ASSOCIATIONS OF ADAM 33 AND eNOS GENE POLYMORPHISIMS IN BRONCHOPULMONARY DYSPLASIA

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ASSOCIATIONS OF ADAM 33 AND eNOS GENE POLYMORPHISIMS IN BRONCHOPULMONARY DYSPLASIA

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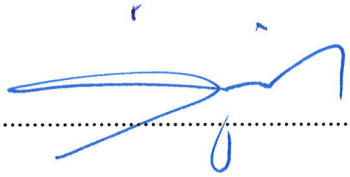
Assist. Prof. E. Çiğdem Kaspar ..... 

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ABSTRACT

ASSOCIATIONS OF ADAM 33 AND eNOS GENE POLYMORPHISIMS IN BRONCHOPULMONARY DYSPLASIA

Bronchopulmonary dysplasia (BPD) is a chronic respiratory disease which can cause perinatal/neonatal lung injury and serious morbidity in premature infants that are born before 37 weeks of gestational age (GA). Small genetic changes of DNA that vary in only one base are known as Single Nucleotide Polymorphisms (SNPs). This type of polymorphism occurs when a single nucleotide (A, T, C, or G) in a specific position in the genome sequence is altered. eNOS (*rs179983*) is an important mediator of physiologic processes in the airways and ADAM33 (chromosome 20, a disintegrin and metalloproteinase domain 33) (*rs 2280090*) is the first gene identified in asthma by positional cloning. The aim of this study was to investigate possible associations between ADAM 33 and eNOS gene polymorphisms in premature infants, as a risk factors for development of BPD. One hundred and twenty two blood samples DNA isolation was carried out using the PureLink^{TM} Genomic DNA Mini Kit and the concentration of the DNA samples was measured by nanophotometer Implen P 300. For the SNP analysis of ADAM33 (*rs 2280090*) and eNOS (*rs1799983*) optimized primers (TaqMan SNP Assays) were used. Real Time Polymerase Chain Reaction (QRT-PCR) was carried out in a CFX96 thermocycler. Chi-square $\chi 2$ test, Fisher's exact test, the odds ratio and confidence intervals were calculated for the comparisons of allelic and genotype frequencies. The results indicated that the AA genotype (p=0,006*; OR 2.54 95% CI 1.179-5.494) of ADAM 33 gene and GG genotype (p=0.000*; OR 1.89, 95% CI 1.514-2.148) of the eNOS gene were risk factors for developing BPD.

ÖZET

ADAM 33 VE eNOS GEN POLİMORFİZİMLERİNİN BRONKOPULMONER DİSPLAZİ İLE İLİŞKİSİ

Bronkopulmoner displazi (BPD) 37 haftalık gestasyonel yaş öncesinde doğmuş olan prematüre bebeklerde perinatal/neonatal akciğer hasarına ve ciddi morbiditeye yol açabilen bir kronik respiratuar hastalıktır. Yalnızca tek bazda değişimin görüldüğü, DNA'daki küçük genetik değişiklikler "Tek Nükleotid Polimorfizmi (SNP)" olarak bilinir. Bu tip polimorfizmler genom sekansındaki belirli bir yerdeki tek bir nükleotid (A, T, C veya G) değiştiği zaman ortaya çıkar. eNOS (*rs179983*) hava yollarında gerçekleşen fizyolojik süreçlerde rol alan önemli bir mediyatördür. ADAM33 (kromozom 20, bir disintegrin ve metaloproteinaz domaini 33) (*rs 2280090*) ise pozisyonel klonlama yöntemiyle astımda tanımlanmış ilk gendir. Bu çalışmanın amacı, premature bebeklerde BPD'nin gelişimine katkı sağlayabilecek risk faktörleri olarak ADAM33 ve eNOS gen polimorfizmleri arasında gerçekleşebilecek olası ilişkileri araştırmaktı. Yüz yirmiiki kan örneği Kanuni Sultan Süleyman, Göztepe ve Ümraniye Hastanelerinden toplandı. DNA izolasyonu PureLink^{TM} Genomik DNA Mini Kit kullanılarak yapıldı ve DNA örneklerinin konsantrasyonları Implen P 300 nanofotometre kullanılarak ölçüldü. ADAM33 (*rs 2280090*) ve eNOS (*rs1799983*)'un SNP analizleri için optimize primerler (TaqMan SNP Assays) kullanıldı. Real Time Polimeraz Zincir Reaksiyonu (QRT-PCR) CFX96 termocycler kullanılarak gerçekleştirildi. Ki-kare $\chi 2$ testi, Fisher kesin olasılık testi, göreceli olasılıklar oranı ve güvenilirlik aralığı alelik ve genotip frekanslarının karşılaştırılmaları için hesaplandı. Sonuçlar ADAM33 geninin AA genotipinin (p=0,006*; OR 2.54 %95 CI 1.179-5.494) ve eNOS'un GG genotipinin (p=0.000*; OR 1.89, %95 CI 1.514-2.148) BPD geliştirmek konusunda risk faktörleri olduğunu gösterdi.

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LIST OF SYMBOLS / ABBREVIATIONS

A	Adenine
ACS	Antenatal Corticosteroid
AD	Atopic Dermatitis
ADAM 33	A Disintegrin and Metalloprotease Domain 33
AOP	Apnea of Prematurity
BAL	Bronchoalveolar Lavage
BPD	Bronchopulmonary Dysplasia
C	Cytosine
Ca^{2+}	Calcium
Calm	Calmodulin
CA	Chorioamnionitis
CI	Confidence Interval
CPAP	Continuous Positive Airway Pressure
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ELBW	Extremely Low Birth Weight Infants
eNOS	Endothelial Cell Nitric Oxide Synthase
FAD	Flavin Adenine Dinucleotide
FAM	6-carboxyfluorescein
G	Guanine
GA	Gestational Age
GST	Glutathione S Tranferases
HC	Healthy Control
HCl	Hydrochloric Acid
IFN- γ	Interferon Gamma
IGF	Insulin Like Growth Factor
IL	Interleukin VLBW Very Low Birth Weight
MGB	Minor Groove Binder
MMPs	Matrix Metalloproteinases
MV	Mechanical Ventilation

NADPH	Nicotinamide Adenine Dinucleotide
NIH	National Institute of Health
NO	Nitric Oxide
NOS	NO Synthase
OR	Odds Ratio
O_2	Oxygen
PCR	Polymerase Chain Reaction
PDA	Patent Ductus Arteriosus
PMA	Postmenstrual Age
RDS	Respiratory Distress Syndrome
SE	Sepsis
SNP	Single Nucleotide Polymorphisms
SPOCK 2	Sparc/Osteonectin, CWCV, and Kazal-like domains proteoglycan 2
T	Thymine
TE	Tris-EDTA
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
Tot. O_2	Total Oxygen
VEGF	Vascular Endothelial Growth Factor
VIC	4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein

1. INTRODUCTION

1.1. WHAT IS BRONCHOPULMONARY DYSPLASIA?

Bronchopulmonary dysplasia (BPD) is a chronic respiratory disease which can cause perinatal/neonatal lung injury and serious morbidity in premature infants, who are born before 37 weeks of gestational age (GA). It affects approximately 10 to 40% of low birth weight infants (VLBW, less than or equal to 1,500 grams) and extremely low birth weight infants (ELBW, less than or equal to 1,000 grams). Nearly two-thirds of infants who develop BPD are ELBW and are less than 28 weeks' gestation at birth [1-4].

BPD was first described by Northway et al, in 1967, as a lung injury in preterm infants resulting from oxygen and mechanical ventilation [5]. This was observed in a series of 32 new born infants with severe hyaline membrane disease or severe respiratory distress syndrome (RDS) [5].

There are three forms of BPD; mild, moderate and severe BPD. According tothe National Institute of Health (NIH) consensus [6], the classification of the severity of BPD dependson the need for oxygen (O_2) over 28 days and 36 weeks postmenstrual age (PMA) for preterm infants with a GA less than 32 weeks (Table 1.1) [6].

Table 1.1. Definition of BPD by NIH [7]

	Gestational Age	
Time point of assesment	<32 weeks	>32 weeks
	36 weeks post-menstrual age or discharge*	>28 days but < 56 days postnatal age or discharge*
Treatment with oxygen	>21% for at least 28 days	>21% days for at least 28 days
Bronchopulmonary Dysplasia		
Mild	Breathing room air at 36 weeks post-menstrual age,or discharge*	Breathing room air by 56 days postnatal age or discharge*
Moderate	Need for <30% O_2 at 36 weeks post-menstrual age, or discharge*	Need for <30% O_2 to 56 days postnatal age or discharge*
Severe	Need for >30 % O_2, with or without positive pressure ventilation or continuous positive pressure at 36 weeks post-mentrual age, or discharge*	Need for >30 % O_2 with or without positive pressure ventilation or continuous positive pressure at 56 days postnatal age or discharge*
* Whichever comes first.		

Northway and his colleagues'definition known as the classical or old BPD is classified as severe BPD. The severe form of BPD is mostly seen in infants who received aggressive ventilation and have had a prolonged exposure to high inspired oxygen concentrations. However with the use of less aggressive ventilation, antenatal corticosteroids andthe use of postnatal surfactants, this old form of BPD has become uncommon and has been replaced by the milder form which occurs in smaller infants who only have mild or no initial respiratory distress [8-11].

Old BPD is characterized by many severe morphologic changes such as; extensive inflammatory and fibrotic changes in the airway and lung parenchyma, atelectasis which is

caused by a blockage of the air passages (bronchus or bronchioles) or by pressure on the outside of the lung and emphysema, which causes damages to the alveoli and smooth muscle hypertrophy in the airways [8,9]. These changes are associated with severe respiratory failure, airway obstruction and pulmonary hypertension [10-14].

The new BPD is referred to as, those premature infants that have only mild initial respiratory failure, spend shorter times with respiratory support and receive ventilation with low pressure and oxygen concentration and also less severe RDS [15]. The milder form of BPD is characterized by inflammatory responses, increased lung fluid, a striking decrease in alveolar septation and impaired vascular development [16-21]. There are also some pathological differences between the old and new form of BPD, surfactant treatment is the main difference between these types of BPD. Table 1.2 represents before and after surfactant therapy. In the new form, rare airway epithelial lesions, fewer, larger and simplified alveoli, rare fibroproliferative changes and mild airway smooth muscle thickening are seen. In the old form, severe airway epithelial lesions, marked airway smooth muscle hyperplasia which causes tumours, decreased alveolarization and surface area, extensive fibroproliferation are observed.

Table 1.2.Differences in pathologic features of old and new BPD [22]

Pre-surfactant (‘‘old ’’)	**Post-surfactant (‘‘new’’)**
Alternating atelectasis with hyperinflation	Less regional heterogeneity of lung disease
Severe airway epithelial lesions (eg. Hyperplasia, squamous metaplasia)	Rare airway epithelial lesions
Marked airway smooth muscle hyperplasia	Mild airway smooth muscle thickening
Extensive, diffuse fibroproliferation	Rare fibroproliferative changes
Hypertensive remodelling of pulmonary arteries	Fewer arteries but ‘‘dysmorphic’’
Decreased alveolarisation and surface area	Fewer, larger and simplified alveoli

1.1.1. Symptoms of BPD

Infants with BPD have symptoms like rapid and shallow breathing, coughing, wheezing or noisy breathing, blue colouring around the lips and nails, poor posture of the neck, shoulders and trunk [23,24].

Abnormalities in lung organogenesis, alveolar septation and vascular development in the distal lung are the other symptoms which can be visualized by a chest radiograph [25,26]. Figure 1.1 shows a chest radiograph of an infant of 24 weeks GA, with BPD showing abnormalities in the lung.

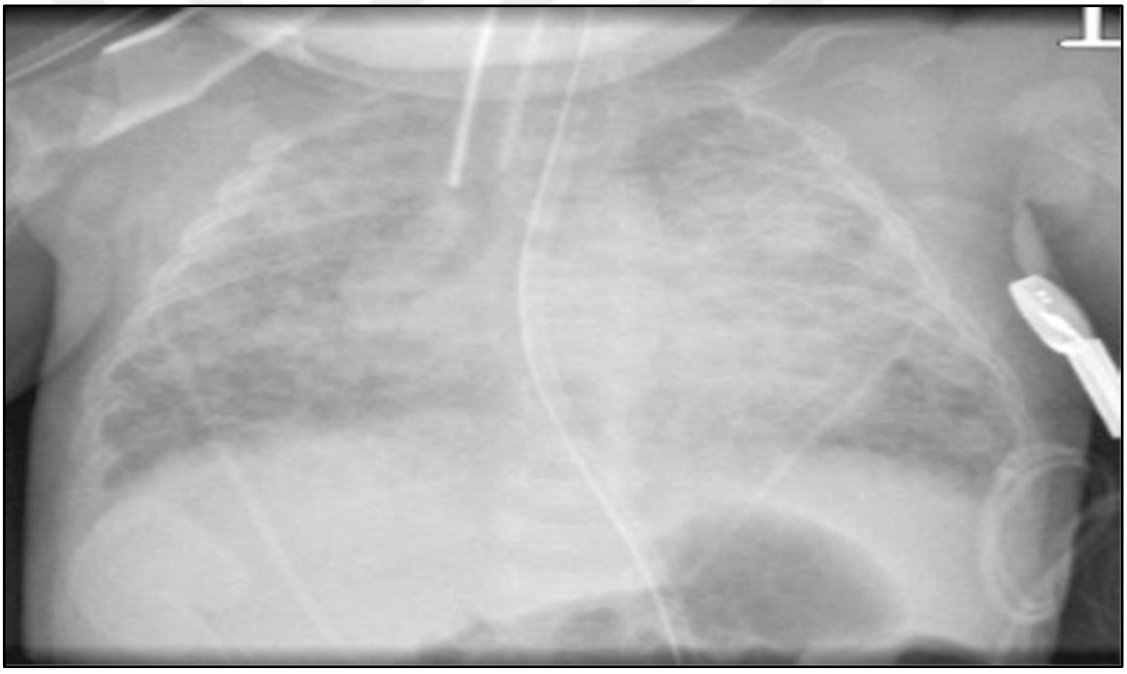

Figure 1.1. Chest radiograph of a 3-month old with BPD [27]

1.2. DIAGNOSIS OF BPD

1.2.1. Oxygen Requirement

The diagnosis of BPD is currently based on the need for supplemental oxygen for at least 28 days after birth, and its severity is classified according to the respiratory support required at 36 postmenstrual weeks [28].

1.2.2. Imaging Techniques

Aggressive ventilation, having long term exposure to high inspired oxygen concentrations causes characteristic changes in lungs and these can be detected by chest radiographs.
Chest radiography determines the severity of bronchopulmonary dysplasia by taking radiographs of the organs inside the chest, such as the heart and lungs. In severe cases of BPD, this test may show large areas of air and signs that the lungs are inflamed or infected. A chest x ray can also detect problems such as a collapsed lung [28].

1.3. RISK FACTORS FOR DEVELOPING BPD

1.3.1. Gestational Age

Gestational age (GA) is one of the most important risk factors for developing BPD. GA refers to the age of infants after birth. If the GA is less than 37 weeks, infants are said to be premature. Premature infants can not breath without ventilation systems because of their immature lungs and as a result ventilation strategies can cause BPD [1].

1.3.2. Birth Weight

Birth Weight (BW) is another important risk factor for BPD. BW refers to the body weight of an infant at its birth. GA is an important criteria for BW because when the GA is less than 37 weeks the BW will be low. If the BW is less than or equal to 1,500 grams it is referred to as very low birth weight (VLBW) and if BW less than or equal to 1,000 grams it referred as extremely low birth weight (ELBW) [4].

1.3.3. Mechanical Ventilation

Mechanical ventilation is necessary for the survival of many preterm infants, but it can injure the lung tissue and is a risk factor for the development of BPD. Mechanical ventilation has an association with risk of barotrauma which is physical damage to body tissues caused by a difference in pressure [29].

1.3.4. Chorioamnionitis

Chorioamnionitis (CA), maternal infection is the most important cause of preterm birth, and severe chorioamnionitis is seen most frequently in preterm deliveries before 30 weeks of GA. CA has been associated with an increased risk of developing BPD. The levels of several inflammatory cytokines have been found to be elevated in fetal cord blood and in the amniotic fluid that is delivered from the mothers to their infants [30,31].

1.3.5. Patent Ductus Arteriosus

Patent Ductus Arteriosus (PDA) is a heart problem due to abnormal blood flow to the heart [32]. It has been shown that infants with RDS, who received a greater fluid intake or did not have diuresis in early life, had a higher risk of developing PDA and therefore BPD [33,36]. There is a strong association between PDA and BPD because, increased pulmonary blood flow can induce neutrophil margination and activation in the lungs and contribute to the progression of the inflammatory cascade [37-39].

1.3.6. Oxygen

Pulmonary surfactant is required for normal lung function throughout post-natal life. Surfactant is a surface-active lipoprotein complex found in the fluid lining the alveolar surface of the lungs which reduces the tension in the alveoli [40]. As a result of surfactant deficiency and/or inadequate respiratory drive, the majority of ELBW infants need supplemental oxygen and assisted ventilation soon after birth to achieve adequate gas exchange. Surfactant therapy has been seen to reduce mortality from respiratory distress syndrome but has not be seen to reduce the risk for BPD [41] .

1.4. STRATEGIES FOR THE PREVENTION OF BPD

BPD is the most common and most studied complication of prematurity. The best-studied strategies for preventing BPD are limiting mechanical injury from assisted ventilation and pharmacologic approaches such as postnatal glucocorticoids, vitamin A, and inhaled nitric oxide [41].

The preterm infants who are exposed to limited oxygen may have minimized lung injury. Targeting lower oxygen saturation in infants who have received supplemental oxygen may protect the lung from oxidative injury. Askie and his colleagues [42] made a randomized trial of routine versus lower oxygen saturation targets in VLBW infants, that remained oxygen dependent at 32 weeks postmenstrual age. The incidence of BPD and need for home oxygen therapy were less in the lower (91 to 94%) oxygen saturation group than in the routine (95 to 98%) group [43].

1.4.1. Mechanical Ventilation Strategies

1.4.1.1. Continuous Positive Airway Pressure (CPAP)

During mechanical ventilation, avoiding excessive tidal volumes may have a protective effect on the lung. It may also help to prevent BPD in at risk infants. For example, application of nasal continuous positive airway pressure (CPAP) soon after birth is associated with less bronchopulmonary dysplasia instead of early endotracheal intubation and mechanical ventilation [44]. Routine usage of CPAP immediately after delivery may avoid the need for intubation in infants with a gestational age of 24 weeks or greater. In one report, mechanical ventilation was avoided in approximately one-third of infants 25 or fewer weeks and nearly 80% of those 28 or more weeks gestational age [44]. Another approach is early surfactant administration and mechanical ventilation for one or two days, followed by extubation and application of CPAP. Using this approach it has been shown that approximately one-quarter of the infants born at less than 27 weeks GA, avoided a subsequent course of mechanical ventilation and these infants were less likely to develop BPD [42].

1.4.2. Pharmacologic Approaches

Research studies using pharmacologic approaches for the prevention of BPD includes the use of caffeine and vitamin A supplementation [44].

1.4.2.1. Caffeine for Apnea of Prematurity

In premature infants, the part of the central nervous system (brain and spinal cord) that controls breathing is not yet mature enough to allow nonstop breathing. This causes large bursts of breath followed by periods of shallow breathing. This is known as apnea of prematurity (AOP). Caffeine is a methylxanthine which is used as a bronchodilator, for treating AOP. The impact of treatment with caffeine on neurodevelopmental outcome has been studied in infants with birth weights from 500 to 1250 g. Infants assigned to receive caffeine had a lower risk of neurodevelopmental impairment [45]. The study by Schmidt and his colleagues showed that there is a possibility that caffeine reduces the exposure to mechanical ventilation and reducing ventilator-induced lung injury [45]. The use of caffeine for the treatment of apnea in prematurity, in extremely low gestational age newborns may reduce the risk of developing BPD [45].

1.4.2.2. Vitamin A

Vitamin A derivatives determine a group of fat-soluble compounds called retinoids which regulate epithelial cell growth. These play an important role in lung disease because they are important in the regulation and promotion of growth and also in differentiation of lung epithelial cells during repair following lung injury [46]. Kennedy et al. observed that preterm infants have low vitamin A levels at birth, and low levels of vitamin A are associated with an increased risk of BPD [47].

1.5. CURRENT THERAPIES FOR BPD

1.5.1. Bronchodilators

Bronchodilators are used for relaxing bronchial muscles by making the airways larger and therefore allowing air to pass through the lungs easier. There are three main groups of bronchodilators such as, beta-agonists, anticholinergics and theophyllines. The effects of increased airway reactivity, increased airway resistance, decreased lung compliance, inhaled beta-agonists (albuterol, salbutamol, ipratropium, terbutaline) have been studied in BPD for their effects on lung function and the need for ventilatory support [48,49] and these medications demonstrated as a short term benefit in reducing airway resistance and improving lung compliance [48].

1.5.2. Corticosteroids

Corticosteroids are mainly used to reduce inflammation. Inflammation leads to the pathogenesis of BPD and the usage of corticosteroid makes physiologic sense, mainly due to their anti-inflammatory properties. Both systemic and inhaled corticosteroids have been studied extensively in preterm neonates for prevention and treatment of BPD [48,49].

1.5.3. Diuretics

Excessive interstitial fluid leads to decreased lung compliance and higher oxygen requirements. To balance the sensitive fluid status of infants with BPD with adequate nutritional intake, diuretics that increase the absorption of fluid from the lung, are often used in infants with BPD[48,49].

1.5.4. Fluid Restriction

Standard management of infants with BPD usually includes moderate fluid restriction (120 to 130 ml/kg/day) to help prevent interstitial alveolar edema. Infants with BPD may be quite fluid sensitive, but the potential benefits of fluid restriction need to be counterbalanced with the ability to provide adequate caloric intake for growth [48,49].

Table 1.3, summarizes the class of drugs, recommended doses and duration treatment for BPD.

Table 1.3. Pharmacological agents in clinical use to prevent/treat BPD[49]

Class of drugs	Recommended dose and duration of treatment	Comments
Caffeine	Caffeine citrate Loading dose: 20-25mg/kg IV/PO Maintenance: 5-10 mg/kg IV/PO Discontinue at least 5-7 days prior to discharge IV (Intravenous), PO (Per OS)	Recomended for treatment of apnea of prematurity and prevention of BPD
Vitamin A	5000 IU intramuscularly 3 times per week, in infants < 1000g for 4 weeks	1 additional infant survived without BPD for every 14-15 infants who received vitamin A
Systemic Corticosteroids	Variable doses and duration (early,late)	Not recomended for early use; consider later use for infants with rapidly deteriorating respiratory status; 2 ongoing trials to evaluate usage of hydrocortisone to prevent BPD
Diuretics	Furosemide: 1mg/kg IV or 2mg/kg PO Hydrochlorothiazide: 20-40 mg/kg/day PO Spironolactone: 2-4 mg/kg/day PO	Loop: use sparingly in early Thiazides/spironolactone: consider for judicious chronic use
Bronchodilators	Guided by clinical response and adverse reactions	Limit use to infants with bronchospasm and acute clinical response

1.6. SINGLE NUCLEOTIDE POLYMORPHISMS (SNP)

Small genetic changes of DNA that vary in only one base are known as Single Nucleotide Polymorphisms (SNPs). This type of polymorphism occurs when a single nucleotide (A, T, C, or G) in a specific position in the genome sequence is altered. As much as 99.9% of the human DNA sequence is similar across populations but there can the difference in the 0.1% of the DNA can make two individuals unique [50, 51] (Figure 1.2).

The position of a SNP is important in determining the nature of effect of the SNP [52,53]. For example, SNP's situated in the coding region of a gene may change an amino acid in the resulting protein, which in turn could directly change the protein structure or function. SNP's that occur in non-coding regions (introns or promoter) do not directly involve amino acid change but they may alter gene expression or function of the protein, or be in linkage disequilibrium with a causative SNP or SNPs. Therefore, SNP's in a non-coding region are also important markers for assessing association with a trait or disease in genetic association studies.

SNP's can act like biological markers to help in understanding the genetic basis of common human diseases [53]. Also SNPs may help to predict an individual's response to certain drugs and susceptibility to environmental factors such as toxins.

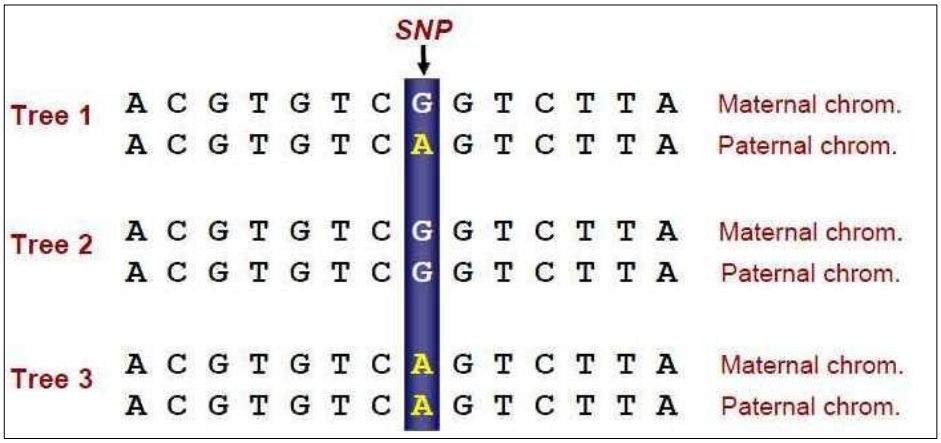

Figure 1.2. Single nucleotide polymorphism occured between in G and A alleles [53].

1.7. GENETICAL FACTORS RELATED TO BPD

1.7.1. Cytokines Associated with BPD

Inflammation, contributed to by antenatal CA and postnatal (local or systemic infections, hyperoxia, ventilator-induced injury) factors, initiates and modifies the process of lung injury in the developing lung [54]. This inflammatory process is dependent upon the effective release and balance of cytokines. An imbalance in these mediators leads to activation of the cellular death pathways in the lung [54].

Several mediators may have direct harmful effects on lung tissue structures by affecting cell integrity and inducing apoptosis, during the inflammatory process. Tumor necrosis factor alpha (TNFα), Interleukin 1 (IL-1), Interleukin 6 (IL-6) and Interleukin 8(IL-8) are important mediators in the early inflammatory response. These cytokines are synthesized by alveolar macrophages, fibroblasts, type II pneumocytes and endothelial cells upon stimulation by hypoxia, hyperoxia, endotoxin, other bacterial cell wall constituents and biophysical factors [55,56].

Kwong and his colleagues observed that, lung inflammatory cells of preterm infants responded with a reduced expression of pro-inflammatory cytokines, when exposed to Interleukin 10 (IL-10) [57]. The imbalance between pro- and anti-inflammatory cytokines can be considered to be an important feature of lung injury [58].

Vascular endothelial growth factor (VEGF) is a relatively specific endothelial cell mitogen that regulates endothelial cell differentiation and angiogenesis, and plays a central role in vascular repair. VEGF is essential for the formation of embryonic blood vessels (vasculature) and plays multiple roles in vascular development and maintenance [59,60]. The absence of VEGF has been seen to result in impaired fetal lung microvascular development [61].

Interleukin 4 (IL-4) is a product of activated T cells, basophils, and mast cells. IL-4 is a potent antiinflammatory cytokine which is able to inhibit monocyte and macrophage production of pro-inflammatory cytokines and chemokines by downregulating many of the inflammatory mediators that are elevated in bronchoalveolar lavage (BAL) fluid in infants with BPD. It may therefore have a protective effect [62].

IL-10 inhibits the synthesis and promoting degradation of pro-inflammatory cytokines such as, tumor necrosis factor (TNF), Interleukin 1 alpha (IL-1α), Interleukin 1 beta (IL-1β), IL-6, IL-8, Interferon gamma (IFN)- γ, IL-12, IL-18, and chemokines. These actions may decrease inflammation, cell injury, and apoptosis [63].

The tissue injury caused by inflammation, follows a phase of repair [64] which leads to an initiation of transforming growth factor-beta (TGF-β), that limits some of the inflammatory reactions and plays a key role in mediating tissue remodeling and repair [64]. If the reparative processes are exaggerated and not adequately localized, fibrosis will appear. This is associated with an increased level of TGF-α and its receptors, an overexpression of which has been shown to result in severe pulmonary fibrosis. In preterm infants with BPD, increased levels of TGF-α have been detected in airway secretions [63].

1.7.2. Gene polymorphisms studied in BPD

Glutathione S Tranferases (GST) are ubiquitous in human organs for providing an important line of cellular defense against reactive oxygen species. It has been observed that GST polymorphisms can alter the detoxification of oxidizing agents which can cause to BPD [65].

Alveolarization requires coordination of extracellular matrix remodeling with epithelial morphogenesis and capillary growth, which involves matrix metalloproteinases (MMPs) that are classified into two major groups according to their subcellular localization: membrane-type MMPs (MT-MMPs) and secreted MMPs. Among secreted MMPs, MMP2 has been seen to play an important role in lung development and repair after injury. MMP2, MMP14, MT3-MMP (MMP16) polymorphisms have been associated with BPD [66].

Hadchouel et al. demonstrated that heterozygous and homozygous genotypes for both MMP16 C/T and MMP16 A/G were found to be associated with a reduced risk of BPD [67].

TNF is one of the principal mediators of the inflammatory cascade response. Elevated levels of TNFα in the bronchoalveolar lavage (BAL) fluid of ventilated preterm infants has seen to be associated with development of BPD [68,69]. High levels of TNFα may promote chronic inflammation by overwhelming counter-regulatory mechanisms, whereas low levels may decrease the risk and severity of BPD [70]. The expression of both TNFα and TNFβ are regulated at the transcriptional level and various SNP have been identified in their respective promoter sequences that could modulate expression. A to G substitutions at positions 308 and 238 for TNFα has been associated with BPD [70].

Kwinta et al. observed an association between the position 460T/C VEGF gene polymorphism and the risk of BPD. They demonstrated that VEGF position 460CC homozygotes had a lower risk than babies with TT or TC genotypes [71]. Hadchouel et al. demonstrated that polymorphism at Sparc/Osteonectin, CWCV, and Kazal-like domains proteoglycan 2 (SPOCK 2) gene was associated with BPD in Caucasian–French and African–French premature infants [72]. Lavoie et al. observed that C allele for polymorphism of SPOCK2 was associated with risk of developing moderate-severe BPD in both Caucasian–French and African–French patients, but it was not associated with mild BPD [73].

Yanamandra and coworkers were reported that the frequency of the IL 10 position 1082 polymorphism in 294 mechanically ventilated VLBW infants, with no significant effect on mortality or the development of BPD [74]. Lin et al. observed a case-control study of IL-4 polymorphisms in 224 Taiwanese preterm infants. There were no significant differences in allelic frequencies of the IL-4 intron 3 or IL-4 promoter polymorphisms between preterm infants who developed BPD and healthy control [75].

Insulin like growth factor IGF (IGF-1) is also involved in growth and injury repair processes in many organs, including the lungs. IGF-1 expression is altered by inflammatory cytokines and oxidants. Kwinta et al. demonstrated that there was no significant association between IGF-1 polymorphism and BPD incidence [74].

There is a relation between BPD and asthma. Asthma is a chronic (long-term) lung disease which is characterized by chronic airway inflammation, hyperresponsiveness, reversible airflow limitation, inflammation and narrowing of the airways. It is the leading cause of morbidity among children [76]. Asthma has similar characteristic symptoms with BPD such as, wheezing, shortness of breath, coughing, and a sensation of tightness in the chest [76,77].

1.8. A DISINTEGRIN AND METALLOPROTEASE DOMAIN- 33 (ADAM 33)

The A disintegrin and metalloprotease domain (ADAM) family of type 1 transmembrane proteins which has been implicated in asthma and bronchial hyperresponsiveness is a subgroup of the zinc-dependent metalloproteinase superfamily and contains over 30 members that are structurally very complex [78,79]. Members of this family are membrane-anchored proteins structurally related to snake venom disintegrins, and have been involved in a variety of biological processes involving cell-cell and cell-matrix interactions, proliferation, differentiation, signaling and apoptosis, inflammatory response including fertilization, muscle development, and neurogenesis [80]. The existence of various ADAM 33 isoforms in human embryonic bronchi and their surrounding mesenchyme shows its contribution to smooth muscle development and function, and these show that ADAM 33 is abundantly expressed in airway smooth muscle, fibroblasts, and myofibroblasts, and plays a role in cell signaling, adhesion and proteolysis [81-83]. Additionally, its presence in mesenchymal tissues is thought to cause ‘‘unusual’’ airway formation that leads to the origins of asthma in early life [84].

Some cytokines such as IL-1 and TNF act broadly to stimulate the inflammatory response, while others act on specific type of immune cells. IL-2 is a key mediator of T cell proliferation and activation. Both IL-4 and IL-2 have been shown to have indirect interactions with the ADAM33 protein and mutations in ADAM33 can promote a hyperreactive response in lung endothelial cells and fibroblasts or affect the inhibition of IL-2 proteins [84].

1.9. ENDOTHELIAL CELL NITRIC OXIDE SYNTHASE (eNOS)

NO (Nitric Oxide) which is produced along with L-Citrulline by the oxidation of L-Arginine and catalyzed by three different isoforms of NOS (NO Synthase), it is a short-lived free radical gas involved in diverse physiological and pathological processes. It consists of 3 types such as neuronal NOS, endothelial NOS and inducible NOS. Type-I nNOS (neuronal NOS) and Type-III eNOS (endothelial NOS) are based on expressing as latent enzymes and require a higher concentration of calcium (Ca^{2+}) for enzyme activity. The three NOS isoforms are encoded by three distinct genes (NOS1, NOS2 and NOS3) located on different chromosomes (12, 17 and 7, respectively), and differentially expressed in different cells [85,86]. All the NOS genes are expressed in airway epithelial cells [87]. NOS1 and NOS3 are largely constitutively expressed, resulting in a low basal synthesis of NO shows limited response to physiological stimuliand they are important for physiological functions in the airways [88]. Type-II iNOS (inducible NOS) is Ca^{2+} independent because it's high affinity for Ca^{2+}/Calm (Calmodulin) provides the enzyme activity, even at basal levels of intracellular Ca^{2+} [89,90]. The catalysis of this reaction requires a number of essential cofactors such as mononucleotide, FAD (Flavin Adenine Dinucleotide), and NADPH (Nicotinamide Adenine Dinucleotide, Reduced) (Figure 1.3).

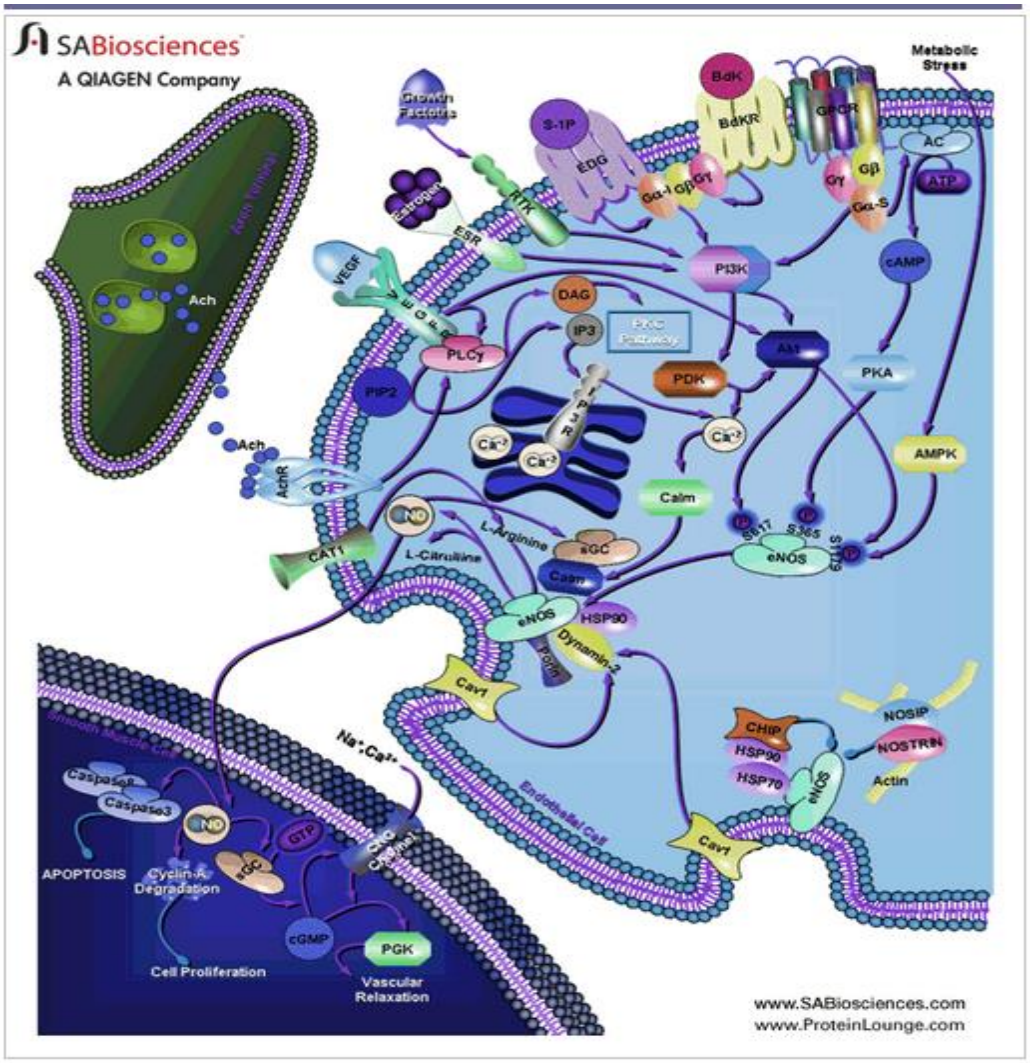

Figure 1.3. eNOS signalling [91]

eNOS is an important regulator of cardiovascular homeostasis because it is the major source of NO production in vascular endothelial cells. eNOS has an important role in blood vessel vasodilatation and blood pressure regulation. In addition, NO released from the endothelium that modulates other processes including platelet aggregation, platelet and leukocyte adhesion to the endothelium, Endothelin-1 generation, vascular smooth muscle cell proliferation, and angiogenesis. Because of the important role of NO in each of these processes, abnormalities in vascular NO production is believed to lead to the pathogenesis of certain vascular diseases such as atherosclerosis and hypertension [92,93].

2. AIM OF THE STUDY

The aim of this study was to investigate possible associations between ADAM 33 and eNOS gene polymorphisms in premature infants as a risk factors for development of BPD.

3. MATERIALS

3.1. SAMPLES

- Human blood sample (Kanuni Sultan Suleyman Hospital, Umraniye Hospital, Goztepe Hospital, Turkey)

3.2. KITS

- DNA isolation kit (Invitrogen, USA)
- TaqMan Universal PCR Master Mix (Applied Biosystems, USA)
- TaqMan SNP Genotyping Assay, ADAM 33, rs 2280090, (Applied Biosystems, USA)
- TaqMan SNP Genotyping Assay, eNOS, rs 179983 (Applied Biosystems, USA)

3.3. EQUIPMENTS

- Vortex (Velp Scientifica, Italy)
- Refrigerator (Arçelik, Turkey)
- Micropipettes, 10 µl, 100 µl, 200 µl, 1000 µl (Eppendorf, USA)
- Micropipette tips, 10 µl, 100 µl, 200 µl, 1000 µl (Expell, Turkey)
- Racks (ISOLAB, Germany)
- Eppendorf tubes, 2ml (ISOLAB, Germany)
- Centrifuge (Mikro 22 R, Hettich, Germany)
- Centrifuge (Allegra, 25 R centrifuge, Beckman Coulter, USA)
- Nanodrop P 300 (Implen, Germany)
- Dry ice
- 50ml falcon tubes (ISOLAB, Germany)
- PCR Microplate, 96 (PCR-96-FLT-C, Axygen, USA)
- Water Bath (Grant, OLS 200, UK)
- Real Time PCR System (Bio-Rad, CFX96, USA)

- Ice machine (Hoshizaki, FM-120DE, Japan)
- PCR tubes (Axygen, 0.2ml, USA)

3.4. CHEMICALS

- Ethanol (≥ 99.8 % Sigma, USA)
- Distilled water
- Tris-EDTA (TE) Buffer (Invitrogen, USA)
- Nuclease free water (Fermentas, USA)

4. METHODS

4.1. STUDY POPULATIONS

One hunderd twenty two blood samples were collected from Kanuni Sultan Suleyman Hospital, Umraniye Hospital and Goztepe Hospital. The inclusion criteria were: 1) preterm birth at 23–33 week gestational age; 2) birth weight 480g-2300g; 3) age of mother 19-34. Detailed perinatal history (birth weight, gestational age, Apgar score at 1and 5 min after birth) and history of treatment in the hospital (mechanical ventilation, oxygen therapy, surfactant treatment, diagnoses) were taken on admission. The study had been approved by the Ethics Committee of Umraniye Hospital.

4.2. DNA ISOLATION

Blood samples were stored at -20^{o}C. Genomic DNA was extracted from 200 µl of blood using PureLink^{TM} Genomic DNA Mini Kit (Invitrogen, USA) (Figure 4.1) according to the manufacturer's instructions.

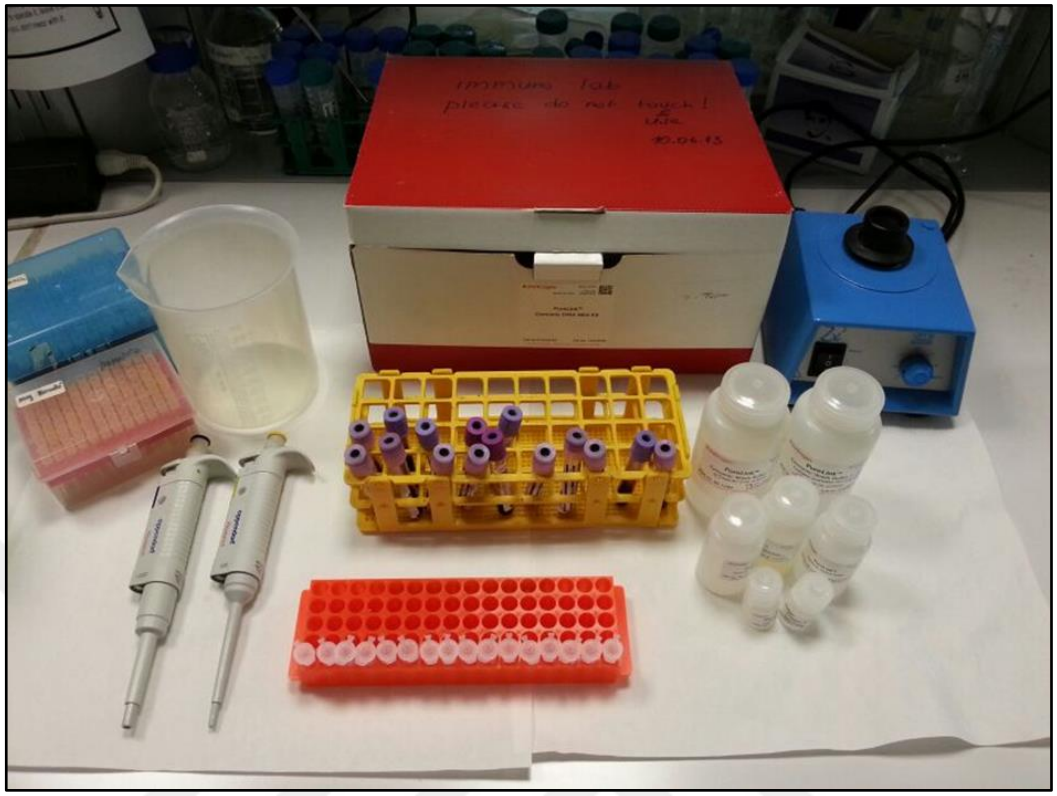

Figure 4.1. DNA isolation with PureLink™ Genomic DNA Mini Kit

4.3. NANOSPECTROPHOTOMETRY

Following DNA isolation the concentration and purity of DNA samples were measured by Nanodrop P 300 (Implen, Germany) (Figure 4.2) at 260 and 280 nm wavelengths. 3 µl PureLink™ Genomic Elution Buffer (Invitrogen, USA) was used as a blank. After setting the blank, 3 µl was taken separately from the DNA sample and measured at 260 nm. The A_{260}/A_{280} ratios showed the purity of the samples. The results between 1.8 and 2.0 is generally accepted as pure for DNA samples [94].

Figure 4.2. Nanodrop P 300 [94]

4.4. TAQMAN SNP GENOTYPING ASSAYS

DNA was diluted to 10ng/µl. 40X SNP primer (TaqMan, USA) was diluted to a 20X working stock with 1X TE buffer (10mM Tris-HCL, 1mM EDTA pH 8.0, made using DNase-free, sterile-filtered water). The mixture was then vortexed and centrifuged (Mikro 22 R, Hettich, Germany) and stored at -20^{o}C in the dark. The SNP Genotyping Assay (TaqMan, USA) contained two TaqMan® MGB probes: one probe labeled with VIC® dye that detects the Allele 1 sequence and the other probe labeled with FAM™ dye detects the Allele 2 sequence. Gene sequences are shown in Table 4.1. The TaqMan Universal PCR Master Mix (TaqMan, USA) was aliqouted to sterile microcentrifuge tubes.

For the SNP analysis of ADAM33 (*rs 2280090*) and eNOS (*rs1799983*) optimized primers (TaqMan SNP Assays MTO, Human SM Assay, USA) were used. The master mix contained 6.25 µl of dH_2O, 12.5 µl TaqMan Universal PCR Master Mix (TaqMan, USA) and 1.25 µl of the primer. Aliquots were pippetted into a 96 well plate and 5 µl of DNA was added to each well. The plates were centrifuged (Allegra, 25 R centrifuge, Beckman Coulter, USA) at 1000 rpm for 1min. Real Time Polymerase Chain Reaction (QRT-PCR) was then carried out in a CFX96 thermocycler (BioRad, USA) (Figure 4.3). Cycling conditions on CFX96 (BioRad, USA) were followed by 10 min at 95^{o}C followed by 40 cycles of 15 seconds at 92^{o}C and 1 min at 60^{o}C [95].

Table 4.1. Context Sequence for QRT-PCR

ADAM 33	GTGCCTCACTCACCCAGGGGCCAGG[A/G]CTGTCCAGTGGCTGTGGGGCCCAAC
eNOS	CCCTGCTGCTGCAGGCCCCAGATGA[G/T]CCCCCAGAACTCTTCCTTCTGCCCC

Figure 4.3. CFX 96 Real Time PCR

4.5. STATISTICAL ANALYSIS

Statistical analysis were performed with SPSS version 21.00 Software (SPSS software,Inc., Chicago,IL). Allelic frequencies and genotype frequencies were determined from BPD patients and healthy controls. Chi-square $\chi 2$ test, the odds ratio, confidence intervals were calculated for the comparisons of allelic frequencies and genotype frequencies. For

demographic variables two independent t test and Mann-Whitney U test was used. Logistic regression and forward conditional method was performed for risk factors of BPD. Power analysis was calculated for genotypes. *p*-value < 0.05* was taken as a statistically significant.

5. RESULTS and DISCUSSION

5.1. STATISTICAL ANALYSIS OF DEMOGRAPHIC DATA

In this study demographic data were gestational age, birth weight, apgar score 1, apgar score 5, mechanical ventilation per day, continuous positive airway pressur per day, duration of oxygen per day. One hundred twenty two infants were included to this study.

Relation of inclusion criteria were investigated for Bronchopulmonary Dysplasia (BPD) and Non Bronchopulmonary Dysplasia (Non BPD) patients. Table 5.1 summarizes the data regarding the frequency distribution in two subject groups.

Table 5.1. Statistical analysis of demographic data between BPD and Non BPD infants

	BPD	Non BPD	*p*-value
GA	26.27 ± 3.93	29.8 ± 2.63	0.000*[a]
BW	888.5 ± 257.2	1439.2 ± 543.3	0.000*[a]
Apgar 1	4.00 (1-7)	5.00 (3-6)	0.000*[b]
Apgar 5	6.00 (3-8)	7.00 (5-8)	0.000*[b]
MV duration (day)	24.93 ± 23.5	8.33 ± 4.42	0.004*[a]
CPAP duration (day)	8.32 ± 7.35	3.44 ± 1.3	0.007*[a]
Tot. O_2 (day)	57.16 ± 40.2	16.33 ± 10.41	0.000*[a]

BPD = Bronchopulmonary Dysplasia, Non BPD = Control, premature babies without BPD, GA= Gestational age, BW= Birth Weight, Apgar1=Apgar score 1, Apgar 5=Apgar score 5, MV duration (day)= Duration time of Mechanical Ventilation, CPAP duration (day)= Duration time of Continuous Positive Airway Pressure, Tot. O_2 (day)= Duration time of total Oxygen,

[*a] was calculated by aritmethically standart deviation,

[*b] was calculated by median and values in brackets shows minimum and maximum values, *p*-Values were calculated by two independent sample t test; *$p < 0.05$ indicates statistically significance.

Gestational age is one of the most important risk factors in the development of BPD. If the baby born prematurely, the possibility of using ventilation system will be higher. Klinger et al. [99], observed that 55.1% of infants with BPD were born at a GA of 24-25 week, 19.6% of babies with BPD were at GA of 26-27 week and 6.4% of infants at GA of 28-29 week in an Israelil population. GA was found statistically significant in this study (p=0.000*).

This study observed similar results for birth weight (p=0.000*) as with GA. This is to be expected since GA and BW are correlated together. Khan et al. [96], also previously observed that BW (p=0.001*) and GA (p=0.001*) were highly associated with BPD infants in the USA.

The apgar score which assesses the health of newborn children immediately after birth, was another inclusion criteria in this study. Khan et al. [96], demonstrated that, apgar 1 (p=0.014*) and apgar 5 (p=0.002*) was highly significant for BPD infants. Similarly in our study, it was observed that the apgar score 1 (p=0.000*) and apgar score 5 (p=0.000*) was found statistically significant.

Mechanical ventilation is necessary for the survival of many preterm infants, but it can injure the lung tissue and is a risk factor for the development of BPD. Mechanical ventilation has an association with risk of barotrauma which is physical damage to body tissues caused by a difference in pressure [29]. In present study, it was demonstrated that duration of mechanical ventilation was associated with development of BPD (p=0.004*).

During mechanical ventilation, avoiding excessive tidal volumes may have a protective effect on the lung. It may also help to prevent BPD in at risk infants. For example, application of nasal continuous positive airway pressure (CPAP) soon after birth is associated with less BPD instead of early endotracheal intubation and mechanical ventilation [44]. Routine usage of CPAP immediately after delivery may avoid the need for intubation in infants with a gestational age of 24 weeks or greater. In one report, mechanical ventilation was avoided in approximately one-third of infants 25 or fewer weeks and nearly 80% of those 28 or more weeks gestational age [44].

In this study we observed that, the duration time of CPAP was higher in infants with BPD (8.32 ± 7.35) and it was found statistically significant (p=0.007*).

The majority of ELBW infants need supplemental oxygen and assisted ventilation soon after birth to achieve adequate gas exchange [41]. Similarly, in our study we demonstrated that the duration time of total oxygen was higher in infants with BPD (57.16 ± 40.2), instead of Non-BPD group (16.33 ± 10.41) and it was found statistically significant (p=0.000*).

5.2. STATISTICAL ANALYSIS OF ALLELE AND GENOTYPE FREQUENCIES IN ADAM33

Distribution of allele and genotype frequencies in ADAM33 were investigated in BPD and Non BPD patients.

Comparison of the A/G allele distribution in ADAM 33 in BPD and Non BPD groups revealed the presence of the A allele to be higher in the BPD group (72.6 %) compared to Non BPD (46.6%). The high significance of the A allele (p=0.000*; OR 2.7, 95% CI 1.576-4.623) reveals an association with susceptibility to developing BPD. Conversely, the G allele showed approximately similar distributions between BPD and Non BPD groups suggesting no statistically significant role for this allele as a risk factor for development of BPD (Table 5.2).

Table 5.2. Distribution of allele frequencies of ADAM33 in Bronchopulmonary Dysplasia

		BPD (N=75)		Non BPD (N=45)			
Gene	Allele	n	n%	n	n%	OR (95% CI)	*p*-value
ADAM33	A	98	72.6	37	24.4	2.7(1.576-4.623)	0.000*
	G	52	49.5	53	50.5		

BPD= Bronchopulmonary Dysplasia, Non BPD= Control, premature babies without BPD, CI= Confidence Interval, OR= Odds Ratio, N= Number of infants, n= Allele count in BPD and Non BPD, n% = Percentage of allele count in BPD and Non BPD,
p-Value calculated using the Pearson Chi-square test;
*$p < 0.05$ indicates statistically significance.

Comparison of genotype distributions between BPD and Non BPD groups revealed the AA genotype to be higher in BPD infants (73.7%) than in Non BPD (26.3%), (p=0,006*; OR 2.54 95% CI 1.179-5.494). The homozygote GG genotype was also observed as highly significant (p=0.004*; OR 0.32 95% CI 0.148-0.710) but associated with health rather than as a risk factor for BPD. Comparison of homozygous and heterozygous genotype showed that, heterozygous GA genotype (p=0.664; OR 1.24, 95% CI 0.461-3.365) was not significant for BPD (Table 5.3). Experimental power analysis were calculated for our statistically significant results and power of AA genotype was found 99% at this study.

Table 5.3. Distribution of genotype frequencies of ADAM33 in Bronchopulmonary Dysplasia

		BPD (N=75)		Non BPD (N=45)			
Gene	Genotype	n	n%	n	n%	OR (95 % CI)	*p*-value
ADAM33	AA	42	73.7	15	26.3	2.54(1.179-5.494)	0.006*
	GG	19	45.2	23	54.8	0.32(0.148-0.710)	0.004*
	GA	14	66.7	7	33.3	1.24(0.461-3.365)	0.664

BPD= Bronchopulmonary Dysplasia, Non BPD= Control, premature babies without BPD, CI= Confidence Interval, OR= Odds Ratio, N= Number of infants, n= Genotype count in BPD and Non BPD, n% = Percentage of genotype count in BPD and Non BPD,

p-Value calculated using the Pearson Chi-square test;

*$p < 0.05$ indicates statistically significance.

BPD is the most common form of chronic lung disease in infancy and preterm infants with BPD have higher airway hyper-responsiveness. To the best of our knowledge, there are no previous studies on the ADAM33 gene polymorphism in relation to BPD. However, ADAM33 polymorphisms have been studied in asthma patient in different populations. Asthma is a complex disease caused by a combination of environmental factors and genetic variability andhas been shown to have similar characteristics, symptoms and inflammatory pathway with BPD [76].

A study by El Falaki et al. [97], demonstrated that, homoyzgous AA genotype of ADAM33 gene was a risk factor for developing asthma. in Egyptian children.

Another study by Chiang et al. [77], also observed a significance in the AA genotype in a Taiwanese population. Another study in caucasians also revealed an association of this gene with asthma, Eerdewegh et al. [98] . These findings are similar to our findings in BPD infants. However, another study by Shuqiang et al. [99], observed that, homozygous AA (p=0.105; OR 3.414, 95% CI 0.705-16.533), GG (p=0.097; OR 0.747, 95% CI 0.529-1.055) and heterozygous GA (p=0.178; OR 1.274, 95% CI 0.896-1.811) genotypes in ADAM 33 were not associated with childhood asthma in a Chinese population.

Other studies on the ADAM33 gene in association with other diseases have also been carried out. A study on atopic dermatitis (AD) (p=0.23) also known as eczema ina Japanese children [100] revealed no association with ADAM 33. Holloway et al.[100], demonstrated that, ADAM33 plays a role in the development of atherosclerosis.In the present study, we found the AA genotype to be significant for susceptibility to developing BPD.

5.3. STATISTICAL ANALYSIS OF ALLELE AND GENOTYPE FREQUENCIES IN eNOS

Based on the known importance of the NOS pathway and association of eNOS with asthma, we inferred that genetic variation in that gene may also have an impact on BPD. The findings of this study demonstrated that the GG genotype in eNOS gene was highly significant for BPD. No investigation of the eNOS gene polymorphism has previously been documented in BPD.

Distribution of allele and genotype frequencies in eNOS was investigated in BPD and Non BPD patients. Table 5.4 and Table 5.5 summarizes the data regarding the frequency distribution for eNOS.

Comparison of the allele frequency distribution revealed the presence of G allele as a highly significant risk factor for development of BPD (p=0.000*; OR 4.07, 95% CI 2.066-8.009) compared to the T allele. The distribution of the T allele in eNOS was found to be similarly distributed amongst BPD (51.9%) and Non BPD (48.1%).

Table 5.4. Distribution of allele frequencies of eNOS in Bronchopulmonary Dysplasia

		BPD (N=75)		Non BPD (N=45)			
Gene	Allele	n	n%	n	n%	OR (95% CI)	*p*-value
eNOS	G	57	81.4	13	18.6	4.07(2.066-8.009)	0.000*
	T	83	51.9	77	48.1		

BPD= Bronchopulmonary Dysplasia, Non BPD= Control, premature babies without BPD, CI= Confidence Interval, OR= Odds Ratio, N= Number of infants, n= Allele count in BPD and Non BPD, n% = Percentage of allele count in BPD and Non BPD,
p-Value calculated using the Pearson Chi-square test;
**p*< 0.05 indicates statistically significance.

Comparison of all 3 genotypes in the eNOS gene showed this gene to be highly statistically significant to BPD (p=0.001*). This study demonstrated that the frequency of the GG genotype (25.3%) of the eNOS gene was higher in babies with BPD rather than TT (53.6%) and TG (59.4%) genotype, when these genotypes were compared with the Non BPD groups. No healthy infants were seen to carry the GG genotype (p=0.000*; OR 1.89, 95% CI 1.514-2.148). The TT genotype (p=0.019*; OR 0.39, 95% CI 0.180-0.870) also displayed a susceptibility for developing BPD. Heterozygous TG genotype (p=0.631; OR 0.63, 95% CI 0.527-2.873) was not associated with the development of BPD. Experimental power analysis was performed for our statistically signifacant results and power of GG genotype was found 99% in our study.

Table 5.5. Distribution of genotype frequencies of eNOS in Bronchopulmonary Dysplasia

		BPD (N=75)		Non BPD (N=45)			
Gene	Genotype	n	n%	n	n%	OR (95 % CI)	*p*-value
eNOS	GG	19	25.3	0	0.00	1.80(1.514-2.148)	0.000*
	TT	37	53.6	32	46.4	0.39(0.180-0.870)	0.019*
	TG	19	59.4	13	40.6	0.63(0.527-2.873)	0.631

BPD= Bronchopulmonary Dysplasia, Non BPD= Control, premature babies without BPD, CI= Confidence Interval, OR= Odds Ratio, N= Number of infants, n= Genotype count in BPD and Non BPD, n% = Percentage of genotype count in BPD and Non BPD,

p-Value calculated using the Pearson Chi-square test;

*$p < 0.05$ indicates statistically significance.

eNOS is an important regulator of cardiovascular homeostasis because it is the major source of NO production in vascular endothelial cells. NO also has multiple roles in the respiratory tract. It relaxes respiratory smooth muscles, acts as a bronchodilator and increases airflow to the lungs. It is also involved in various cytotoxic and proinflammatory activities such as increased airway hyperresposiveness which is an important factor in asthma.

Holla et al. [101], observed that, haplotypes T and C were associated with a lower risk for asthma suggesting that endothelial NOS variants may be one of the factors participating in the protection or susceptibility to asthma in a Czech population.

Other studies on the eNOS gene in relation to other diseases have also been carried out. Santos et al. [102] observed that, homozygous (GG, TT) and heterozygous (TG) genotypes ($p>0.05$ for all comparisons) of eNOS gene were not associated with renal disease in Caucasians with type 2 diabetes.

Essential hypertension is amajor risk factor for coronary artery disease in south East Asians which is significantly associated with the pathophysiology of a variety of vascular disorders such as renal and cardiac failure, occular damage and stroke. In a study by, Srivastavaa et al. [103], it was demonstrated that individuals carrying the T allele are at 2.1 times greater risk for developing essential hypertension.

In another study by, Jang et al. [104], an asociation between the eNOS gene -786T/C and colorectal cancer.

5.4. STATISTICAL ANALYSIS OF RISK FACTORS FOR BPD

Known risk factors for BPD are chorioaminionitis, patent ductus arterious and sepsis. Table 5.6 summarizes the data regarding the frequency distribution in the two subject groups.

Chorioamnionitis (CA), maternal infection is the most important cause of preterm birth, and severe chorioamnionitis is seen most frequently in preterm deliveries before 30 weeks of GA. CA is associated with an increased risk of BPD. In our study we observed that, BPD infants (10/59) and Non BPD patients (3/18) had CA. We demonstrated that CA was not statistically signifacant (p=1.000).

Patent ductus arteriosus (PDA) is a heart problem due to abnormal blood flow. Infants with RDS, who receive a greater fluid intake or do not have diuresis in early life, have a higher risk of PDA and BPD. In a study by Khan et al. [96], observed that PDA (p=0.020*) was highly associated with development of BPD. Conversely, we found that, BPD infants (7/59) and Non BPD patients (4/18) had PDA from 18 infants. We observed that PDA was not significanlty associated with the development of BPD (p=0.272).

Sepsis another risk factor BPD, is caused by severe infection is related with PDA and CA. Although a study by Klinger et al.[105], showed an association between sepsis and BPD. Khan et al. [96] also observed the significance between sepsis and BPD (p=0.003*). In present study, BPD infants (57/59) and Non BPD infants (9/18) had SE. Similar to our study, we demonstrated that sepsis was a risk factor for BPD infants (p=0.000*).

Table 5.6. Statistical analysis of risk factors included chorioamniontitis, patent ductus arteriosus, sepsis in BPD and Non BPD patients**.**

	BPD (N= 59)		Non BPD (N=18)		
	n	n%	n	n%	*p*-value
CA	10	76.9	3	23.1	1.000
PDA	7	63.6	4	36.4	0.272
SE	57	86.4	9	13.6	0.000*

CA= Chorioamniontitis, PDA= Patent Ductus Arteriosus, SE= Sepsis,

BPD= Bronchopulmonary Dysplasia, Non BPD= Control, premature babies without BPD, N= Number of infants, n= CA, PDA and SE count in BPD and Non BPD, n% = Percentage of CA, PDA, SE count in BPD and Non BPD,

p-Value was calculated using the Pearson Chi-square test;

*$p < 0.05$ indicates statistically significance.

5.5. PREDICTION OF BPD

In this study we observed that AA genotype (p=0.006*) of ADAM 33 gene, GG genotype (p=0.000*) of eNOS gene, sepsis (p=0.000*), gestational age (p=0.000*), birth weight (p=0.000*), duration of mechanical ventilation (p=0.004*), duration of CPAP (p=0.007*) and duration of total oxygen (p=0.000*) was statistically significant for developing BPD. The variables which were found statistically significant after a univariate analysis were added to logistic regression as independent variables for multivarial analysis. Prediction of BPD was performed by logistic regression analysis and forward conditional method (Table 5.7).

Table 5.7. Logistic Regression analysis of Bronchopulmonary dysplasia

	BPD (N=75)		
	OR	95% CI	*p*-value
BW	0.99	0.995-0.999	0.016
SE	0.11	0.007-1.583	0.104
Tot. O_2	1.08	1.003-1.182	0.043*

BPD= Bronchopulmonary Dysplasia, BW= Birth Weight, SE= Sepsis, Tot. O_2= Duration of total Oxygen, N= Number of infants, OR= Odds Ratio, CI= Confidence Intervals,
p-Value was calculated using Wald statistics;
*p< 0.05 indicates statistically significance.

In this study, birth weight (p=0.016*; OR 0.99, 95% CI 0.995-0.999) and duration time of total oxygen (p=0.043*; OR 1.08, 95% CI 1.003-1.132) together was found as a predictive variable for developing BPD. The other risk factors were not statistically significant (p>0.05) in logistic analysis.

6. CONCLUSION

In conclusion, the findings of this study show that ADAM 33 and eNOS genes are associated with development of BPD. We found that AA genotype of ADAM 33 gene and GG genotype of eNOS gene were highly significant in BPD infants. The homozygous AA genotype of ADAM 33 gene was associated with demographic data such as gestational age, birth weight, apgar score 1 and 5, maternal age and the use of CPAP in BPD babies. No associations were found with genotypes of eNOS gene and demographic data. In the present study we observed no associations between risk factors and ADAM 33 and eNOS genes.

Future studies should investigate other genes in order to understand the molecular mechanisms underlying the genetic susceptibility for development of BPD. Such studies with a larger cohort would be more advantageous for diagnosis and identification of novel therapeutic drugs.

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