ABSTRACT

The Role of Notch Signaling Pathway in CD4+ CD25 + FOXP3+ T regulatory Cell Balance in Chronic Obstructive Pulmonary Disease

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Notch signaling is essential for airway development and progenitor/stem cell regeneration, regulation, and controlling the fate of airway epithelial cells during differentiation. This signaling pathway also regulates the Th1/Th2 and Th17/ T regulatory (Treg) cells immune balance and has a substantial influence on disease pathogenesis. The impact of the Notch signaling pathway to the pathogenesis and progression of chronic obstructive pulmonary disease (COPD) is not well understood, despite the fact that studies suggest that its dysregulation may lead to various of respiratory diseases. Our current study demonstrated the increased distribution of the Notch receptor on T regulatory cells in healthy smokers, and patients with stable, and exacerbated COPD, as compared to non-smokers also the high level of Th1/Th2 cell ratio in exacerbated COPD patients by flow cytometry. We also studied Notch and Treg/Th17 cell balance by analyzing the expression of Foxp3, RORγt, and Notch downstream genes such as Hes5 and Hey1, in $CD4^{+}$T-helper cells using RT-PCR; At the gene level, no significant differences between study groups were observed. By using the Multiplex Cytokine Assay, we measured the levels of pro- and anti-inflammatory cytokines in COPD patients and found high levels of pro-inflammatory cytokines, as well as a negative correlation between Notch expression and anti-inflammatory cytokines. Together, these data showed that the Notch signaling pathway may deteriorate the Treg cell function as well as may be one of the major drivers in COPD inflammation.

Free Keywords: Notch signaling pathway, T regulatory cells, T cell imbalance, Chronic obstructive pulmonary disease, pathogenesis, inflammation, stable, exacerbated.

ÖZETÇE

Kronik Obstrüktif Akciğer Hastalığında CD4+ CD25 + FOXP3+ T Düzenleyici Hücre Dengesinde Notch Sinyal Yolağının Rolü

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Notch sinyal yolağı başta havayolu gelişimi olmak üzere kök hücre yenilenmesi ve havayolu epitel hücre farklılaşmasının kontrolünde önemli bir role sahiptir. Bu sinyal yolağı aynı zamanda Th1/Th2 ve T düzenleyici/Th17 immün hücre dengesini düzenler ve hastalıklar üzerinde büyük bir etkisi vardır. Çalışmalar, Notch sinyal yolağınındaki düzensizliğin bir çok solunum hastalığına sebep olabileceğini belirtmiştir. Ancak, yolağın kronik obstrüktif akciğer hastalığındaki patogenezi ve seyrindeki rolü henüz aydınlatılmamıştır. Bu çalışmada, sigara içen ve sigara içmeyen sağlıklı donörlerle birlikte stabil ve akut kronik osbtrüktif akciğer hastalığına sahip donörlerden elde edilen periferik mononükleer hücrelerinde Notch sinyal yolağının T düzenleyici hücreler üzerindeki dağılımını ve aktivitesini bununla birlikte Th1/Th2 ve T düzenleyici/Th17 hücre dengesini akış sitometrisi tekniği ile araştırdık. Ayrıca, Th17/T düzenleyici hücre dengelesini Foxp3, RORγt genleri; Notch sinyal yolağı aşağı akışını ise Hes5 ve Hey1 genleri ile RT-PCR anacılığıyla gen düzeyinde analiz ettik. Multipleks Sitokin Testini kullanarak, KOAH hastalarında pro- ve anti-inflamatuar sitokinlerin seviyelerini ölçtük ve yüksek seviyelerde pro-inflamatuar sitokinlerin yanı sıra Notch ekspresyonu ile anti-inflamatuar sitokinler arasında negatif bir korelasyon bulduk. Birlikte, bu veriler Notch sinyal yolunun Treg hücre fonksiyonunu bozabileceğini ve KOAH iltihabında önemli bir rol oynayabileceğini gösterdi.

Anahtar Kelimeler: Notch sinyal yolağı, T düzenleyici hücreler, T hücre dengesi, Kronik Obstrüktif Akciğer Hastalığı, patogenez, inflamasyon, stabil, akut.

Chapter 1:
INTRODUCTION

1.1 CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Globally, chronic obstructive pulmonary disease (COPD) is the third leading cause of mortality and morbidity (Committee, 2022). The Global Initiative for Chronic Obstructive Lung Disease (GOLD) defined COPD as a preventable and treatable condition but not fully reversible, and progressive, lung inflammatory disease(Committee, 2022). It is triggered by an abnormal pulmonary inflammatory response to bacterial and viral infections, as well as environmental factors, including air pollution, dust, and most importantly tobacco smoke. Along with exposures, host factors increase a risk of a person having COPD. This includes genetic abnormalities such as alpha-1 antitrypsin deficiency (Hogg & Timens, 2009). The disease is typically diagnosed with a spirometry test, which not only confirms the diagnosis but also determines the stage of the severity. The first criterion for the diagnosis of COPD is that the forced expiratory volume in the first second (FEV1) ratio to forced vital capacity (FVC) (Committee, 2022). (Ho, Cusack, Chaudhary, Satia, & Kurmi, 2019). Additionally, a decrease in FEV1 indicates airway obstruction and is used to classify disease severity according to GOLD stages **(Table 1)** (Committee, 2022; Ho et al., 2019). The disease can be periodically punctuated by exacerbations (Kim & Aaron, 2018). An exacerbation of COPD is a prolonged deterioration of the patient's status beyond the stable stage and much beyond daily fluctuations that have an acute onset and may require extra therapy in a patient with underlying COPD (O'Donnell & Parker, 2006). The clinical symptoms of COPD exacerbations are extremely variable, reflecting a wide range of heterogeneity in the pathophysiology of COPD in addition to variation in the nature and effect of the provoking agent (Papi, Luppi, Franco, & Fabbri, 2006). Patients with stable COPD experience persistent inflammation throughout the tracheobronchial tree, which is characterized by an elavation in neutrophils, CD8+ T cells, and macrophages in the airway lumen and airway wall, respectively. (Saetta, Turato, Maestrelli, Mapp, & Fabbri, 2001; White, Gompertz, & Stockley, 2003). The inflammatory cellular pattern alterations were seen during exacerbations such as increase in eosinophils, neutrophils, and

inflammatory mediators; cytokines, chemokines, chemokine receptors, adhesion molecules, and markers of oxidative stress. However, studies comparing the underlying mechanisms of stable and exacerbated COPD are still insufficient.

1.2 Risk Factors

Smoking is seen as the major risk factor in respiratory diseases along with COPD (Mannino & Buist, 2007). Studies have shown that smokers have a COPD mortality rate that is at least seven times higher than non-smokers. Smokers were divided into groups according to how many cigarettes they smoked each day, the increase in mortality was found to be associated with the group of mild smokers (1–14 cigarettes/day), while increases in mortality were found to be 10-fold for moderate smokers (15–24 cigarettes/day) and 21-fold for heavy smokers (625 cigarettes/day). As a result, smoking cessation potentially displays a major favorable effect on health and survival prospects. Smokers also have a higher chance of having their FEV1 decrease, with an additional annual FEV1 drop between 7 and 33 ml/year (O'Connor, Sparrow, & Weiss, 1995; Tashkin et al., 1994), some research suggests a connection between the level of smoking and the deterioration in FEV1. Remarkably, following smoking cessation, the rate of FEV1 decrease slows however does not recover to baseline (Fletcher, Peto, Tinker, & Speizer, 1976). In addition, studies demonstrated that the survival curve of those who gave up smoking between the ages of 35 and 44 was barely different from that of non-smokers. While those who ceased smoking between the ages of 55 and 64 had a curve that was nearly identical to smokers', despite being separate, those who gave up their habit between the ages of 45 and 54 showed a curve that was in the middle of those of non-smokers and current smokers. Even people who ceased smoking after turning 65 have a longer life expectancy than those who continue to smoke. In addition, research indicates that present and former smokers had a poorer mean carbon monoxide diffusing capacity than nonsmokers, which was associated with an increased prevalence of respiratory symptoms and diseases .

Air pollution

Air pollution's a strong hazard element for COPD following both short and long-term exposure (Mannino & Buist, 2007). Exposure to biomass fuels such as coal, straw, animal manure, and agricultural leftovers for cooking and heating in poor ventilation dwellings is the leading global risk factor for COPD. In low& middle-income countries, 35% of COPD patients developed the disease due to exposure to indoor smoke from biomass fuels according to World Health Organization (WHO) statistics (Lopez, A.D., et al.,2006). Additionally, according to the WHO, exposure to the indoor smoke is a factor in 36% of deaths from lower respiratory diseases(Lopez, Mathers, Ezzati, Jamison, & Murray, 2006). Moreover, there is outdoor pollution such as particulate matter <10μM (PM_{10}) and $PM_{2.5}$, nitrogen oxides (NO), ozone, and sulfur dioxide (SO_2), and the risk of developing COPD associated with exposure to these outdoor pollutants (Bayram, 2015, 2017). Many studies have reported exposure to outdoor pollutants increases the risk of systemic inflammation, rapid decline in Fev1, and raises the likelihood of developing COPD. It is also highly connected with respiratory morbidity and mortality significantly (Ko & Hui, 2012; Peacock et al., 2011).

1.2.3 Occupational Exposure

Occupational exposure to various dust particles, chemicals, vapours, and fumes is a risk factor for COPD development. It has been reported that work exposures were responsible for 19% of COPD cases in the USA, and 31% of never-smokers had the disease (Hnizdo, Sullivan, Bang, & Wagner, 2002). The rate of occupational exposures might show differences between low&middle-income countries, where occupational exposures may higher than in high-income countries due to less rigorous regulations. The findings of another study revealed that people with COPD or chronic bronchitis were previously exposed to large amounts of gases, dust, vapors or fumes in the workplace (Trupin et al., 2003).

1.2.4 Genetic Factors

Understanding COPD genetics has advanced significantly over the past 50 years. This is mostly because epidemiological studies have been done to find out why some smokers with similar smoking histories get COPD and others don't.The *SERPINA1* gene,

which produces the serine protease inhibitor alfa-1 antitrypsin(AAT-1), has been found seems like the only genetic factor associated with COPD risk that has been thoroughly studied (Stoller & Aboussouan, 2005). AAT-1 deficiency results from a mutation in the *SERPINA1* gene, which is responsible for unrestrained protease activity and the onset of emphysema. A *SERPINA1* anomaly is present in only 3% of the population, indicating that numerous more genetic variants may contribute to the growth of COPD. Furthermore, alpha-2-macroglobulin, vitamin D-binding protein, and blood serotype group genes have been related to an increased risk of COPD. (Sandford, Weir, & Pare, 1997). However, additional research is necessary to elucidate the genetic process causing COPD.

1.3 Pathogenesis of Chronic Obstructive Pulmonary Disease

1.3.1 Chronic Bronchitis

In terms of pathology, COPD has two important pathogenic changes. The first one is long-term inflammation of the bronchi called chronic bronchitis(Kim & Criner, 2013). A high level of mucous production and productive cough are the hallmark signs of chronic bronchitis. Mucus is naturally produced by mucus glands to humidify the airways and trap pathogens(Rogers, 2000). There are other cells throughout the airway that are crucial to producing mucus; these cells are known as goblet cells. In individuals with chronic bronchitis, goblet cells and mucous glands undergo hypertrophy and hyperplasia, which leads to increased production of mucus and its accumulation, as well as the obstruction of airways of patients with chronic bronchitis. As a result of the increase in mucus, the movement of the mucus is difficult by the cilia cells and it is very difficult to swallow or spit the mucus. On the other hand, when the cilia do not beat properly, mucus simply sits in the airways, obstruct the airway and results in inflammation (Maestrelli, Saetta, et al., 2001).

The other significant symptom of patients with chronic bronchitis is air trapping. The term "air trapping" shows reducing the amount of air entering the lungs and air retention during expiration due to airway clogging by mucous plaques. As a result, the oxygen in the air entering the lungs decreases over time and the CO2 concentration increases. This

situation leads to hypoxemia and hypercapnia (Dos Santos Yamaguti et al., 2008). Together, this situation promotes a productive cough in patients with chronic bronchitis.

1.3.2 Emphysema

The other main pathological change in patients with COPD is emphysema, which harms the alveoli and their interstitial walls in the distal lung. (Pahal, Avula, & Sharma, 2018). In this process, both innate and adaptive immune cells such as neutrophils, monocytes, T helper cells produce inflammatory mediators that destroy the lung parenchyma (Caramori, et al 2011). These inflammatory cells release proteases such as elastase and matrix metalloproteinases (MMPs), which destroy the elastic connective tissue of the alveolar walls. Elastic tissue is intended to pull the lungs back to their usual size, but when they lose that elastic recoil, the lungs of patient readily expand rather than being able to recoil and push a lot of the trapped air out. A loss of elastic recoil leads to sacs losing their stretchiness and trapping air instead (Silvers, Petty, & Stanford, 1980). The lungs are no longer efficiently empty, and it becomes more difficult to completely remove all the air. As a result, enlargement of the small airways occur over time. In addition, once the elastases begin to degrade the alveolar tissue that forms the septa, there are no longer any smaller alveoli to expand the surface area. Instead, there is one large ballooned-out inefficient area (Sharafkhaneh, Hanania, & Kim, 2008; Taraseviciene-Stewart & Voelkel, 2008). Together, these all demonstrate how the airway is remodelling as COPD progresses **(Figure 1).**

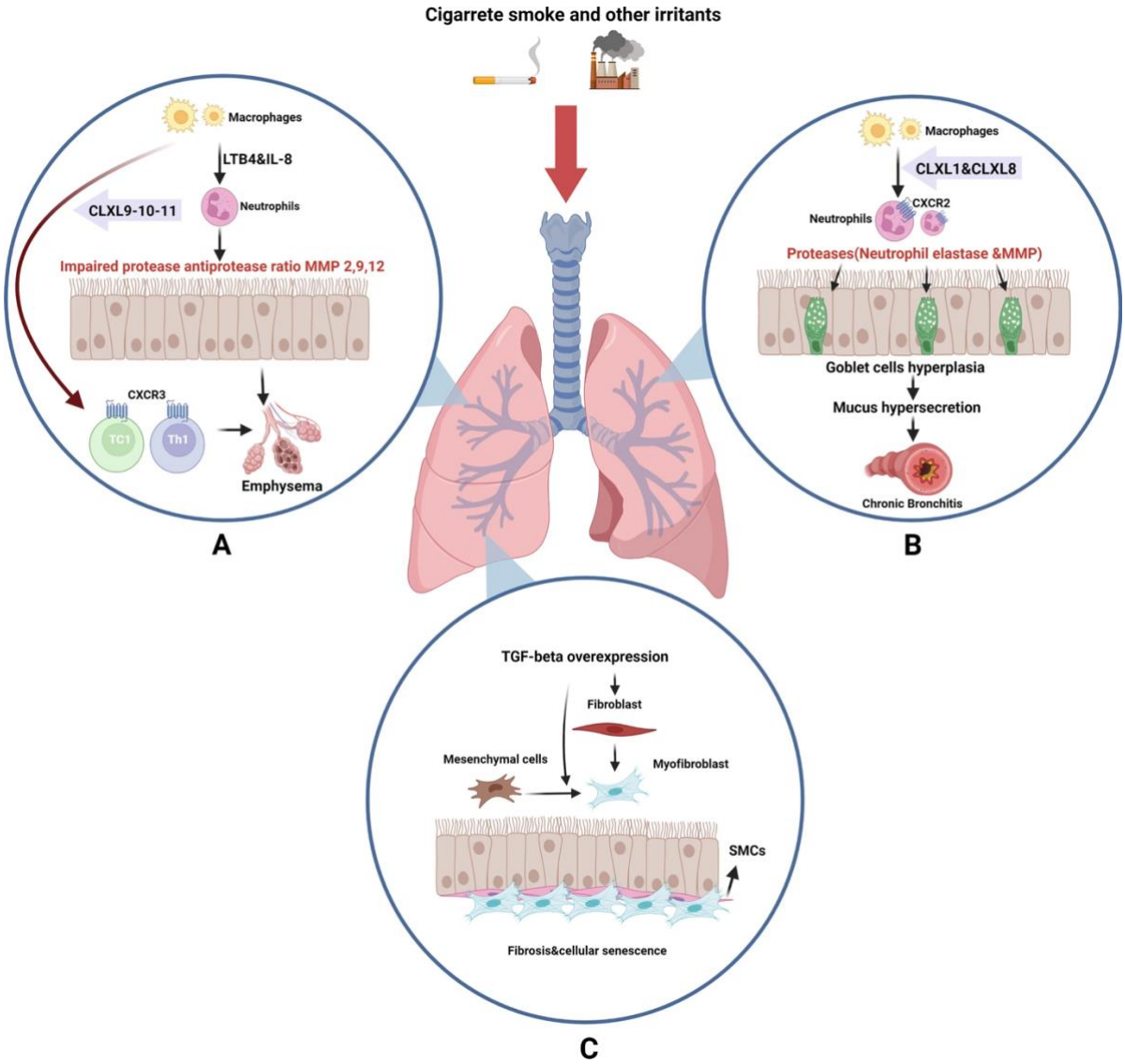

***Figure 1**: Pathophysiology of Chronic Obstructive Pulmonary Disease [Rajabi, Hadi, et al. (2022) Cell Commun. Signal].*

1.4 Immunopathology of COPD and role of inflammatory cells

In COPD, damaged bronchial epithelial cells generate pro-inflammatory mediators which is one of the most important immune processes needed to recruit leukocytes from the bloodstream to the place where inflammation occurs. Neutrophils, macrophages, and lymphocytes are the main cells involved in COPD inflammation(Gaetano Caramori et al., 2016). These inflammatory cells further release cytokines, chemokines, and chemoattractants which pursue the inflammation resulting in an uncontrolled cascade (Chung & Adcock, 2008). These COPD-related inflammatory changes have been

connected to a tissue remodelling and repair process that results in chronic bronchitis and emphysema.

1.4.1. Neutrophils

Neutrophils are the first responders to pathogens for the immune system (Jaillon et al., 2013). In COPD, various inflammatory mediators activate peripheral blood neutrophils, and activated neutrophils migrate to lung tissue. Several mediators, like interleukin [IL]- 8, IL-1, tumor necrosis factor (TNF), and leukotriene B4, regulate its migration to the airways. Elastase, matrix metalloproteases (MMP), and other pro-inflammatory mediators are released into lung tissue, where they promote emphysema and the migration of other inflammatory cells. (Kim, Rogers, & Criner, 2008; Milara, Juan, Peiró, Serrano, & Cortijo, 2012) Studies indicated a significantly increased number of activated neutrophils in the sputums, and bronchoalveolar lavage fluids of severe stable COPD patients compared to mild and moderate patients together with health smokers. They found a positive correlation between the accumulation of neutrophils and the disease severity. For instance, in lower airways, the patients who are in the GOLD stages 3 and 4 have a high number of neutrophils than the Gold 1 and 2 stages as healthy smokers have the least number of neutrophils compared to them(Stockley, 2002). Furthermore, they reported that in blood, the systemic inflammation that is associated with COPD co-morbidities and disease severity is reflected in elevated peripheral neutrophil numbers (Lonergan et al., 2020).

1.4.2. Macrophages

Macrophages are essential components of the innate immune system. They originate from circulating monocytes, which after leaving the circulation, migrate into various body tissues such as parenchyma, alveolar space, and bronchoalveolar lavage (BAL). The two polarizable macrophage types are traditionally activated (M1) and alternatively activated (M2) (C. Zhang, Yang, & Ericsson, 2021). They become polarized into M1 macrophages after being stimulated by lipopolysaccharide (LPS) and cytokines like IFN- γ, and TNF- α. The cytokines and chemokines that are released by these cells include TNF- α, IL-1,

IL-6, IL-12, CXCL9, and CXCL10. The two primary pathways in M1 macrophage polarization are NF-κB and signal transducer and activator of transcription-1 pathways (STAT-1)(Mills, Kincaid, Alt, Heilman, & Hill, 2000). M2 polarization is observed in the presence of cytokines signals such as IL-4, IL-13, IL-10, IL-33, and TGF-β. Notably, only IL-4 and IL-13 directly activate M2 macrophages, whereas other cytokines do not. M2 macrophage activation is thought to occur through the STAT3 and STAT6 pathways (Gordon & Martinez, 2010). Studies indicate that there is no evidence that one type of heterogeneous macrophage dominates in COPD. However, some of these studies show a decreased level of M2 markers in severe COPD patients(Cornwell et al., 2018) and a large expansion of M1 macrophages(McAlinden, 2016). They possess a crucial function in the disease's pathophysiology and are greatly elevated in the patients' airways, lung parenchyma, bronchoalveolar lavage fluid (BALF), and sputum(George & Brightling, 2016). TNF-α, related CXC chemokines, lymphotoxin β4 (LTB4), and monocyte chemotactic peptide (MCP)-1, as well as reactive oxygen species (ROS), are secreted by these COPD-associated macrophages (Barnes, 2004). Additionally, elastolytic enzymes like MMP-2, -9, -12 and cathepsins-K, L,S are secreted by the alveolar macrophages. According to studies, the severity of COPD positively correlates to the amount of macrophage in the airways, similar to the neutrophil counts (Russell et al., 2002). Thus, a augmented monocyte recruitment may result in a rise in macrophage concentration, but it may also be associated with greater lung proliferation and longevity (Hogg, 2004). Several studies have shown an inverse correlation between the number of alveolar macrophages and predicted FEV1 and alveolar macrophages are considerably more amount in COPD patients at GOLD stages III and IV compared to those at GOLD stages I and II, smokers, and non-smokers (Kaku et al., 2014).

1.4.3 Dendritic cells

Dendritic Cells (DCs) are unique antigen-presenting cells (APCs), and they play crucial roles in the initiation, modulation, and maintenance of specific immune responses (Clark et al., 2000) Many diverse DC subsets, including conventional, plasmacytoid, monocyte-derived, etc., are capable of responding to a variety of pathogenic and harmful stimuli by expressing differential sets of pattern recognition receptors, enabling functional specialization of the DCs (Collin & Bigley, 2018). These cells infiltrate the

inflamed bronchi and activate $CD4^+$ T helper cells that separate into various categories through the cytokines they secrete, such as IL-10, IL-12, or IL-23 together with the surface molecules they express, like TNF Superfamily Member4 (TNFSF4) or Inducible T Cell Costimulator-1 (ICOS-l) (Zheng et al., 2018). These procedures have the potential to affect the progression of COPD (Zheng et al., 2018). Studies report that, after smoking cessation, patients with stable COPD have reduced levels of mature $CD83^+$ DCs in their sputum than non-smokers or smokers whose lung function is normal (Tsoumakidou et al., 2009). In addition, when compared to non-smokers, less mature CD83+ DCs existed in the bronchial mucosa of moderate/severe stable COPD patients(Zanini et al., 2014). A study revealed fewer $CD83^+$ $CCR7^+$ DCs and a greater number of $CD1a^+$ DCs in the lower airways of stable COPD patients compared to smokers (Liao et al., 2015), while others have shown no increase in CD1a+ DCs (Demedts et al., 2007). Also, they reported an increase in the overall number of CD83+ DCs in the peripheral lung of patients compared to smokers (Vassallo et al., 2010). Since the significance of diverse subsets of DC in COPD has yet to be understood, during both stable phase and acute episodes, DC-related research is essential to comprehending the pathophysiology of COPD.

1.4.4. Th1, Th2, and Th17 T lymphocyte subsets

In the existence of IL-12 and IFN-,γ naive CD4 + T cells polarize into Th1 cells. These interleukins activate the STAT-4 and T-box expressed in T cells (T-bet) , which are the essential transcription factors that promote Th1 commitment (Zhu & Paul, 2010). These cells preferentially express chemokine receptor CXCR3 as the main marker, and may also express CCR1, 2, and 5 (A Di Stefano et al., 2001). High levels of Th1 response cytokines, such as tumor necrosis factor-alpha (TNF- α) during exacerbations and interferon-gamma (IFN- γ) in emphysema patients, are a major cause of the disease. $CD4^+$ Th1 lymphocytes are essential in COPD inflammation (Yu et al., 2020). Another T lymphocytes that has a role in COPD immunopathology are Th2 cells. These cells secrete a range of of cytokines that affect eosinophil recruitment, mucus production, and B-cell differentiation together with antibody production(Burks et al., 2019). While IL-4, IL-5, and IL-13 are commonly associated with Th2 cells, these can also produce IL-9, IL-10, IL-25, and amphiregulin(C. Wang & Li, 2013). When naive T cells are exposed to IL-4 during T-cell priming, they polarized into Th2 cells. IL-4 stimulates naive T cells to

express GATA3, the master regulator of Th2 differentiation, via the STAT6 pathway. In the excess of Th2 associated with IgE response which also trigger inflammatory in COPD patients(Solleiro-Villavicencio et al., 2015). It has been shown, Th17 cells are crucial for initiating and continuity the inflammatory response in COPD over the past decades by data from clinical and experimental research (Zhao et al., 2018). Th17 cells are determined by the ROR-γT which is the main transcriptional marker with the production of IL-17A, IL-17F, and IL-22(Burks et al., 2019). They are polarized from Naive CD4+ T cells by STAT3 in the presence of IL-6, IL-4, IFN-γ, IL-1β, IL-23, and TGF-β. While IL-17A/F triggers epithelial and endothelial cells, fibroblasts, and macrophages to synthesize CXCL8 which is the primary cytokine for neutrophil recruitment to the inflammation site, the IL-21 stimulates B Cells, $CD8^+$ T Cells, and natural killer cells (Korn, Bettelli, Oukka, & Kuchroo, 2009; Romagnani, 2008). These T helper cells are primarily found in the bronchial mucosa and are linked to the progression of COPD and the increase/recurrence of alveolar collapse (Righetti et al., 2018).

1.4.5 T regulatory lymphocytes

The immune system's capacity to tolerate autoantigens can be compromised by a lack of $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs)(Bhat, Panzica, Kalathil, & Thanavala, 2015; Cosio, Saetta, & Agusti, 2009). Tregs represent 5–10% of CD4+ T cells. At tissue areas where antigen invasion occurs, these cells gather then locally inhibit the immune system by secreting IL-10, IL-35, and TGF-β (Ulbar et al., 2020). FOXP3 intracellular expression is thought to be the most specific human Treg cell marker, and the transcription factor FOXP3 is activated and maintained by the STAT-5 signaling pathway(Isajevs et al., 2009). Both clinical and experimental studies suggest that Tregs play a crucial role in reducing and sustaining the inflammatory response in COPD (Hou & Sun, 2020).

1.4.6 $CD8^+$ T and B lymphocytes

The crosstalk between the T cell receptor (TCR) on $CD8^+$ T cells and the peptide on MHC class-1 of the target cells stimulate CD8+ T cells, which then exert their effects primarily through two mechanisms: cytolytic attack on target cells with granzymes or secretion of interleukins and cytokines including IFN-γ, TNF-α, and IL-2 that can encourage the attraction of additional cells to infection sites (Bevan, 2004). Increased level of CD8+ cells are positively connected with smoking habits and history, the severity of airflow obstruction together with emphysema in relation to airway disorders(Barnes, 2014). More CD8+ cells can be found in the sputum of stable COPD patients compared to those with normal lung function who smoke and to nonsmokers who have never smoked (Tzanakis et al., 2004), and these are highly active and express high perforin levels. (Chrysofakis et al., 2004). In the lung tissue, there is an accumulation of cytotoxic $CD8^+$T cells which promote alveolar epithelium injury and cell death (Tzanakis et al., 2004). It has been demonstrated that patients with COPD in the mild to moderate phase had higher levels of $CD8^+$ T lymphocytes in their blood and lower airways. They also found a negative correlation between tissue and circulating CD4+ and CD8+ T cell counts, as well as the ratio of CD4+/CD8+ T cells, and the FEV1% level in patients with COPD (Barnes, 2014).

Soluble or membrane-bound antigens bind to the B cell receptor (BCR), and then B cells become activated. The activation of BCR initiates a series of downstream signaling cascades that allow B and T cells to interact and send activation signals in both directions(Abbas, Lichtman, & Pober, 2005). B cells that have been activated may go through class switch recombination. They produce IgM and IgD when they are inactive, but once activated, they can also produce IgA, IgE, and IgG while still expressing IgM (Hesslein, Aguila, & Horowitz, 2011). Studies indicate that autoantibodies have been found in the bronchoalveolar fluid of COPD patients which demonstrates the presence of B cells in the COPD (Hogg et al., 2004; John-Schuster et al., 2014). More recently, it is known that B cells can accumulate in patients with COPD and organize into known as tertiary lymphoid follicles (LFs). Researchers have found that as COPD progresses, B lymphocytes infiltrate the small airways of patients and the percentage of these B cells and lymphoid follicles rises (Hogg, 2004). These lymphoid follicles exist in approximately 5% of the small airways of mild-to-moderate COPD patients and healthy

smokers. The airways of smokers with severe and very severe COPD expand by 25–30%. Whereas they are rarely observed in non-smokers(Hogg et al., 2004).

1.5. Immunopathology of COPD exacerbations

COPD exacerbations are periods of clinical deterioration that end up causing significant morbidity and mortality (O'Donnell & Parker, 2006). The most common trigger of exacerbation of COPD is a bacterial or viral infection (Papi et al., 2006). Regardless of the type of infectious agent found, there is an increase in neutrophils in sputum that result in acute respiratory failure. While virus infection is linked to rising in eosinophils in the sputum, this rise in sputum could be indicative of a viral infection during COPD exacerbations. (Aljaafareh, Fakih, & Biswas; Papi et al., 2006). In comparison to stable patients, mild/moderate exacerbated patients have elevated eosinophils, neutrophils, and CCL5 in their bronchial mucosa together with T lymphocytes very late antigen (VLA)-1 and TNF-α. Studies also indicate the increase of eotaxin-1 and CCR3 chemokine receptor expression in this comparison (Aljaafareh et al.; G Caramori et al., 2011; Hogg & Timens, 2009). Whereas, in severe exacerbated patients, there is an increased level of neutrophils in addition to CXCL-5 (ENA-78), CXCL-8 (IL-8), CXCR-1, and CXCR-2 when compare with stable COPD patients (Qiu et al., 2003). Consequently, during COPD exacerbations, there is a considerable rise in the number of inflammatory cells and total inflammation.

1.6 THE BALANCE OF T-REGULATORY/TH17 CELLS IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

The inflammation in COPD develops and progresses because of a disparity between pro-inflammatory and anti-inflammatory immunological responses. Numerous clinical and experimental research has examined the role of Th17/Treg imbalance in the development of COPD (D Lourenço, Ito, Martins, Tibério, & Lopes, 2021). Nevertheless, several part of the lung, COPD stages, or local and systemic indicators have produced inconsistent outcomes. Studies in the early 2000s have proposed that progression and the seriousness of COPD were associated with the decreased level of Treg and increment of Th17 cells (Bedke, Muscate, Soukou, Gagliani, & Huber, 2019; Cosio et al., 2009). In

immunohistochemistry and flow cytometry studies, Zheng et al. (2018) observed an amplification of Th17 cells and a decrement of Treg cells (Zheng et al., 2018). In a different study, smokers and COPD patients' tissue samples demonstrated an increase in Th17 cells, whereas the Treg population of obstructed smokers is reduced in their small airways when compared to healthy smokers (Hou et al., 2013). Conversely, they indicated a rise in the Treg cell population in lymphoid organs in COPDs (Hou et al., 2013). On the other hand, mRNA expression of Th17 cells and ROR-γt in the blood samples of patients with moderate and severe COPD are higher than those in COPD stage-1, and healthy smokers with non-smokers while the level of Treg cells and expression of FOXP3 mRNA is decreased in those patients (H. Wang et al., 2015). Higher level of pro-inflammatory cytokines were also studied by the authors, including IL-6, IL-17A, IL-21, and IL-22, while IL-10 was found to be present at lower concentrations in the blood of subjects with COPD (H. Wang et al., 2015). According to isolated plasma and leukocyte data from the peripheral blood of smokers and patients with various stages of COPD, the gene expression of ROR-γt and TGF-B levels were elevated in patients with COPD stages 3rd and 4th, compared to those in stages 1st and 2nd, and smokers. Despite the fact that STAT5 levels were higher in the COPD 3rd and 4th stages, FOXP3 expression and IL-10 levels decreased in all COPD stages (Lourenço et al., 2021).

However, numerous researches in the literature contradicts these conclusions. In the peripheral blood mononuclear cells (PBMCs) of smokers and COPD patients, for instance, they discovered high level of Th17 and Treg. The increase in Treg was claimed to be long-term cigarette smoke exposure rather than airway obstruction (Vargas-Rojas et al., 2011). In addition, this study indicated a negative correlation between FEV1 and FEV1/FVC values and Th17 cells. Li et al. (2014) evaluated a high level of Treg in the PBMCs of both stable and acute exacerbated COPD patients as opposed to health smokers together with high level of Th17 cell only in the exacerbation COPD group (X.-N. Li, Pan, & Qiu, 2014). However, they discovered a negative correlation between Th17 and Treg level in both COPD exacerbations and stable COPD. Another study indicated an increase in both anti-inflammatory and proinflammatory cytokines together with Treg cells. While the ratio of Treg/IL-17 was normal, they hypothesized that these anti-inflammatory Tregs were unable to inhibit the increase in inflammatory mediators, indicating that these cells are inadequate (Jin et al., 2014).

Due to difficulties in collecting patient specimens, experimental studies are useful for examining the different parts of the lung and local and systemic responses to COPD. Wang et al. (2012) examined the lung tissue with the blood of mice exposed to cigarette smoke (CS) for four and twenty-four weeks, respectively. Chronically exposed mice had higher Th17 in peripheral blood and lung tissue, as well as higher ROR-γt mRNA expression, compared to subacutely exposed and control mice (H. Wang et al., 2012). Also at the beginning, the Treg response was enhanced in the sub-acute CS-exposed group and then diminished in the chronic CS-exposed group compared to the control group. In addition, the prevalence of Treg in both blood and lung tissue, as well as the expression of FOXP3 mRNA, are reduced in the lungs of mice chronically exposed to CS (H. Wang et al., 2012). Another study demonstrated similar results. They observed a high level of Th17 cells, ROR-γt mRNA expression, and IL-17 cytokines while Treg cells, FOXP3, and IL-10 expression levels decreased in the lungs of chronically CS-exposed mice groups compared to the control group (Duan et al., 2016). Ito et al.(2019) indicated that decreases in IL-10^{+}, TGF-β^{+}, and Treg cell numbers have been observed since the onset of COPD and have been linked to decreased lung functional(Ito et al., 2019). Even though the level of Treg cells in the CS-exposed animals eventually reached that of the control group, the decreases in IL-10+ and TGF-+ cell populations proceeded until the end, indicating that immunosuppressive activity is impaired despite the presence of Treg cells in lung tissue (Ito et al., 2019). In the experimental model of COPD exacerbation, inflammatory mediators such as STAT3+ and IL-17 are present alongside anti-inflammatory mediators such as STAT5+ and Treg. Nevertheless, despite the rise in Treg cells, the exacerbated group's lower density of IL-10^{+} cells implies that the failure in IL-10 release was crucial to the exacerbation of the inflammatory response (Cervilha et al., 2019).

1.7 NOTCH SIGNALING PATHWAY

Notch signaling is a highly conserved pathway that serves a variety of physiological activities, including controlling immunological response, cell survival, cell fate, and apoptosis(Kopan & Ilagan, 2009). Jag1 and Jag2, and the Delta-like protein family, which consists of DLL1, DLL3, and DLL4 are Notch ligands. There is also Notch receptor that has three components. One of them is an extracellular component, which is the Notch extracellular domain(NECD). That portion of the receptor binds to the ligand, such as DLL1 (Bray, 2006). There is also a Notch intracellular domain(NICD); the transmembrane domain, which connects the intra- and extracellular domains, is the third component. Ligand must bind to the NECD, and the receptor must first translocate to the plasma membrane, for the notch signaling pathway to become activated (Gerhardt, 2008). In Golgi, the notch receptor precursor undergoes Furin-mediated cleavage (S1), which is required for the formation of the functional heterodimeric receptor. The Fringe family of glycosyltransferases glycosylates Notch, which causes the Notch receptor to migrate to the plasma membrane and interact with a ligand on the surface of the adjacent cell (Schweisguth, 2004). The ligand must then be activated, which is accomplished by a protein called MIB found within the sending cell. MIB ubiquitinates the ligand to become activated. Once the ligand has become ubiquitinated or activated, it binds to the NECD (Moretti & Brou, 2013). In response to the binding of the ligand to the receiving cell's Notch receptor, a protein called ADAM10 cleaves the receptor's extracellular domain. Effectively removing receptor subunit from the receiving cell.This process is known as S2 cleavage (Zhou et al., 2022). Having followed all these, a unique protease known as gamma-secretase cleaves the NICD from the transmembrane region of the receptor. This procedure is referred as S3 cleavage. (Zhou et al., 2022). The NICD is now free within the cytosol after being cleaved off the transmembrane portion of the notch receptor. Then it forms complex with the DNA-binding transcription factor RBP-J, CSL, and a member of the Mastermind-like (MAML) family, which recruits additional transcriptional coactivators (CoA) such as p300. This entire transcriptional activation complex translocates, and functions as a histone acetylase in the nucleus resulting in the transcription of Notch target genes such as Hes and Hey (Miele, 2006). After the notch target genes have been expressed, the notch signalling system can be successfully shut down by downregulating NICD via ubiquitination (Moretti & Brou, 2013). Recent research has revealed the existence of several other Notch signalling pathways known as non-canonical Notch signalling. They indicated that this pathway could occur in either a

ligand-dependent or a ligand-independent manner. Also, signalling can occurs either secretase-dependent or independent. Furthermore, non-canonical Notch signalling is independent of CSL/RBPJ and instead interacts at the cytoplasmic and/or nuclear levels with pathways such as Wnt, PI3K, and mTORC2 (Furkan Ayaz & Barbara A. Osborne, 2014). However, because the non-canonical signalling pathway is not as well characterized as the canonical signalling pathway described above, further research is required to clarify this area.

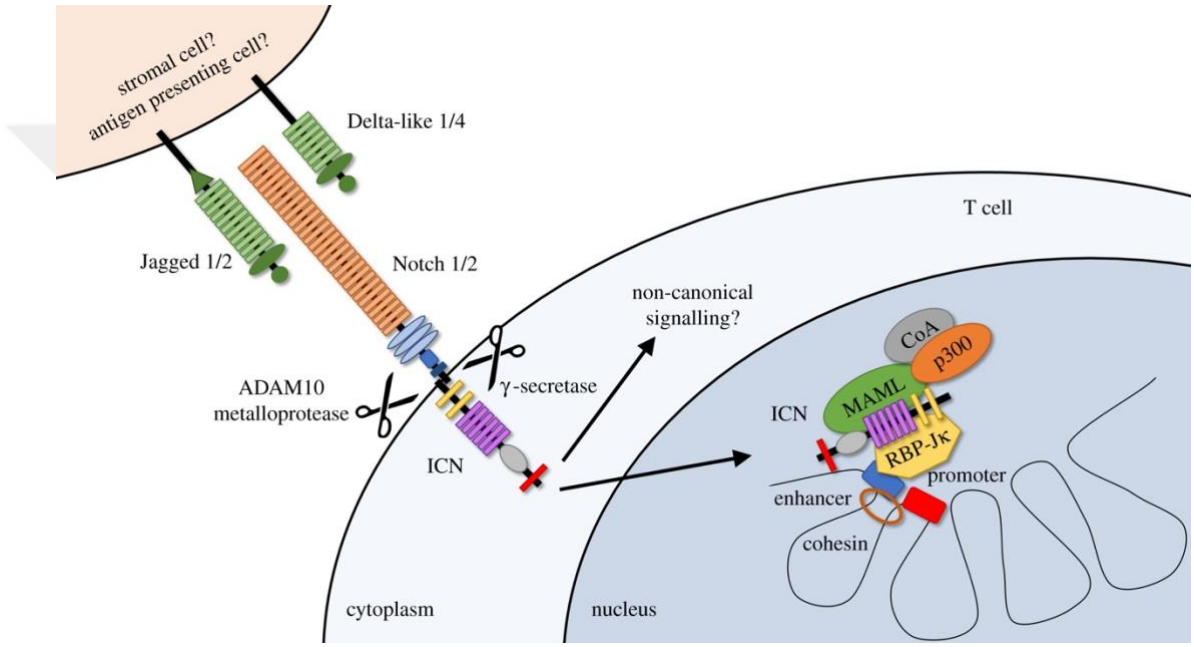

***Figure 2**: Notch signaling pathway [Brandstadter Joshua D. and Maillard Ivan (2019) Open Biol].*

1.7.1 THE ROLE OF THE NOTCH SIGNALING PATHWAY IN THE LUNG

Notch signaling have a crucial role during the development, homeostasis, and regeneration of many organs as well as the lung. It is involved in proximodistal patterning, cell fate selection, and cell proliferation in the mammalian lung (Furkan Ayaz & Barbara A Osborne, 2014). To sustain the airway epithelium's proper function as a barrier of defense against the inhaled environment, Notch is necessary to mediate cell fate choice in epithelial development (Tsao et al., 2016). For instance, in the proximal regions of the lung that consist of the trachea, bronchi, and bronchiole, activation of the Notch is required for the specification and maintenance of cell fate between the club and ciliated cell (Kiyokawa & Morimoto, 2020). In mice studies, activation of the Notch-1 receptor

contributes to the club cell specification. When the club cell population's Notch 1 and Jagged 1 activation is lost, they lose their club cell characteristics and become ciliated cells. In addition, knockout of Notch-1, 2, and 3 genes has revealed that Notch-2 plays a crucial role in the activation of Notch-1-mediated club cell differentiation. Therefore, they say Notch-2 is more crucial than Notch-1 for cell fate choices between the club and ciliated cells. Other in vivo and in vitro studies point out the pathway has also major role in differentiation from the club into goblet cells. Guseh et al. (2009) indicated the expansion of goblet cells through the misexpression of NICD1(Guseh et al., 2009) while Danahay et al. (2015) demonstrated the overexpression of Notch-2 in combination with IL4/IL13 cytokines leads to goblet cell metaplasia (Danahay et al., 2015). Also the differentiation of pulmonary neuroendocrine cells (PNECs) indicated via Notch-mediated lateral inhibition through the Hes1 gene. Hes1- null mouse embryos reveal that the PNEC population increases along with a decline in club cells that express Notch receptors (Vaughan et al., 2015). Besides proximal lung, growing data suggest Notch is also important for the distal area, including alveoli. Concerning a transgenic mouse study, overexpression of NICD-3 inhibits the differentiation of distal progenitors to alveolar cells, and mice overexpressing NICD-1 exhibit similar phenotypes .According to these findings, NICD upregulation inhibits the commitment of developing epithelial cells to alveolar epithelial cells, resulting in cystic epithelium. Additionally, human and animal studies have shown that overexpression of Notch-2 causes aberrant regeneration that frequently generates honeycomb-like histology in those with idiopathic pulmonary fibrosis because of the this fail to trans-differentiation (A. Di Stefano et al., 2001). Under homeostatic settings, lineage-negative epithelial progenitors (LNEP), which are mostly found in small airways and play role in the regeneration of distal lung area, are a minor stem cell population. Severe damage prompts dormant LNEP cells to transition into basal-like cells to repair the injured epithelium, these basal-like cells migrate toward the wound and proliferate. Although trans-differentiation into alveolar epithelial cells is inhibited by Notch activation, it’s necessary to produce basal-like cells from LNEPs(Guseh et al., 2009; Kiyokawa & Morimoto, 2020; Wasnick et al., 2019). These results prove the Notch is essential in lung development, homeostasis, and regeneration.

1.8 THE ROLE OF NOTCH SIGNALING PATHWAY IN COPD

Given the diverse and essential roles of Notch signaling in lung, dysregulation of Notch-related components may be linked to several diseases which including idiopathic pulmonary fibrosis (IPF), and lung cancer (Kiyokawa & Morimoto, 2020). For example, there is increased expression of NICD-1 in highly fibrotic alveolar regions in IPF patients. Additionally, Notch-1expressed cells express alpha smooth muscle actin, which is a myofibroblasts marker, forming a fibrotic foci (Aoyagi-Ikeda et al., 2011). On the other hand, studies indicated Notch-3 expression causes vascular smooth muscle cells proliferation in patients with pulmonary artery hypertension (X. Li et al., 2009). Moreover, this pathway functions primarily as a tumor promoter in non-small cell lung cancer (NSCLC) and as a tumor suppressor in small cell lung cancer (SCLC). Notch signaling-associated genes are often mutated or overexpressed in these two cancer types, even though their roles in oncogenesis are context-dependent. For instance, the Notch1 and Notch3 overexpression were related to considerably lower survival in patients with NSCLC while in a mouse model of SCLC, Because they are relatively resistant to chemotherapy and support neuroendocrine tumor cells trophically, notch-activated SCLC cells may represent a new target for therapy .

Concerning the function of Notch signaling in COPD, Boucher et colleagues (2012) discovered upregulation of NICD-1 and Notch downstream gene HEY2 in patients' submucosal glands and airway epithelium in areas of goblet cell metaplasia (Boucherat, Chakir, & Jeannotte, 2012). Human 3D culture and mice studies demonstrated that Notch signaling, particularly Notch2, governs IL-13-mediated respiratory basal cell differentiation into goblet cells and leads to goblet cell hyperplasia. They suggested that Notch2 might serve as a new therapeutic target for blocking goblet cell metaplasia in human respiratory conditions like COPD (Demedts et al., 2007). In addition, microarray analysis revealed activation of the Notch signaling pathway in rhinovirus-infected COPD cells via upregulation of Notch1-3, and HEY1 gene (Jing et al., 2019). Bodas et al (2021) reported that in vitro studies of human airway epithelium exposed to cigarette smoke revealed that induction of goblet cell differentiation in response to smoking was mediated by Notch3 signaling. (Bodas et al., 2021).

In contrast to these findings, a microarray study of cells from the human epithelium reported that Notch3 and DLL1 expression levels were decreased in COPD patients than

in nonsmokers. Consistent with this observation, the HES5, HEY1/2 genes were downregulated in the COPD group, implying that Notch is effectively suppressed in the COPD patients' epithelium (Tilley et al., 2009). Additionally, Zong and coworkers discovered that Notch 1/2/4 expressions in endothelial cells of COPD patients were downregulated when compared to the control group (Cosio et al., 2009). As a result, given the significance of Notch signaling in lung diseases and the paucity of studies on the association between COPD and Notch, contradictory as they may be, comprehensive studies are necessary to clarify the role of Notch signaling in COPD. Future research should address these questions in order to gain a better understanding of the fundamental role of Notch signaling; the results may reveal novel COPD therapeutic targets.

1.9 THE RELATIONSHIP BETWEEN NOTCH SIGNALING, T CELLS, AND COPD

Along with the effective role of the Notch pathway in regulating lung homeostasis and development, studies also suggest that it regulates T-cell development, maintenance, and activation (Hou & Sun, 2020). As lymphoid progenitors encounter the thymus, they meet ligands on the cortical thymic epithelium, which is required for thymocytes to differentiate into mature T cells via either negative or positive selection (Radtke, 1999; Sambandam et al., 2005; Schwerd et al., 2017; Tan, Visan, Yuan, & Guidos, 2005). During development, thymocytes express Notch1, 2, and 3. According to an in vivo study, only Notch1 is required and sufficient for the commitment of T cell lineage. According to an in vivo study, the absence of Notch 1 halts T cell development and causes severe deficiencies in thymocyte formation. Other hematopoietic lineages remained unaffected despite the lack of Notch signaling (Radtke, 1999). Not only Notch 1, but also Hes1 which is a downstream effector of Notch indicated identical outcomes in the case of disruption of signaling (Tomita et al., 1999). In addition, an overexpression study of a fragment called Notch1- ICN in bone marrow progenitors leads to ectopic T cell development while impairing B cell development, indicating that Notch have a crucial role in directing the T cell fate decision as opposed to the B cell fate decision (Pui et al., 1999). Notch is also involved in the specification of T cell receptors (Deftos & Bevan, 2000). In T cells, the pre-T cell receptor chain gene is demonstrated as a transcriptional

target of Notch signaling(Reizis & Leder, 2002). Notch signals continue to be present until the pre-T-cell receptor checkpoint (Forrester et al., 1990; Wolfer, Wilson, Nemir, MacDonald, & Radtke, 2002). Once a thymocyte successfully passes through the checkpoint, Notch signaling is turned off, and the rest of thymic development is thought to be Notch-independent. When a mature, naive $CD4^+$ T cell enters the periphery and becomes activated the Notch signaling is reactivated. For example, mature naive CD4+ cells express Notch1 and Notch2 receptors, which are upregulated in response to T cell receptor stimulation . Considering all these studies, the molecular mechanism by which Notch regulates the development of early T cells is poorly understood.

Notch pathway plays an important role in subsequent T helper (Th) cell differentiation decisions. However, it is unclear how Notch directs the fate of these various subsets. Early theories on how Notch might influence the maturation of T cells suggested that it functioned as a bipotential switch capable of switching between Th1 and Th2 cells, with varying results depending on the type of Notch ligands involved. They discovered that the forced upregulation of the Dll4 and Jagged1 in APCs resulted in the differentiation of mature CD4+ T cells into Th1 or Th2 cell fate in response to these ligands, respectively (John-Schuster et al., 2014). A more recent hypothesis has been proposed in which Notch promotes T cell differentiation in a non-selective manner. According to this theory, the polarizing environment determines whether Notch signaling promotes Th1 or Th2 responses. They claim that Notch signaling keeps various Th1 and Th2 differentiation gene targets and cytokines consistently expressed (Bedke et al., 2019; D Lourenço et al., 2021). These findings indicate that multiple genes targeting Th1 and Th2 can support a fundamental cell signaling pathway that promotes the differentiation of multiple distinct lineages. It was demonstrated further that Notch is necessary for the maintenance of the Th17 response. According to studies, Dll4 expression on APCs can stimulate CD4+ T lymphocytes by enhancing PI3K signaling downstream of CD28 co-stimulation through an activity pattern similar to costimulatory signals (D Lourenço et al., 2021). This signaling may also control Treg differentiation via Foxp3-dependent processes in addition to Th1, Th2, and Th17. Initially, overexpression studies of Notch suggested that Jagged ligands could promote Treg expansion(Wagner et al.,2003). Indeed, after being exposed to transforming growth factor beta (TGFβ), which induces Foxp3 expression, these cultured T cells with a Treg-like phenotype lost this ability when

γ-secretase inhibitor (GSI), Notch inhibition factor, was added (Samon et al., 2008). Loss-of-function studies in vivo, however, have shown that Notch may serve a distinct purpose in Tregs. They showed that knocking out Notch1 or Rbpj generated a super-regulatory' phenotype (Charbonnier, Wang, Georgiev, Sefik, & Chatila, 2015). To this research, Treg expansion was caused by genetically or pharmacologically inhibiting Notch signaling in vivo during allogeneic bone marrow transplantation (Y. Zhang et al., 2011). Nonetheless, it is clear that more research is needed to fully comprehend the part Notch plays in Th cell differentiation and function, especially in Treg cells.

The effect of the Notch signal on the Treg/Th17 cell balance has also been the subject of many lung-related diseases. For instance, Notch 4 and Wnt-Hippo axis activation on Tregs suppressed their immune response and turned them into Th2 and Th17 cells to promote allergic asthma, according to research on asthmatic humans and asthmatic mice (Harb et al., 2020). Another study revealed that Notch 4 signaling on Treg cells restricts tissue repair mediated by Tregs and induces inflammation in COVID-19 infection (Harb et al., 2021). If we look closely at the studies regarding COPD in the literature, Notch 1/3 and Th1-biased immune responses are higher in COPD patients compared to the healthy group (Lin, Li, Chang, & Lin, 2020) and Notch activation has been shown to regulate Th17 and Treg cell differentiation, as well as disrupt the $CD4^+/CD8^+$ and Th17/Treg balance (Yin et al., 2019). Moreover, an in vivo study by Gu et al (2017) on mice model of COPD reported and increased expression of Notch1/2/3/4 receptors, Hes1/5, and Hey1 mRNA in the T cells leading to upregulation of Th1 and Th17 and an increase in the ratios of Th1/Th2 and Th17/Treg (Gu, Chu, Zeng, Bao, & Liu, 2017). Although studies suggest T cell subsets imbalance in COPD immunopathogenesis, it is not clear whether this has an association with Notch signaling. It is also unknown whether the imbalance of T cells and the dysregulation of Notch pathway contribute to the development and progression of COPD.

Hypothesis

Upregulation of Notch signaling on Foxp3 + CD4+ T-reg cells causes Treg cell imbalance. These all together lead to chronic inflammation in COPD.

Aim

Studies reported Treg cell imbalance in COPD immunopathogenesis and it is unclear whether this is related to Notch signaling. Furthermore, it is uncertain whether Notch signaling dysregulation and T cell subset imbalance plays a role in the severity and exacerbation of COPD. We believe that our research will help us understand the role of Notch signaling and Treg cell imbalance in the pathogenesis and progression of COPD. Moreover, this study may also reveal potential targets for therapeutic intervention.

Our aim was to investigate the distribution and activity of the Notch receptor on T-regulatory cells in disease inflammation from non-smokers, smokers, together with COPD patients in the stable and exacerbated phase of the disease.

Objectives

- To obtain PBMCs from well-defined groups of nonsmokers, smokers, and COPD patients in stable disease and exacerbation.
- To investigate the expression of Notch 1/2/3/4 receptors in Treg cells and their relationship with smoking and COPD.
- To investigate Th1/Th2 balance in PBMCs from COPD.
- To analyze the expression of Hes5, Hey1, Foxp3, and RORγt genes in CD4+ T cells
- To determine the level of pro/anti-inflammatory cytokines in plasma from study subjects.

Chapter 2:

METHODOLOGY

2.1 Study Population

Non-smoker and smoker-healthy control subjects (n = 20 for both), stable subject with COPD and exacerbated subject with COPD (n=10 for both) were recruited for this study **(Table 2)**. Our inclusion criteria: non-smokers who have never smoked or have smoked fewer than 100 cigarettes in their lifetime have no chronic or acute disease of any clinical importance, have normal lung values on spirometry, and have normal chest radiology will be included. Smokers, who have smoked 1 pack a day (1 pack/year) for at least 1 year, who do not have any significant chronic or acute disease, and who have normal lung values in spirometry and normal chest radiology will be included. Clinically stable and exacerbated COPD subject who was diagnosed with COPD according to GOLD criteria (with COPD for at least 1 year) and must have FEV1/FVC ratio <0.7 values on spirometry. Stabil COPD group who never had exacerbations in the last at least 2 months and no lung disease other than COPD. Exacerbation COPD group who had recorded exacerbations in the last 1 month and no lung disease other than COPD. Our exclusion criteria: the groups must not be pregnancy or lactation in females, any clinically relevant medical history of autoimmune diseases or current malignancy (incl. remission for <5 years), any clinically significant/uncontrolled disease including renal, hepatic, gastrointestinal, or cardiovascular diseases, any acute infectious disease within 4 weeks before enrolment; Additionally, the healthy subjects must not include any active or clinically significant lung disease; all COPD patients must not be any active or clinically significant lung disease other than COPD. Laboratory workflow is given in **Figure 3.**

This study was approved by the institutional review board of Koç University (approval number: 2021. 435.IRB2.078). Both the patients and the control group were of Turkish descent. Informed consent was obtained from the patients and control individuals, and the study was carried out by the principles of the Declaration of Helsinki.

Groups	Non-smoker	Smoker	Stable COPD	Exacerbated COPD
Age,years	32.2	35.4	68.7	65.2
Gender, Female/Male	8/12	10/10	2/8	2/8
Smoking, pack/year	-	13.3	50.3	55
FEV1/FVC	-	-	65.02	52.37
FEV1	-	-	52.5	65.5

***Table 2** : Clinical data of the study subjects.*

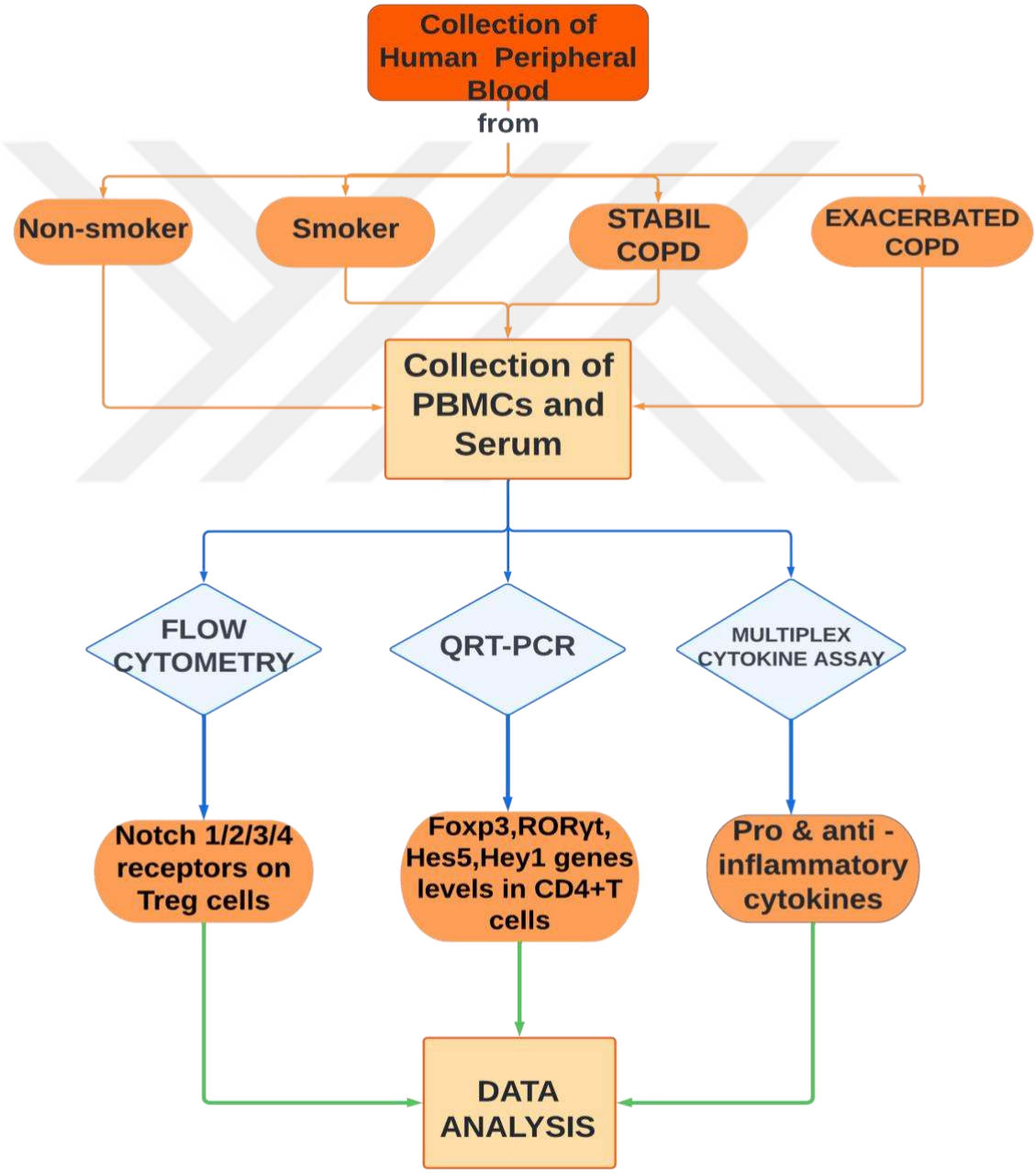

***Figure 3:** Laboratory workflow chart.*

2.2 Sample collection, PBMC isolation, and obtaining plasma

Each subject's 24 milliliter of peripheral blood was collected in EDTA-treated tubes and placed on ice immediately after collection. Blood-containing tubes were centrifuged at 2500 x g for 10 minutes at room temperature (RT), and the plasma was collected in sterile 1.5 ml microcentrifuge tubes. The blood was diluted at a ratio of 1:2 with phosphate buffered saline (PBS, 1X) solution and then, transferred to 50 ml tube containing 15 ml of Lymphoprep ^{TM} Density Gradient Medium, which has the concentration of 1.077 g/ml. (Cat no: 07801, Alere Technologies, Norway, centrifuged in soft acceleration and off deceleration mode, at a speed of 500 x g for 30 minutes at room temperature **(Figure 4a).** Blood cells were divided into layers with a difference in density, and PBMCs which include lymphocytes such as T and B cells were located above the layer of lymphoprep as a buffy coat **(Figure 4b)** After initial centrifugation, the buffy coat was carefully removed and centrifuged at a speed of 500 x g for 5 minutes. After the 2^{nd} centrifugation, the supernatant was aspirated, and PBMCs were incubated for 10 minutes with 1x PharmLyse Erythrocyte Lysing Solution (Cat no:555899, BD Pharmingen, USA) to remove any erythrocytes. As a washing step, PBMCs were washed with 1 ml of PBS (1X, Cat no: L0615-500, Sigma-Aldrich), and the 500 x g for 5 minutes centrifugation together with aspiration step was repeated. Then, 1 ml of PBS was added to the pellet, and the suspension was vortexed thoroughly. Finally, the number of lymphocytes was determined by an automated hematology analyzer (Sysmex, USA) As the last step, PBMCs were frozen in RPMI-1640 medium (Cat no: 12-702F, Lonza, Switzerland) containing %90 fetal bovine serum (FBS, Cat no:16000044 , Thermo Fisher Scientific, USA) and 10% DMSO (dimethyl sulfoxide, Cat no: 67-68-5, Sigma -Aldrich,USA) for further experiments.

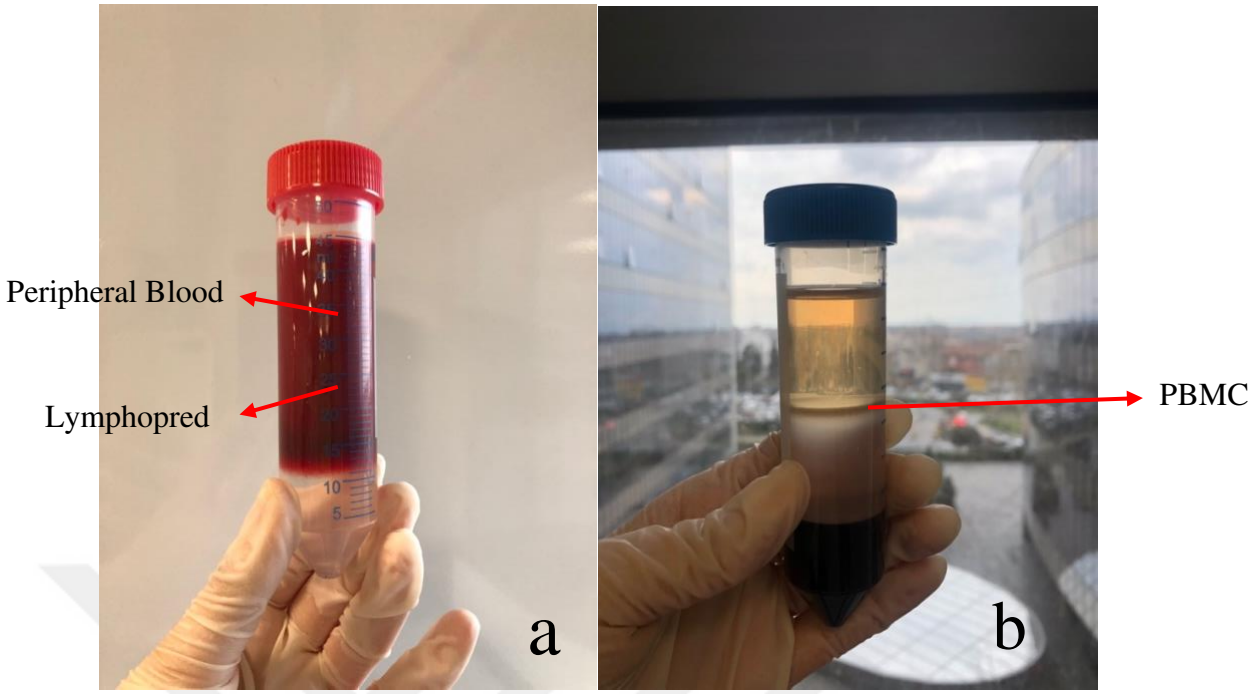

Figure 4: *Human PBMC isolation from peripheral blood. (a) Lymphoprep ™ (1.077), one of the non-smoker's blood respectively, (b) Density gradient formation/ buffy coat after centrifugation.*

2.3 Determination of regulatory T-Cells and Notch Receptors by Flow Cytometry

First, an equal number of PBMCs from each study group were thawed in 1 ml of complete medium including RPMI-1640 medium,10% FBS and 1% Penicillin+Streptomycin (Gibco, USA), centrifuged at a speed of 500 x g for 5 minutes at RT. After the first centrifugation, the supernatant was aspirated, and PBMCs were resuspended with 100 µl of complete medium and 100 µl of PBS. A total of 200 µl samples per subject were divided into two wells of a round bottom 96-well plate for panel-1 (Notch 1 and 2) and for the panel-2 (Notch 3 and 4). Secondly, these cells were stained with antibody named Zombie Dye and incubated for 10 minutes at dark at room temperature to indicate cell viability. They underwent a washing process in which 100 µl of staining buffer (PBS + 0.5% BSA) was applied to them, and then they were centrifuged at 500 x g for 5 minutes. Cell pellets were resuspended in the staining buffer after supernatants were discarded. Then, these cells firstly were stained by cell surface antibodies which are CD3, CD4, CCR4, CXCR3, CD45RA, CD45RO, CD25, and CD127 incubated for 30 minutes at RT in dark. After the washing step with staining buffer and

centrifugation, supernatants were discarded and cells were fixed with True Nuclear Fixation Buffer (Cat no: 424401, Biolegend, USA,) for 1 hour at room temperature in dark and permeabilized with Permeabilization Buffer (Cat no: 424401,Biolegend, USA) for intracellular staining. Anti-Human Foxp3-PE antibody, Helios (BD Biosciences, USA) were added to both two wells of each subject. After all, Notch 1 and 2 antibodies were mixed and added to one well of each subject, while Notch 3 and 4 antibodies were mixed and added to the other. They were incubated in the dark at room temperature for 20 minutes. The cell pellet was resuspended in 500 µl of staining buffer for analysis after being washed with Permeabilization Buffer. Cells were run under Attune NxT (Invitrogen, USA) and analyzed with FlowJo Software (TreeStar, USA). From the CD3+ gate, Tregs were determined as CD4+CD25+CD127-FoxP3+ cells. Our gating strategy has been shown in **Figure 5**. Also, flow cytometry antibody list has given in **Table 3**.

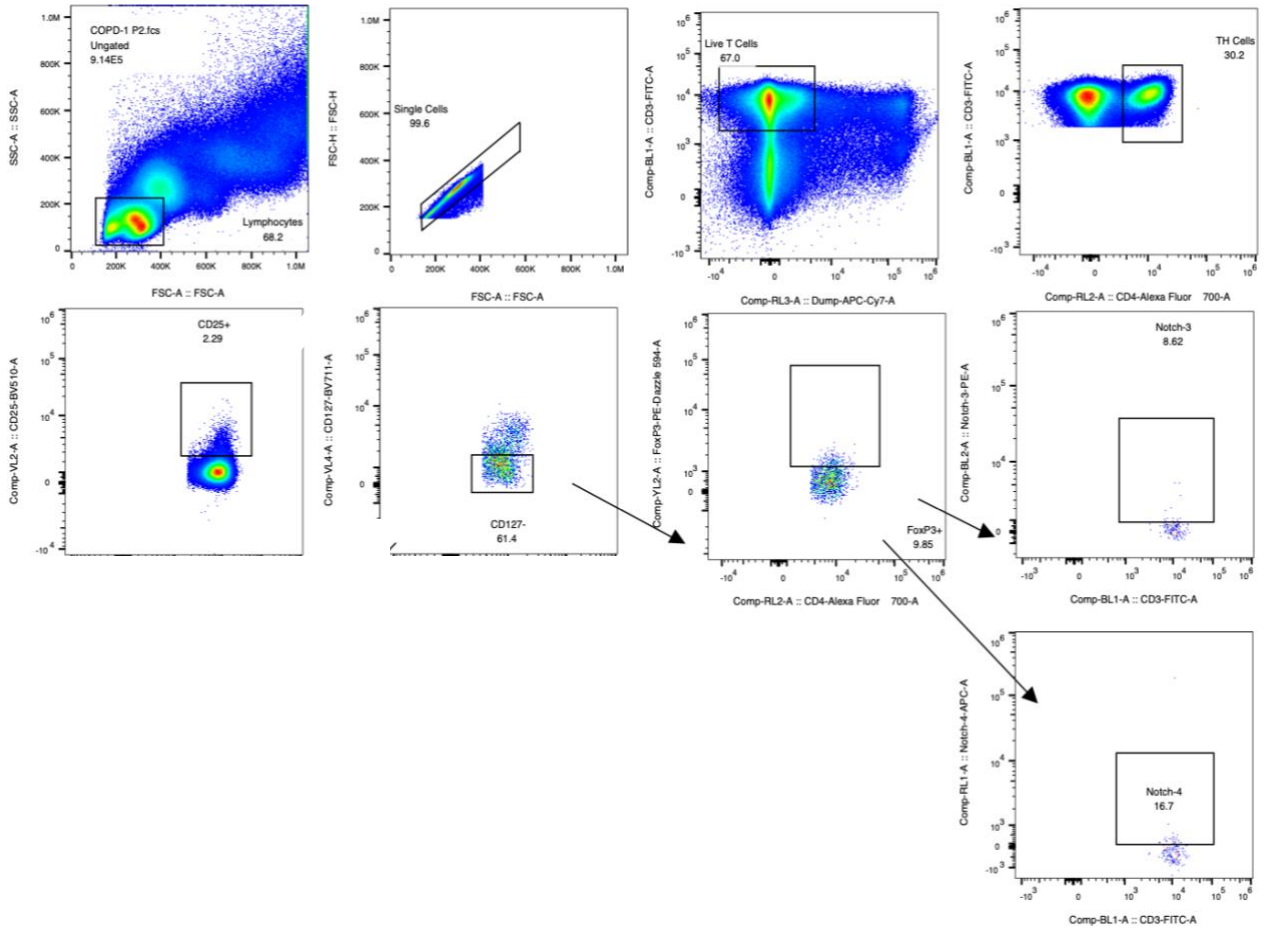

Figure 5: *Flow cytometry gating strategy.*

Marker	Color/Format	Isotype	Clone	Catalog no
CD3	FITC	IgG2a Îº	HIT3a	300306
CD45RO	PE-Cy5	IgG2a Îº	UCHL1	304208
CD45RA	Brilliant Violet 421	IgG2b Îº	HI100	304130
CD4	Alexa Fluor 700	IgG1 Îº	SK3	344622
CD25	Brilliant Violet 510	IgG1 Îº	BC96	302640
CD127	Brilliant Violet 711	IgG1 Îº	A019D5	351328
CCR6	PE-eFluor 610	IgG1 Îº	R6H1	61-1969-42
CD183(CXCR3)	APC-Cy7	IgG1 Îº	G025H7	353721
CD194(CCR4)	PE-Cy5	IgG2a κ	L291H4	359406
FoxP3	PE-Dazzle 594	IgG1 Îº	206D	320126
Notch 1	Brilliant Violet 605	IgG1 Îº	MHN1-519	743908
Notch 2	APC	IgG2a Îº	MHN2-25	348305
Notch 3	Brilliant Violet 605	IgG1 Îº	MHN3-21	745152
Notch 4	APC	IgG2a Îº	MHN4-2	349005

Table 3 : *Flow Cytometry and FACS antibody list*

2.4 CD3+ CD4+ cell sorting from PBMCs

First, an equal number of PBMCs from each study group were thawed in 1 ml of complete medium including RPMI-1640 medium, 10% FBS and 1% Penicillin+Streptomycin (Gibco, USA), centrifuged at the same conditions. After the aspiration of supernatant PBMCs were resuspended with 100 µl of PBS. These cells were stained with Zombie dye and incubated for 10 minutes at room temperature in the dark to determine cell viability. They were washed with 100 µl of staining buffer (PBS + 0.5% BSA) before being centrifuged. The supernatants were discarded, and again the cells were resuspended in the staining buffer then stained with CD3 and CD4 cell surface antibodies. They were incubated at room temperature in the dark for 20 minutes. Following incubation, cells were washed in 2 ml of staining buffer (PBS + 0.5% BSA) before being centrifuged at 500 x g for 5 minutes at room temperature. The supernatant was removed,

and the pellet was resuspended with 100 µl of staining buffer (PBS + 0.5% BSA). The cells were transferred in into a FACS tube which contain 500 µl of staining buffer. Lastly, they were run under BD Aria™ (BD Biosciences, USA), and CD3+ CD4+ T cells were sorted into tubes for further experiments.

2.5 RNA isolation, cDNA synthesis and Quantitative- Real Time Polymerase Chain Reaction (qRT-pPCR)

2.5.1 RNA isolation

To achieve this, each tube that contain sorted CD+ T cells was centrifuged for 5 minutes at 500 x g. Supernatants were removed and RNA isolation from pellets was applied with RNeasy Micro Kit (74004, Qiagen, Germany) in accordance with the manufacturer's standards. The samples were lysed first with 350 µl Buffer RLT on pellets, then homogenized. Ethanol was added to the lysate to improve binding conditions. The RNeasy silica membrane was then loaded with the lysate, where RNA was bound and contaminants were effectively removed.The remaining DNA was removed using a simple on-column DNase treatment. Total RNA was eluted with elution buffer. The total RNA quality and amount were assessed through Qubit RNA HS Assay Kit in accordance with the manufacturer's guidelines (Q32852, Thermo , USA).

2.5.2 Complementary DNA (cDNA) Synthesis

The iScript™ cDNA Synthesis Kit (1708891, Biorad, USA)was used to reverse transcribe RNA into cDNA in accordance with the manufacturer's instructions. By using a 96-well T100 PCR thermal cycler (Bio-rad, USA) device, PCR reactions were carried out. Priming was performed at 25°C for 5 minutes, followed by reverse transcription at 46°C for 20 minutes and 95°C for 1 minute for the inactivation. After incubation, each subject has 120 ng cDNA.

2.5.3 Quantitative Real-Time Polymerase Chain Reaction

cDNA was analyzed by quantitative real-time (qRT-PCR) using QuantiNova SYBR Green PCR kit (Qiagen, Germany) at Applied Biosystem PCR machine (Thermo Scientific) with specific primer sequences which are shown in **Table 4**. Totally, worked in 20 µl of mix in each well and contained 5 µl of SYBR Green I Master, forward (F) and reverse (R) primers at 10 µM working concentration, 10 ng cDNA and nuclease-free water. An initial step at 95 °C for 5 min the sample was subjected to 40 cycles of denaturation for 15 seconds, annealing for 30 seconds, and extension for 30 seconds at 72 °C. Then, a melting curve was obtained by heating in temperature steps of 0.5 °C from 65 to 95 °C. The qRT-PCR experiments were run in duplicate for all samples and genes.

Gene	Primer	Sequence (5'→3')	Amplicon size (bp)
FOXP3	Forward	CTTGAACCCCATGCCACCATC	21
	Reverse	AGGAGTGCCTGTAAGTGGG	19
RORC	Forward	CCTACAATGCTGACAACCGC	20
	Reverse	GGGCTGTGTAGAGGGCAATC	20
IKZF2	Forward	ACTTCTTTCTTTCTCCCTCCGTTTA	25
	Reverse	CATCACCATTTCCAGCTCTGT	21
HEY1	Forward	TCGGCTCTAGGTTCCATGTC	20
	Reverse	CTTGCTCCATTACCTGCTTCTCA	23
HES5	Forward	GCCCCAAAGAGAAAAACCGAC	21
	Reverse	ACGAAGGCTTTGCTGTGCTT	20
GAPDH	Forward	ATGGAAATCCCATCACCATCTT	22
	Reverse	CGCCCCACTTGATTTTGG	18

***Table 4** : Primer sequences used in the quantitative real-time polymerase chain reaction*

2.6 Cytokine Level Determination Assay

Legendplex Human Th Cytokine Panel kit (Biolegend, USA) was used to determine the cytokines in the plasma that we obtained from each study group in accordance with the manufacturer's instructions. Firstly, 25 µl of Matrix Buffer for standards and Assay Buffer (Legendplex Buffer Set/Biolegend, cat no : 740368) for samples were added into wells separately. Secondly, 25 µl of diluted standards were put onto Matrix Buffer while 25 µl of plasma samples were added to the Assay buffer. Then 25 µl of mixed beads were added to all wells. Samples were incubated for 2 hours at RT at 300 rpm. Following incubation, the supernatant was removed, samples were washed and 25 µl of detection antibodies(Human Th Panel detection antibodies/Biolegend, cat no: 741041), which are biotinylated, were added onto samples and incubated for 1 hour at RT at 300 rpm. Without washing 25 µl of Streptavidin-PE was added and incubated for 30 minutes again at the same conditions. As a last step, after removing supernatants and washing steps 150 µl of 1x Wash buffer was added onto samples which are then read on flow cytometer. 1000 fluorochromes per microsphere were used to detect targets. The sample cytokine concentrations were determined by generating a seven-point standard curve, including the blank. The lower limit of detection (LOD) for each cytokine is 24.1 pg/mL, 2.12 pg/mL, 26.9 pg/mL, 2.85 pg/mL, 4.78 pg/mL, 3.56 pg/mL, and 2.47 pg/mL for IFN-γ, IL-2, IL-4, IL-17A, IL-6, IL-22, and IL-10, respectively.

Cytokine	Catalog number
LEGENDplex Human IL-2 capture bead	740717
LEGENDplex Human IL-6 capture bead	740044
LEGENDplex Human IL-1 capture bead	740046
LEGENDplex Human IL-17A capture bead	740546
LEGENDplex Human IL-22 capture bead	740720
LEGENDplex Human IL-4 capture bead	741118
LEGENDplex Human IFN- γ capture bead	740052
LEGENDplex Human TNF- α capture bead	740053

Table 5: *Cytokines which are determined by Legendplex Human Th Cytokine Panel kit.*

2.7 Bioinformatics and Statistical Analysis

All experimental data were evaluated using the statistical software GraphPad Prism. To express all data, the mean, standard error of the mean, median, and range are utilized. A single-factor analysis of variance (ANOVA) was performed to compare several groups. The correlation analysis was computed using the Pearson and Spearman's rank correlation coefficients. A P value of 0.05 represented statistical significance.

Chapter 3:

RESULT

3.1. Peripheral Blood $CD4^+FoxP3^+$ Regulatory T-Cell Population

Smoker subjects had significantly increased peripheral blood $CD4^+FoxP3^+$ regulatory T-cells compared to non-smokers (medians [IQR]= 9.370 [Q1= 7.170-Q2=11.00] vs 6.765 [Q1= 4.890 - Q2=8.860]; n= 20 for both; $p<0.01$). When COPD patients were compared to smokers and non-smokers, those who were stable had significantly more peripheral blood CD4+FoxP3+ regulatory T-cells (medians [IQR]= 13.30 [Q1=11.40-Q3=23.70] vs 9.370 [Q1= 7.170 -Q3= 11.00]; n=10 vs 20; $p<0.01$ and medians [IQR]= 13.30 [Q1=11.40 -Q3=23.70] vs 6.765 [Q1=4.890-Q3=8.860]; n= 10 vs 20; $p<0.0001$). However, there was no significant difference between stable and exacerbated COPD patients ***(Fig 7).***

We did not observe any significant difference in the Mean Fluorescence Intensity (MFI) of FoxP3 between the study groups ***(Fig 7)***.

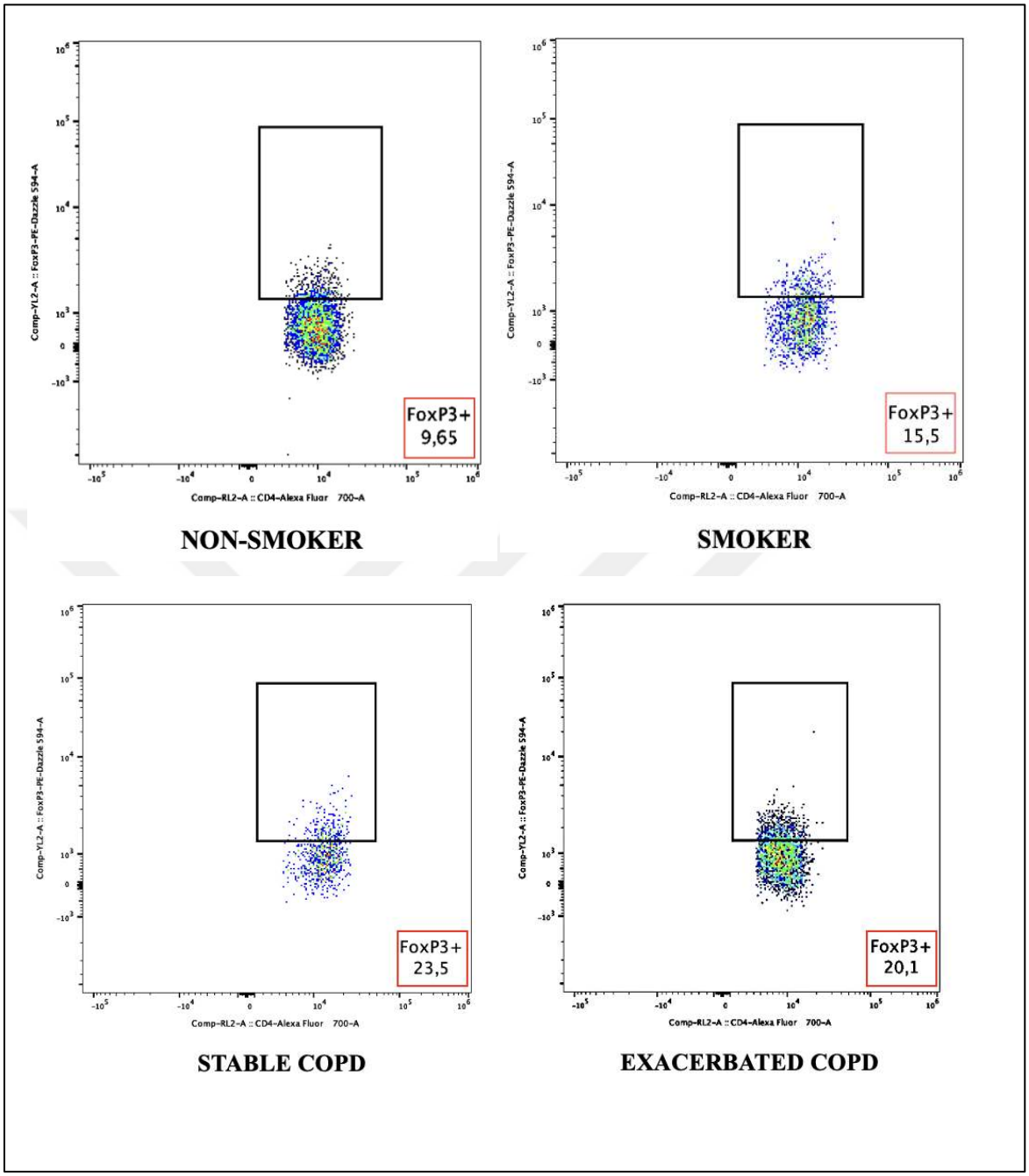

Figure 6: *FoxP3 Expression in Regulatory T-Cells of healthy donors and COPD patients.*

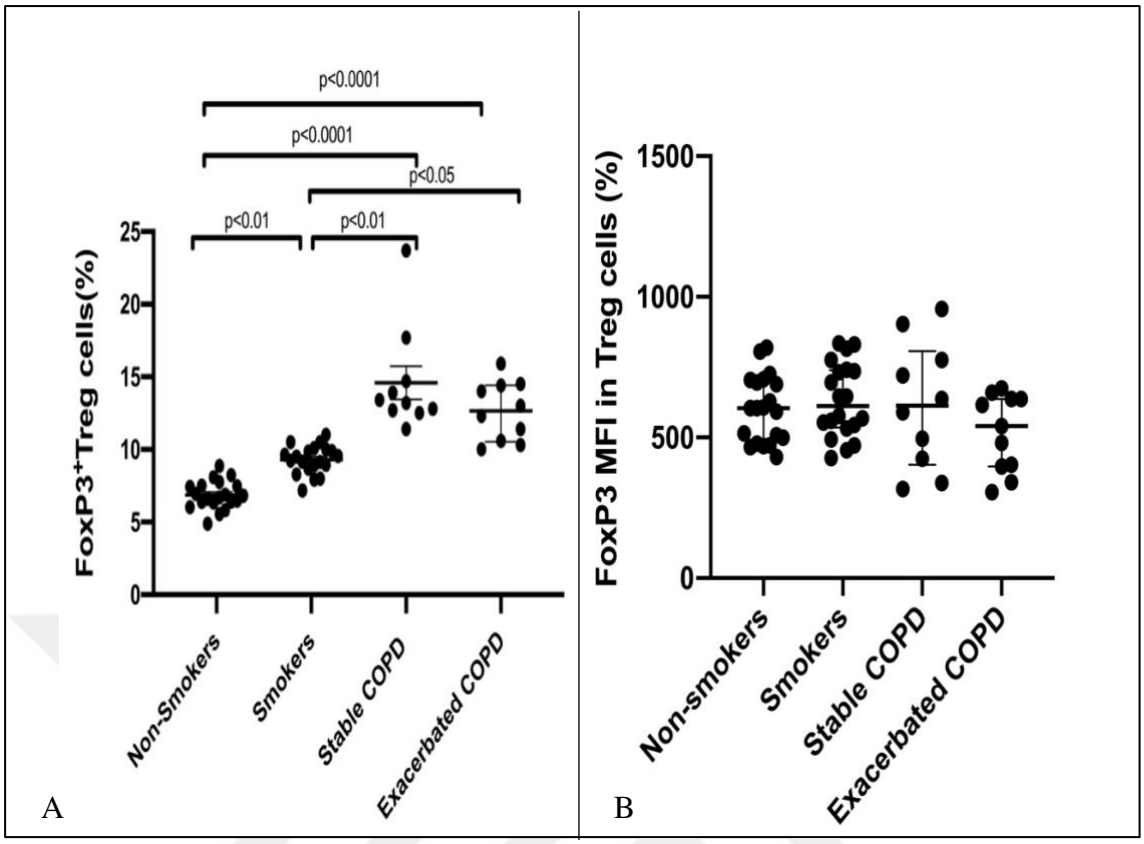

Figure 7: *Expression of Treg FoxP3 (A)$^{+}$ and MFI of FoxP3 surface expression on Treg cells(B).*

3.2. Peripheral Blood FoxP3^{+} Notch^{+} Treg Cell Population

3.2.1 Peripheral Blood FoxP3^{+} Notch1^{+} Treg Cell Population

In comparison to non-smokers, smoker subjects had significantly more FoxP3^{+} Notch1^{+} Treg cells in their peripheral blood (medians [IQR]= 6.835 [Q1=5.960-Q3=9.190] vs 4.475 [Q1=2.040-Q3=11.10]; n=20 for both; $p<0.05$). COPD patients in the stable period had significantly increased FoxP3^{+} Notch1^{+} Treg cells, when compared to smokers (medians [IQR]= 10.70 [Q1=8.800-Q3=17.00] vs 6.835 [Q1=5.960-Q3=9.190]; n=10 vs 20; $p<0.01$) and non-smoker subjects (medians= 10.70 [Q1=8.800-Q3=17.00] vs 4.475 [Q1=2.040-Q3=11.10]; n= 10 vs 20; $p<0.0001$). We also observed that patients with exacerbated COPD had significantly more FoxP3^{+} Notch1^{+} Treg cells than non-smokers (medians [IQR]= 9.785 [Q1=7.140-Q3=11.8] vs 4.475 [Q1=2.040-Q3=11.10]; n=10 vs 20; $p<0.0001$). However, there was no significant difference between stable and exacerbated COPD patients ***(Fig 9)***.

We also observed that MFI of Notch1 was significantly lower in stable (medians [IQR]= 1068 [Q1=789.0-Q3=1268] vs 1431 [Q1=1094-Q3=4215]; n= 10 vs 20; p<0.01) and exacerbated patients (medians= 1014 [Q1=748.0-Q3=1898] vs 1431 [Q1=1094-Q3=4215]; n= 10 vs 20; p<0.01) when compared to non-smokers ***(Fig 9)***.

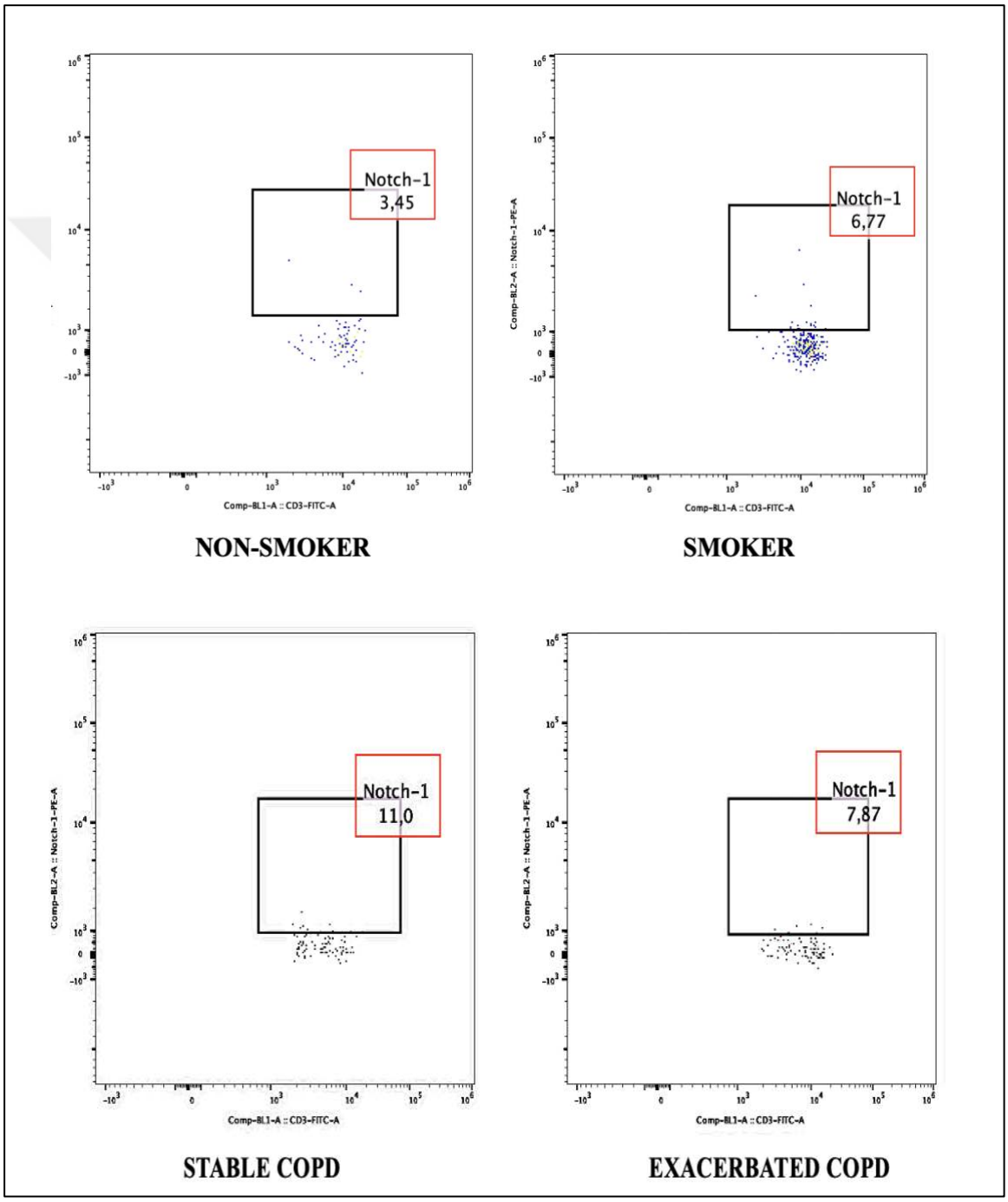

Figure 8: *Notch1 Expression in Regulatory T-Cells of healthy donors and COPD patients.*

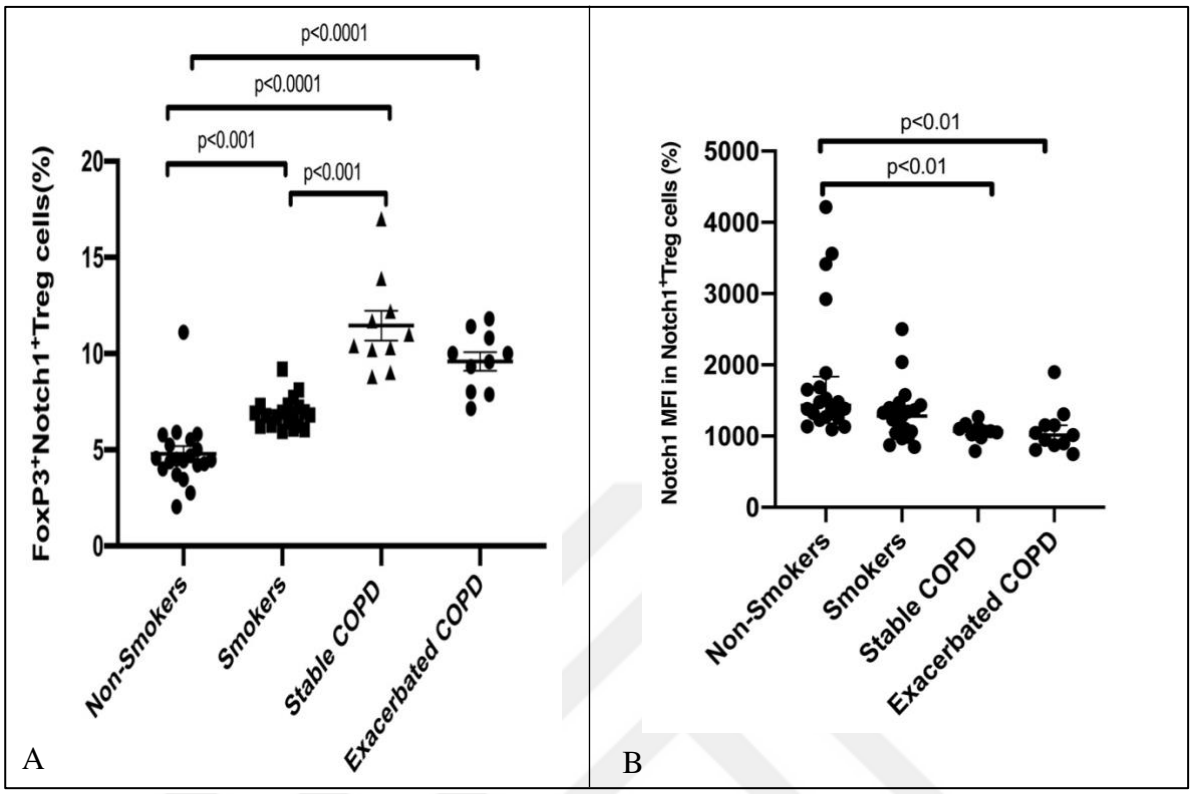

Figure 9: *Expression of Notch1$^+$Treg FoxP3 cell population (A) and MFI of Notch1surface expression on Treg cells (B).*

3.2.2 Peripheral Blood FoxP3$^+$ Notch2$^+$ Treg Cell Population

Compared to non-smokers, smokers had significantly more FoxP3$^+$ Notch2$^+$ Treg cells in their peripheral blood (medians [IQR]= 7.195 [Q1=5.170-Q3=11.00] vs 5.225 [Q1=3.420 -Q3=8.330]; n= 20 for both; p<0.05). COPD patients in the stable period had significantly increased FoxP3$^+$ Notch2$^+$ Treg cells when compared to non-smokers (medians [IQR]= 9,335 [Q1=7.910-Q3=15.90] vs 5.225 [Q1=3.420-Q3=8.330]; n=10 vs 20; p<0.0001). Compared to non-smokers, patients with exacerbated COPD had significantly more FoxP3$^+$ Notch2$^+$ Treg cells (medians [IQR]= 8,780 [Q1=7.140-Q3=16.00] vs 5.225 [Q1=3.420-Q3=8.330]; n=10 vs 20; p<0.0001. However, there was no significant difference between smoker-stable COPD, smoker-exacerbated COPD, and stable-exacerbated COPD patients ***(Fig 11)***.

We did not observe any significant difference in the MFI of Notch2 between the study groups ***(Fig 11)***.

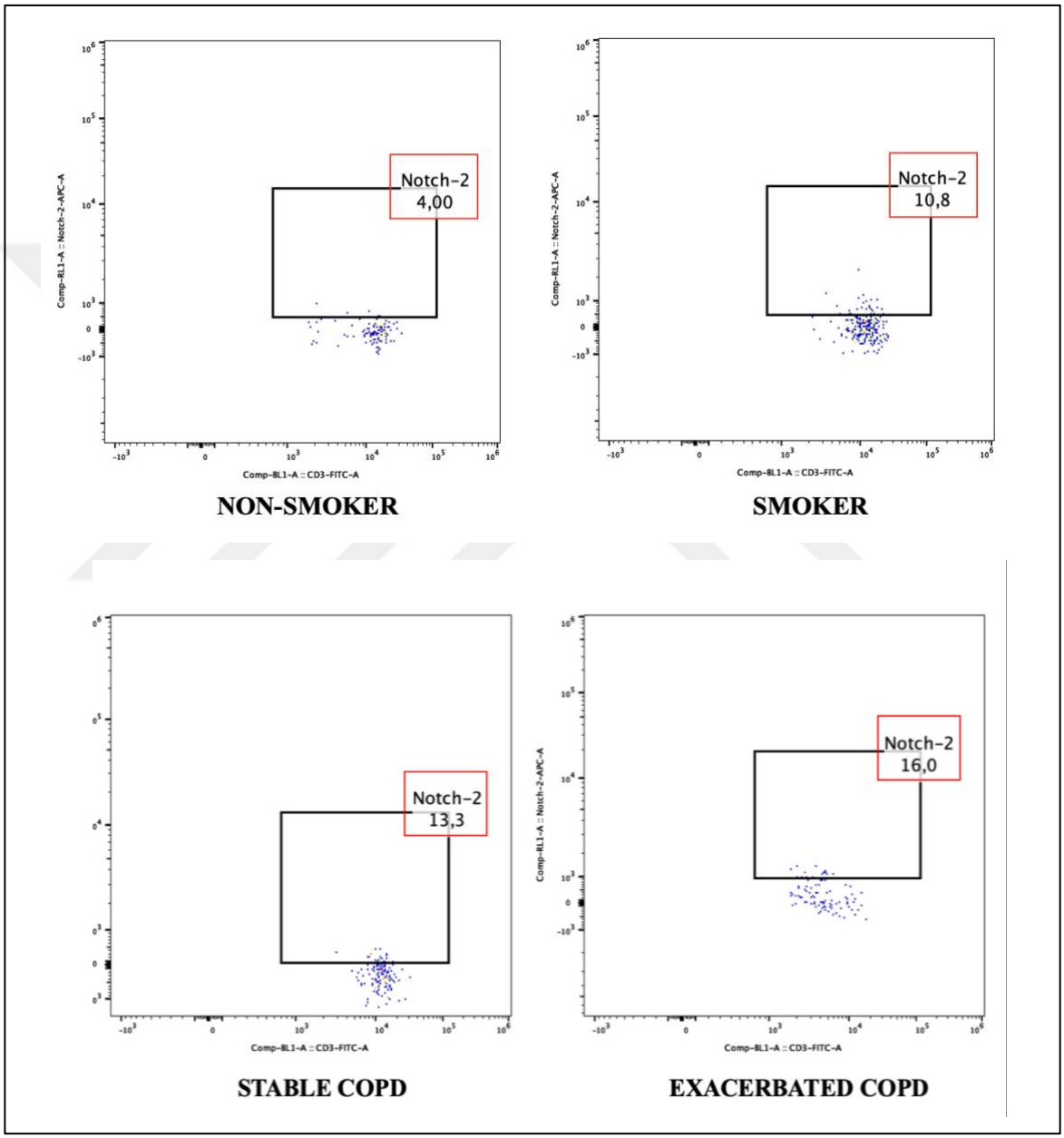

Figure 10: *Notch2 Expression in Regulatory T-Cells of healthy donors and COPD patients.*

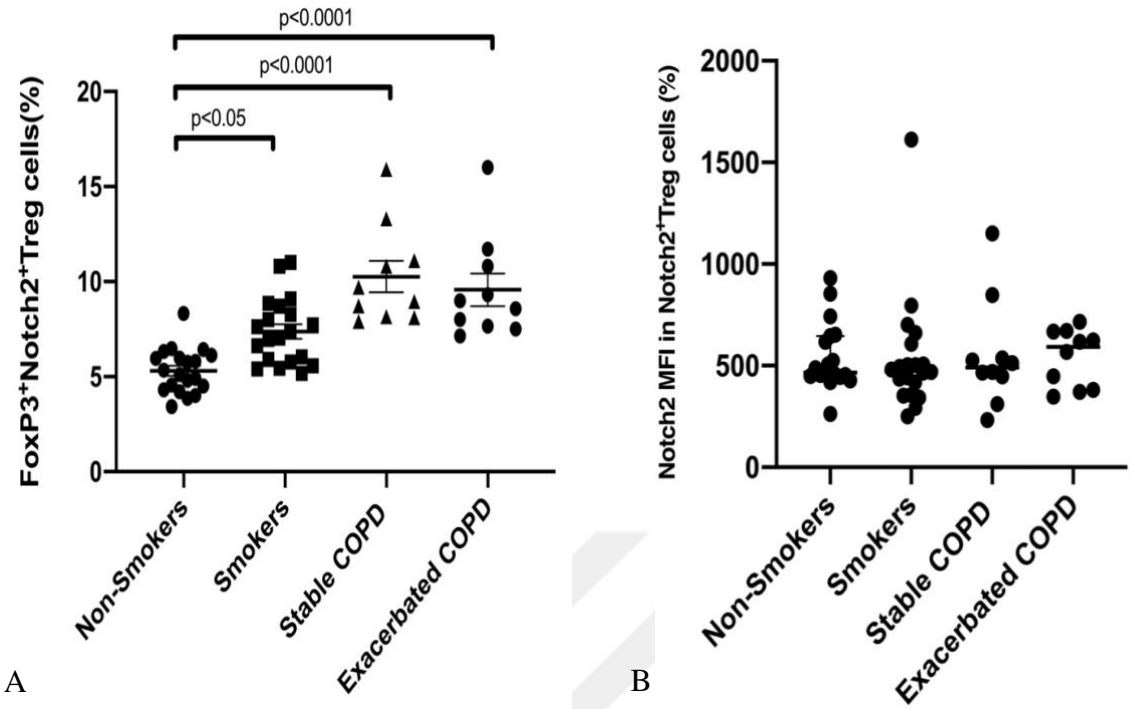

Figure 11: *Expression of Notch2⁺Treg FoxP3 cell population(A) and MFI of Notch2 surface expression on Treg cells (B).*

3.2.3 Peripheral Blood FoxP3⁺ Notch3⁺ Treg Cell Population

Smoker subjects had significantly increased FoxP3⁺ Notch3⁺ Treg cells compared to non-smokers (medians [IQR]= 7.620 [Q1=5.730-Q3=16.70] vs 4.950 [Q1=3.090 - Q3=8.970]; n=20 for both; p<0.001). COPD patients in the stable period had significantly increased FoxP3⁺ Notch3⁺ Treg cells, when compared to non-smokers (medians [IQR]= 9,875 [Q1=7.350-Q3=15.10] vs 4.950 [Q1=3.090-Q3=8.970]; n=10 vs 20; p<0.0001). Also, exacerbated COPD patients had significantly higher FoxP3⁺ Notch3⁺ Treg cells than non-smokers (medians [IQR] = 9,080 [Q1=6.060-Q3=16.30] vs 4,950 [Q1=3.090-Q3=8.970]; n=10 vs 20; p<0.0001). However, there was no significant difference between smoker vs stable COPD, smoker vs exacerbated COPD, and stable vs exacerbated COPD patients ***(Fig 13)***.

We also observed that the MFI of Notch3 in exacerbated COPD patients was significantly lower than in non-smokers (medians [IQR] = 950.0 [Q1=688.0-Q3=1114] vs 1127 [Q1=908.0-Q3=2175]; n=10 vs n=20; p<0.05) ***(Fig 13)***.

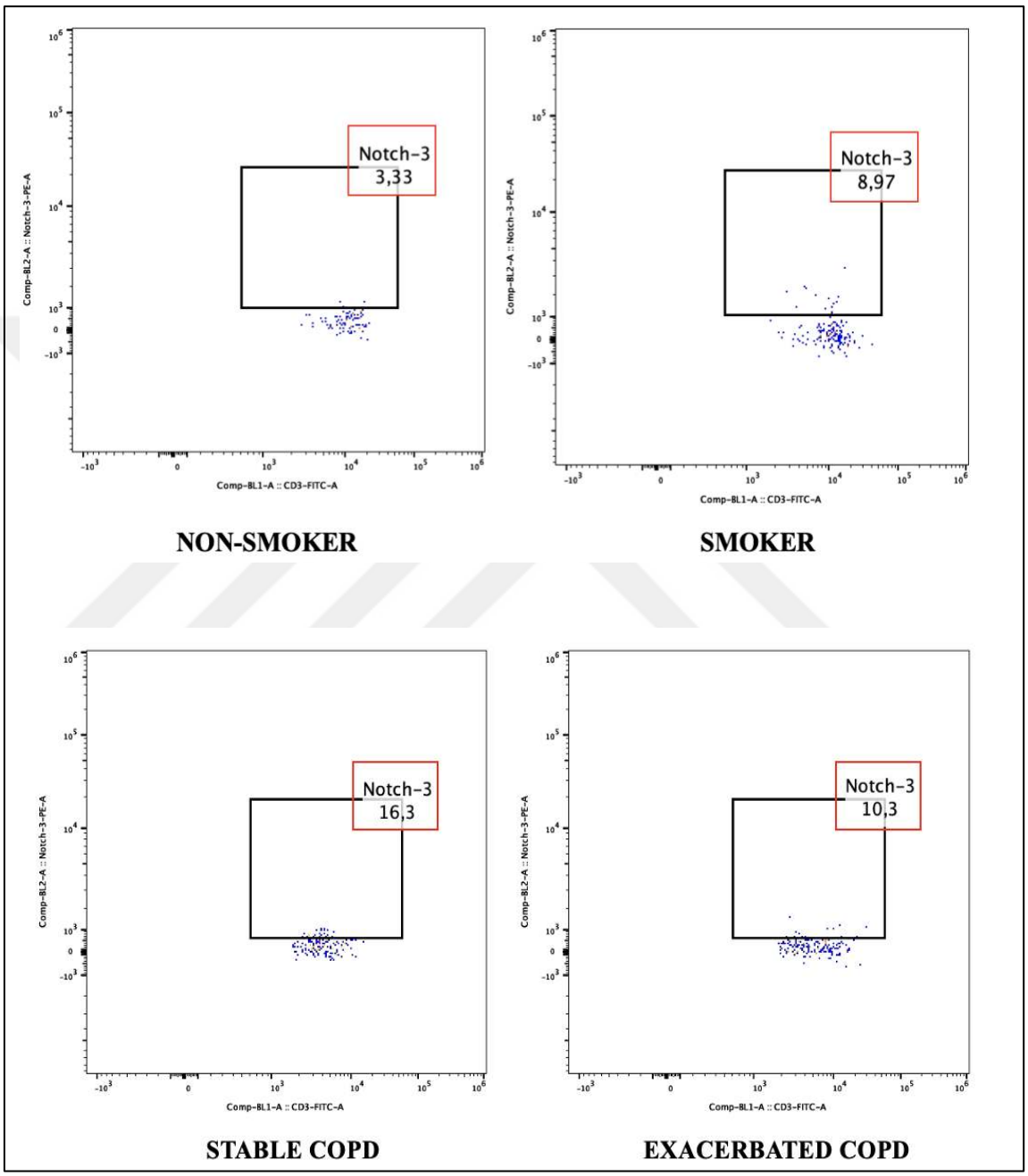

Figure 12: *Notch3 Expression in Regulatory T-Cells of healthy donors and COPD patients.*

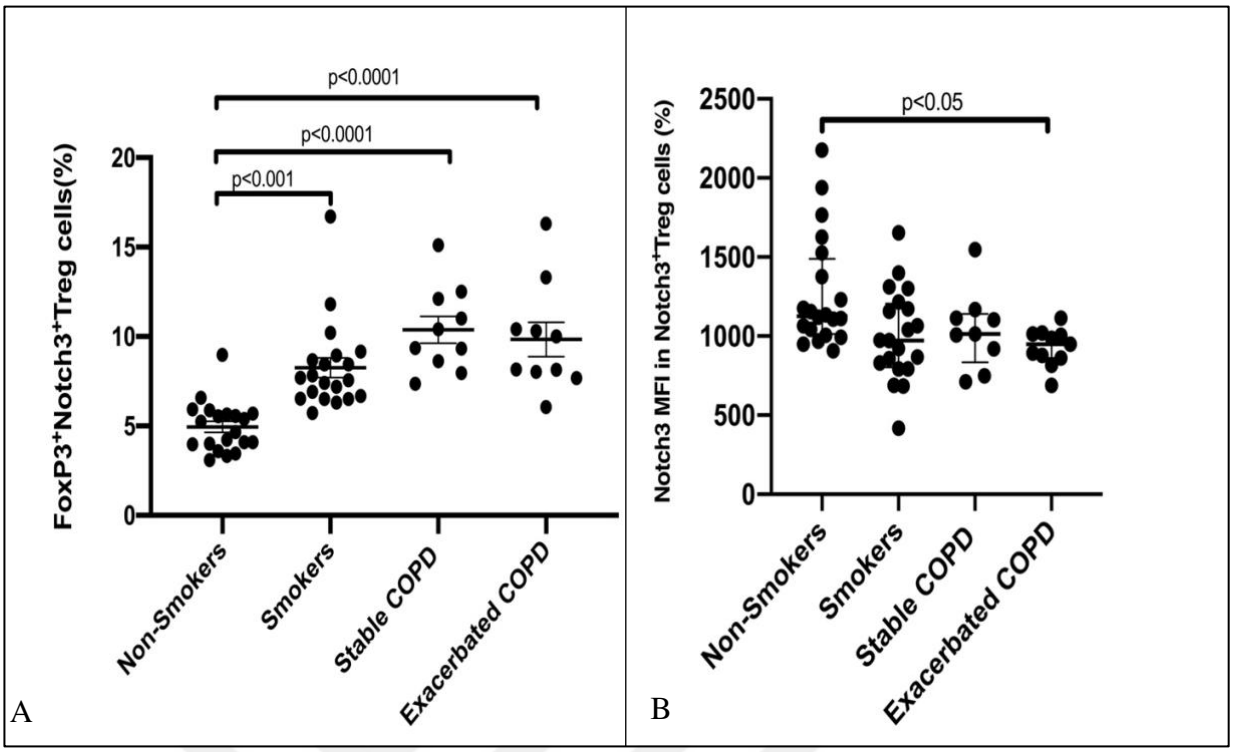

Figure 13: *Expression of Notch3⁺Treg FoxP3 cell population and MFI of Notch3 surface expression on Treg cells.*

3.2.4 Peripheral Blood FoxP3⁺ Notch4⁺ Treg Cell Population

Increased levels of FoxP3⁺ Notch4⁺ Treg cells were found in peripheral blood of smokers (medians [IQR] = 8.420 [Q1=6.400-Q3=16.70] vs 5.005 [Q1=3.590-Q3=8.820]; n=20 for both; p<0.01), when compared to non-smoker subjects. COPD patients in the stable period had significantly increased FoxP3⁺ Notch4⁺ Treg cells comparing to non-smokers (medians [IQR]= 13.75 [Q1=8.610-Q3=23.60] vs 5.005 [Q1=3.590-Q3=8.820]; n=10 vs 20; p<0.01). Furthermore, exacerbated COPD patients had significantly more FoxP3⁺ Notch4⁺ Treg cells, as compared to non-smokers (medians [IQR]= 13.65 [Q1=10.00 -Q3=20.00] vs 5.005 [Q1=3.590 -Q3=8.820]; n=10 vs 20; p<0.0001). However, there was no significant difference between smokers and stable COPD, smokers vs exacerbated COPD, and stable COPD vs exacerbated COPD patients ***(Fig 15)***.

We also observed that MFI of Notch4 was significantly lower in smokers (medians [IQR]= 346.5 [Q1=156.0-Q3=465.0] vs 410.0 [Q1=230.0-Q3=809.0]; n=20 for both;

p<0.05), stable COPD (medians [IQR]= 313.5 [Q1=156.0-Q3=421.0] vs 410.0 [Q1=230.0-Q3=809.0]; n=10 vs 20; p<0.05) and exacerbated COPD patients (medians [IQR]= 257.0 [Q1=167.0-Q3=742.0] vs 410.0 [Q1=230.0-Q3=809.0]; n=10 vs 20; p<0.05), as compared to non-smokers ***(Fig 15)***.

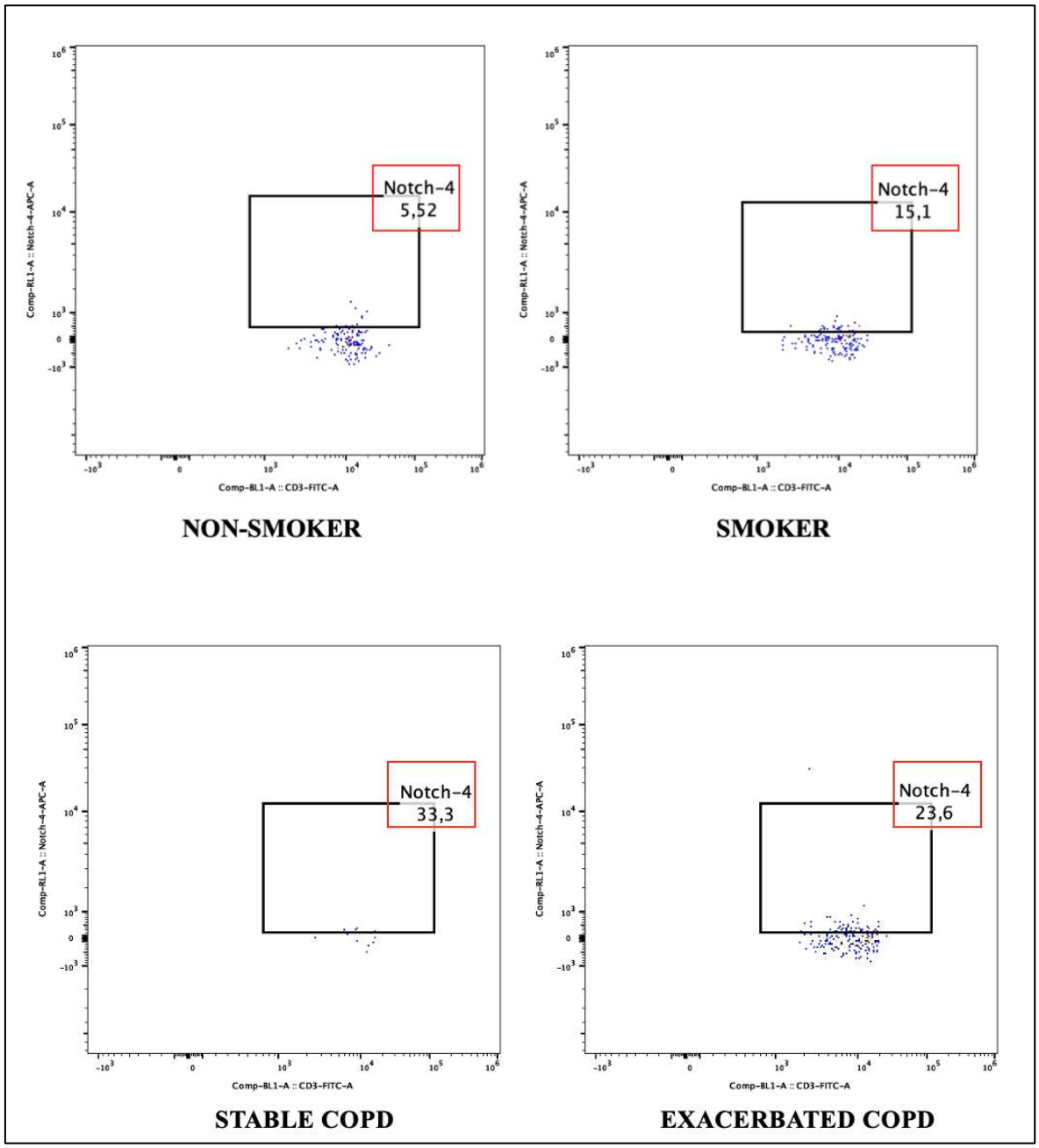

Figure 14: *Notch4 Expression in Regulatory T-Cells of healthy donors and COPD patients.*

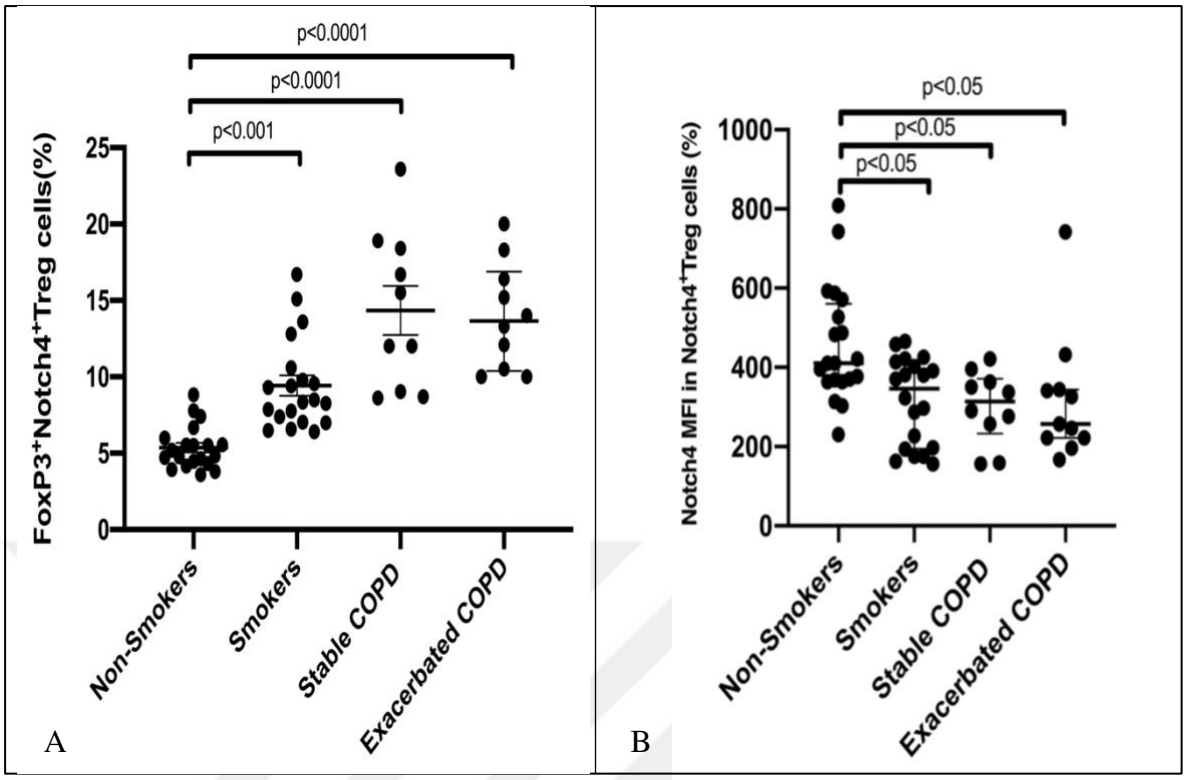

Figure 15: *Expression of Notch4⁺Treg FoxP3 cell population and MFI of Notch4 surface expression on Treg cells.*

3.3. Peripheral Blood CXCR3⁺ TH1 and CCR4⁺ TH2 Cell Population

A significant decrease in CCR4⁺ TH2 cells in stable COPD patients was observed, comparing to smokers (medians [IQR]= 7.780 [Q1=1.700-Q3=18.20] vs 10.40 [Q1=7.270-Q3=20.80]; n= 10 vs 20; $p<0.05$). Also, decreased level of TH2 observed in exacerbated COPD patients against smokers (medians [IQR]= 4.340 [Q1= 1.570-Q3=14.10] vs 10.40 [Q1=7.270-Q3=20.80]; n= 10 vs 20; $p<0.01$) and non-smokers subjects (medians [IQR]= 4.340 [Q1= 1.570-Q3=14.10] vs9.895 [Q1=7.270 -Q3=20.80]; n= 10 vs 20; $p<0.001$) (***Fig 16)***. While there was no significant difference in the CXCR3⁺ Th1 cell population (***Fig 16)***, an increased level of the Th1/Th2 ratio was observed in exacerbated COPD patients compared to non-smokers (medians [IQR]= 9.816 [Q1= 5.638 -Q3=16.62] vs 1.680 [Q1= 0.2217-Q3=3.168]; n= 10 vs 20; $p<0.001$), smokers (medians [IQR]= 9.816 [Q1= 5.638-Q3=16.62] vs 1.800 [Q1= 0.2893-Q3=3.684] n= 10 vs 20; $p<0.001$), and stable COPD (medians [IQR]= 9.816 [Q1= 5.63-Q3=16.62] vs 2.363 [Q1= 1,082 -Q3=5.491]; n= 10 for both; $p<0.01$). Also, increased level of the

Th1/Th2 ratio was demonstrated in stable COPD patients compared to non-smokers (medians [IQR]= 2.363 [Q1= 1,082 -Q3=5.491] vs 1.884 [Q1= 1,082 -Q3=5.491] vs 1.680 [Q1= 0,2217-Q3=3.168]; n= 10 vs 20; p<0.05) **(Figure 17).**

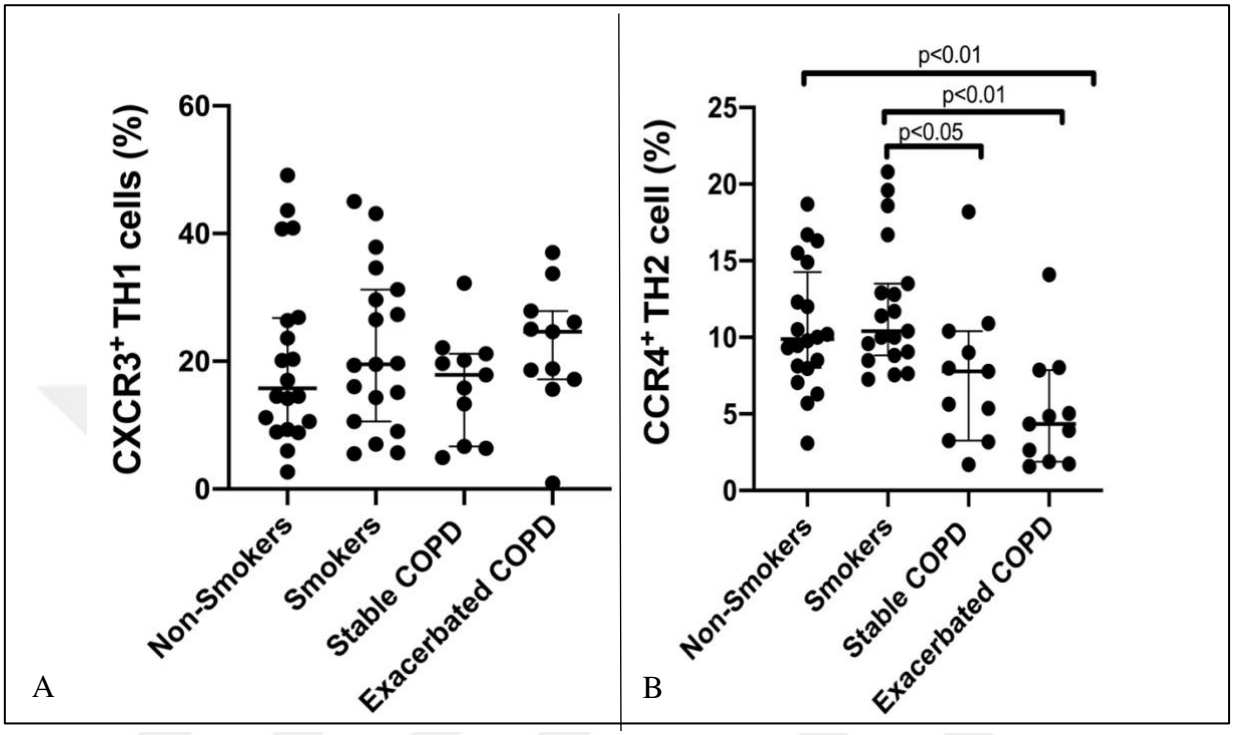

Figure 16: *Expression of CXCR3+ TH1 (A) and CCR4+ TH2 (B) cell population.*

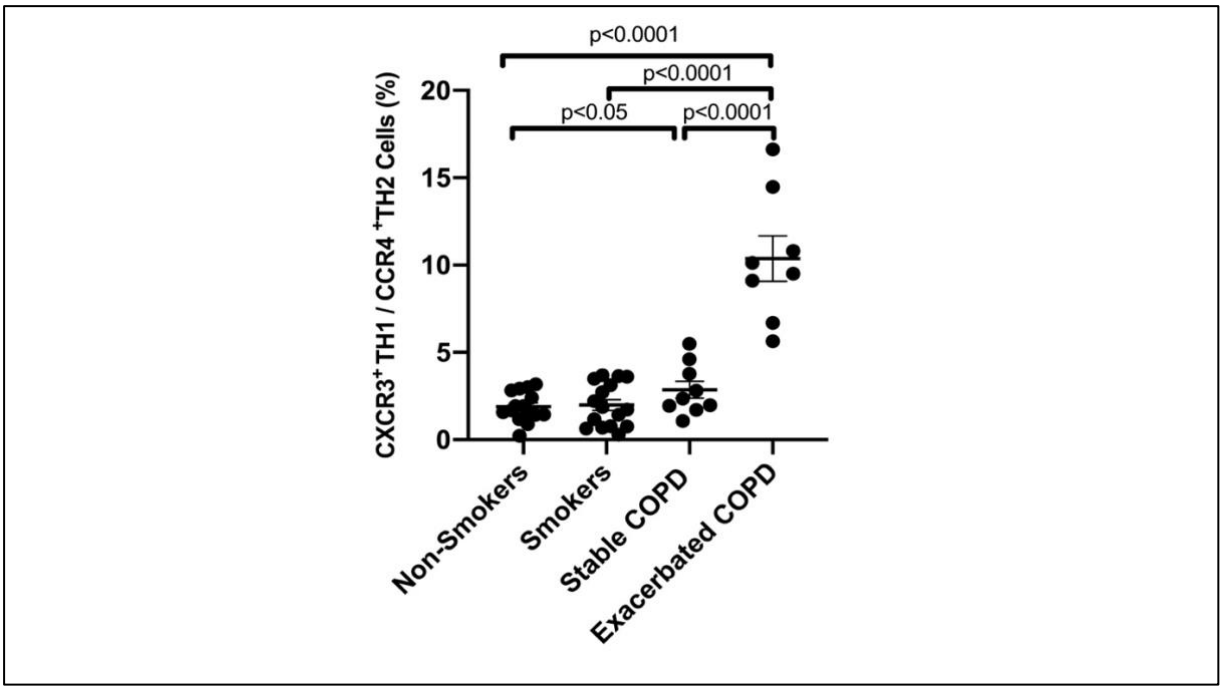

Figure 17: *The ratio of TH1/TH2 cell population.*

3.4. Peripheral Blood CD45RO$^+$ Memory T Cell Population

There was no significant difference in the CD45RO$^+$ memory T cell population between the study groups (n=20 for both healthy controls; n=10 for both COPD patients).

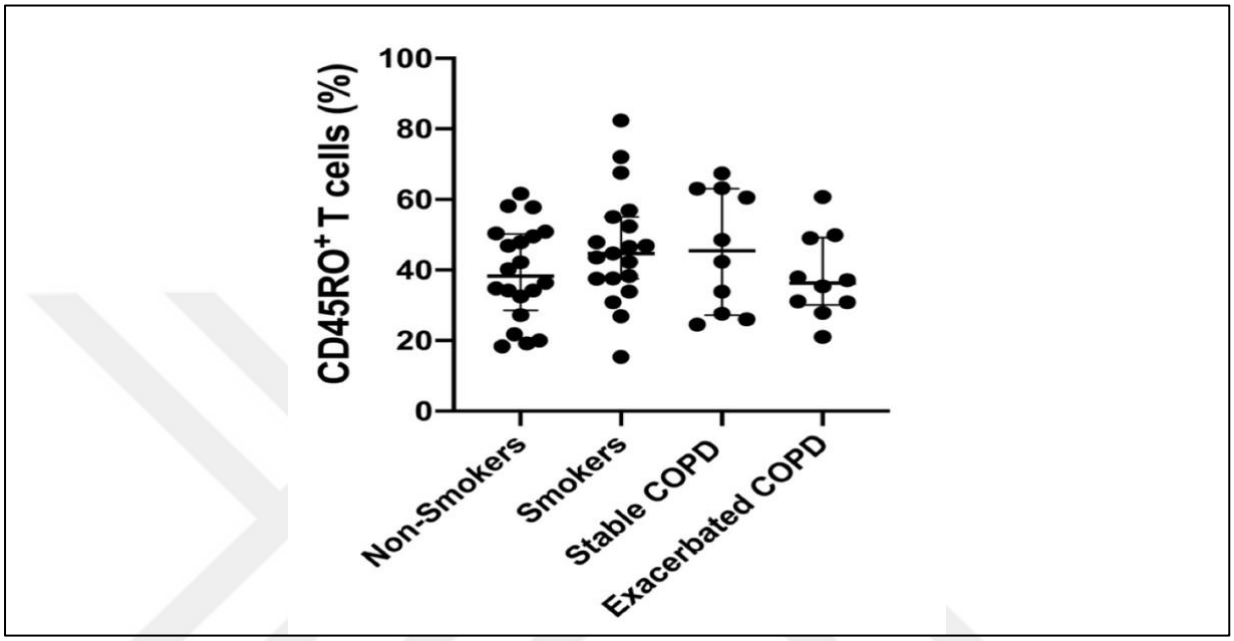

Figure 18: *Expression of the CD45RO$^+$ memory T cell population.*

3.5. Quantitative Real-Time Polymerase Chain Reaction

We did not observe any significant difference in the gene expression level of Foxp3, ROR γ t, and Notch downstream genes; Hes5 and Hey1 in any of the study groups (n=5 for each group of non-smokers, smokers, stable COPD and COPD in exacerbation).

Figure 19 shows the levels of mRNA expression for Foxp3 and ROR γ t in CD4$^+$T cells in study groups, and the mRNA expression for Hes5 and Hey1 mRNA expression in CD4$^+$T cells is given in ***Figure 20.***

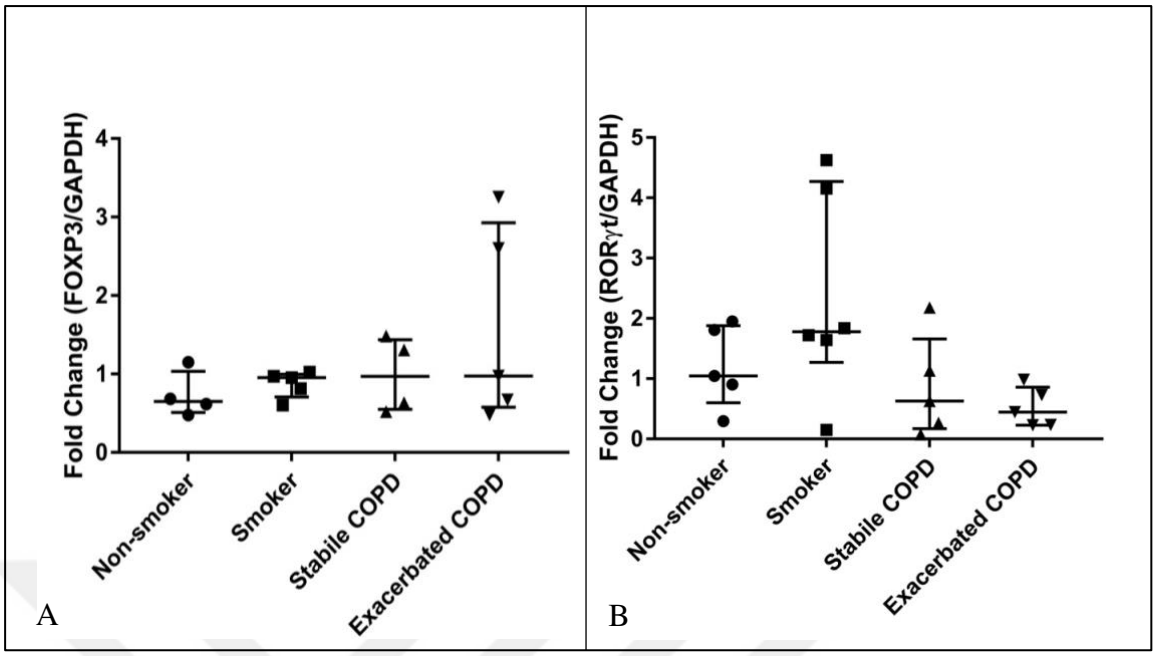

Figure 19: *Levels of mRNA expression for Foxp3 (A) and ROR γ t (B) in CD4⁺T cells.*

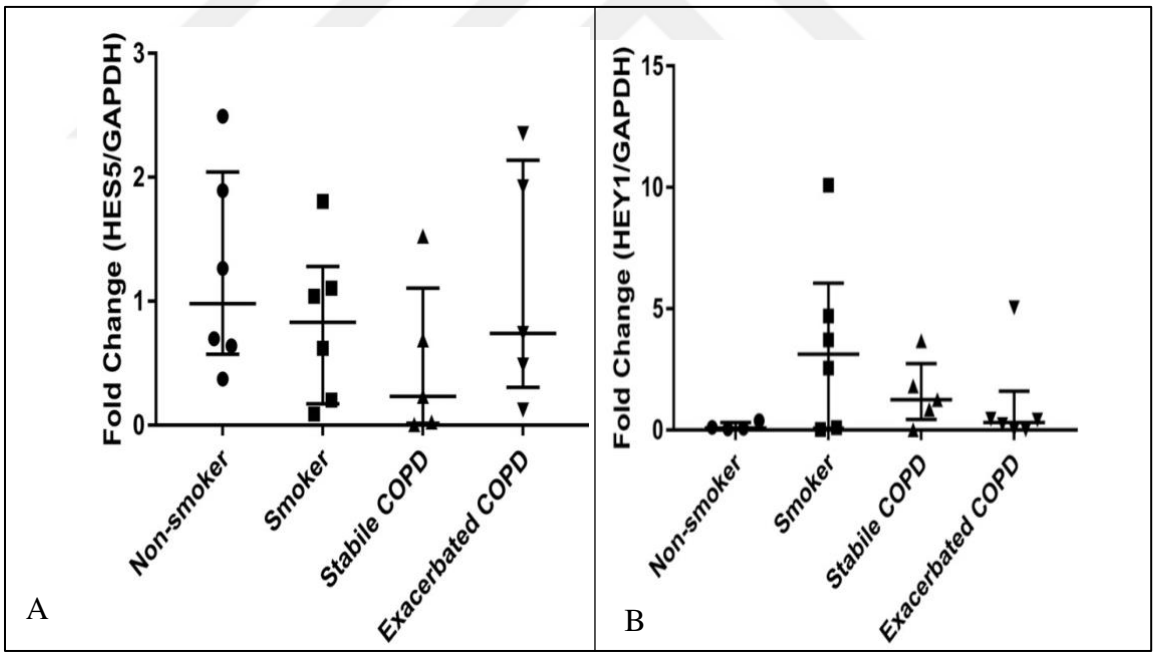

Figure 20: *Levels of mRNA expression of Hes5 (A) and Hey1 (B) in CD4⁺T cells.*

3.6 Cytokine Level Determination Assay Result

3.6.1. Levels of IL-2 and IL-6

A significant increase in IL-2 in stable COPD patients was observed against smokers (medians [IQR]= 21.13 [Q1= 0.00-Q3=86.49] vs 3.785 [Q1= 0.00-Q3=14.99]; n=10 vs 20; p<0.05) and non-smokers (medians [IQR]= 21.13 [Q1= 0.00-Q3=86.49] vs 2.645 [Q1= 0.00-Q3=38.00]; n=10 vs 20; p<0.001). In stable COPD patients, IL-6 level increasing also observed against smokers (medians [IQR]=22,73 [Q1=9.800-Q3=86.49] vs 11,19 [Q1= 0.00 -Q3=37.52; n= 10 vs 20; p<0.05) and non-smokers subjects (medians [IQR] =22,73 [Q1= 0.00-Q3=86.49] vs 10,87 [Q1=0.00-Q3=44.61] n= 10 vs 20; p<0.05) ***(Figure 21)***.

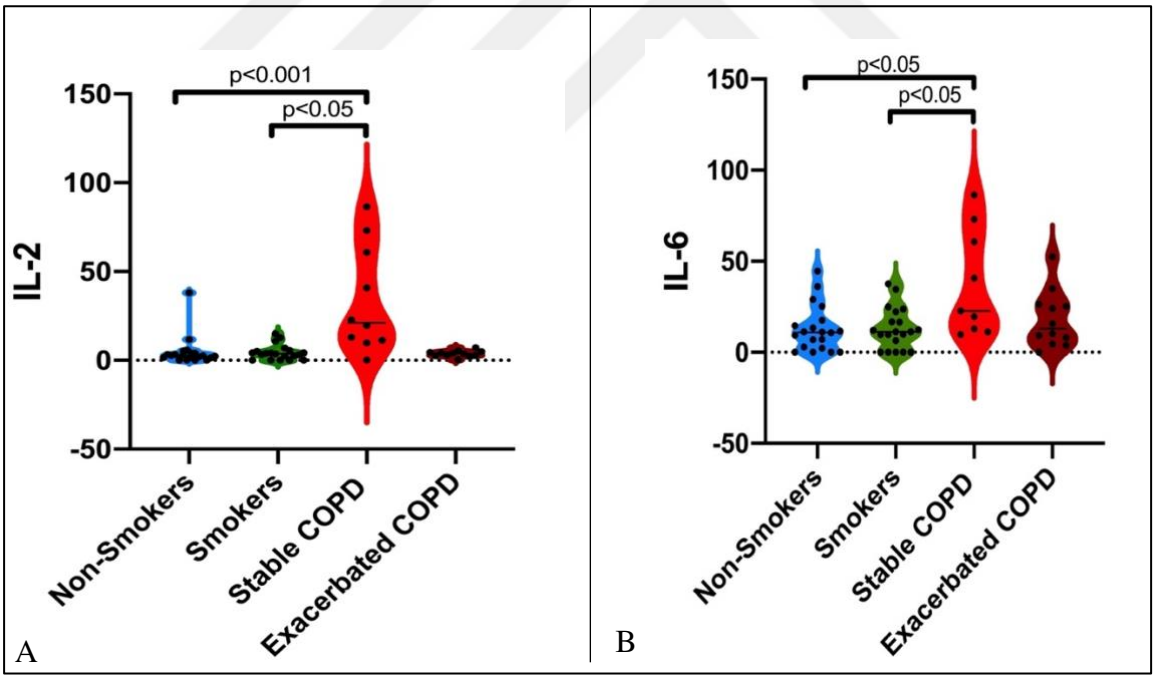

Figure 21: *Levels of IL-2 (A) and IL-6 (B) cytokines in the plasma of each group.*

3.6.2 Levels of IL-17A and IL-22

While there was no significant difference in the level of IL-17A between the study groups, an increased level of IL-22 was observed in stable COPD patients against smokers (medians [IQR]= 31.36 [Q1= 0.00 -Q3=388.9] vs 11.48 [Q1= 0.00-Q3=44.05]; n= 10 vs 20; p<0.05) and non-smokers (medians [IQR]=31.36 [Q1= 0.00-Q3=388.9] vs 10.35 [Q1= 0.00-Q3=57.80]; n= 10 vs 20; p<0.05) ***(Figure 22)***.

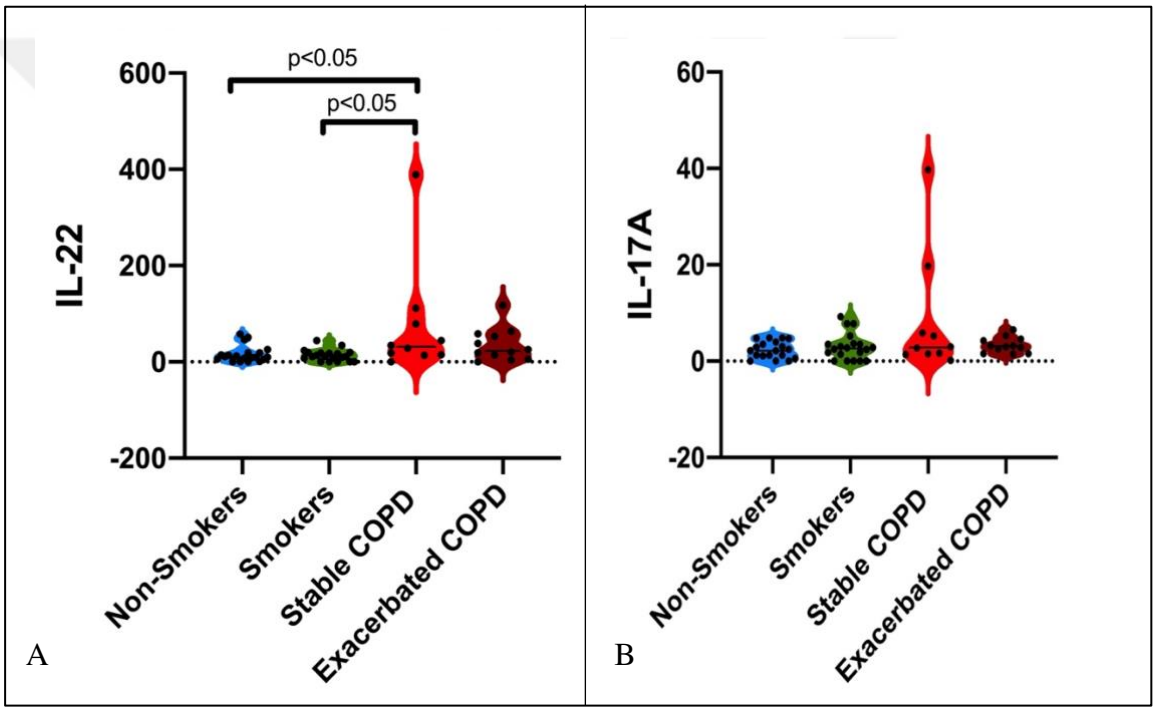

Figure 22: *Levels of IL-22 (A) and IL-17A (B) cytokines in the plasma of each group.*

3.6.3 Levels of IL-10 and IL-4

There was no significant difference in the level of IL-10 between the study groups (Figure 3.6.3) but a significant decrease in IL-4 level was observed in exacerbated COPD patients against stable COPD patients (medians [IQR]= 2.590 [Q1= 2.110-Q3=5.240] vs 3,870 [Q1= 2.840-Q3=8.220]; n=10 vs 10; p<0.05), smokers (medians[IQR]= 2.590 [Q1= 2.110 -Q3=5.240] vs 3.600 [Q1= 2.840 -Q3=5.240]; n=10 vs 20; p<0.05) and non-

smokers (medians [IQR]=2.590 [Q1= 2.110-Q3=5.240] vs 4.755 [Q1= 3.090-Q3=6.990]; n=10 vs 20; p<0.01) ***(Figure 23)***.

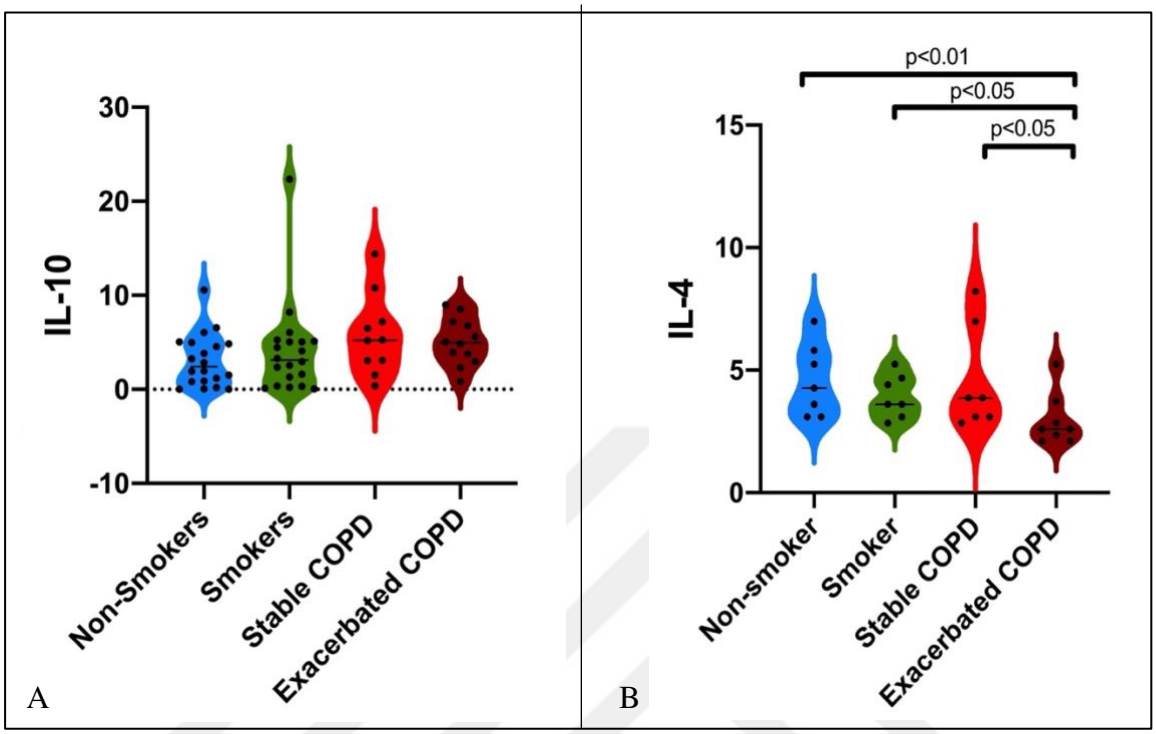

Figure 23: *Level of IL-10 (A) and IL-4 (B) cytokines in the plasma of each group*

3.6.4 Ratio of IL-22/IL-10 and IFN-γ*/IL-4 Cytokine Level*

A significant increase in the ratio of IL-22/IL-10 was observed in stable COPD patients against smokers (medians [IQR]= 7.338 [Q1= 2.107-Q3=27.06] vs 2.410 [Q1= 0.00 -Q3=33.12]; n=10 vs 20; p<0.05) ***(Figure 24)***.Also, IFN-γ/IL-4 cytokine level was increased in exacerbated COPD patients against smokers (medians [IQR]=4.611 [Q1= 3.962-Q3=5.412] vs 4.210 [Q1= 0.3145 -Q3=6.824]; n=8 for both; p<0.01) and non-smokers subjects (medians [IQR]= 4.611 [Q1=3.962-Q3=5.412] vs 3.829 [Q1= 1.941-Q3=5.383]; n=8 for both; p<0.05) ***(Figure 24)***.

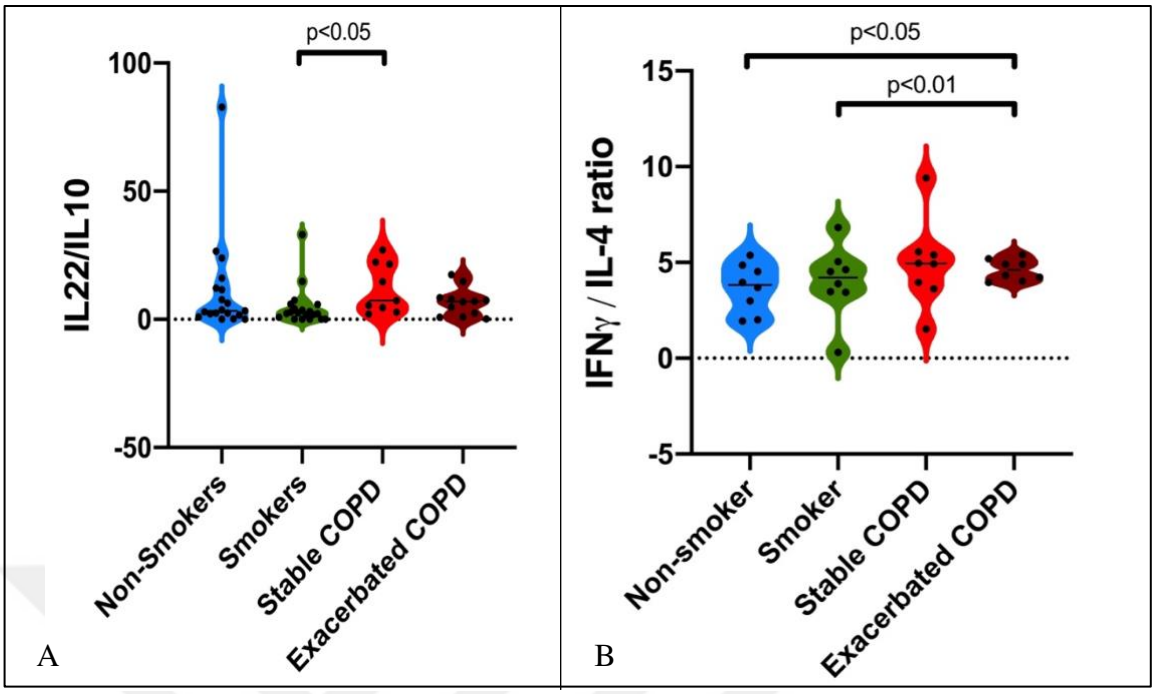

Figure 24: *Level of IL-22/IL-10 (A) and IFN-γ/IL-4 (B) cytokines in the plasma of each group.*

3.7 Correlation Analysis

The proportion of Notch-2 in stable COPD group was negatively correlated with the IL-10 content (r= 0.6; p<0.05) while there was no significant difference between Notch-1/3/4 and IL-10 (**Figure 25**). Also the proportion of Notch-4 in exacerbated COPD group was positively correlated with the IL-2 content but no significant difference observed between Notch1/2/3 and IL-2 (r=0.6; p<0.05)(**Figure 26**).

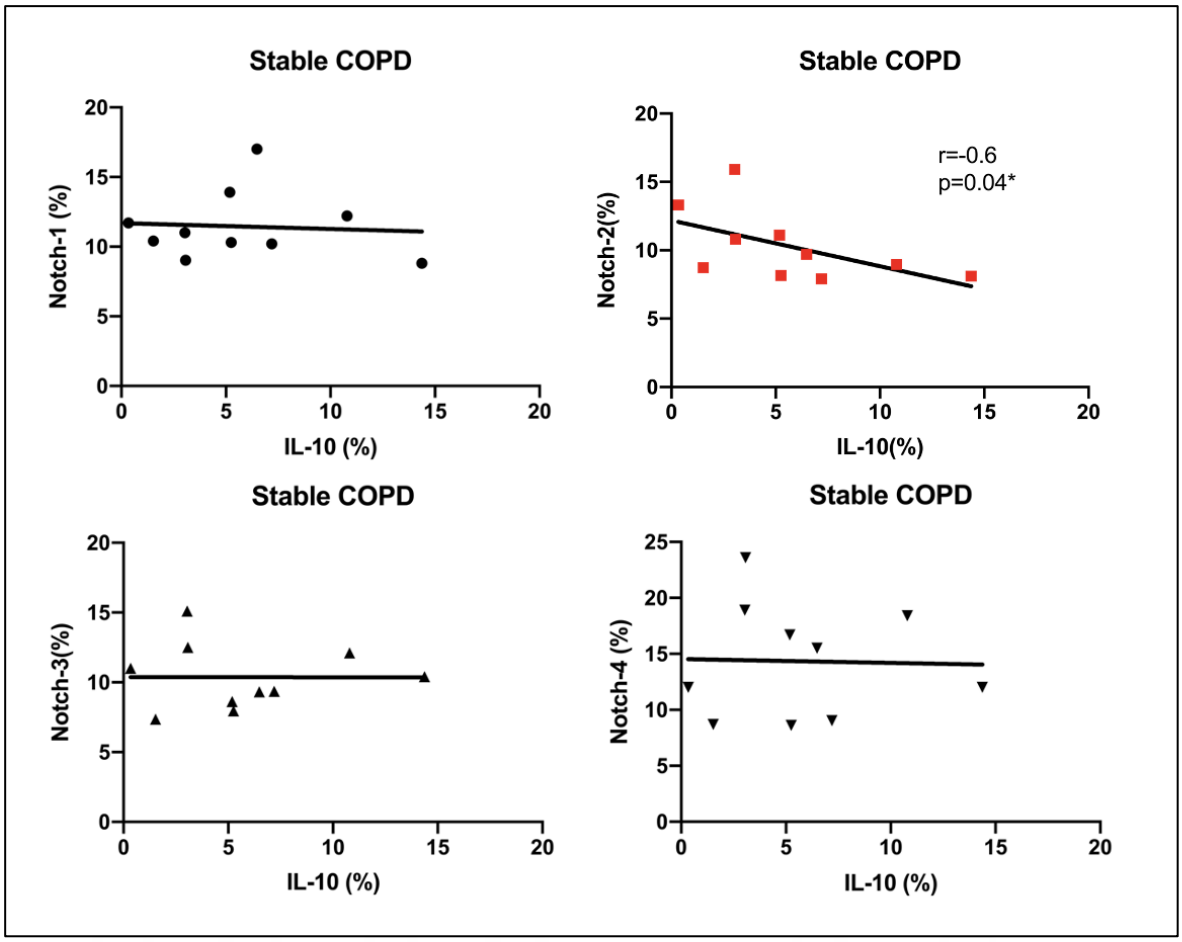

***Figure 25**: Correlation analysis between Notch-1/2/3/4 and IL-10 level in Stable COPD patients.*

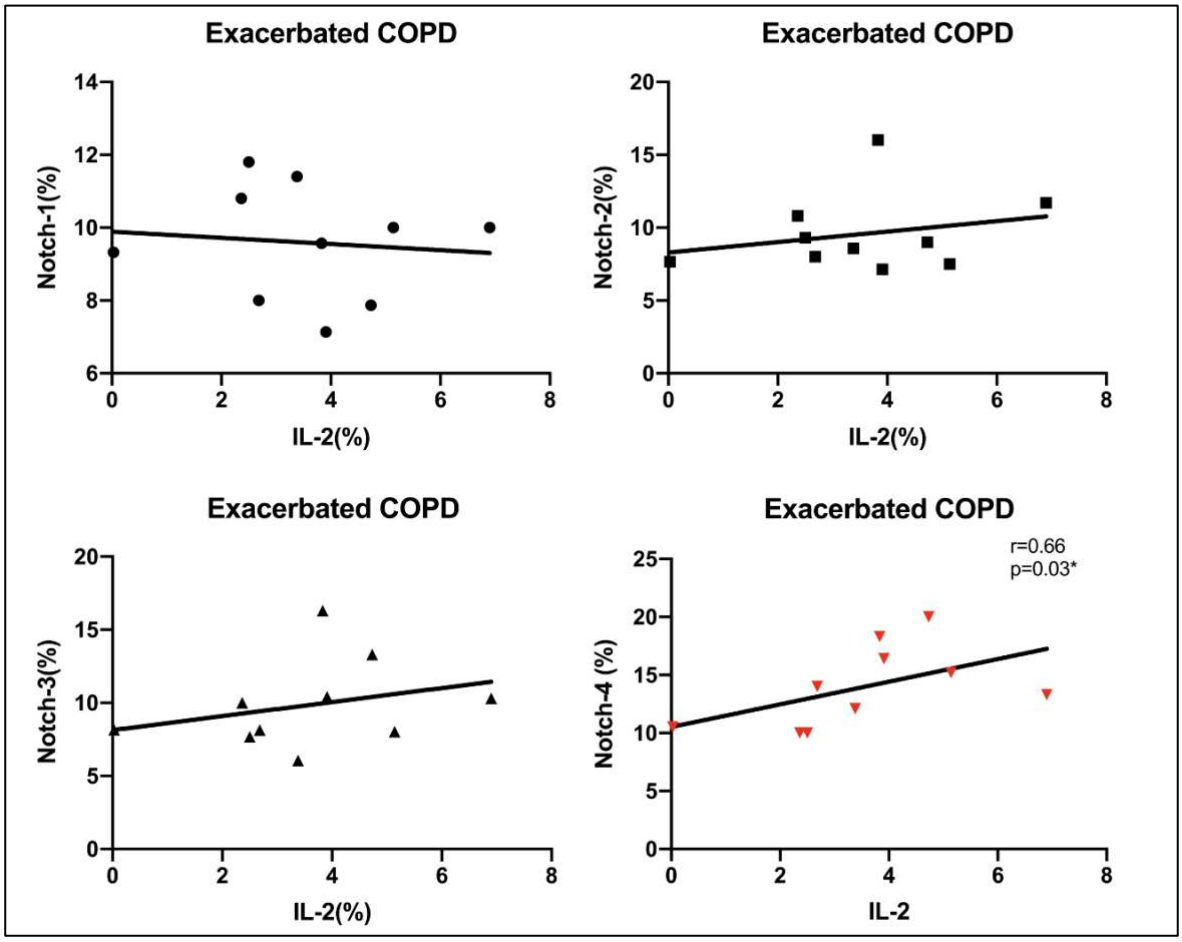

Figure 26: *Correlation analysis between Notch1/2/3/4 and IL-2 level in Exacerbated COPD patients.*

Chapter 4:

DISCUSSION

In this study, we investigated; (i) the expression of Notch receptors on T reg cells and their relationship with smoking and inflammatory parameters in COPD, (ii) Th1/Th2 cell balance together with HES5, HEY1, FOXP3, and RORC (RORγt) gene expression in CD4+ T cells, (iii) the level of pro/anti-inflammatory cytokines in plasma from study subjects. We have demonstrated the increased level of Treg cells and higher distribution of the Notch receptor on Treg cells in healthy smokers, and patients with stable, and exacerbated COPD, as compared to non-smokers together with the high level of Th1/Th2 cell ratio in exacerbated COPD patients by flow cytometry. At the gene level, no significant differences were observed between the study groups. Moreover, we demonstrated high levels of pro-inflammatory cytokines, as well as a negative correlation between Notch expression and anti-inflammatory cytokinesOur findings suggest that an increased surface expression of Notchs on Treg cells leads to an increase in inflammation by impairing Treg cells' function in patients with COPD.

First, we determined the expression of $CD4^+$ $CD25^+CD127^-FoxP3^+$ T reg cells and their relationship to smoking in addition to COPD inflammation by flow cytometric analysis. According to our findings, smoker subjects, stable and exacerbated COPD patients had a high level of Tregs in their PBMCs than non-smokers. While the expression of Tregs in the stable group was the greater than in the others, there was no significant result between the stable and exacerbated groups. These findings are supported by many studies that demonstrated the increased T reg cell expression in smokers and stable COPD patients compared to healthy control groups (Vargas-Rojas et al., 2011; Jin et al., 2014; Cervilha et al., 2019). Also, we demonstrated the mean fluorescence intensity (MFI) of FoxP3 surface expression on T regs of each study group. MFI refers to the relative quantification of a number of antigens on the cell surface. We did not find any significance in our MFI results however, the pattern of a decline in exacerbated COPD patients prompted us to examine the T reg functions. Tregs have various functions, including inhibiting the immunological response, maintaining homeostasis, and self-tolerance. Moreover, it has been demonstrated that Tregs can inhibit T cell growth and

cytokine production. Until now, some functional mediators of Treg suppression have been recognized. For diverse immunological responses, Treg produces inhibitory cytokines such as IL-10, IL-35, and TGF- to prevent T effector cell activation (Kondelkova et al 2010). Additionally, As Tregs mainly release IL-10, has an anti-inflammatory role, we anticipated that IL-10 would rise as the prevelance of Tregs increased. There was no significant difference in IL-10 levels between the study groups, despite the fact that the number of Tregs increased dramatically in the COPD group. Our data suggest that a significantly increase in Treg frequency and no significant change in the level of IL-10 might implicate a functional impairment in Tregs in patients with COPD. In a study by Jin, Yang, et al. on COPD patients in 2014, despite the fact that Tregs were upregulated during exacerbations, it was determined that their capacities are not sufficient because there was no significant difference in IL-10 levels between healthy control groups and patients. (Jin, Yang, et al,. 2014)

According to our flow cytometry results, the Notch-1 level on Treg cells was increased in smokers, and stable patients, and exacerbated in COPD patients as compared to non-smokers. Furthermore, we observed among the Notch receptors, only Notch-1 increased significantly on Treg cells between smokers and stable COPD. We suggest that Notch-1 might be a marker for the pathogenesis of COPD. Boucher et al (2012) showed overexpression of NICD-1 and HEY2 gene in the region of goblet cell metaplasia in COPD patients' the airway epithelium together with their submucosal glands. According to research by Guseh et al. (2009), demonstrated the number of mucous cells containing MUC5AC was elevated following the production of an active form of the Notch1 receptor or the addition of a Notch ligand, while the number of mucous cells was decreased following the addition of a -secretase inhibitor (GSI).

Additionally, we demonstrated that Notch-2 expression in smokers and both COPD subjects was higher compared to non-smokers. They demonstrated the overexpression of Notch-2 in combination with IL4/IL13 cytokines leads to goblet cell metaplasia Danahay, H., et al., (2015). Many in vivo and in vitro studies point out that the Notch signaling pathway has a major role in differentiation from the club into goblet cells (Tsao, P.-N., et al., 2009). According to the literature, Notch signaling may serve as a potential treatment

strategy for avoiding goblet cell metaplasia in human respiratory conditions such as COPD (Demedts et al., 2007). There is accumulating evidence that changes in Notch-3 signaling are crucial to the pathophysiology and progression of COPD. For instance, another study demonstrated that CS exposure activates Notch-3 signaling to promote the development of goblet cell metaplasia in both non-smoker and COPD airway epithelial cells (Bodas, M., et al., 2021). Furthermore, there is no proof that elevated Notch-3 signaling causes COPD-related emphysema, however a recent study demonstrated it does cause Marfan syndrome-related pulmonary emphysema in mice. Emphysematous alterations are a hallmark of the Marfan syndrome mouse model (mgR mice), and their progression is accompanied by an increase in only Notch-3 activation. They basically demonstrated in a mouse model of Marfan syndrome, increased Notch3 signaling causes lung emphysema (Jespersen et al., 2020).

Finally, our findings indicated an increased level of Notch-4 in both COPD study groups as compared to non-smokers. Notch-4 activation was associated with Covid-19 infection and asthmatic inflammation through Treg cell restriction (Harb, H., et al. 2020; Harb, H., et al 2021). Moreover, studies demonstrated Notch 1/3 and Th1-biased immune responses are higher in COPD patients as opposed to healthy subjects (Lin, C.-H., et al. 2020) and in addition to regulating Th17 – Treg differentiation, Notch pathway activation disturb the CD4+/CD8+ and Th17/Treg balance (Yin, X., et al., 2019). In addition, Gu et al (year) on mice model of COPD has indicated the increased expression of Notch1/2/3/4 receptors, Hes1/5, and Hey1 mRNA in the T cells leading to upregulation of Th1 and Th17 and an increase in the ratios of Th1/Th2 and Th17/T reg (Gu, X.-y., et al., 2017). To our knowledge, our study is the first to demonstrate that both patients with exacerbated COPD and stable COPD subjects have a higher overall distribution of Notch on their Treg cells. These results raise the possibility that an imbalance in T reg cells caused by an increase in Notch signaling is involved in the pathogenesis of COPD..

In this study, we also demonstrated the increased level of Th1/Th2 ratio in exacerbated COPD patients which reflects an immune imbalance of inflammatory $CD4^+$ T subsets in diseases. While some of the studies indicate that the imbalance of Th1/Th2 is due to the decreased Th1 and increased Th2 levels(Sun, J., et al 2018), others pointed out Th1/Th2 cell ratio is increase in COPD (Xue, W., et al 2022).In our study, we

analyzed Th1 and Th2 cell levels with flow cytometry. The percentage of Th1 cells did not significantly differ between the study groups, but we found an increasing pattern from nonsmokers to COPD patients with exacerbations. Additionally, the number of Th2 cells decreased in both the stable and exacerbation stages. Together, our results demonstrated that stable and exacerbated COPD patients had a higher Th1/Th2 cell ratio. Moreover, we examined some of the Th1 and TH-2-related cytokines and observed an increase in IL-2 in stable COPD patients and decreased level of IL-4 in exacerbated patients. Furthermore, we demonstrated the IFN-γ/IL-4 ratio which is increased in exacerbated patients. These results support a TH1-like immune response of peripheral blood CD4+ T cells in COPD patients.

Not only the Th1/Th2 balance also, we investigated the Th17/Treg balance according to their cytokine level. As IL-22 cytokine levels increased in stable patients as compared to smokers and non-smokers, there was no significant difference in IL-17A levels between the study groups. Again, in stable COPD patients, the ratio of IL-22/IL-10 was increased significantly in stable group as compared to smokers. In addition, we observed an increased level of another proinflammatory cytokine IL-6, which inhibits the regulatory activity of T cells and promotes the production of IL-17 and IL-22, in stable COPD patients comparing smokers and nonsmokers.

We also investigated the expression of Notch downstream genes; Hes5 and Hey1 to demonstrate the Notch activity and expression of Th17 and Treg master transcription factors; RORγt, and Foxp3 to point out Th17/Treg cell balance at the gene level. According to our RT-PCR results, there were no significant results.

Limitations of this study are the lack of enough PBMCs from the patients and the smaller amount of sorted CD4+ T cells, as compared to healthy controls. Therefore, we performed the gene expression experiment with a much smaller number of patient samples and the mRNA amount of the samples we obtained from those sorted cells was very low. Even if we collected a large number of blood samples, fewer CD4+ cells and mRNA were not enough to detect any change in gene level. In addition, we tried to detect intracellular cytokines, where cells are permeable and antibodies are used to detect cytokine protein within cells by flow cytometry, in order to understand which particular

cells are releasing the cytokine of interest.Nevertheless, despite our efforts, particularly patient PBMCs were not activated, and because there was a limited sample amount available, we assessed cytokine levels from the plasma. Moreover, ideally, both stable and exacerbated samples should be taken from the same patient to eliminate patient parameters. However, due to the limited study time, we were unable to obtain the samples in this manner.

In conclusion, although we could not directly demonstrate the activity of the Notch signaling pathway on T reg cells in our study, for the first time, we demonstrated the increased level of Notch distribution on Treg cells in exacerbated COPD patients together with stable COPD subjects. These findings suggest that an increase in the expression of Notch signaling on T- reg cells may contribute to the Treg cell imbalance in the pathogenesis of COPD. Further studies are required to assess molecular mechanisms under the Notch signaling activity on Treg cells in both stable and exacerbated COPD inflammation. Since there is little published literature on the subject, more study is necessary in this regard.