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Eosinophil and neutrophil extracellular DNA traps in human allergic asthmatic airways

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Abstract

Background—Asthma is a heterogeneous inflammatory airway disorder which involves eosinophilic and non-eosinophilic phenotypes. Unlike in normal lungs, eosinophils are often present in atopic asthmatic airways although a subpopulation of asthmatic subjects predominantly develops neutrophilic inflammation. Recently, it has been demonstrated that eosinophils and neutrophils generate bactericidal extracellular traps consisting of DNA and cytotoxic granule proteins.

Objective—To explore if living eosinophils and neutrophils infiltrating human atopic asthmatic airways actively form extracellular DNA traps *in vivo*.

Methods—Quantitative analysis of eosinophils-releasing DNA was performed in endobronchial biopsies from 20 mild human atopic asthmatics at baseline and after local allergen challenge, and 10 normal subjects. DNA was stained with propidium iodine and major basic protein (MBP) with specific antibody. Differential cell counts and cytokines/chemokines were assessed in bronchoalveolar fluids.

Results—Asthmatic airways were infiltrated with a significantly higher number of eosinophils than normal airways (39.3 ± 4.6 vs. 0.4 ± 0.9 , $p < 0.0001$). All asthmatics but only one control subject expressed eosinophils releasing DNA that colocalized with MBP (33.65 ± 20.33 vs. 0.3 ± 0.9 per hpf, $p < 0.0001$). Four asthmatics mostly expressed neutrophilic inflammation and neutrophil DNA traps. Allergen challenge had no significant quantitative effect on eosinophil or neutrophil DNA traps. Airway eosinophils or DNA traps did not correlate with either BAL levels of IL-5, IFN- γ , or eotaxin, or the provoking doses of methacholine or allergen in asthmatics.

Conclusions—Extracellular DNA traps are generated by eosinophils and neutrophils in human atopic asthmatic airways *in vivo*. The mechanism and role of this new finding will necessitate further investigation.

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Clinical implications—Eosinophil extracellular traps (EETs) (1) are frequently seen in atopic asthma, (2) consist of DNA and granule proteins, and (3) might be a new useful biomarker reflecting eosinophil activation.

Keywords

Atopic asthma; BAL fluid; biomarker; eosinophils; extracellular DNA traps; human; neutrophils

INTRODUCTION

Asthma is a heterogeneous inflammatory airway disorder which involves eosinophilic and non-eosinophilic phenotypes. The latter is frequently subdivided into neutrophilic and paucigranulocytic subtypes.¹ Eosinophils are unique bone marrow-derived granulocytic leukocytes with potent cytotoxic activities and functions linked to both innate and adaptive immunity.² Though eosinophils are mainly tissue leukocytes they are rarely present in normal lungs.³ In contrast, eosinophils are commonly found in asthmatic airways and rise during allergen-induced asthma exacerbation in most atopic asthmatics.⁴ Nonetheless, some atopic asthmatics predominantly develop neutrophilic airway inflammation⁵ and react to allergen with marked airway influx of neutrophils.⁶ Patients with neutrophilic asthma usually have more severe disease and poorly respond to corticosteroids.⁷ Currently, the etiology and pathophysiologic consequences of these phenotypic discrepancies among allergic asthmatics are not well understood.

Recently, it has been reported that eosinophils primed with interleukin (IL)-5 or interferon (IFN)- γ and stimulated with bacterial lipopolysaccharide, eotaxin, or complement 5a (C5a), rapidly release mitochondrial DNA and granule proteins such as major basic protein (MBP) or eosinophilic cationic protein (ECP) to form bactericidal traps in the extracellular space,⁸ which we call in this article "eosinophil extracellular traps" (EETs). It has been also shown, that viable neutrophils primed with granulocyte/macrophage-colony stimulating factor (GM-CSF) and stimulated with TLR4 receptor agonist or C5a release mitochondrial DNA and form neutrophil extracellular traps (NETs).⁹ Active expulsion of mitochondrial DNA by viable eosinophils and neutrophils differs from the previously described microbe-killing extracellular traps generated by dead neutrophils¹⁰ and mast cells¹¹ passively releasing nuclear DNA, histones, and proteases following reactive oxygen species (ROS)-mediated cell damage.¹² Although active release of mitochondrial DNA by viable eosinophils and neutrophils is also ROS-dependent, the process does not lead to cell death.^{8,9} Formation of extracellular traps by eosinophils, neutrophils, and mast cells could be viewed as a double-sided sword providing an important mechanism of host innate immunity against infections and simultaneously causing cell and tissue injury.⁸

In the present study we tested the hypothesis that eosinophils and neutrophils infiltrating airways in human atopic asthmatics would form extracellular DNA traps *in vivo*.

METHODS

Subjects

Twenty mild allergic asthmatics by NAEPP guidelines¹³ and 10 normal volunteers were recruited from the Middle Tennessee community (Table 1). All asthmatic volunteers had positive skin tests with common aeroallergens, methacholine challenge, and inhaled allergen challenge during screening prior to enrollment into the study. Oral antihistamines and inhaled corticosteroids (one patient) were discontinued 48 h prior to procedures. Pregnant women were excluded by urine HCG testing. No volunteer had history of smoking, other concomitant lung disease or a respiratory infection in the 6 weeks preceding the study.

Volunteers consented to our protocol verbally and in writing. The protocol was approved by the Vanderbilt University Committee for the Protection of Human Subjects.

Study protocol

Twenty asthmatic volunteers underwent bronchoscopy with baseline (before allergen challenge) BAL and airway biopsies in the lingua of the left upper lobe followed by segmental allergen challenge (SAC) in a subsegment of the right middle lobe. The allergen challenged subsegment was lavaged and biopsies were obtained 24 h later. At least 3 weeks after the bronchoscopy studies asthmatics underwent methacholine challenge followed by an inhaled allergen provocation the next day. Ten normal volunteers underwent baseline bronchoscopy with collection of BAL and airway biopsies.

Allergy skin test

Prick skin testing was performed with diluent and histamine controls in parallel with a battery of standardized aeroallergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat hair, Bermuda grass, Kentucky bluegrass, fescue meadow grass, orchard grass, redtop grass, ryegrass, sweet vernal grass, timothy grass, short ragweed (Greer Laboratories, Lenoir, NC).

Bronchoscopy studies

Volunteers were fasted overnight. Midazolam 1–2 mg and fentanyl 50–100 mcg were administered intravenously if patients wished conscious sedation. Topical lidocaine (≤ 7 mg/kg) was used for airway anesthesia. BAL was performed using two 50 ml aliquots of normal saline and immediately suctioned back. Four endobronchial biopsies were taken employing disposable alligator forceps (Olympus FB-211D, Olympus, Ltd. Japan) at bifurcations of subsegmental airways, fixed in 4% paraformaldehyde and embedded in paraffin. SAC was done using two incremental doses of the allergen to which the volunteers had positive skin test, each dose in a 5 ml aliquot of normal saline. The first dose of the allergen was 10-fold higher than the concentration that gave 2+ skin reaction on intradermal test (5–8 mm wheal, 20–30 mm erythema). The subsegment was watched for 3 minutes. None of the volunteers developed visible local inflammatory reaction after the first dose of allergen. Thus, in each subject the second dose of the same allergen was instilled at concentration 100-fold higher than the 2+ skin reaction, and the bronchoscope was then removed.¹⁴ Allergen used for challenge included (number of subjects in brackets): *Dermatophagoides pteronyssinus* (4), *Dermatophagoides farinae* (2), cat hair (6), Bermuda grass (1), Kentucky bluegrass (2), timothy grass (1), ray grass (1), and short ragweed (3).

Methacholine challenge

The methacholine (Provocholine, Methapharm Inc., Brantford, Ontario, Canada) challenge was undertaken according to the American Thoracic Society guidelines¹⁵ using a Salter dosimeter (Salter Labs, Arvin, CA) and Flow Screen computerized spirometer (VIASYS Healthcare GmbH, Hoechberg, Germany).

Inhaled allergen challenge

Allergen inhalation challenge was carried out as previously described.¹⁶ Allergen aerosols were generated using a Wright nebulizer connected to a 2-way Hans Rudolph valve mouthpiece (Roxon, Montreal, Canada) attached to a wall oxygen source at 50 psi. Spirometry was performed using Flow Screen computerized spirometer.

Determination of eosinophil and neutrophil DNA traps

EETs and NETs were identified as previously described.^{8,9} Briefly, endobronchial biopsies were paraformaldehyde-fixed and paraffin-embedded. Staining was performed on 4- μ m-thick sections. DNA was stained with propidium iodide (PI). MBP and elastase were detected by indirect immunofluorescence. Polyclonal anti-MBP antibody was a kind gift of Dr. Gerald Gleich (Salt Lake City, Utah), and monoclonal anti-elastase antibody was obtained from DakoCytomation (Glostrup, Denmark). After incubation with primary antibody, tissues were incubated with the appropriate FITC-conjugated secondary antibody (Amersham Pharmacia Biotech, Durbach, Switzerland) and analyzed by confocal laser-scanning microscopy (LSM 510, Carl Zeiss Microimaging, Jena, Germany). To show the extracellular DNA in its full extension, we performed image and data processing as described previously.⁸ It should be noted that this technology allowed identification of the cellular source of the released DNA.⁸ There are currently no fluorescent dyes available that would allow distinguishing between nuclear and extracellular DNA in tissues.

Cell death assay

Cell death was assessed on 4- μ m-thick, paraformaldehyde-fixed, paraffin-embedded lung tissue sections using a TdT-mediated dUTP-biotin nick end-labeling (TUNEL) assay (Roche Diagnostics Corporation, Rotkreuz, Switzerland). Esophageal tissues from patients suffering from eosinophilic esophagitis were employed as a positive control.¹⁷ In these assays, eosinophils were labeled with a monoclonal anti-ECP antibody (EG1, Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden). Slides were analyzed by confocal microscopy.

Fluorescent *in situ* DNA analysis

DNA was assessed on 4- μ m-thick, paraformaldehyde-fixed, paraffin-embedded lung tissue sections using fluorescence-labeled probes (Cy3) for the *Atp6* and *Gapdh* genes using the Label IT labeling kit according to the instructions of the manufacturer (Mirus Bio LLC, Madison, WI).⁸ Hybridization was performed overnight at 42°C using the Label IT fluorescent *in situ* hybridization kit (Mirus).⁸ Slides were mounted by anti-fade DAPI counterstain and analyzed by confocal microscopy.

Measurement of cytokines in BAL

IL-5 and IFN- γ in BAL were measured using 10-plex MSD Multi-Spot assay (Meso Scale Discovery, Gaithersburg, MD) (IL-5, IFN- γ), and GM-CSF and eotaxin by Cytometric Bead Array (CBA) (BD Biosciences, San Jose, CA).

Statistical analysis

A two-tailed Mann Whitney test was used for comparison of asthmatics with healthy controls, Wilcoxon signed rank test for comparisons within the asthmatic group, and Spearman's Rank Correlation Coefficient for correlation analyses, employing software package SPSS (SPSS Inc, Chicago, IL). Data were expressed as means $\pm$ standard error of mean (SEM). Significance was accepted when $p < 0.05$.

RESULTS

Extracellular DNA traps generated by eosinophils is a common phenomenon in atopic asthma

Atopic asthmatic airways were infiltrated with a significantly higher number of eosinophils than normal airways (39.3 ± 4.6 vs. 0.4 ± 0.9 , $p < 0.0001$). Eosinophils were detected in all asthmatic biopsies at baseline although their numbers varied among subjects (range 3–98 eosinophils per 10 hpf, mean 39.3). Small numbers of eosinophils were found in two normal

biopsies (range 1–3 per 10 hpf, mean 0.4). All atopic asthmatics but only one normal subject expressed EETs (33.65 ± 20.33 vs. 0.3 ± 0.9 per 10 hpf, $p < 0.0001$) (Fig 1). The percentage of EETs varied among asthmatics (range 1–92, mean 33 per 10 hpf) but correlated with the total number of eosinophils on biopsies (Spearman coefficient 0.82, $p < 0.0001$). The extracellular DNA co-localized with MBP (Fig 2, A), similarly to the structures previously described in Crohn's disease and intestinal infections.⁸ It should be noted that co-localization cannot always be demonstrated in a two-dimensional-picture. However, when using a z-stack, co-localization at the same extracellular DNA can often be seen, but then on other places of the image, it can no longer be demonstrated. In addition, since it is unlikely that the EETs are pre-formed intracellularly, not all DNA and granule proteins necessarily co-localize in the extracellular space. In fact, extracellular MBP depositions were also seen not adjacent to DNA-releasing eosinophils. In analogy to the NETs, which also contained DNA and granule proteins,¹⁰ we decided to call the structures released by eosinophils "eosinophil extracellular traps (EETs)".

Neutrophil extracellular DNA traps are seen in neutrophilic asthma

Four atopic asthmatic expressed high number of neutrophils and neutrophils generating NETs that exceeded the number of eosinophils and eosinophils generating EETs (61/35 vs. 3/1, 65/47 vs. 16/12, 61/46 vs. 38/38, total neutrophils/NETs vs. total eosinophils/EETs per 10 hpf, respectively). The DNA released by neutrophils co-localized with the granule protein elastase (Fig 2, B).

Extracellular DNA traps appear to be generated by viable eosinophils

Cell death was assessed on selected asthmatic biopsies using the TUNEL technique. Eosinophils were stained with anti-ECP antibody, which also detected extracellular ECP. The morphology of the extracellular ECP structures suggested that ECP was bound to DNA and a component of EETs. In these experiments, we did not obtain any evidence that eosinophils would undergo cell death, suggesting active generation of EETs by living eosinophils (Fig 3, A). This assumption is supported by the observation that the mitochondrial ATP synthase subunit 6 (*Atp6*) gene signal was readily detectable in extracellular DNA released by tissue-infiltrating eosinophils, as assessed by fluorescent *in situ* analysis (Fig 3, B). In contrast, the glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene signal was selectively seen in nuclei (Fig 3, B). Together these data suggest that the DNA is actively released by eosinophils in asthmatic lungs and of mitochondrial but not nuclear origin.

Allergen challenge does not increase eosinophil or neutrophil extracellular DNA traps

Neither, the number of total eosinophils nor eosinophils generating EETs in the asthmatic airways significantly increased following allergen challenge (Fig 4, A). Likewise, neither the number of total neutrophils nor neutrophils generating NETs was significantly higher after allergen provocation (Fig 4, B). In contrast, allergen produced a significant increase in the BAL eosinophils and neutrophils in asthmatics (2.1 ± 0.67 vs. 18.25 ± 5.9 % BAL cells, $p < 0.0002$ and $2.5 \pm .56$ vs. 21.1 ± 4.7 % BAL cells, $p < 0.0003$, respectively). Interestingly, asthmatics with more pronounced neutrophilic airway wall inflammation developed predominantly BAL neutrophilia at 24 h after allergen challenge.

The levels of IL-5 and IFN- γ in BAL significantly increased after allergen challenge although the degree of the response markedly differed among subjects. The increase in allergen-stimulated increase in eotaxin was nearly statistically significant. On the other hand, allergen did not significantly affect generation of GM-CSF in BAL (Fig 5). No correlation was found between the total number of eosinophils or eosinophils generating EETs in biopsies and concentrations of IL-5, IFN- γ , or eotaxin in BAL fluid either before or

after allergen challenge. Likewise, the number of tissue eosinophils or eosinophils generating EETs did not correlate with methacholine PC₂₀ and allergen PC₂₀ in the atopic asthmatics (Table 2).

DISCUSSION

Our study demonstrates that EETs composed of DNA and MBP are almost universally formed in the atopic asthmatic airways. Airway biopsies from some asthmatics showed both EETs and NETs. One patient expressed predominantly extracellular NETs. The DNA-releasing eosinophils and neutrophils had no features of damage or apoptosis and appeared to release mitochondrial DNA, suggesting an active mechanism of extracellular DNA trap formation. In contrast to asthmatic subjects, a small number of EETs was found in one normal volunteer only and none of the normal biopsies expressed NETs. This is a striking finding because all asthmatic patients participating in our study had only a mild persistent or mild intermittent asthma and normal baseline airway function. The presence of EETs and NETs has not previously been described in human atopic asthmatic airways *in vivo*. Presently, however, the specificity of the newly described feature airway inflammation in atopic asthma is unknown because subjects with other inflammatory airway disorders were not involved in our study.

The mechanism of EETs and NETs formation in the atopic asthmatic airways will necessitate additional studies. It has been demonstrated that development of EETs requires priming of eosinophils with IL-5 or IFN- γ and stimulation with lipopolysaccharide, eotaxin, or C5a *in vitro*.⁸ Increased generation of IL-5 is a characteristic feature of airway inflammation in a high proportion of atopic asthmatics.¹⁸ Likewise, increased frequency of IFN- γ producing cells¹⁹ and increased expression of eotaxin²⁰ has previously been described in asthmatics compared to control subjects. In this study, we obtained evidence for increased IL-5, IFN- γ , and eotaxin levels in asthmatic BAL fluid, at least after allergen challenge. Moreover, endotoxin is a natural environmental exposure factor²¹ and C5a is elevated in blood and epithelial lining fluid in asthmatics.²² Similarly, GM-CSF which primes viable neutrophils to generate NETs in response to TLR4 or C5a receptor stimulation *in vitro*⁹ is up-regulated in asthmatic airways.²³ Hence, the airway environment generated by immune and inflammatory responses in atopic asthmatics may favor EETs and NETs formation by viable eosinophils and neutrophils *in vivo*. We found no significant correlation between the number of eosinophils, neutrophils, EET'-s or NET'-s and the levels of IL-5, IFN- γ , eotaxin or GM-CSF in BAL either at baseline or after allergen challenge in our patients. However, these results need to be interpreted with caution because BAL findings not necessarily exactly reflect the tissue environment in the asthmatic airways.²⁴ Moreover, it is likely that the extracellular DNA is rapidly destroyed by tissue nucleases. Different kinetics in the degradation of BAL cytokines and extracellular bronchial DNA could, at least partially, explain the lack of correlation between these parameters.

The physiologic and/or pathophysiologic role of EETs and NETs formation in asthmatic airways is unclear and also will need additional investigations. Asthma is associated with a loss of the structural integrity of airway epithelium and dysfunction of the physical barrier which protects airways from external harmful factors including infections.²⁵⁻²⁸ Thus EETs and NETs, which have a potent bactericidal activity,^{8,9} could serve as an important innate immunity mechanism protecting the airway wall from invasion by infectious factors. On the other hand, cytotoxic cationic proteins released by activated eosinophils may significantly contribute to the pathomechanism of airway injury² and remodeling²⁹ and adaptive immune responses involved in the pathomechanism of atopic asthma³⁰.

We found no correlation between the number of eosinophils or eosinophils generating EETs in airway biopsies and eosinophils in BAL either before or after allergen challenge. Likewise our study did not reveal correlation between the number of eosinophils or eosinophils generating EETs and nonspecific airway hyperresponsiveness to methacholine or airway sensitivity to specific allergen. Previous studies employing humanized monoclonal antibodies against IL-5, mepolizumab, demonstrated marked discrepancy between the BAL and bronchial mucosal eosinophilia in human asthmatics²³. Despite significant reduction in BAL and/or sputum eosinophilia, mepolizumab had no significant effect on airway tissue eosinophilia and airway function in asthmatic patients.³¹ Although a recent study demonstrated reduction of severe asthma exacerbations and improvement in the asthma score in patients with refractory eosinophilic asthma treated with mepolizumab, the role of mucosal eosinophilia in the pathophysiology of specific and nonspecific airway hyperresponsiveness in human asthmatics remains currently unclear^{32,33}.

In summary, in this study we describe formation of eosinophil and neutrophil extracellular DNA traps representing a novel feature of viable eosinophils and neutrophils infiltrating airway wall in human atopic asthmatics *in vivo*. The mechanism of EETs and NETs formation, the physiologic and/or pathophysiologic role of these structures in allergic asthma, as well as the influence of pharmacological drug treatment(s) will necessitate further investigation.

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Abbreviations used

ATP6	ATP synthase subunit 6
BAL	Bronchoalveolar lavage
ECP	Eosinophil cationic protein
EETs	Eosinophil extracellular traps
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HCG	Human chorionic gonadotropin
hpf	High power field
MBP	Major basic protein
NETs	Neutrophil extracellular traps
PC₂₀	Provocative concentration causing 20% fall in forced expiratory volume in 1 second
PI	Propidium iodine
TLR	Toll-like receptor

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Fig 1. Eosinophil and EETs counts per 10 hpf in airway biopsies from asthmatic and control subjects. Values are single numbers as well as mean levels $\pm$ SEM.

Fig 2.

Representative endobronchial biopsies from atopic asthmatics showing EETs and NETs. **A**, EETs generated by eosinophils contain DNA and MBP. Biopsies were from three different patients. In the left panels, infiltrating eosinophils expressing MBP are circled by dashed lines. In the middle panels, examples of extracellular DNA are indicated by arrows. In the right panels, co-localization of extracellular DNA and MBP is circled by dashed lines. **B**, NETs generated by neutrophils contain DNA and elastase. Representative results from one patient are shown. In the left panel, infiltrating neutrophils expressing elastase are circled by dashed lines. In the middle panel, examples of extracellular DNA are indicated by arrows. In the right panel, co-localization of extracellular DNA and elastase is circled by dashed lines. Bars, 10 microM. Control antibodies showed no staining (data not shown).

Fig 3.

Extracellular DNA is released from viable eosinophils. **A**, Positive TUNEL analysis of esophageal biopsy obtained from a patient with eosinophilic esophagitis (EoE, left panel), and asthmatic airway biopsy showing a negative TUNEL assay (right panel). Eosinophils and EETs were identified using anti-ECP antibody (arrows). Lower panels represent magnifications of the indicated fields within the upper panels. **B**, Fluorescent *in situ* analysis of asthmatic biopsies. Positive signals within the extracellular DNA (arrows) were seen with an *Atp6* but not with a *Gapdh* probe. The data in each panel are representative of at least three independent experiments. Bars, 10 μ M.

Fig 4.

No effect of segmental allergen challenge on numbers of EETs and NETs in airway biopsies of atopic asthmatics. **A**, Eosinophils and EETs before and 24 h after allergen challenge. **B**, Neutrophils and NETs before and 24 h after allergen challenge in asthmatics with neutrophil infiltration. Values are single numbers as well as mean levels $\pm$ SEM per 10 hpf. Changes are not significant.

Fig 5. Effect of segmental allergen challenge on cytokine/chemokine concentrations in BAL fluid of atopic asthmatics. The indicated cytokines/chemokines were measured before and 24 h after allergen challenge. Values are single numbers as well as mean levels $\pm$ SEM. P values are indicated.

Table I

Patient demographics, baseline characteristics and history of atopy, asthma, and asthma medication use

Characteristic	Mean/range
Gender, F/M	10/10
Average age (years), F/M	31.7 (23–44)/27.6 (22–29)
Race	
Caucasian	18
African American	2
Family history of atopy	20
History of allergic rhinitis	12
Frequency of daily asthma symptoms	
≤ 2 days/week	15
> 2 days/week but not daily	5
Nighttime awakenings	
≤ 2 days/week	20
> 2 days/week but not daily	0
Baseline spirometry (%predicted)	
FEV ₁ (%predicted)	90 (75–109)
FEV ₁ /FVC	0.69 (0.56–0.76)
Methacholine PC ₂₀ (mg/ml)	5.5 (0.15–10)
Medications	
H1-antihistamine	8
Albuterol ≤ 2 days/week	20
Fluticasone/Salmeterol	1
Leukotriene modifying agents	0

Table II

Relationship between percentages of EETs and NETs in asthmatic airways, provocative thresholds of methacholine and allergen, and levels of cytokines in BAL (correlation coefficient and significance, r and p, respectively)

		Methacholine PC ₂₀	Allergen PC ₂₀	IL-5	IFN- γ	Eotaxin	GM-CSF
EETs	r	-0.43	-0.22	-0.1	-0.43	-0.44	-
	p	0.054	0.34	0.107	0.06	0.11	-
NETs	r	0.56	0.4	-	-	-	-0.86
	p	0.44	0.75	-	-	-	0.33