Cysteinyl-leukotriene levels in sputum differentiate asthma from rhinitis patients with or without bronchial hyperresponsiveness.

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Running title: Cysteinyl-leukotriene levels in sputum from asthmatics

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Abstract

Background: We have previously reported that asthma differs from rhinitis with or without bronchial hyperresponsiveness in perception and degree of lower airway inflammation.

Objective: The aim of the present study was to investigate whether sputum levels of inflammatory markers could further distinguish these patient groups.

Methods: Patients with seasonal allergic rhinitis with or without asthma or bronchial hyperresponsiveness to methacholine were investigated. Induced sputum was performed during as well as off season, and analysed for cysteinyl-leukotrienes, hyaluronan, eosinophilic cationic protein and other inflammatory markers.

Results: Asthmatic patients differentiated from those with rhinitis with or without bronchial hyperresponsiveness in levels of cysteinyl-leukotrienes (geometric mean: 3,3 (lower 95% - upper 95% CI of geometric mean: 1,9-5,1) versus 1,4 (0,9-2,2) and 0,7 (0,3-1,6) pg/μg total protein) and hyaluronan (0,30 (0,22-0,43) versus 0,15 (0,10-0,20) and 0,20 (0,12-0,35) ng/μg total protein) in sputum. The levels of cysteinyl-leukotrienes decreased in sputum from the asthmatic patients, while the levels of hyaluronan remained elevated off-season. Furthermore, elevated levels of eosinophilic cationic protein were noticed among both the asthmatic and rhinitis patients with hyperresponsiveness compared to controls (0,022 (0,014-0,033) and 0,015 (0,011-0,021) compared to 0,010 (0,007-0,014) ng/μg total protein). The level of eosinophilic cationic protein remained elevated off season.

Conclusion: Cysteinyl-leukotrienes are possibly being more related to mast cell mediated inflammation and remodelling, also indicated by increased levels of hyaluronan during and off season. This inflammation may be partly different from the eosinophil driven inflammation.

Keywords: asthma, bronchial hyperresponsiveness, cysteinyl-leukotriene, eosinophilic cationic protein, hyaluronan, rhinitis, sputum

Introduction

In line with the context of asthma as a systemic airway disease, it is well known that asthma and rhinitis often co-exist. Patients with allergic rhinitis have an increased risk of developing asthma (1), and this risk seems to be independent of atopy status (2). Moreover, it is important to control inflammation in the nose in order to achieve optimal asthma control (3). Approximately 50% of patients with allergic rhinitis without any asthma symptoms from the lower airways are hyperresponsive to methacholine (4). The transition from rhinitis only to the development of clinical asthma is probably a gradual one, with bronchial hyperresponsiveness (BHR) representing an intermediate step, associated with increasing lower airway inflammation and an increased risk of future asthma development (5;6). Studies of induced sputum have indicated that rhinitis with BHR represents an intermediate stage between controls and those with rhinitis and clinical asthma, recorded as induced sputum eosinophils or eosinophilic cationic protein (ECP) (7-9). A similar pattern has been found for exhaled Nitric Oxide (FeNO), another non-invasive surrogate marker of lower airway inflammation (10).

Induced sputum is a well established method for measurement of several inflammatory markers in a wide range of inflammatory diseases. It is well known that both the level of ECP and eosinophilic cell count in sputum is higher in asthmatics than in healthy subjects (11;12), with a correlation to disease severity, at least in steroid naïve patients (13). A correlation between eosinophilic cell count and ECP are also shown in several publications (9;14). Additionally, eosinophil cell count has been used as a successful tool to guide asthma treatment adjustment (15-17)).

Cysteinyl-leukotrienes (Cys-LT: LTC_4, LTD_4 and LTE_4) are actively involved in the inflammation seen both in asthma and rhinitis. Inhalation of LTE4 has proven to be a very potent bronchoconstrictor and induces recruitment of inflammatory cell, especially

eosinophils, into the tissue (18). We have previously reported that mast cells, and not eosinophils, probably are a major source for Cys-LT production in the lower airways (19). Cys-LTs are known to be elevated in sputum from asthmatics (20;21), and have additionally been shown to be correlated to eosinophil cell count (22). Moreover, recent animal data indicates that Cys-LTs have a potential role in the process of airway remodelling (23;24). Interestingly, in patients with asthma and concomitant rhinitis, a combination of leukotriene receptor antagonist treatment and steroids provided greater efficacy in reducing airflow obstruction compared to doubling the dose of steroids (25).

Hyaluronan is a glucosaminoglycan, and as such an important part of early connective tissue repair. Elevated levels of HA is commonly seen in bronchoalveolar lavage in patients with fibrosing inflammatory conditions, an thus can be regarded as a potential marker of tissue remodelling (26;27).

The aim of the present study was to measure induced sputum Cys-LT, as well as markers of remodelling and eosinophilic inflammation in sputum from patients with rhinitis with or without BHR, comparing the results with patients with rhinitis and clinical asthma. The data was related to clinical phenotype but also to induced sputum levels of hyaluronan, a glucosaminoglycan present in connective tissue formation, possibly reflecting the degree of tissue remodelling in the lower airways (28). Finally we wished to see whether pattern of lower airway inflammation, measured by induced sputum, changed off season compared to during allergen season in the selected patient groups.

Materials and Methods

Patients

Forty-one adult patients (26 females) with allergic rhinitis (Table 1), age ranging from 18-58 (median 27), were investigated. They all had seasonal symptomatic allergic rhinitis and were investigated both once during pollen season (having symptoms from nose and eyes) as well as off season (at least one month after last reported symptom). All had a positive skin prick test with sensitization to birch, timothy and/or mugwort (ALK Abello, Copenhagen, Denmark)(29). Only those with pure seasonal allergy were investigated, i.e. those with confirmed sensitization to perennial allergens (cat, dog, horse, house dust mite or moulds) were excluded. Sixteen of the patients had concomitant clinically diagnosed mild asthma, according to Global Initiative for Asthma (GINA) standards (30). Another 4 patients were excluded because they were unable to expectorate sputum.

A control group of 13 healthy subjects, age ranging from 19-56 (median 44), who did not report a history of rhinitis or asthma, was recruited and investigated. These subjects had negative skin prick tests and were not hyperresponsive to methacholine.

All subjects were non-smokers without upper respiratory tract infection within three weeks prior to the investigation. Caffeine was withheld four hours before, and strenuous physical exercise was not allowed two hours prior to the investigation.

All patients attended the outpatient clinic of the Department of Lung medicine in Lund. All subjects gave written informed consent, and the ethical committee in Lund approved the study (LU412-03).

BHR test using methacholine

Methacholine challenge test was performed as previously described (31). A MasterScope spirometer, software version 4.5 (Erich Jaeger GmbH, Wurzburg, Germany) was used for

flow-volume spirometry. The test was carried out with tidal volume triggered equipment (Aerosol Provocation System, APS, Erich Jaeger GmbH, Wurzburg, Germany). The APS delivered a cumulative dose of 2000 μg methacholine in five increments, following an initial dose of 0.9 % NaCl. The challenge was discontinued if the FEV_1 declined more than 20 % during the protocol, and the $PD_{20}FEV_1$ was determined. When FEV_1 fell below 80 % of the baseline value or when the total amount of 2000 μg Methacholine was delivered, 400 μg of salbutamol were given to the subject. After ten minutes a new flow-volume spirometry was carried out, to ensure that the subjects were recuperating properly.

Sputum induction

Sputum was induced by inhalation of nebulised isotonic saline solution (0,9 % NaCl) for 0,5, 1, 2 and 4 min, followed by hypertonic solution (4,5 % NaCl) for 0,5, 1, 2 and 4 min. Lung function was measured 1 min after each induction time point, and induction was interrupted if lung function (PEF) was decreased ≥20%. Subjects were asked to rinse their mouth and blow their nose, and try to cough between each dose of nebulised saline. Sputum induction was continued until adequate sample volume was obtained (mean time: 7,8 min, s.d.: 4,4), and there was no difference in sputum induction time among the patient groups.

Sputum processing

Sputum plugs was sorted out, and treated with four volumes of 0,65 mM dithiothreitol (DTT) in PBS for 1 h in 4°C. Additional four volumes of PBS was added, followed by filtration through a 60 μm filter and a final centrifugation (1000 g for 5 min), which separated the supernatant from the cells. The supernatant was frozen until later analysis.

Sputum analysis

Sputum was analysed for cysteinyl-leukotrienes and LTB_4 using EIA (detection limit 13 and 6 pg/ml respectively) from Cayman Chemical (Ann Arbor, MI). Before analysis of subsequent assays, sputum was dialysed to PBS to eliminate the amount of DTT. ECP was measured using UniCap ECP kit (detection limit 0,5 ng/ml, Pharmacia Diagnostics, Uppsala Sweden), IL-8 and IL-13 using Quatikine (detection limit 3,5 and 32 pg/ml respectively, R&D systems, Abingdon, UK), hyaluronan and laminin using ELISAs (detection limit 10 ng/ml for both assays) from Echelon Biociences incorporated (Salt Lake City, UT) and Chemicon International (Temecula, CA) respectively. Total protein concentration was measured using Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). All values were adjusted to the total amount of protein in sputum (and presented as amount per μg total protein) to abolish differences due to sputum heterogeneity.

Samples were run in duplicate with a maximum in-between variation less than 5%. All tests were commercially standardized and further standardisation for the use of sputum analysis was performed.

Statistical analysis

Data is shown as geometric mean (lower 95% - upper 95% CI of geometric mean). As the data could not be assumed to have a normal distribution, nonparametric tests were used. Statistical comparison among the groups was done with Kruskal-Wallis test for unpaired samples, and Mann-Whitney's U-test for unpaired samples was used for comparison between separate groups. Wilcoxon's test was used for paired samples, and Spearman's rho test was used for correlation analysis. A p-value of less than 0,05 was considered significant, and * = $p<0,05$, ** = $p<0,01$ is used.

Results

Sixteen of the 25 rhinitis patients with no concomitant asthma were bronchial hyper-responsive to methacholine.

Cys-LT

There was a significant difference (p=0,002) in the sputum Cys-LT concentration among the groups (Fig. 1A). A minor increase in Cys-LT concentration, but not significant, could be seen from healthy controls (0,68 (0,4-1,1) pg/µg total protein) and patients with rhinitis only (0,69 (0,3-1,6) pg/µg total protein) to patients with rhinitis and BHR (1,4 (0,9-2,2) pg/µg total protein). Moreover, the Cys-LT concentration in the asthmatic group was clearly distinguished (3,3 (1,9-5,1) pg/µg total protein) from the other groups, and significantly increased (p=0,04) in comparison to the patients with rhinitis and BHR.

The Cys-LT concentration in sputum was significantly reduced from pollen season to off season in the group of patients with rhinitis and concomitant asthma (p=0,005; Fig. 2A). This could not be seen in the other patient groups (patients with rhinitis with or without BHR). There was no longer any significant difference between the groups off season.

Hyaluronan

There was a significant difference (p=0,02) in the sputum hyaluronan concentration (Fig. 1B) among the groups. The hyaluronan concentration in the asthmatic group (0,30 (0,22-0,43) ng/µg total protein) was significantly increased (p=0,008) in comparison to patients with rhinitis and BHR (0,15 (0,10-22) ng/µg total protein), as well as the other groups; healthy controls (0,13 (0,07-0,23) ng/µg total protein), patients with rhinitis only (0,20 (0,12-0,35) ng/µg total protein).

There was no significant change in hyaluronan concentration in sputum from pollen season to off season in any of the patient groups (Fig. 2B).

ECP

A similar pattern could be seen for ECP concentration, with a significant difference (p=0,007) in the sputum ECP concentration (Fig. 1C) between the groups. A trend of a minor increase in ECP concentration, but not significant, could be seen from healthy controls (0,010 (0,007-0,014) ng/µg total protein), patients with rhinitis only (0,012 (0,008-0,019) ng/µg total protein), patients with rhinitis and BHR (0,015 (0,011-0,021) ng/µg total protein) to patients with asthma (0,022 (0,014-0,033) ng/µg total protein).

Similar to hyaluronan, there was no significant change in ECP concentration in sputum from pollen season to off season in any of the patient groups (Fig. 2C).

Correlation analysis

There was a significant correlation between Cys-LT and hyaluronan concentration in sputum (p=0,03, r=0,30) during season. There is also a correlation between Cys-LT and ECP concentration (p=0,0004, r=0,47) and ECP and hyaluronan concentration (p<0,0001, r=0,51) during season. The significant correlation between Cys-LT and ECP, and ECP and hyaluronan remained off season.

Other inflammatory mediators

No differences in the concentration of LTB_4, IL-8 and laminin could be observed between the groups (Table 2).

The level of IL-13 was only detectable in a portion of the sputum samples (20 of the patients and 2 of the controls). However, IL-13 was detectable in several of the asthmatic samples, and

we have indication of an increased amount of IL-13 in asthmatics compared to the other groups (data not shown). In those asthmatic patients with detectable levels of IL-13 during season, there was a significant decrease from season to off season (p=0,03).

Discussion

In this study we found that sputum from asthmatics had higher levels of Cys-LT and hyaluronan compared to rhinitis patients with or without BHR, as well as healthy controls. Interestingly, the difference between rhinitis patients with asthma and rhinitis patients with BHR were significant for the level of Cys-LT and hyaluronan, distinguishing the groups from each other. Additionally, Cys-LT were reduced off season compared to season. We could also confirm previous observations of increased concentration of ECP in asthmatics with a trend of gradual increase in concentration levels from healthy controls, rhinitis only, rhinitis with BHR to rhinitis with asthma.

All patients were steroid naive, and in these patients eosinophilic inflammation is known to play a key role. However, other inflammatory pathways are also known to be involved. For example, while eosinophilic inflammation is known to be responsive to inhaled corticosteroids, the steroid effect on mast cell number and activation are less convincing (32). Interestingly, the major cell compartment expressing LTC_4-synthase in asthma is activated mast cells, while eosinophils contribute to a much lesser degree (33). The degree of mast cell activation as well as leukotrienes production is known to be less responsive and even in some cases unresponsive to treatment with inhaled or systemic corticosteroids (34;35). Moreover, leukotrienes as well as activated mast cells are believed to be important players in the inflammatory process leading to tissue remodelling (23;36). In this study we show that the amount of Cys-LT is reduced in sputum from rhinitis patients out off season, while ECP remains high. This might indicate that the mast cells are of importance in seasonal atopic rhinitis giving rise to the amount of Cys-LT during season, while there is a decline off season. On the contrary, eosinophils, releasing ECP, are persistent cells that remain residing in the tissue off season and are a wider feature of the asthma phenotype. There was however a strong correlation between the concentration of Cys-LT and ECP. This indicate a possible

common pathophysiological link other than coming from the same cell source. One explanation could be the eosinophil chemotactic activity by Cys-LT (37).

In addition, mast cells might be the cells distinguishing rhinitis patients with asthma in comparison to those with BHR only. In this investigation we show that the amount of Cys-LT is clearly and significantly differentiating the patients with rhinitis and asthma from those with rhinitis and BHR. The amount of ECP does not significantly differentiate the two groups from each other. ECP are on the other hand known to be affecting BHR, and might play a role in the onset of BHR in rhinitis patients. This might later lead to an asthmatic phenotype, with the addition of mast cells.

The amount of hyaluronan does also distinguish the two groups. This indicates that there is more inflammatory turnover of the connective tissue in rhinitis patients with asthma compared to BHR only, reflecting a more active remodelling process.

In the present study we found a clear correlation between Cys-LT and hyaluronan, during but not off season. This indicates that the two mediators have different time kinetics. While Cys-LT reflects active ongoing inflammation, hyaluronan is believed to reflect tissue matrix turnover, a process that is going on over time and therefore may not change as fast.

There is no apparent difference in the concentration of these sputum markers between patients with rhinitis with or without BHR, as can be seen in comparison to patients with asthma. This clearly distinguishes those with clinically diagnosed asthma from those without. However, there is a slight increase in both ECP and Cys-LT in patients with rhinitis and BHR compared to patients with rhinitis only. This might indicate an initiated inflammatory process in the airways that may later lead to the development of asthma, strengthening our hypothesis that transition from rhinitis only to the development of clinical asthma is probably a gradual one, with bronchial hyperresponsiveness (BHR) representing an intermediate step.

Processing and analyses of sputum are widely discussed, and several issues are important for analysis of mediators (38;39). Dithiothreitol, used for dispersion of the sputum, does affect several assays, and therefore we choose to dialyse our samples prior to protein analysis. In addition, a low concentration of dithiotreitol (0,65 mM) was used as dialysis was not feasible prior to leukotriene analysis due to the small size of leukotrienes. Additionally, to compensate for the lowering of the DTT concentration, the incubation time was increased to 1 h in 4°C (instead of 15-30 min in 4-37°C used in most studies). The levels of inflammatory markers were in the range of previously reported studies. However, sputum samples are very heterogeneous and it is difficult to compare samples. We therefore chose to adjust the concentration of inflammatory markers to total protein concentration to compensate for such differences.

We have found that ECP was increased in patients with rhinitis and asthma which confirms previously reported data. The novel finding was however that those patients with clinical asthma and rhinitis have evidence of Cys-LT driven inflammation and possibly airway remodelling, i.e. more increased connective tissue turnover measured as hyaluronan, distinguishing rhinitis patients with asthma from rhinitis patients with BHR. This suggests that there are two parallel inflammatory patterns where Cys-LT driven inflammation shown in this paper reflects the importance of characterising them both.

Acknowledgement

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Table 1. Demographic and functional characteristics of healthy controls (Ctrl), patients with rhinitis (R), rhinitis with BHR (R+BHR) and rhinitis with asthma (R+A) during season as well as off-season. Age, FEV1 % predicted (p), and PD20 (methacholine) values are given as median (range). Sensitization to birch, timothy and mugwort are shown as number of sensitized patients.

	Ctrl	R	R+BHR	R+A
Male/ Female	12/1	5/4	4/12	6/10
Age	44 (19-56)	27 (22-55)	31 (18-53)	27 (18-58)
FEV1 % p season	107 (80-116)	100 (83-116)	105 (88-138)	100 (62-126)
FEV1 % p off-season		102 (80-113)	103 (85-122)	104 (61-124)
PD20 season	>2000	>2000	670 (100-1884)	164 (55-1443)
PD20 off-season		>2000	931 (87->2000)	565 (33->2000)
Birch	0	5	12	14
Timothy	0	8	15	16
Mugwort	0	2	4	6

Table 2. Concentration of LTB_4, IL-8, Laminin and total protein in sputum from healthy controls (Ctrl), patients with rhinitis (R), rhinitis with BHR (R+BHR) and rhinitis with asthma (R+A) during season. All values are given as geometric mean (lower 95% – upper 95% CI of geometric mean).

	Ctrl	R	R+BHR	R+A
LTB_4 pg/ug tot prot	1,2 (0,8-1,7)	1,9 (1,0-3,5)	1,5 (1,1-2,1)	1,8 (1,0-3,2)
IL-8 pg/ug tot prot	1,1 (0,7-1,7)	0,8 (0,2-3,6)	0,9 (0,5-1,8)	1,0 (0,5-2,1)
Laminin ng/ug tot prot	0,08 (0,07-0,10)	0,10 (0,05-0,18)	0,07 (0,05-0,11)	0,06 (0,04-0,08)
Total protein ug/ml	168 (139-203)	146 (85-252)	158 (119-211)	193 (145-258)

Figure legends

Fig 1. Concentration of Cys-LT (A), hyaluronan (B) and ECP (C) in sputum from patients with rhinitis (R), rhinitis with BHR (R+BHR), rhinitis and concomitant asthma (R+A) and healthy controls (Ctrl). Data are expressed as individual scores and geometric means. K-W=Kruskal-Wallis. ns=non significant.

Fig 2. Concentration of Cys-LT (A), hyaluronan (B) and ECP (C) in sputum during pollen season (s) as well as off season (off s) in patients with seasonal rhinitis (R), rhinitis with BHR (R+BHR) and rhinitis and concomitant asthma (R+A).

Fig 1

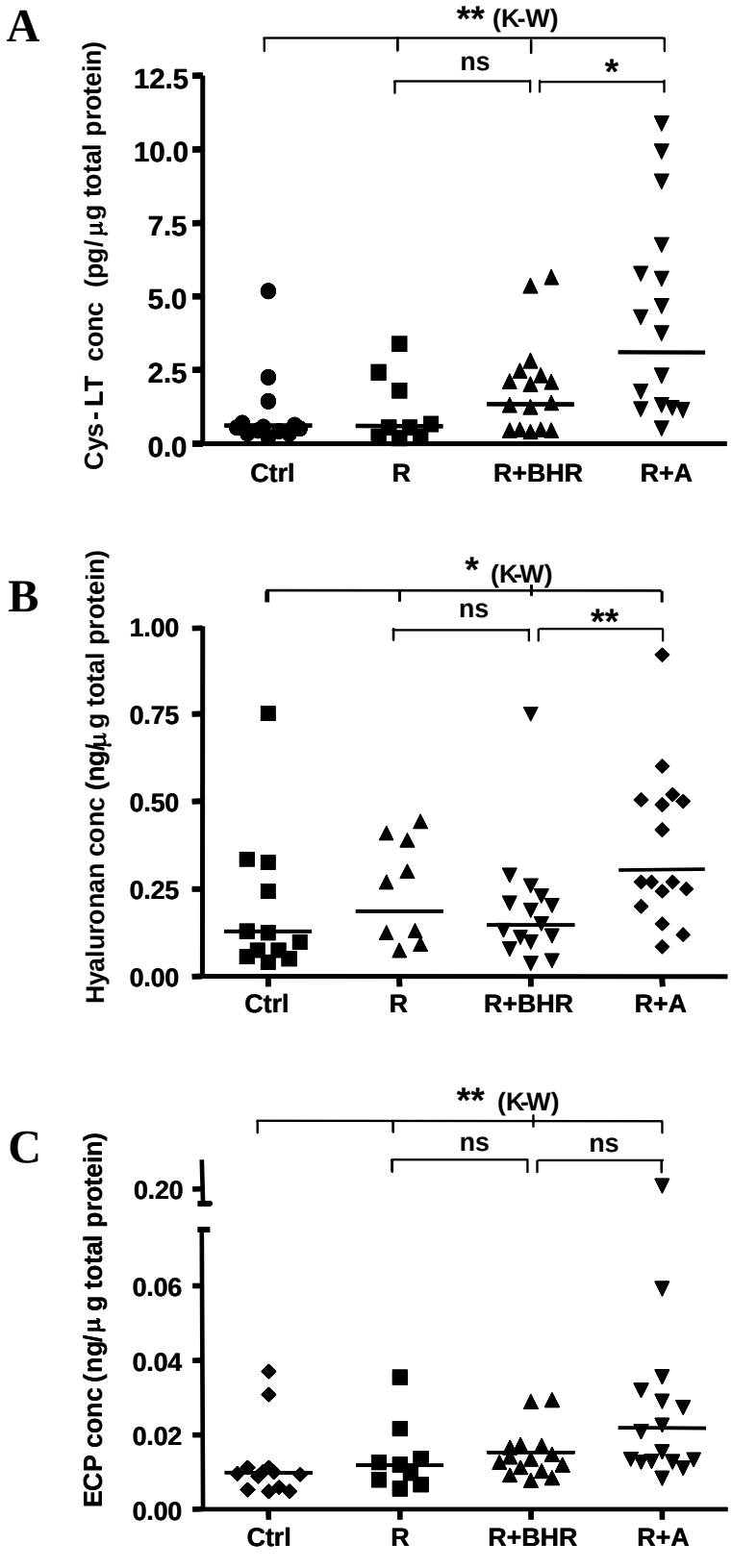
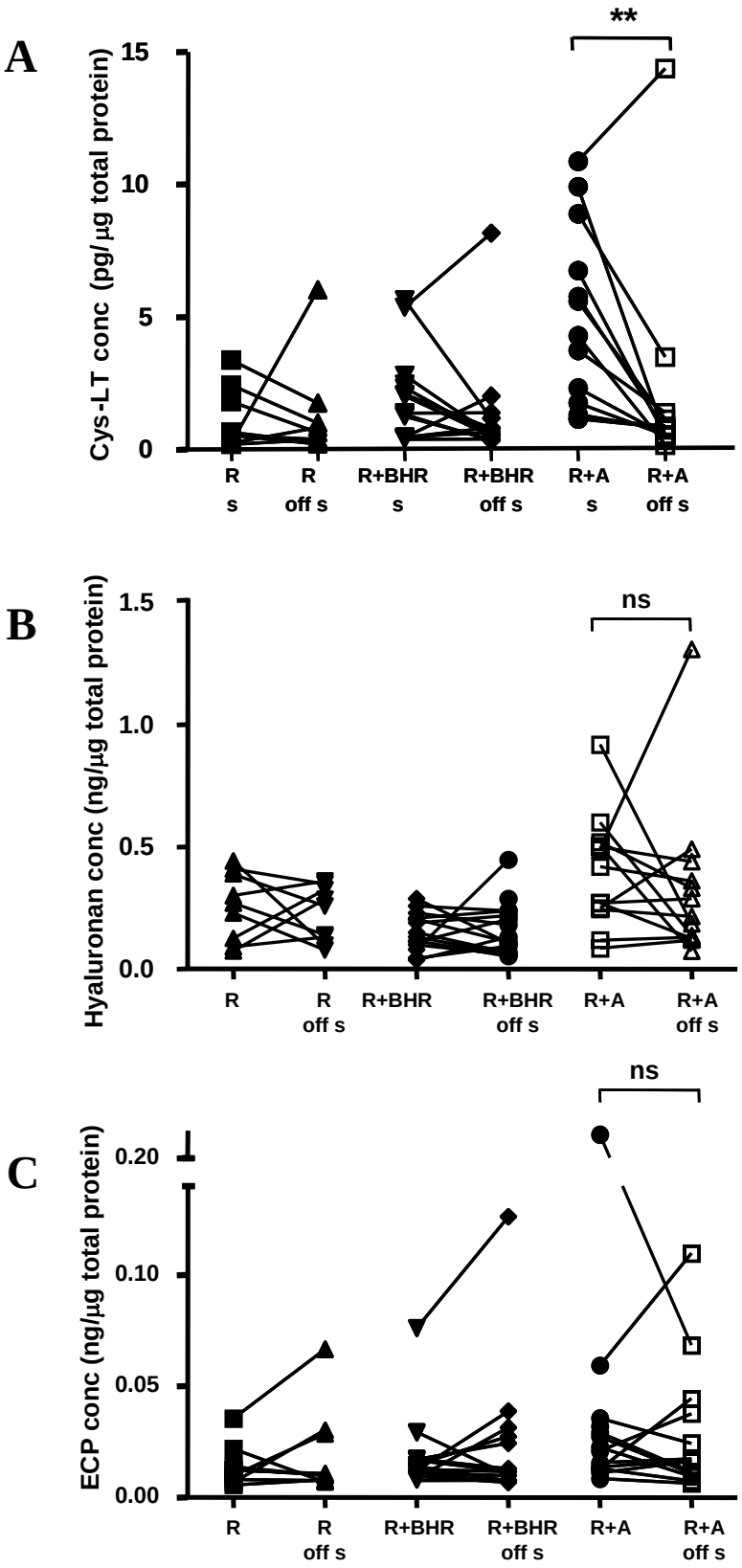

A
** (K-W)
ns
*
Cys-LT conc (pg/μg total protein)
12.5
10.0
7.5
5.0
2.5
0.0
Ctrl
R
R+BHR
R+A
B
* (K-W)
ns
**
Hyaluronan conc (ng/μg total protein)
1.00
0.75
0.50
0.25
0.00
Ctrl
R
R+BHR
R+A
C
** (K-W)
ns
ns
ECP conc (ng/μg total protein)
0.20
0.06
0.04
0.02
0.00
Ctrl
R
R+BHR
R+A

Fig 2


A
**
Cys-LT conc (pg/μg total protein)
15
10
5
0
R s
R off s
R+BHR s
R+BHR off s
R+A s
R+A off s
B
ns
Hyaluronan conc (ng/μg total protein)
1.5
1.0
0.5
0.0
R
R off s
R+BHR
R+BHR off s
R+A
R+A off s
C
ns
ECP conc (ng/μg total protein)
0.20
0.10
0.05
0.00
R
R off s
R+BHR
R+BHR off s
R+A
R+A off s