozygous mutation in a proband from our cohort of severely obese subjects.

F-128: The proband identified with this compound heterozygous mutation in *BBS9* was a 2.5 year old girl with a body weight of 21kg. She developed hyperphagia and started gaining excessive weight at 3 month after birth. The patient presented postaxial polydactyly of both hands, weak eyesight and learning problems.

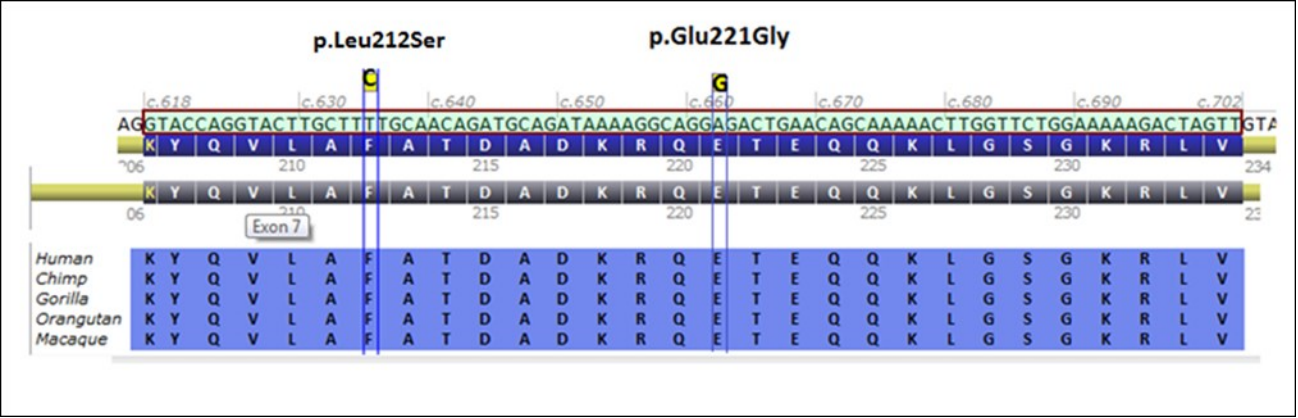


Figure 4.3 Diagrammatic presentation of two heterozygous missense mutations The mutations, Leu212Ser and Glu221Gly in *BBS9*.were identified in exon 7 of proband F-128.

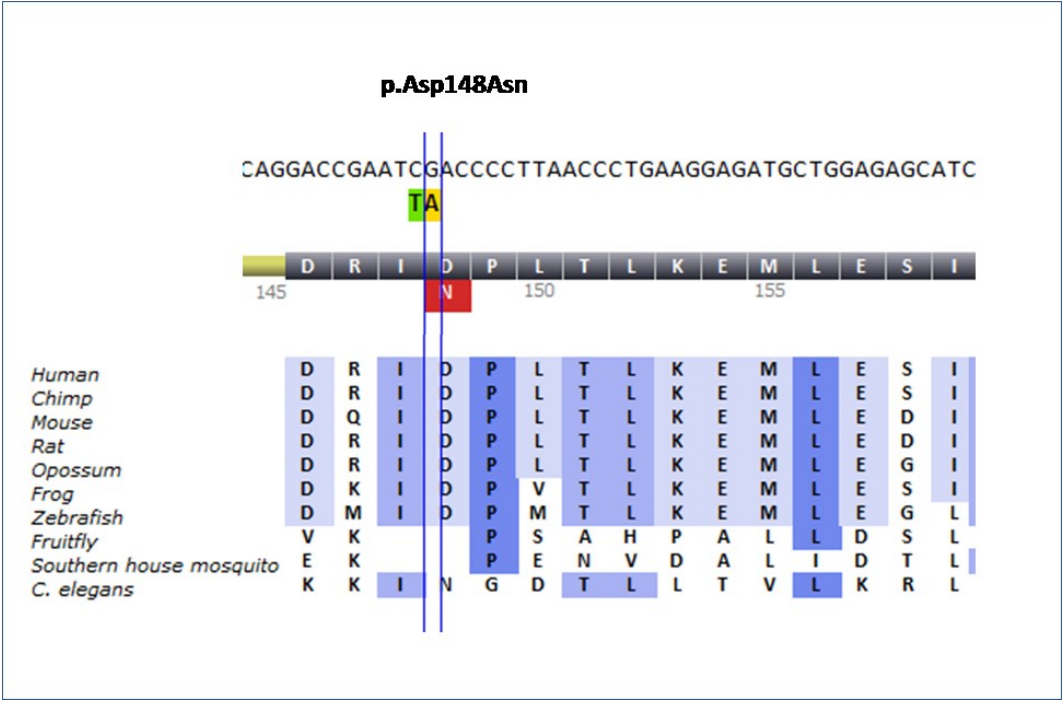


Figure 4.4 Diagrammatic presentation of missense mutation c.442G>A identified in *BBS1*. The mutation was identified in F-129.

4.2.1.4 Bardet-Biedl syndrome 1 (*BBS1*)

c.442G>A (p.Asp148Asn)

This homozygous missense mutation in *BBS1* gene was identified in a female proband and is predicted to be disease causing by Mutation Taster ($P = 1.0$) although tolerated by Sift software (score: 0.5) (Figure 4.4). The mutation is recorded in db SNP database under rs200688985 with a $MAF < 0.01$. The same mutation is reported in subjects from UK and US and shown to be pathogenic [377]. Interestingly, this proband simultaneously carried a loss-of-function homozygous mutation in the *LEPR* gene described in chapter 3 (section 3.3.2.1).

F-129: The proband was the only child and was the outcome of a first cousin union. She weighed 31 kg at the age of 1.4 year with a BMI SDS of 3.4 and was reported hyperphagic. The parents noticed her insatiable hunger soon after she was born and excessive body weight gain when the child was about 3 month old.

4.2.2 Prader-Willi syndrome (PWS) related genomic deletions

Whole genome genotyping followed by copy number analysis by PennCNV identified 2 female probands carrying deletions associated with PWS, One of these deletions is being reported here for the first time. The presence of deletions and their breakpoints were confirmed by carrying out MLPA.

4.2.2.1 4.85 Mb heterozygous deletion

CNV analysis of genotyping data identified a 4.8 Mb heterozygous deletion on chromosome 15 in a proband (Figure 4.5A). Validation of CNV predicted by PennCNV was carried out by MLPA using P343 probemix. The analysis showed deletion of 18 probes in 15q11-13 region associated with PWS. Breakpoint analysis revealed that *MKRN3* was the first deleted gene on centromeric site and

HERC2 was the last deleted gene from the telometric site thus categorizing it as a class II deletion (Figure 4.5B). The mother of the proband was found wild type for the said deletion.

F-87: The proband found to carry this deletion was a 22 years old female with body weight of 101 kg, height of 1.36 m and a BMI of 54. The proband was reported to have a low birth weight but started gaining weight after 1.5 years of age. Her IQ was recoded much lower than normal subject of her age and as per report she has not yet attained puberty.

(A) Log R Ratio and B Allele Frequency plot for 15q11 deletion region in F-87. (B) MLPA of patient using P343 probemix representing 18 probed deletion in 15q11.2-q12 region.

4.2.2.2 3.35 Mb heterozygous deletion

This 3.35 Mb heterozygous deletion was also identified in PWS associated region in chr15 (Figure 4.6A). Screening for duplications and deletions through MLPA analysis by using 343probemix confirmed a heterozygous deletion encompassing nine pairs of primers within the 15q11 (Figure 4.6C). This deletion is part of the conventional interstitial deletions at 15q11-13 that have been associated with PWS syndrome. Moreover, the assay revealed that the said deletion carries distinct breakpoint at the telomeric end. However due to non-availability of the probes at centromeric end, we were unable to access deletion breakpoint at this end.

Copy number changes and CpG island methylation of the 15q11

Methylation specific MLPA (MS-MLPA) with high density of probes at the centromeric end of the 15q11-q13 region was, therefore, used to check for breakpoint copy number deletions as identified by MLPA using P343 probemix. MS-MLPA (ME-028 probemix) used here comprised a total of 48 probes with 32 of these probes specific for PWS critical region of which 13 probes contained a *Hha* I recognition site in their target specific sequence to analyse methylation of the CpG island. The assay confirmed a unique and atypical deletion encompassing 28 of total 32 probes in chromosomal position 15q.11.2 with distinct breakpoints (Figure. 4.6D). The genomic position of the proximal deleted probe was 204,751,54 that lies between BP1 and BP2 and of the proximal deleted probe 232,352,21. Thus, the possible size of the deletion could be approximately 2760067 bp. DNA methylation within 15q11-q13 identified altered methylation pattern in proband (Figure 4.6 C). Identical analyses of rest of the 8 family members revealed normal copy number and methylation status.

F-39: The proband with these unique breakpoints was a 7.5 years old girl weighing 44 kg (BMI SDS: 6.0) born to consanguineous parents. The child had a history of onset of obesity at 6 months of age although she had a low birth weight. The patient was reported hyperphagic, short tempered and with learning disability and other behavioural problems.

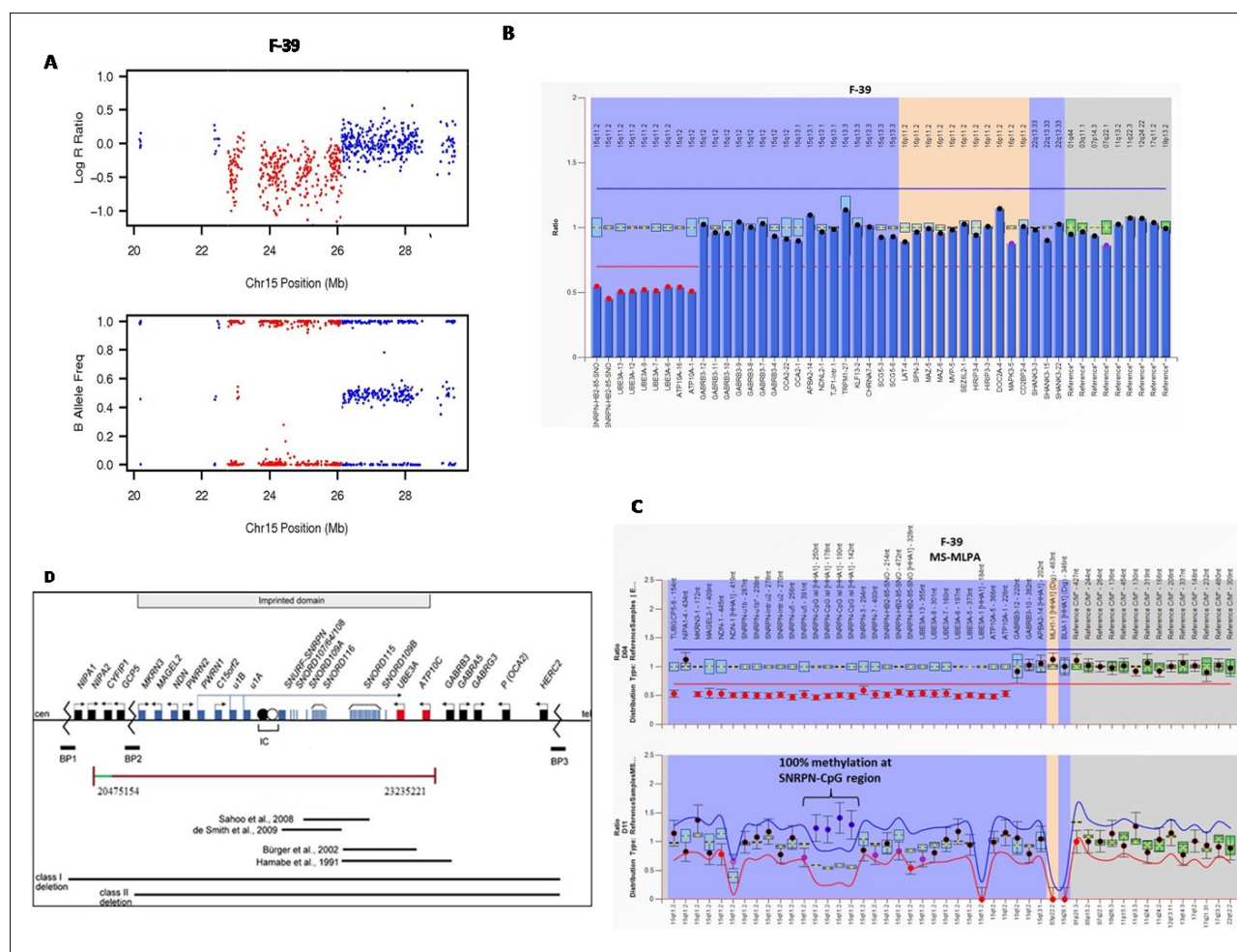


Figure 4.6 Identification and validation of 3.35 Mb deletion in 15q11.2-q12 region. (A) Log R Ratio and B Allele Frequency Plot for 15q11 deletion region. (B) MLPA of patient using P343 probemix representing 9 probed deletion in 15q11.2-q12 region. (C) Upper: MS-PLMA (ME-028 probemix) of patient describing incidence of copy number deletion of 28 probes in PWS/AS region. Lower: Abnormal methylation status at SNRPN-CpG region. (D) Schematic overview of human chromosomal region 15q11q13. Class I and class II deletions and atypical familial deletions in patients with AS and PWS are drawn as horizontal lines (derived from [395]). Red horizontal line depicts deletion breakpoints found in F-39.

4.2.3 1.3 Mb duplication in chromosome 17 – A suspected case of Albright hereditary osteodystrophy (AHO)

A 7 years old girl presented at the hospital had phenotypic features similar to those of patients with AHO. WES analysis failed to identify any mutation in the GNAS region. However, CNV analysis revealed a 1.3 Mb duplication in chromosome 17 (chr17:29060212-30380381) (Figure 4.7A). A previous investigation reports a duplication of 1.3 Mb in 17q11.2 including the *NF1* gene in a patient [396]. This study demonstrated a significant loss of the maternal methylation pattern at all three sites in the GNAS region. The duplication found in our proband was identical to the one reported in this study. To check for differential imprinting in the chromosome 20q13.32 - GNAS locus, MS-MLPA using ME031 probemix was performed. With this procedure we failed to determine any aberrant methylation in one or more sequences within this locus (Figure 4.7B).

F-55: The proband (F-55) was a 7 years old female who was born to non-consanguineous parents. At the time of examination her body weight was 39 kg and a height of 1.01 m. She had a round face, general obesity, short stature, brachydactyly, delayed development of permanent dentition and suffered mental retardation. She was unable to walk by herself, had kyphosis and restricted movement of her right arm and she also had kyphosis. Her mother, elder sister and younger brother were mentally and physically normal but the father had short stature and also suffered kyphosis (Figure 4.8).

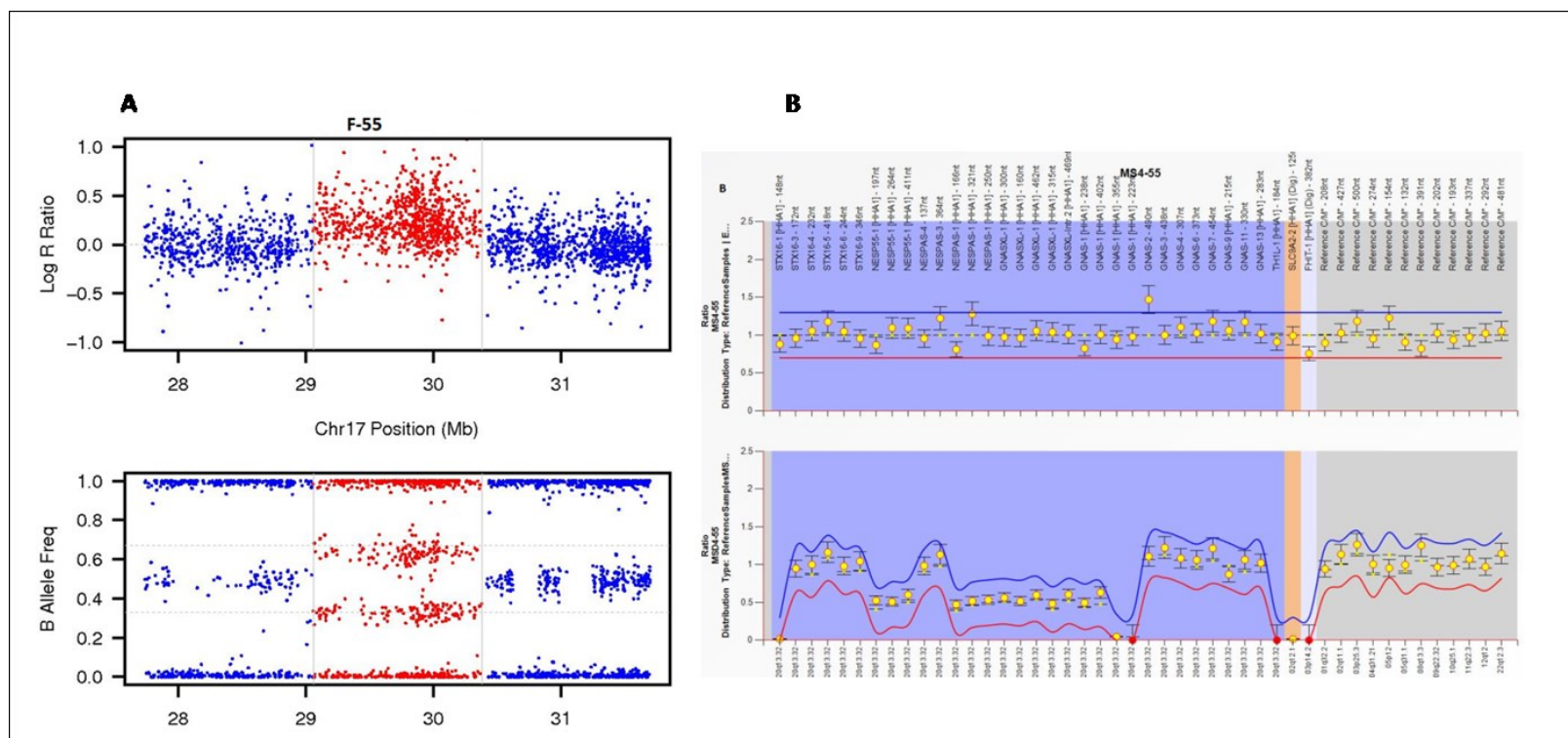


Figure 4.7 Identification and validation of 1.7 Mb deletion in 17q11.2 region.

(A) Log R Ratio and B Allele Frequency Plot for 17q11.2 duplication region. (B) Upper: MS-PLMA (ME031 GNAS probemix) of patient describing absence of copy number change in this region. Lower: Normal methylation status at the GNAS locus at chromosome 20q13.32.



Figure 4.8 Photographs of proband (*F-55*) suspected with AHO and her parents.

4.3 Discussion

This part of study was carried out to identify the genetic variants associated with syndromic type of obesity in subjects from Pakistan. The genetic analysis was carried out by using a combination of techniques including targeted sequencing, whole genome genotyping followed by copy number variation analysis, and MLPA analysis. Here we have identified 4 probands with BBS and 2 probands with PWS. In addition, AHO syndrome was suspected in one of the probands from this cohort.

Targeted sequencing analysis resulted in identification of 6 subjects from 5 unrelated families with causative mutations in *BBS* genes. These included a novel homozygous insertion mutation in *BBS2*, a novel compound heterozygous missense mutation in *BBS9* and a previously reported insertion mutation in *BBS10*, in homozygous state [397]. The variant in *BBS10*, (c.271insT), has been reported in several subjects and considered as one of the commonest mutations found in the *BBS10* gene [397]. Interestingly, one of the probands in addition to a homozygous p.Asp148Asn mutation in the *BBS1* gene also carried a loss-of-function mutation, p.799-1G>T, in the *LEPR* gene that was also found by us in another four unrelated children from the same population, as described in chapter 3 (section 3.3.2.1). The *BBS1* mutation in this proband has previously been reported causative in cohorts from UK and US [377]. The specific impact due to the simultaneous presence of these two variants on the obese phenotype is obviously not clear. However, it may be mentioned that some of the BBS associated features such as polydactyl and vision impairment, were not reported in this proband.

Whereas, one of the probands (*F*- 87) with PWS was shown to carry the more typical type II deletion with BP2-BP3 breakpoints in the PWS associated 15q11 region, the other proband (*F*-39) was found carrying a 3.3Mb intermediate size deletion with distinct breakpoints. The presence of these unique breakpoints was indicated by whole genome genotyping and CNV analysis. MLPA and MS-MLPA, confirmed 3.3 MB interstitially deleted region and methylation defects in SNRNP-CpG region. This deletion and methylation profiling of the subjects validates the presence of PWS in this patient. The proband carrying this deletion had a below average birth weight, early onset obesity and hyperphagia, short stature in relation to age, microcephaly and small hands and feet. The centromeric deletion breakpoint was localized between BP1 and BP2 including GCP5 but spared *NIPA1*, *NIPA2*,

and *CYFIP1*. The latter three genes are widely expressed in the central nervous system, while *GCP5* is primarily expressed in the subthalamus part of the brain [398]. In two previous studies 10 subjects have been identified carrying a deletion between BP1 and BP2, presenting delayed development, dysmorphic features and behavioural problems. Moreover, the deletion was found to be inherited from normal or mildly affected parents [399, 400]. More recently another 83 subjects were identified carrying the same BP1-BP2 deletion [394]. These data indicate the involvement of related genes in neurodevelopmental and motor developmental anomalies. Since a wide range of phenotypes has been observed in subjects carrying a deletion in this region, it is difficult to determine whether all the four non-imprinted genes (*NIPA1*, *NIPA2*, *CYFIP1* and *GCP5*) localized to the interval between BP1 and BP2 contribute towards the PWS phenotype.

The last proximal deleted gene in this subject, was *ATP10A* thus sparing the immediate *GABRB3* and more distal genes. However, this distal cluster of genes not included in the deleted part, was not imprinted. In a subject with PWS identified previously with almost identical distal breakpoints, a low threshold of pain and skin picking behaviour have been observed [239]. A decreased sensitivity to pain along with skin picking behaviour was also reported in our patient with PWS. Previous finding suggests that *GABA* gene may be implicated in pain perception [401] and low threshold of pain observed in PWS patients has been attributed to haploinsufficiency of *GABA* gene receptor [239]. Previous findings in subjects carrying small deletions with normal copy number for *GABA* gene receptors are conflicting and inconclusive [240, 241]. However, our observation of decreased sensitivity to pain in the PWS patient, is consistent with a previous finding suggesting that *GABA* gene may be implicated in pain perception [401].

Hyperphagia and low energy expenditure resulting in excessive adiposity, are the more commonly shared traits in subjects identified with PWS. These features are suggestive of hypothalamic abnormalities that may affect satiety signals. However, genetic and molecular determinants responsible for this function are yet to be elucidated [402-404]. In a previous study the hyperphagic response of PWS patients has been ascribed to elevated levels of the orexigenic gut hormone, ghrelin [405].

It is expected that our finding of a unique intermediate size deletion in one of the probands (*F-39*), in contrast to the previously reported typically larger deletions or microdeletions in 15q11.2 region, can substantially help in predicting association of specific genetic factors with distinct clinical features. However, such an assumption necessitates verification through recording of additional aspects of the phenotype and carrying out relevant clinical tests. No detailed examination on the other proband (*F-87*) with conventional BP1-BP3 deletion and phenotypic comparisons with affected subjects carrying intermediate size deletion has been carried out so far, in an attempt to elucidate the phenotype-genotype relationship in such cases.

One of the probands in our cohort of obese children presented a striking resemblance to the AHO phenotype. Sequence analysis revealed a duplication of a 1.3 Mb region (17q11.2) that has previously been associated with AHO [396]. However, unlike the aforementioned report, our proband carried a normal methylation status. Microduplications in this region that includes the *NF1* gene have also been described in two affected cases [406] associated with mental retardation, cardiac anomalies and dysmorphic features. In the present case, evaluation of calcium and phosphorus in blood and cyclic AMP levels in urine should help in further validation of our findings.

In summary, the present study records a relatively high incidence of Bardt Biedel and Prader Willi syndromes in a Pakistani population, due to rare and novel variations in associated genes and imprinting errors. These findings definitely warrant further genetic and clinical investigations in a larger group of subjects with suspected syndromic obesity. We believe that such efforts while unravelling rare and novel mutations, genes and susceptible genomic regions, would be instrumental in narrowing down the existing genotype-phenotype gap in relation to this complex disease.

5. Chapter five

Hormone Profile of Subjects with Monogenic Obesity

5.1 Introduction

The availability of food and nutritional state of the body are relayed to the hypothalamus *via* multiple afferent signals through hormones, neuropeptides, neurotransmitters and circulating metabolites [272]. These signals are integrated by the hypothalamus and after processing by the brain result in changes in eating behaviour affected by efferent pathways and thus regulate the energy balance [126]. Of the peripheral hormonal signals, leptin, insulin and thyroid hormones, and peptides produced by the gut including ghrelin, peptide YY (PYY), glucagon-like peptide (GLP) 1 and cholecystokinin (CCK), are the main components of the food intake regulatory mechanism [274].

Several studies indicate that plasma levels of ghrelin secreted by oxyntic glands of the stomach and the only orexigenic gut hormone known so far, are elevated before meals and decline postprandially, supporting its role as a meal initiator [407]. Ghrelin action mediated by its receptor, GHS-Rs, triggers neuronal activity of orexigenic neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons and inhibits proopiomelanocortin (POMC) neurons resulting in appetite stimulation [408]. Peripheral ghrelin levels have been shown to be inversely correlated with fat mass and circulating levels of insulin and leptin [371]. Thus, ghrelin levels are lower in subjects with common obesity and higher in anorectic patients as compared to normal lean individuals [371, 409]. Although excessively high ghrelin levels have been associated with excessive appetite in subjects with Prader-Willi syndrome (PWS), yet the role of this orexigenic hormone in hyperphagic response in leptin-melanocortin pathway deficient states [405, 410] has not been elucidated [274].

Anorexigenic peptides, PYY and GLP-1, produced in the L cells of the distal gut act by inhibiting food intake through their effect on the arcuate nucleus [410]. In contrast to ghrelin, levels of PYY and GLP-1 are low in fasting state and rise following meal in proportion to the calories intake [411, 412]. Fasting PYY levels are negatively correlated with the body-mass. Peripheral administration of PYY(3–36) reduces the body weight in ob/ob mice [370]. Similar to PYY, circulating GLP-1 levels are inversely related to the body mass [413]. Exogenous GLP-1 administration reduces appetite and energy

utilization both in normal weight and obese persons [414]. PYY appears to act through a gut-hypothalamic pathway, mediated by its Y2 receptors in the arcuate nucleus to inhibit feeding whereas GLP-1 action mainly involves both the hypothalamus and the brainstem-vagus complex [411, 414].

Whereas the nutritional state of the body is transmitted to the hypothalamus via multiple afferent signals, regulation of food intake and body weight is mediated by the hypothalamic melanocortin pathway. Leptin, besides having long-term role in energy homeostasis, has also been implicated in regulation of food intake through affecting meal-size and appetite [415]. The satiety effect and meal termination response to this hormone, have been attributed to a blocking of NPY neurons and an enhanced POMC/ cocaine- and amphetamine-regulated transcript (CART) signalling [416, 417].

Loss-of-function single-gene mutations disrupting leptin melanocortin pathway result in a disruption of energy homeostasis. This perturbation in energy balance not only results in severe obesity and hyperphagia but also leads to varying degrees of endocrine pituitary dysfunction. This is exemplified in leptin deprived ob/ob mice that exhibit a wide range of phenotypic abnormalities other than severe obesity, such as infertility (hypogonadotrophic hypogonadism) [418], dysregulation of hypothalamo-pituitary-thyroid axis [419, 420] and stunted body growth [421, 422]. These mouse models also show increased adrenal stimulation by ACTH [423] and high circulating glucocorticoid level [424].

It may be pointed out that certain hormones do not show the same trend in leptin deficient humans as observed in mice (described above). For instance the GH axis in humans is not affected by leptin in the same manner or to the same extent as it is in rodents and most of the children with congenital leptin deficiency undergo normal linear growth [156, 425]. Similarly, the functional response of the pituitary-adrenal axis to leptin deficiency in rodents has not been replicated in the human in previous studies [131, 132, 156, 425].

Available data on endocrine profile in leptin and leptin receptor deficient states in the human as reported by various laboratories, are derived mainly from sporadic cases and consequently lack consistency as illustrated by conflicting observations on thyroid gland function in *LEP* mutation carriers [131, 169, 170, 372, 425] or with regard to GH profiles in *LEPR* deficient subjects [131], in various studies.

In this section we describe for the first time the endocrine profile of severely obese subjects with homozygous pathogenic mutations in *LEP*, *LEPR* and *MC4R* genes in a single population, by measuring serum levels of leptin, insulin, thyroid stimulating hormone (TSH) and cortisol. Additionally, leptin levels have also been determined in subjects with heterozygous mutation in the leptin gene and compared with normal wild type individuals to re-address the question of a state of 'partial leptin deficiency' [426] due to the presence of a single functional allele in the former group.

Congenital leptin deficiency also presents a unique model to assess a possible contributory role of gut hormones underlying hyperphagia and lack of satiety that are the most characteristic traits of the disease. We have, therefore, in a separate experiment assessed the fasting and postprandial responses of gut hormones (ghrelin, GLP-1 and PYY) in a group of subjects comprising homozygous, heterozygous and wild type carriers for the leptin gene mutation, c.398delG.

5.2 Results

5.2.1 Hormone levels in subjects with monogenic obesity

Mean hormone concentrations in study subjects are shown in Table 5.1. Leptin levels were <1.0 ng/ml or undetectable in leptin deficient children and were significantly higher in *LEPR* mutants compared to *MC4R* deficient subjects. Also, leptin levels in subjects with heterozygous leptin mutation, similar to those of normal subjects, were robustly correlated with BMI ($r = 0.77$, $P < 0.001$), and B) and ($r = 0.78$, $P < 0.001$) respectively) (Table 5.2 and Figure. 5.1). Insulin levels were not significantly different from each other in the 3 mutant groups and were variable within groups.

Notably, serum cortisol values were significantly increased in leptin and *LEPR* deficient subjects as compared to those with *MC4R* mutations and with wild type sequence. Hypercortisolemia was more pronounced in leptin deficient children compared to the leptin receptor deficient subjects. TSH levels were within the normal range in all subjects and were comparable to the controls.

Table 5.1: Serum hormone levels in children with homozygous mutations in leptin (*LEP*), leptin receptor (*LEPR*) and melanocortin 4 receptor (*MC4R*) genes.

Mutant gene	<i>LEP</i>	<i>LEPR</i>	<i>MC4R</i>
Age range	0.5 - 10	0.6 - 6	0.6-15
years	(n=37)	(n=12)	(n=6)
BMI SDS	5.3±0.2 ^{b*}	5.0±0.4	3.7±0.3
	(n=37)	(n=12)	(n=6)
Leptin ng/ml	< 1.0 ^{a***, b***}	67.2±6.7 ^{c**}	37.5±3.0
	(n=37)	(n=11)	(n=6)
Insulin µIU/ml	13.5±1.9	16.5±2.6	16.8±5.4
	(n=34)	(n=11)	(n=6)
TSH µIU/ml	2.4±0.2	1.9±0.2	1.7±0.2±
	(n=19)	(n=8)	(n=6)
Cortisol µg/dl	26.7±1.3 ^{a**, b***}	19.3±1.4 ^{c*}	10.0±1.6
	(n=29)	(n=10)	(n=6)

Reference values (n=11): age-matched subjects with wild type sequence: BMI SDS: 0.2±0.3; leptin: 2.4±0.3 ng/ml; insulin: 4.7±0.6 µIU/ml; TSH: 3.0 ±0.4; cortisol: 7.9 ±1.4.

Significant difference among mutant groups: ^a *LEP* vs *LEPR*; ^b *LEP* vs *MC4R*; ^c *LEPR* vs *MC4R*;

*P<0.05, **P<0.01, ***P<0.001 (Tukey's multiple comparison test)

^a Mean ±SEM

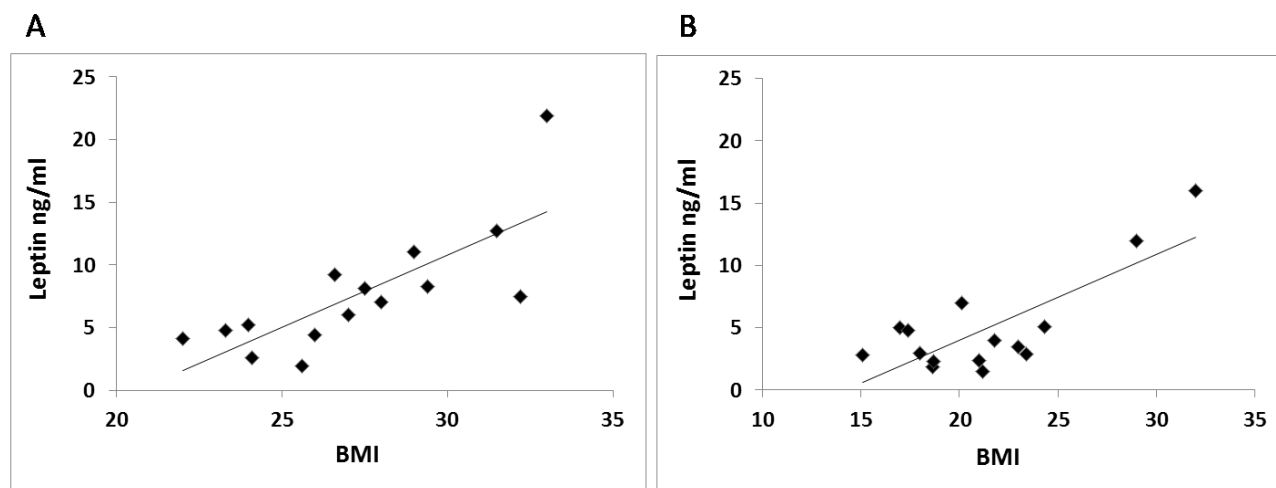


Figure 5.1 Relationship between BMI and leptin.

A) In heterozygous carriers of LEP mutation ($r = 0.77$, $P < 0.001$), and B) in normal lean subjects ($r = 0.78$, $P < 0.001$).

Table 5.2: Age, BMI and leptin levels of heterozygous carriers of leptin gene mutations and wild type controls.

Group	N	Age (years)	BMI	Leptin(ng/ml)
Heterozygous carrier	15	37.9 ± 1.7	27.3 ± 0.9	7.6 ± 1.3
Control (wild type)	15	23.3 ± 1.4	21.4 ± 1.2	4.9 ± 1.0

Mean leptin levels in the two groups were not significantly different: $P = 0.112$ (t test, 2-tailed)

5.2.2 Fasting and postprandial levels of gut hormones

The physical characteristics and hormone levels are shown in Table 5.3 and 5.4. Plasma ghrelin levels in subjects with homozygous *LEP* mutation remained remarkably unchanged following food-intake (344 ± 14 vs 343 ± 13 pg m/L) whereas a significant postprandial fall in ghrelin levels (16-23% of baseline values) was observed in heterozygotes and normal subjects. Mean fasting levels of ghrelin tended to be lower in homozygous mutation carriers compared to the other 2 groups but the differences were not statistically significant among the three groups. The relatively higher mean fasting ghrelin levels and a more acute postprandial fall of the hormone in subjects with wild type sequence, could be attributed to a lower BMI as compared to the heterozygous carriers.

Table 5.3: Physical characteristics of study subjects.

Group	<i>LEP</i> mutants ¹	N	Sex (M : F)	Age (y) Mean±SEM Median (range)	Height(m) Mean±SEM Median (range)	BW (kg) Mean±SEM Median (range)	BMI Mean±SEM Median (range)
I	Homozygous (-/-)	5 ²	1 : 4	18.0±4.5 16(8-30)	1.49±0.07 1.58(1.29-1.67)	108.0±25.6 124(44-161)	45.1±7.4 49.7(26.4-63.3)
II	Heterozygous (-/+)	8	4 : 4	39.8±2.8 37.5(32-52)	1.67±0.03 1.69(1.54-1.81)	77.3±4.5 81(59-91)	27.5±1.3 27(24.1-32.2)
III	Wild type (+/+) (Control)	1 0 ³	8: 2	21.9±1.4 23.5(8-25)	1.56±0.08 1.42(1.24-1.75)	61.6±4.9 63(21-72)	20.2±0.9 20.5(15.1-24.3)

¹Frameshift mutation, c.398delG, in exon 3 of *LEP*.

² 2 subjects were <16 y of age with z-score for BMI 3.34 and 4.07.

³ 1 subject was <16 y of age with z-score for BMI -0.31.

BMI: Body mass index; BW: body weight; M: male; F: female.

Homozygous and heterozygous carriers for *LEP* mutation belonged to two unrelated consanguineous families. The control group (wild type) consisted of unrelated normal subjects.

Table 5.4: Fasting and postprandial hormone and glucose levels.

		Homozygous ¹			Heterozygous ¹			Wild type (Control)		
		Mean±SEM	P value ²	Percent change ³	Mean±SEM	P value	Percent change	Mean±SEM	P value	Percent change
Ghrelin pg/ml	Fasting	344±14	0.327	-0.30%	415±29	0.028	-16%	489±53	0.011	-23%
	Meal	342.8±13			347.4±13			373±24		
PYY pmol/L	Fasting	23.7±5.5	0.219	25%	18.7±3.4	0.011	34%	21.4±4.3	0.003	65%
	Meal	29.6±4.2			25.1±3.2			35.5±4.0		
GLP pmol/L	Fasting	55.9±8.9	0.019	82%	43.7±2.8	0.012	169%	38.0±6.0	0.007	168%
	Meal	93.0±15.8			117.8±20.0			102.6±18.6		
Glucose mg/dl	Fasting	115±30	0.014	40%	101.1±9	0.005	72%	91±4	0.000	63%
	Meal	161±37			186.7±28.3			149±10		

¹ Frameshift mutation, c.398delG, in exon 3 of *LEP*

² P value, paired two-way t test, fasting vs postprandial (meal) levels, significantly different: $P < 0.05$

³ Percent change in postprandial levels

⁴ Not detectable or below the sensitivity level of assay

Differences in fasting plasma PYY levels among the three groups were insignificant. A variable postprandial increase was observed in all the 3 groups (25-66%) but was not statistically significant in leptin deficient subjects (Table 5.4). The mean fasting values of plasma GLP-1 levels though tending to be higher in leptin deficient subjects were not significantly different in the three groups. A significant postprandial rise in GLP-1 levels (82-169%) was observed in all subjects including those carrying homozygous mutation (Table 5.4). However, the increase was minimal in the latter group.

5.3 Discussion

As expected, serum leptin levels were <1.0 ng/ml or non-delectable in subjects with homozygous *LEP* mutations. Two instances have also recently been reported by Wabitsch and co-workers [173, 378] where the aberrant protein encoded by the mutant *LEP* gene was secreted in circulation but lacked bioactivity in subjects carrying a homozygous *LEP* mutation, p.D100Y and p.N103K. In none of our *LEP* mutants we have so far found a similar condition. It may, therefore, be surmised that in the majority of cases, the mutant leptin protein even if released in circulation, is not reactive to antibodies against the normal ligand, and thus escapes detection in immunoassays.

Interestingly, in a previous study leptin levels were reported to be significantly lower in heterozygous carriers of *LEP* mutation as compared to those of normal subjects, resulting in a condition termed as partial leptin deficiency [426]. In the present study, however, we failed to find a significant difference in levels of this hormone between the heterozygous and wild type subjects. It may be pointed out that serum leptin levels are affected by other variables such as age, gender, diurnal variations and nutritional state, independent of the BMI. It is, therefore, rather difficult to carry out a strictly objective comparison between leptin levels measured in heterozygous and wild type subjects especially when the sample size is relatively small and the subjects are drawn from a heterogeneous cohort.

Previously, very high leptin levels have been reported in association with a pathogenic mutation of *LEPR* located in the splice donor site of exon 16 [131]. Excessively increased levels of leptin in this case were attributed to the presence of a truncated *LEPR* protein that lacked both intracellular and transmembrane domains, secreted in blood and capable of binding to circulating leptin in high concentrations. In the present study, hyperleptinemia was observed in majority of

the subjects with homozygous *LEPR* mutations and leptin levels in these subjects were higher than those of MC4R deficient children. However, other studies based on multi ethnic subjects with different *LEPR* variants demonstrate a wide range of circulating leptin concentrations presumably in relation to the extent of adiposity [132, 187, 427].

Mean insulin levels were raised in subjects with *LEP*, *LEPR* and *MC4R* mutations over the control values but were variable in all the three mutant groups. Hyperinsulinemia, therefore, cannot be regarded as a direct outcome of leptin, leptin receptor or MC4R deficiency but is possibly consequent to an increased fat mass and extent of inflammation and hypertension, or due to an independent disposition of diabetic condition. More remarkably, cortisol levels in our subjects were significantly and consistently higher in leptin deficient subjects. Although increased levels of serum cortisol levels were also evident in children with *LEPR* mutations, the severity in this class of mutants was found to be less than that observed in leptin deficient subjects. In sharp contrast to our observations, previous studies indicate that humans with inactivating mutations in *LEP* or *LEPR* have normal cortisol levels and do not show abnormal basal or corticotropin-releasing hormone-stimulated response [156]. Cortisol levels in *MC4R* mutants were not markedly different in the present study, from those of wild type normal weight subjects. TSH levels were unremarkable in all the three mutants groups and were shown to be within the normal range.

In a separate study we assessed the response of gut hormones to food intake primarily to address the possible role of these hormones and especially of ghrelin, in the hyperphagic drive, which is a distinctive behavioural trait of subjects with monogenic obesity. Under normal circumstances ghrelin levels increase during fasting before meals and drop postprandially and its physiological role in appetite stimulation is now well established [407]. Furthermore, peripheral ghrelin administration has been shown to increase food intake in normal subjects [428]. In this study we have shown that ghrelin levels are relatively low and do not undergo a postprandial fall in leptin deficient subjects. On the other hand, in heterozygous individuals, baseline ghrelin concentrations and the acute fall following food intake were almost indistinguishable from that of the controls.

Since ghrelin levels have been shown to be inversely related to fat mass, therefore, relatively low levels of ghrelin in obese subjects could be ascribed to accumulation of body fat and possibly hyperlipidemia [429]. The hypothesis that excessive eating and increase in body fat mass in subjects with common obesity could be partly due to a lack of fall of postprandial ghrelin levels,

has not been substantiated [371]. Interestingly, ghrelin knockout mice or mice lacking GHS-R type 1a receptor have normal appetite and maintain normal body weight demonstrating thereby that a chronic lack of this hormone or its action does not necessarily affect energy homeostasis [430]. In contrast to morbidly obese subjects with congenital leptin deficiency and subjects with common obesity, patients with PWS have markedly increased levels of ghrelin that have been associated with hyperphagia and development of obesity [431].

In this study, responses of PYY and GLP-1 to food intake in leptin gene mutants were not remarkably different from those observed in normal subjects. Majority of studies in subjects with common obesity and PWS patients, also report a postprandial rise in these gut hormones comparable to normal weight individuals [432]. However, as in case of PWS, the hyperphagic response of leptin deficient subjects appears to be refractory to increased levels of gut satiety hormones. Most probably, this refractoriness is not associated with resistance to these peptides but is due to an overriding central orexigenic signal in obese patients. This is evidenced by the observation that infusion of PPY₃₋₃₆ in pharmacological doses reduces the food intake by 30% in obese subjects [433]. Our results in leptin deficient and normal individuals further demonstrate that basal and postprandial peripheral levels of PPY and GLP-1 are not correlated with leptin levels and support the notion that the response of these peptides to food intake is independent of regulation by leptin [434].

In summary, the present data indicate a negligible role if any, of gut hormones in driving the hyperphagic response observed in subjects with congenital leptin deficiency. This supposition is corroborated by experimental data in animal models. Ghrelin deficient and leptin deficient double mutant mice (ghrelin $-/-$ and ob/ob) show little change in food intake and body weight as compared to ob/ob mice [435]. In view of the forgoing, we speculate that hyperphagia in leptin deficient subjects can mainly be ascribed to an over expression of NPY neurons unrestrained due to insufficiency of leptin rather than due to an alteration in the level of gut hormones. Furthermore, our data on hormone profiles suggest that levels of none of the hormones investigated here with the exception of leptin, can be used as reliable biomarkers of leptin, leptin receptor or MC4R deficient states. Undoubtedly genetic analysis remains the only option so far for the diagnosis of early onset severe obesity.

6. Chapter six

Putative genes associated with severe obesity as identified by whole exome sequencing (WES)

6.1 Introduction

Discovery of causative genetic variants associated with disease including obesity, has remained an important and challenging task in the realm of human molecular genetics [436]. In spite of the discovery of a number of pathogenic mutations in genes especially those that directly influence the leptin-melanocortin signalling, the genetic basis of a large percentage of cases with severe obesity is still unaccounted for.

The recent progress in the discovery of novel disease related candidate genes has proceeded hand in hand with the phenomenal advances in analytical technologies such as massively parallel new generation sequencing (NGS) techniques. Of these, whole exome analysis (WES) that investigates protein-coding regions spanning across the whole genome [437] has emerged as a significantly efficient and a time and cost effective technique to identify disease related mutations in known and novel genes particularly those that follow the Mendelian inheritance [438]. The usefulness of WES analysis is emphasised by the fact that about 85% of the disease-related mutations in monogenic disorders have been found in the protein-coding regions (exons and splice sites). Furthermore, in the recent past use of WES has resulted in the discovery of more than 100 such causative genes including those associated with monogenic and syndromic forms of obesity [311, 316, 317] thus also establishing its specificity and potential utility in clinical settings [318, 319].

Use of exome sequencing has proved instrumental in identifying several new forms of Mendelian obesity genes in subjects with obesity. Kabuki syndrome was one of the initial cases in which genetic causality was elucidated by the use of WES and had led to the identification of pathogenic mutations in the MLL2 gene [439]. This discovery was followed by a growing list of causative genes/mutations identified through WES, leading to other forms of syndromic obesity such as Bardet Biedl [440, 441] Alström [442, 443] and Coffin-Siris syndromes [444], as well as to non-syndromic types of obesity [232, 445]. As an adjunct to WES, homozygosity mapping can further facilitate and improve efficiency in identification of recessive pathogenic mutations in consanguineous cases by focusing on genetic variants that lie in a critical locus homozygous only in the affected individual [446].

Here we report novel pathogenic mutations in three candidate genes, *INSIG2*, *ROCK1* and *ADCY3*, in subjects with early onset severe obesity, identified by using WES data analysis and homozygosity mapping.

6.2 Subjects

Forty probands with severe early onset obesity and their family members were selected for this study. All probands were from consanguineous families. Thirty-nine of these subjects were previously screened for 27 obesity associated genes by Sanger sequencing and RainDance targeted sequencing techniques but were found negative. One of the subjects that could not be screened by these procedures due to small amount of the sample was also included in the WES analysis. Interviews were carried out to obtain family history of patients and construct pedigrees spanning at least 3 generations. Written informed consent was obtained in all cases. Anthropomorphic measurements and physical examination were carried out and blood samples were obtained in each case for subsequent DNA extraction and hormone estimations.

6.3 Variant prioritization

In WES performed on a total of 96 samples the achieved mean depth was ~100X (median ~80X). The total coverage was about 69.8Mb and approximately 68.5Mb were covered with a minimum of 8X. Since the subjects were the outcome of consanguineous parentage, therefore, autosomal recessive homozygosity in all the probands for the disease causing mutation(s) was expected. Variants from each family were filtered independently to find specific homozygous mutations in affected individuals. In the initial filtration step, I excluded all variants in heterozygous state. Since the chances of any variant present in publically available databases to be causative/deleterious were rather low, in the next filtration those variants that are enlisted in db SNP and 1000 Genome Project were excluded. Also variants present in homozygous state in the Exome Aggregation Consortium (ExAC) and Exome Sequencing Project (EPS) were excluded. Mutation prediction softwares, Polyphen and MutationTaster, were used to envisage fate of mutation. Variants that were synonymous or predicted to be benign by *in silico* prediction softwares were also ignored.

At the end of filtration steps I was left with a small number of high priority candidate variants. On these variants, I performed filtration based on available biological and functional information.

Careful considerations were given to potentially deleterious variants detected in regions of runs-of-homozygosity. Association of genes in polygenic form of obesity through GWAS, was also taken into account. Biological pathways of genes carrying mutations were studied to find their direct or indirect relevance to the observed phenotypes. Finally, those genetic variants that were likely to be associated with the phenotype were selected for Sanger sequencing for confirmation.

6.4 Whole exome sequencing data analysis - significant findings

The whole exome sequencing of samples from 40 unrelated probands and 56 family members carried out to identify genetic variants/alleles associated with severe obesity resulted in an indication of causative mutations in 3 genes. Although association of these candidate genes with obesity has already been suggested through genome wide association (GWAS) and/or animal studies, their involvement in a predisposition to extreme obesity in the human is being reported here for the first time.

By employing the approach as described in the Material and Methods section 2.3.3, an average of ~16000 high quality variants were generated per individual from paired end reads that were filtered down to less than 10 novel homozygous variations (Table 6.1) through various exclusion steps described above. Here we have described WES results in only 3 probands and their family members whereas data from the remaining affected subjects and their family members is still under process.

Table 6.1: Variants identified in major prioritisation steps applied on exome sequencing of DNA from 3 probands

Sample	F-46	F-103	F-107
Total SNP	14340	13519	14393
Homozygous SNP	5738	5045(3862) ^b	5323 (1395) ^c
Homozygous Novel ^a SNP	41	14	16
SNP after filtration	5	1	3
Total Indels	1300	1309	1345
Homozygous Indels	324	3862(358)	460(117)
Homozygous novel Indels	37	37	20
Indels after filtration	0	4	3

a: Not present in db SNP database

b: Homozygous SNP also present in the obese brother

c: Homozygous SNP not present in the lean mother

6.4.1 insulin induced gene 2 (*INSIG2*)

c.425T>C (p.Ile142Thr)

This mutation was identified in the *INSIG2* gene involving substitution of T to C at position 425 resulting in isoleucine to threonine change at codon position 142. Following the already described filtration strategy we ended up with four novel predicted deleterious homozygous mutations in the proband. These other four genes included NCK-associated protein (*NCKAP5*), family with sequence similarity 96, member A (*FAM96A*), keratin 36, type II (*KRT3*), neural precursor cell expressed developmentally down-regulated 4-like (*NEDD4L*). However, c.425T>C mutation in *INSIG2* gene was the most convincing variant owing to its association with BMI in GWAS and its role in cholesterol and lipid metabolism. The mutation in *INSIG2* gene is predicted to be deleterious by Sift (score: 0.01) and disease causing by MutationTaster (p-value=1). This mutation also lies in the second largest homozygous region in chromosome 2 starting from position 107,673,715 to 155,381,331, spanning 47.7 Mb of ROH region. This mutation that lies in the moderately conserved region (Figure 6.1C) of exon 4 of *INSIG2* gene was also confirmed and validated by PCR amplification from genomic DNA and subsequent Sanger sequence analysis (Fig 6.1B). Whereas this specific mutation was not found within dbSNP variation database or Exome

Sequencing Project (ESP), its presence in heterozygous state in the Exome Aggregate Consortium was documented in only one subject of an unknown origin from a total of 121382 alleles. The other four genes although passed the filtration criteria but none of these had any functional relevance with the phenotype of interest (Table 6.2).

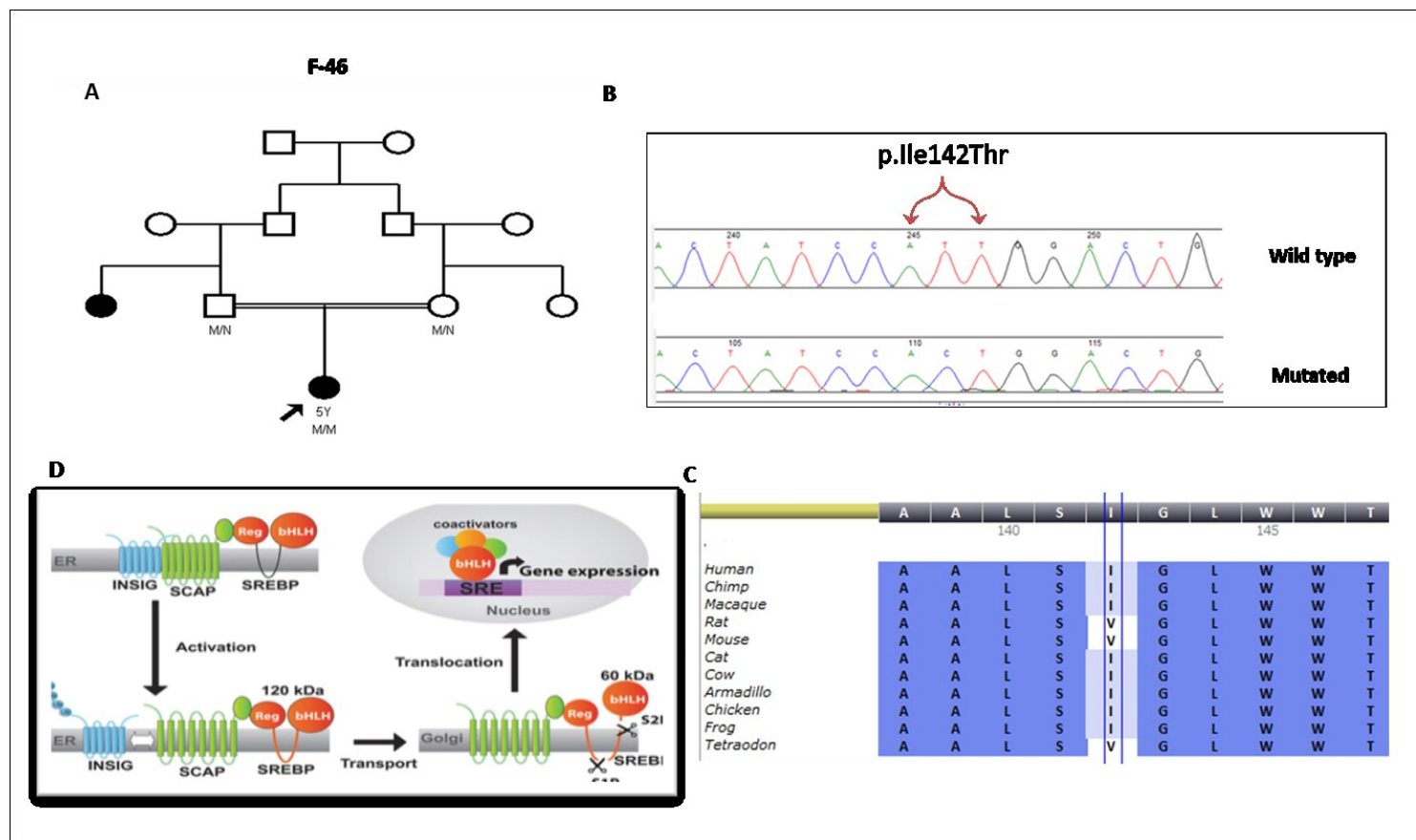


Table 6.2: Potentially pathogenic mutations in homozygous state identified in *F-46* by whole exome sequencing.

Gene	Category	Protein function	Position	cDNA change	Aminoacid change	Mutation Taster	Sift	ROH	ExAC
<i>INSIG2</i>	Missense	Lipid homeostasis	2:118864368	c.425T>C	p.Ile142Thr	Disease causing (p-value: 1)	Deleterious (score: 0.01)	YES	NO
<i>NCKAP5</i>	Missense	Hypersomnia	2:133543192	c.1192G>A	p.Glu398Lys	Disease causing (p-value: 0.99)	Deleterious (score: 0)	YES	NO
<i>FAM96A</i>	Missense	Chromosome segregation	15:64381036	c.139A>C	p.Ile47Leu	Disease causing (p-value: 1)	Deleterious (score: 0)	YES	NO
<i>KRT36</i>	Missense	Keratins to form hair and nails	17:39642711	c.1321A>C	p.Thr441Pro	Disease causing (p-value: 0.849)	Deleterious (score: 0)	YES	NO
<i>NEDD4L</i>	Missense	Degradation of the single-stranded DNA-binding protein	18:55998034	c.878C>A	p.Pro293Gln	Disease causing (p-value: 1)	Deleterious (score: 0.01)	YES	NO

F-46 - clinical characterization

This homozygous missense mutation in *INSIG2* was found in a 5 year old girl with severe obesity. She is the only child of her consanguineous parents from Shakargarh District of Punjab and belongs to the Pathan caste. The proband was reported to start gaining excessive weight at the age of 1 month. Both parents were of normal bodyweight. The father of the affected girl was reported to be diabetic. Physical and clinical data of the proband are presented in Table 6.3.

Table 6.3: Anthropometric and clinical investigation of proband F-46.

ID	F-46
Gender	Female
Age (year)	5
Height (m)	1.06
Weight (kg)	27
BMI SDS	3.6
Leptin (ng/μl)	79.4
TSH (μIU/ml)	1.9
Insulin (μIU/ml)	39.2
Cortisol (μg/dl)	3.8

6.4.2 Rho-associated protein kinase 1 (*ROCK1*)

c.2824G<A (p.Glu942Lys)

Another missense mutation caused by substitution of G with A at coding position 2824 in the *ROCK1* gene resulted in a change from glutamate to lysine at codon position 942. This mutation was identified in the proband and two severely obese siblings (BMI: 59, 42 and 49 respectively) in the homozygous state and in a third sibling (BMI: 37) in heterozygous state (Figure 6.2A). This mutation in exon 24, is predicted to be deleterious according to Sift (score: 0) and disease causing by the MutationTaster (p-value=1). Evidence of pathogenicity was also supported by a high conservation of substituted residues (Figure 6.2C). The said mutation was also confirmed and validated by Sanger sequencing (Figure 6.2B). This mutation is not listed in the dbSNP Variation Database and Exome Sequencing Project (ESP) but is recorded in the Exome Aggregate

Consortium in heterozygous state in 12 subjects of South Asian origin, from a total of 119802 alleles.

Mutations in 4 other genes in the proband also passed the filtration criteria. These genes comprise dachshund family transcription factor 1 (*DACH1*), family with sequence similarity 157, member A (*FAM157A*), arfGAP With GTPase domain (*AGAP3*) and fatty acid desaturase 6 (*FADS6*). Whereas none of the variants identified in these genes provided an obvious explanation for the phenotypes and were also found to be present in homozygous state in ExAC database, *ROCK1* turned out to be the solo highly convincing candidate based on available literature on functional analysis as well as mouse modelling (Table 6.5).

***F-103* - clinical characterization**

This mutation in homozygous state was found in the proband and his two brothers. The proband was initially recruited for the study at the age of 25 years. At this age he weighed 147 kg with a 59 BMI. During a follow-up 2 years later his body weight increased to 185 kg with a BMI of 73. . His two male siblings with BMI 42.2 and 49.1 were also found to carry identical mutation in the homozygous state. Another sibling with a BMI of 37.5 was found heterozygous for this mutation (Table 6.4). The proband was reported to start gaining weight at the age of 5 years. The parents had died earlier and were reported to be overweight and diabetic.

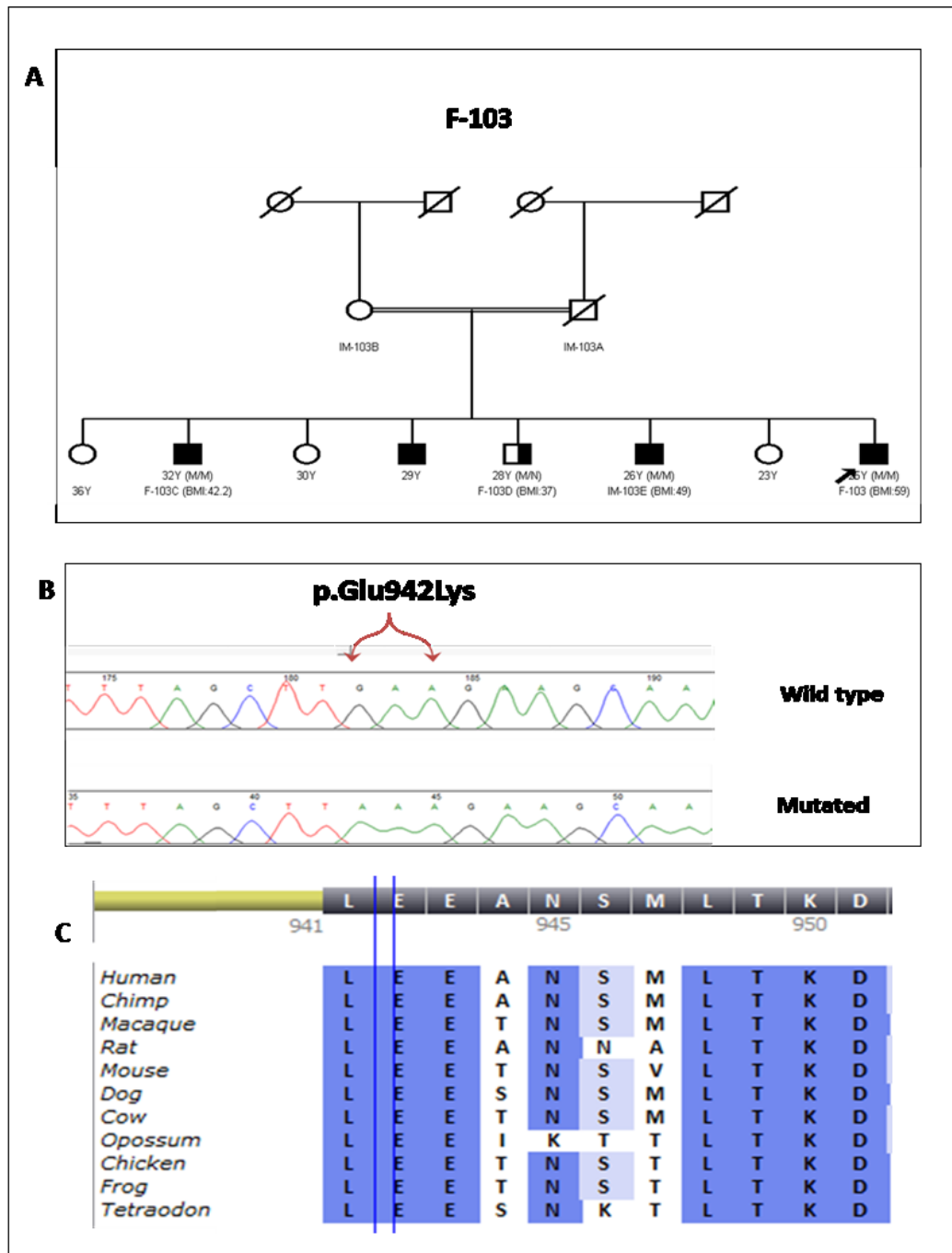


Figure 6.2 Pedigree and mutation in *ROCK1*.

A: Pedigrees of *F-103* with a mutation in *ROCK1*; B: Sequencing scan of *ROCK1* at the site of mutation demonstrating a homozygous missense substitution c.2824G<A C 1 in exon 24. This mutation was identified in homozygous state in *F-103*, *F-103C* and *F-103E*; C: Sequence alignment and conservation of residues across species.

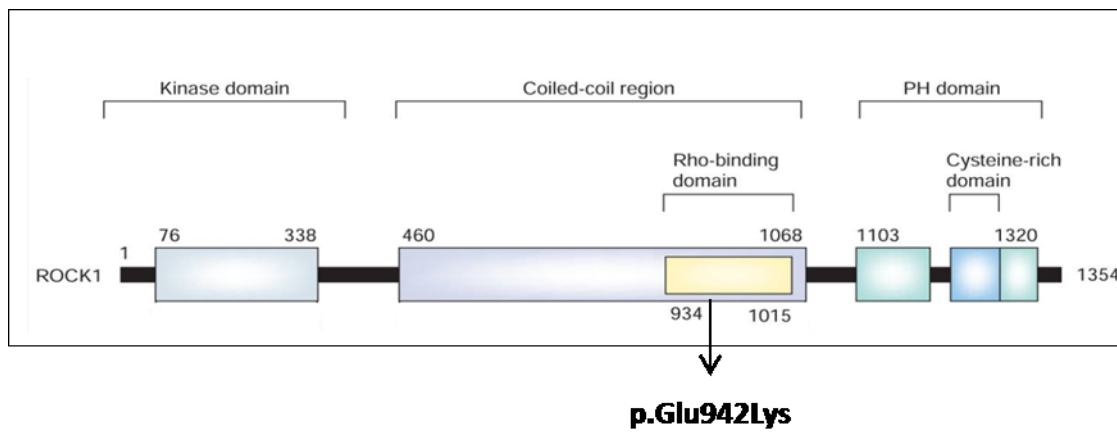


Figure 6.3 Diagrammatic presentation of different domains of ROCK1 protein (Modified from Rikitake and Liao 2005 [447]).

Table 6.4: Anthropometric and clinical investigation of *F-103*.

ID	<i>F-103</i>	<i>F-103C</i>	<i>F-103E</i>	<i>F-103D</i>
Mutation	c.2824G<A (Homozygous)	c.2824G<A (Homozygous)	c.2824G<A (Homozygous)	c.2824G<A (Heterozygous)
Gender	Male	Male	Male	Male
Age (year)	25	32	26	28
Height (m)	1.58	1.6	1.66	1.58
Weight (kg)	147(185) ^A	110	135	94(106)
BMI	59 (73.6) ^B	42	49	37(42)
Leptin (ng/μl)	30	N.A.	N.A.	19
TSH (μIU/ml)	2.1	N.A.	N.A.	0.9
Insulin (μIU/ml)	36.6	N.A.	N.A.	22.7
Cortisol (μg/dl)	7	N.A.	N.A.	12

A: Weight on follow-up

B: BMI on follow-up

Table 6.5: Potentially pathogenic mutations in homozygous state identified in *F-103* by whole exome sequencing.

Gene	Category	Protein function	Position	cDNA change	Aminoacid change	Mutation Taster	Sift	ROH	ExAC
<i>ROCK1</i>	Missense	Insulin and leptin signalling	18:18549166	c.2824G>A	p.Glu942Lys	Disease causing (p-value:1)	Deleterious (score: 0)	NO	Yes (het in 11)
<i>DACH1</i>	Indel	Tumor growth	13:72440659	c.245_251del	p.Gly82Alafs*112	N.A.	N.A.	NO	Yes (homo)
<i>FAM157A</i>	Indel	Unknown	3:197880131	c.210_219del	p.Trp70Cysfs*43	N.A.	N.A.	NO	Yes (homo)
<i>AGAP3</i>	Indel	Synaptic plasticity	7:150783909	c.81dup	p.Gln28Alafs*102	N.A.	N.A.	NO	Yes (homo)
<i>FADS6</i>	Indel	Unknown	17:72889677	c.17_18ins54	p.Met7_Leu350delinsAlaPro*	N.A.	N.A.	NO	Yes (homo)

6.4.3 Adenylyl cyclase 3 (*ADCY3*)

c. 3315delC (p.Ile1106Serfs*3)

This frameshift mutation in *ADCY3* gene was caused by a homozygous one base deletion of G nucleotide at the base position 3315 and was identified in a 10 year old girl. This mutation in last exon (exon 21) of the gene (Figure 6.4D) resulted in truncated protein by introducing a stop codon after two aberrant amino acids starting from 1065 where isoleucine was changed to serine (Figure 6.4B). This mutation which is within the ROH region, was not found in any of the available data bases. Association of this gene with regulation of body weight is supported by several GWAS studies. Variants in other genes that qualified the predetermined filtration criteria included TSPY-Like 6 (*TSPYL6*), Keratin Associated Protein 29-1 (*KRTAP29-1*), Otoferlin (*OTOF*), ATP-Binding Cassette, Sub-Family B (MDR/TAP), Member 4 (*ABCB4*), Alcohol Dehydrogenase, Iron Containing, 1 (*ADHFE1*) (Table 6.4). Due to its recurrent association with BMI and its functional relevance with obesity, *ADCY3* was considered as a potential causative gene. The mother of the proband was heterozygous for this mutation.

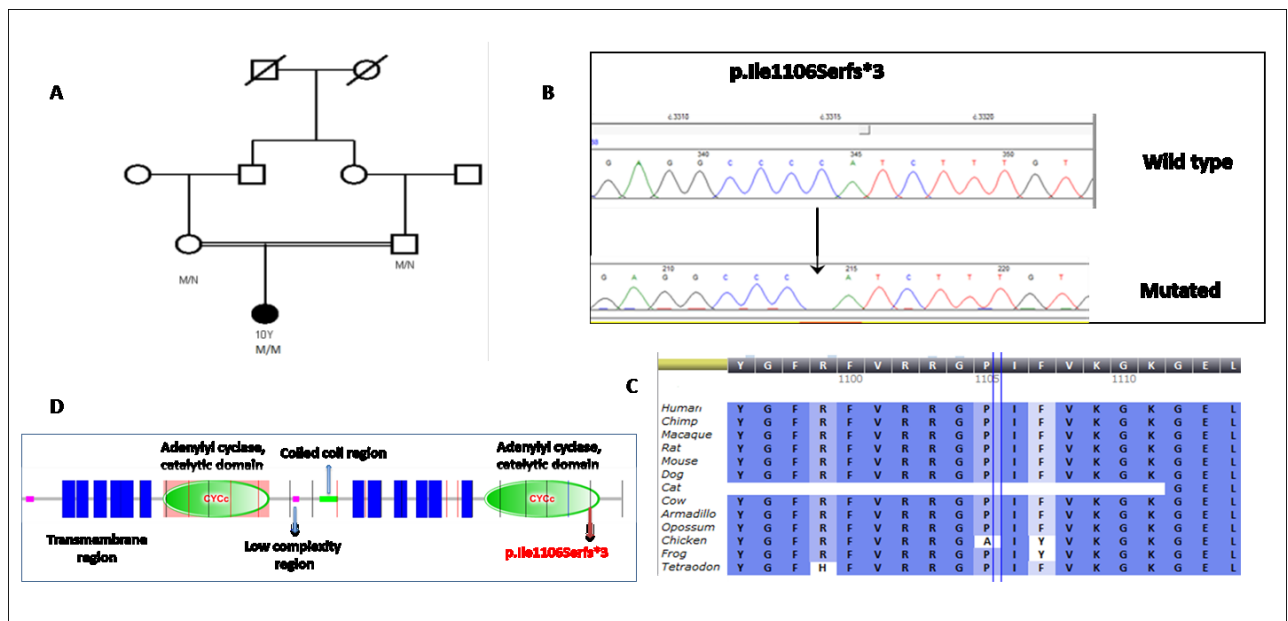


Figure 6.4 Mutation in *ADCY3* and pedigree of affected subject.

A: Pedigrees of *F-107* with mutations in *ADCY3*; B: Sequencing scan of *ADCY3* at the site of mutation demonstrating a homozygous 1 base deletion c. 3315delC in exon 21 disrupting the reading frame. This mutation was identified in homozygous state in *F-107* and in heterozygous state in mother *F-107B*; C: Conservation of amino acid across twelve different species; D: Diagrammatic presentation of different domains on *ADCY3* protein and position of mutation are indicated by red arrow.

Table 6.6: Potentially pathogenic mutations in homozygous state identified in *F-107* by whole exome sequencing.

Gene	Category	Protein function	Position	cDNA change	Amino acid change	Mutation Taster	Sift	ROH	ExAC
<i>TSPYL6</i>	Indel	Unknown	2:54482704	c.580_586del	p.Gly194*	N.A.	N.A.	YES	Yes(Homo)
<i>KRTAP29-1</i>	Indel	Unknown	17:39458574	c.136_151del	p.Ser46Alafs*63	N.A.	N.A.	YES	Yes(Homo)
<i>ADCY3</i>	Indel	Formation cAMP	2:25042921	c.3315del	p.Ile1106Serfs*3	N.A.	N.A.	YES	NO
<i>OTOF</i>	Missense	Recessive deafness	2:26707394	c.1153A>G	p.Asn385Asp	Disease causing	Deleterious	YES	Yes(Het)
<i>ABCB4</i>	Missense	ATP-Binding Cassette	7:87083858	c.337A>G	p.Met113Val	Disease causing	Deleterious	YES	Yes(Het)
<i>ADHFE1</i>	Missense	Oxidation of 4-hydroxybutyrate	8:67364217	c.764C>G	p.Pro255Arg	Disease causing	Deleterious	YES	Yes(Het)

F-107 - clinical characterization

The proband, a 10 year old girl, was the only child of consanguineous parents (Figure 6.4A). She was reported to be hyperphagic soon after birth and started gaining weight at the age of 2 years. She has been slow in learning and at the age of 13 years is in Grade 3. Also, reportedly she has no sense of smell. It is interesting to note that disruption of *ADCY3* gene renders mice unable to detect odour [448]. Physical and clinical data of the proband are presented in table 6.7.

Table 6.7: Anthropometric and clinical investigation of proband F-107.

ID	F-107
Mutation	c.425T>C
Gender	Female
Age (year)	10
Height (m)	1.36
Weight (kg)	57
SDS	3.4
Leptin (ng/μl)	30
Insulin (μIU/ml)	31
TSH (mIU/l)	6.23

6.5 Discussion

In this chapter we have described for the first time pathogenic variants in three candidate genes, *INSIG2*, *ROCK1*, and *ADCY3*, that may be associated with human obesity. These genes were identified by WES analysis of DNA samples from 40 severely obese subjects and their family members, using a rigorous and uniform filtration protocol and selection criteria for variant prioritization.

INSIG2, one of the three genes shown here to be associated with monogenic obesity, was indicated to be associated with BMI through the first GWAS (Farmingham Heart Study) carried out in 2006 [449]. In this study, a SNP rs7566605 located near the 5' end of *INSIG2* was reported to be significantly associated with BMI in both children and adults participants. The finding was replicated in four additional sample sets analysed by GWASs [449] and in some subsequent studies conducted in subjects from diverse populations [450-452]. Moreover significant association of other tagSNPs in *INSIG2* was also reported to be associated with BMI [453]. However, according to some of the follow-up studies the association remains inconclusive [69, 454-456].

A 225 amino acid protein, *INSIG2*, encoded by this gene has been shown to regulate adipogenesis and lipid storage [457] thus nominating *INSIG2* as a strong candidate gene for disposition to obesity [458, 459]. The *INSIG2* protein is located in the endoplasmic reticulum and is an important component of feedback inhibition mechanism of cholesterol biosynthesis in regulation of lipidogenesis. It blocks further cholesterol synthesis in the presence of adequate levels of cellular sterols, by binding to SREBP-cleavage activating protein (SCAP) thus retaining the SCAP/SREBP complex in the ER [460].

INSIG2 is also expressed in adipocytes and its expression increases at the time of adipocyte metabolism suggesting a possible role of this protein in influencing body weight [461]. Furthermore, overexpression of *INSIG2* protein was shown to reduce hepatic lipogenesis in obese Zucker diabetic fatty rats [462]. Further support of involvement of *INSIG2* in body weight regulation comes from *INSIG2* knockout mice that were reported to be heavier than control littermates and had higher concentration of accumulated cholesterol and triglycerides [463]. In the perspective of the aforementioned evidence of functional role of this gene in lipid metabolism, the findings of present study provide the first instance of a pathogenic mutation in the coding region of *INSIG2*, associated with early onset severe obesity in the human.

Another mutation shown to be associated with severe obesity by WES was found in the *ROCK1* gene in three siblings. *ROCK1* encodes the serine/threonine protein that is a major downstream effector of small GTPase RhoA [285]. The encoded protein is implicated in several cellular processes including actin cytoskeleton organization, muscle contraction and cell motility and also implicated in the pathogenesis of metabolic disorders such as hypertension and obesity/diabetes

[464-466]. Role of ROCK1 in central control of energy homeostasis has been attributed to its regulation of leptin action. Emerging data propose that ROCK1 affects leptin action through its mediation of JAK2 activation, which in turn promotes STAT3 activation/dimerization and its transportation inside the nucleus. Whereas, exogenous dose of leptin was shown to enhance ROCK1 activation [285], hypothalamic activity of ROCK1 is reduced in high-fat diet induced obese mice [467]. Also, JAK2 tyrosine phosphorylation is diminished when ROCK1 activity is biologically suppressed in the hypothalamus, suggesting that leptin-induced JAK2 activation is ROCK1-dependent [285].

The role of ROCK1 in leptin signalling and thus body weight regulation was also highlighted by *ROCK1* gene knockout mice. Targeted disruption of *ROCK1* in POMC neurons has been shown to induce hyperphagic obesity by preventing leptin's downstream anorexogenic effects. Additionally, adiposity due to deletion of *ROCK1* in ARC is more emphasised in mice as compared to animals with ROCK1 deficiency in POMC neurons alone. Targeted deletion of *ROCK1* in the AgRP neurons also resulted in increased body fat content and weight gain [285] that could be attributed to a significant decrease in energy expenditure through reduced locomotor activity [467].

In addition to its role in downstream leptin signalling, available evidence in rodents suggests that ROCK1 is a mediator of insulin signalling in the hypothalamus as well as in the peripheral tissues [285, 468, 469]. Treatment with ROCK inhibitors (Y-27632) resulted in insulin resistance by lowering glucose uptake in *in vitro* and *ex vivo* experiments [469]. Also, inhibition of ROCK1 diminishes insulin signalling and subsequent glucose transport in 3T3-L1 adipocytes [469, 470], indicating its positive involvement in insulin signalling and glucose metabolism. An *in vivo* disruption of *ROCK1* has been reported to result in an increase in insulin receptor action proposing its inhibitory role in controlling insulin sensitivity and signalling and a disruption of adipose tissue specific *ROCK1* in diet-induced obese mice, led to an increased insulin signalling and improved insulin sensitivity [471]. These data though somewhat conflicting in different rodent models, comes out clearly to demonstrate a significant role of ROCK1 in influencing metabolic signals that regulate energy homeostasis. It is also suggested that *ROCK1* regulation of glucose metabolism and insulin signalling in peripheral tissues/cells may be differential or conditional to specific metabolic states.

The ROCK protein is comprised of an N-terminal kinase domain (residues 73–356), a central coiled-coil domain (residues 422–1102) which also contains the Rho-binding site towards its end, and a C-terminal pleckstrin homology domain (residues 1118–1317). In the inactive form, the carboxyl terminal of Rho is folded back onto the kinase N terminal kinase domain. This looping helps in maintaining the inactive state of the enzyme [472]. Activation of the protein takes place by binding of the active form of Rho GTPase to the Rho-binding domain of ROCK1 leading to an open (active) kinase domain [472, 473]. Our mutation at position 942 that falls within the dedicated rho-binding domain and thus could have caused a failure in its bonding to RhoA and subsequent activation of kinase (catalytic) domain (Figure 6.3).

Taken together, various lines of evidence, as summarized above, strongly suggest ROCK1 as a central regulator of glucose metabolism and a modulator of leptin action on energy balance. Impaired ROCK1 activity in hypothalamus may contribute to the development/ pathogenesis of central leptin resistance in obesity. Further investigations on the functional impact of this gene in the human could possibly open up new leads to management and treatment of certain forms of obesity and type 2 diabetes [285, 471].

The third obesity associated mutation identified through WES reported in this study was in the coding region of the gene, adenylate cyclase 3 (*ADCY3*) which encodes for an 1144 amino acid protein. This protein is a member of the adenylate cyclase (AC) family and is widely expressed in various human tissues catalysing the production of the secondary messenger, cyclic adenosine monophosphate (cAMP). cAMP is a crucial mediator in eliciting the biological response to a number of neurotransmitters, neuropeptides and hormones including those that regulate energy homeostasis such as α -melanocyte-stimulating hormone (α -MSH), the orexins, GLP-1 and glucagon[474-476] which are important mediator of leptin downstream signalling [477].

ADCY3 is one of a few loci that have shown a recurrent and strong association with obesity through GWAS efforts [102, 104, 478-480] and also with type 1 diabetes [481] and major depressive disorder [482]. In most of the studies *ADCY3* gene was found to be in strong linkage disequilibrium with other neighbouring genes including *EFR3B*, *RJB*, *POMC* and *DNAJC27* [102, 104]. Polymorphisms within *ADCY3* were also shown to confer the risk of obesity susceptibility in Swedish and Chinese Han populations [483-485]. Moreover the identified SNPs in *ADCY3* were found to be associated with expression of *POMC* gene which has a known function in susceptibility

to obesity through its role in regulating melanocortin signalling [102, 104]. Direct evidence of involvement of *ADCY3* gene in body weight regulation comes from animal models. Knockout (*Adcy3*^{-/-}) mice exhibit increased fat mass attributable to low physical activity, hyperphagia and reduced sensitivity toward leptin [486] whereas heterozygous gain-of-function mutations in mice presented increased *Adcy3* activity thus providing protection from diet-induced metabolic derangements [487].

The proband carrying *ADCY3* mutation reportedly lacked sense of smell. It is interesting to note that *Adcy3* knockout mice (*Adcy3*^{-/-}) fail to detect and perceive odour. It was shown that disruption of *Adcy3* causes a complete absence of the electroolfactogram (EOG) response that is normally stimulated by cAMP due to activation of receptors in the olfactory sensory neurons (OSNs) [448]. In summary, information derived from human and from animal models provide convincing evidence that *ADCY3* protein expressed in CNS and adipose tissue could play a significant role in the regulation food intake and energy homeostasis.

A functional characterisation of the mutant genes is necessary to finally induct them in the list of genes associated with monogenic obesity. Interestingly two of the three genes identified here have also been associated with obesity related traits though GWAS, indicating a clear overlap of GWAS identified loci with genes predisposing an individual to extreme form of obesity through single gene mutation. The aforementioned observations strongly recommend screening those gene/loci that have been recurrently associated with obesity related traits through GWAS efforts, in severely obese subjects.

A novel homozygous mutation, p.Glu14Lys (c.40G>A), in *LEPR* gene was also identified through WES. The said mutation was predicted tolerated by Sift with the score of 0.32 and therefore, could not be picked up by stringent filtration criteria. This mutation lies in ROH region (details in chapter 3 section 3.3.2.4). This incidence raises doubts on relying heavily on the *in silico* prediction methods to determine the fate of mutation.

We acknowledge that our findings at present may not be sufficient to definitely implicate these mutations as determinants of the obese phenotype. The significance of our results is limited due to the fact that the incidence of these mutations has not yet been replicated in other obese

individuals from same population. In addition, our results lack confirmation through functional analysis, which should be pursued in future.

In summary, by employing a strategy for whole-exome capture and whole genome genotyping analysis with a goal to identify new causal homozygous mutation, we have identified three genes that might explain the phenotype of the patient with severe early-onset obesity. The present outcome of whole exome analysis underscores the importance of systematic genetic analysis of other inbred populations to unravel novel genetic variants and signalling pathways modulating energy homeostasis.

7. Chapter seven

General Discussion and Conclusions

7.1 General discussion

Relatively frequent presentation of severely obese children at pediatric units in Punjab's hospitals and a general lack of appropriate diagnosis and understanding of the etiology of this form of obesity in the country, prompted us to undertake the present study in 2012. A further incentive to initiate this study was provided by the first report of a loss-of-function homozygous mutation in the human leptin gene resulting in early onset extreme obesity, identified in a family of Pakistani origin residing in the UK [125]. A high degree of consanguinity in the Pakistani population [362, 363] provided a unique backdrop to investigate rare and novel recessive genetic variants associated with large effects. Since working on individuals at the extremes of their phenotype has established its usefulness in the field of genetics [488], we limited our study to subjects with early onset extreme obesity. During the course of this study a large amount of effort went to select and recruit patients and other members of affected families by establishing collaboration with local public hospitals and also by arranging field visits to contact affected families residing in remote villages of central Punjab.

As a result of sequencing analysis of DNA of 175 unrelated probands for 27 known obesity associated genes we were able to identify recessive rare and novel pathogenic mutations in the homozygous state in 54 patients (or families) (32%). Remarkably, of the 21 different mutations identified, 15 were found in three genes, *LEP*, *LEPR* and *MC4R* that directly influence the melanocortin signalling (Figure 7.1). The other six genetic variants identified were associated with syndromic obesity (PWS and BBS). Equally remarkable is a significantly high prevalence of severe obesity incidence due to loss-of-function mutations in the leptin gene in our cohort (20.5%).

Amongst the several afferent peripheral signals that convey energy relevant information to the hypothalamus, leptin secreted by adipocytes, is regarded as the most crucial component for regulating food intake and body weight [189]. This is mainly achieved by leptin's effects on two distinct neuronal populations within the arcuate nucleus that reciprocally regulate energy homeostasis and food intake mediated by the melanocortin pathway [189, 489]. Any disruption of this signalling pathway due to a dysfunction of either leptin or its receptor – that trigger the melanocortin pathway, predominantly results in a most severe form of obesity at an early age [490]. This notion is further strengthened by the observation that the obesity level in 42 leptin

deficient and 11 leptin receptor deficient children identified in our cohort was higher as compared to obese children with melanocortin-4 receptor deficiency (Figure 7.2).

Interestingly, among the 42 leptin deficient subjects from 35 affected families, 35 subjects carried an identical frameshift mutation, c.398delG. Almost all families affected by this mutation belonged to the Arain caste that constitutes a major sub-ethnic group of the Punjab province and has preferred consanguineous marriages between first or second cousins, for many generations [356]. Since this *LEP* variant has not been reported in any other consanguineous population, it may be considered as a founder mutation in this caste. The exceptionally high prevalence of this frameshift mutation compared to the remaining *LEP* mutations in this population could be ascribed due to an earlier establishment of this variant with a founder effect in this specific inbred caste.

In contrast to the relative frequency of pathogenic mutations in the three obesity associated genes determined by this study, in previous investigations based on samples of random populations mainly of European origin, *LEPR* deficiency has been reported to be more prevalent than leptin deficiency [132] and mutations in the *MC4R* have been regarded to cause the most prevalent form of monogenic obesity [201, 203, 204]. Genetic analysis on severely obese subjects from our cohort showed that leptin deficiency due to loss-of-function mutations in the leptin gene is the most prevalent form of severe obesity whereas contribution of *MC4R* mutations to the incidence of monogenic obesity in our population appeared to be minimal.

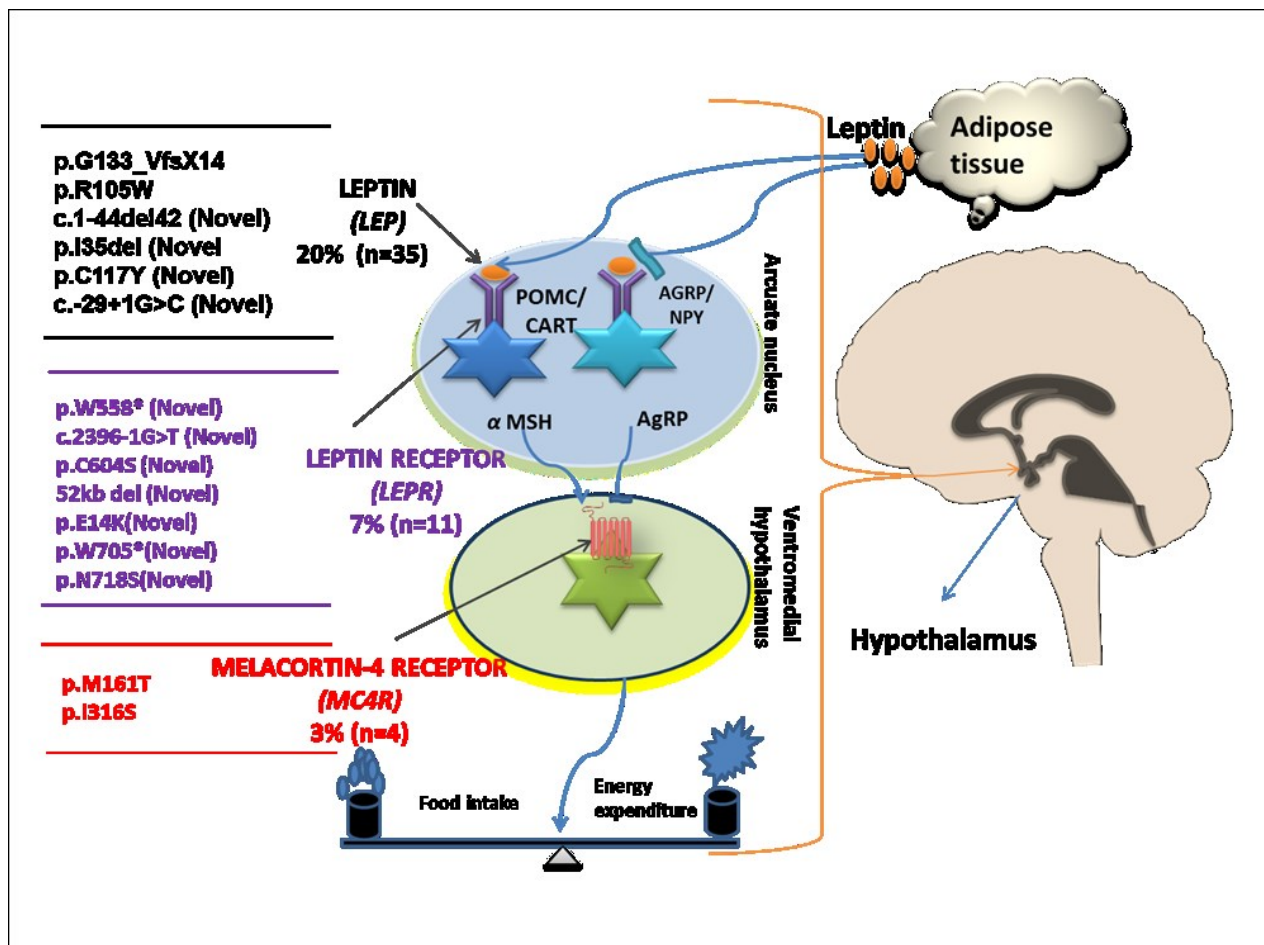


Figure 7.1 Mutations in three genes, playing a key role in leptin signalling and its downstream effect.

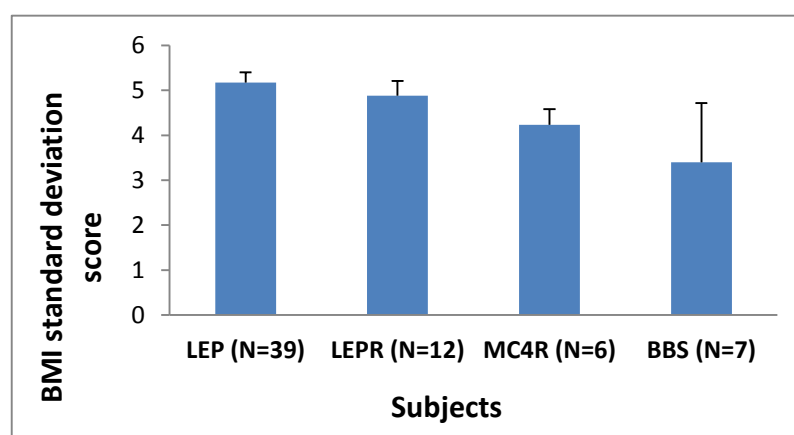


Figure 7.2 Graph showing the BMI standard-deviation score of 4 groups of mutants.

In this study we were unable to distinguish between the clinical phenotypes of leptin and leptin receptor deficient age-matched children. These observations are in contrast to a previous study in humans [132] but corroborate well with findings drawn from mice lacking leptin (*ob/ob*) and leptin receptor (*db/db*) that have been shown to be phenotypically identical [491]. Interestingly, we failed to identify any *MC4R* heterozygous mutant with severe obesity in our study. This observation supports the hypothesis that penetrance of heterozygous *MC4R* mutations is dependent of the obesogenic environment [202]. Also our results though based on a small number of subjects with homozygous *MC4R* mutations suggest that the expression of obesity resulting from *MC4R* deficiency is not only influenced by environmental factors but also in relation to age.

The absence of known or novel mutations in other monogenic obesity associated genes such as *POMC*, *SIM1* and *PCSK1* in our cohort of severely obese subjects is noteworthy. Also, no deletion in a previously established obesity locus, 16p11.2, [256, 257] could be identified. The absence of this deletion in our cohort could be attributable to an age dependent penetrance as seen in heterozygous carriers of this deletion [257] and hence may escape selection at a younger age for inclusion in a cohort of children with early onset severe obesity. It seems advisable, therefore, to investigate 16p deletion in subjects presenting a milder phenotype from this population.

In a previous report partial leptin deficiency has been described in subjects carrying heterozygous loss-of-function *LEP* mutations based on circulating leptin concentrations in a relatively small group sample [426]. Interestingly, in the present study fasting and postprandial leptin levels in heterozygous subjects tended to be comparable with or higher than those of the control subjects and were significantly correlated with BMI highlighting that heterozygosity for *LEP* mutations, may not result in partial leptin deficiency.

Although, recent technological advancements have given an impetus to disease gene identification process, it is obvious from a large proportion of missing or unexplained heritability of obesity that we are still a way off to fill this gap [102] and new susceptibility genes that underlie energy homeostasis have yet to emerge. Existing information derived from genes that have been associated with severe obesity provide unequivocal evidence that severe obesity is an inherited disorder of central regulation of food intake and energy homeostasis. Therefore, investigations on components of centrally located pathways are well justified. The recently identified candidate

genes for obesity such as *MRAP2* [232], *KSR2* [233] and *SH2B1* [269] together with our findings of *ROCK1* and *ADCY3* prompt us to look for yet unidentified genetic variants and molecular pathways predisposing human to the obese phenotype. Furthermore, it can be anticipated that mediators of new centrally located pathways may also emerge that will influence our bodily energy balance by reciprocally influencing leptin melanocortin pathway, such as those involving brain-derived neurotrophic factor and 5-HT, to provide a comprehensive outlook of underlying molecular mechanisms regulating body weight and energy balance [273].

Significantly, GWAS analyses demonstrate that most of the genes associated with Mendelian forms of human obesity (*LEPR*, *POMC*, *MC4R*, *BDNF*, *SH2B1* and *PCSK1*) are also enriched with more common variants associated with relatively small effects on BMI thus predisposing the individual to a milder form of obesity in the general population [101, 110-112]. This overlap is suggestive of shared underlying pathways, disruption of which could predispose an individual to a severe monogenic or a more subtle polygenic form of obesity [113]. Our findings of potentially pathogenic mutations in *ADCY3* and *INSIG2* gene that have previously shown recurrent association with obesity through GWASs together with aforementioned observations, strongly suggest screening of GWAS derived loci in severely obese subjects with the objective to identify novel genetic variants that may be associated with large effects on BMI [114]. Such an approach can significantly help us move from GWAS derived loci to relevant disease susceptible candidate genes.

In addition to rare point and indel mutations, another class of genomic variation that has been given considerable attention in the recent past is the copy number variation (CNV). Several studies have reported deletion and duplications, but these findings in most cases lack replication in other populations. However, an exception is a deletion of 593 kb at 16p11.2 [256, 257] that has also been widely replicated across different populations. It is noteworthy that in previous CNV analyses, consideration has been given only to large deletions due to the notion that that large CNV's are associated with a higher expression of the phenotype. This view underestimates the contribution of small CNV's to the phenotype. In future, careful investigations of small and rare CNV's could contribute in assessing the true importance of CNVs in elucidation of obesity.

Congenital leptin deficiency due to pathogenic mutations in the *LEP* gene is perhaps the only type of obesity where personalised medicine has been shown to be exceptionally successful. Daily

treatment with recombinant leptin in these subjects results in a dramatic reversal of symptoms due to leptin deficiency and a restoration of normal body weight, appetite and endocrine and reproductive functions. Also, clinical trials to manage and treat subjects with *MC4R* mutations that make up a large proportion of subjects with genetically determined obesity in the West, are on way. New small molecule compounds acting as pharmacological chaperones provide novel strategies to improve trafficking of receptor protein to cell membranes in cases of defective surface expression. In addition, pharmacological approaches attempting a direct activation of the downstream signalling of the melanocortin pathway are also under clinical trial [386]. More recently, attempts have been undertaken to generate hypothalamic neurons from pluripotent stem cells that may possibly be used in future to regulate appetite in subjects with monogenic obesity [492].

Life style intervention does not seem to be effective for long term weight management as evident from trials in *MC4R* mutation carrier where intensive life style interventions that successfully induce weight reduction in the initial stages [493] but present problems in maintaining optimal body weight as a long term management. [493]. Such interventions are predicted to be even less helpful in case of leptin and leptin receptor mutation carriers that experience severe hyperphagia.

At the time of discovery of leptin, it was thought that leptin as a satiety factor will prove a breakthrough in the treatment of obese individuals in general. However, it turned out that majority of subjects with obesity, with the exception of leptin deficient subjects, have normal or excessive levels of endogenous leptin and, therefore, addition of an exogenous dose of leptin would prove ineffective. Refractoriness to endogenous or exogenous leptin in obese subjects is generally ascribed to its resistance or tolerance [126, 162]. A number of mechanisms including impaired leptin transport through blood brain barrier and ineffective leptin signalling have been suggested to explain leptin resistance and the possibility that a reversal of these could be helpful in amelioration of obesity, has been contemplated. Pharmacologically targeting SOCS3 and PTP-1B that negatively regulate leptin signalling by reducing STAT3 expression could also contribute to increase leptin sensitivity. Similarly, components that influence transport of leptin across blood brain barrier could be targeted to enhance leptin sensitivity. Recent work by Hung et al provides experimental evidence that ROCK1 that mediates leptin action to regulate food intake and adiposity in leptin responsive neurones (POMC and AgRP), could also act as a novel target to

reduce leptin resistance. [467]. Lastly, it is expected that successful development of effective drugs that directly activate the JAK2/STAT3 signalling pathway may help in the treatment of not only monogenic forms but also common and polygenic types of obesity.

Recently scientists have focused their attention towards another area of brain that controls reward and hedonic aspects for regulation of food. It has been demonstrated that leptin receptors are expressed in the ventral striatum (VS) through which leptin exerts its effect on the mesolimbic-dopamine system to decrease the reward associated with food intake [159, 494]. In rodents, administration of leptin resulted in decreased food intake while a knockdown of LepR in the VTA showed the opposite results. [275]. These findings provide direct evidence of action of a peripheral metabolic signal on the VTA dopamine neurons. It is possible that genes that determine the palatability of food could also be targeted to regulate appetite and eating behaviour for treatment of incentive-related hyperphagia – one of the features contributing to development of extreme and morbid obesity. Here it may be mentioned that pharmacological interventions targeting areas in the brain could be quite challenging and risky as it may impair signalling pathways and associated proteins resulting in other complications.

Another area of research that is making its way in the field of genetics of obesity relates to the investigations on the gut microbiota. The human gut microbiome has been shown to be differentially expressed in gut of obese and lean individuals [26, 495-498]. It is, therefore, feasible that gut microbes could serve as potential nutritional and pharmacological targets to manage obesity.

7.2 Conclusions

By adopting a multi-layered sequencing regimen we have demonstrated a significantly higher incidence of monogenic obesity in Pakistani population than hitherto assessed in multi-ethnic and randomly organised cohorts of subjects with severe obesity. Among 175 subjects with severe obesity we have been able to elucidate genetic causality in 35% unrelated subjects. Of these 21% were shown to carry homozygous mutations in *LEP*, 6.5% in *LEPR* and 2.4% in *MC4R* gene. In addition, 4% of probands were identified with variants resulting in syndromic form of obesity. These findings underscore routine genetic screening particularly of the leptin gene in cases of

early onset severe obesity and hyperphagia as leptin deficiency can be effectively treated through replacement therapy.

Initial findings through WES analysis revealed three new human obesity susceptibility genes in the human. A functional characterization of mutations found in these candidate genes is envisaged. These results further encourage us to carry out WES in the remaining of our probands in which the causal variants have not yet been identified.

Lastly, it may be pointed out that a relatively higher percentage of mutations leading to monogenic obesity in this study could partially be due to our sustained focus on a specific consanguineous population and systematic genetic analysis rather than a higher incidence of mutations in obesity associated genes alone relative to other inbred populations. Such a supposition further emphasises the express need of undertaking comprehensive and systematic screening of cases of severe obesity in other geographical regions where consanguinity is still practiced. Such an enterprise is expected to narrow down the existing gap of missing heritability of obesity.

Investigation on hormones levels provided evidence that gut hormones appear at best, to play a minimal role in inducing hyperphagia in severely obese subjects with congenital leptin deficiency. Moreover the results based on subjects carrying *LEP* gene mutation in heterozygous form indicate that heterozygosity for the *LEP* mutation, does not result in partial leptin deficiency, in contrast to what has been previously suggested.

We hope that a continuation of similar efforts will lead to a better understanding of the genetic mechanisms underlying monogenic and common obesity. Such investigations are important not only in the context of furthering our knowledge of the genetic basis of obesity, but also in the perspective of the emerging era of personalised medicine.

7.3 Future Directions

-Expanding collaboration

The results of the present study encourage us to continue with our quest of finding out the genetic causality of severe obesity in Pakistan's consanguineous population and examine its incidence in

other regions of the country. This will involve planning to increase the size of our cohort and expansion of research collaboration with physicians and hospitals from other cities of Punjab as well as from other provinces. We have already started implementing this plan and new collaboration has been established with the children hospital in Islamabad and the local ethical approval has been obtained.

-Continuation of our efforts of systematic genetic analysis

We contemplate to continue our efforts to find the genetic causality of severe obesity in the Pakistani population especially in those cases in which we have still been unable to determine the genetic basis. This will involve a multi-layered sequencing regimen as used in the present study, and genome-wide genotyping to unravel structural variants. We also aim to inform and educate the public of Pakistan about the disease risks associated with first cousin unions and provide genetic counselling for members of obese families.

-Out-reach of research benefits

The gradual but steady increase in the number of children diagnosed with early onset severe obesity obligates a great deal of ethical and moral responsibility on us to make sure that the patients will be afforded proper personalised care, management and treatment, where feasible. We will continue our efforts to provide leptin replacement therapy in some of the principal local hospitals for treatment of subjects with congenital leptin deficiency.

-Functional analysis of novel pathogenic variations

We plan to carry out the functional analysis of the three obesity susceptibility candidate genes, *INSIG2*, *ROCK1* and *ADCY3*, identified by WES analysis. The functional characterization will be performed in house or through collaboration with other laboratories already engaged in similar analyses.

-Designing of cost effective screening of known genetic variants

We envisage to develop a cost effective and relatively rapid method to screen pathogenic mutations in known susceptibility genes for rapid molecular diagnosis in subjects with severe obesity. The screening method will be based on SNP genotyping by Fluidigm (IFC192.24) using

TaqMan assays. This technique will allow screening of 24 different variants in 96 samples in a single step and should provide an efficient and cost effective method for screening of specific mutations.

-Metabolomic profiling of severely obese children

The relatively high prevalence of monogenic obesity in Pakistani population due to loss-of-function mutations in *LEP* and *LEPR* as reported in this study, provides a unique material to investigate metabolic changes that accompany this type of obesity by carrying out metabolomic profiling of affected and normal subjects. The study of metabolomics allowing simultaneous qualitative and quantitative metabolite profiling offers a powerful tool to reduce the existing genotype-phenotype gap [499]. In a previous investigation, metabolomic profiling in obese leptin and leptin receptor deficient (*ob/ob* and *db/db*) mice, demonstrated significant metabolic differences [500]. The proposed study shall focus on the end metabolites to explore the possibility if any of these metabolic signals could be used as clinical biomarkers for a predisposition to monogenic or other forms of obesity. Preliminary results of a pilot experiment in collaboration with the Department of Surgery and Cancer, ICL, to identify differentially expressed metabolites using mass spectroscopy, indicate significant differences between obese and non-obese groups.

Characterisation of gut microbiota of severely obese subjects

Although studies in *ob/ob* mice have shown a differential gut microbiome composition when compared to their lean counterparts [26], to our understanding no parallel studies have yet been undertaken in human subjects suffering from monogenic forms of obesity. A characterisation of the gut microbiome in leptin and leptin receptor deficient patients and subjects with common form of obesity from the same geographical area is, therefore, in order. Such investigations are expected to provide insights into the role of the gut microbiome in the development of obesity, and may suggest leads to individualised therapeutic options for affected subjects.

8. References

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9. Appendix 1

Supplementary Tables

Table A1: Serum hormone levels in 5 adult subjects identified with homozygous leptin gene (*LEP*) mutation

Age years	BMI	Leptin ng/ml	Insulin μ IU/ml	TSH μ IU/ml	Cortisol μ g/dl
22 $\pm$ 2.6 ^a (<i>n</i> =5)	52 $\pm$ 4 (<i>n</i> =5)	<1.0 (<i>n</i> =5)	23.4 $\pm$ 6.4 (<i>n</i> =4)	5.4 $\pm$ 3.0 (<i>n</i> =3)	31 (<i>n</i> =1)

^a Mean $\pm$ SEM

Table A2: Showing 26 probes from P343 probe mix in 15q region of which 9 probes (shaded grey) are deleted in F-39

No	length	gene	Map view	chr. pos.
1	214	SNRPN-HB2-85	15-022,848294	15q11.2
2	475	SNRPN-HB2-85	15-022,858473	15q11.2
3	256	UBE3A	15-023,136395	15q11.2
4	244	UBE3A	15-023,150555	15q11.2
5	142	UBE3A	15-023,152935	15q11.2
6	160	UBE3A	15-023,167740	15q11.2
7	197	UBE3A	15-023,171919	15q11.2
8	270	ATP10A	15-023,484000	15q12
9	136	ATP10A	15-023,659036	15q12
10	220	GABRB3	15-024,344242	15q12
11	292	GABRB3	15-024,357380	15q12
12	382	GABRB3	15-024,363881	15q12
13	148	GABRB3	15-024,376510	15q12
14	319	GABRB3	15-024,379640	15q12
15	184	GABRB3	15-024,417665	15q12
16	355	GABRB3	15-024,568655	15q12
17	436	OCA2	15-025,770116	15q12
18	445	OCA2	15-026,017939	15q13.1
19	202	APBA2	15-027,196749	15q13.1
20	178	NDNL2	15-027,348739	15q13.1
21	300	TJP1	15-027,900944	15q13.1
22	373	TRPM1	15-029,081415	15q13.3
23	166	KLF13	15-029,452013	15q13.3
24	286	CHRNA7	15-030,191319	15q13.3
25	492	SCG5	15-030,759311	15q13.3
26	427	SCG5	15-030,776133	15q13.3

Table A3: Showing 26 probes from P343 probe mix from 15q region of which 18 probes (shaded grey) are deleted in F-87.

No	Length	Gene	Map view	Chr. pos.
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2	475	SNRPN-HB2-85	15-022,858473	15q11.2
3	256	UBE3A	15-023,136395	15q11.2
4	244	UBE3A	15-023,150555	15q11.2
5	142	UBE3A	15-023,152935	15q11.2
6	160	UBE3A	15-023,167740	15q11.2
7	197	UBE3A	15-023,171919	15q11.2
8	270	ATP10A	15-023,484000	15q12
9	136	ATP10A	15-023,659036	15q12
10	220	GABRB3	15-024,344242	15q12
11	292	GABRB3	15-024,357380	15q12
12	382	GABRB3	15-024,363881	15q12
13	148	GABRB3	15-024,376510	15q12
14	319	GABRB3	15-024,379640	15q12
15	184	GABRB3	15-024,417665	15q12
16	355	GABRB3	15-024,568655	15q12
17	436	OCA2	15-025,770116	15q12
18	445	OCA2	15-026,017939	15q13.1
19	202	APBA2	15-027,196749	15q13.1
20	178	NDNL2	15-027,348739	15q13.1
21	300	TJP1	15-027,900944	15q13.1
22	373	TRPM1	15-029,081415	15q13.3
23	166	KLF13	15-029,452013	15q13.3
24	286	CHRNA7	15-030,191319	15q13.3
25	492	SCG5	15-030,759311	15q13.3
26	427	SCG5	15-030,776133	15q13.3

10. Appendix 2

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