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HTR2C Gene Variant and Salivary Cortisol Levels after Endurance Physical Activity: A Pilot Study

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Keywords
Cortisol · Genotyping · Physical activity · Nutrigenetics · Stress

Abstract
Introduction: The 5-hydroxytryptamine 2C receptor (*HTR2C*) rs6318 polymorphism has been associated with increased sensitivity to stress. This study investigated whether the rs6318 genotype modified the cortisol response to endurance physical activity. ***Methods:*** The *HTR2C* SNP was genotyped in a population of agonistic cyclists, and salivary cortisol levels were measured before and after an endurance competition. ***Results and Conclusion:*** Salivary cortisol levels increased after the competition (from 20.72 ± 12.36 ng/mL to 33.80 ± 21.53 ng/mL; $p = 3.189 \times 10^{-5}$). rs6318 C carriers displayed higher baseline cortisol levels compared to G carriers (26.60 ± 9.35 ng/mL vs. 19.50 ± 12.63 ng/mL; $p = 0.04$). Baseline cortisol levels were able to predict the cortisol response to exercise ($\beta = -0.846$; $p = 1.2 \times 10^{-5}$). Although regression analysis did not identify an association between *HTR2C* genotype and change in cortisol levels, a secondary analysis in which the population was classified by median cortisol changes suggested that they might be weakly associated, thus warranting further investigation.

Introduction

Physical exercise affects circulating levels of cortisol, which is taken up by the liver, adipose tissue, and skeletal muscle, and thus it plays a critical role in exercise capacity and recovery [1]. The contribution of genetic factors to hypothalamic-pituitary-adrenal activity is well established; however, both an intrinsic variability and sensitivity to the environment exist. Morning plasma cortisol heritability is about 30–60% [2]. Recent evidences indicate a nonsynonymous polymorphism in the 5-hydroxytryptamine 2C receptor (*HTR2C*) gene as a predictor of the stress-driven cortisol response. The *HTR2C* gene encodes for a serotonin receptor and contains a polymorphism (rs6318) characterized by a serine to cysteine substitution (Cys23Ser) in the coding region. The frequency of this x-linked nonsynonymous polymorphism in a Toscani population is approximately 16.7% and it has been shown to vary in different ethnicities [3]. The Ser23C allele is constitutively more active than the Cys23G allele [4], and male hemizygotes for the Ser23C allele have a 2-fold increase in plasma cortisol during recall of stressful events compared to G carriers [5]. This study investigated the effect of the rs6318 SNP on cortisol production before and after an endurance physical activity in a homogenous population of trained Italian adult men.

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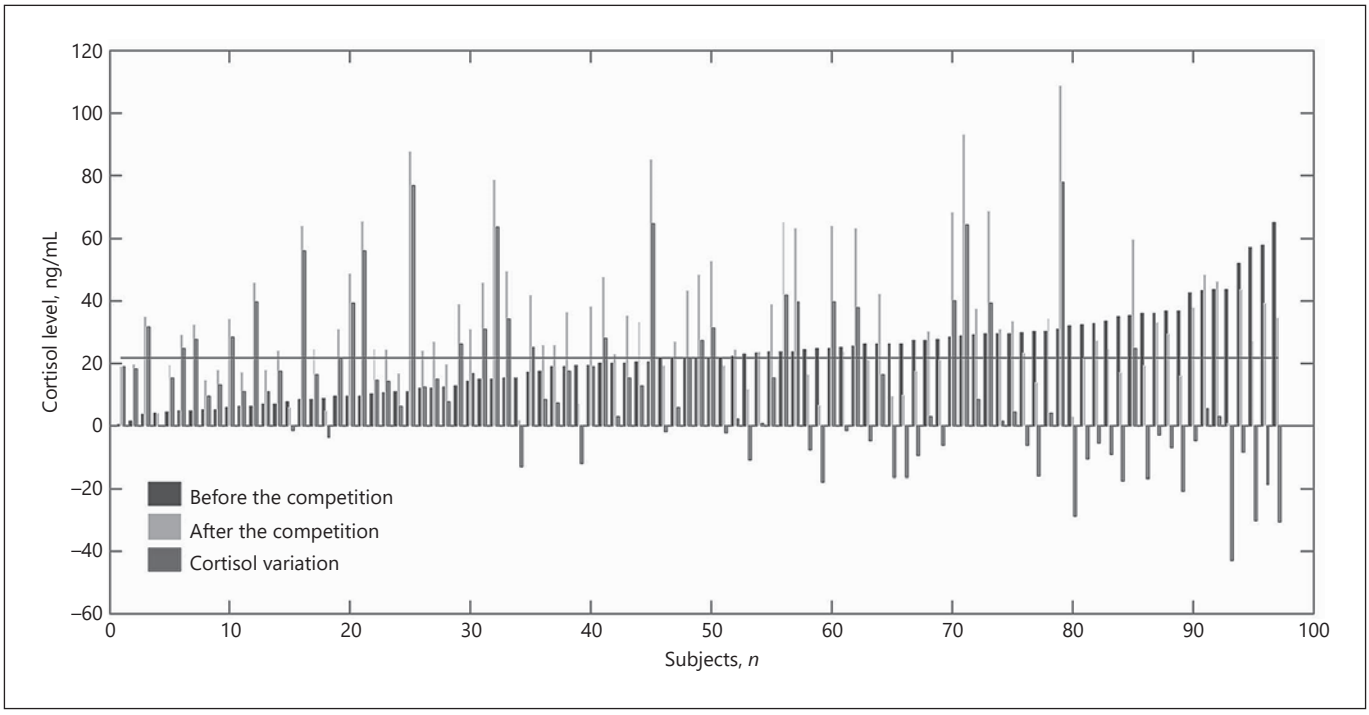

Fig. 1. Bar plot describing the exploratory analysis of cortisol distribution in the total study population. Cortisol levels (ng/mL) before and after the competition, together with the calculated variation of the hormone (Δ-cortisol), are reported for each subject. Subjects are sorted by baseline cortisol level. The average baseline cortisol level is represented by the black horizontal line.

Materials and Methods

Population Recruitment and Sample Collection

Subjects were recruited on a voluntary basis during the cycling race Granfondo Terre dei Varano (a 150-km race in Sibillini National Park). Informed consent was obtained from all of the participants. The Direction of the School of Pharmacy of The University of Camerino approved the study protocol, which is in accordance with the Declaration of Helsinki in its revised edition and with international and local regulatory requirements. Buccal swabs were collected the day before the competition. Saliva samples were collected in the morning before the start of the competition (i.e., baseline) and after completion of the race. Biological samples were numerically encoded to ensure anonymity and they were discarded after the analysis.

Cortisol Assessment

Free cortisol was analyzed from saliva samples using a DRG Salivary Cortisol ELISA Kit (SLV- 2930; DRG Instruments GmbH, Germany). Data are expressed in nanograms per milliliter (ng/mL) at 2 decimal places in accordance with the sensitivity and reproducibility of the assay.

DNA Extraction and Genotyping

Genomic DNA was extracted from buccal swabs and genotyped for the rs6318 polymorphism using a TaqMan SNP genotyping assay (Applied Biosystems, USA), according to the manufacturer's instructions. Fifteen percent of the samples were repeated, showing 100% concordance in genotype call.

Statistical Analysis

Statistical analyses were performed using the SPSS package for Windows, v.20.0 (SPSS Inc., Chicago, IL, USA) and R software. A Shapiro-Wilk test was used to evaluate the normality of data distributions.

Primary analyses consisted of regression models, accounting for age as a covariate, to examine SNP × cortisol associations. Wilcoxon and Pearson χ^2 tests were used as a secondary analysis involving categorical variables. The level of statistical significance was defined by two-tailed p values <0.05. The probability for a null hypothesis that the coefficients have a value of zero (Pr) is for regression analyses.

Results

Demographics

The analyzed population was composed of 128 Italian adult males (aged 42.53 ± 10.22 years). The mean cortisol levels were 20.72 ± 12.36 ng/mL and 33.80 ± 21.53 ng/mL before and after the endurance exercise, respectively (Appendix); 16.9% of the population was hemizygous for the

C allele, while the remaining 83.1% carried the G allele. This result is consistent with available data from the 1000 Genomes Project regarding the minor allele frequency (i.e., 0.167) for rs6318 in a Toscani population [3]. No significant differences in age distribution were detected between C and G allele carriers in the sample ($p > 0.05$). The Appendix shows the complete descriptive statistics for the total population, as well as the population divided by genotype.

Effects of Physical Activity on Cortisol Levels

Results show a significant increase in average cortisol levels after the competition (baseline cortisol: 20.72 ± 12.36 ng/mL, cortisol after exercise: 33.80 ± 21.53 ng/mL; paired Wilcoxon test, $p = 3.189 \times 10^{-5}$) (Appendix). This increase did not occur homogenously in the population. Exploratory analysis revealed that subjects showing the largest increase after the competition had the lowest baseline cortisol levels (Fig. 1). The association between baseline cortisol levels and the change in cortisol levels after the competition (Δ-cortisol) was statistically significant (unpaired Wilcoxon test, $p = 2.433 \times 10^{-5}$).

Effects of HTR2C *Genotype on Cortisol Levels in Response to Physical Activity*

Higher baseline cortisol levels were detected in C-allele carriers compared to G-allele carriers (C-allele carriers: 26.60 ± 9.35 ng/mL, G-allele carriers: 19.50 ± 12.63 ng/mL). Differences in baseline cortisol levels between alleles were significant using a regression model analysis accounting for age as a covariate (regression analysis, Pr = 0.01; $p = 0.04$). Age did not influence this association (regression analysis, Pr = 0.99). When we subsequently divided the population into 2 groups based on median baseline cortisol levels, we observed that 81.8% of C-allele carriers had higher baseline cortisol levels than the median of our analyzed population, compared to only 45.3% of G carriers (Pearson χ^2 test; $p = 0.002$; Table 1).

We next examined whether the rs6318 SNP was associated with cortisol levels after the competition, as well as changes in cortisol levels during the competition (i.e., Δ-cortisol). We did not find a significant association between genotype and cortisol levels after the competition either using a regression analysis (Pr = 0.71; $p > 0.05$) or when we considered the dichotomous distribution of data in our population (Pearson χ^2 test; $p > 0.05$; Table 1). Similarly, a regression analysis did not find a significant association between the *HTR2C* genotype and Δ-cortisol (Pr = 0.32; $p > 0.05$). However, we conducted a secondary analysis in which we divided the population based on the median Δ-cortisol and found a weak association. Specifically, 76.5% of C-allele carriers displayed a decrease in cortisol after the competition with regard to the median Δ-cortisol compared to 47.5% of the G-allele carriers (Pearson χ^2 test; $p = 0.027$; Table 1). Regression analyses revealed that age did not influence cortisol levels after the competition (Pr = 0.38) or Δ-cortisol (Pr = 0.43).

Table 1. Distribution of genotypes in the population

	rs6318 genotype				*p*
	G		C		
	n	%	*n*	%	
Basal cortisol					
Under the median	58	54.7	4	18.2	**0.002**
Over the median	48	45.3	18	81.8	
Cortisol after the competition					
Under the median	49	50.5	8	40	>0.05
Over the median	48	49.5	12	60	
Δ-Cortisol					
Under the median	38	47.5	13	76.5	**0.027**
Over the median	42	52.5	4	23.5	

p values (Pearson's χ^2 test) are shown for each subgroup analysis.

Discussion/Conclusion

The *HTR2C* rs6318 SNP has been previously related to the hypothalamic-pituitary-adrenal axis-mediated response to stress, cardiovascular diseases, and blood cortisol levels [4, 6]. However, no data can be found in the literature regarding the association of this polymorphism with saliva cortisol levels. Considering the influence of physical exercise (both acute and chronic) on changes in cortisol levels [7, 8], saliva has been used in several recent studies to assess variations in this hormone in response to exercise and training instead of blood or urine collection. A high correlation between salivary and serum cortisol levels has been reported, both in basal and exercise conditions [7, 8]. Salivary cortisol reflects the free, biologically active fraction of this hormone [8], and cortisol changes in response to exercise appear to be more pronounced in saliva compared to blood [9]. Therefore, measurements of steroid hormones in saliva can provide a reference for their respective blood concentrations, and they provide useful information with a simple and noninvasive approach.

In this pilot study, we demonstrated that endurance activity (150-km cycling race) increased salivary cortisol

levels, in accordance with previous data reported on plasma cortisol levels [10]. Remarkably, the cortisol response to physical activity in our population was quite heterogeneous, and these differences are partly related to the *HTR2C* rs6318 polymorphism. In particular, compared to G-allele carriers, Ser23C carriers showed increased salivary baseline cortisol levels (as previously reported when measuring plasma levels of this hormone [4, 6]), which in turn were found to predict changes in cortisol levels during exercise. The variation in the cortisol response to physical activity is not completely explained by this one SNP, as indicated by our regression analyses. It is highly probable that other genetic loci, which may also have an impact on cortisol levels, can influence the levels of this hormone both at baseline and during exercise. Furthermore, we acknowledge that our pilot study had a limited sample size and the variability in baseline cortisol measurements could reduce the power of this study. Nevertheless, these results have a potential importance considering that cortisol monitoring in sports could be used to screen a person's stress response to physical exertion [8]. Indeed, evidence from this study supports measurement of cortisol in saliva as a suitable and noninvasive method (comparable to blood) to investigate personalized steroid hormone responses to physical activity. Monitoring hormonal responses to exercise is a useful indicator to avoid overreaching or overtraining. Indeed, a robust increase in resting cortisol levels together with a markedly lower cortisol response after exercise are characteristic of athletes that reported excessive training fatigue or overreaching, probably as a result of "exhaustion" of the HPA axis due to exercise [11]. An interesting parallel can be proposed for the Ser23C hemizygous in our study population. For example, we can speculate that the hyperresponsiveness of the HPA axis that characterizes Ser23C carriers could reflect a potential susceptibility to overreaching by endurance training for these subjects compared to the general population.

These initial data from our pilot study demonstrate that C-allele carriers have significantly higher baseline salivary cortisol levels, which in turn is a major determinant of the dichotomic distribution observed for cortisol variation measured after the competition. Indeed, baseline cortisol levels were found to predict the cortisol response after exercise (regression analysis, $\beta = -0.846$; $p = 1.2 \times 10^{-05}$). Future analyses in a larger population are needed in order to confirm our preliminary results, but our data may suggest that *HTR2C* C-allele carriers, who have higher baseline cortisol levels, tend to have reduced levels of this hormone after physical activity.

In conclusion, knowledge of the genetic determinants related to stress management could be useful to personalize training and optimize performances from both a motivational/psychological perspective and a nutritional perspective. For example, recent investigations have demonstrated that carbohydrate ingestion immediately prior to mental and physical stress may attenuate cortisol responses [12]. Furthermore, given that stress may increase the risk of overreaching and/or cardiovascular events, special attention must be given to individuals genetically prone to cortisol impairments and cardiovascular diseases. Further research should be conducted with the aim of validating the hypothesis emerging from this pilot study in bigger populations and identifying practical advice for an improved management of stress and cortisol responses in terms of nutrition, sport, and mental health precautions, specifically for individuals susceptible to this kind of problems.

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Statement of Ethics

The subjects gave written informed consent. The study protocol was approved by the research institute's committee on human research.

Disclosure Statement

The authors have no conflict of interests to declare.

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Author Contributions

This study was designed by R.G. and L.B. Data were collected and analyzed by L.B., D.F., and M.P. Interpretation of the data and preparation of this paper were undertaken by L.B., D.F., M.P., and R.G. All of the authors approved the final version of this paper.

Appendix

Descriptive Statistics of the Total Population and the Population Divided by rs6318 Genotype

	Subjects, *N*	Min	Max	Mean	SD	Skewness	SE	Kurtosis	SE
Total sample									
Age	128	20	67	42.53	10.22	0.158	0.199	–0.444	0.395
Baseline cortisol level, ng/mL	128	1.40	65.00	20.72	12.36	0.836	0.213	1.073	0.423
Cortisol level after the competition, ng/mL	117	1.74	108.50	33.80	21.53	3.638	0.223	22.543	0.442
Δ-Cortisol, ng/mL	97	–43.03	77.65	11.45	23.69	3.281	0.244	20.045	0.483
C-allele carriers									
Baseline cortisol level, ng/mL	22	1.61	43.36	26.60	9.35	–0.852	0.481	0.888	0.935
Cortisol level after the competition, ng/mL	20	2.86	108.50	34.25	22.58	3.397	0.501	12.885	0.972
Δ-Cortisol, ng/mL	17	–28.87	77.65	5.82	24.69	3.203	0.536	11.440	1.038
G-allele carriers									
Baseline cortisol level, ng/mL	106	1.40	65.00	19.50	12.63	1.140	0.235	1.721	0.465
Cortisol level after the competition, ng/mL	97	1.74	99.08	33.71	21.43	1.010	0.245	0.780	0.485
Δ-Cortisol, ng/mL	80	–43.03	76.72	12.65	23.45	0.372	0.269	0.215	0.532

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