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Metabolism of synthetic steroids by the human placenta

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Running Title: Placental metabolism of steroids

Summary

Pregnant women with asthma are frequently exposed to synthetic glucocorticoids and glucocorticoids are known to reduce fetal growth. The fetus is normally protected from the harmful effects of maternally derived glucocorticoids by the placental enzyme 11β -hydroxysteroid dehydrogenase type 2 (11β -HSD2). Whether 11β -HSD2 inactivates the synthetic glucocorticoids used for asthma treatment during pregnancy (budesonide, beclomethasone dipropionate [BDP] and fluticasone propionate [FP]) remains unknown.

To investigate the relationship between steroid use during pregnancy and fetal growth and development, pregnant women with (n=119) and without asthma (n=84) were followed throughout pregnancy. Data on asthma medication use, neonatal size at birth, placental weight and cord blood cortisol and estriol were collected. Placental tissue samples were collected from non-asthmatic women (n=8) for metabolism studies. Placental 11β -HSD2 activity was determined using BDP, budesonide, FP, prednisolone, dexamethasone and betamethasone as steroid substrates. Steroids and their oxidised metabolites were examined using thin layer chromatography and densitometry.

Placental 11β -HSD2 metabolised BDP, prednisolone, dexamethasone and betamethasone, but not budesonide or FP. No association between the use of inhaled steroids for asthma treatment during pregnancy and alterations in neonatal size, placental weight, gestational age at delivery, or umbilical vein estriol concentrations was demonstrated compared to non-asthmatic women.

Introduction

Pregnant women with asthma are at increased risk of poor pregnancy outcomes [1], including low birth weight [2], preterm delivery [3] and pre-eclampsia [4]. The mechanisms contributing to these outcomes are unknown, but may include poorly controlled asthma [5] or its treatment with inhaled or oral corticosteroids [6]. While there is little evidence in the literature to suggest that inhaled corticosteroids (ICS) are harmful to the fetus [7, 8], non-adherence to ICS medication by pregnant women with asthma for fear of its effect on the fetus remains a major clinical problem [9, 10].

Glucocorticoids are essential for the development and maturation of fetal organs before birth and late pregnancy is characterised by a rise in cortisol levels, which parallels the increased maturity of fetal organs [14]. However, glucocorticoids have potentially adverse effects on the fetus and have been linked to altered fetal programming. Women using prednisone during pregnancy have been reported to have an increase in still-birth, fetal distress, placental insufficiency [15] and low birth weight neonates [16]. Antenatal dexamethasone treatment has been associated with a reduction in birth weight, by as much as 161 g in infants delivered between 30 and 32 weeks [17]. While recent evidence from randomised controlled trials suggests that there is no additional decrease in fetal growth when repeated courses of antenatal steroids are used compared to single doses [18], French *et al.* found that repeated courses of betamethasone were associated with a 9% reduction in birth weight and a 4% reduction in head circumference in preterm infants born prior to 33 weeks gestation [19].

Two isoforms of 11 β -hydroxysteroid dehydrogenase (11 β -HSD) have been cloned and characterised in humans which interconvert glucocorticoids with their 11-keto metabolites (Figure 1). The type 1 enzyme (11 β -HSD1) is NADP(H) dependent, acting primarily as an

oxoreductase, converting cortisone to cortisol. The main functional isozyme in the placental syncytiotrophoblast is 11 β -HSD2, a high affinity, NAD dependent, uni-directional enzyme, catalysing only the dehydrogenase reaction, converting cortisol to cortisone [11]. In the placenta 11 β -HSD2 protects the fetus from the potentially harmful effects of endogenous maternal glucocorticoids [12], by acting as a barrier to protect the fetus from the much higher levels of cortisol found in the mother [13].

Several studies have described a relationship between reduced activity of 11 β -HSD2 and reduced birth weight or intrauterine growth restriction [20, 21]. We have previously found that asthmatic women who do not use inhaled steroids for treatment have smaller female neonates at term [22]. This late gestation reduction in fetal growth was associated with a significant decrease in 11 β -HSD2 activity [22, 23].

It is thought that synthetic steroid derivatives, such as dexamethasone and betamethasone are not extensively metabolised by 11 β -HSD2. In bronchial epithelial cells, Feinstein and Schleimer found that the addition of the 11 β -HSD inhibitor, glycyrrhetinic acid had no effect on the anti-inflammatory activity of beclomethasone dipropionate (BDP), fluticasone propionate (FP) or budesonide, suggesting that they were not metabolised by 11 β -HSD2 [24]. However, in another study using BEAS-2B cells derived from the bronchial epithelium, the addition of the 11 β -HSD inhibitor, carbenoxolone, resulted in an increased potency of dexamethasone by approximately 10 fold [25]. Orsida *et al.* have suggested that the presence of 11 β -HSD2 activity in the lung may serve to re-activate synthetic steroids used as anti-inflammatory agents in diseases such as asthma [26]. Information about the metabolism of synthetic steroid derivatives by 11 β -HSD2 is therefore conflicting.

No studies have been published regarding the placental metabolism of synthetic steroids used for asthma treatment during pregnancy such as BDP, budesonide and FP. If these steroids reach the placenta but are not metabolised into inactive forms, they may have adverse effects on fetal development and may contribute to reduced fetal growth in asthmatic women using inhaled or oral corticosteroid medication. In this paper, we hypothesise that BDP, budesonide, FP, dexamethasone, betamethasone and prednisolone are converted into their 11-keto metabolites by the placental enzyme 11 β -HSD2 and that the use of inhaled steroids by pregnant women with asthma has no effect on fetal growth and development.

Materials and Methods

The studies were approved by the Hunter Area Health Service and University of Newcastle Human Research Ethics Committees. Pregnant women with and without asthma were recruited at the John Hunter Hospital antenatal clinics as part of a prospective cohort study, according to a previously described protocol [22, 23, 27]. Neonatal size at birth (weight, length and head circumference) was recorded in 84 pregnant women without asthma and 119 pregnant women with asthma who used BDP alone (n=29), budesonide alone (n=42), FP alone (n=31) or inhaled corticosteroid (ICS) plus periodic oral steroid (prednisone, n=17) to treat their asthma during pregnancy. The dose of ICS used by the mothers in each trimester was recorded and summarised as the mean daily dose of BDP or equivalent, where 1 µg BDP was considered equal to 1 µg budesonide and 0.5 µg FP [28]. The dose and length of use of oral steroids was not recorded. No women were using long acting β_2 -agonists. Gestational age was calculated from the date of the last menstrual period and the 18 week ultrasound. Preterm delivery was defined as delivery at <37 completed weeks gestation. In a subset of samples, placental weight was recorded and fetal hypothalamic-pituitary-adrenal (HPA) function was examined using measurements of umbilical vein cortisol and estriol as previously described [22].

Metabolism Studies - Placenta Collection

Pregnant women who did not have diabetes, hypertension or asthma and were non-smokers were recruited by midwives from John Hunter Hospital and provided written informed consent for participation. All placentae (n=8) were collected from term, uncomplicated vaginal deliveries. The placenta was collected within 45 minutes of delivery and pieces were snap frozen in liquid nitrogen and stored at -80°C until further use.

Protein extraction

Approximately 1 to 2 g of snap frozen placenta was crushed with a mortar and pestle under liquid nitrogen. Crushed placental tissue was homogenised in 10 volumes of 0.1 M sodium phosphate buffer (pH 7.4) containing protease inhibitors (2 mM ethylenediamine tetra-acetic acid, protease inhibitor cocktail tablets 20/L, 50000 KIU/l trasylol, 100 μ M dithiothreitol, 1 μ M pepstatin A, 1 mM benzamidine, 65000 U/L bacitracin) and 0.25 M sucrose, using a polytron homogeniser (Kinematica AG, Switzerland). The homogenate was centrifuged at 1000 g for 10 minutes to remove cellular debris and the supernatant centrifuged at 100000 g for 60 minutes to obtain the microsomal fraction. The pellet was re-homogenised in 2 ml sodium phosphate buffer containing protease inhibitors and the protein concentration determined by Bradford Assay [29] against a standard curve of bovine serum albumin.

Placental 11 β -HSD2 activity assay

Placental 11 β -HSD2 activity was measured using a modification of the method previously used in our laboratory [22, 23, 30]. Initially, protein concentration (100, 150 and 200 μ g/ml), time (15 minutes, 1, 2, 4, 6 and 24 hours) and steroid substrate concentration (5 μ M, 50 μ M) were optimised. In the final method used, 50 μ M steroid substrate (BDP, budesonide, FP, prednisolone, dexamethasone or betamethasone) was chosen due to the need to visualise the steroid on the thin layer chromatography (TLC) plate. We have previously demonstrated that placental 11 β -HSD2 is saturated by 5 μ M cortisol [22, 23]. Steroids were obtained from Sigma (St Louis, MO, USA), except for FP which was reconstituted from the powder of 45 blisters of a FlixotideTM AccuhalerTM (each blister contained 500 μ g FP) obtained from the hospital pharmacy. To the powder, 2.7 ml water was added and thoroughly mixed, in an attempt to remove the lactose. The mixture was centrifuged at 1000 g for 10 minutes and the remaining solid dissolved in 100 % ethanol to give a final concentration of 10 mM.

The synthetic steroid (50 μ M), cofactor (2 mM nicotinamide adenine dinucleotide, NAD) and sodium phosphate buffer containing protease inhibitors to 1 ml total volume, were pre-incubated for 15 minutes at 37°C in a shaking water bath. 150 μ g of placental protein was subsequently added for 6 hours. The reaction was stopped by the addition of 2 ml ice-cold ethyl acetate. Samples were thoroughly mixed and organic and aqueous layers separated. The organic layer containing steroids was dried under high speed vacuum and steroids were reconstituted in 100 μ l of ethyl acetate. Tubes were rotated for at least 30 minutes, followed by centrifugation at 1000 g for 15 minutes to increase recovery.

A positive control consisted of 3 H-cortisol (approximately 200000 cpm per well, Amersham Biosciences, Buckinghamshire, England), cortisol (5 μ M) and 2 mM NAD in sodium phosphate buffer with protease inhibitors, incubated for 6 hours at 37°C. The negative control consisted of one set of triplicate wells which was incubated for 6 hours without protein added, but containing all other components of the reaction (substrate and cofactor).

Thin-layer chromatography (TLC)

The 100 μ l steroid extract was spotted onto aluminium backed TLC plates (Silica Gel 60 F-254, 20 cm x 20 cm, 0.2 μ M thickness, Alltech, Deerfield, IL, USA). A marker lane with the steroid and its oxidised metabolite standards were included on each TLC plate. The plates were chromatographed in a glass chamber at room temperature, using mobile phases composed of 95:5 (v/v) or 93:7 (v/v) chloroform:methanol. The developing distance was 8 cm from the starting line.

After mobile phase evaporation the components of interest were visualised using UV-light (254 nm). Under these conditions, steroids appeared as black spots against a fluorescent green background. The TLC plates were photographed using a tripod-mounted Minolta 800si camera with a shutter speed of 1 s and aperture of 5.6 with 400 ASA colour film. Developed photographs were scanned and converted to grayscale for densitometric analysis. Densitometry was used to quantify each band using Scion Image software (version 4.0.2 for windows, Scion Corporation, Frederick, MD, USA).

Steroid standards preparation

The oxidised metabolites were prepared according to the method of Shaw and Quincey [31]. Briefly, 20 µg of steroid was incubated for 1 hour at room temperature with 1 ml of 50 % acetic acid/1 % chromium trioxide (w/v). 250 µl of dichloromethane was added twice to extract the steroids. The extract was dried over several days under a fume hood and reconstituted in 20 µl of 100 % ethanol, rotated for 1 hour and centrifuged at 1000 g for 15 minutes to improve recovery. Preliminary experiments using ³H-cortisol indicated that we could obtain >95% yield of oxidised steroid using this method.

Statistical Analysis

Results are presented as median (interquartile range) or mean ± standard error of the mean. Statistical analysis was performed using GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA). ANOVA or the Kruskal-Wallis test was used, along with the appropriate post-hoc tests (Tukey Kramer multiple comparisons test or Dunn's multiple comparisons test). P<0.05 was considered significant.

Results

ICS use for asthma during pregnancy

Table 1 gives the median dose of ICS used in each trimester of pregnancy in the four groups (oral steroids + ICS, BDP alone, budesonide alone and FP alone). ICS use was significantly lower in the BDP group compared to the oral steroid + ICS group in all trimesters (Kruskal-Wallis test and Dunn's multiple comparisons test, $P<0.01$) and compared to the FP group in the first trimester (Kruskal-Wallis test and Dunn's multiple comparisons test, $P<0.05$). In the first and second trimesters, ICS use was lower in the budesonide group compared to the oral steroid + ICS group (Kruskal-Wallis test and Dunn's multiple comparisons test, $P<0.01$).

Effect of ICS use on fetal growth and HPA function

Birth weight was not significantly different between the groups (ANOVA, $P=0.398$). Birth length and head circumference were not significantly different between the groups (Kruskal-Wallis test, $P=0.154$ and $P=0.968$ respectively). Placental weight did not differ significantly between groups (ANOVA, $P=0.547$). Gestational age at delivery was not significantly different between the groups (Kruskal-Wallis test, $P=0.209$). There was one preterm delivery in the control group, one in the budesonide group, one in the FP group and one in the oral steroid + ICS group.

Fetal HPA function was assessed by measuring umbilical vein cortisol and estriol in a subset of samples. There were no differences in umbilical vein estriol concentration between the groups, while umbilical vein cortisol was significantly higher in the BDP group compared to the non-asthmatic group (ANOVA, $P=0.023$, Tukey Kramer multiple comparisons test, $P<0.05$).

Metabolism of synthetic steroids by the placenta

The metabolism of the synthetic steroids was monitored over time using the TLC method (Figure 2). The formation of the 11-keto metabolite of BDP was first observed at 60 minutes (Figure 2a). Other placental metabolites were also present. No 11-keto metabolite was observed for budesonide (Figure 2b) or FP (Figure 2c). The 11-keto metabolite of prednisolone was first observed at 240 minutes (Figure 2d), the 11-keto metabolite of dexamethasone from 120 minutes (Figure 2e) and the 11-keto metabolite of betamethasone from 60 minutes (Figure 2f). Separation of betamethasone, dexamethasone and prednisolone from their metabolites was not sufficient under the conditions chosen to investigate the presence of additional metabolites.

Densitometric analysis indicated that at 6 hours, $11.6 \% \pm 1.0 \%$ of BDP was metabolised by placental 11β -HSD2 into the 11-keto derivative (Figure 3), while an additional $21.9 \% \pm 0.8 \%$ was metabolised into unknown steroid metabolites, represented by 3 additional bands on the TLC plate (Figure 2a). No budesonide or FP metabolites were detected using the TLC/densitometry method. $23.4 \% \pm 1.3 \%$ of prednisolone was metabolised by 11β -HSD2 into the 11-keto derivative and $13.0 \% \pm 1.4 \%$ of dexamethasone and $11.6 \% \pm 0.5 \%$ of betamethasone were metabolised into 11-keto derivatives (Figure 3). Metabolism of prednisolone and betamethasone by 11β -HSD2 was significantly greater than beclomethasone dipropionate and dexamethasone (Figure 3, ANOVA and Tukey Kramer multiple comparisons test, $P < 0.001$).

We have previously measured placental 11β -HSD2 activity in samples collected from pregnant women with and without asthma [22]. When this data was re-analysed based on oral

or inhaled steroid use, there were no significant differences between the groups (data not shown).

Discussion

In this study, we have demonstrated using an *in vitro* enzyme assay and TLC method, that placental 11 β -HSD2 metabolises the synthetic steroids BDP, prednisolone, dexamethasone and betamethasone, but not budesonide or FP. This is the first study to examine placental metabolism of BDP, budesonide and FP, drugs commonly used to treat pregnant women with asthma. Previous studies of placental metabolism of synthetic steroids have been conflicting. Using an *in vitro* method where minces of placental tissue were incubated for 2 hours with cortisol, prednisolone, dexamethasone and betamethasone, Blanford and Murphy found that 67 % of cortisol and 51 % of prednisolone were metabolised, while only 2 % of dexamethasone and 7 % of betamethasone were converted to their 11-keto metabolites [32]. The low metabolism of dexamethasone and betamethasone may have been related to the method used, as another study found that the conversion of dexamethasone and betamethasone to their 11-keto metabolites was increased by using a perfused placenta method as opposed to one with minced placental pieces [33]. In this study, the metabolism of cortisol, prednisolone, dexamethasone and betamethasone were similar [33]. Anderson *et al.* measured betamethasone in maternal plasma, umbilical cord plasma and amniotic fluid, following 3 consecutive days of betamethasone administration to the mother [34]. They found that betamethasone was transferred to the fetus and was measurable in amniotic fluid and umbilical cord blood at a similar concentration to that found in maternal plasma [34]. Using our *in vitro* technique, 25% of prednisolone was converted to prednisone during a 6 hour incubation of placental microsomes. A study of the transplacental passage of prednisolone, where concentrations were measured in maternal and fetal plasma following intravenous infusion, found that concentrations of prednisolone in the fetus were 8-10 fold lower than the mother and the 11-keto metabolite (prednisone) was at a similar concentration in both mother

and fetus [35]. This suggested significant metabolism of prednisolone by the placental 11 β -HSD2 barrier. The results of our study show that 11 β -HSD2 metabolism of prednisolone and betamethasone is significantly higher than that of BDP and dexamethasone, while there was no measurable metabolism of budesonide or FP by the placenta.

There are limitations to the technique we used to measure metabolism. Because of the need to quantify the steroids and their metabolites on the TLC plate, a large concentration of steroid substrate was used (50 μ M), which was ten-fold higher than the saturating concentration we previously used in a similar study of placental 11 β -HSD2 activity [22]. The use of a more efficient separation technique such as high performance liquid chromatography (HPLC) would improve the results obtained from our study. However, other techniques are limited by the detection systems and the availability of the oxidised steroid compounds. In addition, it would be helpful to measure the steroids and their metabolites in the umbilical cord blood to determine whether they are likely to reach the fetal circulation. It is not known whether physiologically active concentrations of the steroids used to treat asthma reach the fetus. However, it is also known that use of these medications in adults can result in systemic side effects such as adrenal suppression [36] and reduced bone mineral density in women [37]. It is possible that in women who use high doses of inhaled or oral steroids during pregnancy, there may be some transfer of these compounds to the fetal circulation. Metabolism of these steroids by placental 11 β -HSD2 may be a mechanism by which the fetus is protected from their adverse effects.

We found that there was more metabolism of prednisolone and betamethasone compared to BDP and dexamethasone, while no metabolism of budesonide and FP was detected. This could be due to the different chemical structures of these steroids. Budesonide and FP both

contain bulky side groups attached to carbons 15 and 16 (budesonide) and carbon 17 (FP). In addition, they are the only steroids of this group without a hydroxyl group on carbon 17. These features may inhibit their binding to 11 β -HSD2. Betamethasone and dexamethasone are stereoisomers, with only the methyl group on carbon 16 in different positions. It is possible that the position of this methyl group leads to altered binding to the enzyme. There are also differences in the halogen molecule on carbon 9. BDP has a chlorine attached, while betamethasone, dexamethasone and FP have a fluorine atom. The natural substrate, cortisol, as well as prednisolone do not contain a halogen in this position. It has been previously suggested that the 9-halogen group provides protection from metabolism by 11 β -HSD2 [38, 39].

In this study, we have found no significant effect of oral or inhaled steroid use for asthma treatment during pregnancy on fetal growth parameters and gestational age at birth, in a relatively small group of women. In addition, there was no effect of ICS use on fetal estriol concentrations, suggesting that these steroids do not alter fetal HPA function. We did, however, find a significant increase in fetal cortisol concentration in the group using BDP, although this was a modest increase compared to the non-asthmatic control group only.

There is extensive evidence for the safety of the major drug classes used to treat asthma during pregnancy, including ICS [7, 40, 41]. A recent Swedish study found that the use of budesonide during pregnancy does not affect gestational age, birth weight, birth length or the rate of still-births or multiple births [7]. This data came from 2968 women who used inhaled budesonide during pregnancy and was compared to 7719 women who used asthma medications other than steroids, and a control population of over 293000 women. The safety of oral steroids for asthma during pregnancy is less clear, with two large prospective cohort

studies recently finding an association between oral steroid use and preterm delivery [2, 3]. Previously, asthmatic women using oral steroids during pregnancy (an average of 10 mg per day, n=70) were found to have a higher incidence of preterm delivery, but no significant increases in congenital malformations were observed compared to general population estimates [42]. Another study found no adverse outcome in 37 infants up to 2 years of age, of asthmatic mothers who used prednisone (5-60 mg per day) during pregnancy [43]. In 1997, oral steroids were found to be independently associated with pre-eclampsia in asthmatic women [40]. Recent epidemiological data demonstrated a significant association between first trimester systemic steroid use and an increased risk of cleft lip (with or without cleft palate) in neonates [44]. Information is lacking on the safety of some of the newer ICS drugs such as fluticasone propionate. In this study we found no difference in size at birth between asthmatic women using BDP, budesonide or FP alone and ICS taken with periodic oral steroids. Recently, Namazy *et al.* studied asthmatic women using BDP, budesonide, FP, triamcinolone or flunisolide and there was no increase in the rate of small for gestational age infants compared to population averages [8].

We conclude that the use of the inhaled steroids, BDP, budesonide and FP for asthma treatment during pregnancy does not result in any significant effects on neonatal size at birth and that the placenta metabolises the synthetic steroids BDP, prednisolone, dexamethasone and betamethasone, but does not metabolise budesonide or FP.

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Figure 1: The reaction catalysed by placental 11 β -HSD2 (a) and chemical structures of synthetic steroids and their 11-keto derivatives (b)

Figure 2: Representative TLC plates showing the metabolism of BDP (a), budesonide (b), FP (c), prednisolone (d), dexamethasone (e) and betamethasone (f) over time (15 minutes, 1, 2, 4, 6 and 24 hours).

Figure 3: Percentage metabolism of each synthetic steroid into the 11-keto metabolite at 6 hours, according to densitometric analysis (A=beclomethasone dipropionate, B=budesonide, C=fluticasone propionate, D=prednisolone, E=dexamethasone, F=betamethasone). * indicates $P < 0.01$ (prednisolone vs beclomethasone dipropionate and dexamethasone), # indicates $P < 0.01$ (betamethasone vs beclomethasone dipropionate and dexamethasone)

Table 1: Dose of ICS used by pregnant women with asthma, and fetal growth and HPA function for each group

	Non-asthmatic (n=84)	Oral steroid + ICS (n=17)	BDP alone (n=29)	Budesonide alone (n=42)	FP alone (n=31)
1 st trimester ICS dose (µg/day)		1000 (800-1600)	100 (0-500)*, **	300 (0-800)	800 (325-1000)
2 nd trimester ICS dose (µg/day)		1000 (800-2000)	400 (100-1000)*	500 (0-1600)***	1000 (500-1000)
3 rd trimester ICS dose (µg/day)		1000 (1000-2000)	500 (200-1000)*	800 (650-1600)***	1000 (500-1000)
Birth weight (g)	3600 (3228-3900)	3440 (3000-3520)	3560 (3270-3880)	3510 (3163-3860)	3460 (3165-4000)
Birth length (cm)	52 (51-53) n=77	51 (48-52) n=17	52 (50-54) n=28	52 (50-54) n=41	52 (49-54) n=26
Birth head circumference (cm)	35 (34-35.5)	34.5 (33.5-35)	34.5 (34-36)	35 (33.5-36)	34.5 (33.3-36)
Placental weight (g)	593 (519-734) n=40	594 (562-666) n=6	681 (505-772) n=10	722 (597-788) n=12	627 (536-723) n=20
Gestational age at birth (weeks)	40 (39-40.8)	39 (38-41)	39.3 (38-40)	39.9 (39-40.8)	39.9 (39-41)
Umbilical vein cortisol (nmol/l)	192 (146-228) n=24	196 (155-220) n=10	278 (160-370)# n=19	155 (120-250) n=10	259 (153-322) n=13
Umbilical vein estriol (nmol/l)	731 (545-903)	572 (496-886)	791 (746-1020)	976 (708-1048)	766 (704-841)

	n=16	n=7	n=17	n=6	n=7
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Values are median (interquartile range)

ICS = inhaled corticosteroid, BDP = beclomethasone dipropionate, FP = fluticasone propionate

* indicates $P < 0.01$ BDP alone vs Oral steroid + ICS (Kruskal-Wallis test & Dunn's multiple comparisons test)

** indicates $P < 0.05$ BDP alone vs FP alone (Kruskal-Wallis test & Dunn's multiple comparisons test)

*** indicates $P < 0.01$ Budesonide vs Oral steroid + ICS (Kruskal-Wallis test & Dunn's multiple comparisons test)

indicates $P < 0.05$ BDP alone vs Non-asthmatic (ANOVA & Tukey-Kramer multiple comparisons test)