Salivary SP-D may be a biomarker reflecting small airway inflammation and asthma exacerbation

Running title: Salivary SP-D in asthmatic children

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Abstract

Background: Noninvasive and child-friendly biomarkers are important tools for understanding the various phenotypes of childhood asthma.

Objective: The aim of this study was to examine the usefulness of salivary surfactant protein (SP)-D in assessing the pathophysiology of childhood asthma.

Methods: We measured salivary concentrations of SP-D and forced oscillation technique (FOT) indexes in 19 healthy controls and 21 asthmatic children. Regression equations for the predictive values of FOT indexes were generated from healthy controls. We analyzed the correlations between salivary SP-D concentration and percentages of the predictive values of FOT indexes, as well as the severity of exacerbation.

Results: We found that salivary SP-D levels were elevated in asthmatic children compared to healthy controls. In the asthmatic children, salivary SP-D levels correlated with the percentages of predicted differences in resistance between 5Hz and 20Hz (%R5-R20), representing the resistance of peripheral airways and with the severity of asthma exacerbation.

Conclusion: Salivary SP-D may reflect asthmatic inflammation in peripheral small airways, and may be a useful marker for monitoring the degree of exacerbation in childhood asthma.

Key words: Asthma, Biomarkers, Children, Forced oscillation technique, Salivary SP-D.

Resumen

Antecedentes: El empleo de biomarcadores no invasivos es una buena herramienta para estudiar la fisiopatología de los diferentes fenotipos del asma infantil.

Objetivo: El objetivo de este estudio fue examinar la utilidad de la proteína salival surfactante (SP) D en la evaluación de la fisiopatología del asma infantil.

Métodos: Se midieron las concentraciones en la saliva de SP-D y se realizaron oscilometrías forzadas de impulsos (FOT) en 21 niños asmáticos y 19 controles sanos. Las ecuaciones de regresión para los valores predictivos de los índices FOT se generaron a partir de controles sanos. Se analizaron las correlaciones entre la concentración de SP-D salival y los porcentajes de los valores predictivos de los índices FOT, así como la gravedad de las exacerbaciones.

Resultados: Se encontró que los niveles en la saliva de la SP-D fueron más elevados en los niños asmáticos en comparación con los controles sanos. En los niños asmáticos, los niveles de SP-D salival se correlacionaron con los porcentajes de las diferencias predichas en la resistencia entre 5Hz y 20Hz (% R5-R20), que representan la resistencia de las vías respiratorias periféricas y la gravedad de la exacerbación del asma.

Conclusión: La SP-D salival puede reflejar la inflamación asmática en las vías respiratorias pequeñas y puede ser un marcador útil para monitorizar el grado de exacerbación en el asma infantil.

Palabras clave: Asma, Biomarcadores, Niños, Oscilometría forzada de impulsos, Proteína salival surfactante D.

Introduction

Bronchial asthma is a common chronic inflammatory airway disease in childhood and is defined by recurrent episodes of wheezing, chest tightness, breathlessness, and coughing, associated with reversible expiratory airflow limitation [1]. Asthma is a heterogeneous disorder, encompassing different phenotypes that can be categorized by various biomarkers, such as induced sputum, exhaled nitric oxide, and lung function [2]. Owing to difficulties in the application of these biomarkers to young children [3], the heterogeneity of childhood asthma and its underlying pathophysiology remain to be clarified. Towards this end, noninvasive and child-friendly biomarkers are required.

Forced oscillation technique (FOT) is a noninvasive method for measuring airway resistance and elastance during quiet tidal breathing [4-6]. Unlike spirometry, FOT does not require active cooperation or forced respiratory maneuvers, and is thus more suitable for young children. FOT has been shown to be more sensitive than spirometry in identifying disturbances of peripheral airway function, and has been successfully used for the assessment of pulmonary function in children who cannot perform spirometry during asthma attacks [7-9]. However, various factors, including age, sex, height, and weight are known to influence FOT parameters [10-13]. Therefore, standardization of FOT indexes with reference values is essential for accurate data acquisition.

Surfactant protein D (SP-D) is a collectin family molecule which, as a component of the innate immune system, plays an important role in pulmonary host defense [14]. The C-terminal lectin head of SP-D suppresses inflammatory cell activation, whereas the N-terminal collagen tail stimulates dendritic cells and macrophages to phagocytose pathogens and apoptotic cells. SP-D also binds to several allergens, thereby modifying allergic responses in the lung [15-17]. In murine asthma models, SP-D attenuates allergen-induced eosinophilia and Th2 cytokine production, resulting in suppression of airway hyper-responsiveness and remodeling [18]. SP-D levels in bronchoalveolar lavage fluid (BALF) have been shown to correlate with the severity of asthma, airway smooth muscle mass, and reticular basement membrane thickness [19], and serum SP-D concentrations are associated with the degree of bronchial inflammation in allergic patients.

SP-D is secreted from salivary glands as well as from Type II alveolar epithelial cells, non-ciliated Clara cells, and alveolar epithelial cells [19, 20]. Of note, glandular tissues in the minor salivary glands of asthmatics exhibit airway-like inflammation [21]. We took

advantage of this fact to develop an easy and noninvasive method of sampling SP-D in the saliva of young children. Salivary levels of SP-D were then correlated with FOT indexes and clinical symptoms. We found that salivary SP-D levels correlated with peripheral airway resistance and were associated asthma exacerbation severity.

Methods

Subjects

We enrolled 21 asthmatic Japanese children and 19 healthy Japanese controls into the study, which was performed at University of Fukui Hospital between 2012-2014. Asthmatic patients were diagnosed by board certified allergists based on the following criteria: (1) more than 3 episodes of wheeze within the previous year, or (2) a hospital admission due to respiratory failure and wheeze within the previous year. Children who had a history of cardiovascular disease, perinatal abnormality, pulmonary infectious disease and/or immunodeficiency were excluded. The severity of an acute asthma attack was assessed by the acute asthma intensity research score (AAIRS) [22]. Enrolled control subjects included healthy children who had undergone preoperative evaluations and in whom asthma and other respiratory diseases had been ruled out. After written informed consent was obtained from guardians, saliva was collected with SalivaBio Children's swab (Salimetrics, Carlsbad, CA), according to the manufacturer's protocol. Briefly, one end of a SalivaBio children's swab was placed under the subject's tongue for 5 minutes. The saturated swabs were inserted into the swab storage tube and centrifuged at 1200 rpm for 7 min at 4°C. Samples (1 – 2 ml) were then stored at -80°C until salivary SP-D was measured. Saliva was collected at least two hours after the last meal. The study was approved by the review board of the University of Fukui Hospital (IRB 20120117).

Forced oscillation technique

Airway resistance at 5Hz and 20Hz (R5, R20), reactance at 5 Hz (X5), resonant frequency (Fres), and area of low reactance (ALX) were measured by Mostgraph-01 (Chest Co. Tokyo, Japan). Mostgraph-01 continuously measures respiratory parameters during whole breath, as well as during the inspiratory and expiratory phases, and detects changes in oscillation frequencies. FOT measurements were performed at the time of saliva collection, and were made in the upright position while the subject wore a nose-clip. During the measurements, the subject's cheeks were supported by the hands of investigators.

SP-D measurement

Salivary SP-D concentrations were measured by ELISA using the SP-D DuoSet (R&D Systems, Minneapolis, MN), according to the company's protocol. Briefly, 96-well

ELISA plates (AGC Techno Glass Co., Tokyo, Japan) were coated with 100 μl/well of 2 μg/ml capture antibody. After blocking with PBS/1%BSA, 50 μl of standard samples, in PBS/1%BSA, or non-diluted salivary samples were added to each well. After washing with PBS/0.05%Tween 20, the plates were incubated with 100 μl/well of 0.5 μg/ml detection antibody, and then with 100 μl/well of horseradish peroxidase-conjugated streptavidin (x 4000 in PBS/1% BSA). The plates were developed with TMB peroxidase substrate solution.

Regression equations for the predictive values of FOT indexes

Linear regression equations for the predicted values of FOT indexes were generated from data collected from the 19 healthy control children. The percentages of predicted FOT values in asthmatic patients were calculated using regression equations, with age, height and weight as independent variables.

Statistical analysis

The data are presented as median values (with the range in parentheses). Unpaired *t* tests and Wilcoxon nonparametric tests were used to compare salivary SP-D levels between asthmatic patients and healthy controls, and to analyze changes in salivary SP-D levels during asthma exacerbations. Correlations between salivary SP-D values and FOT parameters were evaluated using the nonparametric Spearman rho test. A p-value < 0.05 was considered statistically significant.

Results

Baseline characteristics of healthy controls and asthmatic children

We enrolled 19 healthy controls (male: female, 10:9) and 21 asthmatic children (male: female, 13:8) in the study. The median age was 10 years (range 5- 14 years old) in healthy controls and 8 years (range 5- 11 years old) in asthmatic patients (Table 1). Sixteen out of 21 asthmatic children were treated with controller medications, and no patients used systemic corticosteroids with regularity. Fourteen patients had mild persistent asthma, six had moderate persistent asthma, and one had severe persistent asthma, according to the Global initiative for asthma (GINA) 2016 criteria. Salivary samples were obtained during an acute asthmatic attack and during the follow-up convalescent period in 7 out of 12 patients who had had at least one asthma exacerbation during the study period.

Multivariable regression equations for predicted values of FOT indexes

Asthmatic children demonstrated higher FOT indices, with the exception of X5, than controls (Table 2). Previous studies have reported an inverse relationship between resistances and height. More recently, associations between FOT indexes and other growth-associated factors, such as weight and age, have been also demonstrated. Thus, differences in the FOT indices might be due to differences in growth-associated factors between asthmatics and controls rather than the presence or absence of asthma. Furthermore, FOT percentages appear to be more useful biomarkers than absolute FOT values.

Since growth-associated factors do not necessarily correlate with each other, we generated multiple variable regression equations to predict R5, R20, R5-R20, X5, Fres, and ALX values as dependent variables using age, height, and weight of healthy controls as independent variables (Table 3). The multivariable regression equations for the various parameters in a whole breath demonstrated higher R^2 values and coefficients of determination compared with the expiratory and inspiratory phases (data not shown). Thus, predictive values of R5, R20, R5-R20, X5, Fres, and ALX in a whole breath were used in the subsequent analysis.

Salivary SP-D levels correlate with R5-R20 in asthmatic children

SP-D levels in BALF and serum have been shown to reflect the degree of bronchial inflammation in asthmatics [14, 23]. Salivary SP-D levels in asthmatics were higher than in healthy controls ($p< 0.05$) (Figure 1). However, we observed no correlation between salivary SP-D levels and the use of leukotriene receptor antagonists (LTRA) ($p = 0.1268$) or inhaled corticosteroids (ICS) ($p = 0.2187$).

We next analyzed the correlation between salivary SP-D levels and the percentage of predicted values of FOT indexes in all 21 asthmatic children. As shown in Figure 2, salivary SP-D concentrations positively correlated with the percentage of predicted R5-R20 values ($p<0.05$). None of the other FOT indexes were significantly associated with salivary SP-D levels.

Salivary SP-D concentrations increased during acute asthma attacks

Shi et al. demonstrated that increased peripheral airway indexes of FOT, including

R5-R20 and ALX, predict asthma exacerbation [24]. We thus asked whether salivary SP-D levels were associated with the severity of an acute asthma attack, as assessed by AAIRS [22]. As shown in Figure 3A, there was no significant correlation between salivary concentrations of SP-D and AAIRS. However, in each asthmatic child, the SP-D concentrations during an acute asthma attack were higher than during the convalescent period, suggesting that individual changes in salivary SP-D levels reflect a change in asthma severity (Figure 3B). The similar changes in salivary SP-D levels associated with asthma exacerbation was observed in other patients who were not originally recruited in this study subjects (data not shown).

Discussion

We generated regression equations for the predicted values of FOT indexes in Japanese children and demonstrated that salivary SP-D levels increased during asthma exacerbation and positively correlated with %R5-R20, a peripheral airway index of FOT. Thus, salivary SP-D might serve as a non-invasive biomarker for monitoring peripheral airway function and exacerbations of childhood asthma.

Previous studies have demonstrated that height is the most significant predictor of all FOT indexes in children, whereas neither gender nor race has appeared to be an important covariate [10, 11, 13]. In this study, age had the largest absolute β value among the independent variables of the regression equations, indicating that age was the most influential predictive variable of almost all FOT indexes. The limited number of normal healthy controls in our study might explain this discrepancy. Since height and weight have also been shown to influence the regression equations in many FOT indexes, we generated regression equations using height, age, and weight, but not gender, as independent variables.

Asymmetric branching of the bronchial tree may cause discrepancies in the anatomic and acoustic characteristics of the central and peripheral airways, and lung ventilation defects are not uniformly distributed in asthmatic lungs [25], hence some reports have argued that R5-R20 merely represents the frequency-dependence of resistance or the heterogeneity of airway resistance rather than the true resistance of peripheral airways [26]. Historically, however, it has been presumed that R5, R20, and R5-R20 represent the resistances of the whole, central-large, and peripheral-small airways, respectively. In this

context, salivary SP-D levels correlated to %R5-R20 but not %R20, suggesting that salivary SP-D levels are associated with airflow limitation in peripheral airways rather than in central airways.

Serum concentrations of SP-D are used clinically as a non-invasive parameter of the permeability or integrity of the blood-airspace barrier in interstitial lung diseases such as interstitial pneumonia [27]. Serum SP-D has been shown to be elevated in allergic patients with a dual asthmatic response after allergen challenge and more pronounced eosinophilic airway inflammation [23, 28]. Increased permeability of the blood-airspace barrier caused by bronchial inflammation, especially in small airways which have a larger blood-air surface than central airways, may lead to increased serum SP-D in asthmatics [29]. Our findings suggest that elevated salivary SP-D levels in asthmatic children might also be caused by small airway inflammation.

Most large salivary molecules are produced by the salivary gland, whereas smaller molecules are transported from the blood to the saliva by passive diffusion, ultrafiltration, or active transport. SP-D is a 43kDa hydrophilic molecule, and most salivary SP-D is produced by the salivary gland. In fact, salivary SP-D levels do not merely reflect serum levels as there is only a weak correlation between serum and salivary SP-D levels ($r2 = 0.2105$, $p = 0.1822$). Nonvolatile biomarkers in alveolar fluid can be detected in exhaled breath condensate (EBC). The levels detected in EBC are about 10^{-3}-fold less than the levels measured in the alveolar fluid. If the same dilution factor is applied to SP-D, EBC might contain a few hundred pg/ml of SP-D. Since the EBC volume collected during saliva sampling is negligible compared to the volume of saliva, contamination of SP-D by EBC into salivary samples would likely be insignificant.

Wallaert et al. reported that asthmatic patients exhibited T-cell and degranulated mast cell infiltration, and increased basement thickness, in minor salivary glands and bronchial mucosa, suggesting that activated T cells may elicit airway-like inflammation in salivary glands [30]. Allergen challenge increased SP-D levels in BALF, which correlated with the degree of allergic inflammation in asthmatic lungs [31]. Animal studies in transgenic mice have shown that IL-4 and IL-13 increase SP-D expression [32]. Haczku et al. reported that allergen exposure increased SP-D protein levels in an IL-4/IL-13-dependent manner, which in turn, prevented further activation of sensitized T cells [33]. The allergic inflammatory mechanisms whereby alveolar cells release SP-D into BALF may also enhance SP-D secretion from salivary glands, resulting in increased salivary SP-D levels

in asthmatic patients.

Small airway resistance, as assessed by R5-R20, has been shown to be associated with bronchial hyperresponsiveness, as measured by excessive methacholine-induced bronchoconstriction [34]. Shi et al. reported that children with controlled asthma who have increased peripheral airway indices, such as R5-R20 values, are at risk of losing asthma control [35]. Although we found no significant correlation between salivary SP-D levels and AAIRS, which might be due to individual variation, our data suggest that changes in salivary SP-D levels may reflect the degree of asthma exacerbation in each patient. Based on its correlation with R5-R20, salivary SP-D may reflect asthma control status.

In conclusion, salivary SP-D may reflect asthmatic inflammation in peripheral small airways, and may be a useful biomarker for monitoring the degree of exacerbation in childhood asthma.

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Figure legends

Fig 1. Salivary SP-D levels in asthmatic children were higher than those in healthy controls.

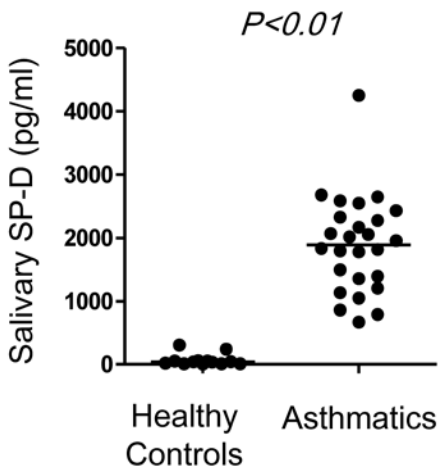

Fig 2. Correlations between salivary SP-D levels and the percentages of predicted values of FOT indexes in asthmatic children. Spearman's correlation coefficients and p-values are indicated. (n= 21)

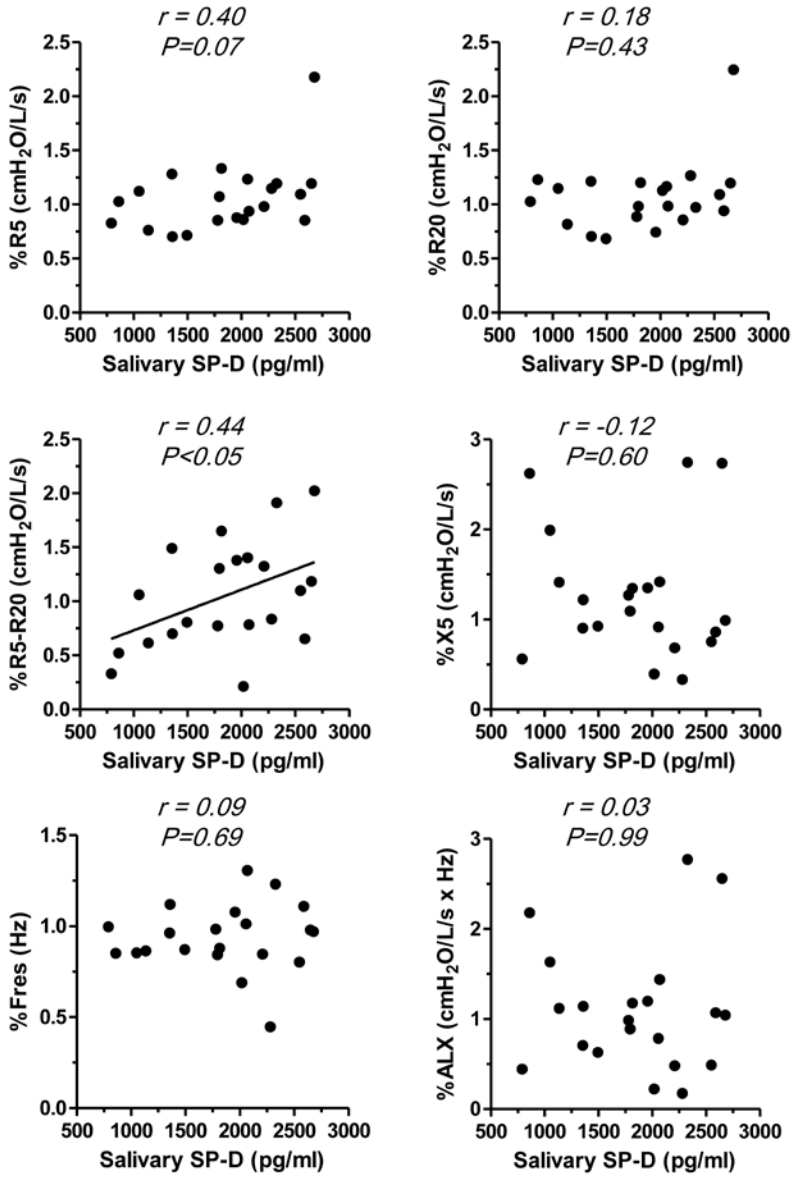

Fig 3. Salivary SP-D levels increase during asthma exacerbation. (A) Correlation between salivary SP-D levels and AAIRS. (B) Individual differences in salivary SP-D levels during convalescence and acute exacerbation in asthmatic children.

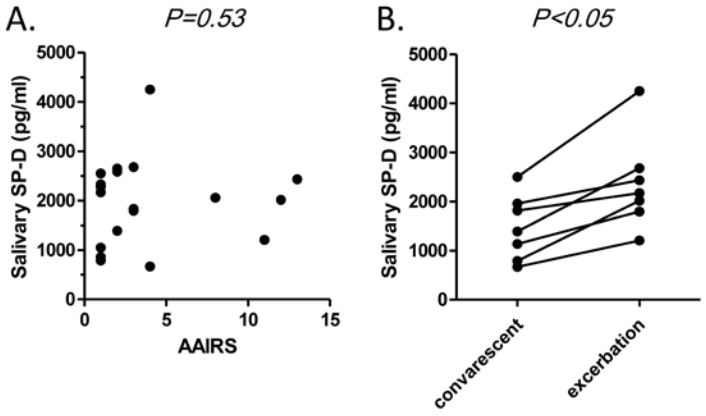

Table 1. Demographics and clinical characteristics of study subjects.

	Controls	Asthmatics	Followed-up asthmatics
N	19	21	7
Age (yrs)	10 (5-14)	8 (5-11)	7 (5-11)
M/F ratio	10:9	13:8	5:2
Weight (kg)	33.9 (14.9-76)	26.3 (17.7-48.6)	19.3 (17.1-45.8)
Height (cm)	140.1 (104.4-168)	128 (104-140.6)	116 (104-144)
Controller			
none	0	5	1
LTRA alone	0	2	0
ICS+LTRA	0	7	4
ICS/LABA	0	1	1
ICS/LABA +LTRA	0	6	1
Asthma severity (GINA) Mild/Moderate/Severe	NA	14/6/1	4/2/1
N of patients with acute asthmatic attack(s)	0	12	7
AAIRS of children with asthmatic attack	NA	2(1-13)	4 (1-13)

median (range), N; number, NA; not applicable, LTRA; leukotriene receptor antagonist, ICS; inhaled corticosteroid, LABA; long- acting beta agonist, GINA; global initiative for asthma 2016, AAIRS; acute asthma intensity research score

Table 2. Absolute values of FOT indexes in healthy controls and asthmatics.

	Controls	Asthmatics
R5 (cm H_2O/L/s)	5.04 [3.42, 6.54]	6.26 [4.61, 7.59]
R20 (cm H_2O/L/s)	3.70 [2.69, 4.84]	4.49 [3.38, 5.92]
R5-R20 (cm H_2O/L/s)	1.19 [0.49, 1.76]	1.6 [1.04, 2.47]
X5 (cm H_2O/L/s)	-1.63 [-1.87, -0.65]	-2.48 [-3.97, -1.46]
Fres (Hz)	12.49 [10.6, 15.98]	15.78 [12.42, 17.1]
ALX (cm H_2O/L/s x Hz)	8.31 [2.66, 13.22]	14.77 [7.2, 30.40]

median [quartile 25%, 75%]

Table 3. Regression equations for predictive values of FOT indexes.

		R5	R20	R5-R20	X5	Fres	ALX
Age	Coef.	-0.4	-0.3	-0.067	0.065	-0.33	-0.509
	β	-0.66	-0.9	-0.234	0.145	-0.185	-0.129
	p	0.24	0.14	0.75	0.82	0.75	0.83
Height	Coef.	-0.04	0	-0.041	0.067	-0.278	-0.672
	β	-0.47	0.01	0.7	-0.496	-1.084	0.616
	p	0.59	0.99	0.4	0.3	0.25	0.23
Weight	Coef.	0.037	0	0.035	-0.039	0.169	0.428
	β	0.345	0.02	-0.999	1.049	0.537	-1.19
	p	0.48	0.14	0.75	0.38	0.31	0.26
Intercept	Coef	13.18	6.83	6.347	-10.15	48.832	93.415
	p	0.04	0.09	0.11	0.06	0.02	0.04
Regression equations for the predicted values		R5= 13.175 -0.395xA -0.041xH +0.037xW	R20= 6.828 +0.328xA +0.001x +0.001xW	R5-R20= 6.347 -0.067xA -0.041xH +0.035xW	X5= -10.153 +0.065xA +0.067xH -0.039xW	Fres= 48.832 -0.330xA -0.278xH +0.169xW	ALX= 93.415 -0.509xA -0.672xH +0.428xW
Adjusted R2		0.61	0.61	0.31	0.49	0.56	0.53

A: Age, W: Weight, H: Height, Coef.: coefficient