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Hypothalamic-pituitary-adrenal axis suppression in asthma: A glucocorticoid receptor polymorphism may protect

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iii. Abstract and keywords

Background: Asthmatic children on corticosteroids can develop hypothalamic-pituitary-adrenal axis suppression (HPAS). Single nucleotide polymorphisms (SNPs) rs242941 and rs1876828 of the corticotrophin-releasing hormone receptor 1 (CRHR1) gene were associated with lower stimulated cortisol (F) levels, whereas rs41423247 of the glucocorticoid receptor (NR3C1) gene was associated with higher basal F levels. The objective of the current study was to confirm whether these three SNPs are associated with HPAS in asthmatic children.

Methods: DNA was extracted from saliva obtained from 95 asthmatic children, who had previously undergone basal F and metyrapone testing. Thirty-six children were classified as suppressed. Non-suppressed children were sub-classified according to their post-metyrapone adrenocorticotropin (PMTP ACTH) level into a middle (106-319 pg/ml) and a high (>319 pg/ml) ACTH response group. TaqMan[®] polymerase chain reaction assays were utilized.

Results: Only rs41423247 was inversely associated with HPAS (OR = 0.27 [95% CI 0.06-0.90]). Its GC genotype was inversely associated with HPAS (log odds = - 1.28, p = 0.021). $\sqrt{\text{PMTP ACTH}}$ was associated with CC (effect size = 10.85, p = 0.005) and GC genotypes (effect size = 4.06, p = 0.023). The C allele is inherited as a dominant trait (effect size = -1.31 [95% CI -2.39 – -0.33; p = 0.012]). In the high ACTH response group, both genotypes affected the PMTP ACTH (effect sizes 1.41 and 15.46; p-values 0.023 and $< 2 \times 10^{-26}$ for GC and CC respectively).

Conclusions: The C allele of rs41423247 was found to be protective against HPAS. CC genotype is associated with the highest PMTP ACTH response.

Key words: Asthma, hypothalamus, pituitary, adrenal, polymorphism, receptors, glucocorticoids, steroids/adverse events, adrenal insufficiency, pituitary-adrenal-function tests

iv. Main text

Key Messages

Rs41423247 (GC) of the NR3C1 gene was found to be inversely associated with hypothalamic-pituitary-adrenal axis suppression (HPAS) in asthmatic children on steroids. The CC genotype is associated with the highest adrenocorticotropin (ACTH) levels. If confirmed, the CC genotype could well be a marker of HPAS protection, thereby allowing for personalized treatment decisions to be made in asthma care.

1 BACKGROUND

Hypothalamic-pituitary-adrenal axis suppression (HPAS) is a well-recognised adverse effect of corticosteroid therapy.^{1,2} Whenever this is prescribed, endogenous cortisol (F) production is reduced. Under basal conditions no untoward effects will be apparent, because the decreased production of F is balanced by the supply of exogenous corticosteroids. In the long term, adrenal glands may atrophy. During a stressful event such as an infection, demand for cortisol may outstrip its exogenous supply. This can precipitate an adrenal crisis, which can result in death. The latter is the most devastating presentation, but occurs rarely.³ Chronic adrenal insufficiency, due to its non-specific clinical features,⁴ is frequently overlooked. For these reasons, it is imperative that HPAS is diagnosed in its subclinical state.

It has been shown that 16.3 % of asthmatic children treated with inhaled corticosteroids (ICS) and nasal steroids (NS) in Cape Town, South Africa, can develop HPAS when assessed with the metyrapone (MTP) test.^{5,6} If partial forms of suppression are included, even two-thirds of children may be affected. A higher body mass index (BMI) and poor adherence was found to be protective against HPAS, while the use of NS predicts it.⁵ Unfortunately suitable screening tests for HPAS are not available.^{7,8} Clinical and biochemical candidate tests include early morning serum F (within the normal reference range) and serum dehydroepiandrosterone sulphate, which correlate poorly with the MTP test. An early morning salivary cortisone has been suggested,⁹ but it has not been evaluated against a gold standard adrenal function test yet and its low positive predictive value would argue against its use. The adrenocorticotropin (ACTH) stimulation test, as a dynamic test, does not fulfil the requirements of a screening test.¹⁰ The only viable alternative would thus be to identify gene variants, which could predict proneness or resistance to developing HPAS.

At the time of planning and executing this study, only one paediatric asthma study, attempting a genetic-HPAS correlation, was identified. In Greek asthmatic children on ICS, homozygotes for the single nucleotide polymorphisms (SNPs) rs242941 (TT) and rs1876828 (AA) of the corticotrophin-releasing hormone receptor 1 (CRHR1) gene were associated with

lower stimulated cortisol (F) levels when tested with the low dose ACTH stimulation test ($0.5\mu\text{g}/1.73\text{m}^2$). Heterozygotes (GC) for rs41423247 (*BclI* restriction length polymorphism) of the glucocorticoid receptor (NR3C1) gene, on the other hand, were found to have higher basal F levels.¹¹ In the quest for universally applicable predictors of HPAS in asthmatic children on corticosteroids, the findings of the Greek study needed to be confirmed by a study, utilizing the gold standard MTP test in another population, to determine whether the SNPs rs242941 and rs1876828 of the CRHR1, and rs41423247 of the NR3C1 gene are associated with HPAS in asthmatic school children on corticosteroids.

2 METHODS

2.1 Participants

Asthmatic children and adolescents, 5-18 years old, treated with ICS and NS at the Lung Institute, Tygerberg and Red Cross Children's Hospitals in Cape Town, South Africa (previously enrolled during a clinic visit), whose hypothalamic-pituitary-adrenal axis (HPA) was assessed with an early morning basal serum F and MTP testing, were re-recruited, if they and their parents were willing to participate in the follow-up study.⁵⁻⁶ HPAS was diagnosed, if either $F < 83 \text{ nmol/l}$ or the post-MTP (PMTP) ACTH $< 106 \text{ pg/ml}$ (23.5 pmol/l), 11-deoxycortisol (11DOC) $< 208 \text{ nmol/l}$ and $11\text{DOC} + \text{C} < 400 \text{ nmol/l}$.^{5,12-14}

2.2 Study design and methodology

A cross-sectional study was performed. Height, weight, sex and age were recorded as measured previously. The BMI z-score (Centre of Disease Control) was computed. Salivary samples were collected with an Oragene DNA collection kit (OG-500). Samples were stored at -20°C . Once all the samples were collected, DNA was extracted. Genotyping for rs242941, rs1876828 and rs41423247 was performed by using TaqMan[®] polymerase chain reaction (PCR) assays. Ethical approval was granted by the ethics committees of both Stellenbosch University and the University of Cape Town. The study conformed to the standards of the Declaration of Helsinki. All participants and their parents gave their informed consent prior to inclusion in the study.

2.3 Statistical analysis

A sample size of 90 was calculated to provide power of at least 80% at a significance level of 0.05 (Appendix A). Analysis was done with the online statistical tool (<https://ihg.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl>) and the R statistical package. Fisher's Exact, Pearson's Chi-square and the Wald's tests were conducted to test for statistical difference of minor allele counts, SNPs and genotypes. PMTP ACTH data were square root transformed and a one-way ANOVA performed. The Wilcoxon test was used for pairwise multiple comparisons of differences in mean $\sqrt{\text{PMTP ACTH}}$ per SNP. Linear analysis for BMI z-score, $\sqrt{\text{PMTP ACTH}}$ and logistic analysis for HPAS were conducted. The best genetic model was selected on the basis of the lowest significant Akaike information criterion.

A total of 96 patients (94 from previous studies^{5,6} and two new patients) were recruited. One child had to be excluded, because of a discrepancy between the basal F level and the MTP test. The demographics, therapy and HPAS status of the 95 enrolled patients are listed (table 1). Only one child was on oral prednisone at the time of testing. According to the basal F and the PMTP ACTH response, 35 children were assigned to the suppressed ($F < 83$ nmol/l or $ACTH < 106$ pg/ml [23.5 pmol/l]), 29 to the middle ($ACTH 106-319$ pg/ml [23.5-70.8 pmol/l]) and 31 to the high ($ACTH > 319$ pg/ml [70.8 pmol/l]) ACTH response group. The respective baseline mean F levels were 243, 280 and 311 nmol/l ($p = 0.039$ between low and high, $p = 0.171$ between low and the middle and $p = 0.455$ between middle and high ACTH response group). There were no statistically significant differences in age, sex, glucocorticoid dose and asthma control between the three groups.

All three SNPs were in Hardy-Weinberg equilibrium (Appendix B). Of these only rs41423247 was associated with HPAS. The rs41423247 (G/C) SNP was inversely associated with HPAS (OR = 0.27 [95% CI 0.08-0.91], $p = 0.028$). Both rs242941 genotype AC and rs1876828 genotype CT were not associated with HPAS.. Similarly, for rs41423247, only the GC and the combined GC+CC genotypes were inversely associated with the suppressed ACTH response group (OR = 0.36 [95% CI: 0.14-0.92, $p = 0.030$] and OR = 0.30 [95% CI: 0.12 - 0.76, $p = 0.009$]). However, the homozygous CC genotype just missed statistical significance, as shown by the wide confidence interval crossing 1.00 (OR = 0.101 [95% CI: 0.005-1.908], $p = 0.041$). The mean $\sqrt{PMTP}$ ACTH of the CC genotype was significantly higher ($p = 0.002$) than for the GC and GG genotypes (fig 1). The respective means and their 95% CIs were 22.59 (16.18-29.00), 16.00 (13.00-19.00) and 11.82 (10.11-13.63). These correspond to real ACTH mean levels of 553.0 pg/ml (122.9 pmol/l) for the CC, 331.0 pg/ml (73.6 pmol/l) for the GC and 187.5 pg/ml (41.7 pmol/l) for the GG genotype.

The frequency of the guanosine (G) allele (the major allele) of rs41423247 was 77%, while the frequency of the cytidine (C) allele (the minor allele) was 23%. The genotype frequencies for GG, GC and CC were 60, 34 and 6% respectively.

BMI was associated with the CC genotype (table 2) while the heterozygous GC was associated with HPAS, independently of BMI (table 3). Both the homozygous CC and the heterozygous GC genotypes were associated with $\sqrt{PMTP}$ ACTH, with CC having a significantly larger impact than GC (table 4). The observed ACTH effect was independent of age, sex, height and weight (and hence of BMI). The higher the ACTH response, the greater and more significant the genotype (CC) effect (table 5).

After correcting for age, sex, height and weight, both the additive and the dominant genetic models were the best fit to describe the protective effect of the C allele on HPAS. The effect size for the additive model was -1.30 (-2.32 – -0.42; $p = 0.007$), for the dominant model, the effect size was -1.31 (95% CI -2.39 – -0.33; $p = 0.012$). Similarly, the effect of the C allele on $\sqrt{PMTP}$ ACTH is best described by an additive (effect size = 4.42 [95% CI 1.77 – 7.07; $p = 0.002$]) and a dominant model (effect size = 4.79 [95% CI 1.54-8.04; $p = 0.005$).

4 DISCUSSION

The SNP rs41423247 (GC) of the NR3C1 gene was found to be protective against HPAS, while no association with HPAS was found with rs242941 and rs1876828 of the CRHR1 gene. In contrast, in Greek asthmatic children basal or stimulated F levels were different for all three SNPs.¹¹ The discrepancy in the findings between results of the two studies can be explained by the use of different adrenal function tests. In the Greek study, the low dose ACTH stimulation test (not the gold standard) was used without defining definitive cut-offs^{1,2} for basal and stimulated F levels. Relying on statistical differences of these levels vis-a-vis their respective genotypes may be misleading. Furthermore, serum F is a very crude test, which is influenced by many variables¹ e.g. by Circadian rhythm. There is no record in Tsartsali et al's paper, whether the basal F levels were taken in the early morning (which is essential). The MTP test utilized in this study is a gold standard adrenal function test with clearly defined criteria for HPAS. Analyses based on this test can be expected to produce valid results.¹

In a recently published genome-wide association study (GWAS) investigating susceptibility to HPAS in asthmatic children, screening was not performed for rs41423247 (GC).¹⁵ Instead rs591118, an intronic variant of the PDGFD gene, was significantly associated with HPAS. This particular SNP was not included in the current study as the study was already completed when the paper reporting rs591118 was published.

In the current study, patients with rs41423247 genotype CC had higher ACTH levels than patients with GC and GG genotypes. Some adults with depression are reported to have hypercortisolism.¹⁶ A study investigating the link between these patients and genetic polymorphism of the NR3C1 gene utilised the dexamethasone/corticotropin-releasing hormone (CRH) test. In this study a poor response to anti-depressive therapy was associated with marginally lower median peak ACTH levels in patients with the CC genotype.¹⁶ The results of this study could however have been confounded by the use of a variable dose of triiodothyronine given to some patients, which could have induced unrecognised adrenal insufficiency in individuals at risk.¹⁷ Tsartsali et al¹¹ and others¹⁸ have reported that the GC genotype was associated with a significantly higher basal F level than the CC genotype. The GG genotype conversely was associated with lower F levels after a dexamethasone suppression test in Dutch elderly individuals.¹⁹ The authors interpreted this as "hypersensitivity" to glucocorticoids. However, on re-analysis of their paper, CC is associated with a significantly higher F level post-dexamethasone, suggesting glucocorticoid resistance of the CC genotype.

Both the HPAS-protective effect and the ACTH response seem to be inherited in an additive and dominant fashion. A similar phenotype is therefore expected with the heterozygous GC and homozygous CC genotype, with the difference that the phenotypic effect of the latter should be more pronounced. Although the overall sample size was probably adequate, the low minor allele frequency of 23% (compared to 38% in Europe²⁰) and the low CC genotype frequency of 6% (compared to 40% in Greek children) limit the interpretation of the study. This resulted in overlapping CIs of the GC and CC genotypes and an incalculable lower bound of the CC CI. However, the additive genetic effect accounted for a large effect size and a highly significant p-value ($<2 \times 10^{-16}$), which even far exceeds the

required significance threshold for a GWAS (5×10^{-8}).²¹ Hence, pending confirmation in a larger study, the most suitable marker for HPAS protection would still be rs41423247 (CC) of the NR3C1 gene.

The biochemical phenotype attributed to both CC and GC genotypes of rs41423247 is akin to the one described in the syndrome of primary generalized glucocorticoid resistance (PGGR).²²⁻²⁴ In this syndrome there is dysregulation of F feedback at hypothalamic and pituitary level, resulting in elevated ACTH and possibly F levels. In the current study, early morning basal F levels of the children with rs41423247 were not elevated (results not shown). Clinical features of this syndrome e.g. hypertension, hirsutism or premature pubarche, were not detected. Hence, the biochemical phenotype of rs41423247 (GC and CC) could represent tissue-specific glucocorticoid resistance.²⁴ More likely, it is just a common variant in the population (40% in our sample) presenting with reduced glucocorticoid sensitivity.²⁴

PGGR is associated with 26 mutations²⁴ and 8 pathogenic SNPS²⁵ in the exome of NR3C1 at 5q31.3, while rs41423247 falls within the intronic regulatory region of the gene,²⁰ confirmed independently by variant browser analysis (result not shown). Mechanistically, impaired promoter function would lead to impaired DNA transcription, RNA translation and protein production.

In a recently published study on asthma children in Turkey, the FEV₁ of children with the GG genotype improved more after a high dose of ICS than children with the CG+CC genotype.²⁶ In the current study, three of five children with the CC genotype had a suboptimal FEV₁ (result not shown). Unfortunately, the number is too small to draw any valid conclusions from it.

In this study population, it was previously shown that children with a higher BMI z-score had higher ACTH levels.⁵ In the current study, an association between CC genotype and BMI z-score could be demonstrated. No association of genotype was found with overweight or obesity (results not shown). In Dutch elderly the GG genotype was associated with a lower BMI¹⁹ in line with the current study. Others have found the opposite.²⁷ The higher BMI z-score associated with rs41423247 is probably mediated through periodically elevated F levels. However, as shown, the BMI z-score does not confound the SNP effect on the ACTH level.

As findings in Greek and South African children (and possibly Turkish children and Dutch adults) are similar, it is likely that the results of this study are generalizable across all populations and ages. Pathophysiological and molecular explanations of the observed phenotype may also be equally applicable to both children and adults. However, the study is limited inadvertently by the small sample of children with the CC genotype. A larger study should therefore be performed to confirm the finding. In addition, a “proof of concept” study should be done (screening asthmatic children for rs41423247 (CC), and when positive, testing them with the MTP test to confirm the phenotype). If confirmed, rs41423247 (CC), as a marker of HPAS protection, should be screened for in asthmatic children on glucocorticoids. This would enable clinicians to choose which children can be safely treated with higher doses of glucocorticoids – a first step towards personalized medicine in asthma therapy.

In conclusion, rs41423247 (GC) of the NR3C1 gene was found to be protective against HPAS. The CC genotype was associated with the highest ACTH levels. The C allele is inherited as an additive and dominant trait. The GC genotype was independently associated with BMI z-score. If confirmed by a larger and a “proof of concept” study, rs41423247 (CC)

could well be a marker of HPAS protection, thereby allowing for personalized treatment decisions to be made in asthma care.

v. ACKNOWLEDGEMENTS

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vii. APPENDIX

A. Sample size calculations

In the previous study 30 patients had hypocortisolaemia or HPAS. The cross-sectional design allowed for an equal number of patients to be analysed across the full spectrum of post-MTP responses i.e. low, midrange and high (30 subjects each). The ACTH mean and standard deviation (SD) were calculated (ACTH mean=330 pg/ml [73.3 pmol/l], SD=280 pg/ml [62.2 pmol/l]) from the previous data. The proportional distribution of the genetic subgroups was derived from the paper of Tsartsali et al, table 4.7.5. Power analysis with a sample size of 90 was done across the three ACTH groups. Significance level was taken as 0.05. A one-way analysis of variance test (ANOVA) was considered for the power analysis. The following mean values were specified under the alternative hypothesis: group 1 (low range) 230 pg/ml (51.1 pmol/l), group 2 (midrange) 330 pg/ml (73.3 pmol/l), group 3 (high range) 430 pg/ml (95.5 pmol/l). This gives a 100 ACTH units difference between two adjacent groups. This is a relative difference of 36% of the SD. $[(100/280) = 0.36]$ which is a conservative expected effect size. Even for unbalanced groups such as $n_1=24$, $n_2=35$ and $n_3=40$, the power will be 81%. Thus, the power is good if the sample size distribution across the genetic subgroups is moderately unbalanced. Power is improved by square root transformations of ACTH to stabilise the within group variances.

B. Table: Tests for deviation from Hardy-Weinberg equilibrium

SNP	Suppressed ACTH level [†] p-value	Middle/high ACTH level [†] p-value
rs242941	0.830	0.580
rs1876828	0.791	0.481
rs41423247	0.445	0.948

SNP, single nucleotide polymorphism

[†] Post-metyrapone test

viii. Tables

TABLE 1 Demographics, therapy and HPAS status

Variable	Amount/type
Total n	95
Boys:girls	49:46
Age, median (range), years	10.0 (5.2-17.0)
Ethnic group, n	
Mixed ancestry	93
Caucasian	1
African	1
BMI z-score, median (range)	-0.05 (-3.10-2.68)
Lung functions [†]	
FEV ₁ , % predicted, median (range)	84.6 (42.0-136.2)
FEV ₁ /FVC, % predicted, median (range)	94.6 (45.0-118.0)
ICS and device, n	
BUD MDI and spacer	85
BUD MDI	7
FP DPI	2
Daily ICS dose/m ² , median (range)	
BUD equivalent, µg	473 (108-1111)
HC equivalent [‡] , mg	5.9 (1.9-13.9)
NS	
BDP	80
BUD	4§
Daily NS dose/m ² , median (range)	
BUD equivalent, µg	122 (54-556)
HC equivalent, mg	1.5 (0.7-6.9)
Total steroid dose/m ² , median (range)	
BUD equivalent, µg	609 (161-1405)
HC equivalent, mg	7.6 (2.0-17.6)
HPAS status, numerator/denominator (%)	
F < 83 nmol/l	6/95 (6.3)
HPAS [§]	23/46 (50)
PMTP ACTH <106 pg/ml (23.5 pmol/l)	35/89 (39)

HPAS, hypothalamic-pituitary-adrenal axis suppression; ICS, inhaled corticosteroids; BUD, budesonide, MDI, metered dose inhaler; FP, fluticasone propionate; DPI, dry powder inhaler; NS, nasal steroid; HC, hydrocortisone; beclomethasone dipropionate; F, cortisol; PMTP, post-metyrapone

[†] Three children had no lung functions recorded

[‡] Eighty micrograms BUD is comparable to 1 mg HC in potency

[§] PMTP ACTH < 106 pg/ml (23.5 pmol/l), 11-deoxycortisol (11DOC) < 208 nmol/l and 11DOC+C < 400 nmol/l

TABLE 2 Linear regression model of BMI z-score versus genotype

Variable	Effect size	95% CI	P
Intercept	-0.83	-1.84 – 0.18	0.110
Rs41423247 (G/C)	0.28	-0.22 – 0.78	0.272
Rs41423247 (C/C)	1.04	0.02 – 2.06	0.049*
Age	0.03	-0.05 – 0.11	0.490
Sex	0.35	-0.13 – 0.82	0.159

* Significant, P, <. 05

TABLE 3 Logistic regression model of HPAS versus genotype

Variable	Log odds	95% CI	P
Intercept	6.21	-0.48 – 13.07	0.068
Rs41423247 (G/C)	-1.28	-2.43 – -0.24	0.021*
Rs41423247 (C/C)	-18.12	Incalc. – 151.41	0.991
Age	0.06	-0.27 – 0.40	0.700
Sex	-0.50	-1.48 – 0.46	0.310
Height	-0.05	-0.12 – 0.03	0.203
Weight	-0.01	-0.11 – 0.05	0.771

HPAS, hypothalamic-pituitary-adrenal axis suppression; Incalc, incalculable

* Significant, P, <. 05

TABLE 4 Linear regression model of $\sqrt{\text{PMTP ACTH}}$ versus genotype

Variable	Effect size	95% CI	P
Intercept	-5.30	-26.17 – 15.58	0.620
Rs41423247 (G/C)	4.06	0.64 – 7.50	0.023*
Rs41423247 (C/C)	10.85	3.43 – 18.28	0.005*
Age	-0.40	-1.47 – 0.67	0.468
Sex	-0.85	-4.06 – 2.35	0.602
Height	0.14	-0.09 – 0.36	0.232
Weight	0.06	-0.15 – 0.26	0.591

PMTP, post-metyrapone

* Significant, P, <. 05

TABLE 5 Multinomial regression model of ACTH response versus genotype

Variable	Effect size	95% CI	P
High ACTH, intercept	-5.46	-13.43 – 2.51	0.180
High ACTH, rs41423247 (G/C)	1.41	0.19 – 2.63	0.023*
High ACTH, rs41423247 (C/C)	15.46	14.17 – 16.75	<2x10 ⁻²⁶ **
High ACTH, age	-0.01	- 0.38 – 0.36	0.960
High ACTH, sex	-0.08	-1.21 – 1.06	0.894
High ACTH, height	0.03	-0.06 – 0.11	0.535
High ACTH, weight	0.03	-0.06 – 0.12	0.559
Middle ACTH, intercept	-8.72	-16.84 - -0.61	0.035
Middle ACTH, rs41423247 (G/C)	1.13	-0.11 – 2.36	0.073
Middle ACTH, rs41423247 (C/C)	14.48	13.19 – 15.77	<2x10 ⁻²⁶ **
Middle ACTH, age	-0.01	-0.47 – 0.28	0.621
Middle ACTH, sex	1.07	-0.08 – 2.22	0.069
Middle ACTH, height	0.07	-0.02 – 0.15	0.123
Middle ACTH, weight	-0.02	-0.13 – 0.09	0.712

* Significant, P, <. 05

** Significant, P, <. 5 x 10⁻⁸

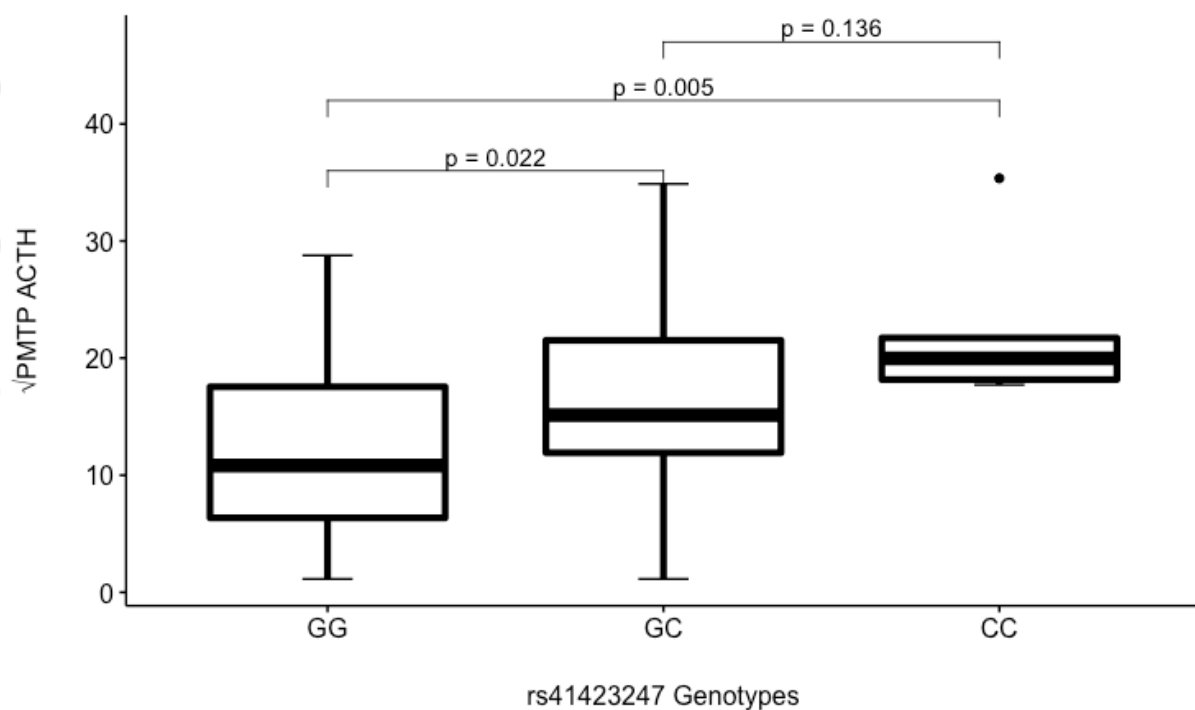


FIGURE 1 Difference in $\sqrt{\text{PMTP ACTH}}$ across genotypes of rs41423247 as analysed by ANOVA