
# Novel approaches to the management of non-eosinophilic asthma

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| Abstract: | Non-eosinophilic airway inflammation occurs in approximately 50% of patients with asthma. It is subdivided into neutrophilic or paucigranulocytic inflammation, although the proportion of each subtype is uncertain because of variable cut-off points used to define neutrophilia. This article reviews the evidence for non-eosinophilic inflammation being a target for therapy in asthma and assesses clinical trials of licensed drugs, novel small molecules and biologics agents in non-eosinophilic inflammation. Current symptoms, rate of exacerbations and decline in lung function are generally less in non-eosinophilic asthma than eosinophilic asthma. Non-eosinophilic inflammation is associated with corticosteroid insensitivity. Neutrophil activation in the airways and systemic inflammation is reported in neutrophilic asthma. Neutrophilia in asthma may be due to corticosteroids, associated chronic pulmonary infection, altered airway microbiome and/or delay neutrophil apoptosis. The cause of poorly controlled non-eosinophilic asthma may differ between patients and involve several mechanism including neutrophilic inflammation, Th2-low or other subtypes of airway inflammation and/or corticosteroid insensitivity as well as non-inflammatory pathways such as airway hyperreactivity and remodelling. Smoking cessation in asthmatic smokers and removal from exposure to occupational agents reduces neutrophilic inflammation. Preliminary studies of 'off-label' use of licensed drugs suggest that macrolides show efficacy in non-smokers with non-eosinophilic severe asthma and statins, low-dose theophylline and PPARγ agonists may benefit asthmatic smokers with non-eosinophilic inflammation. Novel small molecules targeting neutrophilic inflammation, such as CXCR2 antagonists reduce neutrophils, but do not improve clinical outcomes in studies to date. Inhaled PDE4 inhibitors, dual PDE3 and PDE4 inhibitors, p38MAPK inhibitors, tyrosine kinase inhibitors and PI3kinase inhibitors are under development and these compounds may be of benefit in non-eosinophilic inflammation. The results of clinical trials of biological agents targeting mediators associated with non-eosinophilic inflammation, such as IL-17 and TNF-α are disappointing. Greater understanding of the mechanisms of non-eosinophilic inflammation in asthma should lead to improved therapies. |

# Novel approaches to the management of non-eosinophilic asthma

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## ABSTRACT

Non-eosinophilic airway inflammation occurs in approximately 50% of patients with asthma. It is subdivided into neutrophilic or paucigranulocytic inflammation, although the proportion of each subtype is uncertain because of variable cut-off points used to define neutrophilia. This article reviews the evidence for non-eosinophilic inflammation being a target for therapy in asthma and assesses clinical trials of licensed drugs, novel small molecules and biologics agents in non-eosinophilic inflammation. Current symptoms, rate of exacerbations and decline in lung function are generally less in non-eosinophilic asthma than eosinophilic asthma. Non-eosinophilic inflammation is associated with corticosteroid insensitivity. Neutrophil activation in the airways and systemic inflammation is reported in neutrophilic asthma. Neutrophilia in asthma may be due to corticosteroids, associated chronic pulmonary infection, altered airway microbiome and/or delay neutrophil apoptosis. The cause of poorly controlled non-eosinophilic asthma may differ between patients and involve several mechanism including neutrophilic inflammation, Th2-low or other subtypes of airway inflammation and/or corticosteroid insensitivity as well as non-inflammatory pathways such as airway hyperreactivity and remodelling. Smoking cessation in asthmatic smokers and removal from exposure to occupational agents reduces neutrophilic inflammation. Preliminary studies of 'off-label' use of licensed drugs suggest that macrolides show efficacy in non-smokers with non-eosinophilic severe asthma and statins, low-dose theophylline and PPARγ agonists may benefit asthmatic smokers with non-eosinophilic inflammation. Novel small molecules targeting neutrophilic inflammation, such as CXCR2 antagonists reduce neutrophils, but do not improve clinical outcomes in studies to date. Inhaled $PDE_4$ inhibitors, dual $PDE_3$ and $PDE_4$ inhibitors, p38MAPK inhibitors, tyrosine kinase inhibitors and PI3kinase inhibitors are under development and these

compounds may be of benefit in non-eosinophilic inflammation. The results of clinical trials of biological agents targeting mediators associated with non-eosinophilic inflammation, such as IL-17 and TNF-$\alpha$ are disappointing. Greater understanding of the mechanisms of non-eosinophilic inflammation in asthma should lead to improved therapies.

Word count: 299

**Key words**: Airway inflammation; asthma; biological agents; biomarkers; cigarette smoking; corticosteroid insensitivity; eosinophils; neutrophils; small molecules.

## INTRODUCTION

Personalised medicine in asthma aims to individualise treatment using non-invasive biomarkers that predict a beneficial response and/or that identify individuals who are at risk of adverse effects [Agustí *et al.*, 2015]. Several airway inflammatory phenotypes are recognised that help identify a therapeutic response to specific treatments in asthma. For example, eosinophilic airway inflammation, which is usually identified on the bases of sputum or blood eosinophilia, predicts patients with asthma that are likely to obtain a favourable therapeutic response to corticosteroids [Pavord *et al.*, 1999, Little *et al.*, 2000, Green *et al.*, 2002, Bacci *et al.*, 2006, Berry *et al.*, 2007] and to monoclonal antibodies targeting interleukin (IL)-5 [Pavord *et al.*, 2012, Katz *et al.*, 2014, Thomson, 2014]. Type 2 helper T-cell (Th2)-high subtype of asthma is associated with increased epithelial expression of interleukin IL-4, IL-5 and IL-13 [Woodruff *et al.*, 2009, Arron *et al.*, 2013] and is considered to overlap with eosinophilic airway inflammation [Arron *et al.*, 2013]. Evidence from clinical trials suggests that the presence of Type-2 eosinophilic inflammation predicts a therapeutic response not only to corticosteroids [Woodruff *et al.*, 2009], but to monoclonal antibodies targeting specific cytokines such as IL-5 [Bel *et al.*, 2014, Ortega *et al.*, 2014] and IL-13 [Corren *et al.*, 2011]. Many patients with asthma have non-eosinophilic asthma, sometimes associated with neutrophilic inflammation and/or have a Th2-low type of inflammation. Compared to type-2 eosinophilic inflammation there are relatively few interventions available for non-type 2 inflammatory sub-groups. This article aims to discuss the evidence that non-eosinophilic airway inflammation, with or without neutrophic inflammation, is an appropriate target for therapy in asthma and also aims to assess the results of recent clinical trials of licensed drugs, novel small molecules and biologics agents in the treatment of non-eosinophilic asthma.

## IS NON-EOSINOPHILIC AIRWAY INFLAMMATION AN APPROPRIATE TARGET FOR THERAPY IN ASTHMA?

A number of factors need to be considered when attempting to answer the question of whether non-eosinophilic inflammation is an appropriate target for treatment in asthma including the criteria used to define neutrophilic and eosinophilic inflammation, the stability of non-eosinophil inflammation over time, the prevalence of non-eosinophilic inflammation, the strength of evidence for the involvement of non-eosinophilic inflammation in clinical features of asthma and the cause(s) of non-eosinophilic airway inflammation.

### Definition of eosinophilic and neutrophilic airway inflammation

Non-eosinophilic airway inflammation is a term used to describe a subtype of asthma associated with normal numbers of sputum eosinophils. The non-eosinophilic phenotype is subdivided into neutrophilic inflammation, when neutrophil numbers are raised above a defined cut-off level or paucigranulocytic inflammation, when both eosinophil and neutrophil numbers are normal. In addition, some individuals have a mixed type of inflammation, when there is sputum neutrophilia and eosinophilia. Cut-off levels used to define sputum eosinophilia most commonly used are $\geq$2% [Mcgrath *et al.*, 2012, Hastie *et al.*, 2013], >2% [Peters *et al.*, 2014] or $\geq$3% [Schleich *et al.*, 2013, Zhang *et al.*, 2014, Wagener *et al.*, 2015]. A $\geq$3% cut-off is reported to be the most precise value to identify eosinophilic airway inflammation [Simpson *et al.*, 2010]. Sputum eosinophil counts are associated with bronchial tissue eosinophil numbers suggesting that they provide a good indicator of airway eosinophilic pathology [Arron *et al.*, 2014]. The cut-off for a raised sputum neutrophil count is not clearly established with a wide

range of values reported in the literature: >40% (Nair, Gaga et al. 2012; Moore, Hastie et al. 2014), ≥50% (Chaudhuri, Norris et al. 2014), >61% (Simpson, Milne et al. 2009), >65% (Nair et al 2015) and ≥76% (Schleich, Manise et al. 2013). The most appropriate cut-off value that identifies individuals in whom neutrophils are activated and contributing to the pathogenic processes in asthma is not certain. In addition, sputum neutrophils do not correlate with bronchial tissue numbers bringing into doubt their predictive value for identifying neutrophil-induced airway pathology [Arron *et al.*, 2014]. In addition to the presence of non-eosinophilic inflammation, Haldar and Pavord [Haldar *et al.*, 2007] proposed that the criteria for a diagnosis of non-eosinophilic asthma should include objective evidence of airflow obstruction or airway hyperreactivity, a raised asthma control questionnaire (ACQ) score (>1.5) and the absence of a significant smoking history, fixed airflow obstruction or associated bronchiectasis. In the current article, the criteria for non-eosinophilic asthma include the presence of non-eosinophilic inflammation as defined above plus objective evidence of asthma, but the review also includes data from patients with both normal and raised ACQ scores, who have a significant smoking history or who have fixed airflow obstruction.

**Stability of sputum cell counts**

Published data on the long term stability of sputum neutrophil and eosinophil counts is conflicting. Some studies report stable sputum cell counts in patients with mild to severe asthma follow-up over 6 months [Berry *et al.*, 2007], 12 months [Green *et al.*, 2002], 2 years [Jayaram *et al.*, 2006] and 5 years [Simpson *et al.*, 2006, Van Veen *et al.*, 2009]. In contrast, sputum inflammatory cell phenotype changed in 48.6% of patients with severe asthma over 1 year among patients recruited to the BIOmarkers in Severe Chronic AIRway Disease

(BIOAIR) study [Kupczyk *et al.*, 2014]. Similar variability in sputum cell counts has been reported by others [Hancox *et al.*, 2012] and in one study a stable inflammatory phenotype was found in only one third of patients [Al-Samri *et al.*, 2010]. Transient sputum eosinophilia is reported in up to 40% patients with non-eosinophilic inflammation [Bacci *et al.*, 2012, Mcgrath *et al.*, 2012]. The potential for the lack of stability in non-eosinophilic inflammation over time needs to be accounted for in intervention studies targeting sputum inflammatory cell biomarkers.

**Prevalence of non-eosinophilic airway inflammation**

The different cut-off values used to define elevated sputum cell counts, particularly sputum neutrophils, may explain the variation in prevalence figures for non-eosinophilic inflammation between studies. Nevertheless, overall up to 50% of adults and adolescents with stable mild to severe asthma, and in some studies higher proportions, have non-eosinophilic inflammation [Gibson *et al.*, 2001, Green *et al.*, 2002, Simpson *et al.*, 2006, Wang *et al.*, 2011, Mcgrath *et al.*, 2012, Schleich *et al.*, 2013, Moore *et al.*, 2014, Brooks *et al.*, 2016 ]. For example, a review of sputum cytology data from 995 subjects with mild to moderate asthma enrolled in clinical trials undertaken by the Asthma Clinical Research Network (ACRN) reported that non-eosinophilic inflammation (sputum eosinophils <2%) was present in 64% of patients not taking inhaled corticosteroid and 83% of patients taking inhaled corticosteroids. In a sub-group of patients followed up for 6 months, 47% of the inhaled corticosteroid free patients and 72% of those taking inhaled corticosteroids had persistent non-eosinophilic inflammation [Mcgrath *et al.*, 2012]. In a cluster analysis performed on 423 patients recruited to the Severe Asthma Research Program (SARP)

cohort, four asthma inflammatory sub-phenotypes were identified (sputum eosinophilia ≥2%; sputum neutrophilia >40%) [Moore *et al.*, 2014]. Two groups had mild-to-moderate allergic asthma with minimal or eosinophil-predominant sputum inflammation whereas the other two sub-phenotypes had moderate-to-severe asthma with neutrophil-predominant or mixed granulocytic inflammation [Moore *et al.*, 2014]. A study in a small group of adults with stable asthma found 51.7% of subjects had a paucigranulocytic phenotype, 27.6% neutrophilic inflammation and 17.2% eosinophilic inflammation [Wang *et al.*, 2011].

## Involvement of neutrophilic and non-eosinophilic airway inflammation in asthma

Evidence for the involvement of non-eosinophilic inflammation in asthma is based mainly on studies examining the association between sputum inflammatory phenotypes and clinical outcomes in asthma including current symptom control, exacerbations, airflow obstruction and therapeutic response to corticosteroids. Further evidence is provided by reports of local activation of neutrophils and systemic inflammation in neutrophilic asthma.

### *Current symptom control*

The severity of current symptoms is in general similar or slightly lower in non-eosinophilic or neutrophilic subgroups of asthma compared to eosinophilic subgroups [Cowan *et al.*, 2010, Hastie *et al.*, 2010, Wood *et al.*, 2012, Schleich *et al.*, 2013, Baines *et al.*, 2014, Newby *et al.*, 2014, Schleich *et al.*, 2014].

### *Exacerbations*

Sputum neutrophilia is found in up to 80% of exacerbations in adults with asthma [Turner *et al.*, 1995, Fahy *et al.*, 1995 , Lamblin *et al.*, 1998, Green *et al.*, 2002, Jayaram *et al.*, 2006, Maneechotesuwan *et al.*, 2007, Wang *et al.*, 2011], although the predominant sputum cell type can alter during successive exacerbations [D'silva *et al.*, 2007]. Sputum eosinophilia is a better predictor of future exacerbations than sputum neutrophilia [Jatakanon *et al.*, 2000, Leuppi *et al.*, 2001, Kupczyk *et al.*, 2014, Schleich *et al.*, 2014]. For example, a cluster analysis performed on patients recruited to the BIOAIR study identified two clusters with raised sputum eosinophil counts that accounted for 83% of subjects who had 2 or more severe exacerbations during follow-up for 1 year [Kupczyk *et al.*, 2014]. One of these clusters had a mixed inflammatory profile with raised sputum neutrophils (43% percent of patients). A further cluster had a raised neutrophil count and a normal eosinophil count (11% of patients) and a non-eosinophilic paucigranulocytic inflammation was found in only 6% of cases. Patients with severe asthma associated with eosinophil inflammation have more intubations than non-eosinophilic patients [Wenzel *et al.*, 1999].

***Airflow obstruction***

Sputum neutrophilia is association with reduced lung function and based on this finding it has been speculated that airway neutrophils may contribute to the development of persistent airflow obstruction in asthma [Little *et al.*, 2002, Shaw *et al.*, 2007]. Against this hypothesis, a recent cluster analysis of lung function decline and sputum eosinophil count performed in 97 patients with severe asthma identified a non-eosinophilic group in whom the decline in $FEV_1$ was -14 ml per year compared to an eosinophilic group with highly variable eosinophil counts

that had a greater rate of decline in $FEV_1$ of -41 ml per year [Newby *et al.*, 2014]. These findings suggest that eosinophilic inflammation, particularly when there is high variability in eosinophil count, is a greater risk factor for the development of persistent airflow obstruction than non-eosinophilic inflammation. Bronchodilator reversibility and airway hyperresponsiveness are similar in eosinophilic and non-eosinophilic asthma [Berry *et al.*, 2007, Mcgrath *et al.*, 2012], although one study noted greater airway hyperresponsiveness in persistent or intermittent eosinophilic groups [Mcgrath *et al.*, 2012].

***Impaired response to inhaled corticosteroids***

Non-eosinophilic inflammation is associated with an impaired therapeutic response to inhaled corticosteroids [Pavord *et al.*, 1999, Green *et al.*, 2002, Bacci *et al.*, 2006, Berry *et al.*, 2007, Thomson *et al.*, 2009, Mcgrath *et al.*, 2012], although the lack of efficacy may not be complete. Several clinical studies performed in small numbers of patients with non-eosinophilic asthma suggest that this group may obtain some benefit from inhaled corticosteroids although less than that found in eosinophilic patients [Godon *et al.*, 2002, Cowan *et al.*, 2010, Lemière *et al.*, 2011]. Intermittent eosinophilia might be a factor accounting for corticosteroid sensitivity in some of these patients [Bacci *et al.*, 2012, Mcgrath *et al.*, 2012, Suárez-Cuartín *et al.*, 2015].

***Evidence for neutrophil activation in asthma***

There is evidence to suggest that the innate immune system is activated in chronic asthma. Firstly, sputum IL-8 and neutrophil elastase concentrations and innate immune receptors

TLR2, TLR4 and CD14 as well as pro-inflammatory IL-8 and IL1-β gene expression levels are increased in neutrophilic asthma compared to non-neutrophilic asthma [Simpson *et al.*, 2007, Wood *et al.*, 2012]. Secondly, neutrophil activation, as measured by sputum myeloperoxidase (MPO) levels, is positively associated with sputum neutrophil numbers in asthma [Little *et al.*, 2002]. Thirdly, specific sputum gene expression signatures are reported to discriminate eosinophilic asthma from non-eosinophilic asthma as well as to predict a beneficial response to inhaled corticosteroids [Baines *et al.*, 2014]. In this study, non-eosinophilc asthma was identified by increased sputum cell expression of IL1β , alkaline phosphatase, tissuenonspecific isozyme (ALPL ) and CXCR2, whereas eosinophilic asthma was characterised by increased expression of Charcot-Leydon crystal protein or galectin-10 (CLC), carboxypeptidase A3 (CPA3) and deoxyribonuclease I-like 3 (DNASE1L3). The NLRP3 inflammasome is upregulated in neutrophilic asthma and may increase the production of IL-1β [Simpson *et al.*, 2014, Kim *et al.*, 2015]. Anti-inflammatory responses may be impaired in non-eosinophilic asthma based on reduced sputum galectin-3 concentrations, which increases uptake of apoptotic neutrophils and reduced IL-1RA/IL-1ß ratio, and which might increase pro-inflammatory actions of IL-1ß [Gao *et al.*, 2015]. In addition, soluble receptor for advanced glycation end-products (RAGE), which is a pattern-recognition receptor is deficient in BAL samples in neutrophilic asthma [Sukkar *et al.*, 2012]. The T-cell granzyme B pathway, which is though to mediate apoptosis of epithelial cells, might be defective in non-eosinophilic asthma, based on the finding of a higher ratio of the expression of granzyme B to its inhibitor in T cells in this group compared to eosinophilic asthma [Simpson *et al.*, 2014].

Systemic inflammation is increased in patients with neutrophilic airway inflammation. The proportion of patients with elevated CRP, IL-6 and neutrophil elastase concentrations is higher in neutrophilic asthma compared to a non-neutrophic group [Baines *et al.*, 2011, Wood *et al.*, 2012]. Neutrophilic inflammation is associated with increased α-defensin and neutrophil protease gene expression in blood [Baines *et al.*, 2011]. In non-eosinophilic asthma, blood neutrophils released significantly higher levels of IL-8 at rest [Baines *et al.*, 2010]. In a small study, gene expression markers of systemic inflammation were associated with higher BMI, greater history of cigarette smoking, lower FVC% predicted, and increased sputum neutrophils [Fu *et al.*, 2013].

**Potential inflammatory processes leading to non-eosinophilic airway inflammation**

Several inflammatory processes could lead to non-eosinophilic inflammation and airway damage in asthma although the exact immunological mechanisms are unclear (Figure 1) [Trejo Bittar *et al.*, 2015]. Uncertainty in the clinical relevance of experimental animal models of non-eosinophilic inflammation has hampered progress in understanding the involvement of neutrophils and non-eosinophilic inflammation in the pathogenesis of asthma. Stimuli such as viruses, cigarette smoke and pollutants could induce the release of chemoattractants including IL-8 to recruit neutrophils to the airways. In experimental asthma models, the release of IL-17A and IL-17F from activated Th17 cells stimulates the synthesis of neutrophil chemoattractants including CXCL1 and IL-8 from the airway epithelium. [Newcomb *et al.*, 2013]. INF-γ may also be involved in the pathogenesis of severe asthma associated with neutrophilic and eosinophilic inflammation, possibly in part through the release of IFN-γ from Th1 cells [Raundhal *et al.*, 2015 ]. Data from patients with

severe asthma and an experimental murine asthma model implicate high INF-γ immune responses and low secretory leukocyte protease inhibitor expression (SLPI) in airway epithelial cells with airway hyperresponsiveness [Raundhal *et al.*, 2015 ]. Neutrophils are a potential source of oxygen free radicals and enzymes and their ability to activate other airway cell types [Futosi *et al.*, 2013]. Neutrophils in asthma are implicated in causing mucus gland hyperplasia and hypersecretion, airway hyperreactivity and remodelling as well as corticosteroid insensitivity. Interestingly, $Th_1$ and $Th_{17}$ cells may induce airway hyperreactivity and/or remodelling independently of neutrophil activation.

Limited information has been published on the immunopathological characteristics of non-eosinophilic inflammation in asthma compared with other inflammatory airway phenotypes including Th2-low inflammation, Th17-high inflammation or combination of Th2/Th17 profiles. Bronchial biopsy studies of patients with non-eosinophilic asthma and with Th2-low inflammation report reduced sub-mucosal eosinophil numbers and normal sub-epithelial basement membrane thickness in both groups [Wenzel *et al.*, 1999, Berry *et al.*, 2007, Woodruff *et al.*, 2009]. In contrast, bronchial eosinophils numbers and sub-epithelial basement membrane thickness are both increased in eosinophilic asthma and in Th2-high asthma [Wenzel *et al.*, 1999, Berry *et al.*, 2007, Woodruff *et al.*, 2009]. Mast cell numbers are increased in eosinophilic asthma [Wenzel *et al.*, 1999] and Th2-high asthma [Dougherty *et al.*, 2010], whereas mast cells numbers are normal in the sub-mucosal of patients with severe non-eosinophilic asthma [Wenzel *et al.*, 1999] and in the epithelium of non-smoker with Th2-low asthma [Dougherty *et al.*, 2010]. Mast cell numbers in airway smooth muscle are increased in both non-eosinophilic and eosinophilic asthma [Berry *et al.*, 2007]. Bronchial biopsy neutrophil numbers are increased to a similar degree in non-eosinophilic severe asthma and eosinophilic

severe asthma [Wenzel *et al.*, 1999]. Neutrophil numbers in Th2-low asthma have not been reported. A lower proportion of subjects with non-eosinophilic asthma are atopic compared to eosinophilic asthma (18% versus 66%) [Berry *et al.*, 2007] and (58% versus 83%) [Gibson *et al.*, 2001]. Severe asthma associated with neutrophilia has significantly higher sputum levels of Th17-related cytokines (CXCL1, CXCL10 , CCL2, IL-6, and IL-8) compared with severe asthmatics with other inflammatory phenotypes [Manni *et al.*, 2014]. The proportion of Th17 lymphocytes and the ratio of Th17 to regulatory T cells (Treg) in the peripheral blood is greater in patients with non-eosinophilic asthma taking inhaled corticosteroids compared to an eosinophilic asthma group [Furukawa *et al.*, 2015]. Approximately one third of patients with severe eosinophilic asthma have a Th17-high signature that is associated with a Th2-low gene expression profile [Choy *et al.*, 2015]. The number of subjects with non-eosinophilic severe asthma in this study was not sufficient to determine their Th17 profile [Choy *et al.*, 2015]. Taken together, these finding suggest that non-eosinophilic inflammation and Th2-low inflammation in non-smokers with asthma share some similar immunopathological features including normal eosinophil numbers, submucosal mast cell numbers and sub-epithelial basement membrane thickness. There is a need for further studies to establish the similarities and differences in endotypes of non-eosinophilic, Th2-low and Th17 high inflammation to help identify sub-groups of patients for targeted therapies.

**Factors accounting for neutrophilic airway inflammation in asthma**

Several factors either alone or in combination could explain raised sputum neutrophil counts in asthma (Table 1). Firstly, corticosteroids inhibit apoptosis of neutrophils [Cox, 1995] and their use in asthma may contribute to sputum neutrophilia [Saffar *et al.*, 2011]. In addition, Th2-

targeted therapies, including oral corticosteroids may contribute to the development of Th17-high neutrophilic inflammation [Choy *et al.*, 2015, Shum, 2015]. In support of corticosteroids causing neutrophilia in asthma , inhaled corticosteroid withdrawal from patients with moderate asthma resulted in only one subject with neutrophilic inflammation although the reintroduction of inhaled fluticasone for 4 weeks resulted in a raised neutrophil count in only 5% of subjects [Cowan *et al.*, 2010]. In one study of patients with severe oral corticosteroid dependent asthma associated with increased sputum neutrophil number, markers of neutrophil activation including oxidative burst and surface granular receptor expression were similar to patients with mild asthma [Nair *et al.*, 2015]. In the SARP cohort, however corticosteroid use was not associated with sputum neutrophilia, suggesting that continuous corticosteroid exposure may not be the only influence on sputum neutrophil numbers in severe asthma [Moore *et al.*, 2014]. Secondly, co-morbid conditions such bronchiectasis or severe airflow obstructions occurring in association with asthma may result in neutrophilic inflammation. Thirdly, delayed human neutrophil apoptosis has been reported in severe asthma [Uddin *et al.*, 2010], possibly due to epithelial growth factor induced release of mediators with neutrophil chemotactic and anti-apoptotic actions from bronchial epithelial cells [Uddin *et al.*, 2010, Uddin *et al.*, 2013]. Fourthly, macrophage efferocytosis is impaired in non-eosinophilic asthma, which may cause airway neutrophilia [Simpson *et al.*, 2013]. Fifthly, altered airway microbiome has been implicated in airway neutrophilia. Airway colonisation determined by terminal restriction fragment length polymorphism (T-RFLP) analysis is associated with more severe airways obstruction and longer duration of disease as well as neutrophilic airway inflammation and raised sputum IL-8 levels [Simpson *et al.*, 2013, Green *et al.*, 2014]. Taken together, these finding suggest that the cause of airway neutrophilia in asthma is likely to be complex, possibly due to corticosteroid treatment inducing impaired apoptosis of neutrophils and Th17 mediated

neutrophilic inflammation, delayed apoptosis of neutrophils due to epithelial growth factor release and ineffective macrophage efferocytosis of neutrophils as well as an altered airway microbiome.

**Clinical phenotypes associated with non-eosinophilic inflammation**

Non-eosinophilic inflammation, either paucigranulocytic or neutrophilic occurs in a range of clinical phenotypes that account for approximately 50% of adults never or ex-smokers with mild to severe asthma or that have controlled or uncontrolled asthma (Table 2). Non-eosinophilic inflammation, with or without neutrophilic inflammation is commonly found in smokers with asthma [Chalmers *et al.*, 2002, Boulet *et al.*, 2006, Thomson *et al.*, 2013]. A high BMI is associated with non-eosinophilic asthma in some people [Haldar *et al.*, 2008], although others have submucosal eosinophilia [Desai *et al.*, 2013] . Approximately two thirds of cases of occupational asthma due to low molecular weight agents have non-eosinophilic inflammation [Anees *et al.*, 2002], which is associated with a poor asthma prognosis [Lemiere *et al.*, 2014]. Non-occupational-induced asthma that is exacerbated by work exposures is associated with non-eosinophilic phenotype [Lemière *et al.*, 2013]. Additional factors associated with higher neutrophil counts include older age [Brooks *et al.*, 2013], exposure to environmental pollution through living close to car pollution [Wallace *et al.*, 2011], exposure to occupational particulate matter [Simpson *et al.*, 2015] and respiratory infections.

**Biomarkers that can identify non-eosinophilic airway inflammation**

Are there biomarkers that can identify patients with non-eosinophilic airway inflammation? Blood eosinophil numbers are moderately associated with sputum eosinophils [Schleich *et al.*, 2013, Zhang *et al.*, 2014, Wagener *et al.*, 2015]. Using a cut-off for a blood eosinophil count of >0.22 x $10^9$/L [3] [Schleich *et al.*, 2013], >0.26 x $10^9$/L [Zhang *et al.*, 2014] or ≥0.27 x $10^9$/L [Wagener *et al.*, 2015] accurately predicts sputum eosinophilia. In contrast, another study reported that blood eosinophils had a poor predictive value of 47% for sputum eosinophilia (≥3% cut-off) although this was better in severe asthma (71%) [Hastie *et al.*, 2013]. In patients with mild to severe asthma, blood eosinophils were reported to be better than serum periostin and exhaled nitric oxide in identifying sputum eosinophilia [Wagener *et al.*, 2015]. Blood neutrophil numbers has a weak relationship with sputum neutrophil count [Schleich *et al.*, 2013, Zhang *et al.*, 2014] and they have a poor predictive value for sputum neutrophilia (64% or 38% for a cut-off of ≥40% or ≥61% cut-off respectively) [Hastie *et al.*, 2013]. In one study exhaled nitric oxide predicted inhaled corticosteroid response for airway hyperreactivity in non-eosinophilic asthma (area under the curve 0.81), with an optimum cut-off point of 33 ppb [Cowan *et al.*, 2010].

**Which inflammatory phenotype to target?**

In summary, non-eosinophilic airway inflammation is found in approximately 50% of patients with mild to severe asthma. The proportion of this group with neutrophilic inflammation is less certain because of variable cut-off points used in different studies to define neutrophilia. Current symptoms, rate of exacerbations and rate of decline in lung function are generally less severe in non-eosinophilic asthma compared to eosinophilic asthma. Non-eosinophilic inflammation is associated with an impaired response to inhaled corticosteroids. There is some

evidence that neutrophils are activated in the airways of patients with neutrophilic asthma and that biomarkers of systemic inflammation is increased in this group. Neutrophilia in asthma may be due to corticosteroids, associated chronic pulmonary infection, altered airway microbiome and/or delay neutrophil apoptosis, particularly in severe disease. Non-eosinophilic asthma and Th2-low asthma may share some common immunopathological features, but further investigation is required. Due to the lack of effective specific therapies targeting non-eosinophilic inflammation including neutrophilic inflammation there is currently no definitive evidence for the involvement of these inflammatory phenotypes in chronic asthma. Additional pathways may account for poor asthma control in patients with non-eosinophilic asthma including Th1 inflammation, Th17 inflammation, or a combination of Th2 and Th17 inflammation as well as corticosteroid insensitivity (Figure 1). Recent work suggests a reciprocal relationship between Th2 and Th17 pathways in severe disease and that corticosteroid treatment may contribute to the emergence of a Th17-high profile [Choy *et al.*, 2015]. Non-inflammatory mechanisms may also be important in some individuals including airway hyperreactivity and airway remodelling.

## TREATMENTS TARGETING NON-EOSINOPHILIC AIRWAY INFLAMMATION

Many patients with asthma continue to have poorly controlled disease despite treatment with currently available therapies. There is an unmet need for novel treatments that will impact favourably on clinical outcomes in patients with non-eosinophilic inflammation. Non-pharmacological interventions, 'off-label' use of licensed drugs, novel small molecules and biologics agents are being investigated as possible treatments of non-eosinophilic inflammation in asthma (Table 3 and Figure 2).

**Non-pharmacological interventions**

Avoidance from exposure to environmental and occupational pollutants may reduce neutrophilic inflammation in asthma. In a study of smokers with asthma of whom a subgroup quit smoking for 6 weeks the proportion of sputum neutrophils reduced, corticosteroid sensitivity improved and the $FEV_1$ increased compared to those who continued to smoke [Chaudhuri *et al.*, 2006]. After cessation of exposure to occupational agents, neutrophilic inflammation reduced in people in whom their asthma was cured or improved compared to those in whom there was no improvement [Maghni *et al.*, 2004]. Several clinical trials have examined the effect of dietary supplement of vitamin D in asthma, based on the anti-inflammatory and corticosteroid-enhancing actions of vitamin D [Nanzer *et al.*, 2013, Zhang *et al.*, 2014] [Xystrakis *et al.*, 2006]. Two large randomized clinical trials of vitamin D3 supplementation in patients with asthma and vitamin D insufficiency [VIDA and ViDiAs trials), although not selected for specific airway inflammatory cell profiles or corticosteroid insensitivity, reported no improvements in clinical outcomes [Castro *et al.*, 2014, Martineau *et al.*, 2015, Denlinger *et al.*, 2015 ]. Interestingly, vitamin D supplementation reduces eosinophilic inflammation in patients with non-atopic asthma, suggesting that certain inflammatory phenotypes might benefit from vitamin D3 supplementation [De Groot *et al.*, 2015].

**'Off-label' use of licensed drugs**

Several drugs licensed for the treatment of medical conditions other than asthma have been investigated for their efficacy in asthma, including patients with non-eosinophilic inflammation.

Candidate drugs have been chosen usually because of pre-clinical evidence of anti-inflammatory effects that might be relevant to treatment of asthma. Some examples are reviewed below.

***Macrolides***

Macrolides may be of benefit in the treatment of chronic asthma [Reiter *et al.*, 2013], including non-eosinophilic asthma [Simpson *et al.*, 2008], although prescribing macrolides as a long-term treatment increases the risk of adverse drug effects and the development of microbial resistance [Cameron *et al.*, 2012]. The mechanism(s) of action of macrolides in the treatment of airway diseases is not known, but could be due to antibacterial and/or anti-inflammatory actions, which include inhibition of NF-κB and other transcription factors as well as reduction in neutrophil migration and/or function [Culic *et al.*, 2001, Fujitani *et al.*, 2003, Simpson *et al.*, 2008, Cameron *et al.*, 2012, Kobayashi *et al.*, 2013]. Macrolides have additional potentially beneficial properties including anti-viral actions [Gielen *et al.*, 2010, Schögler *et al.*, 2015] and an ability to restore corticosteroid sensitivity by inhibiting the phosphoinositide 3-kinase (PI3K) pathway and restoring histone deacetylase (HDAC)2 activity [Spahn *et al.*, 2001, Kobayashi *et al.*, 2013, Hao *et al.*, 2015] and by attenuating TNFα and IL-17 immune responses [Essilfie *et al.*, 2015]. Two recent exploratory clinical trials have investigated the effects of macrolides in non-eosinophilic asthma. In one trial, smokers with mild to moderate asthma associated with non-eosinophilic inflammation were randomized to azithromycin 250 mg per day or placebo [Cameron *et al.*, 2013]. After 12 weeks, treatment with azithromycin was not associated with improvements in morning PEF, ACQ score, AQLQ score and methacholine $PC_{20}$ compared to placebo and did not alter induced sputum differential counts, bacterial load, *C. pneumonia, M.*

*pneumoniae* seropositivity or upper airways respiratory virus prevalence. In an other randomized controlled trial patients with exacerbation-prone severe asthma received low-dose azithromycin or placebo as add-on treatment to combination therapy of inhaled corticosteroids and long-acting $\beta_2$ agonists for 6 months [Brusselle *et al.*, 2013]. The rate of severe exacerbations and lower respiratory tract infections requiring treatment with antibiotics was not reduced by azithromycin. In a predefined subgroup with non-eosinophilic severe asthma (blood eosinophilia ≤200/µl) there was a reduction in the rate of primary endpoints in azithromycin-treated patients [Brusselle *et al.*, 2013]. Azithromycin improved AQLQ scores, but did change ACQ scores or lung function. Based on these findings, further clinical trials of macrolides in non-eosinophilic severe asthma are indicated. Novel analogues of macrolides have been developed that have enhanced anti-inflammatory properties than current macrolides, such as solithromycin (CEM-101) [Kobayashi *et al.*, 2013, Kobayashi *et al.*, 2013] or that lack anti-bacterial properties, such as the non-antibiotic azithromycin derivative CSY0073 [Balloy *et al.*, 2014].

***Statins***

Statins have pleiotropic immunomodulatory actions [Greenwood *et al.*, 2006] that may of value in the treatment of chronic inflammatory diseases [Greenwood *et al.*, 2006, Hothersall *et al.*, 2006, Yeganeh *et al.*, 2014]. In experimental models of allergic asthma [Mckay *et al.*, 2004, Zeki *et al.*, 2009] and tobacco-smoke-induced lung inflammation [Lee *et al.*, 2005, Davis *et al.*, 2013] statins reduce inflammatory pathways potentially relevant to the pathogenesis of asthma and smoke-induced airway diseases. Statins might also restore corticosteroid sensitivity in asthma [Samson *et al.*, 2006, Maneechotesuwan *et al.*, 2010].

Taken together, these findings suggest that statin treatment may have anti-inflammatory effects in people with asthma including smokers with asthma. A randomized double-blind parallel group trial undertaken in seventy one smokers with mild to moderate asthma associated with non-eosinophilic inflammation compared treatment with atorvastatin 40 mg per day with placebo. After 4 weeks treatment inhaled beclometasone 400 µg per day was added to both treatment arms for a further 4 weeks [Braganza *et al.*, 2011]. At 4 weeks, there was an improvement in ACQ and AQLQ scores with atorvastatin, but not in lung function. There was no significant improvement with atorvastatin and inhaled beclometasone compared to inhaled beclometasone alone in clinical outcome measures at 8 weeks. In a follow-up study the effects of atorvastatin alone and in combination with inhaled corticosteroid was investigated on their ability to suppress the concentration of a range of cytokines, chemokines and growth factors in sputum samples collected during the previous clinical trial [Braganza *et al.*, 2011, Thomson *et al.*, 2015 ]. Sputum mediator concentrations were not reduced by inhaled beclometasone alone. Atorvastatin significantly reduced sputum concentrations of CCL7, IL-12p70, sCD40L, FGF-2, CCL4, TGF-α and MMP-8 compared with placebo and, when combined with inhaled beclometasone, reduced sputum concentrations of MMP-8, IL-1β, IL-10, MMP-9, sCD40L, FGF-2, IL-7, G-CSF and CCL7 compared to ICS alone. Improvements in ACQ and/or AQLQ scores with atorvastatin and inhaled beclometasone were associated with decreases in G-CSF, IL-7, CCL2 and CXCL8. Interestingly, simvastatin suppresses airway IL-17 and upregulated IL-10 in patients with stable COPD [Maneechotesuwan *et al.*, 2013]. Taken together, these findings suggest that short-term treatment with atorvastatin alone or in combination with inhaled beclometasone reduces several sputum cytokines, chemokines and growth factors concentrations unresponsive to inhaled corticosteroids alone in asthmatic smokers with

non-eosinophilic inflammation. There is a need for long-term clinical studies examining effect of statins on exacerbations and airway remodelling in chronic non-eosinophilic asthma.

***Low-dose theophylline***

Low dose theophylline has been shown to restore corticosteroid sensitivity *in-vitro* possibly by increasing HDAC-2 activity, which is suppressed in severe asthma and in smokers with asthma and a similar clinical effect might occur in people with severe disease or who are smokers [Barnes, 2009, To *et al.*, 2010]. Theophylline inhibits oxidative stress dependent PI3K-δ activation and restores corticosteroid sensitivity in PBMCs from patients with COPD [To *et al.*, 2010]. An exploratory clinical trial examined the effects of low dose theophylline added to inhaled beclometasone compared to inhaled beclometasone alone in smokers with asthma associated with non-eosinophilic inflammation [Spears *et al.*, 2009]. The addition of low dose theophylline to inhaled beclometasone, at a dose titrated to provide a 'sub-therapeutic' concentration, resulted in increased efficacy as measured by lung function and suggested the restoration of corticosteroid sensitivity in those treated with the combination. Clinical trials to date have not investigated the therapeutic effects of adding low dose theophylline to patients with severe asthma. A fixed combination of ultra-low dose of theophylline with fluticasone, SKP-2075 (Skepharma), in a dry powder inhaler is under development for the treatment COPD. This combination would potentially be of benefit in the treatment of severe asthma and smokers with asthma, possibly in those people with non-eosinophilic inflammation.

***PPARγ agonist***

In pre-clinical studies peroxisome proliferator-activated receptor-γ (PPARγ) agonists exert anti-inflammatory effects potentially relevant to the treatment of inflammatory airway diseases including asthma and COPD [Spears *et al.*, 2006, Belvisi *et al.*, 2008, Seidel *et al.*, 2012, Stephen *et al.*, 2013, Bourke *et al.*, 2014, Lakshmi *et al.*, 2014, Lea *et al.*, 2014, Donovan *et al.*, 2015 ]. For example, PPARγ agonists reduce eosinophilic and neutrophilic lung infiltration in experimental animal models exposed to allergen or tobacco smoke [Bauer *et al.*, 2010, Lea *et al.*, 2014, Zhao *et al.*, 2014, Morissette *et al.*, 2015]. The oral PPARγ agonist rosiglitazone had a modest effect in attenuating the allergen-induced late asthmatic response [Richards *et al.*, 2010]. A further proof of concept study reported that rosiglitazone compared with inhaled beclometasone dipropionate resulted in improvement in lung function and a borderline reduction in sputum IL-8 concentration in smokers with mild to moderate asthma that was associated with non-eosinophilic inflammation [Spears *et al.*, 2009]. The oral PPARγ agonist pioglitazone is not effective in obese asthmatics [Dixon *et al.*, 2015]. Inhaled PPARγ agonist analogues, such as AD3277 (Pulmagen) are under development for the treatment of chronic inflammatory airway diseases and potentially might be of benefit in non-eosinophilic asthma.

**Novel small molecule drugs**

Novel small molecule inhibitors have been developed for treating neutrophilic/non-eosinophilic asthma including CXCR2 antagonists, FLAP inhibitors, $PDE_4$ inhibitors, dual $PDE_3$ and $PDE_4$ inhibitor and various protein kinase inhibitors.

***CXCR2 antagonist***

CXCR2 receptors are expressed on neutrophils as well as on airway goblet cells, fibroblasts and airway smooth muscle [Chapman *et al.*, 2009]. Ligands for the CXCR2 receptor include the chemokines CXCL8 (IL-8), growth-related protein (Gro)-α, -β, and -γ (CXCL1–3), epithelial-derived neutrophil attractant-78 (ENA-78; CXCL5), granulocyte chemotactic protein-2 (GCP-2; CXCL6) and neutrophil- activating peptide-2 (NAP-2; CXCL7) [Chapman *et al.*, 2009, Campbell *et al.*, 2013]. Activation of CXCR2 receptors result in neutrophil chemotaxis, proteases production, aairway goblet cell hyperplasia, pulmonary blood vessel angiogenesis, collagen deposition and airway smooth muscle contraction and migration [Chapman *et al.*, 2009]. The effects of CXCR2 antagonists have been studied on airway challenges that induce sputum neutrophilia. The CXCR2 antagonist, AZD8309 inhibits LPS-induced airway neutrophilic inflammation in healthy volunteers [Leaker *et al.*, 2013] and the CXCR2 antagonist, SB656933 inhibited *ex vivo* neutrophil activation and ozone-induced airway inflammation in humans [Lazaar *et al.*, 2011]. The CXCR2 antagonist, SCH527123 inhibits ozone-induced neutrophilia in healthy subjects [Holz *et al.*, 2010]. A randomized, placebo-controlled clinical trial of the CXCR2 antagonist SCH527123 administered for 4 weeks to patients with severe asthma and sputum neutrophils > 40% resulted in a reduction of 36.3% in sputum neutrophil percentage, fewer mild exacerbations and a trend towards improvement in ACQ score [Nair *et al.*, 2012]. A clinical trial of the efficacy and safety of a CXCR2 antagonist AZD5069 in severe, uncontrolled persistent asthma reported that the addition of AZD5069 to combination ICS/LABA treatment did not improve clinical outcomes despite a dose-dependent reduction in blood neutrophil counts [O'byrne *et al.*, 2015]. A lack

of improvement in clinical outcome despite a reduction in sputum neutrophil counts was reported with the CXCR2 antagonist AZD5069 in bronchiectasis [De Soyza *et al.*, 2015]. A recent trial of the CXCR2 antagonist Navarixin (SCH527123) in COPD led to significant improvements in $FEV_1$ and reduction in sputum neutrophil count, particularly in current smokers with COPD [Rennard *et al.*, 2015]. The CXCR2 antagonist AZD8309 administered for 4 weeks to patients with moderate to severe COPD was well tolerated with no increase in the rate of infections [Kirsten *et al.*, 2015]. A small-molecule oral CXCR2 antagonists Danirixin (GSK1325756) is undergoing a clinical trial in patients with COPD at risk of exacerbations (ClinicalTrials.gov Identifier: NCT02130193). Oral CXCR2 antagonists could potentially cause neutropenia, and the therapeutic index of these compounds requires careful assessment.

***FLAP inhibitors***

Pro-inflammatory cysteinyl leukotrienes (LTs) are synthesised from arachidonic acid in inflammatory cells by 5-lipoxygenase (LO) and 5-lipoxygenase activating protein (FLAP). FLAP inhibitors such as GSK-2190915 [Evans *et al.*, 2008] prevent the formation of $LTB_4$, which may be of value in the treatment of neutrophilic asthma. GSK2190915 markedly inhibited *ex vivo* calcium ionophore stimulated blood $LTB_4$ formation and urinary leukotriene $E_4$ ($LTE_4$) formation [Bain *et al.*, 2013]. Pre-treatment with GSK2190915 reduces the early and late phase response to allergen challenge and results in a significant reduction of sputum $LTB_4$ levels [Kent *et al.*, 2013]. Despite suppressing the target mediator $LTB_4$, the FLAP inhibitor GSK2190915 has no short-term effect on sputum cell counts or clinical endpoints in smokers and non-smokers with asthma associated with neutrophilic

inflammation (sputum neutrophilia ≥ 50% for one sample and >45% for the other), suggesting that $LTB_4$ suppression alone is inadequate in controlling airway neutrophils in asthma [Chaudhuri *et al.*, 2014]. No active clinical trials of FLAP inhibitors in asthma are currently registered on ClinicalTrials.gov.

***$PDE_4$ inhibitors and dual $PDE_3$ and $PDE_4$ inhibitors***

Phosphodiesterase $(PDE)_4$ inhibitors have immunomodulatory effects on inflammatory cells potentially relevant to the treatment of asthma [Lipworth, 2005, Page *et al.*, 2012, Kim *et al.*, 2015 ]. In an allergen challenge study the oral $PDE_4$ inhibitor roflumilast attenuated the rise in sputum eosinophils and neutrophils numbers after the late asthmatic response [Gauvreau *et al.*, 2011]. High doses of $PDE_4$ inhibitors may be necessary to treat severe asthma, and gastro-intestinal side effects limit their use [Lipworth, 2005, Bateman *et al.*, 2006, Bousquet *et al.*, 2006]. The inhaled administration of $PDE_4$ inhibitors may improve the therapeutic index of $PDE_4$ inhibitors [Chapman *et al.*, 2010, Singh *et al.*, 2010, Nials *et al.*, 2011, De Savi *et al.*, 2014, Moretto *et al.*, 2015]. Inhaled $PDE_4$ inhibitors GSK256066 and CHF6001 both inhibit allergen-induced late asthmatic responses [Singh *et al.*, 2010, Dave *et al.*, 2014] and in patients with moderate COPD inhaled GSK256066 for 4 weeks was well tolerated although there was no inhibitory effect on sputum and blood inflammatory biomarkers [Watz *et al.*, 2013]. The inhaled dual $PDE_3$ and $PDE_4$ inhibitor RPL554 (Verona Pharma) has bronchodilator effects and is well tolerated in patients with asthma and COPD [Franciosi *et al.*, 2013]. In healthy subjects inhaled RPL554 attenuates the neutrophilic response to LPS challenge [Franciosi *et al.*, 2013]. RPL554 is under development for the

treatment of asthma and COPD (ClinicalTrials.gov Identifier**:** NCT02427165 and NCT02542254 respectively)**.**

***Protein kinase inhibitors***

Protein kinases are involved in cellular signalling of pro-inflammatory cytokines in asthma and the inhibition of these kinases may have a role in the treatment of severe asthma associated with non-eosinophilic asthma [Bhavsar *et al.*, 2010, Cohen *et al.*, 2010, Hammaker *et al.*, 2010, Chung, 2011, Guntur *et al.*, 2012]. Several p38MAPK inhibitors restore corticosteroid sensitivity in PBMCs from patients with severe asthma [Bhavsar *et al.*, 2010, Mercado *et al.*, 2012] and COPD [Khorasani *et al.*, 2015]. Clinical trials of p38MAPK inhibitors oral losmapimod (GW856553) and inhaled AZD7624 are register for the treatment COPD (ClinicalTrials.gov Identifier: NCT02299375 and NCT02238483 respectively), although neither are registered for the treatment of asthma. Interestingly, a *post-hoc* analysis of a 6 month clinical trial of oral losmapimod (GW856553) in COPD reported a reduction in exacerbations in a sub-group of patients with a blood eosinophil count $\leq$2% [Marks-Konczalik *et al.*, 2015], which may suggest a preferentially beneficial effect of p38MAPK inhibitors in non-eosinophilic inflammation. A imatinib, a specific ckit tyrosine kinase inhibitor that attenuates airway hyperresponsiveness, inflammation and remodelling in murine model of asthma [Berlin *et al.*, 2005, Rhee *et al.*, 2011] is under development for patients with severe refractory asthma (ClinicalTrials.gov Identifier: NCT01097694). A tyrosine kinase inhibitor masitinib targets c-kit and platelet-derived growth factor (PDGF) receptor improved asthma control in patients with severe corticosteroid-dependent asthma [Humbert *et al.*, 2009] and a further clinical trial underway in patients with severe asthma

treated with oral corticosteroids (ClinicalTrials.gov Identifier: NCT01449162). An alternative therapeutic strategy to silencing c-kit with small interference RNA has been shown to attenuate inflammation in a murine model of allergic asthma [Wu *et al.*, 2012, Wu *et al.*, 2014]. Clinical trials of protein kinase inhibitors have not been studied in patients with sputum inflammatory subtypes such as non-eosinophilic asthma.

***PI3kinase inhibitors***

Low dose theophylline is thought to act, at least in part, through the inhibition of PI3K [Ito *et al.*, 2007, To *et al.*, 2010]. Pre-clinical studies suggest that PI3K-δ inhibitors could potentially reverse corticosteroid insensitivity by increasing HDAC2 activity [Marwick *et al.*, 2009, Marwick *et al.*, 2010] and by reversing fungal-induced steroid resistant airway inflammation through modulation of endoplasmic reticulum stress [Lee *et al.*, 2016]. Selective PI3K inhibitors are being developed as novel therapies for the treatment of chronic inflammatory airway diseases. An inhaled PI3Kδ inhibitor GSK2269557 is undergoing several clinical trials in asthma and COPD. PI3K δ and γ isoforms are involved in inflammatory cell recruitment and activation and dual PI3Kδ/γ inhibitors, such as TG100-115 and IPI-145 reduces airway inflammation induced by allergen or cigarette smoke in murine models [Doukas *et al.*, 2009, Winkler *et al.*, 2013] and restored corticosteroid sensitivity in the smoke model [Doukas *et al.*, 2009]. RV1729, a PI3Kδ/γ Inhibitor has undergone early stage clinical evaluation in asthma and COPD. SH2-containing inositol-50-phosphatase 1 (SHIP1) is an endogenous inhibitor of the PI3K pathway. A SPIP1 activator AQX-1125 reduced the allergen-induced late asthmatic response with a non-significant trend for a reduction in sputum eosinophils and neutrophils [Leaker *et al.*, 2014].

Further development of AQX-1125 is underway for the treatment of COPD (ClinicalTrials.gov Identifier: NCT01954628).

**Biological agents**

Monoclonal antibody blockers of inflammatory cytokines such as IL-17 and TNF-α that activate receptors on the surface of neutrophils have been investigated as treatments for asthma.

***IL-17 blockers***

In pre-clinical studies $Th_{17}$ cells and IL-17 are implicated in causing neutrophilic inflammation and corticosteroid insensitivity [Shen *et al.*, 2011, Newcomb *et al.*, 2013, Chesné *et al.*, 2014]. IL-17 concentrations and expression are increased in BAL, sputum and bronchial biopsy samples in severe patients asthma that correlate with sputum neutrophils. Monoclonal inhibitors of IL-17 are in clinical development [Miossec *et al.*, 2012]. Brodalumab is a human monoclonal antibody that binds with high affinity to human IL-17RA, blocking the biologic activity of IL-17A, -17F, -17A/F heterodimer, and IL-25. A randomized clinical trial of brodalumab in adults with inadequately controlled moderate to severe asthma receiving regular inhaled corticosteroids, but not selected for neutrophilic inflammation, reported no improvement in the primary outcome ACQ score or in lung function and symptom-free days [Busse *et al.*, 2013]. A subgroup with high bronchodilator reversibility demonstrated a borderline improvement an ACQ score. A further clinical trial of brodalumab in inadequately controlled asthma subjects with high bronchodilator reversibility was recently terminated due to a lack of observed efficacy in a pre-specified

interim analysis (ClinicalTrials.gov Identifier: NCT01902290). The results of a preliminary proof of efficacy study of the IL-17A monoclonal antibody blocker secukinumab (AIN457) in patients with uncontrolled asthma was also recently terminated. The investigators report that further investigations would require changes in study design, the use of different endpoints, a different IL-17 antibody or a different patient population (ClinicalTrials.gov Identifier: NCT01478360).

***TNF-α blockers***

Neutralizing TNFα restores corticosteroid sensitivity in a mouse model of neutrophilic airway inflammation [Dejager *et al.*, 2015 ]. Several small clinical studies in severe asthma of the soluble TNF-α receptor blocker etanercept reported beneficial effects on clinical outcomes [Howarth *et al.*, 2005, Berry *et al.*, 2006], whereas larger studies with etanercept [Holgate *et al.*, 2011] and the TNF-α receptor blocker golimumab [Wenzel *et al.*, 2009] did not confirm a consistent beneficial clinical effect. When combined with concerns over increased risk of severe infections and malignancies with TNF-α receptor blocker treatment [Wenzel *et al.*, 2009] it is unlikely that this target will be developed further for the treatment of asthma.

***Other monoclonal antibodies***

Monoclonal antibodies that block IL-1β, for example, canakinumab or block the soluble IL-1 receptor, for example, anakinra [Hernandez *et al.*, 2015] might be of benefit in neutrophilic asthma, although no clinical studies are currently registered. An IL-6 monoclonal antibody blocker toclizumab is licensed for the treatment of rheumatoid arthritis. Toclizumab could

potentially be of benefit in neutrophilic asthma although no clinical studies are registered in asthma.

## Conclusions and future developments

Non-eosinophilic airway inflammation is a term used to describe a subtype of asthma associated with normal numbers of sputum eosinophils. Up to 50% of patients with stable mild to severe never or ex-smokers with asthma have non-eosinophilic inflammation and this inflammatory phenotype is also found in smokers with asthma, some patient with a high BMI or occupational asthma. The non-eosinophilic phenotype is subdivided into neutrophilic inflammation, when neutrophil numbers are raised above a defined cut-off level or paucigranulocytic inflammation, when both eosinophil and neutrophil numbers are normal. The relative proportions of each subtype is uncertain because of variable cut-off points used to define neutrophilia. The most appropriate value that indicates that neutrophils are activated and contributing to the pathogenic processes in asthma is not certain. The severity of current symptoms are in general similar or slightly better in non-eosinophilic or neutrophilic subgroups of asthma compare to eosinophilic subgroups. Sputum eosinophilia is a better predictor of future exacerbations and a greater risk factor for more rapid decline in lung function than sputum neutrophilia. Non-eosinophilic inflammation is associated with an impaired therapeutic response to inhaled corticosteroids. Neutrophilic inflammation is associated with activation of the innate immune system in asthma and systemic inflammation. Several mechanisms either alone or in combination could explain raised sputum neutrophil counts in asthma including corticosteroids, associated chronic sinopulmonary infection, delay human neutrophil apoptosis

due to epithelial growth factor, impaired macrophage phagocytosis and altered airway microbiome. Limited information has been published on the immunopathological characteristics of non-eosinophilic inflammation compared with other inflammatory airway phenotypes including Th2-low inflammation, Th17-high inflammation or combination of Th2/Th17 profiles in asthma. Taken together, the finding suggest that non-eosinophilic inflammation and Th2-low inflammation in non-smokers with asthma share some similar immunopathological features including normal eosinophil numbers, submucosal mast cell numbers and sub-epithelial basement membrane thickness. Due to the lack of effective specific therapies targeting non-eosinophilic inflammation including neutrophilic inflammation there is currently no definitive evidence for the involvement of these inflammatory phenotypes in chronic asthma. Additional pathways may account for poor asthma control in patients with non-eosinophilic asthma including Th1 inflammation, Th17 inflammation, or a combination of Th2 and Th17 inflammation as well as corticosteroid insensitivity (Figure 2). The role of corticosteroid treatment in causing neutrophilic and Th17 inflammation in severe asthma requires further investigation. Non-inflammatory mechanisms may also be important in some individuals including airway hyperreactivity and airway remodelling.

There is an unmet need for novel treatments that will impact favourably on clinical outcomes in patients with non-eosinophilic inflammation. Non-pharmacological interventions, 'off-label' use of licensed drugs, novel small molecules and biologics agents are being investigated as possible treatments of non-eosinophilic inflammation in asthma. Smoking cessation in smokers with asthma and cessation of exposure to occupational agents are associated with a reduction in neutrophilic inflammation. Preliminary data of studies of 'off-label' use of licensed drugs suggest that macrolides show efficacy in non-smokers with non-eosinophilic asthma and

statins, low-dose theophylline and PPARγ agonist may be beneficial in asthmatic smokers with non-eosinophilic inflammation and corticosteroid insensitivity. Further clinical studies are indicated to confirm these findings and to determine the role of these therapies in the management of severe asthma. Novel small molecules targeting neutrophilic inflammation in asthma such as CXCR2 antagonists reduce neutrophil counts, but do not improve clinical outcomes. A FLAP inhibitor did not reduce neutrophils or improve symptoms. Inhaled PDE4 inhibitors and dual PDE3 and PDE4 inhibitors are potential therapies for neutrophilic asthma and a dual PDE3 and PDE4 inhibitors is under development for the treatment of asthma and COPD. Additional small molecule drugs including p38MAPK inhibitors, tyrosine kinase inhibitors and PI3kinase inhibitors are under development for asthma. The development of biological agents to target non-eosinophilic inflammation in asthma has been disappointing to date with the termination of clinical programmes of monoclonal antibodies targeting IL-17 and TNF-α. In the future, the selection of patients with severe asthma and evidence of Th17-high inflammation may be more likely to identify a subpopulation that respond to IL-17 blockers. Long-acting bronchodilators and/or bronchial thermoplasty are possible treatment options for symptomatic patients with paucigranulocytic inflammation in whom there is no evidence of activated inflammatory pathways or corticosteroid insensitivity that could be targeted by specific therapies.

Greater understanding of the mechanisms of non-eosinophilic inflammation in asthma should lead to improved therapies. International collaborative programmes of research investigating pathogenic mechanism of severe asthma have focused mainly on type 2 eosinophilic inflammation. The Unbiased Biomarkers for the Prediction of Respiratory Disease Outcome (U-BOPRED) study [Gaga *et al.*, 2015, Shaw *et al.*, 2015] and the UK Refractory Asthma

Stratification Programme (RASP-UK) [Heaney *et al.*, 2015 ] are designed to identify new phenotypes/endotypes and treatment targets and will hopefully identify new approaches to the treatment of patients with non-eosinophilic asthma.

**Declaration of Conflicting Interests**

In the last three years Professor Thomson has participated in advisory boards and/or received consultancy/lecture fees from Boston Scientific, Genentech, GlaxoSmithKline, Novartis, Respivert, Roche and Takeda and industry-sponsored grant funding to the University of Glasgow from Boston Scientific, Glaxo SmithKline and Novartis for participating in clinical trials.

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**LIST OF ABBREVIATIONS**

ACQ: Asthma control questionnaire

ACRN: Asthma Clinical Research Network

AP-1: Activator protein-1

AQLQ: asthma quality of life questionnaire

BAL: Bronchoalveolar lavage

BDP: Beclomethasone dipropionate

BMI: Body mass index

CAMP: Childhood Asthma Management Program

COPD: Chronic obstructive pulmonary disease

*CRHR1*: Corticotrophin-releasing hormone receptor 1 gene

CCL: Chemokine (C-C motif) ligand

CXCL: Chemokine (C-X-C motif) ligand

CXCR: C-X-C chemokine receptor

eNOS: Endothelial nitric oxide synthase

ERK: Extracellular signal-regulated kinase

FCER2: Low-affinity IgE receptor gene

FGF: Fibroblast growth factor

FLAP: 5-lipoxygenase-activating protein

GILZ: Glucocorticoid-inducible leucine zipper 1

G-CSF: Granulocyte-colony stimulating factor

GLCCI1: Glucocorticoid-induced transcript 1 gene

GOAL: Gaining Optimal Asthma Control

GR: glucocorticoid receptor

GRE: Glucocorticoid-responsive elements

HDAC: Histone deacetylase

HFA: Hydrofluoroalkane

HMG CoA: 3-Hydroxymethyl-3-glutaryl Coenzyme A

IDO: Indoleamine 2, 3-dioxygenase

IFN: interferon

Ig: immunoglobulin

IKKs: IκB kinases

IL: interleukin

JNK: c-Jun N-terminal kinase

LABA: Long acting $\beta_2$-agonists

LPS: lipopolysaccharide

LT: leukotriene

MAPK: Mitogen-activated protein kinase

MKP: Mitogen-activated protein kinase phosphatase 1

MMP: Matrix metalloproteinase

MPO: Myeloperoxidase

NO: nitric oxide

NFκB: Nuclear factor κB

PEF: Peak expiratory flow

PBMC: Peripheral blood mononuclear cell

PDE: Phosphodiesterase

PI3K: Phosphoinositide 3-kinase

PP2A: Protein phosphatase 2A

PPARγ: Peroxisome proliferator-activated receptor-γ

RASP-UK: Refractory Asthma Stratification Programme

RBM: Reticular basement membrane thickness

sCD40L: Soluble CD40 ligand

SHIP1: SH2-containing inositol-50-phosphatase 1

SLPI: Secretory leukocyte protease inhibitor expression

SNPs: Single-nucleotide polymorphisms

STAT: Signal transduction-activated transcription factors

SNP: Single nucleotide polymorphisms

*TBX21*: T-box expressed in T cells21

TGF-α: Transforming growth factor alpha

$Th_{2:}$ Type 2 helper T-cell ($Th_2$)

TNF-α: Tumour necrosis factor α

TLR: Toll-like receptor

U-BOPRED: Unbiased Biomarkers for the Prediction of Respiratory Disease Outcome

**Table 1 Possible factors accounting for neutrophilic airway inflammation in asthma**

- Corticosteroid treatment causing reduced apoptosis of neutrophils and contributing to Th17 mediated neutrophilic inflammation
- Neutrophila associated with chronic sinopulmonary infection and/or bronchiectasis
- Delay human neutrophil apoptosis in severe asthma due to epithelial growth factor induced release of mediators with neutrophil chemotactic and anti-apoptotic actions from bronchial epithelial cells
- Impaired macrophage phagocytosis of neutrophils
- Neutophilia associated with altered airway microbiome

**Table 2 Clinical phenotypes and factors associated with non-eosinophilic airway inflammation in asthma**

Mild to severe asthma in never or ex-smokers (both controlled and uncontrolled)

Smokers with asthma

High BMI (subgroup)

Occupational asthma (subgroup)

Factors associated with higher neutrophil counts

- Older age
- Exposure to environmental pollution
- Respiratory infections

**Table 3 Treatments targeting non-eosinophilic airway inflammation in asthma**

**Non-pharmacological interventions**

Avoidance from exposure to environmental and occupational pollutants

Smoking cessation

Dietary supplementation with vitamin D3

**'Off-label' use of licensed drugs**

Macrolides

Statins

Low-dose theophylline

Peroxisome proliferator-activated receptor-γ (PPARγ) agonists

**Novel small molecule drugs**

***Drugs targeting neutrophilic inflammation***

C-X-C chemokine receptor (CXCR)2 antagonists

5-lipoxygenase-activating protein (FLAP) inhibitors

***Phosphodiesterase (PDE) inhibitors***

$PDE_4$ inhibitors

Dual $PDE_3$ and $PDE_4$ inhibitors

***Protein kinase inhibitors***

p38 Mitogen-activated protein kinase (MAPK) inhibitors

Narrow spectrum kinase inhibitors

Tyrosine kinase inhibitors

***Phosphoinositide 3 (PI3)-kinase inhibitors***

PI3K-δ inhibitors

Dual PI3Kδ/γ inhibitors

***Biological agents***

Interleukin (IL)-17A receptor blockers

IL-17A blockers

Tumour necrosis factor (TNF)-α receptor blockers

IL-1β monoclonal antibody blockers

Soluble IL-1 receptor monoclonal antibody blockers

IL-6 monoclonal antibody blockers

## FIGURE LEGENDS

**Figure 1 Schematic diagram of potential pathways leading to non-eosinophilic inflammation and airway damage in severe asthma**

Several inflammatory pathways could potentially lead to non-eosinophilic inflammation and airway damage in asthma although the exact mechanisms are unclear. Possible pathways are briefly summarized in the schematic diagram. Stimuli such as viruses, cigarette smoke and pollutants could induce the release of chemoattractants including IL-8 to recruit neutrophils to the airways. The release of IL-17A and IL-17F from activated Th17 cells could stimulate the synthesis of neutrophil chemoattractants, such CXCL1 and IL-8 from the airway epithelium. INF-γ may also be involved in non-eosinophilic asthma, possibly in part through its release from Th1 cells. Inflammatory mediators released by neutrophils are implicated in causing mucus gland hyperplasia and hypersecretion, airway hyperreactivity and remodelling as well as corticosteroid insensitivity in asthma. Th1 and Th17 cells may induce airway hyperreactivity and/or remodelling independently of neutrophil activation. *Abbreviations:* CXCL1: chemokine (C-X-C motif) ligand; IFN: interferon; IL: interleukin; LT: leukotriene; MMP: matrix metalloproteinase; MPO: myeloperoxidase; ROC: reactive oxygen species; TNF-α: tumour necrosis factor α

**Figure 2 Targets and potential therapies for treating non-eosinophilic airway inflammation in asthma**

Non-eosinophilic airway inflammation is found in approximately 50% of patients with asthma. The proportion of this group with neutrophilic inflammation is less certain because of variable cut-off points used to define neutrophilia. The higher the cut-off value used to define sputum neutrophilia the greater the proportion of subjects that are classified as having paucigranulocytic inflammation. Pathways that may account for poor asthma control in patients with non-eosinophilic asthma including neutrophilic inflammation, associated inflammatory phenotypes (Th1-high inflammation, Th17-high inflammation, combination of Th2 and Th17 inflammation, mast cell induced inflammation, other inflammatory mechanisms) as well as corticosteroid insensitivity. Non-inflammatory mechanisms such as airway hyperreactivity and airway remodelling may be important in causing symptoms in some individuals. Potential treatments targeting specific pathways are listed in the diagram. Novel small molecules targeting neutrophilic inflammation, such as CXCR2 antagonists reduce neutrophils, but do not improve clinical outcomes. Smoking cessation in asthmatic smokers and removal from exposure to occupational agents reduces neutrophilic inflammation. The results of clinical trials of biological agents targeting mediators associated with non-eosinophilic inflammation, such as IL-17 and TNF-$\alpha$ are disappointing. Preliminary studies of 'off-label' use of licensed drugs suggest that macrolides show efficacy in non-smokers with non-eosinophilic severe asthma and statins, low-dose theophylline and PPARγ agonists may benefit asthmatic smokers with non-eosinophilic inflammation and associate corticosteroid insensitivity. Inhaled $PDE_4$ inhibitors, dual $PDE_3$ and $PDE_4$ inhibitors, p38MAPK inhibitors, tyrosine kinase inhibitors and PI3kinase inhibitors are under development and these compounds may be of benefit in

treating non-eosinophilic inflammation and corticosteroid insensitivity. Long-acting bronchodilators and/or bronchial thermoplasty are possible treatment options for symptomatic patients with paucigranulocytic inflammation in whom there is no evidence of activated inflammatory pathways or corticosteroid insensitivity that could be targeted by specific therapies.

*Abbreviations:* CXCR: C-X-C chemokine receptor; FLAP: 5-lipoxygenase-activating protein; IL: interleukin; PDE: phosphodiesterase; PI3K: phosphoinositide 3-kinase; PPARγ: peroxisome proliferator-activated receptor-γ;

# Novel approaches to the management of non-eosinophilic asthma

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## ABSTRACT

Non-eosinophilic airway inflammation occurs in approximately 50% of patients with asthma. It is subdivided into neutrophilic or paucigranulocytic inflammation, although the proportion of each subtype is uncertain because of variable cut-off points used to define neutrophilia. This article reviews the evidence for non-eosinophilic inflammation being ~~as~~ a target for therapy in asthma and assesses clinical trials of licensed drugs, novel small molecules and biologics agents in non-eosinophilic inflammation. Current symptoms, rate of exacerbations and decline in lung function are generally less in non-eosinophilic asthma than eosinophilic asthma. Non-eosinophilic inflammation is associated with corticosteroid insensitivity. Neutrophil activation in the airways and systemic inflammation is reported in neutrophilic asthma. Neutrophilia in asthma may be due to corticosteroids, associated chronic pulmonary infection, altered airway microbiome and/or delay neutrophil apoptosis. The cause of poorly controlled non-eosinophilic asthma may differ between patients and involve several mechanism including neutrophilic inflammation, Th2-low or other subtypes of airway inflammation and/or corticosteroid insensitivity as well as non-inflammatory pathways such as airway hyperreactivity and remodelling. Smoking cessation in asthmatic smokers and removal from exposure to occupational agents reduces neutrophilic inflammation. Preliminary studies of 'off-label' use of licensed drugs suggest that macrolides show efficacy in non-smokers with non-eosinophilic severe asthma and statins, low-dose theophylline and PPARγ agonists may benefit asthmatic smokers with non-eosinophilic inflammation. Novel small molecules targeting neutrophilic inflammation, such as CXCR2 antagonists reduce neutrophils, but do not improve clinical outcomes in studies to date. Inhaled $PDE_4$ inhibitors, dual $PDE_3$ and $PDE_4$ inhibitors, p38MAPK inhibitors, tyrosine kinase inhibitors and PI3kinase inhibitors are under development and these

compounds may be of benefit in non-eosinophilic inflammation. The results of clinical trials of biological agents targeting mediators associated with non-eosinophilic inflammation, such as IL-17 and TNF-$\alpha$ are disappointing. Greater understanding of the mechanisms of non-eosinophilic inflammation in asthma should lead to improved therapies.

Word count: 299

**Key words**: Airway inflammation; asthma; biological agents; biomarkers; cigarette smoking; corticosteroid insensitivity; eosinophils; neutrophils; small molecules.

## INTRODUCTION

Personalised medicine in asthma aims ~~is~~ to individualise treatment using non-invasive biomarkers that predict a beneficial response and/or that identify individuals who are at risk of adverse effects [Agustí *et al.*, 2015]. Several airway inflammatory phenotypes are recognised that help identify a therapeutic response to specific treatments in asthma. For example, eosinophilic airway inflammation, which is usually identified on the basesd ~~on the presence~~ of sputum or blood eosinophilia, predicts patients with asthma that are likely to obtain a favourable therapeutic response to corticosteroids [Pavord *et al.*, 1999, Little *et al.*, 2000, Green *et al.*, 2002, Bacci *et al.*, 2006, Berry *et al.*, 2007] and to monoclonal antibodies targeting interleukin (IL)-5 [Pavord *et al.*, 2012, Katz *et al.*, 2014, Thomson, 2014]. Type 2 helper T-cell (Th2)-high subtype of asthma is associated with increased epithelial expression of interleukin IL-4, IL-5 and IL-13 [Woodruff *et al.*, 2009, Arron *et al.*, 2013] and is considered to overlap with eosinophilic airway inflammation [Arron *et al.*, 2013]. Evidence from clinical trials suggests that the presence of Type-2 eosinophilic inflammation predicts a therapeutic response not only to corticosteroids [Woodruff *et al.*, 2009], but to monoclonal antibodies targeting specific cytokines such as IL-5 [Bel *et al.*, 2014, Ortega *et al.*, 2014] and IL-13 [Corren *et al.*, 2011]. Many patients with asthma have non-eosinophilic asthma, sometimes associated with neutrophilic inflammation and/or have a Th2-low type of inflammation. Compared to type-2 eosinophilic inflammation there are relatively few interventions available for non-type 2 inflammatory sub-groups. This article aims to discuss the evidence that non-eosinophilic airway inflammation, with or without neutrophic inflammation, is an appropriate target for therapy in asthma and also aims to assess the results of recent clinical trials of licensed drugs, novel small molecules and biologics agents in the treatment of non-eosinophilic asthma.

## IS NON-EOSINOPHILIC AIRWAY INFLAMMATION AN APPROPRIATE TARGET FOR THERAPY IN ASTHMA?

A number of factors need to be considered when attempting to answer the question of whether non-eosinophilic inflammation is an appropriate target for treatment in asthma including the criteria used to define neutrophilic and eosinophilic inflammation, the stability of non-eosinophil inflammation over time, the prevalence of non-eosinophilic inflammation, the strength of evidence for the involvement of non-eosinophilic inflammation in clinical features of asthma and the cause(s) of non-eosinophilic airway inflammation.

### Definition of eosinophilic and neutrophilic airway inflammation

Non-eosinophilic airway inflammation is a term used to describe a subtype of asthma associated with normal numbers of sputum eosinophils. The non-eosinophilic phenotype is subdivided into neutrophilic inflammation, when neutrophil numbers are raised above a defined cut-off level or paucigranulocytic inflammation, when both eosinophil and neutrophil numbers are normal. In addition, some individuals have a mixed type of inflammation, when there is sputum neutrophilia and eosinophilia. Cut-off levels used to define sputum eosinophilia most commonly used are ≥2% [Mcgrath *et al.*, 2012, Hastie *et al.*, 2013], >2% [Peters *et al.*, 2014] or ≥3% [Schleich *et al.*, 2013, Zhang *et al.*, 2014, Wagener *et al.*, 2015]. A ≥3% cut-off is reported to be the most precise value to identify eosinophilic airway inflammation [Simpson *et al.*, 2010]. Sputum eosinophil counts are associated with bronchial tissue eosinophil numbers suggesting that they provide a good indicator of airway eosinophilic pathology [Arron *et al.*, 2014]. The cut-off for a raised sputum neutrophil count is not clearly established with a wide

range of values reported in the literature: >40% (Nair, Gaga et al. 2012; Moore, Hastie et al. 2014), ≥50% (Chaudhuri, Norris et al. 2014), >61% (Simpson, Milne et al. 2009), >65% (Nair et al 2015) and ≥76% (Schleich, Manise et al. 2013). The most appropriate cut-off value that identifies individuals in whom neutrophils are activated and contributing to the pathogenic processes in asthma is not certain. In addition, sputum neutrophils do not correlate with bronchial tissue numbers bringing into doubt their predictive value for identifying neutrophil-induced airway pathology [Arron *et al.*, 2014]. In addition to the presence of non-eosinophilic inflammation, Haldar and Pavord [Haldar *et al.*, 2007] proposed that the criteria for a diagnosis of non-eosinophilic asthma should include objective evidence of airflow obstruction or airway hyperreactivity, a raised asthma control questionnaire (ACQ) score (>1.5) and the absence of a significant smoking history, fixed airflow obstruction or associated bronchiectasis. In the current article, the criteria for non-eosinophilic asthma include the presence of non-eosinophilic inflammation as defined above plus objective evidence of asthma, but the review also includes data from patients with both normal and raised ACQ scores, who have a significant smoking history or who have fixed airflow obstruction.

**Stability of sputum cell counts**

Published data on the long term stability of sputum neutrophil and eosinophil counts is conflicting. Some studies report stable sputum cell counts in patients with mild to severe asthma follow-up over 6 months [Berry *et al.*, 2007], 12 months [Green *et al.*, 2002], 2 years [Jayaram *et al.*, 2006] and 5 years [Simpson *et al.*, 2006, Van Veen *et al.*, 2009]. In contrast, sputum inflammatory cell phenotype changed in 48.6% of patients with severe asthma over 1 year among patients recruited to the BIOmarkers in Severe Chronic AIRway Disease

(BIOAIR) study [Kupczyk *et al.*, 2014]. Similar variability in sputum cell counts has been reported by others [Hancox *et al.*, 2012] and in one study a stable inflammatory phenotype was found in only one third of patients [Al-Samri *et al.*, 2010]. Transient sputum eosinophilia is reported in up to 40% patients with non-eosinophilic inflammation [Bacci *et al.*, 2012, Mcgrath *et al.*, 2012]. The potential for the lack of stability in non-eosinophilic inflammation over time needs to be accounted for in intervention studies targeting sputum inflammatory cell biomarkers.

**Prevalence of non-eosinophilic airway inflammation**

The different cut-off values used to define elevated sputum cell counts, particularly sputum neutrophils, may explain the variation in prevalence figures for non-eosinophilic inflammation between studies. Nevertheless, overall up to 50% of adults and adolescents ~~patients~~ with stable mild to severe asthma, and in some studies higher proportions, have non-eosinophilic inflammation [Gibson *et al.*, 2001, Green *et al.*, 2002, Simpson *et al.*, 2006, Wang *et al.*, 2011, Mcgrath *et al.*, 2012, Schleich *et al.*, 2013, Moore *et al.*, 2014, Brooks *et al.*, 2016 ]. For example, a review of sputum cytology data from 995 subjects with mild to moderate asthma enrolled in clinical trials undertaken by the Asthma Clinical Research Network (ACRN) reported that non-eosinophilic inflammation (sputum eosinophils <2%) was present in 64% of patients not taking inhaled corticosteroid and 83% of patients taking inhaled corticosteroids. In a sub-group of patients followed up for 6 months, 47% of the inhaled corticosteroid free patients and 72% of those taking inhaled corticosteroids had persistent non-eosinophilic inflammation [Mcgrath *et al.*, 2012]. In a cluster analysis performed on 423 patients recruited to the Severe Asthma Research Program (SARP)

cohort, four asthma inflammatory sub-phenotypes were identified (sputum eosinophilia ≥2%; sputum neutrophilia >40%) [Moore *et al.*, 2014]. Two groups had mild-to-moderate allergic asthma with minimal or eosinophil-predominant sputum inflammation whereas the other two sub-phenotypes had moderate-to-severe asthma with neutrophil-predominant or mixed granulocytic inflammation [Moore *et al.*, 2014]. A study in a small group of adults with stable asthma found 51.7% of subjects had a paucigranulocytic phenotype, 27.6% neutrophilic inflammation and 17.2% eosinophilic inflammation [Wang *et al.*, 2011].

**Involvement of neutrophilic and non-eosinophilic airway inflammation in asthma**

Evidence for the involvement of non-eosinophilic inflammation in asthma is based mainly on studies examining the association between sputum inflammatory phenotypes and clinical outcomes in asthma including current symptom control, exacerbations, airflow obstruction and therapeutic response to corticosteroids. Further evidence is provided by reports of local activation of neutrophils and systemic inflammation in neutrophilic asthma.

***Current symptom control***

The severity of current symptoms is~~are~~ in general similar or slightly lower~~better~~ in non-eosinophilic or neutrophilic subgroups of asthma compared to eosinophilic subgroups [Cowan *et al.*, 2010, Hastie *et al.*, 2010, Wood *et al.*, 2012, Schleich *et al.*, 2013, Baines *et al.*, 2014, Newby *et al.*, 2014, Schleich *et al.*, 2014].

***Exacerbations***

Sputum neutrophilia is found in up to 80% of exacerbations in adults with asthma [Turner *et al.*, 1995, Fahy *et al.*, 1995 , Lamblin *et al.*, 1998, Green *et al.*, 2002, Jayaram *et al.*, 2006, Maneechotesuwan *et al.*, 2007, Wang *et al.*, 2011], although the predominant sputum cell type can alter during successive exacerbations [D'silva *et al.*, 2007]. Sputum eosinophilia is a better predictor of future exacerbations than sputum neutrophilia [Jatakanon *et al.*, 2000, Leuppi *et al.*, 2001, Kupczyk *et al.*, 2014, Schleich *et al.*, 2014]. For example, a cluster analysis performed on patients recruited to the BIOAIR study identified two clusters with raised sputum eosinophil counts that accounted for 83% of subjects who had 2 or more severe exacerbations during follow-up for 1 year [Kupczyk *et al.*, 2014]. One of these clusters had a mixed inflammatory profile with raised sputum neutrophils (43% percent of patients). A further cluster had a raised neutrophil count and a normal eosinophil count (11% of patients) and a non-eosinophilic paucigranulocytic inflammation was found in only 6% of cases. Patients with severe asthma associated with eosinophil inflammation have more intubations than non-eosinophilic patients [Wenzel *et al.*, 1999].

***Airflow obstruction***

Sputum neutrophilia is association with reduced lung function and based on this finding it has been speculated that airway neutrophils may contribute to the development of persistent airflow obstruction in asthma [Little *et al.*, 2002, Shaw *et al.*, 2007]. Against this hypothesis, a recent cluster analysis of lung function decline and sputum eosinophil count performed in 97 patients with severe asthma identified a non-eosinophilic group in whom the decline in $FEV_1$ was -14 ml per year compared to an eosinophilic group with highly variable eosinophil counts

that had a greater rate of decline in $FEV_1$ of -41 ml per year [Newby *et al.*, 2014]. These findings suggest that eosinophilic inflammation, particularly when there is high variability in eosinophil count, is a greater risk factor for the development of persistent airflow obstruction than non-eosinophilic inflammation. Bronchodilator reversibility and airway hyperresponsiveness are similar in eosinophilic and non-eosinophilic asthma [Berry *et al.*, 2007, Mcgrath *et al.*, 2012], although one study noted greater airway hyperresponsiveness in persistent or intermittent eosinophilic groups [Mcgrath *et al.*, 2012].

***Impaired response to inhaled corticosteroids***

Non-eosinophilic inflammation is associated with an impaired therapeutic response to inhaled corticosteroids [Pavord *et al.*, 1999, Green *et al.*, 2002, Bacci *et al.*, 2006, Berry *et al.*, 2007, Thomson *et al.*, 2009, Mcgrath *et al.*, 2012], although the lack of efficacy may not be complete. Several clinical studies performed in small numbers of patients with non-eosinophilic asthma suggest that this group may obtain some benefit from inhaled corticosteroids although less than that found in eosinophilic patients [Godon *et al.*, 2002, Cowan *et al.*, 2010, Lemière *et al.*, 2011]. Intermittent eosinophilia might be a factor accounting for corticosteroid sensitivity in some of these patients [Bacci *et al.*, 2012, Mcgrath *et al.*, 2012, Suárez-Cuartín *et al.*, 2015].

***Evidence for neutrophil activation in asthma***

There is evidence to suggest that the innate immune system is activated in chronic asthma. Firstly, sputum IL-8 and neutrophil elastase concentrations and innate immune receptors

TLR2, TLR4 and CD14 as well as pro-inflammatory IL-8 and IL1-β gene expression levels are increased in neutrophilic asthma compared to non-neutrophilic asthma [Simpson *et al.*, 2007, Wood *et al.*, 2012]. Secondly, neutrophil activation, as measured by sputum myeloperoxidase (MPO) levels, is positively associated with sputum neutrophil numbers in asthma [Little *et al.*, 2002]. Thirdly, specific sputum gene expression signatures are reported to discriminate eosinophilic asthma from non-eosinophilic asthma as well as to predict a beneficial response to inhaled corticosteroids [Baines *et al.*, 2014]. In this study, non-eosinophilc asthma was identified by increased sputum cell expression of IL1β , alkaline phosphatase, tissuenonspecific isozyme (ALPL ) and CXCR2, whereas eosinophilic asthma was characterised by increased expression of Charcot-Leydon crystal protein or galectin-10 (CLC), carboxypeptidase A3 (CPA3) and deoxyribonuclease I-like 3 (DNASE1L3). The NLRP3 inflammasome is upregulated in neutrophilic asthma and may increase the production of IL-1β [Simpson *et al.*, 2014, Kim *et al.*, 2015]. Anti-inflammatory responses may be impaired in non-eosinophilic asthma based on reduced sputum galectin-3 concentrations, which increases uptake of apoptotic neutrophils and reduced IL-1RA/IL-1ß ratio, and which might increase pro-inflammatory actions of IL-1ß [Gao *et al.*, 2015]. In addition, soluble receptor for advanced glycation end-products (RAGE), which is a pattern-recognition receptor is deficient in BAL samples in neutrophilic asthma [Sukkar *et al.*, 2012]. The T-cell granzyme B pathway, which is though to mediate apoptosis of epithelial cells, might be defective in non-eosinophilic asthma, based on the finding of a higher ratio of the expression of granzyme B to its inhibitor in T cells in this group compared to eosinophilic asthma [Simpson *et al.*, 2014].

Systemic inflammation is increased in patients with neutrophilic airway inflammation. The proportion of patients with elevated CRP, IL-6 and neutrophil elastase concentrations is higher in neutrophilic asthma compared to a non-neutrophic group [Baines *et al.*, 2011, Wood *et al.*, 2012]. Neutrophilic inflammation is associated with increased α-defensin and neutrophil protease gene expression in blood [Baines *et al.*, 2011]. In non-eosinophilic asthma, blood neutrophils released significantly higher levels of IL-8 at rest [Baines *et al.*, 2010]. In a small study, gene expression markers of systemic inflammation were associated with higher BMI, greater history of cigarette smoking, lower FVC% predicted, and increased sputum neutrophils [Fu *et al.*, 2013].

## Potential inflammatory processes leading to non-eosinophilic airway inflammation

Several inflammatory processes could lead to non-inflammation and airway damage in asthma although the exact immunological mechanisms are unclear (Figure 1) [Trejo Bittar *et al.*, 2015]. Uncertainty in the clinical relevance of experimental animal models of non-eosinophilic inflammation has hampered progress in understanding the involvement of neutrophils and non-eosinophilic inflammation in the pathogenesis of asthma. Stimuli such as viruses, cigarette smoke and pollutants could induce the release of chemoattractants including IL-8 to recruit neutrophils to the airways. In experimental asthma models, the release of IL-17A and IL-17F from activated Th17 cells stimulates the synthesis of neutrophil chemoattractants including CXCL1 and IL-8 from the airway epithelium. [Newcomb *et al.*, 2013]. INF-γ may also be involved in the pathogenesis of-severe asthma associated with neutrophilic and eosinophilic inflammation, possibly in part through the release of IFN-γ from Th1 cells [Raundhal *et al.*, 2015 ]. Data from patients with severe

asthma and an experimental murine asthma model implicate high INF-γ immune responses and low secretory leukocyte protease inhibitor expression (SLPI) in airway epithelial cells with airway hyperrespponsiveness [Raundhal *et al.*, 2015 ]. Neutrophils are a potential source of oxygen free radicals and enzymes and their ability to activate other airway cell types [Futosi *et al.*, 2013]. Neutrophils in asthma are implicated in causing mucus gland hyperplasia and hypersecretion, airway hyperreactivity and remodelling as well as corticosteroid insensitivity. Interestingly, $Th_1$ and $Th_{17}$ cells may induce airway hyperreactivity and/or remodelling independently of neutrophil activation.

Limited information has been published on the immunopathological characteristics of non-eosinophilic inflammation in asthma compared with other inflammatory airway phenotypes including $Th_2$-low inflammation, $Th_{17}$-high inflammation or combination of $Th_2$/$Th_{17}$ profiles. Bronchial biopsy studies of patients with non-eosinophilic asthma and with $Th_2$-low inflammation report reduced sub-mucosal eosinophil numbers and normal sub-epithelial basement membrane thickness in both groups [Wenzel *et al.*, 1999, Berry *et al.*, 2007, Woodruff *et al.*, 2009]. In contrast, bronchial eosinophils numbers and sub-epithelial basement membrane thickness are both increased in eosinophilic asthma and in $Th_2$-high asthma [Wenzel *et al.*, 1999, Berry *et al.*, 2007, Woodruff *et al.*, 2009]. Mast cell numbers are increased in eosinophilic asthma [Wenzel *et al.*, 1999] and $Th_2$-high asthma [Dougherty *et al.*, 2010], whereas mast cells numbers are normal in the sub-mucosal of patients with severe non-eosinophilic asthma [Wenzel *et al.*, 1999] and in the epithelium of non-smoker with $Th_2$-low asthma [Dougherty *et al.*, 2010]. Mast cell numbers in airway smooth muscle are increased in both non-eosinophilic and eosinophilic asthma [Berry *et al.*, 2007]. Bronchial biopsy neutrophil numbers are increased to a similar degree in non-eosinophilic severe asthma and eosinophilic

severe asthma [Wenzel *et al.*, 1999]. Neutrophil numbers in $Th_2$-low asthma have not been reported. A lower proportion of subjects with non-eosinophilic asthma are atopic compared to eosinophilic asthma (18% versus 66%) [Berry *et al.*, 2007] and (58% versus 83%) [Gibson *et al.*, 2001]. Severe asthma associated with neutrophilia has significantly higher sputum levels of $Th_{17}$-related cytokines (CXCL1, CXCL10 , CCL2, IL-6, and IL-8) compared with severe asthmatics with other inflammatory phenotypes [Manni *et al.*, 2014]. The proportion of $Th_{17}$ lymphocytes and the ratio of $Th_{17}$ to regulatory T cells (Treg) in the peripheral blood is greater in patients with non-eosinophilic asthma taking inhaled corticosteroids compared to an eosinophilic asthma group [Furukawa *et al.*, 2015]. Approximately one third of patients with severe eosinophilic asthma have a Th17-high signature that is associated with a Th2-low gene expression profile [Choy *et al.*, 2015]. The number of subjects with non-eosinophilic severe asthma in this study was not sufficient to determine their Th17 profile [Choy *et al.*, 2015]. Taken together, these finding suggest that non-eosinophilic inflammation and $Th_2$-low inflammation in non-smokers with asthma share some similar immunopathological features including normal eosinophil numbers, submucosal mast cell numbers and sub-epithelial basement membrane thickness. There is a need for further studies to establish the similarities and differences in endotypes of non -eosinophilic, $Th_2$-low and $Th_{17}$ high inflammation to help identify sub-groups of patients for targeted therapies.

## Factors accounting for neutrophilic airway inflammation in asthma

Several factors ~~mechanisms~~ either alone or in combination could explain raised sputum neutrophil counts in asthma (Table 1). Firstly, corticosteroids inhibit apoptosis of neutrophils

[Cox, 1995] and their use in asthma may contribute to sputum neutrophilia [Saffar *et al.*, 2011]. In addition, Th2-targeted therapies, including oral corticosteroids may contribute to the development of Th17-high neutrophilic inflammation [Choy *et al.*, 2015, Shum, 2015]. In support of corticosteroids causing neutrophilia in asthma ~~this suggestion~~, inhaled corticosteroid withdrawal from patients with moderate asthma resulted in only one subject with neutrophilic inflammation although the reintroduction of inhaled fluticasone for 4 weeks resulted in a raised neutrophil count in only 5% of subjects [Cowan *et al.*, 2010]. In one study of patients with severe oral corticosteroid dependent asthma associated with increased sputum neutrophil number, markers of neutrophil activation including oxidative burst and surface granular receptor expression were similar to patients with mild asthma [Nair *et al.*, 2015]. In the SARP cohort, however corticosteroid use was not associated with sputum neutrophilia, suggesting that continuous corticosteroid exposure may not be the only influence on sputum neutrophil numbers in severe asthma [Moore *et al.*, 2014]. Secondly, co-morbid conditions such bronchiectasis or severe airflow obstructions occurring in association with asthma may result in neutrophilic inflammation. Thirdly, delayed human neutrophil apoptosis has been reported in severe asthma [Uddin *et al.*, 2010], possibly due to epithelial growth factor induced release of mediators with neutrophil chemotactic and anti-apoptotic actions from bronchial epithelial cells [Uddin *et al.*, 2010, Uddin *et al.*, 2013]. Fourthly, macrophage efferocytosis is impaired in non-eosinophilic asthma, which may cause airway neutrophilia [Simpson *et al.*, 2013]. Fifthly, altered airway microbiome has been implicated in airway neutrophilia. Airway colonisation determined by terminal restriction fragment length polymorphism (T-RFLP) analysis is associated with more severe airways obstruction and longer duration of disease as well as neutrophilic airway inflammation and raised sputum IL-8 levels [Simpson *et al.*, 2013, Green *et al.*, 2014]. Taken together, these finding suggest that the cause of airway neutrophilia in

asthma is likely to be complex, possibly due to corticosteroid treatment inducing impaired apoptosis of neutrophils and Th17 mediated neutrophilic inflammation, delayed apoptosis of neutrophils due to epithelial growth factor release and ineffective macrophage efferocytosis of neutrophils as well as an altered airway microbiome.

**Clinical phenotypes associated with non-eosinophilic inflammation**

Non-eosinophilic inflammation, either paucigranulocytic or neutrophilic occurs in a range of clinical phenotypes that account for approximately 50% of adults never or ex-smokers with mild to severe asthma or that have controlled or uncontrolled asthma (Table 2). Non-eosinophilic inflammation, with or without neutrophilic inflammation is commonly found in smokers with asthma [Chalmers *et al.*, 2002, Boulet *et al.*, 2006, Thomson *et al.*, 2013]. A high BMI is associated with non-eosinophilic asthma in some people [Haldar *et al.*, 2008], although others have submucosal eosinophilia [Desai *et al.*, 2013] . Approximately two thirds of cases of occupational asthma due to low molecular weight agents have non-eosinophilic inflammation [Anees *et al.*, 2002], which is associated with a poor asthma prognosis [Lemiere *et al.*, 2014]. Non-occupational-induced asthma that is exacerbated by work exposures is associated with non-eosinophilic phenotype [Lemière *et al.*, 2013]. Additional factors associated with higher neutrophil counts include older age [Brooks *et al.*, 2013], exposure to environmental pollution through living close to car pollution [Wallace *et al.*, 2011], exposure to occupational particulate matter [Simpson *et al.*, 2015] and respiratory infections.

**Biomarkers that can identify non-eosinophilic airway inflammation**

Are there biomarkers that can identify patients with non-eosinophilic airway inflammation? Blood eosinophil numbers are moderately associated with sputum eosinophils [Schleich *et al.*, 2013, Zhang *et al.*, 2014, Wagener *et al.*, 2015]. Using a cut-off for a blood eosinophil count of >0.22 x $10^9$/L [3] [Schleich *et al.*, 2013], >0.26 x $10^9$/L [Zhang *et al.*, 2014] or ≥0.27 x $10^9$/L [Wagener *et al.*, 2015] accurately predicts sputum eosinophilia. In contrast, another study reported that blood eosinophils had a poor predictive value of 47% for sputum eosinophilia (≥3% cut-off) although this was better in severe asthma (71%) [Hastie *et al.*, 2013]. In patients with mild to severe asthma, blood eosinophils were reported to be better than serum periostin and exhaled nitric oxide in identifying sputum eosinophilia [Wagener *et al.*, 2015]. Blood neutrophil numbers has a weak relationship with sputum neutrophil count [Schleich *et al.*, 2013, Zhang *et al.*, 2014] and they have a poor predictive value for sputum neutrophilia (64% or 38% for a cut-off of ≥40% or ≥61% cut-off respectively) [Hastie *et al.*, 2013]. In one study exhaled nitric oxide predicted inhaled corticosteroid response for airway hyperreactivity in non-eosinophilic asthma (area under the curve 0.81), with an optimum cut-off point of 33 ppb [Cowan *et al.*, 2010].

**Which inflammatory phenotype to target?**

In summary, non-eosinophilic airway inflammation is found in approximately 50% of patients with mild to severe asthma. The proportion of this group with neutrophilic inflammation is less certain because of variable cut-off points used in different studies to define neutrophilia. Current symptoms, rate of exacerbations and rate of decline in lung function are generally less severe in non-eosinophilic asthma compared to eosinophilic asthma. Non-eosinophilic inflammation is associated with an impaired response to inhaled corticosteroids. There is some

evidence that neutrophils are activated in the airways of patients with neutrophilic asthma and that biomarkers of systemic inflammation is increased in this group. Neutrophilia in asthma may be due to corticosteroids, associated chronic pulmonary infection, altered airway microbiome and/or delay neutrophil apoptosis, particularly in severe disease. Non-eosinophilic asthma and Th2-low asthma may share some common immunopathological features, but further investigation is required. Due to the lack of effective specific therapies targeting non-eosinophilic inflammation including neutrophilic inflammation there is currently no definitive evidence for the involvement of these inflammatory phenotypes in chronic asthma. Additional pathways may account for poor asthma control in patients with non-eosinophilic asthma including Th1 inflammation, Th17 inflammation, or a combination of Th2 and Th17 inflammation as well as corticosteroid insensitivity (Figure 1). Recent work suggests a reciprocal relationship between Th2 and Th17 pathways in severe disease and that corticosteroid treatment may contribute to the emergence of a Th17-high profile [Choy *et al.*, 2015]. Non-inflammatory mechanisms may also be important in some individuals including airway hyperreactivity and airway remodelling.

**TREATMENTS TARGETING NON-EOSINOPHILIC AIRWAY INFLAMMATION**

Many patients with asthma continue to have poorly controlled disease despite treatment with currently available therapies. There is an unmet need for novel treatments that will impact favourably on clinical outcomes in patients with non-eosinophilic inflammation. Non-pharmacological interventions, 'off-label' use of licensed drugs, novel small molecules and biologics agents are being investigated as possible treatments of non-eosinophilic inflammation in asthma (Table 3 and Figure 2).

**Non-pharmacological interventions**

Avoidance from exposure to environmental and occupational pollutants may reduce neutrophilic inflammation in asthma. In a study of smokers with asthma of whom a subgroup quit smoking for 6 weeks the proportion of sputum neutrophils reduced, corticosteroid sensitivity improved and the $FEV_1$ increased compared to those who continued to smoke [Chaudhuri *et al.*, 2006]. After cessation of exposure to occupational agents, neutrophilic inflammation reduced in people in whom their asthma was cured or improved compared to those in whom there was no improvement [Maghni *et al.*, 2004]. Several clinical trials have examined the effect of dietary supplement of vitamin D in asthma, based on the anti-inflammatory and corticosteroid-enhancing actions of vitamin D [Nanzer *et al.*, 2013, Zhang *et al.*, 2014] [Xystrakis *et al.*, 2006]. Two large randomized clinical trials of vitamin D3 supplementation in patients with asthma and vitamin D insufficiency [VIDA and ViDiAs trials), although not selected for specific airway inflammatory cell profiles or corticosteroid insensitivity, reported no improvements in clinical outcomes [Castro *et al.*, 2014, Martineau *et al.*, 2015, Denlinger *et al.*, 2015 ]. Interestingly, vitamin D supplementation reduces eosinophilic inflammation in patients with non-atopic asthma, suggesting that certain inflammatory phenotypes might benefit from vitamin D3 supplementation [De Groot *et al.*, 2015].

**'Off-label' use of licensed drugs**

Several drugs licensed for the treatment of medical conditions other than asthma have been investigated for their efficacy in asthma, including patients with non-eosinophilic inflammation.

Candidate drugs have been chosen usually because of pre-clinical evidence of anti-inflammatory effects that might be relevant to treatment of asthma. Some examples are reviewed below.

***Macrolides***

Macrolides may be of benefit in the treatment of chronic asthma [Reiter *et al.*, 2013], including non-eosinophilic asthma [Simpson *et al.*, 2008], although prescribing macrolides as a long-term treatment increases the risk of adverse drug effects and the development of microbial resistance [Cameron *et al.*, 2012]. The mechanism(s) of action of macrolides in the treatment of airway diseases is not known, but could be due to antibacterial and/or anti-inflammatory actions, which include inhibition of NF-κB and other transcription factors as well as reduction in neutrophil migration and/or function [Culic *et al.*, 2001, Fujitani *et al.*, 2003, Simpson *et al.*, 2008, Cameron *et al.*, 2012, Kobayashi *et al.*, 2013]. Macrolides have additional potentially beneficial properties including anti-viral actions [Gielen *et al.*, 2010, Schögler *et al.*, 2015] and an ability to restore corticosteroid sensitivity by inhibiting the phosphoinositide 3-kinase (PI3K) pathway and restoring histone deacetylase (HDAC)2 activity [Spahn *et al.*, 2001, Charron *et al.*, 2007, Kobayashi *et al.*, 2013, Hao *et al.*, 2015] and by attenuating TNFα and IL-17 immune responses [Essilfie *et al.*, 2015]. Two recent exploratory clinical trials have investigated the effects of macrolides in non-eosinophilic asthma. In one trial, smokers with mild to moderate asthma associated with non-eosinophilic inflammation were randomized to azithromycin 250 mg per day or placebo [Cameron *et al.*, 2013]. After 12 weeks, treatment with azithromycin was not associated with improvements in morning PEF, ACQ score, AQLQ score and methacholine $PC_{20}$ compared to placebo and did not alter induced sputum differential counts,

bacterial load, *C. pneumonia, M. pneumoniae* seropositivity or upper airways respiratory virus prevalence. In an other randomized controlled trial patients with exacerbation-prone severe asthma received low-dose azithromycin or placebo as add-on treatment to combination therapy of inhaled corticosteroids and long-acting $\beta_2$ agonists for 6 months [Brusselle *et al.*, 2013]. The rate of severe exacerbations and lower respiratory tract infections requiring treatment with antibiotics was not reduced by azithromycin. In a predefined subgroup with non-eosinophilic severe asthma (blood eosinophilia ≤200/µl) there was a reduction in the rate of primary endpoints in azithromycin-treated patients [Brusselle *et al.*, 2013]. Azithromycin improved AQLQ scores, but did change ACQ scores or lung function. Based on these findings, further clinical trials of macrolides in non-eosinophilic severe asthma are indicated. Novel analogues of macrolides have been developed that have enhanced anti-inflammatory properties than current macrolides, such as solithromycin (CEM-101) [Kobayashi *et al.*, 2013, Kobayashi *et al.*, 2013] or that lack anti-bacterial properties, such as the non-antibiotic azithromycin derivative CSY0073 [Balloy *et al.*, 2014].

***Statins***

Statins have pleiotropic immunomodulatory actions [Greenwood *et al.*, 2006] that may of value in the treatment of chronic inflammatory diseases [Greenwood *et al.*, 2006, Hothersall *et al.*, 2006, Yeganeh *et al.*, 2014]. In experimental models of allergic asthma [Mckay *et al.*, 2004, Zeki *et al.*, 2009] and tobacco-smoke-induced lung inflammation [Lee *et al.*, 2005, Davis *et al.*, 2013] statins reduce inflammatory pathways potentially relevant to the pathogenesis of asthma and smoke-induced airway diseases. Statins might also restore corticosteroid sensitivity in asthma [Samson *et al.*, 2006, Maneechotesuwan *et al.*, 2010].

Taken together, these findings suggest that statin treatment may have anti-inflammatory effects in people with asthma including smokers with asthma. A randomized double-blind parallel group trial undertaken in seventy one smokers with mild to moderate asthma associated with non-eosinophilic inflammation compared treatment with atorvastatin 40 mg per day with placebo. After 4 weeks treatment inhaled beclometasone 400 µg per day was added to both treatment arms for a further 4 weeks [Braganza *et al.*, 2011]. At 4 weeks, there was an improvement in ACQ and AQLQ scores with atorvastatin, but not in lung function. There was no significant improvement with atorvastatin and inhaled beclometasone compared to inhaled beclometasone alone in clinical outcome measures at 8 weeks. In a follow-up study the effects of atorvastatin alone and in combination with inhaled corticosteroid was investigated on their ability to suppress the concentration of a range of cytokines, chemokines and growth factors in sputum samples collected during the previous clinical trial [Braganza *et al.*, 2011, Thomson *et al.*, 2015 ]. Sputum mediator concentrations were not reduced by inhaled beclometasone alone. Atorvastatin significantly reduced sputum concentrations of CCL7, IL-12p70, sCD40L, FGF-2, CCL4, TGF-α and MMP-8 compared with placebo and, when combined with inhaled beclometasone, reduced sputum concentrations of MMP-8, IL-1β, IL-10, MMP-9, sCD40L, FGF-2, IL-7, G-CSF and CCL7 compared to ICS alone. Improvements in ACQ and/or AQLQ scores with atorvastatin and inhaled beclometasone were associated with decreases in G-CSF, IL-7, CCL2 and CXCL8. Interestingly, simvastatin suppresses airway IL-17 and upregulated IL-10 in patients with stable COPD [Maneechotesuwan *et al.*, 2013]. Taken together, these findings suggest that short-term treatment with atorvastatin alone or in combination with inhaled beclometasone reduces several sputum cytokines, chemokines and growth factors concentrations unresponsive to inhaled corticosteroids alone in asthmatic smokers with

non-eosinophilic inflammation. There is a need for long-term clinical studies examining effect of statins on exacerbations and airway remodelling in chronic non-eosinophilic asthma.

***Low-dose theophylline***

Low dose theophylline has been shown to restore corticosteroid sensitivity *in-vitro* possibly by increasing HDAC-2 activity, which is suppressed in severe asthma and in smokers with asthma and a similar clinical effect might occur in people with severe disease or who are smokers [Barnes, 2009, To *et al.*, 2010]. Theophylline inhibits oxidative stress dependent PI3K-δ activation and restores corticosteroid sensitivity in PBMCs from patients with COPD [To *et al.*, 2010]. An exploratory clinical trial examined the effects of low dose theophylline added to inhaled beclometasone compared to inhaled beclometasone alone in smokers with asthma associated with non-eosinophilic inflammation [Spears *et al.*, 2009]. The addition of low dose theophylline to inhaled beclometasone, at a dose titrated to provide a 'sub-therapeutic' concentration, resulted in increased efficacy as measured by lung function and suggested the restoration of corticosteroid sensitivity in those treated with the combination. Clinical trials to date have not investigated the therapeutic effects of adding low dose theophylline to patients with severe asthma. A fixed combination of ultra-low dose of theophylline with fluticasone, SKP-2075 (Skepharma), in a dry powder inhaler is under development for the treatment COPD. This combination would potentially be of benefit in the treatment of severe asthma and smokers with asthma, possibly in those people with non-eosinophilic inflammation.

***PPARγ agonist***

In pre-clinical studies peroxisome proliferator-activated receptor-γ (PPARγ) agonists exert anti-inflammatory effects potentially relevant to the treatment of inflammatory airway diseases including asthma and COPD [Spears *et al.*, 2006, Belvisi *et al.*, 2008, Seidel *et al.*, 2012, Stephen *et al.*, 2013, Bourke *et al.*, 2014, Lakshmi *et al.*, 2014, Lea *et al.*, 2014, Donovan *et al.*, 2015 ]. For example, PPARγ agonists reduce eosinophilic and neutrophilic lung infiltration in experimental animal models exposed to allergen or tobacco smoke [Bauer *et al.*, 2010, Lea *et al.*, 2014, Zhao *et al.*, 2014, Morissette *et al.*, 2015]. The oral PPARγ agonist rosiglitazone had a modest effect in attenuating the allergen-induced late asthmatic response [Richards *et al.*, 2010]. A further proof of concept study reported that rosiglitazone compared with inhaled beclometasone dipropionate resulted in improvement in lung function and a borderline reduction in sputum IL-8 concentration in smokers with mild to moderate asthma that was associated with non-eosinophilic inflammation [Spears *et al.*, 2009]. The oral PPARγ agonist pioglitazone is not effective in obese asthmatics [Dixon *et al.*, 2015]. Inhaled PPARγ agonist analogues, such as AD3277 (Pulmagen) are under development for the treatment of chronic inflammatory airway diseases and potentially might be of benefit in non-eosinophilic asthma.

**Novel small molecule drugs**

Novel small molecule inhibitors have been developed for treating neutrophilic/non-eosinophilic asthma including CXCR2 antagonists, FLAP inhibitors, $PDE_4$ inhibitors, dual $PDE_3$ and $PDE_4$ inhibitor and various protein kinase inhibitors.

***CXCR2 antagonist***

CXCR2 receptors are expressed on neutrophils as well as on airway goblet cells, fibroblasts and airway smooth muscle [Chapman *et al.*, 2009]. Ligands for the CXCR2 receptor include the chemokines CXCL8 (IL-8), growth-related protein (Gro)-α, -β, and -γ (CXCL1–3), epithelial-derived neutrophil attractant-78 (ENA-78; CXCL5), granulocyte chemotactic protein-2 (GCP-2; CXCL6) and neutrophil- activating peptide-2 (NAP-2; CXCL7) [Chapman *et al.*, 2009, Campbell *et al.*, 2013]. Activation of CXCR2 receptors result in neutrophil chemotaxis, proteases production, aairway goblet cell hyperplasia, pulmonary blood vessel angiogenesis, collagen deposition and airway smooth muscle contraction and migration [Chapman *et al.*, 2009]. The effects of CXCR2 antagonists have been studied on airway challenges that induce sputum neutrophilia. The CXCR2 antagonist, AZD8309 inhibits LPS-induced airway neutrophilic inflammation in healthy volunteers [Leaker *et al.*, 2013] and the CXCR2 antagonist, SB656933 inhibited *ex vivo* neutrophil activation and ozone-induced airway inflammation in humans [Lazaar *et al.*, 2011]. The CXCR2 antagonist, SCH527123 inhibits ozone-induced neutrophilia in healthy subjects [Holz *et al.*, 2010]. A randomized, placebo-controlled clinical trial of the CXCR2 antagonist SCH527123 administered for 4 weeks to patients with severe asthma and sputum neutrophils > 40% resulted in a reduction of 36.3% in sputum neutrophil percentage, fewer mild exacerbations and a trend towards improvement in ACQ score [Nair *et al.*, 2012]. A clinical trial of the efficacy and safety of a CXCR2 antagonist AZD5069 in severe, uncontrolled persistent asthma reported that the addition of AZD5069 to combination ICS/LABA treatment did not improve clinical outcomes despite a dose-dependent reduction in blood neutrophil counts [O'byrne *et al.*, 2015]. A lack

of improvement in clinical outcome despite a reduction in sputum neutrophil counts was reported with the CXCR2 antagonist AZD5069 in bronchiectasis [De Soyza *et al.*, 2015]. A recent trial of the CXCR2 antagonist Navarixin (SCH527123) in COPD led to significant improvements in $FEV_1$ and reduction in sputum neutrophil count, particularly in current smokers with COPD [Rennard *et al.*, 2015]. The CXCR2 antagonist AZD8309 administered for 4 weeks to patients with moderate to severe COPD was well tolerated with no increase in the rate of infections [Kirsten *et al.*, 2015]. A small-molecule oral CXCR2 antagonists Danirixin (GSK1325756) is undergoing a clinical trial in patients with COPD at risk of exacerbations (ClinicalTrials.gov Identifier: NCT02130193). Oral CXCR2 antagonists could potentially cause neutropenia, and the therapeutic index of these compounds requires careful assessment.

***FLAP inhibitors***

Pro-inflammatory cysteinyl leukotrienes (LTs) are synthesised from arachidonic acid in inflammatory cells by 5-lipoxygenase (LO) and 5-lipoxygenase activating protein (FLAP). FLAP inhibitors such as GSK-2190915 [Evans *et al.*, 2008] prevent the formation of $LTB_4$, which may be of value in the treatment of neutrophilic asthma. GSK2190915 markedly inhibited *ex vivo* calcium ionophore stimulated blood $LTB_4$ formation and urinary leukotriene $E_4$ ($LTE_4$) formation [Bain *et al.*, 2013]. Pre-treatment with GSK2190915 reduces the early and late phase response to allergen challenge and results in a significant reduction of sputum $LTB_4$ levels [Kent *et al.*, 2013]. Despite suppressing the target mediator $LTB_4$, the FLAP inhibitor GSK2190915 has no short-term effect on sputum cell counts or clinical endpoints in smokers and non-smokers with asthma associated with neutrophilic

inflammation (sputum neutrophilia ≥ 50% for one sample and >45% for the other), suggesting that $LTB_4$ suppression alone is inadequate in controlling airway neutrophils in asthma [Chaudhuri *et al.*, 2014]. No active clinical trials of FLAP inhibitors in asthma are currently registered on ClinicalTrials.gov.

***$PDE_4$ inhibitors and dual $PDE_3$ and $PDE_4$ inhibitors***

Phosphodiesterase $(PDE)_4$ inhibitors have immunomodulatory effects on inflammatory cells potentially relevant to the treatment of asthma [Lipworth, 2005, Page *et al.*, 2012, Kim *et al.*, 2015 ]. In an allergen challenge study the oral $PDE_4$ inhibitor roflumilast attenuated the rise in sputum eosinophils and neutrophils numbers after the late asthmatic response [Gauvreau *et al.*, 2011]. High doses of $PDE_4$ inhibitors may be necessary to treat severe asthma, and gastro-intestinal side effects limit their use [Lipworth, 2005, Bateman *et al.*, 2006, Bousquet *et al.*, 2006]. The inhaled administration of $PDE_4$ inhibitors may improve the therapeutic index of $PDE_4$ inhibitors [Chapman *et al.*, 2010, Singh *et al.*, 2010, Nials *et al.*, 2011, De Savi *et al.*, 2014, Moretto *et al.*, 2015]. Inhaled $PDE_4$ inhibitors GSK256066 and CHF6001 both inhibit allergen-induced late asthmatic responses [Singh *et al.*, 2010, Dave *et al.*, 2014] and in patients with moderate COPD inhaled GSK256066 for 4 weeks was well tolerated although there was no inhibitory effect on sputum and blood inflammatory biomarkers [Watz *et al.*, 2013]. The inhaled dual $PDE_3$ and $PDE_4$ inhibitor RPL554 (Verona Pharma) has bronchodilator effects and is well tolerated in patients with asthma and COPD [Franciosi *et al.*, 2013]. In healthy subjects inhaled RPL554 attenuates the neutrophilic response to LPS challenge [Franciosi *et al.*, 2013]. RPL554 is under development for the

treatment of asthma and COPD (ClinicalTrials.gov Identifier**:** NCT02427165 and NCT02542254 respectively)**.**

***Protein kinase inhibitors***

Protein kinases are involved in cellular signalling of pro-inflammatory cytokines in asthma and the inhibition of these kinases may have a role in the treatment of severe asthma associated with non-eosinophilic asthma [Bhavsar *et al.*, 2010, Cohen *et al.*, 2010, Hammaker *et al.*, 2010, Chung, 2011, Guntur *et al.*, 2012]. Several p38MAPK inhibitors restore corticosteroid sensitivity in PBMCs from patients with severe asthma [Bhavsar *et al.*, 2010, Mercado *et al.*, 2012] and COPD [Khorasani *et al.*, 2015]. Clinical trials of p38MAPK inhibitors oral losmapimod (GW856553) and inhaled AZD7624 are register for the treatment COPD (ClinicalTrials.gov Identifier: NCT02299375 and NCT02238483 respectively), although neither are registered for the treatment of asthma. Interestingly, a *post-hoc* analysis of a 6 month clinical trial of oral losmapimod (GW856553) in COPD reported a reduction in exacerbations in a sub-group of patients with a blood eosinophil count $\leq$2% [Marks-Konczalik *et al.*, 2015], which may suggest a preferentially beneficial effect of p38MAPK inhibitors in non-eosinophilic inflammation. A imatinib, a specific ckit tyrosine kinase inhibitor that attenuates airway hyperresponsiveness, inflammation and remodelling in murine model of asthma [Berlin *et al.*, 2005, Rhee *et al.*, 2011] is under development for patients with severe refractory asthma (ClinicalTrials.gov Identifier: NCT01097694). A tyrosine kinase inhibitor masitinib targets c-kit and platelet-derived growth factor (PDGF) receptor improved asthma control in patients with severe corticosteroid-dependent asthma [Humbert *et al.*, 2009] and a further clinical trial underway in patients with severe asthma

treated with oral corticosteroids (ClinicalTrials.gov Identifier: NCT01449162). An alternative therapeutic strategy to silencing c-kit with small interference RNA has been shown to attenuate inflammation in a murine model of allergic asthma [Wu *et al.*, 2012, Wu *et al.*, 2014]. Clinical trials of protein kinase inhibitors have not been studied in patients with sputum inflammatory subtypes such as non-eosinophilic asthma.

***PI3kinase inhibitors***

Low dose theophylline is thought to act, at least in part, through the inhibition of PI3K [Ito *et al.*, 2007, To *et al.*, 2010]. Pre-clinical studies suggest that PI3K-δ inhibitors could potentially reverse corticosteroid insensitivity by increasing HDAC2 activity [Marwick *et al.*, 2009, Marwick *et al.*, 2010] and by reversing fungal-induced steroid resistant airway inflammation through modulation of endoplasmic reticulum stress [Lee *et al.*, 2016]. Selective PI3K inhibitors are being developed as novel therapies for the treatment of chronic inflammatory airway diseases. An inhaled PI3Kδ inhibitor GSK2269557 is undergoing several clinical trials in asthma and COPD. PI3K δ and γ isoforms are involved in inflammatory cell recruitment and activation and dual PI3Kδ/γ inhibitors, such as TG100-115 and IPI-145 reduces airway inflammation induced by allergen or cigarette smoke in murine models [Doukas *et al.*, 2009, Winkler *et al.*, 2013] and restored corticosteroid sensitivity in the smoke model [Doukas *et al.*, 2009]. RV1729, a PI3Kδ/γ Inhibitor has undergone early stage clinical evaluation in asthma and COPD. SH2-containing inositol-50-phosphatase 1 (SHIP1) is an endogenous inhibitor of the PI3K pathway. A SPIP1 activator AQX-1125 reduced the allergen-induced late asthmatic response with a non-significant trend for a reduction in sputum eosinophils and neutrophils [Leaker *et al.*, 2014].

Further development of AQX-1125 is underway for the treatment of COPD (ClinicalTrials.gov Identifier: NCT01954628).

**Biological agents**

Monoclonal antibody blockers of inflammatory cytokines such as IL-17 and TNF-α that activate receptors on the surface of neutrophils have been investigated as treatments for asthma.

***IL-17 blockers***

In pre-clinical studies $Th_{17}$ cells and IL-17 are implicated in causing neutrophilic inflammation and corticosteroid insensitivity [Shen *et al.*, 2011, Newcomb *et al.*, 2013, Chesné *et al.*, 2014]. IL-17 concentrations and expression are increased in BAL, sputum and bronchial biopsy samples in severe patients asthma that correlate with sputum neutrophils. Monoclonal inhibitors of IL-17 are in clinical development [Miossec *et al.*, 2012]. Brodalumab is a human monoclonal antibody that binds with high affinity to human IL-17RA, blocking the biologic activity of IL-17A, -17F, -17A/F heterodimer, and IL-25. A randomized clinical trial of brodalumab in adults with inadequately controlled moderate to severe asthma receiving regular inhaled corticosteroids, but not selected for neutrophilic inflammation, reported no improvement in the primary outcome ACQ score or in lung function and symptom-free days [Busse *et al.*, 2013]. A subgroup with high bronchodilator reversibility demonstrated a borderline improvement an ACQ score. A further clinical trial of brodalumab in inadequately controlled asthma subjects with high bronchodilator reversibility was recently terminated due to a lack of observed efficacy in a pre-specified

interim analysis (ClinicalTrials.gov Identifier: NCT01902290). The results of a preliminary proof of efficacy study of the IL-17A monoclonal antibody blocker secukinumab (AIN457) in patients with uncontrolled asthma was also recently terminated. The investigators report that further investigations would require changes in study design, the use of different endpoints, a different IL-17 antibody or a different patient population (ClinicalTrials.gov Identifier: NCT01478360).

***TNF-α blockers***

Neutralizing TNFα restores corticosteroid sensitivity in a mouse model of neutrophilic airway inflammation [Dejager *et al.*, 2015 ]. Several small clinical studies in severe asthma of the soluble TNF-α receptor blocker etanercept reported beneficial effects on clinical outcomes [Howarth *et al.*, 2005, Berry *et al.*, 2006], whereas larger studies with etanercept [Holgate *et al.*, 2011] and the TNF-α receptor blocker golimumab [Wenzel *et al.*, 2009] did not confirm a consistent beneficial clinical effect. When combined with concerns over increased risk of severe infections and malignancies with TNF-α receptor blocker treatment [Wenzel *et al.*, 2009] it is unlikely that this target will be developed further for the treatment of asthma.

***Other monoclonal antibodies***

Monoclonal antibodies that block IL-1β, for example, canakinumab or block the soluble IL-1 receptor, for example, anakinra [Hernandez *et al.*, 2015] might be of benefit in neutrophilic asthma, although no clinical studies are currently registered. An IL-6 monoclonal antibody blocker toclizumab is licensed for the treatment of rheumatoid arthritis. Toclizumab could

potentially be of benefit in neutrophilic asthma although no clinical studies are registered in asthma.

## Conclusions and future developments

Non-eosinophilic airway inflammation is a term used to describe a subtype of asthma associated with normal numbers of sputum eosinophils. Up to 50% of patients with stable mild to severe never or ex-smokers with asthma have non-eosinophilic inflammation and this inflammatory phenotype is also found in smokers with asthma, some patient with a high BMI or occupational asthma. The non-eosinophilic phenotype is subdivided into neutrophilic inflammation, when neutrophil numbers are raised above a defined cut-off level or paucigranulocytic inflammation, when both eosinophil and neutrophil numbers are normal. The relative proportions of each subtype is uncertain because of variable cut-off points used to define neutrophilia. The most appropriate value that indicates that neutrophils are activated and contributing to the pathogenic processes in asthma is not certain. The severity of current symptoms are in general similar or slightly better in non-eosinophilic or neutrophilic subgroups of asthma compare to eosinophilic subgroups. Sputum eosinophilia is a better predictor of future exacerbations and a greater risk factor for more rapid decline in lung function than sputum neutrophilia. Non-eosinophilic inflammation is associated with an impaired therapeutic response to inhaled corticosteroids. Neutrophilic inflammation is associated with activation of the innate immune system in asthma and systemic inflammation. Several mechanisms either alone or in combination could explain raised sputum neutrophil counts in asthma including corticosteroids, associated chronic sinopulmonary infection, delay human neutrophil apoptosis

due to epithelial growth factor, impaired macrophage phagocytosis and altered airway microbiome. Limited information has been published on the immunopathological characteristics of non-eosinophilic inflammation compared with other inflammatory airway phenotypes including Th2-low inflammation, Th17-high inflammation or combination of Th2/Th17 profiles in asthma. Taken together, the finding suggest that non-eosinophilic inflammation and Th2-low inflammation in non-smokers with asthma share some similar immunopathological features including normal eosinophil numbers, submucosal mast cell numbers and sub-epithelial basement membrane thickness. ~~Blood neutrophil numbers are a poor predictive for sputum neutrophilia~~. Due to the lack of effective specific therapies targeting non-eosinophilic inflammation including neutrophilic inflammation there is currently no definitive evidence for the involvement of these inflammatory phenotypes in chronic asthma. Additional pathways may account for poor asthma control in patients with non-eosinophilic asthma including Th1 inflammation, Th17 inflammation, or a combination of Th2 and Th17 inflammation as well as corticosteroid insensitivity (Figure 2). The role of corticosteroid treatment in causing neutrophilic and Th17 inflammation in severe asthma requires further investigation. Non-inflammatory mechanisms may also be important in some individuals including airway hyperreactivity and airway remodelling.

There is an unmet need for novel treatments that will impact favourably on clinical outcomes in patients with non-eosinophilic inflammation. Non-pharmacological interventions, 'off-label' use of licensed drugs, novel small molecules and biologics agents are being investigated as possible treatments of non-eosinophilic inflammation in asthma. Smoking cessation in smokers with asthma and cessation of exposure to occupational agents are associated with a reduction in neutrophilic inflammation. Preliminary data of studies of 'off-label' use of licensed drugs

suggest that macrolides show efficacy in non-smokers with non-eosinophilic asthma and statins, low-dose theophylline and PPARγ agonist may be beneficial in asthmatic smokers with non-eosinophilic inflammation and corticosteroid insensitivity. Further clinical studies are indicated to confirm these findings and to determine the role of these therapies in the management of severe asthma. Novel small molecules targeting neutrophilic inflammation in asthma such as CXCR2 antagonists reduce neutrophil counts, but do not improve clinical outcomes. A FLAP inhibitor did not reduce neutrophils or improve symptoms. Inhaled PDE4 inhibitors and dual PDE3 and PDE4 inhibitors are potential therapies for neutrophilic asthma and a dual PDE3 and PDE4 inhibitors is under development for the treatment of asthma and COPD. Additional small molecule drugs including p38MAPK inhibitors, tyrosine kinase inhibitors and PI3kinase inhibitors are under development for asthma. The development of biological agents to target non-eosinophilic inflammation in asthma has been disappointing to date with the termination of clinical programmes of monoclonal antibodies targeting IL-17 and TNF-α. In the future, the selection of patients with severe asthma and evidence of Th17-high inflammation may be more likely to identify a subpopulation that respond ~~to~~ to IL-17 blockers. Long-acting bronchodilators and/or bronchial thermoplasty are possible treatment options for symptomatic patients with paucigranulocytic inflammation in whom there is no evidence of activated inflammatory pathways or corticosteroid insensitivity that could be targeted by specific therapies.

Greater understanding of the mechanisms of non-eosinophilic inflammation in asthma should lead to improved therapies. International collaborative programmes of research investigating pathogenic mechanism of severe asthma have focused mainly on type 2 eosinophilic inflammation. The Unbiased Biomarkers for the Prediction of Respiratory Disease Outcome (U-

BOPRED) study [Shaw *et al.*, 2015 ] and the UK Refractory Asthma Stratification Programme (RASP-UK) [Heaney *et al.*, 2015 ] are designed to identify new phenotypes/endotypes and treatment targets and will hopefully identify new approaches to the treatment of patients with non-eosinophilic asthma.

**Declaration of Conflicting Interests**

In the last three years Professor Thomson has participated in advisory boards and/or received consultancy/lecture fees from Boston Scientific, Genentech, GlaxoSmithKline, Novartis, Respivert, Roche and Takeda and industry-sponsored grant funding to the University of Glasgow from Boston Scientific, Glaxo SmithKline and Novartis for participating in clinical trials.

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**LIST OF ABBREVIATIONS**

ACQ: Asthma control questionnaire

ACRN: Asthma Clinical Research Network

AP-1: Activator protein-1

AQLQ: asthma quality of life questionnaire

BAL: Bronchoalveolar lavage

BDP: Beclomethasone dipropionate

BMI: Body mass index

CAMP: Childhood Asthma Management Program

COPD: Chronic obstructive pulmonary disease

*CRHR1*: Corticotrophin-releasing hormone receptor 1 gene

CCL: Chemokine (C-C motif) ligand

CXCL: Chemokine (C-X-C motif) ligand

CXCR: C-X-C chemokine receptor

eNOS: Endothelial nitric oxide synthase

ERK: Extracellular signal-regulated kinase

FCER2: Low-affinity IgE receptor gene

FGF: Fibroblast growth factor

FLAP: 5-lipoxygenase-activating protein

GILZ: Glucocorticoid-inducible leucine zipper 1

G-CSF: Granulocyte-colony stimulating factor

GLCCI1: Glucocorticoid-induced transcript 1 gene

GOAL: Gaining Optimal Asthma Control

GR: glucocorticoid receptor

GRE: Glucocorticoid-responsive elements

HDAC: Histone deacetylase

HFA: Hydrofluoroalkane

HMG CoA: 3-Hydroxymethyl-3-glutaryl Coenzyme A

IDO: Indoleamine 2, 3-dioxygenase

IFN: interferon

Ig: immunoglobulin

IKKs: IκB kinases

IL: interleukin

JNK: c-Jun N-terminal kinase

LABA: Long acting $\beta_2$-agonists

LPS: lipopolysaccharide

LT: leukotriene

MAPK: Mitogen-activated protein kinase

MKP: Mitogen-activated protein kinase phosphatase 1

MMP: Matrix metalloproteinase

MPO: Myeloperoxidase

NO: nitric oxide

NFκB: Nuclear factor κB

PEF: Peak expiratory flow

PBMC: Peripheral blood mononuclear cell

PDE: Phosphodiesterase

PI3K: Phosphoinositide 3-kinase

PP2A: Protein phosphatase 2A

PPARγ: Peroxisome proliferator-activated receptor-γ

RASP-UK: Refractory Asthma Stratification Programme

RBM: Reticular basement membrane thickness

sCD40L: Soluble CD40 ligand

SHIP1: SH2-containing inositol-50-phosphatase 1

SLPI: Secretory leukocyte protease inhibitor expression

SNPs: Single-nucleotide polymorphisms

STAT: Signal transduction-activated transcription factors

SNP: Single nucleotide polymorphisms

*TBX21*: T-box expressed in T cells21

TGF-α: Transforming growth factor alpha

$Th_2$: Type 2 helper T-cell ($Th_2$)

TNF-α: Ttumour necrosis factor α

TLR: Toll-like receptor

U-BOPRED: Unbiased Biomarkers for the Prediction of Respiratory Disease Outcome

**Table 1 Possible factors accounting for ~~cause(s) of~~ neutrophilic airway inflammation in asthma**

- Corticosteroid treatment causing reduced apoptosis of neutrophils and contributing to Th17 mediated neutrophilic inflammation
- Neutrophila associated with chronic sinopulmonary infection and/or bronchiectasis
- Delay human neutrophil apoptosis in severe asthma due to epithelial growth factor induced release of mediators with neutrophil chemotactic and anti-apoptotic actions from bronchial epithelial cells
- Impaired macrophage phagocytosis of neutrophils
- Neutophilia associated with altered airway microbiome

**Table 2 Clinical phenotypes and factors associated with non-eosinophilic airway inflammation in asthma**

Mild to severe asthma in never or ex-smokers (both controlled and uncontrolled)

Smokers with asthma

High BMI (subgroup)

Occupational asthma (subgroup)

Factors associated with higher neutrophil counts

- Older age
- Exposure to environmental pollution
- Respiratory infections

**Table 3 Treatments targeting non-eosinophilic airway inflammation in asthma**

**Non-pharmacological interventions**

Avoidance from exposure to environmental and occupational pollutants

Smoking cessation

Dietary supplementation with vitamin D3

**'Off-label' use of licensed drugs**

Macrolides

Statins

Low-dose theophylline

Peroxisome proliferator-activated receptor-γ (PPARγ) agonists

**Novel small molecule drugs**

***Drugs targeting neutrophilic inflammation***

C-X-C chemokine receptor (CXCR)2 antagonists

5-lipoxygenase-activating protein (FLAP) inhibitors

***Phosphodiesterase (PDE) inhibitors***

$PDE_4$ inhibitors

Dual $PDE_3$ and $PDE_4$ inhibitors

***Protein kinase inhibitors***

p38 Mitogen-activated protein kinase (MAPK) inhibitors

Narrow spectrum kinase inhibitors

Tyrosine kinase inhibitors

***Phosphoinositide 3 (PI3)-kinase inhibitors***

PI3K-δ inhibitors

Dual PI3Kδ/γ inhibitors

***Biological agents***

Interleukin (IL)-17A receptor blockers

IL-17A blockers

Tumour necrosis factor (TNF)-α receptor blockers

IL-1β monoclonal antibody blockers

Soluble IL-1 receptor monoclonal antibody blockers

IL-6 monoclonal antibody blockers

## FIGURE LEGENDS

**Figure 1 Schematic diagram of potential pathways leading to non-eosinophilic inflammation and airway damage in severe asthma**

Several inflammatory pathways could potentially lead to non-eosinophilic inflammation and airway damage in asthma although the exact mechanisms are unclear. Possible pathways are briefly summarized in the schematic diagram. Stimuli such as viruses, cigarette smoke and pollutants could induce the release of chemoattractants including IL-8 to recruit neutrophils to the airways. The release of IL-17A and IL-17F from activated Th17 cells could stimulate the synthesis of neutrophil chemoattractants, such CXCL1 and IL-8 from the airway epithelium. INF-γ may also be involved in non-eosinophilic asthma, possibly in part through its release from Th1 cells. Inflammatory mediators released by neutrophils are implicated in causing mucus gland hyperplasia and hypersecretion, airway hyperreactivity and remodelling as well as corticosteroid insensitivity in asthma. Th1 and Th17 cells may induce airway hyperreactivity and/or remodelling independently of neutrophil activation.

*Abbreviations:* CXCL1: chemokine (C-X-C motif) ligand; IFN: interferon; IL: interleukin; LT: leukotriene; MMP: matrix metalloproteinase; MPO: myeloperoxidase; ROC: reactive oxygen species; TNF-α: tumour necrosis factor α

**Figure ~~2~~1 Targets and potential therapies for treating non-eosinophilic airway inflammation in asthma**

Non-eosinophilic airway inflammation is found in approximately 50% of patients with asthma. The proportion of this group with neutrophilic inflammation is less certain because of variable cut-off points used to define neutrophilia. The higher the cut-off value used to define sputum neutrophilia the greater the proportion of subjects that are classified as having paucigranulocytic inflammation. Pathways that may account for poor asthma control in patients with non-eosinophilic asthma including neutrophilic inflammation, associated inflammatory phenotypes (Th1-high inflammation, Th17-high inflammation, combination of Th2 and Th17 inflammation, mast cell induced inflammation, other inflammatory mechanisms) as well as corticosteroid insensitivity. Non-inflammatory mechanisms such as airway hyperreactivity and airway remodelling may be important in causing symptoms in some individuals.

Potential treatments targeting specific pathways are listed in the diagram. Novel small molecules targeting neutrophilic inflammation, such as CXCR2 antagonists reduce neutrophils, but do not improve clinical outcomes. Smoking cessation in asthmatic smokers and removal from exposure to occupational agents reduces neutrophilic inflammation. The results of clinical trials of biological agents targeting mediators associated with non-eosinophilic inflammation, such as IL-17 and TNF-$\alpha$ are disappointing. Preliminary studies of 'off-label' use of licensed drugs suggest that macrolides show efficacy in non-smokers with non-eosinophilic severe asthma and statins, low-dose theophylline and PPARγ agonists may benefit asthmatic smokers

with non-eosinophilic inflammation and associate corticosteroid insensitivity. Inhaled $PDE_4$ inhibitors, dual $PDE_3$ and $PDE_4$ inhibitors, p38MAPK inhibitors, tyrosine kinase inhibitors and PI3kinase inhibitors are under development and these compounds may be of benefit in treating non-eosinophilic inflammation and corticosteroid insensitivity. Long-acting bronchodilators and/or bronchial thermoplasty are possible treatment options for symptomatic patients with paucigranulocytic inflammation in whom there is no evidence of activated inflammatory pathways or corticosteroid insensitivity that could be targeted by specific therapies.

*Abbreviations:* CXCR: C-X-C chemokine receptor; FLAP: 5-lipoxygenase-activating protein; IL: interleukin; PDE: phosphodiesterase; PI3K: phosphoinositide 3-kinase; PPARγ: peroxisome proliferator-activated receptor-γ;

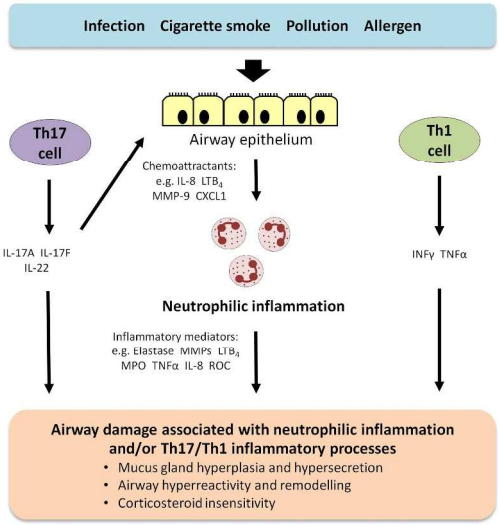


Figure 1 Schematic diagram of potential pathways leading to non-eosinophilic inflammation and airway damage in severe asthma

Several inflammatory pathways could potentially lead to non-eosinophilic inflammation and airway damage in asthma although the exact mechanisms are unclear. Possible pathways are briefly summarized in the schematic diagram. Stimuli such as viruses, cigarette smoke and pollutants could induce the release of chemoattractants including IL-8 to recruit neutrophils to the airways. The release of IL-17A and IL-17F from activated Th17 cells could stimulate the synthesis of neutrophil chemoattractants, such CXCL1 and IL-8 from the airway epithelium. INF-γ may also be involved in non-eosinophilic asthma, possibly in part through its release from Th1 cells. Inflammatory mediators released by neutrophils are implicated in causing mucus gland hyperplasia and hypersecretion, airway hyperreactivity and remodelling as well as corticosteroid insensitivity in asthma. Th1 and Th17 cells may induce airway hyperreactivity and/or remodelling independently of neutrophil activation.
Abbreviations: CXCL1: chemokine (C-X-C motif) ligand; IFN: interferon; IL: interleukin; LT: leukotriene; MMP: matrix metalloproteinase; MPO: myeloperoxidase; ROC: reactive oxygen species; TNF-α: tumour necrosis factor α

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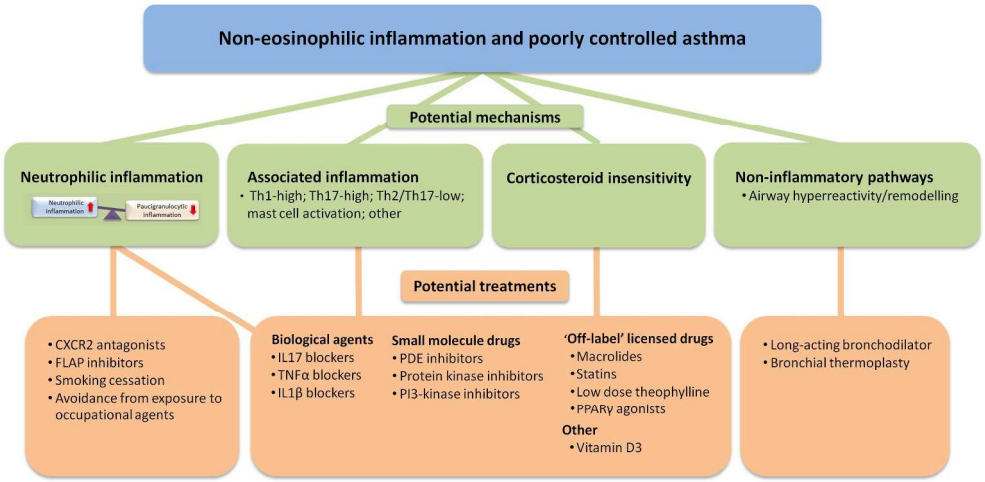


Figure 2 Targets and potential therapies for treating non-eosinophilic airway inflammation in asthma

Non-eosinophilic airway inflammation is found in approximately 50% of patients with asthma. The proportion of this group with neutrophilic inflammation is less certain because of variable cut-off points used to define neutrophilia. The higher the cut-off value used to define sputum neutrophilia the greater the proportion of subjects that are classified as having paucigranulocytic inflammation. Pathways that may account for poor asthma control in patients with non-eosinophilic asthma including neutrophilic inflammation, associated inflammatory phenotypes (Th1-high inflammation, Th17-high inflammation, combination of Th2 and Th17 inflammation, mast cell induced inflammation, other inflammatory mechanisms) as well as corticosteroid insensitivity. Non-inflammatory mechanisms such as airway hyperreactivity and airway remodelling may be important in causing symptoms in some individuals. Potential treatments targeting specific pathways are listed in the diagram. Novel small molecules targeting neutrophilic inflammation, such as CXCR2 antagonists reduce neutrophils, but do not improve clinical outcomes. Smoking cessation in asthmatic smokers and removal from exposure to occupational agents reduces neutrophilic inflammation. The results of clinical trials of biological agents targeting mediators associated with non-eosinophilic inflammation, such as IL-17 and TNF-$\alpha$ are disappointing. Preliminary studies of 'off-label' use of licensed drugs suggest that macrolides show efficacy in non-smokers with non-eosinophilic severe asthma and statins, low-dose theophylline and PPARγ agonists may benefit asthmatic smokers with non-eosinophilic inflammation and associate corticosteroid insensitivity. Inhaled PDE4 inhibitors, dual PDE3 and PDE4 inhibitors, p38MAPK inhibitors, tyrosine kinase inhibitors and PI3kinase inhibitors are under development and these compounds may be of benefit in treating non-eosinophilic inflammation and corticosteroid insensitivity. Long-acting bronchodilators and/or bronchial thermoplasty are possible treatment options for symptomatic patients with paucigranulocytic inflammation in whom there is no evidence of activated inflammatory pathways or corticosteroid insensitivity that could be targeted by specific therapies.
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