ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

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M.Sc. THESIS

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ISTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

DADA2 HASTALARININ PERİFERİK KAN MONONÜKLEER HÜCRELERİNDE TOTAL ADA AKTİVİTESİNİN ANALİZİ

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Turna Demirci, a M.Sc. student of İTU Graduate School student ID 521171122, successfully defended the thesis entitled "ANALYSIS OF TOTAL ADA ACTIVITY IN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs) OF DADA2 PATIENTS", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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FOREWORD

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TABLE OF CONTENTS

Page

ABBREVIATIONS

A : Adenine
ADA : Adenosine Deaminase
ADA1 : Adenosine Deaminase 1
ADA2 : Adenosine Deaminase 2
ADGFs : Adenosine Deaminase-Like Growth Factors
Ala (A) : Alanine
AMP : Adenosine Monophosphate
Arg (R) : Arginine
ATP : Adenosine Triphosphate
CNS : Central Nervous System
Cys (C) : Cysteine
D : Aspartic Acid
DADA2 : Deficiency of Adenosine Deaminese Type 2
ELISA : Enzyme-Linked Immunosorbent Assay
G : Guanine
GDI : Gene Damage Index
Gln (Q) : Glutamine
Gly (G) : Glycine
HSCT : Hematopoietic Stem Cell Transplantation
His (H) : Histidine
DCs : Dentritic Cells
IDGF : Insect-Derived Growth Factor
Ile : Isoleucine
LDGs : Low-Density Granulocytes
Leu (L) : Leucine
Met (M) : Methionine
NE : Neutrophil Elastase
NET : Neutrophil Extracellular Trap
PAN : Polyarteritis Nodosa
PRB : Putative Receptor-Binding

Pro (P) **:** Proline

ROS **:** Reactive Oxide Species

SDS/PAGE **:** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Thr (T) **:** Threonine

Trp (W) **:** Tryptophan

Tyr (Y) **:** Tyrosine

Val **:** Valine

SYMBOLS

°C	**:** Centigrade (Temperature)
nm	**:** Nanometer
nmol	**:** Nanomole
rpm	**:** Rounds per minute (velocity)
xg	**:** Rotor force
µg	**:** Microgram
µL	**:** Microlitre

LIST OF TABLES

Page

LIST OF FIGURES

Page

ANALYSIS OF TOTAL ADA ACTIVITY IN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs) OF DADA2 PATIENTS

SUMMARY

Deficiency of Adenosine Deaminase Type 2 is an autosomal recessive disease caused by biallelic mutations in the *ADA2* gene. It was first defined as monogenic vasculitis syndrome in 2014 as a result of studies conducted by two different groups independently. Although it has been shown that the prevalence of ADA2 Deficiency maybe 4 in 100,000, the prevalence of the disease may differ between ethnic groups, depending on the degree of consanguinity and the presence of founding variants.

Adenosine deaminase is an enzyme involved in the regulation of adenosine homeostasis and purine metabolism by converting adenosine to inosine and 2'-deoxyadenosine to 2'-deoxyinosine. There are two isoforms of adenosine deaminase in humans, and one of them, the 57-kDa homodimer ADA2 protein, is produced by the Adenosine Deaminase 2 (ADA2) gene. The N-terminal portion of the ADA2 protein is responsible for growth factor activity, while the C-terminal portion is responsible for adenosine deaminase activity. In addition to the catalytic domain, the ADA2 protein also has a protein dimerization domain and a cell surface binding domain. ADA2 proteins bind to different cell surfaces via glycosaminoglycan chains and to T cells via adenosine receptors. In this way, it shows both cytokine-like and autocrine-type growth factor properties. Although the ADA2 protein is involved in macrophage polarization, it also has an important regulatory function for neutrophil activation. In addition, it significantly reduces the formation of neutrophil extracellular traps, which are caused by extracellular adenosine and can lead to the activation of proinflammatory cytokines.

Despite the clinical manifestations of DADA2 being very diverse, episodic clinical findings are usually observed in patients with fever and systemic inflammation. The most common type is vasculitis findings. In addition to dermatological and neurological symptoms, it is also rarely defined by renal involvement and gastrointestinal system findings. More than half of patients have attacks of non-infectious fever. Symptoms include recurrent oral and genital ulcers, musculoskeletal symptoms, recurrent abdominal pain, inflammatory bowel disease, and immunodeficiency. Hematologic findings include cytopenia, anemia, and rare bone marrow failure. The diagnosis of the disease is made based on the detection of pathogenic variants on the *ADA2* gene or the measurement of ADA2 activity in serum/plasma. Treatment methods are selected depending on the symptoms and the severity of the disease. Currently, anti-TNF-α is the most common treatment modality, especially for patients with signs of vasculitis. Hematopoietic stem cell transplantation can be used in the treatment of hematological diseases. In addition, although it is not a suitable choice for long-term treatments, fresh frozen plasma infusions are also among the treatments applied.

Enzyme-linked immunosorbent assay (ELISA) is a method used to detect and quantify protein in soluble substances, based on antigen-antibody interaction and measuring enzyme activity by colorimetric analysis.

The purpose of this study was to compare the total adenosine deaminase (ADA) activity in peripheral blood mononuclear cells of patients diagnosed with DADA2 with the control group. 8 patients diagnosed with ADA2 deficiency and 5 healthy individuals were studied. Two of the patients are Syrian and have a G47R/G321E heterozygous mutation. 3 of the patients have G47R homozygous. Total ADA activity was measured in lysates prepared from subjects' peripheral blood mononuclear cells using a colorimetric ADA Activity Assay kit that is a commercial kit. ADA activity was calculated by following the protocol written in the kit, and then the statistical comparison of the results was analyzed by performing the t-test.

The disease-causing variant p.G47R, which occurs in the dimerization domain, affects the stability of the homodimer required for enzyme activity of the ADA2 protein. Therefore, due to the decrease in ADA2 catalytic activity in patients with p.G47R mutation, it is expected that the total ADA activity will be lower than in the healthy group. As a result of statistical analysis, a significant difference was observed in ADA activity ($p=0.0008$). As expected, ADA activity was lower in the patient group compared to the healthy group. In addition, when patients with heterozygous mutations were compared with patients with homozygous mutations, lower ADA activity was observed in patients with heterozygous mutations. In this case, it can be said that the G321E mutation plays an important role in catalytic activity.

DADA2 HASTALARININ PERİFERİK KAN MONONÜKLEER HÜCRELERİNDE TOTAL ADA AKTİVİTESİNİN ANALİZİ

ÖZET

Adenozin Deaminaz Tip 2 Eksikliği, *ADA2* geninde meydana gelen biallelik mutasyonların neden olduğu otozomal resesif bir hastalıktır. İlk olarak 2014 yılında, iki farklı grubun birbirinden bağımsız olarak yapmış olduğu çalışmalar sonucunda monogenik vaskülit sendromu olarak tanımlanmıştır. ADA2 Eksikliği'nin prevalansının 100.000'de 4 olabileceği gösterilmesine rağmen akrabalık derecesi ve kurucu varyantların varlığına bağlı olarak, hastalığın yaygınlığı etnik gruplar arasında farklılıklar gösterebilmektedir.

Adenozin, çeşitli fizyolojik süreçlerin düzenlenmesinden sorumlu temel bir sinyal molekülüdür. Adenozin, fizyolojik koşullarda kanda ve dokularda düşük düzeyde yoğunlaşır, ancak inflamasyon sırasında yüksek miktarda ATP salınımı nedeniyle konsantrasyonu hızla artar. Dengeyi korumak için, adenozin hücrenin içinden dışına taşınabilir. Hücre dışı adenosin, G proteinine bağlı reseptörlerin bir sınıfı olan adenosin reseptörlerini bağlayabilir ve aktive edebilir. Reseptör aktivasyonu, hücre içi siklik AMP seviyesindeki değişiklikler yoluyla çoklu hücre içi süreçleri indükler. Ayrıca adenozin, adenozin konsantrasyonuna ve adenosin reseptörlerinin ekspresyonuna bağlı olarak proinflamatuar veya anti-inflamatuar fonksiyonda da işlev görebilir.

Adenosine deaminaz, adenozini inozine ve 2'-deoksiadenozini 2'-deoksiinozine dönüştürerek adenozin homeostazının ve pürin metabolizmasının düzenlenmesinde görev alan bir enzimdir. İnsanda iki tane adenozin deaminaz izoformu bulunur. Bunlar ADA1 ve ADA2'dir. ADA1, 41 kDa'lık bir monomer proteindir ve asıl görevi, hücre içi adenosini katalize etmektir. ADA1, tüm hücrelerde eksprese edilmesine ek olarak, T ve B lenfositlerinde en yüksek ekspresyona sahiptir. ADA2 proteini ise 57-kDa ve homodimer bir yapıdadır ve 22. kromozomun uzun kolu(22q11.1) üzerinde yer alan ve 10 ekzondan oluşan Adenosine Deaminaz 2 (ADA2) geni tarafından üretilir. ADA2 proteinin N-terminal kısmında sinyal peptidleri olarak işlen gören hidrofobik kalıntılar vardır. Ayrıca N-terminal kısmı büyüme faktörü aktivitesinden sorumludur. Proteinin C-terminal kısmında ise korunmuş ADA katalitik alanı bulunmaktadır ve durum C-terminal kısmın adenozin deaminaz aktivitesinden sorumlu olduğunu göstermektedir. Ayrıca, ADA2 proteininin N-terminal kısmı, α sarmal alanı olarak tespit edilmiştir. Katalitik alana eklenen 50 amino asidin, kemokinlerle çeşitli yapısal özellikleri paylaşan varsayılan bir reseptör bağlama (PRB) alanına katlandığı görülmüştür. PRB alanı, alfa ve beta katına sahiptir. Üç iplikli bir antiparalel beta tabakasından oluşur. İki iplikten oluşan ilmek, korunmuş bir disülfid bağı ile üçüncü ipe bağlanır. Ek olarak, oligosakkarit zincirleri, ADA2 molekülünün üç farklı bölgesinde bulunur. Bunlardan ikisi PRB alanının yakınında bulunurken, üçüncüsü molekülün karşı tarafında bulunur. Oligosakarit zincirleri ayrıca ADA2'yi proteolizden korur. Sonuç olarak, sinyal peptidinin, disülfid bağının ve glikozilasyon bölgelerinin varlığı, ADA2'nin hücre dışı

ortama salgılandığını gösterir. ADA2 proteini, katalitik alanın yanı sıra protein dimerizasyon alanına ve hücre yüzeyi bağlama alanına da sahiptir. Dimerizasyon alanı enzim aktivitesi için gerekliyken ADA2'nin hücre yüzeyine bağlanması, dimerlerin stabilizasyonunu sağlamaktadır. ADA2 proteinleri farklı hücre yüzeylerine glikozaminoglikan zincirleri, T hücrelerine ise adenozin reseptörleri aracılığıyla bağlanır. Bu sayede hem sitokin benzeri hem de otokrin tipi büyüme faktörü özelliği gösterir. ADA2 proteini makrofaj polarizasyonunda görev alamakla beraber, nötrofil aktivasyonu için de önemli bir düzenleyici fonksiyona sahiptir. Ayrıca hücre dışı adenozinin neden olduğu ve proinflamatuar sitokinlerin aktivasyonuna yol açabilen, nötrofil ekstrasellüler tuzakların oluşumunu da önemli ölçüde azalttığı gözlemlenmiştir.

ADA2 Eksikliği'nin klinik belirtileri çok çeşitli olmakla birlikte hastalığın en yaygın klinik görünümü vaskülittir. Genellikle ateşli ve sistemik inflamasyonu olan hastalarda epizodik klinik bulgular gözlenir. Deri ve nörolojik belirtilerle karakterize olmanın yanı sıra, daha az hastada renal ve gastrointestinal bulgular da vardır. Livedoid döküntü, hastaların %73'ünü etkileyen en yaygın kutanöz bulgudur. Ayrıca bazı hastalarda tek klinik bulgudur. Bunun dışında nodüler lezyonlar, Raynaud fenomeni, ülserler, dijital kangren gibi çeşitli cilt bulguları vardır. Hastaların %50-77'sinde nörolojik tutulum mevcuttur. En sık görülen nörolojik olay, genellikle laküner enfarktüs şeklinde olan ve 5 aylıkken bile ortaya çıkabilen iskemik inmelerdir. Akut veya kronik iskemi ile ilişkili başka nörolojik komplikasyonlar da mevcuttur. Bunlar nöbetler, baş ağrısı, ataksi, vertigo, entelektüel ve davranışsal problemler ve omurilik tutulumudur.Daha az yaygın olan hemorajik inmeler de belirtiler arasındadır. Santral sinir sistemi tutulumuna ek olarak, kraniyal sinir felci, nöropati ve mononöritis multipleks dahil olmak üzere hastaların %53'ünde periferik sinir sistemi tutulumu da görülebilir. Ayrıca, birkaç işitme kaybı ve oküler tutulum vakası da bildirilmiştir. Oküler tutulumlar şaşılık, konjonktivit, üveit, papillit ve optik nörit gibi değişkenlik gösterir. Mezenterde oluşan iskemi, iskemik karın ağrısına ve gastrointestinal perforasyona neden olabilir ve ölümcüllük de bildirilmiştir. Ayrıca hastalarda arteriyel anevrizmalar da gözlenmiştir. Diğer belirtiler karında renal arter anevrizması, segmental glomerüloskleroz ve renal amiloidozu içerir. Vaskülit ile ilişkili daha az yaygın semptomlar, testis ağrısı, pankreas enfarktüsü ve pankreatit dahil pankreas tutulumu, pulmoner kanama ve temporal arterittir. Kardiyomiyopati, koroner anevrizmalar ve karditler gibi kardiyak tutulumlar diğer nadir belirtilerdir. Vaskülit bulgularının dışında hastaların yarısından fazlası, enfeksiyöz olmayan ateş ataklarına sahiptir. Tekrarlayan oral ve genital ülserler, kas-iskelet sistemi semptomları, tekrarlayan karın ağrısı, inflamatuar bağırsak hastalığı ve immün yetmezlik de belirtiler arasındadır. Hematolojik bulgular arasındaysa yaygın olarak sitopeni, anemi ve nadiren görülen kemik iliği yetmezliği yer alır.

Farklı fenotipler nedeniyle klinik bulgular tanı için yeterli değildir. DADA2 tanısı, patojenik varyantların saptanmasına ya da serum/plazmadaki ADA2 aktivitesinin ölçümüne bağlıdır. Patojenik varyantlar, Sanger dizilimi veya yeni nesil dizileme ile tespit edilebileceği gibi SNP dizileri, uzun menzilli polimeraz zincir reaksiyonu (PCR), multipleks ligasyona bağlı prob amplifikasyonu gibi diğer moleküler testler de varyant analizi için kullanılabilir. DADA2 hastalarında azalmış veya saptanamayan ADA2 aktivitesi görülmüştür. Bu nedenle, HPLC veya ELISA ile plazma veya serum ADA2 aktivite testinin analizi de tanı için faydalıdır. Tedavi yöntemleri, semptomlara ve hastalığın ciddiyetine bağlı olarak seçilir. Günümüzde en yaygın tedavi yöntemi vaskülit fenotiplerinde yüksek başarı ile anti-TNF-α tedavisidir. En sık kullanılan anti-

TNF ajanları etanercept, adalimumab, infliximab'dır. Anti-TNF tedavisinin yeni iskemik olaylara karşı koruyucu bir etki gösterdiği ve bireysel büyüme ve gelişme gibi diğer hastalık belirtilerinin de tedavi ile düzeldiği gözlemlenmiştir. TNF-α inhibitörleri özellikle vaskülit ve MSS bulgusu olan hastalarda uzun süreli tedavide kullanılabilir. Hematopoietik kök hücre transplantasyonu (HSCT), ciddi hematolojik hastalıkların tedavisinde kullanılabilir. Diğer bir tedavi yöntemi ise taze donmuş plazma infüzyonlarıdır. Ancak, kısa yarılanma ömrü nedeniyle uzun süreli tedavi için uygun bir seçim değildir.Enzim bağlı immünosorbent deneyi (ELISA), çözünür maddeler içindeki proteini saptamak ve miktarını belirlemek için kullanılan bir yöntemdir. ELISA, antijen-antikor etkileşimine dayanır ve enzim aktivitesi kolorimetrik analiz ile ölçülür. Doğrudan, dolaylı ve sandviç ELISA gibi farklı ELISA türleri vardır. Doğrudan ELISA, ilk ve en basit tiptir ve kalitatif ve kantitatif antijen tespiti için uygundur. Yüzeye immobilize edilmiş bir antijen, enzim etiketli bir antikor ile inkübe edilir. Daha sonra eklenen substrat ve enzimin etkileşimi, bir spektrofotometre ile ölçülebilen renk değişikliği ile sonuçlanır. Dolaylı ELISA iki aşamalı bir süreçtir. İlk olarak, konjuge olmayan bir birincil antikor eklenir ve antijen ile inkübe edilir. Daha sonra etiketli ikincil antikor eklenir ve birincil antikora bağlanır. Daha sonra substrat eklenir ve ölçüm yapılır. Dolaylı ELISA, antikor taraması için uygundur. Sandviç ELISA en yaygın tiptir ve eşleştirilmiş antikor çiftleri olarak da adlandırılan iki spesifik antikor gerektirir. Yakalanan bir antikor yüzey üzerine kaplanır ve ilgili bir antijen eklenir, ardından inkübe edilir. Diğer antikor eklenir ve inkübe edilir. Uygun substrat eklendikten sonra ölçülür. Sandviç ELISA, ikinci antikorun etiketlenmesine göre iki tipe ayrılır: Doğrudan veya Dolaylı sandviç ELISA. Sandviç ELISA, çözünür veya düşük konsantrasyonlu antijenlerin saptanması için yararlıdır.

Bu çalışmada DADA2 tanısı almış ve farklı mutasyonlara sahip hastaların periferik kan mononükler hücrelerindeki total adenozin deaminaz (ADA) aktivitesinin sağlıklı kontrol grubu ile karşılaştırılması amaçlanmıştır. Bunun için ADA2 eksikliği tanısı almış 8 hasta ve 5 sağlıklı birey ile çalışılmıştır. Hastalardan 2 tanesi Suriyelidir ve G47R/G321E heterozigot mutasyona sahipken, hastaların 3 tanesi G47R homozigot varyanta sahiptir. Total ADA aktivitesi, Abcam'in kolorimetrik ADA Activity Assay kiti kullanılarak, deneklerin periferik kan mononükler hücrelerinden hazırlanan lizatlarda ölçülmüştür. ADA aktivitesi kit protokolünde yazdığı şekilde hesaplanmıştır ve daha sonra t-test yöntemi ile sonuçların istatistiksel karşılaştırması yapılmıştır.

Hastalığa neden olan ve dimerizasyon domainde meydana gelen p.G47R varyantı, ADA2 proteinin enzim aktivitesi için gerekli olan homodimerin stabilitesini etkiler. Bu yüzden p.G47R mutasyonu taşıyan hastalardaki, ADA2 katalitik aktivitenin azalmasına bağlı olarak total ADA aktivitesinin sağlık grubundan düşük olması beklenmektedir. İstatistiksel analizler sonucunda da ADA aktivitesinde önemli bir fark görülmüştür ($p=0.0008$). Beklenildiği gibi hasta grubunda sağlıklı gruba oranla daha düşük ADA aktivitesi gözlenmiştir. Ayrıca, heterozigot mutasyonu olan hastalar ile homozigot mutasyona sahip hastalar karşılaştırıldığında, ADA aktivitesinin heterozigot mutasyonu olan hastalarda daha düşük olduğu gözlenmiştir. Bu durumda p.G321E mutasyonunun katalitik aktivitede önemli rol oynadığı söylenebilir.

1. INTRODUCTION

1.1 Deficiency of Adenosine Deaminase Type 2 (DADA2)

Deficiency of adenosine deaminase type 2 (DADA2) is an autosomal recessive disease caused by loss-of-function mutations in *ADA2*. DADA2 was first described as monogenic vasculitis syndrome in 2014. In studies conducted independently by two groups, one from NIH and the other from Israel, on patients with similar phenotypes, pathogenic mutations in the *ADA2* gene were identified by whole-exome sequencing analysis [1, 2].

1.1.1 Genetics and epidemology of DADA2

ADA2 gene located on chromosome 22q11.1 and previously known as *CECR1*, Cat Eye Syndrome Chromosome Region 1 gene encodes ADA2 protein. *ADA2* consists of 10 exons (Figure 1.1) extending over 28kb (OMIM).

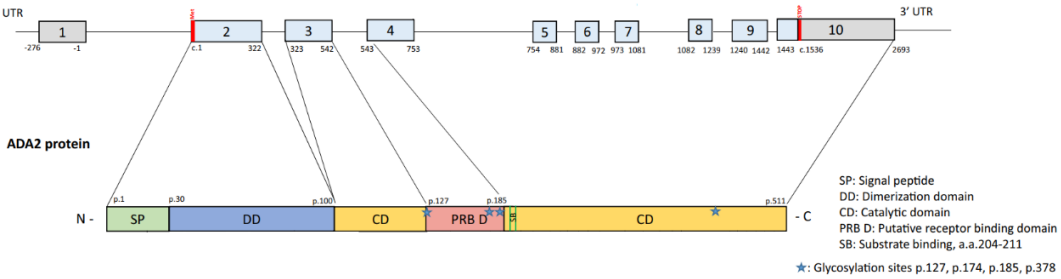

Figure 1.1 : Schematic representation of *ADA2* gene and ADA2 protein [38]. *ADA2* gene consists of 10 exon. The protein has three domain.

Over 300 missense substitutions and indels on the *ADA2* gene are reported in gnomAD databases [3]. There is also numerous copy number variations (CNVs). The threshold of GDI is 13.84 whereas the gene damage index Phred score of ADA2 is 6.29. This shows that the *ADA2* gene is presumably harbored disease-causing mutations [4, 5].

As of 2021, 125 variations have been reported on the Infever database. These are classified as pathogenic(n=17) (Figure 1.2), likely pathogenic(n=72), benign(n=7), likely benign(n=4), uncertain significance(VUS)(n=11), and not classified(n=14) [6].

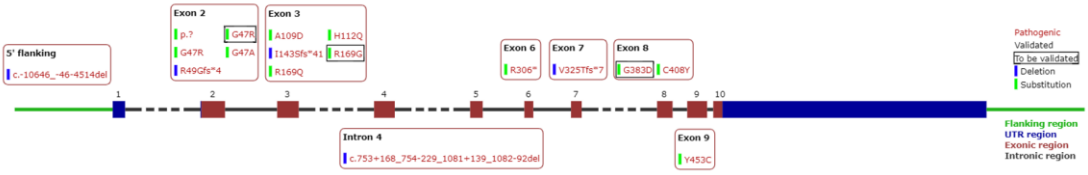

Figure 1.2 : Distribution of pathogenic variants on the ADA2 gene [6]. There are 17 pathogenic variants identified in the Infevers database, including 5 deletions and 12 substitutions. 3 of them need to be validated.

Many of the pathogenic variants were identified in sequence analysis by Zhou et al. This study was conducted on patients with similar clinical manifestations, such as recurrent fevers and early-onset stroke, and their unaffected parents. It was revealed that these people had 28 kb deletion in the 5′ untranslated region and exon 1, p.Y453C, p.R169Q, p.A109D, p.G47A, and p.H112Q pathogenic variants. In addition, a 15-year-old boy from the UK was heterozygous for the p.Met1Thr null allele and p.Ile93Thr. He had a recurrent fever, lacunar strokes, and cutaneous manifestations and died in 2013 because of the complications. The p.I93T variant has been classified as a likely pathogenic while the p.M1T has been classified as a pathogenic. As a result of sequence analysis on Turkish patients with and without a history of stroke, these patients have been reported to have the p.G47R pathogenic variant. The 28kb deletion and the p.M1T in exon 2 affect the signal peptide region of the ADA2 protein and cause altered translation initiation. It has been stated that other variants p.G47R, p.G47A, p.G47R(GC), and p.I93T in exon 2 affect the dimerization domain of the protein. The p.I93T removes an important hydrophobic contact between alpha helices, and so it causes unstabilization in the structure of the dimerization domain. The p.A109D and the p.H112Q variants located in exon 3 affect the catalytic domain, while the other variant p.R169Q in exon 3 affects the putative receptor binding domain. The variant p.Y453C in exon 9 forms a Cys408-Cys453 disulfide bond and thus, it disrupts the structure of the catalytic domain [1].

c.114delG mutation occurring on exon 2 was first reported in a patient with brainstem stroke, livedo racemosa, lymphopenia, low IgG levels, hypertension, and this deletion causes frameshift and insertion of a premature stop codon [7].

The variant p.R169G in exon 3 was detected in a 9-year-old boy with livedo reticularis, recurrent fevers, and ischemic and hemorrhagic strokes. As a result of structure modeling, the p.R169G has been shown to change the putative receptor-binding site in

the ADA2 enzyme, like p.R169Q. In addition, decreased ADA2 activity and ADA2 secretion have been reported in mutant cells [8].

Garg et al. studied the disease course of a patient who developed severe disease phenotypes and died at age 5. When the patient was 1.5 years, the disease started with the complaints of recurrent fever and rash, and the disease continued with the development of ptosis in the right eye and imbalance in walking at the age of 3. While neurological symptoms and lesions compatible with acute infarction were observed at the age of 4.5, the sudden onset of fever, headache, and confusion occurred at the age of 5. Also, intraparenchymal hemorrhage associated with the ventricular system was detected in the right parietal lobe. The patient died 3 days after developing acute respiratory distress syndrome. Sequence analysis revealed that the patient was compound heterozygous for p.Gly47Arg and p.Arg306*. The p.Arg306* occurs on exon 6 and affects the catalytic domain of the protein. In addition, the variant is the first reported nonsense mutation that produces a nonfunctional protein or never produces a protein [9].

Heterozygous deletion of exon 7 (p.Val325Thrfs*7) was reported in a study conducted on a patient with intermittent fever, cutaneous vasculitis, myalgia, and myalgia, and the patient's sister with mild biological inflammation with early-onset, long-term anemia. The p.Val325Thrfs*7 can cause a frameshift and a truncated protein [10].

One of the pathogenic variations occurring on exon 8 and affecting the catalytic domain of the protein is p.C408Y [11]. The other pathogenic variant p.G383D was first reported in a patient together with p.L188P. The p.G383D and p.L188P affect the catalytic domain of the ADA2 protein and may result in the production of non-functional ADA2 protein [12]. Also, it has been reported that the p.G383D altered regulation [6].

According to Infevers database, there are a total of 72 possible pathogenic variants in the ADA2 gene, of which 25 have been confirmed. The likely pathogenic variants generally have an effect on enzymatic activity. The variant p.P251P in exon 4, is formed by a change from guanine at 753 position to adenine and it does not cause to change codon. However, the guanine is located in a donor splicing consensus site, so it has been considered that the p.P251P variant leads to a truncated protein. c.973-2A>G is a rare canonical splicing variant. The p.P251P and c.973-2A>G can affect

mRNA splicing [13]. The other likely pathogenic variant in exon 4 is p.M243R and it affects the catalytic domain of the protein [14]. Another likely pathogenic variant p.R312* in exon 6 causes premature termination, and p.V458D in exon 9 cause to reduce secretion of ADA2.

The compound heterozygous for missense mutations cause the disease in most DADA2 patients. The residue 47 is a hot spot point and five mutations p.G47R, p.G47R GC, p.G47A, p.G47W, and p.G47V occur on the residue 47 [1, 15-19]. Navon Elkan et. (2014) analyzed the three-dimensional protein structure and suggest that "Gly47Arg and Gly47Val may affect the stability of homodimers or their individual subunits, Arg169Gln may alter the receptor-binding domain and, Pro251Leu and Trp264His are likely to affect the active site of the enzyme". Furthermore, the p.His112Tyr variant may disrupt zinc ion coordination within the ADA2 structure and p.Gly321Ala may cause a collision with adjacent amino acids [15]. Donor splicing site mutations occur between the last 5 nucleotides of the exon and the first 15 nucleotides of the intron and they are more pathogenic than the acceptor region variants. Variants in exon-intron junctions are also usually pathogenic [17, 9, 20, 21].

Analysis of allele frequencies of in silico-predicted pathogenic variants has been demonstrated that the prevalence of DADA2 can be as high as 4 in 100,000 [22]. However, its prevalence may also differ in populations concerning the degree of consanguinity and the existence of founder variants. The p.Arg169Gln variant with a carrier frequency of 1: 2100 in the general population [23] is more common in northern European populations, and its carrier frequency is 1:500 in northern European populations [22, 24-26]. The frequency of carrying the p.Gly47Arg variant of the Turkish population is about 1:500, whereas this variant's frequency is 1:10 in Georgian-Jewish populations [22]. p.Thr360Ala is called as Italian variant [27].

Neither a clear genotype-phenotype relationship has been established to date. There is a significant phenotypic variability, which may differ in patients carrying the same variants, even in siblings. However, biallelic homozygous G47R mutations were mostly associated with a vascular phenotype [28]. Also, a homozygous variant p.G47W in exon 2 has been described in an African woman with severe neutropenia, fever, mouth ulcers, and immunological disorders [29].

1.1.2 Properties of ADA2 protein

Adenosine is an essential signaling molecule responsible for the regulation of various physiological processes. Adenosine is concentrated at a low level in blood and tissues at physiological conditions but its concentration is rapidly increasing because of the release of high amounts of ATP during inflammation [30, 31]. To maintain equilibrium, adenosine can be transported from inside to outside of the cell [32]. The extracellular adenosine can bind and activate adenosine receptors which are a class of G protein-coupled receptors [30]. Receptor activation induces multiple intracellular processes through changes in intracellular cyclic AMP level. There are four kinds of adenosine receptors (A1, A2A, A2B, and A3), located on different cell types and expressed in different amounts and combinations. While the activation of A1 and A3 receptors leads to a decrease in intracellular cAMP, the activation of A2A and A2B receptors causes the accumulation of intracellular cAMP [30, 32, 33]. Moreover, adenosine may also function in a pro-inflammatory or anti-inflammatory function, depending on concentration of adenosine and the expression of adenosine receptors [34]. For example, stimulation of A1 and A3 receptors on neutrophils promotes chemotaxis and phagocytosis, while stimulation of A2A and A2B receptors reduces neutrophil responses by inhibiting granule release [35].

Adenosine homeostasis and purine metabolism are regulated by adenosine deaminases converting adenosine and 2'-deoxyadenosine into inosine and 2’deoxyinosine, respectively. There are two adenosine deaminase isoforms for human: ADA1 and ADA2 [36]. 43 years ago, ADA2 was identified as the residual source of adenosine deaminase activity in spleen tissue of a patient with severe combined immunodeficiency (SCID) associated with ADA1 deficiency [37]. ADA1 is a 41-kDa monomer protein, whereas ADA2 is a 57-kDa homodimer protein. The main function of ADA1 is catalyzing intracellular adenosine. In addition to being expressed in all cells, ADA1 has the highest expression in T and B lymphocytes [38]. Deficiency of ADA1 causes Severe Combined Immunodeficiency (SCID) with recurrent infectious because of raising apoptosis of T and B cells [39]. Although ADA1 and ADA2 share a common catalytic activity, the catalytic activity of ADA2 has a lower affinity for adenosine and its optimum pH is 6.9. On the other hand, ADA2 has an essential role in inflammatory situations which have high concentrations of adenosine. ADA2 is also

less sensitive to ADA1-specific inhibitors such as Erythro-9-(2-hydroxy-3- nonyl) adenine (EHNA) and they can be easily distinguished by biochemical assays [38].

N-terminus of ADA2 has short hydrophobic residues [15]. These residues have been determined to function as signal peptides, using the SingnalP program. Afterwords, N-terminal part has been observed to be responsible for growth factor activity [15, 40]. When the ADA2 protein sequence was compared to adenosine deaminase (ADA) obtained from different organisms including human, conserved ADA catalytic domain was detected at the C-terminus of ADA2. This indicates that C-terminus of ADA2 has ADA activity [15]. According to the crystal structures of the human ADA2 apoenzyme and the enzyme in complex with coformycin, two different additional domains of the ADA2 protein have been discovered besides the catalytic domain. These are the protein dimerization domain and cell surface binding domain (Figure 1.1). The dimerization domain which is mediated by an α helical domain has been demonstrated to be necessary for enzymatic activity, and the binding of ADA2 to the cell surface provides stabilization of the dimers. Also, the N-terminal part of ADA2 protein has been detected as the α helical domain. 50-amino acid inserted within the catalytic domain has been seen to fold into a putative receptor-binding (PRB) domain sharing several structural properties with chemokines. The PRB domain has alpha and beta fold. It is constituted of a three-stranded antiparallel beta-sheet. The loop of two strands is linked to the third strand with a conserved disulfide bond. Additionally, oligosaccharide chains are located on three different sites of the ADA2 molecule. Whereas two of them are located near the PRB domain, the third one is located on the opposite side of the molecule. The oligosaccharide chains also preserve the ADA2 from proteolysis. As a result, the presence of the signal peptide, the disulfide bond, and the glycosylation sites indicates that ADA2 is secreted into the extracellular environment [40]. The p.Thr129Pro variant causes ADA2 retention in the endoplasmic reticulum and deficient trafficking of ADA2 to the Golgi by affecting a conserved N-glycosylation consensus residue [41]. This indicates the important of glycosylation on ADA2 secretion. Additionally, ADA2 has two nearly identical monomers and the gel-filtration and SDS/PAGE analyses performed on human and chicken have been shown that ADA2 is secreted as a homodimer [42].

1.1.3 Pathophysiology of DADA2

1.1.3.1 Growth factor activity

ADA2 binds to different cell types through cell surface glycosaminoglycan (GAG) chains of proteoglycans as many cytokines, but it binds to T cells through the adenosine receptors which function as dimers [40, 43]. ADA2 which is secreted by the differentiation of monocyte into DCs and macrophages interacts with T cell, and it induces the proliferation of CD4+ T cells [43, 44]. Besides, it has been observed that the proliferation of CD4 + T cells is facilitated when ADA2 is added to a medium with low monocyte concentration. Furthermore, in the presence of T cells, ADA2-dependent differentiation of monocytes into macrophages occurs, and subsequently, ADA2 stimulates the proliferation of macrophages. Consequently, ADA2 seems both cytokine-like growth factor properties and autocrine-type growth factor properties [43].

On the other hand, the sequence analysis also revealed that human ADA2 is a member of the ADGFs family [36, 45-47]. A family of growth factors sharing sequence similarity with adenosine deaminase (ADA) was first identified in medium from a NIHSape-4 embryonic cell line of flesh fly as an insect-derived growth factor (IDGF) [40]. *Drosophila* homologs of *Sarcophaga* ADA were called adenosine deaminase-like growth factors (ADGFs) because the name IDGF had been used for unrelated Drosophila growth factors [47]. There are six ADGFs responsible for the extrinsic control of tissue growth in *Drosophila*. According to the sequence alignment of ADGFs and ADAs, these proteins have been shared sequence and functional similarity to the ADA enzyme [45]. One of them, ADGF-A, has both catalytic activity and mitogenic activity while the mutant form does not have. Therefore, a null mutation in the *ADGF-A* gene has been caused larval death because of adenosine accumulation in the larval hemolymph [45, 48, 49]. Similarly, the knockout of ADA2 homologs in *Xenopus* (frog) had been led to a reduction in body size and abnormalities of the body axis in the embryos, resulted in developmental disorders [50]. Also, as a result of transgenic expression of ADA2, high rates of embryonic and neonatal mortality and developmental abnormalities such as cardiomegaly had been observed [51].

1.1.3.2 Macrophage polarization

Endothelial-cell activation and increased cytokine levels involved in inflammation response have been shown in DADA2 patients' biopsies obtained from brain and lesion skin. Also, in consequence of defective differentiation of anti-inflammatory M2 macrophages in myeloid U937 cells with knockdown *ADA2* gene, there has been an excessive increase in M1 macrophage differentiation. Moreover, when ADA2-deficient patients' monocytes co-cultured with human dermal endothelial cells, it has been shown impaired endothelial integrity (Figure 1.3) albeit ADA2 does not express in endothelial cells. As a result, deficiency of ADA2 causes the polarization of macrophages influx towards pro-inflammatory (Figure 1.3) [1].

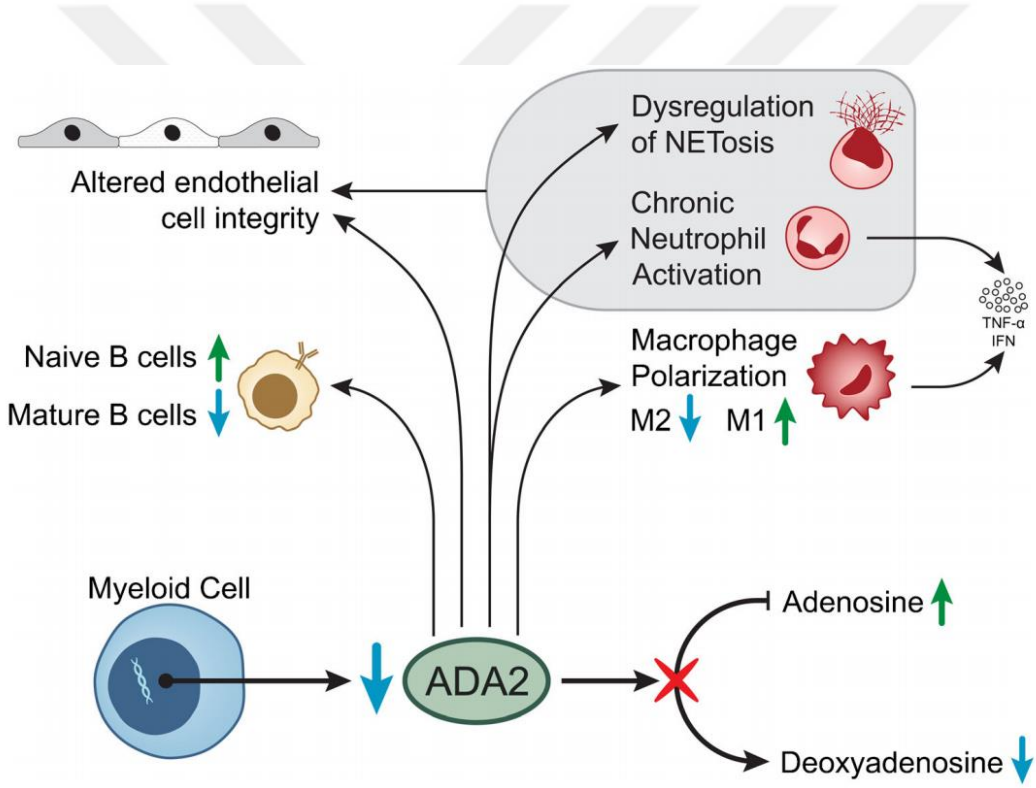

Figure 1.3 : Proposed pathophysiology of DADA2 [15]. In addition to ADA2 catalytic activity, it is associated with growth factor, macrophage polarization, neutrophil activation, and abnormal B cell development.

1.1.3.3 Neutrophil activation

The most common type of granulocyte in human is neutrophils. Neutrophils are an essential part of the innate immune system and are one of the first cells to respond to invading pathogens. During inflammation, neutrophils pass from the blood vessel to the matrix, and secrete proteolytic enzymes. In this way, it increases mobility by

dissolving the intercellular connections and eliminates bacteria and other pathogens through phagocytosis [52]. Neutrophils produce and release adenosine during inflammation and also express adenosine receptors [53].

Up-regulation of interferon-stimulated gene transcripts in peripheral blood have been demonstrated in two DADA2 patients that have biallelic mutations and low serum ADA2 activity. In addition, a significant upregulation of neutrophil expressed genes has been indicated by genome-wide expression sequence analysis, and this result have been supported by qPCR study. As a result of intracellular staining, increased expression of myeloperoxidase (MPO), which is mostly expressed in neutrophil granulocytes, has been observed in peripheral blood mononuclear (PMN) cells [54]. In the light of these results, it has been said that ADA2 has an important regulatory function for neutrophil activation (Figure 1.3).

Another response of neutrophils to pathogens is the formation of neutrophil extracellular trap (NET) which is a form of cell death (Figure 1.4) [35]. It contains a fiber network consisting of chromatin and serine proteases. Reactive oxide species (ROS) are produced by the NADPH oxidase. ROS initiates the breakdown of the granules that allows the release of neutrophil elastase (NE). Then, NE migrates to the nucleus of the neutrophil and leads to histone degradation. The de-condensed chromatin expands until it fills the entire cell. NET is released into the extracellular space through the cell explosion. There, it traps and removes a variety of pathogens [55]. NET formation has a protective function for the host. However, dysregulation of the pathway can cause abnormal endothelial cell function [56].

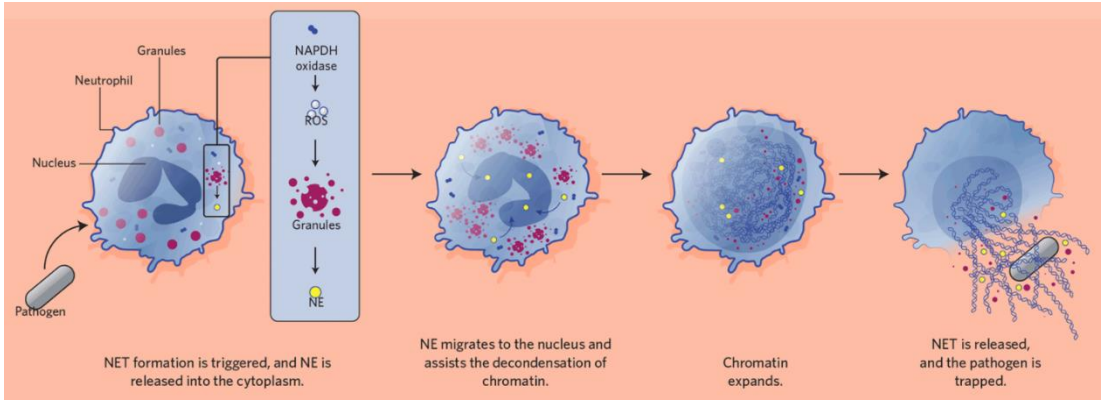

Figure 1.4 : Schematic representation of Neutrophil Extracellular Trap (NET) formation [55].

It was observed that when neutrophils from healthy donors were incubated with different concentrations of adenosine, adenosine significantly increased NET formation. This result raised the question of whether extracellular adenosine increased as a result of ADA2 deficiency could trigger NET formation (Figure 1.3). For this, healthy neutrophils were incubated with adenosine in the presence and absence of recombinant ADA2, and consequently, ADA2 was found to significantly reduce NET formation caused by adenosine. High concentration of extracellular adenosine triggers NET formation via A1 and A3 receptors expressed by neutrophils (Figure 1.5). Consistent with these results, an increased in extracellular adenosine and a large number of NETs in peripheral blood was also detected in DADA2 patients. Additionally, increased circulating low-density granulocytes (LDGs) was observed in patients with DADA2 [35]. LDG has a role in NET formation when there is no stimulus in the environment (Figure 1.5) [57]. In addition, it was observed that interactions between macrophages and neutrophils were important in adenosine-mediated NET formation. After NETs from DADA2 patients were incubated with M1 macrophages, activation of NF-kB followed by upregulation of pro-inflammatory cytokines including TNF-α was observed (Figure 1.5) [57].

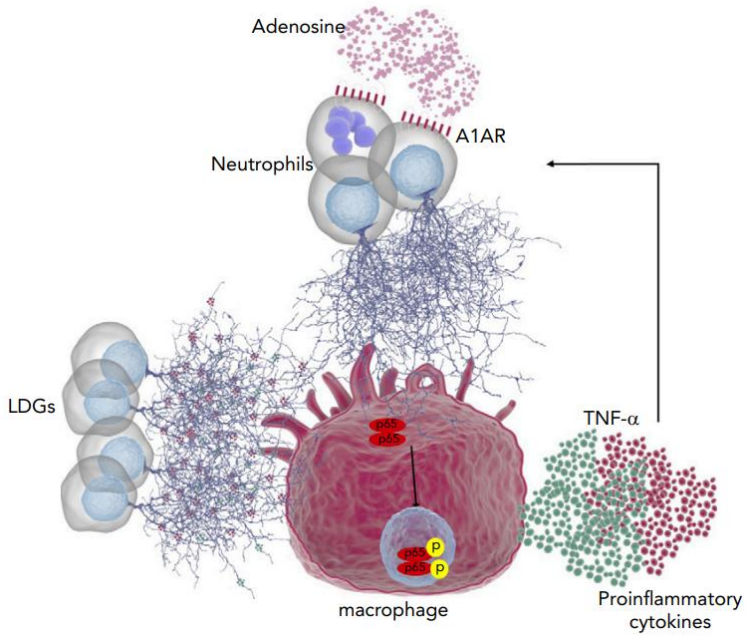

Figure 1.5: Schematic representation of neutrophil and macrophage function in patients [35]. Accumulation of extracellular adenosine and LDGs in patients trigger NET formation which activates macrophage. Then, proinflammatory citokines are activated.

1.1.3.4 Abnormal B cell differentiation

Abnormal B cell development is thought to be a consequence of DADA2 pathogenesis. Memory B cells were observed to be depleted, while these naïve B cells were found at normal or high levels in DADA2 patients (Figure 1.3) [39].

1.1.4 Clinical manifestations

The clinical manifestations of DADA2 vary widely, and most of them have uncertain pathophysiology.

1.1.4.1 Vasculitis

The most common clinical presentation of DADA2 is vasculitis. Usually, episodic clinical findings are observed in patients with fever and systemic inflammation. In addition to being characterized by cutaneous and neurological manifestations, fewer patients have had renal and gastrointestinal findings [1, 2, 15, 27]. Livedoid rash is the most common cutaneous manifestation affecting 73% of patients. It is also only a clinical finding in some patients [58]. Apart from this, there are various skin manifestations such as nodular lesions [2, 27], Raynaud's phenomenon [2, 7, 59], ulcers [15], digital gangrene [2, 58]. Neurological involvements are present in 50–77% of patients. The most common neurological event is ischemic strokes, usually in the form of lacunar infarctions [60]. As a result of the occlusion of small penetrating arteries, there is insufficient blood flow to the subcortical areas of the brain, causing lacunar infarctions [61]. Strokes can occur even at 5 months of age [5]. Additionally, there are other neurological complications associated with acute or chronic ischemia. These are seizures [4, 25], headache, ataxia, vertigo [26], intellectual and behavioral problems [5, 39], and spinal cord involvement [62, 63]. Hemorrhagic strokes have also been reported less commonly [60]. In addition to central nervous system involvement, peripheral nervous system involvement can also be observed in 53% of patients [39], including cranial nerve palsy [2, 26], neuropathy [15, 27], and mononeuritis multiplex [15, 64]. Furthermore, several cases of hearing loss and ocular involvements have also been reported. Ocular involvements vary as strabismus, conjunctivitis, uveitis, papillitis, and optic neuritis [39, 60]. Ischemia occurring in the mesentery can cause ischemic abdominal pain and gastrointestinal perforation, and lethality has also been reported [15, 65]. In addition, arterial aneurysms have also been observed in patients. Arterial hypertension occurs in 20% of patients with renal involvement [5]. The other

manifestations include renal artery aneurysm on the abdomen, segmental glomerulosclerosis, and renal amyloidosis. As a result of imaging and histological examinations, it has been reported that mesenteric and renal aneurysms are not easily distinguished from PAN [39]. Less common symptoms associated with vasculitis are testicular pain [60], pancreatic involvement including pancreatic infarction and pancreatitis [1, 15], pulmonary hemorrhage reported only in two cases [15, 62], and temporal arteritis reported in one patient [66]. Cardiac involvements such as cardiomyopathy [26, 27], coronary aneurysms [2], and carditides [39] are other rare manifestations.

1.1.4.2 Autoinflammation and autoimmunity

More than half of DADA2 patients have non-infectious episodes of fever attacks, the cause of which is uncertain. Fever and acute phase reactants also increase with vasculitis [1, 2, 67]. Other symptoms associated with autoinflammatory include recurrent oral and genital ulcers [59, 13], musculoskeletal symptoms [60], recurrent episodes of abdominal pain, and inflammatory bowel disease [17, 13]. Urticarial rashes may also appear [67].

Autoimmune symptoms are rarely seen. Autoimmune cytopenia such as Coomb's positive hemolytic anemia have been reported [60]. Also, 10% of the patients have transient positive lupus anticoagulants that increase with age [5]. Additionally, antinuclear antibodies (ANA) or anti-neutrophil cytoplasmic antibodies (ANCA) elevated during autoimmune diseases were positive in several patients [60].

1.1.4.3 Hematological

Cytopenia an important finding for the DADA2, even sometimes it is the first manifestation. The first reported cytopenia was lymphopenia which is commonly seen in all clinical phenotypes. Another symptom is anemia. Refractory anemia has been reported in some patients. Pure red cell aplasia (PRCA), hemolytic anemia are other symptoms observed in patients. The rarely reported symptoms include bone marrow failure in the form of neutropenia and pancytopenia [5, 38, 39, 60]. Also, some symptoms affect less than 1% of patients. These include myelofibrosis [1], acute myeloid leukemia [26], and macrophage activation syndrome [1, 26, 68].

1.1.4.4 Immunodeficiency

Despite unclear pathophysiology, immunodeficiency is milder in vasculitis phenotype, while it may be more severe in hematological phenotype [39]. The first study on DADA2 was reported that patients had low IgM levels and mild hypogammaglobulinemia [1]. Currently, at least 25% of patients have hypogammaglobulinemia [38]. Various degrees of antibody deficiency can be seen, which leads to recurrent infections reported in 16% of DADA2 patients. The most common infections are originated from bacteria [39, 60]. However, herpes virus infections have also been noted [17]. Chronic inflammation may develop [69]. In the predominant state of cytopenia, decreased B cells and low class-switched B cells have also been documented in patients [70].

1.1.4.5 Lymphoproliferation

The lymphoproliferation phenotype can include a whole spectrum from benign florid follicular hyperplasia to malignant proliferation. DADA2 patients may commonly have hepatosplenomegaly and lymphadenopathy [5]. Autoimmune lymphoproliferative syndrome (ALPS)-like phenotype was recently reported in 3 patients with the biallelic mutation [26, 71, 72]. In one of these, increased double-negative T cells were also observed [71]. Autoimmune cytopenia related to generalized lymphadenopathy has been observed in some patients, and alpha/beta TCR cells have also been reported [5]. T cell large granular lymphocyte (T-LGL), a rare chronic lymphoproliferative disease, was reported in two patients at the age of 8 and 31 years respectively. T-LGL is characterized by clonal rearrangement of the beta chain and clonal proliferation has been reported in one patient [25]. Additionally, three patients presented Hodgkin's lymphoma have been reported [73, 74].

1.1.5 Diagnosis and treatment of DADA2

Clinical findings are not sufficient for diagnosis because of diverse phenotypes. The diagnosis of DADA2 depends on the identification of pathogenic variants or the measure of ADA2 activity. The pathogenic variants can be detected by Sanger sequencing or next-generation sequencing [39, 60]. However, in some cases small deletions and duplications may not be detected, in that case, the other molecular tests such as SNP arrays, long-range polymerase chain reaction (PCR), multiplex ligation-dependent probe amplification can be used [13]. DADA2 patients have shown reduced

or undetectable ADA2 activity [75]. Therefore, analysis of the plasma or serum ADA2 activity test by HPLC or ELISA is also helpful for diagnosis [5].

Many therapeutic approaches have been reported, and the appropriate method of treatment is selected individually for patients, depending on the symptoms and the severity of the disease [60]. It has been observed that the treatment method used for vasculitis phenotypes failed in hematological and immunological phenotypes [16].

Nowadays, the most common treatment method is anti-TNF-α therapy with high success in vasculitis phenotypes. The most commonly used anti-TNF agents are etanercept, adalimumab, infliximab [60], and TNF-α inhibitors do not have a significant effect on ADA2 enzyme activity levels [76]. It has been observed that anti-TNF therapy features a protective effect against new ischemic events, and the other signs of disease such as individual growth and development are also improved with therapy [5]. TNF-α inhibitors may be used in long-term treatment especially in patients with vasculitis and CNS manifestation. One study reported that two siblings treated with etanercept had a positive response to treatment for over 10 years without relapse [64]. After anti-TNF-α treatment, inflammation was suppressed and B cell function is repaired in patients with mild immunodeficiency [17]. However, the treatment outcomes are unsatisfactory in some patients with severe cytopenia or immunodeficiency [5] furthermore it may increase the risk of infection [60].

Hematopoietic stem cell transplantation (HSCT) can be used in the treatment of severe hematological diseases [5]. The HSCT treatment has been reported to be successful in 21 of 25 ADA2 patients [60]. After HSCT, ADA2 activity returns to normal, and key cytokines decrease. An improvement in all clinical manifestations of the disease has also been shown, including hematological, immunological, and vasculitis [77]. Increased inflammation potential in DADA2 may cause the development of complications after the treatment, leading to serious infections, autoimmune phenomena, and pineal gland bleeding [39]. Also, additional stem cell support had been required in several patients as a result of a decrease in chimerism. Additionally, recurrence of the disease was reported in two patients after transplantation, using heterozygous healthy siblings as donors [77, 78]. In addition to HSCT, which is a definitive treatment for immunodeficiency, immunoglobulin replacement, antibiotics and antivirals can also be used for the treatment [16, 17].

Another treatment method is infusions of fresh frozen plasma. ADA2 activity has been reported to return to normal with this treatment. However, it is not an appropriate choice for long-term treatment because of the short half-life [79].

The success of the treatment and mortality depend on the severity of the disease. Deaths resulting from DADA2 have been reported in 8.2%. While patients with severe hematological symptoms have high mortality, CNS involvement is often the cause of death due to the severity of vasculitis [60].

1.2 Enzyme-Linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) was first described in 1971 by Engvall and Perlmann. ELISA is an immunological assay used to detect and quantify a protein within soluble substances. ELISA is based on antigen-antibody interaction and the enzyme activity is measured by colorimetric analysis. There are different types of ELISA such as direct, indirect, and sandwich ELISA [80]. Direct ELISA is the first and the simplest type and is convenient for qualitative and quantitative antigen detection. An antigen immobilized to the surface is incubated with an enzyme-labeled antibody. The interaction of subsequently added substrate and enzyme results in color change which can be measured by a spectrophotometer. Indirect ELISA is a two-step process. Firstly, an unconjugated primary antibody is added and incubated with antigen. Then labeled secondary antibody is added and bind to the primary antibody. After that, a substrate is added and measurement is performed. Indirect ELISA is suitable for antibody screening. Sandwich ELISA is the most common type and requires two specific antibodies which are also called matched antibody pairs. A captured antibody is coated on the surface and an antigen of interest is added, then incubated. The other antibody is added and incubated. It is measured after adding the appropriate substrate. Sandwich ELISA is divided into two types according to whether the second antibody is labeled: Direct or Indirect sandwich ELISA. The sandwich ELISA is useful for the detection of soluble or low concentration antigens [81].

1.3 Aim of the Study

Adenosine deaminase is responsible for the regulation of adenosine homeostasis and purine metabolism by converting adenosine to inosine and 2'-deoxyadenosine to 2'-deoxyinosine. Humans have two isoforms of adenosine deaminase: ADA1 and ADA2.

Biallelic mutations in the ADA2 gene that produces ADA2 protein cause Adenosine Deaminase Type 2 Deficiency, which is an autosomal recessive disease. Although the catalytic activity of ADA2 has a lower affinity for adenosine, DADA2 patients had decreased or undetectable ADA2 activity in serum or plasma.

The aim of this study is to investigate possible significant differences between the intracellular total adenosine deaminase activities of 8 DADA2 patients and 5 healthy controls. For this, lysates prepared from peripheral blood mononuclear cells of the subjects were analyzed by ADA activity assay. Then, the significance of the differences was determined by Student's t-test, assuming $p<0.05$ as significance.

2. MATERIALS AND METHODS

2.1 Materials

The equipment, chemicals, and the preparation of buffer used in the study are found in Appendix A and B respectively.

2.2 Methods

2.2.1 Sample collection

Blood samples were collected from 8 patients with DADA2 diagnosis in Cerrahpaşa Medical Faculty Pediatric Rheumatology Polyclinic and 5 healthy people as a control group. Two of the patients are Syrian and have the G47R/G321E heterozygous mutation, while the other patients are Turkish and three patients have the G47R homozygous variant and one patient has the homozygous variant not included in the Infevers database. Between 2.5 and 15 milliliters of blood were drawn into EDTA vacuum tubes from each person. Prior to sample collection, subjects' consent was gained by signing an Informed Consent Forms.

2.2.2 Peripheral blood mononuclear cell (PBMC) isolation

PBMCs were isolated within a few hours after the subjects' blood was collected. The procedure followed for PBMC isolation was indicated below:

- The blood sample is placed into a 50ml centrifuge tube. Phosphate Buffered Saline (PBS) is added as much as the amount of blood and the tube is shaken for 15 minutes.
- The lymphocyte separation medium is added to the 15ml centrifuge tube. The blood-PBS mixture is added gently into the tube. The medium and mixture ratio will be 1:2.
- Centrifuge for 20 minutes at 2000 rpm. (acceleration:9, deceleration:1)

- The cloudlike layer between the precipitated erythrocytes and the serum contains cells. These are collected and placed into 15 ml centrifuge tubes.
- PBS is added as much as the amount of cell into the tubes.
- Centrifuge at for 10 minutes 300 xg. (accelerations:9, deceleration:9)
- Supernatant is discarded with a pipette.
- If there is redness in the pellet, add 2-3ml of RBL, centrifuge at 500xg for 10 minutes, and discard the supernatant with a pipette.
- Resuspend the cells in 200ul PBS and measure.
- Cells are resuspended by adding 1 ml of PBS for every 8 million cells. PBS is gradually added by pipetting.
- Centrifuge for 10 minutes at 300 xg (accelerations:9, deceleration:9) and discard the supernatant with a pipette.

2.2.3 Cell lysates

After 1-5x10^6 cells are harvested, cell lysate is performed as stated in the kit protocol (ab211093).

- Wash the cells with cold PBS.
- Centrifuge for 10 minutes at 300 xg and discard the supernatant with a pipette.
- Resuspend cells with 150 – 300 µL cold 1X ADA Assay Buffer.
- Homogenize the cells quickly by pipetting on ice and put into a 1,5ml centrifuge tube.
- Incubate the homogeneous cells on a rotary shaker at 4°C for 15 minutes.
- Centrifuge for 10 minutes at 4°C at 16,000 xg.
- Collect the supernatant and put it in a new pre-chilled tube.
- Keep on ice and measure the amount of protein.Store at -80°C.

2.2.4 Measurement of protein samples with Bradford assay

The protein concentration of samples was measured by Bradford assay. Protein standard dilutions, 0.25µg/µl, 0.5µg/µl, 1µg/µl, 1.5µg/µl, 2µg/µl and 4µg/µl, were prepared using Bovine Serum Albumin (BSA). The Bradford reagent was diluted at

1:5 ratios with proteomic water. Proteomic water was also used ad blank. The protein samples were diluted in 1:3. 5µl standards and samples were distributed into wells of 96 well plates. Then,195 µl Bradford reagent was added and mixed gently with a pipette. The protein concentrations were measured with a microplate reader (BIO-RAD) at an absorbance wavelength of 595 nm. All standards and protein samples were measured in duplicate.

2.2.5 ADA activity assay

Ab212093 Adenosine Deaminase(ADA) Activity Assay Kit was used to measure adenosine deaminase activity.

- All materials were equilibrated to room temperature, and reagents and standard curve dilutions were prepared as written in the user manual.
- The samples were diluted in such a way as to the amount of protein in the well was 5µg.
- Reaction mix and background mix were prepared according to the user manual.
- 50µl standard dilutions, samples and reagent control were distributed into wells of UV transparent plate.
- While 50µl background mix was added into each standard dilutions and 50µl reaction mix was added into each samples and the reagent control.
- The microplate was pre-incubated at 37°C for 5 minutes and then, absorbance was measured at OD = 293 nm on a microplate reader in kinetic mode for 98 minutes at 37°C. All standards, samples, and reagent control were measured in duplicate.

2.2.6 Calculations

Standard curve calculation:

The corrected absorbance values were obtained by subtraction of the mean absorbance value of the blank (Standard #1) is from all the standard readings. The average of duplicate readings is calculated for each standard. Standard curve readings were plotted and the line of the best fir to construct the standard curve was draw.

Measurement of ADA activity in the samples:

Two time points (T1=2 and T2=28) in the linear phase of the reaction progress curves were selected. ΔOD was calculated using the following equation:

$$\Delta OD = (A2 - ABG2) - (A1 - ABG1) \quad (2.1a)$$

Where:

A1 and A2 are absorbance values at selected time points (T1 and T2).

ABG1 and ABG2 are background reagent at selected time points (T1 and T2).

ADA activity of samples was calculated using the following equation:

$$ADA\ Activity = (B / (\Delta T \times M)) \times D \quad (2.1b)$$

Where:

B = amount of Inosine in the sample well calculated from the standard curve (nmol).

ΔT = reaction time (minutes).

M = amount of protein in the well (μg).

D = sample dilution factor.

Statistical analysis:

Statistical analysis was performed with Excel. Unpaired t-test analysis was performed to the ADA activity results and p values were calculated for activity differences between patients groups and healthy controls.

3. RESULTS

The duplicates of standards were averaged after subtracting the absorbance of blank from the absorbance of the standards. A standard curve (Figure 3.1) was generated using the inosine values and averages of standards (Table 3.1).

Table 3.1 : Inosine values and averages of the standards.

Standard #	Inosine(nmol)	Averages of Standards (nm)
1	0	0
2	2	0,0165
3	4	0,055
4	6	0,0805
5	8	0,0795
6	10	0,1155

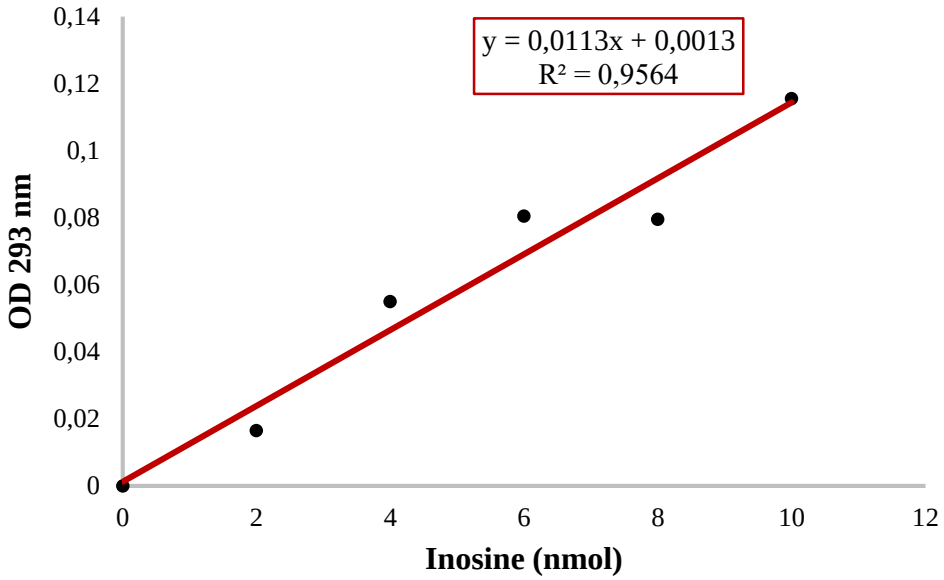

Figure 3.1 : Inosine standard calibration curve.

ADA activity was calculated for the samples by taking time intervals of 2 to 28 minutes that the reaction increased. The results are shown in Table 3.2.

Table 3.2 : Calculations for patients and health controls. P:patients HC:healthy control B:amount of inosine in the sample well**.**

	ΔOD(nm)	B(nmol)	Dilution Factor	ADA Activity(nmol/min/ug)	Mutations
P1	0,0555	4,796460177	13,35	0,492559564	
P2	0,0195	1,610619469	23,1	0,28619469	
P3	0,0445	3,82300885	4,41	0,129688223	p.G47R/p.G321E
P4	0,0435	3,734513274	6,78	0,194769231	p.G47R/p.G321E
P5	0,0375	3,203539823	22,17	0,546326753	p.G47R homozygous
P6	0,029	2,451327434	15,63	0,294724983	
P7	0,0355	3,026548673	30,57	0,711704561	p.G47R homozygous
P8	0,0195	1,610619469	48,15	0,596548673	p.G47R homozygous
H1	0,075	6,522123894	35,22	1,766993873	
H2	0,0655	5,681415929	35,91	1,569381892	
H3	0,046	3,955752212	28,38	0,863671137	
H4	0,053	4,575221239	43,77	1,540441797	
H5	0,0505	4,353982301	43,65	1,461933288	

After calculation of ADA activities, the statistical analyzes were applied to the groups (Table 3.3). ADA activity differences between patient groups and control group were analyzed statistically by Student's t-test.

Table 3.3 : Statistical values for the groups.

	Patients have p.G47R (n=3)	Patients have p.G47R/p.G321E (n=2)	Healthy Controls (n=5)	Patients(n=8)	Healthy Controls(n=5)
Mean	0,618193329	0,162228727	1,440484398	0,406564585	1,440484398
Standard Deviation	0,084786928	0,046019222	0,341463057	0,208617133	0,341463057
Variance	0,007188823	0,002117769	0,116597019	0,043521108	0,116597019
Standard Error of Mean	0,048951756	0,032540504	0,152706921	0,073757295	0,152706921
Confidence Level (95,0%)	0,210622406	0,413466303	0,423982384	0,174408288	0,423982384
95% Confidence Interval	0,41 - 0,83	0 - 0,58	1,02 - 1,86	0,23 - 0,58	1,02 - 1,86

When the ADA activity of all subjects is examined, it is seen that the ADA activity in patients is lower than in healthy individuals (Figure 3.2). This difference is statistically significant according to the t-test (p=0.0008) (Figure 3.3).

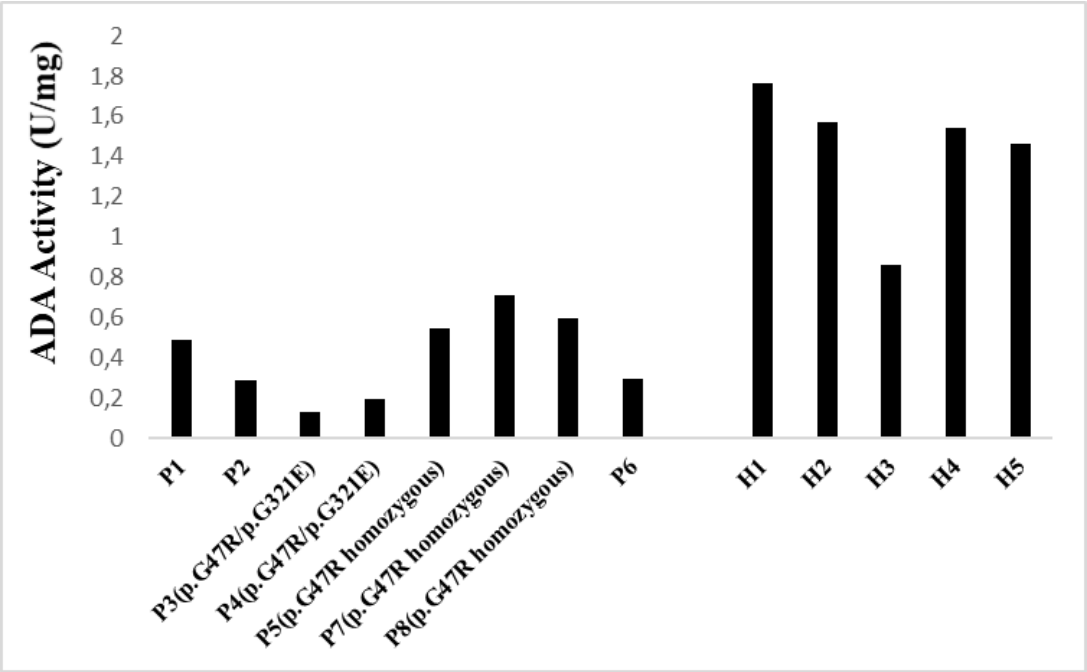

Figure 3.2 : Adenosine Deaminase activity in patients and healthy individuals.

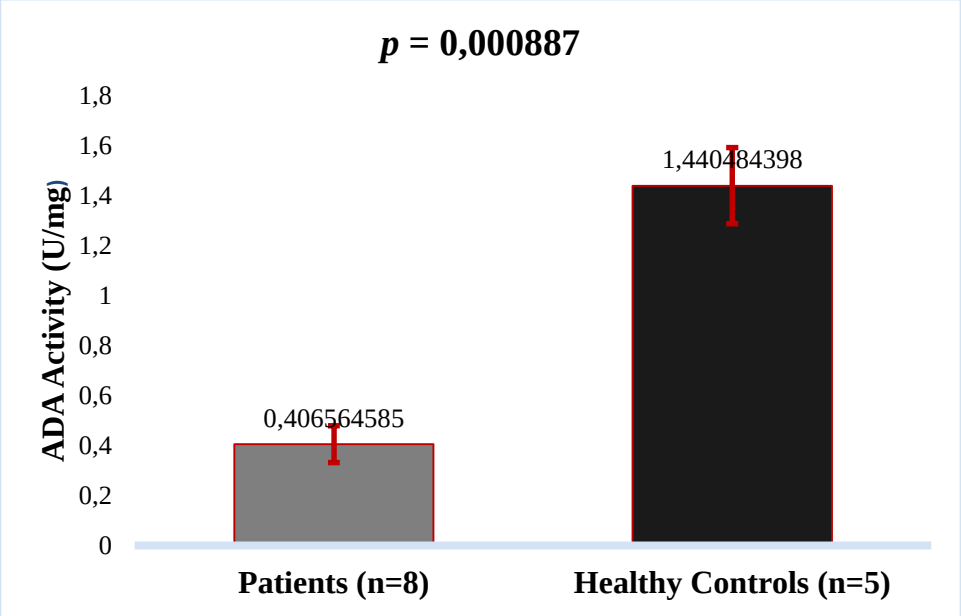

Figure 3.3 : Adenosine deaminase activity for patients and healthy control groups. HC shows a statistically significant higher ADA activity according to patients.

In addition, the ADA activity of patients with p.G47R homozygous mutations and patients with p.G47R/p.G321E heterozygous mutations was compared and the effect of mutations on ADA activity was examined. According to this result, a significant difference was observed in the ADA activity between the patient groups. The heterozygous mutation group has statistically lower ADA activity according to the homozygous mutation group (p=0.004) (Figure 3.4). Furthermore, when the patient

groups were compared with the control group, the ADA activity in patients with heterozygous mutation is lower (p=0.001) than patients with homozygous mutation (p=0.003) (Figure 3.4).

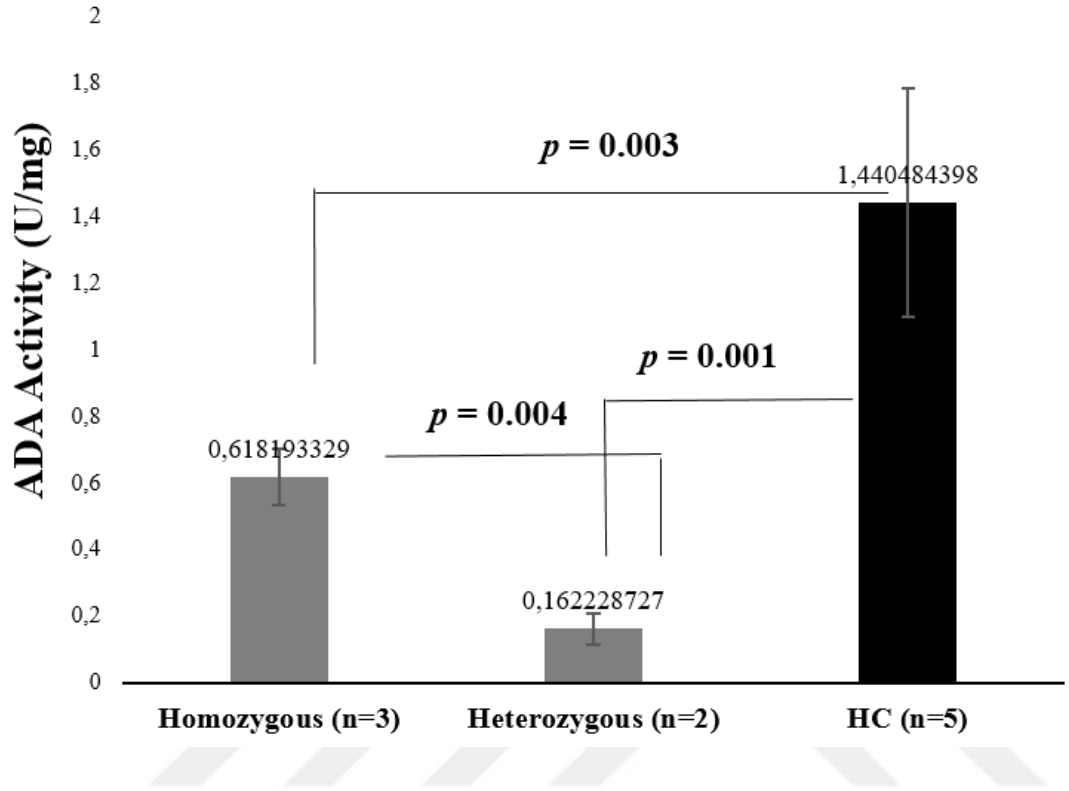

Figure 3.4 : Adenosine deaminase activity for patients who had p.G47R homozygous mutation and p.G321E heterocygous mutation and healthy control groups. ADA activity is statistically significant lower within patient groups according to control group.

4. DISCUSSION

Loss-of-function mutations in ADA2 lead to DADA2 which is an autosomal recessive disease. DADA2 was first described as monogenic vasculitis syndrome in 2014. Although the clinical manifestations of ADA2 Deficiency are very diverse, the most common type is vasculitis findings. More than half of patients have attacks of non-infectious fever. Symptoms include recurrent oral and genital ulcers, musculoskeletal symptoms, recurrent abdominal pain, inflammatory bowel disease, and immunodeficiency [39]. Adenosine deaminase converts adenosine to inosine. There are two isoforms of adenosine deaminase: ADA1 and ADA2. While ADA1 is highly expressed in lymphocytes, ADA2 is mainly produced by monocytes [61], and the highest ADA activity has been found in lymphocytes and monocytes [82]. Peripheral blood mononuclear cells (PBMCs) are composed of lymphocytes and monocytes, mostly lymphocytes.

In this study, total adenosine deaminase (ADA) activity in PBMCs of patients diagnosed with DADA2 was compared with the control group. The disease-causing variant p.G47R occurs in the dimerization domain and affects the stability of homodimers of the ADA2 protein, which is necessary for enzyme activity [41]. Therefore, it is expected that the total ADA activity in patients carrying the p.G47R mutation will be lower than in the healthy group because of a decrease in ADA2 catalytic activity. As a result of statistical analysis, a lower ADA activity was observed in the patient group ($p=0.0008$). However, patients with heterozygous mutations had lower ADA activity than those with homozygous mutations ($p=0.004$). In this case, it can be said that the G321E mutation plays an important role in catalytic activity. In conclusion, as expected, lower ADA activity was observed in the patient groups compared to the healthy group.

In the future, the function of the p.G321E mutation can be further investigated by modelling and cell experiments.

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APPENDICES

APPENDIX A: Equipments

APPENDIX B: Chemicals and Buffer

APPENDIX A

LABORATORY EQUIPMENT

Balance	Precisa 620C SCS
Centrifuges	Beckman Coulter Microfuge® 18
	Beckman Coulter Allegra X-30
Centrifuges Tubes	Greiner Bio-One & Axygen
Distill Water Machine	TKA Wasser-Aufbereitungs
EDTA Blood Tube	VACUTEST-ARZERGRANDE
Freezers	Nuve (-80°C)
High Pressure Steam Sterilizer	TOMY SX-700E
Ice Machine	Scotsman AF10
Magnetic Stirrer	Dragon Lab MS-HS
Microplate Reader	Bio-Rad Benchmark Plus
	BMG LABTECH CLARIOstar
Pipettes	Thermo Scientific
Pipette Tips	Axygen and Thermo Scientific
Plastic Packaging	Parafilm-PECHINEY
Refrigerator	Vestel (+4°C)
Rotator	Stuart
Shaker	Heidolph Doumax 1030

APPENDIX B

CHEMICALS AND BUFFER

Bovine Serum Albumine (BSA)	Sigma&Aldrich
Bradford Reagent	Bio&Rad
Phosphate Buffered Saline (PBS)Tablet	Sigma-Aldrich
Proteomic Water	Applichem&Merck

COMMERCIAL KITS

Ab212093 Adenosine Deaminase(ADA) Activity Assay Kit (Colorimetric) – Abcam

BUFFER

Red Blood Cell Lysis (RBL) Buffer (1X):

Ammonium chloride(NH_4Cl) 8,3 g

Sodium bicarbonate ($NaHCO_3$) 1,01 g

EDTA 0,04 g

Add distilled water to 1 liter

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