

Soluble ST2 (sST2) regulation by Rhinovirus (RV) and 25 (OH) Vitamin-D3 in the blood of asthmatic children

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&The present work was performed in fulfilment of the requirements for obtaining the degree "Dr. med." for Patricia Haag.

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Running Title: ST2 after Rhinovirus and VitD3 in asthma

This work was supported by the European Grant PreDicta at the Friedrich-Alexander-Universität (FAU) Erlangen-Nürnberg, Universitätsklinikum Erlangen and at the Allergy and Clinical Immunology Unit, 2nd Pediatric Clinic, National and Kapodistrian University of Athens.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/cei.13135

Summary

Paediatric asthma exacerbations are often caused by Rhinovirus (RV). Moreover, 25(OH)-Vitamin D3 (=25(OH) VitD3) deficiency during infancy was found associated with asthma.

Here, we investigated the innate immune responses to RV and their possible modulation by 25(OH) VitD3 serum levels in a preschool cohort of children with and without asthma.

The innate lymphoid cell type 2 (ILC2)-associated marker, ST2 was found upregulated in the blood cells of asthmatic children with low serum levels of 25(OH)VitD3 in the absence of RV in their airways. Furthermore, in blood cells from control and asthmatic children with Rhinovirus in their airways, soluble (s) ST2 protein was found reduced. Asthmatic children with low 25(OH) VitD3 in serum and with RV in vivo in their airways at the time of the analysis had the lowest sST2 protein levels in the peripheral blood as compared to control children without RV and high levels of 25(OH) VitD3. Amphiregulin (AREG), another ILC2 associated marker, was found induced in the control children with RV in their airways and low serum levels of 25(OH) VitD3.

In conclusion, the anti-inflammatory soluble form of ST2 also known as sST2, in serum directly correlated with IL-33 in the airways of asthmatic children. Furthermore, Rhinovirus colonization in the airways and low serum levels of 25(OH) VitD3 were found to be associated with downregulation sST2 in serum in paediatric asthma. These data indicate a counter-regulatory role of 25(OH) VitD3 on RV-induced downregulation of serum sST2 in paediatric asthma which is relevant for the therapy of this disease.

Key words: Vitamin D3, Rhinovirus, Interferon Beta, ST2, sST2, ILC2, amphiregulin (AREG), paediatric asthma.

Introduction

Allergic asthma is an increasingly common chronic-inflammatory disease of the airways that affects millions of people worldwide ¹.

While the classical model of allergy-induced airway inflammation focuses on a Th2-driven immune-reaction^{2, 3}, recent studies have noted the role of Group 2 Innate lymphoid cells (ILC2s) in models of allergic asthma ⁴⁻⁶. This newly discovered cell population is able to react directly and immediately to an allergen-induced epithelial cell stimulation without the need for antigen presentation⁶. Once stimulated, ILC2s react with the secretion of IL-5, IL-9 and IL-13, classically known as Th2 cytokines^{5, 7}. In addition, ILC2s release Amphiregulin, a protein belonging to the epidermal growth factor family that has the ability to repair damaged epithelial cells, e.g., after an asthma attack and to promote the growth of normal epithelial cells⁸. AREGB, the gene that encodes Amphiregulin, is a known target gene of 25(OH)-VitD3⁹.

As little is known about how ILC2s can be influenced exogenously, here, we analysed ILC2-associated genes and their regulating factors. During an allergen and Rhinovirus-induced asthma exacerbation *in vivo*, IL-33 and IL-25 (IL-17E)¹⁰ are released from the infected necrotic airway epithelial cells, leading to the activation of IL-33-responsive IL-33R /ST2+ T cells, in general, and specifically, to the activation of ILC2s ¹¹, which are key effector cells releasing Th2 cytokines, such as IL-13 and IL-5, thus linking viral infection and the exacerbation of asthma ¹²⁻¹⁴. ST2 is encoded by IL1RL1, and after differential gene splicing, it can produce a functional membrane-bound receptor (IL-33R or ST2L) or soluble decoy receptor (sST2), depending on the splice variant¹⁵. Moreover, it has been recently demonstrated that 25(OH)-Vitamin D3 enhances the production of the soluble ST2 isoform ¹⁶. Because soluble sST2 neutralizes the effect of IL-33, sST2 is considered an anti-inflammatory factor in asthma^{17, 18}.

Thus, IL-33 neutralization may be a novel therapeutic approach for asthma exacerbations¹².

Vitamin D3, also named cholecalciferol, is a type of vitamin D found in food and is used as a dietary supplement. Cholecalciferol is synthesized in the skin from 7-dehydrocholesterol under the action of ultraviolet UV-B light. Blood levels of this molecule largely reflect the amount of cholecalciferol produced in the skin combined with any vitamin D2 or D3 ingested. The hydroxylation of vitamin D3 in the endoplasmic reticulum of liver hepatocytes to calcifediol (25-hydroxycholecalciferol) occurs by means of a 25-hydroxylase. Hydroxylation in the kidneys of calcifediol to calcitriol is induced by a 1-alpha-hydroxylase (CYP27B1), resulting in the production of the biologically active form 1,25-dihydroxyvitamin D3. Since 1,25(OH)-Vitamin D3 is instable, the detection of 25(OH)-Vitamin D3 is one of the highly discussed therapeutic compounds for allergic asthma¹⁹. It is known that 25(OH)-Vitamin D3-deficient patients show more asthma exacerbations, a longer lasting disease²⁰ and an increased corticosteroid use²¹. Moreover, recent studies demonstrated that 25(OH)-Vitamin D3 supplementation can reduce airway inflammation²² and inflammatory cell proliferation²³.

To analyse the reciprocal influence of genes associated with ILC2s in allergy development and the anti-inflammatory effect of 25(OH)-Vitamin D3, we focused on the influence of 25(OH)-Vitamin D3 in asthma with special interest on ST2¹² by using human blood samples and looking at the immunomodulatory role of serum levels of 25(OH)-Vitamin D3 in asthma, as well as RV-associated asthma in a paediatric cohort of children.

Materials and Methods

All methods in this study were performed in accordance with the relevant guidelines and regulations.

Human Studies

The present study is part of the prospective study PreDicta (Post-infectious immune reprogramming and its association with persistence and chronicity of respiratory allergic diseases). This study was performed on the WP1-cohort set up at the Department of Molecular Pneumology in Erlangen in collaboration with the local Children's Hospital. The study in Erlangen was approved by the Ethics Committee of the Universitäts-klinikum Friedrich-Alexander Universität Erlangen-Nürnberg (Re-No 4435), and it is registered in the German Clinical Trials Register (www.germanctr.de: DRKS00004914). Parents/guardians of all participants gave their informed consent.

Two cohorts of pre-school children (age 4-6 years) with and without asthma were analysed. The recruitment of the subjects, inclusion and exclusion criteria, as well as the timescale for clinical visits and data collection were described recently²⁴⁻²⁶, and patient characteristics are reported in Table 1.

Serum 25(OH)-Vitamin-D3 analysis.

At baseline, 25(OH)-Vitamin-D3 was measured in serum during visits to the Dept. of Paediatrics and Adolescent Medicine of the Friedrich-Alexander Universität Erlangen-Nürnberg (Erlangen, Germany) by using an LC-MS/MS MassChrom Kit from Chromo-systems according to the manufacturer's protocol.

Isolation of peripheral blood mononuclear cells (PBMCs) from children with and without asthma and Rhinovirus infection of PBMCs.

PBMCs were isolated from heparinized blood with Ficoll using density centrifugation at the recruitment time (Baseline 0) (B0=Day 0) and 2 years later at a follow-up visit 4 (F4). PBMCs were then adjusted to a concentration of 1×10^6 viable cells/ml in complete culture medium, as previously described, and parts of them were in vitro challenged or not with Rhinovirus (RV1b) and were cultured for an additional forty-eight-hours to perform RNA gene array assays and further analyses. The growth of RV1b and the description of the RV1b infection itself were previously published in detail elsewhere²⁷.

Gene arrays

To analyse RNA from PBMCs at F4, we performed gene arrays. RNA was reversed transcribed after passing high stringency quality controls. Biotinylated cDNA was prepared according to the standard Illumina protocol from 13.9 ng total RNA using an amplification kit Pico WTA, Lot # 1404311-A/C, followed by labelling 3 µg cDNA using an Encore Biotin IL module kit, Lot # 1212262-D. Using the Illumina iScan Scanner running Illumina iSCAN control software v 3.3.28, Bead Chips were scanned. Illumina Genome-Studio version 2011 was used to analyse the data. Heat maps were generated by performing supervised clustering on normalized expression data using R version 3.1 and library gplots. In the gene array cultured experiments, cells from five control and 7 asthmatic children who were exposed or not exposed to RV1b for 1 h were analysed. Genes related to ILC2 were selected, and the results were reported as log₂ ratio (average signal treated/average signal untreated samples) of statistically significantly regulated genes p equal to or less than 0.01.

RV detection *in vivo*

The detection of rhinovirus in nasopharyngeal swabs from the children was performed at the Department of Virology, University of Turku (Finland). The description of this method was recently published in detail elsewhere²⁷.

Enzyme-linked immunosorbent assay (ELISA).

ELISA kit from Peprtech was used to detect Human IFN β . ELISA from R&D were used to detect IL-33 and sST2 (Duo Set) in human serum. Human TNF-alpha was performed with Duo Set #DY210 von R&D. ELISAs were performed according to the manufacture's protocol.

Human RNA isolation from total blood cells and quantitative Real-Time PCR.

At the Baseline visit, whole blood was collected in Tempus® Blood RNA Tubes (Life Technologies™, GmbH, Darmstadt, Germany), and RNA was extracted with the MagMax for Stabilized Blood Tubes RNA Isolation Kit. Synthesis of cDNA and, consequently, real-time PCR were performed as described for murine cells below with the following primers and sequences: hHPRT (5'-TGA CAC TGG CAA AAC AAT GCA-3', 5'-GGT CCT TTT CAC CAG CAA GCT-3'), hAREGB (5' -GCG AAG GAC CAA TTG GAG CC-3', 5'AGC ATA ATG GCC TGA GCC G-3'), hST2 (5' -CAC GGT CAA GGA TGA GCA AG-3', 5' -GCA GAG CAA GTT AGG TTT GAG-3').

FEV1.

FEV1 (=forced expiratory volume in 1 second) and FVC (=forced vital capacity) were measured at the baseline visit (B0) by using spirometry. After a period of normal breathing, the

participant should inhale at maximal capacity, directly followed by maximal and fast exhalation. The volume exhaled in one second is FEV1. The total exhaled volume is FVC. The ratio FEV1/FVC is reported as FEV1 %.

Statistical analysis

Differences were evaluated for significance (* $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$) by using the parametric -two-tailed Student t-tests were used (Prism version 7 for Windows; GraphPad, La Jolla, CA, USA), unless stated otherwise. Data are shown as the mean values $\pm$ s.e.m. To examine the correlation of two parameters, GraphPad Prism software (version 7 for Windows) was used.

RESULTS

Clinical characteristics of the cohorts of children analysed in this study

In this study, we analysed 22 control children and 24 preschool children with asthma. The clinical data of these cohorts of preschool children are reported in **Table 1**. The average age of controls and children with asthma was 4.7 and 4.9 years, respectively. According to the guidelines, 62.5 % of the children had intermittent, 29.2 % mild-persistent and 8.3 % moderate-persistent asthma. Moreover, 72.7 % of controls and only 45.8 % of the children with asthma had an FEV1 above 100 % (**Table 1**). In 70.8 % of the cases, a viral infection was a triggering factor for the development of the disease. RV was the most common respiratory virus detected in the airways of these children.

Gene arrays from *in vitro* RV-infected PBMCs from pre-school children with asthma revealed the induction of ILC2-associated genes.

Innate lymphoid cells (ILCs) are frequently detected in the lung, although they originate from the bone marrow¹¹. Thus, we reasoned that blood-circulating ILC2 progenitors might be present in the PBMCs of children with asthma. To start to investigate the relationship between circulating ILC2 and RV, we analysed RNA isolated from PBMCs from preschool children with and without asthma, who were recruited in the European study Predicta in Erlangen (WP1-UKER), and cultured the cells for 48 hours after a 1-hour exposure *in vitro* to RV1b, as previously described^{24, 25, 28}. After RNA extraction and RNA quality selection for gene arrays, the mRNA from 7 children with asthma and from 5 control children were analysed for gene arrays as shown in **Figure 1a**. We then selected genes related to the ILC2 pathway that showed a statistically significant RV-dependent upregulation in PBMCs from asthmatic children. As a

control, to create the heat map, we inserted some related genes that were downregulated by the Rhinovirus. Here, we found that several genes inducing ILC2 activation, such as IL-25¹¹, IL2RA⁷ and IL7^{7,29}, and TMED1(IL1RL-1) were upregulated by RV in PBMCs of asthmatic children (**Fig. 1b and Table S1**).

Moreover, we found increased expression of TNF-alpha, a gene stimulated by IL-33³⁰, and IL1B, a gene involved in the activation of IL-33³¹. Finally, we found that RV induced genes that are targets of 25(OH)VitD3, such as Amphiregulin, a gene that interacts with EGR^{9,32} and promotes the growth of normal epithelial cells after cell damage. In contrast, Lactadherin (Mfge8), which is involved in IL-33 signalling, was found to be downregulated by RV in asthma³³.

In summary, the genes upregulated in PBMCs by rhinovirus in this paediatric cohort of asthmatic cases suggest an activation of ILC2 development and a downregulation of genes that cut the viral RNA, such as RNase1.

Furthermore, we analysed which genes were regulated by RV in PBMCs isolated from control children. Here we found an RV-induced upregulation of EGR1, a receptor for Amphiregulin³², in PBMCs. Moreover, NFKBIA (also known as hepatic I κ B α) is increased by RV. This gene plays an important role in persistent inflammation, and its decrease is associated with 25(OH)-Vitamin D3 deficiency³⁴. On the other hand, RXRB, which forms a complex with VDR and would be necessary to activate VDR targets³⁵, is decreased in the PBMCs of control children by RV. In summary, in PBMCs isolated from control children, RV induces genes that are associated with epithelial damage and chronic inflammation. We next looked at RV in the nasal Pharyngeal fluid and 25(OH)VitD3 in the serum of these children. As shown below the heat map, the majority of the asthmatic children analysed had low serum levels of 25(OH)VitD3 and few had RV in the airways (**Fig1b**). By contrast, control children had high (h) serum levels of 25(OH)VitD3 and like asthmatic generally did not have RV in their

airways (Fig 1c). We next confirmed the gene arrays results by measuring TNF alpha in the supernatants of the PBMCs by ELISA and found that PBMCs from asthmatic children released increased levels of TFN-alpha after RV exposure *in vitro* (Fig 1f). Real time PCR showed an upregulation of ST2 in PBMCs isolated from asthmatic children after *in vitro* exposure to RV (Fig 1g).

Reduced Interferon Beta levels in serum and increased ST2 mRNA levels in the blood cells of preschool children with asthma compared to control preschool children

To further investigate the role of *in vivo* RV infection on ILC2, we first looked at IFN-beta, an antiviral gene regulated by Rhinovirus and deficient in the airways of asthmatic subjects³⁶ that recently was found to counter-regulate ILC2³⁷. IFN-beta was found to be downregulated, although not statistically significantly, in the serum of asthmatic preschool children compared to control children (Fig. 2a). Consistent with the upregulation of the ILC2-associated genes after RV challenge, we found in a larger cohort of preschool children with asthma an induction of the ILC2-associated marker ST2 mRNA, in the blood of these preschool children with asthma compared to control children (Fig. 2b). Because ST2 is present in two different spliced variants, we used one set of primers recognizing the total transcript of ST2 (*IL1RL1*) (Fig. 2b), and an ELISA kit recognizing the soluble sST2 (Fig. 2c,d) protein. No difference in sST2 was observed in serum (Fig 2c).

Increased sST2 protein in the NPF of preschool children with asthma with RV in the airways.

We next wanted to analyse sST2 protein in the nasopharyngeal fluid (NPF) of the two cohorts of children at baseline. Here, we found that the asthmatic children had more sST2 in the

airways compared to the control children (**Fig. 2d**). In addition, we found that in serum there is 100 times more sST2 compared to that in the upper airways, indicating that the majority of sST2 is produced by blood cells. These results support the notion that sST2-producing cells are circulating cells rather than cells present in the airways.

Regulation of ST2 by Rhinovirus in the airways of asthmatic children

We next considered the presence of RV in the airways in the above analysed pathway. In the presence of RV in the airways, in control children, IFN β was found to be downregulated in serum, whereas independently from RV, IFN β was always low in the serum from asthmatic children (**Fig. 3a**). *AREG* mRNA was found upregulated in mRNA isolated from total blood cells of control children when Rhinovirus was detected in their airways (**Fig. 3b**). Moreover, in the absence of RV in the airways, in the blood cells from asthmatic children, ST2 mRNA was found upregulated compared to control children without RV in the airways (**Fig. 3c**). These data suggest an ST2 induction associated with factors other than RV.

We then analysed sST2 protein levels in the serum and NPF considering that rhinovirus is present in the airways of these cohorts of preschool children. Although no statistically significant difference was found, as a trend, sST2 protein was reduced in serum and induced in NPF of asthmatic children with Rhinovirus in their airways (**Fig. 3d and e**, respectively). These data indicate that sST2 is induced/recruited in the airways of asthmatic children by RV, indicating a role of sST2 in anti-viral immune responses in the presence of RV in the airways in asthma.

sST2 in serum is directly correlated with IL-33 in the nasopharyngeal fluid of asthmatic children.

Because sST2 neutralizes IL-33, which we found recently induced in the airways of asthmatic children with gram-negative bacteria colonization, we next asked if there was a correlation between sST2 and IL-33 in the airways. We found that serum sST2 protein directly correlated with IL-33 in the airways of asthmatic but not in the airways of control children (**Figure 4a, b**). No significant correlations were found by comparing IL-33 and sST2 protein in NPF (**Fig. 4c, d**).

Vitamin D3 Supplementation and 25(OH)-VitD3 in the serum of asthmatic children.

We further reasoned that many genes we found activated by RV were genes that were downregulated by 25(OH)VitD3, which has a protective role in asthma exacerbations³⁸.

In Germany, Vitamin D3 is given to infants³⁹ to avoid immunological suppression, especially in children who did not receive breastfeeding. By analysing the potential effect of this Vitamin D3 supplementation, we analysed the questionnaire filled by the parents/guardians. Here we noticed that the percentage of children treated with Vitamin D3 supplement (Fluorette: Colecalciferol and fluorid) was higher in the control group compared to the asthma group (**Table 1**). We next analysed the role of cholecalciferol, Vitamin D3, supplementation during infancy on serum 25(OH)VitD3 levels in our cohort of children with or without asthma. Here, by considering 0 as no VitD3 Supplement and 1 as VitD3 Supplement taken as an infant, we found that in contrast to control children (**Fig. Supplementary Fig. 1a and Table 2**), in asthmatic children, increased serum 25(OH)VitD3 correlated with having taken a VitD3 supplement during infancy (**Supplementary Fig. 1b**). In fact in 75% of asthmatic children and in 50% of control children with VitD3 Supplement had high 25 (OH)VitD3

serum levels at the time of recruitment (**Table S2**). Taken together, these data indicate a strong association between asthma development in childhood, insufficient 25(OH)VitD3 in serum, and the lack of VitD3 supplementation as an infant.

Symptomatic asthma exacerbations are associated with increased 25 (OH) VitD3 in serum of asthmatic children.

Wheezing is associated with asthma in the first years of life ⁴⁰. Moreover, RV is the factor that is associated with wheezing in infants ⁴¹. Further, the levels of 25(OH)VitD3 in serum, considering the presence of RV in the airways, was found down/regulated in the serum of control children with RV in their airways and in asthmatic children without RV in their airways (**Fig. 5a**). Moreover, 25(OH)VitD3 was found to be induced in the serum of children during asthma exacerbation when, as we previously reported, RV was present in the airways (**Fig. 5b**). Altogether, these data indicate a role of the serum level of 25(OH)VitD3 in RV induced-asthma development.

Vitamin D Receptor (VDR) is induced by RV and serum 25 (OH)VitD3 level in control children

We next looked for an explanation why 25(OH)VitD3 serum level decreased in serum of control children when RV was detected in their airway. As first, we thought that the host would react to RC by transporting the 25(OH)VitD3 from blood fluid into blood cells. To prove this, we analysed vitamin D3 receptor (VDR) expression in total blood cells. Here we found a significant increase in *VDR* mRNA in control children with RV in the airways as compared to control children without RV in the airways (**Fig 5c**). The same trend was observed for VDR in asthmatic children, although not significant. We then asked if VDR expression would also be influenced by serum level of 25(OH)VitD3. Here we observed a

significant induction of VDR in control children with RV and high serum levels of 25(OH) VitD3 (**Fig 5d**). In asthmatic children we did not have the group with high 25(OH) VitD3 without RV and therefore we cannot make any conclusion. Further extended analyses are required.

IL-33 in the airways directly correlated with 25 (OH) in serum in asthma

We then wanted to investigate the role of 25(OH) VitD3 and the IL-33/ST2 pathway. Here, we started by examining the correlation between 25(OH) VitD3 in serum and IL-33 in the NPF. We found that IL-33 in the NPF was directly correlated with serum 25(OH) VitD3 in asthmatic children (**Fig. 6a,b**) at baseline. This result is in agreement with our previous finding that sST2 directly correlated with IL-33 and with the findings that 25(OH) VitD3 induces sST2.

Low 25-(OH)-Vitamin D3 (^{low} 25(OH) VitD3) levels are associated with lower IFN-beta levels in serum and high AREG in blood cells in asthmatic children

We next asked whether IFN-beta was regulated in children with higher levels of 25(OH)- VitD3 (**Fig. 7a**). Here, we found that control children with higher serum 25(OH) VitD3 levels (higher than 20 ng/ml) had higher serum IFN-beta levels, although this difference was not statistically significant (**Fig. 7a**); asthmatic children always had lower serum levels of IFN-beta irrespective of their serum of 25(OH) VitD3 levels (**Fig. 7a**).

Since IFN-beta limits ILC2 activation, we then analysed AREGB mRNA considering the influence of 25(OH) VitD3 and found that, consistent with an increase in IFN-beta, higher serum levels of 25(OH) VitD3 were associated with a downregulation of AREGB mRNA in blood cells of control children (**Fig. 7b**). By contrast, asthmatic children did not downregulate AREGB when 25(OH) VitD3 was increased (**Fig. 7b**), and at low levels of 25(OH) VitD3 they

had lower AREG compared to the control children with lower 25(OH)VitD3 (**Fig. 7b**). We next considered the presence of RV in the airways and found that IFN-beta induction associated with high 25(OH)VitD3 serum level in control children in the absence of RV in the airways (**Fig. 7c**). AREG was found to be upregulated by RV and low serum 25(OH)VitD3 level in control children (**Fig 7d**).

ST2 mRNA is induced in blood cells of asthmatic children with low 25(OH)-VitD3 serum levels without RV

We next asked about the relationship between ST2 mRNA in blood cells and 25(OH)VitD3 in serum. Here, we found that asthmatic children with low serum levels of 25(OH)VitD3 had significantly higher levels of ST2 mRNA (**Fig. 8a**) compared to the control children with comparable low serum levels of 25(OH)VitD3. By considering the presence of RV in the airways we could confirm the findings that higher ST2 mRNA levels was not associate with the presence of RV *in vivo* but with lower 25(OH)VitD3 serum level. Going back and considering the serum 25(OH)VitD3 levels of the children analysed in the gene arrays, we found that the asthmatic children had all lower levels of serum 25(OH)VitD3 (**Table 2 and number of the children below the Heat maps**) whereas the control children had relatively higher serum 25(OH)VitD3 levels. In conclusion, we found that the level of 25(OH)VitD3 and not RV in the airways is probably the cause of ST2 induction after *in vitro* exposure to RV in PBMCs.

sST2 in serum was reduced by RV *in vivo* in control and in asthmatic children

It has been recently demonstrated that 25(OH)VitD3 enhances the production of the soluble ST2 isoform¹⁶.

Soluble ST2 as mentioned above, is an anti-inflammatory marker able of neutralizing IL-33, the inflammatory cytokine also known as the ligand of ST2. Considering the anti-inflammatory function of 25(OH)VitD3, we next asked about the relationship between 25(OH)VitD3, RV in the airways and sST2. Here we found that the highest levels of the anti-inflammatory isoform sST2 was present in the serum of control children with high levels of 25(OH)VitD3 and without RV in the airways (**Fig 8c,d**). The presence of RV in the airways was the cause of sST2 reduction both in control and in asthmatic children. In this latter also associated with low levels of 25(OH)VitD3 contributed along with RV to the reduction of the sST2 in serum.

In conclusion, we found that ST2 is upregulated in blood cells of asthmatics not by RV but by low levels of serum levels of 25(OH)VitD3. By contrast, the anti-inflammatory sST2 isoform was found downregulated by RV and in asthma also by combined low levels of 25(OH)VitD3.

DISCUSSION

Here, we found that rhinovirus challenge in the airways, which is one of the main factors in asthma exacerbations^{42, 43}, is associated with reduced expression of the anti-inflammatory protein sST2 in the blood cells of children with and without asthma. Moreover, we found that the soluble form of ST2, sST2, was downregulated in the serum of asthmatic children with RV in the airways, and with low serum levels of 25(OH)VitD3. 25(OH) VitD3 deficiency has been associated with asthma as in many inflammatory and autoimmune pathologies^{44, 45}. The highest levels of serum sST2 was observed in the serum of control children without RV in the airways and high serum levels of 25(OH)VitD3. Previous studies showed that ST2 plays an important role in ILC2-driven allergic asthma⁴⁶ and that RV can induce type-2- and ILC2-associated cytokines.⁴⁷ In addition, the role of sST2 was previously reported in the literature of asthmatics in induced sputum⁴⁸. In this study, we first observed by gene arrays, in the peripheral blood of preschool children with asthma, an increased expression of ILC2-related markers such as AREG and confirmed this finding by correlating with the clinical outcomes of these children. Total ST2 mRNA, was found upregulated after in vitro exposure to RV and in the blood of asthmatic children with low serum levels of 25(OH)VitD3 without RV in their airways. sST2 is the ligand of IL-33, and because it is soluble, it can circulate and reach the airways where IL-33 is released. IL-33 has also been described to regulate sST2⁴⁹. Moreover, IL-33 and its receptor and sST2 were largely investigated in asthmatic children^{50, 51}. We, thus, examined the correlation of IL-33 in the airways with sST2. Here, we found that the serum levels of sST2 directly correlated with IL-33 in the airways in asthmatic children. Also serum levels of 25(OH)VitD3 directly correlated with serum sST2 and IL-33 in the airways of asthmatic children.

Asthmatic children who did not receive Vitamin D3 supplementation as infants had more episodes of asthma exacerbation and those who had RV *in vivo* in their airways at the time of the analysis had reduced serum 25(OH)VitD3 levels associated with less sST2 serum levels and with lower AREG, a protein involved in epithelial cell repair, protein levels in the peripheral blood.

RV infection can damage epithelial cells⁵² and delay epithelial cell repair⁵³.

Several studies have shown that 25(OH)VitD3 has the potential to prevent the development of allergic asthma^{54, 55} and even has the ability to reduce asthma exacerbations⁵⁶, so we further investigated its influence on Rhinovirus-induced allergic asthma in preschool children.

RV challenge in the airways is one of the main factors inducing asthma exacerbations⁴². Previous studies showed that ST2 plays an important role in ILC2-driven allergic asthma⁴⁶ and that RV can induce type-2- and ILC2-associated cytokines¹². Since little is known about how this new cell type can be influenced exogenously, we chose to focus on the impact of 25(OH)VitD3 on ST2-driven allergic asthma.

Here, we first found increased ILC2 cell-related markers in the peripheral blood from asthmatic children after RV challenge, in accordance with the results of former studies⁴⁷.

By following up this finding, we found that AREG is induced, whereas IFN-beta, an antiviral interferon that has been recently discovered to inhibit ILC2s, is decreased in the blood of asthmatic children³⁷. We further sub-grouped the children by the presence of RV in their airways *in vivo* and found that, in the absence of RV in the airways, IFN-beta serum levels of asthmatic children were decreased. We further sub-grouped the children according to their serum level of 25(OH)VitD3 and found that only control children with high 25(OH)VitD3 serum levels could produce higher amounts of IFN-beta. In conclusion, IFN-beta is induced by high levels of 25(OH)VitD3 in control children and was found to be reduced in asthma.

To investigate which mechanism is activated by 25(OH)VitD3 in IFN-beta induction, we started to look at ILC2 markers that have inhibitory functions on IFN-beta production. We found that AREGB, which encodes amphiregulin, a protein that promotes the growth and repair of normal epithelial cells in the airways, was found to be induced by in vivo RV infection in control children. This upregulation of AREGB may be activated because of the damage that RV causes to the epithelial cells⁵² in vivo, and as a result, it contributes to epithelial cell repair⁵³. We next considered the effect of the serum level of 25(OH)VitD3 on AREG mRNA levels in blood and found it downregulated when higher serum levels of 25(OH)VitD3 in control children were measured. By contrast, higher serum levels of 25(OH)VitD3 were found to be associated with higher IFN-beta levels in control children. Thus, AREGB is an ILC2 marker, that could be considered as a limiting factor, possibly limiting the IFN-beta production upon RV infection in control preschool children.

We next started to look at ST2 and sST2, which we found upregulated in asthmatic children. ST2 was found to be upregulated in the blood cells of asthmatic children. Considering the presence of RV in the airways, we found a significant induction of ST2 mRNA expression in the blood cells of asthmatic children without RV colonization in the airways. Considering serum levels of 25(OH)VitD3, ST2 mRNA was found to be upregulated in blood cells of asthmatic children with lower levels of 25(OH)VitD3 without RV in the airways. RV in the airways reduced ST2 mRNA. Additional factors could be responsible of the ST2 induction in the absence of RV in the airways in asthmatic children. One possibility is the decrease in IFN-beta production in this group. Moreover, we recently reported that the bacterial colonization of the airways of these children influences ST2. In fact, ST2 was found to be upregulated in asthmatic children with a mixed gram-positive and gram-negative bacterial colonization in their airways²⁵. Finally, high 25(OH)VitD3 serum levels were found to be associated with higher anti-inflammatory sST2 in control children without RV in the airways. The presence of RV in vivo both in control and asthmatic children associated with decreased sST2 serum

levels although in asthmatic children was observed at low levels of serum 25(OH)VitD3 and in control children at high levels of serum 25(OH)VitD3. Thus it is possible that the downregulation of ST2 mRNA observed after RV in asthma relates to sST2.

In summary low levels of 25(OH)VitD3 without RV induced ST2 and low levels of 25(OH)VitD3 with RV reduced sST2. These results explain the apparent discrepancy of the gene arrays which do not recognize the soluble form of ST2.

When we looked at the asthmatic exacerbations, we found them induced in asthmatic children without Vitamin-D3 supplement as infant. Moreover, at the symptomatic visit, a condition always associated with RV in the airways of these children, we found increased serum levels of 25(OH)VitD3. By looking at control children, RV in the airways was always associated with decreased serum level of 25(OH)VitD3, thus suggesting a dysregulation of this system. By looking at the intracellular carrier for 25(OH)VitD3, the Vitamin D3 receptor (VDR), we found it upregulated in control children with RV in the airways and with high levels of serum 25(OH)VitD3. This mechanism seems to make sense as a transport mechanism induced upon 25(OH)VitD3 induction. It remains to be explained why during symptomatic visit with RV in the airways, asthmatic children have higher serum 25(OH)VitD3 levels. One possibility is that in asthmatic children this carrier mechanism is dysregulated.

In conclusion, we suggest that in control children with RV infection, 25(OH)VitD3 is carried intracellularly, where it binds to the VDR. These data are supported by our VDR expression data showing induction of VDR after RV and at high serum level of 25(OH)VitD3 in control children.

Next, we asked if there was a relevant influence from factors present in the infancy of these children of our cohort that could have affected their current status of serum 25(OH)-VitD3, and moreover, the development of allergic diseases. Former studies have shown that lower 25(OH)VitD3 intake increased the risk of developing allergic asthma⁵⁷. Children with asthma

who did not receive Vitamin-D3 supplement as infants, as reported by their parents, had lower serum 25(OH)VitD3 levels.

We further analysed which parameters may have been influenced by the current 25(OH)VitD3 serum level of the children. High 25(OH)VitD3 serum levels were found to be associated with an induction of IFN-beta in the serum of control children, which might indicate a possible synergetic effect of 1α , 25(OH) $_2$ -Vitamin D3 and IFN-beta. By contrast, in asthmatic children, even high 25(OH)VitD3 levels could not induce an anti-microbial IFN-beta-mediated immune response at all, confirming a defect of IFN-beta in the serum of asthmatic children.

AUTHOR CONTRIBUTIONS

S.F. designed the present study. S.F. and P.H. contributed equally to the writing of the manuscript, to the analysis of the Predicta data and to all the experimental aspects of the Predicta study except for the Vitamin D3 measurements, which were performed by M.R. Nikolaos G. Papadopoulos designed the Predicta WP1 project and is the coordinator of the Predicta study. He set up the design of the Predicta study and the questionnaires that were then used by the MP group in Erlangen to analyse the answers of the parents of the children analysed in this study. Additionally, the cell culture studies with PBMCs were designed by N.P. A.K. and T.Z. are the paediatricians who saw most of the children in Predicta WP1-UKER and made the medical diagnosis. T.V. analysed the NPF for respiratory viruses. Anna Graser isolated the PBMCs and did the in vitro RV challenge in the Predicta study. S.T. and N.P. provided the rhinovirus used in these experiments. H.S. and A.B. performed and

analysed the gene arrays. S.T.W., the Chief of the Translational Genomics Core, Personalized Medicine, Cambridge, MA, coordinated the gene arrays and edited the manuscript.

ACKNOWLEDGMENTS

The authors thank all children and their parents/guardians who took part in our Europe-wide study PreDicta. Moreover, the authors are grateful to S. Trump, S. Mittler and B. Klösch at the Division of Molecular Pneumology and to E. Muschiol, I. Jawa and L. Schramm at the Paediatric Pneumology-Allergology, Department of Paediatrics and Adolescent Medicine, Universitätsklinikum Erlangen, Erlangen Children's Hospital in Erlangen for their technical support. Moreover, the authors thank Drs. M. Wölfel, C. Reinhardt, A. Neubert and Prof. Dr. W. Rascher from the Department of Paediatrics and Adolescent Medicine, Universitätsklinikum Erlangen, Erlangen for their assistance in the PreDicta UKER WP-1 study. Furthermore, we thank R. Stergiou from the Allergy and Clinical Immunology Unit of the National and Kapodistrian University of Athens for the RV1b.

This work was supported by the European Grant PreDicta (Post-infectious immune reprogramming and its association with persistence and chronicity of respiratory allergic diseases), in Erlangen and in the other European Center in Athens and by the Department of Molecular Pneumology in Erlangen. This work was also supported by a DFG grant SFB 643/TP 12, and Patricia Haag and Anna Graser were supported by the European Grant Predicta in Erlangen.

COMPETING FINANCIAL INTERESTS

The authors declare no conflict of interest on the matter described in this manuscript.

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Figure legends

Figure 1: Gene arrays of RNA from in vitro RV infected PBMCs of preschool children with asthma revealed the induction of ILC2-associated genes.

(a) Experimental design for analysis after gene arrays of RNA from PBMCs cultured for 1 h in the presence or absence of the RV1b serotype and then cultured for an additional 48 h. Heat maps for mRNA isolated from PBMCs of (b) asthmatic and (c) control children exposed (+RV) or not exposed (–RV) to RV. Differential expression analysis of the regulated genes is shown ($\log_2$ average +RV/average –RV). N asthma=7, n control=5. Horizontal numbers below the heat maps represent the code of the analysed children, the presence (+) or absence (–) of RV or other respiratory viruses (other) in the airways and the levels of serum 25(OH) VitD3 (nd=not determined, l=low inferior of 20 ng/ml and h=high more than 20 ng/ml: Vitamin-D3 = VitD3; American Institute of Medicine recommends 25(OH)VitD3 > 20 ng/ml <http://www.nejm.org/doi/full/10.1056/NEJMe1312291>). (d, e) On the right-hand side of the heat map are the selected genes (red: upregulated, blue: downregulated genes) significantly regulated by RV in asthmatic (upper graph) and in control children (lower graph). (f) Gene arrays were confirmed at protein and qPCR level by TNF ELISA (f) and ST2 at qPCR levels (g).

Figure 2: ST2 pathway is activated in preschool children with asthma.

(a) IFN-beta protein was measured in the serum of control and asthmatic children (n=14, B0) (b) *ST2/HPRT* mRNA values of asthmatic and non-asthmatic children (control n=14, asthma n=16, B0). p=0,005. (c) Soluble ST2 protein in the serum of control and asthmatic preschool children (CN: n=18; A: n=12). (d) Soluble sST2 protein in the nasopharyngeal fluid in control and in asthmatic children (CN: n=21; A: n=24; p= 0.067 paired t test).

Figure 3. ST2 mRNA is induced in blood cells of asthmatic children without Rhinovirus in the airways.

(a) IFN-beta protein of asthmatic and non-asthmatic children sub-grouped by the presence of *in vivo* RV infection in the airways (CN-RV n=5, CN+RV, n=5; A-RV n=7, A+RV n=3). **(b)** *AREGB/HPRT* mRNA values of asthmatic and non-asthmatic children, sub-grouped by *in vivo* RV infection in the airways (control -RV n=6, control +RV n=8, asthma -RV n=5, asthma +RV n=10, B0), p=0,0326 paired t test. **(c)** *ST2/HPRT* mRNA values in asthmatic and non-asthmatic children, sub-grouped by the presence of *in vivo* RV infection in the airways (control -RV n=6, control +RV n=8, asthma -RV n=4, asthma +RV n=10, B0). Control -RV vs asthma -RV, p=0,0152; asthma -RV vs asthma +RV, p=0,0259, two-tailed student's T-test. **(d)**. Soluble ST2 in serum in accordance with the presence of RV (+RV) or not (-RV) in the airways: CN-RV n= 8, CN +RV n=10, A-RV n=6, A+RV n=6. **(e)** sST2 in NPF: CN-RV n=9, CN+RV n=11, A-RV n=11, A+RV n=12.

Figure 4: Serum sST2 directly correlated with IL33 in NPF in asthmatic children.

Linear regressions are reported for sST2 serum and IL-33 in NPF in control **(a)** and asthmatic children **(b)**. **(c, d)** Linear regression for sST2 in NPF and IL-33 in NPF in controls **(c)** and in asthmatic children **(d)**-

Figure 5: Control children with Rhinovirus in the airways have lower 25(OH)VitD3 and higher *VDR/HPRT* mRNA levels while asthmatic children have increased 25 (OH) VitD3 serum levels during symptomatic visit

(a) 25(OH)-VitaminD3 was measured in the sera of children who were subdivided by RV infection in the airways (control –RV n=6, control +RV n=12, asthma –RV n=6, asthma +RV n=11, B0) control –RV vs control +RV, p=0,05; control –RV vs asthma –RV, p=0,009. **(b)** 25(OH) VitD3 in serum: Control B0 n=19; Asthma B0 n=20; Asthma S n= 16; Asthma C n=14, p= 0.0257; A-S versus CN B0, p= 0.0229; A-S versus A B0 Unpaired T-test. **(c).** *VDR/HPRT* mRNA expression in blood cells (CN-RV, n=6,CN+RV n=8, A-RV n=2, A+RV n=5).p=0.014 unpaired t test. **(d)** Considering RV in the airways and serum levels of 25(OH)VitD3 (from left to the right bars: n=1,3,4,2,2,0,1,2); p=0.0384 Unpaired T test.

Figure 6. IL-33 in NPF is directly correlated with serum 25 (OH) Vit D3 in asthmatic children

(a-b) hIL-33 ELISA: NPF B0, VitD3 serum B0; Control n=14; Asthma n=17; Linear Regression.

Figure 7: Low serum 25 (OH) VitD3 levels are associated with reduced serum IFN-beta and induced AREG in the blood cells of control children.

(a) IFN-beta protein in serum was measured in asthmatic and non-asthmatic children with more (^{High} VitD3) or less (^{Low} VitD3) than 20 [ng/ml] 25(OH)-Vitamin-D3. (CN^{low} n=5, CN^{high} n=5, A^{low} n=7, A^{high} n=3, B0). **(b)** *AREGB/HPRT* mRNA values were also measured by qPCR in the children's total blood cells (CN^{low} n=6, CN^{high} n=6, A^{low} n=7, A^{high} n=6, B0). CN^{low} vs. CN^{high}, p=0,003. CN^{low} vs. A^{low}, p=0,028; two-tailed Student's t-test. Considering RV in the airways and the serum level of 25(OH)VitD3 for **(c)** IFN-beta in serum from the left to the right bar n=0,1,5,4,4,1,3,3; **(d)** *AREG/HPRT* mRNA from the left to the right bar n=0,4,6,2,3,1,3,5. P=0.025 paired T test.

Figure 8. Rhinovirus in the airways and low serum level of 25(OH)VitD3 associated with decreased serum level of sST2 in asthmatic children.

(a) *ST2/HPRT* mRNA values analysed in the same children by qPCR (CN^{low} n=6, CN^{high} n=6, A^{low} n=7, A^{high} n=6, B0). CN^{low} vs. A^{low}, p=0,048. Two-tailed unpaired t-test. (b) *ST2/HPRT* mRNA in total blood cells considering RV in the airways and serum levels of 25(OH)VitD3, from the left to the right n=0,5,5,2,3,1,3,5. P= 0.0186 and p=0.0275 Unpaired two tailed t test. (c) sST2 in serum of control and asthmatic children with low and high 25 (OH) VitD3 (CN low n=9, CN high n=8, A low n=9, A high n=3, B0). (d) sST2 in serum considering RV in the airways and 25(OH)VitD3 level (from the left to the right: n=2,4,7,3,5,1,4,2; p=0,0109; p=0.0065; p= 0.0100. Unpaired t test).

Supplementary Figure 1:

Increased serum levels of 25(OH)VitD3 asthmatic preschool children are dependent from Vitamin D3 Supplementation (Fluorette, Vigantolette) as infant .

Serum levels of 25(OH)VitD3 in control (a) and asthmatic (b) preschool children are dependent from Vitamin D3 Supplementation (Fluorette, Vigantolette) as infant (yes 1) or (no 0). VitD3 deficiency is < 20 ng/ml n=5; VitD3 insufficiency 20-30 ng/ml n=12; VitD3 sufficiency > 20 ng/ml n=4.

Table 1: Clinical data of children at baseline visit

Control	Age (years)	Gender	Skin Prick Test	FEV1%	Vitamin D3 Supplement*	Months breastfed
Mean	4,73	f=41% m=59%	neg=90% pos=10%	106,9	Yes=36,4% No=63,6%	5,84
SEM	0,18			2,67		0,88

Asthma	Age (years)	Gender	Asthma Severity**	Phenotype***	Skin Prick Test	FEV1%	Treatment	Vitamin D3 Supplement*	Months breastfed
Mean	4,92	f=37,5% m=62,5%	I=62,5% II=29,2% III=8,3%	v+m=70% u=16,7% a=4,2% e=8,3%	neg=15% pos=85%	102,75	Non-Steroid=20,9% Steroid=79,1%	Yes=20,8% No=79,2%	8,56
SEM	0,15					4,04			1,69

* Vitamin D3 supplement: Vigantolette, Fluorette

** I=Intermittent: FEV1 > 80%; MEF > 65%; symptoms free interval > 2 months; II= Mild Persistent: FEV1 > 80%; MEF > 65 %; symptoms free Interval < 2 months; III= Moderate persistent: FEV1 < 80%; MEF < 65 %; symptoms several days a week; IV= Severe persistent: FEV1 < 60%; Symptoms during the day and night

*** v= virus-induced; a= allergen-induced; e=exersice-induced; u=unresolved; m=mixed (including virus-induced)

Table 2. Analysed data of children at baseline visit

Control	Virus Swab**	25(OH)VitD3***	VDR
208	-	/	/
211	RV ++	16,9	0,94
214	RV ++	13,4	/
215	-	26	0,82
218	RV +++	17,4	/
219	RV +	16,4	/
220	-	22,3	/
221	RV ++	25,5	1,10
222	-	34,1	0,95
226	-	20,3	0,72
227	-	16,5	/
232	RV +++	13,7	1,03
233	RV +, Other	25,9	/
234	RV ++, Other	12,6	/
235	RV ++	14,2	1,30
236	-	/	1,00
237	Other	10,7	/
240	RV+, Other	18,3	1,10
241	RV ++	25,9	1,22
244	/	/	/
245	-	28,4	0,95
246	RV+	24,4	/
Mean		20,15	1,01
SEM		1,46	0,050

Asthma	Virus Swab**	25(OH)VitD3***	VDR
201	RV ++	9,69	/
202	RV ++	/	1,26
203	-	6,72	0,86
204	-	9,85	/
205	-	11	/
206	RV ++	16,8	/
207	-	17,6	/
209	-	/	/
210	Other	11,3	/
212	-	/	/
213	RV ++	17,8	/
216	-	19,9	0,92
217	-	15,8	/
223	RV +++	37,8	/
224	RV ++, Other	24,3	/
225	RV ++, Other	33,5	1,03
228	-	33,5	/
229	RV +++	/	1,89
230	RV +	28,7	0,95
231	Other	11,8	/
238	RV +++	8,5	1,28
239	RV +	13,1	/
242	RV +, Other	19,2	/
243	RV+++	30,6	/
Mean		18,87	1,17
SEM		2,11	0,13

**** Virus Swab:** RV=Rhinovirus; Other= Other respiratory virus (CoV NL6, Flu A, PIV 4, HBoV, RSV B, HEV, PIV 2, MPV, AdV, Flu B)

***** 25(OH) Vitamin D3 ng/ml:** >30 ng/ml= sufficient; 20-30 ng/ml= insufficient; <20ng/ml= deficient

Figure 1

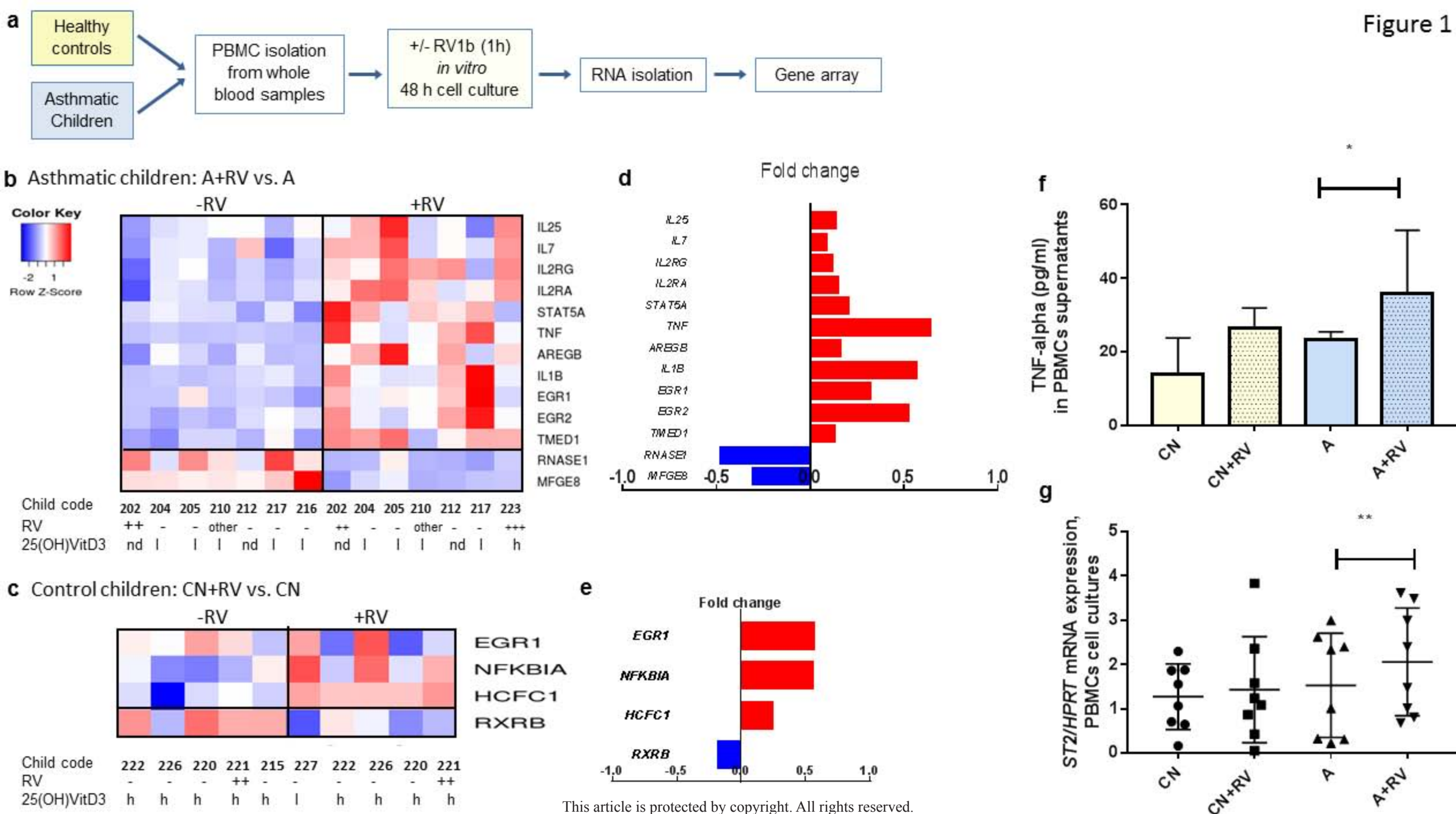


Figure 2

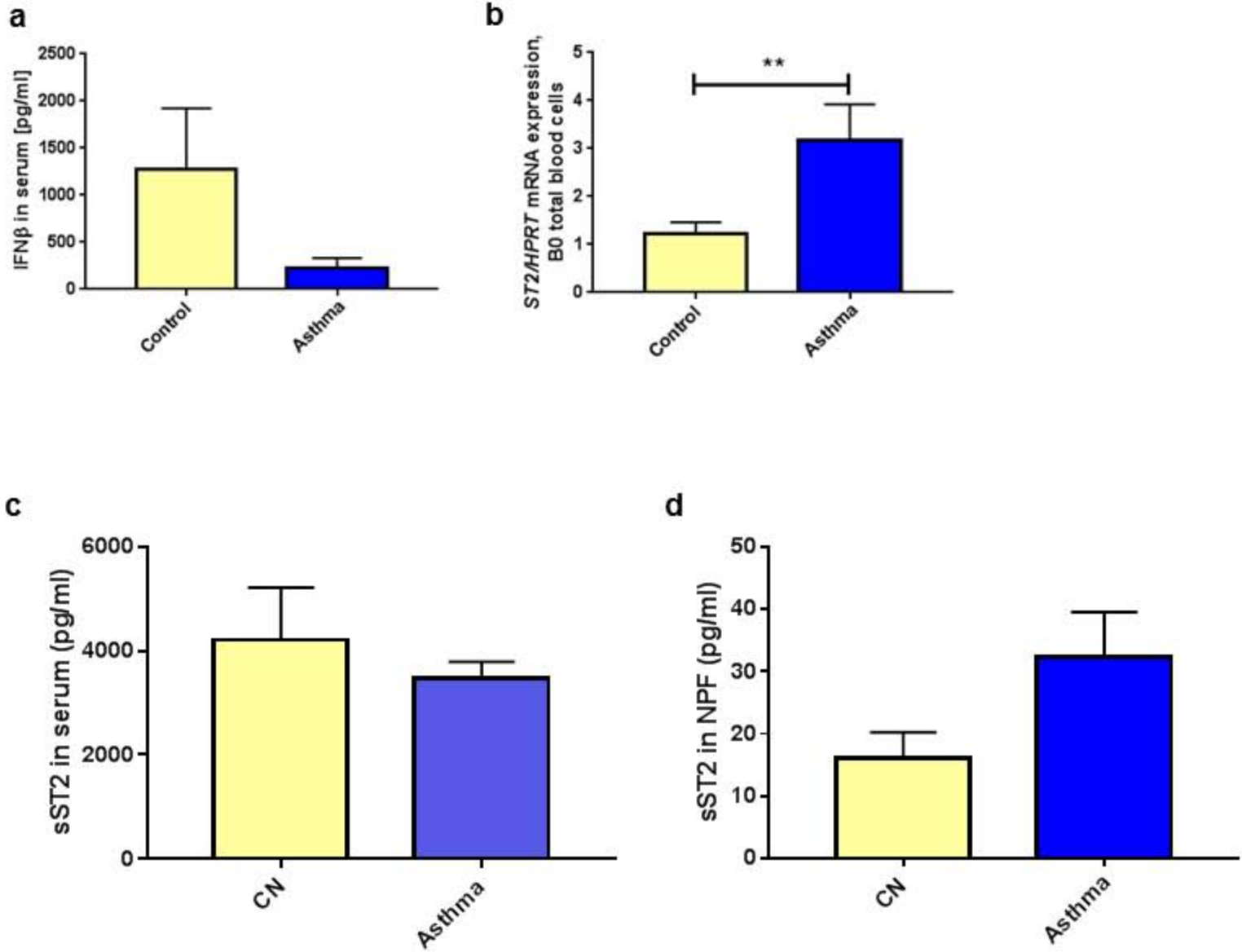


Figure 3

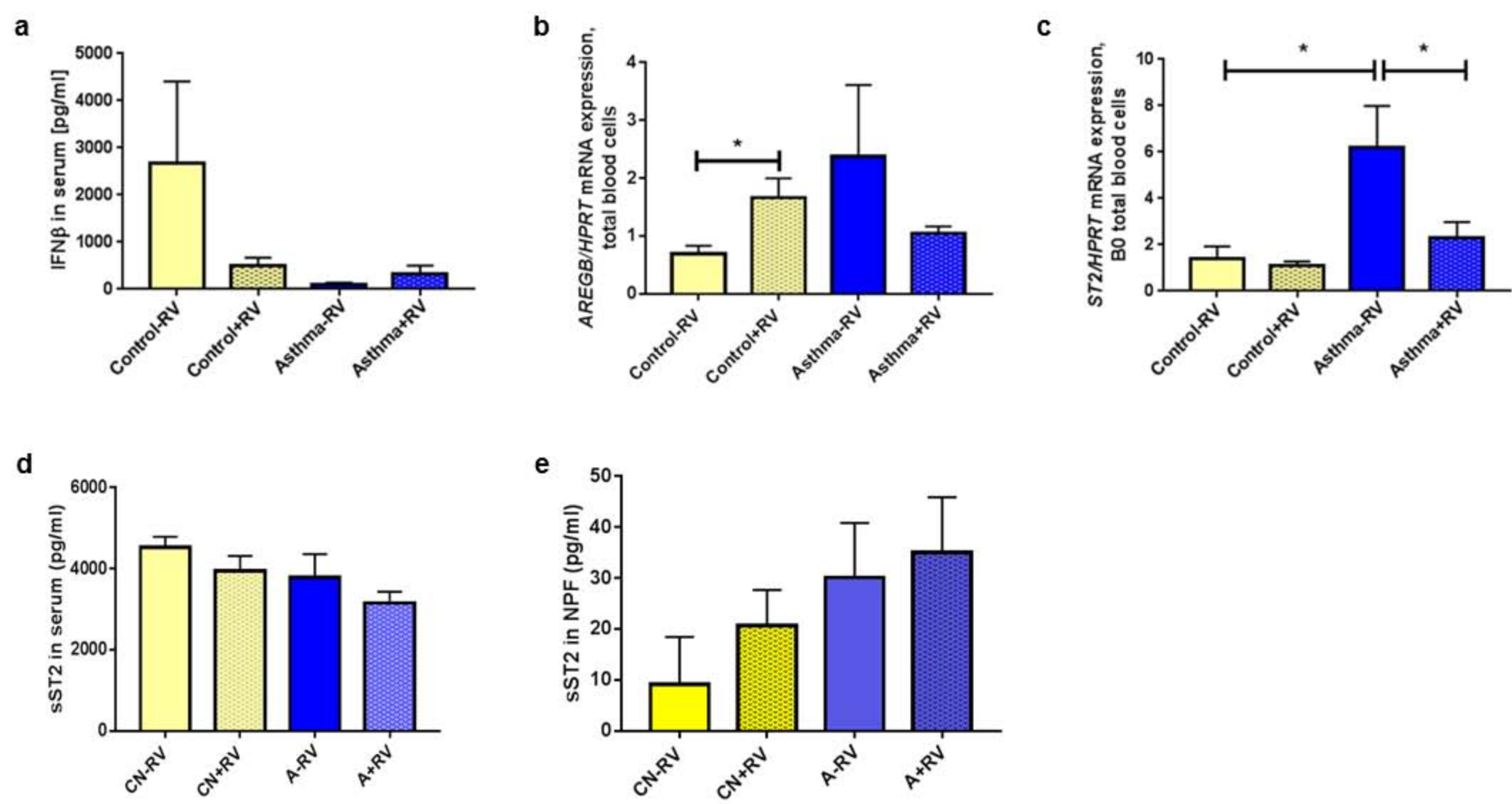


Figure 4

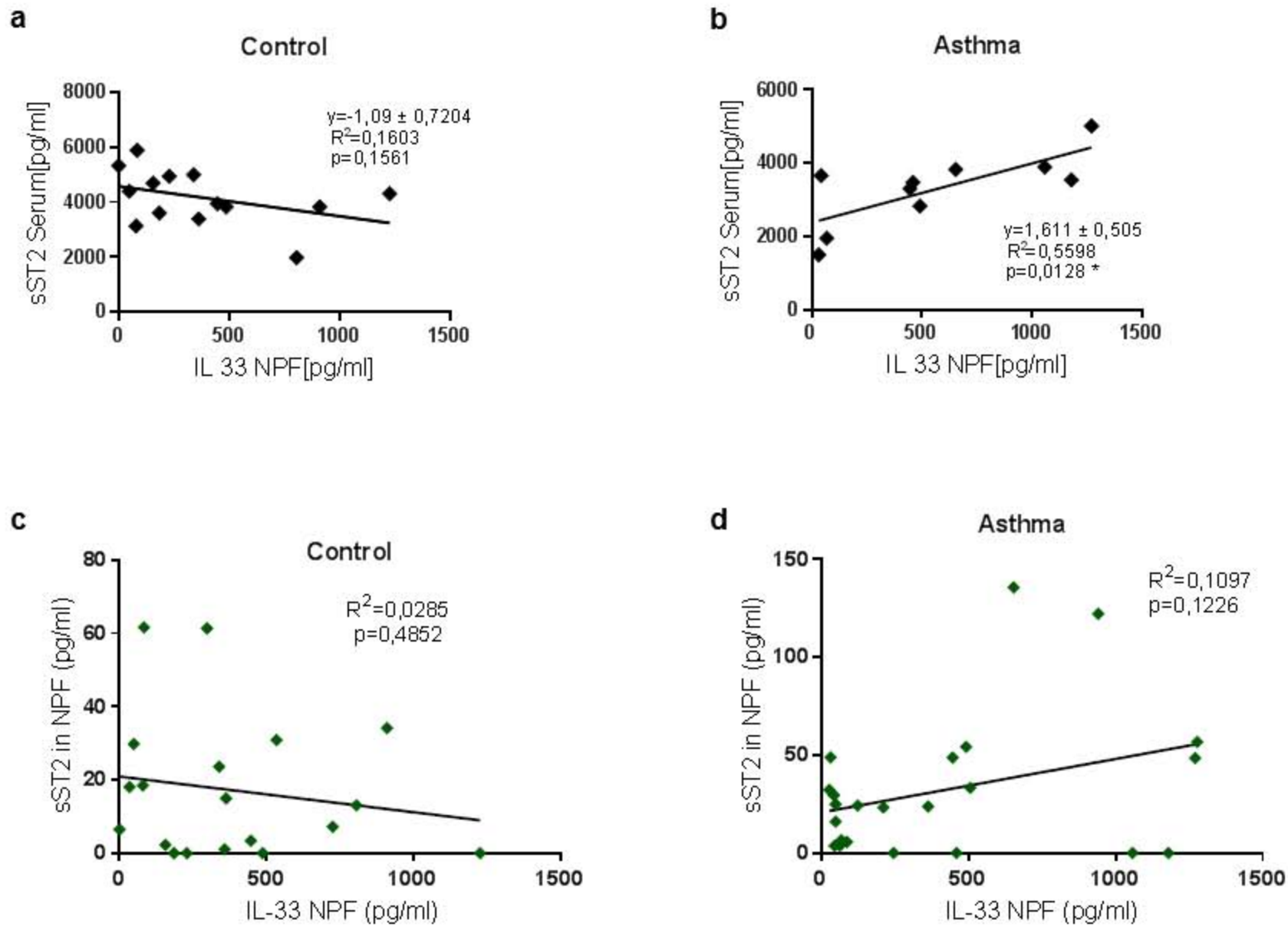


Figure 5

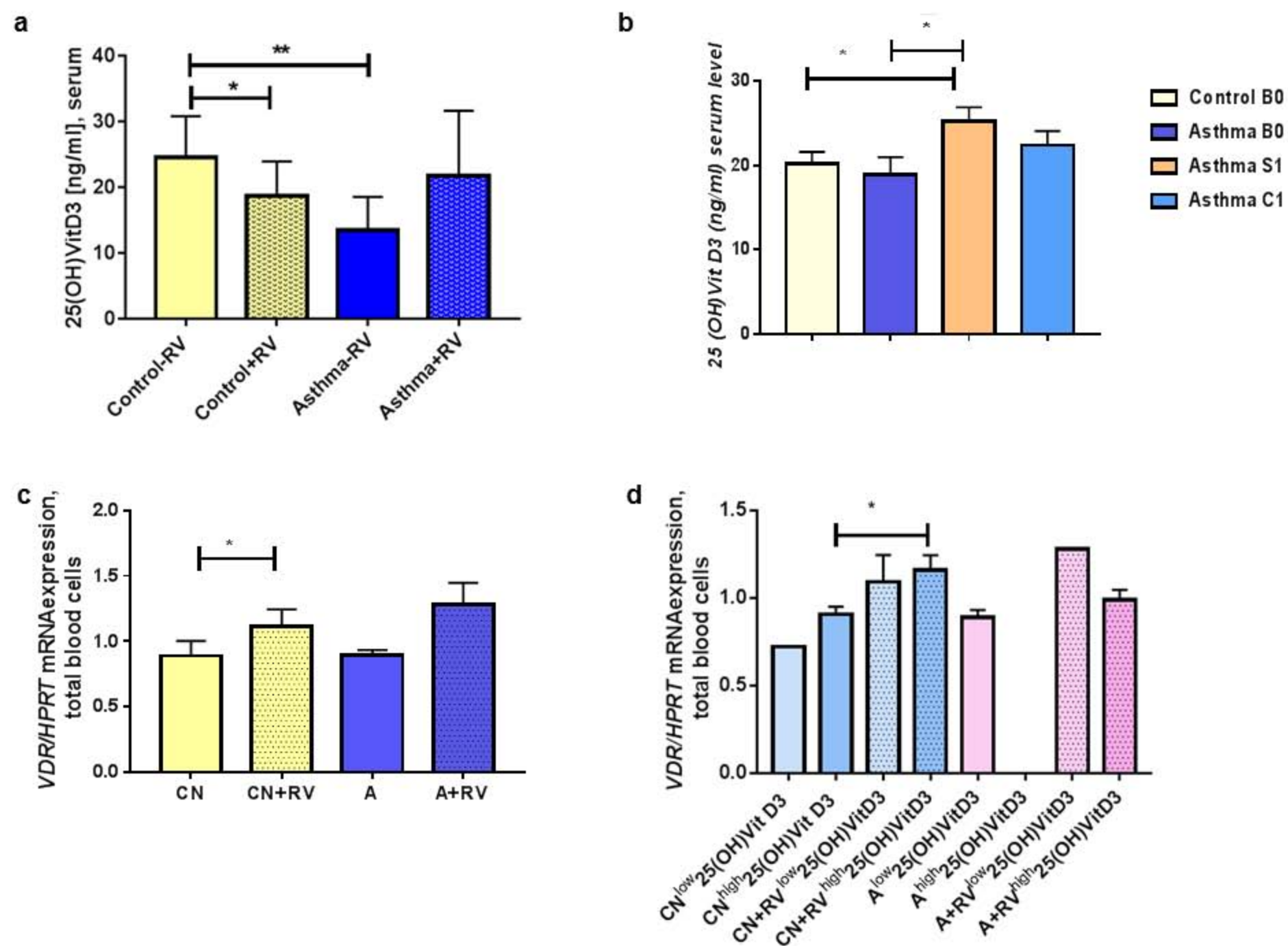


Figure 6

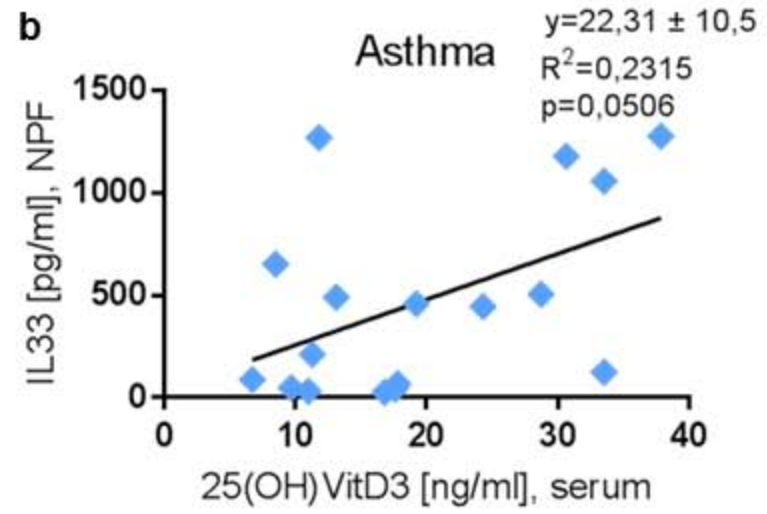
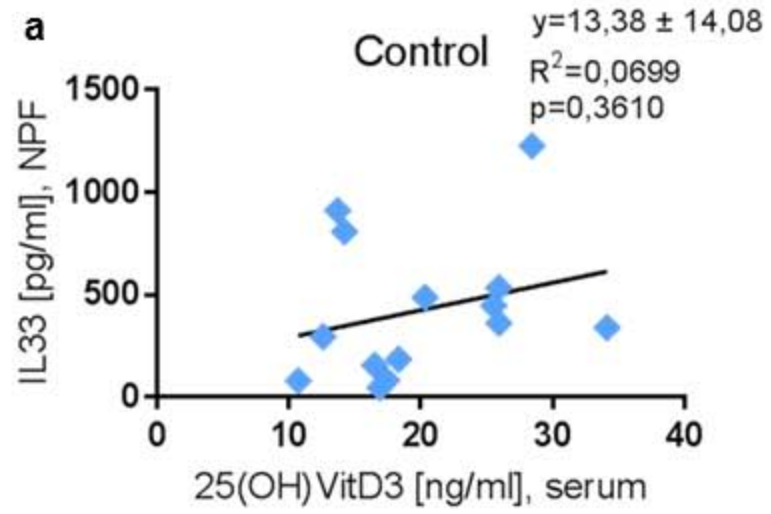
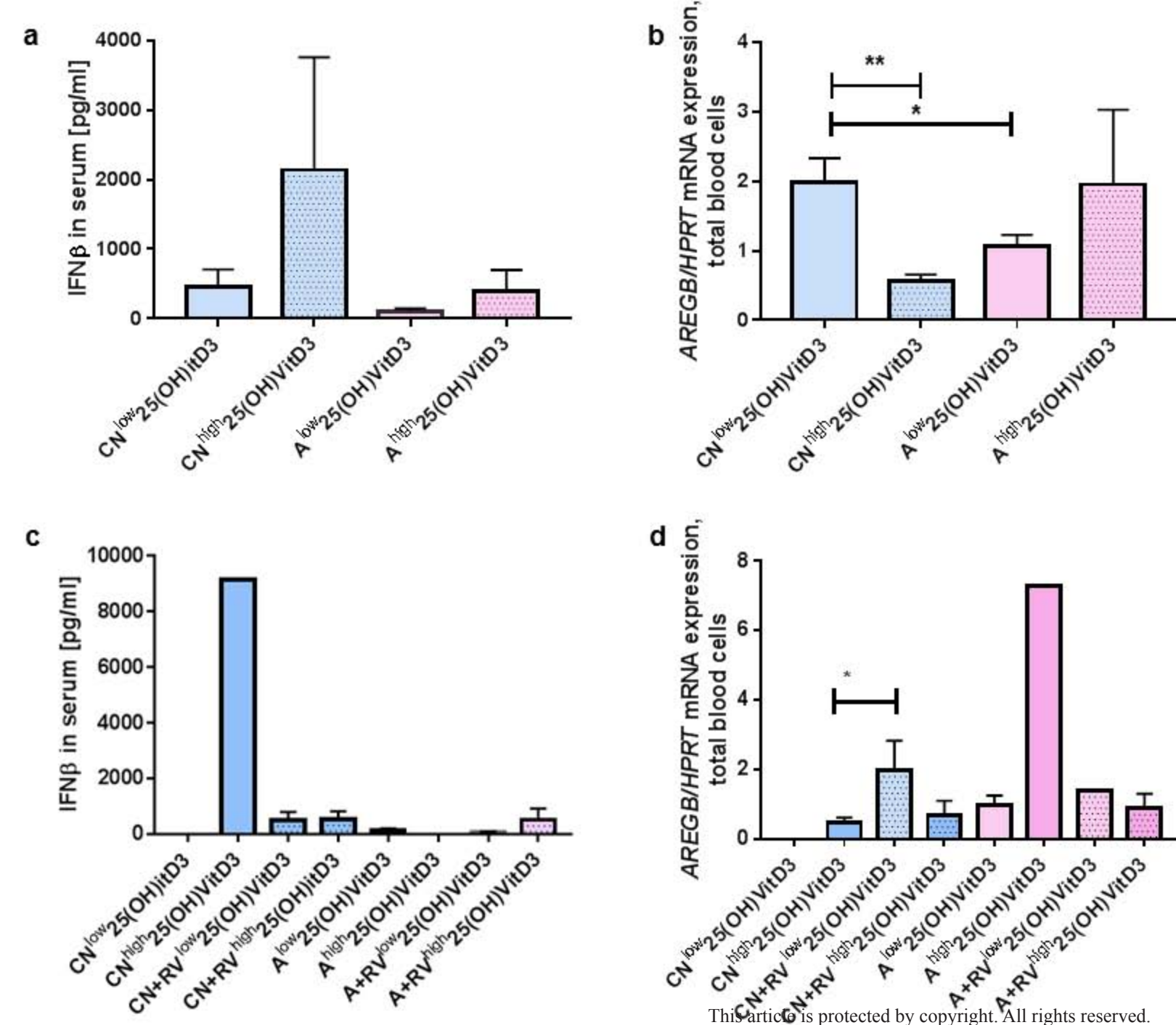
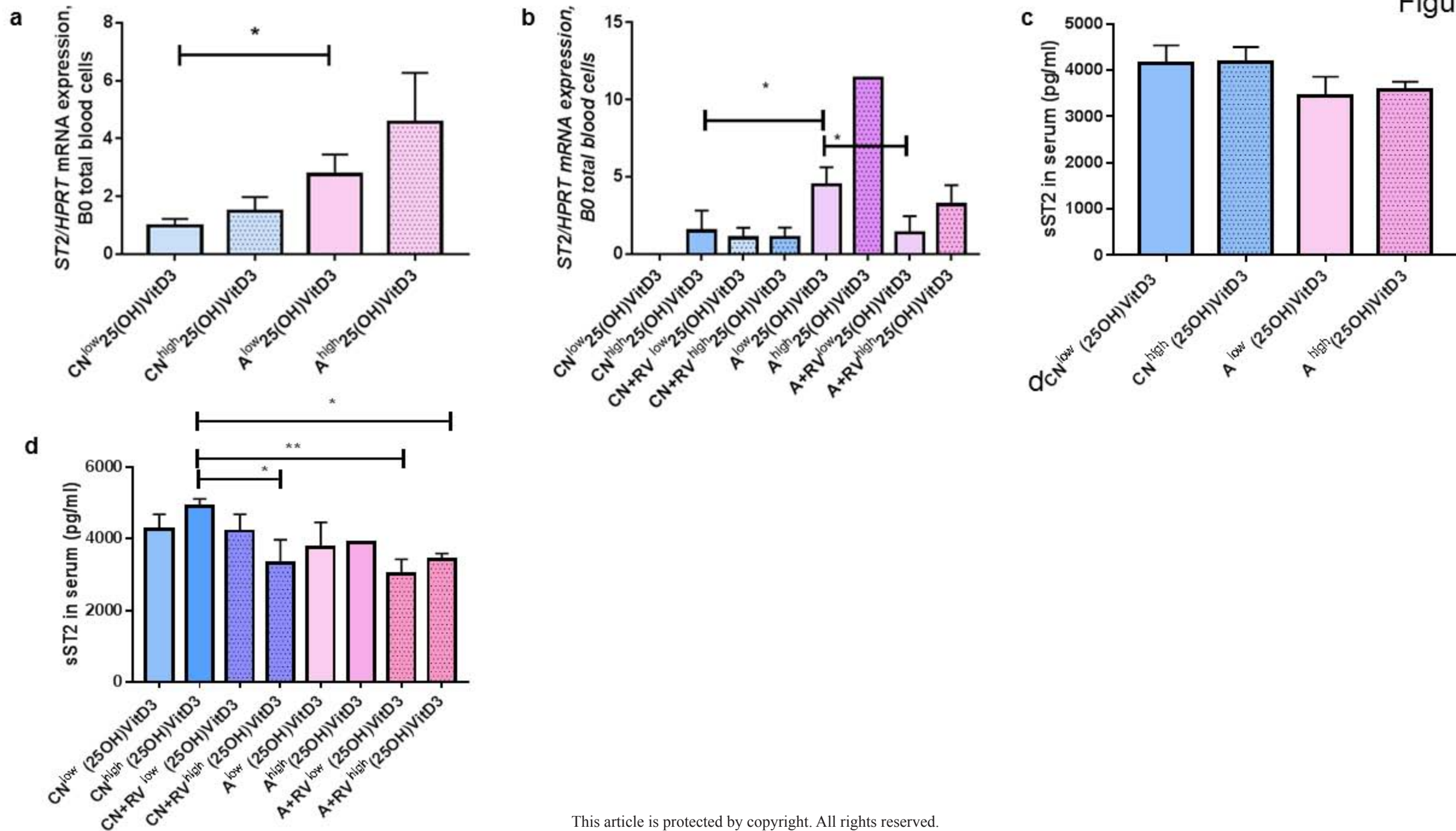
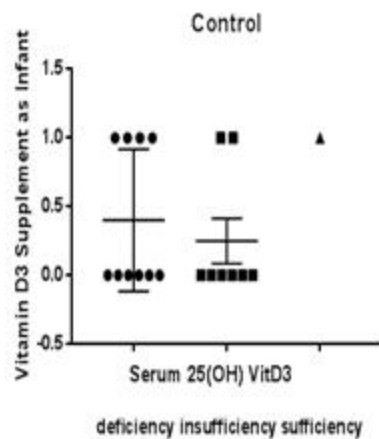


Figure 7

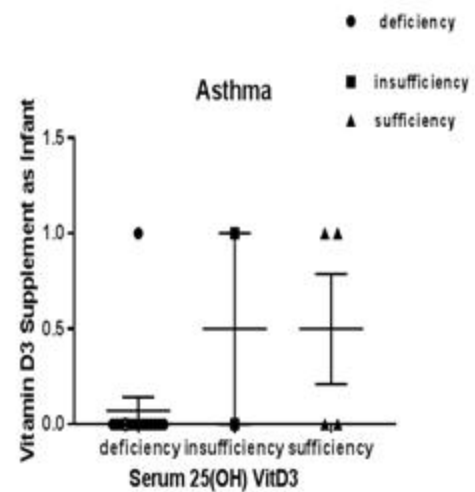




a



b



Supplementary Figure 1

Table S1: IL-C2 related Genes found significantly regulated in gene arrays

Asthmatic Children	
IL25	ILC2 Activator ¹
IL7	B - and T cell development. ILC2 activator. ²
IL2RG	IL2 costimulates ILC2. Against RV infection. ²
IL2RA	IL2 costimulates ILC2. Against RV infection. ²
STAT5A	Mediates the responses of many cell ligands, such as IL2, IL7 ^{3, 4}
TNF	Stimulated by IL 33 ⁵
AREGB	Interacts with EGF, promotes growth of normal epithelial cells; VitD 3 target gene. ^{6, 7}
IL1B	VitD suppresses inflammatory reaction in IL1 β stimulated cells ⁸
EGR1	Receptor for Amphiregulin ⁹
EGR2	Receptor for Amphiregulin ⁹
TMED1	Involved in IL 33 signaling ¹⁰
RNASE1	IL33 release in adipose tissue is mediated by RNase ¹¹
MFGE8	Mfge8 (Lactadherin) suppresses airway hyperresponsiveness by regulating smooth muscle contraction ¹²

Control Children	
EGR1	Receptor for Amphiregulin ⁹
NFKBIA	Is decreased by VitD3 supplementation ¹³
HCFC1	Controls virus gene expression ¹⁴
RXRB	Forms heterodimer complex with VDR to active VDR targets ¹⁵

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Table S2. Analysed data of children at baseline visit

Control	CRP*	Virus Swab**	ST2	AREG	IFN β Swab	IFN β Serum	25(OH)VitD3***	VitD Supplement	sST2 SWAB pg/ml)	sST2 serum (pg/ml)
208	/	-	1,86	1,16	26,33	144,36	/	No	18,80	
211	1,4	RV ++	1,68	1,91	23,092	/	16,9	No	29,85	4397,65
214	0,29	RV ++	0,58	1,43	/	1509,96	13,4	No	6,51	5322,57
215	0,9	-	0,82	0,30	/	9146,35	26	No	11,48	5276,4
218	0,68	RV +++	0,46	3,44	/	831,33	17,4	Yes	61,66	5880,94
219	0,48	RV +	/	/	/	38,86	16,4	Yes	1,08	4491,97
220	0,78	-	/	/	/		22,3	No	7,21	5065,68
221	0,26	RV ++	1,52	0,40	/	1241,19	25,5	Yes	3,46	3938,22
222	0,22	-	3,83	0,60	/	/	34,1	Yes	23,66	4995,03
226	1,25	-	0,82	0,48	77,81	/	20,3	No	0	3823,29
227	21,92	-	/	/	40,244	/	16,5	No	2,30	4692,64
232	0,76	RV +++	0,68	2,51	/	/	13,7	No	34,18	3828,98
233	0,79	RV +, Other	/	/	/	96,72	25,9	No	30,93	
234	1,74	RV ++, Other	/	/	0	46,94	12,6	Yes	61,34	
235	/	RV ++	1,82	1,45	/	/	14,2	No	13,12	1987,17
236	0,11	-	0,51	1,04	/	/	/	Yes	0	4933,61
237	2,16	Other	/	/	/	/	10,7	Yes	18,5	3133,71
240	0,64	RV+, Other	/	1,18	/	79,09	18,3	No	0	3605,98
241	0,36	RV ++	0,63	0,99	/	17,06	25,9	No	14,95	3393,32
244	/	/	/	/	/	/	/	No		
245	0,51	-	0,59	0,59	/	/	28,4	Yes	0	4307,61
246	0,74	RV+	/	/	52,218	777,43	24,4	No	0	2628,51
Mean	1,89		1,21	1,25	36,61	1687,78	20,15	Yes=36,4% No=63,6%	16,14714286	4205,737778
SEM	1,11		0,26	0,24	11,97	1260,39	1,46		4,083339951	238,4376608

Asthma	CRP*	Virus Swab**	ST2	AREG	IFNβ Swab	IFNβ Serum	25(OH)VitD3***	VitD Supplement	sST2 SWAB (pg/ml)	sST2 serum (pg/ml)
201	0,4	RV ++	/	/	/	106,43	9,69	No	24,95	
202	/	RV ++	1,99	1,07	/	830,5	/	No	3,84	
203	1,22	-	3,97	0,77	0	/	6,72	No	5,68	
204	0,31	-	/	/	0	20,72	9,85	No	33,36	3756,24
205	2,13	-	3,66	0,90	0	/	11	No	48,88	1494,05
206	0,69	RV ++	2,41	1,40	0	51,84	16,8	No	32,34	
207	0,13	-	/	/	0	256,40	17,6	No	29,5	3660,58
209	/	-	/	/	/	/	/	Yes	16,01	
210	5,34	Other	1,71	0,33	0	1711,15	11,3	No	23,34	
212	/	-	1,49	1,64	/	18,1	/	No	3,68	
213	0,13	RV ++	/	/	/	63,35	17,8	No	6,58	1953,21
216	0,5	-	5,79	1,30	0	236,76	19,9	No	0	
217	1,01	-	/	/	/	19,34	15,8	No	122,27	3988,1
223	0,63	RV +++	5,29	1,60	/	1313,18	37,8	No	56,82	
224	0,39	RV ++, Other	0,93	0,76	0	/	24,3	Yes	48,84	3302,95
225	/	RV ++, Other	1,88	0,54	/	/	33,5	Yes	24,37	
228	0,37	-	11,38	7,28	/	5,95	33,5	Yes	0	3894,31
229	/	RV +++	0,77	0,74	0	69,69	/	No	23,80	
230	0,69	RV +	7,04	0,65	/	35,6	28,7	No	33,37	
231	1,87	Other	/	/	/	9062,20	11,8	No	48,50	5913,53
238	20,33	RV +++	1,46	1,41	158,665	/	8,5	No	135,79	3831,16
239	0,55	RV +	0,23	1,38	/	/	13,1	Yes	54,27	2830,05
242	0,64	RV +, Other	1,32	/	/	/	19,2	No	0	3475,83
243	2,9	RV+++	0,87	0,90	23,24	197,70	30,6	No	0	3542,81
Mean	2,11		3,07	1,41	16,53	540,17	18,87		32,34491667	3470,235083
SEM	1,05		0,70	0,40	14,37	234,58	2,11		7,174938157	317,6118231

* CRP mg/l: Increase > 5mg/l

** Virus Swab: RV=Rhinovirus; Other= Other respiratory virus (CoV NL6, Flu A, PIV 4, HBoV, RSV B, HEV, PIV 2, MPV, AdV, Flu B)

*** 25(OH) Vitamin D3 ng/ml: >30 ng/ml= sufficient; 20-30 ng/ml= insufficient; <20ng/ml= deficient