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ASSOCIATION OF ANGIOTENSIN CONVERTING ENZYME I/D POLYMORPHISM WITH TRANSIENT TACHYPNEA OF THE NEWBORN

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by

Lolai IKROMZODA

May 2015

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APPROVAL PAGE

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ABSTRACT

Transient tachypnea of neonate (TTN) of the infant is one of the most well-known reasons of early respiratory distress in the immediate neonatal period. There is expanding proof to support the function of activation of the renin angiotensin system over intense lung problem. The aim of this study was to determine association between angiotensin-converting enzyme (ACE) I/D polymorphism, ACE activity and TTN syndrome. Methods: 35 with TTN, 37 control infants were studied for ACE polymorphism and serum ACE activity. Conclusions: The study could not find any difference in I/D allele frequency between control group and TTN group, but ACE activity difference between TTN and control group were significantly different.

Keywords: Angiotensin converiting enzyme,polymorphism,Transitent tachypnea of neonate

ANJEOTENSİN DÖNÜŞTÜRÜCÜ ENZİM I/D POLİMORFİZMASININ YENİ DOĞANLARDAKİ GEÇİCİ TAŞIPNELİ HASTALIĞI İLE İLİŞKİSİ

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ÖZ

Yenidoğan bebeklerin (TTN) geçici taşipnesi sendromu hemen yenidoğan döneminde erken solunum sıkıntısı en iyi bilinen sebeplerinden biridir . Yoğun akciğer problemi üzerinde renin anjiotensin sisteminin aktivasyonu fonksiyonunu desteklemek için bir çok çalışma yapılmıştır. Bu çalışmanın amacı, anjiotensin dönüştürücü enzim (ACE) I / D polimorfizmi, ACE aktivitesi ve TTN sendrom arasındaki ilişkiyi belirlemektir . Yöntem: TTN 35, 37 kontrol bebekler ACE polimorfizmi ve serum ACE aktivitesi için çalışılmıştır . Sonuç : Çalışma ve kontrol grubu TTN grubu arasında herhangi bir I/D allellerinde farkı bulunamadı, ama ACE aktivitesi iki grup arasında farki bulundu, dolayısıyla TTN ve kontrol grup için belirleyeci bir marker olabilir.

Anahtar Kelimeler: Anjeotensin dönüştürücü enzim, polimorfizma, yenidoğan geçici taşinpeli, polimeraz zincir reaksyon

To my Mother-Land

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

ACE I/D	Angiotensin converting enzyme insertion deletion polymorphism
ACE	Angiotensin converting enzyme
BPD	Bronchopulmonary Dysplasia
CFTR	Cystic fibrosis transmembrane receptor
DD	Double deletion
DS	Duchene Syndrome
ENac	Epithelial sodium channel
II	Double insertion
PCR	Polymerase chain reaction
RAAS	Renin- Aldestorone-Angiotensin System
RDS	Respiratory distress syndrome
SNP	Single nucleotide polymorphism
STD	Standard Deviation
TTN	Transient tachypnea neonate

CHAPTER 1

1.1 INTRODUCTION

1.1.1 Transient tachypnea of newborn

Transient tachypnea of the newborn (TTN) was primarily described by Avery and his group (AVERY et al., 1966) back in 1966. In the past, TTN has been considered as a transitory respiratory disturbance as a result of an interruption in alveolar fluid absorption. TTN is described by tachypnea right away after birth, which can be treated fast within 2 to 5 days. In spite of some syndrome, it is commonly recovered and accepted that once TTN clears up there is no farther increased risk for respiratory disease or other long-term results. It has been noted that the probability of having TTN is high in prenatal birth, C-section delivery, and birth of a male infant, while the etiology and pathogenesis of this case are mostly unexplored (Birnkrant et al., 2006; Machado et al., 2011). During late third trimester, fetal lung fluid absorption to interstitial fluid is initiated, and this process continues to infant delivery and any interruption in this physiological event may result to TTN (Aslan et al., 2008). The initial structure of clearance associates with active Na+ transportation to the distal pulmonary epithelium. Meanwhile, by catecholamines stimulations of Cl^- and 2-receptors have been shown to turn the utero-fetal lung from a fluid-secreting to a fluid-absorbing organ. The rise in fetal catecholamine discharge at birth supplies an important channel among-adrenergic stimulation and lung liquid clearance. Nevertheless, fluid is reabsorbed, TTN is a self-limiting disease with no potential adverse squealed (Derbent et al., 2011; Kasap et al., 2008; Silasi et al., 2010; Zeiger et al., 1989). (Fig1.1)

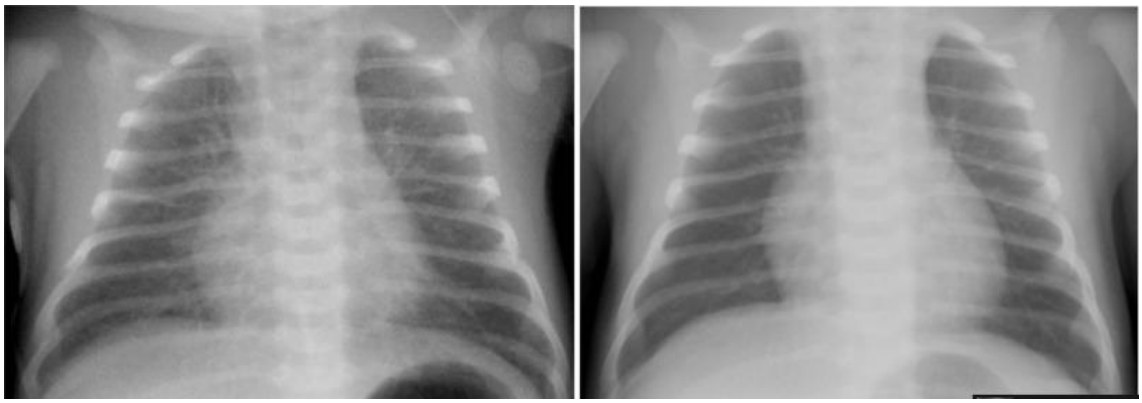


Figure 1.1 Chest Radiography, left TTN, right healthy subject (LearningRadiology).

Studies working on lung mechanic assessments were completed on infants born by either cesarean or vaginal delivery. Milner et al acclaimed that neonates with the same chest circumstances and different delivery type may have different average thoracic gas volume. According to his work, infants mean thoracic gas volume was measured as 32.7 ml/kg for infants born vaginally and 19.7 ml/kg for infants born via cesarean (Kicklighter; Milner et al., 1978; Vyas et al., 1983) with the same chest circumferences. Although the total thoracic volume was in normal range, it was observed that the neonates born via cesarean delivery had still quantity of interstitial and alveolar fluid in contrast to those born vaginally (Silasi et al., 2010).

1.1.2 Angiotensin Converting Enzyme

ACE, angiotensin I and angiotensin II are components of the renin-angiotensin system (RAS), which controls blood pressure by monitoring the volume of fluids in the body. ACE indirectly increases blood pressure in two ways. It converts angiotensin I to angiotensin II which cause vasoconstriction, and degrades bradykinin a potent vasodilator respectively. ACE is produced by cells in the endothelium of blood vessels in the lung and kidney respectively (Goodfriend et al., 2003).

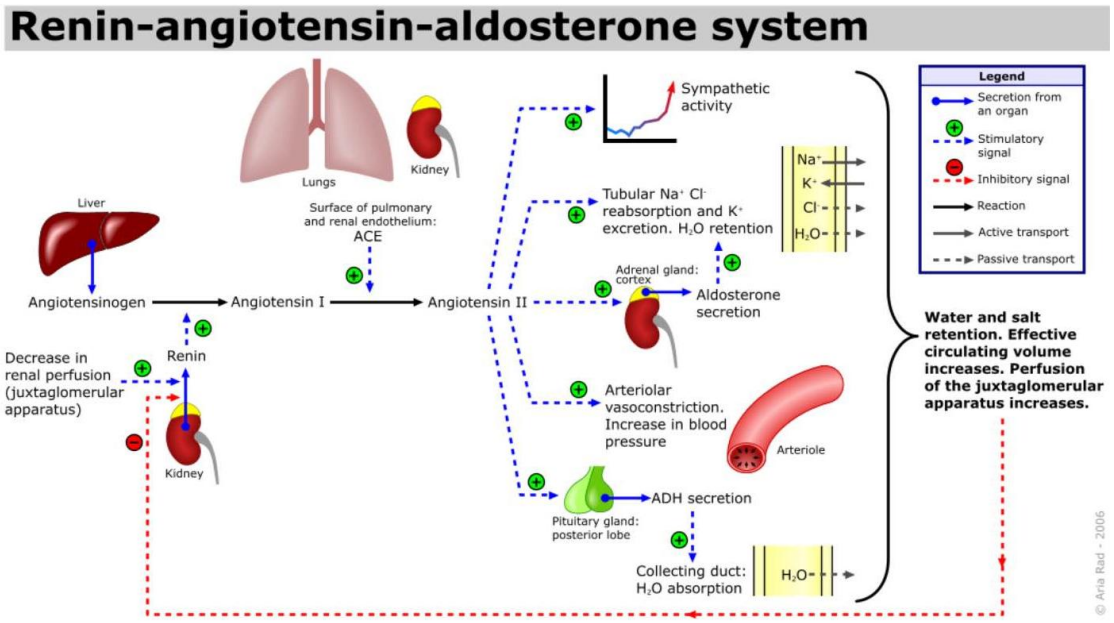


Figure 1.2 Renin-Angiotensin-Aldosteron System scheme (Rad, 2006).

1.1.3 Angiotensin Converting Enzyme Gene

In *Homo Sapiens*, the gene encoding ACE is located on q arm of chromosome 17(17q23) (Mattei et al., 1989; Sayed-Tabatabaei et al., 2006).

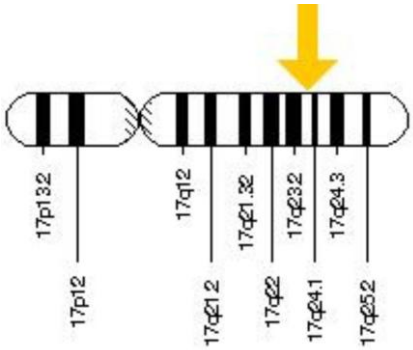


Figure 1.3 Location of ACE gene, chromosome17q (Reference, May 2013).

Total size of gene is about 22 kilobases (kb) nucleotide, consist of 26 exon and 25 introns. In National Center for Biotechnology Information (NCBI) 160 ACE gene polymorphisms are listed and single nucleotide Polymorphisms are majority. Only 34 of SNPs located in coding sequence and 18 SNP are missense mutations. Rigat and his coworkers found insertion and deletion of 287 bp sequence of DNA in intron 16 of ACE gene. In addition, the association of coronary heart disease and stroke, diabetes and nephropathy with ACE polymorphism is studied (Ayada et al., 2014; Borgman et al., 2011; Glenn et al., 2009; Hou et al., 2013; John Baier et al., 2005; Kiss et al., 2014; Parenica et al., 2010; Rigat et al., 1990; Tiret et al., 1992). According to OMIM (Online Mendelian Inheritance of Man), ACE I/D polymorphism has association with many diseases such as, stoke, ischemic, sepsis, hemorrhagic stroke, renal tubular dysgenesis (Table 2.1).

CHAPTER 2

LITERATURE SURVEY ON ACE I/D POLYMORPHISM AND TRANSIENT TACHYPNEA OF NEWBORN

2.1 INTRODUCTION

2.1.1 Transient Tachypnea of Newborn

Transient tachypnea of the Newborn (TTN) is a popular determinant of respiratory distress in the neonatal period, characterized by an oxygen requirement and tachypnea, and occurring in 1- 2% of term babies. The medical path is usually benevolent, revival happening instinctively within a few days (AVERY et al., 1966; Kasap et al., 2008; Kicklighter). Recognized risk factors for TTN consist of premature C- section, those whose mothers were afflicted by maternal diseases, male gender and being a twin (Derbent et al., 2011; Milner et al., 1978; Silasi et al., 2010; Sonderen et al.,2002).

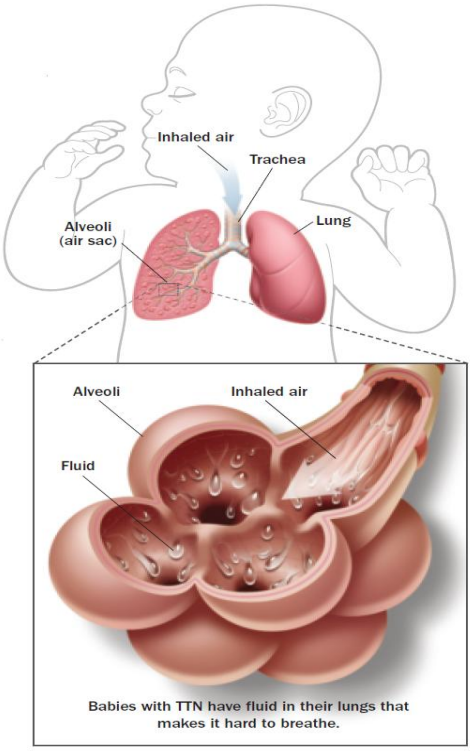


Figure 2.1 Simplified image of newborn lung and alveoli with fluid (Ikaria, 2014).

2.1.2 Causes of TTN

2.1.2.1 Caesarean delivery

Researches exploiting lung mechanic evaluations were done on infants born by either cesarean or vaginal delivery, measured infants with same total thoracic volume but C-section and normal delivery have different gas volumes (Milner et al., 1978) while in another study, epinephrine had a great role in the absorption of fluid from alveolar sack to interstitial fluid . During childbirth an increased level of epinephrine releases, inhibits Cl^- pumps, and promotes Na^+ channels absorption, therefore, net flux of fluid from the lung into the interstitial occurs (Venkatesh and Katzberg, 1997). As a result, in the deficiency of this normal movement in counter-regulatory hormones in the neonate, the removal of pulmonary fluid is restricted (Hooper et al., 2013; Vyas et al., 1983).

2.1.2.2 Maternal asthma and prolonged labor

In a study done in New Jersey, statistical analysis on singleton live birth during 1989-1992 was performed. Large number of newborns were analyzed in two groups, to identify impacts of asthmatic mothers on transient tachypnea of newborns. The outcome of the study defined that, newborns whose mothers with asthma were prone to show TTN, rather than newborns with non-asthmatic mothers. .

2.1.3 Angiotensin Converting Enzyme

Angiotensin I-converting enzyme (EC 3.4.15.1; kininase II) is a dipeptidyl carboxypeptidase that plays a significant function in blood pressure regulation and electrolyte balance by converting angiotensin I into angiotensin II, a potent vasopressor and aldosterone-stimulating peptide (Ramaraj et al., 1998). In addition, ACE degrades bradykinin, a potent vasodilator, indirectly increases blood pressure (François Cambien et al., 1992). Researchers worked generally on the function of angiotensin-converting enzyme in blood pressure monitoring and found that it has different physiological roles in homeostasis. The ACE gene expresses two isozymes: somatic and germinal ACE. Somatic ACE is synthesized in many tissue types including vascular endothelium and renal epithelium whereas germinal one is expressed only in sperm (Ramaraj et al., 1998).

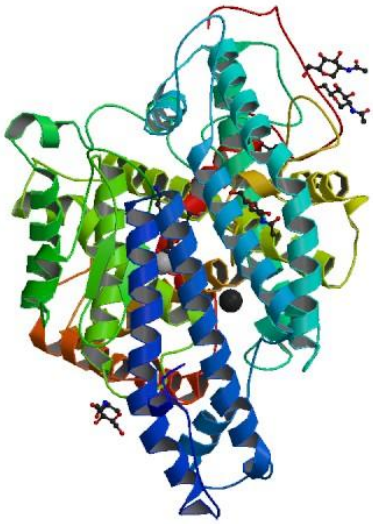


Figure 2.2 ACE Somatic form, with lisinopril drug (Watermeyer et al., 2010).

2.1.4 Genetic disorders related to ACE

2.1.4.1 Cystic fibrosis

Cystic fibrosis results in abnormal mechanism of lung liquid clearance after birth and some other studies consider that temperate and temporary surfactant insufficiency may have function respectively. Genes that have susceptibility to cystic fibrosis may also affect TTN. Especially genes that promotes activation or elevated expression of epithelial sodium channel or the Na(C)-K(C)-ATPase pump, possibly the cystic fibrosis transmembrane conductance regulator (CFTR). Studies done by Arkwright and his coworkers on animals acclaimed a role for keratinocyte growth factor or transforming growth factor alpha, and a minor role for genes regulating aquaporins (Arkwright et al., 2003). But only few studies have analyzed possible combination of TTN with SP polymorphisms or mutations in a population of newborns. Tutdibi and his colleagues examined the relationship of TTN in Turkish and German population with intron 4 polymorphisms of SP-B or heterozygosity for the SP-B 121ins2 mutation. DNA isolation was performed from 83 healthy subjects and 75 TTN subjects respectively. By genotyping and statistical analysis any significant results were not obtained. In conclusion, intron 4variations are not associated with TTN (Tutdibi et al., 2010). Association of TTN with any candidate genes resulting in the syndrome is not accomplished yet.

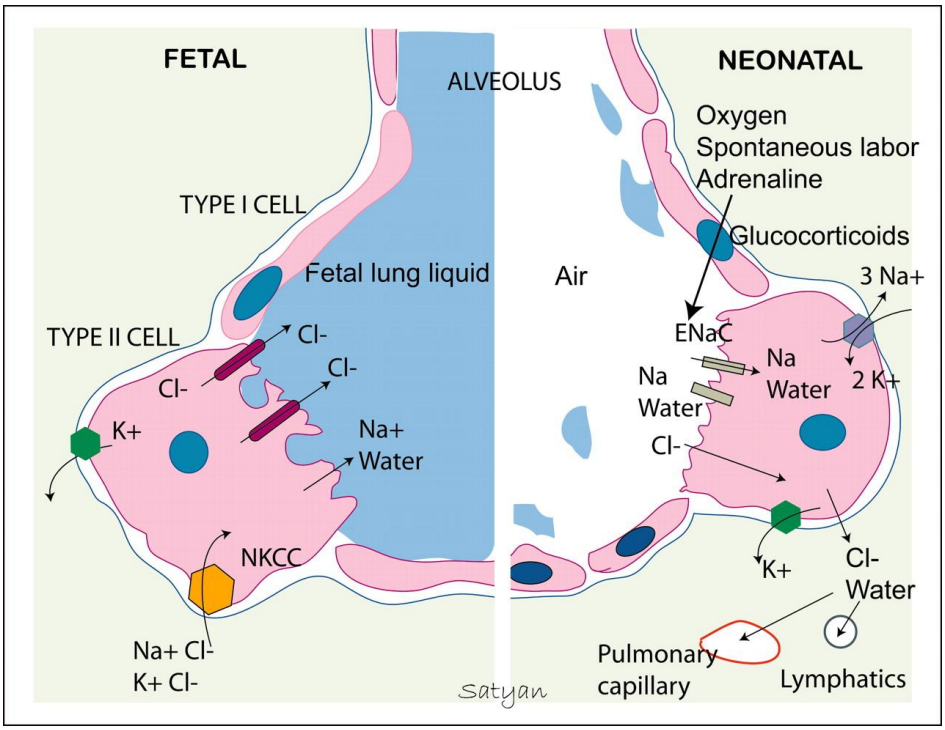


Figure 2.3 Mechanism of Fluid Transport inside lung in fetal and neonatal stages (Review).

2.1.4.2 Heart Failure

Massive treatments of heart failure with ACE inhibitor and beta blockers displays enhancement and subjects with heart failures are cured with both compounds (Exner et al., 2001). In the same study Exner, worked on racial group and noted that black subjects with heart failure had low prediction than white subjects. White subjects with left ventricular function were treated with enalapril represent major decline in the risk of hospitalization but similar result were not obtained among black patient that was not lucid. (Cooper et al., 2006; Flather et al., 2000; Parenica et al., 2010)

2.1.4.3 ACE and Pregnancy

The utilization of ACE inhibitors throughout 2^{nd} -3^{rd} trimester pregnancy periods is not allowed. There is a combination of increased risk of fetopathy is proved, but in the first-trimester consumption of ACE inhibitors had not been connected to a conflicting fetal outcomes. In the result of a study, ACE inhibitors' may be consumed during the first trimester of pregnancy only to threat congenital malformations, however

Copper and his coworkers noted that ACE inhibitors at that period cannot be measured healthy and should be evaded.(Cooper et al., 2006; Flather et al., 2000; Pfeffer et al., 1992)

2.1.4.4 ACE and Alzheimer Disease

Researchers studied the association between the ACE I/D polymorphism and Alzheimer disease in the Japanese population (Hu et al., 1999) and scientists noted that the aggregation of amyloid-beta peptide (A-beta) was prevented by purified specific doses of ACE. Similarly, A-beta cytotoxicity were also highly inhibited by ACE in a rat neural precursor cell line. A 40 amino-acid *A-beta peptide* was cut by ACE from asp7 and ser8, resulted in products of hydrolysis with lower accumulation rate than the original A beta peptide (Hu et al., 2001). Roberds and his group concluded that ACE may modify the susceptibility of AD by degrading A-beta, which prevents the aggregation of amyloid plaques in vivo (Hu et al., 2001; Roberds et al., 2001).

2.1.5 ACE I/D Polymorphism

2.1.5.1 ACE I/D and ACE level in Blood

ACE has an important function in the regulation of homeostasis of the circulatory system, found in the surface of the vascular endothelial as a membrane-bound enzyme or in plasma as a free-synthesized enzyme by circular endothelial cells (François Cambien et al., 1992). Scientists studied inherited similarity for plasma ACE activity in 87 healthy families. The average levels were 34.1, 30.7, and 43.1 in fathers, mothers, and offspring, respectively. Plasma ACE activity was not related with age, height, weight, or blood pressure in parents, but a negative correlation with age was observed in offspring (F Cambien et al., 1988). The conclusion of genetic analysis declared that a major gene may affect the inter-individual variability of plasma ACE while another research studying ACE plasma level suggested that this trait was obviously transmitted dominantly (OKABE et al., 1985). By using all of outcomes from different groups Tiret suggested that the inter-individual variability of plasma ACE was associated with an insertion (I)/deletion (D) polymorphism involving about 250 bp in intron 16 of the ACE gene, the so-called ACE/ID polymorphism. ACE/ID polymorphism was tightly associated with the level of circulating ACE enzyme. The average plasma ACE level of

DD subjects was about twice that of II subjects with ID subjects having intermediate levels (Tiret et al., 1992). Rigat et al. found that ACE insertion matches to a 287bp long Alu repetitive sequence.(Smith et al., 2014; Tiret et al., 1992)

2.1.5.2 ACE I/D level and Blood Pressure

Researchers at the University Oslo did not find any evidence of association of ACE I/D polymorphism with level of systolic or diastolic blood pressure. They examined the combination of ACE I/D polymorphism with systolic and diastolic pressure in two groups of monozygotic twin pairs in which no relation was found regarding to the alteration of blood pressure. In other words, ACEI/D polymorphism has no effect on systolic or diastolic blood pressure (Berge and Berg, 1994). However, Schunkert et al. found that a combination of ACE DD genotype trait with left ventricular hypertrophy, was measured by electrocardiographic standard, may exist. Some epidemiological researches had shown that the deficiency of an increased cardiac workload might be a reason for the left ventricular hypertrophy. D/D genotype was more associated with male subjects than female subjects. When blood pressure measurements were at normal range, D/D genotype could be used as genetic marker in middle-age men with an increased risk of left ventricular hypertrophy. (Schunkert et al., 1994)

2.1.5.3 Smooth muscle proliferation

Cambien et al.(1992), noted that ACE enzyme's another function is to assemble angiotensin I and to catabolize bradykinin while these two peptides were concerned in the modulation of vascular tone and in the proliferation of smooth muscle cells (François Cambien et al., 1992). Soubrier et al. cloned the ACE gene and Tiret et al. confirmed that the main gene effect resulted from an insertion (I)/deletion (D) polymorphism linking about 250 bp situated in intron 16 of the ACE gene, so called ACE/ID polymorphism (Soubrier et al., 1988; Tiret et al., 1992). In addition, Rigat et al. (1990) demonstrated a tight association of ACE with ACE/ID polymorphism. ACE DD polymorphism subjects had two times higher level than ACE II subjects while subjects with ACE ID genotype had intermediate levels. Rigat et al. found that the ACE insertion matches to an Alu repetitive sequence and is 287 bp long (Bonnardeaux et al., 1994; Rigat et al., 1990; Tiret et al., 1992).

2.1.5.4 ACE I/D and Hypoxia relationship

Itoyama et al. worked on severe acute respiratory syndrome (SARS) by ACE I/D genotyping of 44 Vietnamese with SARS and 103 healthy exposed and 50 unexposed controls subjects. SARS subjects were divided into two groups of 22 individual of each according to hypoxemic and non-hypoxemia conditions. As a result, hypoxemic group had higher number of the ACE D allele subjects than non-hypoxemic group whereas another study declared that ACE1 might affect the development of pneumonia in SARS. In conclusion, there was no major diversity among SARS and control subjects (Hamano et al., 2005; Itoyama et al., 2004; Kuba et al., 2005)

2.2 AIM OF THIS STUDY

The aim of this study is to investigate effects of ACE polymorphisms on TTN patients. ACE I/D regions of 35 newborns with TTN and 37 healthy newborns as a control group were analyzed by using PCR and Gene Sequencing technology.

Angiotensin converting enzyme has the most crucial role in Renin- Angiotensin-System, indirectly increases blood pressure resulting in the constriction of blood vessels because converting angiotensin I to angiotensin II can cause to narrow blood vessels (Berge and Berg, 1994; F Cambien et al., 1988). ACE gene is located in 17q arm with size of 22 kb long containing 26 exons and 25 intron and 160 polymorphism which are mostly single nucleotide polymorphisms. (Rigat et al., 1990; Sayed-Tabatabaei et al., 2006). Rigat and his colleagues discovered a polymorphism involving the presence (insertion, I) and absence (deletion, D) of 287 base pair DNA sequence in 16^{th} intron (NCBI SNP ID: rs1799752). The impact of ACE on coronary heart and stroke, lung, kidney and nephropathy is investigated recently (Hou et al, 2013). The effect of ACE I/D polymorphism on sepsis patients is investigated (Baier et al., 2004; Hou et al., 2013; John Baier et al., 2005). The objective of this study is to analyze hypothesis of ACE I/D whether it is associated with TTN patients by using sequencing of I/D region of ACE gene and design primer for PCR amplification. In this study, samples from 35 infants with TTN and 37 infants as a control group are studied for discover the association between ACE polymorphism and serum ACE activity among TTN diagnosed patients.

Table 2.1 List of diseases caused by a mutation in Angiotensin Converting Enzyme (OMIM, 2009).

Number	Phenotype	Mutation	dbSNP
.0001	ANGIOTENSIN I-CONVERTING ENZYME INSERTION/DELETION POLYMORPHISM MYOCARDIAL INFARCTION, SUSCEPTIBILITY TO, INCLUDED MICROVASCULAR COMPLICATIONS OF DIABETES, SUSCEPTIBILITY TO, 3, INCLUDED STROKE, HEMORRHAGIC, SUSCEPTIBILITY TO, INCLUDED STROKE, ISCHEMIC, SUSCEPTIBILITY TO, INCLUDED SEVERE ACUTE RESPIRATORY SYNDROME, PROGRESSION OF, INCLUDED	ACE, INS/DEL	rs4340
.002	ANGIOTENSIN I-CONVERTING ENZYME, BENIGN SERUM INCREASE	ACE, PRO1199LEU	rs121912703
.003	RENAL TUBULAR DYSGENESIS	ACE, TYR266TER	rs121912704
.004	RENAL TUBULAR DYSGENESIS	ACE, 4-BP DEL, 1319TGGA	rs387906576
.005	RENAL TUBULAR DYSGENESIS	ACE, 4-BP DEL, 1319TGGA	rs397514688
.006	RENAL TUBULAR DYSGENESIS	ACE, ARG791TER	rs397514689

CHAPTER 3

MATERIAL AND METHODS

3.1 SUBJECTS

37 healthy newborns (23 female, 14 male) and 35 (17 female, 18 male) TTN patients blood were selected to determine ACE genotype by PCR and further DNA sequencing used to identify different polymorphisms. All of subjects' gestation age was around 36-40 weeks. ACE enzyme activity level was measured from their sera in the Biochemical Laboratory of Sema Hospital. Serum was assessed by a commercial kit for spectrophotometric determination of ACE, based on the method of Cushman et al. (1999) bought from Ben Biochemical Enterprise (USA).

3.1.1 ACE Genotype Determination

Phusion Blood Direct PCR Master Mix (cat# 175 S Thermo Scientific, USA) used for PCR Analysis. Primarily samples (blood) were diluted 1:10. Then, the following primers were used in the assay: forward 5'- CTG GAG ACC ACT CCC ATC CTT TCT –3', reverse 5'- GAT GTG GCC ATC ACA TTC GTC AGA T-3'. The PCR conditions were as follow: the lysis of blood cells at 98 °C for 5 minutes, the denaturation of amplicons at 98 °C for 1 second, annealing at 61 °C for 5 second, the extension at 72°C for 1 second, and final extension at 72 °C for 1 minute. In 20µl of PCR volume, the following components were added with final concentrations of MgCl2 1,5mM, EDTA 2mM, DMSO 5%. PCR products were separated on 2% agarose gel containing 0,5 mg/ml Ethidium bromide. After gel electrophoresis, the bands were visualized under UV Illuminator. PCR product is a 190 bp fragments of the D genotype, and 490 bp is presence of I genotype.(Rigat et al., 1990)

3.2 DATA ANALYSIS

During data analysis, genotypes and ACE level among healthy and TTN subjects were compared and their standard deviation and average were calculated. Chi-square was used to assess normal distributed variables, population frequency of II, DD, and ID genotypes were calculated (Shanmugam et al., 1993).

3.2.1 DNA sequencing and multiple alignment of primers

25 µl of PCR product of patients with highest ACE activity and DD genotypes and healthy samples with lowest ACE activity and II genotypes were sequenced by Sanger sequencing at MediaSantek Laboratory (Turkey). By Chromas Lite, CLC Manin Workbench7.6.1 and multiple alignments were done.

3.2.2 ACE gene and all other polymorphisms

Primarily ACE gene length was checked in NCBI (National Center of Biotechnology) database. The length of the gene is about ~22.000 base pair. Then ENSEMBL database was used to acquire variations. Species Homo sapiens and by confirmation of page, to represent sequence with variation with links it is possible to find out ACE I /D polymorphism with rs 1799752. By determining the sequence it is possible to design primer by different tools such as Primer3, Primer Blast (Sayed-Tabatabaei et al., 2006).

The primers used in this study had been used widely used before. Firstly by Rigat, Shanmugam used same primers to examine ACE I/D polymorphism and later many scientists worked on same primers. (Cambien et al., 1992; Shanmugam et al., 1993)

Table 3.1 Methods, marker description, genomic location, primer sequences, and PCR-RFLP details used to genotype the ACE I/D and other SNPs (Glenn et al., 2009).

Method	Marker description	Genomic location (bp)	Primer	Primer sequences 5'-3'(F/R)	Annealing Temp.(°C)	Restriction Enzymes	Fragment Length	Alleles(bp) [1,2]
PCR method	ACE *I/D* intronic	~58,919,623	ACE-F ACE-R	CTG GAG ACC ACT CCC ATC CTT TCT GAT GTG GCC ATC ACA TTC GTC AGA T	59	None	490/190	(490)/(190)
Insertion-specific PCR	Insertion-specific	~58,919,646	INSERT-F ACE-R	TTT GAG ACG GAG TCT CGC TC GAT GTG GCC ATC ACA TTC GTC AGA T	59	None	408/-	(408)/(-)
Deletion-specific PCR	Deletion specific	~58,919,613	DELETE-F DELETE-R	CCT GCT GCC TAT ACA GTC ACT T TCT GGT AGG GGT TTG AAT GC	64	None	129/-	(129)/(-)

3.2.3 Statistical analysis

3.2.3.1 Chi- Square test

A chi-squared test (χ^2) is used in order to find any statistical difference between observed sample and expected sample number. The sum of squared difference between expected and observed sample number per expected number gives a date which might be used to identify the null hypothesis. Chi square test distribution is used to make assumptions of independent normally distributed data, which is valid in many cases due to the central limit theorem. A chi-squared test can then be used to reject the hypothesis that the data are independent (Greenwood, 1996).

$$\chi^2 = \sum \frac{(O-E)^2}{E} \tag{3.1}$$

O states for observed samples, E expected samples.

CHAPTER 4

RESULTS

4.1 ACE GENE

4.1.1 ACE I/D polymorphism location

ACE I/D polymorphism is located in 16th intron of ACE I/D gene whose sequence was acquired from ENSEMBL data base, as depicted in figure below (each color represents different polymorphisms). 3' UTR states for region in 3' end of RNA with untranslated region is colored in light blue, flagged variants are suspected variants that failed ENSEMBL quality control checks, synonymous variants missense mutations, intronic as name implies intron polymorphism, and stop gained variant are sequence that at least one base codon is changed, resulting in a premature stop codon, leading to a shortened transcript respectively. Next step was to identify ACE I/D polymorphism in the whole sequence. In Figure 4.2 the rs179952 polymorphism in other word ACE I/D polymorphism, 287 base pair of Alu repeat were acquired and by using BLASTN tool of same Database (NCBI), the position of polymorphism inside gene was checked. In addition, primers used previously for ACE I/D polymorphism genotyping was also analyzed by Primer Blast tool and 100% of match was observed.

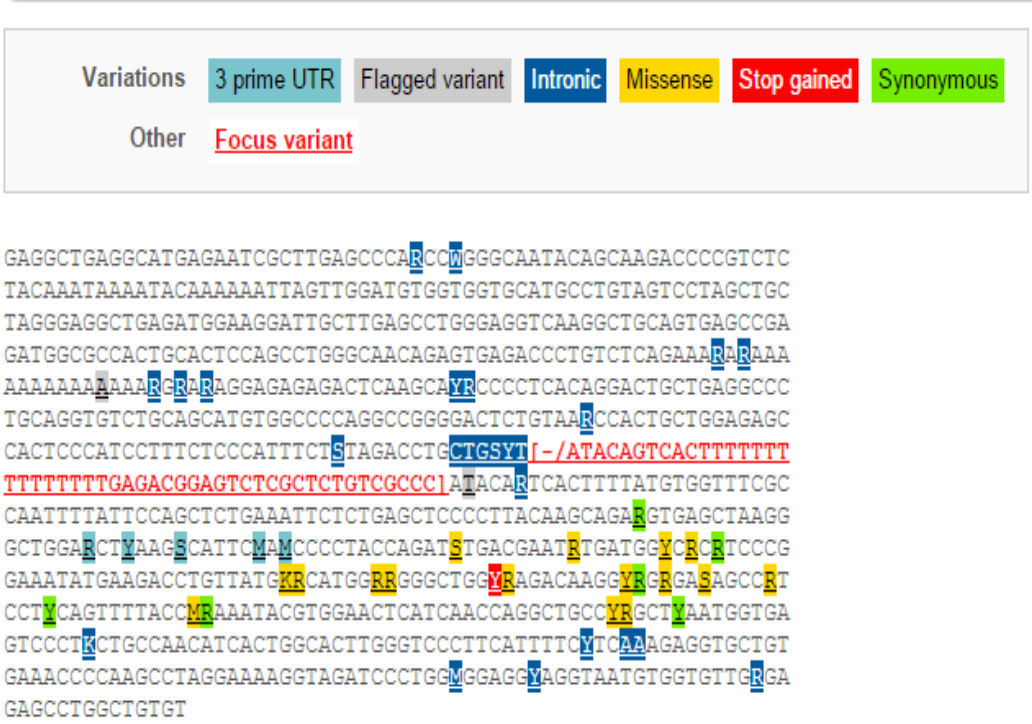


Figure 4.1 16th intron of ACE gene, ACE I/D polymorphism was located in red sequence (ESEMBL).

BLAST/BLAT Genomic Sequence

BLAST/BLAT type	BLASTN
Query location	Query_1 59 to 290 (+)
Database location	3 123517975 to 123518206 (-)
Genomic location	3 123517975 to 123518206 (-)
Alignment score	460
E-value	5e-126
Alignment length	232
Percentage identity	100.00

HSP Location of selected alignment

```
>chromosome:GRCh38:3:123517675:123518506:-1
123518506 TTTAGAGTTTTCAAATATGTTGCATGACATTTGTGTAGCACTTTACAATCTGTAGCAGAG 123518447
123518446 GCTATCTACTGGCGGCAGAGGTCTGTCCCACAGGATTTTAAAAGAACATGGAAGAAAAGC 123518387
123518386 ATGGTGACTGGAGGGTGCATGCCATCTCACACAAGCAGTCAGTGTGCAGCTCAGCTAGAG 123518327
123518326 ATTGCCAGACAGATTCAGTAGTGCAAAATCTAATTTTTTTGAGAAAAGGCAGAAATCTAG 123518267
123518266 ATTTTTAAAATGAGAAATCCCCTAATTTTTAAAAACTGATTCAAATATTTTTTTTTTTT 123518207
123518206 TTTTTTTTTTTTTTTTGAGACGGAGTCTCGCTCTGTCGCCCAGGCTGGAGTGCAGTGGCGG 123518147
123518146 GATCTCGGCTCACTGCAAGCTCCGCCTCCCGGGTTCACGCCATTCTCCTGCCTCAGCCTC 123518087
123518086 CCAAGTAGCTGGGACCACAGGCGCCCGCCACTACGCCCGGCTAATTTTTTGTATTTTTAG 123518027
123518026 TAGAGACGGGGTTTCACCGTTTTAGCCGGGATGGTCTCGATCTCCTGACCTCGTGATCCG 123517967
123517966 CCCGCCTCGGCCTCCCAAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCCAAA 123517907
123517906 TATTTTTAAAGCACTGTACAAGCCCAAACAGAACATCTATGGTCAGGGACCGTAGGTCAC 123517847
123517846 CATTTTGATATTTCTGGTCCAAGAGATGGTGTTCATAGCTTTTTTTCCCCACCTTTTGAA 123517787
123517786 ATTTTTGCAATTATTACACTGTCATAGAACAGAAAGAACCATGACATTTCACAAAATATA 123517727
123517726 TTGCCAGTTTCATGTTCTTTTGACATTTCAGTTACCTTTCTGTCAGTGGTAA         123517675
```

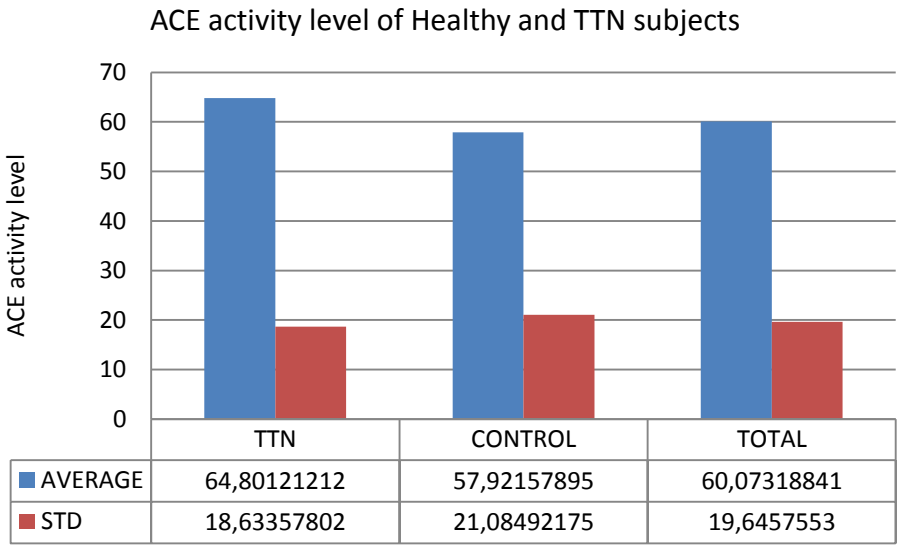
Figure 4.2 ACE I/D polymorphism blast with whole genome, 100% identity was found. Red sequence represents ACE I/D polymorphism (Al-Jafari, 2014).

4.2 ACE LEVEL OF HEALTHY AND TTN PATIENTS

The level of ACE in patients ‘sera were calculated by spectrophotometer analysis at Sema Hospital. The samples for ACE activity were obtained from serum. Serum was assessed by a commercial kit for spectrophotometric determination of ACE, based on the method of Cushman et al. (1999), BEN Biochemical Enterprise (ref # ACE8865). Normal ACE activity level range is determined as 0-52 U/L, and any activity level

higher than this ranged count as high activity level. According to chart below (Table 4.1) average level of ACE in control group is 57.9 U/L, while ACE level in TTN subjects is 64,8 U/L respectively.

Table 4.1 ACE activity level of healthy and TTN subjects, average and standard deviation. Unit: U/L



	TTN	CONTROL	TOTAL
AVERAGE	64,80121212	57,92157895	60,07318841
STD	18,63357802	21,08492175	19,6457553

ACE level amoung subjects were categorized according to genders in TTN samples and control group. Statistically average level of ACE amoung TTN male subjects and healthy subjects were calculated as 65.5, and 59,8, while same condition amoung female subjects were determined as 63,1 and 59,1 respectively (Table 4.2).

Table 4.2 Average ACE activity level in male and female subjects (Unit: U/L).

	Male	Female	Av Male	Av Female
TTN	18	17	65,55	63,17
Healthy	14	23	59,8	58,1

4.3 ACE I/D GENOTYPING BY PCR

Samples from both groups were genotyped by Phusion Blood Direct PCR kit (cat#F-175L, Themo Scientific, Sweden). After gel illumination by UV illuminator, by O' Gene Ruler Experess DNA Ladder length of PCR products is determined. I: stated for insertion while D stated for deletion polymorphism (Fig. 4.3). Difference between I and D genotype is about 300 base pair sequence(Rigat et al., 1990; Tiret et al., 1992).

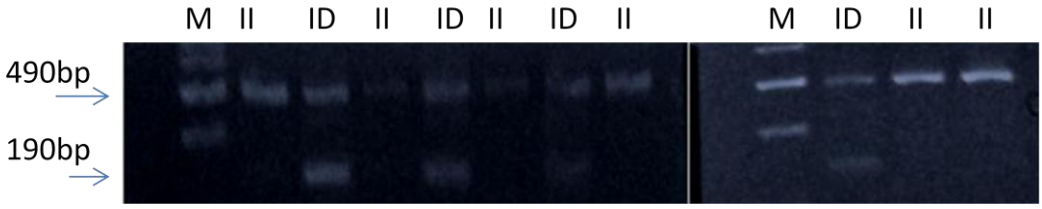


Figure 4.3 2% Agarose gel run at 85V 13 mA for 50 minutes. M demonstrates marker, 490bp insertion and 190 bp bands represents deletions

4.4 STATISTICAL ANALYSIS

4.4.1 Mean value, Chi square test

Statistical values of ACE I/D genotypes from examined samples were calculated, according to gender and different traits. Numbers of subjects from both groups are listed in table below (Table 4.3). The prevalence of II genotype numbers in TTN subjects and

control group are 20% (7 out of 35) and 18% (7 out of 37) respectively while DD genotype prevalence for TTN and control group is 31% (11 out of 35) and 37% (14 out of 37). In addition, the ratio of ID genotypes in TTN samples and control group is 48% (17 out of 35) and 43% (16 out of 37) respectively. Statistical analysis was done on those samples in two condition levels: ACE activity and the prevalence of genotypes. The level of ACE was categorized in two groups: low activity and high activity regarding the reference level whereas the genotypes were categorized as: II (double insertion), ID (heterozygous) and DD (double deletion). The frequency of male and female subjects from both groups is depicted in table below (Table 4.3). Male TTN subjects' and male control subjects' frequencies are 51.4% and 37.8% while female ratio in TTN subjects and control group is 48.5% and 62.1% respectively. TTN subjects with high ACE activity level are 77.1% while in control group it is 51.3%. Hereditarily, the low activity level of ACE for TTN positive and control groups is 22.8% and 48.6% respectively. Table 4.4 summarizes whole results about ACE genotypes in both experimental and control groups (Table 4.4).

Table 4.3 Frequency of gender and ACE Activity level, in TTN and control subjects Frequency percentiles are in brackets.

	Gender		**Level Of ACE**		
	Male	Female	High	Norm	Total
TTN	18(51,4)	17(48,57)	27(77,1)	8(22,8)	35
Control	14(37,8)	23(62,1)	19(51,3)	18(48,6)	37

Table 4.4 Genotype frequency of TTN and control group (frequency percentiles).

	GENOTYPE			
	II	**DD**	**ID**	**Total**
TTN	7(20)	11(31,4)	17(48,5)	35
Control	7(18,9)	14(37,8)	16(43,2)	37

4.4.1.1 Chi square Test

In table below, ACE level of TTN and control group is examined. According to reference level of ACE activity, all subject ACE activity was divided into two groups as high and normal level. The hypothesis, in which both groups are from the same population with high and low ACE activity, was refused according to Chi square test results. In contrast to ACE activity level, ACE I/D genotypes' Chi square value is 0.43, means that the null hypothesis that states this two subject groups are from the same population is accepted (P>0.05)

Table 4.5 Chi square test for TTN and control samples according to genotype and ACE activity level P>0,05.

Samples and Conditions	χ^2	**Critical value**	**Comment**
Chi square test for TTN/control samples according to ACE I/D genotype	0.43	5.991	Hypothesis Accepted
Chi square test for TTN/control samples according to ACE activity level	4,13	3.841	Hypothesis Refused

CHAPTER 5

DISSCUSSION AND CONCLUSION

The ACE I/D polymorphism is one of the most widely studied polymorphism in ACE gene. The human ACE gene is located on Chromosome 17 (17q23) and contains a polymorphism consisting of either the presence (insertion, I) or absence (deletion, D) of a 287 bp Alu repeat in intron 16. Although the disease status can influence serum ACE activity, that depended on the ACE genotype (Shanmugam, 1993). The D genotype is combined with the elevated level of ACE activity in tissues and circulation system, which counts for about 47% of the intra individual variation in plasma ACE activity. In spite of many studies in various of disease states, very few data is known of ACE role in the result of TTN (Glenn et al., 2009; John Baier et al., 2005; Kiss et al., 2014; Satar et al., 2012; Sayed-Tabatabaei et al., 2006). There might be several reasons and relations between TTN and ACE I/D polymorphism one of the potential machinery by which D allele of ACE may persuade the threat of respiratory distress syndrome may be through the generation of higher levels of ACE and its product A-II, which may worsen lung inflammation and hinder with pulmonary vascular remodeling (Kazzi and Quasney, 2005). ACE has important function in inflammatory process, which the ACE inhibitors repress cytokine production in activated mononuclear cells (Baier et al., 2004). The ACE activity is important not only for the pulmonary endothelium, but also in respiratory epithelium (Kasap et al., 2008). The tissue locations that synthesize ACE have displays crucial function in monitoring of the inflammatory response. Unfortunately, researchers that studied the role of genetic factors in TTN newborns are restricted (Constantinescu et al., 1998). The ACE I/D polymorphism frequency for TTN positive group were not different from that for control group. In this study, control group and TTN positive group frequencies are 20% - 18.9% for II, 32%-38% for DD, 48%-43% for ID respectively. Large number of samples might be required to get a more

reliable statistical data. Hussein et al. (2014) found a relation between ACE I/D polymorphism and RDS disease after a study in which they examined 120 premature neonates with RDS and 120 premature control group , genotypic frequency difference between RDS and control group significant, so association of RDS and DD genotype demonstrated-(Hussein et al., 2014). In addition, Yimenincioglu et al (2011) confirmed that high risk of DD genotype is associated with RDS after a study with 101 controls and 100 RDS patient. On the other hand, Satar and his group could not discover any variations in genotype and ACE activity among control and RDS group with subject size of 20 for RDS, 20 for control and 20 for TTN patients. The level of ACE activity importantly varies among subjects who might be a genetically defined.

In our study, serum ACE activity is the highest in DD group as in literature. TTN is a condition characterized by tachypnea that arises shortly after birth but recovers within two to five days. Many different factors may influence TTN such as labor, pulmonary epithelium changes from a chloride- secreting membrane to a sodium absorbing membrane with reversal of the direction of flow of lung fluid. In addition to sodium chloride channels' hormones such as epinephrine, ADH, glucocorticoids and thyroid hormones and other factors directly or indirectly influence fluid absorption interact to increase the Na+ transporting capacity of the epithelium and augments gene expression of epithelial Na+ channel (ENaC). TTN is a multifactorial disease and a plenty of different factors may induce and increase the risk of the disease. As the ACE activity is increased in DD alleles and it might play a role in lung inflammation and interact with pulmonary vascular remodeling (Mehri et al., 2010). TTN may also arise in neonates born after C/S delivery because lung fluid absorption to interstitial fluid has not been started. Since ACE have essential function in inflammatory response, ACE activity of inter-individual genetic variations may be changed in the result of TTN (Mehri et al., 2010). In this study, serum ACE activity is the highest in DD group as in literature, and by examining on chi square test TTN and control group ACE activity had high significance, meaning that ACE activity might be used as marker for ACE patients. Our study could not find any difference in ACE I/D polymorphism I/D frequencies between two subject groups.

There are few articles published about ACE I/D polymorphism and TTN syndrome and in none of articles this result found. Number of subjects in Satar, and in

this study was not as larger as in Hussein, and Yemenicioglu, studies so statistically association of ACE I/D polymorphism with TTN patients was not discovered.

As a conclusion there is an association of ACE activity with TTN but not ACE I/D polymorphism with TTN consequently. Different method might be used to acquire the effect of ACE I/D polymorphism and TTN: 1) to increase the number of subjects in both groups, 2) DNA sequencing, Reverse Transcriptase PCR, because the as a result of insertion polymorphism sequence have 24 nucleotide of mRNA which might affect the rate of angiotensin converting enzyme production.

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