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Macrophage migration inhibitory factor (MIF) gene is associated with adolescents' cortisol reactivity and anxiety

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ABSTRACT

Emerging evidence points to interactions between inflammatory markers and stress reactivity in predicting mental health risk, but underlying mechanisms are not well understood. Macrophage Migration Inhibitory Factor (MIF) is a pleiotropic cytokine involved in inflammatory signaling and Hypothalamus Pituitary Adrenal (HPA) axis stress-response, and has recently been identified as a candidate biomarker for depression and anxiety risk. We examined polymorphic variations of the *MIF* gene in association with baseline MIF levels, HPA axis reactivity, and self-reported anxiety responses to a social stressor in 74 adolescents, ages 10–14 years. Genotyping was performed for two polymorphisms, the -794 CATT5-8 tetranucleotide repeat and the -173*G/C single nucleotide polymorphism (SNP). Youth carrying the *MIF*-173*C and CATT7 alleles displayed attenuated cortisol reactivity when compared with non-carriers. Children with the CATT7-173*C haplotype displayed lower cortisol reactivity to the stressor compared to those without this haplotype. Additionally, the CATT5-173*C and CATT6-173*C haplotypes were associated with lower self-reported anxiety ratings across the stressor. Results extend prior work pointing to the influence of *MIF* signaling on neuroendocrine response to stress and suggest a potential pathophysiological pathway underlying risk for stress-related physical and mental health disorders. To our knowledge, these are the first data showing associations between the *MIF* gene, HPA axis reactivity, and anxiety symptoms during adolescence.

1. Introduction

Adolescence is marked by drastic shifts in neuroendocrine activity and stress physiology which may confer risk for affective disorders (Gunnar et al., 2009; Romeo, 2013; Stroud et al., 2009). The Hypothalamus Pituitary Adrenal (HPA) axis, part of the neuroendocrine system, regulates stress response during acute or chronic stress. Identifying factors that contribute to its dysregulation during adolescence may have important clinical implications. Macrophage Migration Inhibitory Factor (MIF) is a well-established inflammatory marker and regulator of the HPA axis (Dunn, 2000; Turnbull and Rivier, 1995). Emerging evidence in adults implicates MIF in stress-related psychiatric disorders and HPA axis dysregulation. Little is known on MIF's role in stress variability in adolescence, when affective disorders often first emerge. We explore connections between polymorphic variability in the *MIF* gene, HPA axis reactivity and anxiety reports in a normative sample of adolescents.

MIF is one of the first discovered cytokines, originally named for its ability to recruit macrophages to sites of inflammation (Bloom and Bennett, 1966; David, 1966), however its effects are now known to be pleiotropic. MIF is widely expressed throughout the body. High expression is found in the endocrine system, especially the HPA axis (Calandra and Roger, 2003; Fingerle-Rowson et al., 2003; Matsunaga et al., 1999) and throughout the brain, including limbic regions implicated in stress, depression, and anxiety (Bloom and Al-Abed, 2014; Conboy et al., 2011). Among its immune-related functions, MIF acts as a regulator of innate immune- and inflammatory-signaling (Bernhagen et al., 2007; Calandra and Roger, 2003; Mitchell et al., 1999; Savaskan et al., 2012), induces pro-inflammatory cytokines (Calandra and Roger, 2003; Calandra et al., 1994), and counteracts the effects of glucocorticoid signaling (Baugh et al., 2002; Fingerle-Rowson et al., 2003; Flaster et al., 2007).

MIF is also intricately associated with the HPA axis (Bacher et al., 1997; Tampanaru-Sarmesiu et al., 1997; Baugh and Donnelly, 2003) a

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key neuroendocrinological stress response system. MIF is released by the same cells in the pituitary as those that release adrenocorticotropin hormone (ACTH) (Bernhagen et al., 1995; Nishino et al., 1995). Plasma MIF levels follow similar circadian rhythms as plasma cortisol, the end product of the HPA axis (Petrovsky et al., 2003). Interaction between MIF and the HPA axis are well-established, with most work involving in-vitro experiments or pathological conditions involving inflammatory diseases (Calandra and Roger, 2003; Nishino et al., 1995; Tierney et al., 2005). Fewer studies have investigated associations under normative, non-pathological conditions in humans. However, a recent study showed that elevated MIF levels were related to decreased HPA axis response during an acute stressor in healthy adults (Edwards et al., 2010), pointing to a stress-related neuroendocrine and immune interaction involving MIF and the HPA axis.

MIF has recently been investigated as a biomarker of depression, anxiety, and psychological stress. In animal work, *Mif*-knock-out mice showed stress-related increases in anxiety- and depression-like behavior and impairments in hippocampal dependent memory (Conboy et al., 2011). MIF has also been shown to have a role in rodents' increased neurogenesis during fluoxetine treatment, a selective serotonin reuptake inhibitory (SSRI) commonly administered as treatment for depression (Conboy et al., 2011) and was recently found to mediate the anti-depressant effects of voluntary exercise (Moon et al., 2012). Together, these data point to a potential protective effect of MIF in the development of anxiety or stress-related disorders.

In humans, higher baseline serum levels of MIF have been observed in young adults who reported mild to moderate depression symptoms (Hawkley et al., 2006; Edwards et al., 2010), although other studies have not replicated this association (Katsuura et al., 2011). Higher serum MIF levels have also been registered in pregnant women with symptoms of depression after an immune challenge, when compared with pregnant women with no depression (Christian et al., 2010). In clinical settings, depressed patients showed higher baseline MIF levels prior to starting a pharmacological treatment, yet MIF levels did not change over the course of treatment (Musil et al., 2011). Clinically depressed patients who responded to pharmacological treatment have also shown higher MIF mRNA prior to treatment onset, compared to non-responders. MIF declined over the treatment, yet the magnitude of decline was not associated with treatment response (Cattaneo et al., 2013). Recently, circulating MIF was examined in adults with anxiety, depression, stress, and/or adjustment disorders who participated in a randomized controlled trial of mindfulness, a psychological treatment for mental health problems. MIF levels were shown to significantly decrease in the treatment group, when compared to controls, yet the reduction was not associated with symptom improvement (Wang et al., 2018).

In summary, a growing body of evidence implicates MIF as a potential modulator of HPA axis functioning and as a biomarker for anxiety and depression symptoms in healthy adults. However, we are aware of no studies that have investigated such links in earlier developmental periods, such as adolescence, when affective disorders are likely to emerge. Also, no studies have examined whether genetic factors that regulate MIF expression, such as functional polymorphisms of the *MIF* gene, explain inter-individual differences in HPA axis function and behavioral signs of stress in normative adolescents.

To further this line of work, we examined associations between genetic variability in the *MIF* gene, peripheral levels of MIF, HPA axis and self-reported anxiety responses to stress, in a sample of healthy adolescents. *MIF* polymorphisms of interest included the -794CATT5-8 tetranucleotide repeats and the -173*G/C single nucleotide polymorphism (SNP), and haplotypes known to be related to MIF expression (Baugh et al., 2002; Radstake et al., 2005). We expected that adolescents who carried the C allele or higher number of CATT repeats (i.e. CATT 7 or 8) of the *MIF* gene would show reductions in HPA axis reactivity and that this would be associated with lower reports of subjective anxiety. As an exploratory step, we quantified peripheral MIF levels from saliva samples collected prior to the stressor. We expected that individuals with higher expressing alleles would show higher peripheral MIF, and that MIF levels might predict HPA axis reactivity during the stressors.

2. Material and methods

2.1. Participants

The original sample of participants included 104 children (54 female), recruited from a mass mailing list provided from Experian, a credit card company. Families living in New Haven, CT, USA and surrounding towns were targeted for recruitment. Children were considered ineligible for the study if their caregiver, typically their mother, reported that the child in question was being treated for, or carried a diagnosis of psychosis, autism, or bipolar disorder.

The final sample consisted of 74 youth (39 female) from the larger study who provided salivary samples for DNA analyses. Youth in this study ranged from 10 to 17 years of age (M = 13.93, SD = 2.33). In this sample, 64.9% reported their racial/ethnic background as Caucasian (n = 48), 9.5% African American (n = 7), 13.5% Hispanic/Latino (n = 10), 8.1% Asian (n = 6), and 4.1% "Other" (n = 3). In terms of family income, 51.5% (n = 38) reported an annual income of greater than $75,000 and 75.7% ($n$ = 56) of the sample had a parent with a college degree. Most children were living with their biological mothers, 97.3% (n = 72). At the time of the study, 79.8% (n = 59) mothers reported their marital status as "married or in a committed relationship," and 10.8% (n = 8) reported their status is "separated or divorced." Sample characteristics are displayed in Table 1.

2.2. Procedure

Families were initially recruited over the phone. If interested, families were scheduled for a series of laboratory visits. At the first visit, IRB-approved parental permission and child assent were obtained as well as parent report and self-report instruments.

In a second visit, approximately 1–2 weeks later, youth participated in a laboratory assessment that lasted approximately 1 h. Youth were asked to refrain from alcohol or drug use on the day of the laboratory visit, to avoid potential influences on HPA axis or stress reactivity. On the day of the assessment, youth were tested for alcohol and drug intake with a breathalyzer, urine test and self-reported medication use. No participants had evidence of alcohol, and one tested positive for

Table 1
Descriptive Statistics.

Variable	M (SD) or n (%), Total Sample (N = 74)
Gender	
Female	39 (52.7)
Child Age	13.93 (2.3)
Maternal Age	45.90 (6.2)
Maternal Education	3.53 (1.5)
Ethnic/Racial Background	
African American (not of Hispanic origin)	7 (13.5)
Asian	6 (8.1)
Hispanic or Latino	10 (13.5)
White (not of Hispanic origin)	48 (64.9)
Other	3 (4.1)
MIF (ng/ml)	0.62 (1.6)
Baseline cortisol (ug/nl)	0.36 (.1)
Baseline anxiety	1.19 (.8)
Puberty status	2.50 (.6)

Note. Maternal education (1–5, with 1 being HS degree and 5 being graduate degree). Gender and Ethnic/Racial Backgrounds reported as frequencies, all other variables reported as means and standard deviations.

opioids. Two participants in this sample were taking their regular dose of Ritalin at the time of the study. These participants were included in final analyses to maximize power as their inclusion did not alter the results. The sessions began at 4:05 p.m. to control for daily hormonal fluctuations. Placement of an electroencephalography (EEG) sensor net/electrocardiogram (ECG) electrodes (not discussed here) and provision of instructions took ~20 min. Next, eight tasks were administered. First, participants completed a 7-min rest with eyes open (Blood et al., 2015), and second 5-min progressive muscle relaxation following our previous work (Chaplin et al., 2010). Third, a 10-min visual food cue processing EEG task (Wu et al., 2018) was administered followed by the fourth task, a balloon reward EEG task (Crowley et al., 2013, 2014) (10 min). The fifth task was the Trier Social Stress Test for Children (TSST-C, task 2), a well-validated paradigm designed to activate a stress response to a psychosocial stressor (Buske-Kirschbaum et al., 1997) lasting approximately 10 min (more details below). The sixth task was a repeat of the balloon reward task (10-min). The seventh task was a "stress booster" task, administered as a modified version of the TSST (8 min, see below for more details). The 8th task they participated in was a delay-discounting task (Mitchell, 1999), (12 min).

Over the course of the entire session, a total of eight salivary samples and stress ratings were collected (assessment T1–T8). A stopwatch and a written log were used to maintain the timing of assessments including saliva for cortisol and subjective ratings. The first baseline (T1) cortisol assessment and anxiety ratings was taken ~30 min after participants arrived at the laboratory before the tasks started. This sample was labeled as occurring at the "0-min mark". A second baseline (T2) was taken after participants completed the rest, relaxation, food task and first balloon reward task (see above), which occurred at the 39-min mark. The third assessment (T3) was taken after the TSST-C, at the 69-min mark. The fourth assessment (T4) was taken after the completion of the second EEG reward task, at the 89-min mark. The fifth (T5) measurement occurred after the completion of the stress booster and the delay-discounting task at ~109-min mark. Next, three assessments (T6–T8) were collected during a "recovery period", and were spaced 15 min apart. These occurred at the 125-min, 141-min, and 157-min marks, respectively.

2.3. Measures

2.3.1. Trier Social Stress Test – Child (TSST-C) and stress booster

Stress reactivity was assessed using an adapted version of the Trier Social Stress Test for Children (TSST-C). The TSST-C is a widely used social stress task that is useful for eliciting HPA axis activation in children, and is shown to increase salivary cortisol and other physiological indicators of stress, with peak cortisol levels shown around 20 min following the peak stressor (Krishnaveni et al., 2014).

The TSST-C was performed according to instructions provided by Buske-Kirschbaum and colleagues, except that in this study the adolescent prepared and delivered the speech in the same room (rather than a separate "preparation room"). At ~5:44 p.m., two unfamiliar adults (the "judges") entered the laboratory room and told the adolescent that they will have to finish writing a story. They told the adolescent to "make the story as exciting as possible" because they will be "competing against other teenagers." The judges gave the adolescent a story stem (used by Buske-Kirschbaum et al., 1997), and then left the room. The research assistant collected self-reported anxiety, cortisol, and heart rate levels and then told the adolescent to prepare the story for 5 min (from 5:44 p.m. to 5:49 p.m.). At 5:49 p.m., the judges re-entered the room. One judge placed an audio-recorder in front of the adolescent and asked him/her to stand up and recite the story back for 5 min while he/she is audio-taped. If the participant ended their story before 5 min, then they were told to continue. After the 5 min are finished, the second judge asked the adolescent to remain standing and complete a math task ("subtract the number 13 from 1023 over and over as quickly and accurately as possible") for 5 min. In the event of an error, participants heard a buzzer and were told to start from the beginning. The judges were trained research assistants. They were instructed to maintain a neutral expression and not to assist the adolescent during the tasks. During the speech and math tasks, the youth was asked to hold their arm/hand still so as to minimize movement effects on heart rate recordings.

As part of a larger study, participants were "re-stressed" with a stress booster procedure after completing a delay-discounting task. Following procedures similar to the TSST-C numeric subtraction, participants performed a booster TSST-C 22 min after the end of the TSST-C task. This booster lasted 5 min and participants were audio and video recorded as in the previous task. As before, they were observed by a panel of judges and were told that they were competing against other subjects who were also in the same study. Participants were instructed to spell specific words forwards and backwards and were asked to perform to the best of their ability. The initial words used in this booster consisted of 4 letters. If participant made two successive correct answers, they moved on to spelling 5 letter words, progressing in this pattern to 6 letter words, 7 letter words, 8 letter words etc. However, if the participant made an error, they heard a buzzer and did not advance to a longer word group.

At the end of the study, after the final cortisol samples were collected and anxiety ratings were made (see 2.3.2 and 2.4), the participants were congratulated by the judges and experimenters and debriefed (i.e., reminded that all of the adults knew that they were role-playing and had not stolen anything). Audience members immediately adopted a friendly demeanor and the experimenter informed the child that the audience members had been instructed to act in a non-reactive manner as part of the study.

2.3.2. Self-report ratings of anxiety

Self-reported anxiety ratings (ranging from 0 to 10, 0 being "none at all" and 10 indicating "more than ever") were gathered in conjunction with the salivary cortisol collections during 8 time points across the stressor.

2.3.3. Adolescent pubertal status

Adolescents completed the Pubertal Development Scale (PDS) Self Report, while the primary caregiver completed the PDS Parent Report (Petersen, Crockett, Richards, and Boxer, 1988). Both the PDS Self Report and Parent Report contain 5 items scored from 1 to 4. The PDS estimates the pubertal status of the adolescent based on the presence or absence of developmental features such as growth spurt, pubic hair growth and skin changes in both boys and girls, as well as gender specific pubertal changes. Caregiver and self-reports on the PDS were highly correlated ($r = .831$). For the purposes of this study we used the mean of these two measures.

2.4. Cortisol assay

Salivary cortisol was collected to measure HPA axis response and baseline MIF levels. Saliva samples were collected by asking the participant to place a cotton swab between his/her tongue and cheek until this swab was completely saturated (approximately 1–2 min). Samples were immediately stored in a plastic tube, placed in an ice bucket and then stored at −20 °C before being transported to a university laboratory for analysis. Saliva samples were assayed in duplicate following standard radioimmunoassay kits using Coat-A-Count Cortisol Kit (Diagnostic Products Corporation, Los Angeles, CA).

2.5. MIF assay

Circulating MIF levels were measured from saliva samples collected at baseline, using sandwich enzyme-linked immunosorbent assay (ELISA), following a protocol developed by the Bucala Lab at Yale University, which yielded adequate sensitivity and performance criteria

(limit of detection: 0.83 ng/ml, CV%: 3.69) over a range of detection of 0–100 ng/ml. Detailed protocol and assay validity were previously published (Bick et al., 2015).

2.6. Genotyping

DNA collection was performed using Oragene DNA Saliva Collecting kits. The *MIF* gene, located on chromosome 22q11.2, contains several functional polymorphisms in the 5′ promoter region, including a single nucleotide polymorphism (SNP) at position -173 and a CATT5-8 tetranucleotide repeat at position -794. Analysis of the saliva samples for the -173*G/C SNP (rs755622) was carried out using Standard Applied Biosystems Inc. (ABI) Taqman SNP protocols with specially designed primers on the ABI Prism 7900HT. Genotyping of the CATT repeat polymorphism was performed using PCR amplification followed by capillary electrophoresis. The forward primer *MIF*-F -1074 (5′-TGCAG GAACCAAT-ACCCATAGG -‘3) was used with the fluorescently labeled reverse primer, *MIF*-R -728 (5′-AATGGTAAACTCGGGGAC-3′). The PCR conditions were 5x KAPA2G GC Buffer, 0.2 mM dNTP, 4pmoles of each primer, 200 ng DNA, and 1 μL KAPA2G polymerase in a 20 uL PCR reaction. The PCR cycling conditions used were the same as previously described (Baugh et al., 2002). The call rates for the SNP and CATT were 91% and 99%, respectively (i.e. 9 of the samples failed genotyping for the SNP and 1 of the samples failed genotyping for the CATT due to poor quality DNA).

Of the 104 youth in the original sample and that participated in the TSST-C, 74 provided saliva samples as part of DNA collection. Children who provided saliva for DNA collections did not significantly differ from those who did not participate in this portion of the study in terms of age, gender, maternal age or education, cortisol values, and child reported depression and anxiety among children, all $p > .05$.

2.7. Haplotype analyses

Haplotypes were reconstructed with the -173 G/C SNP and -794 CATT5-7 repeats, where 6 haplotype combinations are theoretically possible (5G, 6G, 7G, 5C, 6C, 7C). Haplotype frequencies and inferred probabilities were calculated using PHASE v2.1.1 (Stephens et al., 2001). Haplotype frequencies are presented in Table 2.

Table 2
Genotype and Haplotype Frequencies at 2 *MIF* Polymorphisms.

Genotype	Total Sample (N = 74), n (%)
CATT 55	6 (8.1)
CATT 56	21 (28.4)
CATT 57	5 (6.8)
CATT 66	32 (43.2)
CATT 67	10 (13.5)
-173*C/C	3 (4.2)
-173*C/G	22 (31)
-173*G/G	46 (64.8)
Haplotype	
CATT5-173*G	31 (20.9)
CATT6-173*G	88 (59.5)
CATT7-173*G	0 (0.0)
CATT5-173*C	7 (4.7)
CATT6-173*C	7 (4.7)
CATT7-173*C	15 (10.1)

Note. *MIF*-173 is missing 3 values.

Table 3
Results of PROC MIXED Models for Cortisol and Anxiety Reactivity.

	Cortisol			Anxiety		
	df	F	p	df	F	p
Model 1- MIF-173						
MIF-173	1, 63	6.40	.014	1, 66	3.67	.059
Time	7, 449	13.98	< .001	7, 468	18.68	< .001
Time*MIF-173	7, 449	3.97	< .001	7, 468	1.94	.062
Sex	1, 63	0.17	.681	1, 66	0.00	.980
Ethnicity	1, 63	2.70	.105	1, 66	7.08	.001
Age	1, 63	7.43	.008	1, 66	0.13	.718
Model 2- CATT7						
CATT7	1, 66	8.76	.004	1, 69	0.22	.641
Time	7, 468	7.96	< .001	7, 469	12.88	< .001
Time*CATT7	7, 468	2.21	.032	7, 469	1.28	.256
Sex	1, 66	0.34	.565	1, 69	0.00	.975
Ethnicity	1, 66	2.97	.089	1, 69	5.45	.023
Age	1, 66	5.48	.022	1, 69	0.26	.615
Model 3- CATT6						
CATT6	1, 66	0.16	.690	1, 69	0.98	.327
Time	7, 468	6.00	< .001	7, 469	6.62	< .001
Time*CATT6	7, 468	1.88	.070	7, 469	1.41	.198
Sex	1, 66	0.59	.446	1, 69	0.00	.997
Ethnicity	1, 66	1.77	.187	1, 69	2.50	.118
Age	1, 66	7.04	.010	1, 69	0.16	.695
Model 4- CATT5						
CATT5	1, 66	0.36	.549	1, 69	1.52	.222
Time	7, 468	14.82	< .001	7, 469	19.60	< .001
Time*CATT5	7, 468	0.86	.535	7, 469	1.39	.208
Sex	1, 66	0.59	.445	1, 69	0.01	.907
Ethnicity	1, 66	2.30	.134	1, 69	6.62	.012
Age	1, 66	7.70	.007	1, 69	0.10	.749

3. Results

3.1. Data inspection and handling of missing data

Cortisol data were first examined for normality and extreme values. Three participants had outlying cortisol values at one or more of the eight collection points (T1–T8; defined as > 3 SD above the mean). Each outlying value was winsorized by reassigning a value equal to three SD above the mean. Distributional properties of cortisol and behavioral ratings of stress across the TSST-C were also examined. Given significant positive skew for both variables, a square root transformation was applied to all cortisol values and behavioral ratings of stress. Transformed values were used in analyses.

Preliminary inspection revealed the presence of missing data (with less than 5% of data missing across all study variables). Within anxiety ratings across time points, three participants had one missing value and two participants had two missing values. Within cortisol time points, three participants were missing all values (and were not included in analyses), one participant was missing five values and another was missing two values. Thus, maximum likelihood method was used to account for missing data at random.

3.2. Genotyping

The genotype and allelic frequencies for the two polymorphisms of the *MIF* gene are shown in Table 2. Given the low frequency of individuals who were homozygous for the C allele (n = 3), all analyses compared individuals who were homozygous for the G allele with individuals with one or more copy of the C allele. For the CATT tetranucleotide, there were no individuals with the CATT8 polymorphism in this sample. Therefore, only the CATT5, CATT6, and CATT7 alleles were examined in associations with variables of interest. Based on allelic frequencies for youth in that resulted from genotyping, we compared individuals with no copies of each repeat (CATT5, CATT6,

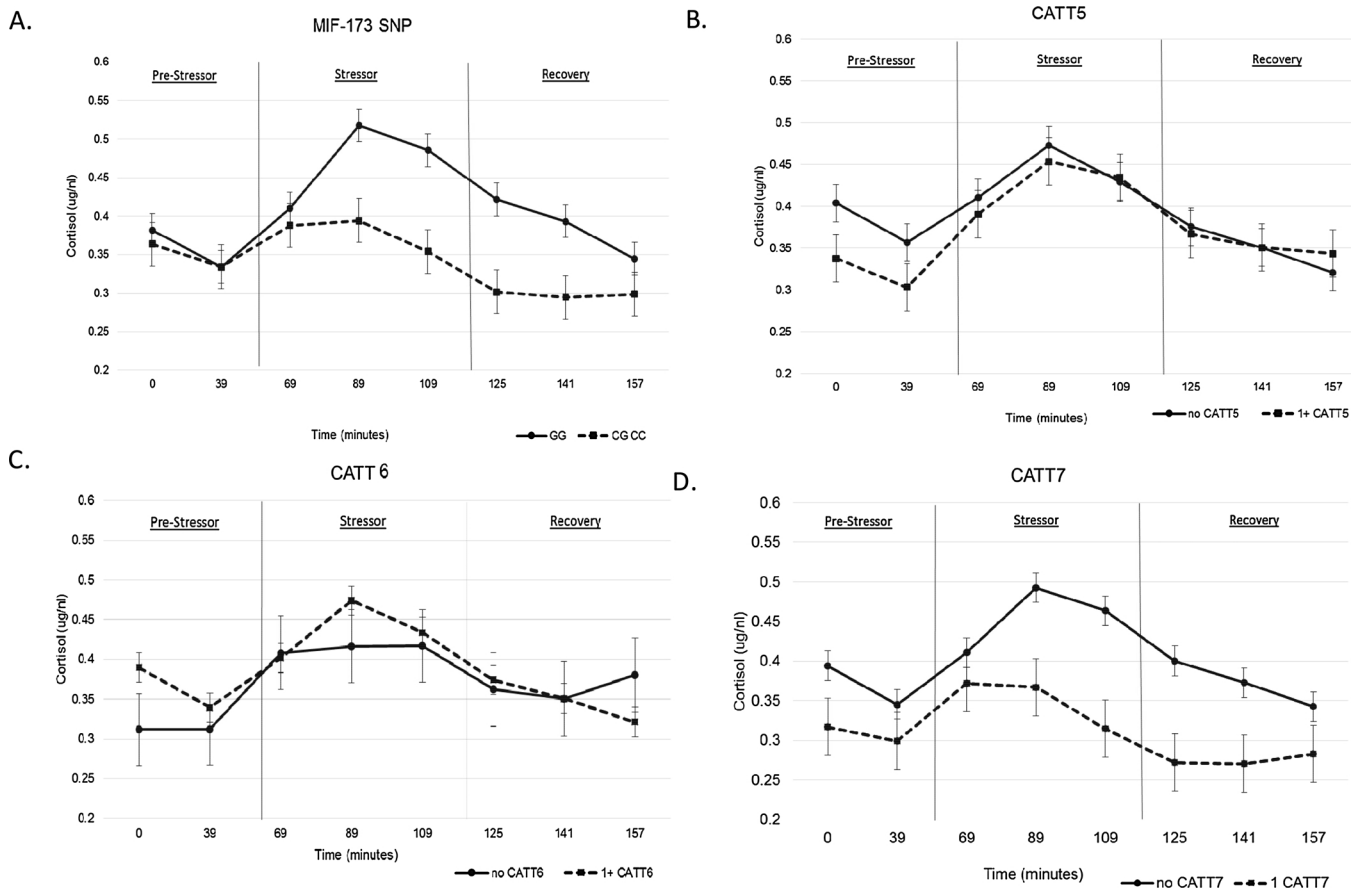

Fig. 1. Saliva Cortisol values across all 8 time points of measurement from baseline (0–39) to stressor (69–109) and recovery (125–157). Data is estimated marginal means and error bars indicate standard error. (Adjusted for sex, age and ethnicity) A) Cortisol Reactivity by *MIF*–173Genotype. Individuals with at least one C allele (CG or CC) showed lower cortisol over time compared to those with GG genotype.
B) Cortisol Reactivity by CATT5 carriers. There were no differences between individuals with no CATT5 alleles (6,6; 6;7) and 1 or more CATT5 alleles (5,6; 5,7; 5,5).
C) Cortisol Reactivity by CATT6 carriers. There were no differences between individuals with no CATT6 alleles (5,5; 5,7) and 1 or more CATT6 alleles (5,6; 6,6; 6,7).
D) Cortisol Reactivity by CATT7 carriers. Individuals with 1 CATT7 allele (5,7; 6,7) showed lower cortisol than those with no CATT7 alleles (5,5; 5,6; 6,6).

CATT7) versus those with one or more copies of each repeat in all analyses.

3.3. Data analytic plan

We examined whether *MIF* genotypes were associated with cortisol levels and anxiety ratings across the session. In our first set of models we examined associations between the *MIF* -173*G/C SNP and dependent variable of interest. Children with one or more C alleles were compared to those with no C alleles (CC and CG versus GG).

In our second set of models we tested associations between each CATT repeat and dependent variables of interest. This was conducted in two parts. In the first step, we tested whether children who held tetranucleotide repeats associated with low expression, CATT5, would differ from those who held repeats associated with higher MIF expression (CATT6 and CATT7). In the second step, we tested whether children who held repeats associated with high expression, CATT7, would differ from those who held repeats associated with lower expression (CATT5 and CATT6).

Next, we examined whether haplotypes moderated the change in cortisol or anxiety over time. To examine the potential interaction between the *MIF* -173 SNP and the CATT repeat polymorphisms on cortisol and anxiety ratings over time, haplotypes were constructed and entered in the PROC MIXED models described below. Each haplotype was analyzed to compare individuals with and without that haplotype (e.g. individuals with CATT5 -173*G haplotype versus those without this haplotype). As there were no participants carrying the G7 haplotype, the G7 haplotype was not included in analyses.

Mixed models were used to test all hypotheses. First, cortisol across the session was entered as the dependent variable, time was entered as a within subjects variable, and genotype was entered as a between subjects factor. Models were run in PROC MIXED in SAS version 9.4. To account for the unevenly spaced time points the spatial power (SP (POW)) covariance structure was used. After running models with cortisol as the dependent variable, models were re-run with anxiety ratings across the stressor as the dependent variable. All model results are in Table 3.

Finally, as an exploratory step, we examined the associations between genetic variability in the *MIF* gene and circulating MIF levels at the baseline time point. In separate linear regressions models, we examined associations between the SNP, each CATT repeat and baseline MIF values. Then, we tested for associations between baseline MIF values and cortisol calculated as the average across the stressor and area under the curve (AUC) to measure cortisol reactivity (Pruessner et al., 2003).

3.4. Preliminary analyses

As a preliminary step, we examined bivariate associations between potential covariates, outcome variables and genotypes. Puberty status was not significantly associated with cortisol ($ps > .09$) or anxiety ($ps > .14$) at any time point. Child age was positively associated with baseline cortisol values, $r = .31$, $p = 0.01$. Ethnicity was associated with baseline anxiety rating, $r = -0.26$, $p = .03$, number of CATT5,

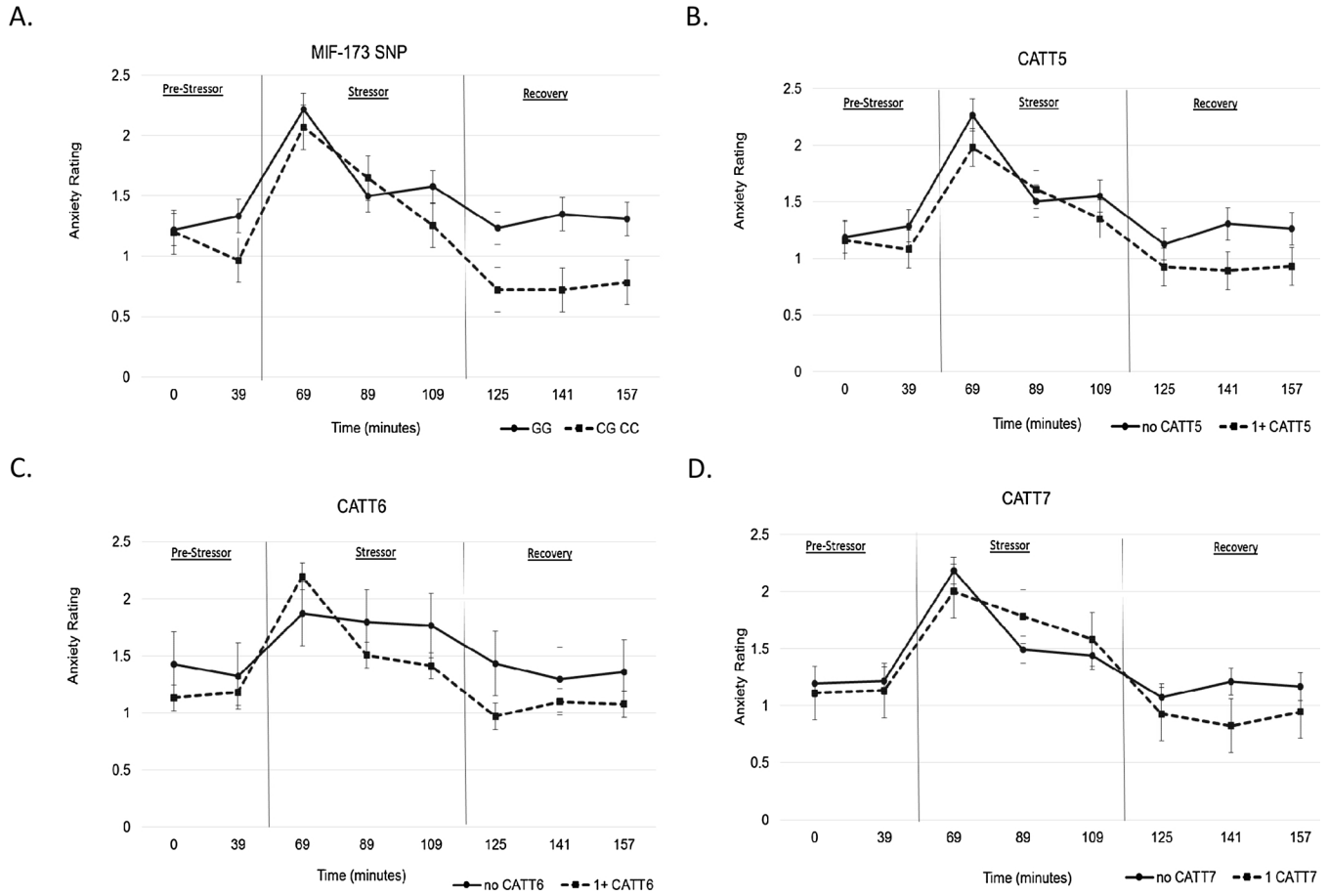

Fig. 2. Self-reported Anxiety ratings across all 8 time points of measurement from baseline (0–39) to stressor (69–109) and recovery (125–157). Data is estimated marginal means and error bars indicate standard error. (Adjusted for sex, age and ethnicity).
A) Anxiety Ratings by *MIF*–173Genotype. There were no differences between individuals with at least one C allele (CG or CC) compared to those with GG genotype. B) Anxiety Ratings by CATT5 carriers. There were no differences between individuals with no CATT5 alleles (6,6; 6;7) and 1 or more CATT5 alleles (5,6; 5,7; 5,5). C) Anxiety Ratings by CATT6 carriers. There were no differences between individuals with no CATT6 alleles (5,5; 5,7) and 1 or more CATT6 alleles (5,6; 6,6; 6,7). D) Anxiety Ratings by CATT7 carriers. There was no difference in anxiety between individuals with 1 CATT7 allele (5,7; 6,7) and those with no CATT7 alleles (5,5; 5,6; 6,6).

CATT6, and *MIF*-173 alleles ($r = -.46, p < .001, r = 0.52$, p $< .001, r = 0.52$, $p < .001$ respectively). This is consistent with previously reported genotypic frequencies in different ethnic samples (Bucala, 2006). Child age, sex, and ethnicity were entered as covariates for all models.

3.5. Primary analyses

3.5.1. MIF-173*G/C SNP

Results of models examining associations between the SNP and cortisol indicated a significant Genotype*Time interaction, $F(7,449) = 3.97, p < .001$, suggesting that *MIF*-173 genotype moderated cortisol reactivity overtime (See Table 3). Post hoc tests revealed that C carriers showed attenuated patterns of cortisol reactivity over the course of the TSST-C when compared with GG carriers, see Fig. 1A.

There was a marginally significant main effect of the *MIF*-173 SNP genotype on anxiety ratings (see Table 3). Follow up analyses revealed that C carriers reported lower ratings of anxiety during the recovery period (T5–T8) of the TSST-C when compared with GG carriers, see Fig. 2A.

3.5.2. CATT tetranucleotide repeat

CATT 5: Results of the second set of models, examining associations between the CATT5 repeat and cortisol values, revealed no significant associations between CATT5 and cortisol reactivity, $p = .535$.

CATT 7: There was a significant interaction effect of the CATT7 repeat with cortisol reactivity $F(4,468) = 2.21, p = .032$ (see Table 3). Post hoc tests revealed that children with one CATT7 allele showed significantly lower cortisol reactivity compared to children with zero CATT7 alleles. Children with one CATT7 allele also showed lower cortisol values during the recovery period (T5–T8) compared to those with zero CATT7 alleles, see Fig. 1D. There was no significant association between CATT7 repeat and anxiety ratings, $p = .256$.

3.5.3. Haplotype analyses

There was a significant interaction effect of the CATT7-173*C haplotype and time, $F(7, 449) = 2.24, p = .030$, indicating that individuals with the CATT7-173*C haplotype exhibited lower cortisol reactivity to the stressor and recovery. There was also a significant interaction between the CATT6-173*G haplotype and time, $F(7,449) = 2.40, p = .020$, such that individuals with the CATT6-173*G haplotype showed greater cortisol reactivity (Fig. 3).

There was also a significant interaction effect between CATT5-173*C haplotype and time on anxiety ratings, $F(7, 468) = 2.87, p = .006$, indicating that individuals with this haplotype exhibited different reactivity and lower recovery to the stressor. The main effect of CATT6-173*C, $F(1,66) = 5.66, p = .020$, was also significant, indicating lower ratings of anxiety across the stressor.

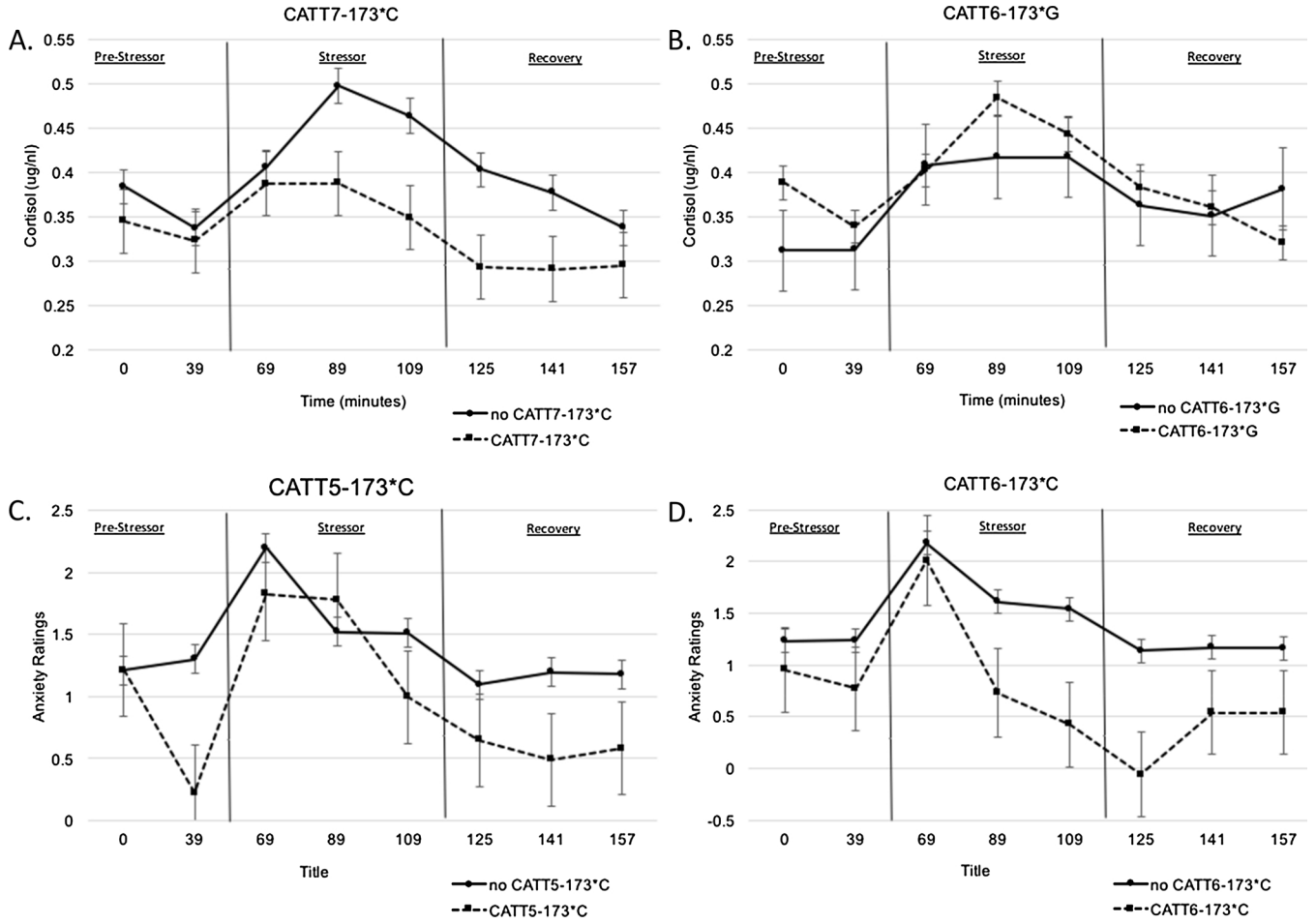

Fig. 3. Cortisol and Anxiety Reactivity by haplotypes. Saliva Cortisol and self-reported anxiety values across all 8 time points of measurement from baseline (0–39) to stressor (69–109) and recovery (125–157). Data is estimated marginal means and error bars indicate standard error. (Adjusted for sex, age and ethnicity).
A) Individuals with CATT7-173*C haplotype exhibit lower cortisol across the stressor compared to those without this haplotype. B) Individuals with CATT6-173*G haplotype exhibit increased cortisol across the stressor compared to those without this haplotype. C) Individuals with CATT5-173*C haplotype exhibit lower anxiety across the stressor compared to those without this haplotype. D) Individuals with CATT6-173*C haplotype exhibit lower anxiety across the stressor compared to those without this haplotype.

3.6. Exploratory models: salivary MIF

There were no significant associations between the *MIF*-173 SNP or CATT6 repeat and circulating levels of MIF ($p = .36$ and $p = .51$ respectively). However, there was a significant association between CATT5 repeat and circulating levels of MIF. Children with two CATT5 alleles displayed lower MIF levels than those with no CATT5 alleles, $B = -0.29$, $p = .04$. There was a marginally significant association between CATT7 repeat and MIF level, ($B = 0.24$, $p = .06$), suggesting a trend of higher MIF levels in participants with one CATT7 allele. There was no association between circulating MIF and average cortisol levels, $p = .75$, or in cortisol reactivity as assessed with AUC, $p = .73$.

4. Discussion

To our knowledge this is the first investigation to examine associations between *MIF* polymorphisms, HPA axis stress reactivity and affective symptoms during a psychological stressor in a sample of healthy adolescents. Data from our study showed that youth with the C allele of the -173*G/C SNP and the CATT7 repeat exhibited lower HPA axis reactivity, as indicated by lower cortisol reactivity during a social stress paradigm, when compared with those with the -173*G allele or lower CATT repeats. The CATT7-173*C haplotype was also associated with lower cortisol reactivity to the stressor and across the recovery period. Interestingly, the C7 haplotype was more strongly associated with average cortisol values than the -173*C SNP or CATT7 repeat alone. The inverse pattern was also observed with youth carrying the CATT6-173*G haplotype displaying higher cortisol in response to the stressor. In terms of behavior, carrying the CATT5-173*C or CATT6*173-C haplotype also predicted lower subjective reports of anxiety. Together, data point to the relevance of polymorphic variants in the *MIF* gene in explaining stress-related variability in HPA axis responding and anxiety. Our exploratory analyses showed that low expressing CATT5 alleles, were significantly associated with lower MIF levels extracted from saliva samples at a baseline assessment. However, salivary MIF levels were not associated with cortisol production during the assessment.

The -173*C allele and CATT7 repeat are variants of the *MIF* gene known to be associated with greater MIF expression (Baugh et al., 2002; Radstake et al., 2005). In our study, individuals with these variants showed lower cortisol values. Therefore, results complement previous research showing inverse associations between circulating MIF, the end product of the *MIF* gene, and cortisol reactivity to acute stress (Edwards et al., 2010). Prior work has established similar patterns of *MIF* genotype and glucocorticoid signaling in cases of autoimmune and inflammatory disorders (Griga et al., 2007; Baugh et al., 2002). However, we know of no studies that have demonstrated similar associations in healthy human populations, especially youth. Our data contribute to

this gap in knowledge, and suggest that polymorphic variations of the *MIF* gene, specifically, carrying the -173*C allele and the CATT7 repeat, known to be associated with higher MIF expression, predicts lower HPA axis functioning in normative populations.

Associations between the *MIF* genotypes and HPA axis reactivity can be interpreted in a number of ways. Given that carriers of the -173*C allele and CATT7 repeat, showed lower overall cortisol production across the assessment relative to those with GG alleles or CATT5-6 repeats, it is possible that low expressing *MIF* variants serve as a protective factor against elevated physiological stress reactivity, known to be associated with increased risk for stress-related disorders in adolescents (Colich et al., 2015; Gunnar et al., 2009; Hankin et al., 2010; Rao et al., 2008; van West et al., 2008). An alternative explanation is that the attenuated HPA axis response observed in individuals with the 173*C and CATT7 variants of the *MIF* gene may signal a general blunting of stress response. Emerging data suggests associations between cortisol hypo-reactivity and risk for anxiety or depressive symptoms (Colich et al., 2015; Hankin et al., 2010; Ouellet-Morin et al., 2011). Post hoc inspections revealed that cortisol values of -173*C carriers did not significantly change relative to their baseline levels at any point during the stressor. In contrast, GG carriers showed cortisol elevations that were significantly higher than baseline values. The largest difference in cortisol reactivity was observed between -173*C and CATT7 carriers and non-carriers at the T4 time point, which followed the initial social stressor and continued into the recovery period.

As part of our study, we also explored salivary MIF in association with *MIF* genotypes and cortisol levels. Our results were consistent with previous work demonstrating increasing CATT repeats associated with increased MIF levels (Baugh et al., 2002). This adds to our prior work in that it suggests that these genetic factors and environmental stressors may modulate MIF levels in pediatric populations (Bick et al., 2015). We found no associations between peripheral MIF levels and cortisol. This may point to limitations of measuring MIF from salivary samples. In this sample, the distribution of MIF values was highly skewed, with many individuals having peripheral MIF values very close to zero. Issues such as sample degradation may have been among the factors that contributed to non-normal values and null results.

Study limitations should be considered when interpreting results. First, data come from a relatively small sample, however, a priori power analyses revealed that we were powered to detect main effects and two-way interactions, as tested in this study. With the size of this sample, we were able to apply a methodologically rigorous paradigm to assess stress reactivity and HPA axis function, which is less feasible in larger sized samples. There were very few cases of carriers of CC alleles, and there were no cases of carriers of the CATT8 repeat. As mentioned, MIF levels were only examined from baseline salivary samples, therefore we did not capture changes in MIF reactivity. Associations between the genotype and salivary MIF response should also be compared with MIF extracted from serum or plasma to provide further validation. Additionally, MIF analyses were conducted after cortisol analyses leading to potential degradation of protein levels.

5. Conclusions and future directions

Despite these limitations, our findings provide preliminary support for the polymorphic variability in the *MIF* gene as an underlying contributor to differences in physiological stress reactivity in youth. Most notable was that -173*C and CATT7 polymorphisms and the -173*C-CATT7 haplotype were associated with blunted cortisol reactivity. Future research should consider if this blunted stress response is specific to the HPA axis or generalized to other stress response systems as well. Finally, the *MIF* gene's potential ability to predispose individuals to alterations in HPA axis may be important for further study of anxiety and depression. The fact that our associations emerged during adolescence suggest that MIF may serve as a candidate marker for stress-related disorders at a point in development where affective mental health problems often first emerge. Future work should continue to examine MIF, HPA axis signaling, and affective symptoms in adolescence.

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