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A study of the relationship between local particulate pollution and exhaled nitric oxide in children with non-allergic asthma admitted to a high-altitude rehabilitation clinic.

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Abstract

Particulate pollution correlates with exhaled nitric oxide (FeNO; a measure of airway inflammation) in children with asthma. Non-allergic asthma represents a very small subset of this group and has different pathophysiological mechanisms from allergic asthma. This study examined the relationship between particulate pollution and FeNO in children with non-allergic asthma admitted to a high-altitude rehabilitation clinic at 1200m in Germany. No correlation was found. I was able to identify non-allergic asthma patients with clinically raised FeNO and to show that this value normalised over the course of a high-altitude admission. As a validation of the above results, I replicated the finding that FeNO decreases significantly during high-altitude rehabilitation in allergic asthma patients.

In Kindern mit Asthmaerkrankung ist ein zeitlicher Zusammenhang zwischen Feinstaubbelastung in der Luft und ausgeatmetem Stickstoffmonoxid (FeNO, ein Zeichen von Entzündungsprozessen in der Lunge) bekannt. Die Überzahl der Kinder, welche an Asthma bronchiale leiden, sind an der allergischen Form erkrankt. Nur sehr wenige Kinder leiden an nichtallergischem Asthma bronchiale. Die nichtallergische Form und die allergische Form unterscheiden sich in der Pathophysiologie. Diese Studie untersuchte das Verhältnis zwischen Feinstaubbelastung und ausgeatmetem FeNO in Kindern mit nichtallergischem Asthma, welche in einer pulmologischen Rehabilitationsklinik bei 1200 Höhenmetern aufgenommen waren. Es zeigte sich kein Verhältnis. In der Kohorte fanden sich aber Kinder mit nichtallergischem Asthma und klinisch erhöhtem FeNO. Bei diesen Kindern zeigte sich während des Klinik-Aufenthalts eine Reduktion im FeNO-Wert. Auch konnte das bereits bekannte Verhältnis zwischen verbrachter Zeit in der Höhe und reduziertem FeNO bei allergischen Patienten repliziert werden.

Part I

Introductory literature review: exhaled nitric oxide in asthma

1 Introduction

1.1 Abbreviations

NO: Nitric oxide

FeNO: Fraction of exhaled nitric oxide

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

NOS: Nitric oxide synthase

iNOS: Inducible nitric oxide synthase

eNOS: Endothelial nitric oxide synthase

nNOS: Neuronal nitric oxide synthase

NADP: nicotinamide adenine dinucleotide phosphate

NADPH: reduced form of NADP

FAD: flavine adenine dinucleotide

FMN: flavine mononucleotide

SNO: S-nitrosothiol

L-NMMA: N5-[imino(methylamino)methyl]-L-ornithine

L-NAME: L-NG-Nitro arginine methyl ester

PGE2: Prostaglandin E2

GINA: Global initiative for Asthma

COX: Cyclooxygenase

1.2 Introduction

In this study, I examine the hypothesis that increased exposure to airborne particle pollutants predicts a raised fraction of exhaled nitric oxide (FeNO) in paediatric patients with non-allergic asthma. A secondary aim is to test whether FeNO decreases during a high-altitude rehabilitation and to investigate whether this is associated with decreased particulate exposure. Finally, I aim to validate my findings by reproducing the known inverse relationship between FeNO and length of high-altitude rehabilitation in allergic asthmatic patients.

I will outline the physiology and mechanisms of action of nitric oxide in the lung and consider the role of particulates in the pathological elevation of FeNO in support of the hypotheses outlined above. The wider physiological effects of NO are beyond the scope of this project. For a detailed review, the reader is referred to Ricciardolo (2004)[77].

1.3 Nitric oxide in the asthmatic human lung

1.3.1 Asthma in children

Asthma is a common chronic non-infectious inflammatory condition of the lung. It is characterised by inflammation of the bronchial epithelium, excess mucous production and hyperreactive bronchial smooth muscle. This causes variable and reversible bronchoconstriction in response to varied triggers. The severity varies from extremely mild to life-threatening.

In children, asthma is a frequent cause of hospitalisation and physician contact. The majority of paediatric asthma exacerbations are triggered by allergies and respiratory infections. A small minority of children with asthma do not have allergies. The cause of their bronchial hyperresponsiveness is unclear [17].

Some patients with asthma have an unusually high number of eosinophils in their lung tissue and bloodstream. Patients with or without allergies can have pulmonary eosinophilia. Pulmonary eosinophilia is associated with exhaled nitric oxide levels in children[18].

1.3.2 Nitric oxide and asthma

Exhaled nitric oxide was found to be higher in asthmatic patients in the early 1990s [8]. It has since come into use a marker for eosinophilic lung inflammation in asthmatic patients, especially in children [27].

Nitric oxide (NO) has many physiological roles, and some pathophysiological effects, in the human lung [76, 77, 45].

1.3.3 Synthesis and regulation of nitric oxide in the human lung

There are three forms of nitric oxide synthase: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) [81]. These are produced by distinct genes located on different human chromosomes (12, 17 and 7 respectively) each with tissue-specific expression. All three isoforms of NOS are present in the respiratory system, and implicated in asthma. NNOS is found in the non-adrenergic, non-cholinergic nervous system, eNOS in the lung vascular endothelium from the nasal mucosa to the bronchi and alveoli, and in the basal membrane of ciliary microtubules. Finally, iNOS is expressed in alveolar macrophages, the epithelium of the proximal and terminal bronchioles, alveolar type II cells, lung fibroblasts, bronchial and vascular smooth muscle, mast cells, chondrocytes, endothelial cells and neutrophils [37, 80, 56, 77].

The substrates for all NO synthases are oxygen and L-arginine. L-citrulline, NO and hydrogen are produced by the conversion of NADPH to NADP+ with FAD and FMN cofactors [13]. L-arginine supply therefore controls the activity of NOS: for example, oral administration of arginine to humans increases exhaled NO in healthy subjects [53, 82]. L-arginine transport is stimulated in part by lipopolysaccharide. Arginine is competitively catabolised by arginase. Finally, NO is formed non-enzymatically from nitrite under acidic conditions, such as when the pH in the airways drops dramatically during an acute asthma attack [43]. NO synthesis and control is summarised in Fig.1.

1.3.4 The many physiological effects of NO on the human lung

NO counteracts several of the pathophysiological mechanisms that cause asthma. It causes smooth muscle relaxation and bronchodilation in the airway itself, and reduces the effects of metacholine provocation on bronchoconstriction [10, 40]. It promotes mucociliary clearance by up-regulating the MUC5AC mucin gene [85] and mediating a cytokine-induced increase in ciliary motility [24, 47, 14, 5].

NO is directly protective against viral infections, a frequent trigger of asthmatic exacerbations, because it denatures a critical viral replication protein (cysteine protease) [42]. In addition, the breakdown products, known as S-Nitrosothiols (SNOs), protect the airway against allergen-induced bronchospasm by altering the gating of epithelial K/Ca channels [75, 3]. In experiments, SNOs increase a short while after allergic antigens are introduced into the lungs bronchoscopically [26]. Severe asthma in children is associated with low concentrations of airway SNO, which contributes to bronchospasm [30]. In cultured human airway cells, NO prevents replication, which is likely to reduce airway remodelling in chronically inflamed lungs [36].

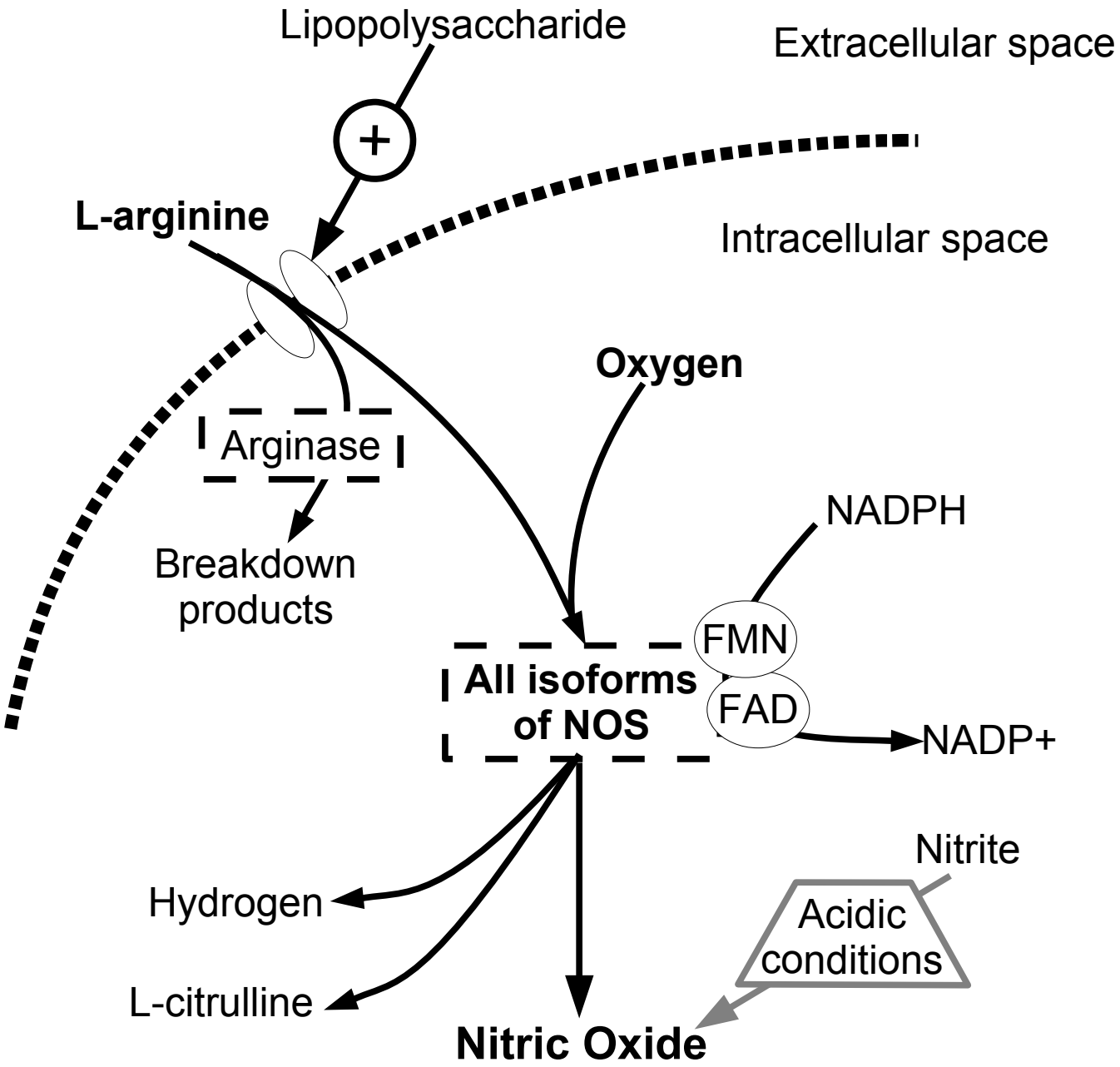
NO mediates neural control of airway patency by controlling the non-cholinergic non-adrenergic bronchodilatory neural pathway (iNANC) [15], the sole neural bronchodilator pathway [77]. This control is crucial in the smooth muscle spasm found in asthmatic individuals.

NO promotes inflammation. This may be useful in a healthy lung, prompting a suitable immune response to pathogens; in an asthmatic lung, though, it is likely to promote hyperreactivity to triggers. iNOS expression and the subsequent increase in NO activates the COX-2 (inducible) isoform to produce a heightened inflammatory response and upregulate the production of prostaglandins [81, 54]. Nonselective NOS inhibitors downregulate this response [81].

Upregulation of iNOS in mouse lung endothelium has a chemoattractive effect on neutrophils and eosinophils [87]. It is hypothesised that this effect also exists in the human lung. This is important because eosinophilic lung inflammation is a

Figure 1: Simplified NO synthesis

All isoforms of NOS use L-arginine as a substrate. The availability of L-arginine (and therefore the production of NO) is upregulated by the effect of lipopolysaccharide on transport across the cell surface membrane. Arginase competes with NOS to break L-arginine down, reducing its supply and thereby downregulating NO production. All NOS isoforms catalyse the production of NO by the transhydrogenation of NADPH to NADP+ using FMN and FAD cofactors, with hydrogen and L-citrulline as byproducts. A further mechanism for the production of NO is the conversion of Nitrite to NO under acidic intracellular conditions. (Image: Jennifer Hobbs, 2018)



hallmark of some types of asthma. NO release was previously believed to be an *effect*, rather than a *cause*, of eosinophilia in these asthmatic patients.

NO also has several other protective effects on the lungs. At high concentrations, it can kill tumour cells, halt viral replication [52, 39], and inhibit the growth of pathogens including *Mycobacterium tuberculosis* [21] and leishmaniasis [86]. Further effects of NO in animal models and humans include pulmonary vasodilation, reduction of histamine release (possibly due to mast cell stabilisation), inhibition of T-lymphocyte proliferation, and regulation of T-cell apoptosis [40, 6, 7, 46].

1.3.5 Pathophysiological effects of NO on the healthy human lung

In addition to its protective functions, NO at high concentrations has several harmful effects. NO and SNOs break down by reacting with superoxide radical to produce peroxynitrite, a strong oxidant which causes direct tissue damage [10, 42]. High concentrations of *inhaled* NO cause vasodilation to the point of risking pulmonary oedema [23, 13, 11]

1.3.6 NO is raised in the asthmatic human lung because of increased iNOS expression

The elevated NO levels seen in asthmatic patients are most likely to be related to increased iNOS expression [76]. Inducible NOS is expressed at low or absent quantities in healthy cells, so little NO is produced despite the presence of L-arginine [94, 67]. By contrast, in areas of chronic inflammation, iNOS is expressed in alveolar macrophages [56]. Macrophages can also be stimulated to produce NO in the absence of inflammation *in vitro* [29]. A baseline level of iNOS expression is found in airway epithelium *in vivo*. This expression is attenuated *in vitro* [32].

Increased iNOS gene transcription and mRNA stability is promoted by a long list of stimuli, including lipopolysaccharide, inflammatory cytokines, chemokines, bacterial toxins, virus infection, allergens, environmental pollutants and hypoxia. This heightened function is not restricted to patients with high numbers of pulmonary eosinophils[4]. Expression of iNOS is downregulated by glucocorticosteroids in animal models[80, 33]. Similar effects have been difficult to demonstrate in humans [77, 94, 20, 29, 37, 47, 52].

Expression of iNOS is lower in asthmatic patients compared to healthy controls, but increases with increasing lung inflammation [94]. 5' promoter regions sensitive to inflammatory cytokines have been identified in the iNOS gene [13]. Increased transcription of the iNOS gene, for example in response to calmodulin, can increase NO production by up to 1000-fold; this upregulation is mediated by highly reactive radicals such as superoxide and peroxynitrite [10]. Significant increases in NO occur over hours, rather than minutes, and effects last days to weeks.

The neuronal and endothelial forms of NOS respond to intracellular calcium increases to produce short, low peaks, insufficient to explain the changes seen in the asthmatic lung. The function of these small variations includes regulating organ blood flow, inhibiting platelet aggregation and leukocyte adhesion, and blocking smooth muscle proliferation. Endothelial NOS is bound inactively to caveolin in the Golgi apparatus until a sufficient increase in intracellular Ca2+ causes dissociation of the two and the eNOS-calmodulin complex then synthesises NO [65]. Oestrogen upregulates this process in human airway endothelial cells [55].

1.3.7 Exhaled nitric oxide (FeNO) is related to allergy and to eosinophilia

Patients with atopic asthma have increased exhaled NO [73, 89, 91] associated with eosinophilic lung inflammation [91, 90, 16] and increased expression of iNOS, exacerbated in severe asthma [93]. Endothelial NOS in the lung is downregulated in asthma exacerbations but this is not sufficient to counterbalance the increase in iNOS activity[76].

Eosinophilia and FeNO are both considered to be related to the Th-2 leukocyte response pathway typical of atopic asthma [68, 83].

1.3.8 FeNO changes in the human lung are not sufficiently explained by eosinophilaemia or by allergy; other mechanisms include infection and exposure to particulate pollution

Eosinophilia does not explain large parts of the variation in FeNO [16, 90]. Neither does allergy: non-allergic patients also show clinically raised FeNO, though it is less pronounced than in allergic patients [78, 79, 44, 51, 38].

Jouaville (2003) found that rhinitis is a better predictor of exhaled NO than atopic status [48].

FeNO increases with exposure to particulate pollutants in children [64, 20], with viral airway infections, bronchiolitis obliterans and chronic obstructive pulmonary disorder (COPD) [41].

Recent evidence suggests that FeNO and eosinophilia are related to two different pathways within the Th-2 response. Interleukin-4 inhibitor dupilumab improves asthma symptoms and FeNO levels, but has no effect on eosinophilia [92]; mepolizumab, an interleukin-5 inhibitor, reduces the rate of asthma attacks and reduces blood and sputum eosinophil count, but has no effect on FeNO [35]. Interestingly, a further interleukin-5 inhibitor (SB-240563) reduces eosinophilia, but does not alter the late asthmatic response or prevent airway hyper-responsiveness to histamine [58]. Asthma attacks and emergency department attendance in young patients (10-35 years) have been found to relate to eosinophilia, but not to FeNO. Nevertheless, an elevation in both is predictive of worsening asthma symptoms [62, 66].

1.3.9 FeNO is a clinically useful measure of airway inflammation

The fraction of exhaled nitric oxide (FeNO) has been adopted as a clinical marker of asthmatic lung inflammation in medical practice [70], especially in paediatric populations where the non-invasive nature of the test is an advantage over alternative diagnostic markers [27].

Raised FeNO has been adopted by the American Thoracic Society and the global GINA guidelines as a predictor of good response to corticosteroid treatment and a warning of worsening asthma control [25, 27, 1, 2, 41, 68, 73], even in patients without an asthma diagnosis presenting with nonspecific respiratory symptoms and insignificant bronchodilator reversibility [74]. However, FeNO does not always fall with increased corticosteroid therapy [72].

Moderate and high-altitude climate therapy has repeatedly been shown to reduce asthma symptoms and FeNO in both adults and children [88, 44, 78]. Speculation on the mechanisms for this has included reduced exposure to allergens in atopic asthmatics [78] as FeNO is more dramatically reduced in atopic patients at high altitude compared to those with non-allergic asthma. However, a reduction in FeNO and an improvement in symptoms is found in both allergic and non-allergic patients [79, 44]. Rijssenbeek-Nouwens et al suggest that mechanisms for improvement in non-allergic patients include reduced pollution and increased UV exposure [78]. Patients with non-allergic asthma offer a unique means of investigating these mechanisms by eliminating the effect of allergen avoidance.

1.3.10 Exposure to particulate pollution predicts airway inflammation as measured by FeNO in both healthy and asthmatic adults

Particulate pollution is classed as airborne particulate matter of <10 microns diameter, and is referred to by the abbreviation PM10. Particles <2.5 microns in diameter are measured as a subgroup of this form of pollution, as these extremely small particles can travel very long distances to cause health problems far from their source. These smaller particulates are referred to as PM2.5. It is likely that PM10 and PM2.5 exposure will cause raised FeNO in children.

Airborne particulates are known to increase FeNO in both healthy and all-cause asthmatic adults. In particular, PM2.5 exposure (as measured by portable PM2.5 measurement devices on individual patients) significantly predicts an increase in FeNO in adults [28], and has been shown to affect methylation status of several relevant genes [95]. In addition, PM 10 exposure during exercise predicts FeNO increase in athletic individuals [57], and both PM10 and PM2.5 exposure correlate with increases in FeNO over short and longer time periods [4][59]. Finally, PM10 exposure predicts FeNO increases in both healthy and asthmatic adults [19]. As particulates appear to increase FeNO even in non-asthmatic individuals, it is to be expected that non-allergic asthmatic individuals will also be affected.

1.3.11 Patients with non-allergic asthma have a significant symptom burden and unclear pathophysiology

Non-allergic asthma is still poorly understood. Fortunately, it is rare in children, though across all age groups approximately 33% of overall asthma patients are affected. It occurs most in adult women, and is thought to be associated with neutrophilic inflammation, with a low or absent Th-2 response [12, 69]. Non-allergic persistent asthma is a more severe and less treatment-responsive disorder than the allergic phenotype [60, 22].

In contrast to the treatment-refractory neutrophilic inflammation found in many adult non-allergic asthma patients, at least some children with non-allergic asthma show eosinophilic lung inflammation with good symptom improvement using inhaled corticosteroids [60]. Blood eosinophilia and FeNO are lower in non-allergic asthmatic children [84], but as both groups show eosinophilic lung inflammation [61], this cannot easily be used to differentiate between the two groups.

Children with perceived non-allergic triggers for symptoms report a significantly lower quality of life than children with allergic triggers [50]. Quality of life has been shown to be significantly worse in adults with non-allergic asthma, compared to allergic asthma [49].

1.3.12 Children with non-allergic asthma are a useful study population to examine the causes of raised FeNO

Particulate exposure predicts worse asthma symptoms, lung function and FeNO in the general paediatric population [64, 20]. In children suffering from non-allergic asthma, symptoms decrease and functional ability increases during high-altitude rehabilitation [78, 79]. It is hypothesised that reduced particulate exposure contributes to this effect [71]. It is important to note that Hajat et al. [34] find that sulfur dioxide, carbon monoxide and nitrogen dioxide pollution predict increased asthma consultations in children, but find no association with particulates; it is possible, therefore, that particulates themselves are not the causal agents.

1.4 Meaningful contribution of this study

This study is the first to review FeNO and particulate pollution exclusively in non-allergic pediatric asthma patients. This subgroup is small and therefore rarely studied in isolation, so represents a unique opportunity to understand the pathophysiology of non-allergic asthma in children.

1.5 Aims of the study

This study aims to test the hypothesis that children arriving at a rehabilitation clinic with a verified physician diagnosis of non-allergic asthma will exhibit a raised FeNO level, and that this is likely to correlate with exposure to inhaled non-allergic irritants (particulate pollution).

A secondary aim is to test whether the known reduction in symptoms during high-altitude rehabilitation correlates with a reduction in FeNO.

A third hypothesis, only to be tested if the first two are supported by the data, is that the reduction in FeNO will correspond to a measurable reduction in particulate pollution exposure over time.

In order to validate the findings of the above, I aimed to replicate the well-known phenomenon that FeNO decreases over time spent in high-altitude rehabilitation in allergic asthma patients.

Part II

Methods

2 Study design

A retrospective study on several years of data was conducted. Patient records between 1.10.2012 and 1.10.2017 of admissions at the moderate-altitude paediatric rehabilitation clinic Alpenklinik Santa Maria (1200m) were reviewed. These dates were selected because 5 full years of admissions were planned for review and the clinic acquired a new FeNO measurement machine during October 2017 so results after that date were not directly comparable to results before it. Ethical approval was granted by the ethical committee of the medical school of the Ludwig Maximilian University of Munich.

2.1 Inclusion and exclusion criteria

Patients were included if they were between 4 and 18 years of age, had had at least one FeNO measurement taken on admission, were discharged from the clinic with a primary or secondary diagnosis of non-allergic asthma (ICD-10 code J45.1) and had a PM10 or PM2.5 pollution measurement taken regularly within 10 km (for PM10 measurements) or 30km (for PM2.5 measurements) of their home postcode. Patients were excluded if they had not had a FeNO measurement, if there was a positive indication that a respiratory allergy could not be excluded (positive or ambiguous respiratory allergy test of any type during or prior to admission: raised IgE with or without identification of a specific sensitisation; positive skin prick test for any respiratory allergen; positive response to nasal or skin provocation using a respiratory allergen) or if they had not had both IgE and skin-prick testing in the course of their medical history (prior to or during admission). If a patient had attended the clinic multiple times during the study period and several of the admissions qualified for inclusion, the latest admission was included and all others excluded.

FeNO change was examined in all non-allergic patients with two FeNO measurements. The effect of pollution on FeNO was intended to be examined in the subset of included patients who had also had two FeNO measurements during the admission, in whom no medication change was made, and in whom PM10 measurements from within 10km of the home postcode were available (as Oberjoch measurement station does not measure PM2.5).

In addition, FeNO measurement data was collected from allergic asthma patients between 01.10.2012 and 01.10.2017 in order to confirm the relationship between time spent at high altitude and reduction in FeNO levels. Inclusion criteria were the diagnosis of allergic asthma (ICD-code J45.0) and the presence of at least two FeNO measurements. It was not possible to discover the exact timing of each FeNO measurement for each patient in this group. Instead, timing was divided into admission (usually within 2 days of arrival), check (usually around 14 days after arrival) and discharge (usually 24-27 days after arrival)

2.2 Measurements

The data recorded for each non-allergic patient was as follows; demographic characteristics (age, sex, treatment on admission, season of admission); Asthma control test level for all patients in whom it was recorded; FeNO measurements for each patient. In addition, the following measurements were obtained: particulate matter at the closest measurement station to their home postcode, within 10 km radius using the average of the month prior to admission; second FeNO measurement if available; any medication changes; and particulate matter (PM 10) in Oberjoch measurement station average over the admission period. For allergic patients, only NO measurements during the course of rehabilitation were recorded, no additional data relating to pollution or demographics was accessed. Patients were irreversibly anonymised immediately following data collection.

2.2.1 Medication coding

Patients were coded as high steroid if they were taking high-dose inhaled steroid for age; High steroid + denotes high-dose steroid and one or more added medications such as a long-acting beta agonist (LABA) or leukotriene receptor antagonist (LRA). The same applies to low steroid, low steroid +, medium steroid and medium steroid +. Patients were coded as "other" if they were taking exclusively LABA or LRA. All patients were prescribed a short-acting beta agonist for immediate relief. Medication changes were coded as increase (increased steroid dose or addition of a LABA or LRA to current regime), decrease (reduction in dose or removal of a LABA or LRA), no change (including patients moved from one steroid to an equivalent dose of another) and unclear (patients moved from a LABA to a LRA with no change in steroid dose, for example). Symptoms were considered to be controlled if the Asthma Control Test score was above 19. Paediatric scoring was used where appropriate.

2.2.2 FeNO measurement

FeNO was measured using a NIOX MINO® handheld electrochemical sensor. This has been shown to be comparable to chemiluminescence analysis of FeNO [63]. Using this sensor, a level at or below 17 ppb is considered clinically normal.

2.2.3 Particulate pollution measurement

Particulate pollution is measured in two ways. The gold standard is to gather particulates on a filter and weigh them (oravimetry). Alternatively, particulates can be counted optically as they pass through the measurement station (optical measurement). Both methods are used in many measurement stations; publically available data tables do not specify which method was used at each station, and it proved impossible to obtain this information.

2.3 Changes to study design during data collection

The original intention was to use particulate matter averages from three weeks prior to hospital admission. Unfortunately, many measurement stations do not provide daily particulate measurement data prior to 2016, instead providing monthly averages on request. In an effort to homogenise the data, monthly averages were used . If a child was admitted in the first week of the month, the previous month's average was used. If he arrived during the middle two weeks, the mean of the month of admission and the previous month; if during the final week in a month, the average for that month was used. For example: for an arrival in the first week in May, the April average was used; if during the second or third week in May, the mean of the April and May averages; and if during the final week in May, the May average was used. An updated ethical review application was submitted to reflect this change.

2.4 Analysis

Statistical analysis was performed using R. Correlations were examined by plotting the data and using Spearman's correlation coefficient. Power analysis indicated a requirement for 13 qualifying participants to find a correlation of strength r=0.5 or above at p=0.05. Categorical comparisons were conducted using the chi-squared test. Post-hoc analyses were conducted using Student's T-test, though the assumptions for this test were not completely met within this dataset. A Bonferroni correction for multiple comparisons was applied. Relationships between demographic data were examined only by graphing the data.

Repeated-measures data from allergic patients was analysed using the lme4 package using a mixed linear model. Mixed linear models provide an estimate of the effect size without allowing for the calculation of a p-value. The results are interpreted in conjunction with the chart of the data. As no exact times for FeNO measurement were available, it was assumed that admission, check and discharge measurements were equally spaced in time to permit analysis. The interdependence of the repeated measures in each patient, combined with the variability in the number of patients undergoing a measurement at each time point, renders more usual statistical methods such as ANOVA inappropriate in this case. Nevertheless, an ANOVA was conducted on a log-transformed subset of the data to provide a rough estimate of statistical significance.

Analysis methods were discussed with the statistics department at LMU.

2.5 Outcomes

The primary outcome was to determine the correlation, if any, between FeNO on admission and particulate matter exposure at home in the month before admission. The secondary outcome, in the subset of patients who had had more than one FeNO measurement, was to investigate whether FeNO falls at altitude in non-allergic asthmatic patients, and if yes, whether this decrease might relate to falling particulate polution exposure. A final objective was to validate our other findings by confirming the relationship between FeNO and time spent at altitude in allergic asthma patients.

Part III

Results

3 Results

3.1 Patient demographic characteristics

The initial search (>3 years of age, discharged between 1.10.2012 and 1.10.2017 with first or second diagnosis non-allergic asthma J45.1) found 473 patients. Of these, 214 were excluded for the following reasons: 61 had a demonstrable respiratory sensitisation, 67 had a non-specific marker of atopy e.g. raised IgE, 83 had no measurement of FeNO during the admission, 2 had no discharge summary and 1 was outside the age range determined for the study. This left 259 patients. Of these, 82 had both negative IgE and skin prick test results.

Some states do not publish historical information on pollution measurement, and emails requesting assistance went unanswered; as a result, several patients were excluded.

PM10 measurements within 30km of the home postcode were available for 49 of the remaining patients; PM10 measurements within 10km were available for only 20. PM 2.5 measurements within 30 km were available for 30 patients, PM 2.5 measurements within 10 km were available for 10 of those. 20 Patients had two FeNO measurements; of these, PM10 measurements were available for only 3. As a result, any analysis of the relationship between pollution change and FeNO change would have been significantly underpowered and such an analysis was not conducted.

Inclusions and exclusions are summarised in Fig.2. Patient demographics are summarised in table 1.

Figure 2: Inclusions and exclusions: non-allergic patients

Of 473 patients identified on initial search, only 82 patients met the baseline inclusion criteria (prick test and IgE test both negative). Only 20 of these patients had two FeNO measurements during the course of rehabilitation. Of these, one further patient was excluded from the final analysis because it was not clear whether the medication was altered between measurements (as this can be assumed to have a significant effect on FeNO).

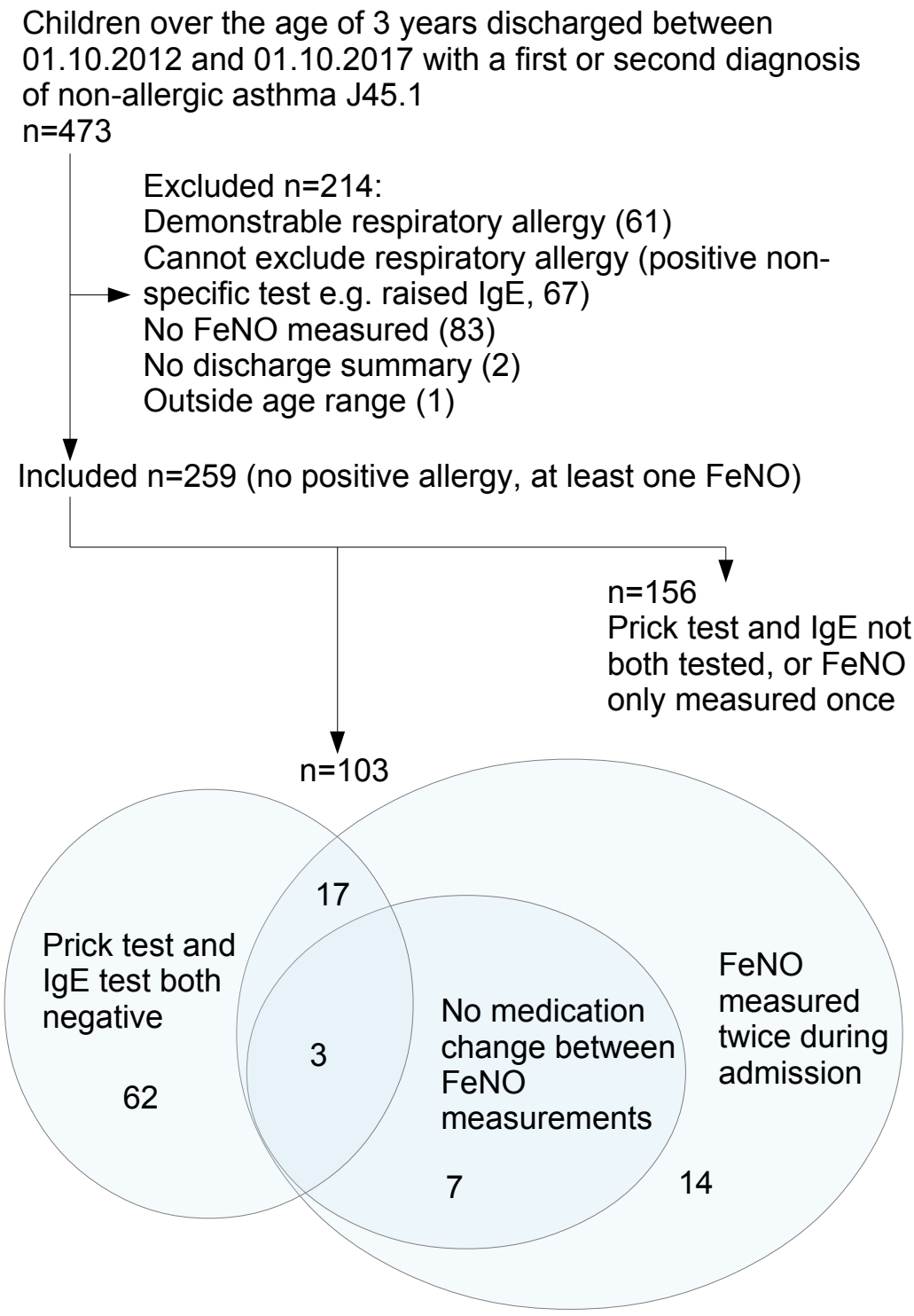


Table 1: Non-allergic patient demographic characteristics

		Negative IgE & skin prick test (n=82)	**PM10 measurement (n=49)**	**PM2.5 measurement (n=30)**	**Two FeNO measurements (n=20)**
Female:Male (%)		37 : 45 (45% : 55%)	23 : 26 (47% : 53%)	11 : 19 (37% : 63%)	11 : 9 (55% : 45%)
Mean age in years (standard deviation)		7.2 (2.5)	7.1 (2.5)	7.6 (2.6)	7.0 (2.2)
Treatment (%)	None	33 (40%)	16 (33%)	9 (33%)	5 (63%)
	Low steroid	11 (13%)	6 (12%)	4 (13%)	3 (15%)
	Low steroid +	18 (22%)	12 (25%)	7 (23%)	7 (35%)
	Medium steroid	5 (6%)	3 (6%)	2 (7%)	2 (15%)
	Medium steroid +	11 (13%)	9 (18%)	7 (23%)	2 (10%)
	High steroid	0	0	0	0
	High steroid +	2 (2%)	1 (2%)	1 (3%)	0
	Other	2 (2%)	2 (4%)	0	0
Asthma Control Test taken (of those, controlled : uncontrolled asthma)		35 (23 : 12)	19 (11 : 8)	14 (9 : 5)	8 (6 : 2)
Season of admission Spring : Summer : Autumn : Winter		25 : 28 : 21: 8	15 : 16 : 11 : 7	8 : 12 : 8 : 2	4 : 9 : 5 : 2
Medication change	None	Not recorded for patients without multiple FeNO measurements			5 (25%)
	Increase				8 (40%)
	Decrease				6 (30%)
	Unclear				1 (5%)

3.2 Primary outcome

There was no significant relationship between at-home particle pollutant exposure and FeNO on admission (PM10 within 10km: n=20, R^2=0.013, p=0.96; PM2.5 within 10km: n=10, R^2=0.01, p=0.98, underpowered) Figs. 3 and 4).

Figure 3: Relationship between PM 10 (within 10 km of the home postcode) and FeNO on admission in patients with non-allergic asthma; n=20
R^2 = 0.013, p=0.96 (non-significant): there is no relationship between PM10 measurement at the home postcode over the 4 weeks before admission, and FeNO on arrival at the hospital.

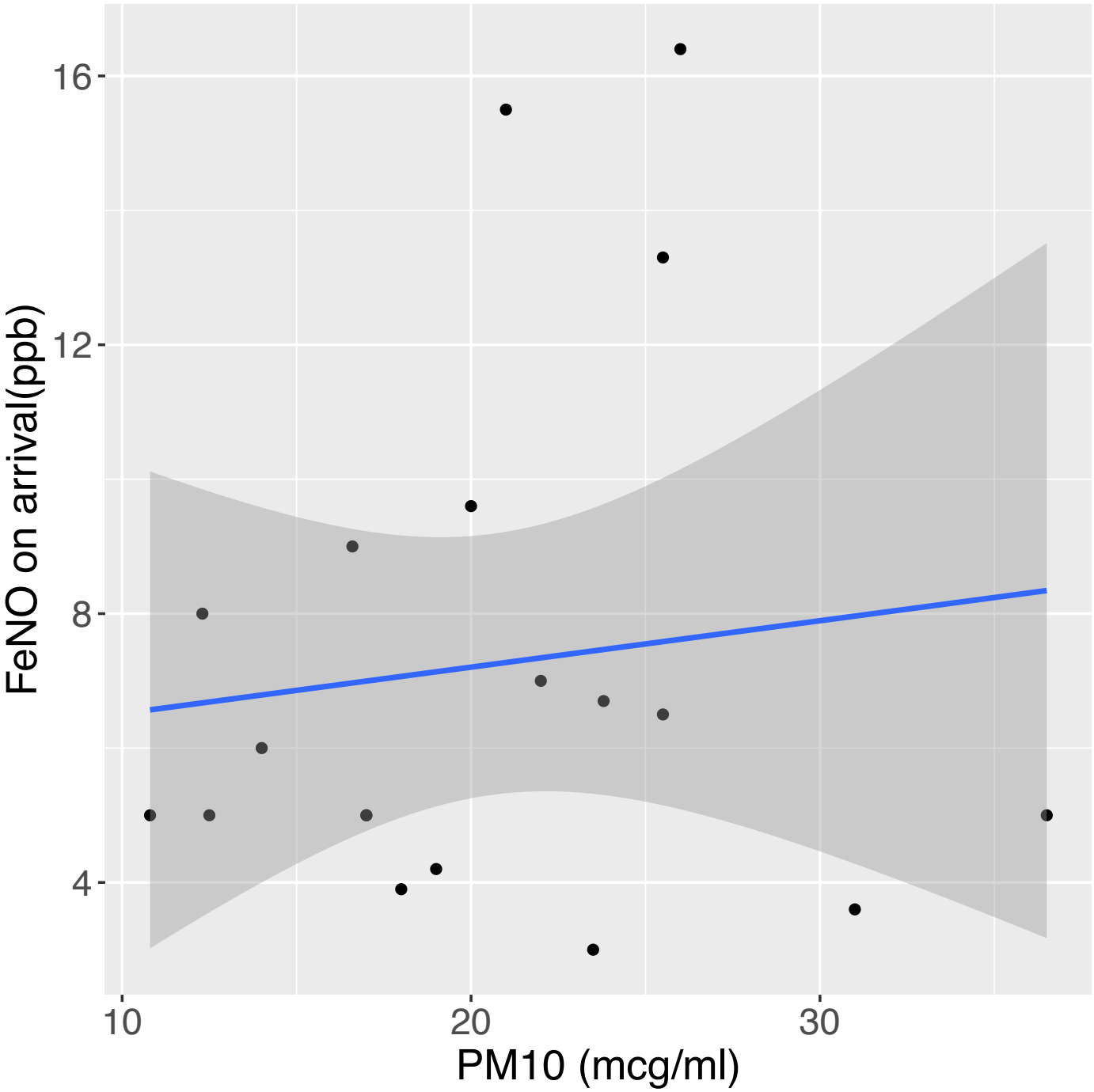
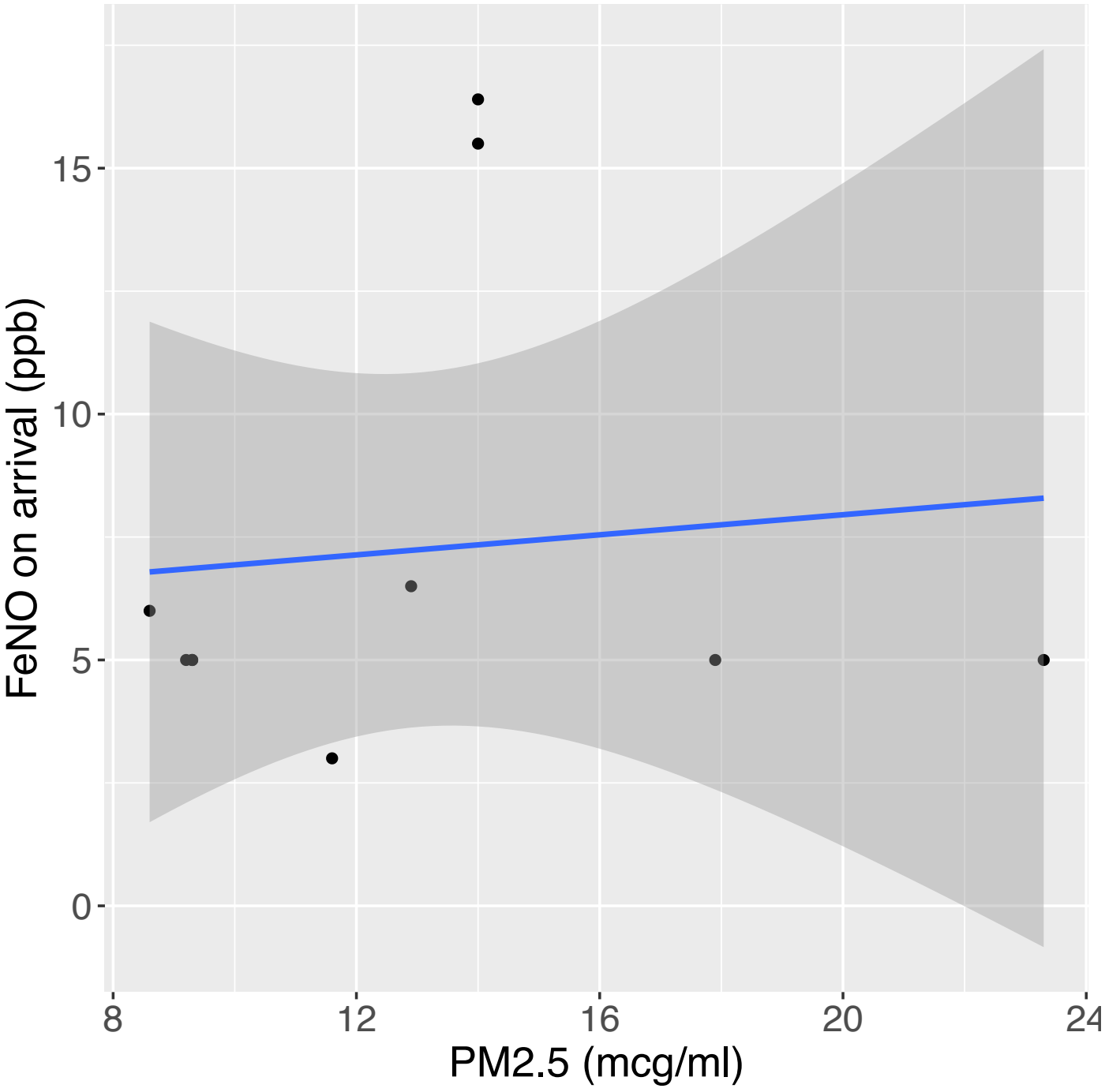


Figure 4: Relationship between PM 2.5 (within 10 km of the home postcode) and FeNO on admission in patients with non-allergic asthma; n=10

R^2 =0.01, p=0.98 (non-significant); underpowered to support or reject the null hypothesis. It is not possible to draw any conclusions about the relationship between exposure to airborne microparticles <2.5 microns in diameter, and FeNO, in the pediatric non-allergic asthmatic population, because there were not enough data points in this 5-year data set.



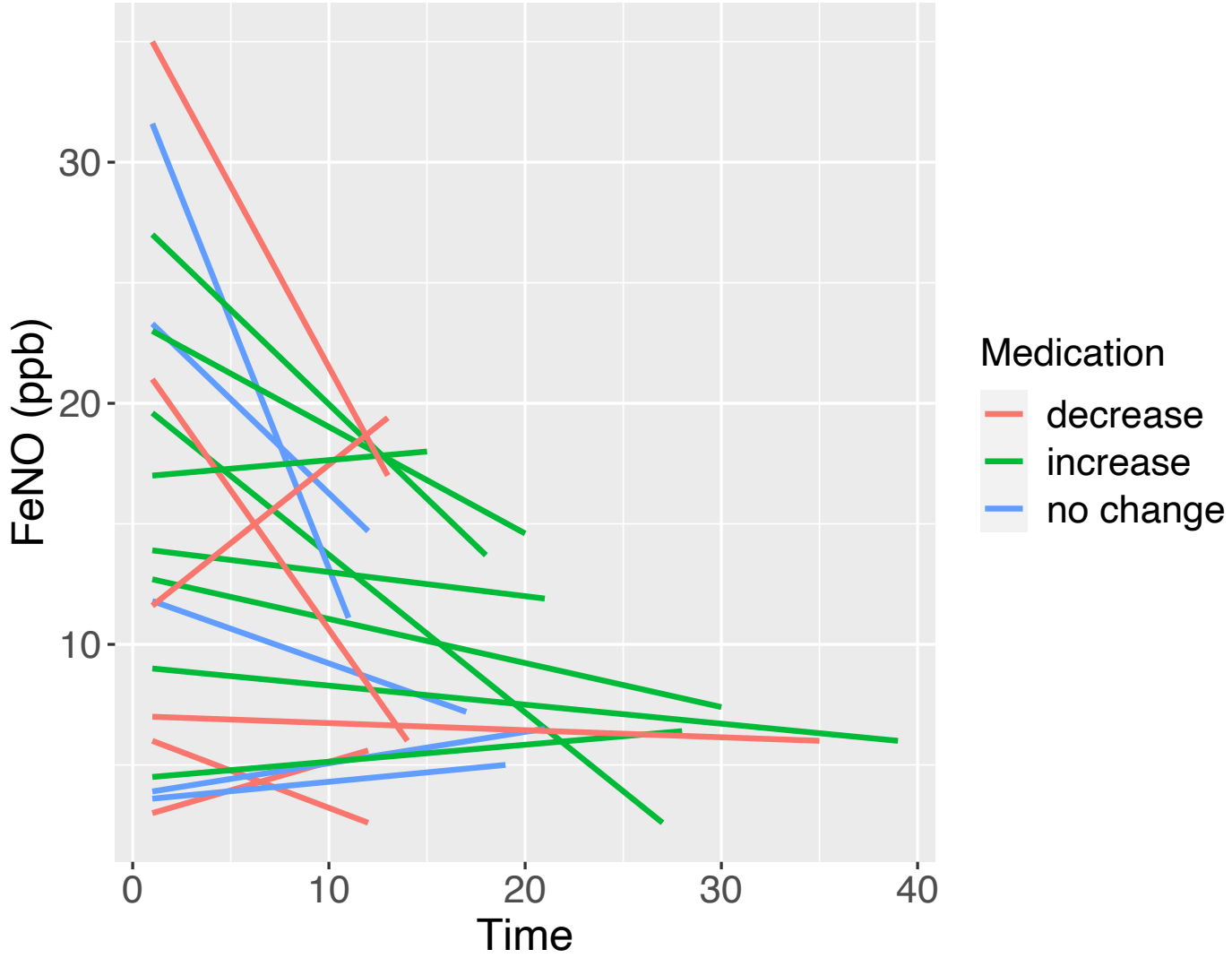
3.3 Secondary outcomes

3.3.1 Patients with non-allergic asthma

Using an appropriate nonparametric test, there was no significant change in the FeNO levels of patients with non-allergic asthma who had two FeNO levels measured during their hospital stay, and for whom medication changes were clearly documented (n=19).

There was a clinically significant improvement (a drop in FeNO level from abnormally high to below 17ppb, into the normal range) for many of the patients, including those whose medication was decreased or held constant. Only one patient shows a clinically significant increase in FeNO during the course of admission. (Fig. 5).

Figure 5: FeNO changes during admission in patients with non-allergic asthma, for whom medication changes were clearly documented (n=19)
Time is measured in days from arrival. Using an appropriate nonparametric test, there is no significant change in FeNO related to time. The charted data shows a clinically significant improvement (a drop in FeNO to below 17 ppb: from abnormally high to clinically normal) for many of the patients, including those whose medication was decreased or held constant. Only one patient, whose medication was decreased during admission, shows a clinically significant increase in FeNO.



The study was underpowered to examine whether changes in FeNO levels might be related to particle pollutant change (n=3; see Fig. 2).

3.3.2 Patients with allergic asthma

For those patients with allergic asthma, 1060 patients were found over the study period who met the inclusion criteria. As the purpose of this analysis was a simple replication and validation of our other findings, demographic and medication data were not collected for this group. There was a highly significant decrease in FeNO levels between admission and first check. It is less clear whether FeNO levels fell significantly further between the check time point and discharge. A box plot of FeNO values by time of test is shown in Figure 6. Fixed effects estimates and residuals of the mixed model used to analyse the data are shown in Table 2 and charted in Fig.7. As the analysis was conducted using a mixed linear model, which does not offer the possibility of computing a p-value, an ANOVA was also conducted on the subset of the data where all three measurements were available, using log-transformed data, in order to provide an approximate estimate of statistical significance. As the assumptions for ANOVA were not met by this dataset, the result should be interpreted with caution. The ANOVA was conducted with only 178 of the available 1060 patients, and was highly statistically significant ($F=52.03$, $p<0.0001$).

Figure 6: Box plot of FeNO measurements over time in patients with *allergic asthma* over the study period (n=1060).
This box plot shows the data of all 1060 allergic asthma patients with 2 or more FeNO measurements within the 5-year study period. The majority of these patients had only 2 FeNO measurements during their hospital stay, at variable times, so there are different numbers of patients in each of the groups. That is to say, some patients with a measurement at admission had a subsequent "check" measurement a few weeks later, but no measurement at discharge; some had a measurement at admission and discharge, but no measurement in between; a small subset had a measurement at all three time points; and several had no admission measurement, but did have a "check" measurement during the course of their hospital stay, followed by a second measurement at discharge. This renders the analysis of this dataset complex; however, the box plot shows a clear trend.
An ANOVA, conducted using the data from only those patients with measurements at all time points (n=178: just under 20% of the available data set) was highly significant, $F=52.03$, $p<0.0001$.

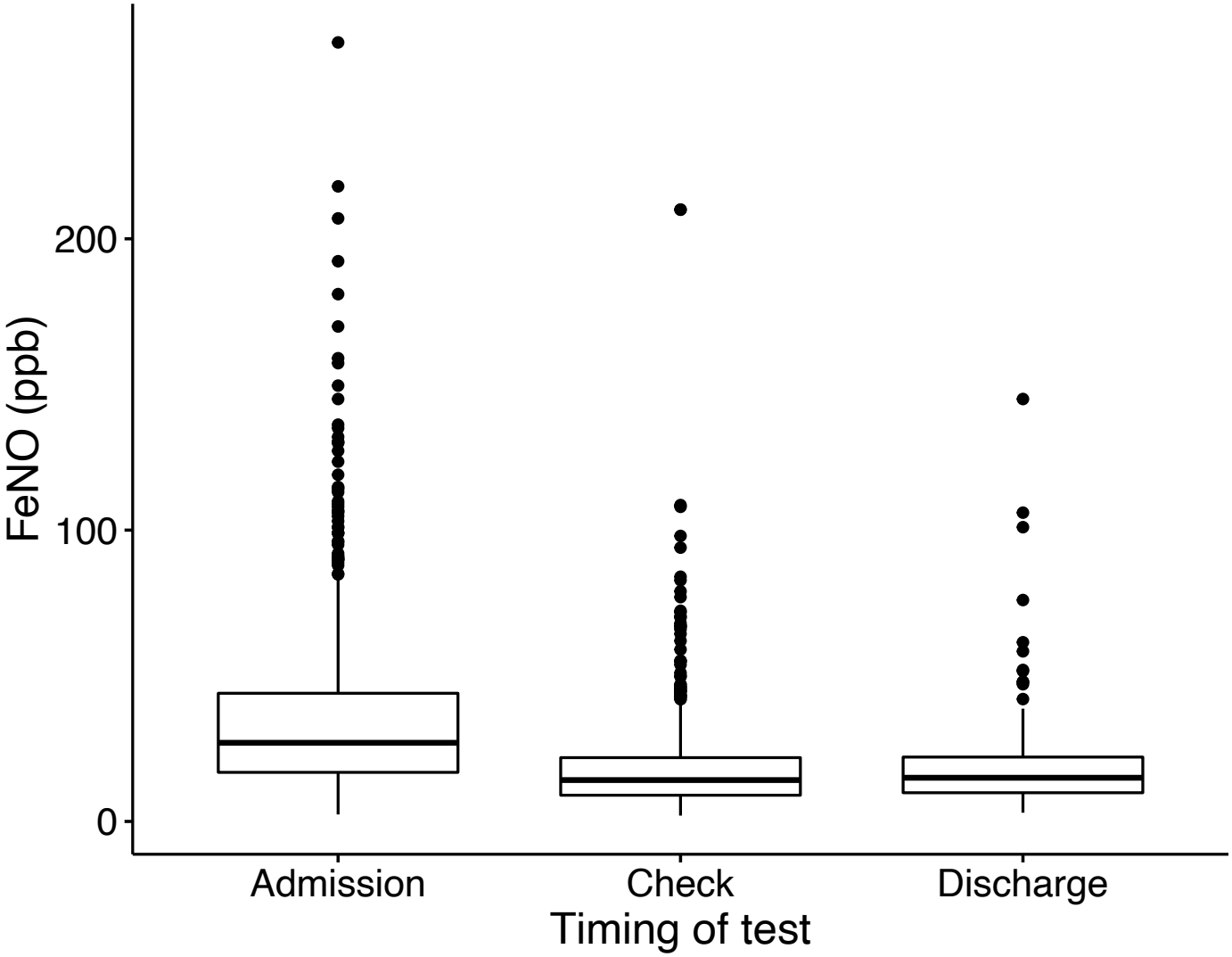


Figure 7: Fixed effects estimates and standard error of the modelled data in patients with *allergic asthma*

Chart of the mixed model calculated using data from allergic patients. In this chart, a transformation was performed on all data points: 20.77 was added to each data point so as to display an entirely positive model, as it is not possible to have a negative FeNO value. It is impossible to calculate a single p-value to represent the probability of obtaining exactly this model given the null hypothesis of no relationship between timing and FeNO. However, the very narrow standard error relative to the large change in expected FeNO suggests that there is a real relationship between timing and FeNO level.

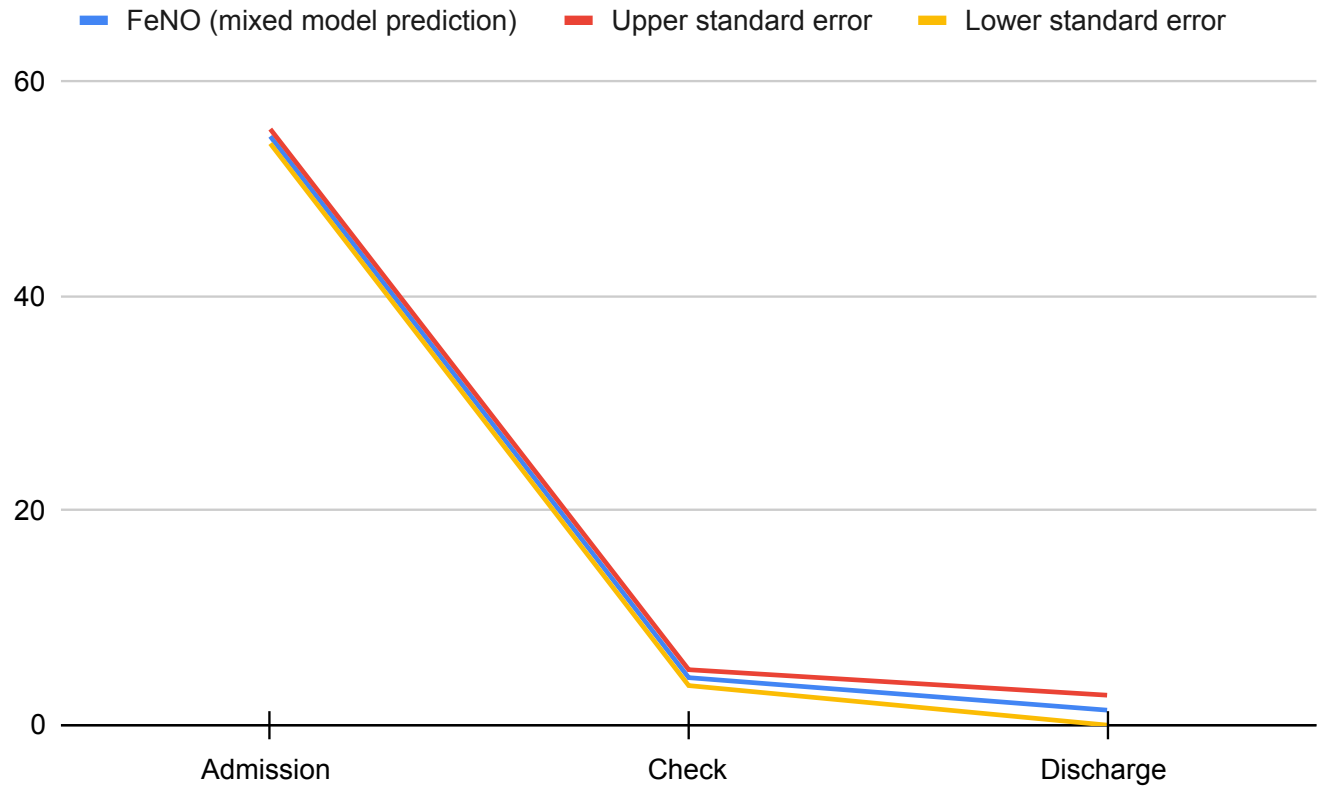


Table 2: Fixed effects estimates and residuals for FeNO measurements over time in patients with *allergic asthma* over the study period

This table should be interpreted in conjunction with Figure 6 (a chart of the data) and Figure 7. (a chart of the resulting mixed mathematical model). In our allergic patients, FeNO starts at a high level, reduces to a much lower value at the "check" time point and reduces still further by the "discharge" time point. The standard error of each value is displayed. The t-value represents the t-test statistic for the modelled data point; however, in a mixed model, the distribution of this statistic is very unlikely *to follow the Student's T distribution. As a result, no p-value can be inferred from the t-value.*

	Estimate	Standard error	t value
Admission	34.1685	0.6748	50.64
Check	-16.3413	0.7408	-22.06
Discharge	-19.3740	1.3960	-13.88

Part IV
Discussion

4 Outcomes

4.1 Primary outcomes

Raised particulate exposure in non-allergic asthmatic patients did not significantly correlate with increased FeNO levels. Those patients at the highest end of particulate exposure did not even have a sub-clinically raised FeNO compared to those at the lowest end.

The strongest non-significant correlation was found between PM10 measurement within 10km and FeNO on admission (R^2=0.013). At this strength of correlation, a power analysis suggests that a sample size of 22733 patients would be required to refute the null hypothesis. As only 20 patients were found during 5 years' admissions at a specialist pulmonology clinic, this participant number seems virtually impossible to achieve; it is certainly reasonable to accept the null hypothesis in the meantime.

The aetiology of raised FeNO in non-allergic asthma patients remains unclear. It is surprising that particulate pollution plays no role.

4.2 Secondary Outcomes

4.2.1 Non-allergic asthma

Very few patients with non-allergic asthma have raised FeNO, but it does exist as a clinical phenomenon in this patient group. Non-allergic asthma patients are a useful study group to examine the pathophysiology of raised FeNO in the asthmatic lung.

One possible reason for raised FeNO on arrival is an infectious asthma exacerbation. However, patients also contract respiratory infections at the high-altitude clinic (an inevitable consequence of the co-habitation of several hundred children in fortnightly rotation), but only one patient developed a clinically raised FeNO during the admission. Unfortunately, the retrospective and irreversibly anonymised nature of this project makes it impossible to determine whether any patients in the study group had respiratory infections during the course of measurement; future prospective studies must include this factor in measurements in order to determine its significance.

The data could not replicate previous findings [78, 44] that FeNO decreases during high-altitude rehabilitation in non-allergic patients. Though the charted data appears to suggest an overall decrease, this effect was not significant using nonparametric testing.

It was impossible to assess whether particulate change had an impact on FeNO change as the study was very underpowered despite the inclusion of 5 years' worth of admissions. Of the few patients who had had both IgE and skin prick testing to exclude allergy, most had only taken one FeNO measurement, and of those with two measurements, many had undergone a medication change. This is perhaps inevitable as the second FeNO measurement only becomes clinically useful after some form of alteration in the patient. In view of the absence of any relationship between particulate pollution and FeNO on admission, though, this may not be a very fruitful avenue for future research.

4.2.2 Allergic asthma

As expected, in patients with allergic asthma, FeNO decreased significantly during high-altitude rehabilitation. This replicates previous results [44, 78, 88]. Many of these patients will have had medication changes (increases or decreases) during the time of admission, as this is an important reason for repeated measurement. These changes may explain some of the observed variation. However, most of the improvement occurs within the first two weeks from admission (Fig.7). This is important because the convention at the clinic is to leave medications unchanged until the first two weeks

of rehabilitation have elapsed, in order to have time to gather all relevant diagnostic tests. As a result the decrease in inflammation may be largely unrelated to medication changes, and is more likely to reflect a reduction in allergen exposure at high altitude [79]. As it was not possible to include specifics related to medication changes and exact timing of FeNO tests, this cannot be shown conclusively in this data set.

5 Limitations of the current study and avenues for further research

5.1 Limitations

Time between FeNO measurements varied widely. A prospective study would naturally choose a fixed interval before the second measurement, at minimum 2 weeks to reflect the latency on FeNO change.

Two statistical analyses were conducted in the awareness that the data sets did not meet the criteria for a valid application of these tests (Student's paired t-test for analysis of the change in FeNO levels in non-allergic asthma patients during the hospital stay, and ANOVA for the same analysis in allergic asthma patients). These tests were conducted for illustrative purposes as the charted data and additional statistical analyses were not considered sufficient. The p-values given here, showing statistical significance, should therefore be treated with caution: the outcomes are better interpreted using the complete charted data and mixed effects model as presented in Figs 5, 6 and 7.

It is likely that particulate exposure is appropriately represented by measurements from stations within 10km [9]; however, local particulate pollution can vary widely and it is possible that this has had a confounding effect. For example, a child living in the countryside might be closest to a particulate station situated on the nearest big motorway - high pollution would be measured at the site, but not necessarily cause problems for the child. Conversely, a child living near fields that are regularly fertilised might be exposed to heavy pollution, but a measurement station in the pedestrian town center 10km away wouldn't reflect this.

In addition, very unfortunately, particulate exposure measurements were not available for those non-allergic asthma patients who presented with a raised FeNO on admission: all patients for whom particulate exposure had been measured within 10km of the home postcode had a clinically normal FeNO. It is possible (though unlikely based on my findings) that there is a relationship between particulate exposure and *clinically* raised FeNO, but there was no data at all in this set which permitted the examination of this hypothesis.

Several German states do not publish historic pollution data, rendering examination of pollution exposure impossible. As a result, children from several large geographic areas were excluded from analysis with respect to pollution.

5.2 Topics for further study

A prospective study might further examine the impact of high-altitude climate therapy on non-allergic asthma patients without medication changes, aiming to ensure good statistical power. Randomisation would be difficult to justify ethically unless the clinician were ambivalent about the need for medication change in selected patients. A further interesting avenue would be to explore the relationship between UV exposure at home and in the alpine environment [31]. Hajat et al. [34] find that sulfur dioxide, carbon monoxide and nitrogen dioxide pollution predict increased asthma consultations in children: it would be interesting to examine any connections between these pollutant gases and lung inflammation in the non-allergic asthmatic population. Exposure to cigarette smoke, exercise and temperature-related triggers are also important future pathways for research [50].

As there were no particulate measurements available for any patients with clinically raised FeNO, it may be worth including particulate exposure as a possible exacerbating aetiological factor in those patients in future research.

Part V
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Part VI

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