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

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48 **Abstract**

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

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

55 **Methods:** 224 asthmatic patients (average age 62.3 years) treated with ICS for at least
56 4 years were enrolled. Annual changes in forced expiratory volume in one second (FEV₁),
57 from at least one year after the initiation of ICS treatment to the time of enrollment or later
58 (average 16.2 measurements over 8 years per individual), were assessed. At enrollment,
59 clinical indices, biomarkers including serum periostin, and periostin gene polymorphisms
60 were examined. Associations between clinical indices or biomarkers and a decline in FEV₁ of
61 30 mL·yr⁻¹ or greater were analyzed.

62 **Results:** High serum periostin levels (≥ 95 ng/mL) at enrollment, the highest treatment
63 step, higher ICS daily doses, a history of admission due to asthma exacerbation, comorbid or
64 a history of sinusitis, and ex-smoking were associated with a decline in FEV₁ of 30 mL·yr⁻¹ or
65 greater. Multivariate analysis revealed that high serum periostin, the highest treatment step,
66 and ex-smoking were independent risk factors for the decline. Polymorphisms of periostin
67 gene were related to higher serum periostin levels (rs3829365) and a decline in FEV₁ of 30
68 mL·yr⁻¹ or greater (rs9603226).

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

71

72 **Clinical implications (25 words)**

73 Serum periostin levels reflect greater FEV₁ decline in asthmatic patients on inhaled

74 corticosteroid treatment. *POSTN* gene polymorphisms may also be helpful for identifying
75 rapid FEV₁ decliners.

76 **Key words**

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

79

80 **Abbreviations**

81 ACT: asthma control test

82 ECP: eosinophil cationic protein

83 FAS I: fasciclin I

84 FEV₁: forced expiratory volume in one second

85 FVC: forced vital capacity

86 hsCRP: high sensitivity C-reactive protein

87 ICS: inhaled corticosteroids

88 IgE: immunoglobulin E

89 IL: interleukin

90 ROC: receiver operating characteristic

91 SNP: single-nucleotide polymorphism

92 TGF- β : transforming growth factor beta

93

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

95 **Capsule summary (32 words)**

96 This is the first study to identify a relationship between high serum periostin and greater
97 annual decline in FEV₁, which sheds new light on serum periostin as a useful biomarker in
98 asthma.

99 Introduction

100 Airway inflammation and remodeling are key features of asthma that have been
101 demonstrated by pathological¹ and radiological findings^{2,3}. Physiologically, patients with
102 asthma show a greater decline in pulmonary function than subjects without asthma⁴. Studies
103 that were mostly conducted in the era before inhaled corticosteroids (ICS) demonstrated that
104 more severe symptoms or severe exacerbations⁵⁻⁷, long-standing asthma⁸, and smoking
105 history^{4,8} were moderate to strong risk factors for greater decline in pulmonary function⁵.
106 Blood and sputum eosinophilia^{9,10} and genetic predisposition¹¹⁻¹³ were also potential risk
107 factors. Owing to early intervention with ICS, however, airway inflammation and the degree
108 of annual decline in pulmonary function have been attenuated in a majority of asthmatic
109 patients¹⁴⁻¹⁶. Meanwhile, a subset of patients still show accelerated decline in FEV₁ and
110 develop irreversible airway obstruction despite adequate treatment^{17,18}. van Veen et al. found
111 that exhaled nitric oxide of 20 ppb or higher is a predictor of accelerated decline in
112 pulmonary function in patients with difficult-to-treat asthma¹⁸. However, other biomarkers for
113 greater decline in FEV₁ despite treatment with ICS remain unknown.

114 The airway inflammation of asthma is classically characterized by infiltration and
115 activation of eosinophils, mast cells, and Th2 cells with several mediators and Th2 cytokines,
116 such as interleukin (IL)-4, IL-5, and IL-13^{19,20}. Periostin, a secreted, 90-kDa, extracellular
117 matrix protein that is induced by IL-4 and IL-13, was originally isolated as an osteoblast-
118 specific factor; it shares structural homology to the insect cell adhesion molecule fasciclin I
119 (FAS I) and binds to fibronectin, tenascin-C, and collagen^{21,22}. In airway epithelial cells
120 collected from patients with asthma, periostin is one of the up-regulated genes²³, and its
121 expression is correlated with thickness of the airway basement membrane²⁴. Takayama et al.
122 clearly demonstrated that periostin is deposited in the airway subepithelial layer in asthmatic
123 patients. Moreover, serum periostin is identified as the single best predictor of airway
124 eosinophilia in patients with severe asthma who remain symptomatic despite maximal ICS

125 treatment²⁵. Therefore, we hypothesized that periostin would be a novel biomarker of
126 Th2/eosinophil-driven airway inflammation and greater decline in pulmonary function, a
127 functional consequence of airway remodeling in patients with asthma.

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

134 **Methods**

135 **For full details see Online Repository**

136 **Patients**

137 Patients with asthma were recruited from nine institutions belonging to the Kinki
138 Hokuriku Airway disease Conference where asthma specialists manage patients. Asthma was
139 diagnosed according to the American Thoracic Society criteria²⁶. From September 2009 to
140 December 2011, patients were enrolled if they had received ICS treatment for 4 years or more,
141 undergone three or more pulmonary function tests when they were stable, and were free from
142 exacerbations for at least one month. The first pulmonary function test was performed at least
143 one year after the commencement of ICS treatment and at 25 years of age or older. Patients
144 who had smoked more than 10 pack-years, smoked in the past one year, or had other
145 pulmonary diseases were excluded.

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

149

150 **Measurements**

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

154

155 **Self-completed questionnaire and clinical indices**

156 The self-completed questionnaire was composed of 4 major items, as presented in
157 Table 1. The Asthma Control Test (ACT)TM was also scored. The treatment step at enrollment
158 was determined according to the Global Initiative for Asthma 2010 guideline²⁷.

159

160 **Pulmonary function**

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

166

167 **Measurement of systemic biomarkers**

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

171 Serum periostin levels were measured using an enzyme-linked immunosorbent assay at
172 Shino-test (Kanagawa, Japan), as described previously²⁸. Pooled serum periostin level data
173 from 66 healthy subjects [mean (SD), 60.7 (16.7) years old, 40 males]^{28,29} were used for
174 comparison with those of asthmatic patients.

175

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

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

182 Genomic DNA was isolated from blood cells using a QIAamp DNA Blood Mini Kit
183 (Qiagen, Tokyo, Japan). SNPs were genotyped using a Taqman genotyping assay according
184 to the manufacturer's instructions (Applied Biosystems, Tokyo, Japan) and analyzed using an
185 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 FEV₁ (Δ FEV₁) 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 Δ FEV₁ were
193 estimated using a generalized linear mixed model with adjustment for sex, height, age at
194 enrollment, and FEV₁ at the first measurement. The institutions were included as random
195 effects in this model. On univariate analysis of Δ FEV₁, 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 software³⁰ to determine spurious significance in multiple testing. The effects
198 on the dichotomous data for a decline in FEV₁ of -30 mL·yr⁻¹ or greater³¹ 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.

209 **Results**

210 **Patients' characteristics**

211 Initially, 233 patients were enrolled in this study, but 9 patients were excluded: 5 with a
212 smoking history of more than 10 pack-years and 4 who did not have enough pulmonary
213 function data available. The demographic data of the remaining 224 patients are presented in
214 Table 2. The mean age at enrollment was 62.3 (13.7) years. Overall, 130 (58%) had onset of
215 asthma at 40 years or older. The average number of measurements of FEV₁, follow-up period,
216 and Δ FEV₁ of 224 patients were 16.2 (13.9) times, 8.0 (4.5) years, and -7.8 (34.6) mL · yr⁻¹,
217 respectively. The distribution of Δ FEV₁ in this population is shown in Figure E1 in the Online
218 Repository. Within 2 years after diagnosis, 46% of patients started ICS treatment. At
219 enrollment, 82% of patients took controllers such as long-acting β_2 agonists, leukotriene
220 receptor antagonists, or sustained release theophylline to achieve adequate asthma control.
221 Based on a questionnaire, adherence to medication was satisfactory; 49% of the participants
222 never and 38% seldom forgot to take ICS or other medications. Based on ACT scores, 50%
223 was totally controlled, and 38% scored from 20 to 24, indicating that they were well
224 controlled at enrollment.

225 Serum periostin levels of asthmatic patients [92.8 (38.4) ng/mL] were significantly
226 higher than those of healthy subjects [39.1 (24.5) ng/mL, $p < 0.001$]. The ROC curve analysis
227 was performed to discriminate patients with asthma who were thought to have refractory Th2
228 inflammation despite long-term ICS treatment from healthy subjects. The highest specificity
229 among the 4 cut-off values tested was achieved at 95 ng/mL (0.985) in the comparison study
230 of 224 asthmatic patients and 66 healthy subjects. Therefore a cut-off value of 95 ng/mL was
231 used to define a high serum periostin group, although it had relatively lesser sensitivity
232 (0.379) (see Figure E2 in the Online Repository). In asthmatic patients, 85 patients (38%) had
233 high serum periostin levels (≥ 95 ng/mL). Of the 85 patients, 40 patients (47%) were on

234 treatment step 4, according to the treatment step classification²⁷, and 9 patients (11%) were
235 on treatment step 5.

236

237 **Associations between serum periostin levels and greater annual decline in FEV₁ and a**
238 **decline in FEV₁ of 30 mL·yr⁻¹ or greater**

239 In an analysis of continuous values of Δ FEV₁, greater decline in FEV₁ 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 FEV₁ (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 FEV₁ 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 FEV₁ of 30 mL·yr⁻¹ or greater [mean -
250 51.8 (18.4) mL·yr⁻¹] and were considered rapid decliners³¹. 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 FEV₁ of 30 mL·yr⁻¹ or greater. High serum
254 periostin (≥ 95 ng/mL) was also associated with a decline in FEV₁ 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 FEV₁ 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 μ g or higher doses of ICS equivalent to fluticasone propionate daily). When

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

264

265 **Serum periostin levels and clinical indices**

266 In 224 patients, serum periostin levels were weakly associated with blood
267 eosinophil counts (Figure 2), serum IgE (Figure 2) and ECP levels ($r = 0.25$, $p = 0.0005$),
268 ICS-untreated period, i.e. period between onset of asthma and the initiation of ICS therapy (r
269 $= 0.16$, $p = 0.01$), daily maintenance doses of ICS at enrollment ($r = 0.13$, $p = 0.05$), and a
270 history of admission due to asthma exacerbation ($r = 0.15$, $p = 0.03$). Serum periostin levels
271 were significantly higher in patients on high-dose ICS ($\geq 1,000 \mu\text{g}$ daily) than in the
272 remaining patients (110.3 ng/mL vs. 89.5 ng/mL , $p = 0.003$). Lastly, serum periostin levels
273 were higher in patients with sinusitis than in those without sinusitis (103.9 ng/mL vs. 88.3
274 ng/mL , $p = 0.007$). Serum periostin levels did not show any seasonal variability or
275 association with age at onset of asthma (data not shown).

276

277 ***POSTN* gene polymorphisms**

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

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

286 in those with the GC/CC genotype (GG 98.7 ng/mL vs. GC/CC 86.1 ng/mL, $p = 0.003$).

287 rs1028728 was not associated with serum periostin levels or with the frequency of rapid

288 decliners, but patients with the TT genotype of rs1028728, 4 patients only, showed no

289 significant decline compared with the AA/AT genotype (AA/AT $-8.6 \text{ mL} \cdot \text{yr}^{-1}$ vs. TT 29.3

290 $\text{mL} \cdot \text{yr}^{-1}$, $p = 0.03$). Rapid decliners were more frequently observed in patients with the minor

291 A allele of rs9603226 than in the GG genotype (GG 16% vs. AG/AA 30%, $p = 0.02$). A

292 marked difference in the frequency of rapid decliners was observed when patients were

293 stratified into the high periostin group [GG of rs9630226 ($n = 37$) 19% vs. AG/AA ($n = 47$)

294 45%, $p = 0.01$].

295 Discussion

296 To the best of our knowledge, this is the first study to identify a relationship between
297 greater decline in FEV₁ and higher serum periostin levels, particularly if they were 95 ng/mL
298 or more, in asthmatic patients on ICS treatment. It was also shown that high serum periostin,
299 together with treatment step 5 and light ex-smoking, was an independent risk factor for a
300 decline in FEV₁ of 30 mL·yr⁻¹ or greater. In addition, polymorphisms of the *POSTN* gene,
301 which encodes periostin, were associated with serum periostin levels and a decline in FEV₁
302 of 30 mL·yr⁻¹ or greater in asthmatic patients. These findings suggest that serum periostin
303 may be a useful biomarker for the development of airflow limitation in asthmatic patients on
304 ICS.

305 In this study, despite long-term treatment with ICS with or without other controllers,
306 23% of asthmatic patients were rapid decliners who showed a decline in FEV₁ of 30 mL·yr⁻¹
307 or greater, for which treatment step 5 was an independent risk factor. Adherence to ICS
308 treatment and the frequency of early intervention with ICS did not differ between rapid
309 decliners and non-decliners, although long-term adherence to ICS was undetermined in the
310 present study. In previous studies of patients who were not treated with ICS, severe
311 exacerbation of asthma contributed to greater annual decline of pulmonary function^{6,7}, but the
312 exacerbation-related greater annual decline disappeared in an early intervention group with
313 ICS treatment in the START study⁶, which might be interpreted to mean that asthmatic
314 patients on ICS treatment have little risk of accelerated FEV₁ decline. However, since the
315 START study originally recruited mild persistent asthmatic patients, its results cannot simply
316 be applied to severe asthmatic patients. As observed in the present study, there would be a
317 subset of asthmatic patients still at risk of greater annual decline of pulmonary function
318 despite intensive treatment for asthma.

319 Persistent eosinophilic airway inflammation is a key process in irreversible airway
320 obstruction¹⁰. Indeed, exhaled nitric oxide of 20 ppb or higher is a risk factor for accelerated

321 FEV₁ decline in patients with difficult-to-treat asthma¹⁸. Studies on novel therapies for
322 refractory eosinophilic asthma, i.e., anti-IL-5 therapy³² and anti-IL-13 therapy³³, revealed that
323 these treatments may reverse airway remodeling when patients are adequately targeted,
324 suggesting the necessity of establishing “companion diagnostics” for this population.
325 According to the most recent study, serum periostin is the single best biomarker reflecting
326 sputum and tissue eosinophilia among several biomarkers, including blood eosinophils and
327 exhaled nitric oxide²⁵. In the current study, the serum periostin level, which was associated
328 with the blood eosinophil count, was the sole biomarker that reflected greater decline in FEV₁.
329 Periostin is secreted by airway epithelial cells^{23,24} and lung fibroblasts²¹ in response to IL-4
330 and IL-13 and is thought to be secreted into the capillary vessels. Downstream of IL-13,
331 which plays a pivotal role in subepithelial airway fibrosis³⁴, airway remodeling³⁵, and steroid
332 insensitivity³⁶, periostin mediates collagen synthesis²⁴ and fibrillogenesis^{24,37} by binding to
333 collagen³⁷ and activates TGF- β ²⁴. In the asthmatic airway, periostin is deposited in the
334 subepithelial layer, colocalizing with collagens I, III, and V, fibronectin, tenascin-C, and
335 periostin itself²¹, which indicates involvement of periostin in airway remodeling in asthma.
336 Collectively, periostin may be a key molecule that links eosinophilic inflammation and
337 remodeling *via* IL-13 in asthmatic airways. Further roles of periostin in allergic inflammation
338 and remodeling in the airways remain undetermined because studies using periostin-deficient
339 mice with acute allergen exposure have yielded conflicting findings³⁸⁻⁴⁰; one study showed
340 that periostin facilitates eosinophil infiltration into the lung³⁸, whereas two other studies^{39,40}
341 suggested protective roles of periostin. Meanwhile, a recent study of a chronic mouse model
342 of atopic dermatitis demonstrated periostin’s role in the chronicity of Th2 inflammation²⁹.

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

347 the airway epithelium was decreased with ICS treatment²³. Rather, the elevation of serum
348 periostin in this population may reflect IL-13-mediated inflammation that is partly refractory
349 to ICS, as was reported in a recent study by Jia and colleagues²⁵. They showed that, in
350 patients with severe asthma who were treated with high doses ICS (> 1000 µg daily),
351 elevation of serum periostin levels was associated with persistent airway tissue eosinophilia,
352 concluding that serum periostin is a systemic biomarker of airway eosinophilia refractory to
353 high-dose ICS²⁵. Providing further support, among patients with moderate to severe asthma
354 who are inadequately controlled despite ICS treatment, patients with high serum periostin
355 levels are likely to benefit from anti-IL-13 antibody, lebrikizumab, treatment³³. The novelty
356 of the present finding is that high serum periostin is an independent risk factor for greater
357 decline in FEV₁, providing the first evidence for the potential association between persistent
358 Th2- or IL-13-driven inflammation refractory to ICS treatment and greater decline in FEV₁, a
359 functional consequence of airway remodeling.

360 Needless to say, current smokers with asthma have more accelerated FEV₁ decline⁴ than
361 those not smoking, and current smoking impairs the therapeutic response to ICS or oral
362 corticosteroids⁴¹. Meanwhile, smoking cessation improves their FEV₁ levels⁴², and ex-
363 smokers with asthma with 10 pack-years or more show an intermediate response to short-
364 term oral corticosteroid treatment, between current smokers and never-smokers⁴¹. In the
365 present study, rather unexpectedly, ex-smoking with 10 pack-years or less was still an
366 independent risk factor for a decline in FEV₁ of 30 mL·yr⁻¹ or greater. It should be recognized
367 that even light ex-smoking increases the risk of airway remodeling in asthmatic patients on
368 ICS, and its underlying mechanisms should be clarified.

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

373 evaluated by otolaryngologists at enrollment. However, considering that periostin is up-
374 regulated in nasal polyp tissue in patients with chronic rhinosinusitis⁴⁵, asthmatic patients
375 with sinusitis may have had severe upper and lower airway inflammation with persistent
376 increases in periostin expression, which may have resulted in a decline in FEV₁ of 30 mL·yr⁻¹
377 or greater. Periostin is a potential molecule that unifies sinusitis and severe asthma.

378 Periostin is encoded on the *POSTN* gene, which is located on chromosome 13q13.3.
379 rs3829365, which is located at the 5'UTR region that may contain sequences to regulate
380 translation efficiency or mRNA stability, was associated with serum periostin levels. This
381 finding suggests that, besides IL-13, a master regulator of periostin, genetic background
382 partly determines periostin levels, although a replication study would be necessary to confirm
383 this. The minor A allele of rs9603226, located 66 bp upstream of exon 21 in the C-terminal
384 region, was associated with a decline in FEV₁ of 30 mL·yr⁻¹ or greater. In periostin, FAS I
385 domains are thought to be primary binding sites to fibronectin, tenascin-C, and collagen V²¹,
386 whereas the C-terminal region in its intact form may down-regulate the binding activity of
387 periostin to these extracellular matrix proteins²¹. We therefore speculate that the minor A
388 allele of rs9603226 might modify the binding activity at the C-terminal region and facilitate
389 airway remodeling, particularly if the airway is in periostin enriched milieu. Further studies
390 are needed to clarify if these SNPs are functional variants.

391 The age of patients in this study appears to be older than in other Euro-American
392 studies^{6,7,14,18,20,23,25}. One reason for the age distribution would be the entry criteria of this
393 study. Another reason would be explained by population aging including population with
394 asthma in Japan. According to a patient survey by the Japanese Ministry of Health, Labour
395 and Welfare in 2008, patients aged 70 to 74 years were the most frequent age group of adult
396 patients with asthma⁴⁶, which is still older than the average age of patients in this study.

397 There are several limitations to the present study. First, since this study was
398 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 , $-7.8 \text{ mL} \cdot \text{yr}^{-1}$. 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 function⁴⁷, 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 $30 \text{ mL} \cdot \text{yr}^{-1}$
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	
<ul style="list-style-type: none"> ▪ 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	
<ul style="list-style-type: none"> ▪ allergic dermatitis ▪ allergic rhinitis ▪ seasonal rhinitis ▪ allergic conjunctivitis ▪ chronic sinusitis 	<ul style="list-style-type: none"> ▪ cardiovascular diseases including ischemic heart disease ▪ gastrointestinal diseases including GERD ▪ collagen vascular diseases including rheumatoid arthritis ▪ diabetes mellitus ▪ pulmonary diseases other than asthma ▪ other diseases including malignancy
Lifestyle and environment	
<ul style="list-style-type: none"> ▪ smoking history ▪ pet breeding ▪ type of occupation 	<ul style="list-style-type: none"> ▪ a highway near the home ▪ age at menopause
Adherence to medication, sputum production, and exacerbations	
<ul style="list-style-type: none"> ▪ 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

558 **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)

559 Data at enrollment are presented unless otherwise stated. Data are expressed as means (SD) except for median
560 (range) for serum IgE. ^{*}Considered atopic when one or more specific IgE antibodies against cat or dog dander,
561 weed, grass, or Japanese cedar pollens, moulds, or house dust mite were positive. [†]Equivalent to fluticasone
562 propionate. [‡]according to the Global Initiative for Asthma 2010 guideline²⁷. [§]0 = never, the details are shown in
563 Table 1. [¶]The first pulmonary function test was performed at least one year after the commencement of ICS
564 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 Δ FEV₁**

	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, μ g	-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, μ g/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 FEV₁ 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 guideline²⁷. [‡]The details are shown in Table 1. [§]Patients were stratified into two groups according to their serum periostin levels: high \geq
569 95 ng/mL, low < 95 ng/mL. ICS: inhaled corticosteroids, C.I.: confidence interval

570 **Table 4. Estimated effects of clinical indices and serum periostin on a decline in FEV₁ of**
 571 **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

572 Estimated effects were adjusted by sex, height, age at enrollment, and FEV₁ at the first measurement.

573 * according to the Global Initiative for Asthma 2010 guideline²⁷.

574 †Patients were stratified into two groups according to their serum periostin levels: high \geq 95 ng/mL, low <

575 95 ng/mL. ICS: inhaled corticosteroids, C.I.: confidence interval

576 ICS daily maintenance dose was excluded from multivariate analysis because of its strong correlation with

577 treatment step.

578

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*

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

581 * defined as patients who showed a decline in FEV₁ of 30 mL·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

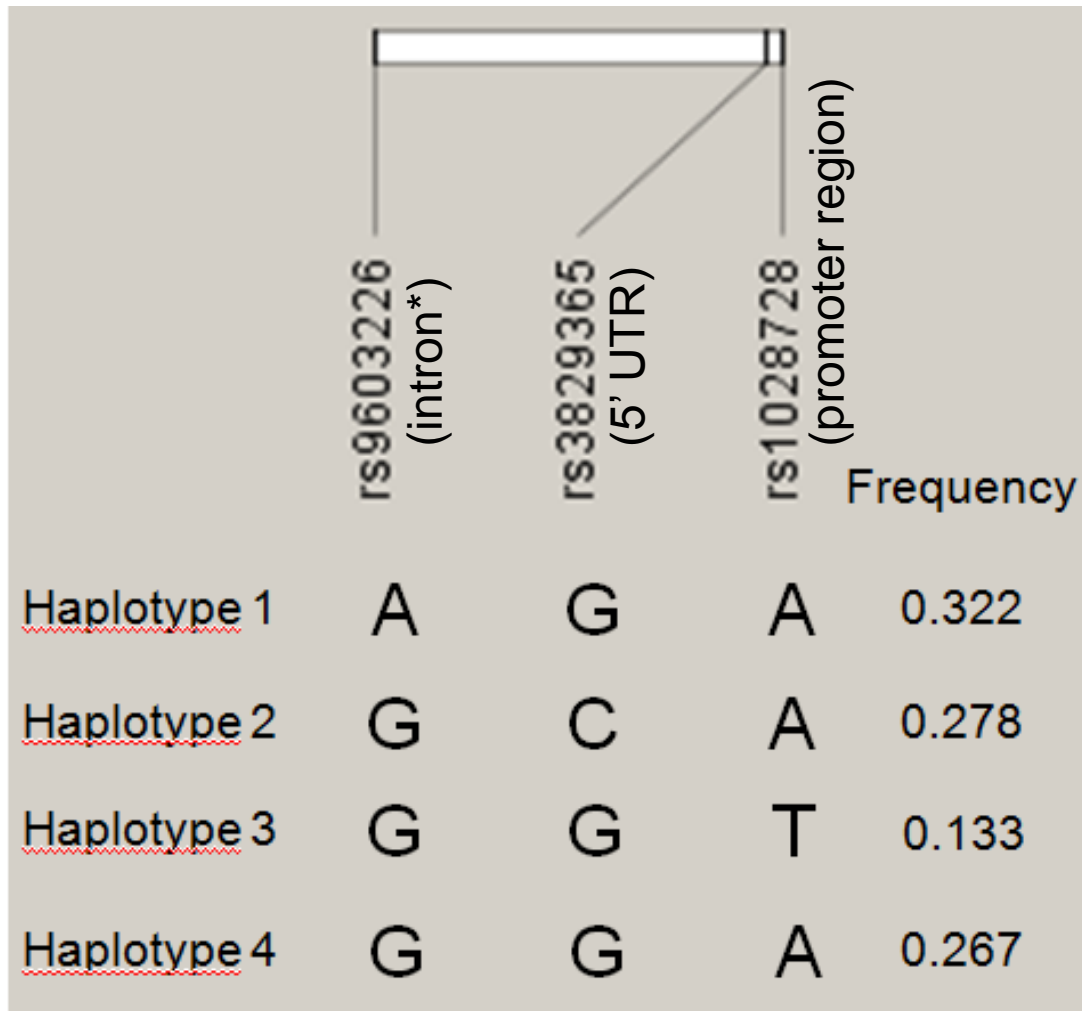


Figure 1.

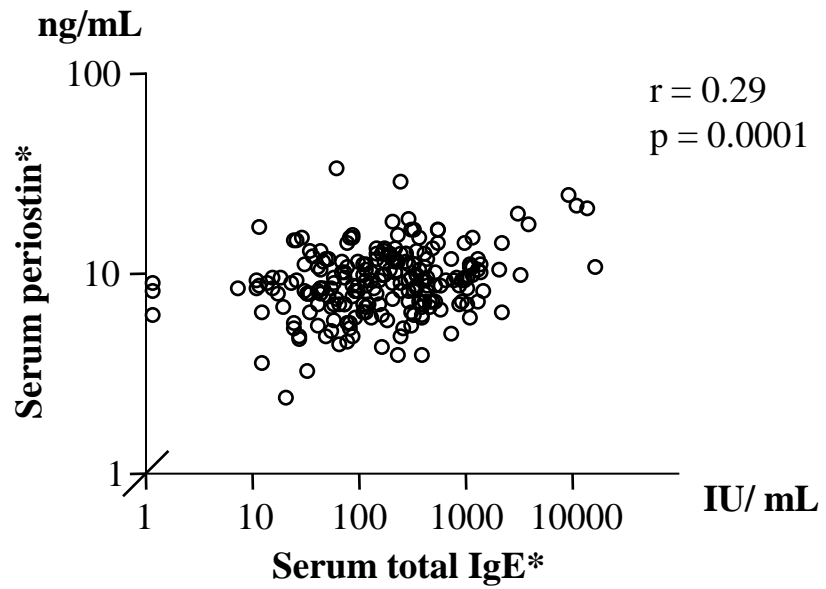
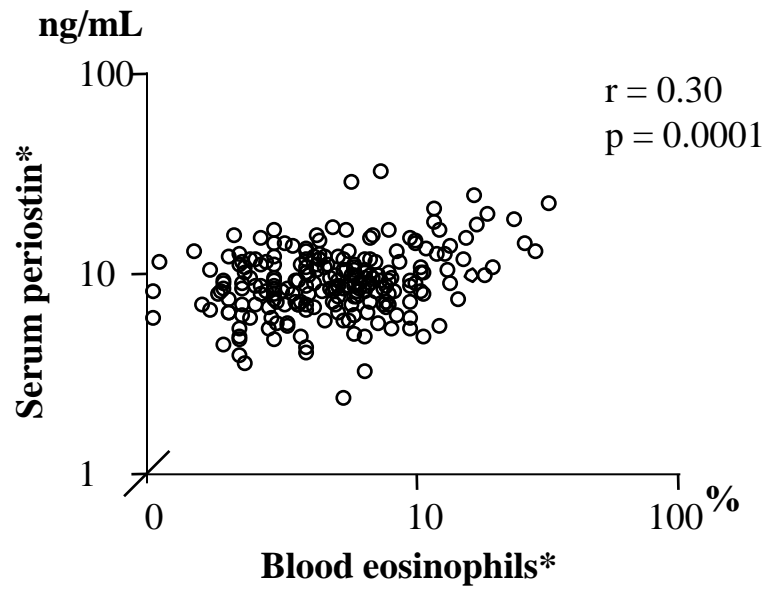


Figure 2.

1 **Online Repository**

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

4

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51

52 **Methods**

53 **Patients**

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

67

68 **Self-completed questionnaire and clinical indices**

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

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

76 Global Initiative for Asthma 2010 guideline^{E2}.

77

78 **Measurement of systemic biomarkers**

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

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

91

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

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

100 region (rs3829365), and at intron 66 bp upstream of exon 21 (rs9603226). The
101 frequencies of the minor alleles in the Japanese population were 0.136 (rs1028728),
102 0.278 (rs3829365), and 0.330 (rs9603226).

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

108

109 **References**

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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.

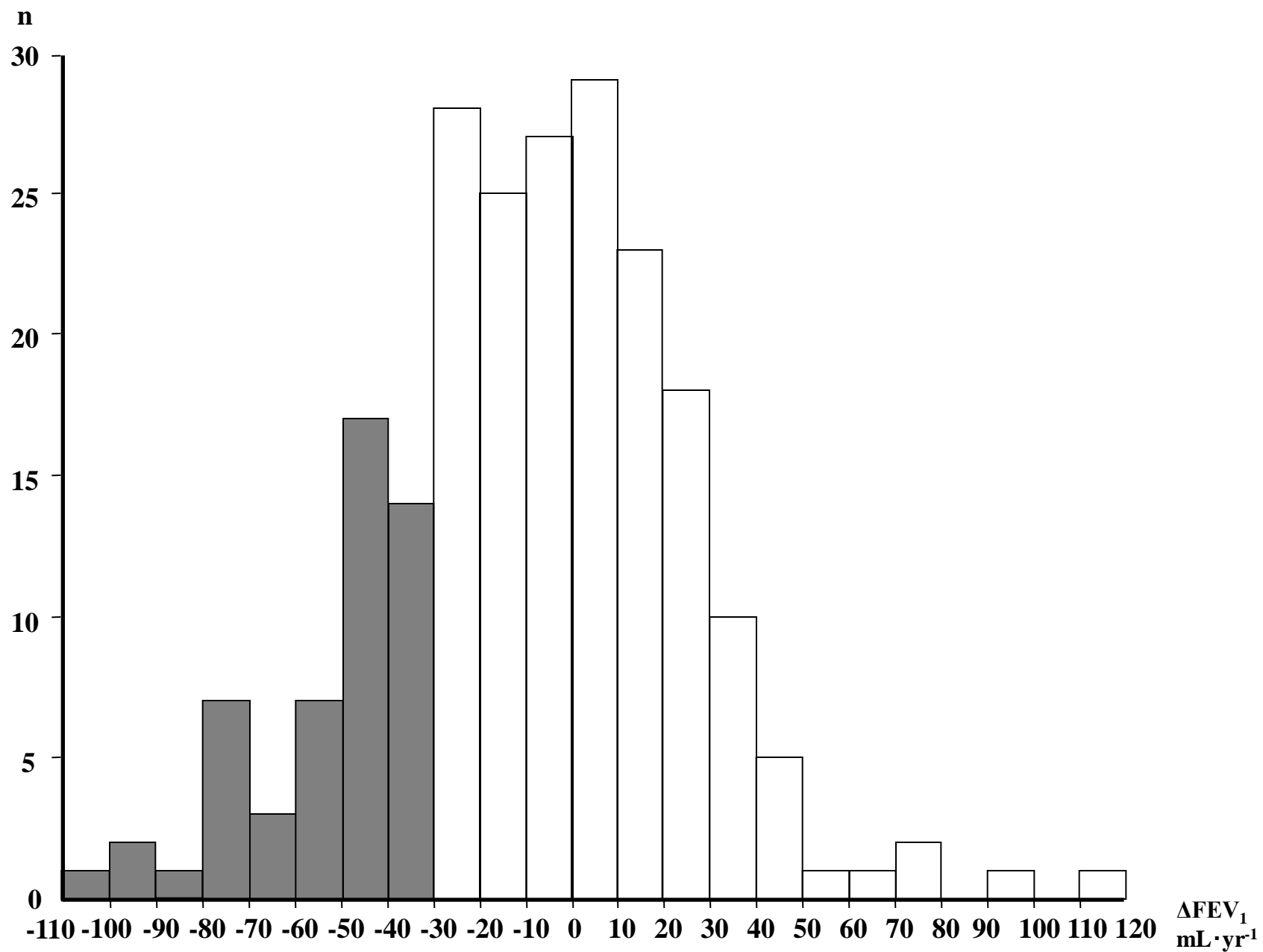


Figure E1.

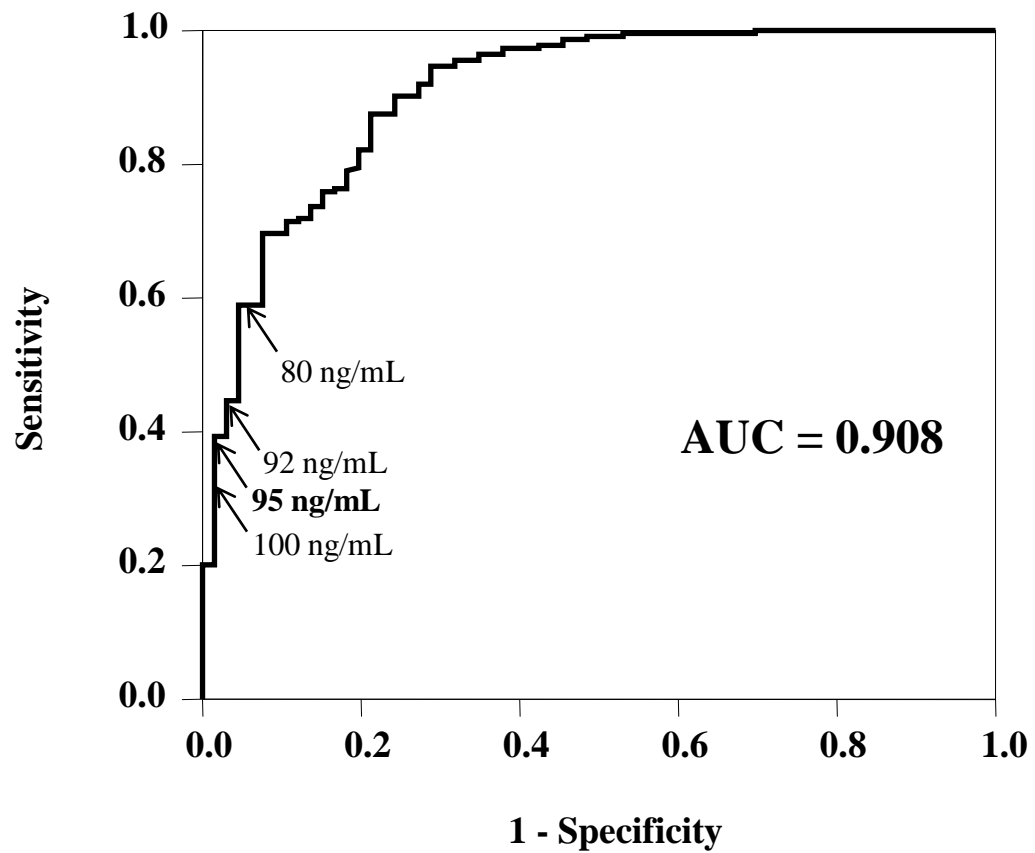


Figure E2.