COL4A3 is degraded in allergic asthma and degradation predicts response to anti-IgE therapy

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A novel, serological biomarker predicts the anti-IgE therapy response in asthmatics. The neo-epitope biomarker C4Ma3 measures the increase of lung collagen 4 degradation in severe exacerbating type 2 asthma and depends on mast cell chymase activity. https://bit.ly/3ejFp7i

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Abstract

Background Asthma is a heterogeneous syndrome substantiating the urgent requirement for endotype-specific biomarkers. Dysbalance of fibrosis and fibrolysis in asthmatic lung tissue leads to reduced levels of the inflammation-protective collagen 4 (COL4A3).

Objective To delineate the degradation of COL4A3 in allergic airway inflammation and evaluate the resultant product as a biomarker for anti-IgE therapy response.

Methods The serological COL4A3 degradation marker C4Ma3 (Nordic Bioscience, Denmark) and serum cytokines were measured in the ALLIANCE cohort (paediatric cases/controls: n=134/n=35; adult cases/controls: n=149/n=31). Exacerbation of allergic airway disease in mice was induced by sensitising to ovalbumin (OVA), challenge with OVA aerosol and instillation of poly(cytidylic-inosinic). Fulacimstat

(chymase inhibitor; Bayer) was used to determine the role of mast cell chymase in COL4A3 degradation. Patients with cystic fibrosis (n=14) and cystic fibrosis with allergic bronchopulmonary aspergillosis (ABPA; n=9) as well as patients with severe allergic uncontrolled asthma (n=19) were tested for COL4A3 degradation. Omalizumab (anti-IgE) treatment was assessed using the Asthma Control Test.

Results Serum levels of C4Ma3 were increased in asthma in adults and children alike and linked to a more severe, exacerbating allergic asthma phenotype. In an experimental asthma mouse model, C4Ma3 was dependent on mast cell chymase. Serum C4Ma3 was significantly elevated in cystic fibrosis plus ABPA and at baseline predicted the success of the anti-IgE therapy in allergic, uncontrolled asthmatics (diagnostic OR 31.5).

Conclusion C4Ma3 levels depend on lung mast cell chymase and are increased in a severe, exacerbating allergic asthma phenotype. C4Ma3 may serve as a novel biomarker to predict anti-IgE therapy response.

Introduction

Asthma significantly impairs health throughout life. More than 235 million individuals are affected worldwide, with a 10% incidence rate in the industrialised world [1, 2]. Asthma is now understood to be a heterogeneous syndrome rather than a single disease, which manifests with chronic airway inflammation and respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough [3]. So-called molecular endotypes may underlie these symptoms, and are thought to drive asthma pathogenesis [4–6]. It is of note that no current phenotype or endotype definition takes into consideration changes of the composition of the extracellular matrix (ECM), nor are these changes an integral part of phenotype or endotype definitions.

Type 4 collagen (COL4) is the most abundant nonfibrillar collagen in the lung, comprised of six genetically distinct isoforms which form heterotrimers [7, 8]. Distribution of the COL4 isoforms varies throughout the body with A1/A2 found in all basal membranes and isoform A3 (which contains tumstatin) mainly found in the lung, oesophagus and kidney [9–12]. In airways of high-risk wheezing children and adult asthmatics, COL4 is less expressed and deposited [13–15]. In particular, one isoform, COL4A3, is reduced 18-fold in lung tissue of asthmatics [14].

The fibrotic response in airway tissue is a balance of fibrosis and fibrolysis. This balance is disrupted in asthma as documented by increased deposition of ECM proteins (*e.g.* thickening of the basal membrane), but also elevated levels of degrading enzymes such as matrix metalloproteinases (MMPs) [16–19]. At the same time, aberrant repair processes also lead to decreased deposition of ECM proteins, such as COL4. Degradation of COL4 can be induced by MMP9 (*e.g.* from neutrophils) [20], cathepsin S [21], MMP2 (*e.g.* from eosinophils) and MMP12 (*e.g.* from macrophages) [22, 23], which are implicated in asthma and cystic fibrosis [16, 19, 24–27].

Mast cell proteases (*i.e.* tryptase and chymase) show different substrate specificities towards COL4. Tryptase is able to activate pro-MMPs, which then are able to degrade COL4 [28], whereas chymase may degrade COL4 directly or *via* latent interstitial collagenases [29, 30]. Mast cells are increased in number, and their type (tryptase$^+$, tryptase-chymase$^+$), tissue distribution and level of activation are critically associated with asthma features [29–31].

Since the discovery of the dramatic reduction of tumstatin in airways of asthmatics, several lines of investigation have established that COL4A3 fragments (*e.g.* tumstatin, CP17, LF15) have additional anti-inflammatory, anti-angiogenic and antimigratory properties in asthma [14, 32–35]. However, the mechanism(s) underlying the diminished levels of COL4A3 and its potential use as a biomarker in asthma have remained elusive to date.

In this study, we set out to delineate the loss of COL4A3 in the asthmatic airways and evaluate its degradation products as biomarker for severe asthma therapy response. We hypothesise that proteolytic cleavage of COL4A3 by an endotype-specific pathway leads to the reduction of tumstatin in asthmatic airways.

Methods

ALLIANCE cohort

Study participants were recruited from multiple centres as described previously, and ethical approval was granted by the local ethics committees [36]. In brief, the following inclusion criteria for children applied in addition to informed consent of either parent or caretaker and the child if aged ⩾8 years: age 6–18 years, term delivery (⩾37 weeks) and active/passive understanding of German. Exclusion criteria have been described previously [36]. Childhood cases are specified as having doctor-diagnosed asthma (age ⩾6 years)

with diagnosis according to current Global Initiative for Asthma guidelines. Healthy controls are defined as children without asthma and otherwise applying the same inclusion and exclusion criteria. Spirometry was performed according to international guidelines (American Thoracic Society/European Respiratory Society). For the adult arm of the ALLIANCE cohort the following inclusion criteria applied in addition to informed consent for participants who were newly recruited during adulthood: age ⩾18 years, active/passive understanding of German and an established diagnosis of asthma according to current guidelines. Participants were allowed to be current or former smokers to avoid significant selection bias; separation criteria for asthma/COPD and further exclusion criteria are described elsewhere [36].

For analysis of the COL4A3 degradation (C4Ma3) we used the core dataset V0_2 of the adult arm from 2017-03-04 and V1_0 of the paediatric arm from 2017-12-09, leading to a total sample size of 742 ALLIANCE study participants. 375 of these had cytokine measurements from serum (175 adults from V0_1 2017-04-03 and 200 children from V1_0 2018-07-17). 16 children were excluded from analysis due to age <5.5 years, nine patients had missing C4Ma3 values, leading to a final sample size of 348. In detail: children ($n_{control}$=34, n_{asthma}=134), adults ($n_{control}$=31, n_{asthma}=149).

The flow chart for the ALLIANCE cohort is shown in the supplementary material.

Animal experiments

Female, 6- to 8-week-old C57/BL6 mice (Charles River, Sulzfeld, Germany) were housed under specific pathogen-free conditions and received ovalbumin (OVA)-free diet and water *ad libitum*. All animal studies were approved by the animal ethics committee from the Department of State, Kiel, Germany.

For precision-cut lung slice experiments, female mice (Balb/c, aged 6–8 weeks) were obtained from Charles River and kept under conventional housing conditions (22°C, 55% humidity, 12 h day/night rhythm). This *in vivo* experimental setup was approved by governmental authority (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES), approval number 33.19-42502-04-15/1937).

For more information on mouse models, cytokine measurements in lung lysates and application of recombinant proteins please see the supplementary methods.

Serological determination of COL4A3 degradation

Degradation of COL4A3 protein was assessed in human EDTA plasma, serum or mouse serum using the neo-epitope specific C4Ma3 competitive ELISA (Nordic Bioscience, Herlev, Denmark). The C4Ma3 ELISA utilises a neo-epitope specific monoclonal antibody to quantify a specific fragment of the COL4A3 generated by MMP2, MMP9 or MMP12 cleavage between amino acid 437 and 438 (438'. PGDIVFRKGP'447) [22]. C4Ma3 was assessed in a blinded manner using double determinations according to the manufacturer (Nordic Bioscience) and all samples were measured within the detection range.

For more detailed methods please see the supplementary methods.

Results

COL4A3 degradation levels are increased in exacerbation-prone allergic asthmatics

Following our hypothesis, we were interested in whether the levels of a highly specific COL4A3 degradation product (C4Ma3, validated, neo-epitope marker [37, 38]) are elevated in patients with asthma. To test this hypothesis, we measured C4Ma3 levels in plasma of adult participants of the ALLIANCE cohort (table 1).

Serum C4Ma3 levels increased with asthma severity, specifically, mild–moderate asthmatics showed an increase of 8% over controls ($p<0.05$; figure 1a), while levels in severe asthmatics were elevated by 15.5% ($p<0.01$; figure 1a). Next, we tested in pairwise comparison serum C4Ma3 levels against several clinical variables (supplementary table S1). Serum C4Ma3 levels were individually significantly associated with sputum eosinophils ($p=0.0007$; figure 1b), one or more exacerbation per week ($p=0.0060$; figure 1c), and exhaled nitric oxide (exNO; $p=0.0104$). In a multivariate analysis (linear regression model), elevated C4Ma3 levels in asthmatics were independently associated with gender ($p=0.0006$), disease duration ($p=0.0006$, log transformed), exNO ($p=0.0023$, log transformed), a clinical marker for allergic airway inflammation and a positive skin-prick test ($p=0.0083$), but not with systemic corticosteroid use or a dose equivalent of inhaled corticosteroids, smoking, age or sputum eosinophils (supplementary table S2).

TABLE 1 Demographics of the ALLIANCE adult cohort

	Controls	Asthma	
		ICS	No ICS
Subjects (n)	31	130	19
Age (years)	40.26	50.82	51.16
Female (%)	64.5	45.4	57.9
Atopy (%)	32.3	73.8	94.7
FEV_1 (% pred)	104.97±7.7	81.46±21.65***	88.78±20.45*
FEV_1/FVC	0.79±0.07	0.64±0.12***	0.66±0.11***
F_{eNO} (ppm)	16.08±6.43	33.16±33.85***	32.56±25.04***
Systemic corticosteroids (n)	0	23	0
Disease duration (years)	NA	22.18±15.61	22.74±17.38
Fluticasone equivalent ($mg \cdot day^{-1}$)	NA	680±500	0
Current smoking (%)	0	7	21
Smoking (pack-years)	0	24.1	36.5

Data are presented as mean or mean±SD, unless otherwise stated. ICS: inhaled corticosteroids; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; F_{eNO}: exhaled nitric oxide fraction; NA: not applicable. *: p<0.05, ***: p<0.001 for asthma (±ICS) *versus* controls.

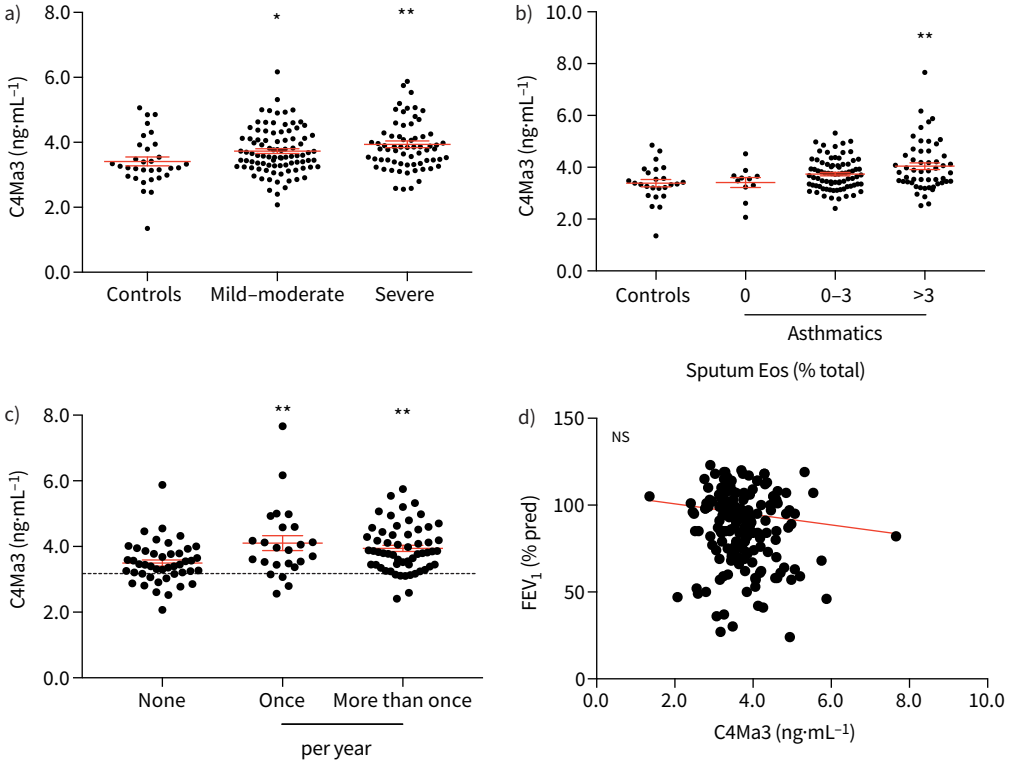

FIGURE 1 C4Ma3 increase is related to asthma severity, exacerbation and sputum eosinophils. a) Sera of $ALLIANCE_{adult}$ study participants were analysed for C4Ma3 levels and participants stratified into controls, mild–moderate and severe asthmatics. C4Ma3 levels significantly increased with severity compared with controls. Kruskal–Wallis and Dunn's post-test; mean±SEM for all graphs, $n_{control}$=31, $n_{mild-moderate}$=88, n_{severe}=61. b) C4Ma3 levels increase with sputum eosinophil (Eos) percentages. Controls and asthmatics were grouped into categories of 0, 0–3, >3% sputum eosinophils and levels of C4Ma3 are displayed. All groups compared with the 0% group using Kruskal–Wallis and Dunn's post-test; mean±SEM for all graphs. c) C4Ma3 is elevated in asthmatics with reported exacerbations. Groups defined as self-reported exacerbations once or more than once per year (n_{none}=45, n_{once}=24, $n_{>once}$=32) and compared with asthmatics with no reported exacerbations (none). Dotted line represents mean level in control subjects. d) Forced expiratory volume in 1 s (FEV_1) % predicted is not significantly associated with C4Ma3 level. NS: nonsignificant. *: p<0.05; **: p<0.01.

C4Ma3 was not significantly associated with forced expiratory volume in 1 s (FEV_1) % predicted (figure 1d), but was associated with airway resistance ($p<0.01$; supplementary figure S1c).

These findings may suggest that elevated COL4A3 degradation is associated with a more severe, persistent allergic phenotype of asthma.

Levels of C4Ma3 in serum are elevated in children with asthma and correlate with serum cytokines in adults

C4Ma3 levels declined from childhood to adolescence and the upper cut-off for C4Ma3 in individuals with no reported lung disease (>20 years) was 4.0 ng·mL^{-1} (99% CI of fitting function; supplementary figure S1d). Asthmatic individuals showed higher C4Ma3 values compared to controls (fitting curve of the controls, figure 2a). Additionally, serum levels of C4Ma3 (age adjusted) of paediatric and adolescent ALLIANCE participants (table 2), were significantly increased in asthma compared to controls (figure 2b; $p<0.05$). This further supports our finding that COL4A3 degradation is a general feature of allergic asthma.

In addition, in adult asthmatics with circulating C4Ma3 levels >4.0 ng·mL^{-1}, significantly elevated levels of interleukin (IL)-9 (figure 2c; $p<0.01$) and IL-13 (figure 2d; $p<0.05$) were detected. No increases were observed in IL-4, IL-5 or eotaxin (supplementary figure S2). Cytokines associated with asthma exacerbations, such as IL-6 (supplementary figure S2; $p<0.01$) and interferon (IFN)-γ (supplementary figure S2; $p<0.05$) were also increased, but not IL-17, IL-12p70 or IL-8. These cytokine data indicate that while clinically COL4A3 degradation is associated with exacerbation, sputum eosinophils and elevated exNO, cytokine levels in serum only partially support an exacerbating type 2 phenotype.

Serum levels of C4Ma3 are increased in mice with acute allergic airway disease

We hypothesised that COL4A3 is degraded in the asthmatic airways due to an allergic airway inflammation and further increased by exacerbation. To test this hypothesis, we analysed mice sensitised

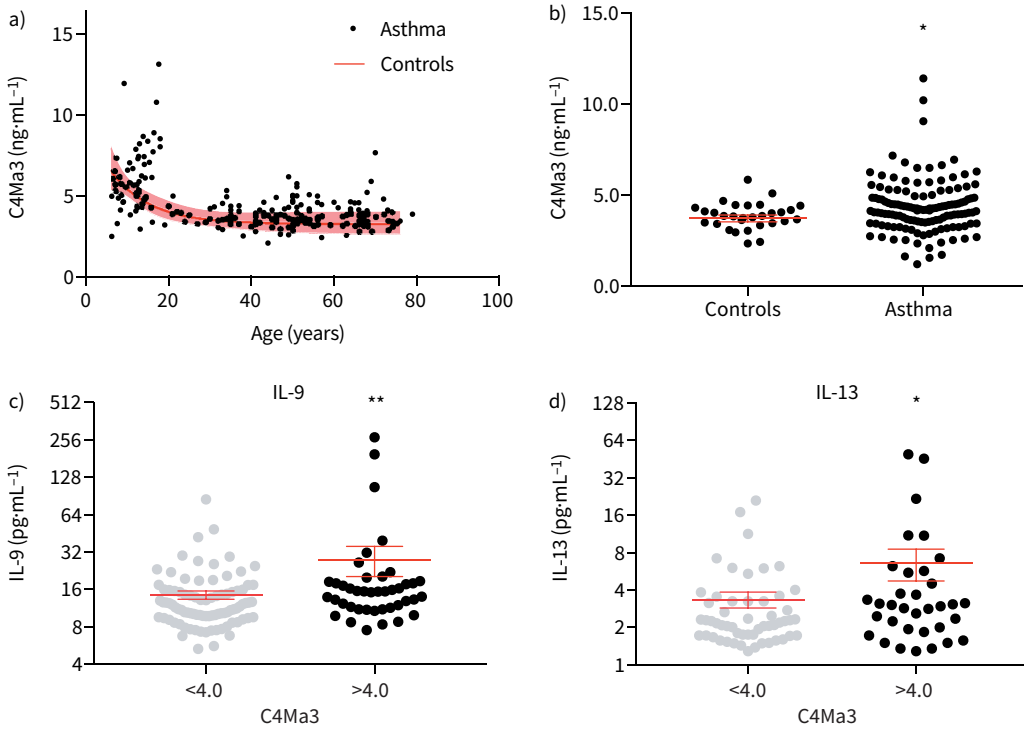

FIGURE 2 COL4A3 degradation correlates is age-dependent and elevated in children with asthma. a) C4Ma3 serum levels in asthmatic individuals vary over age. Fitting function of control individuals (described in figure 1) included for reference. b) Serum levels of C4Ma3 (adjusted for age and centre) were elevated in asthmatic $ALLIANCE_{paediatric}$ patients ($n_{control}$=31, n_{asthma}=130). c) Adult asthmatics with C4Ma3 levels >4.0 ng·mL^{-1} presented with increased serum interleukin (IL)-9 levels (y-axis logarithmic scale). d) Adult asthmatics with C4Ma3 levels >4.0 ng·mL^{-1} also show increased serum IL-13 levels (y-axis logarithmic scale). Mean±SEM for all graphs. *: $p<0.05$, **: $p<0.01$.

TABLE 2 Demographics of ALLIANCE paediatric cohort

	Controls	Asthma	
		ICS	No ICS
Subjects (n)	35	84	50
Age (years)	10.88	11.80	12.46
Female (%)	48.6	32.1	32.0
Atopy (%)	29.0	73.8	94.7
FEV_1 (% pred)	97.02±10.49	92.00±12.34	94.18±13.17
FEV_1/FVC	0.88±0.06	0.82±0.09****	0.82±0.07****

Data are presented as mean or mean±SD, unless otherwise stated. ICS: inhaled corticosteroids; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity. ****: p<0.0001, for asthma (±ICS) *versus* controls.

and challenged with OVA and exacerbated with poly(cytidylic-inosinic) (polyI:C). Significantly elevated serum levels of C4Ma3 were found in OVA animals as compared to PBS (figure 3a; p<0.0001); no further increase followed after neutrophil influx (figure 3a and supplementary figure S3a). The increase of C4Ma3 was independent of the model-allergen used (house dust mite (HDM)) (HDM *versus* PBS, p<0.01; supplementary figure S3b) and lung tissue from HDM-challenged mice *ex vivo* produced significantly more C4Ma3 (supernatant of precision-cut lung slices, p<0.05; supplementary figure S3c). This may suggest that active allergic airway inflammation is key to COL4A3 degradation from lung tissue.

Furthermore, we found C4Ma3 levels significantly correlated with hallmark features of experimental asthma (airway resistance, dynamic compliance, mucus producing cells; supplementary figure S3d,e,f). Bronchoalveolar lavage (BAL) eosinophils correlated significantly with C4Ma3 in serum (r^2=0.55, p=0.0001; figure 3b), similar to our human findings, but neutrophils alone had no effect (polyI:C; figure 3c). Consequently, neutrophil proteases did not correlate with serum C4Ma3 levels (total lung MMP2, MMP9 mRNA levels; supplementary figure S3g,h,i).

However, chymase (mMCPT4) immunostaining (supplementary figure S3j) positively associated with elevated serum levels of C4Ma3 (figure 3d; p=0.0107). No such correlation was found with mast cell tryptase (supplementary figure S3k). We tested the necessity of chymase activity for C4Ma3 level increase by intratracheal instillation of a chymase inhibitor (fulacimstat; Bayer). Fulacimstat (F) did not change baseline levels of C4Ma3 (figure 3e; CTRL *versus* CTRL+F), but it decreased C4Ma3 levels in OVA-challenged animals to baseline levels (figure 3e; OVA+F *versus* CTRL+F; nonsignificant). In the exacerbation group, C4Ma3 remained significantly elevated with fulacimstat (figure 3e; p<0.01). Eosinophil levels increased significantly (figure 3f; p<0.001) in OVA+F animals, but not during exacerbation (figure 3i, supplementary figure S3l; p<0.001). Neutrophils cell counts in exacerbation+F animals were significantly reduced (figure 3f; p<0.01). Interestingly, airway resistance was also significantly reduced in exacerbation+F (supplementary figure S3m; p<0.0001). IL-33 increased significantly in OVA+F and exacerbation+F (figure 3f; p<0.001 and p<0.01, respectively), while keratinocyte-derived chemokine (KC, CXCL1) (figure 3g) only reduced in exacerbation+F (p<0.001). IL-4, -5 and -13 did not show significant changes (supplementary figure S3n–p). IL-6 and IFN-γ reduced significantly exacerbation+F (supplementary figure S3q,r; p<0.05), while eotaxin increased in OVA+F (supplementary figure S3s; p<0.05). There was no difference in the percentage of mucus-producing cells (supplementary figure S3u). Active, recombinant mast cell chymase, instilled intratracheally did not raise circulating C4Ma3 levels (supplementary figure S3t).

Our data establish that COL4A3 degradation (*i.e.* C4Ma3) in the lung is a feature of experimental allergic asthma. Degradation correlated with BAL eosinophil numbers and was not amplified following neutrophil infiltration (exacerbation). Mast cell chymase levels positively correlated with, and chymase inhibition (*in vivo*) prevented COL4A3 degradation. Yet, mast cell chymase alone was insufficient to increase C4Ma3 level. Furthermore, polyI:C-induced COL4A3 degradation during exacerbation may be independent of chymase.

Elevated circulating C4Ma3 levels are a feature of allergic bronchopulmonary aspergillosis and responsive to anti-IgE treatment of uncontrolled asthma

We hypothesised that the severe allergic lung comorbidity known as allergic bronchopulmonary aspergillosis (ABPA) in cystic fibrosis would show significantly elevated levels of circulating C4Ma3.

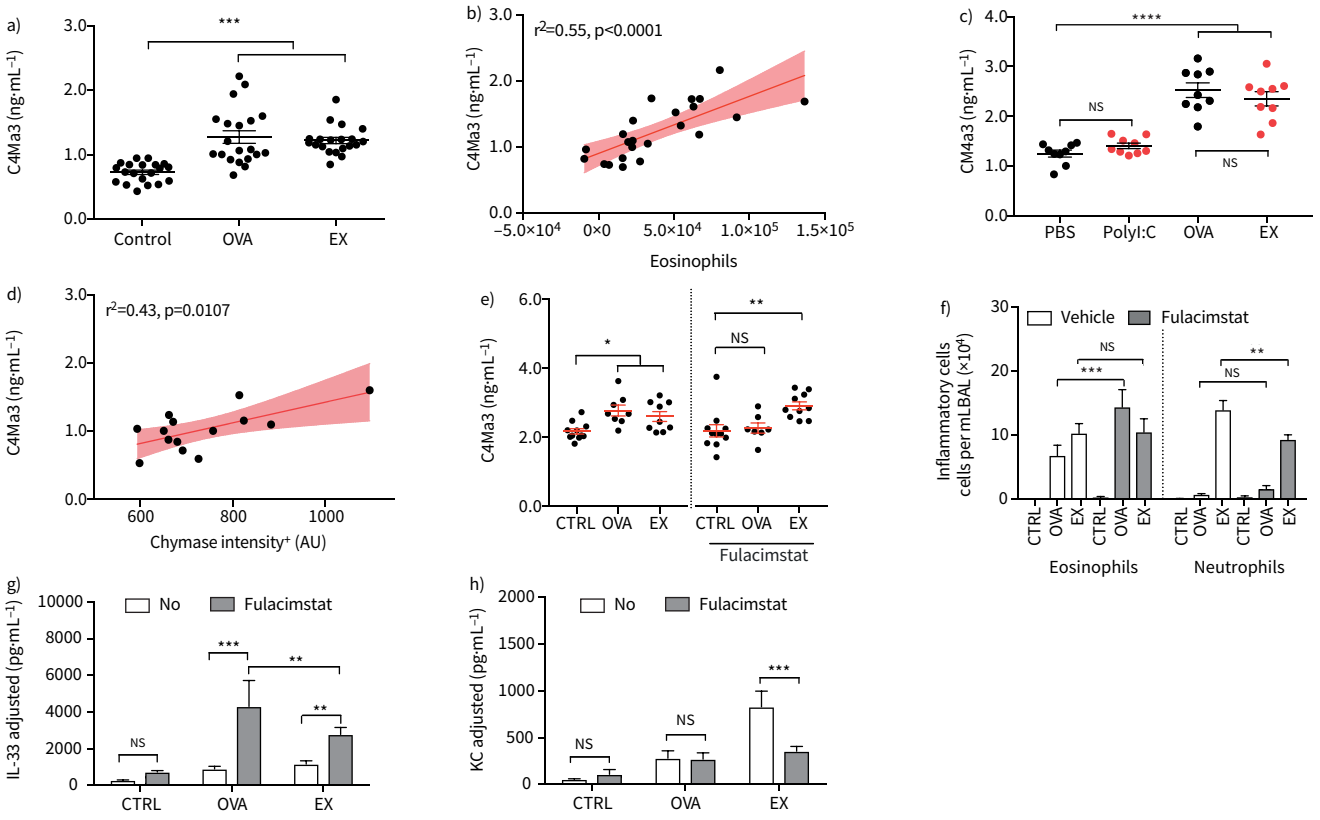

FIGURE 3 Mouse model of acute allergic airway disease shows increased COL4A3 degradation. a) Ovalbumin (OVA)-treated animals have markedly increased serum levels of C4Ma3. COL4A3 degradation product was measured in control (challenge with PBS), OVA and OVA+poly(cytidylic-inosinic) (polyI:C) (exacerbation) animals, n=20 per group. b) Linear regression model of C4Ma3 levels and the respective contribution of eosinophils to the C4Ma3 variance (r^2=0.55, p<0.0001). c) Neutrophils alone or in combination with allergic airway disease are not associated with increased C4Ma3 levels. Control animals (challenged with PBS) are indicated as black, animals that received polyI:C are red, OVA treated as black and exacerbation (OVA+polyI:C) red. n=9 per group. d) Intensity of immunohistological staining of mast cell chymase correlated with C4Ma3 level in serum (r^2=0.43, p=0.0107). e) Fulacimstat treatment of allergic exacerbated asthma model reduces C4Ma3 level in serum. PBS (CTRL), OVA and OVA+polyI:C (EX) animals. n=8–10 per group. f) Bronchoalveolar lavage cytology (of eosinophils and neutrophils) of fulacimstat model. g) Interleukin (IL)-33 cytokine concentrations in lung lysate of fulacimstat model. h) Keratinocyte-derived chemokine (KC, CXCL1) cytokine concentrations in lung lysate of fulacimstat model. IL-33 and KC concentrations were adjusted to total protein content of supernatant of whole-lung lysates (see methods section); n=8–10 per group. AU: arbitrary units; NS: nonsignificant. Data are presented as mean±SEM, unless otherwise stated. Kruskal–Wallis with Dunn's post-test. *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001.

Levels of C4Ma3 in non-ABPA cystic fibrosis should match that of control patients. Cystic fibrosis patients diagnosed with ABPA (supplementary table S3) showed significantly increased C4Ma3 serum levels as compared with non-ABPA cystic fibrosis (p<0.01; figure 4a). In addition, the latter group did not significantly differ in C4Ma3 levels compared with control patients (compare with figure 1c). These findings are in strong support of our hypothesis that mast cells (*i.e.* mast cell chymase) but not neutrophils (*i.e.* MMPs) are central to the COL4A3 degradation in allergic lung inflammation.

Omalizumab neutralises circulating allergen-specific IgE and prevents mast cell activation. In a proof-of-concept study, we analysed 20 severe asthmatic patients who received omalizumab for 6 months (table 3). Based on our data, we anticipated a significant reduction of circulating C4Ma3 level in therapy responders. 10 out of 20 patients clinically responded (>3 points in the Asthma Control Test (ACT) at 6 months) to the anti-IgE therapy. These patients initially had a mean level of 8.87 ng·mL^{-1} C4Ma3, which was significantly reduced to 6.57 ng·mL^{-1} (6 months post-anti-IgE, p<0.01; figure 4b). Nonresponders (ACT <3 points) did not show any change in serum levels of C4Ma3 before and after therapy (5.32 *versus* 5.57 ng·mL^{-1}). Both responder (p<0.0001) and nonresponder (p<0.05) C4Ma3 levels were significantly different from controls at baseline (8.87 ng·mL^{-1} and 5.32 ng·mL^{-1} *versus* 3.76 ng·mL^{-1}, respectively;

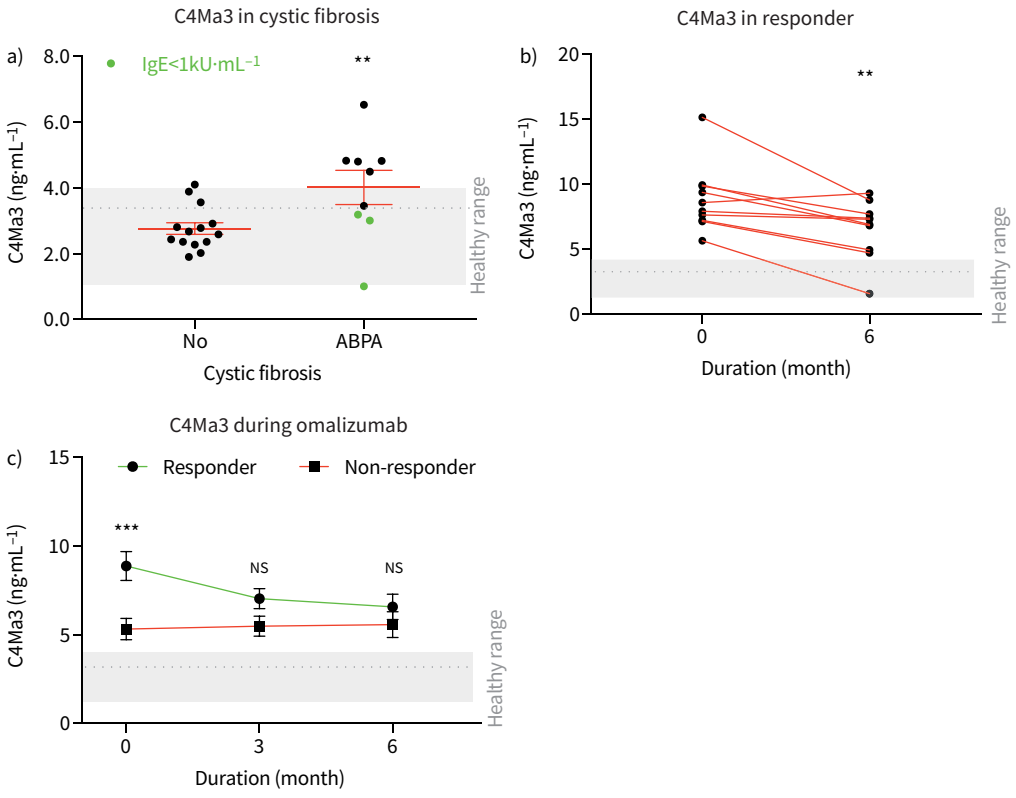

FIGURE 4 C4Ma3 serum levels are elevated in allergic lung disease and are responsive to omalizumab treatment. a) Serum levels of C4Ma3 of cystic fibrosis patients with and without allergic bronchopulmonary aspergillosis (ABPA). Green symbols denote total IgE serum levels <1 kU·mL^{-1}. Analysis was performed with Wilcoxon on ranks of age-adjusted values. b) Serum level of C4Ma3 of asthmatics who respond to omalizumab therapy as measured by an increase of the asthma control test score of ⩾3 points. Patient individual time course from prior (0 month) and at finish of therapy monitoring (6 months). Analysis was performed using Wilcoxon on ranks of age-adjusted values. c) Comparison of average circulating C4Ma3 serum levels in responders and nonresponders during duration of therapy monitoring (0=baseline, 3 months, 6 months). t-test analysis with Holm–Sidak correction for multiple testing. Dotted grey line represents mean level of C4Ma3 in control subjects. Grey shaded area represents the 99% confidence interval of C4Ma3 of control subjects. NS: nonsignificant. **: p<0.01. ***: p<0.001.

TABLE 3 Demographics of omalizumab proof-of-principle trial

	Baseline		3 months		6 months	
	Nonresponders	Responders	Nonresponders	Responders	Nonresponders	Responders
Subjects (n)	9	10	8	10	7	10
Female (%)	67	70				
Age (years)	51.33	43.50				
Smoking (pack-years)	2.33	3.71				
Disease duration (years)	34.78	24.83				
Exacerbation	1.33	1.11				
Total serum IgE (IU·mL^{-1})	640	367				
Eosinophils	445±463	437±206	332±484	393±267	202±132	197±151
FEV$_1$ (% pred)	68.44±11.11	64.19±20.74	67.14±19.87	74.16±21.49	67.00±17.32	80.88±18.75
C4Ma3 (ng·mL^{-1})	5.32±1.83	8.87±2.58**	5.48±1.60	7.04±1.81	5.57±1.91	6.57±2.26
ACT	18.33±6.14	11.50±4.40*	17.00±6.97	18.10±5.45	19.86±5.05	20.10±4.09

Data are presented as mean or mean±SD, unless otherwise stated. FEV$_1$: forced expiratory volume in 1 s; ACT: Asthma Control Test score. *: p<0.05, **: p<0.01 (responder *versus* nonresponder).

supplementary figure S4a). Additionally, responders differed significantly in C4Ma3 level at baseline (month 0, $p<0.001$; figure 4c), but not after 3 or 6 months of treatment when compared with nonresponders. C4Ma3 levels at baseline (0 months) were predictive of therapy success (ACT >3 points) with a diagnostic odds ratio of 31.5 (95% CI 2.35–422.30; supplementary table S4). Using baseline C4Ma3 to predict the treatment success, a receiver operator curve analysis showed an area under the curve of 0.92 (supplementary figure S4). Sensitivity and specificity were 0.9 (95% CI 0.60–0.98) and 0.78 (95% CI 0.45–0.94), respectively.

These data indicate that serum levels of C4Ma3 are elevated in severe allergic lung diseases and can be reduced *via* anti-IgE therapy. Furthermore, high levels of C4Ma3 are indicative of a clinically relevant response to omalizumab therapy in severe asthmatics.

Discussion

To the best of our knowledge, this study is the first to describe the modalities of degradation of COL4A3 in asthma. Since our first report of an 18-fold reduction of COL4A3 expression in asthmatic airways, the fate of this protein has remained elusive [14]. Our investigations into differentially regulated COL4A3 mRNA expression were not conclusive for the diminished levels described previously (data not shown). As part of the basal membrane and the matrikine reservoir of the lung, the loss of COL4A3 has been linked to aggravated inflammation, neovascularisation, goblet cell hyperplasia and increased bronchial hyperreactivity [14, 33, 34]. Here we shed light on the enhanced COL4A3 degradation in patients with asthma (supplementary figure S4c). Importantly, elevated COL4A3 degradation is already a feature of paediatric asthma. Circulating levels of the biomarker C4Ma3 are elevated in ABPA in cystic fibrosis and predictive of the clinical outcome of omalizumab therapy.

We traced the culprit responsible for COL4A3 degradation in an allergic airway disease model to the presence of mast cells. Higher levels of mast cell chymase in tissue of mice correlated with higher level of degradation marker in serum. Balzar *et al.* [29] identified chymase-positive mast cells (M_{TC}) to be significantly elevated in allergic asthma and a feature of severe asthma. Animal models of allergic airway disease have confirmed these findings, and mast cells (*e.g.* M_{TC}) have been implicated in paediatric asthma exacerbation and structural changes of the asthmatic airways [9–12]. Recently, Rønnow *et al.* [38] predicted the mortality of COPD patients in a 3-year follow-up study using C4Ma3 (ECLIPSE study). In the same study, COPD patients with more frequent, hospitalised exacerbations also had higher levels of C4Ma3. A report by Andersson *et al.* [39] identified that in severe (Global Initiative for Chronic Obstructive Lung Disease IV) COPD patients, M_{TC} proportions are elevated in anatomically key lung regions, which negatively correlated with lung function [39, 40]. These data are supportive of our findings, and the detrimental role of M_{TC} in COPD warrants further investigation.

By inhibiting mast cell chymase, we deliver first evidence of the importance of this protease for COL4A3 degradation. In addition, we confirm studies by Waern *et al.* [41], who identified chymase to be crucial for IL-33 degradation. In our model, fulacimstat treatment increased levels of IL-33 in OVA and exacerbating animals. IL-33 has been shown to induce eotaxin release from human lung fibroblasts [41]. In line with this, fulacimstat treatment in our model led to significantly elevated eotaxin levels in OVA mice. Furthermore, Waern *et al.* [41] reported a five-fold increase of BAL eosinophils in a chymase (mouse mast cell protease 4) knockout mouse model of asthma, while we report a two-fold increase after fulacimstat treatment [41, 42]. This may serve as an explanation as to why blocking chymase may lead to reduced C4Ma3 levels, but also eosinophilia. Our findings in the ALLIANCE cohort are in line with our mouse model, as clinical (exhaled nitric oxide fraction [43], eosinophils) and immunological (serum cytokines) measures of a type 2 asthma phenotype correlate with COL4A3 degradation. Additionally, IL-9, a mast cell chemokine and potentiator of allergic airway inflammation [44] was elevated in our C4Ma3-high cohort, as well as IL-13, a hallmark effector phase cytokine in asthma [45, 46]. This may hint at an active allergic inflammation as a prerequisite for COL4A3 degradation in lung tissue.

However, viral response cytokines (IFN-γ and IL-6) were also elevated in C4Ma3-high asthma patients. While we identified an association with increased levels of C4Ma3 in reported exacerbations in asthmatics, this was not reflected in our mouse model of asthma exacerbation.

Instead, inhibiting mast cell chymase in our experimental asthma exacerbation (polyI:C) model led to a slight decrease of neutrophil influx, but did not lower COL4A3 degradation. We observed reduced levels of IFN-γ, KC (CXCL1) and IL-6 in exacerbating and fulacimstat-treated animals, which may explain the lower neutrophil cell count in BAL and the decreased, but not normalised, airway resistance in these animals. However, the lack of COL4A3 degradation may be a result of an alternative pathway. The activity of released mast cell tryptase is not sensitive to inhibition by fulacimstat, and tryptase has been reported to

activate pro-MMP9 (*e.g.* from neutrophils), which can lead subsequently to COL4 degradation [47]. Of note, mast cell tryptase is not selectively released after direct stimulation of TLR3 on mast cells, thus viral infections alone may not suffice to trigger COL4 degradation [48, 49]. This may suggest an alternative pathway, bypassing or acting in concert with the chymase-induced COL4A3 degradation during allergic asthma exacerbation, which warrants further research.

From these initial observations we concluded that an allergic airway disease is required for elevated C4Ma3 level, but not elevated levels of MMPs, as suggested by known canonical degradation pathways [20]. We confirmed this notion in two proof-of-concept studies. Firstly, in cystic fibrosis patients with ABPA, a severe pulmonary allergy, C4Ma3 was significantly elevated. Neutrophil proteases (such as MMP9, MMP2) or MMP12, which are all increased in cystic fibrosis, are capable of generating C4Ma3 [22, 25, 27, 50]. Yet, C4Ma3 levels in serum of cystic fibrosis patients without ABPA were not elevated. In contrast, we only observed elevated C4Ma3 level in cystic fibrosis patients with ABPA, corroborating our previous findings in lung sections of cystic fibrosis patients [14].

Secondly, we analysed serum from patients after an anti-IgE therapy. C4Ma3 levels were elevated in clinical responders at baseline and reduced within 3 months of therapy. Clinical nonresponders did not show any change of C4Ma3. Anti-IgE therapy would be effective in reducing C4Ma3 regardless of whether it originated from a viral induced asthma exacerbation or not. This is highly supportive of our hypothesis, that COL4A3 degradation is a consequence of allergic airway inflammation and can be modified by blocking mast cell activation.

The current study has several limitations. The range of C4Ma3 levels in asthmatics from the ALLIANCE cohort is fairly broad, hence there is a considerable overlap between controls, mild–moderate and severe asthmatics, making distinguishing these groups difficult. We found C4Ma3 to correlate significantly to clinical biomarkers for a type 2 asthma phenotype, but we failed to link all type 2 cytokines (*i.e.* IL-4, IL-5, eotaxin). Instead, a signal from T9 (IL-9) and type 1 cytokines was present in our cohort. Furthermore, we were not able to measure C4Ma3 level during acute asthma exacerbation. Clearly, to better understand the usefulness of C4Ma3 in determining asthma endotypes, larger studies are warranted. Lastly, we used two proof-of-principle studies (ABPA in cystic fibrosis, anti-IgE therapy) to confirm our initial observations and mouse model data. Both studies, besides being highly promising, are of small numbers. In particular, the diagnostic odds ratio of 31.5 for detecting anti-IgE therapy responders prior to therapy is tantalising. It is of utmost importance to verify this result in larger cohorts to reduce current limitations of prediction [51].

In summary, we found the previously reported loss of COL4A3 to be a consequence of increased degradation in both childhood and adult asthma. Increased COL4A3 degradation correlated with more severe disease and asthma exacerbations. Treatment with omalizumab resulted in a decrease of circulating levels of the biomarker C4Ma3, in therapy responder only. High levels of C4Ma3 at baseline where highly predictive of treatment response, whereas low levels comparable to control subjects identified nonresponders. Monitoring C4Ma3, a soluble marker for COL4A3 degradation, in asthma may therefore afford a novel avenue to stratify and monitor anti-IgE therapy.

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