URINARY CC16 AFTER CHALLENGE WITH DRY AIR HYPERPNOEA AND MANNITOL IN RECREATIONAL SUMMER ATHLETES

Running title: Urinary CC16 after bronchial challenge in athletes

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Abstract

Airway epithelial injury is regarded as a key contributing factor to the pathogenesis of exercise-induced bronchoconstriction (EIB) in athletes. The concentration of the pneumoprotein club cell (Clara cell) CC16 in urine has been found to be a non-invasive marker for hyperpnoea-induced airway epithelial perturbation. Exercise-hyperpnoea induces mechanical, thermal and osmotic stress to the airways. We investigated whether osmotic stress alone causes airway epithelial perturbation in athletes with suspected EIB. Twenty-four recreational summer sports athletes who reported respiratory symptoms on exertion performed a standard eucapnic voluntary hyperpnoea test with dry air and a mannitol test (osmotic challenge) on separate days. Median urinary CC16 increased from 120 to 310 ρg·μmol creatinine^{-1} after dry air hyperpnoea (P = 0.002) and from 90 to 191 ρg·μmol creatinine^{-1} after mannitol (P = 0.021). There was no difference in urinary CC16 concentration between athletes who did or did not bronchoconstrict after dry air hyperpnoea or mannitol. We conclude that, in recreational summer sports athletes with respiratory symptoms, osmotic stress *per se* to the airway epithelium induces a rise in urinary excretion of CC16. This suggests that hyperosmolarity of the airway surface lining perturbs the airway epithelium in symptomatic athletes.

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Keywords: asthma, exercise-induced bronchoconstriction, airway hyper-responsiveness, Clara cell, club cell, epithelial injury

Introduction

Airway epithelium is the first barrier to the external environment and thus crucially important for the protection of the internal environment. In asthma, disruption of the epithelial barrier is regarded as one of the primary defects.[1] Through detection of the pneumoprotein club cell (Clara cell) CC16 in extra-pulmonary fluids, strenuous exercise has recently been shown to transiently compromise the integrity of the airway epithelium.[2-5]

Exercise-induced bronchoconstriction (EIB) is highly prevalent in individuals with asthma,[6] but can also be observed in otherwise healthy individuals without any other features of asthma.[7, 8] Endurance athletes are particularly at risk for EIB.[9] In this population, the mechanical stress imposed on the airways by sustained hyperpnoea combined with various environmental stimuli could create a 'chronic wound scenario', whereby repeated injury and repair of the airway epithelium leads to airway hyperresponsiveness (AHR).[10] Further, during hyperpnoea of dry air the water lost by evaporation from the airway surface is replaced by water moving from the epithelial cells, so that the cells become shrunken and hyperosmolar.[11] This change in height and osmolarity in the epithelial cell also occurs in response to a hyperosmolar challenge to the airway surface with mannitol.[12]

Eucapnic voluntary hyperpnoea (EVH) of dry air is the recommended bronchial provocation challenge for EIB detection in elite athletes.[13] In addition to mechanical stress, EVH causes both thermal and osmotic changes in the airways. Osmotic challenge with inhaled mannitol has also been used as a surrogate for exercise to identify EIB.[14, 15]

Bronchial provocation challenge with swimming, but not with mannitol has recently been associated with an increase in urinary CC16 concentration in young elite swimmers.[4] Further,

in another study,[5] serum concentration of CC16 increased after a 1500 m swimming session in well-trained young adult swimmers, but not after recreational pool attendance in children and adults. Taken together these findings suggest that the increased concentration of CC16 in extra-pulmonary fluids following strenuous exercise may be a consequence of mechanical stress on the airway epithelium rather than osmotic stress. Chlorinated by-products found in indoor swimming pools however have the capacity to disrupt the airway epithelium acutely[5] and may damage the club cells.[16] Thus there is uncertainty regarding the respective role of mechanical stress and of osmotic stress on CC16 response during exercise-hyperpnoea in non-swimmers.

The aim of our study was to investigate if urinary concentration of CC16 increased in response to an osmotic stimulus in recreational athletes not engaged in competitive swimming. To this end, urinary concentration of CC16 was measured in a group of recreational summer sport athletes with suspected EIB in two separate conditions: the first, following a 6-min EVH test, and the second, following inhalation of dry powder mannitol to cause the same degree of bronchoconstriction as EVH. Based on previous publications,[2, 4, 17] we proposed that an increase in urinary CC16 will be observed only following EVH.

Materials & methods

Study population

Twenty four recreational summer sports athletes who reported respiratory symptoms, such as cough, wheeze, breathlessness, chest tightness or mucus hyper-secretion, on exertion were recruited. Twelve of the participants had a previous physician diagnosis of asthma (two were diagnosed with childhood asthma only) and five had a previous physician diagnosis of EIB. None had had a bronchial provocation challenge done before. Five participants used inhaled corticosteroids (ICS) daily for at least 6 months (dosage: 400 to 1,000 μg beclomethasone daily or equivalent, including two on combination therapy) and another five used inhaled short-acting beta$_2$-agonists (SABA) alone. All participants trained for a minimum of 3 h *per* week and most (92%) were taking part in some form of competition (but none represented their country at international sporting events). Exclusion criteria were: baseline forced expiratory volume in the first second (FEV_1) <70% predicted, respiratory infection within the last month, current smokers, pregnant women, elite athletes and competitive swimmers. Participants refrained from caffeine− or alcohol−containing−drinks on the test days, and from exercise within 4 hours. ICS were withheld for 12 h, SABA for 8 h and long acting beta$_2$-agonists for 24 h. The study was approved by Brunel University Research Ethics Committee (RE21-08). All participants provided informed written consent.

Study design

Participants were asked to attend the laboratory between 08:00 and 09:30 h. They all completed two experimental visits separated by at least 48 hours, but less than 15 days. During the first visit, an EVH test and a skin prick test were carried out. During the second visit, a mannitol test was performed. During both visits urine samples were collected prior to and after bronchial provocation testing.

Spirometry

Maximal forced vital capacity manoeuvres were carried out at baseline on a MicroLoop spirometer (MicroMedical, Cardinal Health, Basingstoke, UK) according to international guidelines.[18] Predicted normal values were determined from established reference values.[19]

EVH test

The EVH challenge was performed on a EucapSys (SMTEC SA, Nyon, Suisse) according to standard recommendations[20] Participants were required to breathe at a target ventilation rate of 30 times baseline FEV_1 for 6 min while breathing in a dry air mixture at room temperature containing approx. 5% CO_2, 21% O_2 and balance N_2. Forced vital capacity manoeuvres were performed in duplicate at 3, 5, 10, 15, 20, 30 and 60 min after the challenge and the best FEV_1 value was recorded at each time point. A test was considered positive when a ≥10% fall in FEV_1 from baseline was documented over at least two consecutive time points. The % fall was used as index of reactivity of the airways.

Mannitol test

The mannitol test was performed according to the manufacturer's instructions (Pharmaxis Ltd, French Forest, NSW, Australia).[21] In participants negative to EVH, the mannitol challenge was stopped when a 15% decrease in FEV_1 was measured, or when a total cumulative dose of 635 mg had been administered. In those positive to EVH, the mannitol challenge was stopped when the same fall in FEV_1 was attained as during the first visit (all but one athlete had a ≥15% fall in FEV_1 post-EVH and for the athlete with a 14% fall in FEV_1 post-EVH we aimed for a 15% in FEV_1 during mannitol challenge), or when a total cumulative dose of 635 mg had been administered. A test was considered positive when the fall in FEV_1 was ≥15% from the 0 mg dose. The response was expressed as the provoking

dose of mannitol required to induce a 15% fall in FEV_1 (PD_{15}), an index of airway sensitivity. The response–dose ratio (RDR; final percentage fall FEV_1 / total dose of mannitol administered), an index of airway reactivity, was also calculated. Following completion of the test, forced vital capacity manoeuvres were performed at the same time intervals as those after challenge with EVH. The best FEV_1 value at each time point was used in the analysis. During the recovery period, FEV_1 readings were compared to baseline to calculate the % fall in FEV_1.

Urinary CC16

Participants were asked to drink 200 mL of water 1 h before arrival. At commencement of the study visits, participants emptied their bladder and provided a baseline urine sample. Post bronchial provocation testing, urine samples were collected at 30 and 60 min. After each urine collection, 200 mL of water was provided. All samples were stored without addition of preservatives at minus 80°C and were analysed within two months.[22] CC16 was measured using the Human Clara Cell Protein ELISA kit from BioVendor (Modrice, Czech Republic) according to the manufacturer's protocol. The detection limit for CC16 was 20 $\rho g \cdot ml^{-1}$. All samples were analyzed for creatinine using a COBAS 6000 System analyser (Roche Diagnostics) to correct results for dilution.

Atopic status

In eighteen athletes skin prick tests were carried out using standardized allergen extract (ALK, Abello, UK) of house dust mite, timothy grass and cat hair, together with a positive and negative control. A reaction with a wheal of ≥3 mm in diameter was considered a positive test for atopy. In the first six participants allergens extracts were not available at the time of testing.

Data analysis

Sample size requirements were calculated using the data from our previous study; [2] for an alpha of 5% and a beta of 10%, it was expected that at least 21 participants would be needed to detect a reduction of 60% in the rise in urinary CC16 after mannitol compared to after EVH. Athletes were grouped *a posteriori* as AHR^+ (for those positive to EVH and / or mannitol) or AHR^- (for those negative to both EVH and mannitol). CC16 results are presented as peak *versus* baseline, with the peak value as the highest value observed at 30 or 60 min after bronchial provocation challenge. The areas under the time curve (AUC) for CC16 and FEV_1 were calculated from the absolute and relative changes from baseline respectively, during the 60-min observation period after both challenges by using the trapezoidal method. Between-group comparisons were carried out using unpaired t-tests, Mann Whitney tests (for non-parametric variables) or Fishers' exact test (for binomial variables). Urinary CC16 data were not normally distributed. Therefore, within-group comparisons were carried out using Friedman or Wilcoxon tests. Spearman's rank correlation test was used to check for relationships between study variables. All statistical calculations were performed using SPSS 15.0 for Windows (SPSS, Inc., Chicago, IL). Values are means ± SD, unless otherwise stated. The level of significance was set at $P < 0.05$.

Results

Participant characteristics

Twelve participants trained in endurance sports, six in team sports, two in combat sports, three in trampolining / aerobics, and one in rock climbing. Anthropometric, training and baseline lung function data were similar between athletes AHR^+ and AHR^- (Table 1).

Airway response to EVH and mannitol

Eleven athletes (46%) were positive with a sustained 10% fall after EVH. One athlete had a non-sustained bronchoconstriction (maximum fall in FEV_1 of 10% at 10 min recovery only) and was classified as EVH negative. The fall in FEV_1 for the EVH positive group was 25 ± 12% (*versus* 7 ± 2% in the EVH negative group, $P < 0.001$). The ventilation achieved by athletes positive and negative to the test was similar (96 ± 15 *versus* 100 ± 16 $L \cdot min^{-1}$) and was equivalent to 78 ± 6 and 81 ± 10% pred. maximal voluntary ventilation (calculated as 35 times baseline FEV_1), respectively.

Eight athletes (33%) were positive to mannitol with an FEV_1 fall of 21 ± 13% (*versus* 4 ± 3% in negative group, $P = 0.001$), a PD_{15} of 254 ± 169 mg, and a RDR of 0.066 ± 0.035% $\cdot mg^{-1}$ (*versus* 0.005 ± 0.007% $\cdot mg^{-1}$ in the negative group, $P = 0.002$). Three athletes positive to EVH did not reach the 15% threshold after inhaling 635 mg of mannitol. A strong association was found between airway reactivity to mannitol expressed as the RDR mannitol and airway reactivity to dry air hyperpnoea expressed as % fall in FEV_1 post-EVH ($r_s = 0.738$, $P < 0.001$).

Within each study group (athletes AHR^+ and AHR^-) the maximal fall in FEV_1 during the recovery period and FEV_1-AUC were similar between visits (data not shown). Out of the fifteen athletes with current physician-diagnosed asthma and / or EIB, eight (53%) did not have AHR (two of whom were prescribed ICS). Two athletes with physician-diagnosed

childhood asthma were positive to EVH and mannitol. Out of the five athletes using ICS daily for at least 6 months, three still had demonstrable AHR. Three out of five athletes using inhaled SABA alone also still had demonstrable AHR.

Urinary CC16

Three participants AHR^- had urinary CC16 concentrations below detection limits during both tests and were excluded from the CC16 statistical analysis. Two participants AHR^+ had urinary CC16 concentrations below detection point during one visit only (one during the EVH visit and one during the placebo visit); their CC16 values during the alternative visits were kept for analysis. Urinary CC16 data at each time point and the magnitude of change post-challenges did not significantly differ between AHR^+ and AHR^- athletes. Therefore all CC16 results are presented for the study population as a whole.

Baseline urinary CC16 was similar between the two study visits (Table 2). Both bronchial provocation tests caused an increase in urinary excretion of CC16, as shown by the statistically significant difference ($P < 0.05$) between the peak CC16 concentration post-challenges and baseline values (Fig. 1.). No significant difference in the magnitude of change in urinary CC16 was observed between challenges (Table 2). However, over the time-course of recovery, urinary CC16 showed a significant increase both at 30 min ($P = 0.003$) and 60 min ($P = 0.006$) post-EVH, whilst it failed to reach significance after mannitol (Fig. 2). There was also a trend for CC16–AUC to be greater after EVH compared to mannitol ($P = 0.059$) (Table 2).

Significant correlations were found between CC16 values after EVH and mannitol: for peak CC16 r_s was 0.668 ($P = 0.002$), for delta CC16 r_s was 0.568 ($P = 0.011$) and for CC16–AUC r_s was 0.556 ($P = 0.013$). However, no significant association was found between the

maximal fall in FEV_1 post-challenges and the urinary release of CC16. Furthermore, the release of CC16 post-mannitol did not correlate with the total dose of mannitol inhaled.

Discussion

The main aim of this study was to establish whether urinary concentration of the pneumoprotein club cell CC16 was increased after challenge with an osmotic stimulus in recreational summer sports athletes with suspected EIB. Inhaled mannitol was used because, like eucapnic voluntary hyperpnoea, it has been used as a surrogate for exercise to identify EIB.[14] The concentration of urinary CC16 increased after both EVH and mannitol bronchial provocation challenges. This suggests that hyperosmolarity of the airway surface lining *per se* perturbs the functioning of the airway epithelium in symptomatic athletes.

That dry air hyperpnoea leads to an increase in urinary excretion of CC16 confirms our prior findings.[2, 17] In one study we demonstrated that breathing warm humid air during exercise reduced, but did not completely inhibit the increase in urinary CC16 post-exercise.[2] Those findings suggested that, alongside mechanical stress, the thermal and osmotic effects of evaporative water loss during hyperpnoea also play a role in perturbing the airway epithelium. We now extend these findings by reporting that the osmotic challenge mannitol *per se* induces an increase in urinary excretion of CC16. This differs from the urinary CC16 data that were previously obtained post-mannitol in swimmers.[4] In that study, elite youngster swimmers had an increase in urinary CC16 one hour after completion of a swimming test, but not after mannitol. Alongside the possible confounding effect of chlorination by-products on CC16 data,[5, 16] differences such as age of the participants,[23, 24] level of competition and urine collection times could have contributed to the divergence with our current findings.

One limitation of our study is that the population was heterogeneous in terms of medical history and pharmacological treatment. Whilst we acknowledge that asthma and EIB are two distinct entities, EIB is highly prevalent in asthmatic individuals.[6] Furthermore, all our participants with a physician diagnosis of asthma reported exercise-related respiratory symptoms, which are suggestive of EIB. The diversity of medical history ensured that our

population was representative of the athletic population that commonly consult primary care for respiratory disorders, and allowed us to draw some conclusion about the appropriateness of using mannitol to help with the diagnosis of asthma / EIB in recreational summer sports athletes. In keeping with studies performed in elite summer sports athletes (non-swimmers)[14] and in non-athletic mild asthmatics,[25] mannitol had a very high specificity (100%) to detect a positive response to EVH. The sensitivity of mannitol to identify those positive to EVH was however lower (73%), which suggests that in recreational summer sports athletes not engaged in competitive swimming mannitol may be better used to rule-in (rather than to rule-out) the presence of AHR to dry air. We cannot exclude that the long-term use of ICS may have modified club cell biology[26] and / or integrity of the airway epithelium.[27] Following removal in our statistical analysis of the five steroid-treated participants, the significant increase in urinary CC16 remained for both bronchial provocation challenges. Therefore, it is unlikely that ICS usage compromised our overall results.

In our study, the sequence of the challenges was intentional to match the severity of bronchoconstriction for EVH and mannitol, and to avoid differences in local shear deformations and pressure gradients through mucosal folding.[28] This was critical in that the airway epithelium is thought to play a prominent role in transducing mechanical stresses to nearby mesenchymal cells[29] and, therefore, in activating mediator release.[30] Unfortunately, we were not in a position to run an extra bronchial provocation challenge with methacholine, which would have helped to establish the role of airway narrowing *per se* on epithelial perturbations in athletes.

That urinary CC16 increased after both challenges suggests that, in addition to mechanical stress, an increase in osmolarity is a contributing stimulus for CC16 changes observed after EVH. There was no association between the total dose of mannitol administered and the release of urinary CC16 post-challenge, a finding that suggests that sensitivity of the

epithelium to the osmotic stimulus is more important to CC16 release than is dose. CC16 is thought to play a role in reducing inflammation of the airways.[31] Both EVH and mannitol are associated with the release of inflammatory mediators.[32-34] For this reason we believe that the increase in urinary CC16 post-challenges was due, at least partly, to an inflammatory-mediated increase in production / secretion of CC16 by the club cells. Alternatively, the post-challenge increases in urinary CC16 may be explained by an increased leakage of the protein into the bloodstream following permeability changes of the airway epithelium.[35] Changes in ventilation pattern[36] and pulmonary pressure[37] have been shown to increase airway epithelial permeability. Moreover, in conditions of airflow-related shear stress, injury and even death of epithelial cells may occur.[38] During EVH, transient airway epithelial injury could therefore have facilitated the passage of CC16 from the airways to the bloodstream[17] and contributed to the rise of CC16 in urine.

Many athletes routinely engage in sports associated with high ventilatory demands, and often do so in cold dry environments. In these conditions, small airways are likely to be exposed to inadequately conditioned air, which may favour the release of inflammatory mediators of bronchoconstriction.[39] Furthermore, as club cells are mainly localised in the distal airways,[40] CC16 is more likely to be released when small airways get dehydrated. The EVH test, which requires the use of a dry gas mixture and sets up a high target ventilatory flow for the participants, is particularly well-suited to cause dehydration to the small airways. Hyperpnoea with dry air has previously been shown to reduce mucociliary clearance in both the proximal and the peripheral airways.[41] Mannitol, however, is thought to have greater effect on the osmolarity of the proximal airways.[42] A regional difference in the dehydration stress induced in our study may therefore have contributed to a different level of stimulation of the club cells, and may explain i) the lack of significant increase in CC16 at 30 and 60 min post-mannitol and ii) the trend for a greater CC16-AUC after EVH compared to mannitol.

In elite endurance athletes the risk for asthma, AHR and EIB is significantly increased.[9] Moreover, airway remodelling (a direct marker of injury-repair) has been observed in elite cross-country skiers[43] and in elite swimmers.[24] It is therefore tempting to speculate that, similarly to the 'chronic wound scenario' proposed for the pathogenesis of asthma in the general population,[1] elite athletes expose their airways to repeated mechanical, thermal and osmotic stresses that cause disruption of the airway epithelium and lead to structural and functional changes.

In conclusion, this study showed an increase in the concentration of urinary CC16 both after EVH and mannitol bronchial provocation challenges in recreational summer sports athletes who report respiratory symptoms on exertion. It is therefore likely that hyperosmolarity of airway surface lining contributes to the increase in CC16 following dry air hyperpnoea in symptomatic athletes. This strengthens the viewpoint that osmotic changes associated with the conditioning of large volumes of air during strenuous exercise can cause damage to the airway epithelium.[10]

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Conflict of interest

Dr Sandra Anderson is the inventor of the mannitol test used here. The intellectual property is owned by her employer Sydney South West Area Health Service (SSWAHS) who has licensed the commercial rights to Pharmaxis Ltd. She receives a 10% share of the royalties paid to the SSWAHS. Dr Anderson owns shares in Pharmaxis Ltd. that she purchased on the open market and she does not hold any options. The other authors have no conflict of interest to disclose.

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Figure captions

Fig. 1. Club cell (Clara cell) protein (CC16) measured in urine before and after eucapnic voluntary hyperpnoea (a) and mannitol challenge (b) in recreational summer sports athletes with respiratory symptoms on exertion. 'Peak' are the highest values recorded over the 60-min recovery period. Individual values with medians (interquartiles).

Fig. 2. Mean ± SEM urinary excretion of club cell (Clara cell) protein (CC16) in symptomatic recreational summer sports athletes at baseline and over a 60-min period after eucapnic voluntary hyperpnoea (EVH) and mannitol challenge.

Table 1. General characteristics of the study population

	All athletes	**AHR$^+$**	**AHR$^-$**
N (males)	24 (12)	11 (5)	13 (7)
Age (yr)	28 ± 8	27 ± 7	29 ± 10
Height (cm)	172 ± 9	170 ± 9	175 ± 9
Mass (kg)	73.8 ± 12.6	74.2 ± 15.5	73.4 ± 10.1
Weekly training (h)	7 ± 3	7 ± 3	7 ± 4
Training history (yr)	10 ± 8	9 ± 8	10 ± 9
FEV_1 (L)	3.54 ± 0.57	3.54 ± 0.58	3.54 ± 0.59
FEV_1 (% predicted)	95 ± 11	98 ± 12	93 ± 10
FVC (L)	4.72 ± 0.86	4.76 ± 0.78	4.69 ± 0.95
FVC (% predicted)	108 ± 12	113 ± 14	104 ± 9
FEV_1/FVC (%)	75 ± 7	75 ± 8	76 ± 7
FEF_{25-75} ($L \cdot sec^{-1}$)	3.03 ± 0.85	3.00 ± 0.93	3.06 ± 0.81
FEF_{25-75} (% predicted)	69 ± 18	68 ± 19	69 ± 17
Physician diagnosis of asthma/EIB [N (%)]	17 (71%)	9 (82%)	8 (62%)
IBA use [N (%)]	10 (42%)	6 (55%)	4 (31%)
ICS use [N (%)]	5 (21%)	3 (27%)	2 (15)
Atopy [N^+/N total (%)]	12/18 (67%)	6/7 (86%)	6/11 (55%)

Values are means ± SD or N (%). AHR$^+$, athletes with airway hyper-responsiveness to dry air and / or mannitol; AHR$^-$, athletes without airway hyper-responsiveness to dry air and mannitol; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; FEF_{25-75}, forced expiratory flow between 25 and 75% of FVC; EIB, exercise-induced

bronchoconstriction; IBA, inhaled $beta_2$-agonists; ICS; inhaled corticosteroids; N^+, number of athletes with atopy. Between-group comparisons were not statistically significant.

Table 2. Changes in airway calibre and in urinary club cell (Clara cell) protein 16 concentration after bronchial challenge with eucapnic voluntary hyperpnoea (EVH) of dry air or mannitol in 24 symptomatic recreational summer sport athletes (11 with airway hyper-responsiveness).

	EVH	**Mannitol**
FEV_1 values		
Maximal fall of FEV_1, %	10 (7;27)	9 (6;26)
FEV_1–AUC, %·min	339 (189;616)	238 (169;507)
Urinary CC16, ρg·µmol creatinine^{-1}		
Baseline (pre-challenge)	113 (43;242)	87 (44;201)
30 min post-challenge	239 (114;573)**	146 (72;275)
60 min post-challenge	305 (80;666)**	186 (86;297)[a]
Peak post-challenge	305 (124;666)**	186 (100;342)*
Pre- to peak post-challenge (delta)	250 (39;435)	80 (4;198)
CC16–AUC, ρg·µmol creatinine^{-1}·min	6250 (1095;13211)	1860 (-308;5528)[b]

Values are medians (inter-quartile ranges); FEV_1, forced expiratory volume in the first second; AUC, area under the curve; CC16, club cell (Clara cell) protein 16. Urinary CC16 did not significantly differ between athletes with and without airway hyper-responsiveness, therefore data from both groups are presented together. * $P < 0.05$, ** $P < 0.01$, significantly different from baseline; [a] $P = 0.079$ compared to baseline; [b] $P = 0.059$, compared to EVH.

Fig1 TIFF
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Fig. 1a.

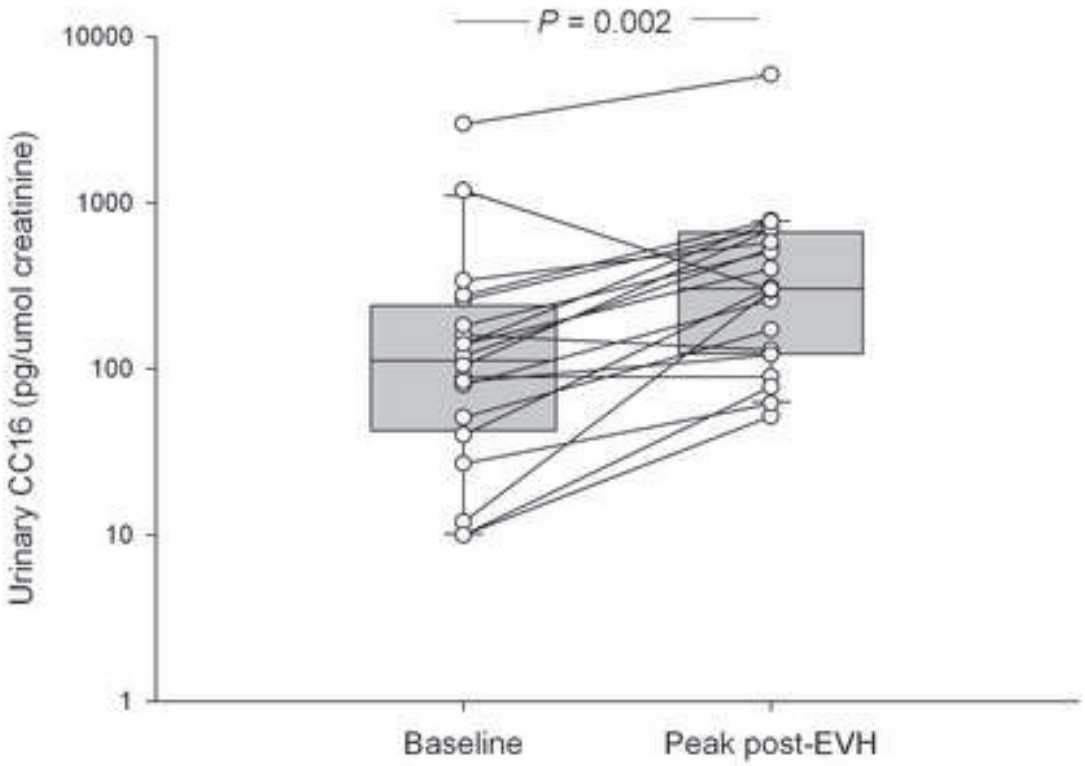

Fig. 1b.

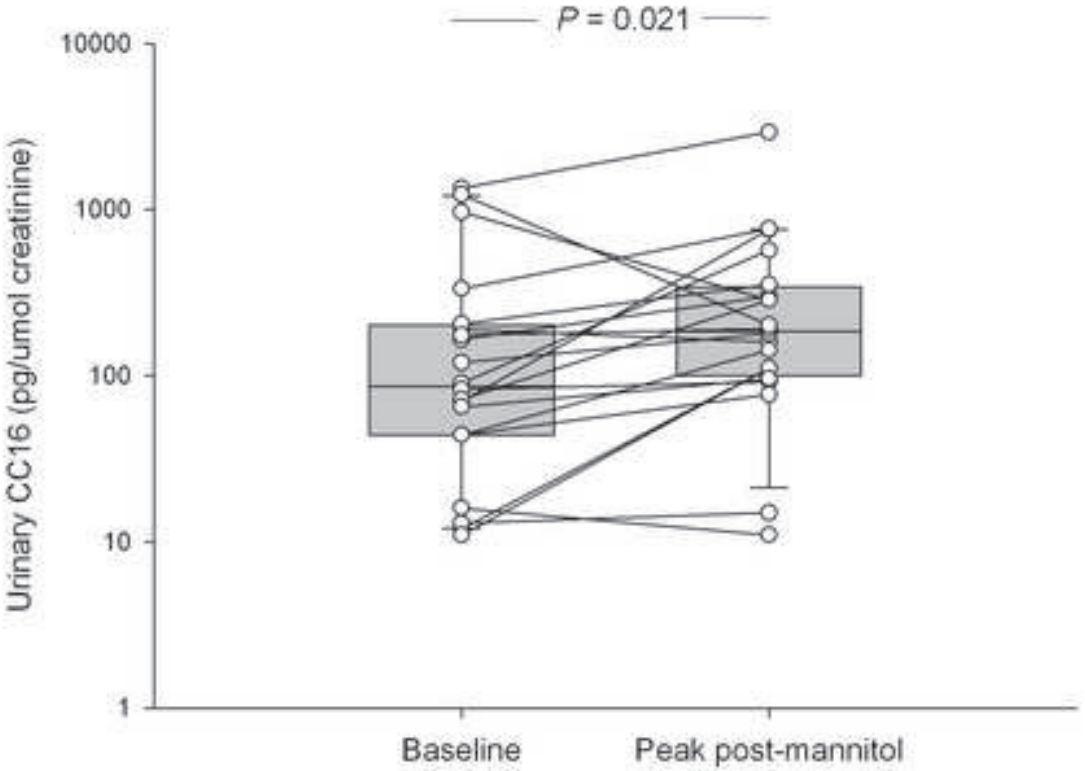

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Fig. 2