RESEARCH ARTICLE

Genetic polymorphisms in the serotonin receptor 7 (*HTR7*) gene are associated with cortisol levels in African American young adults [version 1; peer review: 1 not approved]

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Abstract

Introduction: Serotonin is a neurohormone involved in biological processes, such as behavior and immune function. Chronic psychosocial stressors may cause serotonin release resulting in immune system dysregulation, as evidenced by increased or far decreased levels of cortisol, a blood biomarker of stress and immune function. We hypothesize that genetic polymorphisms in the *HTR7* gene are associated with both hypo- and hyper-cortisolism. **Methods:** The study population included 602 African American subjects between 18-34 years of age, living in Washington, D.C. Five single nucleotide polymorphisms (SNPs) in *HTR7*, rs2420367, rs12412496, rs2185706, rs7089533, and rs7093602 were genotyped by restriction fragment length polymorphism or the TaqMan assay. Statistical analysis, using the program SNPstat, was performed to determine their associations with cortisol measured in the study population. **Results:** While an increased risk of hypocortisolism was found to be associated with rs2420367, rs2185706, and rs7093602 in a gender specific manner, no genotypes could be associated with hypercortisolism. Inversely, a decreased risk of hypocortisolism was found with the haplotype CGGCC (p=0.033), which remained significant in males. When adjusting for gender, females associated with the haplotype AGACC. Hypercortisolism was also associated with a decreased risk for the haplotypes AAACC (p=0.042) and AAGTT (p=0.001). **Discussion:** Based on these results, genetic variation in the *HTR7* gene may contribute to both stress and inflammation, and will provide a new glimpse into stress-related inflammation psychophysiology.

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Introduction

Psychosocial stressors, such as exposure to interpersonal and community violence, may impact immune function (O'Connor *et al.*, 2000), leading to the development of a number of health disparities (Black, 2003; Murali *et al.*, 2007). During exposure to a stressor, many hormones are released in the brain, including serotonin (5-HT). 5-HT is a neurohormone that functions in the regulation of a variety of psychological and physiological processes, including behavior and inflammation (Idzko *et al.*, 2004; Mikulski *et al.*, 2010). Serotonin receptor 7 (*HTR7*) is the most recently described serotonin receptor, and it is found expressed on a number of immune cells, including macrophages and T lymphocytes (Ahern, 2011). Studies indicate that *HTR7* activation results in the production of pro-inflammatory cytokines from immune cells, such as microglial cells, dendritic cells, and monocytes (Dürk *et al.*, 2005; Mahé *et al.*, 2005; Müller *et al.*, 2009). Cytokines, such as interleukin-6 (IL-6), are then involved in the production of a variety of blood biomarkers, including cortisol, the major stress hormone (de Kloet *et al.*, 2005; Howren *et al.*, 2009; Maeda *et al.*, 2010).

Cortisol is involved in the regulation of a number of biological processes, such as cellular metabolism and immune function (Anagnostis *et al.*, 2009; Lundberg, 2005; Webster Marketon & Glaser, 2008). Both hypercortisolism and hypocortisolism have recently been associated with a number of health disparities, including cardiovascular disease and asthma (Buske-Kirschbaum *et al.*, 2003; Manenschijs *et al.*, 2013). During a stress response, cortisol concentration is increased resulting in a shift towards an inflammatory and humoral response (Murali *et al.*, 2007; Straub *et al.*, 2002). Under normal circumstances, this response will be limited and shut off when cortisol levels decrease back to normal levels (Ehlert *et al.*, 2001). If a stressor persists for an extended period of time, the cortisol concentration will continue to increase until the system exhausts its supply (McEwen, 2004). Both cases, whether amplified or depressed, can lead to serious conditions, such as metabolic syndrome and irritable bowel syndrome (Anagnostis *et al.*, 2009; Fries *et al.*, 2005).

An increasing number of single nucleotide polymorphisms (SNPs) in genes for neurohormone receptors have also been linked to a number of health disparities. These diseases include breast cancer, depression, diabetes, and asthma (Deming *et al.*, 2012; Kim *et al.*, 2011; Kring *et al.*, 2009; Lucae *et al.*, 2010). While SNPs in these genes have been identified, there is limited data on the molecular pathways involved in these relationships. With regard to SNPs found in serotonin receptors, the mechanisms used by serotonin receptor 2A (*HTR2A*) in relation to disease state have begun to be identified (Beretta *et al.*, 2008; Snir *et al.*, 2013). It is possible that SNPs within *HTR7* modulate the induction of pro-inflammatory cytokines from immune cells during times of stress, leading to a predisposition towards the development of health disparities. Previous findings prompted us to investigate whether genetic variation in *HTR7* associates with the changes in function of the stress and immune system measured by cortisol levels in African American (AA) young adults.

Methods and materials

Study population

All DNA and sample data were collected during a previous study done between 2010 and 2012 entitled “Gender Differences in the Experience of Violence, Discrimination, and Stress Hormone in African Americans: Implications for Public Health”, which examined the genetic markers for alcohol and depression, violence exposure, and drug use in AA young adults (unpublished study; Jackson L, Shestov M, Abbas M and Saadatmand F). In total, DNA samples from 602 AA individuals living in the Washington, D. C. area were available for genotyping. All individuals, both male and female, ranged between the ages of 18 and 34 years old. In an Audio Computer-Assisted Self-Interviewing (ACASI) survey, only 590 participants provided information concerning behavioral data, including information concerning housing, income, and violence exposure (Table 1). Blood biomarkers, including cortisol (472 out of 602), were determined after blood collection in the morning (Dataset 1; Swanson *et al.*, 2016a). All analyses were performed in a case-control manner for both hypercortisolism (case1, participant cortisol concentration >14 µg/dl; control1, participant cortisol concentration <14 µg/dl) and hypocortisolism (case2, participant cortisol concentration <5 µg/dl; control2, participant cortisol concentration >5 µg/dl). Consent was obtained from each participant in the study, and approval from Howard University’s Institutional Review Board was obtained (approval number, IRB-16-MED-03).

Dataset 1. Raw data for genetic polymorphisms in the serotonin receptor 7 (*HTR7*) gene are associated with cortisol levels in African Americans young adults

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Cortisol measurements were determined in Mic-Gr/dL and recorded as a numerical value or the three values as follows; -9, indicate participants in which no cortisol was measurable in the participant; NA, indicate participants in which blood samples were not taken; √, were taken as case values for analysis. NA for sex and age of participant indicate the participant did not wish to specify. Within SNP genotypes, NA indicates that no genotype was determined.

SNP selection

SNPs in *HTR7* were downloaded from NCBI using the SNP: Gene View (https://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?chooseRs=all&locusId=3363&mrna=NM_019859.3&ctg=NT_030059.14&prot=NP_062873.1&orien=reverse&refresh=refresh) for the comparison of allele frequencies between the CEU, Caucasian descent, and YRI, Yoruban descent, populations. Differences between CEU and YRI populations was of interest as African American’s are likely to possess frequencies that fall between those of Caucasian and Yuroban descent. Four SNPs (rs12412496, rs2185706, rs7089533, and rs7093602) were selected for inclusion in the study based on a large difference in allele frequency between the CEU and YRI populations. The SNP rs2420367 was selected based on no frequency data being recorded with any population besides CEU (Table 2). All of the chosen SNPs were located in the first intron region of the gene, as indicated on NCBI’s SNP: Gene View.

Table 1. Demographic data for the study population of 602 participants.

Demographics include gender, age, housing and income status, and violence exposure.

Characteristic	N	%	Mean (SD)	Range
Gender				
Female	197	32.72		
Male	317	52.66		
Unspecified	88	14.62		
Age, years			21.6	18-34
Housing				
Rent, own, or payed by the government	366	62.03		
Public housing	73	12.37		
Unspecified	151	25.59		
Income source				
Work	380	64.41		
Welfare or public assistance	69	11.69		
Part work part welfare	86	14.58		
Unspecified	55	9.32		
Violence				
Partner violence			0.4 (0.8)	0-6
Community violence			0.7 (0.6)	0-3
Family violence			0.2 (0.3)	0-1
Police action			0.9 (0.5)	0.08-2.91
Police attitude			2.4 (1.0)	0-4
Arrest history			1.9 (1.7)	0-10

Table 2. Summary of SNP data. Listed populations are as follows: YRI- Yoruban descent population listed in HapMap; CEU- Caucasian descent population listed in HapMap; AA- African American population from this study.

SNP	Allele	Allele frequency (%)			Genotype	Genotype frequency (%)			Number genotyped
		YRI	CEU	AA		YRI	CEU	AA	
rs24203767	A	NA	54.16	85.00	A/A	NA	28.33	76.00	516
	C	NA	45.83	15.00	A/C	NA	51.66	18.00	
					C/C	NA	20.00	6.00	
rs12412496	A	43.33	21.66	39.00	A/A	18.33	6.66	21.00	600
	G	56.66	78.33	61.00	A/G	50.00	30.00	37.00	
					G/G	31.66	63.33	42.00	
rs2185706	G	48.66	88.39	52.00	G/G	19.28	78.57	19.00	600
	A	51.33	11.61	48.00	G/A	56.42	19.64	66.00	
					A/A	24.28	1.78	15.00	
rs7089533	C	48.23	88.05	79.00	C/C	20.35	77.87	58.00	593
	T	51.76	11.94	21.00	C/T	55.75	20.35	42.00	
					T/T	23.89	1.76	0.00	
rs7093602	C	49.15	11.66	70.00	C/C	20.33	33.33	48.00	602
	T	50.84	88.33	30.00	C/T	57.62	16.66	43.00	
					T/T	22.03	80.00	9.00	

Genotyping

Genotypes were determined using one of two methods. Restriction fragment length polymorphisms (RFLPs) were used for the genotyping of rs2420367, rs7089533, and rs7093602. Samples were amplified by polymerase chain reaction (PCR) in 96 well plates. The Taq polymerase and dNTP mix utilized for PCR amplification was obtained from Thermo Fisher Scientific (Waltham, MA, USA). SNP primers were supplied by Integrated DNA Technologies (Coralville, IA, USA). The concentration of magnesium chloride was 1.8 mM (rs2420367, rs7089533) and 1.5 mM (rs7093602). A total of 40 cycles were used, in which the annealing temperature was decreased by 2°C every 5 cycles until reaching the optimum annealing temperature of 57°C for each primer set. After confirming proper DNA amplification, the PCR products were digested with the appropriate restriction enzyme. The restriction enzymes, SmlI, AflII, and AvaII, were used at a concentration of 0.2 µL for the three SNPs rs2420367, rs7089533, rs7093602, respectively, (New England BioLabs, Ipswich, MA, USA). Incubation time for SmlI was increased to two hours at 55°C, while incubation for AflII and AvaII remained at the recommended 15 minutes. Visualization of the digestion was done using a 3% agarose gel.

The TaqMan SNP genotyping assay was used to genotype rs12412496 and rs2185706. A volume 1.5 µL of DNA was used, as well as the 10 µL of the prepared master mix as per manufacturer's instructions. The plates were then run using the TaqMan SNP genotyping assay protocol by Applied Biosystems.

Statistical analysis

The genotype and allele frequencies for each SNP were determined using the data generated from all 602 DNA samples. Genotype and haplotype associations were made using the software program SNPstat, which tested the data for Hardy-Weinberg equilibrium, linear and logistic regression, and linkage disequilibrium statistics (<http://bioinfo.iconcologia.net/SNPstats>).

Results

All 602 samples were used for the determination of allele and genotype frequencies within the AA population (Table 2). In determining the haplotype frequencies within the population, the majority of the population possessed one of three haplotypes; AGGCC (18.19%), AGACC (16.72%), and AAGCC (13.22%) ordered by SNPs, rs2420367, rs12412496, rs2185706, rs7089533, rs7093602 (Dataset 2; Swanson *et al.*, 2016a).

Dataset 2. Haplotype frequencies for five SNPs in *HTR7* in African Americans

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SNP order is as follows: rs2420367, rs12412496, rs2185706, rs7089533, rs7093602.

Cortisol, the major glucocorticoid in the stress response, is also an important biomarker of an immune response (Cavigelli & Chaudhry, 2012; Straub *et al.*, 2002). Cortisol is shown to first be involved in the development of an inflammatory response, before eventually acting to depress this response (Elenkov, 2008; Kunz-Ebrecht *et al.*, 2003). It has also been shown to shift the

adaptive immune response towards the humoral response (Cavigelli & Chaudhry, 2012; Murali *et al.*, 2007). As such, it was desired to identify potential associations between both hyper- and hypocortisolism and the five intronic SNPs in *HTR7*. None of the five SNPs were found to be associated with hypercortisolism.

Hypocortisolism was found to be associated with rs2420367, rs2185706, and rs7093602 when adjusting for gender (Table 3 and Table 4). Females showed an association with the genotype A/C (rs2420367) by an 11-fold increase in the risk of hypocortisolism (OR=11.64[1.52-89.24]), when categorizing the interaction first by SNP then by gender (SNP within gender; Table 4). Similarly, males showed a 2-fold increased risk of hypocortisolism with the genotype A/A (rs2420367) when the interaction was categorized first by gender then by SNP (gender within SNP; Table 3). Only males showed an association to hypocortisolism when categorizing in a gender within SNP manner for both rs2185706

Table 3. Associations between hypocortisolism and SNPs within *HTR7*. Data was analyzed as gender within the SNPs.

rs2420367				
		Case	Control	OR
A/A	Female	33	85	1
	Male	32	184	2.26(1.29-3.95)
A/C	Female	1	31	1
	Male	10	38	0.12(0.01-1.02)
C/C	Female	2	11	1
	Male	3	12	0.96(0.13-7.08)
Interaction in trend: 0.016				
rs2185706				
		Case	Control	OR
G/G	Female	5	25	1
	Male	12	49	0.90(0.28-2.89)
A/G	Female	20	94	1
	Male	28	151	1.18(0.62-2.25)
A/A	Female	10	14	1
	Male	5	38	5.23(1.50-18.21)
Interaction in trend: 0.029				
rs7093602				
		Case	Control	OR
C/C	Female	24	71	1
	Male	17	109	2.13(1.06-4.28)
C/T	Female	9	52	1
	Male	23	109	0.87(0.37-2.03)
T/T	Female	3	10	1
	Male	5	23	1.59(0.31-8.11)
Interaction in trend: 0.24				

Table 4. Association between hypocortisolism and SNPs in *HTR7*. Data was analyzed as SNPs within gender.

rs2420367				
		Case	Control	OR
Female	A/A	33	85	1
	A/C	1	31	11.64(1.52-89.24)
	C/C	2	11	2.03(0.42-9.76)
Male	A/A	32	184	1
	A/C	10	38	0.63(0.28-1.41)
	C/C	3	12	0.87(0.22-3.34)
Interaction in trend: 0.0035				

and rs7093602. Males with the genotype A/A (rs2185706) were found to be at a 5-times greater risk (OR=5.23[1.50-18.21]), while those with the genotype C/C (rs7093602) were associated with a 2-times greater risk (OR=2.13[1.06-4.28]; [Table 3](#)). This indicates that some SNPs within *HTR7* are associated with hypocortisolism in a gender specific manner in the AA population.

When analyzing for haplotype associations to hypercortisolism, two haplotypes provided a decreased risk in the population. The haplotype AAACC (p=0.042) was found associated with a 77% decreased risk (OR=0.23[0.06-0.95]; [Table 5](#)), while the AAGTT haplotype (p=0.001) was associated with a 98% decreased risk in the AA population (OR=0.02[0.00-0.21]; [Table 5](#)). When analyzing

Table 5. *HTR7* haplotype associations to cortisol level. SNP ordering for haplotype analysis as follows; rs2420367, rs12412496, rs2185706, rs7089533, rs7093602.

Hypercortisolism					
Haplotype	Frequency	Case	Control	OR	p-value
AGGCC	0.2001	0.0815	0.207	1	
AGACC	0.1518	0.1865	0.15	0.46 (0.10-2.13)	0.32
AAGCC	0.1193	0.0776	0.1206	0.95 (0.17-5.45)	0.96
AAACC	0.1075	0.2505	0.1004	0.23 (0.06-0.95)	0.042
AGGTT	0.0412	0.442	0.0399	0.24 (0.03-1.77)	0.16
AGGCT	0.0408	0.0484	0.0386	0.53 (0.05-6.11)	0.61
CGACC	0.0397	0.0373	0.0375	0.22 (0.03-1.57)	0.13
CGGCC	0.026	0.0227	0.0256	0.80 (0.07-9.29)	0.86
AAGTT	0.0158	0.1492	0.0087	0.02 (0.00-0.21)	0.001
Hypocortisolism					
Haplotype	Frequency	Case	Control	OR	p-value
AGGCC	0.2069	0.1768	0.2103	1	...
AGACC	0.1466	0.1948	0.1378	0.45(0.19-1.08)	0.076
AAGCC	0.1174	0.1158	0.1071	0.58(0.23-1.47)	0.25
AAACC	0.1095	0.1068	0.1226	0.98(0.41-2.32)	0.95
AGATT	0.0673	0.0698	0.0629	0.75(0.23-2.43)	0.63
CGACC	0.0377	0.0398	0.0366	0.84(0.21-3.26)	0.8
AGGCT	0.0373	0.02	0.0475	0.86(0.14-5.32)	0.87
AAATT	0.0304	0.034	0.0293	0.49(0.11-2.25)	0.36
AAGCT	0.0272	0.0367	0.0285	0.39(0.10-1.45)	0.16
CGGCC	0.0248	0.045	0.0203	0.19(0.04-0.87)	0.033
AGACT	0.0193	0.0209	0.0193	0.62(0.10-4.06)	0.62
AAGTT	0.017	0.0524	0.0135	0.27(0.04-1.77)	0.17
CAGTT	0.0162	0.0066	0.0135	1.76(0.18-17.50)	0.63
CAGCT	0.0137	0.0061	0.0166	0.72(0.06-9.04)	0.8
rare	0.0487	...	...	0.47(0.15-1.42)	0.18

the data for hypocortisolism, only the haplotype CGGCC ($p=0.033$) was associated with 79% decreased risk ($OR=0.19[0.04-0.87]$; Table 5).

When adjusting for gender, males remained associated with hypocortisolism by the CGGCC haplotype ($OR=0.01[0.00-0.19]$) providing a 99% decreased risk (Table 6). This relationship held true regardless of the categorization of the interaction. While females did not remain associated with the CGGCC haplotype, a 91% decreased risk was found associated with hypocortisolism by the haplotype AGACC ($OR=0.11[0.02-0.69]$) and by 83% for the grouping of extremely rare haplotypes ($OR=0.17[0.03-0.88]$) (Table 6). These associations held when categorized in a haplotype within gender manner. Due to the low number of individuals denoted as case for both analyses (case1=22; case2=85), the majority of haplotypes were unable to be used for analysis purposes. The results obtained indicate that certain haplotypes are associated with cortisol level in the AA population by decreasing the risk of having either hyper- or hypocortisolism.

Discussion

Despite the existing data on the impact of both genetic and environmental factors on immune function, few studies have examined relationships between these variables, especially in African Americans. We know relatively little about potentially sequential relationships between genetic risk factors, environmental stress, and immune function. In the brain, 5-HT is produced primarily by neurons in the midbrain, especially the dorsal raphe nucleus, which acts to innervate the majority of the brain, allowing 5-HT to modulate the response to stress (Holmes, 2008; Hornung, 2003). *HTR7* is located throughout the brain and periphery, functioning in the regulation of circadian rhythmicity and smooth muscle tone, both in the cardiovascular and gastrointestinal tract. (Abdouh *et al.*, 2004; Hornung, 2003; Mahé *et al.*, 2005) During times of stress, *HTR7* expression is shown to increase and has been linked to depression (Guscott *et al.*, 2005; Holmes, 2008). SNP genotypes and haplotypes in various genes, including serotonin receptors, have also been associated with changes in immune function or disease state (Ikeda *et al.*, 2006; Snir *et al.*, 2013;

Zlojutro *et al.*, 2011). As such, it should follow that SNP genotypes and haplotypes in the *HTR7* gene should also be associated with changes in the stress response, resulting in an effect on immune function. In this paper, we report the allele, genotype, and haplotype frequencies for five intronic SNPs of the *HTR7* gene in an African American population.

During times of stress, cortisol secretion will initially increase, and over time may lead to hypercortisolism if the stressor remains persistent (Fries *et al.*, 2005). In the case of chronic stress, it is possible to reach a state of adrenal exhaustion or hypocortisolism (McEwen, 2004). Both states, hyper- and hypocortisolism, have been associated with a number of stress-related diseases. While hypercortisolism has been shown to contribute to diseases such as depression, heart disease, and type 2 diabetes (Lundberg, 2005; Tse & Bond, 2004), hypocortisolism contributes to post-traumatic stress disorder, asthma, and irritable bowel syndrome (Buske-Kirschbaum *et al.*, 2003; Ehler *et al.*, 2001; Fries *et al.*, 2005; Heim *et al.*, 1999). To test the hypothesis that there would be an association between SNPs in the *HTR7* gene and both hyper- and hypocortisolism, the program SNPstat was used. While hypercortisolism was not associated to any specific SNP genotypes, associations were found to hyper- and hypercortisolism with the haplotypes AAACC and AAGTT, respectively. Both haplotypes provided a decreased risk of 77% and 98%, respectively, and may identify individuals who are less likely to develop hypercortisolism, and the potential resulting health disparities.

An increased risk of hypocortisolism was found to associated with three SNP genotypes in a gender specific manner. For rs2420367, females with the A/C genotype and males with the A/A genotype showed an 11-fold and 2-fold increased risk, respectively. Males, but not females also showed a 5-fold and 2-fold increased risk of hypocortisolism with the genotypes A/A (rs2185706) and C/C (rs7093602), respectively. As such, it is possible to conclude that individuals with these genotypes will be at an increased risk of developing hypocortisolism, and this may also increase the likelihood of developing health disparity diseases.

The haplotype CGGCC was determined to provide a 79% decreased risk of hypocortisolism in the population, and this association remained in males when adjusting for gender. Females however, showed a decreased risk of 78% with the haplotype AGACC. These haplotypes may identify the individuals in the AA population who are at a decreased risk of developing hypocortisolism and associated diseases.

In this work, we demonstrate that genetic variation in *HTR7* influences the production of cortisol in response to chronic stress. While individual SNP genotypes indicate a risk towards the development of hypocortisolism, haplotypes within *HTR7* seem to be protective against both hyper- and hypocortisolism. This protective effect may be a biological indicator of resilience towards maladaptive effects of chronic stress. This supports previous findings that individual genetics are involved in the degree of adaptability seen in response to stress (Feder *et al.*, 2009; Gillespie *et al.*, 2009). To our knowledge, this is the first evidence for a functional link between

Table 6. *HTR7* haplotype associations to hypocortisolism when adjusting for gender. Analysis was performed as a haplotype and gender cross-classification interaction.

Haplotype	Frequency	Female OR	Male OR
AGGCC	0.205	1	0.32(0.02-4.21)
AGACC	0.1495	0.11(0.02-0.69)	0.29(0.03-3.05)
AAGCC	0.1197	0.46(0.10-2.17)	0.16(0.01-1.73)
AAACC	0.1064	0.31(0.08-1.28)	0.59(0.04-7.99)
AGGCT	0.0425	0.08(0.01-1.11)	0.20(0.01-2.78)
AAATT	0.0349	0.26(0.03-2.74)	0.14(0.01-2.27)
CGGCC	0.0253	0.19(0.02-1.71)	0.01(0.00-0.19)
rare	0.0469	0.17(0.03-0.88)	1.32(0.04-46.02)
Interaction p-value: 0.088			

a genetic polymorphism in the *HTR7* receptor gene and immunologically important subphenotypes related to differential levels of a blood biomarker for both stress and the immune response.

Further study is required to investigate this relationship between genetic polymorphisms in *HTR7*, the stress response, and the immune response. Due to the low number of individuals in the case grouping, haplotype analysis was incomplete. By increasing the sample set data with regards to cortisol measurements, a clearer picture of the relationship between these five SNPs and cortisol may be formed. This will enable a more detailed picture of the relationship between *HTR7* polymorphisms and cortisol production. Furthermore, the cytokine shift induced by cortisol during a response to stress suggests that a relationship may exist between these five SNPs and both the inflammatory and humoral response (Maeda *et al.*, 2010; Murali *et al.*, 2007; Pepys & Hirschfield, 2003), and it would be beneficial to analyze whether any association between the five intronic SNPs and biomarkers of the inflammatory and humoral response. This would aid in the understanding of how stress exposure contributes to the development of disease, and the role in which genetics plays in this pathway.

Data availability

Dataset 1. Raw data for genetic polymorphisms in the serotonin receptor 7 (*HTR7*) gene are associated with cortisol levels in African Americans young adults. Cortisol measurements were determined in Mic-Gr/dL and recorded as a numerical value or the three values as follows; -9, indicate participants in which no cortisol was measurable in the participant; NA, indicate participants in which blood samples were not taken; √, were taken as case values for analysis. NA for sex and age of participant indicate the participant did not wish to specify. Within SNP genotypes, NA indicates that no genotype was determined. (doi, 10.5256/f1000research.10442.d146883; Swanson *et al.*, 2016a).

Dataset 2. Haplotype frequencies for five SNPs in *HTR7* in African Americans. SNP order is as follows: rs2420367, rs12412496, rs2185706, rs7089533, rs7093602. (doi, 10.5256/f1000research.10442.d146884; Swanson *et al.*, 2016b).

Author contributions

MA conceived the project and designed the experiments. GS, SM, and AA carried out the experiments. GS performed the statistical analysis and prepared the manuscript. MA, CL, GD, FS, and BW were involved in the revision of the manuscript. All authors agreed to the final content.

Competing interests

No competing interests were disclosed.

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This manuscript by Swanson et al. is a rather simple analysis of a few SNPs in the HTR7 gene checking for association with cortisol levels in a small cohort of African-Americans. But it suffers from numerous severe problems in the description, planning and analysis of the data, to the extent that its major conclusions are not supported by presented evidence anymore after careful consideration of the results.

A) One of the most striking problems is that this manuscript conducts many different statistical analyses, but surprisingly, does not employ any multiple testing correction such as Bonferroni correction. For example, they have 26 different observed haplotypes (out of $2^5=32$ possible haplotypes), and 3 gender analyses (combined, male, female). The appropriate significance threshold after Bonferroni correction would be $0.05/(26 \times 3)=0.00064$, at which level no reported p-values are significant any more. Which means all the reported associations in the manuscript do not stand up to a proper statistical scrutiny. Thus the manuscript, including the title, abstract and conclusions, should actually be rewritten to present this fact that no associations are significant so no association between HTR7 and cortisol can be drawn.

B) The reportedly significant haplotypes for males and female associated with cortisol are very different. Yet no biological explanations are provided as to why that may be the case. Such instances suggest even more that the observed associations could be artifacts of improper statistical analysis.

Problems with the sample description:

C) Since the study referred to while presenting the cohort is an unpublished study, readers can have no further idea about the sample. So it should be described in more detail, possibly in supplement.

D) Is there any information available about ancestry proportions of the samples?

A typical GWAS will include genetic PCs to adjust for population substructure/stratification/admixture while doing association test with SNP genotypes / haplotypes. As the authors mention, these African-Americans are likely admixed, having some European and some African ancestry. Because whole-genome SNP genotypes are not available, it is understood that the authors can't adjust using PCs. But that is a substantial weakness and a possible cause for biased results.

At least some idea about ancestry would help to shed light on the possible degree of substructure in the data.

E) Table 1 provides several variables that the authors seemingly consider to be interesting in the cohort. If so, why aren't any of these variables used in analysis? Otherwise, what is the purpose of presenting them?

Problems with phenotyping:

F) People with health issues, such as tumors in pituitary or adrenal glands, should be excluded, as these issues will affect the normal production of cortisol. Was such data collected in the participants?

G) The choice of cut-offs for defining hypo- and hyper-cortisolism are arbitrary and unexplained. Explanation and reference should be provided.

Problems with the choice of markers:

H) It is not clear why HTR7 specifically was selected to check in contrast with cortisol level. Further biological explanation should be provided.

I) The rationale provided behind the choice of SNPs is rather weak. Not clear why the only SNPs of interest would be those showing a big allele frequency difference between CEU & YRI. As it happens, this rule only ends up selecting some intronic snps. Selecting some functional variants mentioned in the literature, such as non-synonymous SNPs, would be more interesting as such SNPs are more likely to have a biological effect, if any.

J) And even if the SNPs are intronic, they should at least be studied if they have any functional features such as regulatory annotations.

Other problems with statistical analysis:

K) The 'statistical analysis' section is extremely brief and doesn't present any details on the methods or analysis procedure. The various analyses done in the manuscript is very haphazard, without any planned systematic presentation.

L) The authors use the term "adjusted for gender" several times, but it doesn't make sense, compared to the analyses performed.

M) OR for gender within SNP genotype groups doesn't seem relevant as it is not related to the research question.

N) OR for one genotype category AC against AA is significant for one gender in one SNP, but is not true for CC against AA, neither in effect size nor in p-value, and thus the postulated effect of the C allele doesn't seem reliable. Also, wonder why the authors didn't test for allelic effects in addition to genotypic effects.

O) They should provide some power calculations, particularly considering that the statistical analyses actually don't show anything as significant.

Other comments:

P) "as African American's are likely to possess frequencies that fall between those of Caucasian and Yuroban descent." – references should be provided.

Q) "Yuroban" should be Yoruban.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

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