Crucial role for lung iron level and regulation in the pathogenesis and severity of asthma

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ABSTRACT Accumulating evidence highlights links between iron regulation and respiratory disease. Here, we assessed the relationship between iron levels and regulatory responses in clinical and experimental asthma.

We show that cell-free iron levels are reduced in the bronchoalveolar lavage (BAL) supernatant of severe or mild–moderate asthma patients and correlate with lower forced expiratory volume in 1 s (FEV_1). Conversely, iron-loaded cell numbers were increased in BAL in these patients and with lower FEV_1/forced vital capacity (FVC) ratio. The airway tissue expression of the iron sequestration molecules divalent metal transporter 1 (*DMT1*) and transferrin receptor 1 (*TFR1*) are increased in asthma, with *TFR1* expression correlating with reduced lung function and increased Type-2 (T2) inflammatory responses in the airways. Furthermore, pulmonary iron levels are increased in a house dust mite (HDM)-induced model of experimental asthma in association with augmented *Tfr1* expression in airway tissue, similar to human disease. We show that macrophages are the predominant source of increased Tfr1 and Tfr1^{+} macrophages have increased *Il13* expression. We also show that increased iron levels induce increased pro-inflammatory cytokine and/or extracellular matrix (ECM) responses in human airway smooth muscle (ASM) cells and fibroblasts *ex vivo* and induce key features of asthma *in vivo*, including airway hyper-responsiveness (AHR) and fibrosis, and T2 inflammatory responses.

Together these complementary clinical and experimental data highlight the importance of altered pulmonary iron levels and regulation in asthma, and the need for a greater focus on the role and potential therapeutic targeting of iron in the pathogenesis and severity of disease.

Introduction

Clinical and experimental evidence suggests that altered levels of systemic and lung iron and/or iron regulatory molecules are associated with lung inflammation in many diseases, including asthma [1]. Chronic inflammatory responses that underpin many respiratory diseases are associated with decreased systemic iron levels [2, 3]. There is a correlation between low maternal iron status during pregnancy and childhood wheezing, impaired lung function and atopic sensitisation [4]. In addition, low foetal iron is linked with increased susceptibility to eosinophilia in infancy [5]. In a longitudinal study of parents and children, higher umbilical cord iron status was associated with decreased occurrence of wheezing and eczema, while lower iron status was linked to increased risk of atopy [6]. Furthermore, lower serum and exhaled breath condensate iron levels are associated with asthma in children and adults [7–9]. However, plasma iron and malondialdehyde levels, a marker of oxidative stress, are significantly elevated in asthmatic subjects compared to healthy controls [10, 11]. Ferritin levels are increased in the lungs in an experimental mouse model of asthma [12]. Ferritin and iron levels were reduced by intranasal administration of iron chelator complexes in this model, with treatment also attenuating inflammation [12]. By contrast, short-term intraperitoneal injection of iron dextran suppresses hallmark features of asthma in an acute mouse model [13], suggesting that increasing systemic iron levels may protect against disease. However, the potential pathological effects of chronically increasing systemic iron levels and the effects on iron accumulation in the lung on asthma, remain unexplored. Together, these findings provide evidence that asthma is associated with altered iron homeostasis. However, it is unclear whether altered iron levels play roles in pathogenesis or are a consequence of disease. Further clinical and experimental studies investigating the link between iron and asthma are required to better understand the mechanisms involved and address whether increased or decreased pulmonary iron levels are detrimental. In order to address this, we assessed the levels of iron and iron-related gene expression in the airways of patients with severe or mild–moderate asthma and in healthy controls. We show that altered iron metabolism in the airways is associated with asthma. Specifically, reduced extracellular iron levels and increased cellular iron accumulation and airway transferrin receptor 1 (*TFR1*) expression are linked with disease severity, suggesting that iron sequestration/accumulation in cells/tissues may play a role in asthma pathogenesis. We also show that experimental asthma is associated with increased airway *Tfr1* expression that corresponds with increased pulmonary iron accumulation. These findings have led us to hypothesise that increased iron accumulation in the lung plays a key role in driving the key features of asthma. We confirmed this by showing that increased lung iron levels in two murine models of iron overload result in the development of key asthma features similar to that observed in house dust mite (HDM)-induced experimental disease.

Methods

Full details are provided in the supplementary material.

Study approvals

All experiments were conducted with approval of the Human/Animal Ethics Committees of the University of Newcastle (Australia) and the local ethics committees of the Unbiased BIOmarkers in PREDiction of respiratory disease outcomes (U-BIOPRED) clinical centres.

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Clinical analyses

Levels of iron were assessed in bronchoalveolar lavage (BAL) cells from 11 severe asthma patients, 12 mild–moderate asthma patients and 13 healthy controls using Perls' staining with 3,3′-diaminobenzidine tetrahydrochloride (DAB). In the same cohorts, iron regulatory factors were assessed in airway biopsy tissues using quantitative PCR. Colorimetric non-haem iron (NHI) assay was used to measure NHI content in BAL supernatants collected from a second set of severe asthma patients, mild–moderate asthma patients and healthy controls (ten subjects per group). In both sets of donors, asthma severity was categorised based on % predicted forced expiratory volume in 1 s (FEV_1) and asthma severity symptoms·day^{-1}·night^{-1} (tables 1 and 2). The transcriptomic profile of iron regulatory factors was analysed in bronchial brushings of 39 severe asthma patients, 29 mild–moderate asthma patients and 40 healthy controls within the U-BIOPRED cohort [14].

Iron overloaded mice; experimental asthma model; iron quantification; airway inflammation; small airway fibrosis; lung function, flow cytometry analyses of different cell populations in murine lung tissue, human primary ASM and lung fibroblast cell culture, primary bronchial airway epithelial cells (pBECs) cultured at the air–liquid interface (ALI)

Haemochromatosis protein gene (*Hfe*)-deficient (*Hfe*$^{-/-}$) and wild-type (WT) mice on an AKR background (at 36 weeks old) [15] and WT BALB/c mice (at 8 weeks old) were fed a high iron diet (HID), supplemented with 2% carbonyl iron, for 8 weeks and used to model iron overload. HDM-induced experimental asthma was induced in *Hfe*$^{-/-}$ mice and WT BALB/c mice fed a HID, a normal diet (called control chow (CC)) or a low iron diet (LID) (see supplemental figure E1). Iron quantification, airway inflammation, small airway fibrosis, lung function, pulmonary cell population characterisation, and cytokine and extracellular matrix (ECM) responses in human primary airway smooth muscle (ASM) cells and lung fibroblasts, as well as iron-related gene expression in primary bronchial airway epithelial cells (pBECs), were assessed as previously described [16–34] and as outlined in the supplementary material.

Statistics

Comparisons between two groups were performed using a non-parametric Mann–Whitney test. Comparisons between multiple groups were performed using Kruskal–Wallis one-way ANOVA with an uncorrected Dunn's *post-hoc* test. Airway hyper-responsiveness (AHR) data were analysed using two-way repeated measures ANOVA with a Bonferroni *post-hoc* test. Correlation analyses were performed using Spearman's rank correlation.

TABLE 1 Human sample donor characteristics (iron metabolism genes expression in airway biopsy tissues and iron scores in bronchoalveolar lavage (BAL) cells)

Characteristic	Healthy	Mild–moderate asthmatics	Severe asthmatics
Subjects	13	12	11
Age years	53.00±4.729	55.92±4.583	53.73±3.681
Male/female	4/9	6/6	2/9
BMI kg·m^{-2}		31.22±2.302	31.71±4.313
FEV_1 % predicted	102.7±4.494	86.92±2.726**	76±5.389***
FEV_1/FVC	0.817±0.021	0.730±0.016**	0.656±0.049**
ACQ		0.903±0.168	2.166±0.291##
Total cells ×10^6·mL^{-1} BAL	0.170±0.026	0.129±0.021	0.853±0.327*,#
Macrophages ×10^6·mL^{-1} BAL	0.053±0.011	0.068±0.021	0.128±0.031*
Neutrophils ×10^6·mL^{-1} BAL	0.065±0.013	0.031±0.010	0.653±0.311
Eosinophils ×10^6·mL^{-1} BAL	0.002±0.000	0.039±0.017	0.050±0.019*
ICS yes/no	NA	9/3	11/0
ICS dose#	NA	543.30±126.70	800.00±80.90
LABA yes/no	NA	6/6	11/0
LAMA yes/no	NA	4/8	5/6
SABA yes/no	NA	5/7	5/6
OCS yes/no	NA	0/12	2/9

Data are presented as n or mean±SEM. BMI: body mass index; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; ACQ: asthma control questionnaire; NA: not applicable; ICS: inhaled corticosteroids; LABA: long-acting β$_2$-agonist; LAMA: long-acting muscarinic antagonist; SABA: short-acting β-agonist; OCS: oral corticosteroids. #: equivalent to fluticasone (µg·day^{-1}). *: p<0.05 compared to healthy controls; **: p<0.01 compared to healthy controls; ***: p<0.001 compared to healthy controls; #: p<0.05 compared to mild–moderate asthmatics; ##: p<0.01 compared to mild–moderate asthmatics.

Results

In asthma, iron levels in BAL supernatant are reduced, but iron-positive cell numbers are increased

We first assessed cell-free NHI levels and iron-loaded cell numbers in BAL obtained from severe or mild–moderate asthma patients and healthy controls. Iron levels in BAL supernatant were reduced in combined severe and mild–moderate asthma patients compared to healthy controls, with the greatest decrease in severe disease (figures 1a and 1b). NHI levels positively correlate with % predicted FEV_1 but not with FEV_1/forced vital capacity (FVC) (figures 1c and 1d). NHI levels negatively correlate with inhaled corticosteroids (ICS) used in asthma patients (figure 1e). In contrast, severe and mild–moderate asthma patients have increased numbers of iron-positive cells in BAL compared to healthy controls (figures 1f–1h). Iron-positive BAL cell numbers negatively correlate with FEV_1/FVC but not with % predicted FEV_1 (figures 1i and 1j). Together, these novel findings show that decreased extracellular iron levels in the airway lumen, but increased iron in BAL cells, correlates with declines in lung function in asthma, suggesting a relationship between cellular sequestration/accumulation of iron and impaired lung function.

Levels of key iron sequestration molecules are increased in the airways of asthma patients

We next assessed whether levels of iron sequestration molecules (divalent metal transporter 1 (*DMT1*) and *TFR1*) are increased in the airways of asthma patients. *DMT1* and *TFR1* mRNA expression are increased in the airways of asthma patients compared to healthy controls (figures 2a and 2d). In addition, *DMT1* and *TFR1* expression negatively correlates with FEV_1/FVC with similar correlations observed for % predicted FEV_1 (figures 2b, 2c, 2e and 2f). We confirmed that *TFR1* expression is increased using airway brushings from a separate cohort of asthma patients (figure 2g). Importantly, *TFR1* expression is strongly and positively associated with Group 2 innate lymphoid cell (*ILC2*) [35], interleukin 4 *(IL4)*, *IL5* and *IL13* expression (figures 2h–2k). Collectively, these data provide evidence that increased iron sequestration into airway tissues and/or cells due to increased *DMT1* and *TFR1* expression may play a crucial role in the pathogenesis and severity of asthma.

Expression of iron uptake, transport, storage and regulatory factors are altered in the airways and pBECs of asthma patients

We next assessed whether the expression of other iron-related genes is altered in the airways of asthma patients. Severe asthma patients have increased expression of iron uptake molecules (transferrin receptor 2 (*TFR2*), zinc transporter (*ZIP14*) and natural resistance-associated macrophage protein 1 (*NRAMP1*) (p=0.08)) and iron storage molecules (ferritin heavy chain (*FTH*) (p=0.06)) compared to healthy controls

TABLE 2 Human sample donor characteristics (non-haem iron (NHI) quantification in bronchoalveolar lavage (BAL))

Characteristic	Healthy	Mild–moderate asthmatics	Severe asthmatics
Subjects	10	10	10
Age years	58.56±2.724	60.70±5.327	56.00±3.841
Male/female	4/6	5/5	5/5
FEV_1 % predicted	97.5±5.861	89.6±4.759	66.33±7.427**,#
FEV_1/FVC	0.812±0.008	0.741±0.023*	0.745±0.035
ACQ		4.778±0.8127	7.333±3.18
Total cells ×10^6·mL^{-1} BAL	0.110±0.028	0.138±0.027	0.126±0.016
Macrophages ×10^6·mL^{-1} BAL	0.037±0.009	0.031±0.008	0.051±0.013
Neutrophils ×10^6·mL^{-1} BAL	0.025±0.009	0.053±0.022	0.039±0.008
Eosinophils ×10^6·mL^{-1} BAL	0.000±0.000	0.020±0.011	0.029±0.012
ICS yes/no	NA	8/2	10/0
ICS dose#	NA	432.0±110.4	1063±174.2
LABA yes/no	NA	5/5	10/0
LAMA yes/no	NA	2/8	6/4
SABA yes/no	NA	4/6	4/6
OCS yes/no	NA	0/10	0/10

Data are presented as n or mean±SEM. BMI: body mass index; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; ACQ: asthma control questionnaire; NA: not applicable; ICS: inhaled corticosteroids; LABA: long-acting β$_2$-agonist; LAMA: long-acting muscarinic antagonist; SABA: short-acting β-agonist; OCS: oral corticosteroids. #: equivalent to fluticasone (μg·day^{-1}).*: p<0.05 compared to healthy controls; **: p<0.01 compared to healthy controls; #: p<0.05 compared to mild–moderate asthmatics.

(figures 3a–3d). The levels of *NRAMP1*, *FTH*, ferritin light chain (*FTL*) and iron regulatory protein 1 (*IRP1*) expression are significantly higher in the airways of mild asthma patients compared to healthy controls (figures 3c–3f). Hepcidin (*HAMP*) expression is decreased in the airways of severe asthma patients compared to healthy controls (p=0.06) (figure 3g). Interestingly, *FTL* is lower in severe asthma patients compared to mild asthma patients (figure 3e). Notably, there is no significant change in iron exporter ferroportin (*FPN*) expression in any of the groups (figure 3h). Together these data show that factors associated with increased intracellular iron uptake and storage are largely increased in the airways of asthma patients, which may explain the decreases in extracellular iron levels in BAL supernatant and increased iron accumulation in cells/tissues.

To explore which cells are associated with altered iron in the airways, we next assessed expression of *DMT1*, *TFR1*, *TFR2*, *ZIP14*, *NRAMP1*, *FTH*, *FTL*, *IRP1*, and *FPN* in pBECs obtained from asthmatic patients and healthy controls cultured at the air–liquid interface (ALI). As with the mRNA expression changes observed in airway tissue biopsy and bronchial brushings, we observed that *TFR1* and *TFR2* expression are significantly increased in the pBECs of asthmatic patients (figures 3j and 3k). There is no significant change in expression of any other factors (figures 3i and 3l–3q).

Iron levels and Tfr1⁺ macrophages are increased in HDM-induced experimental asthma and increased iron induces pathological responses in ASM cells and lung fibroblasts

We next determined whether the levels of iron and regulatory factors are similarly altered in HDM-induced experimental asthma (see supplemental figure E2) and whether exogenous iron alters cytokines and ECM responses in primary human ASM cells and lung fibroblasts. We also show that HDM-induced experimental asthma is associated with the accumulation of NHI in lung tissue (figure 4a) and with iron accumulation mostly in macrophages around the airways (figure 4b). We found increased numbers of iron-positive cells in BAL (figure 4c) and increased expression of *Tfr1* in the airways (figure 4d), as well as increased transferrin levels in BAL supernatant (figure 4e). Together, our clinical and experimental data provide evidence for increased iron accumulation in lung tissues and cells in asthma that are either playing a role in, and/or are a consequence of, the pathogenesis of asthma. We next assessed the cellular source of increased *Tfr1* using flow cytometry. We show that the number of Tfr1⁺ cells is increased in HDM-challenged mice and that these cells are predominantly alveolar and interstitial macrophages (figure 4f). These data suggest that HDM-induced experimental asthma is characterised by an increase in macrophages that sequester iron. To determine whether these Tfr1⁺ macrophage populations have a different phenotype, we measured expression of *Il10*, transforming growth factor beta (*Tgfb*), interferon gamma (*Ifng*) and *Il13* in Tfr1⁺ and Tfr1⁻ macrophages in HDM-induced experimental asthma and saline-challenged controls (figures 4g–4j). We show that Tfr1⁺ macrophages from HDM-challenged mice have increased *Il10* and *Il13* expression compared to those from saline-challenged controls, as well Tfr1⁻ macrophages from both saline-challenged and HDM-challenged groups (figures 4g and 4j). This suggests that iron-sequestering Tfr1⁺ macrophages may play a pathologic role in asthma by promoting Type-2 (T2) inflammatory responses. To further characterise the potential effects of increased extracellular iron levels in tissues, we also exposed human ASM cells and fibroblasts to increasing concentrations of ferric ammonium citrate (FAC). Increasing concentrations of FAC increase pro-inflammatory cytokine production (IL6 and IL8) and/or ECM gene expression (tenascin C (*TNC*)) by these cells (figures 4k–4p).

Hfe⁻/⁻ iron overload mice have increased iron levels in the lung associated with increased features of asthma

We next assessed the consequences of increased iron accumulation in lung tissues and cells on the pathogenesis and severity of key features of asthma using murine models of iron overload. In the absence of HDM treatment, sham phosphate-buffered saline (PBS)-treated *Hfe⁻/⁻* mice, which overload iron systemically (*e.g.* in the liver) (figure 5a), have significantly higher lung iron levels compared to sham-treated WT controls (figure 5b). Whilst HDM increases iron levels in the lungs of WT mice compared to sham-treated WT controls, it does not further enhance levels in *Hfe⁻/⁻* mice (figure 5b). Increased iron in all groups is predominantly deposited in macrophages surrounding the airways and in the alveoli (figure 5c).

We also demonstrate that the increased iron accumulation observed in sham-treated *Hfe⁻/⁻* mice is associated with increases in leukocyte numbers in BAL and lung interleukin 13 (*Il13*) expression, which lead to small airway fibrosis and AHR compared to sham-treated WT controls (figures 5d, 5e and 5j–5m). Indeed, these features are similar to those observed in HDM-treated WT controls. HDM treatment in *Hfe⁻/⁻* mice increases the severity of all of the disease features compared to HDM-treated WT controls (figures 5d, 5e and 5j–5m). Together, these data demonstrate that pulmonary iron accumulation alone can drive key features of asthma and that a combination of iron overload and HDM treatment may contribute to more severe features of experimental asthma.
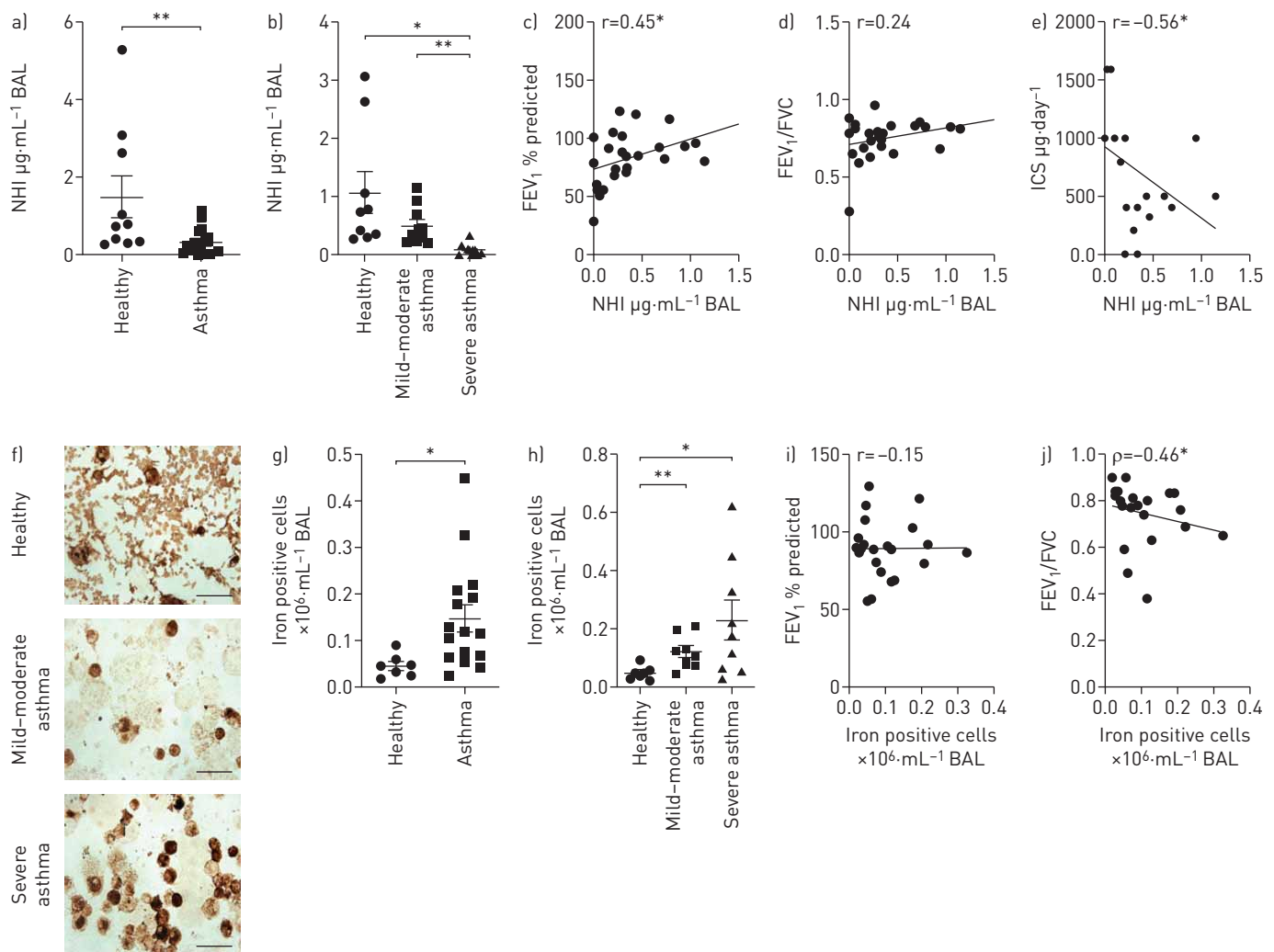
FIGURE 1 Iron regulation in the airways is altered in asthma. a, b) Non-haem iron (NHI) levels were measured in bronchoalveolar lavage (BAL) supernatant collected from patients with severe or mild–moderate asthma and healthy controls (n=10 per group). c) NHI levels were correlated with % predicted forced expiratory volume in 1 s (FEV_1) and d) FEV_1/forced vital capacity (FVC). e) NHI levels were also negatively correlated with inhaled corticosteroid (ICS) use. f–h) BAL cells were visualised using Perls' staining enhanced with 3,3′-diaminobenzidine tetrahydrochloride and iron-positive cells were enumerated (n=8–10). Correlation between i) iron-positive cells and % predicted FEV_1 and j) FEV_1/FVC was determined and are represented as Spearman's rank correlation coefficient (Spearman's rho (r)). Data are presented as mean±SEM, unless otherwise stated. Scale bars=50 µm. *: p<0.05 compared to respective controls; **: p<0.01 compared to respective controls.

Diet-induced iron accumulation also increases key features of asthma

To confirm our findings with $Hfe^{-/-}$ mice we next assessed the effects of HID-induced iron overload on disease features. Similar to our findings in $Hfe^{-/-}$ mice, WT mice fed a HID, which overload iron systemically (figure 6a), have increased pulmonary iron levels compared to mice fed CC (figure 6b). Furthermore, as with findings in $Hfe^{-/-}$ mice, diet-induced iron overload increases lung iron levels following HDM treatment, with similar levels observed in HDM-treated mice on both a HID and a CC diet (figure 6b). Consistent with findings in $Hfe^{-/-}$ mice, iron accumulation in the lungs of mice fed a HID is associated with airway fibrosis and AHR in the absence of HDM treatment (figure 6h–6k). HID-induced overload increases the severity of HDM-induced experimental asthma, with small airway fibrosis and AHR increased in HDM-treated mice fed a HID compared to those fed a CC diet (figures 6h–6k). There were some discrepancies observed between the effects on $Hfe^{-/-}$ mice compared to HID fed mice. HID-fed mice did not exhibit increases in BAL leukocyte numbers, but did have elevated tissue eosinophil numbers in HDM-induced experimental asthma (figures 6c–6e). These data confirm that increased iron accumulation in the lung can drive the pathogenesis and increase the severity of key asthma features.
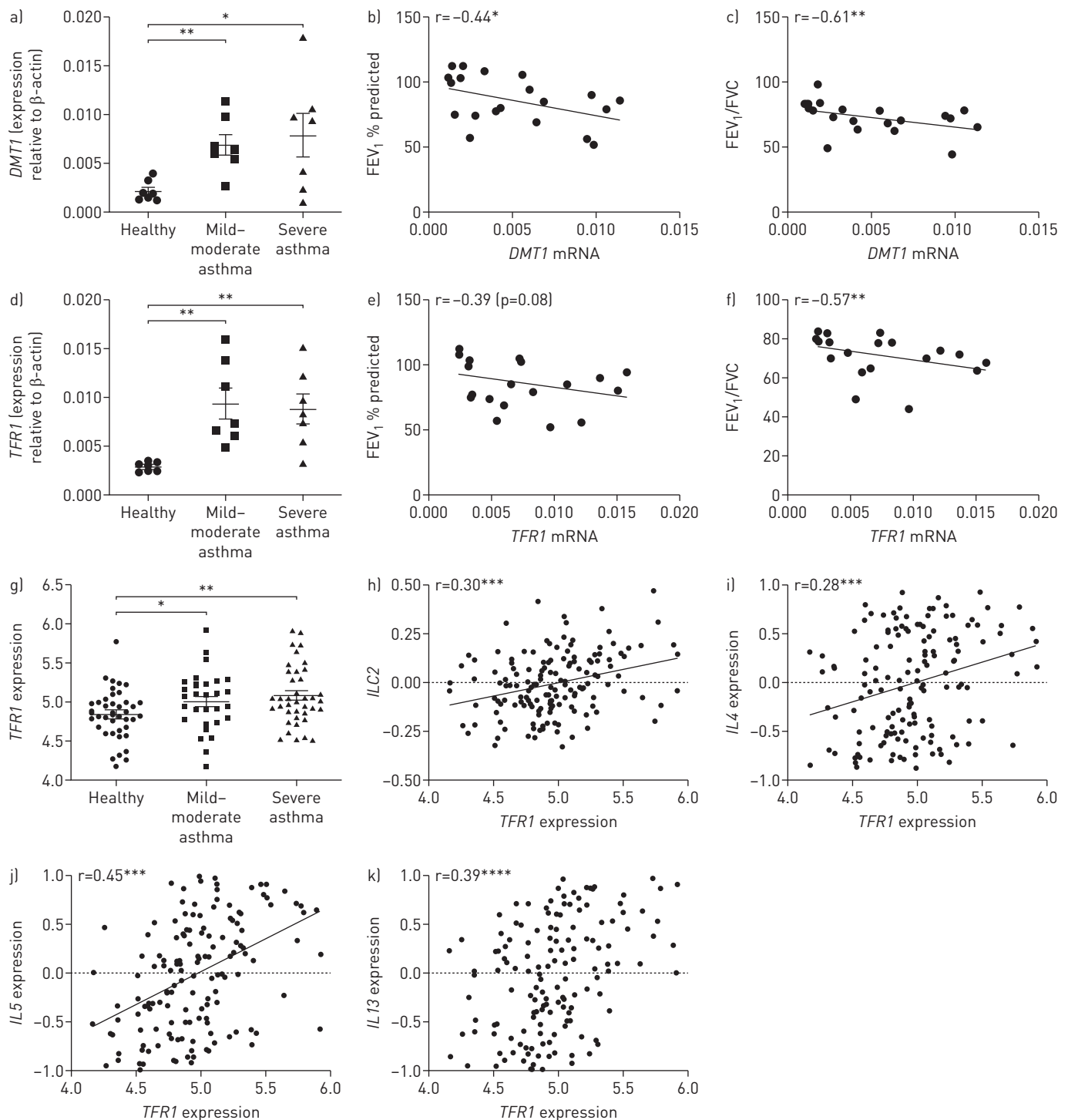
FIGURE 2 Increased divalent metal transporter 1 (*DMT1*) and transferrin receptor 1 (*TFR1*) expression in the airways correlates with impaired lung function in asthma patients. a, d) *DMT1* and *TFR1* mRNA expression was quantified in the airway tissues collected from seven severe asthmatic patients, seven mild–moderate asthmatic patients and six healthy controls using quantitative PCR (relative to the reference gene β-actin). b, e) *DMT1* and *TFR1* expression levels negatively correlate with % predicted forced expiratory volume in 1 s (FEV_1) and c, f) FEV_1/forced vital capacity (FVC). Correlations for each comparison are represented as Spearman's rank correlation coefficient (Spearman's rho (r)). *TFR1* mRNA expression in the bronchial brushings of asthma patients from within the Unbiased BIOmarkers in PREDiction of respiratory disease outcomes (U-BIOPRED) cohort are shown in (g). *TFR1* expression was positively correlated with h) Group 2 innate lymphoid cell (*ILC2*) gene signature, i) interleukin 4 (*IL4*), j) *IL5* and k) *IL13* expression. Data are presented as mean±SEM, unless otherwise stated. *: p<0.05 compared to respective controls; **: p<0.01 compared to respective controls.
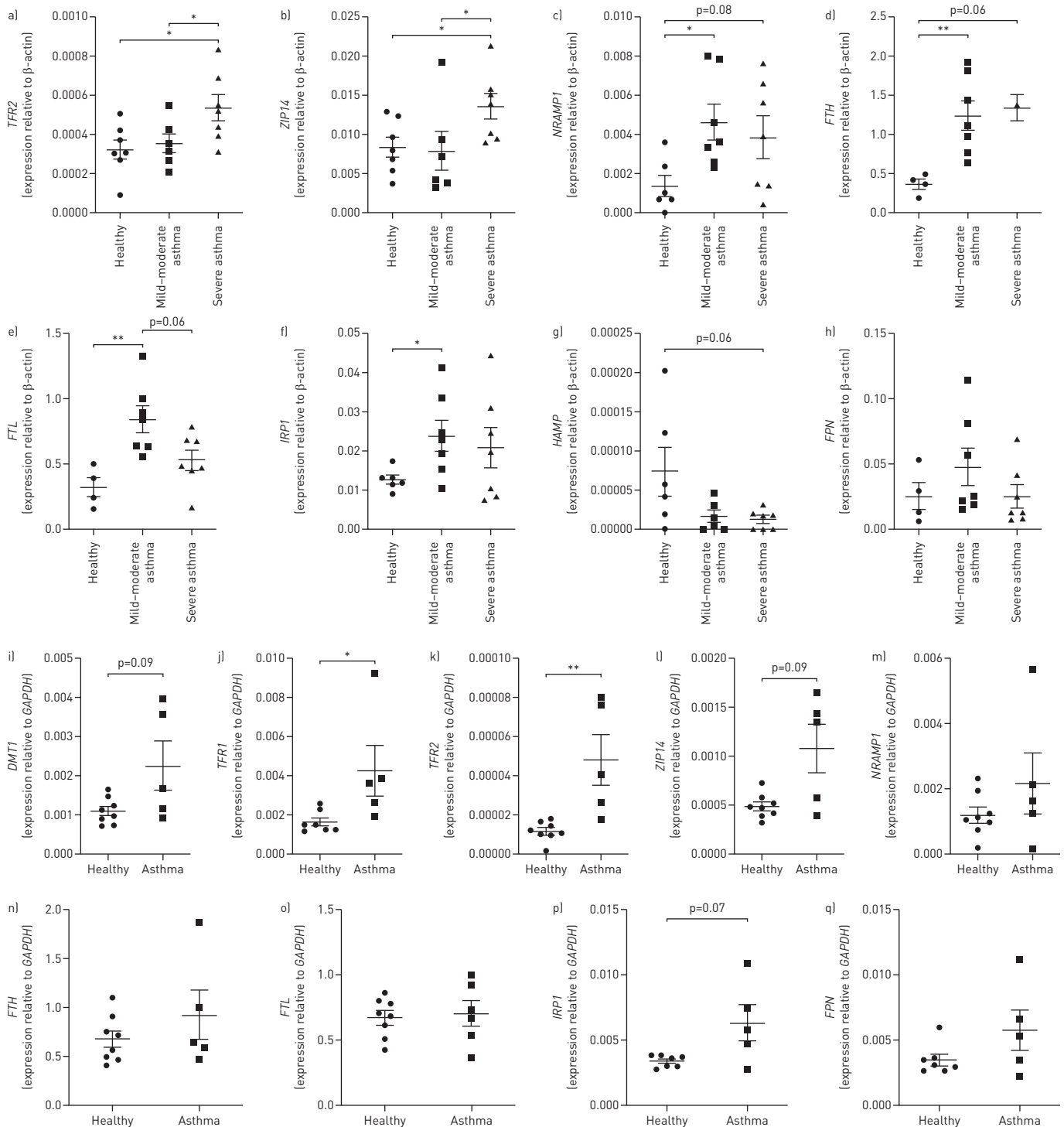
FIGURE 3 Altered expressions of iron regulatory factors in the airways of asthma patients. The mRNA expression of a) transferrin receptor 2 (*TFR2*), b) zinc transporter (*ZIP14*), c) natural resistance-associated macrophage protein 1 (*NRAMP1*), d) ferritin heavy chain (*FTH*), e) ferritin light chain (*FTL*), f) iron regulatory protein 1 (*IRP1*), g) hepcidin (*HAMP*) and h) iron exporter ferroportin (*FPN*) was assessed in the airway tissues collected from seven severe asthma patients, seven mild–moderate asthma patients and six healthy controls using quantitative PCR. The mRNA expression of i) divalent metal transporter 1 (*DMT1*), j) transferrin receptor 1 (*TFR1*), k) *TFR2*, l) *ZIP14*, m) *NRAMP1*, n) *FTH*, o) *FTL*, p) *IRP1* and q) *FPN* was also assessed using quantitative PCR in primary bronchial epithelial cells obtained from seven severe asthma patients and eight healthy controls that had been cultured at the air–liquid interface (ALI). Expression was relative to the reference gene β-actin and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) for each group, respectively. Data are presented as mean±SEM. *: $p < 0.05$ compared to the respective controls; **: $p < 0.01$ compared to the respective controls.
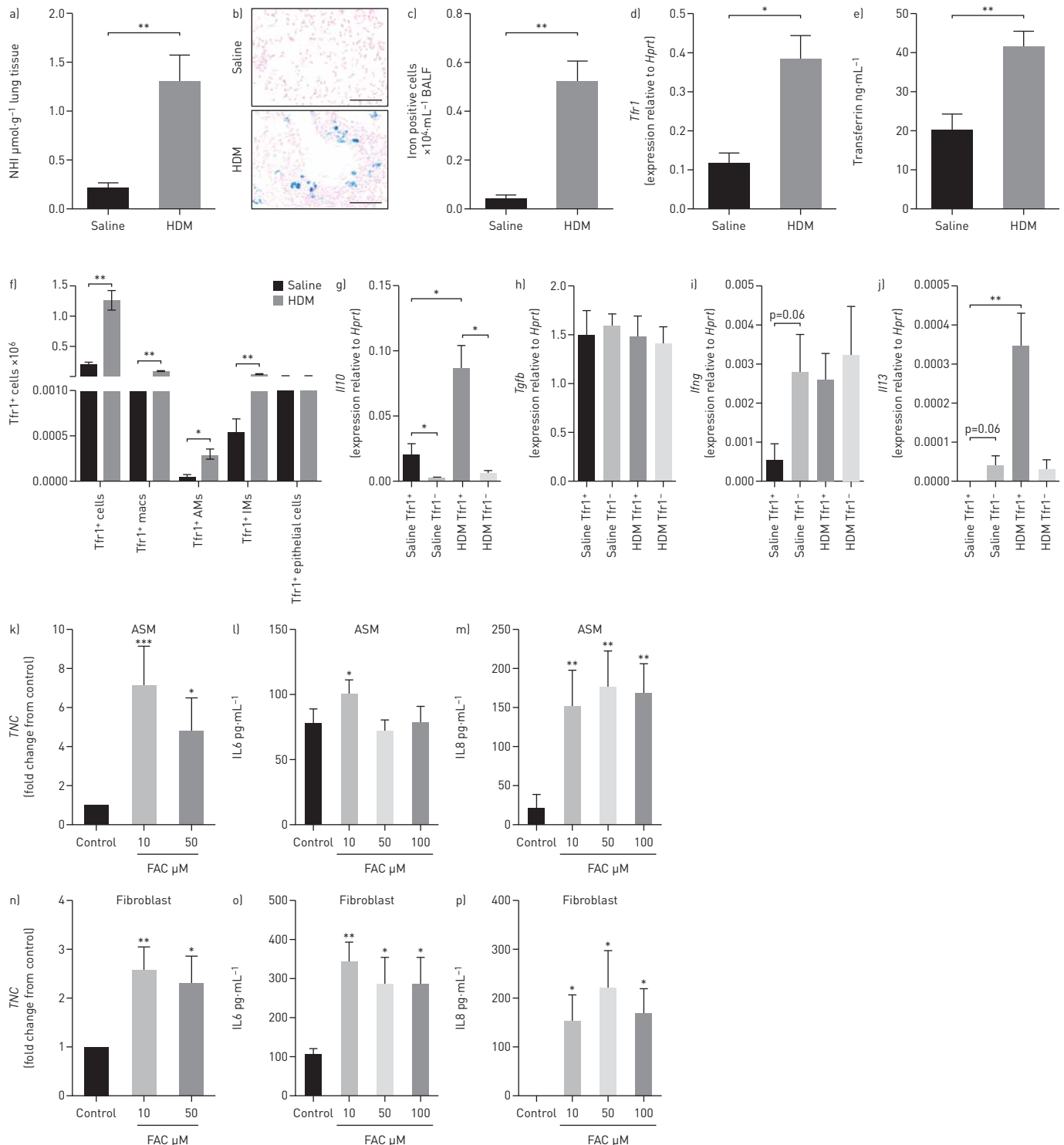
FIGURE 4 Iron levels are increased in the lung in house dust mite (HDM)-induced experimental asthma. a) Wild-type (WT) BALB/c mice at 6–8 weeks old were intranasally administered HDM antigen for 5 days·week^{-1} for 6 weeks and then major features of HDM-induced experimental asthma were assessed. Non-haem iron (NHI) content was measured in the lung homogenates. b) Localisation of iron in the lung was assessed in lung sections using Perls' staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB). c) Iron-positive cells were enumerated in bronchoalveolar lavage (BAL) cells using Perls' staining. d) *Tfr1* expression was measured in airway tissues using quantitative PCR. e) Transferrin levels were measured by enzyme-linked immunosorbent assay (ELISA). f) Tfr1$^+$ cells were sorted using fluorescence-activated cell sorting (FACS) analysis. Expression of g) interleukin 10 (*Il10*), h) transforming growth factor beta (*Tgfb*), i) interferon gamma (*Ifng*) and j) *Il13* was assessed in sorted Tfr1$^+$ and Tfr1$^-$ macrophages in the presence and absence of HDM-induced experimental asthma using quantitative PCR. k–m) Levels of tenascin C (*TNC*) and interleukin 6 (IL6) and IL8 were measured in ferric ammonium citrate (FAC)-treated airway smooth muscle (ASM) cells and n–p) lung fibroblast cells using quantitative PCR and ELISA, respectively. Data are presented as mean±SEM (n=6–8), pooled from two repeat experiments. Scale bars=50 µm. BALF: bronchoalveolar lavage fluid; mac: macrophage; AM: alveolar macrophage; IM: interstitial macrophage; *Hprt*: hypoxanthine phosphoribosyltransferase. *: p<0.01 compared to PBS controls; **: p<0.001 compared to PBS controls; ***: p<0.0001 compared to PBS controls.

Interestingly, mice fed a LID, which had reduced liver iron levels (figure 6a), did not have reduced lung iron levels in the absence of HDM treatment (figure 6b). However, mice fed a LID did have reduced lung iron accumulation in HDM-induced experimental asthma (figure 6b), which is associated with decreases in total leukocyte numbers in BAL and mucus secreting cell (MSC) numbers around the airways (figures 6c, 6f and 6g). The decrease in lung iron levels has no effect on HDM-induced airway fibrosis or AHR (figures 6h–6k).

Together, these data show that the relationship between lung iron levels and the pathogenesis and severity of asthma is complex. Nevertheless, these data provide new evidence that increased iron accumulation in the lung plays key roles in driving the characteristic features of asthma.

Discussion

Clinically, both low [7, 8] and high [10, 11, 36] systemic iron has been reported in asthma. In most clinical studies, iron levels are assessed systemically in serum/plasma but not locally in the lung. Indeed, only one study has been reported showing that low iron in exhaled breath condensate is associated with asthma [9]. However, it is unclear whether altered iron levels play a role in pathogenesis of disease, or if they are a consequence of it. To investigate the potential role of iron level and/or regulation in the lung in the pathogenesis and severity of asthma, we first assessed iron levels in the BAL supernatant and cells of patients with mild–moderate asthma and severe asthma compared to healthy controls. We show, for the first time, that NHI levels in BAL supernatant are markedly lower in asthma patients compared with healthy controls. Significantly, when the asthma patients are separated into mild–moderate and severe groups, NHI levels are significantly further reduced in patients with severe asthma. In contrast to the decreased iron levels in BAL supernatant, we show that the number of iron-loaded cells are increased in the BAL of patients with mild–moderate asthma and severe asthma. Both the levels of extracellular iron in supernatant and iron-loaded cell numbers in BAL correlate with lung function, with higher extracellular iron levels associated with better lung function and higher iron-positive cells associated with worse lung function. This is the first report showing correlations between airway iron homeostasis and key lung-function parameters. Taken together, our data suggest that lower extracellular iron in the airways and higher intracellular iron in the airways are associated with asthma and are linked with disease severity.

Our clinical data, which suggests that asthma is associated with increased iron sequestration/accumulation into cells and/or tissues, is supported by our findings that *DMT1* and/or *TFR1* expression is significantly increased in the airways in clinical and experimental asthma and correlates with impairment of lung function. In the lung, iron is mostly imported into cells by TFR1, as well as by DMT1 and NRAMP1 [1, 37–39]. Thus, increased expression of *DMT1* and *TFR1* may contribute to the decreased extracellular iron and increased intracellular iron that we observe in BAL of asthma patients. These changes may be a consequence of chronic inflammatory responses that underpin disease. Inflammation is a well-known promoter of iron sequestration into cells, predominantly due to cytokine-mediated modification of hepcidin responses [40]. A previous study showed that liver specific $Hepc^{-/-}$ mice had increased iron in the lung similar to Hepc globally deficient mice, suggesting that systemic hepcidin plays a predominant role in regulating iron levels in the lung [41]. However, studies are still required to determine whether hepcidin regulation occurs at a subtle level locally within the lung. IL6 also enhances TFR1 responses and iron uptake in hepatocytes [42]. TFR expression is upregulated in the lungs of rats exposed to lipopolysaccharide [43]. We extend these findings by showing that *TFR1* expression is associated with increased T2 inflammatory gene (*IL4*, *IL5* and *IL13*) expression in the asthmatic airways. We also show that Tfr1$^+$ macrophages are increased in the lungs during HDM-induced experimental asthma and that these cells display a phenotype of increased *Il13* expression. We also show that exogenous iron increases pro-inflammatory cytokine and ECM responses in human ASM cells and/or lung fibroblasts. Together these data suggest that increased extracellular iron levels in lung tissue may drive disease through increasing pathological responses in ASM cells and fibroblasts. Our data suggest that increased iron sequestration by Tfr1$^+$ macrophages, perhaps as a protective response to increased extracellular iron levels and/or innate pro-inflammatory responses in the asthmatic airway, may result in the induction of T2 inflammatory responses that play a key role in the pathogenesis of asthma.

Whilst our initial findings suggest strong links between the sequestration/accumulation of iron into cells/ tissues and the pathogenesis and severity of asthma, they do not show whether changes in iron homeostasis that result in increased iron accumulation in cells is a driver or consequence of disease. To investigate the roles of pulmonary iron accumulation in disease, we employed a complementary suite of murine models of iron overload and chronic HDM-induced experimental asthma. We show that both genetic ($Hfe^{-/-}$ mice) and dietary (HID fed mice) models of iron overload result in significant accumulation of iron in lung tissues. This iron accumulation is similar to that observed in HDM-induced experimental asthma. Furthermore, iron overload induced pulmonary iron accumulation results in the
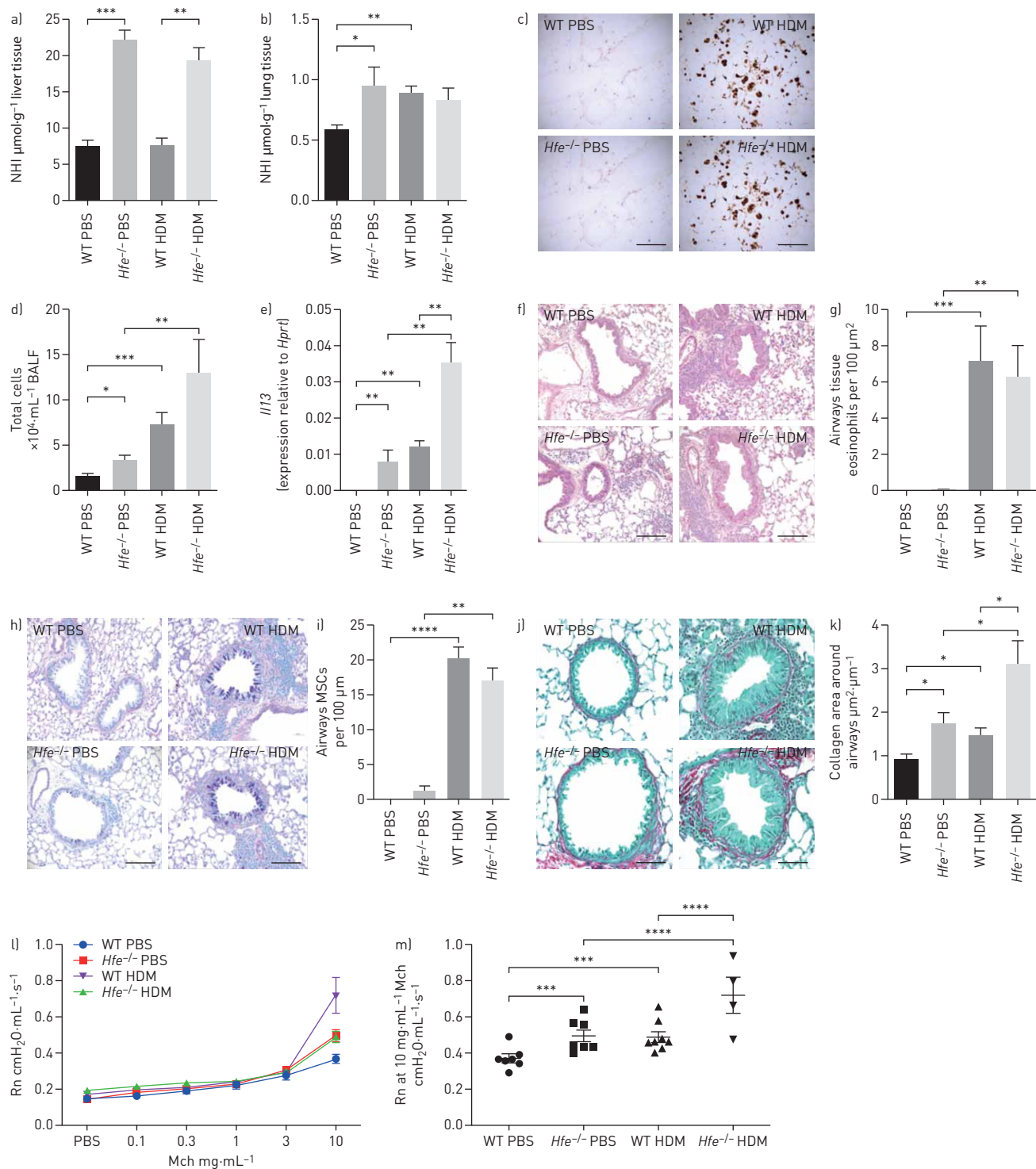
FIGURE 5 Absence of the haemochromatosis protein gene (*Hfe*) increases the severity of house dust mite (HDM)-induced experimental asthma. HDMs were administered to wild-type (WT) AKR and *Hfe*$^{-/-}$ mice at ~36 weeks old for 5 days·week^{-1} for 6 weeks and then the hallmark features of experimental asthma were assessed. Levels of non-haem iron (NHI) were measured in the a) liver and b) lung. c) Localisation of iron in the lung was assessed in lung sections using Perls' staining with 3,3′-diaminobenzidine tetrahydrochloride (DAB). d) Total leukocytes were enumerated in processed bronchoalveolar lavage (BAL). e) Interleukin 13 (*Il13*) mRNA expression was measured by quantitative PCR. f, g) Tissue eosinophilic inflammation was quantified in chrome salt fixation-stained lung sections. h, i) Mucus secreting cells (MSCs) were enumerated around the airways in periodic acid–Schiff (PAS)-stained lung sections. j, k) Area of collagen deposition surrounding the basement membrane of small airways was determined in Sirius Red stained lung tissue sections, in six to eight airways per mouse, using *ImageJ* (version 1.47, NIH, Bethesda, MD, USA). l) Airway hyper-responsiveness (AHR), in terms of central airway resistance (Rn), was measured in response to increasing concentrations of nebulised methacholine (Mch). m) Rn at 10 mg·mL^{-1} Mch was determined from AHR curves. Data are presented as mean±SEM (n=6–10), pooled from two repeat experiments. Scale bars=50 µm. BALF: bronchoalveolar lavage fluid. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001.

presentation of many of the key features of asthma, including airway inflammation, T2 cytokine (IL13) production, airway fibrosis and AHR. Together, these data provide strong evidence that increased iron levels in tissue play a key functional role in the pathogenesis and increased severity of asthma.

To conclude, complementary clinical and experimental studies show a strong relationship between iron level and regulation, and the pathogenesis and severity of asthma. We show, for the first time, that altered levels of iron and iron-related gene expression in the airways of patients with asthma are linked with lung function, with evidence for increased iron accumulation into tissues being detrimental for disease. Using murine models, we show that increased lung iron accumulation drives the pathogenesis and severity of key features of asthma. Due to the small amounts of tissue available (or lack of available RNA) we were not able to measure iron levels in airway tissue biopsies, or iron regulatory gene expression in BAL cells. We also used bronchoscopy samples from two different cohorts. These are limitations of the current study. Nevertheless, our findings highlight the need for further studies to investigate the mechanisms that underpin the effects of iron dysregulation on the pathogenesis and severity of asthma, as well as to explore and test novel iron-targeted therapies. Given the interactions that occur between microbes and iron, and the role of infection in asthma, further studies are also required to explore the inter-relationship between microbiomes, iron levels/regulation, mucosal immunity and asthma.

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