MOLECULAR CHARACTERIZATION OF OBESITY AND METABOLIC SYNDROME GENES IN SELECTED PAKISTANI FAMILIES

A thesis submitted in the fulfilment of the requirements for the degree of Doctor of Philosophy

In

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By

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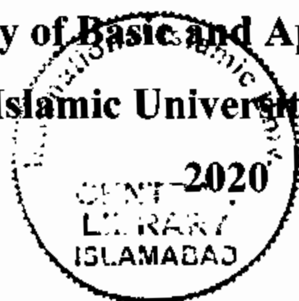
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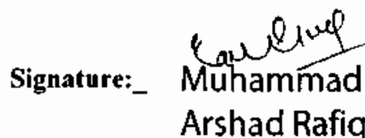

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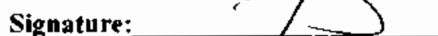

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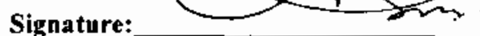

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I, **Robina Khan Niazi** hereby declare that research work of this Ph.D. thesis, entitled **"Molecular characterization of obesity and metabolic syndrome genes in selected Pakistani families"** is solely my research work with no significant contribution from any other person. It is further declared that the research work presented in this dissertation has not been submitted for any other degree or qualification to any other university.

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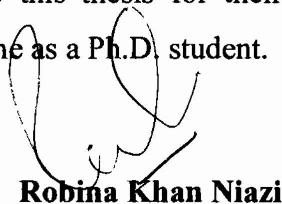

Robina Khan Niazi

Dedicated To

My Beloved Parents

List of Publications

This thesis is based on the following two peer reviewed publications in international journal and three manuscripts are under preparation:

1. **Niazi RK,** Gjesing AP, Hollensted M, Have CT, Grarup N, Pedersen O, et al. Identification of novel LEPR mutations in Pakistani families with morbid childhood obesity. **BMC Medical Genetics. 2018; 19(1):199.**
2. **Niazi RK** and Gjesing AP, Hollensted M, Have CT, Borisevich D, Grarup N, et al. Mutation screen of 31 selected genes involved in monogenic forms of obesity in consanguineous Pakistani families with early-onset obesity segregating as an autosomal recessive trait. **BMC Medical Genetics. 2019; 20:152.**
3. **Niazi RK,** Gjesing AP, Hollensted M, Have CT, Grarup N, Pedersen O, et al. Whole exome sequencing revealed the homozygous variant in *QSOX2* gene with known consanguineous Pakistani origin (in preparation).
4. **Niazi RK,** Gjesing AP, Hollensted M, Have CT, Grarup N, Pedersen O, et al. Whole exome sequencing revealed novel homozygous variant in *LTBP3* gene underlying obesity in a consanguineous Pakistani family (in preparation).
5. **Niazi RK,** Gjesing AP, Hollensted M, Have CT, Grarup N, Pedersen O, et al. Whole exome sequencing revealed homozygous variant in *KISS1R* gene underlying obesity in a consanguineous Pakistani family (in preparation).

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List of Abbreviations

ACTH	Adrenocorticotropic hormone
ADA	American diabetes association
ADCY3	Adenylate cyclase 3
ADRB	β adrenergic receptor
AGRP	Agouti-related protein
ALMS1	Alstrom syndrome protein 1
α-MSH	Alpha-melanocyte-stimulating hormone
ARL6	ADP ribosylation factor like GTPase 6
BBS	Bardet-Biedl syndrome
BBSome	Bardet-Biedl syndrome protein complex
BDNF	Brain-derived neurotrophic factor
β-LPH	β-lipotropin
BMI	Body mass index
BWA	Burrows wheeler aligner
CADD	Combined annotation dependent depletion
CART	Cocaine and amphetamine related transcript
CCDC28B	Coiled-coil domain containing 28B
CCK1	Cholecystokinin1
CDC	Centers for disease control and prevention
CEP290	Centrosomal protein 290
CNVs	Copy number variations
CPE	Carboxypeptidase E
CREBBP	CREB binding protein
CVD	Cardiovascular disease
DXA	Dualenergy X-ray absorptiometry
EAC	Esophageal adenocarcinoma
ExAC	Exome aggregation consortium
EP300	E1A binding protein P300
ERC	Ethical review committee
FDA	Food and drug administration
FHS	Framingham heart study
FMR1	Fragile X mental retardation 1
GnomAD	Genome aggregation database
GATK	Genome analysis toolkit
GBDS	Global burden of disease study

GNAS	Guanine nucleotide binding protein, alpha stimulating
GnRH	Gonadotrophin releasing hormone
GWAS	Genome-wide association studies
HCC	Hepatocellular carcinoma
HDLc	High density lipoprotein cholesterol
IR	Insulin resistance
IER3IP1	Immediate early response 3-interacting protein 1
JAK	Janus kinase
KISS1R	kisspeptin 1 receptor
LDLc	Lowdensity lipoprotein cholesterol
LEP	Leptin
LEPR	Leptin receptor
LTBP3	Latent transforming growth factor beta binding protein 3
MAF	Minor allele frequency
MAPK	Mitogen-activated protein kinase
MC4R	Melanocortin 4 receptor
MI	Myocardial infarction
MKKS	McKusick-Kaufman syndrome
MKS1	MKS transition zone complex subunit 1
MRAP2	Melanocortin 2 receptor accessory protein 2
MetS	Metabolic syndrome
MSH	Melanocyte stimulating hormones
NGS	Nextgeneration sequencing
NPY	Neuropeptide Y
NTRK2	Neurotrophic tyrosine kinase receptor type 2
OA	Osteoarthritis
OLETF	Otsuka long evans tokushima fatty
PC1	proprotein convertase 1
PCRs	Polymerase chain reactions
PCSK1	Proconvertase 1
PHF6	PHD finger protein 6
PIMS	Pakistan institute of medical sciences
PK	Proteinase k
POMC	Proopiomelanocortin
QC	Quality control
QSOX2	Quiescin Q6 sulfhydryl oxidase 2
SDS	Standard deviation score
SH2B1	Src homology 2 B adapter protein 1

SIM1	Single minded 1
SNPs	Single nucleotide polymorphisms
STAT	Signal transducer and activator of transcription
T2DM	Type 2 diabetes mellitus
TE	Tris EDTA
TMEM67	Transmembrane protein 67
TRIM32	Tripartite motif containing 32
UCP	uncoupling protein
TTC8	Tetratrico peptide repeat domain 8
VCFs	Variant calling files
VPS13B	Vacuolar protein sorting 13 Homolog B
WES	Whole exome sequencing
WHO	World health organization

Abstract

Genetic screening of the Pakistani population, known to possess a high degree of consanguinity is an important tool for the identification of deleterious mutations in genes implicated in monogenic disorders. Therefore, sequencing of consanguineous Pakistani families with severe early-onset obesity represents a powerful method of identifying homozygous novel mutations, to the discovery of mutations in genes causing early-onset obesity. This genetic exploration will be helpful for the treatment of genetic obesity through discovering gene targets.

The genetic analysis presented in this dissertation, comprises the clinical and molecular analysis of 25 families with early onset obesity, segregating in an autosomal recessive manner. After clinical characterization of each family, a genetic investigation using targeted re-sequencing, chip-based genotyping and whole exome sequencing techniques, was carried out to search for the underlying obesity genes carrying the damaging sequence variants.

The application of targeted re-sequencing, which is a high-throughput and cost-effective approach was aimed to examine the prevalence of known monogenic forms of obesity. The targeted re-sequencing data was combined with chip-based genotyping, enabling the identification of rare and potentially novel causal variants co-segregating with obesity. The analysis of sequencing data with respect to the three most damaging genes *LEP, LEPR* and *MC4R* revealed that only two of the 25 examined probands carried homozygous recessive mutations and both mutations were positioned in *LEPR*. One was a frame-shift mutation (p.Ser1090Trpfs*6) in family OB4 and the other was a missense mutation (p.Pro892Arg) in family OB25.

Additionally, targeted re-sequencing of the coding and flanking regions of 31 selected genes known to be involved in monogenic forms of obesity (excluding *LEP*, *LEPR* and *MC4R)* was performed in the remaining 23 probands from Pakistani families with severe early-onset obesity segregating as an autosomal recessive trait. Only one compound heterozygous proband was identified in family OB15, carrying two variants in *BBS9/PTHB1* (c.223C>T; p.R75X and c.1441C>T; p.R481X), which cause Bardet-Biedl Syndrome.

The probands (n=17) in which no mutations were identified with targeted re-sequencing were subjected to whole exome sequencing (WES), performed as a means to identify obesity-causing mutations in the coding regions of the genome in consanguineous families OB3, OB5, OB7, OB14, OB21 and OB23. The application of exome sequencing identified the obesity causing sequence variants in candidate genes in homozygous condition, segregating in an autosomal recessive heritance in families OB5, OB21and OB23. However, exome sequencing failed to identify the variants segregating with obesity within families OB3, OB7 and OB14, hence; whole genome sequencing has been suggested to perform.

During the genetic investigation of family OB5, the application of exome sequencing identified the novel frameshift mutation c.94_104delCGGCTGCCGCG (p.Arg32fs) in homozygous condition in the candidate gene *QSOX2,* which causes premature truncation of the QSOX2 protein. In family OB21, a novel stop gain mutation c.2277C>A (p.Cys759*) in homozygous condition was found in *LTBP3* as a candidate gene. It is predicted that this mutation resulted in the truncation of the protein and is thus responsible for a loss of function of the LTBP3 protein. In family OB23, the novel homozygous frameshift mutation c.525_532delGCCGGTGC (p.Pro176fs), which truncates the KISS1R protein, was identified in *KISS1R* as a candidate gene. The pattern of segregation analysis showed autosomal recessive inheritance within these families.

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1. Introduction

Obesity is a complex chronic global medical condition affecting people at all ages, ethnicities, nationalities and sexes [1]. This diseaseis responsible for resulting the global deaths due to its close relationship with a number of comorbid serious health conditions [2].

Adipose tissue in humans has different forms such as subcutaneous fat, visceral fat, yellow bone marrow and is found underneath the skin, in the region of internal organs, in bone marrow and in breast tissue. Originally adipose tissue was just considered to be an energy storage organ, however, after the discovery of leptin and its extensive biological functions in the year 1995, there developed the concept of adipose tissue as an endocrine organ [3]. Based on color, adipocytes are classified as white, brown and pink. White adipocytes store lipids and are involved in releasing fatty acids during episodes of fasting. Brown adipocytes use lipids and glucose in the context of thermal homeostasis. Pink adipocytes are presently identified in mouse as subcutaneous fat deposit throughout pregnancy and lactation processes [4]. Recently adipose tissue was identified to produce numerous bioactive peptides, known as a dipokines that has an affective adipocyte role in an autocrine and paracrine regulation with an additional involvment in additional metabolic pathways [5-7].

These adipokines (also known as adipose tissue-derived cytokines) affect a variety of physiological functions including food intake, energy homeostasis, nutrient metabolism, coagulation and inflammation as well as cardiac and blood pressure parameters [8].

1.1 Definition of obesity

World health organization (WHO) declared obesity like a medical state where an unnecessary and potentially health impairing buildup of fat in adipose tissue to an degree so as to can damage healthiness through a quantity of non-communicable illness [2]. An excess body fat accumulation outcomes in an disproportion between energy consumption and outflow [9]. Adipocyte hypertrophy and/or hyperplasia which explains the increase in size and rise in cell quantity, respectively, are two means through which the abnormal

accumulation of fat in body results. However, in a clinical background and for practical purposes, obesity is addressed like a surplus body weight instead of excess body fat, because measuring weight is less difficult than the straight measurement of fat. Therefore, body mass index (BMI) is supposed as a surrogate percentage of fat accumulation and calculated as a ratio of two anthropometric measurements commonly used; weight of a person in kilograms to his/her height in meter squared (kg/m^2), that remains fixed regardless of age and gender and it is greatly correlated to percentage body fat and an entirety body fat [10-12].

In pediatric background, the age-dependent changes in body composition in children and adolescents are obvious during their growth and have to be accounted for. According to WHO, a child or a set of children can be matched up to with the help of three different systems to the reference population: percentiles, percent of median and Z-scores (standard deviation scores) [13].Therefore, WHO has developed growth charts to measure overweight and obesity in infants and younger children age up to five and for young individuals between five and 19 years [14].

Moreover, the criteria used to define childhood obesity is different in different regions, for instance, in the United States, most studies made on paediatric obesity or overweight are supported on the growth charts made public by centers for disease control and prevention (CDC) during 2000. As CDC definition, children with >85th and >95th percentiles, BMI are to be considered overweight and obese, respectively [15]. Similarly in consideration of age-dependent changes in body composition in children and adolescents, the measured BMI is therefore, standardized to a reference population of similar age and sex, thereby obtaining a BMI standard deviation score (SDS), where the value zero corresponds to the mean and thus the 50th percentile of the reference population [13].

Although BMI SDS is an easily applied measure when assessing obesity in children, in many instances, it is also a rudimentary measure, as it does not distinguish between lean and fat mass or account for variations in body fat distribution. Other more refined methods to measure lean and fat body mass take account of dual-energy X-ray

absorptiometry (DXA) which may give a more precise estimation of obesity, as compared to BMI [16].

1.2 Classification of obesity

According to the classification system developed by WHO, the BMI cut-offs; >25 kg/m^2 and >30 kg/m^2 are used to represent overweight and obesity, respectively and they vary between populations, as the influence of BMI on related comorbidities is known to differ across different ethnicities [17, 18]. In adults, healthy weight is defined as a BMI between 18 and 25 kg/m^2, overweight is a BMI between 25 and 29 kg/m^2, obese is a BMI of >30kg/m^2 and morbidly obese is a BMI of >40 kg/m^2. Hence, the three obesity categories are classified as: obese class I includes the BMI= 30.00-34.99 kg/m^2, obese class II includes the BMI= 35.00-39.99 kg/m^2 and obese class III includes the BMI ≥40.00 kg/m^2 [19]. More classes include very severe obesity, known as super-obese with BMI of ≥50 kg/m^2 and super super-obese with BMI of ≥60 kg/m^2 reported in subjects [20].

The BMI cut-off point indicates the projection towards the increased risk of health complications. Subjects with a BMI >40 kg/m^2 are at a significantly greater danger for diabetes and other complex medical circumstances than those with a BMI in the range of 30-35 [21]. Due to higher lifetime medical costs, such individuals have a much shorter life expectancy [22].

The Asian populations at low values of BMI develop negative health impacts than Caucasians therefore, some nations for their population redefined obesity, for instance, Japan has defined obesity with BMI >25 kg/m^2 [23], however, China considers a BMI of >28 kg/m^2 [24].

1.3 Epidemiology of obesity

Due to high prevalence throughout the previous three decades, the epidemic of obesity has dramatically increased globally and emerged with major health challenges in several divisions of the world that affects approximately every feature of the lives of

patients and human life especially in children and adolescents[25-27]. Globally there is an expectation that by the year 2030, the obesity epidemic has the strength to influence 1.2 billion people [28]. According to the report in 2016, above 1.9 billion people of aged 18 years adults and older were overweight (39%) and above 650 million were obese (13%) and this huge figure gives reflection about high incidence of obesity that has two fold since 1980 [19]. Within pediatric or childhood obesity, 41 million children ranged <5 were overweight or obese while over 340 million children and adolescents with ages ranged between 5-19 years old, were overweight [19].

Although developed countries have a higher rate of prevalence for obesity [25], it is also a serious ground of apprehension in the developing world, with a foresee sharp increase in adult obesity that subsequent to the threat factor of type 2 diabetes mellitus (T2DM) [29, 30].

Within Asia, pediatric obesity imparts a serious public health crisis [31]. Global burden of disease study (GBDS) shared the report in 2013 that from the Asian countries, Pakistan lies on position ninth among the 10 most obese countries worldwide for both adults and children [25, 32, 33].

1.4 Obesity and Metabolic Syndrome

Obesity participates an essential and causal position into the growth of the metabolic syndrome (MetS) [34-36]. Globally every year, a large number of individuals lost their lives due to obesity and its related health disorders [37]. The obesity-related comorbidities have long been observed in adults, they are occurring ever more often in children [38]. MetS, also called multiple risk factor syndromes refer to the co-existence of several risk factors by the same person and is an emergent therapeutic dilemma in developed countries [39-41]. However, the occurrence of MetS is also growing in developing countries [42] including regions, for instance, Asia [43].

The most common contributor to the increase in MetS is an excessive accumulation of body fat [44]. Obesity for being a major metabolic risk factor, has given an umbrella titles for numerous disorders and syndrome [45, 46] in adolescents and in children

including; insulin resistance, T2DM, hypertension, cardiovascular disease, dyslipidemia, fatty liver disease, sleep apnoea and certain kinds of cancer [47-56].

1.5 Genetic Basis of Obesity

The etiology of obesity is multifarious and may be result of many reasons, such as physical activity, nutritional intake, environmental (parental lifestyle/behaviours) and social structures, metabolic and genetic factors [57]. The genetic influences play a powerful role in persons with severe and early-onset obesity that probably progress towards the serious clinical complications [58]. Moreover, the genetic studies in humans to discover the significant molecular knowledge for energy homoeostasis may facilitate to goal safe and specific drug development [59].

Currently, in consequent to a single gene defects, severe human obesity has presented a sight into lasting regulation of body weight. Therefore, an identification and understanding of heritable single-gene disorders with specific traits is the best way to identify with the genetic basis of the disorder at functional level. The hereditary genetic differences predisposing to obesity ranges from 30-60% to 60-80% in studies of family and twin, respectively [60, 61], mostly showing autosomal recessive pattern of inheritance. However, a great deal of effort is still necessary to reveal unexplored genetic source to obesity [62].

The genetics of obesity is mainly divided into non-syndromic and syndromic obesity. Non-syndromic obesity is further divided into monogenic obesity genes including *LEP (leptin), LEPR (leptin receptor), POMC (proopiomelanocortin), MC4R (melanocortin 4 receptor), PC1(proprotein convertase 1), NPY (neuropeptide Y), SIM1(singleminded1)* and polygenic obesity including β adrenergic receptor genes; *ADRB1, ADRB2, ADRB3* and uncoupling protein genes; *UCP1*, *UCP2, UCP3.* The syndromes in obesity showed the pleiotropic and chromosomal rearrangement [59]. Understanding the molecular reasons of the predisposition of obesity proved as a significant condition to recognize the underlying pathways that involved in an advancement of obesity.

Pakistani population is unique for being great in mass, extremely consanguineous with large pedigrees and is hereditarily varied due to ethnically diverse [63]. For these reasons, this study is made in selected Pakistani families with autosomal recessive inheritance to explore the rare recessive syndromic and non-syndromic monogenic obesity forms with damaging mutations. Moreover, the findings of the current study with recessive mode of inheritance are very important to value the molecular pathophysiological means in the predisposition of obesity. Hence, this study with the identification of the causative genetic factors would impart contribution to the improvement of obesity prevention, management and treatment across the world.

2. Literature Review

Obesity and overweight for being the most significant contributors to enhance the risk for the advancement of chronic medical complications, are so widespread that they are substituting the more conventional public health concerns, for instance, under nutrition and contagious diseases since long [64].

2.1 Obesity-Related Disorders

Some of the major obesity-associated health impairing disorders are discussing below:

2.1.1 Insulin Resistance and T2DM

Obesity participates an essential role in mounting the risk of developing insulin resistance (IR), which is accompanied by an insulin secretory defect and it consequent into T2DM [65]. T2DM is considered as the most devastating among obesity complications. It is characteristically a constant disease that affects with a ten year shorter living expectancy [66]. According to WHO, diabetes will be known as the seventh leading source of death by the year 2030 [67].

Since obesity and overweight play an significant responsibility in the development of T2DM amongst other diabetes [68, 69] hence, among the obese people, the succession of T2DM takes place more frequently and is evident from the fact that T2DM is five to six times more regular in obese individuals than in those with standard weight [70]. Obesity and T2DM often occur in parallel and studies found that about 60-90% of patients with T2DM were obese [71, 72].

A genetic factor plays a job in the onset of obesity and T2DM, however, as apparent from the information that not all obese individuals develop T2DM [73]. Similarly, within a definite population, normal individuals could have T2DM which propose that factors of genetic and/or environmental also take part in its progression [73]. Moreover, obesity is ethnic based and hence 30% surplus body fat is correlated with 30% of cases in descent of Chinese and Japanese, 60-80% of cases in descent of European and

African and 100% of cases in Pacific Islanders and Pima Indians. However, a high waist-hip ratio is frequently present among those who are not obese [74, 75].

Addressing the complications led by diabetes, it enlarges the possibility of cardiac disease and stroke, which revealed 50% of people die of them with diabetes [76]. Furthermore, diabetes is also among the foremost ground of kidney failure [67].

There is a piece of strong and reliable facts that obesity management can hold-up the development from prediabetes to T2DM [77, 78] and may be beneficial in the treatment of T2DM [79-81]. Among overweight and obese patients with T2DM, modest and persistent weight loss has been given away to get better glycemic control that consequently decreases the need for glucose-lowering medications [82-84]. The American Diabetes Association (ADA) most recently provided "Standards of Medical Care in Diabetes" that could be helpful in the management of obesity for the treatment of T2DM [85].

2.1.2 Obesity and Hypertension

According to the studies, upto 70% of cases of hypertension are attributed due to obesity [86]. The incidence and an increase of hypertension, defined as systolic pressure >140 mmHg or diastolic pressure >90 mmHg has a direct relationship with obesity when assessed by BMI [87]. The linear relationship between hypertension and BMI has been observed from studies in different ethnical populations throughout the world [87-89]. Framingham Heart Study (FHS) accounted the risk assessment of hypertension and declared 65% of hypertension in women and 78% in men that might be due to excess weight gain [90].

The close association involving obesity and hypertension has attribution in the amplification of the occurrence of hypertension in adolescents and in children as well [91-93]. Hypertension in childhood is a sign of permanent high blood pressure in young adulthood and this condition is a foremost danger for cardiovascular disease which is associated with cardiovascular morbidity [94-96]. Obesity-related hypertension directs to numerous other complications, for instance, atherosclerosis, a trial fibrillation, diseases of

cerebrovascular and coronary artery, congestive heart failure, left ventricular hypertrophy and renal insufficiency [97-100].

Moreover, the control of hypertension becomes difficult to attain because once the hypertension due to obesity is ascertained in adults, the control is linked with therapy of weight loss [52].

2.1.3 Obesity and Cardiovascular Disease

Obesity is a self-determining reason for causing cardiovascular disease (CVD), moreover, its administration decreases the frequency of ischemic heart disease [101-105]. This is possibly due to the excess adipose tissue on the body affecting chemical responses in the body, possibly leading to insulin resistance (T2DM), which has the risk of development into CVD [106].

CVD could be defined by including angina, heart failure, myocardial infarction (MI) and sudden cardiac death. Obesity (particularly central obesity) is among the adaptable risk causes affecting the occurrence of first MI in the course of all ethnic groups and gender [107].

2.1.4 Obesity and Dyslipidemia

Dyslipidemia covers the conditions of lipoprotein metabolism and is an important component of the MetS [108]. Obesity-related dyslipidemia includes elevated levels of triglycerides, decreased levels of high-density lipoprotein cholesterol (HDLc) and normal or slightly increased levels of low-density lipoprotein cholesterol (LDLc) with increased small and dense LDL particles [109]. All these components have been shown to be atherogenic [110]. Hence, dyslipidemia is a broadly acknowledged risk aspect for the advancement of CVD [110].

Moreover, a good correlation has been found between triglycerides and insulin resistance with the influence of triglycerides in the pathway of insulin action that results into insulin resistance and might grounds hyper triglyceridemia [111]. Since dyslipidemia (hyper triglyceridemia) is linked with insulin resistance in T2DM hence, causes metabolic syndrome in obesity [112, 113]. The administration of obesity-related dyslipidemia of the

MetS included LDL cholesterol that has remained the primary target of lipid-lowering therapy, raising HDL levels is now an important secondary target to reduce CVD risk [114, 115]. Other methods of control are lifestyle modifications with weight loss through exercise, using dietary fibers, weight loss medications can improve dyslipidemia which reduce CVD risk and when necessary, obese individuals should be targeted for intense lipid-lowering therapy [115].

2.1.5 Obesity and Osteoarthritis

Osteoarthritis (OA) is a clinical syndrome of joint pain with dysfunction sourced by joint degeneration and affects more individuals in comparison with other joint disease [116]. Obesity has an association with hand, knee and hip osteoarthritis [117-121]. However, obesity involved to have occurrence and development of OA, with the strongest association being at the knee [122]. Moreover, it is one of the most crucial possibility factors for osteoarthritis in both men and women [120]. A study on subjects with a BMI >30 kg/m^2 were 6.8 times more expected to build up knee OA than controls with normal-weight [123].

Symptoms of OA and progression towards the complexity of disease can be slowed by weight loss [122]. It is especially administered to improve pain with playing a role in obese individuals with knee OA [124].

2.1.6 Obesity and Cancer

Excess body weight also showed linkage to the increased risk for a number of malignancies [125-127]. Due to an association with obesity, the elevated numbers of fatality from hepatocellular carcinoma (HCC), colorectal, esophageal and breast cancer among female malignancies explained an increase of 1.52-fold in men and 1.62-fold in women in an account of relative risk of cancer-related death [127]. Moreover, obesity contributes a critical character in the progression of other cancer types, including thyroid [128], prostate [129], endometrial, ovarian and cervical [130, 131].

Although the systematic and molecular mechanisms of obesity with certain types of cancer are complex, however, obesity-induced complications, for instance, enlarged lipid

build up, adipokines, inflammation, insulin resistance, estrogen and gut microbiota could put in to carcinogenesis [132].

2.2 Genetics of Obesity

From previously two decades, the genetic research on obesity has been the main focus [133]. Recently, the rapid developments due to next generation sequencing technologies have brought an enhancement in our understanding of the genetic powers in the development of obesity [134, 135]. The first evidence about the influence of genetics in obesity risk has been presented through establishing an association between weight and genetics in a study that was executed on 514 veteran twin pairs [136].

Due to the genetic contribution twin, family and adoption studies indicated the widely accepted high range i.e. 40-70% for heritability of BMI [137, 138]. The genetic studies based on the phenotypic variations between individuals have indicated that obesity in parents is among the foremost risk factors for both childhood and adult obesity [139]. The possibility of childhood obesity increased twice when both parents are obese [140]. Most of the genes are correlated to food ingestion and regulation of energy equilibrium, thus are implicated in the vulnerability of obesity [141]. For the reasons of characteristics on genetic and phenotypic, three distinct forms of obesity have been identified; monogenic; syndromic and non-syndromic obesity and polygenic (common) obesity.

2.2.1 Monogenic Obesity

Monogenic obesity, also recognized as a Mendelian form of obesity is very rare, severe and usually with early-onset obesity [142]. Monogenic obesity affects 5% of the population and is consequent from the modification of a single gene [143]. Studies based on an extreme obese individuals with consanguineous background, have been demonstrated to be a very thriving method in identifying mutations in monogenic obesity causing genes that are supposed to cause Mendelian obesity [142, 144].

Two types of monogenic inheritance of obesity could be established on the basis of phenotypic etiology; non-syndromic and syndromic. Generally, these Mendelian forms of

obesity are considered by an extreme phenotype of morbid obesity with early-onset [145].

2.2.1.1 Non-Syndromic Forms of Monogenic Obesity

Several monogenic obesity-related mutations particularly of the genes encoding appetite regulating proteins have been revealed to cause autosomal recessive and dominant forms of obesity. These mutations are rare and accountable for causing an early-onset severe phenotype of morbid obesity with hyperphagia and other endocrine disorders [146]. Many alterations in obesity genes are implicated in the leptin-melanocortin signalling pathway present in the hypothalamus that contributes a key function in the hypothalamic regulation of food intake and energy expenditure [147].

Genes involved in Non-Syndromic Forms of Monogenic Obesity

Generally, mutations in *LEP*, *LEPR* and *MC4R* represent the most common cause of monogenic forms of obesity and mutations within these genes have been demonstrated to cause early-onset morbid childhood obesity in probands of various ethnicities [148-153].

The other genes implicated in monogenic forms of obesity are *brain-derived neurotrophic factor* (*BDNF*), *neurotrophic tyrosine kinase receptor type 2* (*NTRK2*), *proconvertase 1* (*PCSK1*), *POMC, Src homology 2 B adapter protein 1* (*SH2B1), melanocortin 2 receptor accessory protein 2* (*MRAP2)* and *SIM1*. The damaging mutations in these genes displayed recessive inheritance except *MC4R* that exhibit both recessive and co-dominant with penetrance power depending on the ethnic group [152].

These well known ten genes mutations explaining upto 10% of cases with the phenotype of early-onset extreme obesity [145, 154, 155] and all are involved in the leptin-melanocortin signaling pathway.

Leptin-melanocortin pathway is activated when leptin is secreted by the adipose tissue and binds to the leptin receptor (LEPR) that is present in the surface neurons in the arcuate nucleus of the hypothalamus [156]. The signal is then propagated through the POMC/cocaine and amphetamine-related transcript (CART) and melanocortin system,

which regulates satiety and energy homeostasis [146]. While POMC/CART neurons synthesize anorexigenic peptide alpha-melanocyte-stimulating hormone (α-MSH), a distinct group of neurons synthesizes the orexigenic peptide; neuropeptide Y (NPY) and agouti-related protein (AGRP), which act as an inhibitors of MC3 and MC4 receptors [157]. The derived peptide nature of POMC depends on the endoproteolytic-type enzyme present specific in the brain region. In the anterior pituitary, the PCSK1 enzyme produces adrenocorticotropic hormone (ACTH) and β-lipotropin (β-LPH), while in the hypothalamus the combined presence of PCSK1 and PCSK2 control the production of α-, β-, γ- MSH (melanocyte-stimulating hormones also known as melanotropins or intermedins) and β-endorphins [146]. The protein encoded by the *MC4R* gene is a membrane-bound receptor and a member of the melanocortin receptor family [158]. The protein interacts with ACTH and MSH hormones and is mediated by G proteins. Due to deleterious mutations in leptin-melanocortin pathway, they results into the disruption of the pathway that ultimately results in obesity. The most common genes involved in leptin-melanocortin pathway implicating the obesity are discussing below;

Leptin

Leptin (*LEP*) gene mapped on chromosome 7q31.2, was the first to identify as the monogenic obesity gene and it causes severe obesity due to leptin deficiency in both mice and humans [149]. This gene encodes leptin, a 16-KD protein-hormone secreted by white adipocytes and binds to LEPR, which regulates energy expenditure through hypothalamic neurons [156, 159].

This protein operates in signalling pathway that can reduce food intake and/or regulate energy balance. Over the subsequent research, it has been proved that leptin deficiency is heritable and responsible for excessive early-onset obesity [160].

Summarizing the spectrum of mutations displayed by *LEP*, most of the mutations, including the first mutation were reported from Pakistani population (Table 2.1). The other mutations were reported from populations including Turkey [161], Turkmenistan [162], Egypt [163], Austria [164] and India [165]. A consanguineous Turkish pedigree displayed a second mutation, c.313C>T resulting to a missense mutation, p.R105W in *LEP* described congenital leptin deficiency in three individuals [161]. The third mutation,

c.422C>G led to a missense mutation, p.S141C was reported in two individuals belonging to Turkmenistan [162]. The fourth mutation, c.309C>A resulted into a missense mutation of the protein, p.N103K was reported in two children from a consanguineous Egyptian family [163]. The fifth mutation, c.215T>C resulting to a missense mutation of the protein, p.L72S was revealed in a child of an Austrian family with non-consanguinity [164]. The sixth and seventh mutations were identified from consanguineous Pakistani families [166]. The eighth mutation, c.163C>T led to a nonsense mutation of the protein, p.Q55* [165].

Leptin Receptor

Leptin receptor (*LEPR)* gene mapped on chromosome 1p31.3 encodes the LEPR protein and is involved in leptin actions and regulation of fat metabolism. LEPR is a member of the gp130 family of cytokine receptors, which stimulate gene transcription via activation of cytosolic signal transducer and activator of transcription proteins (STAT), predominantly in the hypothalamic neurons [167]. It has six isoforms (LEPRa-f), yet, leptin signalling action is primarily mediated by the long LEPRb expressed in the hypothalamus [167-169] by means of JAK-STAT (Janus kinase-signal transducer and activator of transcription) pathway [170]. The short forms are expressed in a number of tissues including the adrenal gland, kidney, lung and choroid plexus [171, 172].

In humans, inactivation of leptin receptor and leptin deficiency, both results in related clinical phenotypes including severe early-onset hyperphagic obesity with a rapid weight increase during the first few months of life [134, 150, 156]. In 1998, the first mutation with LEPR deficiencies was identified in a family of Kabilian descent with morbid obesity [150]. Later on, *LEPR* with more mutations in homozygous or compound heterozygous conditions have been reported in populations with different ethnical backgrounds, for instance, Algerian [150], Bangladeshi [153], Dutch [173], Egyptian [174], French [175-177], German [178],Guinean [159], Iranian [153], Norwegian [153], Portuguese [176], Southern European [153], Sudanese [159], Turkish [153, 176, 178], Turkmenistanian [179], White (UK) [153] and Pakistani [134, 151, 180].

Until now, 38 mutations have been discovered in *LEPR* thus far. These mutations comprised of 13 missense and 25 mutations which resulted into the truncation of LEPR protein were deletions, duplications, insertions or nonsense [178].

Melanocortin 4 Receptor

Melanocortin 4 Receptor (MC4R) gene mapped on chromosome 18q21.3 with single exon, which encodes 332-amino acid. It is a G-protein linked receptor (seven-transmembrane), significantly entailed in food intake and controls energy balance [181]. It is expressed mainly in the hypothalamus, which interacts with ACTH and MSH hormones through G proteins [181-183]. Similar to the deficiencies of LEP and LEPR, during the first year of life, MC4R deficiency has been linked with hyperphagia, increased fat and lean mass, increased linear height, increased bone mineral density and severe early hyperinsulinemia [184, 185], although some of these associations remain controversial [186, 187].

The prevalence of damaging mutations in heterozygous, compound heterozygous and homozygous conditions are 0.5-5.8% and has been reported in morbidly obese children and adults in different ethnicity globally [188, 189]. Among the ethnical groups, the highest prevalence of *MC4R* homozygous mutations is being reported in cohorts from Pakistani descent with inbred background [151, 184]. The obesity severity is less severe in *MC4R* heterozygous carriers as reported in Europeans [152] than the *MC4R* homozygous carriers [190].

2.2.1.2 Syndromic Forms of Monogenic Obesity

Syndromic monogenic forms of obesity are very rare. Many of them are distinguished by severe and early-onset obesity linked with other specific features, including alterations in hormone levels or dysmorphic characteristics, such as organ developmental deformities [191, 192]. Although some syndromic forms of obesity, including Alström syndrome is an autosomal recessive disorder caused by mutations in *ALMS1(Alstrom syndrome protein 1)* gene and is not characterized by developmental delay [193]. Many other syndromic forms of obesity are linked with varying degrees of mental retardation, including Prader-Willi syndrome caused by either deletion of a segment of paternal copy

of chromosome 15q11.2-q12 or the entire paternal chromosome 15 [193], SIM1 syndrome caused by disruption or deletion at chromosome 6q [194] and WAGR (W; wilms tumor, A; aniridia, G; genito-urinary abnormalities, R; range of developmental delays) syndrome caused by a deletion at chromosome 11p13 or a deletion in *BDNF* which encode brain-derived neurotrophic factor protein [195]. Moreover, Bardet-Biedl syndrome (BBS) is an autosomal recessive disorder caused by mutations within 21 loci, fragile X syndromecaused by disruption of the *FMR1* (*fragile X mental retardation 1*) gene, Cohen syndrome (COH), an autosomal recessive disorder caused by mutations in *COH1* and Albright's Hereditary Osteodystrophy, which is an autosomal dominant disorder caused by mutations in *GNAS1 (guanine nucleotide binding protein, alpha stimulating 1)* are all pleiotropic disorders linked with developmental delay [193]. These rare syndromic forms of obesity may be instigated by either autosomal, X-linked chromosomal abnormalities or distinct genetic defects [143, 192, 196].

Generally,79 obesity syndromes including genes of *ARL6* (*ADP ribosylation factor like GTPase 6*)*/BBS3*, *CCDC28B* (*coiled-coil domain containing 28B*), *CEP290* (*centrosomal protein 290/ BBS14,CREBBP* (*CREB binding protein*), *EP300* (*E1A binding protein P300*), *GNAS*, *IER3IP1* (*immediate early response 3-interacting protein 1*), *MKKS* (*McKusick-Kaufman syndrome*)*/BBS6*, *MKS1* (*MKS transition zone complex subunit 1*), *MRAP2 (melanocortin 2 receptor accessory protein 2*), *NTRK2* (*neurotrophic receptor tyrosine kinase 2*)*/TRKB*, *PHF6* (*PHD finger protein 6*), *TMEM67* (*transmembrane protein 67*)*/ JBTS6*, *TRIM32* (*tripartite motif containing 32*), *TTC8* (*tetratricopeptide repeat domain 8*)*/BBS8* and *VPS13B* (*vacuolar protein sorting 13 homolog B*)*/COH1* have been reported in the literature, among which 19 have been fully genetically elucidated, 11 partially, 27 have been mapped to a chromosomal region and for the remaining 22, neither the gene/s nor the chromosomal location/s have yet been identified [197]. For the unraveled syndromes, 68 diseased genes have been found and among them, 59 genes have been replicated as well as confirmed in independent studies [197].

2.3 Prevalence of the Most Common Non-Syndromic Monogenic Forms of Obesity in Pakistani Population

Worldwide, consanguineous unions have been practiced in many populations for several generations due to their social and economic benefits [198-200] . Pakistani population has the utmost rate of consanguinity worldwide, with a frequency of 60-76% [201, 202]. In families with a known history of consanguineous marriages, the degree of homozygosity in family members will be on average 11% and consanguinity thereby increases the risk of family members suffering from autosomal deleterious recessive disorders [202]. Genetic screening of consanguineous families with severe early-onset obesity has led as a powerful method of identifying homozygous mutations causing early-onset obesity and has enabled the identification of rare damaging variants in monogenes for instance; *LEP*, *LEPR* and *MC4R* [149, 150, 203].

Therefore, due to having high consanguinity, the high prevalence of single-gene severe early-onset child obesity disorders in most common obesity genes; *LEP*, *LEPR* and *MC4R* have been reported from Pakistani population and it is as high as up to 30% [151] which was previously reported to be 3-5% in European descents [204] (Table 2.1).

In humans, the first mutation in *LEP* was carried by two cousins of eight and two years old respectively, from a consanguineous Pakistani background and they carried a deletion (c.398*del*G) which resulted into frameshift mutation, p.G133V*fs**15, causing congenital leptin deficiency [149]. The other novel mutations in homozygous state; c.104_106*del*TCA, c.481_482delCT and a known mutation c.398*del*G, were identified in nine carriers from a cohort of 25 individuals with Pakistani origin [166]. A study on a cohort of 62 unrelated children from consanguineous Pakistani background revealed the identification of same homozygous frameshift mutation, p.G133V*fs**15 (c.398*del*G) in nine probands and one proband with a mutation involving deletion of three base pairs (I35del), all characterized with early-onset and severe obesity [190]. Another study in Pakistani consanguineous background pedigrees revealed the identification of *LEP* mutations, c.1-44*del*42 in a 17 months male child and c.350G>a (p.C117Y) identified in 18 months old boy proband [151].

Similarly, *LEPR* mutations in Pakistani population include, homozygous mutations, c.2396-1G>T (p.799-1G>T) in a 14 months old female proband and c.1675 G>A (p.Trp558*) in a seven months old female proband have been reported for the first time in the Pakistani population, characterized by early-onset morbid obesity with hyperphagia [169]. Moreover, two novel mutations with 1.3 kb and 58.8 kb homozygous deletions were identified in an eight months child and c.1810T>a (p.C604S) was identified in one and five years old siblings with severe obesity [151].

Hence, the identified mutations in two genes; *LEP* and *LEPR* are ethnic specific and the prevalence of monogenic obesity caused by mutations within these two genes is as high as >20% in Pakistani study populations with obesity [134, 151, 190]

Within Pakistani population, for the first time a pathogenic mutation p.M161T (c.482T>C), was identified in the homozygous condition in *MC4R*, in a six month old girl [190]. Similarly, another homozygous mutation in *MC4R*, p.I316S (c.947T>C) in a 15 years old individual, was reported from the same population group [190]. Both, *MC4R* homozygous or heterozygous carriers were consequence with early-onset severe obesity, though the level of severity depends on different ethnic groups [152, 205].

Most recently, the consanguineous Pakistani population identified with loss-of-function mutations (c.3315*del*, c.2578–1G>A and c.191A>T) in homozygous condition in novel monogenic form of obesity i.e. *ADCY3* gene encoding adenylate cyclase 3 in children of ages 15 and six years old, respectively with severe obesity (Table 2.2) [206]. This study also identified the compound heterozygous mutations (c.1268*del* and c.3354_3356*del*) in *ADCY3* in a child of severely obese of age 11 years from a non-consanguineous family of European-American descent.

Thus, Pakistani inbred population becomes a very important sample tool to explore the genetic contributors of monogenic obesity that leads towards the identification of novel pathways and physiological mechanisms underlying to the progression of obesity.

2.4 Prevalence of Rare Non-Syndromic Monogenic Forms of Obesity in Pakistani Population

Other genes involved in leptin-melanocortin pathway are *POMC, PCSK1, MC3R, SH2B1, BDNF, NTRK2, SIM1* and *MRAP2.* To date, no prevalence has been reported in these genes from Pakistani population. The only investigation was performed in *POMC* gene in a case-control cohort study, based on 475 adult Pakistani individuals and identified a single heterozygous obese carrier with p.R236G mutation (0.4%) [207]. This negative prevalence of homozygous and compound heterozygous deleterious mutations even in Pakistani population, which is famous for being source of genetic findings of many rare genetic disorders for reasons of having unique characteristics such as being diverse in ethnicity (genetically diverse), considered cousin marriages as a society norms and having pedigrees of large size, explains that damaging mutations in these single gene obesity are very rare in humans [208].

2.5 Prevalence of Syndromic Forms of Monogenic Obesity in Pakistani Population

Pakistani families with an elevated rate of consanguinity have showed the way to the identification of various case reports within heritance of autosomal recessive obesity syndromes in this population.

2.5.1 Bardet-Biedl Syndrome

The Bardet-Biedl syndrome (BBS) is a rare autosomal recessive ciliopathy disorder causing the phenotypes including hypogonadism, developmental delay with learning difficulties, speech and vision impairment and severe childhood obesity [209]. Regarding disease causing BBS, 21 genes have been known [210-212], particularly several novel mutations discovered in consanguineous Pakistani families.

The summary of the homozygous variants causing BBS identified in consanguineous Pakistani families is given in Table 2.3.

2.5.2 Alström Syndrome

Alström syndrome (ALMS) is a rare autosomal recessive ciliopathy with approximately 300 known cases [213]. The clinical manifestations include retinal dystrophy, hearing impairments, early-onset obesity, insulin resistance and T2DM [213]. ALMS has been mapped to chromosome 2p13.1 [214] and six unrelated families displayed mutations in *ALMS1* [215]. From Pakistani population, a consanguineous family also reported with the identification of a large multiexonic (exons 13-16) genomic deletion of 41.2 kb – chr2:73,772,326-73,813,435 within the *ALMS1* gene [216].

2.5.3 Cohen Syndrome

Cohen syndrome (COH) identifies with the primary clinical manifestations including central obesity along with lack of ocular, neural and muscular strength. Mutations in the *COH1/VPS13B* gene segregate as an autosomal recessive disorder and result in a rare COH. Among the Pakistani population, three families with consanguineous inbred displayed principal primary phenotypes of COH and sometimes with the addition of secondary phenotypes of intellectual disability and in some cases autistic-like traits [217]. Within these three families, the two families revealed with the identification of *VPS13B* with a one base pair deletion (p.Phe2293Leu*fs**24) resulting in premature protein truncation and the third family revealed with a deletion of exons 37–40 (p.Gly2177Ala*fs**16) leading to a truncated protein. These reported mutations were identified in homozygous condition with a co-segregation pattern in affected individuals [217].

2.6 Next Generation Sequencing

The applications of high throughput next generation sequencing (NGS) knowledge have transformed the field of human genetic diseases in epigenetic, genomic and transcriptomic fields [218, 219]. It is practiced to sequence the genomes as whole genome sequencing (WGS) or coding regions as whole exome sequencing (WES) or targeted panels by sequencing the selected genes [218]. For patient care in clinics, the use of NGS has been proved as a diagnostic means for recognition of mutations in disease

causing genes particularly in autosomal diseases [220]. After the identification of novel genes or mutations implicated in causing genetic disorders they are often further validated in an animal model, which are mostly used to provide experimental evidence and to support the new discoveries.

2.7 Monogenic Obesity Models

A vital purpose of obesity genetics research is the discovery and understanding of monogenic obesity. The studies performed in obesity models explained the significant breakthrough in recognizing the significant mechanisms involved in energy homeostasis within human obesity. Moreover, an animal model for human obesity and its co-morbidities was developed for developing new therapies and means of prevention for obesity [221]. Summary of animal monogenic genetic models in the monogenic obesity which display the phenotypes of hyperphagia, decreased energy expenditure, hyperglycemia and insulin resistance is given in Table 2.4.

Table 2. 1 The spectrum of novel homozygous mutations in the most common obesity genes from Pakistani Population.

Gene	Mutation	Age (yrs)	Sex	Reference
LEP	c.398*del*G (p.G133V*fs**15)	2.0	M	[149]
	c.104_106*del*TCA (p.I35*del)*	0.7	F	[166]
	c.481_482*del*CT (p.L161G*fs**10)	1.6	M	
	c.1-44*del*42	1.5	M	[151]
	c.350G>a (p.C117Y)	1.6	M	
LEPR	c.2396-1G>T (p.799-1G>T)	1.2	F	[169]
	c.1675 G>A (p.Trp558*)	0.7	F	
	1.3 kb, and 58.8 kb deletion	0.8	M	[151]
	c.1810T>a (p.C604S)	1.0	M	
MC4R	c.482 T>C (p.M161T)	0.6	F	[190]
	c.947 T>C (p.I316S)	15.0	M	

Table 2. 2 The spectrum of homozygous mutations in the new obesity gene *ADCY3* from unrelated Pakistani families.

Gene	Mutation	Type of mutation	Sex	Age (yrs) of probands	Reference
ADCY3	c.3315*del*	frameshift	F	15.0	[206]
	c.2578–1G>A	a splice-site	M	06.0	
	c.191A>T	missense	M	06.0	

Table 2. 3 The spectrum of homozygous variants causing BBS identified in unrelated consanguineous Pakistani families.

Sr. No.	Gene	Variant	Type of mutation	Phenotypes of disorder	Reference
1	*BBS12*	p.S701X	nonsense	The mild phenotype of postaxial polydactyly and late-onset retinal dysfunction (night blindness) and without renal or genital anomalies, obesity, mental retardation or learning difficulties	[222]
2	*BBS3*	c.281T>C; p.Ile94Thr	missense	Obesity, hypogonadism, intellectual disability/mental retardation, vision impairment.	[223]
3	*BBS10*	c.1075C>T; p.Gln359*	nonsense	Obesity, hypogonadism, intellectual disability/mental retardation, vision impairment.	[223]
3	*BBS10*	c.1958_1967*del* (deletion of 10 nucleotides)		Abnormal liver functioning and bilateral basal ganglia calcification	[224]
4	*BBS1*	c.47+1G>T	splice site	Obesity, retinitis pigmentosa, polydactyly, and learning difficulties.	[225]
5	*BBS1*	c.442G>A; p. Asp148Asn	missense	Obesity, retinitis pigmentosa, polydactyly, learning difficulties, and speech problems.	[225]
6	*BBS9*	c.299*del*C; p. Ser100Leu*fs**24	frameshift	Obesity, synpolydactyly, intellectual disability, retinitis pigmentosa, and renal abnormality.	[226]

Table 2. 4 Summary of animal monogenic models in monogenic obesity which display the phenotypes of hyperphagia, decreased energy expenditure, hyperglycemia and insulin resistance.

Model Name	Mutation	Hyperphagia	Decreased energy expenditure	Hyperglycemia	Insulin resistance	Reference
ob/ob mouse	Lep^{ob}/Lep^{ob} (leptin deficiency)	yes	Yes	yes	yes	[227-229]
db/db mouse	Lep^{db}/Lep^{db} (leptin receptor)	yes	Yes	yes	yes	[227, 230, 231]
s/s mouse	disrupted STAT3 signal of leptin receptor	yes	Yes	no	mild or late	[232, 233]
Zucker rat	mutated leptin receptor (fa/fa)	yes	Yes	yes	yes	[234, 235]
Koletsky rat	mutated leptin receptor (null mutation)	yes	Yes	yes	yes	[230, 236-240]
ZDF rat	mutated leptin receptor (fa/fa)	yes	Yes	yes	yes	[241]
Wistar Kyoto fatty rat	Zucker /fa/fa x Wistar-Kyoto	yes	Yes	yes	yes	[242]
POMC knockout mouse	POMC deficiency	yes	Yes	yes	yes	[243, 244]
POMC/AgRP double knockout mice	POMC and AgRP deficiency	yes	Yes	yes	yes	[245]
MC4R knockout mouse	melanocortin 4 receptor	yes	Yes	yes	yes	[246]
MC4R knockout rat	melanocortin 4 receptor	yes	Yes	yes	yes	[247]
MC3R knockout	melanocortin 3	yes	yes	yes	mild or	[248]

mouse	receptor				late	
MC4/MC3 receptor double knockout mouse		yes	Yes	yes	yes	[249]
Ectopic agouti expression	agouti overexpression	yes	Yes	yes	yes	[250, 251]
AgRP overexpression	AgRP overexpression	yes	Yes	mild or late	yes	[252]
Carboxypeptidase E (CPE) mutation	Disruption of prohormone processing	yes	Yes	mild or late	Mild or late	[253, 254]
Otsuka Long Evans Tokushima Fatty rat (OLETF)	CCK1 receptor knockout	yes	Yes	yes	yes	[255-258]

3. Aims and Objectives of Study

The general aim of current research thesis is to investigate, discover and understand the genetic contribution entailed in the etiology of childhood obesity with Pakistani origin. This investigation will help us to establish genotype-phenotype correlation, genetic counselling, awareness about issues and knowledge associated with marriages between closely related individuals. Furthermore, based on molecular diagnosis, the identification of rare and damaging variants predisposing to obesity holds the promise of future development of novel therapeutic options and personalized medicine.

The specific aims and objectives of the study were included:

1. The identification of diseased genes, causing obesity in patients.
2. The identification of pathogenic variants.
3. The identification of the association of pathogenic variants in identified genes with their clinical phenotypes.
4. The exploration of the inheritance pattern of the disease.
5. In silico analysis of the identified pathogenic variants in genes.
6. To understand how genetics contribute to the pathogenicity of the disease.

4. Materials and Methods

4.1 Ethical Approval and Study Participants

For working on the study, ethical approvals were attained from the Ethical Review Committee (ERC), International Islamic University, Islamabad, Pakistan and Ethical Review Board (ERB) from Pakistan Institute of Medical Sciences (PIMS) Hospital, Islamabad, Pakistan. Twenty-five families originated from different regions of Pakistan, were recruited and examined at PIMS Hospital, Islamabad. All the families were visited and clinical information was collected through standardized questionnaire. The families were educated in their local languages and written informed permissions were taken from the affected members and/or their guardians for clinical and molecular analysis of the disorder and presentation of the data including photographs for publications if needed. The parents or guardian were interviewed for obtaining information relevant to the consanguineous nature, clinical history and construction of the pedigree. Pedigrees were drawn according to the protocol described by Bennett et al. [259]. For phenotypes, documentation photographs of the affected individuals were obtained using a digital camera.

Fourteen of the included families had known consanguineous marriages. The selection of the families was based on three criteria including 1) BMI of probands $\geq$30 kg/m^2; 2) Probands displaying obesity onset before five years of age; and 3) Parents of the probands with BMI $\leq$25 kg/m^2, consistent with an autosomal recessive mode of inheritance.

4.2 Clinical Examination

Through interview sessions, information was recorded about the age of obesity onset (years), other major chronic diseases (if any); metabolic disorder(s) running in the family, eating habits, physical activity, along with obesity-related co-morbidities. Waist circumference (cm) and height (cm) were measured with a non-elastic plastic tape with the participant standing in an upright position without shoes. Weight (kg), without shoes

and in light clothes, was measured to the nearest 0.1 kg on a digital scale. From these measures, BMI was calculated as the weight in kilograms divided by the square of the height in meters (kg/m^2) and using the LMS method [260], a BMI SDS was calculated based on a WHO reference population [261]. Clinical characteristics of the families are presented in Table 4.1.

4.3 Blood Sampling

Approximately 3-5 mL venous non-fasting blood samples from affected and un-affected family members were collected in 8.5 mL vacutainer tubes (BD Vacutainer® ACD, Franklin Lakes NJ, USA) and in 10 mL EDTA containing vacutainer set (BD Vacutainer® K3 EDTA, Franklin Lakes NJ, USA), by venipuncture with the help of 5 mL (BD 0.60 mm X 25 mm) and 10 mL (BD 0.8 mm X 38 mm 21) syringes, attached usually with a butterfly (BD Vacutainer, Franklin Lakes NJ, USA) especially in children of age below five years.

4.4 Genomic DNA Extraction

DNA was extracted from blood samples from n=36 affected and n=88 unaffected family members. Genomic DNA was primarily extracted using the standard phenol-chloroform method [262]. However, in some families, the extraction of genomic DNA from blood was also carried out using a commercially available kit.

4.4.1 Phenol-Chloroform Method

In phenol-chloroform method of DNA extraction, 0.75mL blood and equal volume of solution A (10 mM Tris (pH 7.5), 1% v/v Triton X-100, 5 mM $MgCl_2$, 0.32 M Sucrose) was mixed in a 1.5 mL tube (GEB, Torrance, CA, USA) and kept at room temperature for 15-20 min. The centrifugation was held at 13,000 rpm for 60 seconds, supernatant was disposed of and pellet was re-suspended in 500 µL of solution A. Centrifugation was repeated and again the pellet was re-suspended in 500µL of solution B (2 mM EDTA pH 8.0, 10 mM Tris pH 7.5, 400mM NaCl), with addition of 25µL of

10% SDS and 8-12 µL of proteinase K (PK) and incubated the tubes overnight at 37°C. On the following day 500 µL fresh mixture of equal volumes of phenol and chloroform: isoamylalcohol (24:1 ratio) was get ready in the microcentrifuge tube, mixed uniformly and centrifuged for 10 min at 13,000 rpm. Two clear phases were observed; the upper aqueous phase was picked and placed into a new microcentrifuge tube. Again, 400 µL chloroform: isoamylalcohol mixture was added to the microcentrifuge tube and after inverting several times the tube was centrifuged for 10 min at 13,000 rpm. The upper phase was again transferred to a new microcentrifuge tube and DNA was precipitated with 55 µL of sodium acetate (3M, pH 6) and 0.5 mL chilled isopropanol. After inverting several times, the tube was centrifuged for10 min at 13,000 rpm. The supernatant was disposed of and pellet of DNA was washed with ice-cold 70% ethanol. Centrifugation of the tube was again carried out at 13,000 rpm for 10 min. Ethanol was discarded and the DNA pellet was dried in vacuum concentrator 5301 (Eppendorf, Hamburg, Germany) at 30/45 °C. The dried DNA pellet was dissolved in 130-150 µLTris-EDTA (TE) buffer (Sigma-Aldrich, St Louis, MO, USA) and incubated overnight at 37 °C for almost 24 hours. On the next day, the DNA was ready to quantify with the help of spectrophotometer.

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4.4.2 DNA Extraction by Kit

In addition to the phenol-chloroform method, the extraction of genomic DNA from a small amount of blood was also carried out using commercially available kit i.e. the QIAamp DNA Mini Kit (Qiagen, Germany) by following manufacturer's instructions.

The step of protocol were to pipette 200 µL blood and was mixed with an equal amount of lysis buffer B3 and 20 µL proteinase K (10 mg/mL) in an Eppendorf tube of 2mL. To mix the mixture, vortexed the tube for 10 seconds. The tube was incubated in a water bath at 55 °C for 10 min. Next, this mixture was mixed with 200 µL chilled ethanol (96-100%) and transferred to the labeled column assembled in waste collecting tube, followed by centrifugation for 60 seconds at speed of 10,000 rpm. Then the column was kept in another collection tube and washed twice with 500 µL ethanol added wash buffer that followed by centrifugation at 10,000 rpm for 60 seconds. Next empty spin was given at 13,000 rpm for 2.5 min to dry the column. Subsequently, 80-100 µL elution buffer was

added at the center of the column after keeping it in a new tube. Centrifugation was performed at the 13,000 rpm for 3 min.

4.5 DNA Quantification

The DNA was quantified by Nanodrop ND-1000 spectrophotometer (Life Technologies, USA) at an optical density of 260 nm.

4.6 Targeted Re-Sequencing

The probands (n=25), one proband from each family and four additional affected individuals (OB2-6, OB4-8, OB4-9 and OB4-10) underwent targeted re-sequencing. Using a chip-based customized nucleotide probe, targeted re-sequencing was performed to examine the coding regions of most common genes; *LEP, LEPR* and *MC4R* (Paper I). Additionally, the same chip-based customized nucleotide probe was used for capturing genomic DNA of the coding regions of 31 selected genes involved in monogenic forms of obesity (*ADCY3, ALMS1, ARL6, BBS1, BBS2, BBS4, BBS5, BBS7, BBS9, BBS10, BBS12, BDNF, CCDC28B,CEP290, CREBBP, EP300, GNAS, IER3IP1, MKKS, MKS1, MRAP2, NTRK2, PCSK1, PHF6, POMC, SH2B1, SIM1, TMEM67, TRIM32, TTC8* and *VPS13B*) (Paper II).

4.6.1 Target Region Capture and Next Generation Sequencing

The use of a gene testing panel based on a target region capture system was coupled with NGS technology. The method for target region capture has been extensively explained previously [263]. According to the manufacturer's standard cluster generation and sequencing protocols, the final captured DNA libraries were sequenced using the Illumina HiSeq2000 Analyzers as PE 90 bp reads [263], providing an average coverage depth for each sample of at least 100-fold. Only variants having a minimum mean depth of 20x were included.

4.6.1.2 Data Filtering and Analysis

Image analysis, error estimation and base calling were performed using the Illumina pipeline (version 1.3.4) with default parameters. Indexed primers were used to identify the different samples in the primary data. During performing the quality control

(QC) and by using a local dynamic programming algorithm, all unqualified reads were removed. The remaining reads were aligned to the reference human genome UCSChg19 using Burrows-Wheeler Alignment Tool (BWA-0.5.9). Next, SNPs and indels were identified using SOAPsnp software 2.0 and SAMtools v1.4 [264, 265].

4.6.2 Variant Selection in Targeted Re-Sequencing

Variants were considered possibly pathogenic if: 1) they were variants with minor allele frequency (MAF) <0.1 % in publically available databases [266, 267]; 2) coding non-synonymous variants or splice variants located up to 3 nucleotides into the intron/exon boundary; 3) having minimum depths of 20x; and 4) a allelic ratio between 0.4-0.6 for heterozygous mutations (Paper II).

Families were recruited based on the presence of affected children from non-affected parents both from families with known and without known consanguinity. Thus, three different genetic inheritance patterns may likely exist in the recruited families: 1) recessive, 2) compound heterozygotes and 3) heterozygous *de novo* heritance. The latter mode of inheritance was not considered despite that causal heterozygous mutations have been suggested for some of the selected genes [268-271], as an authentication of such potentially causal heterozygous *de novo* mutations is complex and sequencing information from both parents is warranted.

Pathogenicity of the variants was evaluated by using *in silico* annotation online tools especially using combined annotation dependent depletion (CADD) score where a PHRED-scaled CADD score above 10 predicts pathogenicity in top 10 percentile of all variants and a score above 20 predicts the top 1 percentile [272].

During the functional annotation of the most common obesity genes, the identified variants were annotated according to the transcripts 1) LEP: NM_000230; 2) LEPR:

NM_002303.5; and 3) MC4R: NM_005912. For the rare obesity genes, the identified variants were annotated according to the transcripts 1) ALMS1: NM_015120; 2) BBS7: NM_176824; 3) BBS9: NM_014451.3; 4) BBS10: NM_024685; 5) CEP290: NM_025114; 6) CREBBP: NM_001079846; 7) EP300: NM_001077489; 8) PCSK1: NM_000439; 9) POMC: NM_000939; and 10) VPS13B: NM_017890.

4.7 Chip Genotyping for Pakistani Participants

Illumina Infinium Human CoreExomeBeadChip (CoreExomeChip) genotyping was performed in *n*=124 individuals from 25 families of Pakistani origin using Illumina's HiScan system (Illumina, San Diego, CA, USA) at the laboratory facilities of the Novo Nordisk Foundation Center for Basic Metabolic Research at Symbion, Copenhagen, Denmark. The standard pipeline using the Genotyping module (version 1.9.4) of Illumina Genome Studio software (version 2011.1, Illumina) was used for the genotype calling. The pipeline yielded 551839 genetic variations, which entered our QC pipeline. The Illumina final report was converted to plink format using custom scripts and aligned with the positive strand of the GRCh37 reference [273].

Initial QC removed 13762 single nucleotide polymorphisms (SNPs), which had high amounts of missing genotype calls (more than 5%). We did not remove SNPs deviating from Hardy-Weinberg equilibrium as is otherwise usual, since thesevariants may be of interest given the specific mode of data sampling. Furthermore, we had little power to remove such SNPs given our limited sample size. Seven individuals having more than 5% missing genotype calls were removed. In addition, six individuals with extreme levels of negative inbreeding (indicated by an F-statistic of less than -0.05, for common (MAF >2%) or rare (MAF ≤2%), SNPs were removed. However, all of these six individuals were also removed due to high missingness. Four samples, one of which was a genetically identical duplicate of another sample, were removed due to inconsistent gender assignments. We used principal component analysis to detect outliers and verify the genomic proximity of related samples. This analysis was performed both with and without additional samples from 1000 Genomes [267]. No ethnic outliers were detected

and to a large extent, families grouped into differentiable clusters. We also applied Identity-By-Descent analysis to verify the pedigrees. Two samples were removed due to the discrepancy between the genetic information and the pedigree obtained from the family.

4.8 Chip Genotyping for Danish participants

Illumina Infinium Human CoreExomeBeadChip (CoreExomeChip) genotyping was performed in n=298 individuals from 61 families of Danish origin using Illumina's HiScan system (Illumina, San Diego, CA, USA) at the laboratory facilities of the Novo Nordisk Foundation Center for Basic Metabolic Research at Symbion, Copenhagen, Denmark.

The standard pipeline using the Genotyping module (version 1.9.4) of Illumina Genome Studio software (version 2011.1, Illumina) was used for the genotype calling. In these families, participants were excluded in the case of disagreement between questionnaire information on the relationship and actual genotype resemblance. Individuals with a low call rate, those with a mislabelled sex and individuals with a high discordance rate to previously genotyped SNPs were also excluded [274].

4.9 Homozygosity Mapping and Co-Segregation Analysis

Based on genotyping, runs of homozygosity was determined in each Pakistani family using the "homozyg" command in PLINK [275] and haplotypes were constructed per family using MERLIN 1.1.2 [276].

4.10 Relatedness Analysis

Based on genotyping the inbreeding coefficient, which estimates the probability of a random locus in related individuals being identical by descent, was calculated using the "het" command using PLINK [275].

4.11 Statistical Analysis

Comparison of inbreeding coefficients was performed using a student's t-test in R software (version 3.2.3; R Foundation for Statistical Computing, Boston, MA, USA).

4.12 Whole Exome Sequencing

Genomic DNA samples of affected individuals were subjected to whole exome sequencing (WES) and the sequencing was carried out on HiSeq4000 sequencer at BGI Europe Genome Center. According to the manufacturer's standard cluster generation and sequencing protocols, the final captured DNA libraries were sequenced using the Agilent V6 (60M) on HiSeq4000 platform as PE 150 bp reads by lane for library construction, with100x depth per sample.

4.12.1 Selection of Probands

In a cohort of families (n=22), the probands n=17along with additional affected individuals (OB2-5, OB3-4, OB6-5 and OB14-7) were selected for whole exome sequencing (WES).The selected probands from families were OB1-5, OB2-6, OB3-6, OB5-5, OB6-6, OB7-3, OB8-3, OB9-5, OB11-5, OB12-4, OB13-6, OB14-5, OB17-3, OB19-4, OB21-4, OB23-5 and OB24-10. The genomic DNA samples of probands; OB1-5, OB2-6 and OB6-6 with additional affected individuals; OB2-5, OB6-5, were received degradation, therefore, the proper sequencing and analysis were not taken.

4.12.2 Analysis of WES Data

4.12.2.1 Reads: pre-processing, alignment and post-processing

Illumina Universal Adapter read-through contaminations and low-quality (Q<=10) ends of reads were removed from raw sequencing data using cut adapt to reduce the error rate in reads.

Resulting reads were aligned to the human reference genome GRCh38 (localized and unlocalized primary contigs, excluding alternative haplotypes contigs) using BWA [277].

To prevent bias in interpreting variants coverage, overlapping reads from one pair were merged using bamUtil clip Overlap and duplicate reads were removed using SamtoolsRmdup.

4.12.3 Quality Control

Analysis of reads quality was performed for QC with the following steps that were taken into consideration.

- Fast quality control (FastQC) reports are PASS for all samples hence no problems during sequencer run received.
- Sex has been determined using the ratio between reads coverages of sex chromosomes hence, all samples had sex as expected hence no obvious samples swaps.
- >95% of reads are mapped to the human genome hence no significant contamination,
- 78-86% of reads are mapped to the target area of 60Mb SureSelect V6 Human Exome hence, had no enrichment problems.
- >100x average coverage of target, even after pre-processing: filtering out low quality reads and possible read duplicates hence sequencing was done deep enough.
- >97% of target regions are covered with at least 20x and were calculated for each sample, calculated with bedtools and samtools, hence no serious bias in enrichment or PCR stages that would skew the coverage to prefer particular regions.

In conclusion, for most of the samples, the QC was ok.

4.12.4 Mutations Calling and Annotation

Mutations were called using Genome analysis toolkit (GATK) [278] Haplotypecaller and GenotypeGvariant calling files (VCFs). Autosomes were called as diploid, sex chromosomes were called according to sex defined on QC step and mitochondrial DNA mutations were called as ploidy=10 to estimate possible cases of heteroplasmy.

Called mutations were filtered for quality. To prevent errors in calling, mutations covered with <20 reads were removed. To filter false calls in regions of the genome, where read mapping is problematic, mutations with MQ (mapping quality) <30 or MQRankSum <-6 or >1 were removed.

Annotation was done, using:

- Affected gene – dbNSFP, where available, otherwise SnpEff first annotation.
- Gene/protein effect and impact – SnpEff first annotation.
- Prediction about pathogenicity:
    - CADD score – dbNSFP, where available.
    - SIFT score – dbNSFP, where available, otherwise SIFT4G annotation where available.
- Frequencies of mutation:
    - ExAC
    - gnomAD
- Databases info about mutation:
    - ClinVar
- Databases info about affected gene:
    - OMIM
    - DISEASES
    - Whether the affected gene was included in the 250-genes panel.

All annotation, except for information from databases about affected genes, was done in a phenotype-independent manner.

4.12.5 Prioritization of Variants

Mutations were prioritized, from the highest to the lowest rank:

1. Covered with at least 20x reads.
2. In known exome areas in genome and padding.
3. Mutation's frequency is less than 1% or less than 3% or less than 5%.
4. Homozygotes.

5. The variant is present in a homozygosity region in patient and not in parents.
6. Cross check with available healthy patients:
    a. Not homozygous in any known healthy adult from available data.
7. Not in known highly-variable areas (for example; HLA-region – several haplotypes with a significant difference in protein coding sequences exist, but they are not pathogenic/benign, they are just haplotypes).
8. Not in known repeat areas (for example; simple repeats – AGAGAGAGAG...AG for 10-100 and more nucleotides, because many processing problems occur in such areas, creating non-existing "mutations" to be called or real mutations to be wrongly annotated) – this criterion is still weakly controlled and a lot of insertions and deletions with length divisible by 3 are still observed in output.
9. Mutation makes a high impact on protein sequence according to SnpEff.
10. SIFT and CADD predict mutation as pathogenic or no prediction or contradicting results.
11. Mutation is described in clinvar as pathogenic or not described in clinvar.
12. *Gene* is described in OMIM or DISEASES as causative for any disorder with high confidence or low confidence or no description or contradicting description.
13. *Gene* is one of the genes that we know from the literature are associated with obesity.

Hence, the candidate variants were selected based on phenotype-genotype correlation, gene function, expression and nature of variant. These variants were further validated by segregation analysis to check the mode of heritance in all participated members of the family using chip-based genotyping data.

Table 4. 1 Clinical characteristics of the families involved in the study.

Family ID	Subject ID	Gender	Age at enrolment (years)	Age at obesity onset (years)	Height (cm)	Weight (kg)	BMI (kg/m²)	BMI SDS	Waist circumference (cm)	Family history of obesity	Obesity-related co-morbidities	Family-related disorders
OB1	OB1-4	Male	17.74	<5	164.5	96.1	35.5	3.02	N/A	No	Hyperphagia, hypertension	Hypertension
	OB1-5	Female	9.93	<5	121.9	98.0	66.0	4.78	N/A		Hyperphagia	
OB2	OB2-5	Female	23.14	<5	154.9	97.0	40.4	3.67	111.7	No	Hyperphagia, hypertension	Hypertension
	OB2-6	Male	9.50	<5	111.8	40.0	32.0	3.92	81.2		Hyperphagia	
OB3	OB3-4	Male	30.10	<5	173.7	99.3	32.9	1.03	106.6	Yes	Hyperphagia, hypertension	None
	OB3-5	Male	12.26	<5	143.2	78.1	38.1	3.61	96.5		Hyperphagia	
	OB3-6	Female	10.02	<5	137.1	81.9	43.6	4.16	101.6			
OB4	OB4-8	Female	8.29	40 days	140.2	75.0	38.2	4.40	111.7	No	Hyperphagia	None
	OB4-9	Male	10.39	40 days	140.2	80.2	40.8	4.18	111.7			
	OB4-10	Male	8.03	40 days	115.8	55.0	41.0	5.39	99.0			
OB5	OB5-5	Female	12.28	<3	137.1	75.2	40.0	3.63	91.4	Yes	Hyperphagia, hypertension	Diabetes, hypertension, asthma, heart disease
OB6	OB6-5	Female	17.03	<3	152.4	67.1	28.9	1.93	86.3	Yes	None	None
	OB6-6	Male	14.94	<5	164.5	95.3	35.2	3.12	106.6		Hyperphagia	
OB7	OB7-3	Male	15.12	<5	162.5	77.0	29.2	2.36	101.6	Yes	Hypertension, gynaecomastia	Diabetes, hypertension, heart disease,

												nephropathy
OB8	OB8-3	Female	6.70	<5	101.6	32.0	31.0	4.48	78.7	Yes	Dyslipidaemia	Diabetes, hypertension
OB9	OB9-5	Male	23.36	<5	177.8	132.0	41.8	3.11	111.7	Yes	Hyperphagia, hypertension	Diabetes, hypertension, asthma
OB10	OB10-4	Female	22.77	~5	162.5	81.0	30.7	2.10	111.7	No	Polycystic ovary, hypertension	Cardiovascular disease, hypertension
OB11	OB11-5	Male	16.95	<5	172.7	108.0	36.2	3.12	111.7	Yes	Hyperphagia	Diabetes, hypertension
OB12	OB12-3	Male	29.92	~5	170.2	115.0	39.7	2.03	114.3	Yes	Diabetes, hypertension	Diabetes, hypertension, Osteoporosis, arthritis
	OB12-4	Male	27.19		162.5	104.0	39.4	2.32	106.6		Gout, ulcer, epilepsy, hypertension	
OB13	OB13-6	Male	13.21	<5	154.9	70.1	29.2	2.68	99.0	Yes	Hypertension, nephropathy, chronic fatigue	Asthma
OB14	OB14-5	Female	13.69	<5	157.5	80.3	32.4	2.78	109.2	Yes	Hyperphagia	Diabetes, hypertension
	OB14-6	Male	26.08	~5	177.8	115.0	36.4	2.07	124.4		Hypertension	
	OB14-7	Male	19.28		172.7	105.0	35.2	2.91	119.3		Hypertension	
OB15	OB15-5	Male	16.29	~2	152.4	76.0	32.7	2.73	104.1	No	Developmental delay, CVD	None
OB16	OB16-4	Male	13.11	~2	162.5	66.0	25.0	2.02	86.3	Yes	Hyperphagia	Diabetes
OB17	OB17-3	Female	0.66	3 months	76.2	20.0	-	7.37	58.4	No	Hyperphagia	None

OB18	OB18-3	Female	23.33	<5	162.5	89.0	33.7	2.64	109.2	Yes	Hypertension	Diabetes, hypertension, heart disease, nephropathy
OB19	OB19-4	Male	15.32	<5	139.7	92.1	47.2	3.93	106.6	No	Hyperphagia	Hypertension
OB20	OB20-4	Male	19.03	~5	162.5	82.0	31.1	2.26	104.1	No	Hyperphagia	None
OB21	OB21-4	Male	19.95	<5	142.2	85.0	42.0	3.66	114.3	Yes	Heart problem, hyperphagia	None
OB22	OB22-3	Male	20.19	~5	177.8	122.0	38.6	3.22	119 3	No	Hypertension	None
OB23	OB23-5	Male	30.11	~5	177.8	115.0	36.4	1.57	124.4	Yes	Hypertension, varicose vein problem	Hypertension, diabetes, nephropathy, metabolic syndrome
OB24	OB24-10	Male	11.18	1.5	137.1	53.0	28.2	2.98	96.5	No	Hyperphagia, fatigue, continuous head movement, weak eye sight	Nephropathy, asthma, cardiovascular disease, arthritis

5. Results

5.1 Application of Targeted Re-Sequencing

Twenty-five probands from 25 familial cases of obesity were subjected to targeted re-sequencing. During the analysis of targeted re-sequencing data, we identified two causative and damaging mutations in the *LEPR* gene in two probands which were novel. The two novel identified mutations are given below;

5.1.1 Paper I: Identification of novel *LEPR* mutations in consanguineous Pakistani families with morbid childhood Obesity

The targeted re-sequencing data was combined with chip-based genotyping, enabling the identification of rare and potentially novel causal variants co-segregating with obesity. When analyzing the sequencing data for *LEP* and *MC4R,* no coding variants were identified. However, in *LEPR,* eight coding variants were identified, of which two were synonymous, five were missense and one was a frame-shift mutation. Identified variants were classified as potentially damaging if they were: 1) non-synonymous; 2) homozygous due to the recessive mode of inheritance and 3) are in the general population, with a MAF below 0.1% [266, 267]. Two identified variants fulfilled these criteria; one was a frame-shift variant (p.Ser1090Trpfs*6) and the other was a missense mutation (p.Pro892Arg). The pathogenicity of the missense variant was assessed by assigning CADD score [279].

5.1.1.1 Frameshift mutation (Ser1090Trpfs*6)

In family OB4 (Figure 5.1), the novel homozygous frameshift mutation c.3260AG (p.Ser1090Trpfs*6) which truncates the LEPR protein, was identified in exon 20 of the *LEPR*. This frameshift was the result of a deletion of AG at nucleotide position 66102459 to 66102461. This variant is overlapping within the homozygosity region on chromosome 1p31.1 from position 55397406 to 75241971. This homozygous region was only shared among affected family members.

Affected members in family OB4 (Figure 5.2) originated from the Rajanpur district of the Punjab province in Pakistan, had a very homogenous phenotype comprising of hyperphagia and rapid weight gain with subsequent morbid obesity. This severe form of phenotype was evident in several affected family members, including two females OB4-7 and OB4-8 aged 12 and 8 years, respectively, as well as two male first cousins OB4-9 and OB4-10 aged 10 and 8 years, respectively. At the time of recruitment of the family, the proband OB4-7, aged 12 years, weighed 145.0 kg with a BMI SDS of 4.48 and a BMI of 62.8 kg/m^2 (Table 5.1). On follow-up, at the age of 14 years with the pubertal stage of Tanner IV [280], the proband developed diabetes with a C-peptide level of 0.92 nmol/L yet without menarche. Moreover, the sibling (OB4-8) and two other affected members (OB4-9, OB4-10) were in the pre-pubertal stage of Tanner I [280, 281]. The severity of the mutation was evaluated based on gender, as a gender-specific effect of LEPR has previously been suggested [282]. However, we did not find any influence of the gender of the carriers on the effect of the mutation (Table 5.2).

5.1.1.2 Missense mutation (p.Pro892Arg)

The novel missense mutation c.2675C>G (p.Pro892Arg) located in exon 20 in *LEPR*, was identified in proband OB25-4 of family OB25 (Figure 5.3). This variant is located in position 66101875 within a homozygous region from position 59653630 to 91206170 on chromosome 1p31. Born of consanguineous marriage (Figure 5.4), the proband presented with a normal birth weight of 3.0 kg but after two months she rapidly gained weight and attained the weight of 18.0 kg at the age of one year, which corresponds to a BMI SDS of 6.49 (Table 5.1). In addition, the proband suffered from diabetes and displayed developmental delay. The proband was also in the pubertal stage of Tanner I [280]. When annotated using online bioinformatics tools, p.Pro892Arg was predicted to be damaging and had a CADD score of 28 [279], strongly suggesting the mutation to be deleterious.

5.1.2 Paper II: Mutation screen of 31 selected genes involved in monogenic forms of obesity in families with Pakistani origin

We investigated the level of relatedness in families with and without known consanguinity using the inbreeding coefficient. This estimates the probability of a random locus in related individuals being identical by descent. We found that families with known consanguinity had a mean inbreeding coefficient of 5.6% (SD: 4.5) in contrast to families without consanguinity having a mean inbreeding coefficient of 3.2% (SD: 3.0) ($p=0.003$). However, when comparing non-consanguine Pakistani families to Danish out bred families having an inbreeding coefficient of -1.02% (SD: 0.648), the inbreeding coefficient of non-consanguine Pakistani families was still significantly higher ($p=4*10^{-13}$). Thus, recessive inheritance-patterns are likely to exist in Pakistani families with both known and unknown consanguinity.

Thirty-one genes were selected based on their known causal involvement in childhood obesity and among the probands, we identified a total of 31variants located in *ALMS1*, *BBS7*, *BBS9*, *BBS10*, *CREBPB*, *EP300*, *PCSK1*, *POMC* and *VSP13B* fulfilling the criteria for being possibly pathogenic (28 missense and three nonsense) (Table 5.3).

Due to a large number of consanguineous families and the high level of relatedness in families without known consanguinity, we searched for homozygous recessive variants. Yet, no homozygous pathogenic carriers were found.

Subsequently we investigated the presence of probands carrying two heterozygous mutations within the same gene and found four potentially compound heterozygous probands: 1) OB1-5 carrying the p.K1992E and the p.L2520S in *ALMS1*; 2) OB2-5 carrying the p.S872L and p.K140R in *CEP290*; 3) OB8-3 carrying the p.T1512I and p.G1890X also in *CEP290*; and 4) OB15-5 carrying the p.R75X and p.R481X in *BBS9* (Table 5.4).

OB1-5 (Figure 5.5) carrying two rare missense variants in *ALMS1*, display hyperphagia in addition to severe early-onset obesity. Sequencing of the mother (OB1-2) and the affected brother (OB1-4) revealed that the variantsfound in the proband (OB1-5) was not carried by the affected brother, nor was the mother a carrier of any of the two *ALMS1*

variants found in the proband. In addition, the affected individuals in OB1 did not present withthe phenotypic characteristicsof Alstrom syndrome such as retinal degeneration, hearing loss, diabetes mellitus, dilated cardiomyopathy, urological dysfunction, pulmonary, hepatic and renal failure. Thus, neither co-segregation nor phenotypic presentation suggests that the two variants found in OB1-5 in *ALMS1* are causal for childhood obesity in OB1.

The probands in family OB2 (Figure 5.6) and OB8 (Figure 5.7) are each having two mutations in *CEP290*. OB2-5carried the p.K140R and p.S872L missense mutations and the proband in OB8 (OB8-3) is carrying the p.T1512I missense mutation and the p.G1890X nonsense mutation. The functional prediction of the p.K140R variant based on the CADD score indicate only a minor impaired functionality (CADD score:14.6, Table 5.4).This lack of presumed functionality is supported by the lack of clinical characteristic in OB2 of patients with *CEP290* mutations such as retinal degeneration, hypogonadism, polydactyly, renal dysfunction and MR [283]. Subsequent sequencing of *CEP290* in the parents (OB2-1 and OB2-2) and brother (OB2-6) of OB2-5 revealed that both variants present in the proband were inherited from the father but not from the mother in whom none of the two variants were present (Table 5.4).

The p.G1890X variant found in OB8-3 has previously been found to cause Joubert syndrome-related disorders (JBTS) in a homozygous manner in a Turkish family [284]. The JBTS affects the central nervous system (brain and spinal cord), retina and kidney and it is inherited in an autosomal recessive manner. Moreover, a high CADD scorewere found for both the missense and nonsense variants (26.5 and 36, respectively) supporting a highly pathogenic nature of these two mutations. Yet, sequencing of *CEP290* in the parents (OB8-1 and OB8-2) of proband OB8-3 showed that the variants found in OB8-3, both were inherited from the mother. This lack of co-segregation was also supported by the proband (OB8-3) not displaying any of the symptoms characteristic of the syndromes related to *CEP290* mutations such as JBTS, thus, we do not believe OB8-3 is suffering from *CEP290* related obesity.

The proband in OB15 (Figure 5.8) is carrying the two nonsense mutations p.R75X and p.R481X both very likely highly deleterious mutations. Patients with BBS caused by

homozygous or compound heterozygous mutations in *BBS9* are characterized by obesity, polydactyly, renal anomalies, retinopathy and mental retardation. OB15-5 presents with a large number of the primary BBS phenotypes including hypogonadism, developmental delay with learning difficulties, speech and vision- impairment in addition to severe childhood obesity (Figure 5.9). Moreover, sequencing of *BBS9* in the parents (OB15-1 and OB15-2) of OB15-5revealed that p.R75X was inherited from the mother and p.R481X was inherited from the father. Therefore, the proband OB15-5 is likely a patient with BBS due to compound heterozygous mutations in *BBS9*.

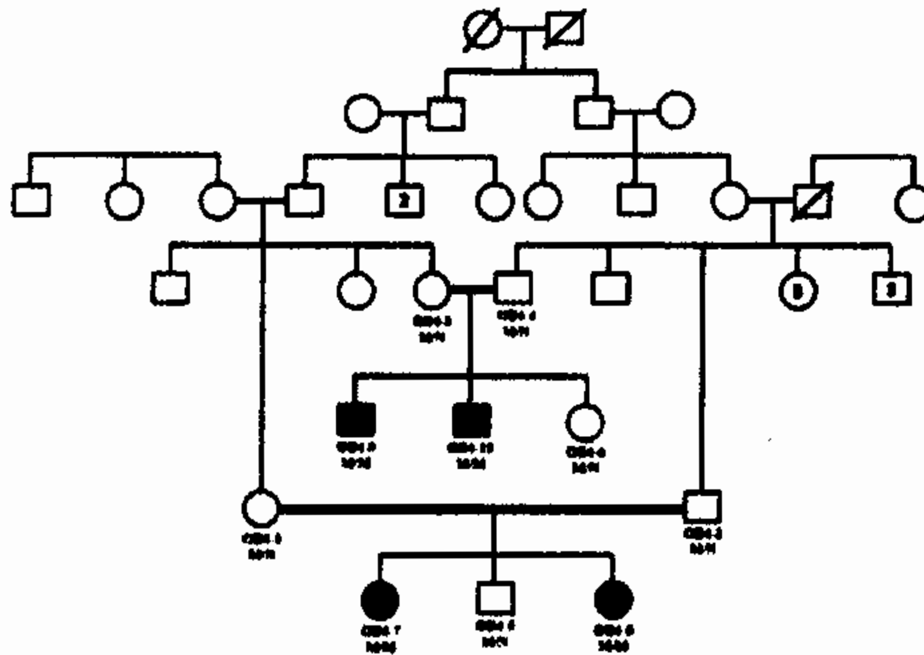

Figure 5. 1 Pedigree of Family OB4 identified with a frameshift mutation c.3260AG (p.Ser1090Trpfs*6) in LEPR. Circles and squares represent female and male family members, respectively. Filled symbols indicate affected members. Symbols with slash indicate deceased family members and double lines between symbols show cousin marriages. Numbers within symbols indicate the number of siblings of the same gender. M and N represent the mutated and the normal (wild type) allele, respectively.

Figure 5. 2 Pictures of affected individuals; (a) OB4-7, (b) OB4-8, (c) OB4-9 and (d) OB4-10 of Family OB4 identified with frameshift mutation (p.Ser1090Trpfs*6).

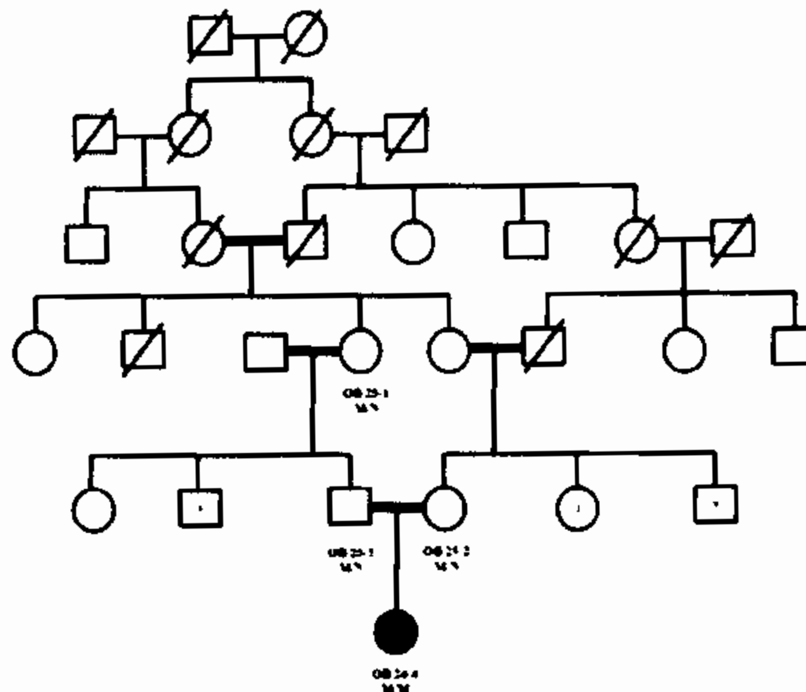

Figure 5. 3 Pedigree of Family OB25 identified with a missense mutation c.2675C>G (p.Pro892Arg) in *LEPR*. Circles and squares represent female and male family members, respectively. Filled symbols indicate affected members. Symbols with slashed indicate deceased family members and double lines between symbols show cousin marriages. Numbers within symbols indicate the number of siblings of the same gender. M and N represent the mutated and the normal (wild type) allele, respectively.

Figure 5. 4 Picture of affected individual OB25-4 of Family OB25 identified with missense mutation (p.Pro892Arg).

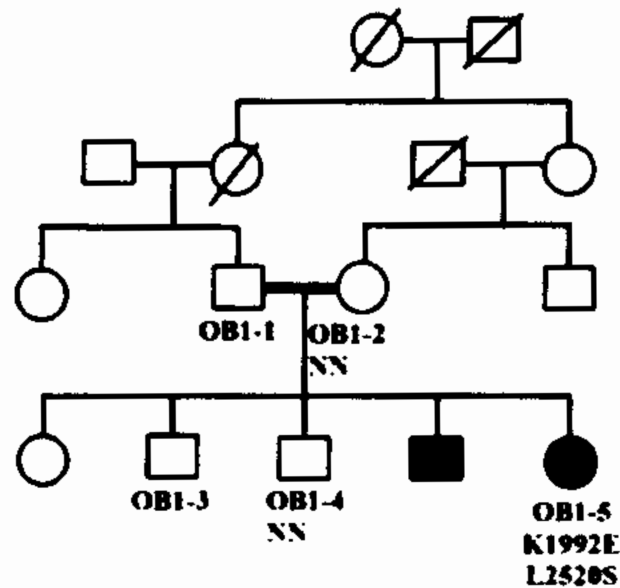

Figure 5. 5 Pedigree of Family OB1 carrying two rare missense variants; p.K1992E and the p.L2520S in *ALMS1.* Circles and squares represent female and male family members, respectively. Filled symbols indicate affected members with obesity. Slashed symbols indicate deceased family members and double lines between symbols show cousin marriage.

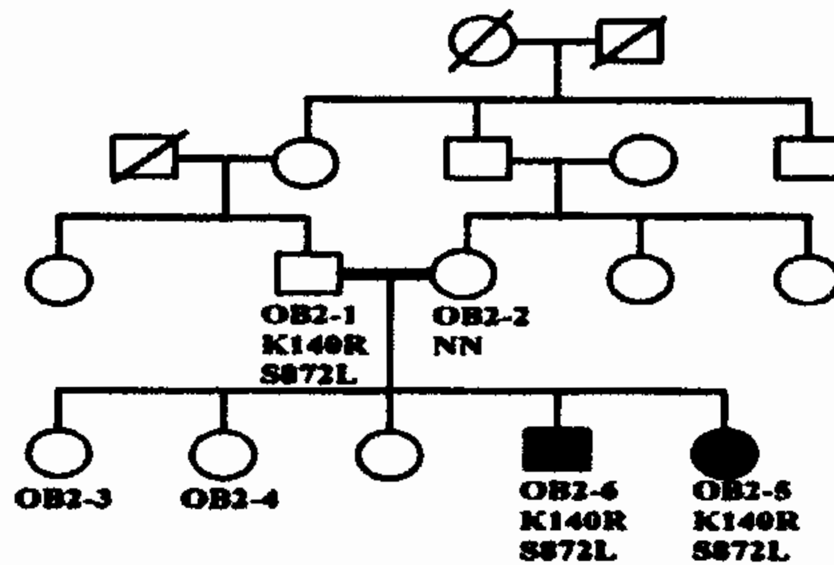

Figure 5. 6 Pedigree of Family OB2, both affected individuals carrying the p.S872L and the p.K140R missense mutations in *CEP290*. Circles and squares represent female and male family members, respectively. Filled symbols indicate affected members with obesity. Slashed symbols indicate deceased family members and double lines between symbols show cousin marriage.

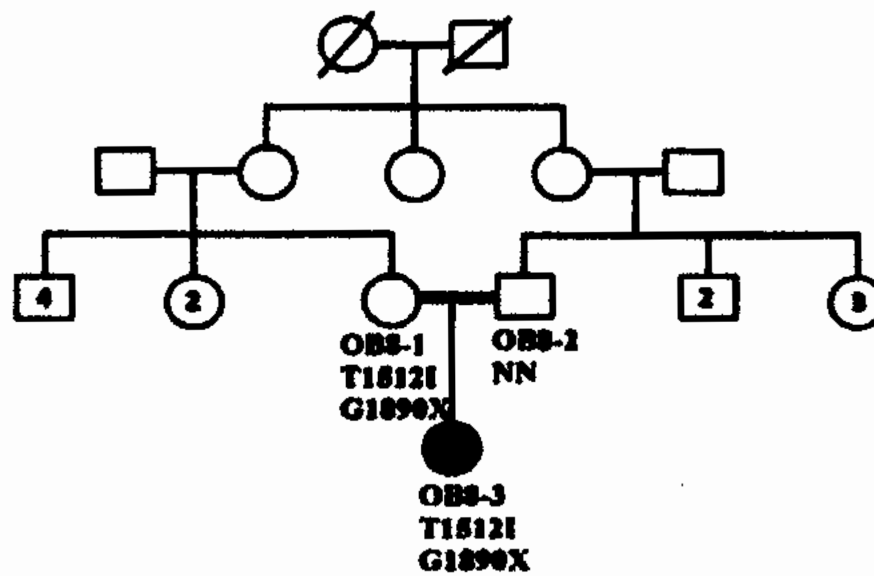

Figure 5. 7 Pedigree of Family OB8 carrying the p.T1512I and p.G1890X missense mutations in *CEP290.* Circles and squares represent female and male family members, respectively. Filled symbols indicate affected member. Symbols with slashed indicate deceased family members and double lines between symbols show cousin marriages. Numbers within symbols indicate the number of siblings of the same gender. M and N represent the mutated and the normal (wild type) allele, respectively.

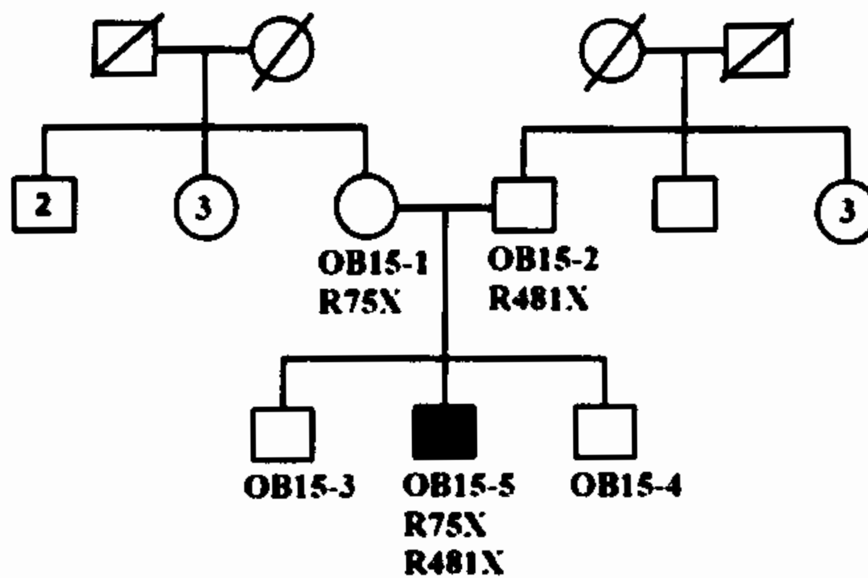

Figure 5. 8 Pedigree of Family OB15 identified with compound heterozygote mutations; p.R75X and p.R481X in *BBS*. Circles and squares represent female and male family members, respectively. Filled symbols indicate affected member with obesity. Symbols with slashed indicate deceased family members and double lines between symbols show cousin marriages. Numbers within symbols indicate the number of siblings of the same gender. M and N represent the mutated and the normal (wild type) allele, respectively.

Figure 5. 9 (a) Picture of an affected individual OB25 of Family OB25 identified with compound heterozygote mutation (p.R75X and p.R481X), (b) feet with no additional finger and (c) hands with no additional finger.

Table 5. 1 Clinical characteristics of probands with homozygous LEPR mutations.

	Proband OB4-7	**Proband OB25-4**
Family ID	OB4	OB25
Gender	Female	Female
Age at enrolment (years)	12.18	1.03
Age at obesity onset	40 days	2months
Height (cm)	152.0	76.2
Weight (kg)	145.0	18.0
BMI (kg/m^2)	62.8	31.0
BMI SDS	4.49	6.49
Waist circumference (cm)	137.0	73.6
Family history of obesity	No	No
Related comorbidities	Diabetes	Diabetes, dyslipidemia, hepatic and renal function disorder
Mutation type	Frameshift	Missense

Table 5. 2 Gender-stratified analysis of BMI, weight and waist of homozygous carriers of the *LEPR* p.Ser1090Trpfs*6 mutation in family OB4.

	BMI (kg/m^2)	zBMI	Weight (kg)	Waist (cm)
Female carriers				
OB4-7	62.8	4.49	145.0	137.0
OB4-8	38.2	4.40	75.0	111.7
Mean (SD)	50.5 (17.4)	4.44 (0.062)	110.0 (49.5)	124.4 (17.9)
Male carriers				
OB4-9	40.8	4.18	80.2	111.7
OB4-10	41.0	5.39	55.0	99.0
Mean (SD)	40.9 (0.15)	4.79 (0.85)	67.6 (17.8)	105.4 (8.98)
P-value*	0.6	0.7	0.4	0.3

*Evaluated using a t-test.

Table 5. 3 List of identified 35 rare variants in heterozygous condition for being possibly pathogenic variants in 31 probands in 23 Pakistani families. The highlighted variants in bold are those which may contribute as possible disease causing compound heterozygous mutations.

Sr. No.	Variant	Type of mutation	rs Number	MAF (gnomAD)	CADD score	Number of proband carriers
Gene (transcript)	*ALMS1*(NM_015120)					
1	p.E3929G	Missense	rs771370411	0.00002031	28.7	OB9-5
2	p.T707A	Missense	rs571389435	0.0002607	9.91	OB17-3
3	**p.K1992E**	**Missense**	**.**	**0**	**24.3**	**OB1-5**
4	p.S2415G	Missense	.	0.000008128	26.1	OB21-4
5	p.T1088R	Missense	rs556855697	0.0003710	19.2	OB3-6
6	p.S1896L	Missense	rs757128530	0.000008140	22.4	OB10-4
7	p.R2344Q	Missense	rs759366425	0.00002443	12.97	OB6-5
8	**p.L2520S (chr2:73682310)**	**Missense**	**rs776090716**	**0.00001807**	**27.7**	**OB1-5**
Gene (transcript)	*BBS2*(NM_031885)					
9	p.S70N	Missense	rs4784677	0.9944 (0.0056)	12.57	OB22-3
Gene (transcript)	*BBS4*(NM_001252678)					
10	c.A720T	Missense	rs147202164	0.001541	10.27	OB3-6
Gene (transcript)	*BBS7*(NM_176824)					
11	p.E348G	Missense	rs575431546	0.00008955	25.8	OB3-6
Gene (transcript)	*BBS9*(NM_014451.3)					

12	p.T12A	Missense	rs4498440	0.004257	11.32	OB11-5
13	p.C270S	Missense	rs763742314	0.00004879	20.6	OB18-3
14	p.E713V	Missense	rs61764068	0.0007593	28	OB10-4
15	p.A672T	Missense	rs751173437	0.00001220	24	OB9-5
16	**p.R75X**	**Nonsense**	**rs775081992**	**0.00002438**	**37**	**OB15-5**
17	**p.R481X**	**Nonsense**	**rs748601675**	**0.000004075**	**40**	**OB15-5**
Gene (transcript)	*BBS10* (NM_024685)					
18	p.R422Q	Missense	rs138961848	0.0003574	12.57	OB9-5
Gene (transcript)	*CEP290*(NM_025114)					
19	p.R20H	Missense	.	0	23.4	OB21-4
20	p.E905D	Missense	.	0	24.1	OB18-3
21	**p.S872L**	**Missense**	**rs373341530**	**0.0002599**	**24.7**	**OB2-6** **OB2-5**
22	**p.T1512I**	**Missense**	**.**	**0**	**26.5**	**OB8-3**
23	**p.K140R**	**Missense**	**rs750776051**	**0.000008128**	**14.6**	**OB2-6** **OB2-5**
24	**p.G1890X**	**Nonsense**	**rs137852832**	**0.00009589**	**36**	**OB8-3**
Gene (transcript)	*CREBBP*(NM_001079846)					
25	p.T879A	Missense	NA	0	8.825	OB12-4
Gene (transcript)	*EP300*(NM_001077489)					
26	p.N248S(only found in	missense	rs762095513	0.00004874	8.69	OB17-3

	NM_00142 9)					
27	p.R321P	Missense	.	0	35	OB17-3
28	p.P414R	Missense	rs559714658	0.0009946	9.372	OB9-5
29	p.P79S	Missense	.	0	21.6	OB2-6
30	p.S268L	Missense	rs566198679	0.00001639	15.85	OB24-10
31	p.R155C	Missense	rs755050269	0.00008312	21.2	OB20-4
Gene (transcript)	*PCSK1*(NM_000439)					
32	p.N127I	Missense	rs574780528	0.0002275	21.3	OB2-6 OB2-5
33	p.T366M	Missense	rs369762633	0.00004072	23	OB5-5
Gene (transcript)	*POMC*(NM_000939)					
34	p.E30D	Missense	rs758258712	0.00001219	24	OB7-3
Gene (transcript)	*VPS13B*(NM_017890)					
35	p.T962M		rs547184348	0.0002167	27.1	OB13-6

Table 5. 4 Probands carrying two heterozygous variants within the same gene.

Family ID	Proband ID	Gender	Gene	Identified variants	Primary phenotype of the patient	Secondary phenotypes of patients	Phenotypic characteristic of patients with syndromes related to investigated gene	Haplotype co-segregation with phenotype
OB1	OB1-5	F	*ALMS1*	p.K1992E p.L2520S	Hyperphagia	NA	ALMS: retinal degeneration, hearing loss, diabetes mellitus, dilated cardiomyopathy, urological dysfunction, pulmonary, hepatic and renal failure	No
OB2	OB2-5	F	*CEP290*	p.S872L p.K140R	Hyperphagia, hypertension	NA	Joubert syndrome: brain abnormalities, molar tooth sign, hypotonia, ataxia, hyperpneaorapnea, ocular motor apraxia	No genotypes available
OB8	OB8-3	F		p.T1512I p.G1890X	Dyslipidemia	NA		Yes
OB15	OB15-5	M	*BBS9*	p.R75X p.R481X	Hypogonadism, Mental retardation, Obesity, Vision impairment.	Speech impairment, hypertension.		Yes

5.2 Application of Whole Exome Sequencing

With the application of whole exome sequencing (WES) technique, the families; OB3, OB5, OB7, OB14, OB21 and OB23 with known consanguineous background and solid findings are included in this thesis. Exome data was combined with chip-based genotyping, enabling the identification of rare and potentially causal variants co-segregating with disease.

5.2.1 Paper III: Whole exome sequencing revealed homozygous variant in *QSOX2* gene with known consanguineous Pakistani origin

In family OB5 (Figure 5.10), the proband OB5-5 was the product of consanguineous marriage with two lean siblings and parents and these five family members (OB5-1, OB5-2, OB5-3, OB5-4 and OB5-5) were sampled for genetic analysis. At the time of recruitment of the family, the proband OB5-5 of aged 12 years, weighed 75.2 kg with a BMI SDS 3.63 and a BMI of 40.0 kg/m^2 and other anthropometric features about the proband are presented in Table 4.1.

During the analysis of exome data, the novel homozygous frameshift mutation c.94_104delCGGCTGCCGCG; p.Arg32fs which, cause premature truncation of the QSOX2 (quiescin Q6 sulfhydryl oxidase 2) protein, revealed in exon 1 of *QSOX2* gene that mapped on chromosome 9q34.3. Parents and the normal siblings are heterozygotes for this mutation and the co-segregation analysis showed that the variant found in OB5-5 is located inside the homozygosity region, which is compatible with autosomal recessive heritance. Moreover, mutation c.94_104delCGGCTGCCGCG; p.Arg32fs in *QSOX2* was not found in any of the available public databases; 1000 Genomes, genome aggregation database (gnomAD) and exome aggregation consortium (ExAC) hence, considered the mutation as a novel.

Previously, there is no finding of *QSOX2* gene in obesity. Therefore, experimental studies need to be performed to explore the pathway of *QSOX2* as a candidate gene, involved in the development of obesity in humans.

5.2.2 Paper IV: Whole exome sequencing revealed novel homozygous variant in *LTBP3* gene underlying obesity in a consanguineous Pakistani family

In family OB21 (Figure 5.11), the proband OB21-4 was the product of consanguineous marriage along with lean parents (OB21-1, OB21-2) and sibling (OB21-3). At the time of recruitment and examination, the proband OB21-5 of aged 20 years, weighed 85.0 kg with a BMI SDS 3.66 and BMI of 42.0 kg/m^2. The other anthropometric features about the proband are listed in Table 4.1.

In this family, in relation to phenotypes with the gene function, a novel homozygous stop gain mutation (c.2277C>A; p.Cys759*) resulted in the truncation of LTBP3 (latent transforming growth factor beta binding protein 3) protein and was found in exon 16 of *LTBP3* gene mapped on chromosome 11. Segregation of the variant c.2277C>A in *LTBP3* among the sampled four lean individuals (OB21-1, OB21-2, OB21-3, OB21-4) and an affected individual (OB21-5) in the pedigree revealed that the proband was homozygous for the variant c.2277C>A while the parents and the lean siblings were heterozygote for this mutation, segregating with obesity. Mutation effect prediction tool such as CADD score predicted the variant as disease causing and damaging. Additionally, the frequency of mutation (c.2277C>A; p.Cys759*) in *LTBP3* is not found in any of the publically available databases, hence, we are going to report for the first time.

The current findings of the carrier with stop gain variant in *LTBP3* displayed the phenotypes of acromicric dysplasia, dental anomalies and geleophysic dwarfism in addition to obesity (Figure 5.12). Hence the current findings are consistent to the phenotypes previously known characteristic to *LTBP3* mutations such as acromicric dysplasia, dental anomalies and geleophysic dwarfism [285, 286]. However, in current study the proband also displayed obesity which has not been reported before besides other previously known phenotypes characteristics to *LTBP* gene. Therefore, it would be important to discover the underlying pathway directly or indirectly involved in causing

obesity within *LTBP3*. Thus, experimental studies need to be performed for the discovery of *LTBP3* as a gene involved in causing obesity.

5.2.3 Paper V: Whole exome sequencing revealed homozygous variant in *KISS1R* gene underlying obesity in a consanguineous Pakistani family

In family OB23 (Figure 5.13), the proband OB23-5 was also the product of consanguineous marriage with lean parents (OB23-1, OB23-2) and siblings (OB23-3, OB23-4). In this family, the homozygous frameshift mutation c.525_532delGCCGGTGC (p.Pro176fs) which truncated the kisspeptin receptor (KISS1R) protein and identified at position 919889 in exon 4 of the *KISS1R* gene mapped on a location to19p13.3. Based on genotyping of family members showed that the lean parents and two siblings (OB23-1, OB23-2, OB23-3, OB23-4, respectively) were heterozygotes for this mutation, while the variant found in an affected individual (OB23-5) was located inside the homozygosity region, which is compatible with autosomal recessive pattern of inheritance.

At the time of recruitment and examination, the proband OB23-5 of aged 30 years, weighed 115.0 kg with a BMI SDS 1.57 and BMI of 36.4 kg/m^2. The other anthropometric features about the proband are listed in Table 4.1. Additionally, the affected individual had no secondary sex characteristics i.e. no mustaches and no beard with hypogonadism (Figure 5.14).

Humans and mice with mutations in *KISS1* (encode neuropeptide kisspeptin) and its receptor, *KISS1R* genes show impaired puberty, hypogonadism and infertility [287-289].The current findings of *KISS1R* frameshift mutation responsible for causing hypogonadism in addition to obesity which is consistent with previously known phenotypes of hypogonadism in humans and obesity in female mice. *KISS1R* frameshift mutations are known to cause hypogonadotropic hypogonadism and precocious puberty [290, 291]. Besides hypogonadism, more importantly, we have identified the male patient with obesity for the first time in humans.

Thus, the current identification reflects the involvement of kisspeptin signalling pathways as a novel regulator of obesity and metabolism besides governing reproduction.

5.2.4 Family OB3

Family OB3 (Figure 5.15) originated from Charsada district of KPK province of Pakistan. On the basis of the information given by the family, pedigree was drawn. In total, individuals including three affected (OB3-4, OB3-5, OB3-6) and three unaffected (OB3-1, OB3-2, OB3-3) were sampled for genetic analysis. The proband OB3-4 being a born of a consanguineous marriage, with an additional affected individual OB3-6 was subjected to the sequencing for exome. At the time of recruitment of the family, the proband OB3-6, aged ten years, weighed 81.9 kg with a BMI SDS 4.16 and BMI of 43.6 kg/m^2. The other anthropometric features about the affected individuals in the family are presented in Table 4.1.

Family with having the consanguinity and prediction of the autosomal recessive form of inheritance within the pedigree, annotated variants were filtered for homozygosity in exome data with a MAF <0.01 which has to be shared by the affected individuals only. The selected variants in homozygous condition were searched on the basis of matching of phenotypes with the gene function to find the association of the obesity phenotype with obesity or metabolic diseases genes.

However, no coding pathogenic variant in homozygous condition was identified in any relevant obesity or metabolic gene to which the phenotypes of the affected individuals were matched. Hence, no causal gene for obesity was identified with the application of exome sequencing in the current case. This result suggests us the WGS which, may lead to the identification of pathogenic variant in deep intronic or variant in the promoter region, directly or indirectly show an association with obesity phenotype.

5.2.5 Family OB7

Family OB7 (Figure 5.16) originated from Rawalpindi district of Punjab province of Pakistan. On the basis of given information, pedigree was drawn. In total, individuals including one affected (OB7-3) and two unaffected (OB7-1, OB7-2) were sampled for genetic investigation.

In family OB7, although the proband (OB7-3) is not the product of consanguineous marriage with lean parents however, his parents belong to the same caste i.e. Syed and

the family has trend to have marriages within the caste only. Hence, the case could be considered as with a consanguineous background. At the time of recruitment of the family, the proband OB7-3, aged of 15 years, weighed 77.0 kg with a BMI SDS 2.36 and BMI of 29.2 kg/m^2 and his weight was progressively increasing with the passage of time inspite of with regular exercise and diet control. The other anthropometric features about the proband are presented in Table 4.1.

On the basis of the prediction of the autosomal recessive form of disease from the pedigree and consideration of consanguinity, exome data was filtered for homozygous variants. All the suspected coding variants in homozygous condition were searched in available databases of human genetic variations. On the basis of relationship of phenotypes with the gene function, no pathogenic variant was found in the homozygous region in any related obesity or other metabolic genes. The identified homozygous variants neither matched with the observed phenotypes displayed by the proband nor segregate within the family members.

Hence, no causal gene for obesity was found in the coding regions with the application of exome sequencing. The pathogenic variant might be present in the noncoding region which was not captured by exome sequencing. This result suggests us the whole genome sequencing which might lead to the identification of deep pathogenic intronic variant or pathogenic variant in the promoter region associated with disease phenotype which was not possible to capture by the exome sequencing

5.2.6 Family OB14

Family OB14 (Figure 5.17) originated from Attock district of KPK province of Pakistan. On the basis of information, pedigree was drawn. In total, individuals including three affected (OB14-5, OB14-6, OB14-7) and four unaffected (OB14-1, OB14-2, OB14-3, OB14-4) were sampled for genetic inference.

In this familial case, with the known consanguineous background, the proband OB14-5 with an additional affected individual, OB14- 7 was subjected to the sequencing for WES. At the time of recruitment of the family, the proband OB14-5, aged about 14 years old, weighed 80.3 kg with a BMI SDS 2.78 and BMI of 32.5 kg/m^2. The other

anthropometric features about the affected individuals in the family are presented in Table 4.1.

On the basis of the prediction of the autosomal recessive form of disease and consanguinity in pedigree, exome data was filtered for homozygous variants. All the suspected coding variants in homozygous condition were searched in available databases of human genetic variations. On the basis of relating the phenotypes with the gene function, no pathogenic variant was found in the homozygous region in any obesity related genes and not in any metabolic gene. Based on chip genotyping segregation analysis, the identified homozygous variants were also not segregate within the family members.

No causal gene for obesity was found with the application of exome sequencing in this case. The current result reflects that the pathogenic variant might be present in the noncoding region that could be captured by whole genome sequencing hence, suggesting us the whole genome sequencing which may lead to the exploration of deep pathogenic intronic variant or pathogenic variant in the promoter region associated with etiology of obesity.

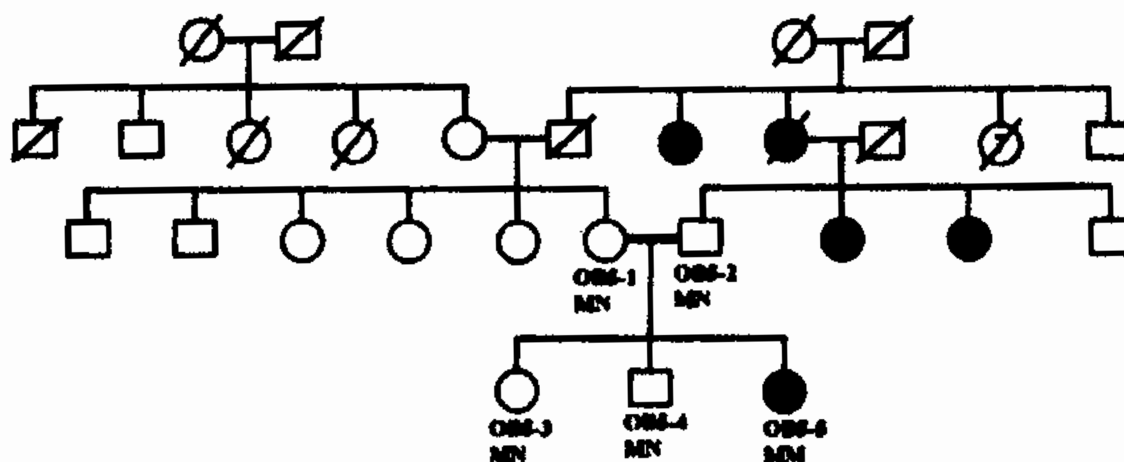

Figure 5. 10 Pedigree of Family OB5 identified with frameshift mutation c.94_104delCGGCTGCCGCG (p.Arg32fs) in *QSOX2*. Circles and squares represent female and male family members, respectively. Filled symbols indicate affected members with obesity. Symbols with slashed indicate deceased family members and double lines between symbols show cousin marriages. Numbers within symbols indicate the number of siblings of the same gender. The allotments of IDs are given to those members who are participated in a study

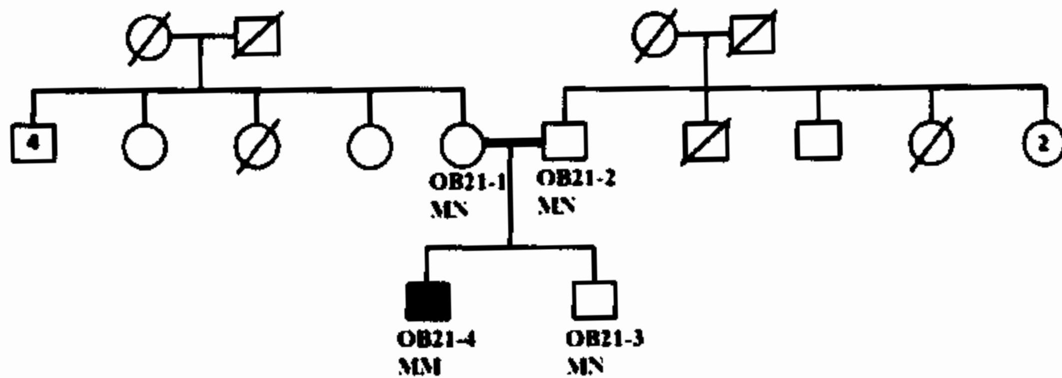

Figure 5. 11 Pedigree of Family OB21 identified with stop gain mutation c.2277C>A (p.Cys759*) in *LTBP3*. Circles and squares represent female and male family members, respectively. Filled symbols indicate affected members with obesity. Symbols with slashed indicate deceased family members and double lines between symbols show cousin marriages. Numbers within symbols indicate the number of siblings of the same gender.

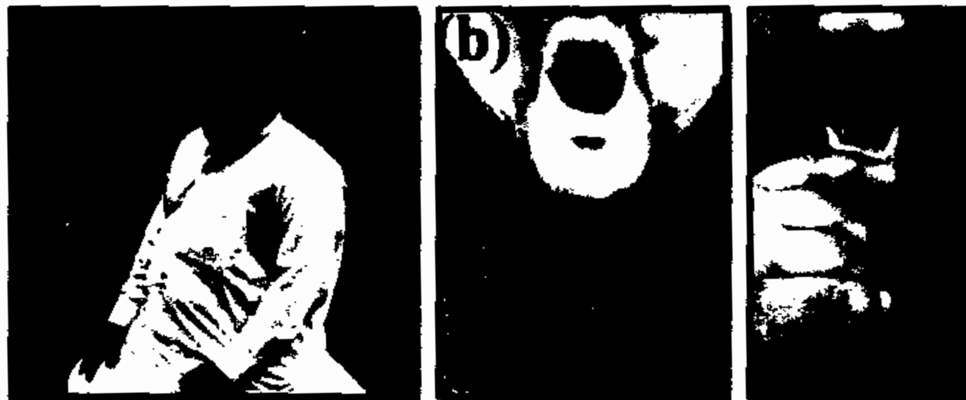

Figure 5. 12 Picture of an affected individual of Family OB21 identified with a stop gain mutation (p.Cys759*) with phenotypes of (a) acromicric dysplasia, (b) a small mouth and (c) dental anomalies.

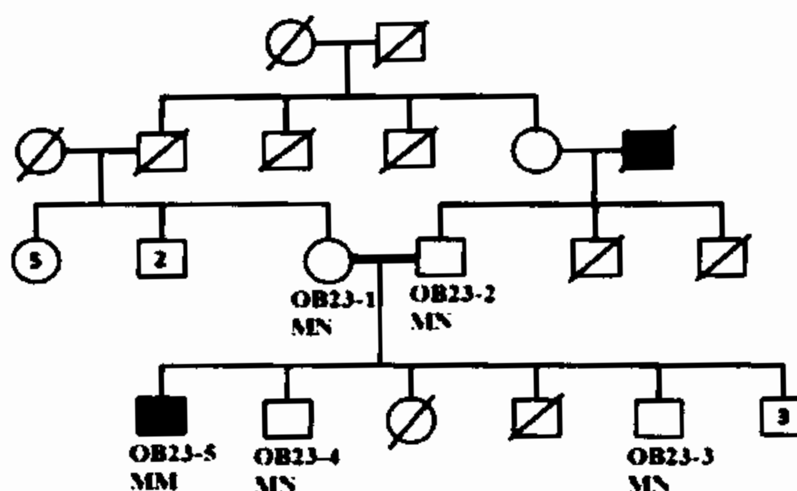

Figure 5. 13 Pedigree of Family OB23 identified with frameshift mutation c.525_532delGCCGGTGC (p.Pro176fs) in *KISS1R*. Circles and squares represent female and male family members, respectively. Filled symbols indicate affected members with obesity. Symbols with slashed indicate deceased family members and double lines between symbols show cousin marriages. Numbers within symbols indicate the number of siblings of the same gender.

Figure 5. 14 Picture of an affected individual of Family OB23 identified with a frameshift mutation (p.Pro176fs).

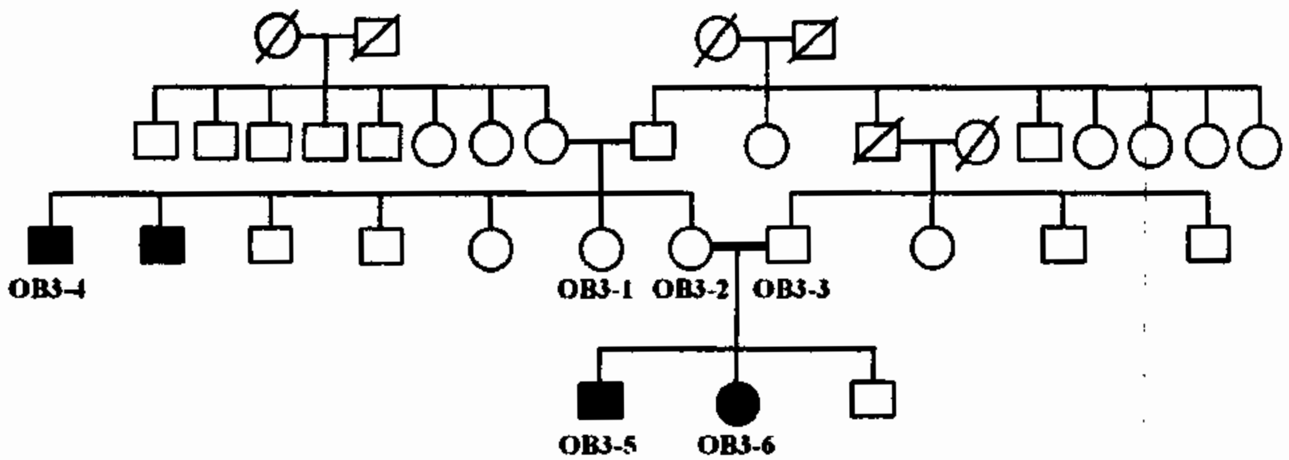

Figure 5. 15 Pedigree of Family OB3 has no findings with an application of whole exome sequencing. Circles and squares represent male and female members of the family, respectively. Filled symbols indicate affected members with obesity and unfilled symbols indicate lean members. Slashed symbols indicate deceased members and double lines between symbols show cousin marriage. Symbols with given Ids are those who participated in the study.

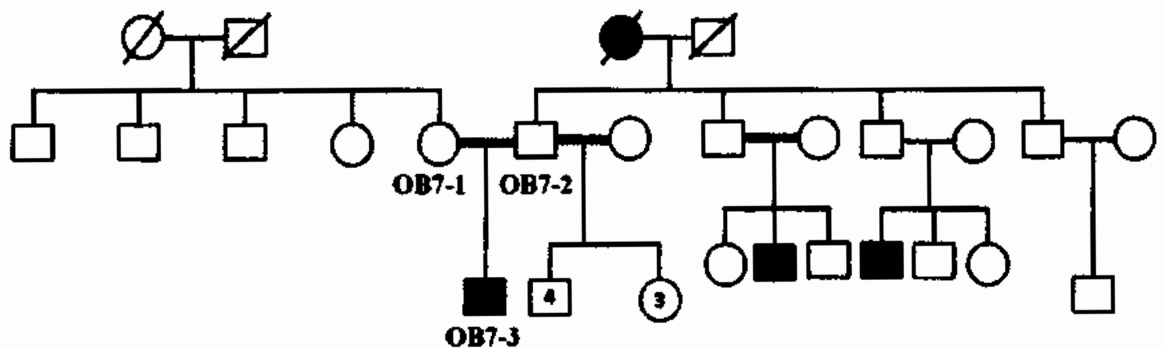

Figure 5. 16 Pedigree of Family OB7 has no findings with an application of whole exome sequencing. Circles and squares represent male and female members of the family, respectively. Filled symbols indicate affected members with obesity and unfilled symbols indicate lean members. Slashed symbols indicate deceased members and double lines between symbols show cousin marriage. Symbols with given Ids are those who participated in the study.

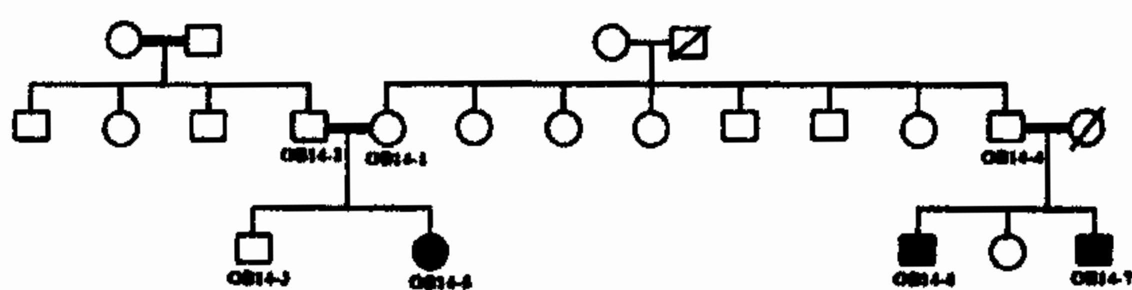

Figure 5. 17 Pedigree of Family OB14 has no findings with an application of whole exome sequencing. Circles and squares represent male and female members of the family, respectively. Filled symbols indicate affected members with obesity and unfilled symbols indicate lean members. Slashed symbols indicate deceased members and double lines between symbols show cousin marriage.

6. Discussion

The overall purpose of this thesis was to contribute to exploration, diagnosis and understanding of the genetic etiology in cases with severe early-onset childhood obesity in consanguineous and non-consanguineous Pakistani families. The current investigation provides possible assistance in devising and improving strategies for the prevention and treatment for this major global health problem worldwide.

6.1 Paper I

In the present study, we employed targeted re-sequencing which is a high-throughput and cost-effective approach. This was performed in order to reveal the genetic etiology in cases with severe early-onset childhood obesity. The sequencing data were analyzed with respect to the three main genes involved in monogenic forms of obesity, i.e. *LEP, LEPR* and *MC4R*. Given the high prevalence of *LEP, LEPR* and *MC4R* mutations previously reported in Pakistani populations [151], a similar prevalence of causal variants within these genes was expected. However, only two of the 25 examined probands carried homozygous recessive mutations and both mutations were positioned in *LEPR*.

LEPR mutations have previously been reported to influence the risk of developing severe early-onset obesity, hypogonadotropic hypogonadism and hypothalamic hypothyroidism [156, 292], which is similar to the clinical characteristics of leptin deficiency [156]. Furthermore, in mice, *Lepr* mutations have been found to influence the susceptibility of type 2 diabetes [293]. Hypogonadotropic hypogonadism in LEPR-deficient individuals may be due to a defect both at the hypothalamic and at the pituitary level [173]. However, hypogonadism may change with time in age as in the case of spontaneous pubertal development and a natural pregnancy [156].

Both of the novel *LEPR* mutations identified in the current study are positioned in the intracellular domain of LepRb, which is involved in energy homeostasis, glucose metabolism, fertility, growth and the action of insulin [294, 295]. Upon binding, leptin activates the LepRb through the mediation of multiple signalling pathways including

phosphorylation of cytoplasmic tyrosine kinases of Janus Kinase 2 (JAK2), conscription of signal transducer and activator of transcription 3 (STAT3) and mitogen-activated protein kinase (MAPK) cascade [230, 294, 296, 297]. In both humans and mice, there are multiple forms of LepRb, including short intracellular domain forms ranging from 32 to 40 amino acids and the long form of 303 amino acids, which is predominantly expressed in the hypothalamus [295, 298-301]. The missense mutation (p.Pro892Arg), identified in a family OB25, is located in the Box 1 motif which is important both for leptin-dependent JAK2 activation through the mediation of signalling by the intracellular domain and for the physiologic actions of leptin [299, 302].The CADD score indicates the pathogenicity level and the score of the identified mutation (p.Pro892Arg) is 28, which indicates this is a very likely disease-causing variant. The second mutation i.e. frameshift (Ser1090Trpfs*6), identified in the family OB4, is located in the long intracellular domain of LepRb and has sequence motifs resulting in the truncation of the domain, thereby suggesting a dysfunctional effect on its intracellular signal-transducing capabilities.

The highly deleterious nature of the identified two mutations (p.Pro892Arg, Ser1090Trpfs*6) is consistent with clinical conditions of hyperphagia, rapid weight gain and extreme obesity as observed in proband OB4-7. In both probands, the mutations were found in a homozygous state and based on genotyping of family members, the homozygous region was found to be shared among affected individuals only, while parents of the probands were heterozygous carriers. Thus, when combined, our results strongly indicate that the identified mutations are causal.

Previously it has been observed that boys in the Pakistani population are more prone to obesity than girls [63]. In addition, Iranian consanguineous families have revealed that LEPR deficiency may be more severe in females compared to males [282]. However, in OB4 where the mutation was found in both affected boys and girls, the mutation showed the same level of severity irrespective of gender.

Increasing knowledge of genetic factors involved in childhood obesity leads to an improved understanding of the genetic etiology of this disorder. For this purpose, the Pakistani population is unique due to its large size, a high number of families with

known consanguineous marriages and the high frequency of large pedigrees [63]. Especially the identification of rare, damaging variants predisposing to obesity holds the promise of future development of novel therapeutic options and personalized medicine based on molecular diagnosis [156, 303]. In the case of congenital leptin deficiency caused by deleterious *LEP* mutations, hormonal leptin therapy has proved to have dramatic treatment effects, successfully decreasing the body weight and hyperphagia of the carriers [304, 305]. Recently, treatment with mechanism-based therapy using a MC4R agonist (setmelanotide) in two patients with damaging *POMC* mutations completely reversed hyperphagia and induced a remarkable weight loss while normalizing insulin sensitivity [306]. Albeit no effective drug therapies are currently available for LEPR deficient individuals, treatment of dysfunctional POMC with MC4R-agonist suggests its efficacy in other monogenic defects of the hypothalamic leptin-melanocortin pathway including *LEPR* deficient patients [306]. Hence, the treatment with setmelanotide might be effective in the treatment of probands with non-functional LEPR, as identified in our study.

6.2 Paper II

In the next step, targeted re-sequencing of the coding and flanking regions of 31 selected genes known to be involved in monogenic forms of obesity (excluding *LEP*, *LEPR* and *MC4R)* was performed in 23 probands from Pakistani families with severe early-onset obesity segregating as an autosomal recessive trait. One compound heterozygous proband was identified carrying two variants in *BBS9/PTHB1* (c.223C>T; p.R75X and c.1441C>T; p.R481X in Exon 3 and Exon 14, respectively) causing BBS.

Homozygous and tri-allelic variants in BBS genes have been reported to cause BBS phenotypes in Pakistani population [226, 307, 308], but no prior *BBS9* compound heterozygous patients have been reported in non-consanguineous Pakistani families.

Bardet-Biedl syndrome protein complex (BBSome) is a central entity of ciliogenes and it has 10 subunits from BBS1 to 10 [309]. BBS9, a 99-kDa protein is one of the components of the BBSome and it has a possible role in associating other subunits [310]. Studies of BBS9 function in knock down mouse and zebrafish have revealed its

significant role in cilia biogenesis [311]. In our study, the identified variant, p.R75X is positioned in the N-terminal domain and the p.R481X is positioned in the C-terminal half of the PTHB1 protein. The full-length BBS9 contains 887 amino acids, thus, termination of the protein after only 75 and 481 amino acids, respectively, is not surprisingly detrimental for the function of the protein due to nonsense mediated decay or production of the truncated protein [312]. Hence, we believe that these loss-of-function mutations in *BBS9* may be responsible for structural abnormality in cilia due to reduced integrity of BBSome proteins complex.

Previous studies examining the genetic causes of severe early-onset obesity in Pakistani families have mainly focused on a few genes most often linked to monogenic forms of obesity, i.e. *LEP*, *LEPR* and *MC4R* [134, 149, 151, 166], yet more recently, a mutation screen of multiple genes was performed in 39 unrelated children with severe obesity from consanguineous Pakistani families[134]. The study included 21 of the 31 genes examined in the present study and similar to our findings, no casual mutations in homozygous conditions were identified [134]. Many of the genes (*CCDC28B, CREBBP, EP300, IER3IP1, MRAP2, PHF6, SH2B1, TMEM67, VPS13B),* selected in the present study were, therefore, screened for the first time in Pakistani families with the aim of assessing the prevalence of damaging mutations conferring early-onset obesity. However, our findings indicate that mutations within the selected 31 genes are not a common cause of severe early-onset obesity in the Pakistani population.

For syndromic forms of obesity, no personalized treatment approach has been identified and the affected individuals are generally advised to follow current general treatment approaches, including increased physical activity, psychomotricity and a restricted diet [313]. Yet, administration of pharmacological chaperones and potent MC4R agonists, tested in human cells and obese rhesus macaques, reverses obesity by reducing body weight, insulin resistance and improving cardiovascular without any side effects [314, 315]. These novel MC4R agonists and pharmacological chaperones may prove to be useful in the treatment of other forms of obesity [314, 316]. Deeper knowledge of the genes involved in obesity may hold the prospect of enabling us to design novel and

personalized approaches for the prevention, management and treatment of monogenic forms of obesity [317].

6.3 Paper III

In medical sciences, the application of WES has been proved a cost-effective method with the detection of disease-causing variants and helpful for the treatment of genetic diseases through discovering gene targets [219, 318]. Sequencing of whole exome is performed by capturing and sequencing of the coding regions of genome at a deeper level [219]. Due to the effectiveness in disease diagnosis and their treatment, whole exome or genome may be adopted as a routine clinical laboratory procedure upto next two decades [219].

The identification of genes implicating in development of obesity is important for the development of novel therapeutic strategies. During the genetic investigation of a familial case of Family OB5, the application of WES identified the frameshift mutation c.94_104delCGGCTGCCGCG; p.Arg32fsin homozygous condition which caused premature truncation of the QSOX2 protein, located in exon 1 of the *QSOX2* gene that mapped on chromosome 9q34.3.The mutationc.94_104delCGGCTGCCGCG; p.Arg32fs in *QSOX2* gene was not found in any of the publically available databases hence, we are the first to report. The pattern of segregation analysis in a family showed autosomal recessive inheritance.

QSOX2 is a member of the sulfhydryl oxidase/quiescin-6 (Q6) family and is known to regulate the sensitization of neuroblastoma cells for IFN-gamma-induced cell death [319]. *QSOX2* gene consists of 12 exons and encodes a protein of 698 amino acids with mass of 77.33 kDa. This gene has domains such as a putative signal peptide (amino acids 1–50), a PDI-like TRX domain (amino acids 76–147), an ERV1 domain (amino acids 408–540), a conserved *N*-glycosylation motif (amino acid 266), Q6-like regions and a C-terminal transmembrane domain. In our study, the identified frameshift mutation locates in a PDI-like TRX domain of *QSOX2*.

Through genome-wide association studies (GWAS), several variants within *QSOX2* gene have been identified, which showed an association with phenotypes and diseases such as blood protein levels (rs10858248) with p-value $2.00e^{-50}$ [320], crohn's disease (rs10781499) with a p-value $6.00e^{-30}$ [321], three variants (rs7849585, rs10858250, rs12338076) identified for height with a p-values $1.00e^{-29}$, $7.00e^{-23}$, $2.00e^{-8}$, respectively [322-324] and inflammatory bowel disease (rs10781499) with a p-values $5.00e^{-36}$ [321], ulcerative colitis (rs10781499) with a p-value $2.00e^{-16}$ [321] and waist circumference adjusted for body mass index (rs11103390) with a p-values $5.00e^{-7}$ [325]. Additionally, there is a missense variant (p.S391L, rs60980157) in *QSOX2* which has been an association with T2DM with a p-value of $3.19e^{-16}$ and several intron variants within this gene associating with waist circumference (http://www.type2diabetesgenetics.org).

In our current study, the proband has been identified with frameshift mutation in homozygous condition in coding region of *QSOX2* gene for causing obesity in addition to severe hyperphagia. Based on the genotyping of the family, the other lean members were heterozygous. Therefore, the mode of recessive inheritance and the association of *QSOX2* gene with metabolic disease such as T2DM and obesity variable such as waist circumference support *QSOX2* as a candidate and interesting gene for etiology of obesity. However, functional study would be important to perform to explore the understanding of the underlying pathway that is responsible for the development of obesity.

6.4 Paper IV

With the application of exome sequencing in the familial case of OB21and based on relationship of phenotypes with the gene function, a stop gain mutation c.2277C>A; p.Cys759* in homozygous condition has been found in *LTBP3* as a candidate gene. This is predicted that the mutation resulted in loss of function of the LTBP3 protein which, resulted after the truncation of LTBP3 protein within *LTBP3*. The pattern of segregation analysis showed autosomal recessive inheritance. The mutation (c.2277C>A; p.Cys759*) in *LTBP3* gene was not found in any of the publically available databases hence, would to report for the first time.

LTBP3 is one of the member of LTBPs family (LTBP1, LTBP2, LTBP3 and LTBP4) and LTBPs are known for targeting and activation of TGF-β particularly LTBP3 that regulates the bioavailability of TGF-β in chondrocytes in mice [326].The overall structure of all known LTBPs is similar and can be divided into four parts, the N-terminal region, the following hinge domain, the central cluster of EGF-like repeats and the C-terminal TGF-b binding region [327]. In our study, the identified mutation is positioned in EGF domains.

Among humans, the first mutation (c.2322C > G; p.Tyr774*) within *LTBP3,* in homozygous state was identified in consanguineous Pakistani population with display of phenotypes such as short stature, vertebral and skull bone alterations and oligodontia [328]. The second mutation (p.Cys620Trpfs*171) was identified in two female patients of ages 18 years and 2 months and 15 years and 3 months with a non-consanguineous family background of Emirati origin [329]. Both patients displayed clinical phenotypes of short stature, oligodontia and mitral valve prolapse in addition to BMI 26.8 kg/m^2 and BMI 21.3 kg/m^2, respectively. In our study, we identified the third mutation c.2277C>A; p.Cys759* within *LTBP3* with clinical phenotypes of short stature, dental anomalies, acromicric dysplasia, geleophysic dysplasia in addition to early-onset obesity. At the age of 19 years and 9 months, the mutation carrier was142.2 cm tall with weight 85 in kg, hence, having BMI 42.03 kg/m^2. Mutation effect prediction tool such as CADD score predicted the variant as disease causing and damaging.

As we identified the mutation in addition to early-onset obesity and the cause of the observed obesity phenotype is unclear, thus it is important to discover the underlying pathway involved in causing obesity within *LTBP3*.The studies on patients with mutations within *LTBP3* have not reported with the obesity phenotype previously, therefore, whether the obesity phenotype occurs as a primary or secondary with consequent to obesity, remains to be determined.

6.5 Paper V

With the application of exome sequencing, the proband (OB23-5) is identified with frameshift mutation c.525_532delGCCGGTGC (p.Pro176fs) in homozygous condition, which truncated the KISS1R protein. Based on genotyping information, the identified variant was located inside the homozygosity region and received an autosomal recessive pattern of inheritance.

KISS1R earlier known as *GPR54*, encode a galanin-like G protein-coupled receptor [330] that binds neuropeptide kisspeptin (metastin), a peptide encoded by the metastasis suppressor gene KISS1which is expressed in the hypothalamus and has been implicated in the neuroendocrine regulation of gonadotrophin-releasing hormone (GnRH) secretion [331-333]. Mutations in this gene are known to involve in the regulation of endocrine function and play a role in the onset of autosomal recessive hypogonadotropic hypogonadism, infertility and impaired puberty in human and mice [287-289]. In addition to GnRH neurons, KISS1R is expressed in other brain areas and peripheral tissues including metabolic tissues like fat, liver and pancreas [331, 333, 334], which suggests that kisspeptin has an additional uncharacterized functions beyond reproduction.

The current findings of proband with *KISS1R* frameshift mutation showed phenotype of hypogonadism in addition to obesity and going to report for the first time in male human. To address the etiology of obesity, leptin signalling is already known to associate with hypogonadotropic hypogonadism [150] however, we do not find direct evidences that KISS system affects leptin signalling but there are opposite evidences, that leptin affects KISS system [335].

To elucidate the potential metabolic roles for kisspeptin signaling, a study was made in mice lacking kisspeptin signaling (*Kiss1r* KO mice) which revealed the female mice *Kiss1r* KO with increased weight gain without hyperphagia and decreased locomotion, reflects reduced metabolism or energy expenditure [336]. The findings showed that, other than stimulating the reproduction, the alterations in kisspeptin system is also an

important player directly or indirectly in obesity, energy imbalance, locomotion and glucose regulation or metabolic dysfunction [336].

In humans, the current newly discovery of homozygous variant of kisspeptin signaling system with obesity and hypogonadism in male would extends our understanding of the relationship between reproduction and energy balance. Moreover, it may be helpful in providing novel insight into various metabolic diseases, such as diabetes, polycystic ovary syndrome and obesity.

6.6 Families OB3, OB7and OB14 with No Findings

Affected individuals in three families (OB3, OB7 and OB14) showed marked clinical features of obesity including elevated BMI with no signs and symptoms of neurological complications. The patients were born of phenotypically normal parents, hence showing autosomal recessive mode of inheritance. In order to hunt down a disease causing sequence variant, exome sequencing was carried out for the DNA sample of at least one affected individual from each family (OB3-6, OB7-3and OB14-5 from family OB3, OB7 and OB14, respectively). The variants were filtered out on the basis of clinical relevance and associated functions of the genes. Downstream analysis was performed by excluding intergenic variants, intronic variants and variants occurring in the untranslated regions (UTRs). While bearing in mind the autosomal recessive mode of inheritance, homozygous frameshift variants, missense variants, stop gained stop lost variants in the exonic regions and splice site variants were extensively targeted. Besides, potential variants in genes which are most likely involved in weight gain were carefully ascertained. However, no potentially pathogenic variant was detected in those genes. Heterozygous mutations were also screened in previously known obesity related genes to ascertain possible compound heterozygosity but still no significant results were obtained.

Additional affected individuals (OB3-4 and OB14-7) were also submitted to exome sequence analysis to hunt down a common defective variant among those patients.

However, the probands showed no pathogenic sequence variants in the previously known or novel genes.

In conclusion, exome sequencing failed to identify disease causing mutations underlying obesity in the aforementioned three families. These results suggest that the failure to discover a genetic cause of obesity may be due to technical limitations of our study design or the occurrence of the pathogenic sequence variant in non-exonic regions. If not, the disease causing mutation may be somatic in nature and not detectable by our approach. Alternatively, obesity is genetically heterogeneous and our cohort was not sufficient to reveal the causative genes. Future studies like whole genome sequencing and pyro-sequencing should consider designs where exonic and non-exonic regions are seen and apply a sufficient sequencing depth so that also low-grade somatic mosaicism can be detected. Besides, epigenetic studies may be helpful in understanding the regulation of expression levels of candidate genes that may open a new path towards understanding the pathophysiology of obesity phenotypes in the affected individuals included in this study.

7. Conclusions and Future Perspectives

7.1 Conclusions

From the findings of papers I to V, we can conclude that:

1. With the application of targeted re-sequencing in Pakistani families, two novel mutations implicating in *LEPR* including a frameshift and a missense were identified in probands with severe early-onset obesity. Both of these mutations were found in a homozygous state. The findings demonstrated the effectiveness of targeted re-sequencing to identify novel damaging mutations and this approach may, therefore, be utilized in clinical testing or diagnosis of different known monogenic forms of obesity with the aim of optimizing obesity treatment.
2. Mutations within the 31 selected genes do not seem to be a cause of severe early-onset obesity in families with Pakistani origin and did not reveal the presence of homozygous obesity causing variants. However, compound heterozygote *BBS9* mutations were identified within one proband with clinical supports, indicating that compound heterozygosity must not be overlooked when looking for recessive disorders.
3. The whole exome genotyping explored the identification of homozygous variant in *QSOX2* gene in a selected Pakistani family. Previously *QSOX2* gene is known as the causative gene in diabetes. In the current study, it has been identified as a candidate gene to cause obesity in a patient with known consanguineous background. However, an experimental study would be important to perform that would be helpful in understanding the function of gene with underlying molecular pathways involved in obesity etiology.
4. Whole exome sequencing revealed novel homozygous variant in *LTBP3* as a candidate gene underlying obesity in a consanguineous Pakistani family. However, an experimental study is important to perform which would be

helpful in understanding the function of gene, with underlying molecular pathways that involved in obesity etiology.

5. Whole exome sequencing revealed homozygous variant in *KISS1R* underlying obesity in a consanguineous Pakistani family. In the current study, it has been identified as a candidate gene to cause obesity in a patient with the known consanguineous background. However, an experimental study would be important to perform for understanding the function of gene and underlying molecular pathways involved in obesity development.
6. The families which are not identified with obesity causing genes with the application of exome sequencing are suggested for WGS. WGS might be helpful in identification of deep pathogenic intronic variants or pathogenic variants in the promoter region associated with disease phenotype and its etiology.

7.2 Future Perspectives

The future perspectives of the current thesis study may involve:

1. To plan the functional study of the candidate genes those which were identified through exome sequencing with previously known phenotypes in addition to obesity. Such investigation will help us to understand better about the pathways underlie in obesity etiology in Pakistani population.

2. To complete the analysis of exome data with interpretation and validation of those families which were subjected to exome sequencing but are not included in this thesis.

3. The families which are not solved by exome sequencing would be planned to subject for the WGS as this approach is the most efficient choice to investigate the genetic variants including CNVs (copy number variations), small insertions/deletions or SNPs and other structural variants found in coding and non-coding regions from whole genome in a single dataset with coverage of both rare and common variants.

4. To establish meetings and programmes for genetic counselling in families. Such activities would be the source of providing awareness about issues and would spread knowledge associated with marriages between closely related individuals.

5. The possible treatment and observational studies in patients from selected Pakistani families with the identification of damaging variants would be planned to initiate the control trials on them. The purpose of the control trials would to check the efficacy of the FDA (food and drug administration) approved and prescribed drug/s (if available) by reversing the obesity phenotype into normal lean. However, keeping in view the extremely high costs of conducting clinical trials and several issues related to ethical and technical, it is not always feasible and possible to conduct one.

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Appendix A: Ethical Approval Letters from Institutes

International Islamic University, Islamabad
Director(Office of Research, Innovation & Commercialization)
Tel: 051- 9258091 Fax: 051-9258072
E-mail: abdul.hammed@iiu.edu.pk

December 11, 2018

To,
Dr. Asma Gul,
Associate Professor,
Chairperson Deptt. of Biological Science

Subject: Approval of the thesis title "Molecular characterization of obesity and metabolic syndrome genes in selected Pakistani families" by the Ethical Review Board

Dear Dr. Asma Gul,

The Institutional Ethical Review Board has reviewed and discussed application of Ms. Robina Khan Niazi to publish the above mentioned study in the Department of Biological Sciences with yourself as the Research Supervisor.

The Board approves the study to be conducted in the presented form. None of the supervisor and co-supervisor participating in this study took part in the decision making and voting procedure for this study.

The Institutional Ethics Committee expects to be informed about the progress of the study, any changes occurring in the course of the study, any revision in the protocol and information informed consent and ask to be provided a copy of the final report.

Yours Sincerely,

Dr. Abdul Hameed
Director ORIC,
International Islamic University Islamabad,
Pakistan

SHAHEED ZULFIQAR ALI BHUTTO MEDICAL UNIVERSITY
PIMS ISLAMABAD - 44000

PROF. JAVED AKRAM
Vice Chancellor
Shaheed Zulfiqar Ali Bhutto
Medical University, Islamabad

Chairman
Ethics Review Board

PROF. DR. ALI JAWA
Secretary
Ethics Review Board

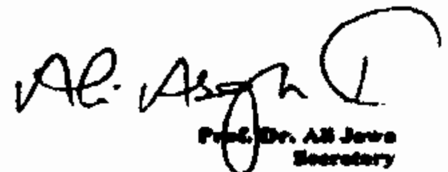
No. F. 1-1/2015/ERB/SZABMU/ Dated : 3-01-2017

Miss Robina Khan Niazi
P.hD Scholar
International Islamic University
Islamabad.

Subject: MOLECULAR CHARACTERIZATION OF OBESITY AND METABOLIC SYNDROME GENES IN PAKISTANI POPULATION

Thank you for submitting your research proposal to the Ethical Review Board. After evaluation of your project, an unconditional permission is given to proceed with this project.

However, the committee reserves the right to discontinue the research study if reports are received regarding causation of undue risks/hazards to study subjects.



Prof. Dr. Ali Jawa
Secretary

Sector G-8/3, Islamabad 44000, Pakistan.
e-mail : [illegible] www.pims.gov.pk

Appendix B: Patient Clinical History Form

Name:______________________________ DOB:____________ Date:____________

PATIENT HISTORY FORM

Patient Name:______________________________ ☐**Male** ☐**Female**
DOB:____________ **Age:** __________ **Marital Status:** ☐**S** ☐**M** ☐**D** ☐**SEP**
Occupation:______________________________
Spouse/Significant Other's Name__________________ **Phone #**______________

Height	Weight	Waist Circumference

Your Family History:

	SevereObesity	Obesity	Normal
Father			
ParentalGrandfather			
ParentalGrandMother			
Father's Brothers			
Father's Sisters			
Mother			
MaternalGrandfather			
MaternalGrandmother			
Mother's Brother			
Mother's Sisters			
Your Brothers			
Your Sisters			
Your Sons			
Your Daughters			

Please check which of the following conditions that your blood-related family members have experienced. Please indicate by circling which family member was affected.

Diabetes (fasting/random)	Mother	Father	Other__________
Hypertension	Mother	Father	Other__________
Heart Disease	Mother	Father	Other
Obesity	Mother	Father	Other
Osteoporosis	Mother	Father	Other__________
Seizure	Mother	Father	Other__________
Anemia	Mother	Father	Other
Asthma	Mother	Father	Other__________
Arthritis	Mother	Father	Other__________
Thyroid Disease	Mother	Father	Other__________
Kidney problems	Mother	Father	Other
Early Death	Mother	Father	Other__________
Lipid/Cholesterol Problems	Mother	Father	Other__________
Cancer (type) __________	Mother	Father	Other__________

Neurological
Disorder (Parkinson's) Mother Father Other____________________
Alzheimer's Disease Mother Father Other____________________
Other Mother Father Other

Your Medical History:

When was your last physical exam with your primary care physician?
Medications:
List all medications (including over-the-counter medications, vitamins, herbs & diet aids):__________

Drug Name	Dose	Time Per Day	Purpose

List all medications not tolerated: (describe reaction)
____

Musculoskeletal:
_____Arthritis _____Back Pain _____Spinal Disc problems
_____Knee Pain _____Gout _____Fibromylagia
Chronic Fatigue Fractured hip, wrist or spine (circle)
_____Other______________________________________
Nervous System:
Neuropathy Parkinson's disease Epilepsy/seizures
_____Dizziness _____Headache/Migranes _____ Stroke (CVA)
_____Other Neurological Disorders______________________
Serous Problems with memory or difficulty thinking
Gastrointestinal:
_____Ulcers _____Reflux/GERD _____Hernia
_____Crohn's Disease _____Ulcerative Colitis _____Hemorrhoids
_____Colon Polyps _____Diverticulosis _____Hepatitis
_____Liver Disease _____Gallbladder Disease _____Dental Problems
Change in Bowels Other
Cardiac:

_____ Angina/Chest Pain _____ Congestive Heart Failure _____ Heart Attack
_____ Coronary Artery Disease (_____year)
Heart Murmur Irregular or Rapid Heart Beat
_____ High Cholesterol_______High Triglycerides _____ High Blood Pressure

Endocrine/Hormonal Dysfunction:
Diabetes (fasting/random reading) Infertility Irregular periods
_____Hypothyroidism _____Hyperthyroidism _____Excessive hot/cold
_____Visual Changes _____Voice Changes_________Eating Disorder
Abnormal hair growth Increase in thirst or urination Growth hormone deficiency/resistance__________Polycystic ovarian cancer_________
Other____

Blood Disorders:
_____Anemia _____Bleeding or clotting problems
_____Iron Deficiency _____Other_______________________________

Vascular:
_____ Leg Ulcers _____ Edema (Swelling of Legs)
_____ Peripheral Vascular Disease

Genitourinary:
_____Kidney disease _____Prostate disease _____Difficulty urinating
_____Kidney Transplant _____ year
Frequent bladder or kidney infections
_____Other____

OB / GYN:
Number of pregnancies Number of Children
Date of onset of menses_____________________________
Date of onset of menopause (if applicable)_______________

Cancer:
Cancer Type: ____________________________ Year: _____
Treatment: ____

Do you have any other health concerns?________________________________
____
Are you currently exercising? Yes, No If yes, what type and how often?
____

If you have ever received any kind of Physical or Occupational therapy please list the condition and date of treatment below:
Condition_______________________________Dates________________________
Condition Dates

Patient Signature **Date**

Prepared By: Robina Khan Niazi

Appendix C: Consent form taken from Patient/Guardian in Families

RESEARCH CONSENT FORM

I voluntarily agree to participate in this genetic study because I am a member of the family with genetic condition. The purpose of this study is to identify the genetic basis of this disorder, and the information will be used for research study.

The known discomforts/ risk of the study are only those involved with giving a blood sample.

By taking part in this study I understand that I will not receive payment from any party.

I understand that my images/ photos taken may be required for publication in medical journal as part of an article, which may be seen by general public as well as medical professionals.

Furthermore, I understand that my non-identified genetic and clinical information will be submitted to secure databases of research data, so that other qualified researchers can then apply to use my research data for other genetic studies.

I read and understand the above information.

Name of subject: ______________________________

Gender: ______________________________

Signature of the patient/ guardian: ______________________________

Family ID: ______________________________

Signature of Investigator: ______________________________

Date: ______________________________

Appendix D: Publications

Niazi et al. BMC Medical Genetics (2018) 19:199
https://doi.org/10.1186/s12881-018-0710-x

BMC Medical Genetics

RESEARCH ARTICLE Open Access

Identification of novel *LEPR* mutations in Pakistani families with morbid childhood obesity

Robina Khan Niazi[1,2,3], Anette P. Gjesing[1], Mette Hollensted[1], Christian Theil Have[1], Niels Grarup[1], Oluf Pedersen[1], Asmat Ullah[4], Gulbin Shahid[4], Wasim Ahmad[4], Asma Gul[2] and Torben Hansen[1*]

Abstract

Background: Mutations in the genes encoding leptin (*LEP*), the leptin receptor (*LEPR*) and the melanocortin 4 receptor (*MC4R*) are known to cause severe early onset childhood obesity. The aim of the current study was to examine the prevalence of damaging *LEP*, *LEPR* and *MC4R* mutations in Pakistani families having a recessive inheritance of early onset obesity.

Methods: Using targeted resequencing, the presence of rare mutations in *LEP*, *LEPR* and *MC4R* was investigated in [illegible] families suspected of having autosomal recessive early onset obesity. Segregation patterns of variants were assessed based on chip based genotyping.

Results: Homozygous *LEPR* variants were identified in two probands. One carried a deletion [illegible] resulting in the frameshift mutation p.[illegible], and the second carried a substitution [illegible] resulting in the missense mutation p.Pro[illegible]Arg. Both mutations were located within regions of homozygosity shared only among affected individuals. Both probands displayed early onset obesity, hyperphagia and diabetes. No mutations were found in *LEP* and *MC4R*.

Conclusions: The current study highlights the implication of *LEPR* mutations in cases of severe early onset obesity in consanguineous Pakistani families. Through targeted resequencing, we identified novel damaging mutations and our approach may therefore be utilized in clinical testing or diagnosis of known forms of monogenic obesity with the aim of optimizing obesity treatment.

Keywords: Early onset obesity, Hyperphagia, Leptin, Leptin receptor, Melanocortin 4 receptor, Monogenic obesity, Pakistani families, Targeted resequencing

Background

Currently, childhood obesity is considered one of the most serious public health challenges of the twenty first century. The prevalence of childhood obesity is increasing at an alarming rate, affecting high income as well as low- and middle-income countries, and the number of overweight and obese children below the age of five is estimated to be 41 million (http://www.who.int/topics/obesity/en/).

A strong genetic factor is evident in the etiology of obesity, with heritability estimates ranging from 40 to 70% [1–3]. Genetic defects disrupting the leptin-melanocortin signaling pathway very often result in severe early onset obesity and hyperphagia [4–6], and the genes most commonly involved in monogenic forms of obesity are part of this pathway, including *leptin* (*LEP*), the *leptin receptor* (*LEPR*), and the *melanocortin 4 receptor* (*MC4R*) [7–9]. Leptin is a 16-KD hormone secreted by white adipocytes which binds to LEPR and regulates energy expenditure through hypothalamic neurons [10]. LEPR is a member of the cytokine receptor family with six isoforms (LEPRa-f), yet, leptin signaling is primarily mediated by the long

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Niazi et al. BMC Medical Genetics (2019) 20:152
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BMC Medical Genetics

RESEARCH ARTICLE Open Access

Screening of 31 genes involved in monogenic forms of obesity in 23 Pakistani probands with early-onset childhood obesity: a case report

Robina Khan Niazi[1,2†], Anette Prior Gjesing[2*†], Mette Hollensted[2], Christian Theil Have[2], Dmitrii Borisevich[2], Niels Grarup[2], Oluf Pedersen[2], Asmat Ullah[1,2], Gulbin Shahid[3], Ifrah Shafqat[1], Asma Gul[1] and Torben Hansen[2]

Abstract

Background: Consanguineous families display a high degree of homozygosity which increases the risk of family members suffering from autosomal recessive disorders. Thus, homozygous mutations in monogenic obesity genes may be a more frequent cause of childhood obesity in a consanguineous population.

Methods: We examined 23 probands from 23 Pakistani families displaying autosomal recessive obesity. We have previously excluded mutations in MC4R, LEP and LEPR in all probands. Using a chip based target region capture array, 31 genes involved in monogenic forms of obesity were screened in all probands.

Results: We identified 32 rare non-synonymous possibly pathogenic variants (29 missense and three nonsense) within the 31 selected genes. All variants were heterozygous, thus no homozygous pathogenic variants were found. Two of the rare heterozygous nonsense variants identified (p.R5X and p.R483X) were found in BBS9 within one proband, suggesting that obesity is caused by compound heterozygosity. Sequencing of the parents supported the compound heterozygous nature of obesity as each parent was carrying one of the variants. Subsequent clinical investigation strongly indicated that the proband had Bardet Biedl syndrome.

Conclusions: Mutation screening in 31 genes among probands with severe early onset obesity from Pakistani families did not reveal the presence of homozygous obesity causing variants. However, a compound heterozygous carrier of BBS9 mutations was identified, indicating that compound heterozygosity must not be overlooked when investigating the genetic etiology of severe childhood obesity in populations with a high degree of consanguinity.

Keywords: Autosomal recessive, Bardet Biedl syndrome 9, Compound heterozygous, Early onset obesity, Monogenic obesity, Pakistani families, Consanguinity

Background

Worldwide, the prevalence of obesity has risen more than tenfold during the past four decades and approximately124 million children and adults, aged five to 19 years old, were obese in 2016 [1]. Obesity is one of the major risk factors for metabolic syndrome, including arterial hypertension, cardiovascular disease, diabetes mellitus, dyslipidemia and cancer [2, 3]. The etiology of obesity comprises both environmental and genetic factors, with a heritability of body mass index (BMI) between 40 to 70% [4, 5].

In rare monogenic forms of obesity, disruption of a single gene is the cause obesity and individuals typically display severe early-onset obesity along with hyperphagia and endocrine disorders [6, 7]. Most of the causative proteins in monogenic forms of obesity are acting in the hypothalamic leptin-melanocortin signalling pathway,

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