ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

AGEING, CARDIOVASCULAR DISEASES AND CANDIDATE GENE POLYMORPHISIMS

M.Sc. Thesis by
Selçuk DAŞDEMİR, B.Sc.

Department : Advanced Technologies
Programme: Molecular Biology and Genetics & Biotechnology

Supervisor: Assist. Prof. Dr. Eda Tahir TURANLI

AUGUST 2008

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521061218

Date of submission : 5 August 2008

Date of defence examination: 7 August 2008

Supervisor (Chairman): Assist. Prof. Dr. Eda TAHİR TURANLI

Members of the Examining Committee : Assoc. Prof. Dr. Arzu KARABAY KORKMAZ (ITU)

Prof. Dr. A. Nazlı Başak (BU)

AUGUST 2008

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

YAŞLANMA, KALP DAMAR HASTALIKLARI VE ADAY GEN POLİMORFİZMLERİ

YÜKSEK LİSANS TEZİ

Selçuk DAŞDEMİR

521061218

Tezin Enstitüye Verildiği Tarih : 5 Ağustos 2008

Tezin Savunulduğu Tarih : 7 Ağustos 2008

Tez Danışmanı: Yrd.Doç.Dr. Eda TAHİR TURANLI

Diğer Jüri Üyeleri : Doç.Dr. Arzu KARABAY KORKMAZ (İTÜ)

Prof. Dr. A. Nazlı Başak (BÜ)

AĞUSTOS 2008

ACKNOWLEDGEMENTS

Firstly, I would like to thank to my supervisor Assist. Prof. Dr. Eda TAHİR TURANLI for the patience, support and the opportunities she provided me. I have learned many things that could provide advantages me in all of my career from her during this project. I want to thank her for encouraging me to establish this thesis, which could not have been accomplished without her.

I would also like to thank to Prof. Dr. Hasan YAZICI for implementing and supporting this project; Prof. Dr. Tanju BEĞER, Prof. Dr. Deniz ERDİNÇLER, and Dr. Aslı ÇURGUNLU for clinical evaluation of the healthy elderlies.

I would like to thank to Dr. Murat BOLAYIRLI for his meticulous work in biochemical tests and his help in measuring serum clusterin levels and the collaborating nurses from Geriatrics polyclinic of Cerrahpaşa, Gül ÖZKESKİN and Nefise YURTSEVER for their kind help in collecting the blood samples. I would also like to thank to Istanbul Technical University Medico department for collecting serum samples from our young controls.

I would like to thank to Istanbul University and Istanbul Technical University for the financial support they provided for this project.

I would also like to thank to the authorities of TUBITAK (2228 programme) and gratefully acknowledge the financial support they provided me for my graduate studies.

I would like to thank my lab partners, Esra KARACA, Sinem Karaman and Gökçe ÇELİKYAPI for their collaboration and help in setting up our lab from ground and their help in my experiments.

August 2008 **SELÇUK DAŞDEMİR**

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ABBREVIATIONS

APOE	: Apolipoprotein E
APOJ	**:** Apolipoprotein J
CHD	: Coronary Heart Disease
CLU	: Clusterin
COPD	: Chronic Obstructive Pulmonary Disease
CRP	: C-Reactive Protein
CVD	: Cardiovascular disease
DGGE	: Denaturing Gradient Gel Electrophoresis
DNA	: Deoxyribonucleic Acid
EDTA	: Ethylenediaminetetraacedic acid
ETOH	: Ethanol
FH	: Familial Hypercholesterolemia
HDL	: High-Density Lipoprotein
hsCRP	: High-sensitivity C-Reactive Protein
LDL	: Low-Density Lipoprotein
LDLR	: Low Density Lipoprotein Receptor
MEFV	: Mediterranean Fever (gene)
RE	: Restriction Enzyme
RF	: Rheumatoid Factor
RFLP	: Restriction Fragment Length Polymorphism
SIRT3	: Sirtuin 3
SLE	: Systemic Lupus Erythematosus
VNTR	: Variable Number of Tandem Repeats

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AGEING, CARDIOVASCULAR DISEASES AND CANDIDATA GENE POLYMORPHISMS

SUMMARY

Ageing is a multifactorial phenomenon and affected from genetic and environmental factors. It is the process of systems' deterioration with time and involves many changes at biochemical and morphological levels. Association of genes with successful ageing is started to become clear in recent years. In an initial study about successful ageing, Payami and his friends have shown that the risk of becoming senile in relatives of successfully aged people is lower than relatives of Alzheimer patients and people in normal population. A more recent study which has been done on centerineans has shown that the absence of M694V mutation on MEFV gene, which is an inflammatory gene, could be related to living longer and healthly. It has been shown that centenarian offspring has less cardiovascular disease (CVD) and ischaemic heart disease when compared with age-matched controls. It also has been demonstrated that cancer is delayed in families of centenarians.

Cardiovascular disease (CVD) is the most common reason of illness and death in the world, both in developed and developing countries. It is observed that protection from CVD enables to live longer and healthy. From pervious studies, it is also known that there is a significant relationship with inflammation and CVD. Thus, a person without choronic inflamatory diseases have lower risk for CVD.

In our project, we have analysed the association of APOE, LDLR, SIRT3 and APOJ/Clusterin genes' polymorphisms in an elderly population (N=170) without a major chronic or acute disorder including inflammatory and heart diseases. These polymorphisms have been previously associated either with healthy aging or heart diseases. Our hypothesis was to test the frequency of the protective alleles between the elderly population and the historic controls from general population. We also did some serum analyses in this sample and RF (rheumatoid Factor), hsCRP (high sensitivity-C reactive Protein) and clusterin levels were analysed and the latter was also compared with young controls (N=38) without a major disorder. After finding variations on the clusterin serum levels we have searched for polymorphism and/or mutations on APOJ gene using Denaturing Gradient Gel Electrophoresis (DGGE) method. Our results indicate that there are not associaton between SIRT3 and LDLR genes polymorphisms and longevity. We have seen a positive correlation between protective ε2 allele of APOE gene with longevity ($P<0.0001$). We have also detected a positive correlation between serum clusterin levels and longevity in males ($P=0.0005$). We have seen an association between serum clusterin levels and hsCRP levels ($P=0.001$), and we have shown a positive correlation between LDLR C1959T polymorphism with hypertension ($P=0.042$).

YAŞLANMA, KALP DAMAR HASTALIKLARI VE ADAY GEN POLİMORFİZMLERİ

ÖZET

Yaşlanma, multifaktoriyel bir olaydır ve genetik ve çevresel faktörlerden tarzından etkilenir. Zamanla sistemlerin bozulması sürecidir ve biyokimyasal ve morfolojik düzeyde birçok değişikliği içerir. Son yıllarda, bir takım genlerin sağlıklı yaşlanma ile ilişkisi açıkca belirlenmeye başlanmıştır. Payami ve arkadaşları sağlıklı ve uzun yaşamış yaşlıların akrabalarındaki bunama riskinin serbest toplumdaki ve Alzheimer hastalarının akrabalarındaki riskten daha az olduğunu göstermiştir. Yüzyıllık yaşlılarda yapılan daha yeni bir çalışma, iltihapla ilişkilendirilen MEFV inflamasyon genindeki M694V mutasyonu olmamasının daha uzun ve sağlıklı yaşamla ile ilgili olabileceğini göstermiştir. Sağlıklı yaşlıların çocuklarının daha az kalp damar hastalığı (KDH) ve iskemik kalp hastalığı riski taşıdıkları yaşıtlarıyla karşılaştırılarak gösterilmiştir. Ayrıca, sağlıklı yaşlıların ailelerinde kanserin ortaya çıkışının da geciktiği gösterilmiştir.

Kalp damar hastalığı (KDH) bütün dünyadaki gerek gelişmiş gerekse gelişen devletlerde en yaygın hastalık ve ölüm nedenidir. KDH' dan korunmuş olmanın sağlıklı ve uzun yaşamaya imkan tanıdığı gözlemlenmiştir. Önceki çalışmalardan KDH ile inflamasyon arasında önemli bir ilişki olduğu bilinmektedir. Bu yüzden, kronik inflamatuvar hastalıkları olmayan bir insan KDH için daha az risk taşıyor olabilirler.

Biz bu projemizde; APOE, LDLR, SIRT3 ve APOJ/Clusterin genlerindeki polimorfizmlerin sağlıklı yaşlanma ile ilişkisini kalp hastalığı da dahil olmak üzere herhangi bir kronik inflamatuvar hastalığı olmayan yaşlı bir grupta araştırdık (N=170). Bu genlerin özelliği önceki çalışmalarda kalp damar hastalıklarıyla ve sağlıklı yaşlanmayla ilişkilendirilmiş olmalarıdır. Bizim hipotezimiz, bu genlerdeki koruyucu allellerin sıklığının genel populasyonla karşılaştırılmasıdır. Ayrıca serum RF (romatoid faktör), hsCRP (yüksek duyarlıklı c-reaktif protein) ve clusterin düzeylerini belirledik ve yaşlı grubumuzun serum Clusterin seviyelerini herhangi bir önemli hastalığı bulunmayan genç bir kontrol grubuyla (N=38) karşılaştırdık. Serum clusterin düzeyleri arasında bir farklılık bulduktan sonra, APOJ geni üzerindeki mutasyon ve/veya polimorfizmleri Denaturing Gradient jel elektroforezi yöntemi ile araştırdık. Sonuçlarımız LDLR ve SIRT3 genlerindeki polimorfizmlerin sağlıklı yaşlanma ile ilişkili olmadığını gösterdi. APOE genindeki koruyucu e2 alleli ile sağlıklı yaşlanma arasında pozitif bir ilişki gördük ($P<0.0001$). Aynı zamanda, erkeklerde serum clusterin düzeyleri ile sağlıklı yaşlanma arasında pozitif bir ilişki belirledik ($P=0.0005$). Serum clusterin düzeyleri ile serum hsCRP düzeyleri arasında bir ilişki gördük ($P=0.001$) ve LDLR C1959T polimorfizmi ile hipertansiyon arasında pozitif bir ilşki gösterdik ($P=0.042$).

1. INTRODUCTION

1.1. Ageing and Longevity

Ageing is a multifactorial phenomenon that is influenced both by genetic and environmental factors. It is the process of systems' deterioration with time and involves many changes at biochemical and morphological levels. Helfand and Rogina identify ageing as an inevitable consequence of being a multicellular organism, associated with a random, passive decline in function, leading to a global loss of homeostasis over time and an increase in mortality [1, 2].

Many theories have been suggested to understand presence of senescence that explain when it occurs, and what are the biological processes responsible for it. These theories can be divided into two main classes: Biochemical and evolutionary. The biochemical theory invokes damages on DNA, cells, tissues, and organs and connects senescence with imperfections in the biochemical processes. A kind of this biochemical imperfection is the presence of free radicals that can cause death of the cells or may even lead to cancer. On the other hand, the evolutionary theory explains senescence as a competitive result of the reproductive rate, mutation, heredity, and natural selection [1]. The evolutionary theory clearly predicts that intrinsic decline in function caused by the accumulation of damage, particularly at the molecular level and and eventually gives rise to age-related frailty and disease. At the molecular level, evidence suggests that several of the most important mechanisms involve damage to macromolecules. An increase in DNA damage is associated with ageing, which implicates the importance of DNA repair mechanisma at the rate of ageing processes [2]. When species with different longevity are compared, it has been seen that there is a linear relationship between longevity and DNA repair [3]. The loss of telomeric DNA is also seen in many human somatic tissues with ageing, and as a result there is a decline in cellular division capacity with ageing [4]. For example humans have higher capacity for DNA repair, stress resistance and shown to live longer than any other mammal [5].

It is suggested that there is a connection between molecular stress and ageing and is explained by the accumulation of mitochondrial DNA (mtDNA) mutations with age [6]. Heat shock proteins (HSPs) have previously been shown to be important in longevity because they help to sequester and if possible restore denatured proteins. When a decline occurs in their function, damaged proteins are increased in cells and many diseases including Cataract, Alzheimer's disease and Parkinson's disease may develop [7]. Ageing is a non-adaptive process and, therefore, is not programmed. The genes have not evolved to cause damage to accumulate, and the evolution of ageing can therefore be understood only as a side-effect of other causes of evolutionary change [8].

Ageing is widespread amongst animal species and the rate of aging is species dependent. In Figure1.1, the total life span and rate of senescence in different organisms is seen [9]. There are different factors that affect ageing in different spieces. Mutations in genes encoding constituents of the insulin/insulin-like growth factor (IGF)-like signalling (IIS) pathway can extend lifespan of the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and the mouse *Mus musculus* [10]. Dietary restriction is seen to reduce the date rate of Drosophila [11]. Some organisms, like the freshwater Hydra, show no signs of ageing at all [12]. Others like the Pacific salmon age all at once, just as soon as their once-in-a-lifetime chance of reproduction has come and gone. In the case of the Pacific salmon, the rapid postreproductive death of the adult appears to be driven by sex hormones. If a salmon has its gonads removed, it cannot of course reproduce, but it lives much longer [13]. Numerous single gene mutations, as well as some pharmacologic interventions, have been identified that extend the life span of *C. elegans* [14]. Ageing in the yeast *Saccharomyces cerevisiae* this is defined by the replicative life span, which is measured by the number of times an individual cell divides, and the lifespan in yeast is controlled by the coordination of metabolic activity and resistance to stress. Some 19 genes have been shown to play a role in determining yeast longevity most of which possess human homologues. These genes encode a wide variety of biochemical functions [15].

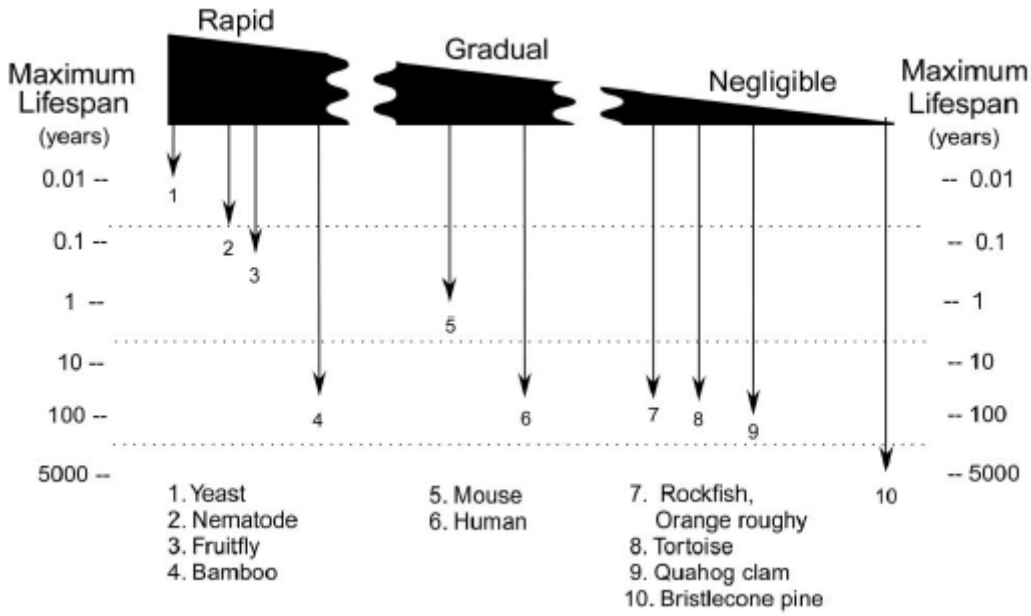

Figure 1.1 Comparison of life spans and rate of senescence in spieces ranging yeast to bristlecone pine [9].

Successful aging is being free from disability (absence of diseases like heart disease, diabetes, chronic inflamatory diseases), having good physical and cognitive functioning and participating in social and productive activities [16]. Centenarians are the best example of successful aging. Centenarians do not have major age-associated diseases, and are in good mental and physical condition [17]. Studies on centenarians show that genetic factors play an important role in longevity. It has been shown that centenarian offspring has less cardiovascular disease (CVD) and ischaemic heart disease when compared with general age-matched controls [18, 19]. It also has been demonstrated that cancer is delayed in families of centenarians [20].

1.2. Genetics of Ageing

There is a lot of evidence that show genes affect ageing. For example, mutations on several genes affect the length of life in some organisms [21]. For instance, it has been seen that a mutation in the gene encoding Prophet-of-Pit-1 (PROP), a pituitary transcription factor that regulates the secretion of growth hormone and insulin production increases the length of life in a Mouse strain [22]. Nine classes of genes that affect aging is identified in C. *elegans* [23]. Specific mutations in *daf-2*, *daf-16*, *daf-23*, *age-1* and *clk-1* genes of *C. elegans* increase the nematode's life-span three-tofive-fold [24]. In yeast, sir–2, a nicotinamide adenine dinucleotide (NAD) dependent histone deacetylase, has been found to slow down aging [25]. Over-

expression of Cu/Zn super oxide dismutase can increase the maximum life-span of transgenic *Drosophila* up to 48% [26]. It has been recently identified that a mutation in the mouse SHC gene (*p66shc*) is associated with a 30% longer life-span [27]. In a study that used microarray technology identified groups of genes whose activities, when upregulated by the insulin/IGF-I pathway, promote either ageing or longevity in nematode worms [28]. It has been shown that an increase in P53 activity as a result of a deletion of a portion of one copy of the P53 gene is associated with cancer prevention but also accelerated aging in mice [29].

Studies in humans also show that genes play both role in ageing and longevity. In a study done in 310 centenarians and as controls 164 adults aged 20–70, a polymorphism (DD genotype) in Angiotensin-converting enzyme (ACE) gene has been found significantly more frequent in centenarians than in the controls indicating the beneficial effect of the genotype in maintaining human survival [30]. In another study, the case-only approach is applied to detect the interaction effect on longevity between the renin (REN) gene and the mitochondrial haplotypes in 157 Italian centenarians. It has been reported that a highly significant interaction between REN allele 10 (one of the five polymorphic alleles of REN defined by the number of short tandem repeats) and mitochondrial haplotype H is related to longevity [31]. In an another study, V/V (I405V) genotype of cholesteryl ester transfer protein (CETP) gene is found to be associated with human longevity [32].

There are also genetic diseases that show premature aging in their pathology such as, Werner's syndrome (WS), which is a rare autosomal recessive disorder characterized by premature aging. It is caused by mutation of WS gene that causes genomic instability Individuals with this syndrome typically develop normally until they reach puberty. Following puberty they age rapidly, so that by age 40 they often appear several decades older. People affected by Werner syndrome usually do not live past their late forties or early fifties, often dying from the results of cancer or heart disease [33]. The other disorder about premature aging, Hutchinson–Gilford, is caused by mutations in lamin A. The people diagnosed with this disease usually have small, fragile bodies like those of elderly people. Later the condition causes wrinkled skin, atherosclerosis and cardiovascular problems [34].

Classical genetic studies attempt to identify the relative contribution of genetic and environmental components to the observed phenotype, such as family and twin

studies. These studies are important to understand the heritability (the ratio of the total genetic variance to the total phenotypic variance) patterns of a given trait.

In a family study that is done in four succesfully aged families show that there are many siblings achieving exceptional longevity in them. Average life span in these families ranging from 96-106 years old. In these families 50% of children reach extreme old age [35].

Twin studies, that compare the frequencies of desired traits in identical (monozygotic) twins to fraternal or nonidentical (dizygotic) twins, are performed in order to estimate the relative contributions of genes and environment. The studies that have been done in the Scandinavian twins show the heritability of life expectancy to be 20–30% [36].

A study which has been done in cohorts of Danish, Finnish and Swedish twins born between 1870 and 1910 comprising 20,502 individuals followed until 2003–2004 shows interesting results. Mean lifespan for monozygotic (MZ) twins increases 0.39 [95% CI (0.28, 0.50)] years for every year his co-twin survives past age 60 years. This rate is significantly greater than the rate of 0.21 (0.11, 0.30) for dizygotic (DZ) ones. In addition to this, It has been found that having a co-twin surviving to old ages substantially and significantly increases the chance of reaching the same old age and this chance is higher for MZ than for DZ twins. The relative recurrence risk of reaching age 92 is 4.8 (2.2, 7.5) for MZ males, which is significantly greater than the 1.8 (0.10, 3.4) for DZ males [37]. These studies implicate the importance of genetic factors but also the envrionment appears to have a substantial role in longevity.

1.3. Influence of Cardiovascular disease (CVD) and Inflammation on Ageing

The frequency of coronary disease, hypertension, heart failure and stroke increase exponentially with advancing age. While epidemiologic studies have discovered that aspects of lifestyle and genetics are risk factors for these diseases, age, per se, confers the major risk. Thus, being free from cardiovascular disease at advanced age increase the probability of longevity [38].

Cardiovascular disease (CVD) is the most common reason of illness and death in the world, both in developed and developing countries [39]. It refers to the class of diseases that involve the heart and/or blood vessels(arteries and veins) [40].

Many risk factors, such as Hypercholesterolemia, age, sex, hypertension, diabetes, smoking, oxidized-low-density lipoproteins (LDL), small and dense LDL, homocysteine and inflammatory cytokines increase the frequency of atherosclerosis which is associated with the hardening and loss of elasticity of artery walls [41, 42, 43, 44].

Abnormal levels of lipoproteins are linked to atherosclerosis, increasing of low-density lipoprotein (LDL) and decreasing of high-density lipoprotein (HDL) cholesterol levels promote risk of atherosclerosis [45].

CVD is a multifactorial disease, so genetic factors and environmental conditions are both important in for developing cardiovascular disease [46].

Many genes such as apolipoprotein E (APOE), angiotensin I converting enzyme 1 (ACE), plasma cholesteryl ester transfer protein (CETP), paraoxonase 1 (PON 1) have been associated with CVD. They are also related to successful ageing [16].

Inflammation play a central role in the pathophysiology of atherosclerosis. Inflammatory cells such as monocytes/macrophages and T cells are found in the atherosclerotic lesions. Inflammatory cytokines such as Interferon-γ, Tumor Necrosis Factor-α (TNF-α), Interleukin (IL)-1 and IL–6 takes role in the development of atherosclerosis [47, 48]. IL–6 leads to plaque growth by stimulating endothelial activation, vascular smooth muscle cell proliferation and leukocyte recruitment. It is also important for the production of triglycerides which could influence the apperance of CVD. TNF-α increases vascular permeability during devolopment of atherosclerosis by stimulating expression of adhesion molecules and major histocompatibility complex (MHC) proteins. TNF-α is involved in obesity-related insulin resistance and prothrombotic effects. IL–10 has anti-atherogenic effects like causing inhibition of adhesion of LDL-activated monocytes to endothelium and down-regulation of fibrinogen biosynthesis [49].

Alzheimer's disease, Parkinson's disease, cancer, amyotrophic lateral sclerosis, atherosclerosis, myocardial infarction, rheumatoid arthritis and type 2 diabetes are controlled by chronic inflammatory mechanisms and they are all majorly age-associated diseases [50].

According to that there is a reduction in response to environmental stimuli with age. Mortality rate increases (92-fold for heart disease, 43-fold for cancer, greater than

100-fold for stroke, greater than 100-fold for chronic lung disease and 89-fold for pneumonia and influenza) in old people due to reduced capacity of immune system [51]. Therefore, inflammation which is an evolutionarily conserved stress response, is very important for the length or quality of life in older adults. It has been shown that cardiovascular morbidity and mortality rates are about double in chronic obstructive pulmonary disease (COPD) cohorts compared to the general population [52]. A typical autoimmune disease, systemic lupus erythematosus (SLE) is associated with an up to 50 times increased risk of CVD [53]. Experiments which has been done on animals show that immune reactions can modulate atherosclerosis development [47].

1.4. Genetic Studies on Human Longevity and Candidate Genes

Genes that are involved in immune response/infammation, response to stressors including oxidative stress, insulin/IGF1 signalling pathway have all been associated with increased longevity in the literature. There have been identified polymorphisms in those candidate genes and their frequencies were found to be significantly different in centenarians when compared to younger controls [54]. For instance, IL6 gene and Thrombin-Activatable Fibrinolysis Inhibitor (TAFI) gene have been associated with longevity in males [55, 56]. Also another gene for C-reactive protein (CRP) is found to be involved in the regulation of human longevity [57].

Another one is Toll like receptor TLR4 gene and its +896G+ polymorphism has been shown to be significantly associated with lower frequency of Acut Myocardial Infarction [58].

A recent study on cententerians has shown that the absence of M694V mutation on MEFV gene, which has role in inflammation, could be related to live longer and healthy [59]. Another study point out that the polymorphisms of insulin like-growth factor 1 receptor and phosphoinositide 3-kinase genes could be related to long life [60].

There are many more molecular genetic studies of successful aging and many genes have been associated with longevity. Angiotensin I converting enzyme 1 (ACE), plasma cholesteryl ester transfer protein (CETP), cytochrome P4501A1 (CYP1A1), klotho (KL), microsomal triglyceride transfer protein (MTP), paraoxonase 1 (PON1),

apolipoproteins A-I (APOA1), A-IV (APOA4), C-III (APOC3), and E (APOE), interleukins 1α(IL1A), 1β (IL1B), 2 (IL2), 6 (IL6), 8 (IL8), 10 (IL10), and 12 (IL12), human leukocyte antigens A (HLA-A), B (HLA-B) and DRB1 (HLA-DRB1), transforming growth factor β1 (TGFB), tumor necrosis factor A (TNFA), sirtuin 3 (SIRT3) genes have been associated with longevity in previous studies [16].

In our study of successfully aged population free from chronic disease we have chosen to analyse four candidate genes, namely APOE, LDLR, APOJ and SIRT3. These are explained in more deatil in sections below.

1.4.1. Apolipoprotein E (ApoE) and Diseases

Apolipoprotein E (apoE) is a 299-amino acid glycoprotein which has role in binding of lipoprotein particles to the low density lipoprotein (LDL) receptor and the apoE receptor, so it has an important role in lipid metabolism [61, 62].

The association of apolipoprotein E (apoE) gene polymorphism with many diseases such as coronary artery disease [63], Alzheimer's disease [64], coronary heart disease [65] has been shown in previous studies.

Three common alleles, called ε2, ε3, and ε4, encode three major apo E isoforms; ε2, ε3 and ε4. There are six alternatives: three homozygous phenotypes, ε2/2, ε3/3, ε4/4, and three heterozygous phenotypes, ε3/2, ε4/3, and ε4/2 [66]. These genotypes are caused by substitutions in amino acids at residue 112 and residue 158. The ε3 isoform has cysteine and arginine at position 112 and 158, respectively. The ε4 has arginine and the ε2 has cysteine at both sites [67].

It has been shown that individuals who have ε4 alleles have decreased levels of high density lipoprotein (HDL) and increased level of low density lipoprotein (LDL) which increases the risk of coronary heart disease, and diminishes longevity [68]. In contrast, ε2 allele is related to lower LDL and total cholesterol [69]. In another study, ε4 has been found to be associated with higher serum total cholesterol and LDL cholesterol levels while ε2 is related to lower serum total cholesterol and LDL cholesterol levels in Turkish population [70].

ε4 allele has been also associated with increased risk for Alzheimer's disease [71]. ε2 allele has a positive effect on longevity. Frequency of ε2 is high in healthy individuals. On the other hand, ε4 allele has a negative effect on longevity. Frequency of ε4 is low in healthy individuals [72].

We hypothesized that there should be a decreased frequency of the E4 (ε4) allele and an increased frequency of the E2 allele (ε2) in our elderly population without chronic inflammation including heart disease.

1.4.2. Low Density Lipoprotein Receptor (LDLR) and Diseases

The low density lipoprotein receptor (LDLR) is a cell surface receptor that plays an important role in cholesterol homeostasis by removing low density lipoprotein (LDL) particles from the plasma via receptor-mediated endocytosis [73].

LDLR is located on chromosome 19 at 19p13.1-p13.3 [74], over 1000 mutations have been identified in this gene [75].

LDLR receptor controls plasma cholesterol levels by the way of interacting between the LDL [76]. If there is a problem, low density lipoprotein (LDL) cholesterol levels increase and this is big risk factor for coronary heart disease (CHD) and cardiovascular disease (CVD) [77].

Mutations at the LDLR gene cause dysfunction of the receptor and lead to familial hypercholesterolemia [78]. Familial hypercholesterolemia (FH) is an autosomal dominant disease with increased total cholesterol and low density lipoprotein (LDL) cholesterol affecting one in 400–500 people in the general population [79, 80]. More than 600 different, but rare, mutations in the LDLR have been associated with familial hypercholesterolaemia, and AvaII RFLP (restriction fragment length polymorphism) is widely studied polymorphism of LDLR gene which has been found to be associated with familial hypercholesterolemia [81, 82]. AvaII RFLP is present in exon 13 and C to T change at 1959 bp creates the site for AvaII [83].

In our Project, we have looked the LDLR C to T change at 1959 bp in exon 13 in our elderly group who are free from chronic inflamatory diseases. We expect to see frequency of protective C variant more common than the other T allele.

1.4.3. Apolipoprotein J/ Clusterin (CLU) and Diseases

Apolipoprotein J/ Clusterin (CLU) (otherwise called apolipoprotein J, sulfated glycoprotein–2, or testosterone-repressed prostate message- 2 among others) is a secreted glycoprotein and highly conserved. It is formed by two 40 kD sub which hold together by disulphide bonds [84].

Many cells including epithelial and neuronal cells secrete clusterin constitutively and it circulates in all cell body fluids like serum, plasma and urine [85].

ApoJ/clusterin functions include lipid transport, sperm maturation, complement defense, regulation of apoptosis, tissue remodeling, membrane protection, and promotion of cell-cell or cell–substratum interactions [86]. Moreover, CLU has been shown to function as an extracellular chaperone that stabilizes stressed proteins in a folding-competent state [87].

In human plasma ApoJ forms a high density lipoprotein (HDL) complex with apolipoprotein A-1 (apoA1) , cholesteryl ester transfer protein (CETP) and paraoxonase, so CLU may have function in the regulation of lipid transport [88].

Clusterin is upregulated in injured organs in many severe physiological disturbances states including Alzheimer's disease, atherosclerosis, myocardial infarction, and kidney degenerative diseases [89, 90]. It is also induced in prostate and seminal vesicle carcinogenesis, ovarian cancer, pancreatic cancer, breast carcinoma [91, 92, 93]. In addition, CLU serum levels increase significantly in diabetes type II and during development of coronary heart disease or at myocardial infarction [94].

It has been shown that apoJ/clusterin-deficient mice exhibit enhanced inflammatory severity and sequelae in an autoimmune myocarditis model, so it may serve an anti-inflammatory role under some conditions [95].

Clusterin has been cloned as a senescence-induced gene and its upregulation during both replicative senescence and stress-induced premature senescence has been shown in human cells [96]. Clusterin expression is reduced in lymphocytes of healthy centenarians [97].

Because of these properties clusterin could be a biomarker of human aging and senescence. To investigate that we determined serum clusterin levels in our healthy

eldery group (n = 115) and in a control young group (n= 38) without a major disorder.

1.4.4. Sirtuin 3 (SIRT3) and Diseases

The Sirtuin 3 (SIRT3) gene lies at the telomeric terminal on 11p15.5 chromosome (www.ncbi.nlm.nih.gov/omim: MIM 604481; contig NT_035113). It is a human homologue of the SIR2 genes [98]. Proteins involved in SIR2 gene familiy (nicotinamide dinucleotide phosphate (NAD+) -dependent protein deacetylases) are implicated in chromatin structure, transcriptional silencing, metabolism and aging in organisms ranging from yeast to humans [99]. The proteins in this family also has been associated with inhibition of cardiac hypertrophy and enhanced cardiacmyocyte survival [100].

SIRT3 has been shown that regulates the acetylation level and activity of acetyl-coenzyme A synthetase 2, which could has a role in energy production in mammals under starvation conditions [101]. SIRT3 is reduced in muscle of diabetic animals [102], induced in white and brown adipose tissue in response to calori restriction and its overexpression affects expression of genes involved in mitochondrial function [103].

A variable number of tandem repeats (VNTR) polymorphism of a 72-bp repeat core in intron 5 of the SIRT3 gene and presence of either a GATA3 or a delta-EF1 regulatory site has been associated with longevity in males [104].

In our Project, we also look VNTR polymorphism in intron 5 of the SIRT3 gene to search its association with longevity in our study population.

1.5. Aim of The Study

In this project, we have searched the association for APOE, LDLR, SIRT3 and APOJ genes' polymorphisms in our elderly population free from chronic inflammatory disease including CVD. We hypothesized that there should be more protective alleles of these genes in our elderly population compared to historic controls, so they could come to old age without CVD or any major heart disease related inflammatory syndromes.

2. MATERIALS AND METHODS

2.1. Materials and Laboratory Protocols

2.1.1. Used Equipments

The laboratory equipment used during this project is listed in Appendix A.

2.1.2. Used Chemicals, Enzymes, Markers and Buffers

The chemicals, enzymes and markers used are given in Appendix B together with their suppliers. The compositions and preparation of buffers and solutions are given in Appendix C.

2.1.3. Used Kits

The kits used and their suppliers are given in Appendix D.

2.2. Selection Criteria of the Elderly group without chronic inflammation

An elderly group free of chronic inflammatory disease was chosen among the patients from the outpatient clinics of the division of of Geriatrics of the Cerrahpasa Medical Faculty (CMF). A rheumatologist and a geriatrician selected an elderly group free of chronic inflammatory disease among the outpatients of the geriatric division medicine. The patients were over 60, did not have a history of any chronic inflammatory disease among those who attended clinic for routine health control examinations or follow up visits for hypertension, osteoporosis, osteoarthritis, hyperlipidemia or hypercholesterolemia (N=170). Sample selection has started two years ago and until now 170 elderly has registered into our database. CMF ethics committee approved the study protocol and oral informed consent was obtained from all participants.

2.3 Collection and Storage of Blood Samples

The peripheral blood samples were collected from the patients in vacuum tubes containing EDTA. The samples were kept at −20 ^{o}C for short term storage (1-2 weeks), and in −80 ^{o}C for longer terms of storage.

2.4 DNA Isolation from Human Whole Blood

The DNA isolation was done by Magtration System 8Lx Instrument, with the kit provided by the supplier (Precision System Science). Approximately 4 mL of blood sample was used for each isolation. The isolated DNA was kept at -20 °C after the working solutions were done (50 ng/μL) and the DNA samples were kept at + 4 °C.

2.5 DNA Amount, Purity and Working Solution Calculations

The concentration and purity of the isolated DNA is calculated by using the absorbance values measured at 260, 280 and 320 nm. The concentration of the DNA is calculated with the equation 2.1 and the purity of the DNA samples are calculated with the equation 2.2 given below:

$$DNA\ Concentration\ (ng/\mu L) = (A_{260} - A_{320}) \times 50 \times Dilution\ Factor \tag{2.1}$$

$$DNA\ Purity = \frac{(A_{260} - A_{320})}{(A_{280} - A_{320})} \tag{2.2}$$

2.6 Sample Collection For Clusterin Analysis

Serum was isolated conventionally from the blood samples. Biovendor human clusterin ELISA kit was used to measure serum clusterin levels. ELISA analysis was done in Centre Biochemistry lab of Cerrahpasa Medical Faculty. Initially, an approximately equal numbers of female (N=46) and males (N=40) were chosen from

the database based on their entry into the database. After finding variation in serum the clusterin levels and positive correlation with hsCRP levels, a second set of people were chosen based on the top %5 high clusterin and high hsCRP (N=6) with the lowest %5 low clusterin and low hsCRP levels (N=6). These individuals were further studied for Denaturating Gradient Gel Electrophesis (DGGE) in order to find polymorphisms and/or mutations with in the APOJ gene. Finally, a group of young people (N=38) without known diseases were chosen from the students in Molecular Biology and Genetics department of Istanbul Technical University in Turkey. Their serum clusterin levels were compared with elderly population.

2.7 The Principle of Human Clusterin ELISA Test

The test kit is a solid phase enzyme immunometric assay (ELISA) in microplate format, designed for the quantitative measurement of human Clusterin in serum, plasma and CSF. This ELISA utilises two anti-human Clusterin Mouse monoclonal antibody and human serum based calibrator.

Calibrators, quality controls and diluted samples are incubated in microtitration wells coated with first anti-human Clusterin monoclonal antibody. After a thorough wash, biotin-labelled second anti-human Clusterin monoclonal antibody is added to the wells and incubated with the immobilized antibody-Clusterin complex. After one-hour incubation and a next washing step, streptavidin-horseradish peroxidase (HRP) conjugate is added and incubated for half an hour. After last washing step, the conjugate bound is allowed to react with the substrate (H2O2- tetramethylbenzidine - TMB). The reaction is stopped by addition of acidic solution, and absorbance of the resulting yellow product is measured spectrophotometrically at 450 nm. The absorbance is proportional to the concentration of Clusterin. A calibration curve is constructed by plotting absorbance values versus Clusterin concentrations of calibrators, and concentrations of unknown samples are determined using this calibration curve.

2.8 C-reactive protein (CRP)

C-reactive protein (CRP) is a plasma protein, an acute phase protein produced by the liver [105]. CRP is an inflammatory marker may be present in increased levels in the blood in patients at risk for cardiovascular disease [105]. hsCRP (high sensitivity-C

reactive Protein) assays are used to measure blood CRP levels [105]. hsCRP is measured by immunonephorometry which is an application of nephelometry to the quantification of antigen or antibody. The technique depends on the light-scattering properties of microparticles [106].

2.9 Rheumatoid Factor (RF)

Rheumatoid Factors (RF) are auto antibodies that are usually produced in particular inflammatory diseases, mainly in Rheumatoid Arthritis (RA). On the other hand, RF production in the normal elderly people, in immunized normal people and in individuals with chronic inflammation or autoimmune conditions is also reported [107, 108]. RF is also measured by immunonephorometric techniques.

2.10. Polymerase Chain Reaction (PCR)

PCR was used to amplify the target DNA sequences on LDLR, SIRT3, APOE and APOJ genes which contained the related SNP regions [109, 104, 110, 111]. A standard mixture of PCR (except from the primers) was used to amplify the regions containing the related SNPs of our candidate genes.

Table 2.1 Standard PCR mixture

Ingredient	Stock Concentration	Volume	Final Concentration
Taq Buffer	10X	2 µL	1X
$MgCl_2$	25 mM	1.5 µL	1.5 mM
Forward Primer	10 pmol/µL	1 µL	0.5 µM
Reverse Primer	10 pmol/µL	1 µL	0.5 µM
dNTP mix	2 mM	0.4 µL	40 µM
Taq Polymerase	5 U/µL	0.2 µL	0.05 U/µL
dH_2O	–	7.9 µL	–
Template DNA	50 ng/µL	2 µL	100 ng
Q Solution	5X	4 µL	1X
FINAL	20 µL		

2.10.1. Oligonucleotide Primers

The oligonucleotide primers used in this study were selected from previous studies [109, 104, 110] and are given in the table 2.2 below. They are confirmed on Amplify 3X software [112] that they actually bind to the related sequence on APOE, SIRT3 and LDLR genes. The efficiency of binding and the amplicon sizes are also determined with this software.

It is also important to analyze the primer sets for dimer formation. The hairpin, heterodimer and self dimer analysis of the primer sets are done and confirmed with the SciTools on the IDT DNA website [113].

Table 2.2 Oligonucleotide primers

SNP	Primer Sequence	Amplicon Size	Reference
LDLR	F-5'-GTCATCTTCCTTGCTGCCTGTTTAG-3'	228 bp	[109]
LDLR	R-5'-GTTTCCACAAGGAGGTTTCAAGGTT-3		
APOE	E2mut (5' ACT GAC CCC GGT GGC GGA GGA GAC GCG TGC)	318 bp	[110]
APOE	E3 (5' TGT TCC ACC AGG GGC CCC AGG CGC TCG CGG)		
SIRT3	F-5'-TTCCTGAAGCTGGGTACA-3'	399-759	[104]
SIRT3	R-5'-CATTCACCTTCCCAAAGTGG-3'		

2.10.2. PCR Optimization

The PCR reactions usually need to be optimized for higher efficiency, amplification of the correct target and determination of appropriate annealing temperature for different primer sets. In this thesis, Mg^{2+} titration, Touchdown PCR and Gradient PCR methods were applied to optimize the PCR conditions. For PCR reaction of APOE gene, we additionally use DMSO and GC melt in order to dissolve GC rich regions of it. We also use Q solution for PCR reaction of APOE, SIRT3 and APOJ gene to prepare a better buffer condition for taq polymerase enzyme.

2.10.3. Magnesium (Mg^{2+}) Titration

Mg^{2+} is the cofactor of Taq DNA polymerase, as well as many DNA polymerases. The amplification efficiency changes within a scale of low to high concentrations of Mg^{2+}. Frequent observations about the effect of Mg^{2+} concentration on PCR includes the decrease in amplicon amount with low concentrations, and unspecific amplification of non-target regions with high concentrations. In order to obtain the appropriate amount of Mg^{2+} for a PCR reaction, Mg^{2+} titration should be applied. This procedure is usually done by setting up PCR mixtures that only differ in Mg^{2+} concentration. A used procedure for determination of Mg^{2+} concentration of PCR is given in table 2.3 below.

Table 2.3 Magnesium titration

Ingredient	1.5 mM	2.0 mM	2.5 mM	3.0 mM	3.5 mM
$MgCl_2$ (25 mM)	1.2 µL	1.8 µL	2.4 µL	3.0 µL	3.6 µL
ddH_2O	12.2 µL	11.6 µL	11.3 µL	11.0 µL	10.4 µL
F. Primer	1 µL	1 µL	1 µL	1 µL	1 µL
R. Primer	1 µL	1 µL	1 µL	1 µL	1 µL
10X buffer	2 µL	2 µL	2 µL	2 µL	2 µL
Taq	0.2 µL	0.2 µL	0.2 µL	0.2 µL	0.2 µL
dNTP	0.4 µL	0.4 µL	0.4 µL	0.4 µL	0.4 µL
Template	2 µL	2 µL	2 µL	2 µL	2 µL

2.10.4. Gradient PCR

When appropriate annealing temperature is not known for a primer set, gradient PCR might be used to determine the optimum annealing temperature for a PCR reaction. In gradient PCR, the thermo cycler is set in such a way that the wells in the thermo cycler block have a gradient of temperature from high to low for the annealing phase of the cycles. So, a set of identical PCRs can be run in the same block with different annealing temperatures. When the PCR products are viewed with agarose gel electrophoresis, the PCR mixture that has the nearest annealing temperature to the optimum temperature should give the best band intensity. In this thesis, the gradient PCR was used to optimize the annealing temperature of APOJ PCR which is amplified for DGGE analysis, a gradient from 50 to 55 was used and the best bands were observed in 55 °C, and this temperature is used as the annealing temperature of promoter region of APOJ PCR for the rest of the studies.

2.10.5. PCR Cycle Conditions

The PCR conditions were modified according to the desired amplicon.

2.11. PCR Conditions of LDLR

The standart PCR mixture of LDLR PCR is shown in the table 2.4 below.

Table 2.4 Standard PCR mixture for LDLR

Ingredient	Stock Concentration	Volume
Taq Buffer	10X	2 µL
$MgCl_2$	25 mM	1.2 µL
Forward Primer	10 pmol/µL	1 µL
Reverse Primer	10 pmol/µL	1 µL
dNTP mix	2 mM	2 µL
Taq Polymerase	5 U/µL	0.2 µL
dH_2O	–	9.6 µL
Template DNA	50 ng/µL	3 µL
FINAL		20 µL

The cycle conditions of LDLR PCR are shown in the table 2.5 below.

Table 2.5 PCR cycle conditions for LDLR

Repeat Number	Degree	Time	Phase
1	94 °C	3 minutes	Initial Denaturation
35	94 °C	30 seconds	Denaturation
	60 °C	30 seconds	Annealing
	72 °C	30 seconds	Extension
1	72 °C	5 minutes	Final Extension

2.12. PCR Conditions of APOE

The standart PCR mixture of APOE PCR is shown in the table 2.6 below.

Table 2.6 Standard PCR mixture for APOE

Ingredient	Stock Concentration	Volume
Taq Buffer with $MgCl_2$	10X	2 µL
DMSO	25 mM	0.25 µL
Forward Primer	10 pmol/µL	1 µL
Reverse Primer	10 pmol/µL	1 µL
dNTP mix	2 mM	2 µL
Taq Polymerase	5 U/µL	0.2 µL
dH_2O	–	6.55 µL
Template DNA	50 ng/µL	3 µL
Q Solution	5X	4 µL
FINAL		20 µL

The cycle conditions of APOE PCR are shown in the table 2.7 below.

Table 2.7 PCR cycle conditions for APOE

Repeat Number	Degree	Time	Phase
1	94 °C	3 minutes	Initial Denaturation
40	94 °C	10 seconds	Denaturation
	65 °C	30 seconds	Annealing
	72 °C	30 seconds	Extension
1	72 °C	7 minutes	Final Extension

2.13. PCR Conditions of SIRT3

The standart PCR mixture of SIRT3 PCR is shown in the table 2.8 below.

Table 2.8 Standard PCR mixture for SIRT3

Ingredient	Stock Concentration	Volume
Taq Buffer	10X	2 µL
$MgCl_2$	25 Mm	1.2 µL
Forward Primer	10 pmol/Ml	1 µL
Reverse Primer	10 pmol/µL	1 µL
dNTP mix	2 Mm	2 µL
Taq Polymerase	5 U/µL	0.2 µL
dH_2O	–	5.6 µL
Template DNA	50 ng/µL	3 µL
Q Solution	5X	4 µL
FINAL	20 µL	

The cycle conditions of SIRT3 PCR are shown in the table 2.9 below.

Table 2.9 PCR cycle conditions for SIRT3

Repeat Number	Degree	Time	Phase
1	94 °C	1 minutes	Initial Denaturation
35	94 °C	1 minutes	Denaturation
	59 °C	1 minutes	Annealing
	72 °C	1 minutes	Extension
1	72 °C	5 minutes	Final Extension

2.14. Agarose Gel Electrophoresis of PCR Products

The right percentage of an agarose gel is important for observing the PCR bands. In this thesis, all of the PCR products' amplicon sizes are in a range between 195 to 360 bp, which can be seen on a 1% agarose gel. In order to observe the PCR products of the APOE and LDLR polymorphisms, 1% mini or midi gels were prepared. For SIRT3 1.5% mini or midi gels were prepared. Mini gels were prepared with 0.5 g or 0.75 g agarose and 0.5 µg/mL ethidium bromide added into 50 mL of 1X TBE buffer, which was diluted from 10X stock TBE. Similarly, %1 and %1.5 midi gels were prepared with 1.5 g or 2.25 g agarose and 0.5 µg/mL ethidium bromide added into 150 mL of 1X TBE buffer. 5 µL of PCR product was mixed with 1 µL 6X loading dye and loaded into the wells. Gene ruler 1 kb (Fermentas) and Gene ruler low range (Fermentas) markers (can be seen in appendix B) were used as DNA ladder in order to calculate the lengths of the PCR products. The gels were run in 1X TBE buffer, at 120V with power supplier, for at least 30 minutes. The gels were observed under UV light with a transilluminator, and pictures were taken with UV PhotoMW software.

2.15. Restriction Enzyme Digestion of PCR Products

The restriction enzyme digestions of the PCR products were done according to the protocol provided by supplier of the enzymes. Restriction enzyme digestions were also performed and confirmed in electronic environment with EnzymeX software [114]. The general protocol is given in table 2.10 and the restriction enzymes and expected fragment sizes are given in table 2.11 below.

Table 2.10 Restriction Enzyme Digestion mixture

Ingredient	**1X amount**
10X reaction buffer	2 µL
ddH_2O	7.5 µL
Restriction Enzyme (10 U/µL)	0.5 Ml
Amplicon	10 µL
Final Volume	**20 µL**

The restriction enzyme mixture containing PCR products were kept at 37 °C overnight for digestion. Digestion and the RE are inactivated by application of the mixtures to 80 °C for 30 minutes.

Table 2.11 Restriction Enzymes used for each polymorphism and expected fragment sizes

SNP	Restriction Enzyme	Amplicon bp	Expected Fragments
APOE	Afl*III*	318	ε4: 295 bp
			ε2 and ε3: 231/232 bp
APOE	Hae*II*	318	ε2: 267 bp
			ε3 and ε4: 231/232 bp
LDLR	Ava*II*	228	Wild Type: 1 fragment (228 bp)
			Mutant: 2 fragments (141+ 87 bp)

2.16 Agarose Gel Electrophoresis of Restriction Enzyme Digested PCR Products

In this thesis, the RE digested bands are observed in agarose gels of concentrations between 2% to 4%, according to the size of the products and the minimum band size difference that can be discriminated. LDLR restriction enzyme digested products were viewed in 2%, APOE restriction enzyme digested products were viewed in 4% and SIRT3 PCR products were viewed in 1.5% mini or midi agarose gels. In all of the restriction analyses 10 µL of RE digested PCR product is mixed with 2 µL of 6X loading dye and loaded into the wells. The gels are run in 1X TBE buffer, at 120V, for at least 30 minutes. The gel pictures are taken with UV PhotoMW software.

2.17. Genotyping

The genotyping was done by analyzing the agarose gel photos of the RE digested PCR products for APOE and LDLR genes and PCR products for SIRT3. Gene (allele and genotype) frequencies were calculated by counting. Low range marker (Fermentas) was added to decide the band sizes along with positive controls. This was done separately and blindly by myself and by my advisor Dr. Eda Tahir Turanlı.

2.18. Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing Gradient Gel Electrophoresis (DGGE) is a sensitive technique for separation of different changes in DNA sequence. Single base pair substitutions, insertions and deletions can be detected by DGGE with high efficiency [115]. DNA molecules denaturate at different melting temperatures which is called Tm (melting temperature) when they subjected to an acrylamide gel which has a gradient with an increasing concentration of denaturant [116]. Urea and formamide are used to form a denaturing environment. The DNA molecules that contain one or more different nucleotides have different melting temperatures, and they could be separated efficiently on gel. Differences in the amount of denaturation between DNA strands cause different migration patterns along the gel. Each of them will be seen as a separate band after visualization of gel under UV via using ethidium bromide or SYBR-Gold [117].

2.18.1. PCR For DGGE Analysis

Primers were selected to cover all coding regions and several noncoding regions of the APOJ gene in order to find polymorphisms and/or mutations with in the APOJ gene. The list of primers for PCR reaction of CLU is given in table 2.12. We determine these primers via fast PCR program and previous studies [111].

Table 2.12 APOJ gene primers

PRİMER NAME AND SEQUENCE	REGION	PRODUCT SIZE	TM (ºC)
CLU-PROM1517F: GGACGGATGCATGACCTAAG	PROMOTER	130 bp	55.4
CLU-PROM1517R: CTTGTCCAAGCCACATCCTC			55.9
CLU-PROM1141F:TAGAGGGGATGGGCAGTGT	PROMOTER	157 bp	57.9
CLU-PROM1141R: CGACTCACCCACAGACAAGA			56.6
CLU-E1-F: **GC**AGGCTGTCTAGCTGTTCCCA	EXON1	260 bp	61.5
CLU-E1-R: TCTGACCTCATCACCCTGTG			56.3
CLU-E2-F: TCTTCCTCTCGCCTCATTCT	EXON2	275 bp	55.2
CLU-E2-R: **GC**AGTGGGATGGTCAAGGCA			60.3
CLU-E3-F: **GC**CCAGCCTTGTGTCTTCCTGTA	EXON3	270 bp	61.2
CLU-E3-R: ACTCAGAGCCTTGGCCACT			58.9
CLU-E4-F**: GC**CTCCTAACTGTGCCCATGCT	EXON4	505 bp	61.9
CLU-E4-R: ATGAGCTTCCACCCCTTCTC			56.7
CLU-E5-F**:** GCATGGCTGGATGACTGAC	EXON5	156 bp	56.2
CLU-E5-R: CGACTCACCCACAGACAAGA			56.6
CLU-E6-F: TCACTTGCGTTTCTTCCATC	EXON6	395 bp	53.1
CLU-E6-R: **GC**CTGCCGTGTGATAAATGCTC			58.9
CLU-E7-F: GCATGCATTTTGACCACAGT	EXON7	289 bp	54.3
CLU-E7-R: **GC**AAAGGCCCGCCTGCTTAC			62.1
CLU-3UTR1-F: TGTTTTACTTTGGAGGATAACTGTTT	UTR1	230 bp	53.1
CLU-3UTR1-R: **GC**TCGAGTGTTAGAGTGCAGGA			58.6
CLU-3UTR2-F: **GC**ACGTCACCAAGTAACCAGGC	UTR2	240 bp	60.8
CLU-3UTR2-R: CGGAAGCAGCAACTCAACA			55.9
CLU-INTRON2182-F: GTGTCTGGGCCCACTCTG	INTRON1	154 bp	58.2
CLU-INTRON2182-R:CTGGGAGCTGGGCTAAGG			57.8
CLU-INTRON2317-F: ACTTTCGGCTGTTGCATTTC	INTRON2	157 bp	54.2
CLU-INTRON2317-R: AAACCGTCAGCTCACAGGAG			57.2

2.19 PCR Conditions of APOJ

The cycle conditions of APOJ PCR are shown in the table 2.13 below.

Table 2.13 PCR cycle conditions for APOJ

Repeat Number	Degree	Time	Phase
1	94 °C	5 minutes	Initial Denaturation
40	94 °C	30 seconds	Denaturation
	53-59 °C	45 seconds	Annealing
	72 °C	45 seconds	Extension
1	72 °C	5 minutes	Final Extension

The standart PCR mixture of APOJ PCR is shown in the table 2.14 below.

Table 2.14 Standard PCR mixture for APOJ

Ingredient	Stock Concentration	Volume
Taq Buffer with $MgCl_2$	10X	2 µL
Forward Primer	10 pmol/Ml	1 µL
Reverse Primer	10 pmol/µL	1 µL
dNTP mix	2 Mm	2 µL
Taq Polymerase	5 U/µL	0.2 µL
dH_2O	–	6.8 µL
Template DNA	50 ng/µL	3 µL
Q Solution	5X	4 µL
FINAL		20 µL

Only annealing temperature is different for different regions of the APOJ PCR (see table 2.15).

Table 2.15 Anneling temperatures of APOJ PCRs

APOJ PCR	Annealing temperature
APOJ 1517, E1, E2, E5 and E6	55 °C
APOJ 1141 and 2317	56 °C
APOJ E3	53 °C
APOJ E4, UTR2 and 2182	57 °C
APOJ E7	59 °C

2.18.2. DGGE Conditions

For DGGE, the PCR products subjected to electrophoresis for 4 hours at 120 v, 60 °C a 7–8% polyacrylamide gel containing a 30–70% denaturing gradient in TAE buffer. After electrophoresis gel is visualizised under ultraviolet illumination via using SYBR-Gold.

2.18.3. Sequencing

For sequence PCR, we use PCR products as template for sequence PCR. They subjected to cycle sequencing in both directions using the BigDye version 3.0 sequencing kit. After that, they are purified and given to machine for direct sequencing.

2.20. Statistical Analysis

2.20.1. Association studies

Studies concerning genetic association aim to test if there are differences between two groups (e.g. diseased subjects and healthy controls) in candidate genes or in whole genomes. Genetic association studies may take one of two approaches, either family-based, or population based. The former employ the family of tests known as Transmission Disequilibrum Tests (TDT) which detect association in the presence of linkage. Other studies do not focus on families where a praticular disease is segregating, instead samples of affected and unaffected individuals are drawn from

the population, and the frequency with which certain alleles are present in each of these groups is tested for association with a disease [118]. In this thesis we compared the elderly group gene frequencies with appropriate historic controls from the literature.

2.20.2. Chi-square analysis

Chi-square test (χ^2 test) is any statistical hypothesis test in which the test statistic has a chi-square distribution when the null hypothesis is true, or any in which the probability distribution of the test statistic (assuming the null hypothesis is true) can be made to approximate a chi-square distribution as closely as desired by making the sample size large enough. It is a non-parametric analysis and the best-known situations in which the chi-square distribution are used are the common chi-square tests for goodness of fit of an observed distribution to a theoretical one, and of the independence of two criteria of classification of qualitative data [119].

2.20.3. Odds ratio

It is defined as the ratio of the odds of an event occurring in one group to the odds of it occurring in another group, or to a sample-based estimate of that ratio. These groups might be men and women, an experimental group and a control group. Odds ratio calculations were done with the online tool from Simple Interactive Statistical Analysis (SISA) [120]. Odds ratio is generally used to compare the probability of a certain event in two groups. An odds ratio of 1 implies that the condition (or event) is equally possible in both of the case and the control groups [121].

2.20.4. Student t-test

A t-test is any statistical hypothesis test in which the test statistic has a Student's t distribution if the null hypothesis is true. It is applied when sample sizes are small enough that using an assumption of normality. It is a parametric and quantitative analysis [122].

2.20.5. P-value

P value (the level of significance) accounts for the probability of any difference observed between two data sets is only by chance. As a result, the lower the p value, the more evidence for rejecting the null hypothesis. In this thesis, the obtained data was designated into levels of significance according to the p value intervals:

If $P>0.05$, there is no significant difference

If $P<0.05$, the difference is significant

If $P<0.01$, the difference is highly significant

If $P<0.001$, the difference is extremely significant

All the tests were done using SPSS software (version 15.0).

3. RESULTS

3.1. DNA Isolation Results

In this thesis, Magtration System 8Lx DNA isolation procedure was followed for all the samples. The average DNA concentration obtained from Magtration System 8Lx instrument and kit is 261.2 ng/µL and average A_{260}/A_{280} value is 1.8 (N=155).

3.2. Demographic Data of the Elderly Group without Chronic Inflamatory Diseases

The healthy elderly group in this study was made up of mostly women (75.1%) and had an average age of 74.125 (SD=6.553). Among the healthy individuals who were free from any chronic or acute illnesses we observed the presence of hypertension, osteoporosis, and osteoarthrosis (Table 3.1). Majority of the sample had normal levels of hsCRP and RF values.

Table 3.1 Demographic properties of the Elderly Group

Healthy Elderly N=162		**Percentage**	
Gender	Female Male	75.1 24.9	
Hypertension		73.9	Female 73.5% Male 75%
Osteoporosis		52.8	Female 62.8% Male 22.5%
Osteoarthrosis		29.1	Female 32.2% Male 20%
Averages		**Female**	**Male**
Average Age: 74.125 (95% C.I=67.57-80.67, SD=6.553)		73.608	75.675
N=138		**hsCRP < 5 IU/mL** 91%	**RF < 15 mg/UL** 88%
hsCRP < 5 IU/mL + RF < 15 mg/UL = 83 %			

3.3. Association Analyses: Comparison of Elderly Candidate Genes With Historic Controls

3.3.1. LDLR RESULTS

Elderly group allele and genotype results for LDLR C1959T in exon 13 are given in table 3.2 below.

Table 3.2 LDLR C1959T Allele Frequencies

SNP	Healthy elderly group (N=168)
LDLR	C: 0.70 (117 of 168) T: 0.30 (52 of 168) CC: 77 (168) CT: 81 (168) TT: 10 (168)

The elderly group genotype/allele data was compared with historic controls from the literature (see table 3.3). We hypothesize to see protective C allele frequencies more in the elderly group compared to historic controls.

Table 3.3 LDLR C1959T Polymorphsim – comparison with historic controls

SNP	The healthy elderly group of genetic analysis (N=168)	Bertolini et al, 1992 Italian population (General, Healthy) (N= 183) [123]	Chaves et al, 1991 Spanish population (General, Healthy) (N= 61) [124]
LDLR	C: 0.70 T: 0.30	C: 0.552 T: 0.448	C: 0.625 T: 0.375
	Not significant		

There were no significant differences between healthy elderly group with historic controls. We further subdivided the sample for, gender; presence of hypertension, osteoporosis, and osteoarthrosis and searched if there was any association between these factors with the polymorphism.

We found a significant difference (P=0.042) with presence of hypertension and the frequency of C1959T allele of LDLR gene. In other words, the risk T allele is more commonly and significantly present in people who have hypertension than the people without hypertension (see Table 3.4).

Table 3.4 LDLR C1959T Polymorphsim - Hypertension

		LDLR		
		CC	**CT/TT**	**Total**
Hipertansiyon	No	25	16	41
	Yes	52	70	122
Total		77	86	163
X^2=4.147		P=0.042		

3.3.2 APOE RESULTS

Elderly group allele and genotype results for APOE are given in table 3.5 below.

Table 3.5 APOE polymorphism results in our elderly group

SNP	**The elderly group (N=151) (percentage)**
APOE	E2: %37 (113) E3: %50 (153) E4: %12 (36) E2/E2 %4.6 (7) E2/E3 %52.3 (78) E2/E4 %14.7 (22) E3/E3 %18.7 (28) E3/E4 %9.3 (14) E4/E4 0 (0)

We except to see ε2 alleles of APOE gene higher and ε4 alleles of APOE gene lower in our elderly group. When we compared our results with historic controls, we found that, the protective ε2 allele frequency is higher in our group as we assumed (x^2=20.73, p<0.0001). However, there is not a significant difference between ε4 allele frequencies. The observed allele frequencies are given in table 3.6 below.

Table 3.6 APOE Polymorphism analysis – comparison with historic controls

SNP	The elderly group (N=151) (percentage)	Turkish Controls (94 healthy individuals) [125]	Longevity Galinsky Cambridge, UK (N=282) [126]
APOE	E2: 113 (%37) E3: 153 (%50) E4: 36 (%12)	E2: 12 (%13.2) E3: 64 (%68.1) E4: 18 (%18.7)	E2: 22 (%8) E3: 225(%80) E4: 33 (%12)
	X^2=20.73, p<0.0001		

3.3.3 SIRT3 RESULTS

Elderly group allele and genotype results for SIRT3 VNTR polymorphism in intron 5 of the gene are given in table 3.7 below.

Table 3.7 SIRT3 polymorphism results in the elderly group

The healthy elderly group of genetic analysis (N=166)		
SIRT3	Allele frequencies 1:0.58, 2:0.15, 3: 0.11, 4: 0.11, 5: 0.03, 6: 0	Genotype frequencies 1/1:95 2/2:24, 3/3:10 1/2:2, 2/3:2, 3/4:14 1/3:0, 2/4:0, 3/5:1 1/4:1, 2/5:0, 4/4:9 1/5:0, 4/5:5, 5/5:3

As the increased repeat number is associated with longevity, we hypothesized that there should be more of the repeat in our group than historic controls. The observed allele frequencies are given in table 3.8 below. There was no significant difference on VNTR polymorphism in intron 5 of the SIRT3 gene between the elderly population and historic controls.

Table 3.8 SIRT3 Polymorphism analysis – comparison with historic controls

The healthy elderly group of genetic analysis (N=166)		Italians 20- to 80-year-old subjects (N= 703) [104]
SIRT3	**1:0.58** 2:0.15 3: 0.11 4: 0.11 5: 0.03 6: 0 **Sum: 0.42**	**1: 0.41** 2: 0.12 3: 0.20 4: 0,25 5: 0.04 6: 0.04 **Sum: 0.59**

3.3.4. RESULTS OF SERUM CLUSTERIN LEVEL

Serum Clusterin levels were compared between different groups. A significant difference was found between elderly group and young controls (P= 0.01) (see table 3.9). As we expected, we found serum clusterin levels lower in the elderly group than in the young group.

Table 3.9 Serum clusterin levels- comparison with young controls

	Groups	N	Minimum	Maximum	Mean (µg/mL)	Std. Deviation
Serum Clusterin Level (µg/mL)	Elderly	115	41.67	440.40	186.3956	86.83414
	Young	38	128.01	333.30	210.9079	54.56490
T=2.091	P= 0.01					

We also compared males and females within and between groups. In the elderly group there was a significant difference among the females and males (higher in the females (p= 0.01) (Table 3.10).

Table 3.10 Serum Clusterin Levels-Gender in Elderly Group

	Gender	**N**	**Mean** (µg/mL)	**Std. Deviation**	**Std. Error Mean**
Serum Clusterin Level (µg/mL)	Male	39	160.3646	62.69081	10.03856
	Female	76	199.7536	94.53366	10.84375
	T=5.97 p=0.01				

On the other hand, the same correlation was not seen among the young group (Table 3.11).

Table 3.11 Serum Clusterin Levels-Gender in Young Group

	Gender young	**N**	**Mean** (µg/mL)	**Std. Deviation**	**Std. Error Mean**
Serum Clusterin Level (µg/mL)	Male	18	222.0533	60.54144	14.26975
	Female	20	200.8770	47.90520	10.71193
	Not significant				

As it was expected comparison of only males serum clusterin levels between and young and old groups revealed a highly significant difference (males having lower clusterin levels in the elderly) (P= 0.0005) (Table 3.12).

Table 3.12 Serum clusterin levels- comparison with young controls in males

	Groups	**N**	**Mean** (µg/mL)	**Std. Deviation**
Serum Clusterin Level (µg/mL)	Elderly	39	160.3646	62.69081
	Young	18	222.0533	60.54144
T=3.557	P= 0.0005			

In contrast, we could not see a difference between these two groups when we compare only females (see table 3.13)

Table 3.13 Serum clusterin levels- comparison with young controls in females

	Groups	**N**	**Mean** (µg/mL)	**Std. Deviation**
Serum Clusterin Level (µg/mL)	Elderly	76	199.7536	94.53366
	Young	20	200.8770	47.90520
	Not Significant			

We also compared serum clusterin levels with hsCRP levels, which is an important inflammation marker. We found a positive correlation (p= 0.001) between serum clusterin levels and hsCRP positivity. Serum level of clusterin is significantly higher in people whose hsCRP levels are above 3 IU/mL. Results of these analyses are given in table 3.14.

Table 3.14 Serum Clusterin Levels- comparison with serum hsCRP levels

	hsCRP	**N**	**Mean** (µg/mL)	**Std. Deviation**	**Std. Error Mean**
Serum Clusterin Level (µg/mL)	Under 3 IU/mL	70	157.3141	62.70578	7.4947
	Above 3 IU/mL	44	233.8814	99.64733	15.0224
	T=12.23, P=0.001				

3.4. DGGE RESULTS

After finding positive correlation with increased clusterin levels with hsCRP we wanted to search for polymorphisms/mutations along the CLU gene using DGGE. First we analysed the promoter region and selected 6 people from each group (people having highest and lowest hsCRP+clusterin levels) and performed the analysis. Our

preliminary analysis revealed differences among the samples (Figure 3.1). However further analyses are needed to understand the type of variation, such as sequencing.

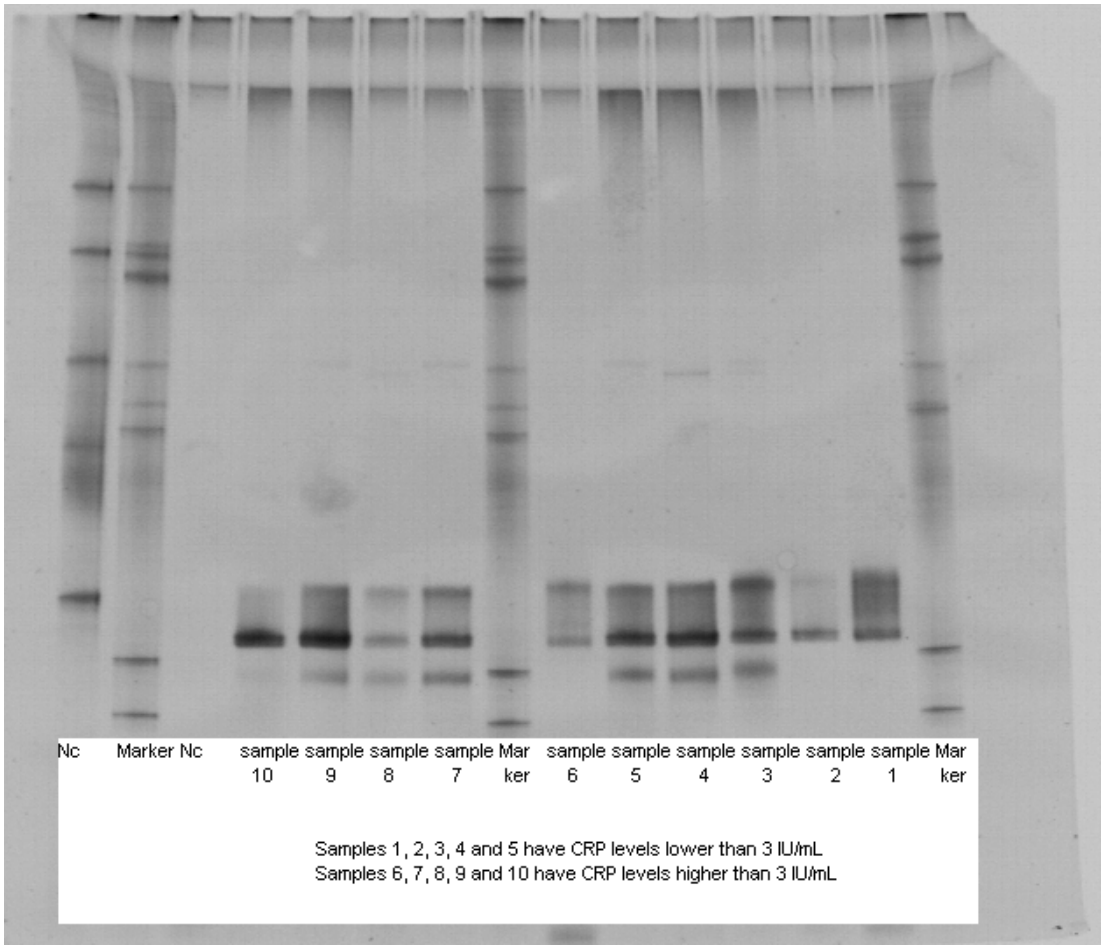

Figure 3.1. Results of promoter region for APOJ

4. CONCLUSION & DISCUSSION

Our research on the elderly population free from chronic inflammatory diseases has initially started to search for MEFV gene mutations and compare their frequencies with historic controls. To this end we have collected samples from elderly population over the two years (N=170) from Cerrahpasa Medical School. Our initial hypothesis was the less frequent occurance of MEFV mutations in the healthy elderly population was rejected. We did not see differences in their frequencies between elderly and Turkish historic controls [127]. We wanted further analyse the genetic properities of this population.

Cardiovascular Diseases (CVD) account for about 50% of all deaths worldwide. CVD causes deaths almost twice of cancer and 10 times of accidents [128].16.7 million deaths occur because of CVD per annum (WHO). Inflammation plays an important role in CVD. Many diseases such as chronic obstructive pulmonary disease (COPD) and systemic lupus erythematosus (SLE) that are related to inflammation, increase the risk of CVD [28, 29]. Thus, being free from inflammation may decrease the risk of CVD and increase the chance of survival at old ages. We have searched this hypothesis in an elderly population free of conditions associated with chronic inflammation.

We therefore investigated the associations of APOE, APOJ, LDLR and SIRT3 genes in our elderly group. The association of these genes with CVD have been shown in previous studies. We expected to see the frequency of protective alleles more common in the elderly group. A positive correlation between APOE ε2 allele and longevity is found from our study as we expected when we compare our elderly group with historic controls (x^2=20.73, $p<0.0001$). The positive effect of ε2 allele on longevity has been show in previous studies [42]. However, we have detected a negative correlation between APOE ε4 allele and longevity. In previos studies, it has been seen that ε4 allele has a negative effect on longevity. Frequency of ε4 is low in healthy individuals [42]. In addition, a study by Heijmans et all in 2000, has shown the deleterious effect of ε4 homozygosity on mortality at old ages [2], and there is

not a person with ε4/ε4 phenotype in our group that confirm this data. It has been shown that mutations at the LDLR gene cause dysfunction of the receptor and lead to familial hypercholesterolemia [48]. We hypothesize to see the protective C1959 allele LDLR higher in our elderly group. However, this study should be compared with a Turkish control group to have a more reliable data. In subgroup analysis, we found a significant difference (F= 4.417, P=0.042) with presence of hypertension and the frequency of LDLR C1959T polymorphism. In other words, the risk T1959 allele is more commonly and significantly present in people who have hypertension than the people without hypertension. It is reported that a variable number of tandem repeats (VNTR) polymorphism of a 72-bp repeat core in intron 5 of the SIRT3 gene and presence of either a GATA3 or a delta-EF1 regulatory site has been associated with longevity in males [74]. We have not seen a positive association between VNTR polymorphism in intron 5 of the SIRT3 gene and longevity. We will also look the presence of either a GATA3 or a delta-EF1 regulatory site by direct sequencing. High serum clusterin levels is associated with many diseases like Alzheimer's disease, atherosclerosis, myocardial infarction, diabetes type II and coronary heart disease [59, 60, 64]. Therefore, we hypothesized to see lower serum clusterin levels in our elderly group than in our young group without any disorders. We have detected a significant difference between serum clusterin levels when we compared our healthy elderly group with healthy young group (F=2.091, P=0.01). Serum clusterin levels was lower in elderly group as we expected. We found a significant difference (F= 5.97, P= 0.01) when we compared males and females in elderly group. Serum clusterin levels were higher in females than in males in the elderly group. We have seen a positive correlation between serum levels of clusterin and longevity in males (F= 3.557, P=0.0005). However, we have not seen this effect in females. As expected there was no significant difference in clusterin levels in the young group. Subgroup analysis in our elderly group has indicated that there is a positive correlation between serum clusterin levels and hsCRP positivity (F=12.23, P=0.001). This data confirms our suggestions. hsCRP positivity increases the risk of CVD, and higher serum clusterin levels is also associated with increased CVD risk. We have seen that people with hsCRP positivity have increased serum clusterin levels in our elderly group. Variations on clusterin levels will be further analysed at the DNA level.

In future, families of the individuals can be collected to do family based association studies or linkage analyses. We could also analyze the risk of CVD in siblings of elderly group to detect if protective alleles of our genes are higher between them. These studies can be better done using highthroughput analysis such as microarray techniques. We should also look serum clusterin levels in a more large group to test our significant results that we detected about male longevity. We could also look effect of high serum clusterin levels on mortality at old ages.

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APPENDIX A

LABORATORY EQUIPMENT

Balances	Precisa 620C SCS
	Precisa BJ 610C
Centrifuges	Sigma 1-13 B. Braun International
	Allegra 25R Centrifuge Beckman Coulter
Electrophoresis equipments	E – C mini cell primo EC320
Gel Documentation System	UVI PhotoMW Version 99.05 for Windows
Pipettes	Gilson Pipetman 20 µL 200 µl, 1000 µl
	Thermo Finnpipette 10 µL,
pH meter	Mettler Toledo MP220
Spectrophotometer	PerkinElmer Lambda25 UV/VIS Spectrometer
Thermo cycler	Applied Biosystems GeneAmp PCR
	System 2700
	Corbett PalmCycler
	Techne FTGENE 5D
Transilluminator	Biorad UV Transilluminator 2000
Vortex	Herdolph Reax top

APPENDIX B

CHEMICALS

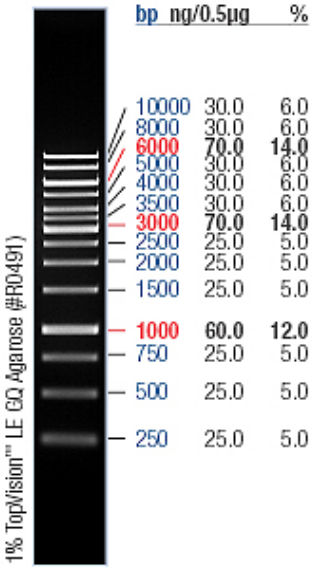
Agarose	AppliChem
Boric acid	Amresco
dNTP	Fermentas
EDTA	AppliChem
Ethanol	Riedel-de Haën
EtBr	Amresco
$MgCl_2$	Fermentas
$Na_2HPO_4.7H_2O$	Riedel-de Haën
NaH_2PO_4	Riedel-de Haën
NaOH	Riedel-de Haën
Primers	IDT DNA
NaCl	Carlo Erba
Tris Base	Amresco
10X PCR Buffer	Fermentas
	Qiagen
	Roche

ENZYMES

Afl*III*	Roche
AvaI*I*	Fermantas
Hae*II*	Roche

MARKERS

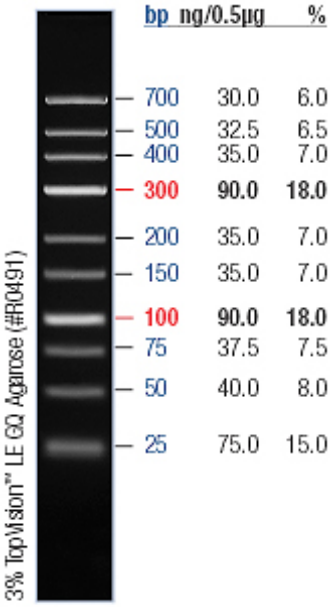
Gene Ruler™ 1 kb DNA Ladder Fermentas



bp	ng/0.5µg	%
10000	30.0	6.0
8000	30.0	6.0
6000	**70.0**	**14.0**
5000	30.0	6.0
4000	30.0	6.0
3500	30.0	6.0
3000	**70.0**	**14.0**
2500	25.0	5.0
2000	25.0	5.0
1500	25.0	5.0
1000	**60.0**	**12.0**
750	25.0	5.0
500	25.0	5.0
250	25.0	5.0

0.5µg/lane, 8cm length gel,
1X TAE, 7V/cm, 45min

Gene Ruler™ DNA Ladder Low Range Fermentas



bp	ng/0.5µg	%
700	30.0	6.0
500	32.5	6.5
400	35.0	7.0
300	**90.0**	**18.0**
200	35.0	7.0
150	35.0	7.0
100	**90.0**	**18.0**
75	37.5	7.5
50	40.0	8.0
25	75.0	15.0

0.5µg/lane, 8cm length gel,
1X TBE, 5V/cm, 1h

APPENDIX C

BUFFERS

TE Buffer

Tris base	10 mM
EDTA	1 mM

Add ddH_2O to 1 liter and adjust the pH to 8.0

TBE (Tris-Borate-EDTA) Buffer (10X)

Tris base	108 g
Boric Acid	55 g
EDTA	40 ml (0.5 M, pH 8.0)

Add ddH_2O to 1 liter and adjust the pH to 8.0

Mini Agarose Gel (1%)

Agarose	0.5 g
TBE buffer (1X)	50 mL

Add 1.5 µL EtBr (final concentration: 0.5 mM) before pouring the gel into tray.

Midi Agarose Gel (1%)

Agarose	1.5 g
TBE buffer (1X)	150 mL

Add 4.5 µL EtBr (final concentration: 0.5 mM) before pouring the gel into tray.

Mini Agarose Gel (2%)

Agarose	1 g
TBE buffer (1X)	50 mL

Add 1.5µL EtBr (final concentration: 0.5 mM) before pouring the gel into tray.

Midi Agarose Gel (2%)

Agarose	3 g
TBE buffer (1X)	150 mL

Add 4.5µL EtBr (final concentration: 0.5 mM) before pouring the gel into tray.

Mini Agarose Gel (4%)

Agarose	2 g
TBE buffer (1X)	50 mL

Add 1.5µL EtBr (final concentration: 0.5 mM) before pouring the gel into tray.

Midi Agarose Gel (4%)

Agarose	6 g
TBE buffer (1X)	150 mL

Add 4.5µL EtBr (final concentration: 0.5 mM) before pouring the gel into tray.

APPENDIX D

USED KITS

DNA Isolation 8 Lx Magtration® Genomic DNA kit

Human Serum Clusterin Biovendor

CURRICULUM VITAE

Selcuk Dasdemir was born in Bakirkoy, in 1984. He graduated from Kasgarlı Mahmut High School and enrolled to the Istanbul University-Cerrahpasa Medical Faculty, Biomedical Sciences department in 2002. He finished Biomedical Sciences department in 2006 and started to MSc degree education in Molecular Biology - Genetic and Biotechnology Program in Istanbul Technical University.