ASSOCIATIONS OF IL-33 AND IL-18R1 GENE POLYMORPHISMS IN BRONCHOPULMONARY DYSPLASIA

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ASSOCIATIONS OF IL-33 AND IL-18R1 GENE POLYMORPHISMS IN BRONCHOPULMONARY DYSPLASIA

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ABSTRACT

ASSOCIATIONS OF IL-33 AND IL-18R1 POLYMORPHISMS IN BRONCHOPULMONARY DYSPLASIA

Bronchopulmonary dysplasia (BPD) is a chronic respiratory disease common in premature babies, and is associated with increased mortality and significant long-term cardiorespiratory and neurodevelopmental sequelae. The gestational age (GA) is less than 34 weeks with the infants who are BPD, and their birth weight (BW) is less than 1500 grams. Single Nucleotide Polymorphisms (SNPs), are small genetic changes of DNA, which occur in only one base, these polymorphisms happen when a single nucleotide (A, T, C, or G) in a specific position in the genome sequence changes. IL-33 is the 11th member of the interleukin 1 (IL-1) cytokine family, and it was first characterized as a protein expressed by the endothelial cells associated with the lymph node. It has been shown that IL-33 is expressed at higher levels in asthmatic patients than healthy patients, and in mouse models of asthma than wild-type mice. IL-18R1 is the ligand binding chain of IL-18 receptor complex, and in various study groups has been found to be associated with asthma and BPD. The purpose of this study was to investigate possible associations between gene polymorphisms in IL-33 and IL-18R1 and developing BPD in the Turkish population. DNA was isolated from a hundred and twenty two blood samples was done using the PureLink^{TM} Genomic DNA Mini Kit and then the concentration of the DNA samples was measured by nanophotometer Implen P 300. IL-33 (*rs10975519*) and IL-18R1 (*rs1974675*) optimized primer (TaqMan SNP Assays) were used for the SNP analysis, and CFX96 thermocycler was used to perform the Real Time Polymerase Chain Reaction (QRT-PCR). By using SPSS Software 21 and performing the Chi-square $\chi 2$ test, the odds ratio (OR) and confidence intervals (CI) were calculated to compare allelic and genotype frequencies. The results showed that the C allele (p=0.0043*; OR 1.727, 95% CI 1.014-2.940) of the IL-18R1 gene was more protective against the development of BPD.

ÖZET

IL-33 VE IL-18R1 GEN POLİMORFİZİMLERİNİN BRONKOPULMONER DİSPLAZİ İLE İLİŞKİSİ

Bronkopulmoner displazi (BPD) prematüre bebeklerde rastlanan kronik bir solunum hastalığıdır, uzun süreli kardiyorespiratuvar ile nörogelişimsel sekeller ile ilgilidir ve sonucunda ölüm gözlenebilir. Bu bebekler genelde 34 haftalık gestasyonel yaş öncesinde doğuyor ve doğdukları andaki ağırlıkları da 1500 gramın altında oluyor. Tek Nükleotid Polimorfizmi (SNP), DNA'nın tek bir bazında gerçekleşen değişikliklerdir, bu polimorfizmler genom sekansındaki spesifik bir pozisyonda tek bir nükleotid (A, T, C veya G) değiştiğinde gerçekleşir. IL-33 interlökin 1 (IL-1) sitokin ailesinin 11. üyesidir, ve ilk olarak lenf düğümleriyle ilgili olan endotel hücreleri tarafından ekspres edilen bir protein olarak karakterize edilmiştir. Yapılan araştırmalarda, astım hastalarında hasta olmayan insanlarda daha fazla IL-33 bulunduğu ve astım olan farelerde de normal farelerden daha fazla IL-33 bulunduğu gösterilmiştir. IL-18R1, IL-18 reseptör kompleksinin ligand bağlanma zinciridir, ve yapılan değişik popülasyon gruplarındaki çalışmalarda astım ve BPD ile ilişkili olduğu gösterilmiştir. Bu çalışmanın amacı IL-33 ve IL-18R1 gen polimorfizimlerinin Türk popülasyonunda BPD'nin gelişmesinde bir rolü olup olmadığını bulmaktır. 122 kan örneğinden PureLink^{TM} Genomic DNA Mini Kit'ini kullanarak DNA izole edildi ve daha sonra izole edilen DNA'ların konsantrasyonları Implen P 300 nanofotometre kullanılarak ölçüldü. SNP analizleri (TaqMan SNP Assays) için optimize edilmiş IL-33 (*rs10975519*) ve IL-18R1 (*rs1974675*) primerleri kullanıldı ve CFX96 thermocycler Real Time Polimeraz Zincir Reaksiyonunu (QRT-PCR) gerçekleştirmek için kullanıldı. Göreceli olasılıklar oranı ve güvenilirlik aralığı, alelik ve genotip frekanslarının karşılaştırılmalarını yapabilmek için hesaplandı. Bu hesaplama SPSS Software 21 programından yaralanarak ve Ki-kare $\chi 2$ testini uygulayarak yapıldı. Sonuçlar IL-18R1 genindeki C allelinin (p=0.0043*; OR 1.727, 95% CI 1.014-2.940) BPD hastalağının gelişmesine karşı koruyucu bir etkisi olduğunu göstermektedir.

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LIST OF SYMBOLS/ ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
BHR	Bronchial Hyper-responsiveness
BW	Birth Weight
BPD	Bronchopulmonary Dysplasia
CA	Chorioamnionitis
CAP	Caffeine for Apnea of Prematurity
CCL2	CC-Chemokine Ligand 2
CCL3	CC-Chemokine Ligand 3
CPAP	Continuous Positive Airway Pressure
CXCL2	CX Chemokine Ligand 2
DAMPs	Damage Associated Molecular Patterns
DNA	Deoxyribonucleic acid
EAE	Experimental Autoimmune Encephalomyelitis
F-7	Coagulation Factor VII
FGF4	Fibroblast Growth Factor 4
GA	Gestational Age
GR	Glucocorticoid Receptor
GST	Glutathione S-Transferase
GWA	Genome Wide Association
GWAS	Genome Wide Association Study
HDAC2	Histone Deacetylase 2
HLA	Human Leukocyte Antigen
HMGB1	High-Mobility Group Box 1
IFN-γ	Interferon Gamma
IL-1	Interleukin-1
IL-1β	Interleukin-1 beta
IL-1RAcp	Interleukin-1 Receptor Accessory Protein
IL-1RAP	Interleukin-1 Receptor Accessory Protein
IL-1RL1	Interleukin-1 Receptor-Like 1
IL-1Rrp	Interleukin-1 Receptor-Related Protein

IL-3	Interleukin-3
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-13	Interleukin-13
IL-18	Interleukin-18
IL-25	Interleukin-25
IL-33	Interleukin-33
IL-AcPL	Interleukin Accessory Protein Like
iNO	Inhaled Nitric Oxide
IRAK	Interleukin-1 Receptor Associated Kinase
IQ	Intelligence Quotient
JNK	Jun Amino-Terminal Kinases
KITLG	Kit Ligand
LBT4	Leukotriene B4
MAPK	Mitogen-Activated Protein Kinases
MBL-2	Mannose Binding Lectin 2
MIF	Macrophage Migration Inhibitory Factor
MMP16	Matrix Metalloproteinase 16
mRNA	Messenger Ribonucleic acid
MYD88	Myeloid Differentiation Primary Response Gene 88
MV	Mechanical Ventilation
NCPAP	Nasal Continuous Positive Airway Pressure
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NF-HEV	Nuclear Factor From High Endothelial Venules
NICHD	National Institute of Child Health and Human Development
NICU	Neonatal Intensive Care Unit
NOD	Non-Obese Diabetic
O_2	Oxygen
PCR	Polymerase Chain Reaction
PDA	Patent Ductus Arterious
PEEP	Poor Positive and Expiratory Pressure
PGD_2	Prostaglandin D_2
PMA	Postmenstrual Age

PNA	Postnatal Age
PRR	Pattern Recognition Receptors
RCT	Randomized Control Trials
RDS	Respiratory Distress Syndrome
ROS	Reactive Oxygen Species
SNP	Single Nucleotide Polymorphism
SP-B	Surfactant Protein B
SPOCK2	Testican 2
SOD	Superoxide dismutase
STAT	Signal Transducer and Activator of Transcription
T_H1	T Helper Cell 1
T_H2	T Helper Cell 2
TIR	Toll/ Interleukin-1 Receptor Superfamily
TLR	Toll Like Receptor
TLR4	Toll Like Receptor 4
TNF	Tumour Necrotic Factor
TNF-α	Tumour Necrotic Factor α
TRAF	Tumour Necrotic Factor Receptor Associated Factor
TSPL	Thymic Stromal Lymphopoietin
VDR	Vitamin D Receptor
VEGF	Vascular Endothelial Growth Factor
VLBW	Very Low Birth Weight

1. INTRODUCTION

1.1. BRONCHOPULMONARY DYSPLASIA

Pulmonary diseases, which result from neonatal respiratory disorders, are referred as chronic lung diseases [1][2]. Bronchopulmonary dysplasia (BPD) is the most common chronic lung disease, therefore BPD is one of the most common complications of preterm infants. This disease has various challenges [3], and its long-term sequela is not just present in the lungs of the infants, but also in many other of their organ systems.

Before the 1960s and mechanical ventilation was introduced as a treatment, the preterm babies who had respiratory distress syndrome (RDS), which is a breathing disorder, either survived but without respiratory morbidity, or worse died within the first week after birth.

In 1967, Northway and his colleagues [4] characterized the development of a new chronic disease in a group of preterm babies with severe respiratory distress syndrome, who had been exposed to high concentrations inspired oxygen and aggressive ventilation [2][4][5]. They represented four stages of BPD, which were defined by the results of characteristics and radiographs. These were defined in four stages; RDS (2-3 days), Regeneration (4-10 days), Transition to Chronic Disease (11-20 days), Chronic Lung Disease (> 1 month) [6].

The examinations of the lung tissues of these babies showed that there are vascular adjustments, changing areas of alveolar inflation, dying bronchiolitis, and pulmonary fibrosis (Figure 1.1.) [3].

In standard, old BPD appeared in preterm babies who had developed severe RDS, and who were treated with high oxygen concentrations and ventilation pressures. The average gestational age (GA) of these babies was 32 weeks, and the average birth weight (BW) was 1,900 g. The hallmarks of this form of BPD were inflammation of the airway, hypertrophy of the smooth muscle and fibrosis [3].

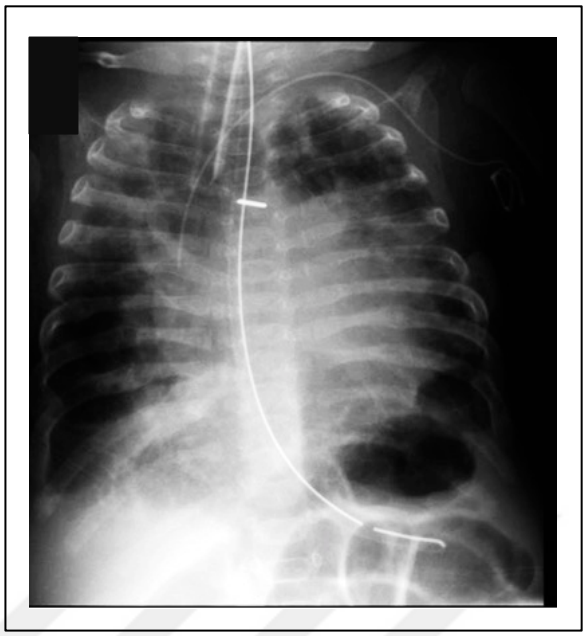

Figure 1.1. Chest radiograph of an infant with old form of BPD [5]

Over the years with the introduction of new measures to prevent and treat RDS, the old severe form of BPD is less seen [2][5]. Instead, BPD is now seen mostly in preterm babies with extremely low BW, whose pulmonary vasculature is not developed enough and lungs are immature [5].

In June 2000, The National Institute of Child Health and Human Development organized a workshop to characterize the definition of BPD and lung damage in preterm babies. They identified holes in information about development of the lung, the best clues of results for infants with BPD, and resolved preferences for future research [7]. As a result of this workshop, severity-based definition of BPD was introduced [3][7][8], and BPD was classified in to three groups; mild, moderate and severe (Table 1.1.).

The radiographic images of babies with this new form of BPD, usually occur of misty background and glass opacity, this is cleared when treated with surfactant and becomes a misty opacification by a couple of weeks and usually to a quite even pattern of off-colour, interstitial, opacities (Figure 1.2.) [5].

Table 1.1. Definition of BPD: Diagnostic Criteria [7]

Gestational Age	**≥ 32 weeks GA**	**< 32 weeks GA**
Assessment Time Point	28 days < PNA <56 days or discharge from hospital	36 weeks PMA or discharge from hospital
Treatment with oxygen >21 per cent for at least 28 days plus		
Severe BPD	Need for ≥30 per cent O_2 and/or positive pressure, at 56 days PNA or discharge from hospital	Need for ≥30 per cent O_2 and/or positive pressure, at 36 weeks PMA or discharge from hospital
Moderate BPD	Need for <30 per cent O_2 at 56 days PNA or discharge from home	Need for <30 per cent O_2 at 36 weeks PMA or discharge from hospital
Mild BPD	Breathing room air by 56 days PNA or discharge from hospital	Breathing room air at 36 weeks PMA or discharge from hospital

The babies, who are delivered several weeks before even the alveolarization begins and are at risk for developing the new form of BPD, usually have been seen to have mild RDS when born [2]. Though at this early developmental stage, the development of alveolarization and pulmonary microvascular growth can even get affected by essential exposure of injurious factors [2]. As a result, the normal structural development of the lung can be vanished by, the development of fewer and larger alveoli and smaller surface for gas exchange.

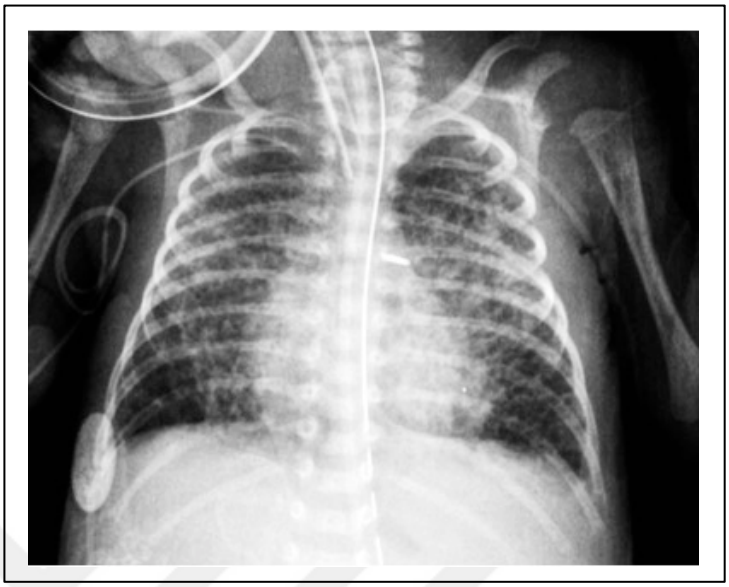

Figure 1.1. Chest radiograph of an infant with the new form of BPD [5][9]

It has also been reported by Husain and his colleagues [10], that there has been an evolutionary histological change in the new form of BPD. In the old form of BPD; the airway epithelial lesions are severe, the fibroproliferation is extensive, the alveoli and internal surface area is decreased, there is hyperinflation and there is vascular hypertensive lesions [10][11]. In the new form of BPD; the alveoli are simplified and larger, the airway epithelial lesions are negligible, the vascular hypertensive lesions are less severe compared to the old form of BPD, there are decreased dysmorphic capillaries, and the fibroproliferation is variable [10][11].

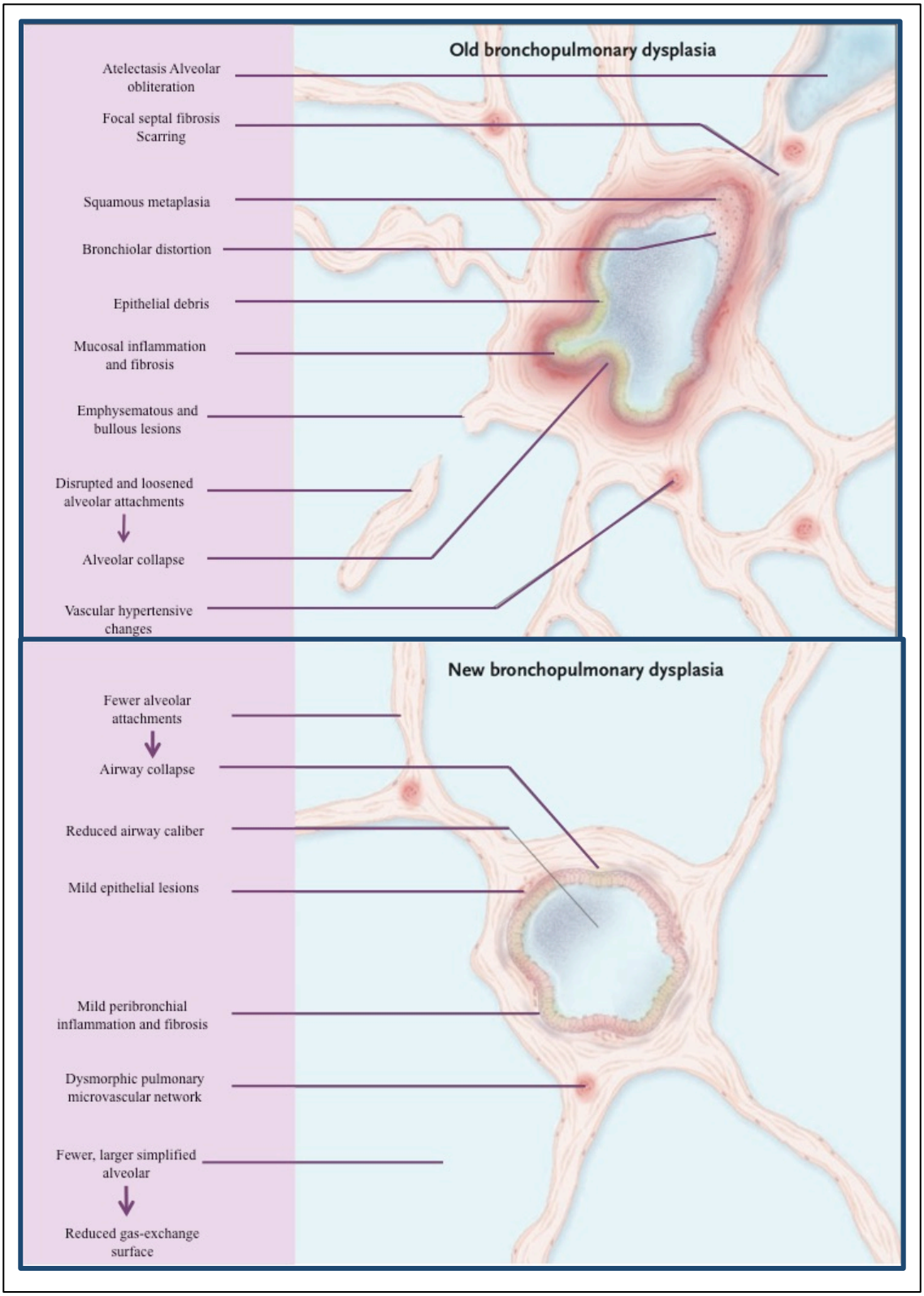

Figure 1.2. Histological differences between Old and New BPD [2]

1.1.1. The Incidence of BPD

The incidence of BPD differs depending on the definition used [6][12]. In 2005, a study done by the National Institute for Child Health and Human Development (NICHD) Neonatal Research Network demonstrated that, the incidence of BPD, which is defined as supplemental oxygen requirement at 36 weeks PMA [13], decreases with increasing birth weight [14]. BPD had an incidence of 52 per cent in infants with BW 501-750g, 34 per cent among infants with BW of 751-1000g, 15 per cent among infants with BW of 1001-1200g, and seven per cent in infants with BW of 1201 and 1500g [13][14].

1.2. PATHOGENESIS OF BRONCHOPULMONARY DYSPLASIA

Besides having an extremely immature lung, the pathogenesis of the new BPD involves, prenatal and/or postnatal inflammatory responses, pulmonary volutrauma, and decrease in oxygen levels due to treatment [15]. Premature infants have immature lungs [3], and many causes alone or in combination are likely to be damaging to the immature lung.

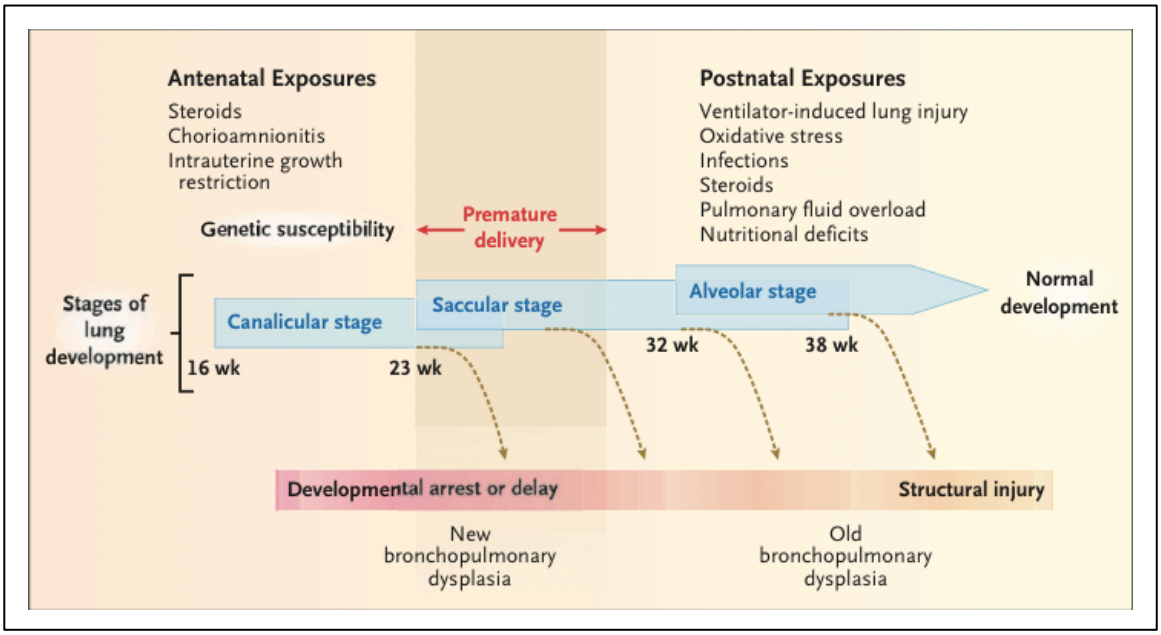

Figure 1.3. Stages of lung development, potentially damaging factors, and types of lung injury [2]

Various types of pulmonary damage may appear subject to the timing, length, and period of exposure to these factors (Figure 1.4) [2]. Factors such as oxygen toxicity, mechanical ventilation, ureaplasma, chorioamnionitis, Patent Ductus Artetiousus (PDA), surfactant deficiency, nutrition and genetic factors are have been implicated as crucial factors in pathogenesis of BPD.

1.2.1. Chorioamnionitis

Chorioamnionitis (CA), which is an inflammation of the fetal membranes, has been suggested in many studies to have a strong association with the development of BPD. The association is hard to evaluate, since the diagnosis of CA has many definitions [8]. For instance, clinical CA might only show acute inflammatory differences, while histological CA has may show chronic infection.

In a study done in 2005 by Demsey and colleagues [16], with 392 infants born at ≤ 30 weeks PMA, it was shown that there was a significant reduction in the incidence of RDS in the presence of CA, and that histologic CA increased the risk of premature delivery.

Even though, there are many clinical studies, which have shown the association of CA with the development of the lung [8], it is still uncertain whether CA has a direct impact on impaired lung development and BPD [17].

1.2.2. Hypoxia, Hyperoxia, and Oxygen Toxicity

BPD has been the best example for the outcome of oxygen toxicity in preterm babies, since it was described by Northway and colleagues [4][18]. Even though it is a disease caused by various factors, it is mainly caused by exposing the premature lungs with high levels of oxygen and as a result the production of reactive oxygen species (ROS), which damage the development of the immature lungs (Figure 1.5) [19][20].

The new form of BPD is a result of treatment with mechanical ventilation with high concentrations of oxygen to the neonatal intensive care unit (NICU), which leads to hyperoxia but on the other hand has increased the survival rate of preterm infants with RDS [19]. This exposure to hyperoxia remains one of the principle factors for the

development of BPD. Without consideration of GA, when changing from quite a hypoxic environment at birth to an *in utero* room, the air will always be hyperoxic to the infant [19]. Though, this extreme environmental change is manageable due to evolutionary-dependent mechanisms, which are also related to the development of the respiratory system. Unfortunately, the lungs of preterm infants are structurally and functionally immature, and therefore are often not developed enough to breath oxygen, and require oxygen therapy to prevent respiratory distress and hypoxia in tissue [19]. Therefore, the supplemental oxygen exposes the lung to hyperoxia. Even though, it is necessary to give supplemental oxygen in the neonatal period to support life, it has been shown with new-born animals that when exposed to hyperoxia during the growth and development of the lung, the lung was injured and had similar pathologic findings as seen in human BPD [19][21][22].

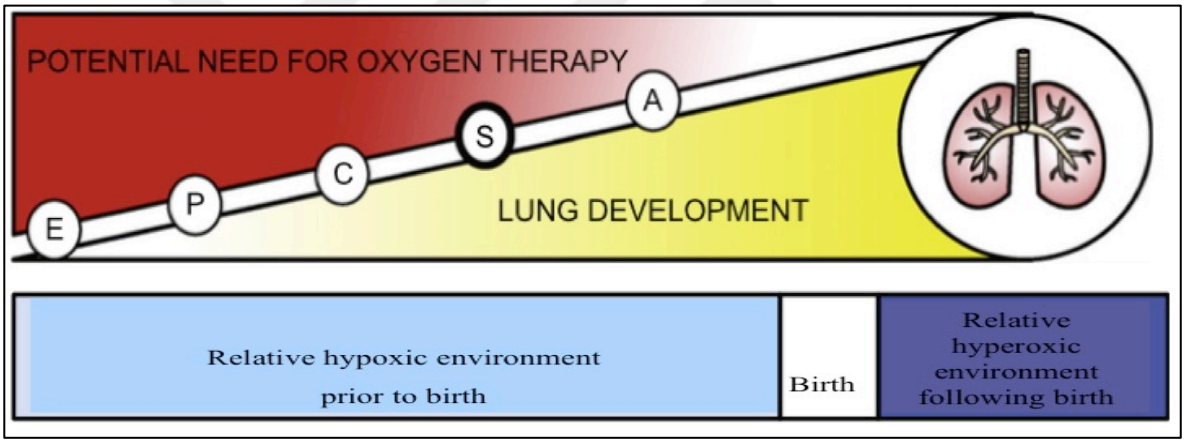

Figure 1.4. Supplemental oxygen requirements depending on the lung development [19]

Lung development is divided into five different stages, including the embryonic (E), pseudoglandular (P), canalicular (C), saccular (S), and alveolar (A) periods. With the exception of the alveolar period lung development primarily occurs in a relative hypoxic environment in utero until it transitions to a relative hyperoxic environment at birth. Lung development is disrupted when preterm infants, whose lungs are often in the saccular stage (bold) and developing under low oxygen conditions, transition into room air or are exposed to therapeutically elevated levels of oxygen.

The respiratory tract epithelium is exposed to 21 per cent oxygen, because of where it is located anatomically. Therefore, the cells that compose the lung are the first line to be effected by oxygen-induced injury [19]. This oxygen-induced injury, in other words “oxygen toxicity”, is regulated by ROS. ROS does have some benefits in many intracellular signaling pathways, but excess ROS results with oxidative stress of the cell, lipid peroxidation leading to cellular and tissue injury, protein oxidation and DNA damage [23].

Premature infants are almost always at risk for sustaining lung injury, which is caused by oxidative stress, because in humans the defences against antioxidants develop late in gestation. Therefore, in order for the lung to function properly, it is important to have adaptive response mechanisms, because it is hard and critical to balance the oxygen toxicity in a developing lung [19].

1.2.3. Mechanical Ventilation

Figure 1.5. Mechanical ventilation in a preterm infant [24]

Mechanical ventilation is life saving for premature infants with respiratory failure, but the damaged tissue and inflammation caused by it lead to chronic lung diseases [25][26].

In 1967, Taghizadeh and Reynolds [27], observed that the use of excessive high peak airway pressures during mechanical ventilation causes mechanical disruption to the lung, which is a very critical factor in the development of BPD [11].

It has been confirmed with animal models that, constant alveolar collapses and lung parenchymal injuries occur, when ventilating is done with poor positive and expiratory

pressure (PEEP) [28][29][30]. Albertine and colleagues [31] studied with preterm lambs and showed that the lung development was disrupted and pulmonary histopathologic changes were produced after using prolonged mechanical ventilation. The changes were very similar to those seen in the lungs of preterm infants who die with BPD [11][31]. Studies done with premature baboons showed that when baboons are ventilated with high frequency oscillations there is reduction in air leak, increase in survival and improvement in maintenance of the alveolar architecture compared to the premature baboons, which are ventilated with conventional ventilation [29][32][33].

Avoiding mechanical ventilation may reduce the risk of lung injury and the incidence of BPD [8].

1.2.4. Surfactant Deficiency

Pulmonary surfactant is a surface-active phospholipid protein complex, which is formed by type II alveolar cells and lines a thin film at the air-water interface of the lung. It increases pulmonary compliance, prevents atelectasis at the end of expiration and facilitates the recruitment of collapse airways. Deficient pulmonary surfactant in premature infants may lead to RDS. Surfactant therapy is applied to prevent and cure this situation.

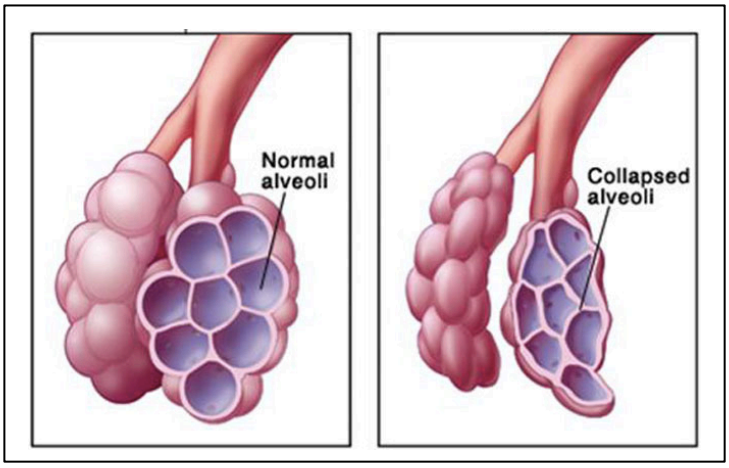

Figure 1.6. Comparison of normal and collapsed alveoli [34]

Studies have shown surfactant abnormalities in premature infants with BPD [35][36][37].

1.2.5. Infection

In utero inflammation and late respiratory morbidity association has encouraged the investigation for causative infectious organisms [29].

Genital mycoplasmas, especially *Mycoplasma hominis* and *Ureaplasma urealyticum* (Uu), have been shown to cause preterm delivery and to colonize and stimulate inflammation in the lower respiratory system [29][38].

Uu is a microorganism, which is generally isolated from the amniotic fluid in preterm births, and it is also the main pathogen detected in airway secretions just after birth [40]. Many studies suggest that the colonization of *Ureaplasma* in the respiratory tract is associated with the development of BPD [39]. Thus, its potential role in the development of BPD still remains uncertain.

1.2.6. Patent Ductus Arteriosus

Patent ductus arteriosus (PDA) is a congenital disorder in the heart, wherein an infant's ductus aretiosus fails to close after birth. The role of the PDA in the evolution of BPD remains uncertain, though the presence of PDA has been associated with higher mortality and the usage of mechanical ventilation and therefore a higher risk of developing BPD [8]. During PDA, the pulmonary blood flow is increased and the physiological compensation is lost. This leads to edema and damage to the endothelial tissue [41].

1.2.7. Nutrition

The possible outcomes of bad nutrition on the respiratory function have been considered as a possible aetiology of BPD for a while [42], since the normal lung development is dependent on sufficient nutrition [29]. Studies done with animal models have shown that fetal or early neonatal nutritional restriction reduces alveogenesis with enlarged alveoli and thickened septa for age.

1.2.8. Genetic Factors

Recent studies have shown genetic susceptibility for BPD is as a potential factor in the development of BPD [11]. Genes, which express proteins that are important for the regulation of inflammation, development of vascularization and synthesis of surfactant [2], are likely to be associated with the development of BPD. The association between genetic factors and BPD is further discussed in section 1.6.

1.3. THE TREATMENT AND MANAGEMENT OF BRONCHOPULMONARY DYSPLASIA

Treating the evolving BPD is challenging because of the complex act and balance between the risk factors [43] and since there are many factors contributing to the development of BPD a multidisciplinary method is important for an efficient treatment [44]. Avoiding and limiting the injury to the lung caused by supplemental oxygen and positive pressure ventilation, controlling the fluid balance, avoiding infection and providing the optimal nutrition are the general treatment goals [43].

There are many therapies used in the management of BPD, but these therapies are still not evidence based [45][46]. Although common treatments such as surfactant, caffeine therapy, antenatal steroids, optimization of nutrition, targeted oxygen saturation goals, vitamin A therapy and protective ventilation strategies have helped to improve the outcomes of BPD and are supportive, they have also changed the course of BPD [47][48].

1.3.1. Vitamin A

A group of fat-soluble compounds called retinoids is defined by the derivatives of vitamin A. These compounds play a critical role in lung diseases, because they are important in regulating and the promoting differentiation and growth of the lung epithelial cells [49]. It has been shown in animals that vitamin A deficiency results with abnormal lung growth, which is followed by hypoxia [50]. Further on, preterm infants have low vitamin A levels at birth, and low vitamin A levels have been shown to be a risk factor for developing BPD [3][51][52].

In a trial done in 1987, vitamin A supplementation showed a clear benefit in reducing BPD [53], further on these findings were supported in a larger study in 1999, in which infants with a BW < 1,000 g were given vitamin A as a prophylactic supplementation [42][54]. Treatment with vitamin A is one of the only few methods that has been proven to show an effect on reducing the presence of BPD [5].

1.3.2. Caffeine

Caffeine is a methylxanthine and it is used for the treatment of apnea during prematurity [49]. Even though the mechanisms are unclear, this treatment has been shown to decrease the risks of BPD by increasing the respiratory muscle performance, the CO_2 chemoreceptor responsiveness, and the central nervous system excitability [8][55][56][57]. During the international Caffeine for Apnea of Prematurity (CAP) trial, which compared caffeine to placebo given within the first 10 days after birth, the infants who had the treatment had a lower incidence of BPD (defined by oxygen need at 36 weeks PMA).

1.3.3. Corticosteroids

Corticosteroids (also known as glucocorticosteroids, glucocorticoids or just steroids) are among the most widely used drugs in the world and are effective in many inflammatory and immune diseases [58]. They are a class of chemicals, which are produced in the adrenal cortex, such as steroid hormones.

During chronic inflammatory diseases, such as asthma, various inflammatory genes are activated. Corticosteroids can suppress these genes by recruiting histone deacetylase-2 (HDAC2) and binding the activated glucocorticoid receptors (GR) to the co-activators. [58].

Since inflammation plays an important role in the pathogenesis of BPD, the use of corticosteroid makes physiologic sense because of their anti-inflammatory properties [47].

1.3.4. Inhaled Nitric Oxide

Inhaled nitric oxide (iNO) is a selective pulmonary vasodilator and does not have short-term side effects; therefore infants with hypoxemic respiratory failure and pulmonary hypertension are commonly treated with it. [5][59].

Clinically there have been many randomized control trials (RCT), which have assessed the effect of iNO treatment for BPD, though these results have not been reliable [5]. An observational study done by Donohue and colleagues [60], failed to show a statistically significant difference in the incidence of BPD at 36 weeks, though the study did show a slight difference in favour of iNO therapy on the outcome of death.

Table 1.2. A summary of strategies to prevent or ameliorate BPD [45]

Methods which have been confirmed to treat BPD	**Methods which have shown short-term help on lung function, but no confirmed benefit on BPD**	**Methods with no confirmed benefit**
Caffeine	Diuretics	Erythromycin
Targeting lower SpO	iNO	α1-Proteinase inhibitor
Vitamin A		SOD
Prophylactic surfactant		
Corticosteroids		

1.4. LONG TERM OUTCOMES OF BRONCHOPULMONARY DYSPLASIA

It is known and shown that BPD survivors have lifelong morbidities. Children, who have survived BPD, have higher rates of educational, cognitive and behavioral disabilities, and reduced lung function. Even during the first year after birth, the infants affected with BPD have up to 50 per cent higher rates of rehospitalization [61].

1.4.1. Neurodevelopmental Outcomes

In neurodevelopmental outcomes, the independent effect of BPD is hard to prove, given that most of the infants have additional medical complications and that most of the studies have been cross-sectional [61]. Even though these infants have a risk of neurodevelopmental damage, BPD is also an additional risk factor [61][62]. The most common neurological outcome of BPD is cerebral palsy, which is a neurosensory problem. Other than cerebral palsy some infants with BPD have motor, visual and auditory problems [63][64], have lower average IQ [64][65][66][67][68][69], delayed speech and language learning [70], behavioral problems [65][67], more attention problems, memory and learning deficits [71].

1.4.2. Pulmonary Outcomes

During late adolescence, significant impairment and deterioration in the function of the lung is observed in patients with BPD. [61][72] [73]. These ages (late teens and early twenties) are the oldest ages that have been reported to have lung function impairment.

A study was done by Northway and his colleagues in 1990 [74], to show the respiratory long-term outcomes of BPD. They compared 26 subjects, who were around 18 years old, had old BPD, and born between 1964 – 1973; to 26 age-matched controls who had not been ventilated and who had similar BW and GA; and 53 age-matched normal subjects who had also not been ventilated, not born premature, not have a history of a chronic lung diseases and were non-smokers. As a result they saw that; 68 per cent of the BPD subjects had airway obstruction, which was reversible but fixed in 24 per cent, had reduction in

airway variables reflecting airflow, 25 per cent of them had one or more severe abnormalities in lung function, they had more limitation of exercise capacity, episodes of pneumonia, long-term medication use and wheezing, compared to the other two groups. This study indicated that subjects with BPD have poor respiratory functioning and more respiratory illnesses in their later life than those of similar BW and GA who did not have BPD [74][75].

1.5. BRONCHOPULMONARY DYSPLASIA AND ASTHMA

Asthma is a complex respiratory disease, which is caused by the interaction between genetic and environmental factors [76][77]. In 1980, Nickerson and Taussing [79], reported that airways with a genetic predisposition becomes highly reactive after the treatment of neonatal lung disorders and this then contributes to the development or/and progression of BPD [78][80]. Nickerson and Taussing [79], were the first to suggest that family history of asthma may be associated with BPD in neonates.

Their publication is largely cited in the neonatal field [80], and many other studies have been carried out to investigate this hypothesized association. However, there are not many studies, which confirmed Nickerson and Taussing's hypothesis. In 1995 Hagan and colleagues studied with preterm infants, that were born between 1987 and 1988, but did not find a family history of asthma to be associated with BPD or any other kind of chronic lung disease of prematurity [80][81]. A study done by Evans and colleagues in 1995 [80], done with neonates with BW <1,501g and born between 1988 and 1991, also failed to confirm an association between family history of asthma and BPD.

Increasing smooth muscle contractions is a common characteristic that BPD and asthma both share, besides in asthma smooth muscle might have a role in developing the asthmatic phenotype [78][82][83]. The definition of asthma, a condition characterized by allergic-induced bronchospasm [78], suggests that considerable overlap may exist between the airway pathology of asthma and BPD. They both contain elements of reversible airway obstruction, bronchial hyperresponsiveness and airway inflammation [78]. They both tend to cluster in families and share wheezing phenotypes. Overlap exists between asthma and

BPD, with regard to the presence of elevated concentrations of airway inflammatory mediators concurrent with reduced levels of anti-inflammatory activity [78].
Wheezing is a common finding among children with BPD, which arises the question whether BPD is a phenotypic variant of asthma. More carefully managed and well-controlled studies should be done to prove Nickerson and Taussing's hypothesis, though it is commonly accepted that one way or another there is a relationship between BPD and asthma.

1.6. BRONCHOPULMONARY DYSPLASIA AND GENETICS

In addition to environmental factors, the results of twin studies have pointed out that genetic factors have also an important role in the pathogenesis of BPD, and that BPD could be heritable.

1.6.1. Single Nucleotide Polymorphisms

A single nucleotide polymorphism (SNP) is a single base change in a DNA sequence, which usually has an alternative of two possible nucleotides at a specific position [84]. These changes can be used to compare genomic DNA sequences in different individuals [85]. SNPs are predicted to occur at 1 out of every 1,000 bases in the human genome, which makes them highly abundant [85][86][87].

Serious consequences might occur to an individuals phenotypic characteristics depending on where the SNPs occur [85]. A SNP in the coding region of a gene would change the structure or function of the protein to be encoded by the gene [85]. This kind change in a protein is an enough cause of most of the known dominantly or recessively inherited monogenic disorders [85]. A SNP in the regulatory region of a gene might have an influence on increasing the risk of a common disease. Though, most of the SNPs to be known are located in the non-coding regions of the genome, therefore have no direct known impact on the phenotypic characteristics of an individual [85], and are being used as useful markers in evolutionary studies and population genetics [88][89].

1.6.2. Heritability of BPD

Many different studies have been done to reveal the heritable factors influencing the risk of BPD in premature babies. These studies include twin studies, genome wide association (GWA) studies, familial aggregation, and candidate gene studies.

Twin studies are very effective studies to discover genetic versus environmental factors of complex human diseases [90], since they are brought up in the same environment and dizygotic twins share 50 per cent of their genetic information and monozygotic twins share 100 per cent of their genetic information. Parker and colleagues [91] were the first to use twins to investigate the possibility that the development of BPD is affected by genetic factors. For this study, they analyzed 108 twin pairs of infants with BW $\leq$ 1,500 g. They observed that when BPD developed in the first-born twins (n = 23), it also developed 65 per cent (n = 15) in the second born twins, and if BPD was not developed in the first-born twins (n = 85), it only developed eight per cent (n = 7) in the second born twins. BPD status in the first-born twins was found to be a statistically significant predictor of BPD in the second born twins (adjusted OR = 12.3, $p < 0.001$), after they adjusted other risk factors (BW, GA, sex, RDS, pneumothorax, PDA, birth order and Apgar scores).

In 2006 Bhandari and his colleagues [92] used 450 twin pairs to determine the heritability of BPD, and showed that genetic factors account for 53 per cent of the variance in the liability for BPD after controlling the environmental risk factors.

In a Canadian based study in 2008, Lavoie and colleagues [93] used 318 dizygotic twins and showed a higher heritability, 79 per cent, for moderate to severe BPD in infants with GA $\leq$ 30 weeks.

These twin studies suggest that genetics factors play an important role in the development of BPD. The heritability of BPD is estimated to be between 50 per cent and 80 per cent among preterm infants [94].

1.6.3. Candidate Genes

The development of the respiratory system begins at 28 days of gestation, though branching occurs later [95]. There are many genes, which finely regulate this process and the balance between pro- and anti- inflammatory cytokines [96]. Therefore, most candidate gene association studies of BPD have focused on the genes involved in lung development, pro- and anti- inflammatory cytokines, sepsis, tissue repair, cell injury and death, and oxygen toxicity [96][97]. A list of the candidate gene studies is shown in Table 1.3.

Table 1.3. Summary of candidate gene association studies of BPD

Pathway	Associated Gene	References
Lung development: alveolarization, vasculogenesis	*ACE*	Kazzi and Quasney 2005 [98]
	F-7	Hartel *et. al.* 2006 [99]
	MMP-16	Hadchouel *et. al.* 2008 [100]
	VEGF	Mailaparambil *et. al.* 2010 [12]
	SPOCK-2	Hadchouel *et. al.* 2011 [101]
	FGF-4	Rezvani *et. al.* 2013 [102]
	KITLG	Huusko *et. al.* 2014 [103]
	VDR Fok-1	Koroglu *et. al.* 2014 [104]
Innate immune system and inflammatory responses	*GST*	Manar *et. al.* 2004 [105]
	SP-B	Rova et. al. 2004 [106], Hallmman *et. al.* 2007 [107], Zhang *et. al.* 2015 [23]
	TNF-α	Kazzi *et. al.* 2004 [108], Mailaparambil *et. al.* 2010 [109]
	MBL-2	Hilgendorff *et. al.* 2007 [110]
	TLR-10	Mailaparambil *et. al.* 2010 [12]
	MIF	Prencipe *et. al.* 2011 [111]
	HLA	Rocha *et. al*. 2011 [112]
	TLR-5	Sampath *et. al.* 2012 [113]
	TLR-4	Lavoie *et. al*. 2012 [114]
	NFK-β-I-α	Ali *et. al.* 2013 [115]
	TLR-6	Winters *et. al*. 2013 [116]

1.7. INTERLEUKIN – 33 (IL-33)

Interleukin-33 (IL-33) was first named as NF-HEV (nuclear factor from high endothelial venules), since it was known to interact with the nuclear chromatin in endothelial cells [117][118][119]. In 2005 it was rediscovered by Schmitz and colleagues [120], who used computational tools, while searching for Interleukin-1 (IL-1) family members. This study also revealed that IL-33 has the closest amino acid sequence homology to IL-18 within the IL-1 family [118][122].

IL-33 is the 11th member of the IL-1 cytokine family and is a ligand for the ST2 receptor, which is also known as Interleukin-1 Receptor-like 1 (IL-1RL1) [121]. ST2 is a member of the Interleukin-1 Receptor (IL-1R) family, is a member of the Toll-like receptor (TLR)/IL-1R (TIR) superfamily [122]. This receptor has 3 isoform proteins by differential splicing (Figure 1.8). The membrane-bound ST2, which contributes to the biological effects of IL-33; the soluble ST2, which inhibits IL-33 from binding to the membrane-bound ST2; and a variant ST2.

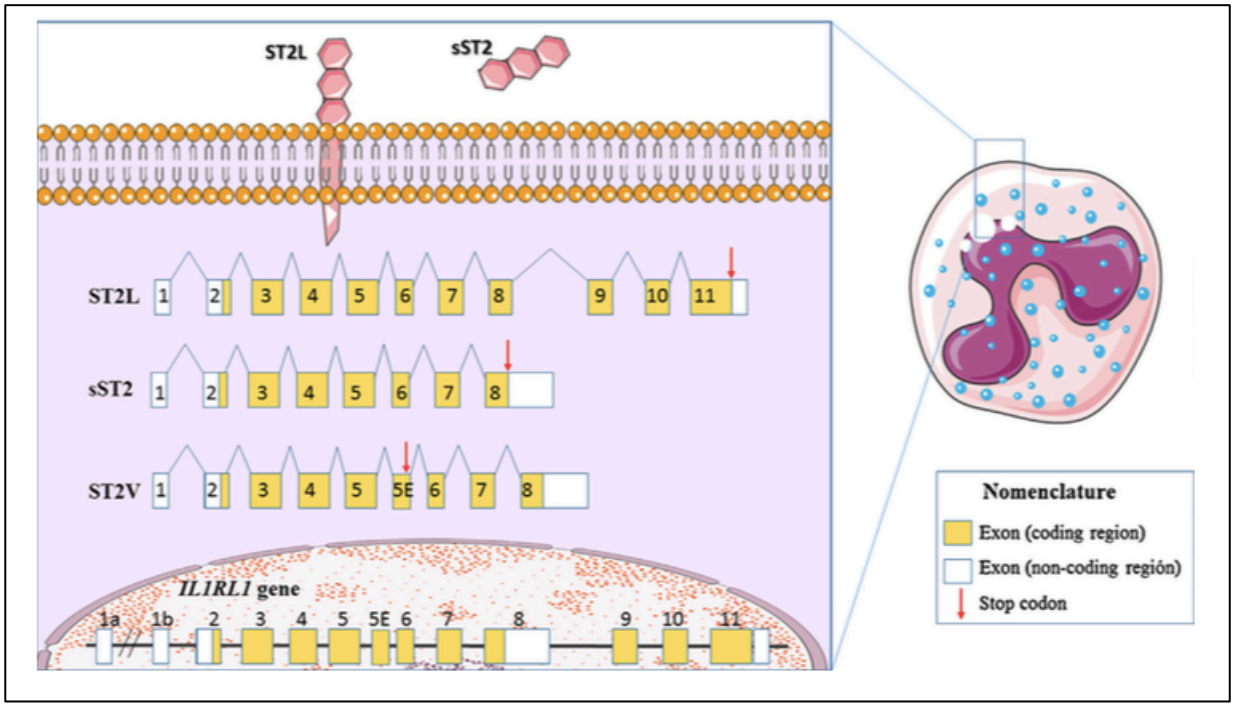

Figure 1.7. Coding regions of the isoforms of ST2 [129]

IL-33 mRNA is expressed by a variety of cells in human and mice. It is mainly expressed by fibroblasts, adipocytes, endothelial cells, epithelial cells and smooth muscle cells, and is released when cells undergo necrosis. Therefore unless there is a pro-inflammatory stimulus, IL-33 localizes full-length in the nucleus [118][122]. The amino terminus of IL-33, which also has a chromatin-binding domain, mediates this localization. The carboxyl terminus is the IL-1 like domain, and mediates the cytokine activity (Figure 1.9) [123]. The full-length IL-33 is the biologically active form of IL-33.

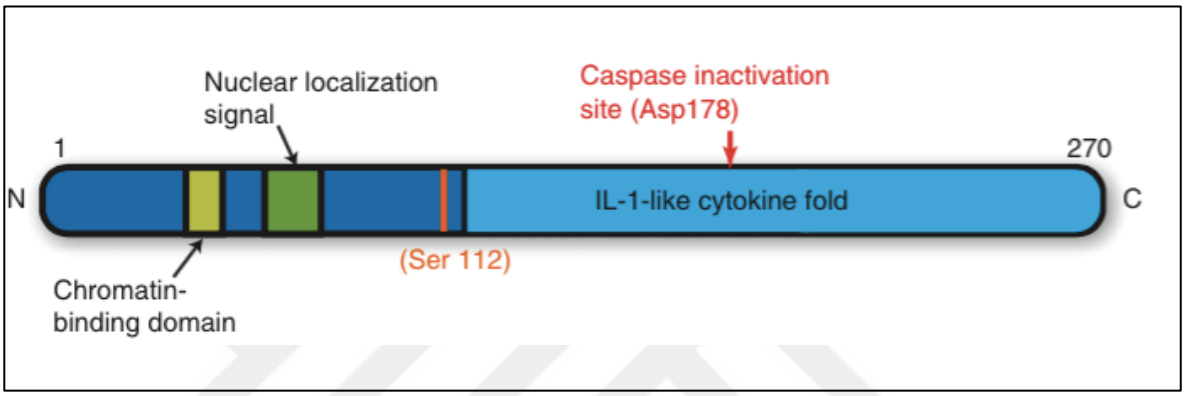

Figure 1.8. Representation of the IL-33 protein [123]

IL-33 is cleaved by caspase 3 and caspase 7 during apoptosis, which is suggested to be a mechanism gained evolutionary, to restrict the release of biologically active full-length IL-33 during apoptosis (Figure 1.10) [122].

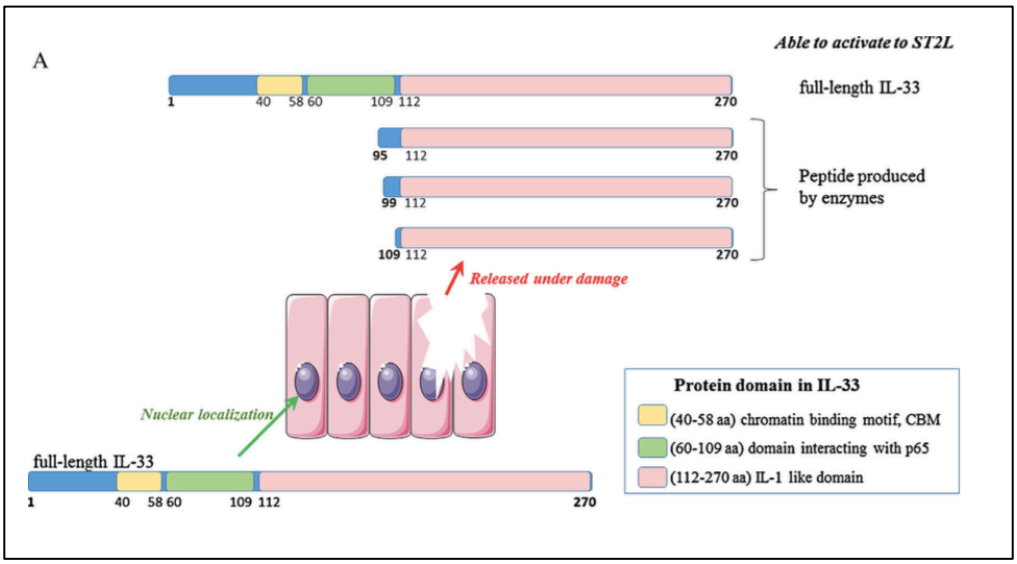

Figure 1.9. Variants of IL-33 [129]

1.7.1. IL-33 Signaling Pathway

The binding of IL-33 to a heterodimeric receptor complex, which consists of ST2 and IL-1R accessory protein (IL-1RAP), recruits the myeloid differentiation primary response protein 88 (MYD88) complex, which leads to the activation of nuclear factor -κβ (NF-κβ) and mitogen activated protein kinase (MAPK) pathway.

The activation of NF-κβ also leads to the degranulation of IgE primed mast cells and the production of IL-1β, IL-3, IL-6, TNF, CXC- chemokine ligand 2 (CXCL2), CC-chemokine ligand 2 (CCL2), CCL3, prostaglandin D_2 (PGD_2) and leukotriene B_4 (LBT_4) (Figure 1.11) [122].

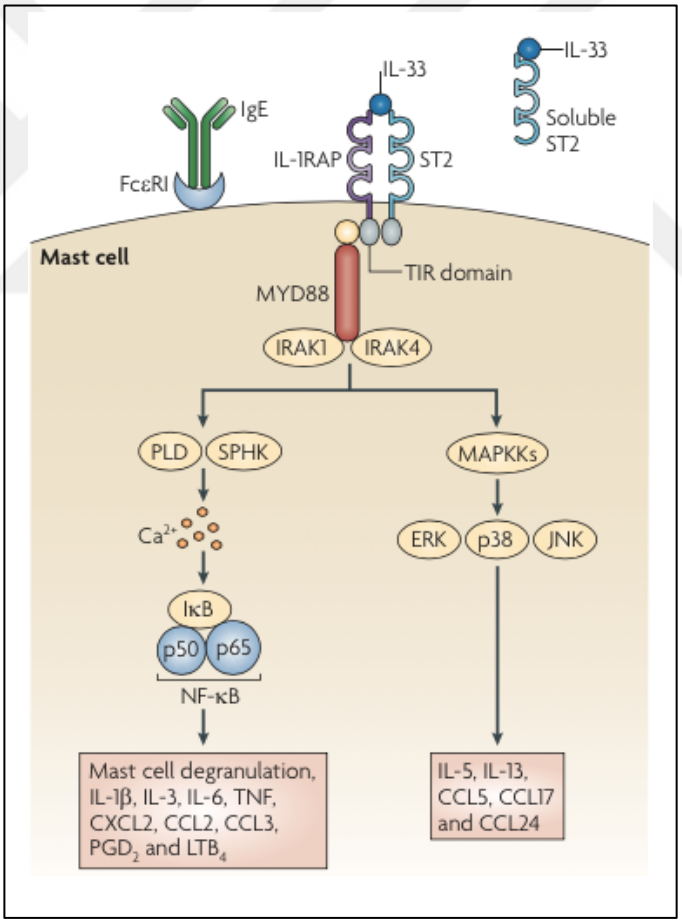

Figure 1.10. IL-33 signaling pathway [122]

1.7.2. Target Cells of IL-33

There are many cell types, which express membrane-bound ST2 (Figure 1.12). The expression of this form of ST2 was first found in T helper 2 (T_H2) cells, and not T helper 1 (T_H1) cells [122][124][125], therefore the role of IL-33 was first studied in T cells. It was found that when T_H2 cells were treated with IL-33, IL-5 and IL-13 production was enhanced [120], and IL-33 polarized naïve T cells, which led to the production of IL-5 and IL-13 independently of IL-4 [122][126], the major cytokine that leads to the differentiation of naïve T cells to T_H2 cells [118].

As described in the previous section, IL-33 induces pro-inflammatory cytokines and chemokines by mast cells. It triggers the degranulation of IgE-primed mast cells and enhances them to mature and survive [122]. Through the induction of IL-33, basophils produce various cytokines and chemokines, such as IL-13, IL-6 and IL-4. The secretion of these cytokines and chemokines enhance the basophil adhesion, survival, integrin expression, degranulation and chemotaxis. [122][127][128]. IL-33 has been shown to induce eosinophils *in vivo* [120], and activate them to produce superoxide and CXCL8. It upregulates the expression of adhesion molecules, which enhances eosinophil adhesion and survival [127].

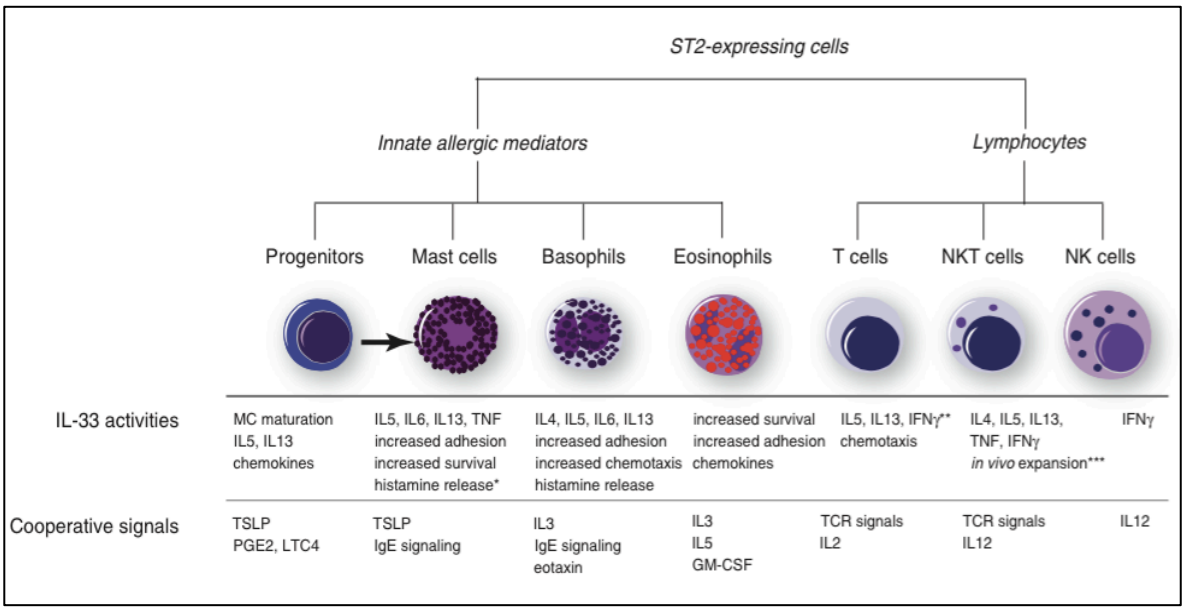

Figure 1.11. Cellular activities of IL-33 [123]

Mast cells, basophils and eosinophils have important roles in allergy responses. This suggests that IL-33 has a critical role in allergy, septic shock and asthma [122].

1.7.3. IL-33 as an Alarmin

In tissue injury during infection or trauma, necrotic cells release damage associated molecular patterns (DAMPs), also called alarmins, to alert acquired-type immune cells as a danger signal [130][131]. For example, high-mobility group box 1 (HMGB1), which is a nuclear factor, is released by dying necroctic cells and acts as a pro-inflammatory cytokine [119][130]. Like HMGB1, IL-33 is also a nuclear factor. In addition, IL-33 is released from dying damaged cells, which signals innate immune system cells that there are potentially harmful pathogens and IL-33 is expressed in cells of many blood barriers. These have led to the suggestion that IL-33 is an alarmin.

1.7.3. The Role of IL-33 in Diseases

There are many studies, which have implicated that the IL-33 signaling pathway is associated with various diseases. IL-33 has two different roles in diseases; it is associated with host-protection against infections by promoting T_H2 cells [122], and it can lead to mast cell and T_H2 cell mediated inflammatory diseases, such as bowel disease.

The major influence in the evolution of the immune system is the need to fight infection [122]. Thus, it is possible that IL-33 evolutionarily maintained a role in defense against pathogens. Up till now, there have been many studies, which have identified the role of IL-33 against pathogens such as *Leishmania major* [124], *Toxoplasma gondii* [156], *Nippostrongylus brasiliensis and Trichinella spirals* [157], *Schistosoma mansoni* [158] [159], *Trichuria muris* [132], *Pseudomonas aeruginosa* [160], *Mycobacterium tuberculosis* [161], *Leptospira* [162] and some viruses such as respiratory syncytial virus infection [133] and the dengue virus infection [134].

IL-33 has been found to be associated with cardiovascular diseases [142][143][144][145] [146], skin disorders, central nervous system inflammation [147][148][149][150], pain [151], allergy [141] and other inflammatory diseases such as arthritis [152][153][154][155].

There is an accumulation of evidence that reveals cytokines contributing to carcinogenesis, by having pro- or anti- tumour roles. This would depend on the balance between various inflammatory mediators and the phase of the tumour development [129][163]. In endothelial cells, IL-33 induces morphological differentiation, migration and proliferation, and a viable effect on angiogenesis [124][164][165]. Further on, IL-33 expression is absent in tumours, while it is expressed in endothelial cells of the healthy tissue [166]. Thus, IL-33 might be playing an important role in tumour angiogenesis and tumour escape from the immune system.

1.7.4. IL-33 and Asthma

Up till now asthma has been characterized as a T_H2 cells mediated disease, but therapeutic approaches targeting T_H2 cells responses are not satisfying. Therefore a new view has been proposed. Recent studies are investigating the significance of pulmonary epithelium in propagating and directing allergic responses [207]. Epithelial cells are the sources of IL-33, thus the IL-33/ST2 signaling pathway is thought to play a key role in the pathogenesis of allergy and asthma [207][208][209].

The first line of host defense against allergens and inhaled infectious organisms are airway epithelial cells [207][210]. These cells express pattern recognition receptors (PRRs), which can detect danger signals in the environment. Once these receptors are activated, the epithelial cells release chemokines and cytokines, including high levels of IL-33, which then alert and activate the immune system (Figure 1.13) [207].

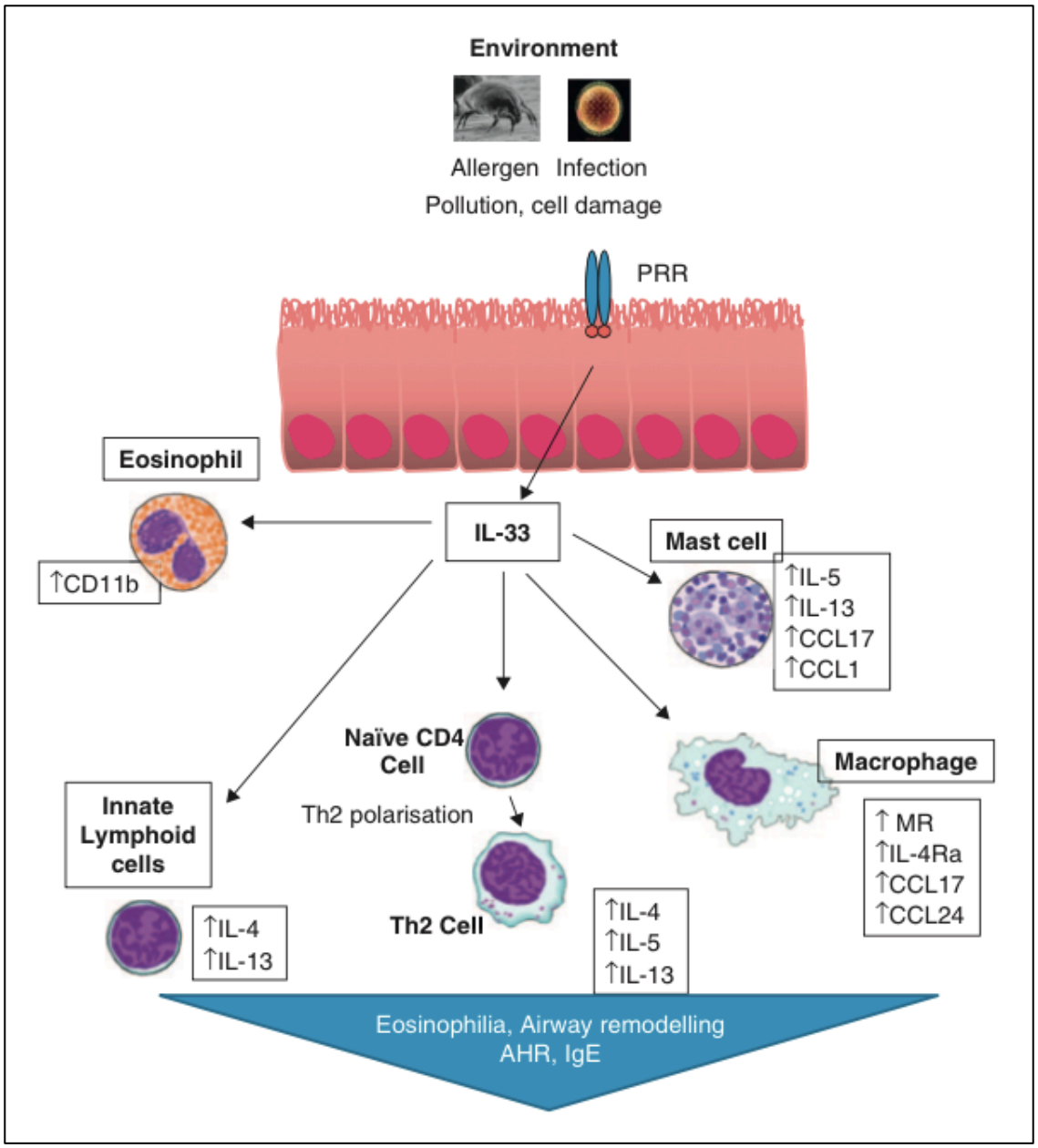

Figure 1.12. IL-33 in asthma [205]

Recent studies have shown that the master regulators of the IL-33 pathway are dendritic cells [207]. Toll-like receptor-4 (TLR4) signaling induces the release of IL-33 by airway epithelial cells, as well as IL-25 and thymic stromal lymphopoietin (TSPL), which then recruit and activate dendritic cells. Activated dendritic cells promote allergen specific T_H2 responses. Studies [210][211][212][213][214][215][216] have proven that dendritic cells express ST2, and the binding of IL-33 and ST2 triggers their proliferation and maturation, and triggers them to trigger activated T cells. (Figure 1.14)

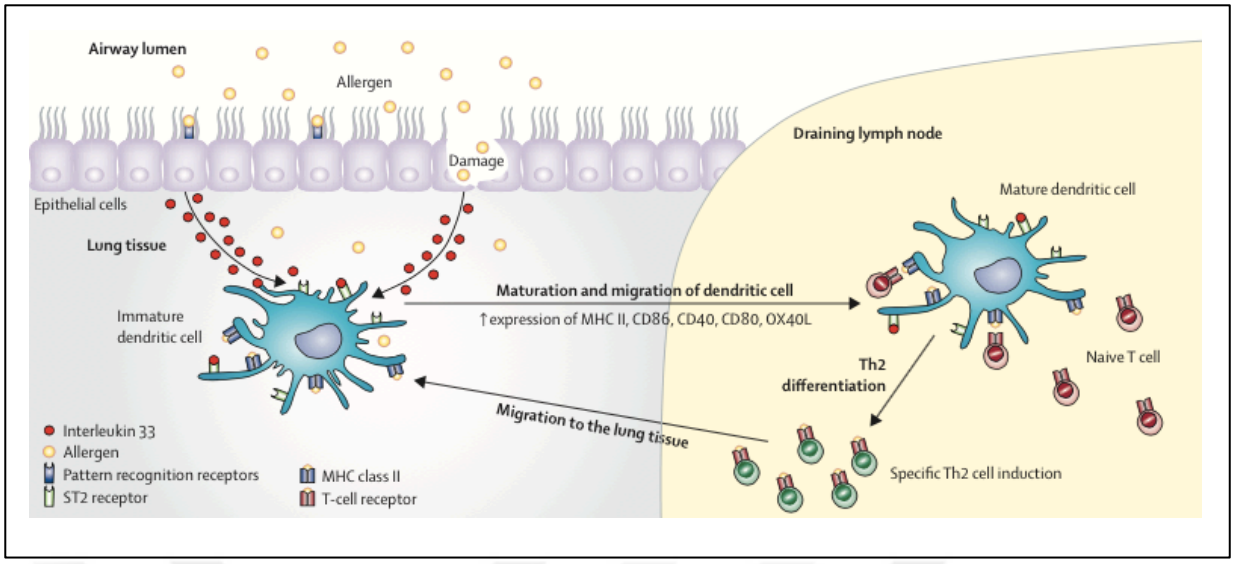

Figure 1.13. Role of IL-33 activated dendritic cells in asthma [207]

Studies done with animals, particularly mice, have shown that asthma is attenuated when IL-33 and/or ST2 is blocked, IL-33 expression increases in experimental and clinical asthma, and that when IL-33 is administrated some features of asthma is induced [125][126][135][136][137][138][139][140].

Asthma is a complex disease and results from the interaction of environmental and genetic factors. After identifying the key role of IL-33 in development of asthma, the IL-33 and IL-1RL1 genes have become susceptible genes in the development of asthma. There have been many SNP studies, including GWAS done, regarding this question. A summary of polymorphisms in IL-33 and IL-1RL1 associated with asthma is given in Table 1.6.

Table 1.4. A summary of polymorphisms in IL-33 and IL-1RL1 associated with asthma

Gene	SNP	Association	Population	Reference
IL-1RL1 (ST2)	rs6543116	Childhood Asthma	Australia	[217]
IL-1RL1 (ST2)	rs1420101	Eosinophils and Asthma	Mixed populations	[218]
IL-1RL1 (ST2)	rs1861246	BHR and Asthma	Dutch	[219]
IL-1RL1 (ST2)	rs1921622	BHR and Asthma	Dutch	[219]
IL-33	rs3939286	Eosinophils and Asthma	Mixed populations	[218]
IL-33	rs1342326	Asthma	European and North American	[220]
IL-33	rs2381416	Asthma	Mixed population	[221]
IL-33	rs928413	Asthma	Italian	[222]
IL-33	rs2066362	Asthma	European and North American	[220]
IL-33	rs16924159	Asthma	White (Non-Hispanic)	[223]
IL-33	rs12551256	Asthma	White (Non-Hispanic)	[223]
IL-33	rs7025417	Asthma	White (Non-Hispanic)	[223]

1.8. INTERLEUKIN – 18 RECEPTOR 1 (IL-18R1)

Like IL-33, Interleukin-18 (IL-18) is also a member of the IL-1 family, and was first originally identified as an IFN-γ-inducing factor [167][168] from T_H1 cells in 1995 (Figure 1.15).

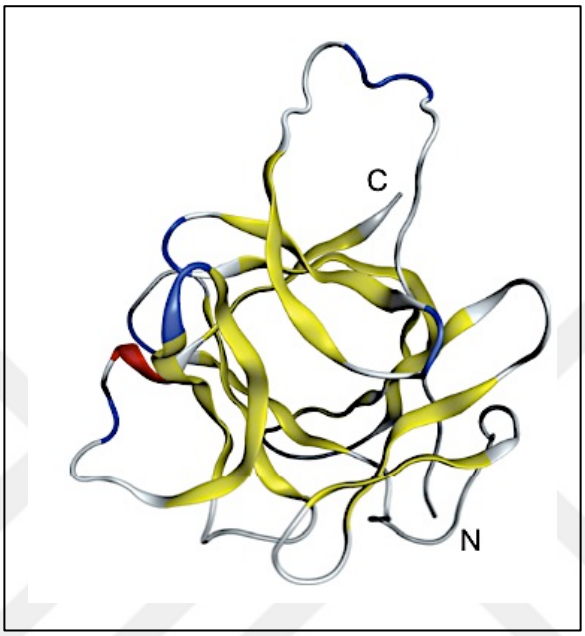

Figure 1.14. Structure of IL-18 [167]

IL-18 mRNA is expressed by a variety of cells in human and mice. High levels of IL-18 mRNA expression were first observed in macrophages, including Kupffer cells [169][170][171]. Further studies showed that IL-18 is not only produced by immune system cells, but it is also produced by non-immune system cells [172]. As a response to stimuli, keratinocytes [173], dendritic cells [174], human peripheral blood mononuclear cells [175], osteoablastic stromal cells [176], intestinal epithelial cells [177] and airway epithelia [178] also express IL-18.

IL-18 is synthesized as a biologically inactive 24-kDa precursor, pro-IL-18 [172]. Pro-IL-18 is cleaved by caspase-1 into an 18-kDa mature and active form. Caspase-1 is found in various cell types and like other caspases, it is first produced in a biologically inactive form [172], pro-caspase-1. Pro-caspase-1 is activated by an inflammasome (Figure 1.16).

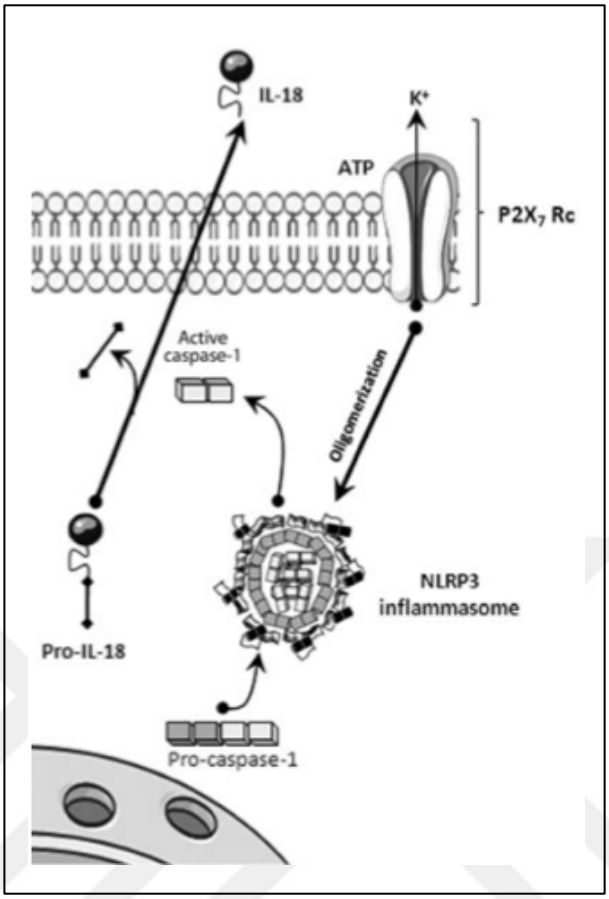

Figure 1.15. Activation of IL-18 by caspase-1

1.8.1. Interleukin-18 Receptor (IL-18R)

Once IL-18 is released, it binds to its receptor IL-18R. The IL-18R system has a very similar system to the IL-1R system [168][172], and it is a member of the IL-1R/Toll-like receptor (TLR) superfamily. There are two subunits, which compose the receptor complex. These are the IL-1R1 subunit, which is the ligand binding subunit, and the IL-1R accessory protein (IL-1RAcp), which is the signal transducing subunit.

In 1997, Torigoe and colleagues purified the IL-18 binding receptor from the Hodgkin's disease cell line [179], and determined that the ligand binding receptor of IL-18 was IL-1 receptor-related protein (IL-1Rrp). A further study done in 1998 [180] concluded that, even though IL-RAcP-like protein (IL-AcPL) does not bind to the ligand it self, it is required for the IL-18 signalling, by forming a high affinity heterotrimetric complex with IL-1Rrp and IL-18. Therefore, the IL-18R complex consists of a ligand binding chain, IL-

Rrp, which is also termed as IL-18Rα or IL-18R1, and a signal transducing chain, AcPL, which is also termed as IL-18Rβ (Figure 1.17).

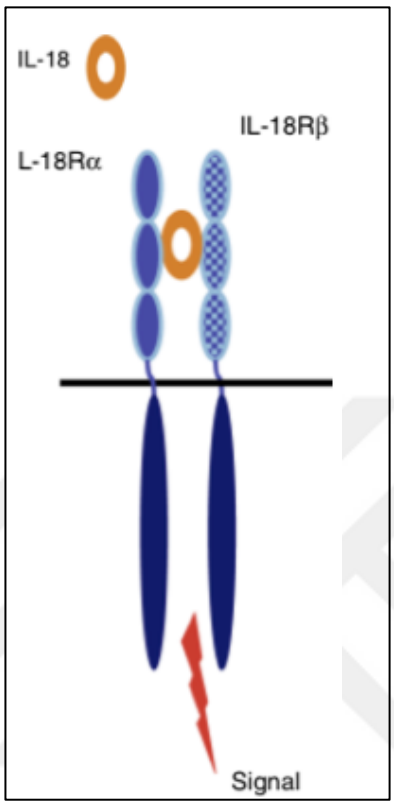

Figure 1.16. The IL-18R complex [168]

To determine the role of IL-18R1, a study was done in 1999 by generating IL-18R1 deficient mice [181]. When triggered with IL-12 and Ag, Naïve T cells develop into T_H1 cells. As a result the T_H1 cells express both low and high affinity IL-18R and with the binding of IL-18 to the receptors, produce IFN-γ [172]. Though, IL-18 did not bind to the IL-18R1 deficient mice's T_H1 cells and did not activate the JNK and NFκB pathways.

IL-18R1 is highly expressed on T_H1 cells, which could be used as a surface marker for T_H1 cells. Further on, IL-18R1 mRNA has been detected in many different types of organs in mice; lung, spleen, intestine, thymus, prostate, heart, placenta, colon and recent studies have also discovered IL-18R1 in the brain [182]. Further on, weak expression of IL-18R1 mRNA has been detected in the ovary and the testis [172]. Erythroid, myeloid, megakaryotic and monocytoid cell lines have also been shown to express IL-18R1 mRNA [183]. Therefore, it could be said that IL-18R1 mRNA is widely but not universally located in the body [172]

1.8.2. IL-18R1

IL-18R1 is expressed on various types of immune system cells and non-immune system cells; naïve T cells, T_H1 cells, macrophages, mast cells, B cells, natural killer cells, basophils, neutrophils, epithelial cells, endothelial cells, chondrocytes, synovial fibroblasts and smooth muscle cells [168]. It is important to note that IL-18R1 is highly expressed on the surface of T_H1 cells, but not on the surface of T_H2 cells, which means that IL-18R1 could be used as a cell surface marker to differentiate T_H1 and T_H2 cells.

There have been two recent studies, which suggested that IL-18R1 might have IL-18-indepent functions. A study done in 2006 [184] showed that IL-18R1-deficient mice were resistant to and not affected by experimental autoimmune encephalomyelitis (EAE), while IL-18-deficient mice were affected by EAE. This result suggested an involvement of another IL-18R1 ligand other than IL-18 in this inflammation. Further on, they observed that generation of pathogenic IL-17 producing T_H cells required the engagement of IL-18R1 on antigen-presenting cells, while IL-18 was not necessary.

In a second study done in 2006 [185], the responses of IL-18-deficient pancreatic islets and IL-18R1-deficient pancreatic islets were compared in mice with allograft rejection. The results of this study showed differences between IL-18-deficient cells and IL-18R1-deficient cells, and suggested that IL-18R1 is used by an inflammation suppressing signal. It is still unclear whether a second ligand for IL-18R1 exists and what it might be.

1.8.3. The IL-18R Signaling Pathway

The IL-18R complex recruits MyD88, IRAK and TRAF6. This recruitment is similar to the recruitment of IL-1 and IL-33, and it leads to the activation of JNK, NFκB, MAPK, STAT and p38 pathways (Figure 1.18). Although, the JAK/STAT signalling pathway is not activated by IL-18 in T_H1 cells [186].

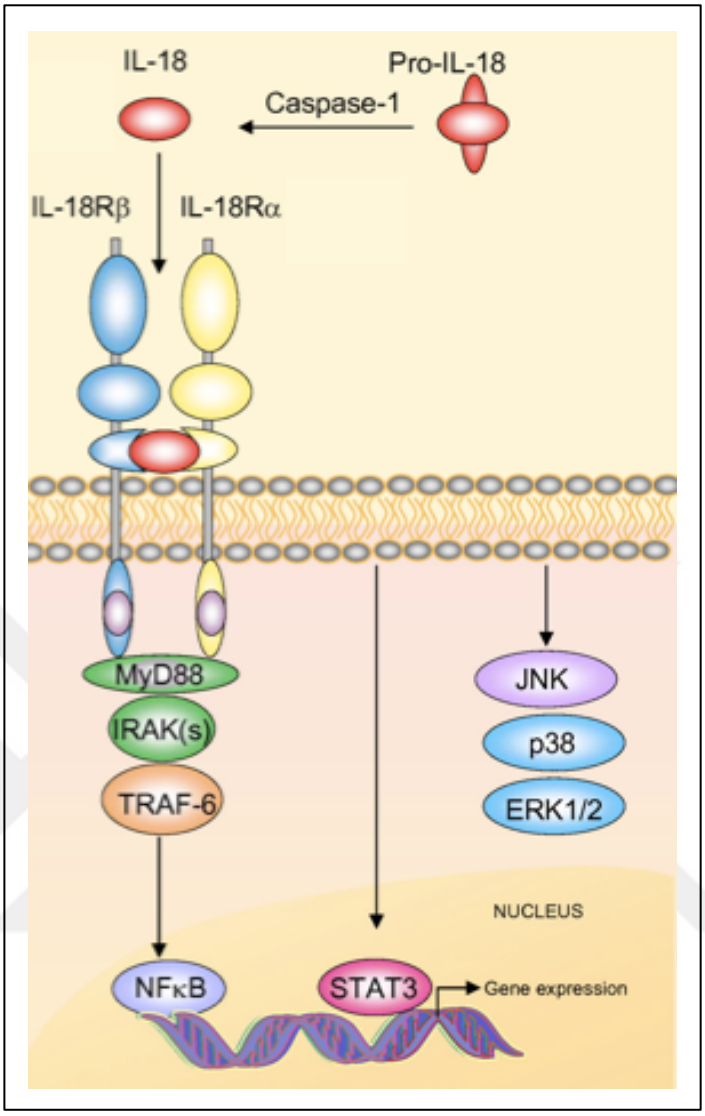

Figure 1.17. The IL-18R mediated signaling pathway [187]

1.8.4. The Role of IL-18 and IL-18R1 in Diseases

IL-18 plays an important protective role in host defense, though the over expression of IL-18 is toxic and may lead to various diseases since it is an important pro-inflammatory cytokine and is expressed not only by the immune system cells, but also by the non-immune system cells (Figure 1.19).

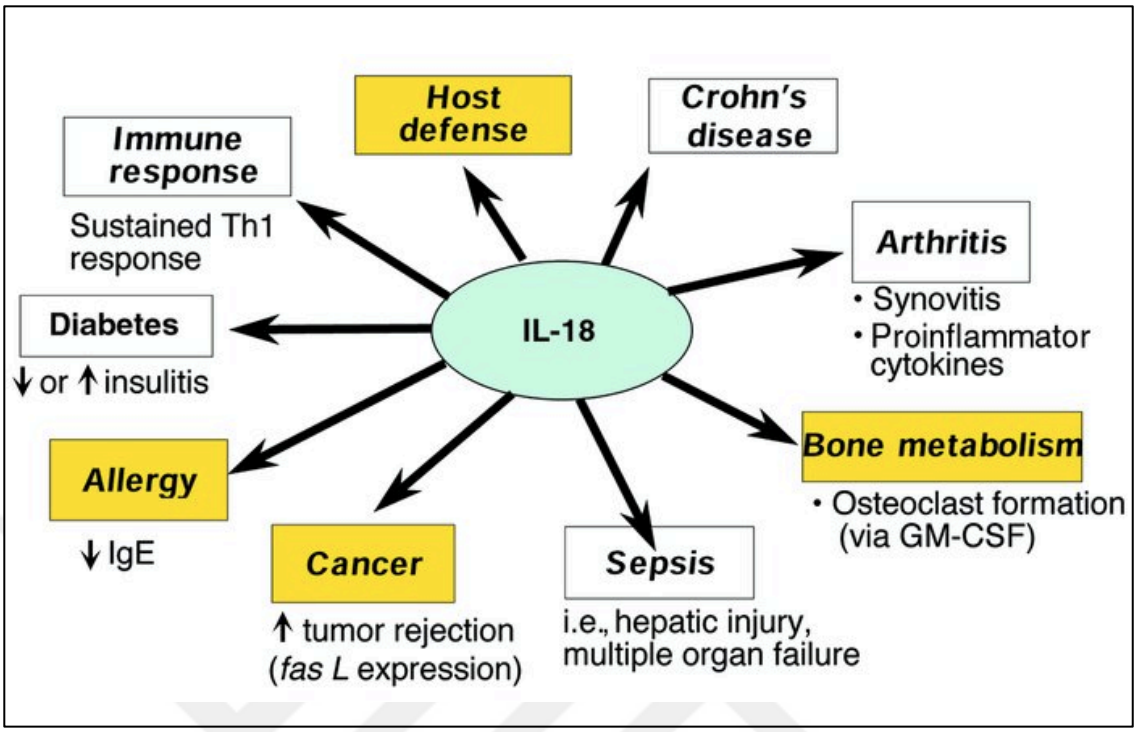

Figure 1.18. Summary of diseases associated with IL-18 [188]

IL-18 and IL-18R have been shown to take part in the development of diabetes in non-obese diabetic (NOD) mice [189], rheumatoid arthritis [190], liver diseases [191][192][193], Chron's disease [194][195], and multiple sclerosis [184]. In addition, high levels of IL-18 have been detected in the serum and plaques of psoriasis patients [196][197] [198].

1.8.5. The Role of IL-18 and IL-18R1 in Asthma

The synergism of IL-18 and IL-12 activates T_H1 driven immune response, which leads to the production of IFN-γ and inhibition of IgE expression [172][199] [200]. But IL-18 alone activates the T_H2 cell driven immune responses, which leads to the production of IL-4 and IL-13. IL-4 and IL-13 stimulate the expression of IgE [172][199] [200]. Therefore, IL-18 is an important regulator of IgE production (Figure 1.20).

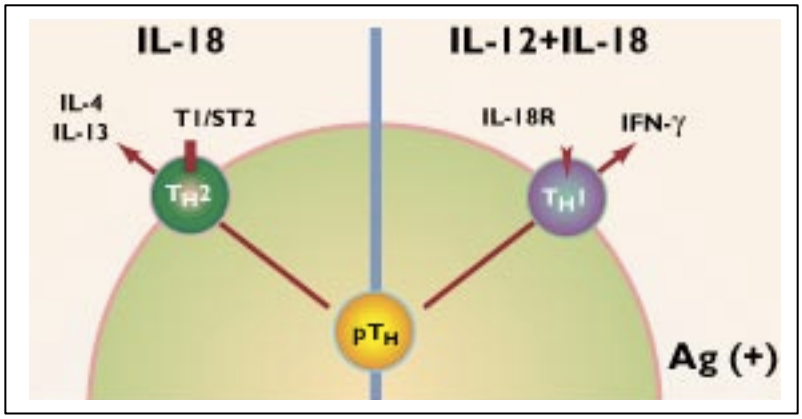

Figure 1.19. Action of IL-18 on T cell [200]

The chronic inflammatory airway disease asthma is associated with high levels of IgE and the infiltration of allergen specific T_H2 cells [172]. In 1998, Hofstra [201] and colleagues treated a mouse model of allergic asthma with IL-12/IL-18 and observed that the treatment inhibited the development of T_H2 cells. This inhibition led to the inhibition of eosinophilic infiltration and airway hyperresponsiveness, and decreased IgE levels.

Recently, there have been many studies done to discover the genetic background of asthma, and IL-18 and IL-18R1 have been attractive genes. Even though a genome wide association study (GWAS) done in 2013 showed [76] no association between IL-18R1 and asthma, there have been many other SNP studies, which have shown the opposite. A summary of polymorphisms in IL-18 and IL-18R1 associated with asthma is given in Table 1.5.

Table 1.5. A summary of polymorphisms in IL-18 and IL-18R1 associated with asthma

Gene	SNP	Association	Population	Reference
IL-18R1	rs377166	Asthma	European	[202]
IL-18R1	rs12999364	Asthma	Dutch	[203]
IL-18R1	rs1035130	Asthma	Dutch	[203]
IL-18R1	rs1558627	Asthma	Dutch	[203]
IL-18R1	rs2270297	Asthma	Dutch	[203]
IL-18	rs5744247	Asthma severity	Japanese	[204]
IL-18R1	rs3213733	Asthma	Turkish	[205]
IL-18R1	rs1362348	Asthma	Danish United Kingdom Norwegian	[199]

2. AIM

The purpose of this study was to investigate possible associations between IL-33 and IL-18R1 gene polymorphisms and the development of BPD in Turkish population.

3. MATERIAL

3.1. SAMPLES

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

3.2. KITS

- DNA Isolation kit (Invitrogen, USA)
- TaqMan Universal PCR Masyrt Mix (Applied Biosystems, USA)
- TaqMan SNP Genotyping Assay, IL-33, rs 10975519 (Applied Biosystems, USA)
- TaqMan SNP Genotyping Assay, IL-18R1, rs 1974675 (Applied Biosystems, USA)

3.3. EQUIPMENT

- 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)
- 50ml falcon tubes (ISOLAB, Germany)
- PCR tubes (Axygen, 0.2ml, USA)
- PCR Microplate, 96 (PCR-96-FLT-C, Axygen, USA)
- Ice
- Vortex (Velp Scientifica, Italy)
- Centrifuge (Mikro 22 R, Hettich, Germany)
- Centrifuge (Allegra, 25 R centrifuge, Beckman Coulter, USA)
- Nanodrop P 300 (Implen, Germany)

- Refrigerator (Arçelik, Turkey)
- Water Bath (Grant, OLS 200, UK)
- Real Time PCR System (Bio-Rad, CFX96, USA)
- Ice machine (Hoshizaki, FM-120DE, Japan)

3.4. CHEMICALS

- Ethanol (≥ 99.8 per cent Sigma, USA)
- Distilled water
- Tris-EDTA (TE) Buffer (Invitrogen, USA)
- Nuclease free water (Fermentas, USA)
- Phosphate Buffered Saline (PBS)

4. METHODS

4.1. STUDY POPULATIONS

One hundred and twenty two blood samples were collected from Goztepe Hospital, Umraniye Hospital and Kanuni Sultan Suleyman Hospital. The inclusion criteria of the study was; gestational age less then 34 weeks. The perinatal history (GA, BW, Apgar score one and Apgar score) and the history of treatments applied while in hospital (surfactant treatment, diagnoses, mechanical ventilation, oxygen therapy) were accepted, following approval from the Ethics Committee of Umraniye Hospital.

4.2. DNA ISOLATION

PureLink™ Genomic DNA Mini Kit (Invitrogen, USA) was used to perform this part of the experiment. Blood Samples were (200 µL) transferred to an eppendorf tube (1.5 mL). PureLink™ Proteinase K (Invitrogen, USA) (20 µL) was added to each sample. Then, PureLink™ RNase (Invitrogen, USA) (20µL) and each sample was vortexed for five sec and incubated at room temperature for two min. Following incubation, PureLink™ Genomic Lysis/Binding Buffer (Invitrogen, USA) (200µL) was added to each sample and vortexing was done for five sec. Each sample was incubated at 55℃ for 20 min.

When incubation was over, 96 per cent Ethanol (200µL) was added to each sample, vortexing was done and then the samples were transferred to PureLink™ Spin Columns (Invitrogen, USA) which were in collection tubes. The tubes were centrifuged for 1 min at 10000g and room temperature.

Following centrifugation, the collection tubes were discarded, each spin column was transferred to new collection tubes and PureLink™ Wash Buffer 1 (Invitrogen, USA) (500µL) was added to each of them. The samples were then centrifuged for one min at 10000 g and room temperature. The collection tubes were discarded, each spin column was transferred to new collection tubes and PureLink™ Wash Buffer 2 (Invitrogen, USA)

(500µL) was added to each sample. The samples were centrifuged for 3 min at maximum speed and room temperature. The collection tubes were then discarded and the spin columns placed in Eppendorf tubes. PureLink™ Genomic Elution Buffer (Invitrogen, USA) (50µL) was added to each sample, incubated for one min at room temperature, and the samples were centrifuged for 1 min at maximum speed and room temperature. The tube now was containing purified genomic DNA. A second elution step was performed by placing the spin columns in new Eppendorf tubes, adding PureLink™ Genomic Elution Buffer (Invitrogen, USA) (50µL), incubating for one min at room temperature, and centrifuging the samples for one and a half min at maximum speed and room temperature. The tubes were containing purified genomic DNA. So the spin columns were discarded and the purified DNA was kept at -20℃ till next usage.

4.3. NANOSPECTROPHOTOMETRY

The purity of the DNA samples was measured using Nanodrop P300 (Implen, Germany) and its programme Nano2000, at 260nm and 280nm wavelengths.

The Nanodrop P300 (Implen, Germany) was gently cleaned with distilled water before starting. Before starting measuring the samples, one µL of PureLink™ Elution Buffer (Invitrogen, USA) was measured as blank. Then, one µL of each sample was measured in order at 340 nm. After each sample was measured, the Nanodrop P300 (Implen, Germany) was gently wiped with a dry piece of tissue paper. The concentration of DNA (C), and the A260, A280 and $^{A260}/_{A280}$ values were taken.

The purity of DNA is determined by the $^{A260}/_{A280}$ value. This value needs to be between one point 8 and two. If below or above, this value is indicative of contamination or the DNA is not 100 per cent pure.

4.4. TAQMAN SNP GENOTYPING ASSAYS

Each DNA sample was diluted to 10ng/µl by mixing it with DNase-free, sterile-filtered water. 40X SNP primer (TaqMan, USA) was diluted to a 20X working stock by mixing it with 50µL of 1X TE buffer. Further on the mixture was vortexed and centrifuged (Mikro 22 R, Hettich, Germany) and then stored at -20^{o}C in a dark environment. The SNP Genotyping Assay (TaqMan, USA) had two TaqMan® MGB probes: a probe labelled with VIC® dye that detects the Allele 1 sequence and another probe labelled with FAM™ dye that detects the Allele 2 sequence. The TaqMan Universal PCR Master Mix (TaqMan, USA) was aliquoted.

IL-33 (rs10975519) and IL-18R1 (*rs1974675*) optimized primers (TaqMan SNP Assays MTO, Human SM Assay, USA) was used for analysing the SNPs. The reaction mix was prepared by adding six point twenty five µl of dH_2O, one point twenty five µl of the primer and 12.5 µl TaqMan Universal PCR Master Mix (TaqMan, USA). 20µL of the prepared reaction mix was added in each well of a 96-well plate, and then five µL of the DNA samples were added on it. The plates were centrifuged (Allegra, 25 R centrifuge, Beckman Coulter, USA) at 1000 rpm for one min. CFX96 thermocycler (BioRad, USA) was used to carry out the Real Time Polymerase Chain Reaction (QRT-PCR). 10 min at 95^{o}C continued by 40 cycles of 15 seconds at 92^{o}C and a min at 60^{o}C were the cycling conditions used on the CFX96 (BioRad, USA).

4.5. STATISTICAL ANALYSIS

SPSS version 21.00 Software (SPSS software,Inc., Chicago,IL) was used to perform statistical analysis. Genotype frequencies and allelic frequencies of BPD patients and healthy controls were determined. The odds ratio, Chi-square $\chi 2$ test and the confidence intervals were calculated to compare the genotype frequencies and allelic frequencies. *p*-value < 0.05* was taken as being statistically significant.

Two independent tests, the t test and the Mann-Whitney U test were used for demographic variables and for the risk factors of BPD logistic regression and forward conditional method was used. *p*-value < 0.05* was taken as being statistically significant.

5. RESULTS AND DISCUSSION

5.1. STATISTICAL ANALYSIS OF DEMOGRAPHIC DATA

In this study the demographic data analysed were gestational age, birth weight, apgar score 1, apgar score 5, mechanical ventilation per day, continuous positive airway pressure per day, duration of oxygen per day. One hundred and twenty infants were included in this study.

The relation of the inclusion criteria was studied for a group of premature babies with BPD, the BPD group, and premature babies without BPD, the control group. Table 5.1 summarizes the demographic data in the two study groups.

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

	BPD	**Control**	***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, 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 independent sample t test,

*[b] was calculated by Mann-Whitney U test; **p*< 0.05 indicates statistically significance.

GA is one of the strongest cause to be associated with the pathogenesis of BPD. There have been many studies to show the association of GA with the development of BPD [9][10][11][12]. It has been observed that the severity of BPD is in proportion to GA and BW [9][13][14]. A study done in Israel to investigate the perinatal risk factors for BPD showed that BPD occurred in 13.7 per cent of preterm very low BW (VLBW) infants, who were alive at 36 weeks PMA [230]. This percentage increased from 4.1 per cent among infants of 30-32 GA to 50 per cent among infants of 24-25 weeks GA. In 2011, Laughon et. al. concluded in their study that GA is the strongest risk factor in the pathogenesis of BPD, and that the severity of BPD is proportional to BW and GA.

The findings of this study support all the other studies that have worked on the association of GA and BW with the pathogenesis of BPD. In this study GA (p= 0.000) and BW (p=0.000) were both found to be statistically significantly associated with the development of BPD.

The apgar score is a test done to assess the health of a newborn, it compromises heart rate, respiration, colour, muscle tone and reflexes [231]. The apgar score reported one minute after birth is termed as Apgar 1, and the score reported five minutes after birth is termed at Apgar 5. A study done in 1991 estimated that each lower point on the Apgar score system increases the infants with VLBW chance to develop a chronic lung disease by 26 per cent [232]. A study done in 2011 showed that there is a 16 per cent increase in developing BPD with each lower point on the Apgar score system [232]. In our study, similar observations were made and it was observed that the apgar score 1 (p=0.000*) and apgar score 5 (p=0.000*) were found to be statistically significantly associated with the development of BPD.

Although mechanical ventilation is an important treatment for very preterm infants, the use excessive tidal volume leads to lung injury, including BPD. In 1976, Taghizadeh and Reynolds [27] proposed that the excessive airway pressures given during mechanical ventilation causes trauma to the lung, and this disruption is the most important factor associated with the development of BPD. Many more studies have been done, which support their suggestion [31][233][234][235][236]. In this study, we observed that the duration of mechanical ventilation (p=0.004) and the duration of continuous positive airway pressure (p=0.007) were statistically significant in the pathogenesis of BPD.

Research has shown that excessive exposure of oxygen leads to oxygen toxicity, which leads to the development of reactive oxygen species, stopping the lung to grow and inducing the inflammatory cascade [3]. In a recent clinical study, two groups of preterm infants (GA 24-28 weeks) were used to observe the results of using less oxygen [237]. One group was treated with 30 per cent of oxygen, while the other group was treated with 90 per cent of oxygen. It was observed that the incidence of BPD had reduced 50 per cent in the 30 per cent oxygen group. Similarly in our study we found that the total duration time of oxygen (p=0.000) is statistically significant with the development of BPD.

5.2. STATISTICAL ANALYSIS OF ALLELE AND GENOTYPE FREQUENCIES IN IL-33

The distributions of allele and genotype frequencies in IL-33 were investigated in 76 premature babies with BPD, the BPD group, and 43 premature babies without BPD, the Control group.

Within the control group, 69.8 per cent of the patients carried allele C, and 30.2 per cent of the patients carried allele T. Within the BPD group, 73.0 per cent of the patients carried allele C, and 27.0 per cent of the patients carried allele T. Comparison of the C/T allele distribution in IL-33 in the BPD group and the Control group patients revealed that neither allele is significantly associated with BPD (p= 0.591; OR 0.852, 95 per cent CI 0.476-1.527) (Table 5.2).

Table 5.2. Distribution of allele frequencies of IL-33 in Bronchopulmonary Dysplasia

		BPD		**Control**			
Gene	**Allele**	**n**	**n%**	**n**	**n%**	**OR(95%CI)**	***p*-value**
IL-33	C	111	73.0	60	69.8	0.852(0.476-1.527)	0.591
	T	41	27.0	26	30.2		

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.

Within the BPD group 15.8 per cent of the patients and within the control group 23.3 per cent of the patients carried the TT genotype (p=0.313; OR 1.616, 95 per cent CI 0.632-4.131). Within the BPD group 61.8 per cent of the patients and within the control group 62.8 per cent of the patients carried the CC genotype (p= 0.918; OR 1.041, 95 per cent CI 0.481-2.254). Within the BPD group 22.4 per cent of the patients and within the control group 14.0 per cent of the patients carried the heterozygous TC genotype (p=0.264; OR 0.563, 95 per cent CI 0.203-1.557). The comparison of the genotype distribution of IL-33 in the BPD and the Control group revealed that neither genotype is a risk factor for the development of BPD (Table 5.3).

Table 5.3. Distribution of genotype frequencies in IL-33 in BPD

		BPD		**Control**			
Gene	**Genotype**	**n**	**n%**	**n**	**n%**	**OR(95% CI)**	***p*-value**
IL-33	TT	12	15.8	10	23.3	1.616(0.632-4.131)	0.313
	CC	47	61.8	27	62.8	1.041(0.481-2.254)	0.918
	TC	17	22.4	6	14.0	0.563(0.203-1.557)	0.264

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

IL-33 is a member of the IL-1 cytokine family, which also includes IL-18. Many studies have determined that IL-33 is a mediator of airway inflammatory diseases, including asthma. A summary of polymorphisms in IL-33 associated with asthma is shown in Table 1.16. A study done in 2014 revealed that SNPs in the IL-33-IL1RL1 pathway is associated with wheezing phenotypes and asthma in childhood [224]. Although IL-33 was identified as an asthma susceptibility gene in GWAS [225], a study done to define the contribution of

SNPs in IL-33 in clinical endpoints revealed that SNPs in IL-33 was not associated with asthma [76]. Therefore, to come to a certain conclusion further studies should be done.
Even though Nickerson and Taussing [79] showed an association between family history of asthma and BPD, there are some studies done, that did not come to the same conclusion. These studies failed to confirm Nickerson and Taussing's conclusion [226][227]. Therefore, it is still not clear whether there is a direct association between asthma and BPD. If there is no association between the two diseases, it would be expected not to find an association between SNPs in IL-33 and the development of BPD.

This study failed to find any kind of association of IL-33 with the development of BPD. This also may be due to different cohort patients. Further studies are required in order to investigate this association.

5.3. STATISTICAL ANALYSIS OF ALLELE AND GENOTYPE FREQUENCIES IN IL-18R1

The distributions of allele and genotype frequencies in IL-18R1 were investigated in 77 premature babies with BPD, the BPD group, and 45 premature babies without BPD, the Control group.

The control group showed 36.7 per cent of the patients carried allele T, and 63.3 per cent of the patients carried allele C. The BPD group showed 50 per cent of the patients carried allele T, and 50 per cent of the patients carried allele C. A comparison of the T/C allele distribution in IL-18R1 in the BPD group and control group showed the C allele is statistically significantly associated to be protective against the development of BPD (p= 0.043). Therefore, it is revealed that the presence of the C allele is 1.727 times more protective against the development of BPD compared to the T allele (Table 5.4).

Table 5.4. Distribution of allele frequencies in IL-18R1 in BPD

		BPD		**Control**			
Gene	**Allele**	**n**	**n%**	**n**	**n%**	**OR(95%CI)**	***p*-value**
IL-18R1	T	77	50	33	36.7	1.727(1.014-2.940)	0.043*
	C	77	50	57	63.3		

BPD= Bronchopulmonary Dysplasia, Control= Premature babies without BPD,
CI= Confidence Interval, OR= Odds Ratio, N= Number of infants, n= Allele count in BPD and Control groups, 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.

In the BPD group 45.5 per cent of the patients and 57.8 per cent of the control group displayed the CC genotype (p=0.189; OR 1.642, 95 per cent CI 0.782-3.450). Nine point one per cent of the BPD group and 11.1 per cent of the control group carried the heterozygous CT genotype (p= 0.119; OR 0.542, 95 per cent CI 0.250-1.175). In the BPD group 45.5 per cent of the patients and in the control group 31.1 per cent of the patients had the TT genotype (p=0.718; OR 1.25, 95% CI 0.372-4.1999). The comparison of the genotype distribution of IL-18R1 in the BPD and the Control group revealed that neither genotype was statistically significant as a risk factor for the development of BPD (Table 5.5).

Table 5.5. Distribution of genotype frequencies in IL-33 in BPD

		BPD		**Control**			
Gene	**Genotype**	**n**	**n%**	**n**	**n%**	**OR(95% CI)**	***p*-value**
IL-18R1	CC	35	45.5	26	57.8	1.642(0.782-3.450)	0.189
	CT	7	9.1	5	11.1	0.542(0.250-1.175)	0.119
	TT	35	45.5	14	31.1	1.25(0.372-4.199)	0.718

BPD= Bronchopulmonary Dysplasia, Control= Premature babies without BPD, CI= Confidence Interval, OR= Odds Ratio, N= Number of infants, n= Genotype count in BPD and Control groups, 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.

IL-18R1 is a receptor of IL-18, and has shown to be associated with various diseases including allergy and asthma. A summary of SNPs in IL-18R1 associated with asthma is given in Table 1.5. An SNP study investigated the association between TIM1, TSLP and IL18R1 with childhood asthma in a Turkish population, they revealed that IL-18R1 is significantly associated with asthma patients in the Turkish population [205]. Though, as discussed in section 4.2, it is still unclear whether asthma and BPD are associated.

Inflammatory response is due to the regulation of cytokine gene expression and secretion, and there are various cytokines stimulated by IL-18, which have also been found to be associated with the pathogenesis of BPD, such as TNF-α [108][109]. A study carried out in 2011 concluded that there was no direct association of polymorphisms in IL-18 with preterm birth and the development of BPD [228]. Though, an SNP study investigated the role of SNPs in IL-18R1 and IL-18RAP in development of BPD in African-American infants showed that SNPs in these two genes were associated with the development of BPD in the study population [229].

In this study we failed to show an association between genotype distributions of IL-18R1 and the development of BPD, though we revealed an association between the C allele and development of BPD in the Turkish population. Therefore, we propose that there is an association between SNPs in IL-18R1 and the pathogenesis of BPD, although further investigations must be done.

5.4. STATISTICAL ANALYSIS OF RISK FACTORS FOR BPD

BPD has a multifactorial ethology and it has been established many times that chorioamnionitis (CA), patent ductus arteriosus (PDA) and sepsis (SE) are contributors to the development of BPD. Table 5.6 summarizes the statistical analysis of CA, PDA and SE in 59 premature babies with BPD, the BPD group, and in 18 premature babies without BPD, the control group.

Table 5.6. Statistical analysis of risk factors included chorioamnionitis, patent ductus arteriosus, sepsis in the BPD group and Control group

	BPD (N= 59)		**Control (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, Control= 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 Fisher's exact test;
**p*< 0.05 indicates statistically significance.

It has been shown that chorioamnionitis, a maternal intra-uterine inflammation, is one of the leading causes of very preterm delivery. Although many studies have shown that chorioamnionitis is associated with the development of BPD, no definite association has been shown. In 2013 Masmonteil suggested that chorioamnionitis could not be definitely considered a risk factor for BPD [17]. Similar to Masmonteil's research, we have also found that CA (p=1.000) is not associated with the development of BPD.

Patent ductus arteriosus, a congenital heart defect, has also been shown to be associated with the development of BPD in many studies, although its direct role remains controversial. The confusion may be due to the need of mechanical ventilation during the presence of PDA. This study failed to show a contribution of PDA ($p=0.272$) in the development of BPD.

Sepsis is an inflammatory response to an infection. Many studies have shown that sepsis increase the risk of BPD in premature infants. A study done in 2011 showed that infants with increasing severity of BPD were more likely to have sepsis [230]. In this study we observed that sepsis ($p=0.000$) is statistically significantly associated with the development of BPD.

6. CONCLUSION

The results of this study show that an SNP in the genotypic distribution (CC, TC, TT) of IL-18R1 is not associated with the development of BPD, while the C allele instead of the T allele IL-18R1 is more protective against the development of BPD. There were no associations found between the genotypes of IL-33 (CC, TT and TC) and the risk factors for developing BPD in the Turkish population.

In order to fully understand the genetic risk factors, which lead to the development BPD, future studies should be done with other genes. These studies should be carried out with larger populations; this would lead to a more accurate diagnosis and identification of novel therapeutic drugs.

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