CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Association of TNF-α G308A (rs1800629) Polymorphism with Obesity Induced Diabetes and Cardiovascular Disorder

by

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A thesis submitted in partial fulfillment for the degree of Master of Science

in the

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Dedicated to Almighty ALLAH and the Holy Prophet Muhammad (P.B.U.H)and
My Loving Family

CERTIFICATE OF APPROVAL

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Abstract

Obesity a multi-factorial pandemic disease is emerging as a great threat in Pakistani population. Gender, age, urbanization, and sedentary life style, unhealthy dietary habits, and reduced physical activity are the key factors considered for high prevalence of obesity in recent years. According to global survey on obesity, Pakistan is the ninth most obese nation among list of "fattest countries". The rapid increase of obesity is an outcome of environment, genetics and hormones . Tumor necrosis factor alpha (TNF-α) is a cytokine involved in systemic inflammation and its polymorphism G-308A has been reported to be associated in fat deposition, insulin resistance, type 2 diabetes and cardiovascular disorders. This study is designed to understand the association of TNF-α G308A in obesity and obesity induced diseases focusing on type 2 diabetes and cardiovascular disorders. In this regard random sampling was performed. Anthropometric measurements of 283 subject was collected from Kahuta region. Genotyping was done via Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR)-RFLP assay. Results indicate the prevalence of obesity was 24%, prevalence of diabetes was 13% while the incidence of CVD was found to be 2%. Frequency of obesity-induced diabetes was 16.4% with the p-value of 0.411 statistically non-significant while for obesity-induced CVD frequency was 44.8% with a p-value of 0.000 shows highly significant association. CRP association for both obesity and CVD shows p-value of 0.000 highly significant whereas CRP with diabetes p-value 0.293 shows a non-significant association. Non-significant association of TNF-α G308A polymorphism with obesity, diabetes, and CVD was notice. Furthermore, we observed allele 'A' is 60% frequently distributed while allele 'G' is 40%.The current research revealed that lack of association of polymorphism with obesity, diabetes, and CVD is more likely to be with insufficient power. Polymorphism are usually interrelated as well as co-inherited so it is difficult to draw conclusions on small scale sample size, hence it is suggested as future direction that there must be a comprehensive study of TNF-α promoter region by collecting large scale sample population in different ethnic groups.

Contents

List of Figures

List of Tables

Abbreviations

BMI	Body Mass Index
Bp	Blood Pressure
BSR	Blood Sugar
CAD	Coronoray Artery Disease
CHD	Coronary Heart Disease
CRP	C Reactive Protein
CVD	Cardiovascular Diseases
HLA	Human Leukocyte Antigen
HPA	Hyoothalmic Pituaitary Adrenal
IL	Interleukin
LPL	Lipoprotein Lipase
MI	Myocardial Infarction
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphisms
SPSS	Statistical Package of Social Science
TC	Total cholesterol
TNF-α	Tumor Necrosis Factor Alpha
WHO	World Health Organization

Chapter 1

Introduction

1.1 Background

Obesity is defined as an increase in total body fat resulting from imbalance between calories intake and their consumption by the body. Genetics, environment, socioeconomic status and life style play a significant role in developing obesity. Gender, age, urbanization, and sedentary life style, unhealthy dietary habits, and reduced physical activity are the key factors considered for high prevalence of obesity in recent years [1]. Sedentary work nature, enhanced use of computers, the convenience of various transportation modes within the cities and overuse of private cars explains for the reduced physical activities. In addition to this reluctance in walking and playing as a result of disturbed law and order situation and street crime has also contributed [2].

Obesity is a worldwide pandemic as Almost 2.1 billion people are suffering worldwide. According to WHO, Global prevalence of obesity has increased three times since 1975 and it is reported that more than 1.9 billion adultsareoverweight among which 650 million are categorized as obesemore alarmingly, 41 million kids even below the age of five are either overweight or obese while 340 million children and adults of age between 5-19 years are obese [3]. According to global survey on obesity, Pakistan is ninth most obese nation among list of "fattest

countries" [4]. Although, obesity is a major health issue in Pakistan, but it got attention recently. Fat rich Pakistani cooking as well as variations in life style, are considered among the major causes of obesity in Pakistan.

It is also well documented that people who have obesity, are vulnerable to health issues and develop diseases such as, Hypertension, Dyslipidemia, Type 2 diabetes, Coronary heart disease, Hypercholesterolemia, Gallbladder disease, Osteoarthritis, Stroke, Sleep apnea, Asthma and even cancer (endometrial, breast, colon, kidney, gallbladder, and liver) [5-8]. Mental illness such as major depressive disorder (MDD) and anxiety are also associated with obesity. Obesity may also lead to general body pain and difficulty with physical functioning [9,10]. Figure 1.1 summarise association of various diseases with obesity.

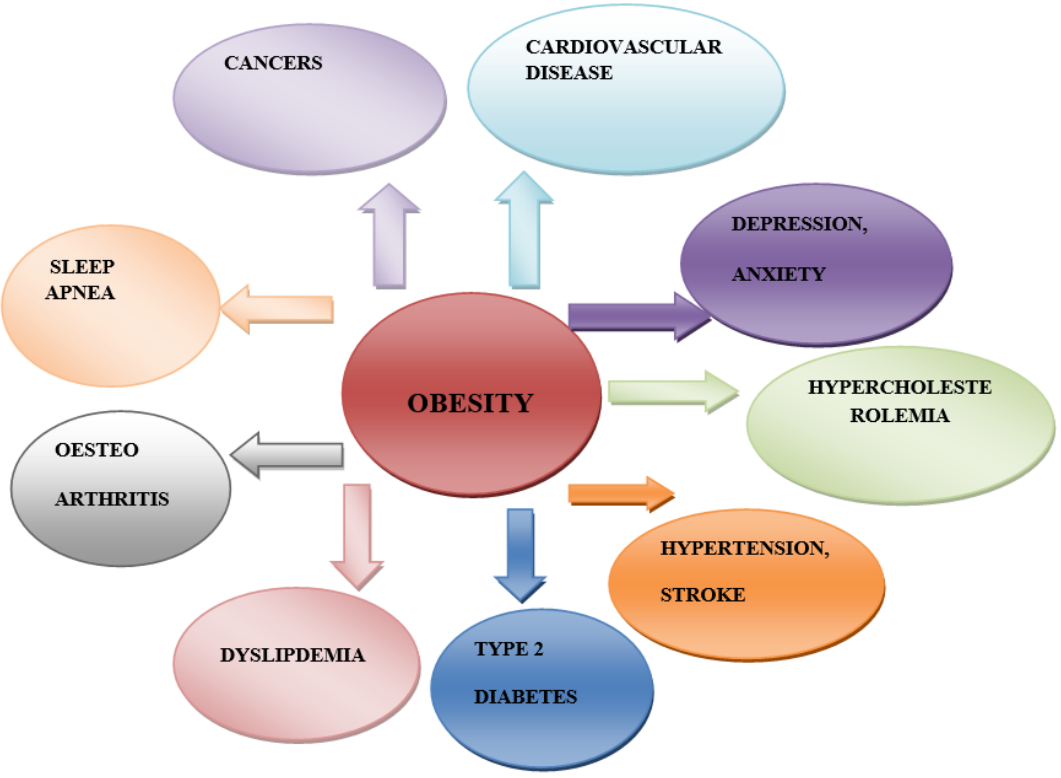

FIGURE 1.1: Association of Various Diseases with Obesity [5-10].

As non communicable multi factorial disease, the onset of obesity is subjected to a composite interaction among environment and genetics, in addition to which a number of hormones such as adipokines regulate the onset of obesity.

Obesity may be linked with variety of clinical complications including diabetes or insulin resistance, dyslipidemia, hypertension, and high levels of fibrinogen and C-reactive protein, all of them increases the risk of cardiovascular diseases [11,12]. Inflammatory pathways arecentral in onset of obesity induced diabetes and cardiovascular diseases.The elevated levels of IL-6, C-reactive protein and TNF-α, and decreased levels of adiponectin and interleukin-10, results due to increased fat content and results in activation of a inflammatory pathway which triggers endothelial dysfunctioning as well as insulin resistance, ultimately causing diabetes and atherosclerosis [13-15].

Obesity is a major contributor in development of hyperglycemia and insulin resistance. These two conditions are foundational to diabetes type II. Globally 180 million people are affected by diabetes. It is expected that the number of patient may reach upto 300 million by 2025 [16]. Increased blood glucose level indicates obesity induced type 2 diabetes, results from increase glucose make up in the liver (gluconeogenesis and glycogenolysis) and low glucose uptake by muscle.[17,18]. Adipocytes release free fatty acids which interfere with glucose uptake by peripheral tissues. In addition adipocytes also act as endocrine organs and produce cytokines such Tumor Necrosis Factor alpha (TNFα), Interleukin-6 (IL-6) and Interleukin 1-β (IL-1β)which are involved in Insuline resistance and chronic inflammation.

Any abnormal situation associated with low insulin function and low blood glucose level together and anti-inflammatory effects resulting in muscle wasting,enhanced infection tendency, and increased inflammatory respons. Similarly, obesity can also alter the function of heart by increasing the risk factors such as hypertension, dyslipidemia, and glucose intolerance. Other than the changes in metabolism, accumulation of adipose tissue can alter the structure and function of heart in general. The cytokines such as TNFα, IL-6 and C-reactive Proteins also play significant role in pathogenesis and are considered as biomarkers of coronary damage. Therefore, mortality and morbidity of cardiovascular has been reported to be elevated in individuals who are overweight and have chronic inflammation pathways activated [19]. The major

risk factor for cardiovascular disorder is abdominal obesity i.e. visceral fats [20]. The increase risk of hypertension and consistent increase leading towards stroke, myocardial infarction (MI), heart attack, and aortic aneurysm, and also a important source of chronic kidney failure. Slight elevation of arterial pressure leads to enhance the risk of heart problems [21].

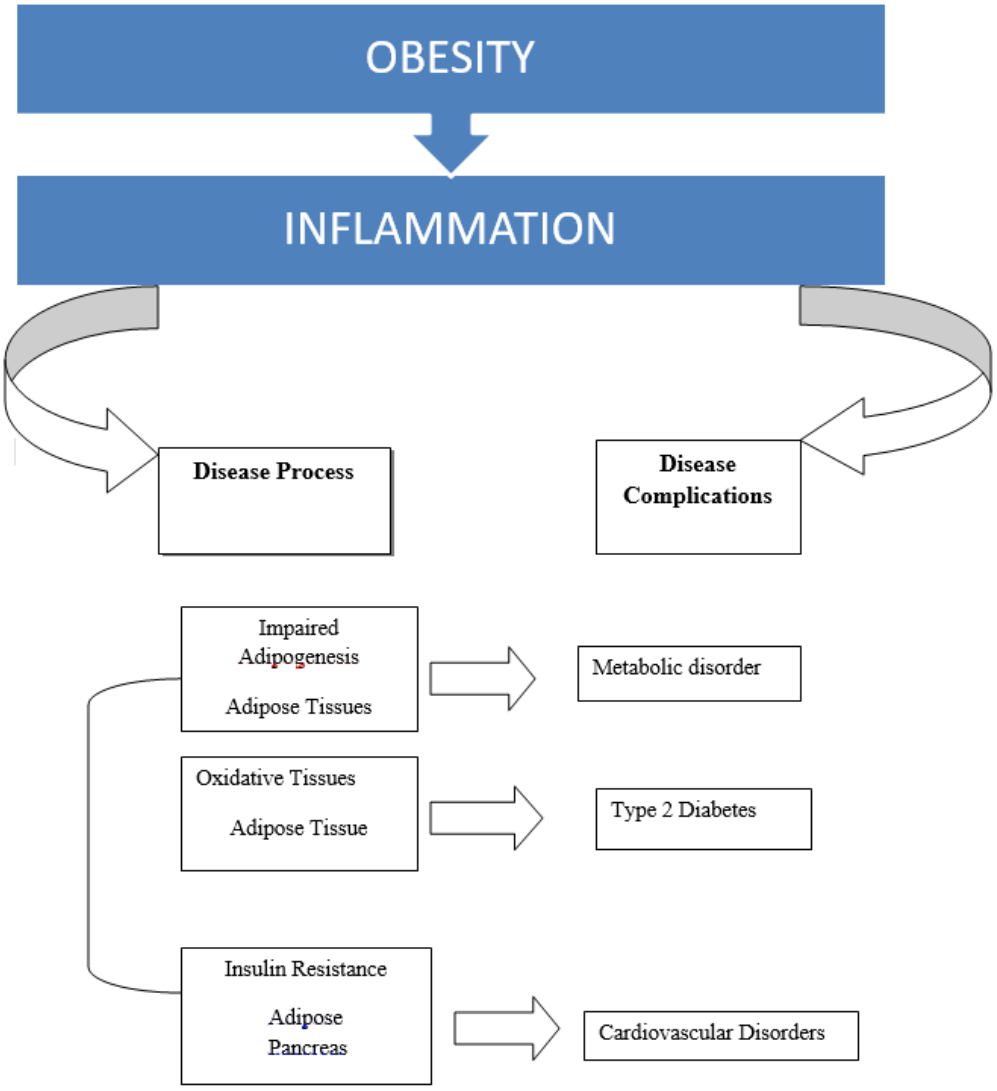

FIGURE 1.2: Pathways Involved in Obesity Induced Diabetes and Cardiovascular Disorders.

Mobilization of stored fats is significant in providing the metabolic support to immune system during combat with infections. During a basic inflammatory response, catabolic pathways are favoured and anabolism is suppressed. This integeration of immune system and metabolic pathways is critical in maintaining

health condition but in case of chronic matebolic overload i.e. obesity, complications emerge and result in various disease conditions (Figure 1.3) The first identified link between inflammation and obesity is TNF-α.

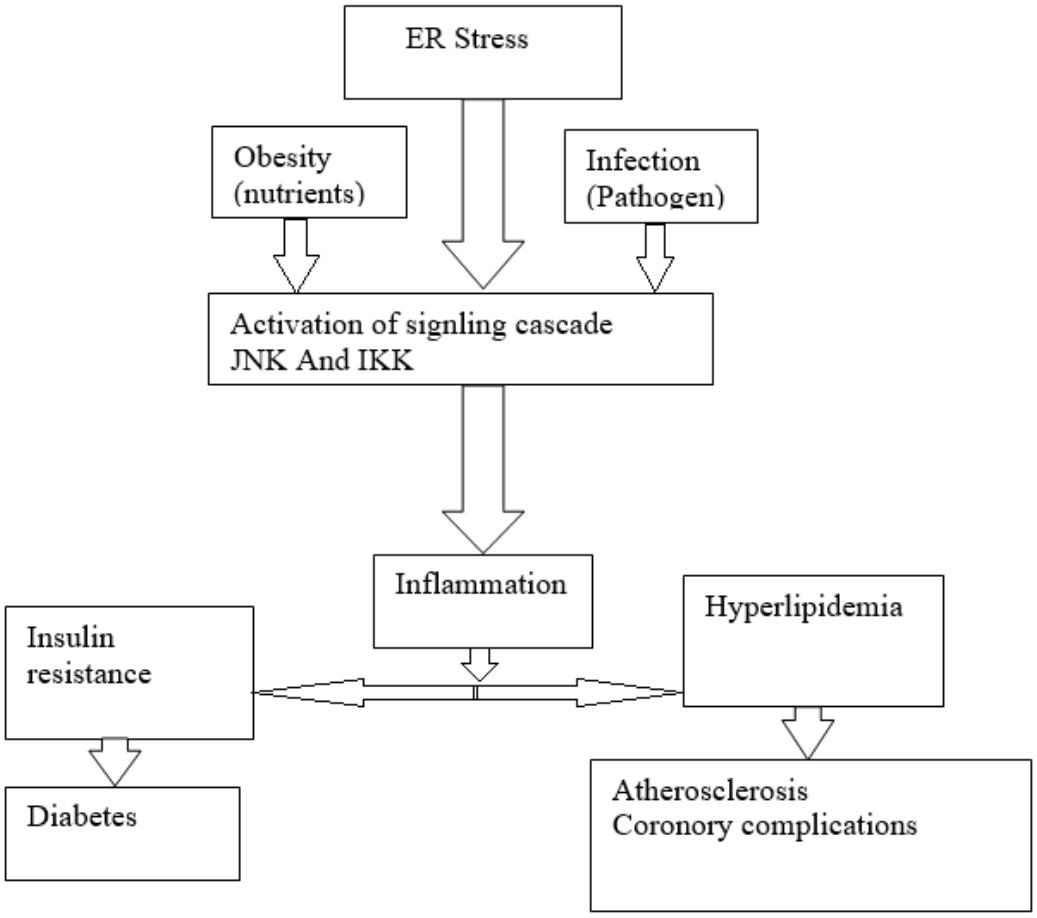

FIGURE 1.3: Inflammation a Key Link Between Obesity Diabetes and Cardiovascular Disorder

Tumournecrosis factor- alpha (TNF-α) is a multidimensional factor which trigger various pathways including increase growth, growth retardation, angiogenesis, cell toxicity, inflammation, and immune modulation [21]. Multiple functions in fat metabolism, insulin resistance, coagulation and endothelial functions are reported to be associated with TNF α. [22]. This cytokine is one of the most typical pro inflammatory cytokine and its various gene variants or polymorphism are being studied in various populations are found to be involved in causing ailments related to inflammation and lipid metabolism. Out of eight most common variants rs1800629 also referred as TNF-308 polymorphisms is widely

studied. The polymorphism is G308A, where G is the common allele and is associated with higher levels of TNF expression.

The allele has been found more frequent among obese individuals [23]. Genotyping results performed on various populations revealed the association of polymorphism with accumulation of body fats in women [24]. TNF-α is expressed in and secreted by adipose tissue, its effect correlate with adiposity Therefore, TNF-α have been recommended as a hopeful remedy for insulin resistance and type 2 diabetes[25]. In the inflammation process of atherosclerosis TNF-α is alsoa input cytokine. Research advocate 308GA TNF-α gene polymorphism may possibly add to cardiovascular hazard in patients suffering from type 2 diabetes and it could be represented as valuable projecting indicator for cardiovascular disease in type 2 diabetic women [26]. Therefore, genotyping information of TNF α, especially in context of the polymorphism G308A, in Pakistani population can lead to have a better idea of genetic predispositions for onset of obesity and its outcomes in form of diabetes and cardiovascular diseases.

1.2 Aims

Tumor Necrosis Factor alpha (TNF-α) is a cell signaling cytokine concerned with obesity and can induce inflammatory pathways resulting in Diabetes and Cardiovascular disorders. This study was designed with an aim to explore association of G308A polymorphism of TNFα with obesity induced diabetes and cardiovascular disorder.

1.3 Objectives

The study is designed with following major objectives:

1. To determine the prevalence of obesity and obesity induced diabetes and cardiovascular disorder.

2. To determine association of G308A polymorphism in TNF-α with obesity induced diabetes and cardiovascular disorders.

Chapter 2

Literature Review

Obesity, now considered as worldwide pandemic, acts as foundation for onset of various co-mordities. These co-morbidities are in fact outcome of the damages conferred by obesity. This chapter covers the basis of obesity and its role in onset o diabetes and cardiovascular disorder, the later part of chapter focuses on role of TNF α gene and its polymorphisms G308A in obesity induced Diabetes and Cardiovascular disorders.

2.1 Obesity

Obesity is a condition of the increased fat mass. A substitute marker for fat mass is the body (BMI), which is measured by a standard procedure as weight in kilograms divided by heigh in square meters. Body Mass Index of 25–29 kg/m2 is called overweight and BMIs greater ($\geq$30 kg/m2) would be considered as obesity [35]. Moreover obesity is defined as raise in body mass crossing the boundaries of body requirement resulting in an unnecessary fat buildup [27-36].

Obesity is one of the major issues of twenty first century not only globally but also in Pakistan but unfortunately it was not being noticed and only in past few years this issue has been raised when research reveals the list of "fattest countries" and Pakistan was at ninth number (out of 194 countries) in stipulations of its flabby

population [37]. It has been found that one-out of every four Pakistani adults as being overweight [38,39] but now obesity has become pandemic and recent report of WHO shows Pakistan being the ninth most obese nation in the World. Root causes of Obesity in Pakistan were found to be sedentary life style and unhealthy fat rich dietary system [37]. Research indicates that people existing in urban areas of Pakistan are likely to be at more risk of being obese due to their life style rather rural areas individuals are less prone for being overweight or obese. Interestingly Pakistani women also suffering higher rates of obesity than men. It has been found that not only for obesity but in case of diabetes prevalence Pakistani nation has the peak percentage in South Asia [40]. This shows that obesity induced diabetes is common in Pakistan.

2.1.1 Risk Factors of Obesity

Multiple risk factors contributes towards obesity including environmental, socioeconomic and genetic factors. Major risk factors include age, gender, urbanization, unhealthy dietary habits, dynamical lifestyle reduced physical activity are the most contributive factors for the raised prevalence of obesity not only in Pakistan but worldwide as well [41].

It has been proposed that eating at "fast food" restaurants was positively leads towards obesity as compared to this vegetable consumption and physical activity will help to reduce weight [42]. Sleep deprivation plays contributory role towards weight gain , as found in middle-aged women. Reduced sleep may cause menopause and that is correlated with weight gain, additionally, night-time eating common in women also cause them weight gain [43].The mechanism by which sleep deprival might affect weight are uncertain but it has been found that limited sleep may lead to daytime exhaustion and therefore reduced physical activity. Furthermore experimental studies put forward the fact of being obese as found that lack of sleep may cause alterations in serum leptin and ghrelin levels provokes hunger and appetite leading to weight gain [44].

2.1.2 Genetic Predispositions of Obesity

Obesity is a complicated illness, consequential variety of both genetic and environmental factors. Advancement in quantitative genetics, genomics and bioinformatics has played a significant role towards genetic and molecular basis of obesity [45]. Hereditary factors such as, family history, genetic predisposition, and ethnicity have a significant role in the pathogenesis of obesity. Various genetic components play their roles as some individuals have the predisposition of gaining more weight than others when triggered by sedentary lifestyle thus, they cover an increased risk of rising obesity and associated disorders depicted in (Figure 2.1).

About 60 genes have been reported to be linked by GWAS through obesity-related traits, including MC4R, PCSK1, BDNF, FTO, POMC, SH2B1, MTCH2, KCTD15, LEPR, NEGR1, and NTRK2 [46-52]. Table 2.1 show a list of few obesity associated genes and their impact (Figure 2.1).

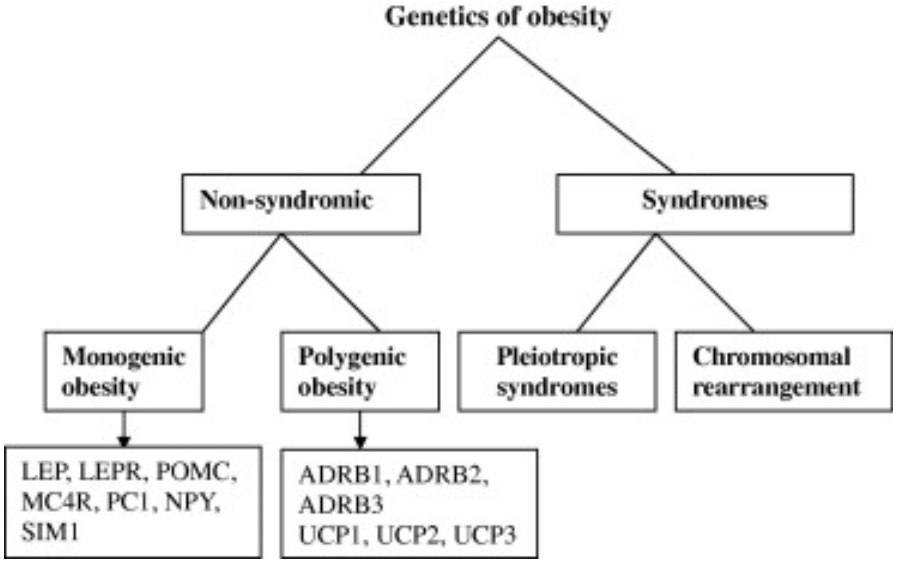

FIGURE 2.1: Genetics Predisposition of Obesity

2.1.3 Co-morbidities of Obesity

Obesity is considered as mother of all diseases and obese people are at greater risk for mounting various medical issues counting insulin resistance and type 2 diabetes mellitus, dyslipidemia, cardiovascular disease, hypertension, stroke, hypercholesteremia, sleep apnea, gallbladder disease, hyperuricemia , osteoarthritis, gout, and certain forms of cancers. As soon as the body mass index exceeds beyond 30 kg/m2, obesity and obesity-related disease contribute significant role towards mortality. To improve health standards and reduce the frequency of obesity-related disease, exercises to weight loss, even if moderate could be beneficial [53]. Despite the fatal disorders some psychological problems has been reported in obese individuals like depression anxiety and binge-eating disorder. Last but not least people suffering with obesity may face social stigmatization and prejudice and severely obese people suffer with psychological and physiological issues and they may have negative impact not only at their quality of life but also quantity, as obesity shortens the life span. [54].

2.2 Tumor Necrosis Factor Alpha TNF - α

The term Tumor necrosis factor (TNF) was firstly coined by O'Malley et al. (1962), was initially reported to induce apoptosis. It was reported that this molecule is considered to be concerned with regulation of many vital processes for example growth, cellular differentiation, proliferation, and defense mechanism. Multiple cells counting macrophages, neutrophils, monocytes, NK cells, and T-cells are involved in the production of tumor necrosis factor (TNF) [27]. This cytokine when over-expressed reports the production of left ventricular dysfunction, ,pulmonary oedema cardiomyopathy and many other diseases [28,29]. Recently it has been observed the levels of circulating TNF-α are high in patients with unstable rheumatoid arthritis (RA) , angina, and autoimmune disorders [30]. The proinflammatory cytokine TNF-α have significant function in the susceptibility of several diseases and shows variety of functions in lipid

metabolism, insulin resistance, coagulation and endothelial functions. Despite these functions TNF is also contributing major role in coronary heart disease (CHD) [31].

2.3 Polymorphisms in TNF-α

Elahi et al. 2009 reported many TNF-α polymorphisms including -376 (G/A), -1031 (T/C), -857 (C/A), -863 (C/A), -162 (G/A), -851 (C/T), -419 (G/C), -308 (G/A), -49 (G/A) and -238 (G/A) . It has been observed that the position of TNF-α encoded gene found on chromosome 6 between the HLA-B and HLA-DR genes [27].

Along with the reported polymorphisms, the $-$G308A TNF-α promoter polymorphism have been linked several fold with the expansion of autoimmune diseases on the other hand there is minute or no association was observed for other TNF-α gene polymorphisms with autoimmune disorders[32]. A study has been conducted in a German caucasian population to know the expression of Tumor necrosis factor-α (TNF-α) in adipocytes and it was observed that the cytokine depict strong association with obesity along with insulin resistance, furthermore it was concluded that A allele of TNF-α gene (G$-$308A) polymorphism in the 5$'$-flanking region is more common in overweigh and obese individuals rather in normal weight or underweight individuals, hence it has been well defined that expression of reported cytokine influence insulin resistance and fat mass [33]. Another study from British Caucasian population from East Midlands reveals involvement of TNF-α (G$-$308A) polymorphism among coronary heart disease, it was concluded that allele (TNF-α $-$308 GA or GG) has a high prevalence for coronary heart disease [34].

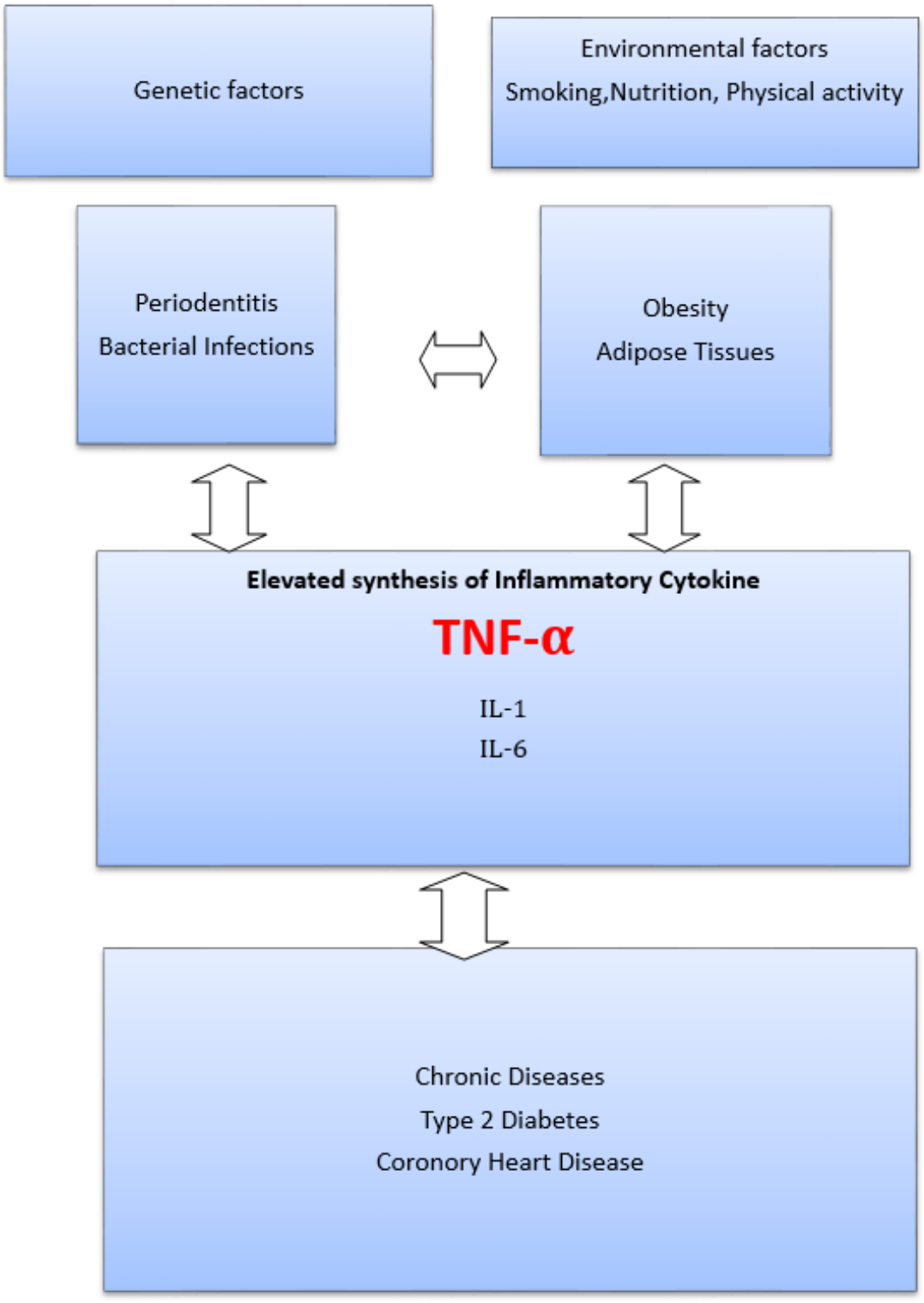

FIGURE 2.2: Pathways of obesity induced TNF-α in pathogenesis of chronic diseases.

2.3.1 Role of TNF - α G308A Polymorphism in Obesity

Obesity along with insulin resistance are the outcomes of increased level of Tumor necrosis factor-α (TNF-α) primarily found in adipocytes. A study has been conducted in German Caucasian residents to know the relationship of TNF-α gene (G-308A) among obesity and insulin resistance and it was found that an A allele of a polymorphism of the TNF-α gene (G-308A) is more common in subjects with obesity as compared to Individuals with normal weight[55]. Research reveals sufficient evidence about tumor necrosis in the development of obesity which triggers diabetes mellitus and it was found that polymorphism -308 G/A of TNF gene was related to increase in body weight. Genotyping was done and it was found that the gene at promoter region of TNF alpha was found to accumulate body fats in women [56].

2.4 Diabetes

Diabetes is a fatal, metabolic syndrome accomplished by increased levels of blood glucose (or blood sugar), leads towards co morbidities such as severe harm to the heart, blood vessels, kidneys, nerves, even eyes, a major cause of blindness. World Health Organization (WHO) reported only three forms of diabetes mellitus are recognized i.e. type-1, type-2 and gestational diabetes. Among them widespread is type 2 diabetes, usually found in adults occurs when the body become either completely resistant to insulin or do not make an adequate amount of insulin. In last of three decades the incidence of type 2 diabetes has risen radically rather type 1 diabetes, in earlier times acknowledged as juvenile diabetes considered to be persistent during this state pancreas produces minute or no insulin. Research revealed that diabetes and obesity are correlated therefore it is targeted worldwide to minimize incidence of diabetes and obesity by 2025 [57].

Number of individuals suffer with diabetes was 108 million in 1980 as compared to this it has been alarmingly risen to 422 million in 2014 and global incidence

of diabetes between adults over 18 years of age has risen from 4.7% to 8.5% during the time period from1980 to 2014. Prevalence of diabetes has become more promptly arisen in low-income developing countries causing co morbidities as well as mortality,while it has been observed in 2015 that 1.6 million deaths were directly caused by diabetes. WHO projects that diabetes will be the seventh leading cause of death in 2030. Balanced diet, maintaining a normal BMI, regular physical activity,avoiding smoking and regular screening are key points to avoid or else delay the onset of type 2 diabetes.The Diabetes Prevalence Survey of Pakistan conducted in 2016-17 revealed that 16.98% of the total population had diabetes mellites [58,59]. On the contrary, the IDF 2017 Atlas reported that 7.6 (6.5%) million people go through diabetes in Pakistan [60]. According to WHO 2017 report Pakistan ranked 7th for diabetes.

2.4.1 Risk Factors of Diabetes

The possibility for being diabetic varies for type 1 ,type 2 and gestational diabetes. For Type1 diabetes determinants comprise family record, race, autoimmune disease, environmental factors such as exposure to toxins and chemical compounds, infections. An imbalance in intestinal micro biota,The 'hygiene hypothesis', Not breastfeeding, Drinking cow's milk, Birth weight and infant growth. Risk factors for type 2 diabetes for example genetics and lifestyle are remarkable in its development. Among these factors few of them such as family history, age and ethnicity are unavoidable, as compared to this we possess choices to control over lifestyle hazard related to weight diet, and exercise habit. Being overweight or obese is also a prime risk factor for developing type 2 diabetes. This review summarize that family history and ethnicity are also the contributing risk factors for type 2 diabetes as reported that people of South Asian descent who are more prone to have diabetes up to six times while upto three times in African and Africa-Caribbean people. In USA, black and Hispanic natives have much more chances of diabetes than non-Hispanic white. Elevated cholesterol, high blood pressure, background of gestational diabetes, reduced

physical activity, smoking, depression, stroke and polycystic ovarian syndrome are also the risk factor for type 2 diabetes. Gestational diabetes is progressively more familiar complication of pregnancy and a large proportion of women who experience it are either overweight or obese. Therefore, weight control earlier than and during pregnancy lessen risk of developing gestational diabetes. Additional menace reported for gestational diabetes comprise pre-diabetes, high blood pressure, ethnicity, hormonal disorder, and early onset of pregnancy etc [61].

2.4.2 Genetic Predispositions of Diabetes

A tendency to develop type 1 diabetes is passed throughout generations, but the heritage map is unidentified. The exact reason for type 1 diabetes are unidentified although several risk factors has been determined. Commonly Type 1 diabetes is well thought-out to be an autoimmune disorder. On behalf of unfamiliar reasons our own immune system itself damages the beta cells meant for producing insulin and then sign and symptoms of type 1 diabetes appear. Regarding genetic predispositions HLA genes, including HLA-DQA1, HLA-DQB1, and HLA-DRB1, have several variations, and individuals having definite grouping of these variations is called a haplotype. Certain HLA haplotypes are connected with a elevated risk of developing type 1 diabetes, among them HLA-DQA1, HLA-DQB1, and HLA-DRB1 gene variations resulting in the uppermost risk. It has been observed that the variants are also found in general population but only 5% individuals bearing these variants develop type 1 diabetes. Ecological factors and variations in other genes are additional contributors thought to enhance the expansion of this multifaceted disease [62].Type 2 diabetes does not shows clear pattern of inheritance, although many affected individuals have at least one close family member blood relation with the disease and the risk chances for type 2 diabetes increases with the increased figure of affected family unit. Genetics not alone increase the susceptibility but standard of living for example eating and exercise habits plays a contributive role

in inception of disease [63]. The incidence of type 2 diabetes has considerable variation from one to other geographical region mainly due to the variations in environmental and behavioral risk factors such as, sedentary life style, unhealthy diet, hypertension, and consumption of some drugs. Genetic background also has a significant part in the progress of type-2 diabetes as having a familial background of diabetics substantially increases the risk up to 25%. Various genetic components play their roles in increasing the risk of developing type-2 diabetes. Researchers identified approximately 70 genes with strong association to type-2 diabetes across the globe, 29 of which are affiliated to East- and South-Asian populations.[64], [65] Some of these genes include; MC4R, PPARG, FTO, TCF7L2, NOTCH2, WFS1, JAZF1, HHEX, SLC30A8, CDKAL1, and NOTCH2. However, FTO gene is one of the potential factors that affect the genetic susceptibility of type-2 diabetes in diverse populations [66,67].

TABLE 2.1: List of Genes Associated With Type 2 Diabetes

Sr. No	Genes	Impact	Reference
1	MC4R	Alters food behavior related endophenotypes e.g. rs17782313 or rs12970134 SNP	(Xi et al. 2012; Taylor et al. 2011)
2	FTO	Affects food intake and energy balance of body e.g. rs1421085 or rs9939609 SNP	(Steemburgo et al. 2013; Yang et al. 2017)
3	HNF4A	Functions in intracellular modelling and post-receptor signaling, strong association with type 2 diabetes	(Ali 2013; Sun, Yu, and Hu 2014)

Table 2.1 continued from previous page

Sr. No	Genes	Impact	Reference
4	ST6GAL1	Involved in post-translational modifications by glycosylation, strong association with type 2 diabetes e.g. rs16861329 SNP	(Kooner et al. 2011; Sun, Yu, and Hu 2014)
5	VPS26A	Altered encoding of transport protein, strong association with type 2 diabetes e.g.rs1802295 SNP	(Ali 2013; Kooner et al. 2011)
6	GRB14	Changes in binding to insulin receptors and insulin-like growth-factor receptors, strong association with type 2 diabetes e.g.rs3923113 SNP	(Kooner et al. 2011; Sun, Yu, and Hu 2014)
7	KCNJ11	Misregulation of insulin secretion by beta-cells is powerfully linked with type 2 diabetes	(Sun, Yu, and Hu 2014)

2.4.3 Role of TNF - α G308A Polymorphism in Diabetes

Tumor Necrosis Factor α (TNF-α) has significant effects on lipid metabolism in the perspective of acute inflammation, as in case of sepsis. It has been observed that elevated production of TNF-α is strongly concerned with obesity induced insulin resistance and the pathogenesis of type 2 diabetes. Experiments were performed on adipose tissues taken from obese humans or rodents. Results indicates that inserting TNF-α to animals may persuade insulin resistance, while

neutralization of TNF-α can perk up insulin sensitivity. Prominently same results from knockout mice lacking TNF-α or its receptors have recommended that TNF-α has a role in regulating in vivo insulin sensitivity on the other hand if TNF-α is missing in mice only partial protection against obesity induced insulin resistance is observed. Several mechanism including down regulation of genes for normal insulin function were associated with the metabolic effects of TNF-α which further effect insulin signaling while current facts propose that that suppressing TNF-α in type 2 diabetic subjects was not enough to cause metabolic improvement, yet possibility of cytokine for common metabolic disturbances such as a dyslipidemia and insulin resistance [68]. A research was conducted to elaborate the function for tumor necrosis factor α (TNFα) -G308A polymorphism for causing type 2 diabetes as well as cardiovascular disorder. Number of subjects choosen for study was 664 aged 85 years and above and it was found that reported polymorphism is connected with cardiovascular disorder and type 2 diabetes. Studies revealed that the -G308A TNF promoter polymorphism was related with the incidence of old age diabetes (P=0.006).The observed results for the risk of diabetes between homozygous for the A-allele was different from G-allele. It was found that A-allele was likely to be 4.6-fold (95% CI, 1.6-13.3) elevated than G-allele, moreover it was found by tremendous research of 10 year follow up that promoter polymorphism was not responsible of all causes of mortality including cancer, infectious diseases and cardiovascular disorders. Additionally TNFa and TNFc microsatellite genotypes were observed however not coupled with morbidity or mortality. It was concluded that the -G308A polymorphism is strongly associated with the menace of diabetes on the other hand excluding cardiovascular mortality [69].

2.5 Cardiovascular Disorders

Cardiovascular diseases that is also known as CVD is that class of diseases that involves blood or heart vessels [70]. CVD includes coronary artery diseases or CAD that can be myocardial infarction and angina that are commonly known as

heart attacks [71]. Some other cardio vascular diseases include hypertensive heart diseases, heart arrhythmia, rheumatic heart diseases, strokes, cardiomyopathy, thromboembolic disease, congenital heart diseases and many more [71,72]. It has been reported by WHO that cardiovascular disorders are the number 1 cause of death worldwide. In 2016, 31% of all global deaths having approximate number of 17.9 million people were because of cardiovascular disorders, Out of reported deaths 85% were as a result of stroke and heart attack [32]. In the United States annually round about 610,000 people die because of heart disease that's one in every four deaths. Heart problem is the important cause of death for both gender . Coronary heart disease (CHD) is the universal type of heart complaint, killing more than 370,000 people yearly [73]. WHO latest report published in 2017 tell us the figure for deaths in Pakistan reaches dues to cardiovascular problem was 265,051 or 21.76% of the total deaths.

2.5.1 Risk Factors of Cardiovascular Disorders

Risk factors of cardiovascular Disorders various health conditions, your age, family history and your lifestyle may increase your risk for heart disease. It has been reported that about 47% Americans suffering either from one of the contributing risk factors for cardiac problem including high blood pressure, high cholesterol and smoking [74].

2.5.2 Genetic Predispositions of Cardiovascular Disorders

Genetic predispositions of cardiovascular Disorders family history, twin studies, animal models and gene association studies hold up a hereditary foundation for coronary artery disease (CAD) [76]. Genes add to CAD expansion and progression, depending on environmental basis for risk factor modification and lifestyle choices. Ancestors record is the most excellent marker of a predisposition to CAD and more enhancement is probable by means of biochemical and DNA testing. LDL cholesterol, homocysteine and lipoprotein are the inherited cardiovascular risk

factor might be customized, such as early recognition of CAD might lead to earlier involvement for genetically vulnerable folks [36].

2.5.3 Role of TNF- α G308A Polymorphism in Cardiovascular Disorders

Tumor Necrosis Factor-α (TNF-α) play important role in inflammation process causing atherosclerosis. Despite its special effects on insulin resistance, lipid metabolism and endothelial function, it may be concerned in coronary heart disease (CHD). A study was conducted in a Mediterranean non-diabetic and type 2 diabetic populations to know about the association of polymorphism in Coronary heart diseases [75]. Total number of 241 CHD patients were taken for study and number of control group was 207, type 2 diabetic patients were 106 with CHD and 135 were type 2 diabetic patients without CHD were evaluated. RFLP-PCR technique was used to analyze single nucleotide polymorphism. It was observed by genotypic analysis that coronary heart disease having type II diabetes shows larger prevalence of reported polymorphism the -308 TNF-α A allele (40.6%) than controls (23.2%) as compared to this patients exclusive of type 2 Diabetes Mellitus shows (28.5%) (P=0.0056). The probability proportion of coronary heart disease in type 2 diabetic patients in occurrence with G-308 TNF-α an A allele was 2.86. This divergence was reported mainly in diabetic women for carriers A allele. These results propose that -308 TNF-α gene polymorphism may add to coronary heart disease risk in patients having type 2 diabetes and it could comprise functional projecting marker for coronary heart disease in women with type 2 diabetes [77].

In a Pakistani population a control familial study was conducted to estimate the relationship between TNF-alpha gene promoter SNPs at -308 and -238, and occurrence of coronary artery disease. Study was divided into two phases, for the first phase 150 patients were selected with 150 individuals with control group while second phase comprises genetics of vulnerable alleles from 88 families with CAD affected offspring. Biochemical analysis was done, while enzyme-linked

immunosorbent assay technique was used to asses serum TNF-alpha concentrations. PCR-RFLP method was used for genotyping of SNPs . Elevated serum TNF-alpha and hs-CRP were observed from CAD vs. controls ($P < 0.0001$; for both). In this case control study it was found that SNP was considerably related with the possible susceptibility of coronary artery disease CAD [78]. The results confirmed a significant link of polymorphisim allele A at -308 and CAD ($P = 0.0035$), whereas the -238 SNP was not related with the disease. Haplotype A–G of the TNF-alpha gene at -308GG>A and -238G>A showed higher frequency in the experimental group as compared to control group ($P < 0.05$). Furthermore data showed transmission of the disease susceptible allele A at TNF-alpha-308 from parent to affected offspring in a trio-family study ($P < 0.0001$). The existing research conclude that the TNF-alpha-308G>A polymorphism is associated with coronary artery disease in the observed residents In addition, It has been reported for the first time that the TNF-alpha-308A allele was notably related with the ancestral CAD for our high risk population [79].

Chapter 3

Materials and Methods

3.1 Methodology and Techniques

In order to achieve objectives of the project, Methodology summarize in figure 3.1 was designed with following major steps.

3.1.1 Selection of Location

Kahuta with population of 2.5 million was selected as sampling location for research conduction. The reason behind selection of this region was that it is one of the regions with multi ethnic diversity in Pakistan as people from all over the Pakistan are resident here.

3.1.2 Ethical Approval

This research was ethically approved from bioethical review committees Department of Bioinformatics and Biosciences, Capital university of Science and Technology. Informed Patient consent was also prescribed compulsory before sample collection.

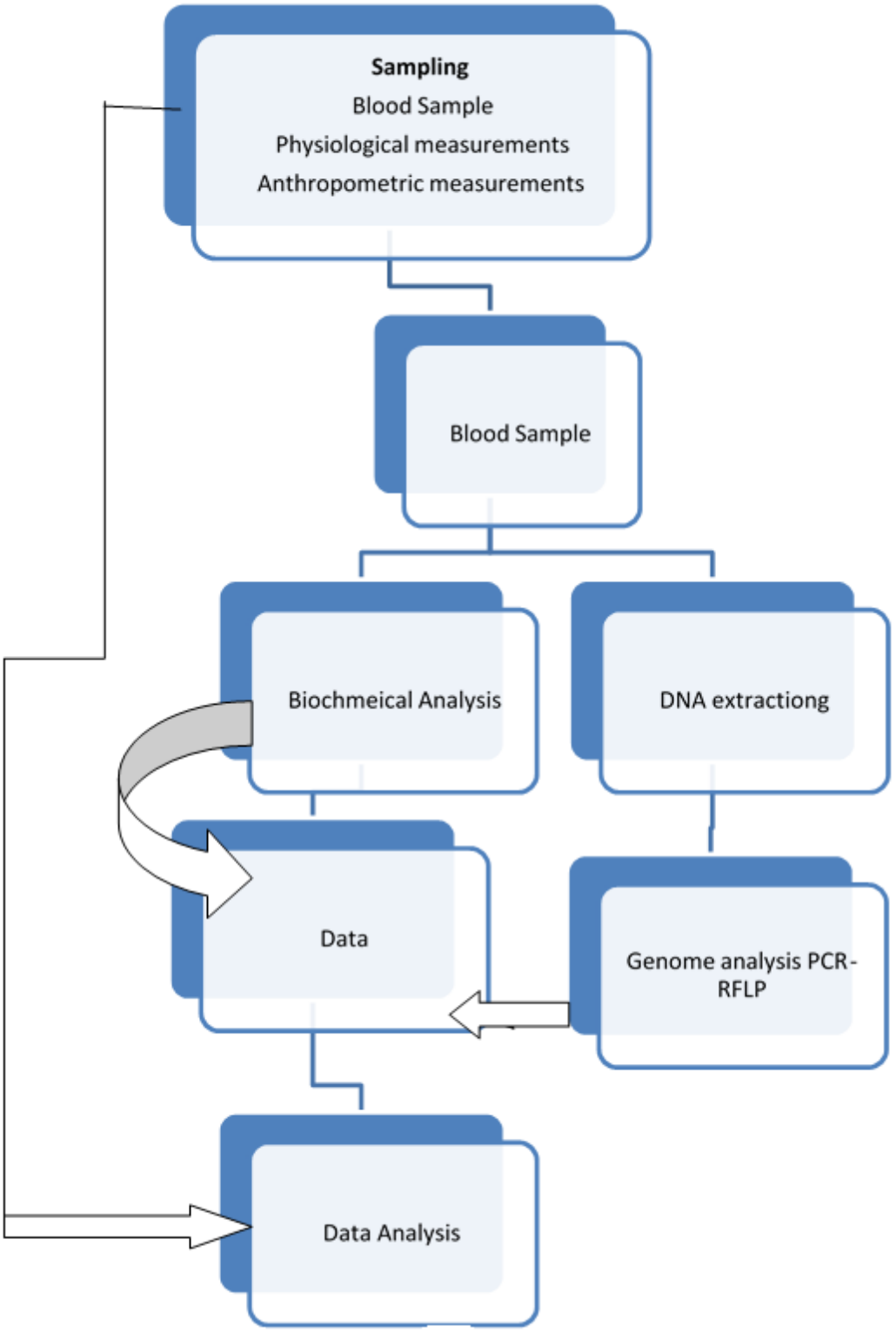

FIGURE 3.1: Methodological steps to determine association of G308A polymorphisim of TNF α with obesity induced diabetes and Cardiovascular disorders

3.1.3 Inclusion and Exclusion Criteria

The inclusion criteria for designed study were healthy individuals of age 18 and above living in Kahuta region, rather pregnant cases and physical injured or individuals with infections were excluded. A data collection form for collection of information were designed by review of literature and in consultation with physician.

3.1.4 Sampling and Testing Equipment

The equipment used for sampling and testing were blood sugar reagent kit(Merck), cholesterol reagent kit(Merck), BD syringes, alcohol swabs, facemasks, gloves, Vacutainers(Red, Purple and Gray Cap), Stethoscope, Measuring Tape, Glucose meter kit, test tubes, micropipettes, micropipettes tips (Blue and Yellow), Eppendrof tubes with holder, Water Bath, Incubator, Centrifuge Machine and PCR machine.

3.1.5 Sample Collection and Size Calculation

Technique used for sample collection was random from adults of all ages and ethnicities excluding people with major physical abnormalities. Random selection depends upon the size of population, area, and physical and chemical samples were collected from the people.For the calculation of regional prevalence and correlation, the formula used to calculate sample size was as below:

$$\text{Sample Size} = \frac{Z^2 * p\,(1-p)/e^2}{Z^2 * p\,(1-p)/e^2 N} \qquad \text{Equation: 1}$$

In the above formula, N is the population size, e is margin error and Z is the z-score the number of standard deviations. About 381 samples were collected by using above formula. Margin error was 5%, confidence level found was 95% and z- score was 1.96.

3.2 Anthropometric Measurements

Anthropometric measurements are commonly used to evaluate the size, shape, composition and other measurements related to human body. The most universal anthropometric dimensions include weight, height, abdominal circumference, and skin fold measurements as per the standards' I used following measurements as per requirement of my project.

3.2.1 Weight Measurement

An analog weight measuring device was used to measure weights of subjects before measuring weight machine was calibrated and 0 error was removed by adjusting pointer to exactly zero. Weight measuring machine was placed on a hard flat surface. Subjects were asked to remove extra clothing like heavy jackets, shawls, and shoes. They were asked to stand straight on both feet so that equal force was applied on the machine. Readings were taken twice and mean was calculated for each subject. Measured weight was recorded on each questionnaire in Kilograms.

3.2.2 Height Measurement

10-meter rod was purchased and fixed straight with a wall from floor subjects were asked to remove shoes and high heels in case of the female patient. They were asked to stand straight with face direction not too lower or high. A steel ruler was used to press hairs of subjects and note down the exact height in inches. Height was recorded on a questionnaire of each subject in inches.

3.2.3 Body Mass Index Calculation

Body Mass Index was calculated using formula Kg/m2 in Microsoft excel registered version. Subjects were classified into six categories such as underweight, normal,

overweight, obese class 1, obese class 2, and obese class 3 as per recommendations of WHO.

3.2.4 Blood Pressure

Blood Pressure was measured by means of a blood pressure measuring device. The device was calibrated with manual mercury-based BP device with the consultation of a doctor. No stethoscope was required for digital measuring of BP. Subjects were asked to sit calm and take rest for five minutes. Two readings were obtained and the average was calculated and recorded on a questionnaire of each subject and readings were recorded as diastolic/ systolic.

3.2.5 Waist Circumference

Waist circumference was measured with a simple measuring tape around the waist.

3.3 Blood Sampling and Testing

Subject were asked to sit relaxed, a suitable site for vein puncture to collect blood, by tiding the tourniquet 3 to 4 inches above was selected for insertion of the syringe on the subject arm or back side of Hand.

After putting gloves vein was palpated. The vein was selected, cleaned in a circular motion, after the area was cleaned, it was touched or palpated again.

Subjects were asked to close the fist and avoid pumping the fist. Subject arm was firmly gripped and the needle was inserted into the lumen of the vein. syringe was filled for 5cc blood. Tourniquet was removed first, than needle from the patient's arm was removed using a swift and backward motion. Alcohol swab was placed as a precautionary measure to avoid scar. 5ml blood from each subject was collected in 5 cc Syringe. 1 ml blood was stored in red-capped clot activator vacutainer

for cholesterol and 2 ml Blood was stored in grey capped vacutainer containing sodium fluoride and potassium oxalate for Glucose test. For DNA extraction 2ml blood was stored in Purple cap anticoagulant EDTA vacutainer.

3.3.1 Sample Preparation

Blood samples collected in red and gray capped vacationer centrifuged for 5-10 min at 800 rpm to separate serum. Using micropipette 100 microliters of serum and cholesterol and glucose reagent poured and mixed in different disposable test tubes. Samples were then incubated in a water bath for 5-10 min.

3.3.2 Biochemical Analysis

Two biochemical test were performed on blood samples.

3.3.3 Blood Glucose Measurement

Blood glucose level was measured by glucometer. A very simple method involve pricking finger to get a drop of blood on a blood sugar measuring strip. The strip was inserted into the machine and results were displayed on the screen hardly in seconds.

3.3.4 Total Lipid Profiling

Cholesterol level was measured in order to check the total lipid profile of individuals. Lipid tests were then performed on blood samples to identify the low-density lipoproteins, high-density lipoproteins and triglycerides profile in the body.

3.4 PCR-RFLP Assay

3.4.1 DNA Extraction

DNA was extracted from the samples by using salting out method through following given procedure. Some of the reagents that will be used in this procedure are given in (Table 3.1).

3.4.2 RBCs Lysis

Lysis of RBCs will be done by adding 50 μl of 1x Triton-X and 900μl TKM 1 to 300 μl blood inside an autoclaved 1.5 ml eppendorf. This mixture was put for incubation at 37 °C for 5 minutes for the breakdown of red blood cells. Cells were further centrifuged for 3 minutes at 8000 rpm after that supernatant was discarded. Same step was repeated 3-4 times by decreasing amount of 1x Triton-X till RBCs lysis got complete. At the end white pallet containing white blood cells WBCs was obtained.

3.4.3 Cell Lysis

300μl of TKM 2 and 40 μl of 10% SDS was added to the pellet obtained through RBCs lysis. After thorough mixing, the mixture was incubated for 5 minutes at 37 °C. In the last part 100 μl of 6M NaCl was added and the mixture was vortexed to precipitate the proteins. The obtained cells were centrifuged further for 5 minutes at 8000 rpm.

Table 3.1: Composition of Reagents

Sr. No	Reagent Name	Composition	Volume Prepared
1	TKM 1 BUFFER (LOW SALT BUFFER)	Tris HCI=0.605g KCI=0.372g Mgcl2 =1.016g EDTA=0.372g Mixed them well in 500 ml of water and adjust the pH at 7.6.	500 ml
2	TRITON-X	100% triton-x=0.1 ml Distilled water= 9.9 ml Mixed well.	10 ml
3	TKM 2 BUFFER (HIGH SALT BUFFER)	Tris HCI= 0.121 g KCI= 0.74 g Mgcl2 =1.203g EDTA=0.467g Mixed them well in 100 ml of water and adjust the pH at 7.6.	100ml
4	SODIUM DODECYL SULPHATE (SDS)	SDS= 1g Distilled Water = 10ml Mixed well.	10ml
5	6M NaCl	Nacl= 8.765 g Distilled water= 25 ml Add NaCl in 25 ml of distilled water and mixed well to completely dissolve.	25 ml

Table 3.1 continued from previous page

Sr. No	Reagent Name	Composition	Volume Prepared
6	THE BUFFER	Tris HCI= 0.30g EDTA= 0.009g Distilled water= 100ml Add both chemicals in 100ml of distilled water and adjust its pH to 8.0	100ml

3.4.4 DNA Precipitation

The supernatant obtained in the last step was poured carefully in new eppendorf tubes containing 300 μl of isopropanol. Precipitation was done by inverting the eppendrof gradually Further, the eppendorf were centrifuged for 10 minutes at 8000 rpm to settele down the DNA. The supernatant was removed, and 100μl of 70% ethanol was added to the Eppendorf and mixed slightly to get rid of surplus salts. Eventually the tubes were centrifuged for 5 minutes at 8000 rpm to pellet down the DNA. The supernatant was removed and DNA was air dried. Following the step of careful drying, 50 μl of buffer(TE) was added to soften the DNA. DNA was preserved at 40 c for years without disintegration.

3.4.5 Primer Designing / Preparation

Standard protocol of primer prepration was used by adding 20µl pimer and 80µl water in mini eppendrof and could be stored at chilling temperature in refrigerator. To determine the polymorphism presence primer was designed using primer3as Forward F- 5'-AGG CAA TAG GTT TTG AGG GCC AT-3' and reverse primer as R- 5'-CAT CAA GGA TAC CCC TCA CAC TC-3' Fig 3.1

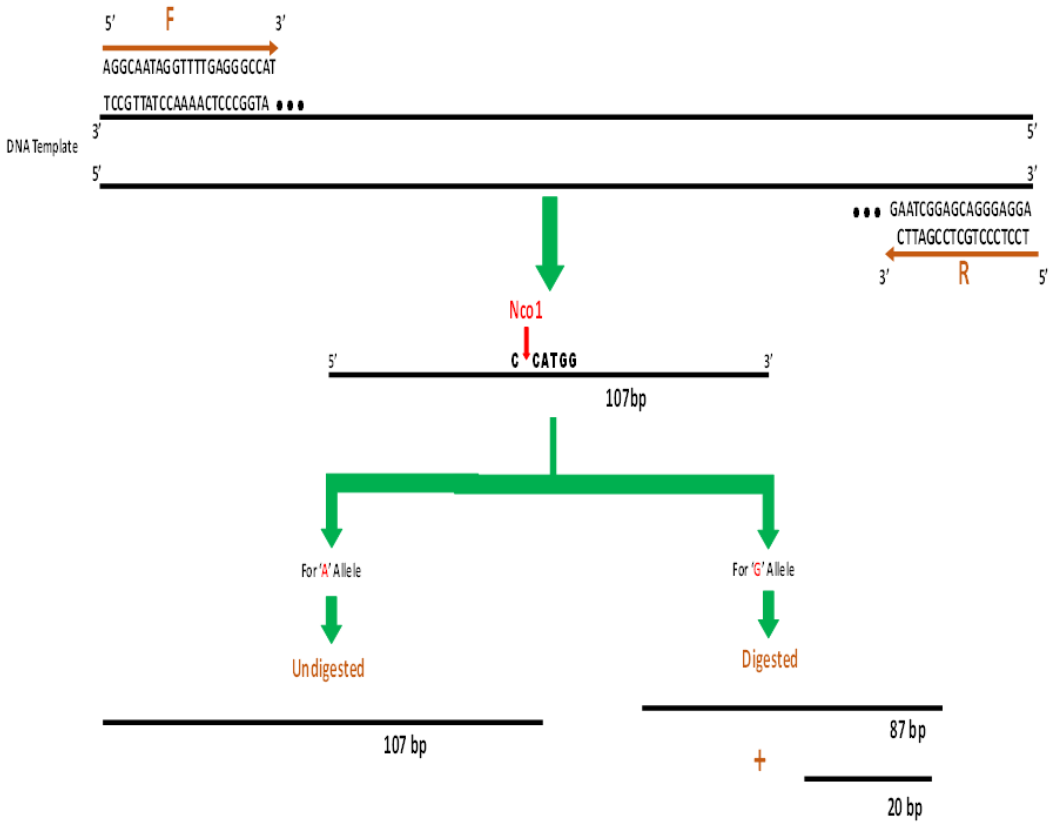

FIGURE 3.2: Shows designed primer for Polymorphisim TNF-α 308G/A.

3.5 PCR Optimization

PCR protocol Optimized to analyze several samples in single PCR cycles. PCR products aere analyzed for quality on Agarose Gel. The PCR recipe is given below in table 3.2. This PCR recipe will make a total of 10μl that will be added into the PCR tubes and placed into the PCR machine by setting its temperature of denaturation, annealing, and extension. Firstly temperature of 94℃ was set for 2 minutes, secondly again 94℃ was adjusted for 1 minute, thirdly 58.5℃ was set for 40 seconds and then 72℃ for 30 seconds.Final extension of 72℃ for 10 minutes was done and hold for 40℃.

TABLE 3.2: PCR Recipe For The PCR Optimization

Material	**Quantity**
10X reaction buffer	1µl
dNTPs	1µl
Taq polymerase	0.6µl
$MgCl_2$	1µl
Forward primer	0.8µl
Reverse primer	0.8µl
DNA	1.5µl
H_2O	4.6µl

3.5.1 Ezyme Digestion

After getting PCR product enzyme digestion was done according to optimized procedure by taking 10 µl PCR product, 18 µl nuclease free water was added with the quantity of 2µl tango buffer and 1.5 µl NcoI enzyme. Product was mixed gently and incubated for 3hours.

3.6 GEL Electrophoresis

After incubation Gel electrophoresis was done to visualize the banding patterns of TNF-α G308 polymorphism to analyze the association of obesity induces type 2 diabetes and cardiovascular disorders.

3.7 Statistical Analysis

The statistical analysis was done by the use of SPSS software. Prevalence in the form of a percentage, pie charts, and bar charts are plotted using Ms excel. chi-square test was used to determine the significant association of a variable with each other. The correlation was calculated to determine the association of inflammation with obesity and its co-morbidities. Odds ratios and relative risk were calculated to determine the exposure of odds outcome. Significance is defined as p-value 0.05. following mathematical equation that was used to calculate the genetic variation of a population at equilibrium $p2 + 2pq + q2 = 1$

Chapter 4

Results and Discussion

The study was designed with major objectives to determine the prevalence of obesity and obesity induced diabetes and cardiovascular disorder and to find association of G308A polymorphism in TNF-α with obesity induced diabetes and cardiovascular disorders. Kahuta multi ethnic region located at the distance of 25 Km from Rawalpindi and Islamabad was selected for designed study. Three ethically approved free medical camps were arranged in both rural and urban areas for research and sample collection. Furthermore study was conducted over a period of eight months from June 2018 to January 2019. Total no of 400 samples were collected but evaluated samples were 283 because rest of the samples data were inadequate. Percentage for both gender were almost equal. Out of 283 Individuals 142 were male and 141 were females.

4.1 General Characteristics of Population

General characteristics of population are given in (Table 4.1).

Table 4.1: Showing Statistical Analysis of General characteristics of Population

	Age	Height(Meters)	Weight (Kg)	BMI
N	277	283	283	283

Table 4.1 continued from previous page

	Age		Height(Meters)	Weight (Kg)	BMI
Mean	40.68		1.63	69.41	26.30
Std. Deviation	16.30		.121	16.041	5.901
CRP		Blood Pressure		Cardiovascular	
+ve	-ve	High BP	Normal BP	CVD Presence	CVD Absence
88	195	111	172	40	243

4.1.1 Prevalence of Obesity

Total no of subjects were 283, out of them 216 were non obese and 67 were obese. In kahuta region prevalence of obesity is 24% (Figure 4.1) While Non obese population was found to be 76%. Obesity was determined by the comparison of the classification of obesity according to WHO (BMI) and Asia-Pacific (BMI) as shown in table 4.2

TABLE 4.2: Classification of Obesity According To WHO (BMI) and Asia-Pacific (BMI)

	WHO (BMI)	Asia Specific (BMI)
Underweight	>18	>18
Normal	18.5-24	18.5-22.9
Overweight	25-29.9	23-24.9
Obese	≥30	≥25

According to Pakistan Bureau of Statistic (2017) 72.5% of Kahuta population lies in rural areas and as per our results obesity is low in rural areas due to their lifestyle and these results correlates with the results of Nutright Blog Obesity in Pakistan (2017) The Alarming stats 2017 [77].

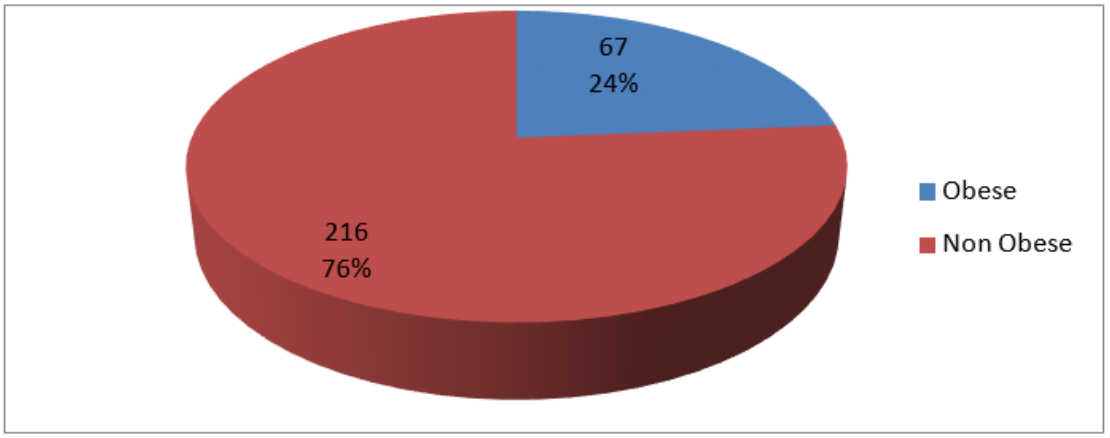

FIGURE 4.1: Percentage of Obese and Non Obese Population

4.1.2 Prevalence of Diabetes

Out of 283 individuals 13% were diabetic while 87% were non diabetic. Diabetes was determined by random blood sugar. Diabetes was determined by fasting Blood glucose and random blood glucose. During sampling random glucose was taken as it is independent of whatever is been eaten before. Its normal range lies between 100 mg/dL (5.6 mmol/L). More than100 mg/dL is considered indication of diabetes.

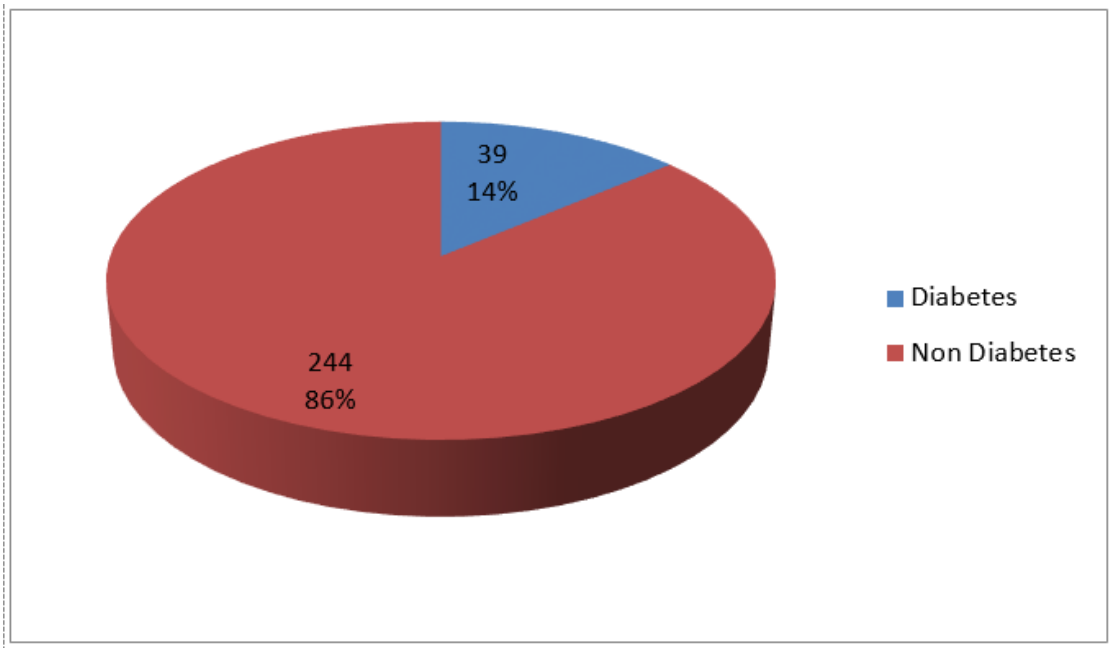

FIGURE 4.2: Percentage of Diabetic and Non Diabetic Individuals

4.1.3 Prevalence of Cardiovascular Disorder

Prevalence of cardiovascular in Kahuta region is 27% and non cardiovascular patients were 72%. WHO 2014 reported for Pakistan that 19% of total deaths are only because of cardiovascular disorder in all ages and both genders and this ratio is increasing day by day as the mortality rate due to cardiovascular disorder was 29% in 2018 as per new findings of WHO.

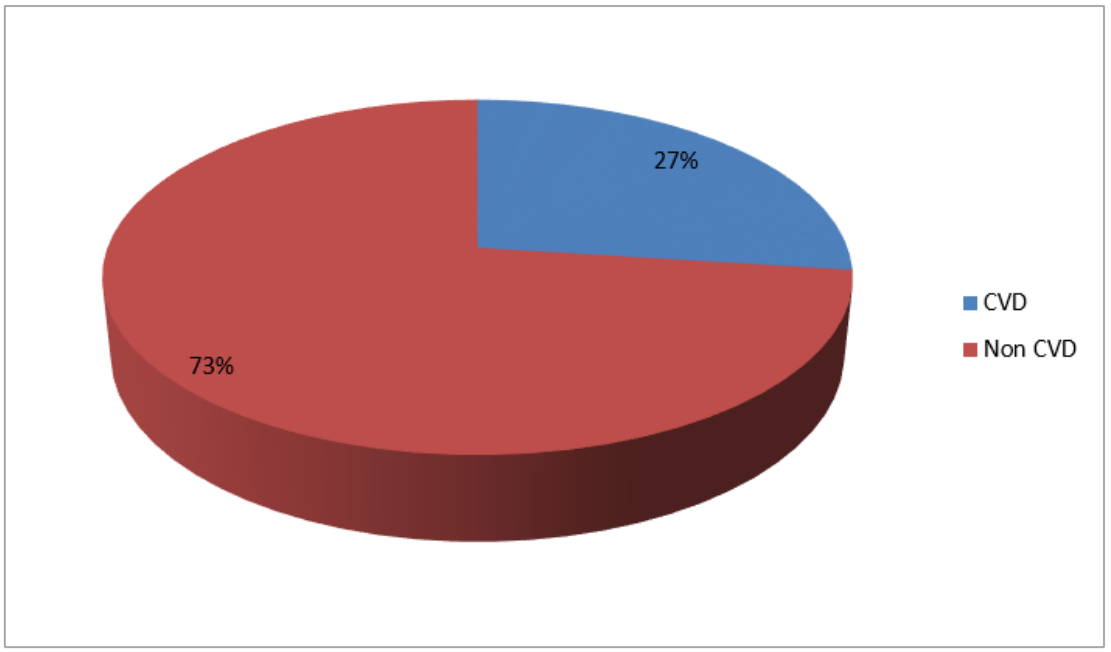

FIGURE 4.3: Percentage of CVD And Non CVD Individuals

4.1.4 Prevalence of Obesity Induced Diabetes

Total no of subjects were 283, out of them 216 were non obese and 67 were obese. Frequency for obesity induced diabetes was 16.4% and non obese population was also found with 12.5% diabetic patients. Chi-square test revealed the presence of p-value of 0.411 which was suggesting non significant association between obesity induced diabetes. Body mass index has a strong relationship to diabetes and insulin resistance.

Diabetes mellitus affects more than 180 million people around the world, and the number of patients is probable to raise to 300 million by 2025. Obesity-associated

TABLE 4.3: Prevalence of Obesity Induced Diabetes

		Diabetes	**Total**	
		No	**Yes**	
Non Obese	**Count**	189	27	216
	Expected Count	187	29	216
	% within Obesity	87.50%	12.50%	100.00%
Obese	**Count**	56	11	67
	Expected Count	58	9	67
	% within Obesity	83.60%	16.40%	100.00%
Total	**Count**	245	38	283
	Expected Count	245	38	283
	% within Obesity	86.60%	13.40%	100.00%

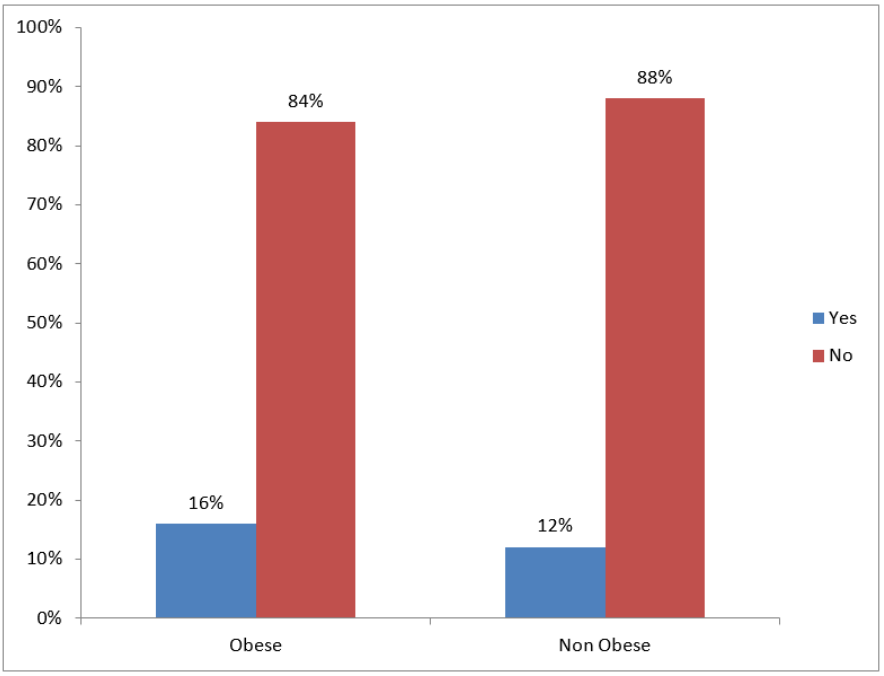

FIGURE 4.4: Percentage of Obesity Induced Diabetes. x -axis represent obese and non obese individuals either with presence or absence of diabetes while y-axis represent percentage of population.

type 2 diabetes accounts for 90–95% of all diagnosed diabetes in adults [78] (Table 4.3).

4.1.5 Prevalence of Obesity Induced Cardiovascular Disorder

Total no of subjects were 283, out of them 216 were non obese and 67 were obese. Frequency for obesity induced CVD was 44.8% and non obese population was also found with 22.7% cardiac patients. Chi-square test revealed the presence of p-value of 0.000 which was suggesting significant association between obesity induced cardiovascular problems. In Pakistan, 30 to 40% of all death is caused by CVD.

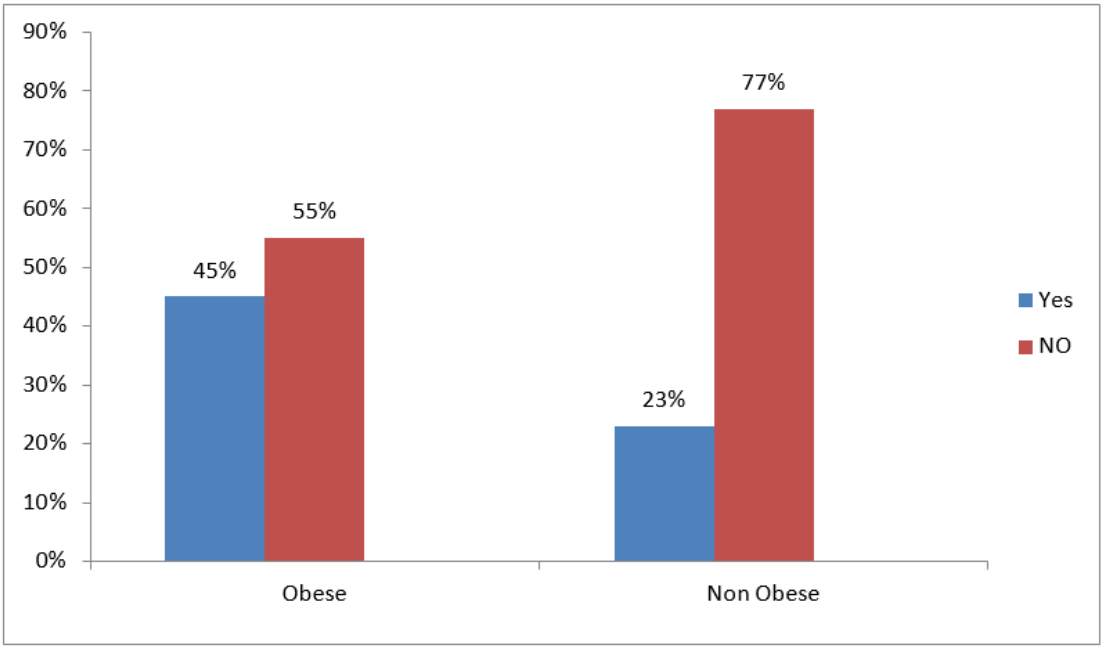

FIGURE 4.5: Percentage of obesity induced Cardiovascular Problem. x -axis represent obese and non obese individuals either with presence or absence of cardiovascular problem while y- axis represent percentage of population.

The CHD death in Pakistan has reached about 200,000 per year that is 410/100,000 of the total population. CVD mortality and morbidity has been shown to be elevated in individuals that are overweight, particularly with central deposition of fat [79- 81].

TABLE 4.4: Statistical Analysis of Obesity Induced Cardiovascular Disease.

	CVD Probability	**Total**		
			No	Yes
Non Obese	Count	167	49	216
	Expected Count	155.7	60.3	216
	% within Obesity	77.30%	22.70%	100.00%
Obese	Count	37	30	67
	Expected Count	48.3	18.7	67
	% within Obesity	55.20%	44.80%	100.00%
Total	Count	204	79	283
	Expected Count	204	79	283
	% within Obesity	72.10%	27.90%	100.00%

4.1.6 C-Reactive Protein

C-reactive protein (CRP) is a marker of inflammation in the body produced by liver cells. CRP levels of 1 mg per liter or lower are considered low risk for cardiovascular disease. CRP levels of 1-3 mg per liter are considered moderate risk for cardiovascular disease and greater than 3 mg per liter are considered high risk for cardiovascular disease.CRP levels more than 10 mg per liter may put forward an acute coronary process, such as heart attack acute myocardial infarction. Out of 283 individuals 88 were showing positive CRP while 195 were having negative CRP constitute percentage of 31% and 69% (Figure 4.6).

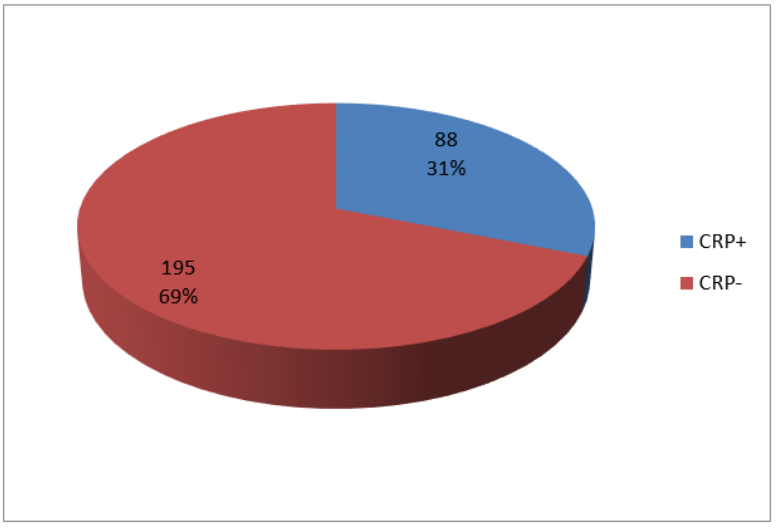

FIGURE 4.6: Percentage of Positive and Negative Cases of C-Reactive Protein

TABLE 4.5: Percentage of Positive and Negative CRP in Obese and Non Obese Population

	C-Reactive Protein		Total
	positive	negative	
Obese	34	31	65
Non obese	53	163	216
Total	88	195	283

4.1.7 C-reactive Protein Vs Obesity

Out of 283 selected samples 88 were having positive CRP while 195 were found to be with negative CRP. Further categorizing from obese and non obese population 34 individuals comprising 52% with obesity were CRP positive while while 31 respondents comprising 47% were showing negative results. On the other hand from non obese population 53 individual with 25% shows positive CRP while 163 individual having 75% are with negative CRP.

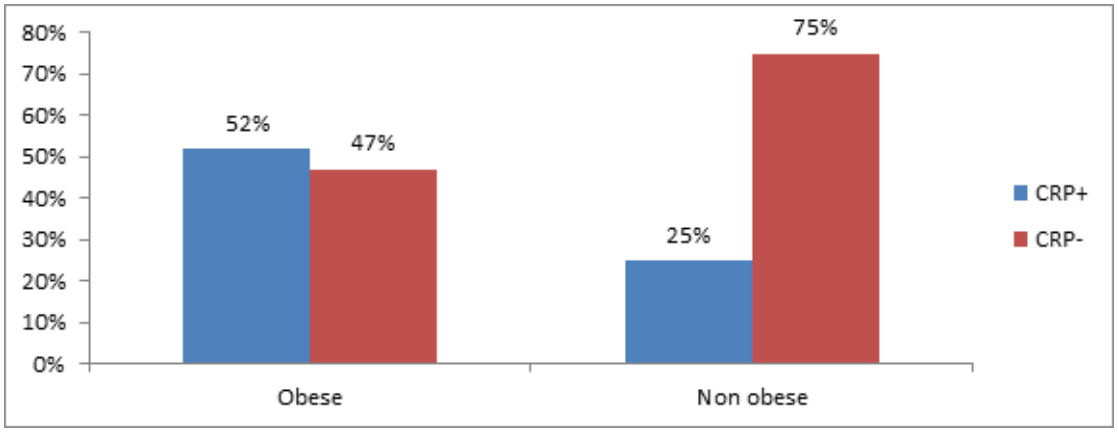

FIGURE 4.7: Positive and Negative C-Reactive Protein in Obese and Non-Obese Population. x -axis represent obese and non obese individuals either with positive or negative CRP while y- axis represent percentage of population.

Chi square vale is 0.000 which is less than 0.05 and its showing significant association of obesity with CRP.

4.1.8 CRP Vs Diabetes

Out of 283 selected samples 88 were having positive CRP while 195 were found to be with negative CRP. Further categorizing from diabetic and non diabetic

population 14 individuals consist of 16% with diabetes were CRP positive while 74 respondents consist of 84% were showing negative results making total of 100%. On the other hand from non diabetic population 25 individual with 13% shows positive CRP while 170 individual having 87% are with negative CRP. Chi square value reveals p value of 0.293 showing non significant association

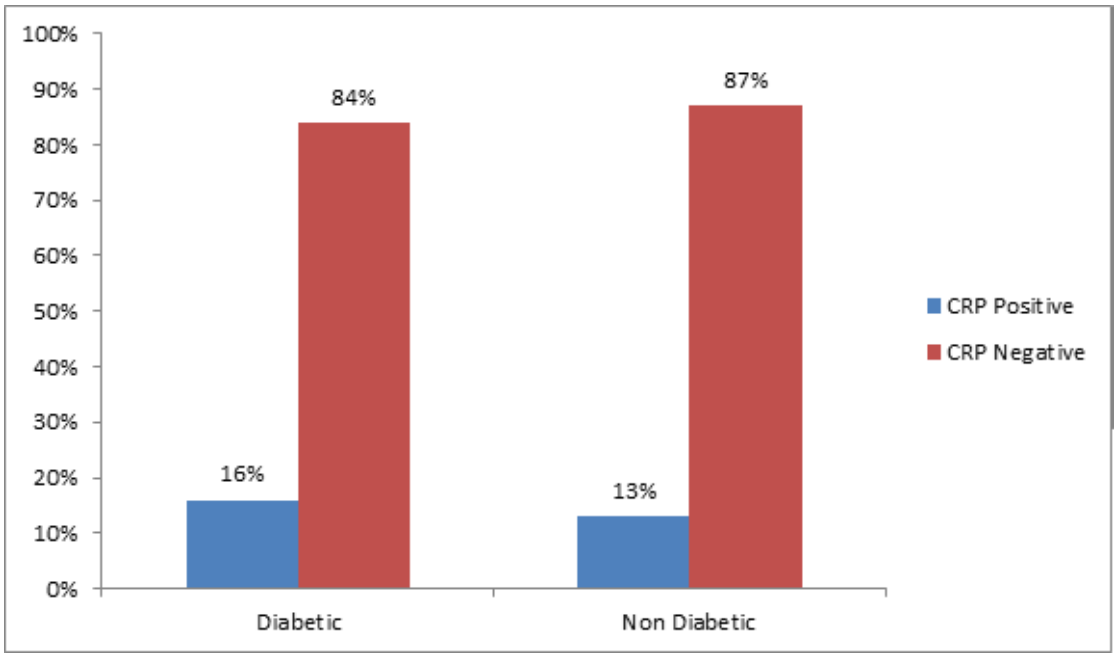

FIGURE 4.8: Positive and Negative C-Reactive Protein in diabetic and Non-diabetic Population. x -axis represent diabetic and non diabetic individuals either with positive or negative CRP while y- axis represent percentage of population.

TABLE 4.6: Showing Positive and Negative CRP in Diabetic and Non Diabetic Population

C-Reactive Protein			
	positive	negative	Total
Diabetes	14	74	88
Non diabetic	25	170	195
Total	38	244	283

TABLE 4.7: Showing CRP Induced Cardiovascular Problem

CRP	CVD Probability		Total
	yes	no	
Positive	43	45	88
Nagative	37	158	195
Total	80	203	283

4.1.9 CRP Vs CVD Probability

CRP could be the marker to determine cardiovascular problem. The evaluated samples having cardiovascular problem was 80 and out of these samples it was found that 43 individuals comprise 54% were showing CRP positive results while 37 individuals comprise 46% were showing CRP negative results. Moreover it was compared with 203 non cardiac patients and I observed that out of 203 samples,45 individuals cover 22% were showing CRP positive results while the rest 158 cover 78% were showing CRP negative results as shown in table 4.6. Chi square vale is 0.000 which is less than 0.05 and its showing significant association of CRP with cardiovascular problem.

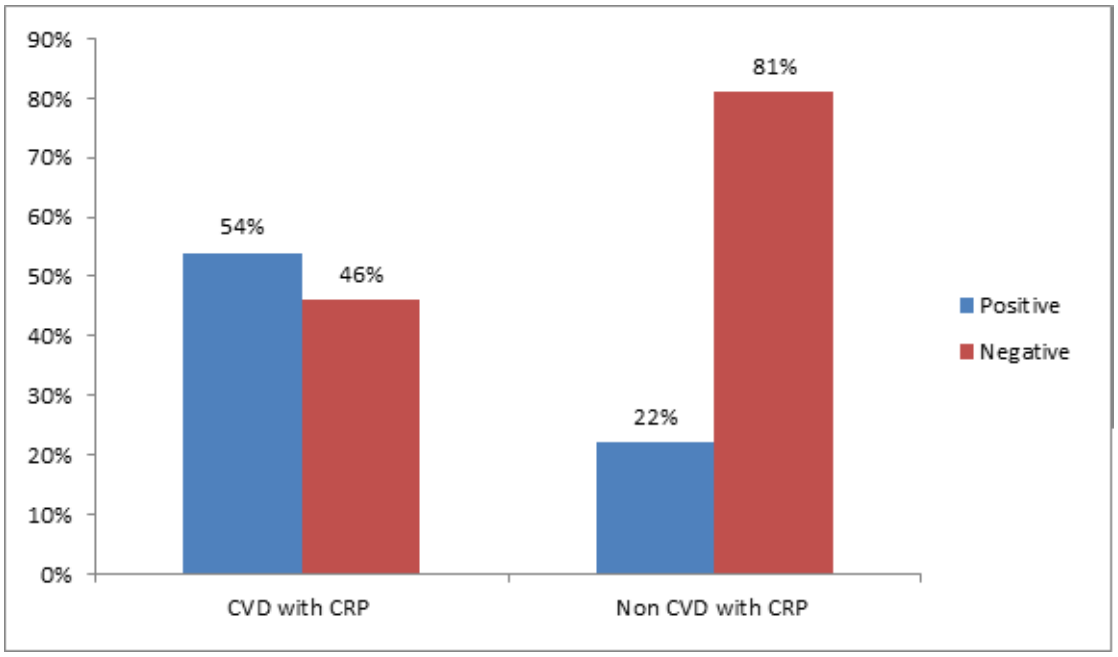

FIGURE 4.9: CRP Induced Cardiovascular Problem Probability. x -axis represent cardiac and non cardiac individuals either with positive or negative CRP while y- axis represent percentage of population

4.2 Prevalence of G308A Polymorphism

The prevalence of polymorphism in overall population was found to be with following percentage with alleles 43% for AA, 33% for AG, GA and 24% for GG (Table 4.8).

TABLE 4.8: Showing Prevalence of Risk Alleles

Polymorphisim	No	Percentage
AA	121	42.8
AG,GA	95	33.6
GG	67	23.7
Total	283	100.0

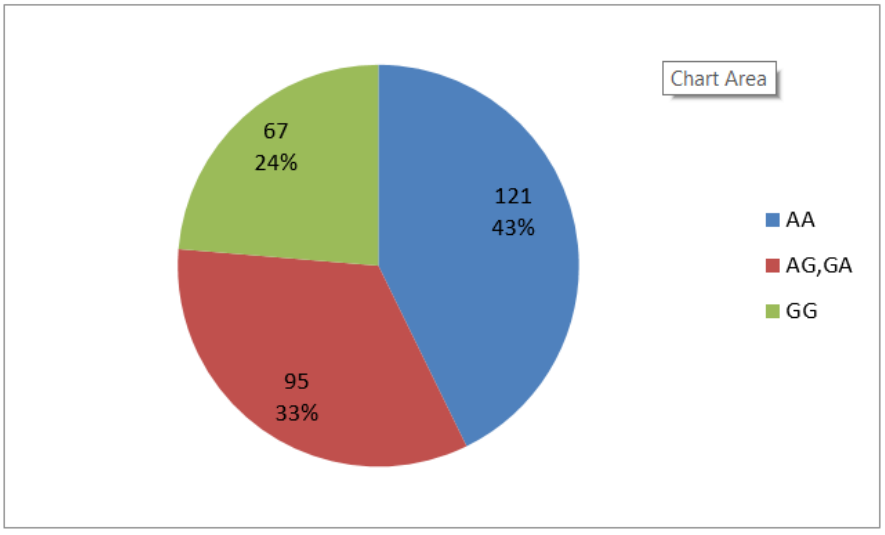

FIGURE 4.10: Prevalence of Risk Alleles in a Population.

4.2.1 Association of Polymorphism with Obesity

The prevalence of G308A for obese individual was 46% for AA allele while AG,GA was 28% and GG was found to be 25%. While for non obese individuals AA constitute 42% while AG,GA have 35% while GG comprises 23%. Chi square vale is 0.062 which is greater than 0.05 and its showing non significant association of obesity with G308A.

TABLE 4.9: Polymorphism with its Alleles in Obese and Non obese Population.

Count		G08A			Total
		AA	AG, GA	GG	
Obesity	Obese	31	19	17	67
	Nonobese	90	76	50	216
Total		121	95	67	283

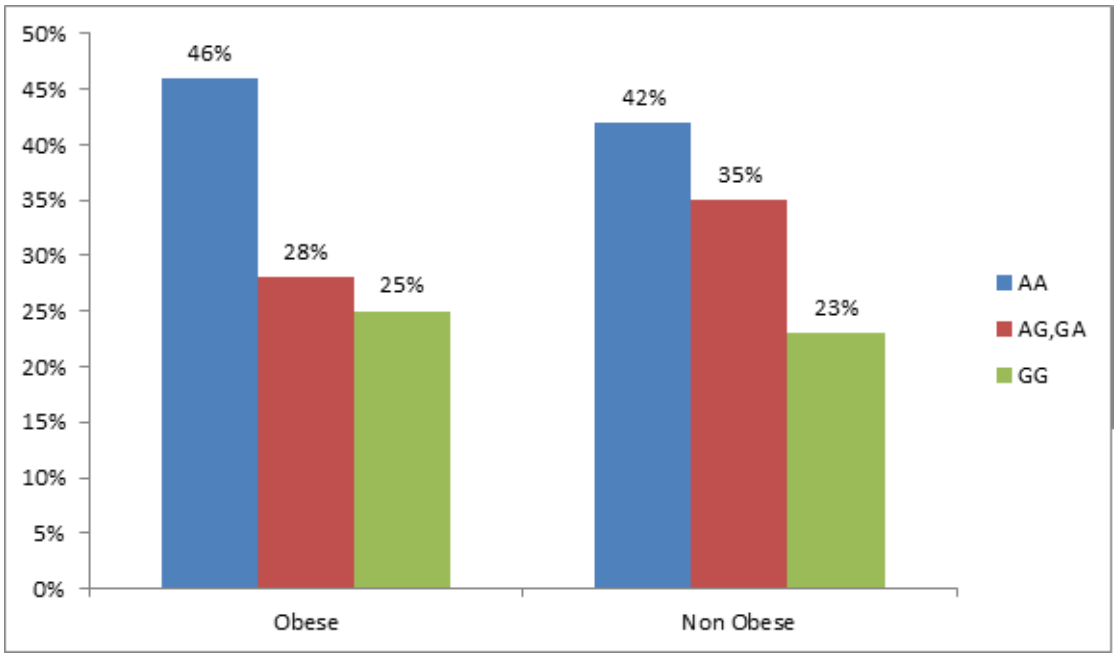

FIGURE 4.11: Percentage of Alleles in Obese and Non obese Population. x -axis represent obese and non obese individuals with Allele probability while y-axis represent percentage of population.

4.2.2 Association of Polymorphism with Diabetes

The prevalence of G308A for diabetic individual was 47% for AA allele while AG,GA was 29% and GG was found to be 24%. While for non diabetic individuals AA constitute 42% while AG,GA have 34% while GG comprises 24%. Chi square vale is 0.759 which is greater than 0.05 and its showing non significant association of diabetes with G308A (Table 4.10).

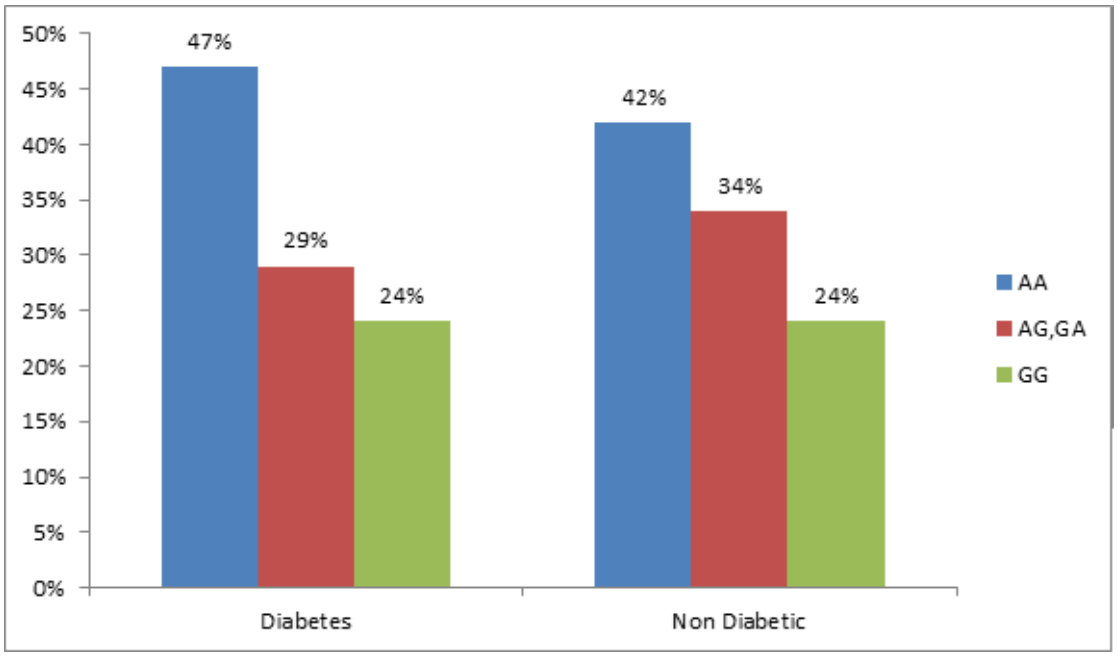

FIGURE 4.12: Percentage of Allele in Diabetic and Non-diabetic pop- ulation. x -axis represent diabetic and non diabetic individuals with Allele probability while y- axis represent percentage of population.

TABLE 4.10: Polymorphism with its Alleles in Diabetic and Non Diabetic Population.

Count		G308A			Total
		AA	AG, GA	GG	
Diabetes	Yes	19	11	9	39
	No	102	84	58	244
No		121	95	67	283

4.2.3 Association of Polymorphism with Cardiovascular Disorder

The prevalence of G308A for cardiovascular individual was 35% for AA allele while AG,GA was 35% and GG was found to be 30%. While for individuals having no any cardiac problem,allele AA constitute 44% while AG,GA have 33% while GG comprises 23%.

Chi square vale is 0.479 which is greater than 0.05 and its showing non significant association of Cardiovascular disorder with G308A. Chu et al conducted a studied on TNF polymorphism and risk of CHD and MI and it was found that AA genotypes in the G-308 A (rs1800629)polymorphism of the TNF-α gene did

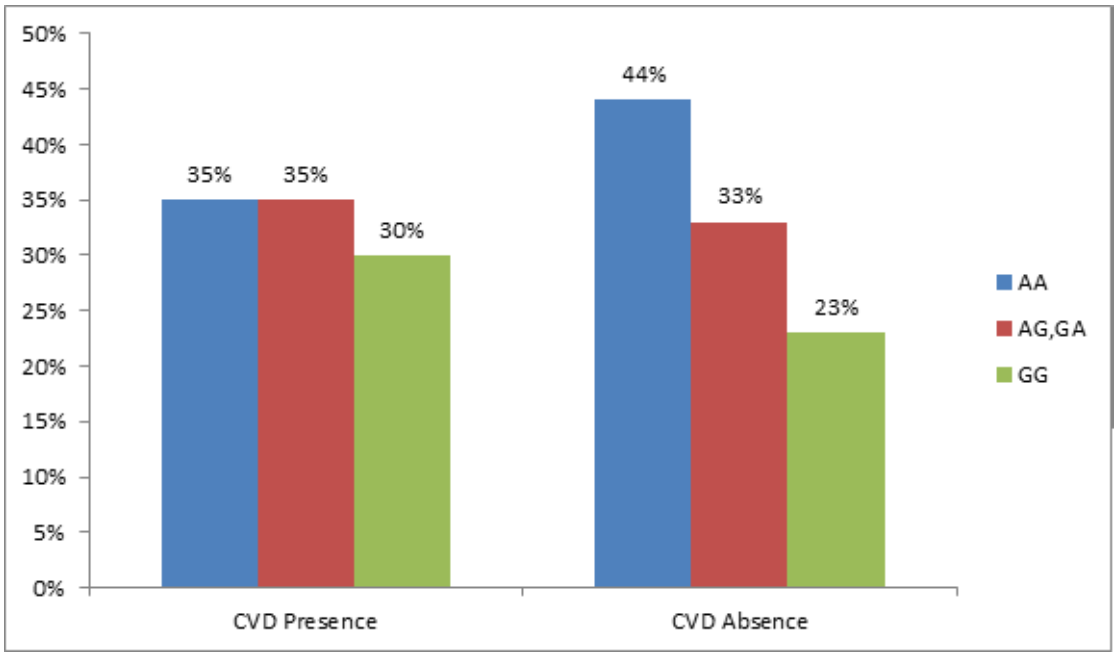

FIGURE 4.13: Percentage of Alleles in Cardiac and Non Cardiac Patients. x -axis represent Cardiac and non cardiac individuals with Allele probability while y- axis represent percentage of population.

TABLE 4.11: Polymorphism with its Alleles in Cardiac and Non Cardiac Population.

Count		G308A			Total
		AA	AG, GA	GG	
CVD (Question)	yes	14	14	12	40
	No	107	81	55	243
Total		121	95	67	283

not occur more frequently in CHD/MI patients than in controls. Study showed no association between the G-308 A (rs1800629) polymorphism of the TNF-α gene (presence of A allele) and CHD/MI in the Chinese Han population [82]. The above mentioned results truly matches with our results depicted in figure.

4.3 Allele Frequency

Overall frequency of Allele "A" and "G" is depicted in figure as we observed that number of 'A' allele is more frequently distributed as compared to 'G' allele. For 283 individual no of Alleles are 566 and out of this number I found that "A" Allele is 337 comprising 60% and "G" Allele is 229 making 40% (Figure 4.14)

$$The\ frequency\ of\ an\ allele = \frac{Total\ no\ of\ copies\ of\ that\ allele\ in\ population}{Total\ no\ of\ copies\ of\ all\ alleles\ of\ gene}$$

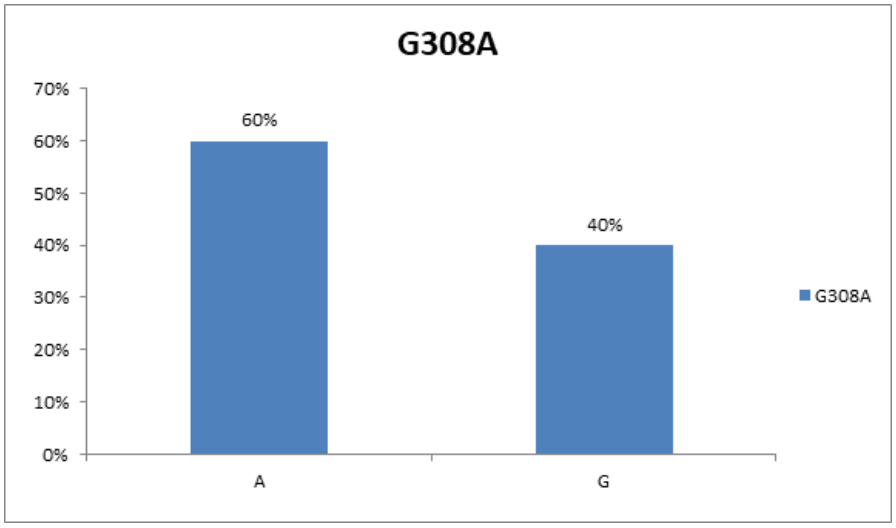

FIGURE 4.14: Frequency of allele 'A' and ' G within overall sample size. x -axis represent 'A' and 'G' allele while y- axis represent percentage of population.

No of A allele is 337 and divided with 566 it makes the value of p as 0.59 and G alleles are 229 and they are divided by 566 total alleles and they makes q as 0.40 and hence P+q=1, 0.59+0.40=1

4.3.1 Frequency of 'A' and 'G' Allele in Obese and Non Obese Individuals.

Total no of "A" Allele in obese individuals are 81 and "G" Allele are 53 in number and for non obese individuals "A" alleles are 256 and "G" alleles are 176.

The percentage of 'A' allele for obese individuals was found to be 14% and for non obese was 45% while percentage of 'G' allele was 9% for obese and 31% for non obese. Both 'A' and 'G' allele comprise 100% following the allele frequency equation as p+q=1

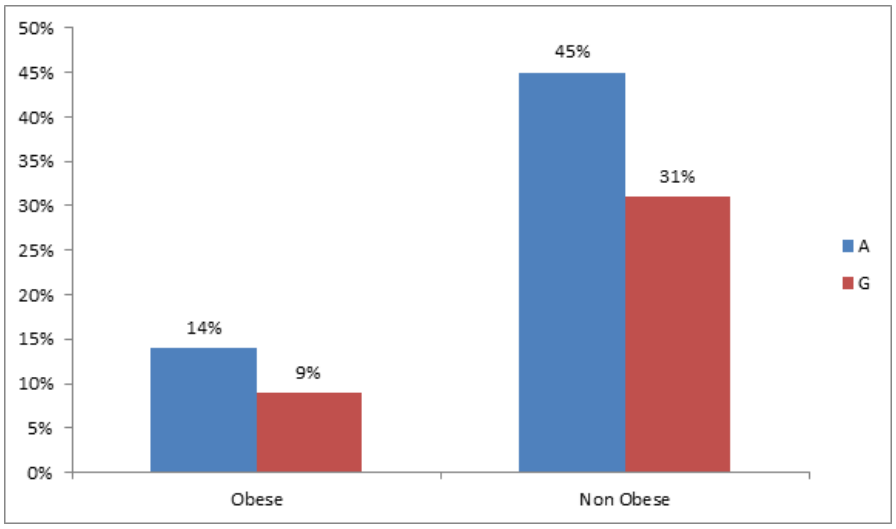

FIGURE 4.15: Frequency of 'A' and 'G' Allele in obese and non obese individuals. x -axis represent 'A' and 'G' allele in obese and non obese individuals while y- axis represent percentage of population.

4.3.2 Frequency of 'A' and 'G' Allele in Diabetic and Non Diabetic Individuals

Total no of "A" Allele in diabetic individuals are 49 and "G" Allele are 29 in number and for non diabetic individuals "A" alleles are 288 and "G" alleles are 200. The percentage of 'A' allele for diabetic individuals was found to be 9%

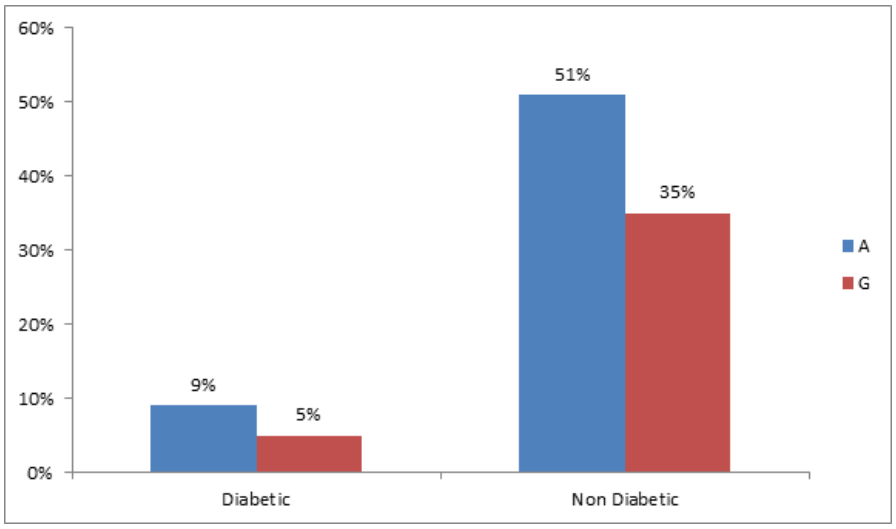

FIGURE 4.16: Frequency of 'A' and 'G' Allele in diabetic and non-diabetic individuals. x -axis represent 'A' and 'G' allele in obese and non obese individuals while y- axis represent percentage of population.

and for non diabetic was 51% while percentage of 'G' allele was 5% for diabetic

individuals and 35% for non diabetic. Both 'A' and 'G' allele comprise 100% following the allele frequency equation as p+q=1

4.3.3 Frequency of A and G Allele in Cardiac and Non Cardiac Individuals.

Total no of "A" Allele in cardiac individuals are 42 and "G" Allele are 38 in number and for non cardiac individuals "A" alleles are 295 and "G" alleles are 191.

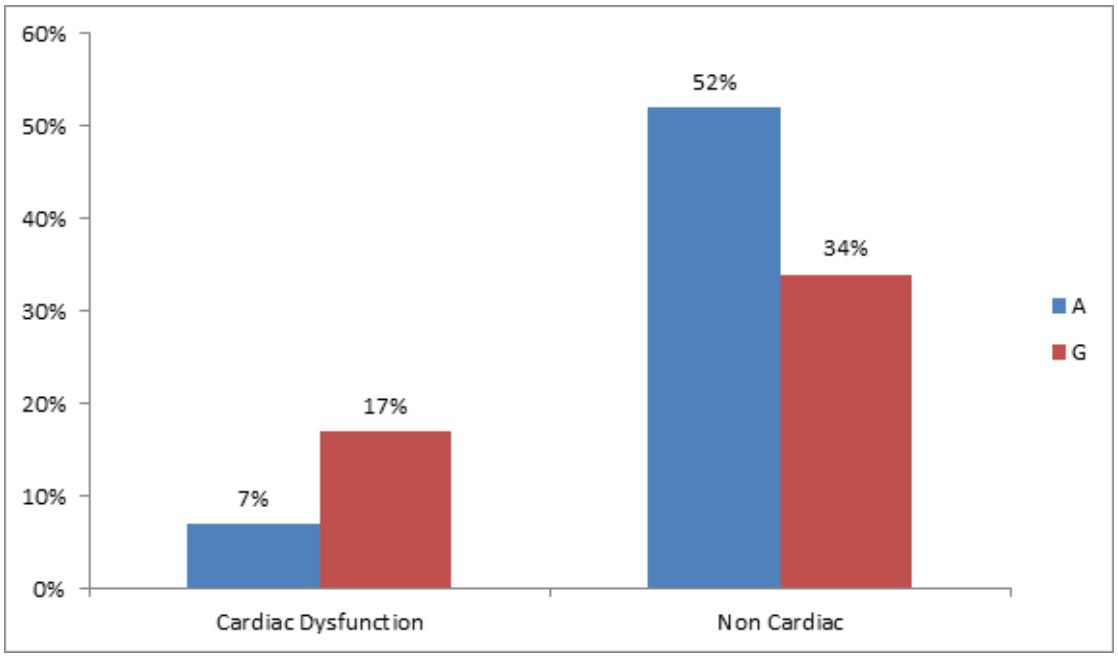

FIGURE 4.17: Frequency of A and G Allele in Cardiac and Non-cardiac Individuals. x -axis represent 'A' and 'G' allele in cardiac and non cardiac individuals while y- axis represent percentage of population.

The percentage of 'A' allele for cardiac individuals was found to be 7% and for non cardiac was 52% while percentage of 'G' allele was 17% for cardiac individuals and 34% for non cardiac individuals. Both 'A' and 'G' allele comprise 100% following the allele frequency equation as p+q=1.

4.4 Discussion

The total evaluated samples in the stu.0dy were 283, out of which percentage of both genders were almost same as 142 (50%) were male and 141 (49.82%)were females. It was found that the mean age of the subjects was 40.68 and BMI mean value was estimated to be 26.30. BMI propose that community of Kahuta region lies in the category of overweight and they are not obese so serious preventive measure are required for them. Blood pressure of 111 individuals comprises 39% was normal while 172 individuals comprises 61% was found to be high but on the other hand Blood Random Sugar levels were observed above Normal.

Mean value of BMI represents that Kahuta natives were overweight but not lies in the category of being obese but comparing with WHO BMI classification criteria almost 6% subjects were underweight,42% were normal weight,28% were overweight while rest of the subjects lies under three sub classes of obesity likewise 13% individuals were in Obese Class I because of greater BMI than 25,8 % subjects were under Obese Class II and remaining 3% were categorized under Obese Class III. BMI greater than 30 were considered to be Obese and consequently frequency of obesity was 24% in the studied population and it was found that risk of obesity in females is greater than males .It has been reported by NCBI that overweigh and obesity are more common in females as compared to males in developing countries. Tanzil, S., & Jamali, T. (2016) reported that in Pakistan obesity is more common in females of all age groups than males hence there should be some serious preventive steps to control this pandemic at population level. Prevalence of diabetes was found to be 14% and the prevalence of CVD was found to be 27% in Kahuta population but risk of CVD was greater in males as compared to females. Frequency of Obesity induced diabetes was 16.4% and p value was found to be 0.411 shows non significant association. Frequency of obesity induced CVD was found to be 44.8% and chi square reveals p value of 0.01 showing significant association of obesity induced CVD.

Out of 283 samples it was found that 88 (31%) were CRP positive and 195 (69%) were showing negative results. Association of CRP was found for obesity,

diabetes and CVD and it was found that obesity and CVD shows significant association with CRP while there is non significant association of CRP was found with diabetes. SAH Bokhari (2015) also reported significant association of CVD with inflammation in South-Asia population from 2001 - 2012.

The prevalence of TNF α G308A polymorphism in overall population was found to be with following percentage with alleles 43% for AA, 33% for AG,GA and 24% for GG. Association of Polymorphisim was checked and compared with p value and it was found that polymorphism shows non significant association with obesity,diabetes and CVD. It was found that overall frequency of 'A' allele was 60%more in population as compared to 'G' allele which was 40%.

Chu, H., Yang, (2012). et all reported no significant association of polymorphism with coronary heart disease in Chinese Hans population. Ghodsian, N., et all (2015) reported non significant association of polymorphism with diabetes , CVD and hypertension.

Chapter 5

Conclusions and Recommendations

In my current study 283 individuals were examined, different parameters like anthropometric, biochemical and PCR-RFLP were considered. It was concluded that obesity is pandemic in Pakistan and it might induce metabolic diseases and chronic inflammation. The burden of obesity and obesity induced diseases like cardiovascular disorders as well as type II diabetes is higher and there is need to reduce the risk factor of disease to avoid mortality and morbidity rate. Avoidance from unhealthy diet and life style modification may reduce the risk of disease. Genetic determinant must be revealed causing obesity, genetic screening techniques and gene therapy is the best future perspective to monitor and reduce the disease frequency.

Concerning about the association of TNF α polymorphism G308A It is concluded that there is association between obesity and inflammation but no significant association of polymorphism was found between obesity induced diabetes and CVD with inflammation. It was also observed that most of the samples were heterozygotes and frequency of A allele was significantly higher than G allele.

Limitations of present study must be acknowledged as there was inadequate sample size and it was further reduced when PCR–RFLP was performed to check the association of polymorphism between obesity, diabetes and cardiovascular disorders. Thus lack of association is more likely to be with insufficient power. Polymorphism are usually interrelated as well as co inherited so it is difficult to draw conclusions on small scale sample size, hence it is suggested as future direction that there must be comprehensive study of TNF-α promoter region by collecting large scale sample population in different ethnic groups.

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Appendix A

PIDNo:

QUESTIONNAIRE FOR RESEARCH PROJECT

Project Title: Association of TNF-α G308A (rs1800629) Polymorphism with Obesity Induced Diabetes and Cardiovascular Disorder.

Investigator(s): Capital University of Science & Technology, Expressway, Kahuta Road, Zone-V, Islamabad. PHONES: +92-51-2512800-1, +92-51-4486700-4, FAX NUMBER: +92-51-4486705 UAN: +92-51-111-555-666 Extensions: 123,280,0

Instructions:

1. This survey form may contain words that are new to you. If you read any words that are not clear to you, please ask the person who gave you this form to explain them to you.
2. Your records will be kept confidential and will not be released without your consent except as required by law.
3. Your identity will be kept private.
4. If the results of this study are written in a scientific journal or presented at a scientific meeting, your name will not be used.

5. Your initials _____ indicate your permission to be identified by name in any publications or presentations.

6. If you do not want to be acknowledged by name in any publications or presentations, please initial here_____.

7. The data will be stored in a locked file cabinet.

8. Your signed consent form will be stored in a cabinet separate from the data.

9. Your decision to take part in this research study is entirely voluntary.

10. You may refuse to take part in or you may withdraw from the study at any time without penalty or loss of benefits to which you are normally entitled.

11. You may be asked to leave the study for any of the following reasons.

12. Failure to follow the Project Director's instructions.

13. A serious adverse reaction which may require evaluation,

14. The Project Director thinks it is in the best interest of your health and welfare; or

15. The study is terminated.

16. You may wish to discuss this with others before you agree to take part in this study.

17. If you have any questions about the research now or during the study, please contact: _____

BIODATA: (This information provided by Patient will be confidential)

First Name: _____ Mid Name: _____

Last Name: _____ DOB _____ Gender:_____

Age _____ Contact No: (Office) _____

Home: _____ Email:_____

Permanent Address:

Address: _____

1. ANTHROPOMETRIC MEASUREMENT

Weight (kg)	
Height (m)	
BMI (kg/m2)	
Blood Sugar mmol/L	
Total cholesterol (TC)	
Triglycerides (TG) (mmol/l)	
HDL-C (mmol/l)	
LDL-C (mmol/l)	
CRP	
Myoglobin	
CK-MB	

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; CRP C Reactive Protein; CK-MB, Creatine Kinase-MB.

2. OBESITY, CVD AND DIABETES COMPLAINTS

High blood pressure	Yes	No	Diabetes	Yes	No
CVD	Yes	No	Eating disorder	Yes	No

3. FAMILY HISTORY

Obese Persons in family

Father	Sister	Uncle	Mother's Sister
Mother	Brother	Aunty	Mother's Brother

4. PHYSICAL ACTIVITY

Morning walk	Evening walk	Work at home	Outing

5. DIETARY HISTORY

Breakfast	Lunch	Dinner

6. SOCIAL AND PERSONAL HISTORY

Do you have children? No / Yes - How many?	
Education:	
Marital status:	Single / Married /Separated / Divorced
Job:	Part time /Full time

7. MEDICAL/CLINICAL HISTORY

Medication to control obesity	Yes / No
Diet plan to control obesity	Yes / No
Any surgery if yes when or for what	Yes / No
Medicins using for any other diesease	Yes / No
Smoking or counsuption of anyother tobaco product	Yes / No

8. SAMPLES

Blood Sample: _____

Thank you for completing the questionnaire please return it to _____ **Department of Health and Life Scinece, Capital University of Science and Technology, Islamabad.** If you have any concerns regarding this research please contact me or my supervisor in the first instance.

Consent

I have read the above description of this research study. I have been informed of the risks and benefits involved, and all my questions have been answered to my satisfaction. Furthermore, I have been assured that any future questions I may have will also be answered by a member of the research team. I voluntarily agree to take part in this study. I understand I will receive a copy of this consent form,

_____ _____

Subject Signature Date

Appendix B

Marital Status	Medication	Surgury	Tattooing /Piercing	DPCO	Addiction	G308A
Married	NO	YES	NO	NO	Smoking	GA
Unmarried	NO	NO	NO	NO	NO	GG
Unmarried	NO	NO	Piercing	NO	NO	GG
Married	NO	NO	NO	NO	Smoking	GA
Married	NO	NO	NO	NO	NO	AA
Married	YES	YES	Piercing	NO	Smoking	AA
Unmarried	NO	NO	NO	NO	NO	GA
Unmarried	NO	NO	Piercing	NO	NO	GG
Married	NO	YES	Piercing	NO	NO	AA

Appendix C

FIGURE 1: Medical Camp at Dr. Javed's Clinic

FIGURE 2: Medical Camp at Dr. Javed's Clinic

FIGURE 3: Medical Camp at Dr. Javed's Clinic

FIGURE 4: Medical Camp at Dr. Javed's Clinic

FIGURE 5: Medical Camp at Dr. Javed's Clinic

FIGURE 6: Medical Camp at Nogran Village

FIGURE 7: Prepared Samples After Centrifugation to Separate Blood Serum

FIGURE 8: Biochemical Testing at Lab

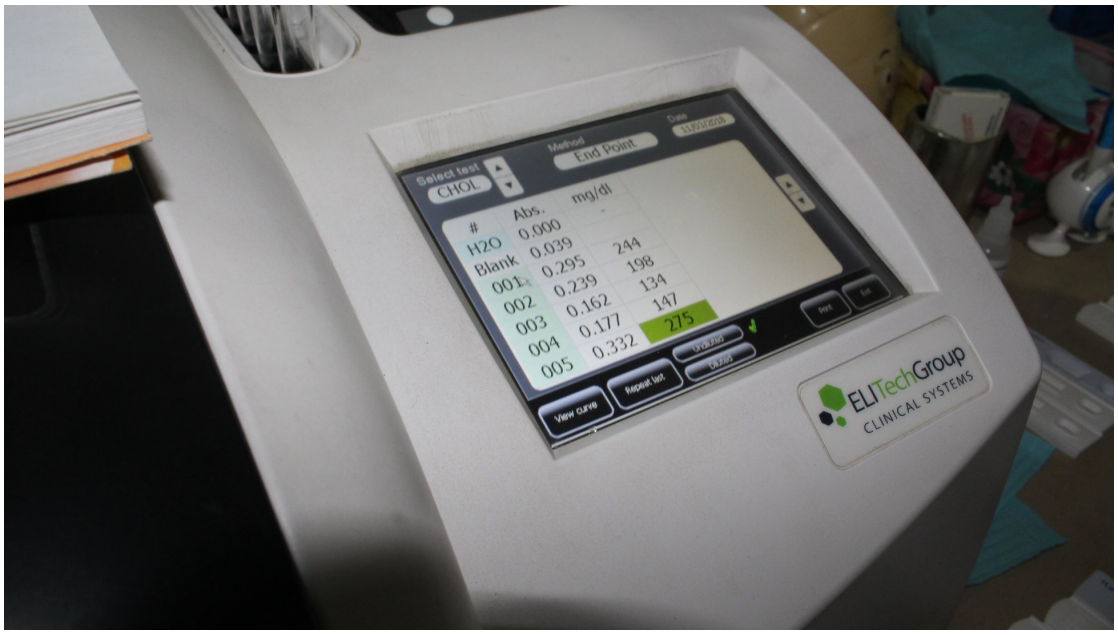

FIGURE 9: Biochemical Testing at Lab

FIGURE 10: Biochemical Testing at Lab

FIGURE 11: Lab Work

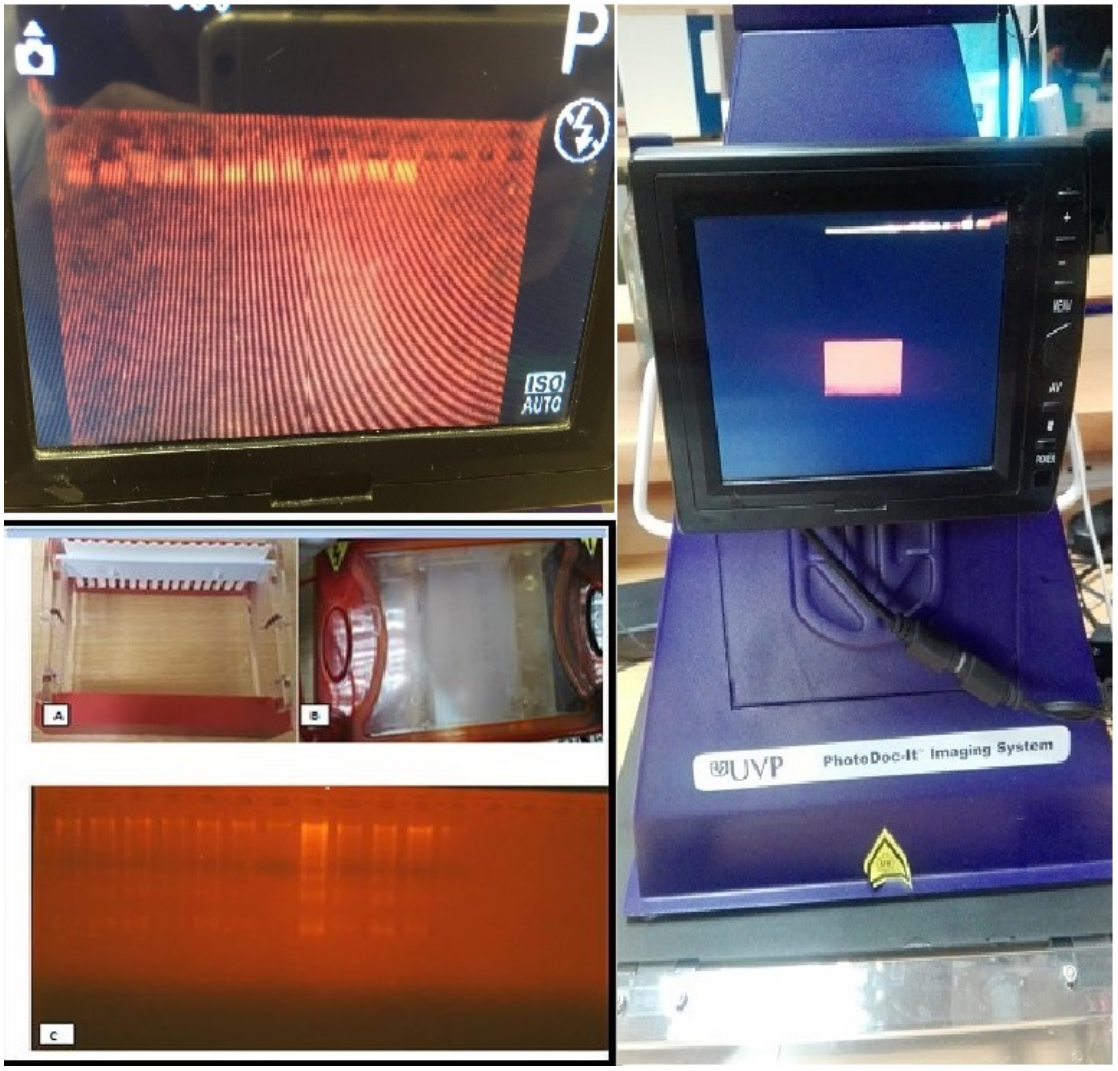

FIGURE 12: PCR Analysis and Gel Electrophoresis