Review Article

COPD: immunopathogenesis and immunological markers

ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a disease of the lungs characterised by progressive and irreversible airflow limitation associated with chronic inflammation. Despite extensive research, the immunopathogenesis of COPD is still not fully elucidated. In this review, we outline the current understanding of the pathophysiology of COPD with a particular focus on chronic inflammation and the role of inflammatory cells such as neutrophils and macrophages in the disease, describe the exhaled breath condensate, a novel method of detecting inflammatory biomarkers, and suggest novel biomarkers to better characterise the immunopathogenesis of COPD.

Keywords: COPD; biomarkers; exhaled breath condensate; microRNA.

1. INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a disease state characterised by airflow limitation that is progressive, irreversible and associated with an inflammatory response to noxious particles and gases [1]. It is the sixth leading cause of death in the world and is expected to become the third leading cause of mortality in the year 2020 [2].

Currently, COPD consists of three main pathophysiological phenotypes:chronic bronchitis, emphysema and small airway disease [2]. Chronic bronchitis is caused by excess production and secretion of mucus by goblet cells. This culminates in epithelial remodelling and obstruction of small airways which leads to worsening of airflow obstruction and changes in airway surface tension predisposing to collapse [3]. Emphysema is caused by the degradation of elastin fibres and components of the extracellular matrix due to unregulated proteolysis resulting in irreversible damage to the lung parenchyma [2, 4].

Currently, much research is ongoing to find new biomarkers to diagnose COPD and better understand itspathophysiology.

This review explores the current understanding of the pathophysiology of COPD, with reference to the inflammatory cells involved such as neutrophils and macrophages. This review will also describe the exhaled breath condensate, an innovative method of identifying inflammatory markers, and proposes novel biomarkers to better characterise the immunopathogenesis of COPD.

2. PATHOPHYSIOLOGY OF COPD

The pathophysiology of COPD is still not well understood although severaltheories have been postulated in an attempt to describe it. Currently, 4 main mechanisms are described.

1. Chronic inflammation of the airways due to the influx of inflammatory cells into the lungs in response to cigarette smoke(Fig. 1).
2. Oxidative stress
3. Imbalance between proteolytic and anti-proteolytic activity culminating inlung tissue destruction
4. The apoptosis of lung structural cells has been postulated as a crucial upstream event in the development of COPD[5].

2.1 Chronic inflammationof the airways

COPD is mainly caused by exposure to noxious gases (usually cigarette smoke) or particles culminating in inflammation and remodelling in the large and small airways, and the destruction of lung parenchyma [6]. Currently, the inflammation in COPD is thought to consist of two phases: a phase involvingthe innate immune response, whereby a danger signal such as damage-associated molecular patterns (DAMPs) triggers inflammation, and a subsequent phase involving the acquired immune response[7, 8].

2.1.1 Innate immunity stage

Cigarette smoking introduces oxidants into the lungs which then activate pattern recognition receptors expressed in innate immune cells such as alveolar macrophages, dendritic cells and lung epithelial cells.Furthermore, oxidative damage by cigarette smoke has been postulated to cause DAMPs to be released from the injured epithelial cells [8].

Upon activation, these innate immune cells producevarious chemotactic factors that recruit inflammatory cells to the lungs. These includeCXC-chemokine ligand 1 (CXCL1) and CXCL8 (aka IL-8), which acts via CXC chemokine receptor 2 (CXCR2) and CC-chemokine receptor 2 (CCR2) to recruit neutrophils and monocytes (which subsequently differentiate into lung macrophages), CC-chemokine ligand 2 (CCL2), which binds toCCR2 to recruit monocytes, and CXCL9, CXCL10 and CXCL11,which binds to CXCR3 to recruittype 1 cytotoxic T (Tc1) cells and Th1 cells[9, 10]. Tc1 and Th1 cells then release interferon (IFN)-γwhich stimulates further release of CXCR3 ligands, culminating in a persistent inflammatory state[11].

In addition, oxidative damage by cigarette smoke culminates in the activation of the transcription factor nuclear factor-kB (NF-kB) and activator protein 1 (AP-1) in airway epithelial cells and macrophages [12, 13]. The activated transcription factors result in the transcription of downstream inflammatory cytokines such as tumour necrosis factor α (TNF-α), interleukin-6 (IL-6) and interleukin-8 (IL-8)which then recruit neutrophils to further amplify the inflammatory process [12]. The disease severity correlates with themagnitude of inflammation as evident by the presence of inflammatory cells[14].

Neutrophils and macrophages release oxidants and proteolytic enzymessuch as neutrophil elastase (NE) and maxtrix metalloproteinase-9 (MMP-9) which breakdown elastin and collagen in lung matrix [8]resulting in tissue damage. They also release cytokines capable of further amplifying the inflammatory response process[15].

The role of neutrophils and macrophages in COPD and the mediators that they produce will be discussed in greater detail in the subsequent sections.

2.1.2 Adaptive immunity stage

In addition to neutrophils and macrophages, a role has been suggested for B cells, lymphoid aggregates and CD8+ T cells in the chronic inflammatory process of COPD. This occurs especially in small airways, and the degree of inflammation positively correlates with disease severity[16].CD8+ T cells and natural killer cells release the proteolytic enzymes perforin and granzyme B which are toxic to lung tissue cells[17][18].

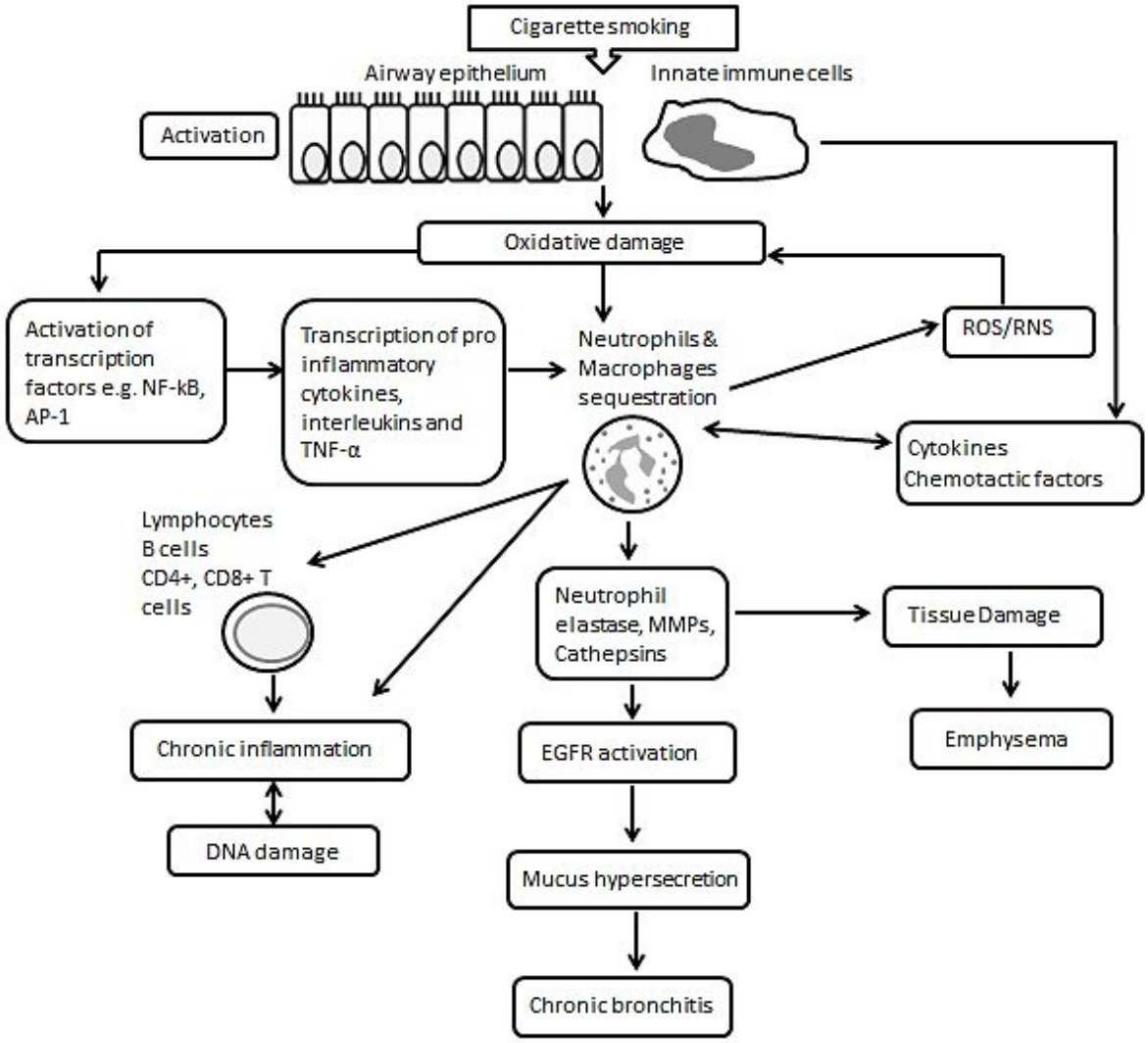

Fig. 1.Smoking as the major cause of chronic inflammation in the immunopathogenesis of COPD.Adapted from [9, 11, 19, 20].

2.1.3 Persistence of chronic inflammation in COPD

Even after smoking cessation,it is thought that chronic inflammation persists in COPD. The inflammatory process could be possibly sustained by defective antimicrobial responses resulting inmicrobial colonization or low-grade infections[21, 22]. Furthermore, the dysfunctional regulation of tolerogenic immune mechanisms could result in autoimmune reactions which subsequently culminate in chronic inflammation [7, 23].

In addition, the chronic inflammation in COPD could be explained by cumulative DNA damage as there is a substantial amount of information that supports the association between DNA damage and chronic inflammation.Inflammatory cells produce reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can cause serious DNA damage such as double-strand breaks, oxidation and nitration [24].
Aoshiba and colleagues (2013)have suggested a two-hit hypothesis explaining howthe inflammation in COPD becomes chronic. The first hit occurs from a danger signal such as DAMPs which initiates the inflammationand the second hit is when the inflammation perpetuates due to DNA damage. This hypothesis explainsthat the vicious cycle between DNA damage and inflammation causes the inflammation to

progressively worsenin COPD patients. In addition, the inflammation in COPD remains even after smoking cessation due to the persistence of DNA damage [24].

2.1.4 Role of Neutrophils in COPD

COPD is often thought to be a disease principally caused by neutrophils. Several studies show that neutrophils are found primarilyin the lumen of both small and large airways and also in bronchial epithelium, glands and airway smooth muscle bundles from sputum, bronchoalveolar lavage (BAL) [16] and bronchial biopsy specimens from COPD patients[25, 26].

Bronchial biopsy specimens have shownan increase in sub-epithelial neutrophils in severe COPD when compared to mild COPD,which in turn was higherthan in smokers without COPD[27]. Moreover, the number of neutrophils found in the sputum seemed to positively correlate with lung function decline over time[28]. In addition, reduced spontaneous apoptosis of peripheral blood neutrophils was observed in patients with an acute exacerbation of COPD [29].

Neutrophils are known to produce reactive oxygen metabolites, proteases [30], inflammatory cytokines, lipid mediators [31] and antibacterial peptides [32]and are associated with lung tissue destruction in emphysema and mucous cell metaplasia in chronic bronchitis [20](Fig. 2).

Neutrophils produce proteases/metalloproteaseswhich include NE and MMPs with gelatinase and collagenase activity (MMP-8, MMP-9) and their proteolytic potential have been investigated by several studies [33]. Metalloproteases are activated from their inactive preforms by proteolysis after exocytosis and are capable of breaking down structural components of the extracellular matrix which include collagens, proteoglycans, fibronectin, gelatin and laminin[34].

Apart from the ability to degrade extracellular matrix, NE can also stimulate mucin production and secretion. The proteolytic cleavage of transforming growth factor α (TGFα), a ligand of epidermal growth factor receptor, by NE induces mucin production. Increased mucus production and defective mucociliary clearance culminates in airway obstruction in COPD patients[19].

A number of different signals recruit neutrophils to the airways. Elevated levels of neutrophilicchemoattractants such as CXCL8 aka IL-8, leukotriene B4 (LTB4) [35], CXCL1 (aka growth-related oncogene-α, GRO-α) [36]and CXCL5 (epithelial neutrophil activating protein 78, ENA-78) [37]have been found in the airways of COPD patients. Activation of CXCR2, a high affinity receptor to which several chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL8) bind, induces chemotaxis of neutrophils [38].

A study conducted by Milaraand colleagues(2011) contributednovel insights into the role of neutrophils in COPD. It was demonstrated that subjects who developed severe early onset (age < 56 years) COPDhadpersistently elevated neutrophil count in the peripheral circulation despite years of smoking cessation, compared to age-matched controls without COPD. Furthermore, these neutrophils are highly activated with enhanced chemotaxis, and exhibit increased production of elastase and ROS when stimulated in comparison to controls. Lastly, these activated neutrophils are also more resistant to apoptosis[39]. This may help explain the disease progression in COPD even after smoking cessation.

Neutrophils activated by cigarette smoke are less deformable as a result of conversion of G-actin into F-actin. Several studies demonstrate that these stiffer neutrophils tend to be sequestered principallyin the capillaries of the upper lung regions which are locationstypical for smoking-related centrilobular emphysema [40-42]. The prolonged transit times of these activated neutrophils through the lung allows more time for proteases to be released to cause alveolar walldamage [43].

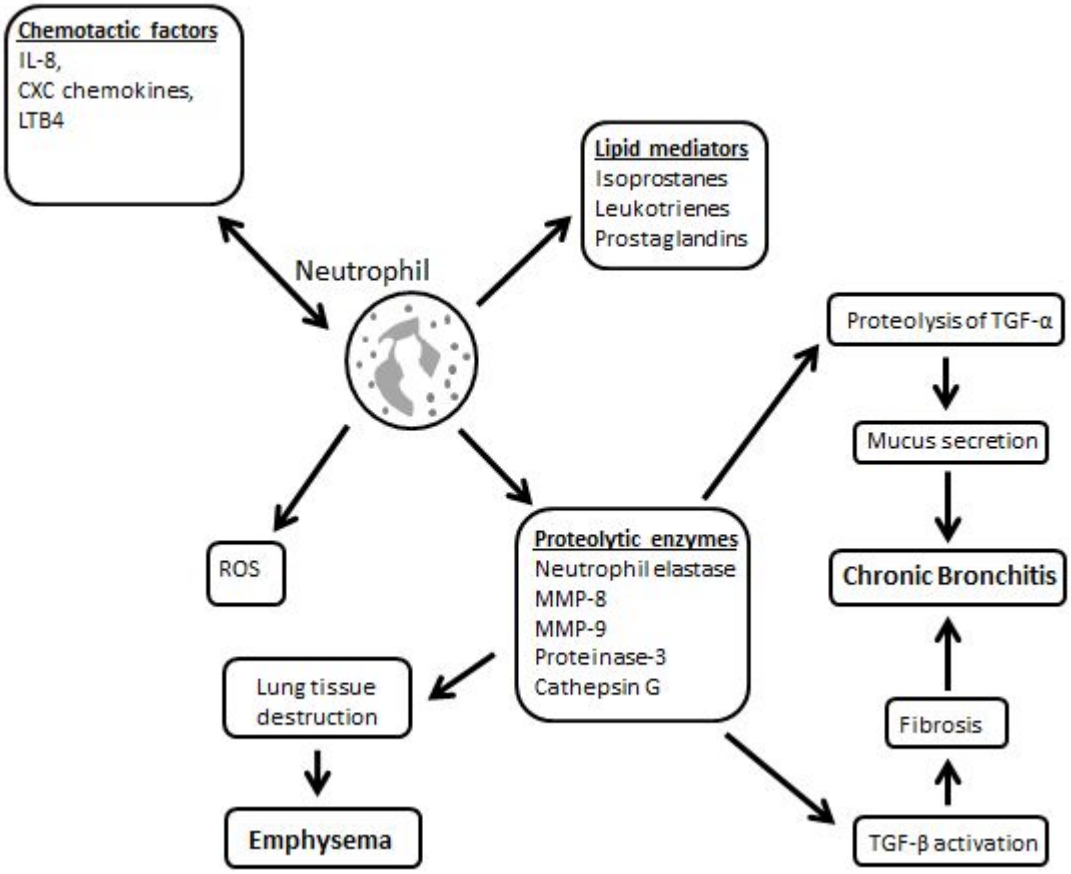

Fig. 2.Role of neutrophils in COPD. Adapted from: [19, 30, 31, 38, 44-46].

2.1.5 Role of Macrophages in COPD

Alveolar macrophages (AM) can secrete several inflammatory mediators such as reactive oxygen and nitrogen species, lipid mediators, growth factors, cytokines and chemokines(Fig. 3).They have both pro-inflammatory and anti-inflammatory functions in the respiratory tract and may be activated by various stimuli such as cigarette smoke, endotoxin, pro-inflammatory cytokines and immune stimuli. Generally, AMs from COPD patients demonstrate a higher production of inflammatory mediators than that of normal smokers, which in turn is higher than that of non-smokers [9].AMs are activated by cigarette smoke to release inflammatory mediators, such as TNF-α, IL-8,[47] and leukotriene (LT) B4 [11].

AMsoriginate from circulating monocytes which migrate to the lungs in response to chemoattractants such as CXCL1 acting on CXCR2 and CCL2 (aka MCP1) acting on CCR2 [48].

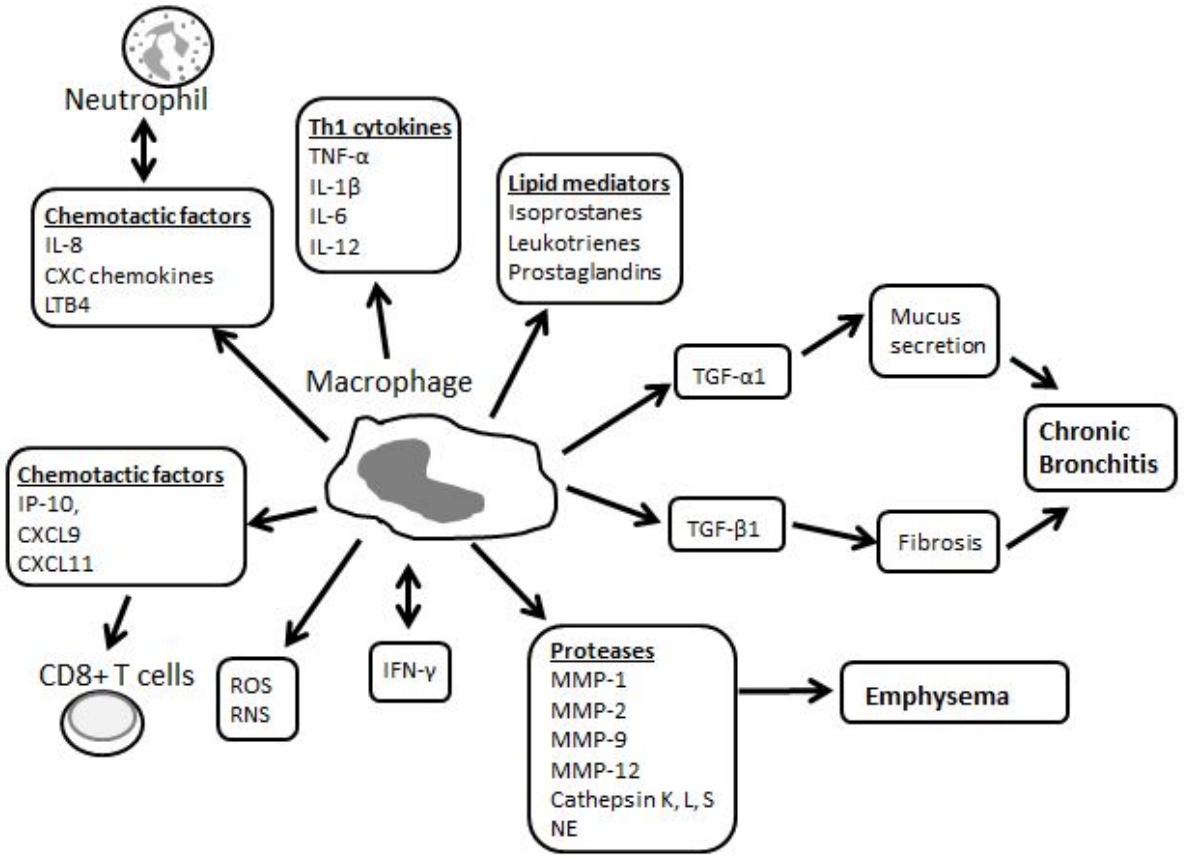

Fig. 3.Role of macrophages in COPD.Adapted from: [9-11, 19, 44-47, 49].

Table 1 shows the inflammatory mediators produced by macrophages and their role in COPD.

Inflammatory mediators	Existing literature
Growth Factors	Human AMs express transforming growth factor-β1 (TGF-β1) and TGF-β3[50].In COPD patients, there is an increased expression of TGF-β1 in airway macrophages[51]. TGF-β1 induces fibrosisand may be responsible for the fibrosis and narrowing of peripheral airways in COPD[45, 46].Furthermore, TGF-β1activates MMP-9, which then further activates TGF-β1.It is thought that MMP-9 may be able to mediate the proteolysis of TGF-β-binding protein which could account for the physiological release of TGF-β1.This phenomenon could demonstratea connection between emphysema and small airway fibrosis in COPD. [52]. Furthermore, TGF-β1 has been shown to be able to downregulate β2-adrenoceptors [53].
Proteases	Macrophages produce MMP-1 [11], MMP-2, MMP-9, MMP-12, cathepsins K, L and S and NE taken up from neutrophils [49, 54].These proteases damage the alveolar wall attachments culminating in lung parenchymal destruction, collapsed small airway lumens and reduced alveoli recoil [11]. MMP-9 seems to be the mainelastolytic enzyme secreted by alveolar macrophages in COPD patients [55, 56]. It is also highly expressed in lungs with emphysema,particularly at areas where macrophages gather[57]. MMP-12 (macrophage metalloelastase) is thought to be necessary for the release of activated TNF-α by alveolar macrophages and it plays a vital role in cigarette smoke-induced emphysema in mice [58]. It has been shown that the Th1 producedchemokinesIP-10/CXCL10 and MIG/CXCL9 interact with the CXCR3 receptorfound in alveolar macrophages to up-regulateMMP-12 production[59]. MMP-12 is the proteinase that is highly involved in mouse models of

	emphysema[60, 61]. However, there are conflicting studies about the role of MMP-12 in human emphysema[62, 63].

Table 1.Macrophage-producing inflammatory mediators of interest in COPD.

Activated macrophages also play a role in the destruction of lung parenchymal by inducing oxidative stresswhich is a direct signal for apoptosis of epithelial and endothelial cells [64]. Another signal for apoptosis in COPD is the loss of cell contact with the ECM caused by the degradation of the matrix by proteolytic enzymes[4, 65].

From the aforementioned studies in both human subjects and murine models, it is evident that macrophages play an active role in the destruction of lung parenchyma and the airways. However, the exact pathways and the key mediators have not yet been identifiedcompletely [11].

2.2 Oxidative Stress

Oxidative stress is another mechanism involved in the pathogenesis of COPD in whichan excessive production of reactive oxygen species overwhelm the antioxidant defence mechanisms [66]. Oxidants are produced bycigarette smoking or are released from inflammatory leukocytesand alveolar epithelial and endothelial cells[67]. Oxidative stress can cause cell dysfunction or apoptosis and lung extracellular matrix damage[5].

As mentioned above, oxidants contribute to the inflammatoryprocess in COPD by activating the transcription factor NF-κBwhich leads to the transcription of pro-inflammatorygenes [12][13].In addition to its contribution to the inflammatory process, oxidants also react readily withpolyunsaturated fatty acids of cell membranes to form lipid peroxidation products such as hydroperoxides[68], endoperoxide and aldehydes such as ethane, pentane, malondialdehyde[69]and 4-hydroxy-2-nonenal which are highly reactive [70]. Lipid peroxidation damages the cell membrane leading to cell destruction[68]and LPPs react with DNA to cause adduct formation [71].

2.3 Imbalance between proteolytic and anti-proteolytic activity

2.3.1 α1-antitrypsin (A1AT) deficiency

A1AT deficiency is a known risk factor for COPD. A1AT inhibits NE and therefore protects the lung from NE-induced damage[72]. Anti-proteinases such as α-1-proteinase-inhibitor (α-1-PI) and anti-leukoproteaseare inactivated by oxidants [69], leading to a proteinase/anti-proteinase imbalance which culminates in the destruction of lung elastin and connective tissue thereby causing emphysema [73].

2.4 Apoptosis

Apoptosis is suggested as the fourth mechanism to explain the pathogenesis of COPD. The imbalance between apoptosis and replacement of alveolar epithelial and endothelial cells in the lung has been thought to contribute to the lung tissue destruction in response to cigarette smoke, resulting in emphysema [5].

The various mechanisms are strongly interrelated in the pathogenesis of COPD and do not function separately. For instance, oxidative stress contributes to the proteinase and antiproteinase imbalance by inactivating antiproteinases, whereas an accumulation of apoptotic cells results in secondary necrosis and can amplify ongoing lung inflammation [8].

3. NOVELBIOMARKERS TO CHARACTERISE THE IMMUNOPATHOGENESIS OF COPD

Currently, the immunopathogenesis of COPD is still not fully understood. Increasing evidence suggests that either local or systemic samplingof biological molecules known as biomarkers can aid in better understanding the pathophysiological mechanisms of COPD [74]. The identification of biomarkers for COPD could help develop better methods to classify the different disease phenotypes, facilitate earlier diagnosis and to monitor response to novel therapeutic treatment in early clinical studies [45, 75].

4. EXHALED BREATH CONDENSATE AS A TOOL FOR SAMPLING BIOMARKERS

Exhaled breath condensate (EBC) is an emerging non-invasive technique that can detect biomarkers in various lung diseases. EBC is produced by the cooling of exhaled breath vapour and it contains water vapour and aerosolised particles which are produced by the airway lining fluid. EBC allows the investigation

of the composition of the airway lining fluid which may provide a sample of inflammatory mediators from inflammatory lung conditions [44].

Several studies demonstrate the utility of EBC to detect a broad range of organic and inorganic compounds including small inorganic molecules (H_2O_2, pH and nitric oxide related biomarkers), lipid mediators (8-isoprostane, leukotrienes and prostaglandins), small proteins (cytokines and chemokines) and nucleic acid derivatives (Table 2). These clinically relevant compounds are either due to chronic inflammation of the respiratory tract or acute oxidative stress or both. However, the majority of these compounds are of minute concentrations which may affect the accuracy of their detection in EBC[76].

The utility ofEBC to sample biomarkers has several advantages. It is non-invasive, inexpensive [77], does not affect or aggravate an ongoing pulmonary inflammatory process[78], conveniently performed and highly reproducible [79].

EBC possesses the potential to be utilised for diagnosing COPD, disease phenotying, evaluating treatment responseas well as defining patient's prognosis[80]. For instance, EBC can be utilised to measure airway inflammation which allowsthe monitoring of response to anti-inflammatory treatment. It may also permit early interventions for COPD patients before the occurrence of symptom development and lung function decline [81, 82].

However, the disadvantages of EBC include salivary contamination which may affect EBC measurement [77, 78].Furthermore, the collected condensate is not anatomic-site specific as the precise location where aerosol particles are derived from the lower respiratory tract and the relative contribution of the various sites to the particles is still unknown[79].

The table below summarises the variety of biomarkers studied in EBC of COPD patients. Studies on certain biomarkers such as TGF-β, MMP-8, neutrophil elastase and miR-223 have not been carried out yet and remains a potential area of exploration.

Category	Biomarker	Findings in COPD patients	Studies
pH	pH	Lower	[83, 84]
Reactive oxygen species	Hydrogen peroxide	Increase	[85]
Reactive nitrogen species	Nitric oxide	Higher	[86]
	Nitrite (NO_2^-)	Elevated	[87]
	Nitrate	No significant difference	[86]
	Peroxynitrite	Higher	[88]
	Nitrosothiols	Higher	[87]
Cytokines	TNF-α	Increased	[89]
	IL-1β	Increased in exacerbation	
	IL-6	IL-6 increased	[90]
	IL-8	Increased in exacerbation	[89]
	IL-10	Increased in exacerbation	
	IL-12p70	Increased in exacerbation	
	IL-17	No difference	[91]
Collagenase	MMP-9 TIMP-1	Increase in COPD exacerbation	[92]
	Neopterin	No significant difference	[93]
	IP-10	No significant difference	[93]
	8-IP	Elevated in COPD	[76]
	Malondialdehyde	Increased	[94]
Arachidonic acid derivatives	PGE2 LTB4	Increased	[95]
	Prostaglandin F2-alpha	No significant difference	
Nucleic acids	microRNAs	Lower expression of Let-7a, miR-328, miR-21in COPD	[96]

281 **Table 2.Summary of EBC biomarkers studied in COPD patients.**

Recently, microRNAs have been an area of interest in identifying novel biomarkers for COPD.

5. MicroRNAs

MicroRNAs (miRNAs) are small noncoding RNAs comprising 20 to 25 nucleotides that are expressed in bodily fluids and tissue. They are emerging as potential biomarkers that are vital in the regulation of inflammation [96]. miRNAs control gene expression by initiating mRNA degradation or inhibiting mRNA translation [97].

There is increasing literature suggesting that there is abnormal expression of specific miRNAs in certain lung diseases such as COPD [98].

In a study comparing the miRNA expression profile of bronchial epithelial cells from never-smokers and smokers, 28 miRNAs were found to be differentially expressed. In particular, miR-218 was thought to be important in modulating epithelial gene expression following cigarette smoke exposure [99].

Another study showed that miR-638 was upregulated in emphysema. miR-638 is thought to respond to oxidative stress byculminating in an accelerated lung aging response and dysfunctional ECM repair [100].

5.1 MicroRNA-223

MicroRNA-223 is myeloid-specific and was shown to down regulatemyeloid progenitor proliferation and granulocyte differentiation and activation [101].

In a study by Fazi, et al., the authors have identified that miR-223 is an important modulator of human myeloid differentiation that is specifically expressed in myeloid cells. In addition, miR-233 is upregulated during retinoic acid mediated granulocytic differentiation of acute promyelocyticleukemia cells both in vivo and in vitro. Both overexpression and knockdown experiments show the relevant role of miR-223 in the differentiation process. For the first case, there was a twofold increase in the cells committed to the granulocyte-specific lineage, whereas decreased miR-223 levels resulted in the opposite effect [102].

Detection of miRNA-223 in human EBC for COPD patients has not been carried out yet and hence remains a potential area for exploration. The following presents current studies done on miR-223 in relation to COPD.

5.1.1 Murine studies

miR-223 has been known to target Mef2c, a transcription factor that promotes myeloid progenitor proliferation. miR-223-deficient granulocytes demonstrate hypermaturity, are more sensitive to activating stimuli and show stronger fungicidal activity. miR-223 mutant mice was observed to develop increased tissue damage and inflammatory lung pathology after endotoxin challenge as a result of neutrophil hyperactivity [101].

Another study showed that environmental cigarette smoke led to the downregulation of miR-223 expression in the lungs of rats.

5.1.2 Human lung tissue samples

However, there is a conflicting study which showed that miR-223 was increased in expression by nearly threefold in lung tissue samples from COPD patients compared with smokers without airflow limitation[103].A possible reason could be due to the difference in genetic makeup in humans and mice and thus more studies on miR-233 could be done especially in human subjects.

As neutrophils play an important role in the immunopathogenesis of COPD, and miRNA-223 is essential in neutrophil production and development, the role of miRNA-223 in COPD remains a

potential area of interest. This could also pave the way for novel therapeutic strategies for the disease.

7. CONCLUSION

Despite extensive research carried out for many decades, the immunopathogenesis of COPD and the exact mechanisms of the disease are still not fully understood. EBC could be utilised as a non-invasive method to diagnose COPD and aid in better understanding the immunopathogenesis of COPD by the identification of novel biomarkers. More studies could be done on microRNAs in relation with COPD.

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