



Letter

Human tissue kallikrein 9 in asthmatic patients

The number of patients with asthma is expected to increase to 100 million by 2025¹; however, there is not a single generally accepted or validated biomarker of asthma that has been implemented clinically.² Human tissue kallikreins (KLKs) were initially recognized as kinin-generating enzymes. Kinins stimulate the release of cytokines, which play an important role in the pathophysiology of asthma.³ There are many reports that indicate implication of the KLK-kinin system in the pathogenesis of asthma. With this information in mind, we designed the current study to evaluate the potential of human tissue KLK9 (hK9) as biomarker of asthma. To the best of our knowledge, there is no published scientific report regarding the expression of hK9 in the sera of asthmatic patients. However, elevated KLK-like activity has been noticed in the bronchoalveolar lavage fluid of patients with mild atopic asthma.⁴ Similarly, hK1 has been observed to be upregulated in murine and cynomolgus or nonhuman primate models of chronic asthma.⁵ None of the other member of the KLK family has been studied in asthma.

To quantify hK9 in the sera of nonasthmatic individuals and asthmatic patients, we used an anti-hK9 monoclonal antibody-based highly specific and sensitive time resolved immunofluorescence back titration enzyme-linked immunosorbent assay,⁶ which is more economical than the reported sandwich enzyme-linked immunosorbent assay method.⁷ Principle and optimization of the method have been previously discussed,⁶ and cost-effectiveness of the approach makes it more suitable for clinical implementation. Serum samples were collected from asthmatic patients who presented at the Bahawal Victoria Hospital, Bahawalpur, Pakistan, after getting due consent. Patients included in the study had either childhood history of asthma or asthma diagnosed via spirometry. With the use of a spirometer, obstruction (ratio of forced expiratory volume in 1 second to forced expiratory volume <70%) was documented and improvement in the ratio of forced expiratory volume in 1 second to forced vital capacity was noted by administering 12% or 200 mL of a bronchodilator (eg, short-acting β -agonists). Serum samples taken from nonasthmatic volunteers were used as controls. Information about lifestyle, food pattern, family history of disease, and medication was also documented (eTables 1 and 2). To document true or impartial behavior of the biomolecule being evaluated as a biomarker of asthma, patients with hepatitis B virus, hepatitis C virus, human immunodeficiency virus, or any other infectious disease were excluded from the main cohort.

All statistical analysis was performed using SPSS statistical software, version 18.0, and MedCalc statistical software, version 12.2.0.0. Extended statistical analysis that included receiver

operating characteristic (ROC) curve analysis, logistic regression analysis (both univariate and multivariate), and scatterplots helped to determine the predictive power of hK9 expression to predict the risk of developing asthma. The significance level of the area under the curve (AUC) in ROC curve analysis⁸ was helpful to evaluate the discriminating power of KLK protein expression to discriminate diseased patients from nonasthmatic individuals (eFig 1, eFig 2, and eTable 3).

A few asthmatic individuals were found to have either no expression or undetectable expression level of hK9 (Table 1). Data regarding the percentage positivity ratio (i.e., percentage of patients found positive for the expression) of hK9 in asthmatic patients' and nonasthmatic individuals' serum samples are summarized in Table 2. To determine the significance of hK9 expression level variations between nonasthmatic individuals and asthmatic patients, hK9 quantification data of all the asthma patients ($n = 74$) were analyzed taking nonasthmatic individuals

Table 1
Serum hK9 concentrations in asthmatic patients and nonasthmatic controls

Group	No. of tested samples	hK9 Concentration, $\mu\text{g/L}$			P value
		Range	Mean (SD)	Median	
Asthmatic patients					
Females	23	0.114–2.100	0.846 (0.500)	0.856	<.001 ^a
Males	25	0.064–1.425	0.630 (0.390)	0.573	<.001 ^b
HCV- or HBV-positive asthmatic patients					
Females	7	0.138–0.856	0.489 (0.303)	0.647	<.001 ^a
Males	15	0.165–1.658	0.685 (0.406)	0.623	0.06 ^c
Total asthmatic patients	70	0.064–2.100	0.699 (0.433)	0.655	<.001 ^a
Patients using medicine	32	0.114–2.100	0.782 (0.468)	0.723	<.001 ^a
Patients using no medicine	38	0.064–1.658	0.629 (0.395)	0.635	<.001 ^a
Nonasthmatic controls					
Females	48	0.748–5.668	2.983 (1.070)	2.724	.18 ^b
HCV- or HBV- positive females	5	0.531–6.385	3.072 (2.174)	2.377	
Males	51	0.102–7.958	3.108 (1.509)	2.961	
HCV- or HBV-positive males	8	0.216–4.039	2.241 (1.202)	2.337	
Total nonasthmatic controls	112	0.102–7.958	3.047 (1.310)	2.874	

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; hK9, human tissue kallikrein 9.

^aP value with respect to nonasthmatic female controls was calculated by the independent sample *t* test.

^bP value with respect to nonasthmatic male controls was calculated by the Mann-Whitney test.

^cP value with respect to HCV- and HBV-positive females was calculated by the independent sample *t* test.

^dP value with respect to HCV- and HBV-positive males was calculated by the Mann-Whitney test.

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Table 2
Age positivity ratios of the study participants

Group	Age positivity ratio, %
Nonasthmatic controls (n = 99)	
Females (n = 48)	100 (n = 48/48)
Males (n = 51)	100 (n = 51/51)
Asthmatic patients (n = 74*)	
Asthmatic females (n = 25)	92 (n = 23/25)
HBV- or HCV-positive asthmatic females (n = 8)	87 (n = 7/8)
Asthmatic males (n = 26)	96 (n = 25/26)
HBV- or HCV-positive asthmatic males (n = 15)	100 (n = 15/15)

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus.

*This sample size includes 4 asthmatic patients with human tissue kallikrein 9 levels below the detection limit.

(n = 99) as controls without any fractionation of patients and nonasthmatic controls into sex, age, or any other parameter-based subgroups. Demographic data revealed that 45.94% of the total patients included in the study were using different types of medicines, such as bronchodilators and anti-inflammatory drugs (Table 1). Serum hK9 levels in patients using medicines and patients taking no medicines were noticed to be statistically insignificant. The hK9 levels were significantly down-regulated ($P < .001$) in both the patient groups (mean [SD], 0.699 [0.433] $\mu\text{g/L}$) compared with the nonasthmatic controls (mean [SD], 3.047 [1.310] $\mu\text{g/L}$; Table 1). Thus, the serum hK9 level has the potential to be used as a biomarker for asthma.

Sex-specific controls were used for the comparison with the diseased patients. Both male and female asthmatic patients exhibited significantly down-regulated expression of hK9 ($P < .001$) compared with sex-specific nonasthmatic controls. Results of the ROC curve analysis revealed that hK9 expression level quantification well discriminates the asthmatic females (AUC = 0.974; $P < .001$) and males (AUC = 0.949; $P < .001$) from the sex-specific nonasthmatic controls (eFig 2). Bivariate analysis was performed to determine the interdependency of hK9 expression level along with the age of patient. hK9 proved to be an independent marker with significant ability ($P < .001$) for prediction of the presence of asthma and discrimination of both male and female asthmatic patients from the sex-specific nonasthmatic controls (eTables 3 and 4).

Asthmatic patients included in this study ranged in age from 11 to 70 years. Considering the level of hK9 in different age groups of male and female asthmatic patients, it became obvious that hK9 down-regulation is not particularly associated with any age group (eTable 5). In asthmatic female patients of all ages, the hK9 expression level was significantly lowered ($P < .005$ or $.05$). Regarding the effect of family history, hK9 expression down-regulation in asthmatic patients was independent of the family history of disease. Patients of both sexes had highly significant down-regulation of hK9 with either a positive ($P < .0001$) or negative family history of asthma ($P < .001$ for males and $P < .001$ for female asthmatic patients; eTable 6). In the same way, duration of the disease seems to have no effect on serum hK9 expression because hK9 was down-regulated in all asthmatic patients irrespective of the duration of disease (eTable 7).

To the best of our knowledge, this study is the first to evaluate the potential of hK9 as a biomarker of asthma, although there is some evidence that the KLK-kinin system plays an important role in inflammatory airway diseases, including asthma.⁹ In both male and female asthmatic patients, significant down-regulation of hK9 expression level was noticed compared with the sex-specific groups of nonasthmatic controls. Although data are too pre-

liminary and limited because of small samples, based on current evidence the hK9 down-regulated expression level may serve as a good diagnostic tool for the prediction of asthma. Variation in the concentration of hK9 has no association with age, and a weak negative but statistically nonsignificant correlation exists between serum concentration of hK9 and age in nonasthmatic controls and asthmatic patients (eTable 6). Both male and female asthma patients with positive and negative family histories of the disease had significantly reduced expression levels of hK9 in the serum (eTable 7). Thus, we have observed statistically significant down-regulation ($P < .001$) of hK9 in the sera of asthmatic patients compared with nonasthmatic controls; however, there is need to extend the study on the large scale to explore the role of hK9 in asthma and validate it as biomarker.

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Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jana.2015.01.008>.

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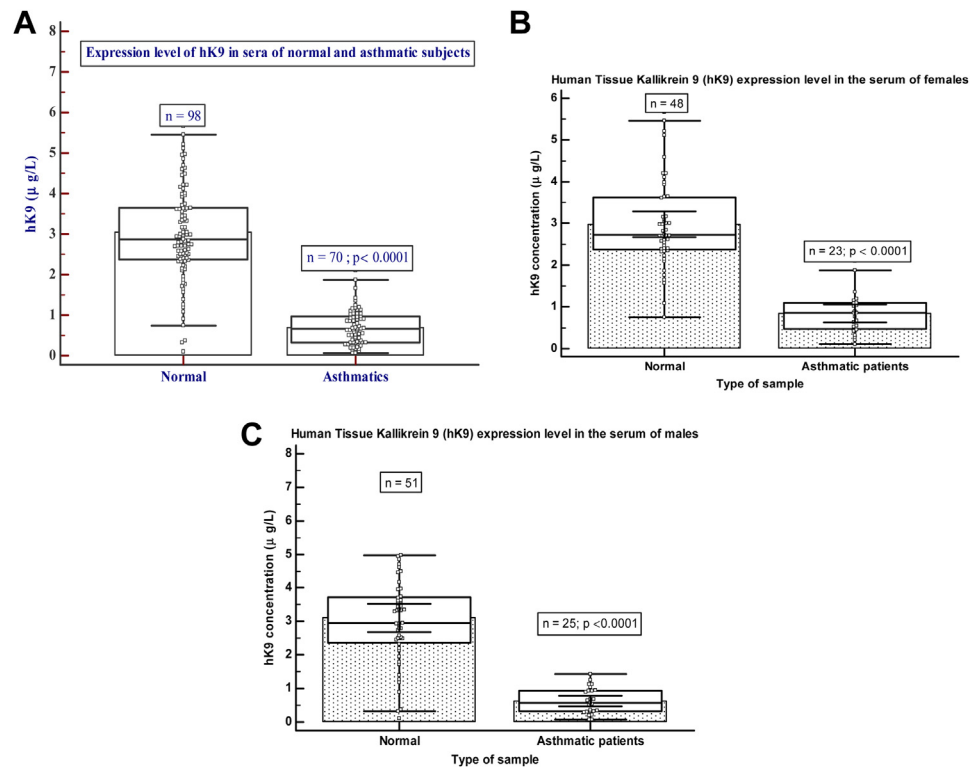
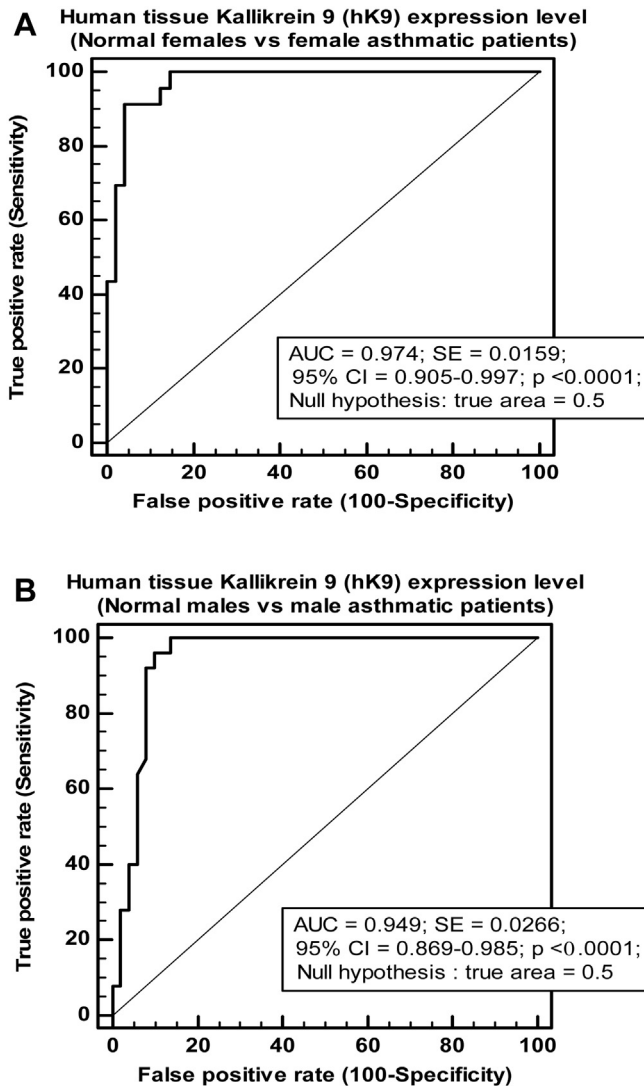


Figure 1. Comparison of human tissue kallikrein 9 (hK9) concentration in the serum of nonasthmatic and asthmatic study participants. A, Total study participants; B, female participants; C, male participants. Horizontal line indicates the range; central box, the values from the lower to upper quartile (ie, 25th to 75th percentile); middle line of the central box, median; and horizontal line inside the central box, 95% confidence interval of the mean. Dotted area indicates error bars for the mean, and the small box along the horizontal line directly corresponds to the distribution of samples' with respect to serum hK9 concentration.



eFigure 2. Receiver operating characteristic curve analysis of human tissue kallikrein 9 (hK9) quantification for discrimination between nonasthmatic and asthmatic females (A) and males (B). The area under the curve (AUC) was analyzed by Hanley and McNeil. CI indicates binomial exact confidence interval.

eTable 1

Summary of demographics, socioeconomic status, and medical history of the asthmatic patients

Parameter	Finding
Demographics	
No. of patients	74
Age, y	
Range	9–70
Mean (SD)	30.25 (16.36)
Median	26.00
Sex, %	
Male	51.0
Female	49.0
Occupation, %	
Personal business	7.80
Laborer	27.50
Housewife	39.20
Student	23.50
None	2.00
Residential status, %	
Rural	66.70
Urban	33.30
Educational status, %	
Quranic	23.50
Primary (grade 5)	19.60
Middle (grade 8)	7.80
Matric (grade 10)	7.80
Graduation (grade 14)	5.90
Illiterate	35.40
Marital status, %	
Single	43.10
Married	54.90
Widow	2.00
Marriage, %	
Interfamily	13.80
Intrafamily	86.20
Parents are cousins, %	
Yes	88.20
No	11.80
Family marriage trend, %	
Interfamily	5.80
Intrafamily	92.20
Mixed	2.00
Socioeconomic status	
Monthly income RS, %	
<5,000	80.40
5,000–10,000	17.60
>20,000	2.00
Lifestyle and habits	
Exercise, %	
Regular	7.80
Occasional	2.00
Not any	90.20
Food pattern, %	
Normal food	13.70
Vegetables	86.30
Smoking, %	
Yes	11.80
No	88.20
Passive smoking, %	
Yes	5.90
No	94.10
Drinking, %	
Yes	0.00
No	100.00
Sleep pattern	
Nighttime sleep	
<4 hours	19.60
5–8 hours	68.60
8–12 hours	11.80
Daytime sleep	
1–2 hours seldom	13.70
>2–≤4 hours seldom	3.90
Not any	82.40
Pets, %	
Yes	49.00
No	51.00
Health and medical history	

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eTable 1 (continued)

Parameter	Finding
Duration of disease, %	
Since birth	15.70
<1 year	13.70
>1–≤5 years	37.30
>5–≤10 years	23.50
>10 years	9.80
Family member with disease, %	
Yes	51.00
No	49.00
Relative with disease, %	
Yes	43.10
No	56.90
History of surgery, %	
Yes	15.70
No	84.30
Type of medication, %	
Allopathic	54.80
Allopathic and alternative	41.20
Alternative only	2.00
Not any	2.00
HBV and HCV infection status, %	
HBV positive	4.10
HCV positive	25.70
Both HBV and HCV positive	1.30
Negative	68.90

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; RS, Pakistan rupee.

eTable 2

Summary of demographics, socioeconomic status, and medical history of non-asthmatic controls

Parameter	Finding
Demographics	
No. of subjects	116
Age, y	
Range	11–68
Mean (SD)	30.42 (12.00)
Median	26.00
Sex, %	
Male	51.00
Female	49.00
Occupation, %	
Government employee	25.50
Private organization employee	2.00
Personal business	3.90
Laborer	17.60
Housewife	10.80
Student	40.20
Residential status, %	
Rural	38.20
Urban	61.80
Educational status, %	
Primary (grade 5)	8.80
Middle (grade 8)	7.80
Matric (grade 10)	12.70
Intermediate (grade 12)	14.70
Graduation (grade 14)	14.70
Postgraduation (grade 16)	22.50
Illiterate	18.60
Marital status, %	
Single	55.90
Married	41.10
Widow	1.00
Divorced	2.00
Marriage, %	
Interfamily	14.30
Intrafamily	85.70
Parents are cousins, %	
Yes	59.80
No	40.20
Family marriage trend, %	
Interfamily	36.30
Intrafamily	54.90
Mixed	8.80
Socioeconomic status	
Monthly income RS, %	
<5,000	19.60
5,000–10,000	25.50
10,000–20,000	24.50
>20,000	23.50
Not any	6.90
Lifestyle and habits	
Exercise, %	
Regular	10.80
Occasional	8.80
Twice or thrice in a week	6.80
Not any	73.50
Food pattern, %	
Normal food	42.10
Normal food and fast food	2.90
Fast food	1.00
Meat	10.80
Vegetables	41.20
Vegetables and rice	1.00
High carbohydrate	1.00
Smoking, %	
Yes	14.70
No	85.30
Passive smoking, %	
Yes	15.60
No	84.40
Drinking, %	
Yes	2.00
No	98.00

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eTable 2 (continued)

Parameter	Finding
Sleep pattern	
Nighttime sleep, %	
<4 hours	6.90
5–8 hours	63.70
8–12 hours	28.40
Not any	1.0
Daytime sleep, %	
1–2 hours seldom	32.40
>2–≤4 hours seldom	10.80
>4–≤6 hours	2.90
>6–≤8 hours	1.00
Not any	52.90
Pets, %	
Yes	13.70
No	86.30
Health and medical history	
Family history of breast cancer, %	
Yes	1.00
No	99.00
Family history of asthma and allergy, %	
Yes	12.70
No	87.30
Type of medication, %	
Allopathic	38.20
Alternative	1.00
Homeopathic	1.00
Unani	3.90
HBV and HCV infection status, %	
Yes	12.10
No	87.90

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; RS, Pakistan rupee.

eTable 3

Serum hK9 concentrations in asthmatic patients and nonasthmatic controls^a

Group	No. of tested samples	hK9 concentration, $\mu\text{g/L}$			P value
		Range	Mean (SD)	Median	
Asthmatic patients					
Females	23	0.114–2.100	0.846 (0.500)	0.856	<.001 ^b
Males	25	0.064–1.425	0.630 (0.390)	0.573	<.001 ^c
Total asthmatic patients	48	0.064–2.100	0.699 (0.433)	0.655	<.001
Nonasthmatic controls					
Females	48	0.748–5.668	2.983 (1.070)	2.724	
Males	51	0.102–7.958	3.108 (1.509)	2.961	
Total nonasthmatic controls	99	0.102–7.958	3.047 (1.310)	2.874	

Abbreviation: hK9, human tissue kallikrein 9.

^aData were evaluated at a 95% confidence interval.

^bP value with respect to nonasthmatic female controls was calculated by the independent sample *t* test.

^cP value with respect to nonasthmatic male controls was calculated by the Mann-Whitney test.

eTable 4Logistic regression analysis of hK9 expression for predicting the presence of asthma^a

Variable	Crude odds ratio	SE	95% CI	P value	Coefficient	Overall model fit P value
Females						
Univariate analysis						
hK9	0.0280	0.878	0.0050–0.1564	<.001	–3.577	<.001
Age	1.0108	0.016	0.9787–1.0439	.51	0.011	.52
Bivariate analysis						
hK9	0.0282	0.874	0.0051–0.1566	<0.0001	–3.567	<.001
Age	1.0161	0.032	0.9541–1.0822	0.6190	0.016	<.001
Males						
Univariate analysis						
Log (hK9)	0.0054	1.148	0.0006–0.0515	<0.0001	–5.216	<.001
Age	0.9910	0.023	0.9473–1.0367	0.6949	–0.009	<i>P</i> = 0.6928
Bivariate analysis						
Log (hK9)	0.0053	1.148	0.0006–0.0503	<0.0001	–5.239	<.001
Age	0.9809	0.034	0.9173–1.0489	0.5726	–0.019	<.001

Abbreviations: CI, confidence interval; hK9, human tissue kallikrein 9.

^aData were evaluated at a 95% confidence interval.**eTable 5**Variations in serum hK9 concentration in asthmatic patients with respect to sex and age^a

Group	No. of tested samples	hK9 concentration, $\mu\text{g/L}$			P value
		Range	Mean (SD)	Median	
Female asthmatic patients					
Aged 11–20 years	5	0.197–1.158	0.742 (0.416)	0.903	<.001
Aged 21–30 years	7	0.318–2.100	0.888 (0.605)	0.668	<.001
Aged 31–40 years	3	0.270–1.876	1.084 (0.803)	1.105	.01
Aged 41–50 years	3	0.114–0.856	0.498 (0.372)	0.523	<.001
Aged 51–60 years	3	0.550–1.021	0.778 (0.236)	0.764	.003
Aged 61–70 years	2	1.103–1.360	1.231 (0.182)	1.231	.02
Total female asthmatic patients	23				
Female nonasthmatic controls					
Aged 11–20 years	4	2.151–3.646	2.894 (0.623)	2.889	
Aged 21–30 years	20	1.085–5.668	3.267 (1.409)	2.900	
Aged 31–40 years	12	0.748–4.202	2.597 (0.815)	2.667	
Aged 41–50 years	7	2.092–3.993	2.735 (0.626)	2.534	
Aged 51–60 years	2	2.692–3.155	2.923 (0.327)	2.923	
Aged 61–70 years	3	2.978–4.106	3.363 (0.644)	3.005	
Total nonasthmatic female controls	48				
Total female patients	71				
Male asthmatic patients					
Aged 11–20 years	10	0.064–1.425	0.624 (0.443)	0.518	<.001
Aged 21–30 years	4	0.319–1.249	0.773 (0.410)	0.762	.004
Aged 31–40 years	9	0.065–0.935	0.554 (0.333)	0.637	.048
Aged 41–50 years	1		1.133		
Aged 51–60 years	1		0.313		
Total male asthmatic patients	25				
Male nonasthmatic controls					
Aged 11–20 years	7	2.586–4.847	3.697 (0.718)	3.635	
Aged 21–30 years	30	0.373–4.968	2.936 (1.195)	2.904	
Aged 31–40 years	7	0.102–7.348	2.832 (2.422)	2.799	
Aged 41–50 years	4	1.388–7.958	4.346 (2.735)	4.020	
Aged 51–60 years	3	1.958–2.941	2.448 (0.491)	2.446	
Total nonasthmatic male controls	51				
Total male patients	76				

Abbreviation: hK9, human tissue kallikrein 9.

^aData were evaluated at a 95% confidence interval, and *P* values were calculated with respect to nonasthmatic male controls by the Mann-Whitney test.

eTable 6Correlation of hK9 and family history of asthma^a

Group	No. of tested samples	hK9 concentration, $\mu\text{g/L}$			P value
		Range	Mean (SD)	Median	
Asthmatic patients with positive family history					
Females	16	0.114–2.100	0.864 (0.493)	0.879	<.001 ^b
Males	18	0.064–1.425	0.617 (0.420)	0.605	<.001 ^c
Total asthmatic patients	34				
Asthmatic patients with no family history					
Females	7	0.270–1.876	0.806 (0.555)	0.668	<.001 ^b
Males	7	0.279–1.133	0.666 (0.326)	0.528	<.001 ^c
Total asthmatic patients	14				
Nonasthmatic controls					
Females	48	0.748–5.668	2.983 (1.070)	2.724	
Males	51	0.102–7.958	3.108 (1.509)	2.961	
Total nonasthmatic controls	99				
Total patients	147				

Abbreviation: hK9, human tissue kallikrein 9.

^aData were evaluated at a 95% confidence interval.^bP value with respect to nonasthmatic female controls was calculated by the independent sample *t* test.^cP value with respect to nonasthmatic male controls was calculated by the Mann-Whitney test.**eTable 7**Evaluation of hK9 as prognostic marker of asthma^a

Group	No. of tested samples	hK9 concentration, $\mu\text{g/L}$			P value
		Range	Mean (SD)	Median	
Duration of disease in asthmatic females					
Since birth	1		2.100		
<1 year	3	0.450–1.103	0.740 (0.332)	0.668	<.001 ^b
1–5 years	12	0.197–1.193	0.724 (0.359)	0.742	<.001 ^b
>5–10 years	5	0.764–1.876	1.185 (0.445)	1.021	<.001 ^b
>10 years	2	0.114–0.414	0.264 (0.212)	0.264	<.001 ^b
Total asthmatic females	23				
Duration of disease in asthmatic males					
Since birth	6	0.064–1.039	0.503 (0.348)	0.518	<.001 ^c
<1 year	5	0.313–1.425	0.851 (0.517)	0.951	.002 ^c
1–5 years	4	0.329–1.133	0.732 (0.463)	0.733	.004 ^c
>5–10 years	7	0.211–0.923	0.560 (0.288)	0.573	<.001 ^c
>10 years	3	0.065–0.935	0.546 (0.442)	0.637	.009 ^c
Total asthmatic males	25				
Nonasthmatic controls					
Females	48	0.748–5.668	2.983 (1.070)	2.724	
Males	51	0.102–7.958	3.108 (1.509)	2.961	
Total nonasthmatic controls	99				
Total patients	147				

Abbreviation: hK9, human tissue kallikrein 9.

^aData were evaluated at a 95% confidence interval.^bP value with respect to nonasthmatic female controls was calculated by an independent sample *t* test.^cP value with respect to nonasthmatic male controls was calculated by the Mann-Whitney test.