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Urinary excretion of lipid mediators in response to repeated eucapnic voluntary hyperpnea in asthmatics

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Abstract

Exercise-induced bronchoconstriction displays refractoriness manifested as a decreased response to repeated exercise challenge within hours. The refractoriness may be attenuated by inhibition of the biosynthesis of prostaglandins (PG). The aim of the study was to determine which PGs and other lipid mediators are excreted during the refractory period.

First, 16 subjects with mild stable asthma performed two repeated 4 min challenges with eucapnic volountary hyperpnea (EVH) 1 and 3 hours apart. There was a similar degree of refractoriness in both protocols (about 15% protection). The one hour interval was too short to study mediator excretion because the urinary levels did not return to baseline before the second challenge. With the 3 hour protocol, there was increased urinary excretion of cysteinyl-leukotrienes (CysLTs) and metabolites of the mast cell product PGD_2 after both challenges.

Next, another 8 subjects performed two 6 min challenges with EVH 3 hours apart which produced a greater bronchoconstrictor response than the 4 min protocol(30.0±5.4% vs 17.7±1.5%; p=0.0029) and a greater degree of refractoriness (about 30%) Analysis by UPLC-MS/MS confirmed excretion of the bronchoconstrictor CysLTs and PGD_2 during both challenges. In addition, there was increased excretion of the bronchoprotective PGE_2, and also of the main metabolite of PGI_2.

This is the first report of excretion of PGE_2 and PGI_2 during the refractory period to EVH challenge, suggesting that they may mediate the refractoriness. Maintained excretion of PGD_2 and LTE_4 following the repeat challenge argues against mast cell mediator depletion as the mechanism of refractoriness.

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Introduction

Exercise is a common trigger for an attack of asthma and a majority of asthmatic subjects experience bronchoconstriction after exercise.(4) The narrowing of the airways is thought to be caused by dehydration and a transient hyperosmolarity of the airway surface which causes release of bronchoconstricting mediators from mast cells and other cells in the airways.(2) In support of hyperosmolarity being the mechanism triggering exercise-induced bronchoconstriction (EIB), similar airway responses are seen following inhalation of mannitol,(8, 22) and eucapnic voluntary hyperpnea (EVH)(5, 32). These challenges are therefore being used as surrogates for exercise to diagnose potential for EIB.(17) The bronchoconstricting mediators released into the airways are rapidly removed by the circulation and are excreted into the urine.(20, 27) Using enzyme immuno assay (EIA) methodology, we have previously demonstrated increased urinary excretion of cysteinyl leukotrienes (CysLTs) and the prostaglandin (PG) D_2 metabolites following exercise(28), EVH(18, 19), and mannitol(22), respectively. Whereas CysLTs may be biosynthesised in many inflammatory cells, PGD_2 is almost exclusively produced by the mast cells and its release therefore provides objective evidence of mast cell activation. Previous knowledge about mediator excretion into the urine following EIB has however been restricted to those two lipid mediators.

Here we report on an extended spectrum of mediators using a newly developed platform for mass spectrometry enabling us to simultaneously study the urinary excretion of metabolites of CysLTs, PGD_2, PGE_2, $PGF_{2\alpha}$, thromboxane A_2 (TXA_2), as well as multiple isoprostane species.(6) The methodology was applied to establish the profile of lipid mediators generated in response to repeated EVH challenges. When challenge by exercise, or with EVH/mannitol, is repeated within 4 hours, a decrease in the bronchoconstrictor response is observed.(11) This decreased responsiveness to repeated challenges is called refractoriness

and its duration the refractory period. The occurrence and the degree of refractoriness decrease continuously with increasing time between the challenges.(11) The mechanisms behind this protective response remain unclear. We hypothesise that identification of endogenous molecules that mediate refractoriness may help to define new targets for treatment of airway obstruction.

We have previously observed that the subjects who were most refractory to repeated challenge with mannitol had the highest levels of CysLT and the PGD_2 metabolite 11β-Prostaglandin (PG) $F_{2\alpha}$ during the refractory period.(22) This finding suggested a decreased responsiveness to the released mediators at the level of the airway smooth muscle as one possible mechanism in refractoriness.(21, 22) Because non-steroidal anti-inflammatory drugs (NSAIDs) almost completely abolish refractoriness (26), it has been proposed that PGE_2 may be of importance for the development of refractoriness.(24, 26, 36) However, there is to date no direct evidence for release of PGE_2 *in vivo* during the refractory period.

Because the occurrence of refractoriness depends upon the time between the challenges and because the urinary excretion of mediators also has time-dependent kinetics, our study first defined the optimal conditions for this combined study of the bronchoconstrictor response and the urinary excretion of alleged mediators of bronchoconstriction and refractoriness. After the establishment of a suitable experimental design, it was possible to provide the first evidence of increased excretion of PGE_2 following EVH challenge. The study also discovered increased urinary excretion of metabolites of PGI_2 during the refractory period, possibly adding yet another potential endogenous protective factor to consider.

Materials and Methods

Study design

The urinary excretion of mediators is delayed compared to the airway response necessitating us to determine the optimal interval between challenges to detect refractoriness while still being able to study the urinary mediator excretion. Therefore, we performed an initial study (study 1) to compare two different intervals between challenges. In this first study 16 asthmatics were recruited to perform repeated 4 min EVH challenge either 1 or 3 hours apart, in a randomised cross-over design.(figure 1)

During screening the subjects underwent a physical examination, skin prick test, and spirometry. A 4 min EVH challenge was performed using a slight modification of a protocol published by Smith et al.(35) Subjects who met the inclusion criteria of a maximum fall in FEV_1 ≥10% were included in the study. On the two study days, following baseline spirometry, repeated challenge with 4 minutes of EVH (challenge I and challenge II) was performed. Urine samples were collected 30 minutes before, immediately before the start of the first challenge and then hourly until 240 minutes after the first challenge. Lung function was monitored repeatedly. In order to be able to calculate a percentage protection, data were analysed per protocol excluding the subjects who did not achieve a 10% fall in FEV_1 on the first challenge on a particular study day. For the one hour protocol, 5 of the 16 and for the 3 hours protocol 1 of 16 subjects did not achieve a fall in FEV_1 ≥10% following challenge I,

Based upon this initial range-finding study, study 2 was designed. Now, a 6 minute EVH challenge was performed and only subjects who had a maximum fall in FEV_1≥15% were included. Nine subjects met the inclusion criteria, but one subject was excluded from analysis because of asthma deteriorating between the screening visit and the study day, indicating that she did not meet the inclusion criteria of having stable mild asthma. Thus 8 subjects were eligible for further analysis. The screening day was performed in the same way as for study 1

with the exception of 6 min EVH instead of 4 min EVH. During the study day the subjects performed repeated challenge with 6 minutes of EVH 3 hours apart. Urine samples were collected 30 minutes before, immediately before the start of the first challenge and then every hour until 300 minutes after the first challenge.

Subjects

Non-smoking subjects with mild and stable asthma were eligible for participation. Asthma was defined by at least one of three criteria; response to asthma treatment, episodic wheezing and variation in lung function over short periods of time. To be included the subjects had to display baseline $FEV_1 \geq 70\%$ of predicted value. Study subjects only used asthma medications as needed and were allowed to have used short acting β_2-agonist only during the month before the study. Exclusion criteria included respiratory tract infection within the last six weeks before inclusion. Subject characteristics are presented in table 1.

All included subjects gave their written informed consent and the study was approved by the local ethics committee (Karolinska Institutet regional ethics committee Dnr 03-127, Ethics board Stockholm 2012/1277-32).

EVH

Hyperpnea with dry , room temperature air containing 5% carbon dioxide was performed through a low-resistance, one-way valve in the sitting position (Ailos Asthma Test®, Karlstad, Sweden) (5, 32). The target ventilation was 35 x FEV_1 x 0.75 (L/min) and was maintained for 4 or 6 minutes.

Lung function

Lung function (FVC and FEV_1) was measured according to the American Thoracic Society criteria, using a wedge spirometer (Vitalograph®, Buckingham, UK). FEV_1 was measured in duplicate before EVH, immediately after, at 2, 5, 10, and then every 10 minutes during 1 hour following each challenge. On each occasion the highest value of two FEV_1 measurements was registered. The fall in FEV_1 was calculated in percent of the pre-challenge value.

Skin prick test

Skin prick test was performed during screening using the following allergens; birch, timothy, mugwort, dog, cat, horse, dermatophagoides pteronyssinus, farinae, *cladosporium* and *alternaria* (Soluprick SQ, ALK, Denmark). A positive response was defined as a measurable wheal of ≥3 mm in the absence of any equivalent reaction in the control test.

Urinary mediators

After collection, urine samples were stored at -70°C until analysis. All urine samples were analysed for creatinine using the modified Jaffe colorimetric method.(22) LTE_4, 11β-$PGF_{2\alpha}$, 8-isoprostane-$PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, PGE_2, TXB_2 and tetranor-PGDM were analysed using enzyme immunoassay kits commercially available (Cayman Chemical, Ann Arbor, Michigan) as previously described.(22) In addition, the urine samples from study 2 were analysed using ultra-performance liquid chromatography triple quadrupole mass spectrometry (UPLC-MS/MS) as described by Balgoma et al.(6), with the exception that prostacyclin metabolites not were detected due to a technical error. All levels of mediators were corrected for dilution using creatinine and expressed as ng/mmol creatinine.

Statistical analysis

All data are presented as mean value ± standard error of the mean (SEM), unless otherwise stated. Statistical significance was determined using paired t-test to identify differences in the maximum fall in lung function and to compare baseline with peak mediator excretion. The Wilcoxon signed rank test was performed for data that were not normally distributed. Correlations were calculated using Pearson product moment correlation. Significance was defined as the commonly accepted level of p-value of <0.05. All statistical analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

Study 1 - Defining optimal time between challenges

Subject characteristics of the 16 subjectss included in this first part of the study are presented in Table 1. Skin prick test was positive in 13 of the subjects. There were no significant differences in pre-challenge lung function (FVC and FEV_1) or exhaled NO levels on the different study days (data not shown).

Airway response - For the one-hour protocol (n =11) the mean maximal decrease in FEV_1 was 19.6±2.4 % after EVH challenge I and 15.4±1.2 % following challenge II one hour later ($p = 0.06$). For the three-hour protocol (n = 15) the mean maximal decrease in FEV_1 was 19.5±2.1 % after challenge I and 16.3±2.5 % after challenge II three hours later ($p = 0.02$) (table 1). These results correspond, to an attenuation of 15 % when challenge II was performed after one hour, and 19 % attenuation when challenge II was done three hours after challenge I. No correlation was found between % fall following challenge I and the protection afforded for neither of the 1 hour nor the 3 hour study day. Comparing the degree of protection between the 1 hour and 3 hour protocols for the 11 subjects who had data from both study days we found no differences; the mean protection was 15±9% and 15±8% respectively.

Urinary CysLT and PGD_2 excretion - with the 1 hour protocol (n=11) there were no significant differences in the excretion (ng/mmol creatinine [±SEM]) of LTE_4 or 11β-$PGF_{2\alpha}$ after either of the challenges (figure 2Aand 2C).

In contrast, for the 3 hour protocol (n=15) 11β-$PGF_{2\alpha}$ increased significantly both after challenge I (29±4 vs 24±4 ng/mmol creatinine, p=0.0054) and after the challenge II (28±3 vs 24±3 ng/mmol creatinine, p=0.0209) (figure 2B). Also, there was an increase of LTE_4 after challenge I (40±3 vs 32.5±3 ng/mmol creatinine, p=0.0011) and a strong tendency for an increase after challenge II (39±4 vs 33±3 ng/mmol creatinine, p=0.0582) (figure 2D).

Repeatability of the EVH challenge - The agreement between challenges was in general good, however in a few patients the standard deviation was sometimes large, with differences >20% when comparing the maximum percentage fall in FEV_1 following challenge I. Bland-Altman plots for demonstration of the variability revealed that the larger the fall in FEV_1 the greater the variability becomes. (7) There were however no significant differences between the screening day and the study days (table 1).

Study 2 – extended analysis of urinary mediator excretion

From the results of study 1 it was obvious that the one hour interval between the challenges was too short and unsuitable because the mediator levels do not return to baseline within this time frame. Also, since the timing of the peak excretion of mediators after a challenge differ between subjects it is not satisfactory with only one sample following challenge. Therefore another 8 subjects were recruited to perform repeated EVH 3 hours apart. In order to increase the probability to catch the peak excretion following the second challenge, a sampling of urine was added at 300 minutes after the first challenge. Also, to try to enhance the response, we chose to use 6 minutes of EVH instead of 4 minutes and included only subjects with a fall in $FEV_1 \geq 15\%$ at screening. Subject characteristics are presented in table 1. Skin prick test was positive in in 7 of the subjects

Airway response - The maximum fall in FEV_1 following challenge I was 30.0 ±5.4% and 21.2 ±4.5 % following challenge II, p=0.0076 (Table 1). The mean degree of protection was 30 % and all subjects showed some degree of protection, with the range being 2% to 70% () Thus, overall the bronchoconstriction induced in study 2 was greater than in study 1 (30.0±5.4% vs 19.5±2.1%. p=0.0029), indicating the 6 min EVH to be more powerful than the 4 min.

Extended urinary mediator analysis using both UPLC-MS/MS and EIA- The results of the extended analysis are presented in table 2 (UPLC-MS/MS), in table 3 (EIA) and in figure 3A-J (UPLC-MS/MS and EIA). Of the 30 metabolites in the platform, 13 were found at measureable levels. The most abundant mediator was tetranor-PGEM (~3500 ng/mmol creatinine) followed by the main isoprostane metabolite 8,12-iso-$PF_{2\alpha}$-VI (~500 ng/mmol creatinine). The tetranor metabolite of PGD_2 displayed levels around 200-250 ng/mmol creatinine, whereas the levels of $PGF_{2\alpha}$ were around 100 ng/mmol creatinine. Metabolites of thromboxane were slightly less abundant and CysLTs were found in the lowest concentrations of all measured compounds. In general, the levels detected in EIA were higher compared to the levels found using UPLC-MS/MS. There was however a good correlation between the EIA and UPLC-MS/MS results.

PGD_2 metabolites – Measuring the peak excretion of 11β-$PGF_{2\alpha}$ using EIA the increase from baseline did not reach significance following challenge I. Following challenge II there was however a significant increase from baseline (table 3, figure 3A). The levels of 11β-$PGF_{2\alpha}$ were back to baseline before challenge II. Using UPLC-MS/MS, while the early metabolite 11β-$PGF_{2\alpha}$ was undetectable, its metabolite 2,3-dinor-$PGF_{2\alpha}$ was found in similar concentrations as the 11β-$PGF_{2\alpha}$ values indicated by the EIA. The 2,3-dinor-$PGF_{2\alpha}$ increased to an equal magnitude after both challenges indicating a similar level of mast cell activation (table 2, figure 3B). The levels of these two early PGD_2 metabolites returned to baseline before challenge II was initiated.

The EIA for tetranor-PGDM, a later and more abundant metabolite of PGD_2, showed increased levels following challenge I, but failed to reach significance following challenge II (table 3, figure 3C). Conversely, with UPLC-MS/MS, the increase of tetranor-PGDM failed to reach significance following challenge I, but increased significantly following challenge II (figure 3D).

Cysteinyl leukotrienes – Using EIA, for LTE_4 there was an increase from baseline following the challenge I, but not following challenge II (table 3, figure 3E). The levels of LTE_4 were still somewhat elevated from baseline before challenge II making interpretations of excretion following challenge II more difficult. In UPLC-MS/MS, LTE_4 was the only CysLT that could be detected (table 2). Most of the subjects displayed increased levels following challenge I with a strong tendency for the whole group although not statistically different. The levels returned to baseline before challenge II, and significantly increased levels were seen following challenge II (figure 3F).

Thromboxanes and Isoprotanes – TXB_2 and its metabolites, the isoprostanes and $PGF_{2\alpha}$ failed to display consistent increases irrespective of whether analysed by EIA or UPLC-MS/MS. (table 2 and table 3).

Prostaglandin E_2 – The EIA for PGE_2 showed increased concentrations following challenge I, but not following challenge II (table 3, figure 3G). In UPLC-MS/MS the levels of PGE_2 were significantly increased following both of the challenges, with no differences between the two peaks (table 2, figure 3H). Tetranor-PGEM increased significantly following challenge I whereas the increase following challenge II failed to reach significance (table 2, figure 3I). There were no differences in the levels of urinary PGE_2 between male and female subjects, whereas the levels of PGEM were significantly higher in male subjects (The females are #22, 23, and 24 in figure 3I). The peak levels of PGE_2 or PGEM after the first or second challenge did not correlate with the degree of refractoriness.

Prostacyclin – Using the EIA clear increases were seen of the prostacyclin metabolite 6-keto-$PGF_{1\alpha}$ following both the challenges (table 3, figure 3J). This metabolite was not included in the UPLC-MS/MS. The peak levels of PGI_2 after the first or second challenge did not correlate with the degree of refractoriness.

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Discussion

In this study we report on extended analysis of mediator excretion during the refractory period following repeated EVH challenge. For the first time in this setting for exercise-induced bronchoconstriction, we could demonstrate increased urinary excretion of metabolites of PGE_2 and PGI_2 following EVH. In contrast, the levels of metabolites of thromboxane and isoprostanes remained unchanged, indicating specificity in the excretion of eicosanoids. Using the mass spectrometry platform we also documented increased levels of CysLTs, and metabolites of PGD_2 following the EVH challenge, thus replicating and validating by mass spectrometry our previous findings using EIA only.(18, 19) The increased urinary excretion of those two bronchoconstrictive mediators following both of the two repeated EVH challenges are similar to our previous findings of mediator excretion following repeated mannitol inhalation challenge.(22) Taken together, the main findings support that excretion of mast cell mediators is maintained also during the second challenge, and that there is significant excretion of two lipid mediators with bronchoprotective properties during the refractory period, namely prostacyclin and PGE_2. It is noteworthy that the urinary levels of the main metabolite of PGE_2 were higher than for any of the other lipid mediators detected in the urine.

The primary eicosanoids are potent biologically active mediators, however, they are troublesome to measure since they are very rapidly metabolized and cleared from the circulation (34). Also, following withdrawal of blood it has been shown that e.g. TXB_2 can be generated *ex vivo* (29). This often makes the measurement of these primary compounds very difficult, and the interpretation of such data ambiguous. Urine has emerged as a non-invasive alternative and metabolism and urinary excretion of these compounds have been extensively studied.(23, 38) The use of urinary excretion of eicosanoid metabolites is now well-established.(10) As we used a mass spectrometry platform which has previously been

346 applied to study the urinary mediator excretion following allergen challenge (9), the current
347 results allow for comparison of differences and similarities with respect to the patterns of
348 excretion in response to these two different indirect triggers of bronchoconstriction.
349 Following allergen challenge the levels of CysLT, and metabolites of PGD_2 and TXB_2 were
350 all increased but there was no increase in levels of PGE_2 or its metabolites. For the
351 bronchoconstrictive mediators PGD_2 and CysLT, the results in this EVH study are thus
352 concordant with the findings in the allergen inhalation challenge study, whereas we could not
353 find significant increases of TXB_2 or its metabolites following EVH. The discrepancies in
354 mediator excretion following EVH and allergen challenge respectively, suggests differences
355 in the cells activated by the different challenges.

356 For PGE_2 we observed increases of both the primary mediator and its most abundant
357 metabolite tetranor-PGEM. This is distinctly different to what was found following allergen
358 challenge.(9) The finding of PGE_2 excretion following EVH but not allergen challenge might
359 be explained by the mechanisms of the challenges. The reactions to both of the challenges are
360 initiated by mast cell activation as evident by the uniform excretion of PGD_2, but whereas the
361 allergen challenge is an IgE-dependent specific mast cell activation, EVH activates mast cells
362 through changes in osmolarity.(2) The change in local tissue osmolarity is likely to induce
363 excretion of PGE_2 from other cells in the airways and in particular from airway epithelial
364 cells.(14, 16) In contrast, there was no increase in the excretion of $PGF_{2\alpha}$ which is consistent
365 with previous findings in plasma following exercise challenge (3), and, again underscores the
366 specificity of the pattern of excretion of lipid mediators.

367 The observation that pre-treatment with inhalation of PGE_2 has been shown to inhibit
368 the response to exercise challenge(25) as well as the inhibiting effect of NSAID pre-treatment
369 on the development of refractoriness(24, 26, 36), has led to the speculation PGE_2 is the key
370 mediator of refractoriness. However, there are no previous reports on increased excretion of

371 PGE_2 following exercise challenge in asthmatics, rather a decrease in the levels of PGE_2 has
372 been seen in induced sputum.(13) Increased levels have been seen in male subjects following
373 exercise in exhaled breath condensate (EBC) but the same was not seen in female
374 subjects.(31) As lipid mediators in EBC to a significant extent may reflect salivary
375 admixture, the data are inconclusive.(12)
376 In our study, the excretion of the abundant metabolite of PGE_2, tetranor-PGEM,
377 increased following challenge I but not following challenge II. This supports increased
378 excretion of PGE_2 from the lung following EVH challenge. Interestingly we also found that
379 primary PGE_2 increased in the urine following both of the challenges. The current concept is
380 that the kidney itself is exclusive source of urinary PGE_2. This view is based on previous
381 metabolic studies showing rapid metabolism of systemic PGE_2. Thus, primary PGE_2 was
382 only seen in the urine following renal artery infusion, but not after brachial vein infusion. (38)
383 However, the metabolic studies were done with relatively low doses of PGE_2 and it is likely
384 that the systemic load of PGE_2 following massive excretion from the airways during the EVH
385 challenge is much greater, explaining that a small proportion is excreted in the urine un-
386 metabolised.
387 Comparing the levels of urinary mediators between EIA and UPLC-MS/MS for LTE_4,
388 there were good correlations but absolute values were generally higher in EIA. For 11β-
389 $PGF_{2\alpha}$, however, this metabolite was not at all detected in UPLC-MS/MS, but rather the
390 levels in EIA seemed to correspond to the levels of 2,3-dinor-$PGF_{2\alpha}$, the metabolite to which
391 the antibody is cross-reactive. What is actually measured with the EIA for 11β-$PGF_{2\alpha}$
392 therefore appears to be 2,3-dinor-$PGF_{2\alpha}$. This has previously been noted in work from our
393 group,(27) and confimed by others,(15) but the commonly used term has still been 11β-$PGF_{2\alpha}$
394 because this is the name of the antibody in the commercially available kit. For the other
395 mediators analyzed both using EIA and UPLC-MS/MS there was in general a good agreement

between the methods as can be seen by similar patterns of excretion (figure 3) and good correlations. In the Bland-Altman analysis(7) it was evident that with increasing concentration the discrepancies between the EIA and UPLC-MS/MS results became larger.

We performed an initial study in order to optimize the conditions for mediator analysis during the refractory period. From previous studies we know that refractoriness is greater the sooner after the first challenge the second challenge is performed.(11) However, since there is a lag between the excretion of mediators in the lung and the excretion in urine we needed to extend the interval. Considering most mediators were back to baseline, and refractoriness was still found, the 3 hours interval was sufficient. The subjects in study 2 displayed from no to almost complete protection (2-70%) at the second challenge, which is in line with previous findings that the degree of refractoriness is indeed a continuous response.(22)

Concerning the repeatability of the EVH challenge Bland-Altman analysis(7) revealed a high variability between challenges, which is in line with findings by Price and colleagues.(30) Considering that the between study day difference for several subjects were >10%, the question arises about the usability of the EVH challenge as a predictive tool in the diagnosis of EIB. The number of subjects with large differences was however smaller for the 6 minute protocol (Figure 6) which also caused a greater response. This makes the 6 min challenge more suitable for diagnosis and drug intervention trials.

In conclusion, the consistent finding of increased levels of the bronchoconstrictive mediators following two repeated eucapnic hyperventilation challenges makes decreased mediator excretion following the second challenge an unlikely mechanism of refractoriness. Our findings lend further support to the importance of PGE_2 and possibly also PGI_2 in the development of refractoriness, and provide circumstantial support for our previous suggestion of decreased responsiveness at the level of the airway muscle.(21, 22) The next step will need

to be specific interventions with e.g. subtype specific PGE_2 receptor (EP) agonists to define the mechanisms in greater detail. We have recently found that low doses of PGE_2 via EP2 receptor activation has a long-lived inhibitory effect on mast cell dependent constriction of human small airways.(33) A better understanding of the mechanism of this unique natural protective mechanism may aid in the search for new treatment targets in asthma.

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Disclosures – none

Figure legends:

Figure 1. Study design. EVH = eucapnic volountary hyperpnea. U = urine sampling.

Figure 2. *Mediator excretion during study 1*
Levels of urinary mediators 11β-$PGF_{2\alpha}$ ng/mmol creatinine in urine during 1 hour (A) and 3 hour (B) protocol. LTE_4 ng/mmol creatinine during 1 hour (C) and 3 hour (D).

Figure 3. *Extended analysis of the mediator excretion during study 2*
EIA and UPLC-MS/MS data. All values are presented as ng/mmol creatinine.

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581
582

Table 1. Screening and Study day results

Study 1

Patient No.	Screening					Study day - 1 hour protocol			Study day - 3 hours protocol		
	Sex	***Age***	***FEV_1 (L)***	***FEV_1 (% predicted)***	***EVH***	***EVH I***	***EVH II***	***% Protection***	***EVH I***	***EVH II***	***% Protection***
1	F	35	3.9	111	-11.0	-14.5	-15.0	-3	-14.8	-15.4	-4
2	F	52	2.3	86	-12.0	-8.3*	-11.0	*	-10.1	-2.6	74
3	F	25	2.5	74	-15.5	-11.9	-17.4	-46	-15.4	-17.7	-15
4	F	28	3.3	94	-29.3	-39.7	-23.9	40	-32.5	-27.7	15
5	M	43	3.2	84	-12.9	-19.5	-18.5	5	-14.4	-10.8	25
6	F	34	3.3	72	-23.4	-17.0	-13.3	22	-38.7	-41.1	-6
7	M	28	3.4	90	-42.0	-26.7	-12.2	54	-20.4	-12.2	40
8	M	27	4.7	104	-16.8	-19.1	-18.8	2	-20.7	-19.5	6
9	M	44	3.5	70	-16.5	-21.5	-14.5	33	-18.0	-13.9	22
10	M	27	4.6	102	-28.7	-18.8	-15.3	19	-19.7	-24.0	-22
11	M	39	4.4	104	-21.6	-10.8	-11.2	-4	-19.0	-12.5	34
12	M	39	3.0	91	-17.8	-7.5*	-16.3	*	-28.0	-21.1	25
13	M	44	4.7	117	-12.9	-6.5*	-8.8	*	-11.1	-8.2	26
14	M	41	3.8	93	-13.7	-15.5	-9.1	41	-17.2	-5.5	68
15	M	47	3.6	93	-12.0	-5.9*	-12.7	*	-2.8*	-4.8	*
16	M	43	5.1	101	-35.5	-9.3*	-13.3	*	-12.1	-12.9	-7
Mean±SEM		**37±2**	**3.7±0.2**	**93±3%**	**-20.1±2.3%**	***-19.6*±2.4%**	**-15.4±1.2% (NS)**	**15±8%**	**-19.5±2.1%**	**-16.3±2.5% £**	**19±7%**

Study 2

Patient No.	Screening					Study day		
	Sex	***Age***	***FEV_1 (L)***	***FEV_1 (% predicted)***	***EVH***	***EVH I***	***EVH II***	***% Protection***
17	M	25	5.2	103	-17.6	-16.7	-11.9	29
18	M	22	5.1	99	-27.1	-23.4	-18.1	23
19	M	24	3.8	82	-39.5	-41.9	-29.7	29
20	M	22	5.2	108	-55.8	-62.2	-45.5	27
21	M	24	4.9	101	-16.8	-19.7	-16.6	16
22	F	30	2.4	94	-26.6	-30.0	-29.4	2
23	F	47	3.0	96	-18.1	-18.2	-9.7	47
24	F	33	3.9	102	-18.7	-28.1	-8.4	70
Mean±SEM		**28±3**	**4.0±0.4**	**98±3%**	**-27.5±4.9%**	**-30.0±5.4%**	**-21.2±4.5% $**	**30±7%**

The percentage change of baseline value. * = Subjects excluded from the refractoriness calculations (<10% fall from baseline following challenge I). M = Male. F = Female. FEV_1 = forced expiratory volume in 1 second. EVH I = the first EVH (eucapnic voluntary hyperpnea) challenge, EVH II = the second EVH challenge after one or three hours. EVH I vs EVH II (NS = non-significant; £ = p=0.0195; $ = p=0.0076)

Table 2. UPLC-MS/MS ng/mmol creatinine

	Baseline 1	Peak 1	Baseline 2	Peak 2
PGD_2	Not detected			
11β-$PGF_{2\alpha}$	Not detected			
2,3-dinor-$PGF_{2\alpha}$	60±14	88±18*	56±13	89±20£
Tetranor-PGDM	202±30	234±23	190±21	240±20£
PGE_1	Not detected			
13,14-dihydro-15-keto-PGE_1	Not detected			
PGE_2	17±3	33±5*	19±4	25±4£
13,14-dihydro-15-keto-PGE_2	Not detected			
Tetranor-PGEM	3193±1015	3435±966*	3087±932	3957±1016
$PGF_{2\alpha}$	107±18	161±41	154±43	164±34
13,14-dihydro-15-keto-$PGF_{2\alpha}$	Not detected			
Tetranor-PGFM	Not detected			
TXB_2	14±6	15±5	13±5	13±3
11-dihydro-TXB_2	8±2	9±2	7±2	9±1
2,3-dinor-TXB_2	68±18	81±17	59±14	83±18
8-iso-$PGF_{2\alpha}$	26±2	33±4	29±5	33±4
2,3-dinor-8-iso-$PGF_{2\alpha}$	147±7	184±15	163±18	187±10£
8,12-iso-$iPF_{2\alpha}$-IV	430±48	615±116	547±96	577±61
LTB_4; LTB_4 metabolites$	Not detected			
LTC_4; **LTD_4**	Not detected			
LTE_4	7±1	10±1	6±2	12±3£
EXC_4; **EXD_4**; **EXE_4**	Not detected			

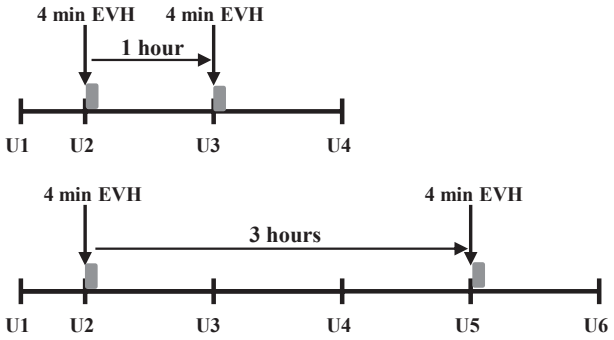
*= significantly different from 1st baseline ($p<0.05$)
£ = significantly different from 2nd baseline ($p<0.05$)
$ = 6-trans-LTB_4; 20-OH-LTB_4; 20-CO2H-LTB_4

Table 3. Enzyme immunoassay ng/mmol creatinine

	Baseline 1	Peak 1	Baseline 2	Peak 2
PGD_2				
11β-$PGF_{2\alpha}$	65±15	80±16	58±15	71±14£
Tetranor-PGDM	738±93	874±112*	815±98	880±103
PGE_2	49±7	77±9*	54±7	60±9
TXB_2	182±26	214±32	197±39	199±31
8-iso-$PGF_{2\alpha}$	92±12	101±10	83±8	94±9£
PGI_2				
6-keto-$PGF_{1\alpha}$	158±23	210±38*	155±22	188±21£
LTE_4	45±7	65±8*	53±10	57±7

*= significantly different from 1st baseline ($p<0.05$)
£ = significantly different from 2nd baseline ($p<0.05$)

Study 1



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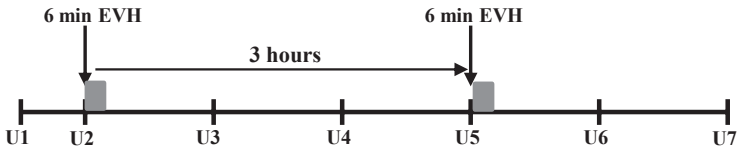
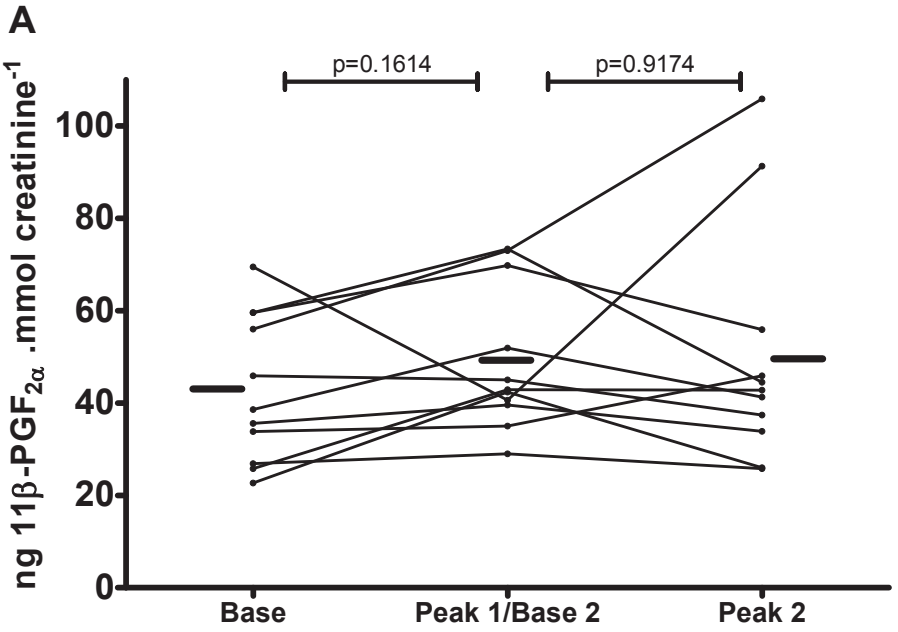
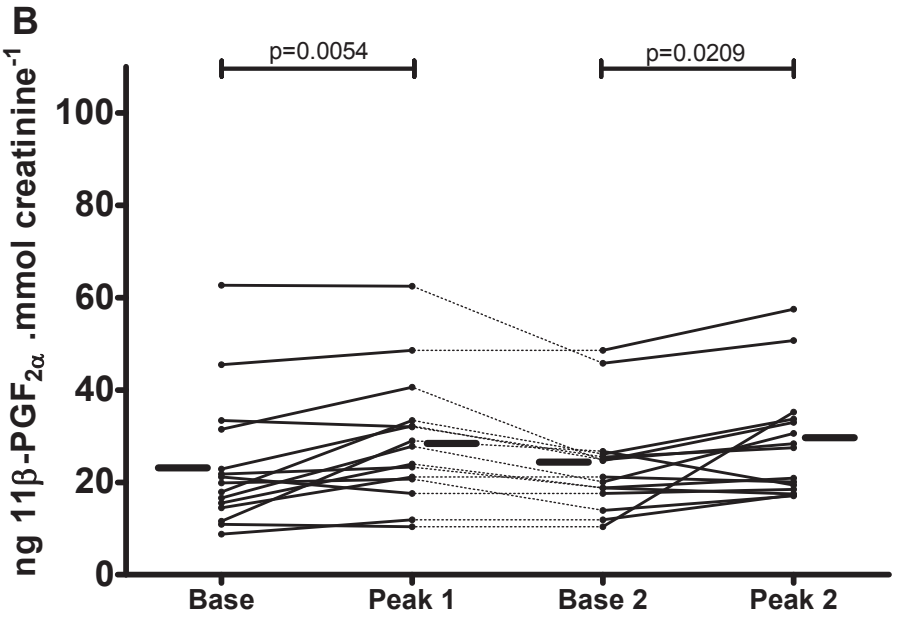
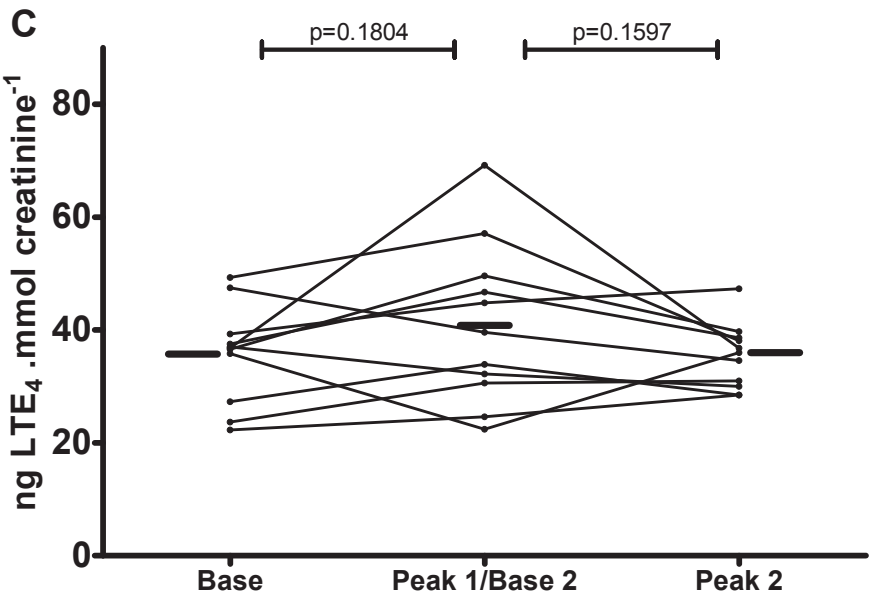
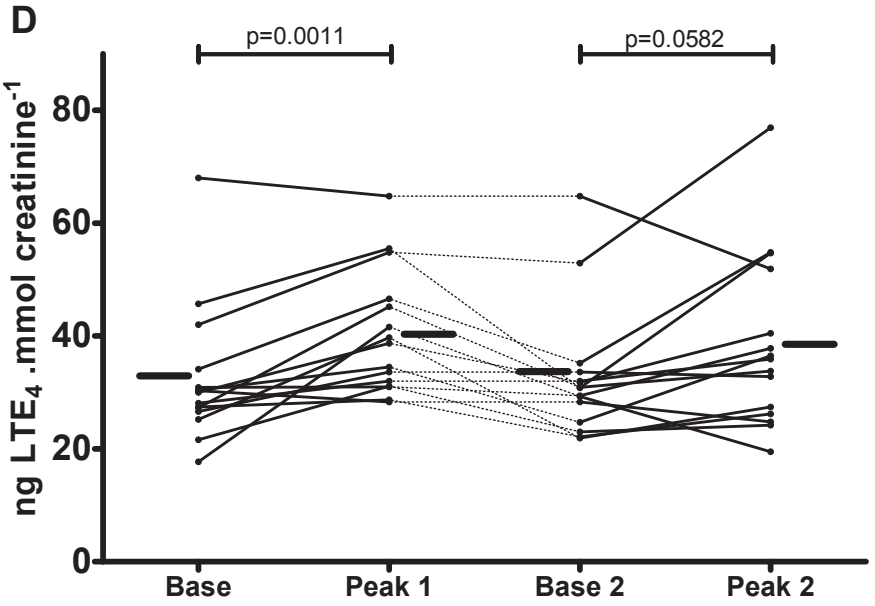
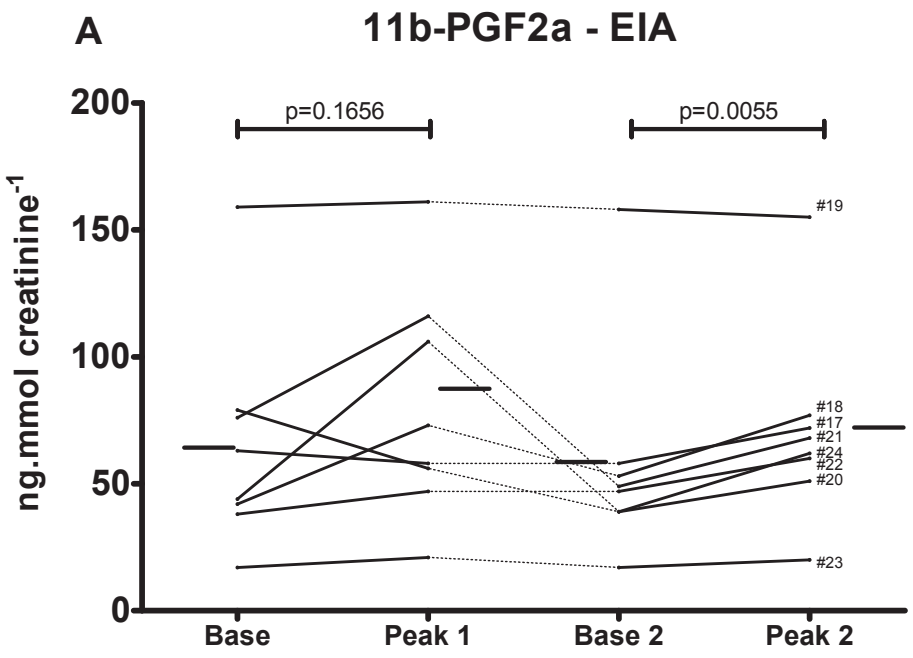


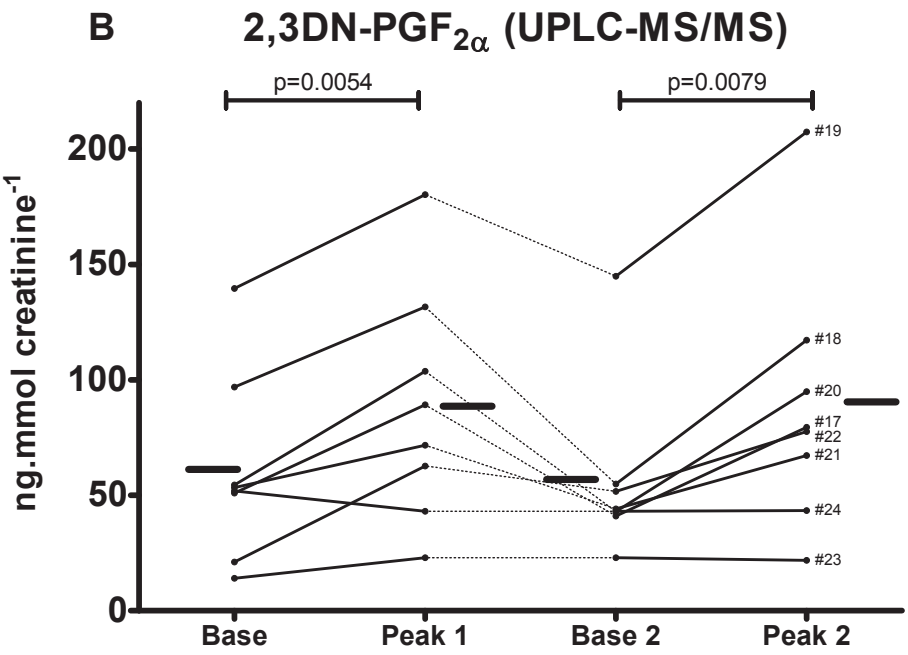

A
p=0.1614
p=0.9174
ng 11β-PGF2α .mmol creatinine-1
100
80
60
40
20
0
Base
Peak 1/Base 2
Peak 2


B
p=0.0054
p=0.0209
ng 11β-PGF2α .mmol creatinine-1
100
80
60
40
20
0
Base
Peak 1
Base 2
Peak 2


C
p=0.1804
p=0.1597
ng LTE4.mmol creatinine-1
80
60
40
20
0
Base
Peak 1/Base 2
Peak 2


D
p=0.0011
p=0.0582
ng LTE4.mmol creatinine-1
80
60
40
20
0
Base
Peak 1
Base 2
Peak 2


A
11b-PGF2a - EIA
ng.mmol creatinine-1
200
150
100
50
0
p=0.1656
p=0.0055
#19
#18
#17
#21
#24
#22
#20
#23
Base
Peak 1
Base 2
Peak 2


B
2,3DN-PGF2α (UPLC-MS/MS)
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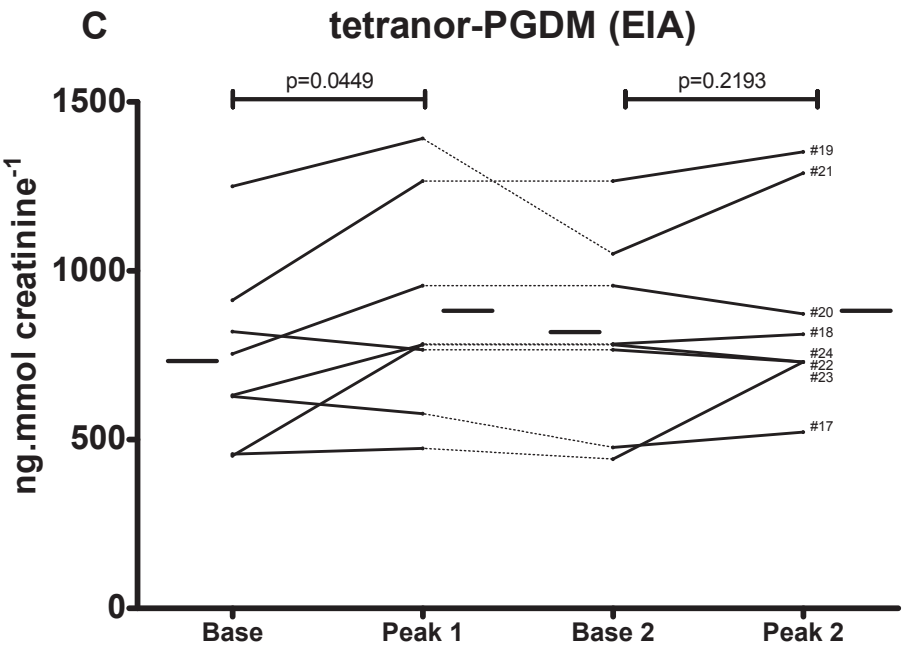
p=0.0079
ng.mmol creatinine-1
200
150
100
50
0
#19
#18
#20
#17
#22
#21
#24
#23
Base
Peak 1
Base 2
Peak 2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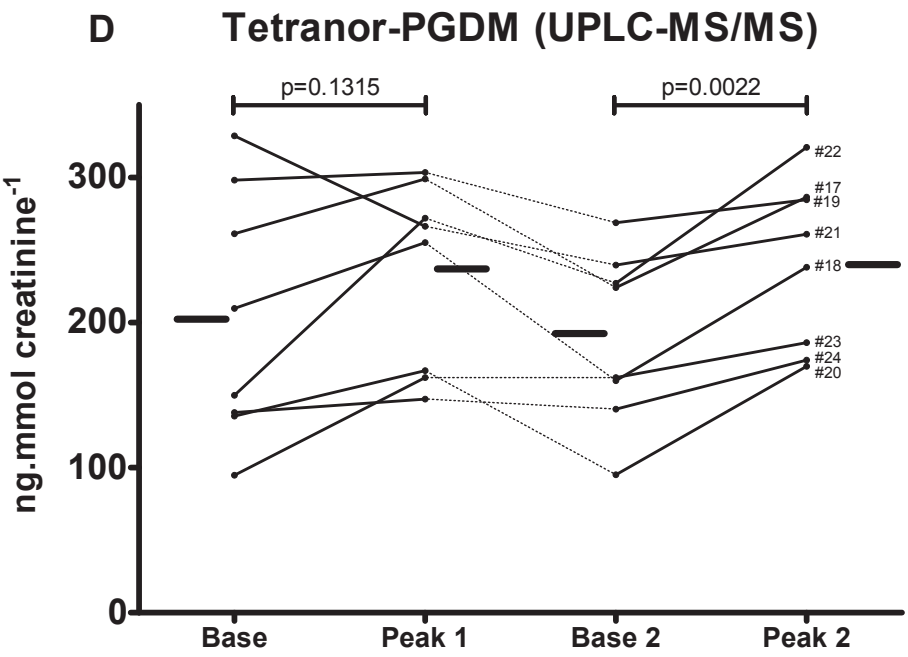
p=0.2193
1500
1000
500
0
ng.mmol creatinine-1
#19
#21
#20
#18
#24
#22
#23
#17
Base
Peak 1
Base 2
Peak 2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Tetranor-PGDM (UPLC-MS/MS)
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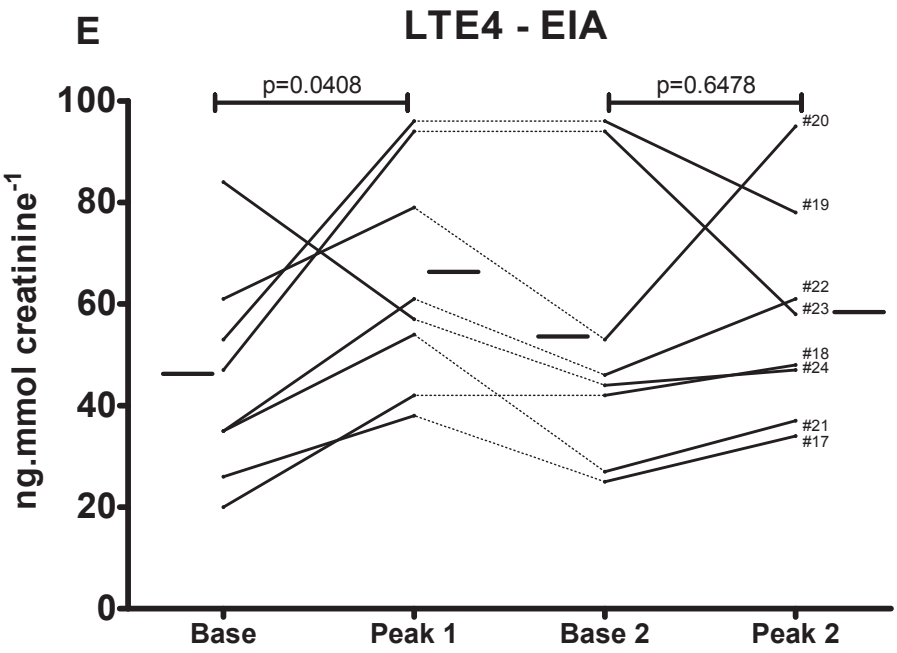
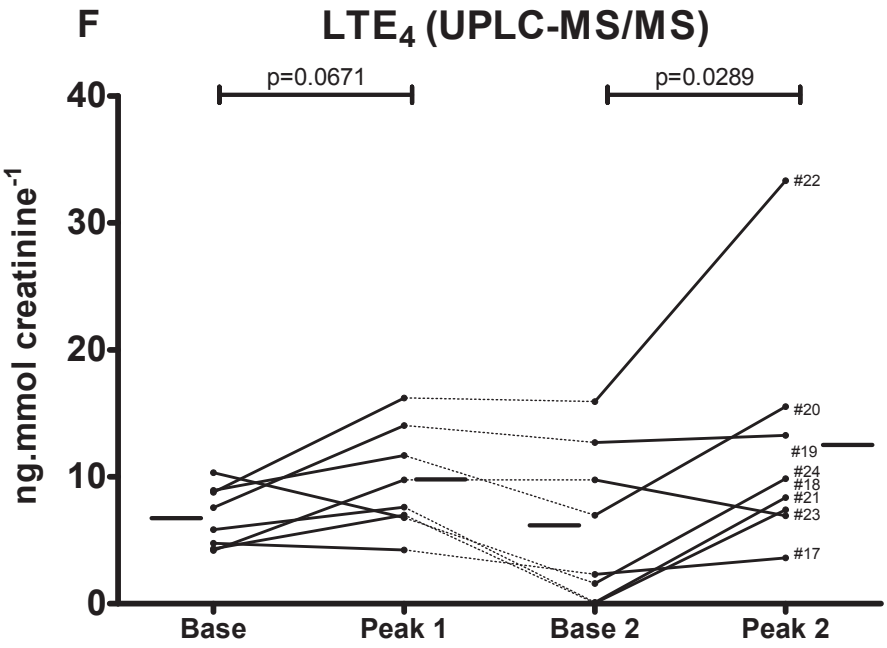
p=0.0022
ng.mmol creatinine-1
300
200
100
0
Base
Peak 1
Base 2
Peak 2
#22
#17
#19
#21
#18
#23
#24
#20


E
LTE4 - EIA
p=0.0408
p=0.6478
100
80
60
40
20
0
ng.mmol creatinine-1
#20
#19
#22
#23
#18
#24
#21
#17
Base
Peak 1
Base 2
Peak 2


F
LTE4 (UPLC-MS/MS)
p=0.0671
p=0.0289
40
30
20
10
0
ng.mmol creatinine-1
#22
#20
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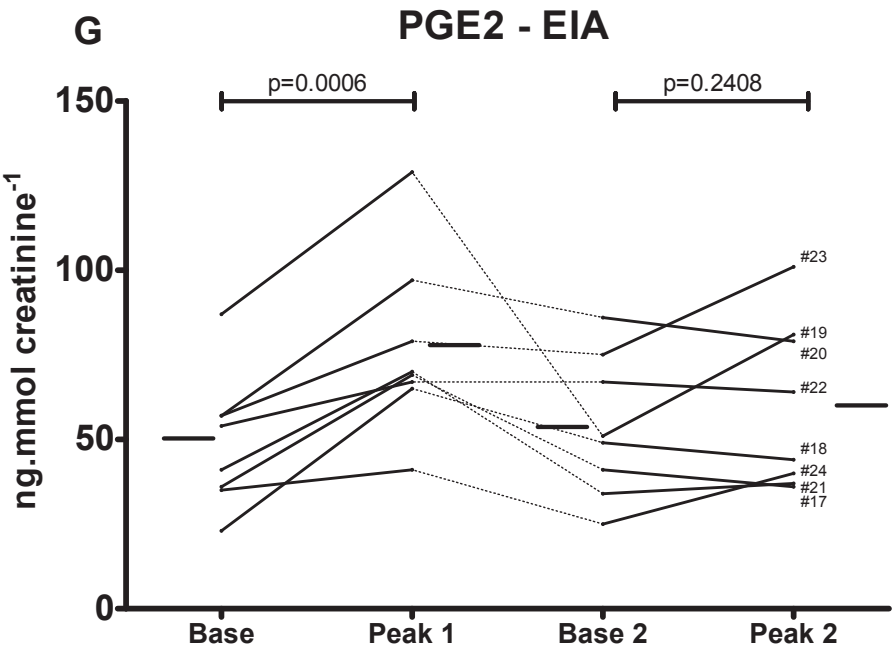
#21
#23
#17
Base
Peak 1
Base 2
Peak 2


G
PGE2 - EIA
p=0.0006
p=0.2408
150
100
50
0
ng.mmol creatinine-1
#23
#19
#20
#22
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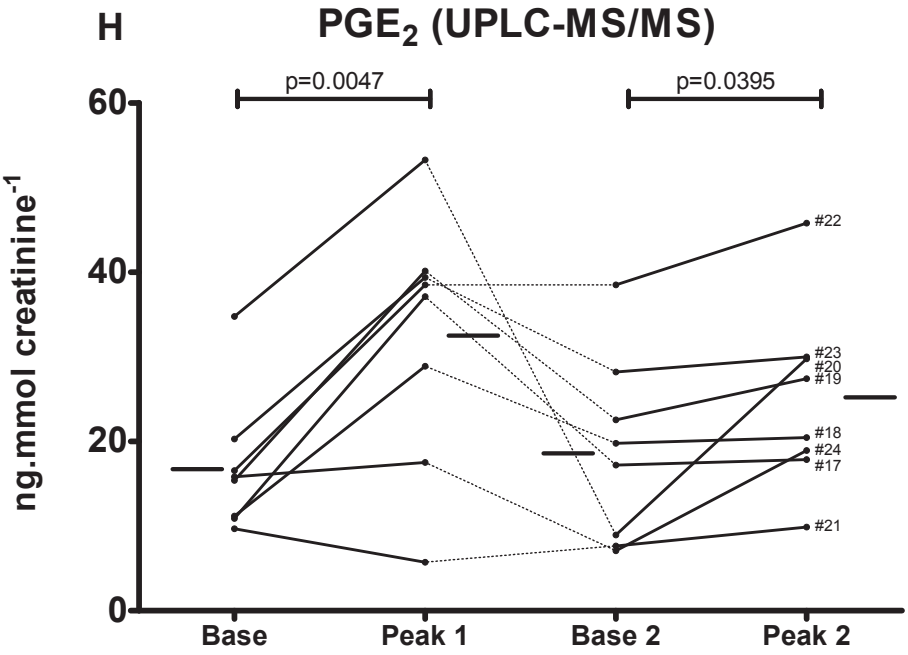
#24
#21
#17
Base
Peak 1
Base 2
Peak 2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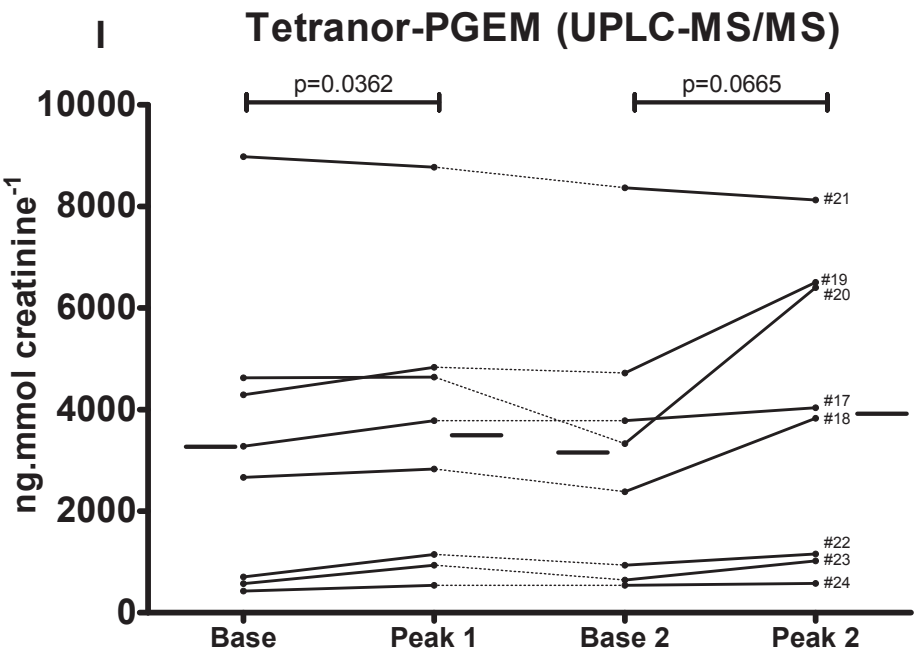
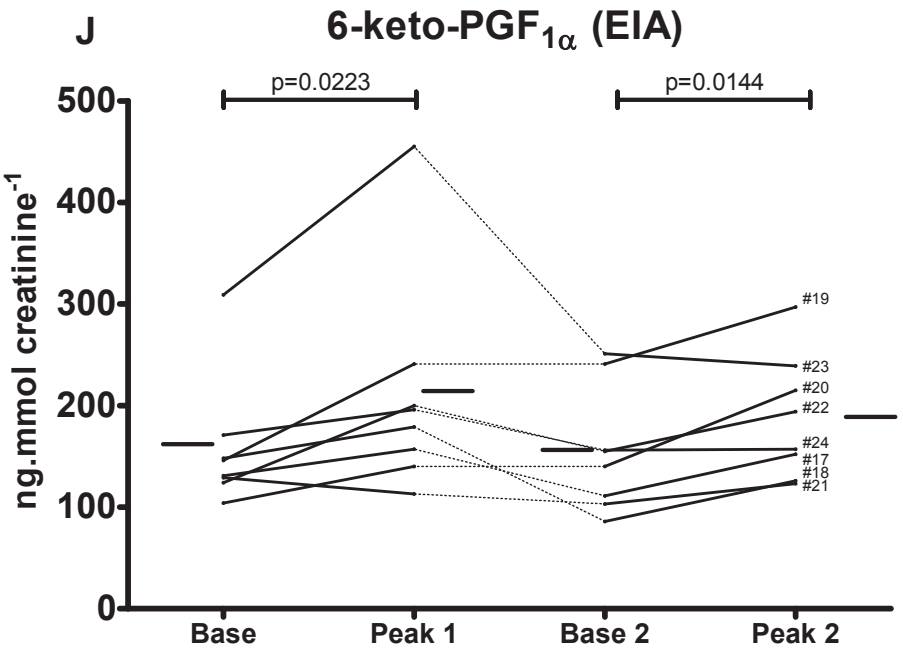
PGE2 (UPLC-MS/MS)
p=0.0047
p=0.0395
ng.mmol creatinine-1
60
40
20
0
Base
Peak 1
Base 2
Peak 2
#22
#23
#20
#19
#18
#24
#17
#21

I


Tetranor-PGEM (UPLC-MS/MS)
p=0.0362
p=0.0665
10000
8000
6000
4000
2000
0
ng.mmol creatinine-1
#21
#19
#20
#17
#18
#22
#23
#24
Base
Peak 1
Base 2
Peak 2


J
6-keto-PGF1α (EIA)
p=0.0223
p=0.0144
ng.mmol creatinine-1
500
400
300
200
100
0
#19
#23
#20
#22
#24
#17
#18
#21
Base
Peak 1
Base 2
Peak 2