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Increased periostin associates with greater airflow limitation in patients receiving inhaled corticosteroids

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Abstract

Background: Periostin, an extracellular matrix protein, contributes to subepithelial thickening in asthmatic airways, and its serum levels reflect airway eosinophilic inflammation. However, the relationship between periostin and the development of airflow limitation, a functional consequence of airway remodeling, remains unknown.

Objective: To determine the relationship between serum periostin levels and pulmonary function decline in asthmatic patients on inhaled corticosteroid (ICS) treatment.

Methods: 224 asthmatic patients (average age 62.3 years) treated with ICS for at least 4 years were enrolled. Annual changes in forced expiratory volume in one second (FEV_1), from at least one year after the initiation of ICS treatment to the time of enrollment or later (average 16.2 measurements over 8 years per individual), were assessed. At enrollment, clinical indices, biomarkers including serum periostin, and periostin gene polymorphisms were examined. Associations between clinical indices or biomarkers and a decline in FEV_1 of 30 $mL \cdot yr^{-1}$ or greater were analyzed.

Results: High serum periostin levels ($\geq$ 95 ng/mL) at enrollment, the highest treatment step, higher ICS daily doses, a history of admission due to asthma exacerbation, comorbid or a history of sinusitis, and ex-smoking were associated with a decline in FEV_1 of 30 $mL \cdot yr^{-1}$ or greater. Multivariate analysis revealed that high serum periostin, the highest treatment step, and ex-smoking were independent risk factors for the decline. Polymorphisms of periostin gene were related to higher serum periostin levels (rs3829365) and a decline in FEV_1 of 30 $mL \cdot yr^{-1}$ or greater (rs9603226).

Conclusions: Serum periostin appears to be a useful biomarker for the development of airflow limitation in asthmatic patients on ICS.

Clinical implications (25 words)

Serum periostin levels reflect greater FEV_1 decline in asthmatic patients on inhaled

corticosteroid treatment. *POSTN* gene polymorphisms may also be helpful for identifying rapid FEV_1 decliners.

Key words

Asthma, inhaled corticosteroids, lung function decline, periostin, *POSTN* gene polymorphism, sinusitis, treatment step

Abbreviations

ACT: asthma control test

ECP: eosinophil cationic protein

FAS I: fasciclin I

FEV_1: forced expiratory volume in one second

FVC: forced vital capacity

hsCRP: high sensitivity C-reactive protein

ICS: inhaled corticosteroids

IgE: immunoglobulin E

IL: interleukin

ROC: receiver operating characteristic

SNP: single-nucleotide polymorphism

TGF-β: transforming growth factor beta

Total word counts for the text and the abstract are 3800 and 258 words, respectively.

Capsule summary (32 words)

This is the first study to identify a relationship between high serum periostin and greater annual decline in FEV_1, which sheds new light on serum periositin as a useful biomarker in asthma.

Introduction

Airway inflammation and remodeling are key features of asthma that have been demonstrated by pathological[1] and radiological findings[2,3]. Physiologically, patients with asthma show a greater decline in pulmonary function than subjects without asthma[4]. Studies that were mostly conducted in the era before inhaled corticosteroids (ICS) demonstrated that more severe symptoms or severe exacerbations[5-7], long-standing asthma[8], and smoking history[4, 8] were moderate to strong risk factors for greater decline in pulmonary function[5]. Blood and sputum eosinophilia[9, 10] and genetic predisposition[11-13] were also potential risk factors. Owing to early intervention with ICS, however, airway inflammation and the degree of annual decline in pulmonary function have been attenuated in a majority of asthmatic patients[14-16]. Meanwhile, a subset of patients still show accelerated decline in FEV_1 and develop irreversible airway obstruction despite adequate treatment[17, 18]. van Veen et al. found that exhaled nitric oxide of 20 ppb or higher is a predictor of accelerated decline in pulmonary function in patients with difficult-to-treat asthma[18]. However, other biomarkers for greater decline in FEV_1 despite treatment with ICS remain unknown.

The airway inflammation of asthma is classically characterized by infiltration and activation of eosinophils, mast cells, and Th2 cells with several mediators and Th2 cytokines, such as interleukin (IL)-4, IL-5, and IL-13[19, 20]. Periostin, a secreted, 90-kDa, extracellular matrix protein that is induced by IL-4 and IL-13, was originally isolated as an osteoblast-specific factor; it shares structural homology to the insect cell adhesion molecule fasciclin I (FAS I) and binds to fibronectin, tenascin-C, and collagen[21, 22]. In airway epithelial cells collected from patients with asthma, periostin is one of the up-regulated genes[23], and its expression is correlated with thickness of the airway basement membrane[24]. Takayama et al. clearly demonstrated that periostin is deposited in the airway subepithelial layer in asthmatic patients. Moreover, serum periostin is identified as the single best predictor of airway eosinophilia in patients with severe asthma who remain symptomatic despite maximal ICS

treatment[25]. Therefore, we hypothesized that periostin would be a novel biomarker of Th2/eosinophil-driven airway inflammation and greater decline in pulmonary function, a functional consequence of airway remodeling in patients with asthma.

In this study, the effects of biomarkers and clinical indices on greater annual decline in pulmonary function in asthmatic patients on ICS treatment were examined, with the specific aim of determining the association between serum periostin levels and pulmonary function decline. Polymorphisms of the *POSTN* gene, which encodes periostin, were also examined on the hypothesis that *POSTN* gene polymorphisms may affect serum periostin levels.

Methods

For full details see Online Repository

Patients

Patients with asthma were recruited from nine institutions belonging to the Kinki Hokuriku Airway disease Conference where asthma specialists manage patients. Asthma was diagnosed according to the American Thoracic Society criteria[26]. From September 2009 to December 2011, patients were enrolled if they had received ICS treatment for 4 years or more, undergone three or more pulmonary function tests when they were stable, and were free from exacerbations for at least one month. The first pulmonary function test was performed at least one year after the commencement of ICS treatment and at 25 years of age or older. Patients who had smoked more than 10 pack-years, smoked in the past one year, or had other pulmonary diseases were excluded.

This study was approved by the ethics committee of each participant institution and was registered in the UMIN Clinical Trials Registry (Registry ID UMIN000002414). Written informed consent was obtained from all participants.

Measurements

At enrollment, patients underwent a work-up that included answering a self-completed questionnaire, spirometry, and blood tests. After enrollment, spirometry was repeated at least 6 months later for up to 12 months.

Self-completed questionnaire and clinical indices

The self-completed questionnaire was composed of 4 major items, as presented in Table 1. The Asthma Control Test (ACT)™ was also scored. The treatment step at enrollment was determined according to the Global Initiative for Asthma 2010 guideline[27].

Pulmonary function

Spirometry was performed using an electrical spirometer, which was calibrated once a week, at each institution. Spirometry data were obtained only when patients were stable. To determine pulmonary function on daily medications, ICS and other controllers, including long-acting β_2 agonists, leukotriene receptor antagonists, or slow-release theophylline, were not withdrawn before spirometry.

Measurement of systemic biomarkers

Blood eosinophil and neutrophil counts, and serum levels of total immunoglobulin E (IgE), specific IgE against common inhaled allergens, eosinophil cationic protein (ECP), high sensitivity C-reactive protein (hsCRP), and periostin were determined.

Serum periostin levels were measured using an enzyme-linked immunosorbent assay at Shino-test (Kanagawa, Japan), as described previously[28]. Pooled serum periostin level data from 66 healthy subjects [mean (SD), 60.7 (16.7) years old, 40 males][28,29] were used for comparison with those of asthmatic patients.

Haplotype analysis, DNA extraction, and genotyping of the *POSTN* gene

A total of 47 single-nucleotide polymorphisms (SNPs) in the region of the *POSTN* gene and its upstream, total 39 kb, was captured in the HapMap Japanese data set. Haplotype analysis identified 4 major haplotypes and 2 minor haplotypes. Two minor haplotypes were grouped into the closest major haplotype, and 3 tag SNPs that determined the 4 haplotypes were identified (Figure 1).

Genomic DNA was isolated from blood cells using a QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan). SNPs were genotyped using a Taqman genotyping assay according to the manufacturer's instructions (Applied Biosystems, Tokyo, Japan) and analyzed using an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems).

186

187 **Statistical analysis**

188 Statistical analyses were performed using JMP version 9.0 (SAS Institute Inc., Tokyo,
189 Japan). Annual changes in FEV_1 (ΔFEV_1) were estimated for each subject by fitting a least-
190 square regression line to all of his/her all available data points. Receiver operating
191 characteristic (ROC) curve analysis was performed to determine a serum periostin cut-off
192 value for asthmatic patients. The effects of serum biomarkers or other indices on ΔFEV_1 were
193 estimated using a generalized linear mixed model with adjustment for sex, height, age at
194 enrollment, and FEV_1 at the first measurement. The institutions were included as random
195 effects in this model. On univariate analysis of ΔFEV_1, the adjusted p value, i.e., q value,
196 which was a measure of significance in terms of the false discovery rate, was obtained using
197 R and QVALUE software[30] to determine spurious significance in multiple testing. The effects
198 on the dichotomous data for a decline in FEV_1 of -30 $mL \cdot yr^{-1}$ or greater[31] were similarly
199 estimated using a generalized linear mixed model by IBM SPSS Advanced Statistics 19
200 (SPSS Inc., Tokyo, Japan). Multivariate analysis was performed using variables with $p < 0.10$
201 on univariate analysis, except for ICS daily maintenance dose because of its strong
202 correlation with treatment step. On multivariate analysis, the periostin level was considered
203 as a dichotomous variable (high or low) instead of a continuous variable**.** Correlation
204 coefficients between serum periostin levels and clinical indices were estimated by fitting
205 least-square regression lines to data, in which institutions were included as random effects.
206 Unpaired *t*- and Chi-square tests were performed for comparisons of continuous and
207 dichotomous variables, respectively. When data were not normally distributed, they were log-
208 transformed. Data are presented as means (SD). P values ≤ 0.05 were considered significant.

Results

Patients' characteristics

Initially, 233 patients were enrolled in this study, but 9 patients were excluded: 5 with a smoking history of more than 10 pack-years and 4 who did not have enough pulmonary function data available. The demographic data of the remaining 224 patients are presented in Table 2. The mean age at enrollment was 62.3 (13.7) years. Overall, 130 (58%) had onset of asthma at 40 years or older. The average number of measurements of FEV_1, follow-up period, and ΔFEV_1 of 224 patients were 16.2 (13.9) times, 8.0 (4.5) years, and -7.8 (34.6) $mL \cdot yr^{-1}$, respectively. The distribution of ΔFEV_1 in this population is shown in Figure E1 in the Online Repository. Within 2 years after diagnosis, 46% of patients started ICS treatment. At enrollment, 82% of patients took controllers such as long-acting β_2 agonists, leukotriene receptor antagonists, or sustained release theophylline to achieve adequate asthma control. Based on a questionnaire, adherence to medication was satisfactory; 49% of the participants never and 38% seldom forgot to take ICS or other medications. Based on ACT scores, 50% was totally controlled, and 38% scored from 20 to 24, indicating that they were well controlled at enrollment.

Serum periostin levels of asthmatic patients [92.8 (38.4) ng/mL] were significantly higher than those of healthy subjects [39.1 (24.5) ng/mL, $p < 0.001$]. The ROC curve analysis was performed to discriminate patients with asthma who were thought to have refractory Th2 inflammation despite long-term ICS treatment from healthy subjects. The highest specificity among the 4 cut-off values tested was achieved at 95 ng/mL (0.985) in the comparison study of 224 asthmatic patients and 66 healthy subjects. Therefore a cut-off value of 95 ng/mL was used to define a high serum periostin group, although it had relatively lesser sensitivity (0.379) (see Figure E2 in the Online Repository). In asthmatic patients, 85 patients (38%) had high serum periostin levels ($\geq$ 95 ng/mL). Of the 85 patients, 40 patients (47%) were on

234 treatment step 4, according to the treatment step classification[27], and 9 patients (11%) were
235 on treatment step 5.
236
237 **Associations between serum periostin levels and greater annual decline in FEV_1 and a**
238 **decline in FEV_1 of 30 mL·yr^{-1} or greater**
239 In an analysis of continuous values of ΔFEV_1, greater decline in FEV_1 was associated
240 with higher serum periostin levels at enrollment, treatment step 5, lower ACT scores,
241 incomplete adherence to medications, comorbid or a history of sinusitis, and comorbid
242 diabetes mellitus (Table 3). When patients were stratified into two groups according to their
243 serum periostin levels, high serum periostin (≥ 95 ng/mL) was also associated with greater
244 decline in FEV_1 (Table 3). Of these, high serum periostin was significant after controlling for
245 multiple testing using the false discovery rate ($q = 0.03$, data not shown in Table 3).[30]
246 Multivariate analysis revealed that greater decline of FEV_1 was solely associated with high
247 serum periostin (≥ 95 ng/mL) (estimated effect -5.39, 95% confidence interval -10.0 to -0.77,
248 $p = 0.02$).
249 Fifty-two patients (23%) showed a decline in FEV_1 of 30 mL·yr^{-1} or greater [mean -
250 51.8 (18.4) mL · yr^{-1}] and were considered rapid decliners[31]. When adjusted by confounders,
251 higher serum periostin levels at enrollment, treatment step 5, a history of admission due to
252 asthma exacerbation, higher ICS daily doses, comorbid or a history of sinusitis, and ex-
253 smoking were associated with a decline in FEV_1 of 30 mL·yr^{-1} or greater. High serum
254 periostin (≥ 95 ng/mL) was also associated with a decline in FEV_1 of 30 mL·yr^{-1} or greater
255 (Table 4). On multivariate analysis, high serum periostin (≥ 95 ng/mL), treatment step 5, and
256 ex-smoking were independent risk factors for a decline in FEV_1 of 30 mL·yr^{-1} or greater
257 (Table 4).
258 Of the 224 patients, 19 patients were on treatment step 5, and 36 patients took high-
259 dose ICS (1,000 μg or higher doses of ICS equivalent to fluticasone propionate daily). When

patients were stratified into the high periostin group, the average ΔFEV_1 of patients on treatment step 5 (n = 9) was -41.0 (49.3) $mL \cdot yr^{-1}$, and 7 of them (78%) had excess decline; the average ΔFEV_1 of patients on high-dose ICS (n=18) was -34.3 (39.4) $mL \cdot yr^{-1}$, and 11 of them (61%) had a decline in FEV_1 of 30 $mL \cdot yr^{-1}$ or greater.

Serum periostin levels and clinical indices

In 224 patients, serum periostin levels were weakly associated with blood eosinophil counts (Figure 2), serum IgE (Figure 2) and ECP levels (r = 0.25, p = 0.0005), ICS-untreated period, i.e. period between onset of asthma and the initiation of ICS therapy (r = 0.16, p = 0.01), daily maintenance doses of ICS at enrollment (r = 0.13, p = 0.05), and a history of admission due to asthma exacerbation (r = 0.15, p = 0.03). Serum periostin levels were significantly higher in patients on high-dose ICS (≥ 1,000 μg daily) than in the remaining patients (110.3 ng/mL *vs.* 89.5 ng/mL, p = 0.003). Lastly, serum periostin levels were higher in patients with sinusitis than in those without sinusitis (103.9 ng/mL *vs.* 88.3 ng/mL, p = 0.007). Serum periostin levels did not show any seasonal variability or association with age at onset of asthma (data not shown).

***POSTN* gene polymorphisms**

Associations between polymorphisms of the *POSTN* gene, which encodes periostin, and both serum periostin levels and pulmonary function decline were then investigated. In one patient, DNA quality was insufficient for genotyping; thus, 3 tag SNPs of the *POSTN* gene were analyzed in 223 patients. All genotyped data were in Hardy-Weinberg equilibrium. The frequencies of the 3 tag SNPs and analysis results using dominant and recessive models for serum periostin levels and a decline in FEV_1 of 30 $mL \cdot yr^{-1}$ or greater are presented in Table 5.

Serum periostin levels were higher in patients with the GG genotype of rs3829365 than

in those with the GC/CC genotype (GG 98.7 ng/mL *vs.* GC/CC 86.1 ng/mL, p = 0.003). rs1028728 was not associated with serum periostin levels or with the frequency of rapid decliners, but patients with the TT genotype of rs1028728, 4 patients only, showed no significant decline compared with the AA/AT genotype (AA/AT -8.6 mL・yr^{-1} *vs.* TT 29.3 mL・yr^{-1}, p = 0.03). Rapid decliners were more frequently observed in patients with the minor A allele of rs9603226 than in the GG genotype (GG 16% *vs.* AG/AA 30%, p = 0.02). A marked difference in the frequency of rapid decliners was observed when patients were stratified into the high periostin group [GG of rs9630226 (n = 37) 19% vs. AG/AA (n =47) 45%, p = 0.01].

Discussion

To the best of our knowledge, this is the first study to identify a relationship between greater decline in FEV_1 and higher serum periostin levels, particularly if they were 95 ng/mL or more, in asthmatic patients on ICS treatment. It was also shown that high serum periostin, together with treatment step 5 and light ex-smoking, was an independent risk factor for a decline in FEV_1 of 30 mL·yr^{-1} or greater. In addition, polymorphisms of the *POSTN* gene, which encodes periostin, were associated with serum periostin levels and a decline in FEV_1 of 30 mL·yr^{-1} or greater in asthmatic patients. These findings suggest that serum periostin may be a useful biomarker for the development of airflow limitation in asthmatic patients on ICS.

In this study, despite long-term treatment with ICS with or without other controllers, 23% of asthmatic patients were rapid decliners who showed a decline in FEV_1 of 30 mL·yr^{-1} or greater, for which treatment step 5 was an independent risk factor. Adherence to ICS treatment and the frequency of early intervention with ICS did not differ between rapid decliners and non-decliners, although long-term adherence to ICS was undetermined in the present study. In previous studies of patients who were not treated with ICS, severe exacerbation of asthma contributed to greater annual decline of pulmonary function[6,7], but the exacerbation-related greater annual decline disappeared in an early intervention group with ICS treatment in the START study[6], which might be interpreted to mean that asthmatic patients on ICS treatment have little risk of accelerated FEV_1 decline. However, since the START study originally recruited mild persistent asthmatic patients, its results cannot simply be applied to severe asthmatic patients. As observed in the present study, there would be a subset of asthmatic patients still at risk of greater annual decline of pulmonary function despite intensive treatment for asthma.

Persistent eosinophilic airway inflammation is a key process in irreversible airway obstruction[10]. Indeed, exhaled nitric oxide of 20 ppb or higher is a risk factor for accelerated

FEV_1 decline in patients with difficult-to-treat asthma[18]. Studies on novel therapies for refractory eosinophilic asthma, i.e., anti-IL-5 therapy[32] and anti-IL-13 therapy[33], revealed that these treatments may reverse airway remodeling when patients are adequately targeted, suggesting the necessity of establishing "companion diagnostics" for this population. According to the most recent study, serum periostin is the single best biomarker reflecting sputum and tissue eosinophilia among several biomarkers, including blood eosinophils and exhaled nitric oxide[25]. In the current study, the serum periostin level, which was associated with the blood eosinophil count, was the sole biomarker that reflected greater decline in FEV_1. Periostin is secreted by airway epithelial cells[23, 24] and lung fibroblasts[21] in response to IL-4 and IL-13 and is thought to be secreted into the capillary vessels. Downstream of IL-13, which plays a pivotal role in subepithelial airway fibrosis[34], airway remodeling[35], and steroid insensitivity[36], periostin mediates collagen synthesis[24] and fibrillogenesis[24, 37] by binding to collagen[37] and activates TGF-β[24]. In the asthmatic airway, periostin is deposited in the subepithelial layer, colocalizing with collagens I, III, and V, fibronectin, tenascin-C, and periostin itself[21], which indicates involvement of periostin in airway remodeling in asthma. Collectively, periostin may be a key molecule that links eosinophilic inflammation and remodeling *via* IL-13 in asthmatic airways. Further roles of periostin in allergic inflammation and remodeling in the airways remain undetermined because studies using periostin-deficient mice with acute allergen exposure have yielded conflicting findings[38-40]; one study showed that periostin facilitates eosinophil infiltration into the lung[38], whereas two other studies[39, 40] suggested protective roles of periostin. Meanwhile, a recent study of a chronic mouse model of atopic dermatitis demonstrated periostin's role in the chronicity of Th2 inflammation[29].

In the present study, patients on high-dose ICS showed higher serum periostin levels than the other patients. Although a longitudinal study is needed to determine responses of serum periostin levels to ICS treatment, we do not think that the high serum periostin levels in patients on high-dose ICS were induced by ICS treatment, because periostin expression in

the airway epithelium was decreased with ICS treatment[23]. Rather, the elevation of serum periostin in this population may reflect IL-13-mediated inflammation that is partly refractory to ICS, as was reported in a recent study by Jia and colleagues[25]. They showed that, in patients with severe asthma who were treated with high doses ICS (> 1000 μg daily), elevation of serum periostin levels was associated with persistent airway tissue eosinophilia, concluding that serum periostin is a systemic biomarker of airway eosinophilia refractory to high-dose ICS[25]. Providing further support, among patients with moderate to severe asthma who are inadequately controlled despite ICS treatment, patients with high serum periostin levels are likely to benefit from anti-IL-13 antibody, lebrikizumab, treatment[33]. The novelty of the present finding is that high serum periostin is an independent risk factor for greater decline in FEV_1, providing the first evidence for the potential association between persistent Th2- or IL-13-driven inflammation refractory to ICS treatment and greater decline in FEV_1, a functional consequence of airway remodeling.

Needless to say, current smokers with asthma have more accelerated FEV_1 decline[4] than those not smoking, and current smoking impairs the therapeutic response to ICS or oral corticosteroids[41]. Meanwhile, smoking cessation improves their FEV_1 levels[42], and ex-smokers with asthma with 10 pack-years or more show an intermediate response to short-term oral corticosteroid treatment, between current smokers and never-smokers[41]. In the present study, rather unexpectedly, ex-smoking with 10 pack-years or less was still an independent risk factor for a decline in FEV_1 of 30 $mL \cdot yr^{-1}$ or greater. It should be recognized that even light ex-smoking increases the risk of airway remodeling in asthmatic patients on ICS, and its underlying mechanisms should be clarified.

Chronic sinusitis is a well-known comorbidity with severe asthma[43, 44]. In the present study, rapid decliners were more frequently observed in asthmatic patients with sinusitis than those without sinusitis on univariate analysis, and their periostin levels were higher than in patients without sinusitis. In the present study, polypoid lesions in the sinuses were not

evaluated by otolaryngologists at enrollment. However, considering that periostin is up-regulated in nasal polyp tissue in patients with chronic rhinosinusitis[45], asthmatic patients with sinusitis may have had severe upper and lower airway inflammation with persistent increases in periostin expression, which may have resulted in a decline in FEV_1 of 30 $mL \cdot yr^{-1}$ or greater. Periostin is a potential molecule that unifies sinusitis and severe asthma.

Periostin is encoded on the *POSTN* gene, which is located on chromosome 13q13.3. rs3829365, which is located at the 5'UTR region that may contain sequences to regulate translation efficiency or mRNA stability, was associated with serum periostin levels. This finding suggests that, besides IL-13, a master regulator of periostin, genetic background partly determines periostin levels, although a replication study would be necessary to confirm this. The minor A allele of rs9603226, located 66 bp upstream of exon 21 in the C-terminal region, was associated with a decline in FEV_1 of 30 $mL \cdot yr^{-1}$ or greater. In periostin, FAS I domains are thought to be primary binding sites to fibronectin, tenascin-C, and collagen V[21], whereas the C-terminal region in its intact form may down-regulate the binding activity of periostin to these extracellular matrix proteins[21]. We therefore speculate that the minor A allele of rs9603226 might modify the binding activity at the C-terminal region and facilitate airway remodeling, particularly if the airway is in periostin enriched milieu. Further studies are needed to clarify if these SNPs are functional variants.

The age of patients in this study appears to be older than in other Euro-American studies[6,7,14,18,20,23,25]. One reason for the age distribution would be the entry criteria of this study. Another reason would be explained by population aging including population with asthma in Japan. According to a patient survey by the Japanese Ministry of Health, Labour and Welfare in 2008, patients aged 70 to 74 years were the most frequent age group of adult patients with asthma[46], which is still older than the average age of patients in this study.

There are several limitations to the present study. First, since this study was observational in nature, ICS doses and numbers or types of controllers were not fixed during

399 the follow-up period. Controllers such as long-acting β_2 agonists were not withdrawn at
400 pulmonary function testing to evaluate function on daily medications, which may have
401 resulted in the small average ΔFEV_1, -7.8 mL $\cdot$ yr^{-1}. Meanwhile, averages of 16.2
402 measurements of FEV_1 and 8.0 years of follow-up were satisfactory for a longitudinal
403 analysis of pulmonary function[47], and ΔFEV_1 was normally distributed. Secondly, serum
404 biomarkers were measured only once at enrollment, but the significant associations between
405 *POSTN* gene polymorphisms and serum periostin levels or a decline in FEV_1 of 30 mL$\cdot$yr^{-1}
406 or greater may circumvent the inherent insufficiency of single measurement of serum
407 periostin. Thirdly, most of the clinical information, including smoking history and chronic
408 sinusitis, was based on a self-completed questionnaire, which might be biased by recall
409 memory. Despite these limitations, the current findings may provide directions for future
410 research.

411 In conclusion, serum periostin appears to be a useful biomarker that reflects the
412 development of airflow limitation in patients on prolonged treatment with ICS. *POSTN* gene
413 polymorphisms may also be helpful for identification of rapid decliners.

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553
554

555 **Table 1. Contents of the self-completed questionnaire**

Asthma-related history ・family history of asthma ・age of asthma onset ・history of pediatric asthma ・history of admission due to asthma worsening or exacerbation ・aspirin hypersensitivity ・asthma deterioration at the working place

Comorbidity or a history of the following diseases	
・allergic dermatitis	・cardiovascular diseases including ischemic heart disease
・allergic rhinitis	・gastrointestinal diseases including GERD
・seasonal rhinitis	・collagen vascular diseases including rheumatoid arthritis
・allergic conjunctivitis	・diabetes mellitus
・chronic sinusitis	・pulmonary diseases other than asthma
	・other diseases including malignancy

Lifestyle and environment	
・smoking history	・a highway near the home
・pet breeding	・age at menopause
・type of occupation	

Adherence to medication, sputum production, and exacerbations ・How often do you forget to take inhaled corticosteroids or other medications? 0: never, 1: seldom, 2: sometimes, 3: often, 4: always ・How often do you produce sputum? 0: never, 1: once in a few days, 2: every morning, 3: every morning and daytime ・How often did you receive systemic steroids due to asthma exacerbations during the recent 6 months? 0: never, 1: once, 2: twice or more

556 GERD: gastro-esophageal reflux disease

557

Table 2. Patients' characteristics

Sex (males/ females), n	53 / 171
Age at enrollment, years	62.3 (13.7)
Age at asthma onset, years	42.0 (19.0)
Body mass index (kg/m^2)	23.1 (3.5)
Smoking history (never), n	181
Atopic predisposition[*], %	70
Pediatric asthma (none/ recurrent/ persistent), %	81 / 8 / 11
Disease duration, years	20.2 (14.5)
ICS-untreated period, years	9.2 (13.1)
ICS daily maintenance dose[†], μg	525 (318)
Number of other controller medications, n	1.4 (1.2)
Treatment step (2/ 3/ 4/ 5)[‡], %	16 / 27 / 49 / 8
Sputum production (0/ 1/ 2/ 3)[§], %	54 / 20 / 8 / 18
Asthma Control Test, points	22.6 (3.5)
History of admission due to asthma, n (%)	78 (35)
Allergic rhinitis, n (%)	129 (58)
Chronic sinusitis, n (%)	65 (29)
Blood neutrophils, %	60.1 (10.0)
eosinophils, %	5.2 (4.9)
Serum IgE, IU/mL	180 (0 - 16000)
periostin, ng/mL	92.8 (38.4)
high sensitivity C-reactive protein, mg/L	1341 (3147)
eosinophil cationic protein, μg/L	15.1 (29.3)
FEV_1 at the first measurement, L[¶]	2.11 (0.69)
%predicted FEV_1 at the first measurement, %	91.9 (19.2)
FEV_1/ FVC at the first measurement, %	73.9 (9.8)
FEV_1 at enrollment, L	2.04 (0.73)
%predicted FEV_1 at enrollment, %	97.4 (22.2)
FEV_1/ FVC at enrollment, %	72.2 (10.0)
Reversibility at enrollment, %[#]	3.8 (6.0)

Data at enrollment are presented unless otherwise stated. Data are expressed as means (SD) except for median (range) for serum IgE. [*]Considered atopic when one or more specific IgE antibodies against cat or dog dander, weed, grass, or Japanese cedar pollens, moulds, or house dust mite were positive. [†]Equivalent to fluticasone propionate. [‡]according to the Global Initiative for Asthma 2010 guideline[27]. [§]0 = never, the details are shown in Table 1. [¶]The first pulmonary function test was performed at least one year after the commencement of ICS treatment and at 25 years of age or older. [#]n = 206, airway reversibility to 200 μg of inhaled salbutamol.

565 **Table 3. Estimated effects of clinical indices and biomarkers on ΔFEV$_1$**

	Estimates	95% C.I.	p value
Smoking history, ex *vs.* never	-8.48	-20.2, 3.27	0.16
Atopic predisposition	-1.10	-6.29, 4.09	0.68
Disease duration, years	-4.79	-18.4, 8.86	0.56
ICS-untreated period, years	0.10	-0.24, 0.45	0.65
ICS daily maintenance dose, μg	-0.01	-0.03, 0.001	0.07
Number of other controller medications, n	-0.36	-4.21, 3.49	0.86
Adherence to medication, incomplete *vs.* complete*	-4.56	-9.08, -0.04	0.05
Treatment step, 5 *vs.* 2-4†	-7.77	-15.7, 0.13	0.05
Sputum production, never *vs.* others‡	0.99	-3.53, 5.51	0.67
Asthma Control Test, points	1.53	0.29, 2.77	0.02
History of admission due to asthma	-4.49	-9.45, 0.46	0.08
Aspirin hypersensitivity	-6.52	-20.0, 6.98	0.34
Asthma deterioration at the working place	-12.2	-54.4, 30.0	0.57
Allergic rhinitis	-1.21	-5.88, 3.45	0.61
Allergic dermatitis	4.51	-1.51, 10.5	0.14
Chronic sinusitis	-10.1	-19.8, -0.27	0.04
Ischemic heart disease	3.41	-16.6, 23.4	0.74
Hypertension	-3.79	-9.12, 1.53	0.16
Dyslipidemia	-3.67	-9.42, -2.06	0.21
Diabetes mellitus	-8.03	-15.4, -0.67	0.03
Gastro-esophageal reflux disease	-3.85	-9.89, 2.19	0.21
Malignancy	-3.44	-26.0, 19.1	0.76
Post-menopause	5.05	-14.2, 24.3	0.60
Pet breeding	-0.28	-12.6, 12.0	0.96
Log blood neutrophils, %	-7.40	-69.1, 54.3	0.81
eosinophils, %	-0.67	-1.60, 0.27	0.16
Log serum IgE, IU/mL	-2.85	-9.74, 4.04	0.42
periostin, ng/mL	-29.1	-56.2, -1.97	0.04
high sensitivity C-reactive protein, mg/L	-1.88	-9.85, 6.10	0.64
eosinophil cationic protein, μg/L	-4.47	-15.7, 6.81	0.44
Periostin group, high *vs.* low§	-6.96	-11.4, -2.51	0.002

566 Estimated effects were adjusted by sex, height, age at enrollment, and FEV$_1$ at the first measurement. * "Complete", when patients answered
567 that they never forgot to take ICS or other medications; "incomplete", the remaining cases. †according to the Global Initiative for Asthma
568 2010 guideline[27]. ‡The details are shown in Table1. § Patients were stratified into two groups according to their serum periostin levels: high ≥
569 95 ng/mL, low < 95 ng/mL. ICS: inhaled corticosteroids, C.I.: confidence interval

Table 4. Estimated effects of clinical indices and serum periostin on a decline in FEV_1 of 30 mL·yr^{-1} or greater

	Univariate analysis			Multivariate analysis		
	Estimates	95% C.I.	p value	Estimates	95% C.I.	p value
Treatment step, 5 *vs.* 2-4*	1.63	0.51, 2.60	0.004	1.24	0.078, 2.30	0.04
History of admission due to asthma	1.09	0.37, 1.90	0.003	0.70	-0.11, 1.50	0.09
ICS daily maintenance dose, μg	0.001	0.00, 0.002	0.01	-		
Chronic sinusitis	0.82	0.11, 1.53	0.03	0.61	-0.15, 1.37	0.12
Smoking history, ex *vs.* never	0.87	-0.002, 1.74	0.05	0.98	0.030, 1.93	0.04
Log serum periostin, ng/mL	2.96	0.78, 5.13	0.008	-		
Periostin group, high *vs.* low†	1.03	0.33, 1.72	0.004	0.87	0.11, 1.63	0.03

Estimated effects were adjusted by sex, height, age at enrollment, and FEV_1 at the first measurement.

*according to the Global Initiative for Asthma 2010 guideline[27].

†Patients were stratified into two groups according to their serum periostin levels: high ≥ 95 ng/mL, low < 95 ng/mL. ICS: inhaled corticosteroids, C.I.: confidence interval

ICS daily maintenance dose was excluded from multivariate analysis because of its strong correlation with treatment step.

579 **Table 5.** Frequencies of 3 tag SNPs and analysis results using dominant and recessive models
580 for serum periostin levels and frequency of rapid decliners*

					Serum periostin levels		Frequency of rapid decliners	
					p value		p value	
Tag SNP	Genotype	n (%)	Allelic	n (%)	Dominant†	Recessive‡	Dominant†	Recessive‡
rs1028728	AA	164 (74)	A	383 (86)				
	AT	55 (25)	T	63 (14)	0.40	0.46	0.17	0.14
	TT	4 (2)						
rs3829365	GG	113 (51)	G	316 (71)				
	GC	90 (40)	C	130 (29)	0.003	0.70	0.40	0.33
	CC	20 (9)						
rs9603226	GG	107 (48)	G	311 (70)				
	AG	97 (44)	A	135 (30)	0.80	0.33	0.01	0.81
	AA	19 (9)						

581 * defined as patients who showed a decline in FEV_1 of 30 $mL \cdot yr^{-1}$ or greater
582 † Assuming that heterozygotes have the same increased risk as minor homozygous genotypes.
583 ‡ Assuming that heterozygotes have no increased risk.
584

585 **Figure legends**

586 Figure 1. Three tag SNPs that determine 4 major haplotypes of the *POSTN* gene and

587 haplotype frequencies in the Japanese population are presented.

588 *at intron 66 bp upstream of exon 21

589

590 Figure 2. Relationships between serum periostin levels and blood eosinophil counts (left) or

591 serum IgE levels (right).

592 Presented in logarithmic scales on both the X- and Y-axes.

593

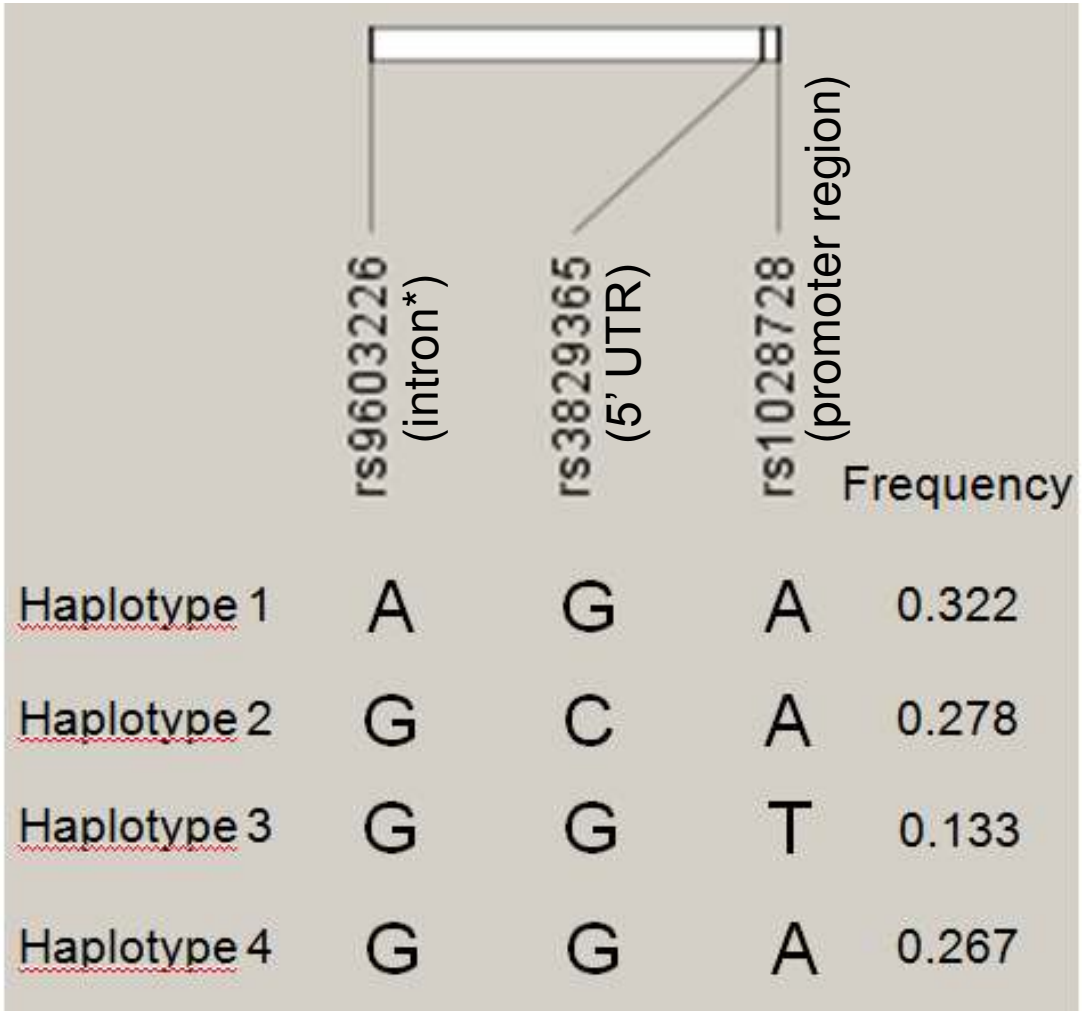
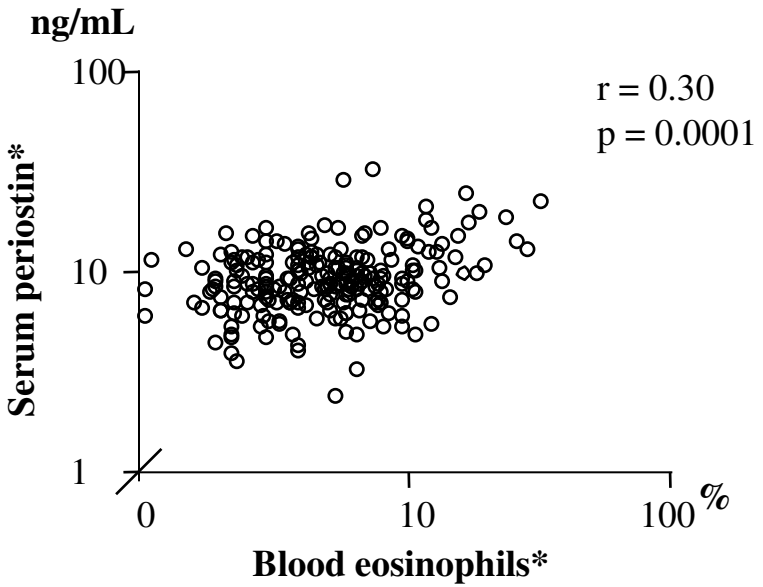


Figure 1.



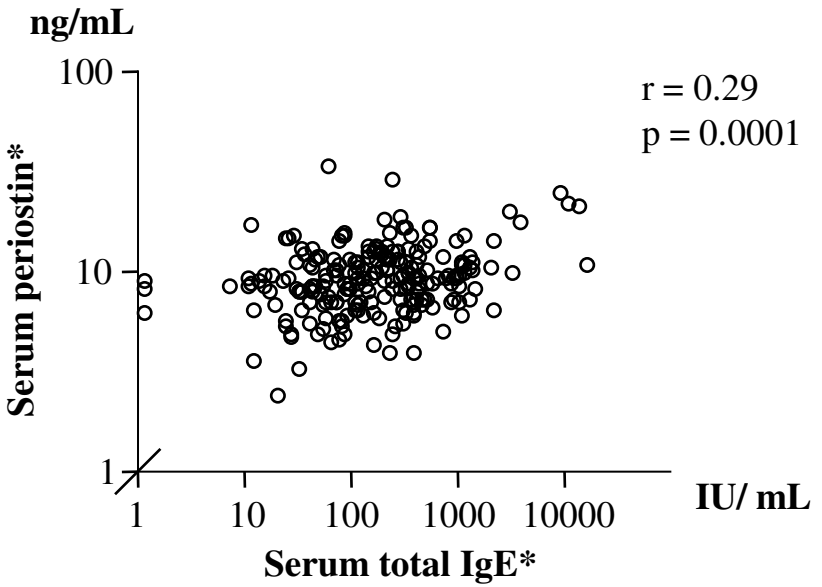


Figure 2.

Online Repository

Increased periostin associates with greater airflow limitation in patients receiving inhaled corticosteroids

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Methods

Patients

Patients with asthma were recruited from nine institutions belonging to the Kinki Hokuriku Airway disease Conference where asthma specialists manage patients, including six university hospitals, two satellite general hospitals, and one satellite clinic. Asthma was diagnosed according to the American Thoracic Society criteria[E1] on the basis of a history of recurrent episodes of wheezing and chest tightness with or without cough and documented airway reversibility to a bronchodilator or hyper-responsiveness to inhaled methacholine. From September 2009 to December 2011, patients were enrolled if they had received ICS treatment for 4 years or more, undergone three or more pulmonary function tests when they were stable, and were free from exacerbations for at least one month. The first pulmonary function test was performed at least one year after the commencement of ICS treatment and at 25 years of age or older. Patients who had smoked more than 10 pack-years, smoked in the past one year, or had other pulmonary diseases were excluded.

Self-completed questionnaire and clinical indices

The self-completed questionnaire was composed of 4 major items, as presented in Table 1.

Adherence to ICS or other medications, frequency of sputum production, and requirement for systemic corticosteroids during the last 6 months were graded as shown in Table 1. The Asthma Control Test (ACT)™ was also scored. Duration of ICS treatment and details on medication at enrollment were recorded from medical charts by patients' physicians. The treatment step at enrollment was determined according to the

Global Initiative for Asthma 2010 guideline[E2].

Measurement of systemic biomarkers

Blood eosinophil and neutrophil counts, and serum levels of total immunoglobulin E (IgE) (ImmunoCAP® total IgE, Phadia K.K., Tokyo, Japan), specific IgE against common inhaled allergens (ImmunoCAP® specific IgE), eosinophil cationic protein (ECP) (ImmunoCAP® ECP), high sensitivity C-reactive protein (hsCRP) (CardioPhase® hsCRP, Siemens Healthcare Diagnostics K.K., Tokyo, Japan), and periostin were determined.

Serum periostin levels were measured using an enzyme-linked immunosorbent assay at Shino-test (Kanagawa, Japan), as described previously[E3]. Briefly, two rat anti-human periostin monoclonal antibodies (SS18A and SS17B) were used. SS18A and SS17B are antibodies against the first and fourth FAS I domains, respectively. Intra- and inter-assay coefficients of variation ranged from 1.31% to 2.54% and 1.49% to 2.01%, respectively.

Haplotype analysis, DNA extraction, and genotyping of the *POSTN* gene

A total of 47 single-nucleotide polymorphisms (SNPs) in the region of the *POSTN* gene and its upstream, total 39 kb, was captured in the HapMap Japanese data set with minor allele frequencies > 0.10. Pairwise tagging was performed at $r^2 > 0.8$ using a tagger in Haploview 4.2 software. Haplotype analysis identified 4 major haplotypes and 2 minor haplotypes. Two minor haplotypes were grouped into the closest major haplotype, and 3 tag SNPs that determined the 4 haplotypes were identified (Figure 1). These 3 tag SNPs were located at promoter region (rs1028728), 5'UTR

region (rs3829365), and at intron 66 bp upstream of exon 21 (rs9603226). The frequencies of the minor alleles in the Japanese population were 0.136 (rs1028728), 0.278 (rs3829365), and 0.330 (rs9603226).

Genomic DNA was isolated from blood cells using a QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan). SNPs were genotyped using a Taqman genotyping assay according to the manufacturer's instructions (Applied Biosystems, Tokyo, Japan) and analyzed using an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems).

References

E1. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, November 1986. Am Rev Respir Dis 1987; 136:225-44.

E2 Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) 2010. Available from: Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) 2010. Available from: http://www.ginasthma.org.

E3. Okamoto M, Hoshino T, Kitasato Y, Sakazaki Y, Kawayama T, Fujimoto K, et al. Periostin, a matrix protein, is a novel biomarker for idiopathic interstitial pneumonias. Eur Respir J 2011; 37:1119-27.

124 Figure legends

125

126 Figure E1. The distribution of ΔFEV_1 in the study population

127

128 Figure E2. ROC curve analysis of serum periostin levels comparing asthmatic patients
129 and healthy subjects, in which the cutoffs of 95 ng/mL, 80 ng/mL, 92 ng/mL, and 100
130 ng/mL are presented with arrows.

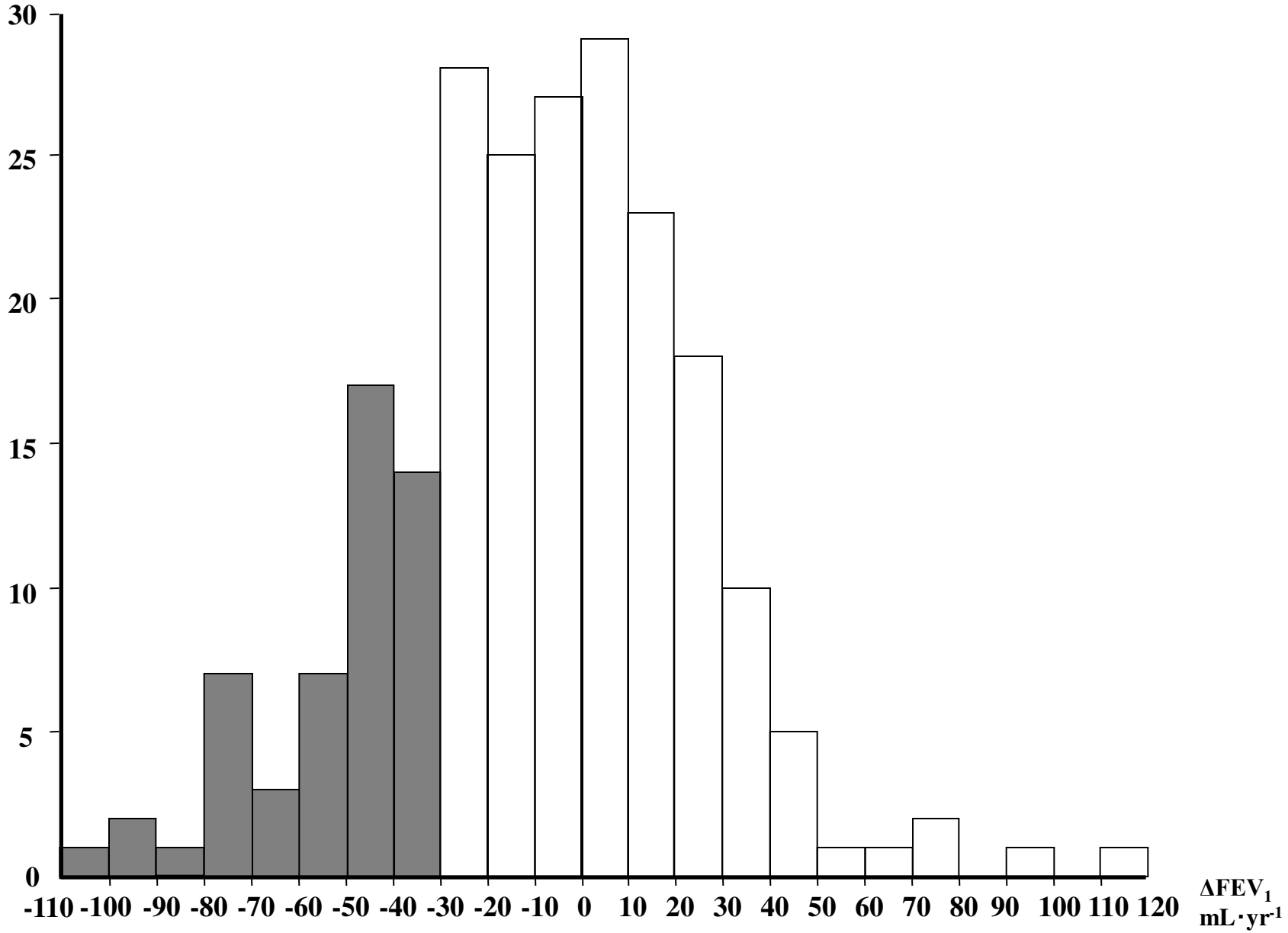

Figure E1.

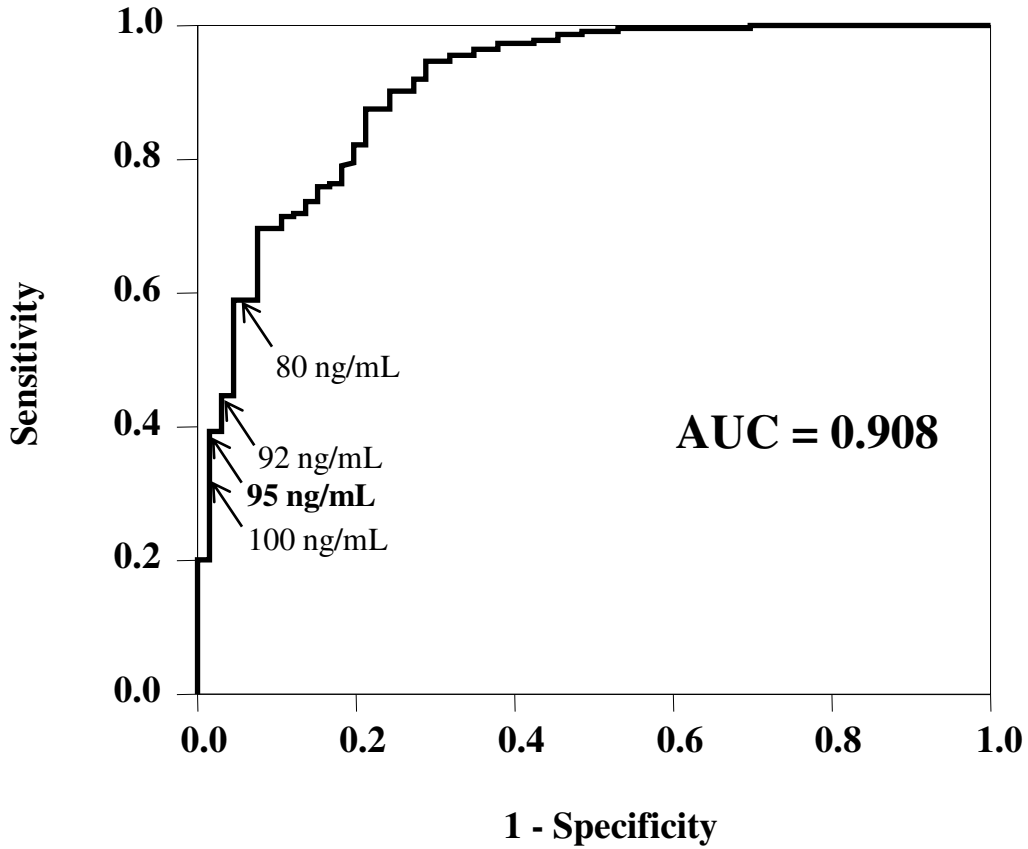

Figure E2.