

AHR and Exhaled NO in asthmatic children

Airway Hyperresponsiveness to Mannitol and Methacholine

and Exhaled Nitric Oxide in Children with Asthma

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Key words

Exhaled nitric oxide, Airway hyperresponsiveness, Mannitol dry powder, Methacholine, Childhood asthma

INTRODUCTION

Asthma is a chronic, heterogeneous, chronic disease characterized by airway inflammation and hyperresponsiveness (AHR). [1,2] Notably, AHR can be assessed in patients via bronchial challenge tests, which are classified as ‘direct’ or ‘indirect’ methods according to theoretical differences in the mechanisms of action. Direct bronchoconstrictor stimuli, such as methacholine, histamine, or prostaglandins, directly target their receptors on airway smooth muscle and endothelial cells to induce airway narrowing. [3] In contrast, indirect stimuli, such as exercise, adenosine monophosphate (AMP), or mannitol dry powder (MDP) indirectly induce airway narrowing via the release of inflammatory mediators after mast cell stimulation. [4] However, the different mechanism underlying AHR to mannitol and methacholine are both related to airway inflammation.

Fractional exhaled nitric oxide (FeNO), for which protocols have been developed and defined since 1991, is currently used as a noninvasive, inexpensive, and reliable indicator of the airway inflammatory status in patients with asthma. [5-8] In allergic asthma, airway inflammation results from the activation of mast cells and antigen-specific type 2 T-helper cells, leading to the production of cytokines such as interleukin (IL)-4, IL-5, and IL-13. IL-4 and IL-13 subsequently promote the upregulation of epithelial inducible NO synthase expression via STAT-6-mediated signaling. [9] Although FeNO and AHR represent different aspects of airway inflammation and structural airway changes, the relationship between clinical markers of airway inflammation and AHR in patients with asthma might have clinical implications. We therefore investigated the relationships of airway responsiveness to MDP and methacholine with FeNO levels in children

with asthma according to inhaled corticosteroid (ICS) treatment status.

METHODS

Subjects

For this study, children aged 5-17 years with mild to moderate asthma were recruited from the allergy outpatient clinics of Severance Children's Hospital. The inclusion criteria were physician-diagnosed asthma and a history of recurrent wheezing and/or dyspnea that was resolved with a bronchodilator. The exclusion criteria included a history of recent respiratory infection (within the last 4 weeks), history of recent surgery, history of heart disease that could impose risks during bronchial challenges, history of any health condition considered inappropriate for participation in this study, and a pre-bronchodilator predictive value of forced expiratory volume in 1 sec (FEV₁) of < 70%.

Forty-five children were taken asthma medicine regularly in past years; of these, 32 used ICSs and 13 had leukotriene modifiers. Accordingly, total subjects were divided into a steroid-treated group (n = 32) and steroid-naïve group (n = 102). No children had been using regular anti-inflammatory treatment within 1 week preceding the study. The clinical severity of asthma was based on the recommendations of the Global Initiative for Asthma. [12] All children underwent bronchial challenge testing with both methacholine and MDP from March 2010 to February 2013. Bronchial challenge testing with methacholine was performed on the first day, and inhaled MDP was used on other days after an interval of at least 24 hours. This study was approved by the Institutional Review Board of Severance Hospital.

Pulmonary function tests

Spirometry was performed according to the European Respiratory Society recommendations. [10] FEV₁ and forced vital capacity (FVC) were measured using a Jaeger MasterScreen IOS (Jaeger, Wurzburg, Germany), which was calibrated weekly. All measurements were performed in duplicate. Pulmonary function was calculated from the best curve.

Methacholine and MDP challenge tests

The bronchial challenge test with methacholine was performed according to standardized procedures. [11] Methacholine was diluted in saline to the following concentrations: 0.075, 0.15, 0.31, 0.62, 1.25, 2.5, 5, 10, 25, and 50 mg/mL. Cumulative doses of up to 16 mg/mL were administered with a Spira dosimeter (Spira Respiratory Care Center Ltd., Hameenlinna, Finland), using the dosimetric method previously described by Yan et al. [12] A positive challenge response was defined as a 20% decrease in FEV₁ (PC₂₀ methacholine) after the inhalation of 16 mg/mL of methacholine or less.

A bronchial challenge with MDP (Aridol®; Phamaxis Ltd., French's Forest, NSW, Australia) was administered according to the manufacturer's recommendations, and the FEV₁ was recorded in line with current guidelines. [13] The MDP capsule dose started at 0 mg and increased to a total cumulative dose of 635 mg. Each capsule was placed in the inhalation device, and a hole was made by pressing the device button before inhalation. The FEV₁ was measured after 60 s after a deep inhalation of MDP. A 15% decrease in FEV₁ (PD₁₅ MDP) to a cumulative MDP dose of ≤ 635mg was considered a positive response indicative of AHR. [14,15] If the FEV₁ did not decrease by more than 15% until the last dose, the test was considered negative. The cumulative

dose of MDP (PD₁₅ MDP) was calculated via interpolation of a log-linear dose-response curve.

FeNO measurement

Fractional exhaled nitric oxide (FeNO) was measured online (rate, 0.05 L/s) using a Nitric Oxide Analyzer (NIOX MONO®; Aerocrine AB, Solna, Sweden). The mean of three acceptable readings was taken, and each reading was separated by an interval of at least 30 s of relaxed tidal breathing according to the American Thoracic Society guidelines. [16] All children refrained from eating nitrate-rich foods for 2 hours before the measurement of FeNO. This parameter was measured prior to spirometry or bronchial challenge tests.

Total and specific serum IgE and blood eosinophil counts

Serum total and specific IgE levels were measured using a fluorimmunoenzyme assay (ImmunoCAP®; Thermo Fisher Scientific Inc. Phadia AB, Uppsala Sweden) according to the manufacturer's instructions. A specific IgE test was performed with common inhalant allergens in Korea including Birch, Ragweed, *Dermatophagoides pteronyssinus*, *Dermatophagoides farina*, *Alternaria species*, *Blattella germanica*. Atopy was defined as specific IgE levels to at least 1 of allergen ≥ 0.7 kUA/L or total IgE ≥ 150 IU/mL. Peripheral blood eosinophils were counted in blood samples containing EDTA using an automated hematology analyzer (NE-8000; Sysmax, Kobe, Japan). Blood sample collection and serum preparation were performed according to the manufacturers' instructions.

Skin prick test

Skin prick test were performed with 44 inhalant allergens. The allergens were supplied by Allergopharma (Reinbek, Germany). Normal saline was used as a negative control, and a 0.1% histamine solution was used as a positive control. Atopy was defined as at least one positive reaction with wheal diameter 3 mm or larger.

Statistical analysis

Continuous variables with normal distributions are described as means and standard deviations (SDs); otherwise, variables are presented as medians and interquartile ranges (IQRs). The maximal percentage decrease in FEV₁ from the baseline after methacholine and MDP administration was calculated. In addition, the response-dose ratio (RDR) values for methacholine and MDP were calculated as the percentage of FEV₁ decrease at the last dose divided by the cumulative dose. Comparisons between groups used Student's t-test for normally distributed variables, the Mann-Whitney test with Bonferroni's correction for non-normally distributed variable, and the chi-square test or Fisher's exact test for categorical variables. Spearman's rank correlation was used for correlation analyses. SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) was used to perform all statistical analyses. P-values of <0.05 were considered statistically significant.

RESULTS

Subject characteristics

One hundred thirty-four children (89 males, 45 females) were enrolled. The median age was 9 years, and atopy was observed in 88.1% of total subjects. The clinical characteristics of the

steroid-treated and steroid-naïve groups of children are shown in Table 1. There were no significant differences in age, sex ratio, atopy prevalence, serum total IgE level, blood eosinophil count, and body mass index (BMI) between 2 groups. However, the median FeNO level was significantly higher in the steroid-treated group (74.2 ppb) than in the steroid-naïve group (31.0 ppb; $p < 0.001$; Figure1).

The pulmonary function parameters of the 2 groups are presented in Table 2. There were no significant differences in the percent of FEV₁ predicted value, FVC predicted value, and FEF₂₅₋₇₅ predicted value. However, the groups differed significantly in the percent of FEV₁/FVC predicted value [steroid-treated group vs. steroid-naïve group (mean $\pm$ standard deviation), 81.84 ± 13.49 vs. 91.86 ± 10.65 ; $p < 0.001$]. In addition, a marginal difference was observed in the change in FEV₁ after bronchodilator inhalation [steroid-treated group vs. steroid-naïve group (mean $\pm$ standard deviation), 11.15 ± 10.22 vs. 7.09 ± 6.40 ; $p = 0.047$].

AHR to methacholine and MDP

One-hundred children (74.6%) had positive results to MDP with a geometric median PD₁₅ of 189.7mg. Response to methacholine was positive in 90 (67.2%), with geometric median PC₂₀ of 5.29mg/mL. Among 134 subjects, 72 (53.7%) responded to both MDP and methacholine. Twenty-eight (20.9%) children had only positive results to MDP. In 18 children, response to MDP was negative but was positive to methacholine. Supplement Table 1 display characteristics of groups according to results of two bronchial provocation tests. There is no difference in age, sex, blood eosinophil count, FeNO level.

The median PC₂₀ methacholine and PD₁₅ MDP values did not differ between the steroid-

treated group (5.51 mg/ml and 150.8 mg, respectively) and the steroid-naïve group (8.55 mg/ml and 324.5 mg, respectively; $p = 0.338$ and 0.076). However, significant difference in the median RDR MDP was observed between the steroid-treated group and steroid-naïve group (0.131% fall/mg vs. 0.056% fall/mg; $p = 0.004$). The median RDR methacholine did not differ significantly between the steroid-treated and steroid-naïve group [4.22% fall/(mg/ml) vs. 2.67% fall/(mg/ml); $p = 0.226$; Table 2].

Correlation between FeNO levels and AHR

In the overall subject group, FeNO was significantly associated with PD₁₅ MDP ($r = -0.28$, $p = 0.005$). However, no correlation was observed between FeNO and PC₂₀ methacholine. Similarly, FeNO associated significantly with RDR MDP ($r = 0.299$, $p = 0.003$) in the total subjects group, whereas no correlation was detected between FeNO and RDR methacholine [Figure 2 (a)]. However, FeNO was associated with both the serum total IgE level and peripheral blood eosinophil count in the total subjects group [$r = 0.211$, $p = 0.04$ and $r = 0.288$, $p = 0.005$, respectively; Figure2 (b), (c)].

In the steroid-naïve group, the FeNO level correlated with RDR MDP ($r = 0.237$, $p = 0.049$), but not with RDR methacholine. In contrast, the FeNO level did not correlate with either parameter in the steroid-treated group (Figure 3), and no correlations of FeNO with the peripheral blood eosinophil count and serum total IgE level were observed in either steroid-treated or steroid-naïve group.

DISCUSSION

The mechanisms underlying AHR to MDP and methacholine might be related to airway inflammation via different processes.[3,4] Cytotoxic chemokines, such as eosinophil cationic protein, might enhance the access of methacholine to smooth muscle cells via the basement membrane. [17] Additionally, the response to methacholine has been shown to depend partly dependent on airway caliber; specifically, AHR to methacholine has been reported in non-asthmatics with a low airway caliber, including healthy children and chronic obstructive pulmonary disease patients. [10] Meanwhile, mannitol is an osmotic stimulus that induces the release of inflammatory mediators from inflammatory cells in the bronchial mucosa, thus causing airway narrowing; accordingly, challenge test with MDP depend critically on the presence of inflammatory cells in the airway. [18-20] Challenge tests with MDP have become more popular in recent years because of the ease of administration and the association of this type of challenge with exercise-induced bronchoconstriction and, to a lesser degree, clinical asthma. [21-23] In addition, challenge test with MDP does not require extensive equipment and has been proven safe in children. [24] Numerous studies of adult patients with asthma have identified relationships between AHR to indirect stimuli and FeNO; specifically, FeNO exhibits a stronger correlation with indirect stimuli than with direct stimuli in this population. [25-29] Additionally, among asthmatic adults, FeNO correlates significantly with indirect AHR in atopic subjects, but not in non-atopic subjects. [25] To date, several studies on the relationships between FeNO and AHR to indirect stimuli in children have been reported. In atopic children with asthma, a previous study demonstrated that increased FeNO was closely associated with exercise-induced bronchoconstriction, and a recent study reported a closer association of FeNO with AHR to AMP than with AHR to methacholine in steroid-naïve asthmatic children. [26,30] In this study of

asthmatic children, we focused on the relationship between clinical markers of airway inflammation and AHR. We confirmed these previous findings in asthmatic children on the basis of the correlation between FeNO level and RDR MDP but not RDR methacholine.

AHR to mannitol correlates more strongly with eosinophilic airway inflammation and improves rapidly with inhaled corticosteroid treatment. [28,31] Because ICS use reduces sensitivity to the mannitol challenge test, the present study divided the subject into steroid-treated and steroid-naïve groups of children with asthma to compare the influence of anti-inflammatory treatment on the FeNO level and AHR. [31] Accordingly, a significant association between FeNO and AHR to MDP was observed in the steroid-naïve group, but not in the steroid-treated group. Similarly, previous study found no correlation between the FeNO level and indirect AHR in steroid-treated asthmatics, whereas FeNO was found to be significantly associated with indirect AHR in an earlier study of steroid-naïve young adults with atopic asthma. [25,32] Porsbjerg et al. demonstrated a stronger correlation between FeNO and indirect stimuli than direct stimuli in steroid-naïve adult asthma patients. [29] We believe that our study results corroborate these earlier findings.

The FeNO level is associated with several factors, such as spirometry and bronchial provocation tests, which transiently reduce FeNO levels. [33,34] Accordingly, FeNO was measured prior to spirometry or bronchial challenge tests in the present study. Additionally, a previous report observed a dose-dependent reduction in the FeNO level after treatment with ICS or systemic corticosteroids. [35] In contrast, the present study observed a significantly higher median FeNO level in the steroid-treated group (74.2 ppb) than in the steroid-naïve group (31.0

ppb). All subjects quitted regular anti-inflammatory treatment within 1 week preceding the study. Children in steroid-treated group had past history of regular ICS use. Unfortunately, there was no data about anti-inflammatory treatment for asthma in this study group. Among the steroid-treated group, only 9 subjects used ICSs regularly prior to bronchial provocation tests within the last 1 month. Still, an ICS washout period of 1 month or more might possibly have affected the FeNO levels of the 23 subjects in the steroid-treated group. Additionally, steroid-treated group had lower percent of predicted FEV1/FVC than steroid-treated group in pulmonary function test. The differences in the subjects' asthma phenotypes and the cell types underlying airway inflammation must be considered; for example, among the inflammatory phenotypes of asthma, the eosinophilic phenotype has been associated with FeNO. [36,37]

There are many studies about the utility of bronchial methacholine and mannitol challenge tests in various populations. The sensitivity shown by bronchial challenge test with indirect stimuli is lower than that with direct methods.[38,39] On the other hand, bronchial challenge test with MDP showed high diagnostic specificity for asthma in young adults.[23] On the other hand, bronchial challenge test with MDP showed high diagnostic specificity for asthma in young adults [40] A previous study showed that AHR to mannitol were poorly correlated with AHR to methacholine in children with asthma. [41] This study showed that 20.9 %, 13.4 % had only positive response to MDP and methacholine. However, there is no difference in clinical characteristics and pulmonary function between 2 groups. It was possible to Response to indirect stimuli has been associated with higher levels of sputum eosinophils in asthmatics. [42] Porsbjerg et al suggest that inflammatory subtypes of asthma are related to AHR to mannitol. [37] This result is likely due to difference in inflammatory subtype and control status of asthma.

We note that several limitations should be considered when interpreting our findings. First, all participants were referred to a tertiary hospital for a diagnosis of asthma. We cannot rule out the subjects' medication history with respect to short-term corticosteroid treatment, and the dose and duration of ICS treatment might have varied among these subjects. Second, we were unable to exclude the effects of passive smoking exposure, which causes both acute and chronic reductions in FeNO levels. [43] Third, we did not compare the airway inflammation statuses and sputum profiles according to asthma severity in this group of children. Further studies are needed to confirm the relationship between the underlying airway inflammation mechanism and degree of AHR.

CONCLUSION

In conclusion, this study supports previous findings that the FeNO level, as a measure of airway inflammation, is more closely associated with the degree of response to MDP than with the degree of response to methacholine in steroid-naïve children.

The authors have no conflicts of interest to declare.

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The authors have no conflicts of interest to declare.

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Table1. Clinical Characteristics of the total subjects

	n = 134
Age, y*	9 (7 - 11)
Male, n (%)	89 (66.4)
Atopy, n (%)	118 (88.1)
Total IgE, IU/ml*	338.0 (163.0 - 755.0)
Blood eosinophil, /ml*	470 (220 - 700)
BMI, kg/m ² *	18.0 (17.0 - 21.0)
Airway responsiveness	
AHR to mannitol	100 (74.6 %)
AHR to methacholine	90 (67.2 %)
AHR to mannitol and methacholine	72 (53.7 %)
PD ₁₅ to mannitol (mg)	189.7 mg (71.0 - 324.5)
PC ₂₀ to methacholine (mg/mL)	5.29 mg/mL (2.18 - 8.41)

* Data are presented as medians (interquartile range) IgE, immunoglobulin E; BMI, body mass index. Serum total IgE ≥ 150 IU/mL or sIgE > 0.70 kU/L in immuno CAP assay or at least 1 positive skin prick test

Positive reaction to mannitol: PD₁₅ < 635 mg

Positive reaction to methacholine: PC₂₀ < 16 mg/mL

Table2. Pulmonary function parameters and airway hyperresponsiveness statuses of 2 study groups

	Steroid-treated (n = 32)	Steroid-naïve (n = 102)	P-value
FEV ₁ , % predicted	93.23 ± 14.50	96.97 ± 13.71	0.192
FVC, % predicted	100.34 ± 12.25	97.90 ± 10.94	0.379
FEV ₁ /FVC, % [†]	81.84 ± 13.49	91.86 ± 10.65	<0.001
dFEV ₁ , % [*]	11.15 ± 10.22	7.09 ± 6.40	0.047
FEF ₂₅₋₇₅ , %	66.15 ± 21.50	75.57 ± 24.78	0.057
Atopy, n (%)	30 (93.8)	88 (86.3)	0.356
FeNO, ppb [†]	74.2 (40.8-95.4)	31.0 (15.1-57.4)	<0.001
RDR methacholine, %/(mg/ml)	4.22 (0.94-16.07)	2.67 (0.88-6.32)	0.226
RDR MDP, %/mg [*]	0.131 (0.053-0.383)	0.056 (0.024-0.113)	0.004

Data are presented as means ± standard deviations or median (interquartile range).

FEV₁, forced expiratory volume in 1s; FVC, forced vital capacity; dFEV₁, the difference in FEV₁ after β₂-agonist inhalation; FEF₂₅₋₇₅, forced expiratory flow between 25% and 75%; RDR, response-dose ratio.

Student's t-test or Mann-Whitney test used comparing steroid-treated and steroid-naïve groups.

^{*} P-values <0.05,

[†] P-values <0.001

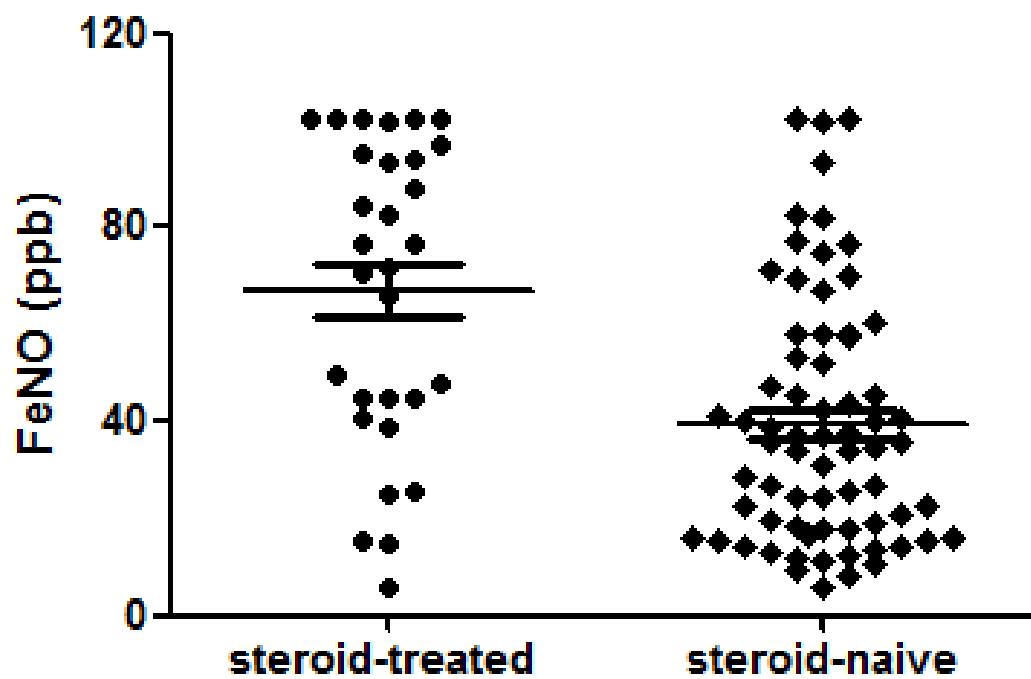


Figure 1. FeNO levels in steroid-treated and steroid-naïve study subjects. The Mann-Whitney test was used to compare the groups (* $P < 0.0001$). FeNO levels were significantly higher in the steroid-treated group than in the steroid-naïve group.

FIGURE 2

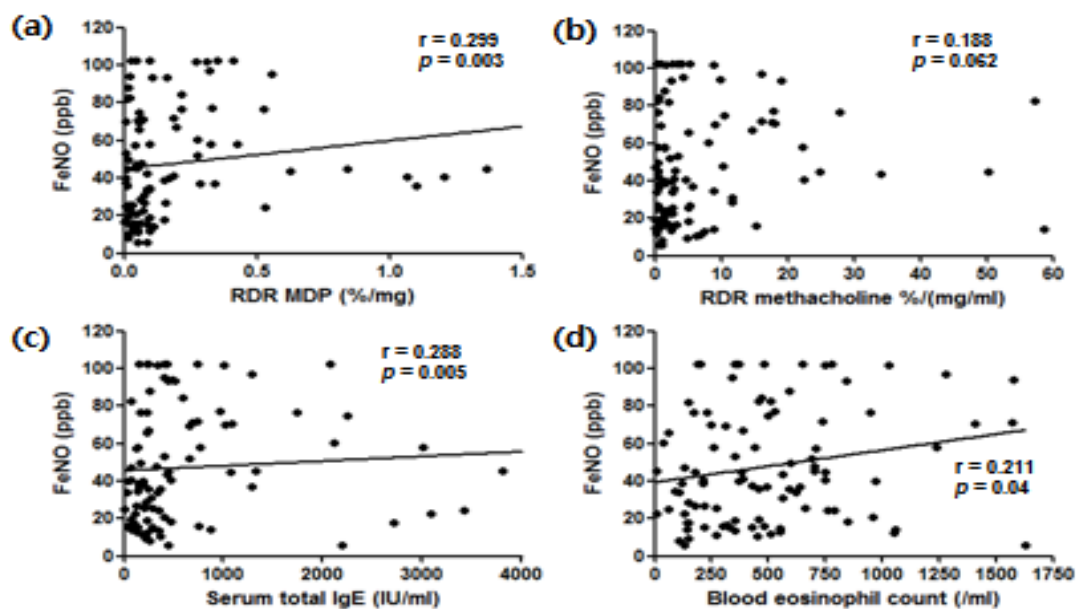


Figure 2. Scatter plot of all subjects. (a) Association between the fractional exhaled nitric oxide (FeNO) level and response-dose ratio (RDR) MDP. (b) Association between FeNO level and RDR methacholine. (c) Association between FeNO level and serum total IgE level. (d) Association between FeNO level and blood eosinophil count.

FIGURE 3

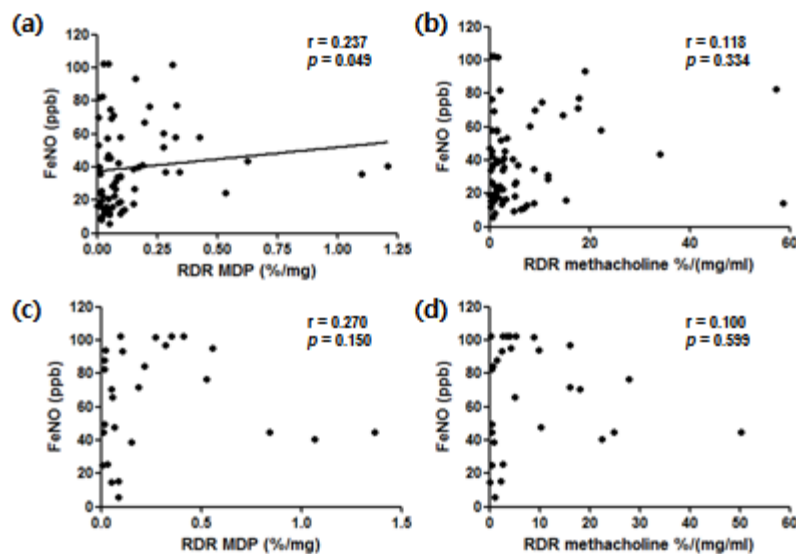


Figure 3. Scatter plot of the groups. (a) Association between the FeNO level and RDR MDP in the steroid-naïve group. (b) Association between the FeNO level and RDR methacholine in the steroid-naïve group. (c) Association between the FeNO level and RDR MDP in the steroid-treated group. (d) Association between FeNO level and RDR methacholine in the steroid-treated group.