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Early View

Original research article

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

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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 (pediatric cases/controls: 134/35; adult cases/controls: 149/31). Exacerbation of allergic airway disease in mice was induced by sensitizing to 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 (CF, n=14) and CF with allergic broncho-pulmonary aspergillosis (ABPA, n=9) as well as severe allergic, uncontrolled asthmatics (n=19) were tested for COL4A3 degradation. Omalizumab (anti-IgE) treatment was assessed by 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 CF plus ABPA and at baseline predicted the success of the anti-IgE therapy in allergic, uncontrolled asthmatics (diagnostic odds ratio 31.5). **Conclusion:** C4Ma3 level 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.

Key words: Allergic Asthma, Exacerbation, Collagen 4, Remodelling, Mast Cell Chymase, anti-IgE therapy, biomarker

Introduction

Asthma significantly impairs health throughout life. More than 235 million individuals are affected worldwide, with a 10% incidence rate in the industrialized world (Global Atlas of Asthma, EAACI 2013, WHO, 2018). Asthma is now understood as 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.[1] So-called molecular endotypes may underly these symptoms, and are thought to drive asthma pathogenesis.[2-4] It is of note, that no current phenotype or endotype definition takes changes of the extracellular matrix composition into consideration; 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 forms heterotrimers.[5, 6] 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 lung, oesophagus, and kidney.[7-10] In airways of high-risk wheezing children and adult asthmatics, COL4 is less expressed and deposited.[11-13] In particular, one isoform – COL4A3 – is reduced 18-fold in lung tissue of asthmatics.[12]

The fibrotic response in airway tissue is a balance of fibrosis and fibrolysis. This balance is disrupted in asthma; 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).[14-17] At the same time, aberrant repair processes also lead to decreased deposition of ECM-proteins, such as COL4. Degradation of COL4 can be induced via by matrix-metallo-proteinase (MMP) 9 (e.g. from neutrophils) [18], cathepsin S [19], MMP2 (e.g. from eosinophils), and MMP12 (e.g. from macrophages) [20],[21], which are found implicated in asthma and cystic fibrosis.[14, 17, 22-25]

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 [26], whereas chymase may directly or via latent interstitial collagenases degrade COL4.[27, 28] Mast cells are increased in numbers and their type (Tryptase^{+}, Tryptase-Chymase^{+}), tissue distribution, and level of activation are critically associated with asthma features.[27-29]

Since the discovery of the dramatic reduction of tumstatin in airways of asthmatics, several lines of investigations have established, that COL4A3 fragments (e.g. tumstatin, CP17, LF15) have additional anti-inflammatory, anti-angiogenic, and anti-migratory properties in asthma.[12, 30-33] 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 hypothesize that proteolytic cleavage of COL4A3 by an endotype-specific pathway leads to the reduction of tumstatin in asthmatic airways.**

Methods:

Cohorts

ALLIANCE

Study participants were recruited multi-centric as described previously and ethical approval was granted by the local ethics committees.[34] In brief, the following inclusion criteria for children applied in addition to informed consent of either parent or caretaker and of the child if aged 8 years or older: age 6 to 18 years, term delivery (≥ 37 weeks); active/passive understanding of German. For exclusion criteria see reference.[34] Childhood cases are specified as having doctor-diagnosed asthma (age ≥ 6 years) with diagnosis according to current GINA-guidelines. Healthy controls are defined as children without asthma and otherwise applying the same in- and exclusion criteria as above. Spirometry was performed according to international guidelines (ATS/ERS). 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, see here [34] for separation criteria asthma/COPD. Further exclusion criteria are described elsewhere.[34]

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 pediatric arm from 2017-12-09 leading to a total sample size of 742 ALLIANCE study participants. 375 of these had cytokine measurements from the serum (175 adults from V0_1 2017-04-03 and 200 children from V1_0 2018-07-17). Further we had to exclude 16 children from analysis due to an age < 5.5 years leading to a final sample size of 336. In detail: For children ($n_{control}$=34, n_{asthma}=134), for adults ($n_{control}$=31, n_{asthma}=149).

See supplement for the flow chart for ALLIANCE cohort.

Animal Experiments

Animals

Female, 6- to 8-week old C57/BL6 (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, 6–8 weeks) were obtained from Charles River (Sulzfeld, Germany) and kept under conventional housing conditions (22°C, 55% humidity, and 12 h day/night rhythm). This *in vivo* experimental setup was approved by governmental authority (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, LAVES, approval no. 33.19-42502-04-15/1937).

For more information on mouse models, cytokine measurements in lung lysates and application of recombinant proteins please see 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 utilizes a neo-epitope specific monoclonal antibody to quantify a specific fragment of the COL4A3 generated by MMP-2, -9, or -12 cleavage between amino acid 437 and 438 (438'.PGDIVFRKGP'447)[20]. C4Ma3 was assessed in a blinded manner using double determinations according to the manufacturer (Nordic Bioscience, Herlev, Denmark) and all samples were measured within the detection range.

For more detailed methods please see supplementary methods.

Results:

1. *COL4A3 degradation levels are increased in exacerbation-prone allergic asthmatics*

Following our hypothesis, we were interested if the levels of a highly specific COL4A3 degradation product (C4Ma3, validated, neo-epitope marker [35, 36]) 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-to-moderate asthmatics showed an increase of 8% over controls ($p<0.05$, fig. 1a), while levels in severe asthmatics were elevated by 15.5% ($p<0.01$, fig. 1a). Next, we tested in pairwise comparison serum C4Ma3 levels against several clinical variables (supplement table 1). 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 ICS, smoking, age or sputum eosinophils (supplement table 2**)**. C4Ma3 was not significantly associated with FEV1% predicted (figure 1d) but airway resistance ($p<0.01$, supplement figure 1a).

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

2. *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 cutoff for C4Ma3 in individuals with no reported lung disease (> 20 years) was 4.0 ng/mL (99% CI of fitting function, supplement figure 1b). Asthmatic individuals showed higher C4Ma3 values compared to controls (fitting curve of the controls, figure 2a). Serum levels of C4Ma3 (age adjusted) of pediatric and adolescent ALLIANCE participants (table 2), were also 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, 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 and Eotaxin (supplement figure 2). Cytokines associated with asthma exacerbations such as IL-6 (supplement figure 2, $p<0.01$) and IFN-γ (supplement figure 2, $p<0.05$) were also increased, but not IL-17, IL-12p70 or IL-8. These cytokine data indicate that whilst clinically COL4A3 degradation is associated with exacerbation, sputum eosinophils and elevated exNO, cytokine levels in serum only partially support an exacerbating type 2 phenotype.

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

We hypothesized 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 sensitized and challenged with ovalbumin (OVA) and exacerbated with 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 supplement figure 3a). The increase of C4Ma3 was independent of the model-allergen used (HDM) **(**HDM vs. PBS, $p<0.01$, supplement figure 3b) and lung tissue from HDM challenged mice *ex vivo* produced significantly more C4Ma3 (supernatant of PCLS, supplement figure 3c, $p<0.05$). This may suggest that active allergic airway inflammation is key to COL4A3 degradation in from lung tissue.

Further, we found C4Ma3 levels to significantly correlate with hallmark features of experimental asthma (airway resistance, dynamic compliance, mucus producing cells, supplement figure 3 d, e, f). 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 MMP-2,-9 mRNA levels, supplement figure 3g,h,i).

However, chymase (mMCPT4) immuno-staining (reference: supplement figure 3j) positively associated with elevated serum levels of C4Ma3 (figure 3d, $p=0.0107$). No such correlation was found with mast cell tryptase (supplement figure 3k). We tested the necessecity of chymase activity for C4Ma3 level increase by intra-tracheal instillation of a chymase inhibitor (Fulacimstat, Bayer). Fulacimstat (F) did not change baseline levels of C4Ma3 (figure 3e, CRTL vs. CTRL+F), but it decreased C4Ma3 levels in OVA challenged animals to baseline levels (figure 3e, OVA+F vs. CTRL+F, n.s.). 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, supplement figure 3l, $p<0.001$). 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 (supplement figure 3m, $p<0.0001$). IL-33 increased significantly in OVA+F and exacerbation+F (figure 3f, $p<0.001$ and $p<0.01$ respectively), while KC (figure 3g) only reduced in exacerbation+F ($p<0.001$). IL-4, -5, -13 did not show significant changes (supplement figure 3n-p). IL-6 and IFN-γ reduced significantly exacerbation+F (supplement figure 3q,r, $p<0.05$), while eotaxin increased in OVA+F (supplement figure 3s, $p<0.05$). There was no difference in the percentage of mucus producing cells (supplement figure 3u). Active, recombinant mast cell chymase, instilled intra-tracheally, did not rise circulating C4Ma3 levels (supplement figure 3t).

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 increase C4Ma3 level. Further, PolyI:C induced COL4A3 degradation during exacerbation may be independent of chymase.

4. *Elevated circulating C4Ma3 levels are a feature of allergic broncho-pulmonary aspergillosis and responsive to anti-IgE treatment of uncontrolled asthma*

We hypothesized that the severe allergic lung co-morbidity known as allergic broncho-pulmonary aspergillosis (ABPA) in Cystic Fibrosis would show significantly elevated levels of circulating C4Ma3. Levels of C4Ma3 in non-ABPA CF should match that of control patients. CF patients diagnosed with ABPA (supplement table 3) showed significantly increased C4Ma3 serum levels as compared with non-ABPA CF ($p<0.01$, figure 4a). In addition, the latter group did not significantly differ in C4Ma3 levels compared with control patients (compare 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 neutralizes circulating allergen specific IgE and prevents mast cell activation. In a proof-of-concept study, we analyzed 20 severe, asthmatic patients, who received Omalizumab for six months (table 3). Based on our data, we anticipated a significant reduction of circulating C4Ma3 level in therapy responders. Ten out of 20 patients clinically responded (>three points in the asthma control test (ACT) at 6 month) to the anti-IgE therapy. These patients initially had a mean level of 8.87 ng/mL C4Ma3, which was significantly reduced to 6.57 ng/mL (six months post-anti-IgE, $p<0.01$, figure 4b). Non-responders (ACT <3 points) did not show any change in serum levels of C4Ma3 before and after therapy (5.32 vs. 5.57 ng/mL). Both, responders' ($p<0.0001$) and non-responders' ($p<0.05$) C4Ma3 levels were significantly different from controls at baseline (8.87 ng/mL and 5.32 ng/mL vs. 3.76 ng/mL, respectively, supplement figure 4a). Additionally, responder differed significantly in C4Ma3 level at baseline (month 0, $p<0.001$, figure 4c) not after three or six months of treatment when compared with non-responders. C4Ma3 levels at baseline (0 months) were predictive of therapy success (ACT>3) with a diagnostic odds ratio of 31.5 (CI: 2.35 to 422.30, supplement table 4). Using baseline C4Ma3 to predict the treatment success, a Receiver-Operator-Curve Analysis showed an area under the curve of 0.92 (supplement figure 4). Sensitivity and specificity were 0.9 (CI: 0.60 to 0.98) and 0.78 (CI: 0.45 to 0.94), respectively.

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

Discussion:

This study, to the best of our knowledge, 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.[12] Own investigations into differentially regulated COL4A3 mRNA expression were not conclusive for the diminished levels we 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, neo-vascularization, goblet cell hyperplasia, and increased bronchial hyperreactivity.[12, 31, 32] Here we shed light on the enhanced COL4A3 degradation in asthmatics (supplement figure S4c). Importantly, elevated COL4A3 degradation is already a feature of pediatric asthma. Circulating levels of the biomarker C4Ma3 are found elevated in ABPB in CF and predictive of the clinical outcome of an 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 and colleagues identified chymase^{+} mast cells (M_{TC}) to be significantly elevated in allergic asthma and a feature of severe asthma.[27] Animal models of allergic airway disease have confirmed these findings and mast cells (e.g. M_{TC}), have been implicated in pediatric asthma exacerbation and structural changes of the asthmatic airways.[7-10] Recently, Rønnow and colleagues predicted the mortality of COPD patients in a three year follow-up study using C4Ma3 (ECLIPSE study).[36] In the same study, COPD patients with more frequent, hospitalized exacerbations also had higher levels of C4Ma3. A report by Andersson *et al.* identified, that in severe (GOLD IV) COPD patients, M_{TC} proportions are elevated in anatomically key lung-regions, which negatively correlated with lung function.[37,38] This data is 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 and colleagues, who identified chymase to be crucial for IL-33 degradation.[39] 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.[39] In line with this, Fulacimstat treatment in our model led to significantly elevated eotaxin levels in OVA mice. Further, Waern et al. reported a five-fold increase of BAL eosinophils in a chymase (mouse mast cell protease 4) knock-out mouse model of asthma, while we

report a two-fold increase after Fulacimstat treatment. [39, 40] This may serve as an explanation as to why blocking chymase may lead to reduced C4Ma3 levels but also to eosinophilia. Our findings in the ALLIANCE cohort are in line with our mouse model, as clinical (FeNO [41], 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 [42] was elevated in our C4Ma3-high-cohort, as well as IL-13, a hallmark effector phase cytokine in asthma.[43, 44] 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 and IL-6 in exacerbating and Fulacimstat treated animals, which may explain the lower neutrophil cell count in BAL and the decreased but not normalized 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.[45] 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.[46, 47] This may suggest an alternative pathway, by-passing 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.[18] We confirmed this notion in two proof-of-concept studies. Firstly, in CF patients with ABPA, a severe, pulmonary allergy, C4Ma3 was significantly elevated. Neutrophil proteases (such as MMP9, MMP2) or MMP12, which are all increased in CF, are capable of generating C4Ma3.[20, 23, 25, 48] Yet, C4Ma3 levels in serum of CF patients without ABPA were not elevated. To the contrary, we only observed elevated C4Ma3 level in CF patients with ABPA, corroborating our previous findings in lung sections of CF patients.[12]
Secondly, we analyzed 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 non-responder did not show any change of C4Ma3. Alos, 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 CF, anti-IgE therapy) to confirm our initial observations and mouse model data. Both studies, besides being highly promising, are of small number. Especially, the diagnostic odds ratio of 31.5 for detecting anti-IgE therapy responders prior to therapy is tantalizing. It is of utmost importance to verify this result in larger cohorts to reduce current limitations of prediction.[49]

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 non-responders. 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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Conflict of Interest Disclosure Statement

MW declares no conflict of interest; MP declares no conflict of interest; JMBS, SR, DJL are employees of Nordic Bioscience; MAK is a full time employee and stock holder of Nordic Bioscience; UZ declares no conflict of interest. BO declares no conflict of interest; JD declares no conflict of interest; TB declares no conflict of interest; LL declares no conflict of interest; MWeg declares no

conflict of interest; JKB declares no conflict of interest; IK declares no conflict of interest. AMD declares no conflict of interest. EVM declares no conflict of interest. EVM received consulting fees from from European Commission, Tampereen Yliopisto, University of Edinburgh, Nestec S.A., University of Veterinary Medicine, Vienna, Chinese University of Hongkong, Research Center Borstel - Leibniz Lung Center, OM Pharma S. A., Pharmaventures Ltd., Peptinnovate Ltd., Turun Yliopisto, Helsingin Yliopisto, Chinese University of Hongkong, Imperial College London, Universiteit Utrecht, Universität Salzburg, Österreichische Gesellschaft f. Allergologie u. Immunologie, HiPP GmbH & Co KG; She received fees for speaking from The American Academy of Allergy Asthma & Immunology, British Society for Immunology, Medical University of Vienna, Schweizerisches Institiut für Allergie- und Asthmaforschung, Howard Hughes Medical Institute, University Hospital Erlangen, Margaux Orange, Deutsche Akademie der Naturforscher Leopoldina e.V., Hannover Medical School, American Thoracic Society, Inc., European Academy of Allergy and Clinical Immunology, Mundipharma Deutschland GmbH & Co. KG, DOC Congress SRL, ITÄ-Suomen Yliopisto, Interplan - Congress, Meeting & Event Management AG, INC, Ökosoziales Forum Oberösterreich, Imperial College London, WMA Kongress GmbH, University Hospital rechts der Isar, European Respiratory Society, HAL Allergie GmbH, PersonalGenomes.org, Nestlé Deutschland AG, Universitätsklinikum Aachen, SIAF - Swiss Institute of Allergy and Asthma Research, Deutsche Pharmazeutische Gesellschaft e. V.; Verein zur Förderung der Pneumologie am Krankenhaus Großhansdorf e.V., Pneumologie Developpement, Mondial Congress & Events GmbH & Co. KG,, Volkswagen Stiftung, Böhringer Ingelheim International GmbH, Hanson Wade Ltd., DSI Dansk Borneastma Center; Also she received author honoraria from Elsevier Ltd., Springer-Verlag GmbH, Schattauer GmbH, Georg Thieme Verlag, Springer Medizin Verlag GmbH; In addition, Dr. von Mutius has a patent Application number LU101064, Barn dust extract for the prevention and treatment of diseases pending, a patent Publication number EP2361632: Specific environmental bacteria for the protection from and/or the treatment of allergic, chronic inflammatory and/or autoimmune disorders. with royalties paid to Protectimmun GmbH, a patent Publication number EP 1411977: Composition containing bacterial antigens used for the prophylaxis and the treatment of allergic diseases. licensed to Protectimmun GmbH, a patent Publication number EP1637147: Stable dust extract for allergy protection licensed to Protectimmun GmbH, and a patent Publication number EP 1964570: Pharmaceutical compound to protect against allergies and inflammatory diseases licensed to Protectimmun GmbH. CV declares no conflict of interest. AF declares no conflict of interest. OD declares no conflict of interest. URJ declares no conflict of interest. GH declares no conflict of interest and is a consultant for Novartis and Sanofi. GUM declares no conflict of interest. YL declares no conflict of interest. MJ declares no conflict of interest. CH declares no conflict of interest. KR declares not conflict of interest; he received grants by by Boehringer Ingelheim and personal fees from AstraZeneca, Novartis, Sanofi, Regeneron, Roche, Chiesi Pharmaceuticals outside the submitted work. BS declares no conflict of interest. MJ

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Author contributions: MW, MP, MK designed the experiments, performed analysis and wrote the manuscript. TB, JMBS, SR planed the analysis of the degradation fragment and performed the analysis. LL, MWeg planned, performed the mouse models. OD and KS designed and performed the experiments with precision-cut lung slices. GUM und URJ recruited and analyzed the omalizumab treated asthmatic patients. BO provided RV-16 and edited the manuscript. CV, AF, MVDB, JKB, UZ, DJL extensively reviewed and edited the manuscript. OF, TB, AMD, BS, CH, KR, EVM, GH, MK designed the ALLIANCE cohort recruitment scheme, recruited participants, collected specimens and edited the manuscript. IK analyzed data and edited the manuscript. JD and YL performed and analyzed the HDM mouse models. All authors approved the final version of the manuscript.

Additional Files

Supplementary Methods and Supplementary Figure Legends
Supplementary Tables 1-4
Supplementary Figures 1-4

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Figure Legends

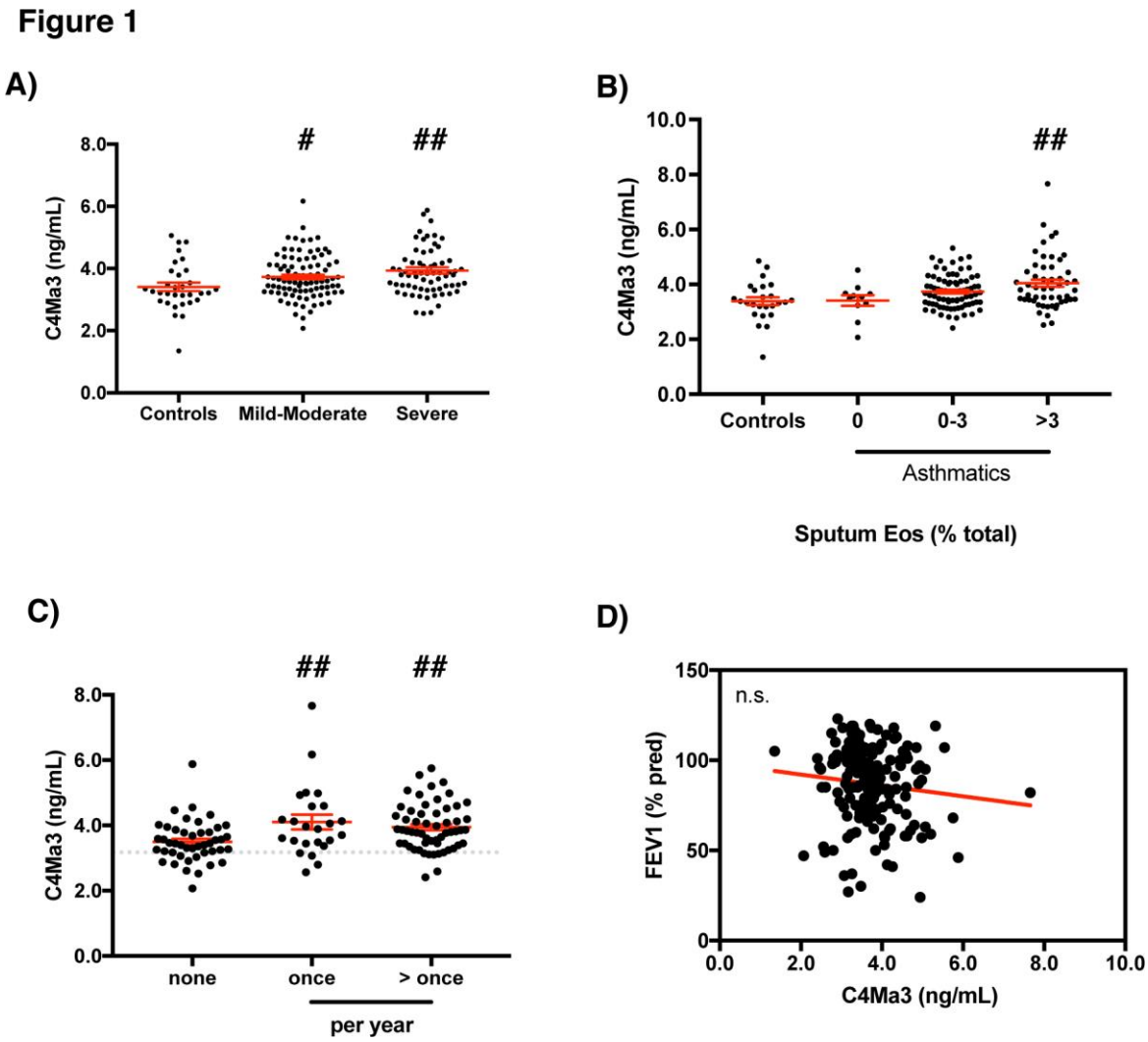

Figure 1: C4Ma3 increase is related to asthma severity, exacerbation and sputum eosinophils. A) Sera of ALLIANCE$_{ADULT}$ study participants were analyzed for C4Ma3 levels and participants stratified into controls, mild-moderate, and severe asthmatics. C4Ma3 levels significantly increased with severity compared with controls. Kruskal-Wallis and Dunn's post te
st and Mean±SEM for all graphs, $n_{control}$=31, n_{m-m}=88, n_{severe}=61. B) C4Ma3 levels increase with sputum eosinophil percentages. Controls and asthmatics were grouped into categories ranging from 0, 0-3, >3% sputum eosinophils and levels of C4Ma3 are displayed. All groups compared with the 0% group with Kruskal-Wallis and Dunn's post test. Mean±SEM for all graphs, ###p<0.001. 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 grey line represents mean level in control subjects. ##p<0.01 D) FEV1% predicted is not significantly associated with C4Ma3 level.

Figure 2

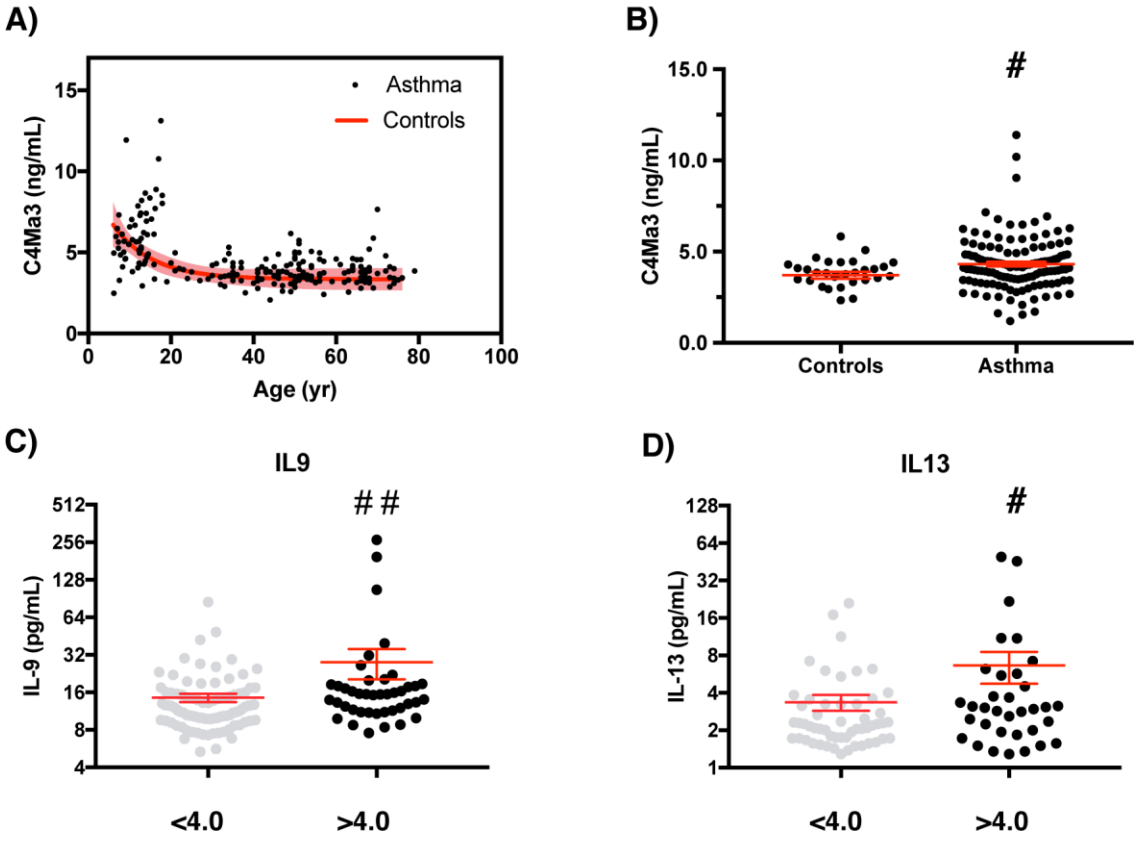

Figure 2: COL4A3 degradation correlates is age-dependent and elevated in children with asthma. A) C4Ma3 serum levels in asthmatic individuals vary over age. Fitting function of control individuals (red, see above) included for reference. B) Serum levels of C4Ma3 (adjusted for age and center) were elevated in asthmatic ALLIANCE$_{PEDIATRIC}$ patients ($n_{control}$=31, n_{asthma}=130). C) Adult asthmatics with C4Ma3 levels >4.0 ng/mL presented with increased serum IL-9 levels (y-axis logarithmic scale). D) Adult asthmatics with C4Ma3 levels >4.0 ng/mL also show increased serum IL-13 levels (y-axis logarithmic scale). Mean±SEM for all graphs, #p<0.05, ##p<0.01.

Figure 3

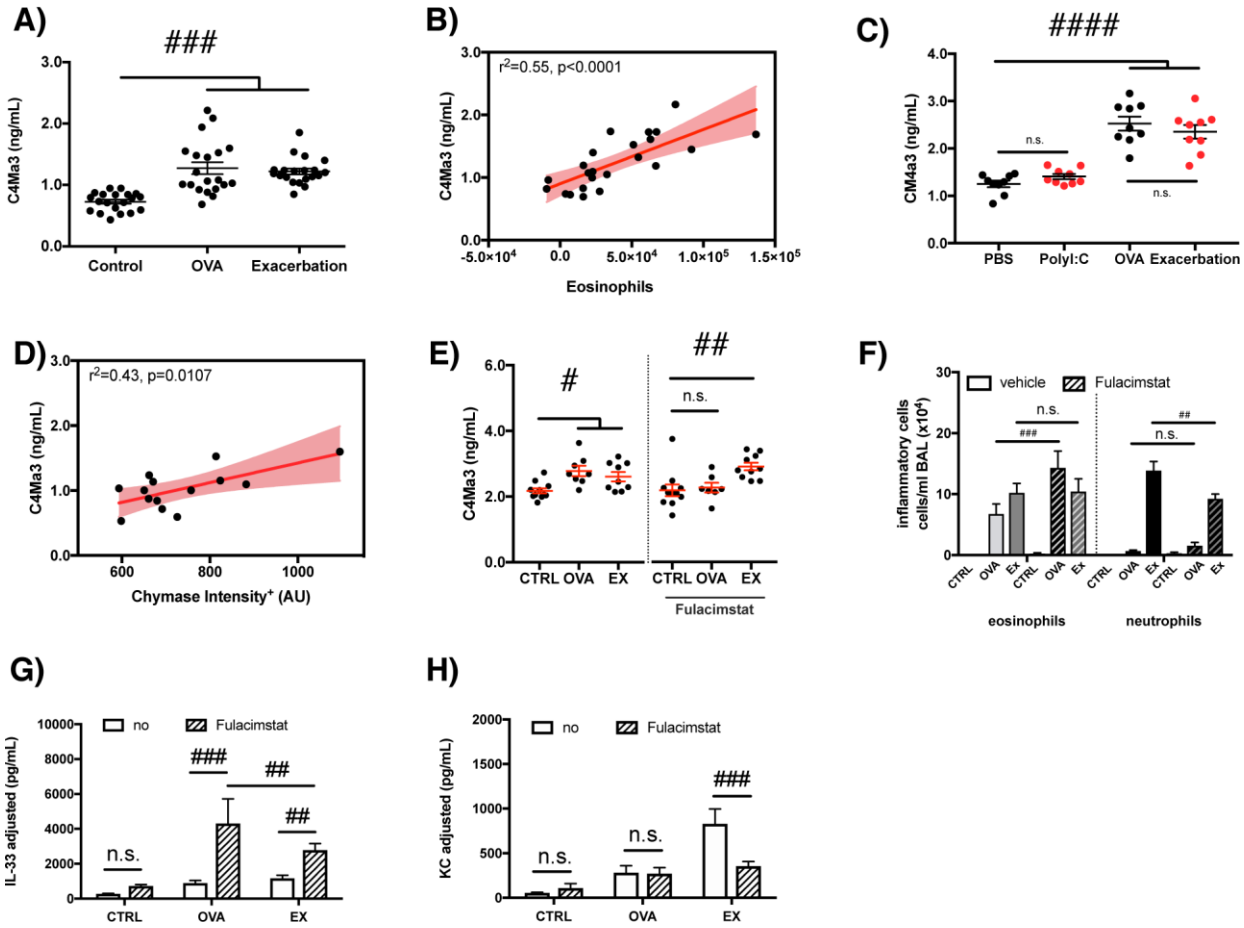

Figure 3: Mouse model of acute allergic airway disease shows increased COL4A3 degradation. A) OVA treated animals have markedly increased serum levels of C4Ma3. COL4A3 degradation product was measured in control (challenge with PBS), OVA and OVA+PolyI:C (Exacerbation) animals, n=20 per group. B) Linear 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 immuno-histology staining of mast cell chymase correlated with C4Ma3 level in serum (r^2=0.43, p=0.0107, AU= Arbitrary Units). 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) Broncho-alveolar lavage cytology of (eosinophils and neutrophils) of Fulacimstat model. G) IL-33 cytokine concentrations in lung lysate of Fulacimstat model. H) KC 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), n=8-10 per group. Unless otherwise specificed: Mean±SEM. Kruskal-Wallis with Dunn's post test, n.s. = not significant, #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001.

Figure 4

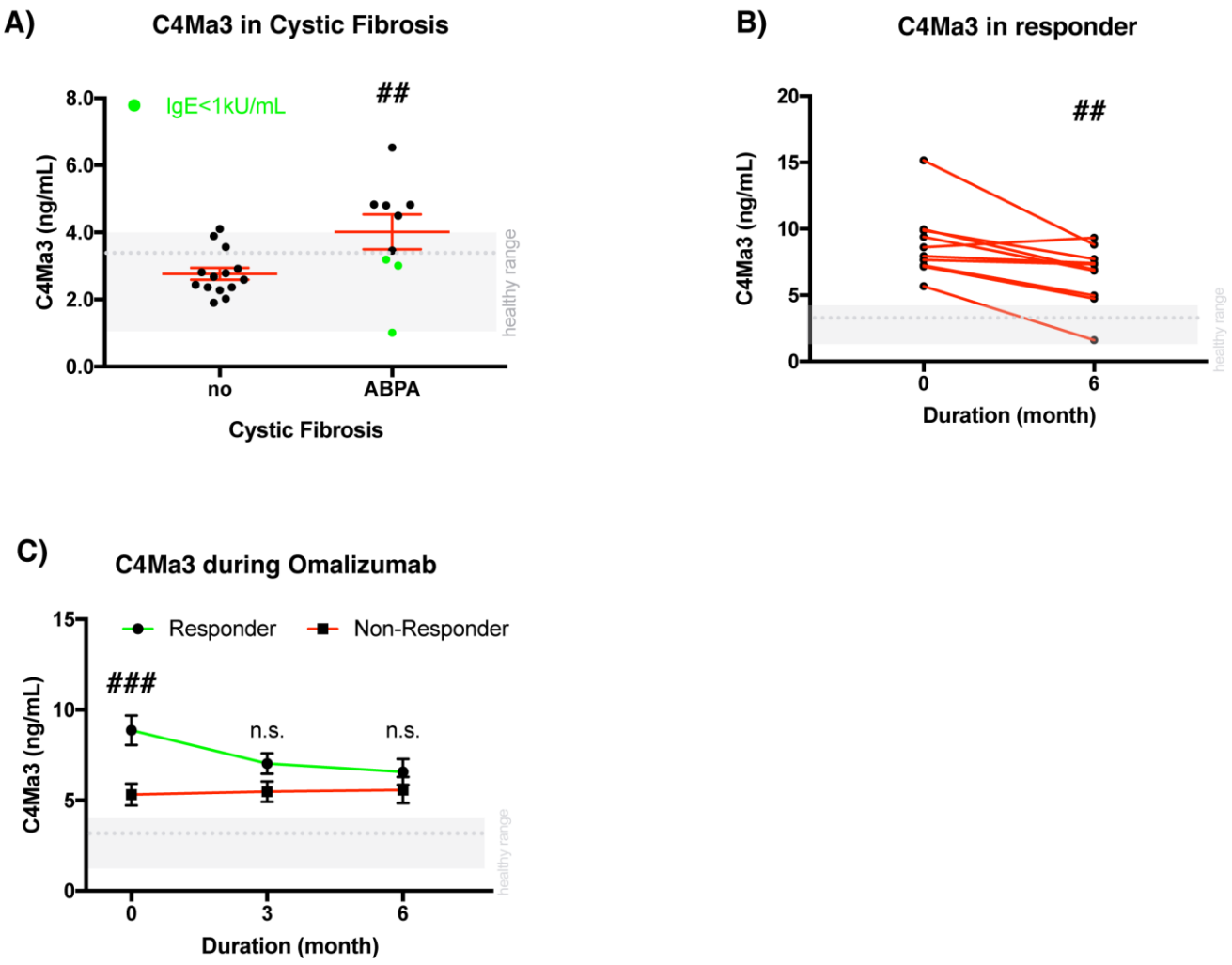

Figure 4: C4Ma3 serum levels are elevated in allergic lung disease and are responsive to Omalizumab treatment. A) Serum levels of C4Ma3 of cystic fibrosis patients with and without allergic broncho-pulmonary aspergillosis (ABPA). Green symbols denote total IgE serum levels <1kU/mL. Analysis was performed with Wilcoxon on ranks of age-adjusted values ##p<0.01. 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 or more. Patient individual time course from prior (0 month) and at finish of therapy monitoring (6 months). Analysis was performed with Wilcoxon on ranks of age-adjusted values ##p<0.01. C) Comparison of average circulating C4Ma3 serum levels in responder and non-responder during duration of therapy monitoring (0 = baseline, 3 months, 6 months). T-test analysis with Holm-Sidak correction for multiple testing; ###p<0.001, n.s. = not significant. Dotted grey line represents mean level of C4Ma3 in control subjects. Grey shaded are represents the 99% CI of C4Ma3 of control subjects.

Tables

Table 1: Demographics of ALLIANCE adult cohort:

	Controls	Asthma	
ICS	NA	Yes	No
N	31	130	19
Age	40.26	50.82	51.16
Female (%)	64.5	45.4	57.9
Atopy (%)	32.3	73.8	94.7
FEV1% pred	104.97 (7.7)	81.46 (21.65)**	88.78 (20.45)*
FEV1/FVC	0.79 (0.07)	0.64 (0.12)**	0.66 (0.11)**
FeNO (ppm)	16.08 (6.43)	33.16 (33.85)**	32.56 (25.04)**
Systemic CS	0	23	0
Disease Duration	NA	22.18 (15.61)	22.74 (17.38)
Fluticasone Equivalent (mg/day)	NA	680 (500)	0
Smoking (% current, pack years)	0, 0	7, 24.1	21, 36.5

** $p<0.001$, * $p<0.05$ for asthma (± ICS) vs. controls. Percentage atopy, age, percentage women, FEV1% predicted (pred) and FEV1/FVC are displayed as mean. Values in brackets are standard deviation.

ICS = inhaled corticosteroids, NA = not applicable, N = number, FEV1% = Forced expiratory volume in 1 second (percentage of predicted), FEV1= Forced expiratory volume in 1 second, FVC = Forced vital capacity, FeNO = exhaled nitrogen-oxide, Sytemic CS = systemic corticosteroids.

Table 2: Demographics of ALLIANCE pediatric cohort:

Disease status	Controls	Asthma	
ICS	NA	Yes	No
N	35	84	50
Age	10.88	11.80	12.46
Female (%)	48.6	32.1	32.0
Atopy (%)	29.0	73.8	94.7
FEV1% pred	97.02 (10.49)	92.00 (12.34)	94.18 (13.17)
FEV1/FVC	0.88 (0.06)	0.82 (0.09)***	0.82 (0.07)***

***p<0.0001, for asthma (± ICS) vs. controls. Percentage atopy, age, percentage women, FEV1%predicted and FEV1/FVC are displayed as mean. Values in brackets are standard deviation. ICS = inhaled corticosteroids, NA = not applicable, N = number, FEV1% = Forced expiratory volume in 1 second (percentage of predicted), FEV1= Forced expiratory volume in 1 second, FVC = Forced vital capacity.

Table 3: Demographics of Omalizumab proof-of-principle trial:

	Baseline		3 Month		6 Month	
Responder Type	Non-Responder	Responder	Non-Responder	Responder	Non-Responder	Responder
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				
Tot. IgE (IU/mL)	640	367				
Eosinophils	445 (463)	437 (206)	332 (484)	393 (267)	202 (132)	197 (151)
FEV1% 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)	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)

Baseline, follow-up 3 and 6 months. *p<0.5, **p<0.01 (responder vs. non-responder); Age, percentage women, FEV1%predicted, ACT are displayed as mean. Values in brackets are standard deviation. N = number, FEV1% = Forced expiratory volume in 1 second (percentage of predicted); Tot. IgE = total serum IgE; ACT = asthma control test score.

Supplement

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

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Supplement methods:

Animal Experiments

Animal Treatment Protocol

OVA and PolyI:C model:

Mice were sensitized to OVA by three intraperitoneal (i.p.) injections of 10 µg of OVA (OVA grade VI, Sigma, Deisenhofen, Germany) adsorbed to 150 µg of aluminum hydroxide (Imject alum, Thermo, Rockford, Illinois, U.S.) on days 1, 14 and 21. Mice were exposed three times to an OVA (OVA grade V, Sigma) aerosol (1% wt/vol in PBS) on days 26, 27 and 28 in order to induce acute allergic airway inflammation. PolyI:C challenge was introduced on day 28 with 200µg in 50µL PBS via aerosol.

Chymase Inhibitor treatment:

50µg Fulacimstat (BAY1142524, MCE, Germany) was administered intra-tracheally on days 25 (prior to OVA challenge), 26, 27 and 28 via aerosol. Fulcamistat was dissolved in dimethyl-sulfoxide (DMSO, Sigma, Germany) at a concentration of 500µg/mL and diluted in PBS for inhalation. Vehicle for inhalation: 10% DMSO in PBS.

All animals were sacrificed by cervical dislocation under deep anesthesia on day 29. Negative control animals were sham-sensitized to PBS and subsequently challenged with OVA aerosol (PBS group).

HDM model:

Female 8-12 weeks old WT B57BL/6 mice were sensitized by i.p. injection of 10 µg HDM extract from *Dermatophagoides pteronyssinus* (Greer Laboratories Inc., Lenoir USA, Lot-#: 262538) in 100 µl PBS on day 0 and 7. This was followed by an intra-tracheal (i.t.) allergen challenge with 100 µg HDM in 50 µl PBS on day 14 and 21.[1] Mice were sacrificed 72 hours after the last allergen challenge for blood sampling and organ removal. The study was approved by the Schleswig-Holstein state authorities (V 242 - 24572/2018 (44-5/18)).

Recombinant human Chymase treatment

Balb/C mice were given 10µg recombinant human mast cell chymase (active, Sigma Aldrich, Germany) in 50µL PBS and sacrificed 24h later..

Precision-cut-lung-slices

Human lung slices

Human lung lobes were obtained from patients who underwent lobectomy for lung cancer. Experiments were approved by the ethics committee of the Medical School Hannover (MHH,

Hannover, Germany) and are in accordance with The Code of Ethics of the World Medical Association (number 2701-2015). Human PCLS were sliced into approx. 300 µm thick lung sections as described before. [2] After PCLS preparation, two PCLS per well were incubated overnight with either 1% allergic plasma or culture medium. Allergic plasma was removed the next day and replaced by culture medium for 24h. Then collected supernatant was stored at −80°C for C4Ma3 analysis.

Mouse lung slices

Balb/c mice were sensitized by intranasal application of 25µg house-dust mite (HDM) in 50 µL of PBS (control group received PBS only) for four days per week, over four weeks, and used 24h after the last challenge for the preparation of precision-cut lung slices. Lungs from HDM-sensitized or PBS-treated mice were inflated using 1.5% agarose/medium solution and polymerized on ice. Slices of 350 µm were cut in 4°C cold EBSS, using an automatic oscillating tissue slicer (OTS 5000, Warner Instruments, CT, USA) and transferred into a medium filled petri dish. The medium was exchanged at least four times every 30 min for 2–3h to remove cell debris under cell culture conditions (37°C, 5% CO_2, and 100 % humidity). Two PCLS per well in duplicates were cultured in medium or stimulated with 100 µg/mL polyI:C for 48h, supernatants of duplicates were pooled and stored at −80°C for C4Ma3 analysis.

Bronchoalveolar Lavage

Lungs were flushed with 1 ml of sterile ice-cold PBS containing protease inhibitor (Complete, Roche, Basel, Switzerland) via a tracheal canula, and obtained cells were counted using a Countess automated cell counter (Life Technologies, Darmstadt, Germany). 50 µl-aliquots of lavage fluids were cytospun, stained with Diff-Quick (DADE Diagnostics, Unterschleissheim, Germany) and cells were differentiated microscopically according to morphologic criteria.

Determination of Airway Responsiveness

24 h after the last OVA challenge airway responsiveness was assessed by performing a methacholine (MCh, acetyl-β-methyl choline, Sigma, St. Louis, MO, USA) provocation test, while central airway resistance was recorded using a Buxco FinePoint R/C system (DSI-Buxco Electronics, Sharon, CT, USA). Briefly, mice were anaesthetized with ketamin and xylazin and neuromuscular activity was blocked with pancuronium bromide (1 mg/kg; Sigma). Tracheostomized mice were ventilated mechanically through a tracheal canula that was attached to the FinePoint R/C system. Airflow and transpulmonary pressure were recorded continuously to calculate lung resistance (RL) (cm H_2O/mL/s) and dynamic lung compliance (mL/cm H_2O) in each breath cycle. Mice were allowed to stabilize for

5 min before MCh provocation testing with increasing concentrations of MCh (3.125, 3.25, 12.5, 25, 50, and 100 mg/mL) aerosolized for 5 min each. Baseline pulmonary parameters were assessed with aerosolized phosphate-buffered saline (PBS). Expressed results comprised the mean absolute values of the responses of lung resistance recorded during 5 min after the inhalation of each MCh aerosol. Mice received terbutaline (10 µg/kg; terbutaline-hemisulfate, Sigma) intranvenously to solve MCh-induced broncho-spasm prior BAL and preparation of the lung.

Lung Histology

Lungs were fixed *ex situ* with 4% (wt/vol) paraformaldehyde (PFA) via the trachea under constant pressure, removed and stored in 4% PFA. Fixed lung tissues were embedded in paraffin. Subsequently, 2 µm tissue sections were stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS), respectively. Photomicrographs were recorded by a digital camera (DP-25, Olympus, Tokyo, Japan) attached to a microscope (BX-51, Olympus) at 40- and 100-fold magnification using Olympus cell^A software. For mucus quantification, systematic uniform random samples of the lungs were prepared according to standard methods including the orientator technique. The surface area of mucin-containing goblet cells (Sgc) per total surface area of airway epithelial basal membrane (Sep) and the volume of PAS-stained epithelial mucin (Vmucin) per Sep were determined using a computer-assisted stereology tool box (newCAST, Visiopharm, Hoersholm, DK) according to the following formulas:

$$\frac{S_{gc}}{S_{ep}} = \frac{\Sigma_{Igc}}{\Sigma_{Iep}} \text{ and } \frac{V_{mucin}}{S_{ep}} = LP \cdot \Sigma \frac{P_{mucin}}{2} \cdot \Sigma_{Iep}$$

where ∑Igc is the sum of intersections of test-lines with goblet cells, ∑Iep is the sum of all intersections of test-lines with the epithelial basal membrane, ∑Pmucin is the sum of all points hitting mucin and LP is the test-line length at final magnification.

Immunohistochemistry

Paraffin embedded mouse lungs were cut at 4µm. Antibody against MMP9 (1/200) (Abcam (ab38898)), antibody against mMCPT4 (1/200) (Biomol (M2414-20)) and antibody against Tryptase (1/100) (R&D (MAB1937)). Sections were deparaffinised and EDTA buffer (MMP9), Tris-HCL buffer (Chymase) and Tris-EDTA buffer (Tryptase) and heating (8-min boiling in microwave oven) were used as antigen-retrieval methods. Endogenous peroxidase activity was blocked by incubation with 0.3% (volume/volume) H_2O_2 for 30 min. Subsequently, sections were incubated with a primary antibody diluted in PBS supplemented with 1% (weight/volume) bovine serum albumin for 1 hour at room temperature. Sections were incubated with a secondary antibody (1/100) (DAKO) for 30 min at room temperature. After each incubation step, sections were rinsed in PBS for 5 min. Peroxidase activity was demonstrated by application of 3,3'- diaminobenzidine (Sigma, St. Louis, MO, USA)

containing 0.03% H_2O_2 for 15 min. Sections were counterstained with Mayers haematoxylin, dehydrated and mounted with mounting medium (Merck, Darmstadt, Germany). A non-specific antibody as a negative control was used. The stained slides were scanned on a Hamamatsu scanner and analyzed on the Aperio ImageScope software. The positive pixel count v9 Algorithm was used under default settings.

Lung Lysate

Lungs were dissected from mice and snap-frozen in liquid nitrogen. Deep frozen lungs were homogenized with mortar and pestle. Lung powder was transferred into RIPA buffer, incubated for 40 minutes and centrifuged for 20 minutes at 4 °C. Protein containing supernatants were collected. Protein concentrations were determined with Pierce BCA Protein Assay Kit according to manufacturer's guidelines (Thermo Fisher Scientific, MA, USA).

Mesoscale Cytokine Assays

IL-4, -5, -6, -8, -13, Eotaxin, and IFN-γ concentrations were measured with U-Plex Assay Kit according to manufacturer's guidelines (Meso Scale Diagnostics, MD, USA). Cytokine concentrations were normalized to protein concentrations as determined by Bicinchonic Acid (BCA, Pierce, ThermoFisher, Germany).

Serological determination of COL4A3 degradation

A competitive ELISA using a monoclonal antibody raised against a degradation fragment of the COL4A3 was used to assess C4Ma3 levels in human EDTA plasma or mouse serum by the following procedure: Streptavidin-coated microtiter plates (cat. no. 11940279, Roche Diagnostics, Hvidovre, Denmark) were coated with 100µL/well of 1.25ng/mL biotinylated peptide (PGDIVFRKGP-K-biotin) diluted in assay buffer (25mM PBS-BTB, 2g/L NaCl, pH 7.4) and incubated for 30 minutes. Standard peptide (PGDIVFRKGP), quality control samples, or samples of interest (20µL/well) were added in double determinations. Subsequently, 100 µL/well of 100ng/mL HRP-labeled monoclonal antibody diluted in assay buffer was added, and plates were incubated for one hour. Following incubation, 100µL/well of 3,3',5,5'-tetramethylbenzidine (TMB) was added and plates were incubated for 15 minutes in the dark. To stop the enzyme reaction of TMB, 100µL 0.1% sulphuric acid was added and the absorbance was measured at 450nm with 650nm as the reference using an ELISA reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). All incubation steps were performed at 20°C with shaking at 300rpm and followed by five washes (20nM TRIS, 50mM NaCl, pH 7.2). The standard peptide had a starting concentration of 100ng/mL and was diluted 2-fold to create an 11

points calibration curve with the last point consisting of assay buffer only. A calibration curve was plotted using a 4-parametric mathematical fit model. Each ELISA plate included five quality control samples to monitor intra- and inter-assay variation.

BioRad Bioplex cytokine measurements

Serum levels of 27 cytokines (BioRad, Munich, Germany) were measured using Bio-Plex MAGPIX Multiplex Reader (Bio-Rad, Munich, Germany) according to manufacturer's recommendations. In brief, samples were defrosted at 4°C and 25µL was pipetted in doublets per patient. Concentration range varied for each cytokine and can be obtained from biorad.com. Standard curve fitting was performed using Bio-Plex Manager MP software (Bio-Rad, Germany, Version 1.0.0.03). A five parameter (5PL) fit with logistic weighting based on logarithmic coefficient of variance was used throughout. Panel of ten serum cytokines and chemokines reflective of type 1 (IL-12p70, IFN-γ, IL-17A, IL-6, IL-8) or type 2 (IL-4, IL-13, IL-5, eotaxin, IL-9) profiles. Only concentrations in range were used for further calculations. For statistics all cytokine data were log-transformed and rank based non-parametric tests were used (including correction for multiple comparison).

Supplement figure legends

Supplemental Figure S1: A) ALLIANCE cohort flow chart B) Treatment protocol OVA/PolyI:C with and without Fulacimstat; Mice were sensitized to OVA by three intraperitoneal (i.p.) injections of 10 µg of OVA (OVA grade VI, Sigma, Deisenhofen, Germany) adsorbed to 150 µg of aluminum hydroxide (Imject alum, Thermo, Rockford, Illinois, U.S.) on days 1, 14 and 21 as previously described [3]. Mice were exposed three times to an OVA (OVA grade V, Sigma) aerosol (1% wt/vol in PBS) on days 26, 27 and 28 in order to induce acute allergic airway inflammation. PolyIC challenge was introduced on day 28 with 200µg in 50µL PBS via aerosol. Chymase Inhibitor treatment: 50µg Fulacimstat (BAY1142524, MCE, Germany) was administered intra-tracheally on days 25 (prior to OVA challenge), 26, 27 and 28 via aerosol. Fulcamistat was dissolved in dimethyl-sulfoxide (DMSO, Sigma, Germany) at a concentration of 500µg/mL and diluted in PBS for inhalation. Vehicle for inhalation: 10% DMSO in PBS. All animals were sacrificed by cervical dislocation under deep anesthesia on day 29. Negative control animals were sham-sensitized to PBS and subsequently challenged with OVA aerosol (PBS group). C) Levels of C4Ma3 in asthmatics were plotted against the effective airway resistance. Linear regression fit quality is represented by r_s (Spearman coefficient) D) C4Ma3 serum levels decreased in control individuals over age. Fitted line displays exponential decrease function with 95% confidence intervals for fitting, r^2=0.48, red bands indicate 95% CI.

Supplemental Figure S2: Type 1 and type 2 serum cytokine levels of asthmatic patients with <4ng/mL or >4ng/mL C4Ma3 level. Y-axis log_2 transformed; Analysis was performed with Wilcoxon on ranks of log_{10} transformed cytokine values with FDR p-value correction. A) Serum IL-4 levels, B) Serum IL-5 levels, C) Serum eotaxin levels, D) Serum IL-8 levels, E) Serum IL-12p70 levels, F) Serum IL-17 levels, G) Serum IL-6 levels, H) Serum IL-9 levels, I) Serum IL-13 levels, J) Serum IFN-γ levels. n.s. not significant, $p<0.05$, $**p<0.01$.

Supplemental Figure S3: A) BAL cytology of control, OVA, and exacerbation groups. Total cell numbers are displayed, macrophages (clear), lymphocytes (light grey), eosinophils (dark grey), neutrophils (black), n=16 per group. B) Serum of mice sensitized and challenged intra-nasally with house-dust mite allergen and compared to PBS challenged littermates was analyzed using the C4Ma3 neo-epitope assay for COL4A3 degradation. PBS n=8; n=9 HDM; Mean±SEM; Kruskal-Wallis with Dunn's post test ** $p<0.01$; C) Comparison of C4Ma3 release from allergic lung sections. Supernatant of 48h incubation of precision-cut lung slices from mice sensitized and challenged intra-nasally with house-dust mite allergen and compared to PBS challenged littermates. PBS n=5; HDM n=5; Mean±SEM; Kruskal-Wallis with Dunn's post test, * $p<0.05$ D) Lung function (resistance) measurement mice. C4Ma3 was log transformed (Spearman $r_s=0.34$, $p<0.01$). E) Compliance negatively correlates (Pearson correlation coefficient r) with increasing levels of C4Ma3 serum values in mice. Due to non-normal distribution C4Ma3 values were log transformed. F) Bronchial surface coverage with mucus producing cells as assessed by CAST ($r^2=0.60$, $p<0.001$). G) Total lung expression of MMP2 (-ΔCt vs. β-actin (ACTB) expression, lower -ΔCt values are representative of lower expression) in correlation with C4Ma3 serum level in mice. No significant (n.s.) correlation was observed. H) Total lung expression of MMP9 (-ΔCt vs. β-actin (ACTB) expression, lower -ΔCt values are representative of lower expression) in correlation with C4Ma3 serum level in mice. No significant (n.s.) correlation was observed. I) Immuno-histology for MMP9 in lung sections in correlation with C4Ma3 serum level in mice. No significant (n.s.) correlation was observed. J) mMCPT4 histology in mouse lung sections. Paraffin-embedded mouse lung sections were stained for mMCPT4. All sections counterstained with haematoxylin. Boxed areas are magnified and black arrows indicate mMCPT4 positive cells. Overall specific staining intensity was calculated according to positive pixel count algorithm v9 from Aperio ImageScope software. Scale bar: 20µm K) Immuno-histology for mast cell tryptase in lung sections in correlation with C4Ma3 serum level in corresponding mice. No significant (n.s.) correlation was observed. L) Broncho-alveolar cytology (macrophages, lymphocytes, eosinophils and neutrophils) of Fulacimstat treatment model. M) Airway resistance of Fulacimstat treatment model. Indicated in red: Treated animals; significant indicator * denotes comparison between PBS and exacerbation groups; # indicates comparison between treated and vehicle groups. N) IL-6 protein

concentration in lung lysate (pg/mL) adjusted to total protein content of sample. # $p<0.05$ O) Recombinant human mast cell chymase was administered intra-tracheally to naïve mice and after 24h serum levels of C4Ma3 were measured. No significant increase detected. P) IL-4 protein concentration in lung lysate (pg/mL) adjusted to total protein content of sample. Q) IL-5 protein concentration in lung lysate (pg/mL) adjusted to total protein content of sample. # $p<0.05$ R) IL-13 protein concentration in lung lysate (pg/mL) adjusted to total protein content of sample. S) Eotaxin protein concentration in lung lysate (pg/mL) adjusted to total protein content of sample. # $p<0.05$, ### $p<0.001$ T) IFN-γ protein concentration in lung lysate (pg/mL) adjusted to total protein content of sample. # $p<0.05$, ### $p<0.001$ U) Percentage of mucus producing cells covering basal membrane of bronchus. ### $p<0.001$. Unless otherwise specified: Mean±SEM. Kruskal-Wallis with Dunn's post test, n.s. = not significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****, #### $p<0.0001$.

Supplemental Figure S4: C4Ma3 serum levels at baseline in asthmatics vs. controls prior anti-IgE therapy and Receiver-Operator-Curve of C4Ma3. A) Baseline serum level of C4Ma3 of asthmatics, who did (Res) or did not (Non-Res) respond to Omalizumab therapy as measured by an increase of the asthma control test score of 3 points or more after six month. Controls (n=31) were compared to Res (n=10) and Non-Res (n=9) with Kruskal-Wallis on ranks and Dunn's post test; # $p<0.05$, #### $p<0.0001$ B) Receiver-Operator-Curve of C4Ma3 for prediction of responders (increase of 3 points or more) after a six month intervention with anti-IgE therapy. Sensitivity vs. 1-Specificity. AUC=0.92; C) Proposed Model of chymase activity leading to degradation of COL4A3 in asthma. In allergic asthma, epithelial derived interleukin (IL-) 33 drives via mediators (dashed line) the expansion of T helper type 2 cells (Th2), which produce e.g. IL-4 and IL-13 that drive the B-cell class switch to allergen-specific Immunglobuline (Ig) E. Release of IgE and exposure to allergen leads to the activation and degranulation of mast cells. Th2 cells also produce IL-5, which is responsible for recruitment of eosinophils to the lung tissue. An additional mechanism for the recruitment of eosinophils is the expression of eotaxin by lung fibroblasts after IL-33 stimulation. After allergen exposure, mast cell degranulation leads to the release of mast cell proteases such as chymase (or the murine homologue mMCPT-4), which not only degrades IL-33 and thereby limiting its aggravating effects but also leads to COL4A3 degradation and possible depletion of the matrikine (tumstatin) reservoir. The degradation fragments (e.g. C4Ma3) are detectable in serum and a marker of mast cell chymase activity in the lung. Inhibition of mast cell chymase with Fulacimstat stops the degradation of IL-33 and COL4A3, and results in an increase of eotaxin expression and elevated eosinophilia. In addition, because the IL33 increase does not directly affect Th2 cytokines (IL-4, IL5, IL-13) in the acute model, the association between serum levels of COL4A3 degradation product, eosinophila and Th2 cytokines is lost. Blocking mast cell activation with anti-IgE therapy may also result in the inhibition of COL4A3

degradation, but subsequently the overall allergic inflammation is reduced and the correlation between COL4A3 degradation product and allergic inflammation in the lung is maintained.
In asthma exacerbations such as after a viral infection (or via PolyI:C in our model), IL-6 and IL-8 are produced by the epithelium via Toll-like-receptor 3 activation. IL-6 is known to aggravate the underlying allergic inflammation boosting eosinophila, mucus production and bronchial hyperresponsiveness. IL-8 is a potent chemokine for neutrophils, which are a source of (pro-) matrix-metallo-protease (MMP9). Blocking of chymase in an acute asthma exacerbation leads to the increase of IL-33 and continuous expression of eotaxin. Therefore, no change in eosinophilia occurs. At the same time and because of an unknown mechanism, IL-6, IL-8 and to some degree IL-13 are reduced. This results in a reduction but not normalization of bronchial hyperresponsiveness and neutrophilia. In this scenario the inhibition of chymase is advantageous as compared to the non-exacerbated state (see above). The presence of neutrophil derived (pro-)MMP9 and another mast cell protease (tryptase), which are known to act in concert to degrade COL4 [4] is however not impacted by inhibition with Fulacimstat. This may therefore lead to unchanged levels of the COL4A3 degradation product in serum (indicated by dashed lines in the model). The treatment with anti-IgE however affects the release of both mast cell proteases and consequently, degradation of COL4A3 in asthma exacerbation would be inhibited. In this case, the COL4A3 fragment again serves as a biomarker the severity of the inflammation in the lung. The inhibition of the degradation has several beneficial effects. COL4A3 harbors a matrikine (tumstatin) which has been shown to stop inflammation-induced angiogenesis [5, 6], which is strongly increased in asthma and believed to contribute to the stiffness of the airways. Furthermore, tumstatin decreases the infiltration of eosinophils and neutrophils and decreases mucus production, leading to reduced tissue damage and airway narrowing.[6, 7] Last but not least, COL4A3 is an important constituent of the basal membrane of the lung. Blocking the degradation of COL4A3 would prevent this particular form of basal membrane remodeling.

Supplementary references:

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Supplemental Tables

Supplemental table S1: Univariate analysis of association of clinical variables

Variable	CC	p-value
Sputum Eosinophils (log, percentage)	0.27	**0.0007**
Exacerbations (no, one, > one)	0.25	**0.0060**
Exhaled NO (log concentration, ppm)	0.20	**0.0104**
Blood Eosinophils (log, absolute numbers)	0.14	0.0704
Gender (m/f)	0.14	0.0709
disease duration (log years)	0.14	0.0862
Cummulative sIgE (log sum)	0.08	n.s.
Age (years)	0.06	n.s.
Number of sensitisations	0.05	n.s.
Atopy (y/n)	-0.03	n.s.
Systemic Corticosteroids (y/n)	0.03	n.s.
Smoking packyears (log years)	-0.05	n.s.
Fluticasone Equivalent (log dose, mg)	0.00	n.s.

CC =pairwise correlation coefficient; Exhaled NO = exhaled nitric oxide;

Supplemental table S2: Summary of Linear Regression Model:

Variable	Estimate	SE	t Ratio	p-value
Gender (m/f)	0.769	0.208	3.71	0.0006*
disease duration (log years)	0.412	0.111	3.7	0.0006*
Exhaled NO (log concentration, ppm)	0.473	0.146	3.25	0.0023*
Atopy (y/n)	0.832	0.300	2.78	0.0083*
Systemic Corticosteroids (y/n)	0.597	0.309	1.94	0.0599
Smoking packyears (log years)	0.111	0.099	1.12	0.2696
Sputum Eosinophils (log, percentage)	0.020	0.022	0.9	0.3746
Age (years)	0.002	0.009	0.2	0.8458
Intercept	-0.100	1.262	-0.08	0.9373
Fluticasone Equivalent (log dose, mg)	-0.004	0.152	-0.03	0.9799

*indicates significant variable in linear regression model. SE = standard error of estimate; Linear regression model characteristics: r^2=0.51, p=0.0002. Exhaled NO = exhaled nitric oxide; Estimates indicate direction and quantity of effect size.

Supplemental table S3: Cystic Fibrosis Patients with and without allergic broncho-pulmonary aspergillosis:

Cystic Fibrosis	Allergic Broncho-Pulmonary Aspergillosis	
	Yes	No
N	9	14
Women (%)	66	43
Age (years)	18.07 (7.93)*	9.94 (9.94)
FEV1% pred	61.89 (25.94)**	97.5 (14.68)
IgE (IU/mL)	1481 (1246) ***	36 (50.83)
Highest IgE (IU/mL)	2394 (1433)****	83 (137)

N = number, FEV1% = Forced expiratory volume in 1 second (percentage of predicted); IgE = total serum IgE; BMI = body mass index; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 (yes vs. no; value in brackets are standard deviations.

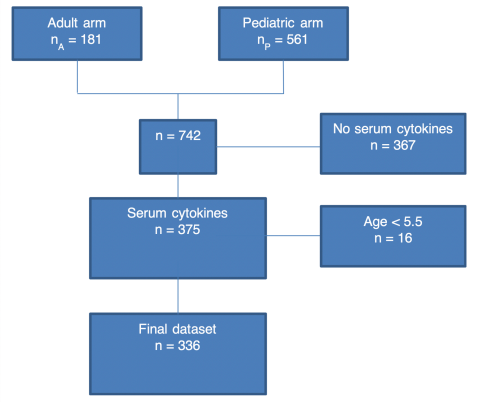
Supplemental table S4: C4Ma3 baseline levels predict therapy response type:

Responder Type	Responder	Non-Responder
N	10	9
Predicted Responder	9	2
Predicted Non-Responder	1	7
Specificity	0.90	95% CI: 0.60 to 0.98
Sensitivity	0.78	95% CI: 0.45 to 0.94
DOR	31.5	95% CI: 2.35 to 422.30

DOR: Diagnostic Odds Ratio.

Supplement Figure S1

A)



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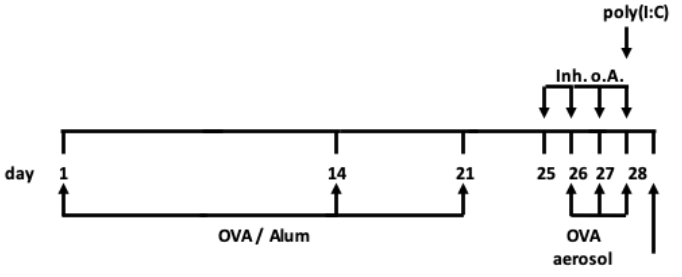

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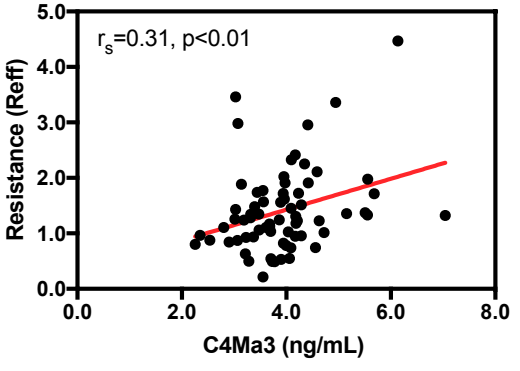

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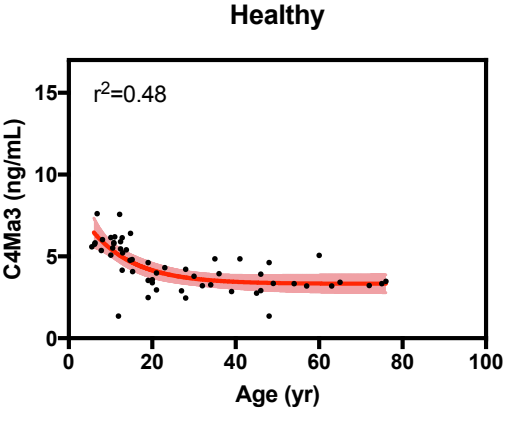
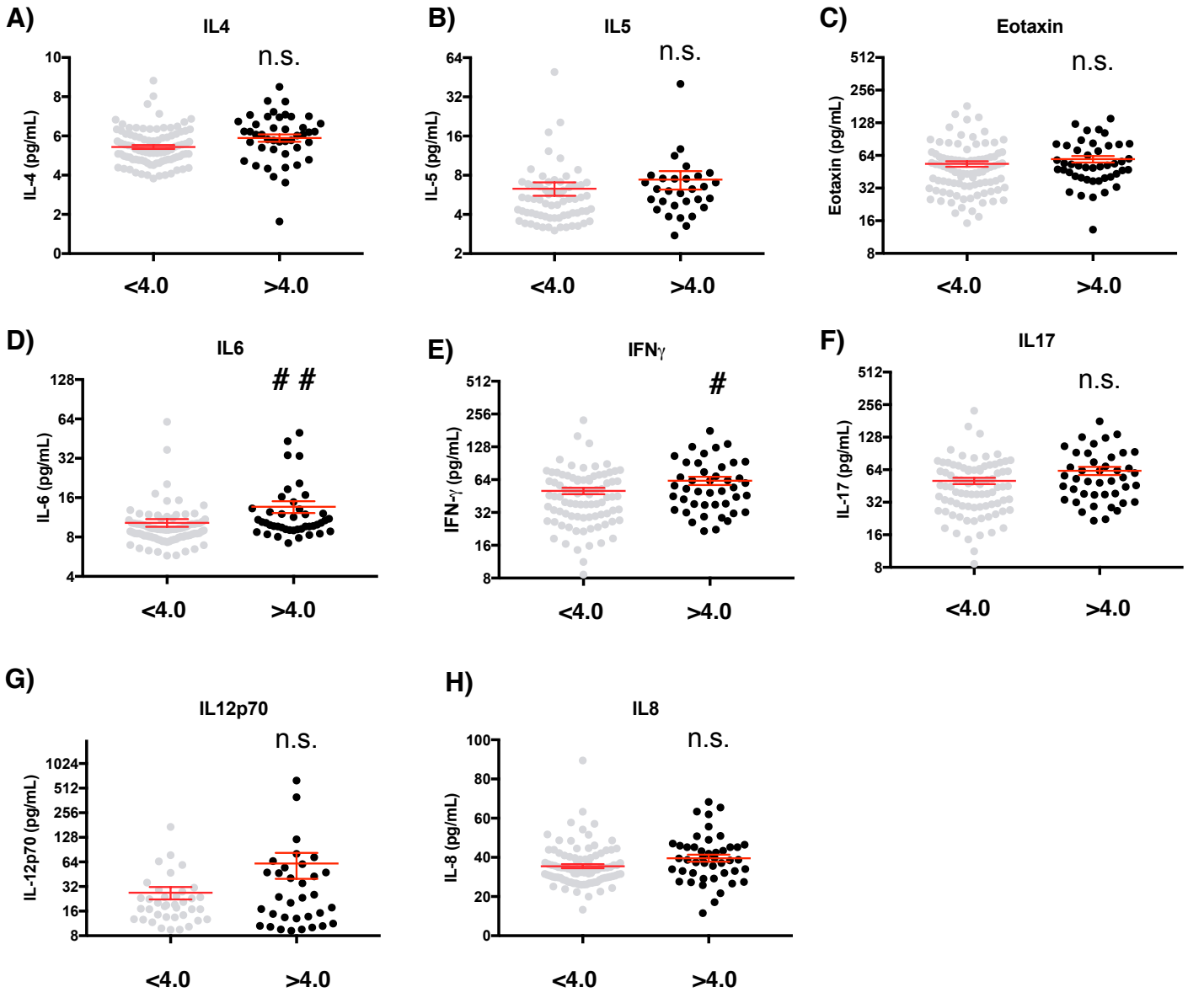
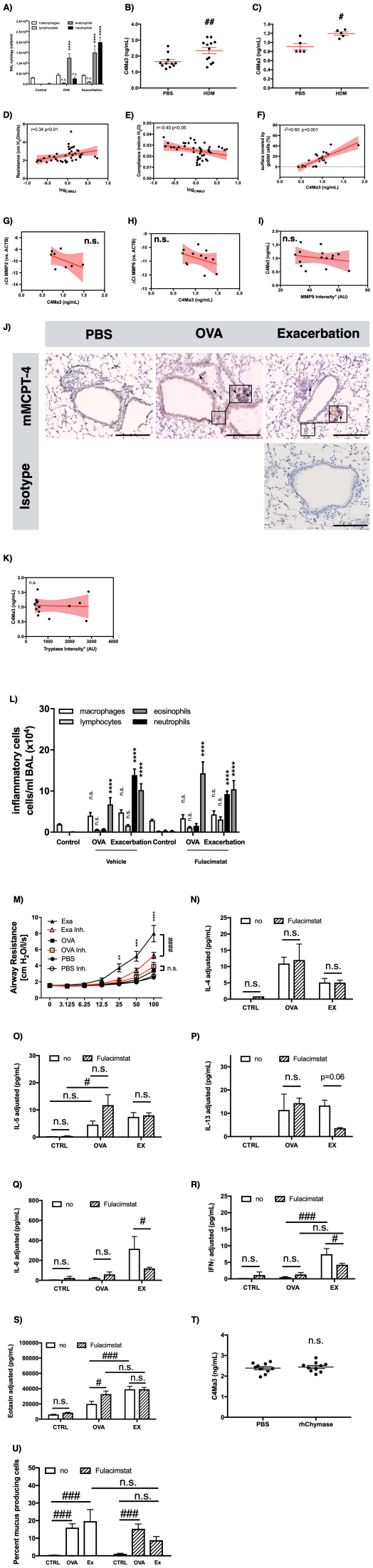


Supplement Figure S2

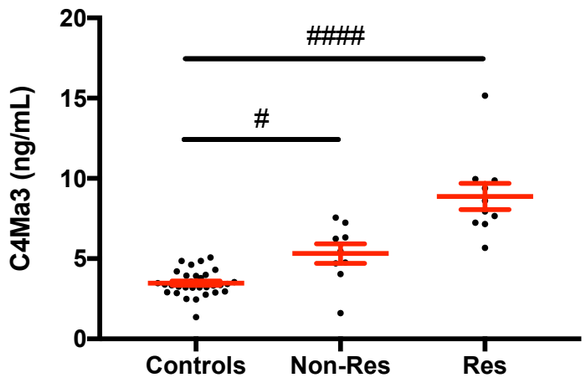


Supplement Figure S3



Supplement Figure S4

A)



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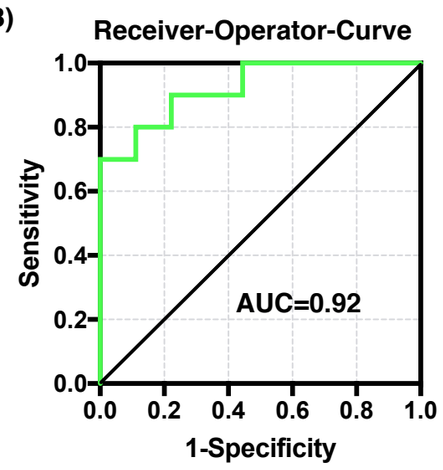

C) Chymase degradation of COL4A3