OCTOBER 2019 M.Sc. in Biochemistry Science and Technology HUDA SHERZAD AHMED

REPUBLIC OF TURKEY

GAZİANTEP UNIVERSITY

GRADUATE SCHOOL OF NATURAL & APPLIED SCIENCES

ASSOCIATION OF KIR2DL4 GENE POLYMORPHISMS WITH OBESITY

M.Sc. THESIS

IN

BIOCHEMISTRY SCIENCE AND TECHNOLOGY

BY

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Supervisor

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Co-Supervisor

Asst. Prof. Dr. Deniz MIHÇIOĞLU

by

Huda SHERZAD AHMED

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This is to certify that we have read this thesis and that in our consensus/majority opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Huda SHERZAD AHMED

ABSTRACT

ASSOCIATION OF KIR2DL4 GENE POLYMORPHISMS WITH OBESITY

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M.Sc. in Biochemistry Science and Technology
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Obesity is one of the most important health problem which threatening public health and spreading rapidly. Obesity results from the excessive amount of fat accumulation in the body. The relationship between obesity and function of immune system was shown by previous studies. KIR genes encode receptors regulating cytotoxicity of Natural Killer cells and cytokine manufacturing. The KIR2DL4 gene is an atypical member of the KIR gene family; it differs from the other members in term of cellular location, ligand specificity and protein function. The aim of this study was to investigate the relationship between KIR2DL4 polymorphisms and obesity. Fifty obese patients (BMI >30) and 50 normal weight (BMI< 30) control subjects participated to the study. Blood samples were collected from the subjects, and after DNA isolation PCR- RFLP method was used for genotyping of polymorphisms. Allele and genotype frequencies were calculated by direct counting and genotype distributions in the groups were compared by chi-square analysis. Results of the study indicated that there is no statistically significant association between obesity and rs649216, rs660773 polymorphisms ($p > 0.05$), however rs660437 C allele might be a risk factor for development of obesity ($p = 0.004$).

KeyWords: Obesity, KIR2DL4, Polymorphism, PCR-RFLP

ÖZET

KIR2DL4 GEN POLİMORFİZMLERİNİN OBEZİTE İLE İLİŞKİSİ

SHERZAD AHMED, Huda
Yüksek Lisans Tezi, Biyokimya Bilimi ve Teknolojisi
Danışman: Prof. Dr. Filiz ÖZBAŞ GERÇEKER
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58 sayfa

Obezite halk sağlığını tehdit eden ve hızla yayılan en önemli sağlık problemlerinden birisidir. Obezite vücutta aşırı yağ birikimi sonucu ortaya çıkmaktadır. Obezite ile immün sistem işlevi arasındaki ilişki önceki çalışmalar ile gösterilmiştir. KIR genleri, Doğal Öldürücü hücrelerin sitotoksisitesini ve sitokin üretimini kontrol eden reseptörleri kodlamaktadır. KIR2DL4 geni KIR gen ailesinin sıra dışı üyesidir; hücresel yerleşim, ligand özgüllüğü ve protein işlevi açısından diğer üyelerden farklılık göstermektedir. Bu çalışmanın amacı; KIR2DL4 gen polimorfizmleri ile obezite arasındaki ilişkinin araştırılmasıdır. Çalışmaya 50 obez hasta (BMI >30) ve 50 normal kilolu (BMI< 30) kontrol birey dahil edilmiştir. Bireylerden kan örnekleri alınmış, DNA izolasyonu sonrasında PCR-RFLP yöntemi kullanılarak polimorfizmlerin genotiplendirilmesi yapılmıştır. Allel ve genotip frekansları direk sayım yöntemi ile hesaplanmış ve gruplardaki genotip dağılımları ki-kare analizi ile karşılaştırılmıştır. Çalışmanın sonuçları rs649216, rs660773 polimorfizmleri ile obezite arasında istatistiksel olarak anlamlı bir ilişki olmadığını ($p>0.05$) ancak rs660437 C allelinin obezite gelişimi için risk teşkil edebileceğini göstermiştir ($p =0.004$).

Anahtar Kelimeler: Obezite, KIR2DL4, Polimorfizm, PCR-RFLP

’Dedicated to my family’’

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LIST OF SYMBOLS

α	Alpha
β	Beta
χ^2	Chi Square
∞	Infinity
μ	Micro

LIST OF ABBREVIATIONS

AIM	Apoptosis Inhibitor of Macrophage
BMI	Body Mass Index
bp	Base Pairs
CHD	Coronary Heart Disease
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
FTO	Fat mass and Obesity-Associated Protein
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigens
Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariant Natural Killer T cells
ITIM	Immunoreceptor Tyrosine-Based Inhibition Motifs
KIR	Killer Cell Immunoglobulin-like Receptors
LDL	Low Density Lipoprotein
LEP	Leptin
LEPR	Leptin Receptor
MC4R	Melanocortin 4 Receptor
MHC	Major Histocompatibility Complex
min	Minute
ml	Milliliter
MUFA	Monounsaturated Fatty Acid
Na_2EDTA	Sodium EDTA
ng	Nanogram
NK	Natural Killer
NPY	Neuropeptide Y

PC1	Proprotein Convertase 1
PCR	Polymerase Chain Reaction
POMC	Proopiomelanocortin
PUFA	Polyunsaturated Fatty Acids
RE	Restriction Enzymes
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minute
sec	Seconds
SNPs	Single Nucleotide Polymorphisms
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
Taq	Thermus aquaticus
TBE	Tris Borate EDTA
TNFα	Tumor Necrosis Factor Alpha
Tregs	Regulatory T Cells
UCP	Uncoupling Proteins
UV	Ultra Violet
V	Volt
WHO	World Health Organization
WHR	Waist to Hip Ratio

CHAPTER I

INTRODUCTION

1.1 Obesity

Obesity is growing quickly in many parts of the world and has become one of the main issues of public health (Hruby et al., 2015). Obesity is defined as the result of accumulation of excessive amounts of fat in the body. Obesity is the consequence of genetic, behavioral, environmental, physiological, social, and cultural variables resulting in energy imbalance and encouraging unnecessary accumulation of fat (Rachete et al., 2003). Obesity is characterized by 3 or more of the following components; high level of blood glucose, plasma triglycerides, blood pressure, low plasma HDL, high plasma LDL and cholesterol. The Centers for Disease Control and Prevention (CDC), showed that obesity levels in the United States have steadily risen over the previous two decades, increasing from 19.4% in 1997 to 24.5% in 2004, 26.6% in 2007, 33.8% in 2008 and 35.7% in 2010. In the United States, child obesity also exceeds 17%; indeed, about 70% of obese teenagers grow up to be obese, which in turn improves their general mortality later in life (Nicklas et al., 2001). The greatest frequency of obesity is noted in the United States, Europe, Middle East, and the smallest frequency in Africa and East Asia (Figure 1.1).

1.2 Diagnosis of Obesity

The methods used for the measure of obesity and fat of body are Body Mass Index (BMI), waist to hip ratio (WHR), fat distribution, skinfold thickness, densitometry and bio-impedance. BMI and WHR are the most common methods that are used (Chan et al., 2003). BMI is determined by dividing of body weight to the height square in meters. BMI is below 18.5 defines the underweight. BMI ranges from 18.5 to < 25 means normal weight and from 25.0 to < 30 is accepted as overweight. When BMI is 30.0 or higher, this indicates the presence of obesity. Obesity is often classified into 3 groups; classs 1 (BMI 30 to < 35), class 2 (BMI 35- 40) and class

(BMI 40 or higher). Obesity in Class 3 is also called as "extreme" or "severe" obesity (CDC, 2017).

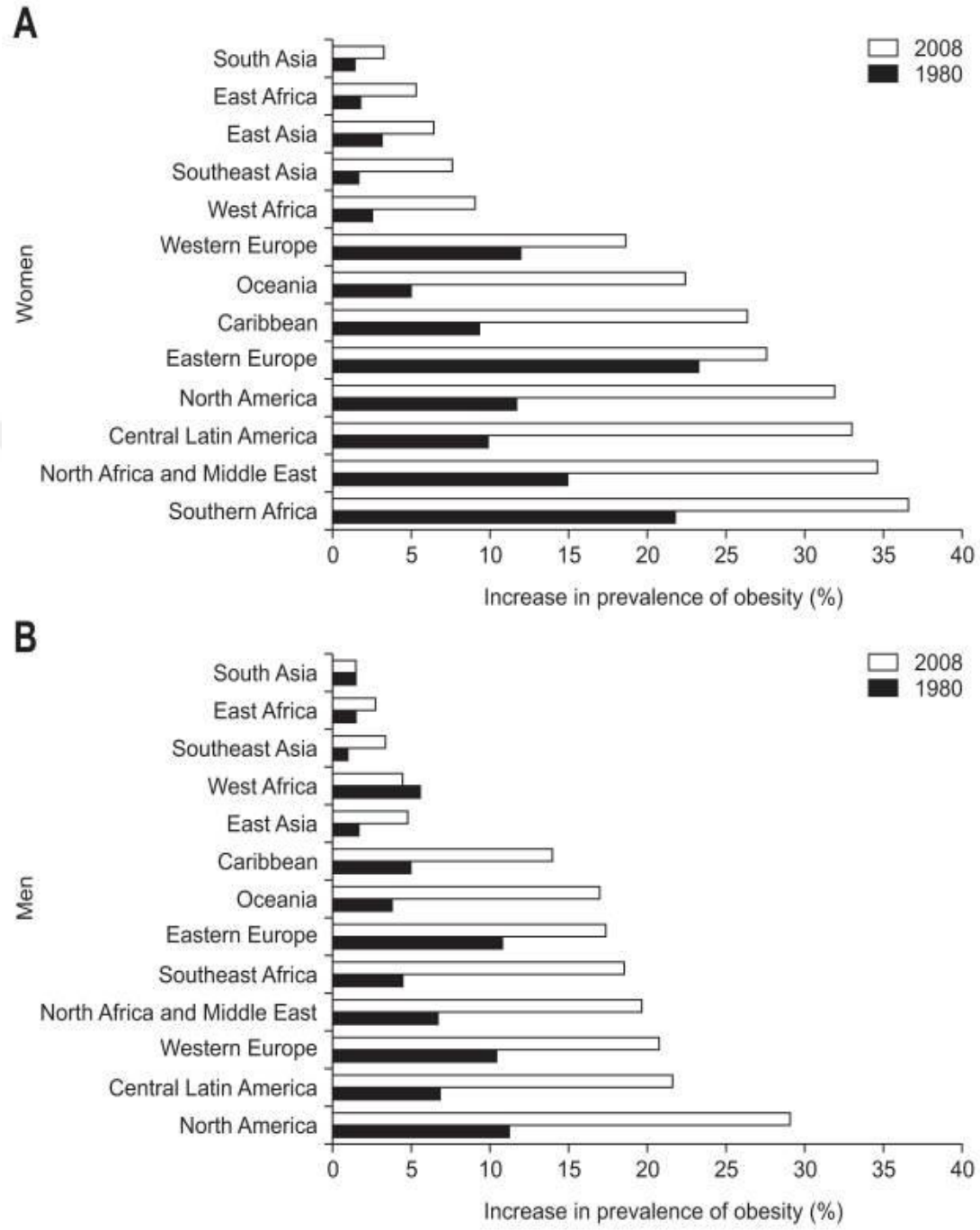

Figure 1.1 Obesity prevalence in male and female (Nam 2017)

In distinct gender and ethnicity groups, the validity of BMI as measured by the reference technique was well proved. BMI also acts as an indicator of the incidence and mortality of disease predictions. BMI was discovered to have a strong specificity, but low sensitivity to diagnosis of obesity (Romero-Corral et al., 2008).

WHR is a more specific measure of body fat (abdominal fat) distribution, although less frequently used (Brown, 2009). WHR can be calculated by dividing waist circumference by hip circumference. It can be used to classify forms of the body into two principal classifications: android obesity or abdominal obesity (visceral fat) apple in shape and gynoid obesity (pear in shape). Android obesity is more prevalent among men and is due to abdominal obesity however gynoid obesity is more prevalent among women (Ashwell, 2009). Android fat distribution is considered more harmful than gynoid fat distribution because of the accumulation of fat in the profound abdominal area around the visceral organs. For a female, having a pear shape implies that their proportion is less than or equal to 0.8. They are apple-shaped if their proportion is higher than 0.8. For men, they are pear-shaped if their ratio is less than or equal to 1.0, but they are an apple-shaped form if the ratio is greater than 1.0. (WHO, 2008). This hidden fat can cause development of several diseases.

1.3 Obesity Related Disorders

The adverse health impacts of obesity are well recorded (Wikstrand et al., 1993). Body weight is directly linked to the growth of cardiovascular disorders (CHD), regardless of traditional risk variables. Recent studies have reported that overweight and obese individuals have 2-and 3.6-fold enhanced risk of CHD. A weight gain of up to 11 kg raises the risk of disorder 1.6-fold, while a weight gain of 11-19 kg enhanced risk 1.9-fold. The risk of CHD rises 3-fold compared to the danger in lean topics when the BMI crosses 29 kg / m^2 (Willet et al., 1995).

Excessive weight gain, particularly when associated with enhanced visceral adiposity, is a significant cause of hypertension, representing 65% to 75% risk of main (vital) hypertension in humans. Increased reabsorption of renal tubular sodium negatively affects pressure natriuresis and plays a major role in hypertension (Reaven, 2002).

Heart attack is happening when the body decrease or blocks blood flow to coronary artery. Coronary arteries provide blood flow to the heart muscle. They can get narrow. Excess accumulation of substances including cholesterol and fat results to the development of plaques and this part of cardiac muscle cannot get enough oxygen (MNT, 2018).

Obese people have a greater risk for type 2 diabetes, characterized by insulin-resistance. This is a disease of consistently high blood glucose levels. Fat tissue cells have to store more nutrients in obese individuals than they can tolerate. The stress in these cells causes an inflammation that discharges proteins called cytokines. Cytokines then inhibit insulin receptor transmissions, resulting in the cells gradually becoming insulin resistant. Insulin enables cells to use energy with glucose (sugar). In the presence of insulin resistance, the body cannot transform the glucose into energy and end up with a consistently elevated rate of blood glucose (HP, 2017).

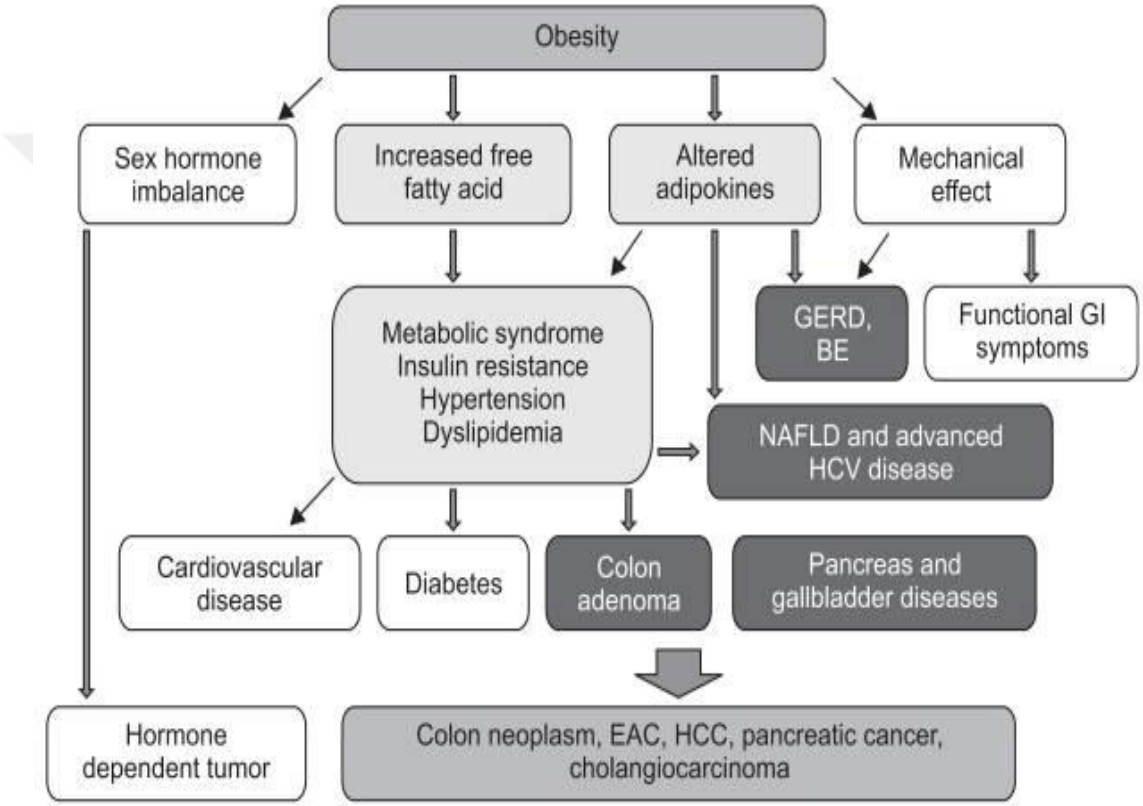

Figure 1.2 Medical impacts of obesity (Nam 2017)

Overweight or obese individuals are also at higher risk of getting osteoarthritis. In a research of twin, middle-aged females, the increased risk osteoarthritis was estimated to raise by 9-13% for each kilogram, at least two-thirds of patients with osteoarthritis were defined as obese (Cicuttini et al., 1996).

Gallstone development risk is improved 2.7-fold in females with BMIs over 40 kg / m^2 compared to females with BMIs below 24.9 kg / m^2 and increased 2.3-fold in males (Everhart et al., 1999).

By decreasing complete the volume of lung and capacity of functional residual, obesity impacts the respiratory system. Abdominal obesity creates stress on the diaphragm, reducing the lung's capacity to grow during inspiration, while chest fat accumulation decreases chest cavity (Ray et al., 1983).

The connection between enhanced body weight and the risk of sleep apnea has been verified by many cross-sectional studies. Approximately 40% of obese people have substantial sleep apnea and 70% of patients with sleep apnea are obese (Vgontazas et al., 1994). Peppard et al. (2000) showed that a 10% increase in body weight was related to a 6-fold enhanced risk of sleep apnea.

Asthma in the overweight and obese population appears to be more common. In one research, it was noted that the incidence of asthma was 38% greater in overweight patients and 92% greater in obese patients. No variations were investigated in gender, but the symptoms of asthma improved with an increase in BMI (Beuther et al., 2007).

Obesity is correlated with menstrual defects such as having periods longer than 36 days, abnormal cycles and virile hair growth with facial hair. It has also been reported that the risk of subsequent ovulatory infertility is enhanced by high BMI at age 18, even in females who are not deemed obese (Rich-Edwards et al, 1994). In pregnancies influenced by obesity, spontaneous abortion is more prevalent and this can happen in pregnancies arising from natural conception and therapy with assisted reproductive technology. Obesity has also been shown to significantly decrease fertility in the people and to significantly decrease pregnancy rates (Norman et al., 1998; Wang et al., 2000).

Obesity, especially abdominal obesity, is connected with Polycystic Ovary Syndrome (PCOS) (Franks, 1995). Two significant variables lead to PCOS in obese females, high estrogen production relative to their lean counterparts, and low sex hormone binding globulin manufacturing, which is followed by a large free biologically active fraction of estradiol. In the expression of PCOS, insulin resistance is regarded crucial and is strongly linked with abdominal obesity. Decreasing insulin levels directly or indirectly increases ovarian activity (Pettigrew et al., 1997)

The American Institute for Cancer Research and the World Cancer Research Fund revealed that compelling proof exists for a relationship between obesity and pancreatic, colorectal, postmenopausal breast, endometrial, kidney and esophageal cancers. They also discovered that excess abdominal fat increases the danger of pancreatic, endometrial, and postmenopausal breast cancer (Freedland et al., 2007).

Abnormalities in the response of DNA damage pathway associated with high BMI have been recorded. In young women, a reverse connection was discovered between BMI and nucleotide excision repair ability. Obesity was identified to change repair of double strand breaks caused by genotoxic agents. Obesity related improved production of reactive oxygen species could modify the response to DNA damage by impacting gene expression in repairing DNA. It has been recorded that DNA repair enzyme was inhibited by the oxidative stress (Figure 1.1). In obese individuals, differential expression of genes associated to stress response and toxic agent is observed (Wlodarczyk and Nowicka, 2019).

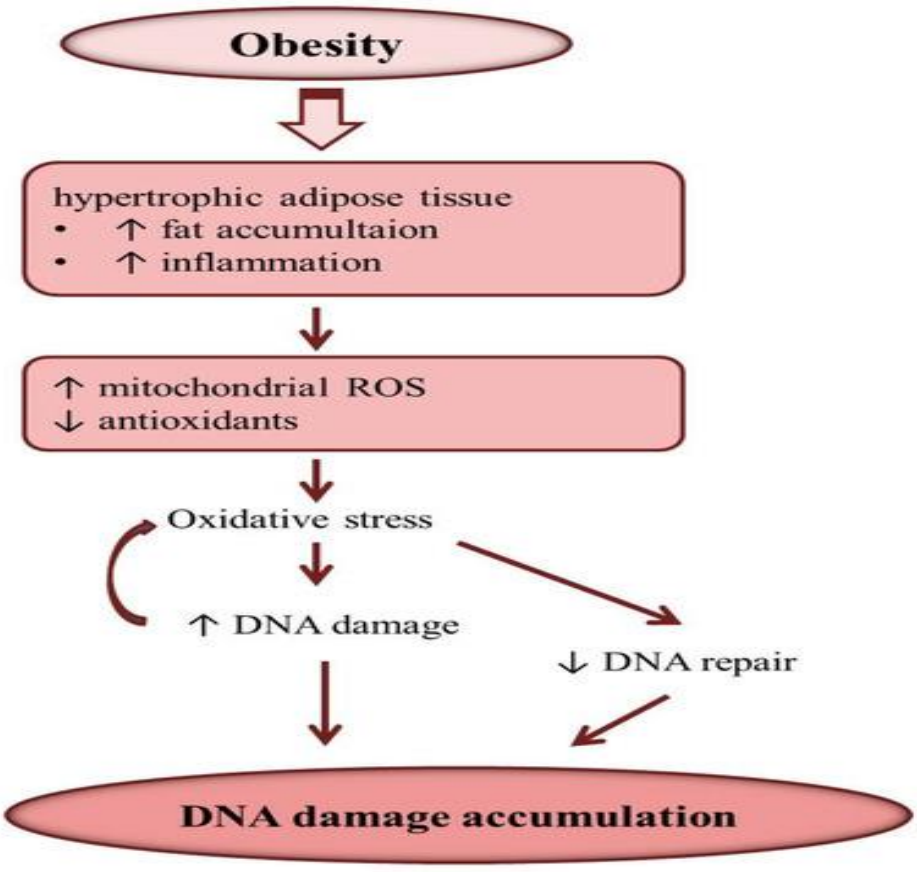

Figure 1.3 The impact of obesity in DNA damage (Wlodarczyk and Nowicka, 2019)

1.4 Genetics of Obesity

Obesity is classified into groups on the basis of suspected etiology (Herrera and Lindgren, 2010). Monogenic obesity is associated with loss-of-function mutations in a single gene. Leptin gene plays an important role in body weight management by reducing food intake and improving energy expenditure (Wasim et al., 2016). Deficiencies in the manufacturing of leptin cause early and serious hereditary obesity, lack of leptin circulation, and hyperphagia. Leptin operates by the LEPR. People with LEPR functional defects have phenotypic resemblances with LEP-deficient patients, such as fast weight gains with serious hyperphagia and endocrine defects in the first few months of life (Le Beyec et al., 2013). These patients are unable to benefit from recombinant leptin therapy because the receptor does not react to its ligand; serum leptin concentrations are high in these instances (Dubern et al., 2012).

Proopiomelanocortin (POMC) gene plays role in nutrition behaviour, with leptin strongly regulating the expression of POMC (Cheung et al., 1997). A frame shift mutation in the POMC gene was observed to cause function loss, leading in early obesity, adrenal insufficiency and red hair pigmentation (Flickinger et al., 1994).

Melanocortin 4 Receptor (MC4R) gene have an important effect in regulating the spending of appetite and energy, its dysfunction creates hyperphagia, impaired satiety. Severe obesity is found to be correlated with homozygous mutations (Hainerova et al., 2013).

Mutations of Proprotein Convertase 1 (PC1) gene primarily result serious early onset obesity, impaired processing of prohormones and hypocortisolaemia. Another characteristic is dysfunction of the small intestine, which can result from the incorrect maturation of propeptides in the PC1-secreting cells along the stomach (Farooqi et al., 2005).

In fasting or hypoglycaemia cases, Neuropeptide Y (NPY) secretion is inhibited after intake of food. Many studies indicated greater concentrations of NPY and body fatness for the Pro7 allele carriers. The polymorphism of Leu7Pro in the NPY gene tends to be involved in regulating lipid metabolism (Van Rossum et al., 2006).

In polygenic or common obesity affecting the general population, there is an interaction between several polymorphic genes.

β-adrenoceptor gene families (ADRB1, ADRB2, ADRB3) are extensively researched candidate genes for their involvement in regulating power spending (Shawky and Sadik, 2012).

In thermogenesis Uncoupling protein UCP1, UCP2 mediates mitochondrial proton leakage which releases energy stores as heat and thus affects the effectiveness of energy metabolism. It was suggested that uncoupled proteins behave as energy metabolism regulators. They are fatty acid transmembrane carriers in the mitochondria promoting proton exchange and because of their function in regulating fuel metabolism, UCP2 and UCP3 are regarded as candidate genes for obesity (Ochoa et al., 2004).

In syndromic obesity, individuals characterized by organ-specific developmental defects mental retardation and dysmorphic properties and categorized into two classes: pleiotropic syndromes and chromosomal rearrangements.

Bardet-Biedl Syndrome (BBS) it is autosomal recessive disorder characterized by central obesity, polydactyly, dystrophy of the rod-cone, learning disabilities, hypogonadism and kidney defects (Chiang et al., 2006).

GNASI mutations contributes to Albright's hereditary osteodystrophy characterized by obesity, circle shaped face, short, many hormone resistance and ossification of ectopic tissue (Bell et al., 2005).

Main features of Borjeson, Forssman and Lehmann Syndrome is obesity, mental retardation, hypogonadism, epilepsy, gynaecomastia (Lower et al., 2002).

Cohen Syndrome is an autosomal recessive disease with obesity, mental retardation, retinochoroidal dystrophy and microcephalus (Chandler et al., 2003).

Alstrom Disease is an autosomal recessive disease defined by childhood obesity connected with chronic hyperglycaemia, hyperinsulinemia and neurosensory defect. Other characteristics could be dilated cardiomyopathy, hepatic disorder, hypothyroidism, developmental delay (Shawky and Sadik, 2012).

Fragile X Syndrome is distinguished by mental retardation, macroorchidism, big ears, macrocephalus, prominent jaw, loud jocular speech and obesity (Shawky and Sadik, 2012).

Ulner Syndrome, comes with ulnar abnormalities, delayed puberty and hypoplastic nipples due to defects in the TBX3 gene situated at 12q24.1 (Bamshad et al., 1997).

Wilson – Turner Syndrome is associated by X-linked mental retardation, obesity, gynaecomastia, difficulty of expression, emotional variability, tapering fingers and feet (Wilson et al., 1991).

Mehmo Syndrome is X-linked disorder with mental retardation, epileptic seizures, hypogenitalism, microcephalus and obesity (Leshinsky-Silver et al., 2002).

Prader-Willi Syndrome is the most common syndrome with an incidence of about one in 25000 births and a population incidence of one in 50000, with obesity, hyperphagia, hypotonia, mental retardation. It is generally triggered by the absence of the paternal section 15q11.2-q12 (Shawky and Sadik, 2012).

Sim-1**,** is characterized with hyperphagia, early-onset obesity and translocation among 1p22.1 and 6q16.2, that interfere the SIM-1 gene on 6q chromosome (Faivre et al., 2002).

1.5 Immune System and Obesity

The immune system prevents the body against microorganisms by means of innate or natural (non-specific) immunity and acquired (particular) immunity requiring prior exposure to pathogens (Abbas et al.,2000). To create an integrated defense, these two main branches operate in a coordinated manner. Stimulation of T-helper cells generate cytokines by antigen (IL-2), which encourages the development and macrophage differentiation in addition to the interaction between T-helper and B-cells due to manufacturing of B-cells by immunoglobulin (Lomas et al., 2002). Leptin, adiponectin and pro-inflammatory cytokines are the main adipose derived immunomodulatory adipokines (Fantuzzi, 2005). During obesity Adiponectin levels were decreased and change natural killer cell cytotoxicity and cytokine production by human myeloid cell (Wolf et al., 2004). Conversely, TNFα, IL-6 and IL-1b are excessively produced in obese white adipose tissue (Tilg et al., 2006). Cytokines

could be excreted into blood and may have distal impacts, though, it continues to be clarified precisely how chronic manufacturing of these cytokines affects cellular immunity. Chronic exposure to pro-inflammatory cytokine could desensitize immune cells during an actual infection to inflammatory reactions (Ziegler-Heitbrock et al., 1994). The pleiotropic impact of leptin on immune cell activity are greatly diverse and complicated (La Cava et al., 2004). Nearly all innate immune system cells express the leptin receptor isoform, obRb, needed for leptin signaling (Lord et al., 1998).

Immune cells and adipocytes are neighbors in lean adipose tissue and communicate to keep homeostasis and regulate the processing and storage of adipocyte lipids. Invariant Natural Killer T cells (iNKT) cells, eosinophils, regulatory T cells (Tregs), B-cells generating IgM, and alternately activated M2 macrophages were the primary resident immune cells. IL-10 manufacturing by iNKT, Tregs, and M2 macrophages as well as eosinophil development of IL-4 is essential for the conservation of a tolerogenic setting. There is a reduction of iNKT cells and Tregs throughout adipose growth in obesity and a phenotypic shift in M2 to M1 macrophages that accumulates between overloaded and ruptured adipocytes (Figure 1.2) (Exley et al., 2014).

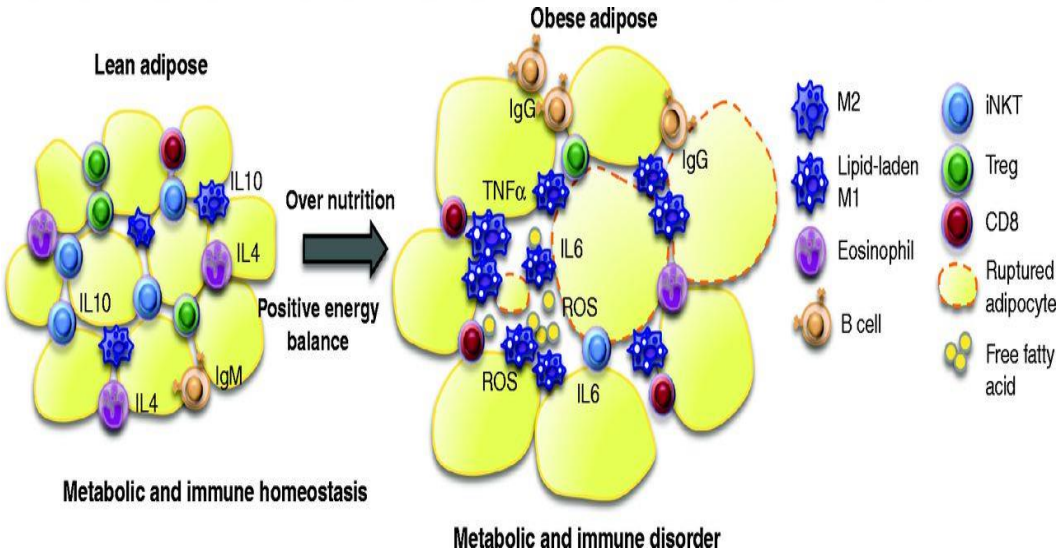

Figure 1.4 Adipose tissue in lean and obese individuals (Exley et al., 2014)

In monocytes, leptin controls the production of IL-6, IL-12 and TNFα pro-inflammatory cytokines and activity of phagocyte (Loffreda et al., 1998). In healthy people's polymorphonuclear neutrophils, induced leptin chemotaxis, reactive oxygen

species production and oxidative capability is affected (Caldefie-Chezet et al., 2003). Leptin signaling affects natural killer cells (NK) proliferation, differentiation, activity and activation aspects. (Zhao et al., 2003). The function of leptin is important for the cells of innate immunity, because almost all cells of innate immunity are disabled with the lack of intact leptin signal (Justin et al., 2012). Leptin signaling similarly affects the adaptive arm of immunity responses (Papathanassoglou et al., 2006). Throughout T-cell maturation leptin is a significant source of signals of pro-survival to single-positive and double-positive thymocytes. (Howard et al., 1999). Leptin perform a major part in lymphopoiesis and myelopoiesis since ob / ob had about 60% as many nucleated cells in the bone marrow comparable to wild-type controls (Claycombe et al., 2008). Leptin can boost T-cell production in the appearance of a polyclonal stimulator and it can modify activation marker expression on CD4 + and CD8 + T-cells (Martı´n-Romero et al., 2000). Leptin has deep impacts on cell function by acting as an immune cell metabolism regulator (Maciver et al., 2008).

Furthermore, obese people have already shown to have enhanced predisposition to infections, bacterial infection (such as periodontal inflammation, nasal carriage of Staphylococcus aureus and gastric infection by Helicobacter pylori) (Perdichizzi et al.,1996). A preliminary search also showed that obesity is linked to herpes simplex virus 1 disease measured by seropositivity (Karjala et al., 2007). Regarding the enhanced risk of multiple types of microbial infections, few are understood about how obesity can change antimicrobial drug pharmacokinetics (Hanley et al., 2010). Obesity is correlated with bad antibody reaction to the plasma vaccine for hepatitis B (Weber et al., 1985).

In the last decades, the importance of nutritional lipids on immune function has been studied extensively (Calder, 1998). Dietary fat can boost cancer incidence by disturbing the immune system's tumor control processes. Animal studies show that obesity and consumption of high fat diet negatively impact on immune response and increase the risks of severe infections. Dietary lipids are especially relevant throughout the maintenance of polyunsaturated fat tissue levels, such as linoleic acid that couldn't be manufactured in body and is needed by lymphocytes for proper function. A reduced incidence of atherosclerosis was correlated in those who consume diets rich in monounsaturated fatty acids (MUFAs) detected in animal meat

like red meat, whole-milk goods, seeds, and high-fat fruit including olives and avocados (Yaqoob, 1998). Very little interest was given to the immune system impacts of MUFAs. Immune cells are an inherent feature of the inflammatory incidents engaged in atherosclerosis but evidence suggests that a diet rich in MUFAs results in a reduction in expression of intercellular adhesion molecule-1 (ICAM-1), a protein engaged in leukocyte – leukocyte adherence and also in leukocyte endothelial cell adherence. It also appears that MUFAs reduce expression of another associated protein, the main leukocyte fibrinogen receptor (Mac-1), that could perform a part in inflammation pathophysiology. MUFAs, on the other hand, do not tend to affect NK cell activity or T-lymphocyte proliferative response. Though, several reports revealed reduced proliferation of T-lymphocytes and reduced cytotoxic T-lymphocyte activity following a high-fat diet in n-6 PUFAs (Calder, 1998). It has also been discovered that NK cell cytotoxic activity is smaller. There was a reverse linear relationship between the concentration of the ratio of oleic acid: linoleic acid as well as the cytotoxic function of spleen NK. It was also demonstrated that consumption of n-6 PUFAs decreases output of IL-2 and IFN-g. Diets that are rich in n-3 PUFAs were associated with cell-mediated immune response inhibition, but the processes are still uncertain. N-3 PUFAs have been observed to prevent the role of human antigen-presenting cells involved in antigen identification. This is assisted by discoveries that diets rich in n-3 PUFAs have reduced ability to display antigens to autologous lymphocytes linked to reduced cell adherence molecules expression. Incidentally, this information indicates a prospective mechanism for the positive impact of n-3 PUFAs in the therapy of rheumatoid arthritis, an autoimmune modification connected with high MHC class II expression and antigen-presenting cell adhesion molecules. In addition, diets high in n-3 PUFAs during infection with Listeria monocytogenes have been recorded to generate greater serum levels of IFN-g. IFN-g strongly controls inducible nitric oxide synthase (iNOS) in order to promote macrophagemedia death of intracellular pathogens and the expression of type I and II MHC molecules to promote targeted immune response.

Epidemiological data promote the concept that obesity is linked to changes in immune function, yet for some parameter's information are controversial (Samartı´n and Chandra, 2001). Fat mass rises in obesity. Both adipocyte and immune cell that massively infiltrate adipose tissue excrete elevated adipokine concentrations, which

are accountable for a pro-inflammatory state and the Th17/Treg equilibrium in addition, obesity is correlated within the adipose tissue with lower B-regulatory and unchange NK cells.

Under obesity conditions, blood concentrations of Apoptosis Inhibitor of Macrophage (AIM) rise. Firstly, AIM induces lipolysis and thus saturated fatty acids are produced. In addition, saturated fatty acids can stimulate an inflammatory NLRP3 that secretes IL-1β and IL-18, both of which are engaged in autoimmune disorder pathogenesis. Secondly, AIM forms immune complexes with autoantigen-associated natural autoreactive IgM, supporting retention on follicular dendritic cell. The next introduction of autoantigens to follicular B-cells causes IgG autoantigens being produced. Obesity was discovered to encourage a profile of Th17, a subset of immune-mediated disorders pathogenesis. The Western diet, partly liable for obesity, could also trigger dysbiosis, a change of the gut microbiota, leading to modulation of additional intestinal immune responses as well as subsequent deregulation of the Th17/Treg equilibrium. Obese individuals have a greater incidence of deficiency in vitamin D. Lower concentrations of vitamin D were correlated with enhanced Th17 cells, B-cells, antibodies secretion and decreased Treg cells (Figure 1.3) (Versini et al., 2014).

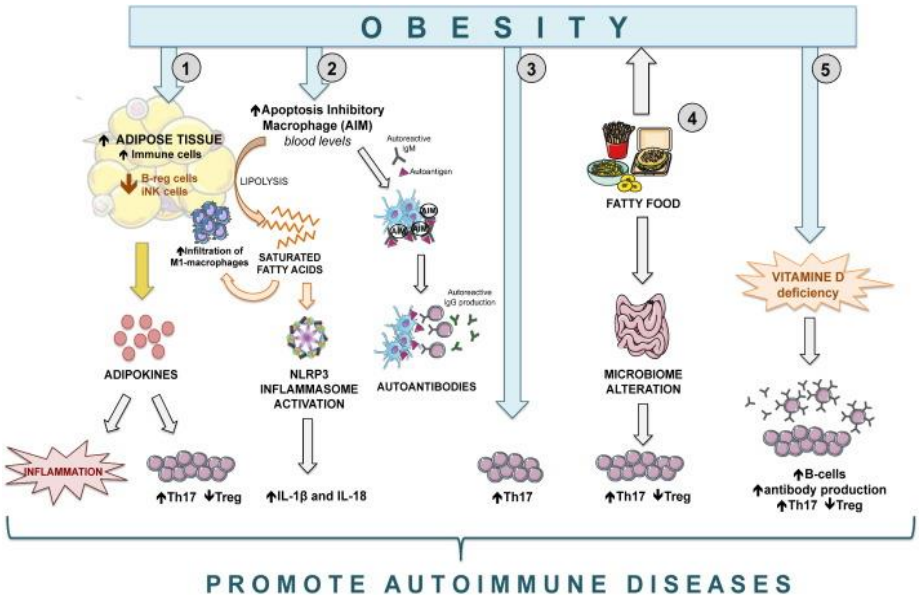

Figure 1.5 Mechanisms promoting autoimmune diseases in obesity (Versini et al., 2014)

One of the issues in evaluating immune function in obese people would be that the impact of obesity on the immunity could be covered by existence of dyslipidemia and hyperglycemia. Findings that exclude obese people with diabetes, hyperlipidemia or insulin resistance might erase these confounding factors. As with animal models, many studies confirm a reduced lymphocyte ability to proliferate in response to activation of mitogen. Insulin receptor formation on T lymphocytes is decreased in obese individuals after in vitro stimuli (Nieman et al., 1999). So, this reduced expression might also play a part in the defect of the activities of T-lymphocytes. Recent surveys show that leukocyte and lymphocyte subgroups are high in obese people (Nieman et al., 1999). Other research, however, reported T-lymphopenia in obese people (Tanaka et al., 1993). This lymphopenia appears to be associated with greater BMI and manufacturing of in vivo TNF-a. Obesity in older males and females is linked with a decreased activity of natural killer cells. An adverse correlation has been created between body fat as well as the activity natural killer cell in older females and adult males (Nieman et al., 1999). A beneficial connection was noted in infants between body weight and lower respiratory tract inflammation. Possible explanations for the increased prevalence would include mechanical variables affecting pulmonary functions and defective immune condition associated to the function of cell-mediated immunity and phagocytes. Obesity did not affect monocyte and granulocyte phagocytosis, whereas higher concentration of activated monocyte, basal monocyte as well as basal granulocyte oxidative bursts were found in obese individual. In addition, there's several investigations that evaluate the immune response after losing weight or dietary deprivation in obese individuals, indicating that immune impairment can be fixed with appropriate weight control. Following a mild restriction on energy, obese individuals have a smaller mitogen-stimulated response proliferation and a reduced oxidative monocyte burst and NK cell amounts, but not the amounts of T- and B-cell (Nieman et al., 1996). Whereas the overall number of lymphocytes after a weight loss program did not alter, the response of T-lymphocytes to distinct mitogens and B-lymphocyte blastogenesis were elevated at the end of the nutritional restriction era (Tanaka et al., 2001). Fasting blood glucose and triglyceride levels were mildly decreased throughout weight loss period. These findings indicate that an increase in the physiological environment throughout weight loss can lead to an enhancement in

immune function. After individuals have reached and retained normal weight, a beneficial impact on the immune response may be noted over the long term.

Further study in this region is warranted before significant findings can be reached because the impacts of short-term weight loss on immunity in obese individuals have been evaluated by most study. In addition, it is necessary to address the interacting impact of psychological stress modifications with losing weight on immune function. These findings show that some function of the host defense mechanism in the obese person tend to be enhanced by dietary limitation.

Natural killer (NK) cell is cytotoxic lymphocytes, which is essential element of innate immune system. Jost and Altfeld (2013) suggest that NK cells were involved in the regulation of viral infection, acute myeloid leukemia as well as neuroblastoma, herpes viruses, poxviruses and human immunodeficiency virus (HIV) (Costello et al., 2002).

NK cell activation is defined by the interplay for both inhibitory and activating signals transmitted from a range of cell surface receptors that include the immunoglobulin-like killer cell family (KIR) in humans (Moradi et al., 2015). The human killer cell immunoglobulin-like receptors (KIR; also recognized as CD158) which is a class of transmembrane glycoproteins expressed on NK cell as well as a subgroup of T cell (Vilches et al., 2002). KIR regulates the growth, resistance and activation of NK cells (Caligiuri, 2008). As well as KIRs contribute to the immunoglobulin class, as well as two or three extracellular immune globulins are structurally differentiated as domains. KIRs primarily acknowledge MHC type I alleles such as HLA-A, HLA-B and HLA-C classes (Pende et al.,1996). Two functionally different KIR sets are available: inhibitory and activating. Every set has the same extracellular domain, thus connecting each set to the same ligands. Furthermore, due to variations in transmembrane and cytoplasmic domain or intracellular domain, inhibitory response is signaled by one class of KIRs and the activity response is signaled by the other class of KIRs, after attaching to identical MHC group I alleles (Biassoni et al., 1996). Selvakumar et al (1997) revealed that the Natural Killer receptor family of KIR, found on chromosome 19p13, involves 14 members as well as a number of allelic variations, six of those are inhibitory also six are activator. These are monomeric (single chain) receptors either with 2 (KIR2D) or

3 immunoglobulin-like domains (KIR3D), that can be further classified into long cytoplasmic tails (L) and short cytoplasmic tails (S). The long tail KIRs produce an inhibitory transmitter, however, the short tail KIRs produce an activation transmitter. The inhibitory signal outcome from the existence of immunoreceptor tyrosine-based on immunoreceptor tyrosine (ITIMs) in the long tail receptor cytoplasmic domains.

Table 1.1 KIR Genes and Associated HLA Ligands (Campbell and Purdy, 2011)

KIR Genes	HLA ligands
KIR2DL1	HLA-C Group 2
KIR2DL2	HLA-C Group 1, and some HLA-C Group 2 and HLA-B
KIR2DL3	HLA-C Group 1, and some HLA-C Group 2 and HLA-B (weaker affinity
KIR2DL4	HLA-G
KIR2DL5	unknown
KIR3DL1	Bw4 epitopes among HLA-B allotypes and some HLA-A
KIR3DL2	Certain HLA-A allotypes
KIR3DL3	unknown
KIR2DS1	HLA-C Group 2
KIR2DS2	HLA-C Group 1
KIR2DS3	HLA-C Group 1
KIR2DS4	HLA-C (some of both Group 1 and 2), A*11
KIR2DS5	Unknown
KIR3DS1	HLA-Bw4

1.6 KIR2DL4 Gene

KIR2DL4 is atypical KIR which varies by other family members in terms of cellular location, signaling, specificity of ligands, as well as protein function. The structure of KIR2DL4 is unique from many KIRs (besides KIR2DL5) in that domain D1 is unavailable; alternatively, it still has a domain structure D0-D2. KIR2DL4 have

inhibitory as well as signaling domains in a unique way (Faure et al., 2002). It is also known as "framework gene," since all haplotypes are available. In other words, KIR2DL4 includes a single cytoplasmic motif domain of tyrosine- based inhibition immunoreceptor and a fundamental arginine residue throughout the transmembrane domain. Rather than combining with DAP12, though, KIR2DL4 uses the chain from FceR to transmit stimulating signals (Kikuchi-Maki et al., 2005).

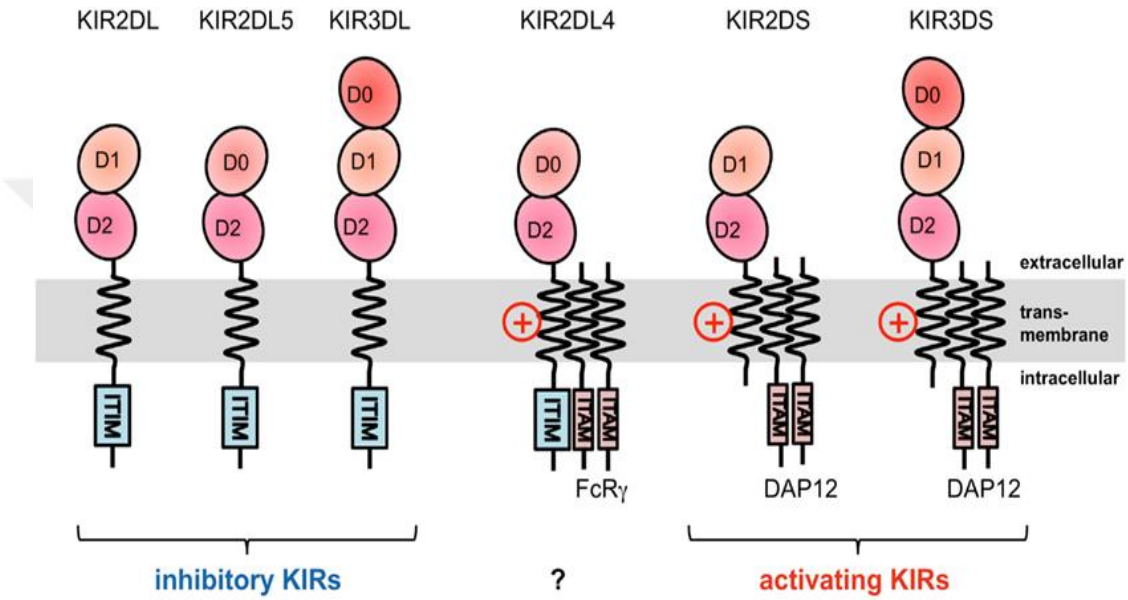

Figure 1.6 Different configurations of the KIRs (Kuroki et al., 2012)

KIR2DL4 has an ITIM pattern, yet it connects with the FcRγ chain. KIR2DL4 mainly signals from endosomes (Rajagopalan, 2010). KIR2DL4 has been the only KIR to be transcribed into practically most of the analyzed human IL-2 cultured NK cell clones. Though, receptor expression on surface of newly isolated peripheral blood NK cells is limited to the small sub-set CD56 as well as uterine NK cells, but IL-2 culture upregulates surface expression on nearly all cells within 2 weeks (Goodridge et al., 2003).

In addition, prevalent polymorphisms in a substantial portion of the human population inhibit surface expression completely (Goodridge et al., 2007). Unlike other active or inhibitory members of the KIR group who control cytotoxicity of NK cells and cytokine manufacturing, KIR2DL4 induces potent cytokine output but poor

cytotoxicity (Rajagopalan et al., 2001). Interestingly, KIR2DL4 has structural features of active as well as inhibitory KIR by having single cytoplasmic ITIM as well as a charged residue of transmembrane arginine (Cantoni et al., 1998). KIR2DL4 could communicate materially with FceRI-π transmembrane adaptor protein, that is thought to mediate via arginine transmembrane residue to transmit activating signals. (Kikuchi-Maki et al., 2005). The ITIM domain, on the other hand, can activate Src homology domain involving tyrosine phosphatase 2 (SHP-2) and can mediate negative signals (Faure et al., 2002).

There is little information about signal transduction pathways caused by KIR2DL4 to generate functional responses. KIR2DL4's only recognized ligand is soluble-HLA-G, generated by trophoblast cells derived from fetus. Signals that cause uterine NK cell activation is unclear, however in this mechanism, KIR2DL4 connection with HLA-G can perform a significant part. KIR2DL4 is expressed in several decidual / placental, yet not peripheral, NK cells throughout pregnancy (Ponte et al., 1999). Both rodents and humans have restricted cytolytic capacity in uterine NK cells, and yet generate cytokines such as IFN-α as well as TNF-α (Van der Meer et al., 2004). IFN-π generated by uterine NK cells of the mouse was involved in supporting the remodeling of the maternal artery vasculature needed for ordinary pregnancy (Ashkar et al., 1999). Controlled surface expression, divergent composition, distinctive functional characteristics, and evolutionary preservation indicate, special biological functions for KIR2DL4 throughout pregnancy.

1.7 Aim of the Study

The aim of this study is to analyze rs660773 G>A (intron 7), rs660437 C > A (intron 7) and rs649216 C >T polymorphisms in KIR2DL4 gene in obese and normal weight control group and consequently to search for an association of these polymorphisms with obesity.

CHAPTER II

LITERATURE REVIEW

Vgontzas et al. (1994) studied sleep apnea in 200 obese females and 50 obese males (BMI ≥ 45.3) and 128 control group. It was shown that obese males and femals were at extreme risk for sleep apnea.

In a cohort study, approximately 135,000 male construction employees were involved and a positive correlation was found between weight, body mass index (BMI) and lean body mass (LBM) with the prostate cancer risk (Andersson et al.,1997)

Nieman et al. (1999) showed that immune function differed between 116 obese and 41 non-obese individuals and obese people had high leukocyte and lymphocyte range, suppressed mitogen-induced lymphocyte proliferation (an index of T- and B-cell function), increased monocyte and granulocyte phagocytosis and oxidative burst activity, and normal NK cell activity.

Obesity was reported to be a major health risk for the creation of osteoarthritis (OA) in hand and knee joints, with a substantial increase in risk of OA per kg of body weight by 9-13% (Cicuttini et al., 2001).

Middle-aged African American females have greater SAT depots, adjusted for overall body fatness, but with respect to VAT, they do not differ from white women. The intensity of the abdominal fat / metabolic risk relation is enhanced by ethnic differences in this connection (Lovejoy et al., 2001)

Yiannakouris et al (2001) explored the impact of exonic LEPR gene polymorphisms and showed that Q223R polymorphism was an essential predictor of 5% variation in body composition. The distributions of allele and genotype frequencies of other two polymorphisms were not significantly different in control and obese group.

Morimoto et al. (2002) studied the risk of postmenopausal breast cancer in 85917 females (50-79 years old). They reported that obesity was a significant risk factor for postmenopausal breast cancer, but only among females who had never took hormone replacement therapy (HRT).

Tsai et al. (2004) found an important correlation between abdominal adiposity and gallstone disease frequency. The abdominal circumference and WHR could be used to estimate the risk of developing gallstones irrespective of the body mass index as indicators of abdominal adiposity.

Droyvold et al. (2005) showed the relationship between high level of BMI and hypertension. They suggested that the elevated BMI has an independent impact on the change in systolic and diastolic blood pressure in both female and male. Individuals having high BMI value also have a higher risk of hypertension.

A research by 218 non-smoking male showed that non-alcoholic fatty liver disease (NAFLD) is correlated to exercise deficiencies and high BMI independently (Church et al., 2006).

Pischon et al. (2006) suggested that obesity is associated with renal cell carcinoma irrespective of female's fat distribution, while low hip circumference is linked to the increased male's risk of renal cell carcinoma.

Santin et al. (2006) demonstrated that there was no relationship between KIR gene and T1DM in Basque population.

Reeves et al. (2007) reported that 5% of all cancers in postmenopausal female in UK, results from being overweight or obese. They concluded that increased BMI is correlated with a substantial increase in cancer risk.

A cross-sectional research with 7078 samples was conducted. Subjects had upper endoscopy throughout health checks to demonstrate visceral obesity and metabolic syndrome as a risk for reflux esophagitis. It was reported that metabolic syndrome is linked to reflux esophagitis and abdominal obesity, particularly visceral obesity, is a significant risk for reflux esophagitis (Chung al., 2008).

Li et al. (2009) demonstrated that obesity is a risk factor for pancreatic cancer. They studied 841 pancreatic adenocarcinoma patient and 754 control subjects and found that early adulthood obesity is linked to higher risk of pancreatic cancer and a younger age for onset of disease. Obesity at an old age in patients with pancreatic cancer was associated with reduced overall survival.

In a cohort study, Munger et al. (2009) examined the association of multiple sclerosis and body size in females from US. It has been shown that the obese females were more likely to develop multiple sclerosis.

The incidence of colorectal adenomas increased in both genders with an increased level of visceral adipose tissue. Waist circumference was found to be correlated with colorectal adenomas in male, but not in female. High visceral adipose tissue level was positively correlated with multiple adenomas (Nam et al., 2010).

In a cross-sectional study of US adults diagnosed with diabetes, the researchers demonstrated a link between rising obesity and increased diabetes prevalence (Nguyen et al., 2010)

Ozturk et al. (2012) studied the KIR gene polymorphisms in 33 breast cancer patients and 77 control subjects. They observed the presence of frame genes in both groups and activation of KIR2DS1 were much greater in breast cancer group than in control group.

In Brazil, Vairo et al. (2012) studied the variability of KIR genes in individuals with Gaucher disease and explored no significant variations in the frequency of the KIR genes between the patient and control groups.

A meta-analysis from 24 prospective researches with 41791 cases, reported that obesity is not linked to the risk of gastric cancer. However, there was a positive relationship between high BMI and the risk of gastric cardiac cancer, but not with gastric non-cardiac cancer (Chen et al., 2013).

Le Page et al. (2013) determined the KIR2DL4 genotypes in two groups of children and they found that KIR2DL4 9A/10A polymorphism has no effect on asthma.

Smith et al. (2014) studied the KIR genes and their ligands in childhood acute lymphoblastic leukemia. They include 212 patients and 231 control subjects to the study and showed that the KIR genotype was substantially correlated with enhanced disease incidence.

Erken et al. (2015) investigated the KIR genotype distribution in Familial Mediterranean Fever (FMF) patients and observed that the KIR genes were found in all patients and controls. In addition, KIR2DP1 was found in all FMF patients and 95% of control. Other than KIR2DS4, the frequency of activator KIR genes were smaller than 58 percent, whereas inhibitory genes KIR2DL1 and KIR3DL1 were common in all samples.

In non-hispanic white children, novel genetic connections have been identified between INSIG2 and obesity/LDL. The influences of polymorphisms in INSIG2 on lipid regulation could be essential in the growth of obesity (Kaulfers et al., 2015).

KIR2DL4 gene polymorphisms were studied in 100 patients with preeclampsia and 100 healthy pregnant females and it was found that the KIR2DL4 gene single nucleotide polymorphism is not associated with pre-eclampsia susceptibility. However, the reduced KIR2DL4 quantity may be associated with pre-eclampsia occurrence (Wang et al., 2015).

In a meta-analysis, 470 colorectal cancer patients and 483 control cases were investigated and it was found that KIR2DS5, KIR2DL4, KIR3DL2, KIR3DL3 and both pseudogenes were present in nearly all individuals (Ghanadi et al, 2016).

In China, He et al. (2016) investigated the expression of KIR2D (L1, L3, L4, S4) and KIR3DL1 in non-small cell lung cancer and they observed a high expression in patients.

Hirose et al. (2016) investigated Leptin's function in hypertension linked to obesity among 348 teenager male (15–17 years of age) and 165 men (40–59 years of age).

Nowak et al, (2016) investigated the polymorphisms of KIR2DL4, HLA-G, LILRB1 genes in spontaneous miscarriage in 277 couple samples. They could not associate KIR2DL4, LILRB1 or HLA-G genotype with the risk of spontaneous abortion in both women and their partners.

In Saudi Arabia, Al-Sharif (2017) analyzed 100 obese asthma patients and 100 non-obese asthma patients, and discovered a high connection between inflammatory cytokines, immune system reaction and obesity in asthma patients.

Bylinska et al. (2017) studied the effects of HLA-G, KIR2DL4, LILRB1 and LILRB2 gene polymorphisms on endometriosis disease. They included 276 endometriosis patients and 314 healthy females to the study and found no impact of KIR2DL4 of polymorphism on disease. The findings supported the role of HLA-G and LILRB1, LILRB2 polymorphisms in endometriosis.

Chathoth et al. (2017) stated that UCP1 and NPC1 gene polymorphisms could be related with obesity. They found that UCP1 gene promoter polymorphism has significant connection with obesity. In contrast, polymorphisms of NPC1 were not found to be a major risk for obesity.

Dar et al. (2017) searched for the connection between obesity and rheumatoid arthritis (RA). They studied 11406 RA patients and 54701 controls and showed that obesity is strongly related to RA.

Liu et al. (2017) investigated the connection of KIR gene polymorphisms with type 1 diabetes mellitus susceptibility. They studied 2076 patients with T1DM and 1976 healthy individuals and found a negative association with KIR2DL4 and other KIR genes.

Martinez-Barquero et al. (2017) investigated five polymorphisms in IL18RAP gene linked to the IL-18 adipogenic signaling pathway. They observed that the rs7559479 was related to greater BMI and a higher risk of obesity.

The association of obesity and the risk of systemic lupus erythematosus was investigated among females and a considerably enhanced risk of SLE was found among obese females compared to ordinary BMI females (Tedeschi et al., 2017).

Almeida et al. (2018) explored that BMI was strongly correlated with MC4R rs17782313 and FTO rs9939609, with mild and very weak impacts, respectively, suggesting a quite limited effect to childhood obesity. LEPR rs1137101 and PPARG-2 rs1801282 had low and moderate adverse impacts on BMI, respectively, and may provide slight protection against obesity in children.

Bähr et al. (2018) demonstrated that there is no substantial alteration in the incidence of monocytes, B lymphocytes, or natural killer T cells, but T lymphocyte frequency is considerably elevated in obese people.

Wang et al. (2018) studied 9 SNPs selected in the genes SLC15A1 and PLA2G16 in 611 individuals and discovered that they were not related to obesity.

Tupikowska-Marzec et al. (2019), found that the FTO gene rs9939609 polymorphism is correlated with psoriasis and more serious inflammation. They determined a risk allele related with enhanced intensity of BMI, WHR and insulin. Holding the FTO rs9939609 risk allele could potentiate insulin resistance as a result of coexisting obesity in patients diagnosed with PS.

CHAPTER III

MATERIAL AND METHODS

3.1 Material

Fifty normal (BMI<30) and 50 obese (BMI>30) individuals participated to the study. Samples were obtained from Department of General Surgery, Faculty of Medicine, SANKO University. Bloods samples were collected in EDTA tubes and stored at +4°C until DNA isolation.

This study was approved by the Ethics Committee of Gaziantep University and supported by Scientific Research Projects Governing Unit of Gaziantep University (Project no: FEF.YLT.18.22).

3.2 Methods

3.2.1 DNA Isolation

Invitrogen by Thermo Fisher Scientific Pure Link Genomic DNA mini kit was used to isolate DNA from the blood samples.

1. ≤200 µL samples collected in EDTA tubes were added to the eppendorf tubes.
2. Firstly 20 µL Proteinase K was added to the eppendorf tubes, then 20 µL RNase was added. The vortex was used in order to mix well (10-20 sec) and the samples were incubated for 2 minute.
3. 200 µL PureLink ® Genomic Lysis / Binding Buffer was added and blended with vortexing for protein digestion, then the samples were incubated at 55 °C for 10 minutes.
4. 200 µL 96–100% of ethanol were added to the tubes then blended by vortexing for 5 seconds.
5. For binding of DNA, ~700 µL of lysate was added to the PureLink® Spin Column. Then samples were centrifuged at 10.000 rpm for 1 min. After that,

the collection tubes were removed and the spin columns were placed into clean collection tubes.

6. For the purpose of washing DNA, 500 μL wash buffer 1 was added to the column then the centrifuged at room temperature at 10.000 rpm for 1 minute. Then 500 μL wash buffer 2 was added to the column and centrifuged at maximum velocity for 3 minutes.
7. 60 μL of PureLink® Genomic Elution Buffer was added to the column and tubes were incubated for 1 minute. Then the samples were centrifuged at maximum velocity for 1 minute. DNA was collected in tubes. This process was done again to obtain more DNA.
8. DNA concentrations were determined by using Nanodrop device. Purified DNA samples were kept at -80 °C until use.

3.2.2 Amplification of KIR2DL4 by PCR

In this study; rs649216-9571 C >T, rs660437-9769 C >A (intron 7) and rs660773-9797 G >A (intron 7) polymorphisms were studied in both obesity and control groups.

Table 3.1 Primer sequences used for PCR amplification of KIR2DL4 gene

GENE	PRIMER SEQUENCE	PCR PRODUCT
KIR2DL4	F 5' -CCT GCA TGC TGT GAT TAG GTA C-3' R 5 '-CGT GAG GAT ACA GTT CAC AAT -3'	297 bp

Table 3.2 Reaction components for PCR amplification of KIR2DL4 gene

Component	**Volume (**µl)
10xTaq buffer with $MgCl_2$	5
dNTP mix	4
Primer KIR2DL4-F	1
Primer KIR2DL4-R	1
DNA sample	2
Taq DNA polymerase	0.2
H_2O	36.8
Total volume	50

Table 3.3 Program used for PCR amplification of KIR2DL4 gene

	1	**2**			**3**	**4**
No. of cycle	1	35			1	-
Temperature (°C)	94	94	55	72	72	4
Time	5 min	40 sec	40 sec	40 sec	5 min	∞

3.2.3 Agarose Gel Electrophoresis

1. 2 g of agarose was measured and mixed by 100 mL 1xTBE.
2. Mixture was heated in microwave until the agarose was completely dissolved.
3. Agarose solution was let cool down then ethidium bromide added to the gel.
4. The agarose gel was poured to gel tray and left for 45 minutes. The combs removed from the gel after polymerization.
5. 5 µL loading dye and 8 µL PCR product were mixed and loaded into the wells of gel carefully without spilling the sample into the adjacent wells.
6. The first well of gel was filled with ladder (Gene ruler 50 bp DNA ladder) and other wells filled with samples.
7. The gel tank was closed carefully and run at 120 Volt for 30 minutes.
8. For separation of restriction fragments the samples were electrophoresed for 40 minutes in 100 Volt.
9. The electrophoresis products were visualized by UV light.

3.2.4 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) technique is used to detect DNA fragments after digestion with restriction enzymes.

Table 3.4 Restriction enzymes used in the study

Polymorphism	Restriction Enzyme
rs649216-9571 C >T	Earl
rs660437-9769 C > A (intron7)	BspMI
rs660773-9797 G >A (intron7)	HpyCH4III

For the analysis of rs649216-9571 C >T polymorphism, the samples were incubated at 37°C for 5 hours and restriction products were separated by agarose gel electrophoresis (3%, at 100V for 30 minutes).

For the analysis of rs660437-9769 C > A polymorphism, the samples were incubated at 37°C for 16 hours and restriction products were separated by agarose gel electrophoresis (4%, at 80V for 75 minutes).

For the analysis of rs660773-9797 G>A, the samples were incubated at 65°C for 3 hours and restriction products were separated by agarose gel electrophoresis (3%, at 90V for 60 minutes).

Table 3.5 Genotyping of KIR2DL4 polymorphisms by RFLP

Genotypes	Restriction product/s (bp)
rs649216-9571 C >T	
TT	297
CT	297 253 44
CC	253 44
rs660437-9769 C > A	
CC	297 150 147
CA	297 147 103 47
AA	147 103 47
rs660773-9797 G>A	
AA	297
GA	297 286 11
GG	286 11

3.3 Analysis of Data

Allele and genotype frequencies were calculated by direct counting. Genotype distributions of polymorphisms in patient and control group were compared by chi-square (χ^2) analysis. “SPSS 22.0 for Windows” program was used for statistical analysis.

CHAPTER IV

RESULTS

4.1 Demographic and Clinical Data

Fifty obese and 50 control individuals participated to this study. Age, gender, weight, height and BMI values of patients and control subjects were recorded (Table 4.1 and Table 4.2).

After isolation, DNA concentrations were measured by Nanodrop. DNA concentrations of obese group were ranged between 31.6 and 267 ng/µl, and the average concentration was 77.60 ng/µl. The DNA concentrations of control group were ranged between 27.56 and 399.6 ng/µl and the average concentration was 71.46 ng/µl.

The female/male ratio in both patient and control group was 1:1. Twenty-five men and 25 women were included in each group.

The ages of patients ranged between 18-68 years, while the mean age was 41.64 ± 10.62. The age in control group were between 18-60 years, the mean age was 41.64 ± 10.62.

The weights of patients were between 76-154 kg with the mean weight of 114.3 ± 18.68. The weights of control subjects ranged between 44.93-88.98 kg, with the mean value of 60.46 ± 8.94.

The heights of patients were between 2.4-3.42 m^2, with the mean height of 2.86 ± 0.25. Control subjects have the heights ranged between 2.34-3.42 m^2, with the mean value of 2.77 ± 0.26.

The BMI of patients were between 30.1- 64.16 kg\$m^2$ (mean value: 40.02 ± 6.6) while the control individuals had BMI between 18-26 kg\$m^2$ (mean value: 21.7 ± 2.04) (Table 4.3).

Table 4.1 Age, gender, and BMI information of obese group

Number	Age	Gender	Weight\kg	Height/m^2	BMI kg\m^2
1	32	F	87	2.62	33.2
2	60	F	82	2.56	32.03
3	20	M	90	2.99	30.1
4	45	F	88	2.65	33.2
5	28	F	86	2.72	31.61
6	32	F	88	2.75	32
7	39	M	120	3.38	35.5
8	23	F	80	2.65	30.18
9	33	M	90	2.99	30.1
10	38	M	92	2.89	31.83
11	68	M	95	2.82	33.6
12	35	M	120	3.24	37
13	37	F	112	2.65	42.26
14	47	F	92	2.4	38.33
15	18	M	105	3.42	30.7
16	20	F	110	2.56	42.96
17	47	F	76	2.4	31.6
18	38	F	123	2.89	42.56
19	46	F	103	3.42	30.11
20	33	F	114	2.56	44.53
21	42	F	154	2.4	64.16
22	48	M	119.7	2.85	41.9
23	38	M	125.9	3.02	41.68
24	41	M	136	3.13	43.45
25	42	M	119	2.92	40.75
26	44	M	133	3.2	41.56
27	56	M	125	3	41.66
28	47	F	111.6	2.68	41.64
29	48	F	120	2.85	42.1
30	46	F	141.5	2.68	52.79
31	58	F	113.7	2.82	40.31
32	40	F	113	2.65	42.64
33	38	M	119	2.92	40.75
34	44	F	121	2.89	41.86
35	44	M	136.2	3.2	42.5
36	44	F	124.8	3.09	40.38
37	26	F	144	2.85	50.52
38	38	F	121	2.7	44.81
39	38	M	125	2.8	44.64
40	48	M	105.2	2.59	40.6
41	58	M	145	2.85	50.87
42	45	M	120	2.9	41.37
43	45	M	122	3.02	40.39
44	44	M	133	3.2	41.5
45	58	M	114	2.8	40.71
46	45	M	139	3.09	44.98
47	58	M	116	2.8	41.42
48	48	M	111.7	2.52	44.3
49	40	F	128.3	3.16	40.5
50	32	F	124.4	3.02	41.1

Table 4.2 Age, gender and BMI information of control group

Number	Age	Gender	Weight kg	Height m^2	BMI kg\m^2
1	18	F	47.43	2.49	19
2	32	M	62.65	3.13	20
3	36	F	49.92	2.49	20
4	32	M	60.87	3.2	19
5	44	F	58.56	2.78	21
6	20	F	49.86	2.62	19
7	19	M	59.91	2.49	24
8	32	F	46.83	2.46	19
9	38	M	60.55	3.02	20
10	47	F	60.62	2.75	22
11	32	F	55.61	2.52	22
12	20	M	64.14	2.78	23
13	46	F	65.69	2.85	23
14	42	F	58.88	2.56	23
15	40	F	58.56	2.78	21
16	59	F	50.48	2.65	19
17	34	F	54.22	2.46	22
18	22	M	54.49	3.02	18
19	19	F	44.93	2.49	18
20	40	F	55.25	2.4	23
21	44	F	65.84	2.99	22
22	50	M	55.77	2.7	20
23	34	M	46.81	2.34	20
24	49	M	66.47	2.89	23
25	45	M	65.84	2.99	22
26	44	M	88.98	3.42	26
27	56	M	73.96	2.95	25
28	52	F	51.2	2.56	20
29	42	F	64.91	2.82	23
30	49	F	53.79	2.68	20
31	60	F	63.2	2.52	25
32	45	F	66.47	2.89	23
33	38	M	54.92	2.49	22
34	50	M	60.2	3.16	19
35	42	F	71.4	2.85	25
36	48	F	48.67	2.43	20
37	32	F	63.58	2.89	22
38	42	F	69.36	2.89	24
39	43	M	66.6	3.02	22
40	49	F	59.89	2.72	22
41	60	M	66.6	3.02	22
42	48	M	54.43	2.59	21
43	48	M	77.44	3.09	25
44	49	M	78.62	3.27	24
45	58	M	55.77	2.78	20
46	52	M	64	2.56	25
47	48	M	65.79	3.13	21
48	58	M	59.61	2.59	23
49	48	M	70.43	3.06	23
50	52	M	53.09	2.52	21

Table 4.3 Mean values of obese and control groups

VARIABLES	OBESE GROUP	CONTROL GROUP
Age (years)	41.64 ± 10.62	41.64 ± 10.62
Weight (kg)	114.3 ± 18.68	60.46 ± 8.94
Height (m^2)	2.86 ± 0.25	2.77 ± 0.26
BMI (kg/ m^2)	40.02 ± 6.6	21.7 ± 2.04

4.2. Analysis of KIR2DL4 Gene Polymorphisms by PCR-RFLP Technique

4.2.1 Analysis of rs649216- 9571 C > T Polymorphism

PCR product were digested with Earl restriction enzyme. The products were separated by agarose gel electrophoresis and visualized under UV light.

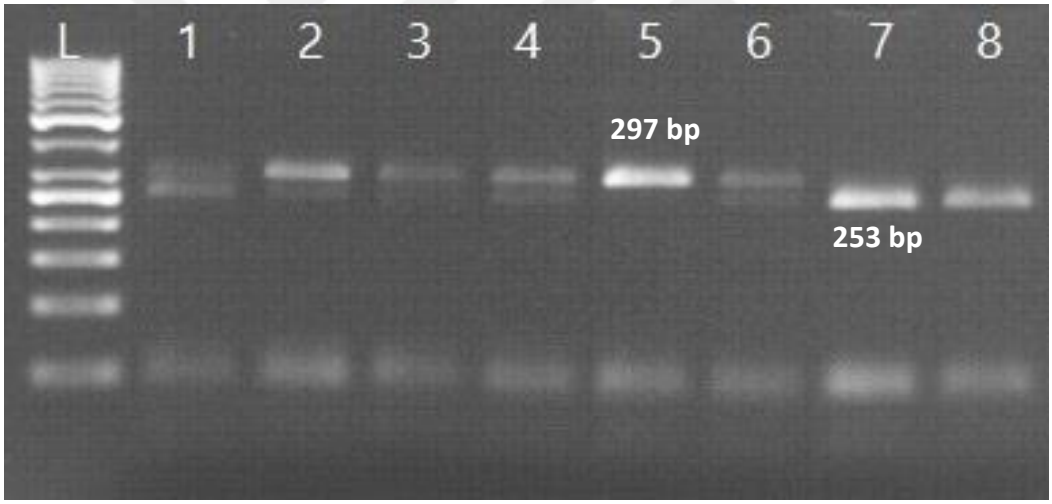

Figure 4.1 Genotyping of KIR2DL4 rs649216 polymorphism

L : 50 bp DNA Ladder

1, 2, 3, 4, 6 : CT (297, 253, 44 bp)

5 : TT (297/297 bp)

7, 8 : CC (253/253 bp)

Table 4.4 Comparison of rs649216 – 9571 C > T genotype distribution

Genotype	Control (n=50) n (%)	Patients (n=50) n (%)	χ^2 (p*)
TT	17 (34)	14 (28)	4.35 (0.114)
CT	25 (50)	19 (38)	
CC	8 (16)	17 (34)	

The genotype frequencies for KIR2DL4 rs649216 – 9571 C > T polymorphism was compared between patient and control group and no significant difference was observed (p>0.05).

4.2.2 Analysis of rs660437- 9769 C > A Polymorphism

KIR2DL4 PCR products were digested with BspMI enzyme and the products were electrophoresed on 3% agarose gel and visualized under UV light.

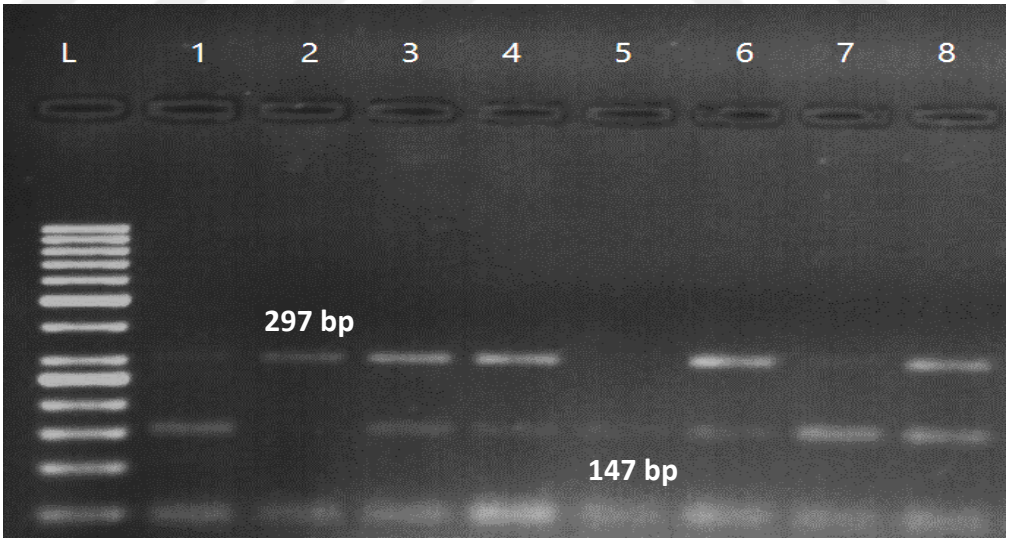

Figure 4.2 Genotyping of KIR2DL4 rs660437 polymorphism

L : 50 bp DNA Ladder

1, 3, 4, 6, 7, 8 : CC (297, 150, 147 bp)

2 : undigested PCR product

5 : AA (147, 103, 47 bp)

Table 4.5 Comparison of rs660437– 9769 C > A genotype distribution

Genotype	**Control (n=50) n (%)**	**Patients (n=50) n (%)**	**χ^2 (p*)**
CC	12 (24)	22 (44)	**10.87 (0.004)***
CA	1 (2)	8 (16)	
AA	37 (74)	20 (40)	

The genotype frequencies for **KIR2DL4 rs660437– 9769 C > A** polymorphism were compared between patient and control group and a significant difference was found (χ^2=10.87, p=0.004).

The frequency of CC genotype was 24% in control group, while it was 44% in patients. AA genotype was seen in 74% of the control group, however the frequency of this genotype in patients was 40%. CA genotype frequency was significantly higher in patient group (16%) than in control group (2%).

4.2.3. Analysis of rs660773 – 9797 G > A Polymorphism

PCR products were digested with HpyCH4III enzyme. The restriction products were separated by agarose gel electrophoresis and visualized under UV light.

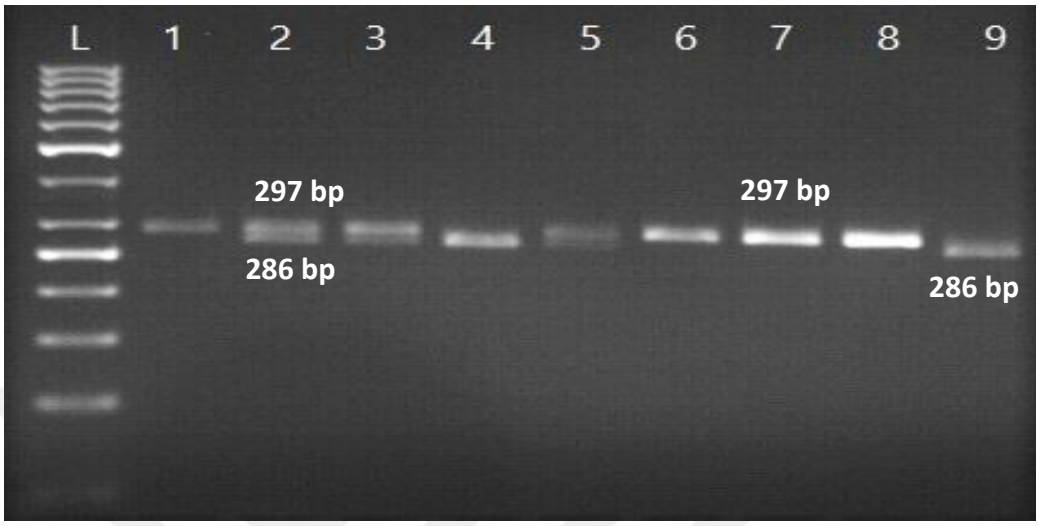

Figure 4.3 Genotyping of KIR2DL4 rs660773 polymorphism

L : 50 bp DNA ladder

1, 6, 7, 8 : AA (297/297 bp)

2, 3, 5 : GA (297, 286, 11 bp)

4, 9 : GG (286, 11 bp)

When the genotype frequencies for KIR2DL4 rs660773 – 9797 G > A polymorphism was compared between patient and control group, no significant difference was found ($p>0.05$).

Table 4.6 Comparison of rs660773 – 9797-G > A genotype distribution

Genotype	**Control (n=50) n (%)**	**Patients (n=50) n (%)**	**χ^2 (p*)**
AA	18 (36)	28 (56)	5.01(0.082)
GA	19 (38)	10 (20)	
GG	13 (26)	12 (24)	

CHAPTER V

DISCUSSION

Obesity, resulting from excessive intake of food, absence of physical activity and genetic susceptibility is accepted as one of the most important health problems of the 21st century. It is also a significant risk factor for many other diseases such as; T2DM, hyperlipidemia, hypertension, cardiac disorders and different types of cancers.

The overall energy expenditure of obese individuals is higher than their normal counterparts owing to the energy needed to preserve an enhanced body mass. Genetic influences are difficult to elucidate and identification of the responsible gene/s is not easily achieved by pedigree studies.

The etiopathogenesis of obesity has not been clearly understood yet. It is accepted that both genetic and environmental factors contribute to the susceptibility to the disease. So far, many studies have been performed to determine the genetic components leading to obesity, but the molecular mechanisms of disase development could not be clearly understood yet.

Leptin seems to be the most famous gene having role in obesity. It is secreted by the adipose tissue and acts as an endocrine signal in different tissues (Marti et al., 1999). Leptin is involved in immuneregulation and this is effected by fasting and overeating.

Leptin receptors are also important, they have a role in food intake and energy expenditure (Trayhurn et al., 1999). Yiannakouris et al. (2001) explored the impacts of 3 prevalent exonic polymorphisms (K109R, Q223R and K656N) in the leptin receptor (LEPR) gene. Q223R polymorphism was reported to be an important predictor of 5% of the variation in the structure of the body. On the other hand, allele and genotype frequencies of K109R and K656N polymorphisms were found to be not sigificantly different in control and obese group.

The majority of the genes found to be related to obesity are the genes associated with energy metabolism. Insulin-induced gene 2 (INSIG2) gene is one of them. Kaulfers et al. (2015) found a significant connection between the rs12464355 polymorphism of INSIG2 gene and LDL in children. Due to impacts on lipid regulation, polymorphisms in INSIG2 could be essential in obesity development.

The Uncoupling Protein 1 (UCP1) gene is regarded to be an obesity candidate gene, because of important associations of rs1800592 and rs3811791 UCP1 promoter polymorphisms with obesity (Chathoth et al., 2018). It was reported that these polymorphisms effects the function of the mitochondrial membrane and mediates proton leakage leading to deregulation of energy expenditure, thermogenesis and reduction in oxidative stress.

Martínez-García et al. (2013) analysed 22 obesity-related gene loci in Spanish populations and found that only the Fat Mass and Obesity-associated (FTO) gene was clearly associated with BMI.

Later, Khan et al. (2018) worked on FTO gene polymorphisms and found that the rs9939609e “AA” genotype is related with greater values of BMI. In another study, Tupikowska-Marzec et al. (2019) found that FTO gene rs9939609 polymorphism could potentiate insulin resistance and obesity.

Obesity has been related to different conditions but also to immune dysfunction (Bovill et al., 1996; Sweettman et al. 1997). Gottschlich et al. (1993) reported that the incidence and severity of infectious disase in burn obese patients is higher than in burn lean individuals. Bacteriaemia and clinical sepsis were observed more frequently in obese patients compared to non-obese ones.

Genetic animal models were also used to understand the relationship between obesity and immunocompetence and the results suggested the presence of an association (Chandra et al., 1980; Tanaka et al., 1998). Both malnutrition and overeating have influence on the immune function (Chandra et., 1999).

IL-18 regulates the immune response and is expressed in cells involved in chronic inflammation, autoimmune diseases and several cancers and infectious diseases. Its concentration reported to be increased in obese people suggesting its role in obesity and metabolic syndrome by increasing adipogenesis (Wood et al., 2005). Martínez-

Barquero et al. (2017) studied IL18RAP gene polymorphisms related to the IL-18 adipogenic signaling pathway and demonstrated that rs7559479 AG-GG genotypes are related with high BMIs.

There are several reports in both humans and animal models suggesting the linkage between adipose tissue and immunocompetent cells. NK cell function is regulated by positive or negative signals produced by the interaction of KIRs with their ligands on target cells. These data and knowing the importance of KIRs for immune function lead us to investigate the role of KIR2DL4 polymorphisms in the development of obesity.

KIR2DL4 is a member of the Killer Cell Ig-like receptor (KIR) family, the gene is positioned on chromosome 19p13.4. It is expressed in almost all human NK cells and differs from the other KIRs in some aspects such as; cellular localization, ligand specificity and structure. KIR2DL4 has a high degree of conservation; it has a low value of polymorphism among KIR genes. This might be a possible reason of limited number of studies on KIR2DL4 polymorphisms in the literature. In one of these studies; Le Page et al. (2013) reported that KIR2DL4 9A/10A polymorphism has no significant relation with asthma.

SNPs of the KIR2DL4 gene were evaluated in pre-eclampsia patients and no significant difference was found in genotype distributions and allele frequencies in patients and controls. But, expression analysis indicated that KIR2DL4 mRNA level is significantly lower in placenta tissues with pre-eclampsia than those with normal pregnancy (Wang et al., 2015).

He et al. (2016) investigated the expression level of KIR2D (L1, L3,L4, S4) and KIR3DL1 in non-small cell lung cancer and discovered high expression of KIR2D (L1, L3, L4, S4) and KIR3DL1 in non-small cell pulmonary cancer (NSCLC) tumor cells and tumor infiltrating lymphocyte (TIL). Besides, positive expression of KIR2D (L1, L3, L4, S4) and KIR3DL1 on tumor cells or TILs in NSCLC patients was linked with weak prognosis.

The role of HLA-G, LILRB1 and KIR2DL4 gene polymorphisms in spontaneous miscarriage was studied by Nowak et al. (2016). They observed a high number of KIR2DL4 9A/10A genotypes in male partners of miscariage group than the control

group, however no association was found between KIR2DL4 polymorphism and spontanous abortion susceptibility in female.

In the present study, 3 polymorphisms of KIR2DL4 gene (rs660773 - 9797 G > A, rs660437 - 769 C> A, rs649216 - 9571 C> T) were analysed and compared in obese and control group. When the genotype frequencies of the polymorphisms were compared between the patient and control group, no significant differences were obtained for rs660773 and rs649216 ($p>0.05$). However, the genotype frequency distribution of rs660437 in patients was significantly different from that of control group ($p=0.004$).

The result of this study suggested that rs660437 C allele could be a risk factor for obesity, while rs660773 and rs649216 alleles seem to have no effect on the development of the disorder.

To the best of our knowledge, this is the first study elucidating the role of KIR2DL4 gene on obesity. Although this is an original study, it has several limitations. First of all, the number of subjects in the groups was not so high. Due to the limited time to finish the project, the number of patient could not be increased more. Furthermore, the protein expression of cell surface KIR2DL4 could not be examined in this study due to budget limitations. The effect of rs660437 C allele on protein expression and function remains to be identified, which might be a future direction in this field. Furthermore, the clinical implications of obesity in immunity need to be clarified with new comprehensive studies.

CHAPTER VI

CONCLUSION

The study of KIR2DL4 gene polymorphisms (rs660773, rs660437 and rs649216) in obese patients and control group indicated that there is a statistically significant difference in genotype distribution of rs660437 polymorphism between two groups and therefore it can be stated that this polymorphism could be a risk factor for obesity. However, the other polymorphisms were not found to be associated with this condition.

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