PTSD and the Klotho Longevity Gene:

Evaluation of Longitudinal Effects on Inflammation via DNA Methylation

Supplementary Materials

Supplementary Methods

DNA Extraction. DNA isolation was achieved with a Qiagen AutoPure instrument and Qiagen reagents; DNA concentrations were normalized using the Quant-iT™ PicoGreen dsDNA fluorescent assay (Invitrogen). TaqMan® RNase P Detection assay (Applied Biosystems Assay, Life Technologies, Carlsbad, CA) with fluorescence detection on a 7900 Fast Real Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, CA) was used per the manufacturer's instructions to determine DNA quality and quantity. Bisulfite conversion for all samples (including cases, controls, and technical replicates) were completed at the same time in the same batch.

Genotyping. DNA was first whole-genome amplified, fragmented, precipitated and resuspended and then hybridized on Illumina HumanOmni2.5-8 beadchips for 20 hours at 48ºC per manufacturer's instructions (Illumina, San Diego, CA). A single-base extension followed by a multi-layered staining process was conducted following hybridization. The Illumina iScan System was used to image the beadchips, with results processed using Illumina GenomeStudio v2011.1 software containing the Genotyping v1.9.4 module.

Ancestry PCs. Principal components (PCs) to estimate ancestry and ancestry substructure within the white, non-Hispanic participants (for use as ancestry covariates) were computed based on 100,000 randomly chosen common (minor allele frequency >5%) SNPs using PLINK version 1.9 (Chang, Chow et al. 2015).

DNA Methylation Data Processing Pipeline. GenomeStudio was used to output individual-level background-corrected probe data and idat files. DNAm data were cleaned with the CpGassoc package and the ChAMP package in R (R Development Core Team, 2008). Probes that did not meet a detection p-value threshold of 0.001 were set to missing. One chip

had 7 out of 8 failed samples (>10% missing) and thus these data were discarded and samples were rerun on a new chip, resulting in no samples with >10% missing data. No samples had intensity < 50% of the experiment-wide mean or intensity <2,000 arbitrary units (AU). Cross hybridizing probes (i.e., between autosomes and sex chromosomes) were excluded (Chen et al., 2013; n=44,132) as were 977 "underperforming" EPIC probes included in Illumina Product Quality Notification PQN0223 Dated April 19, 2017.

White Blood Cell Count Estimation. As is standard, proportional white blood cell (WBC) counts were estimated from the methylation data using the R minfi package (Aryee et al, 2014), as applied to work with EPIC chip data (Fortin et al., 2017). This algorithm yields estimated proportions of CD4 and CD8 T-cells, natural killer (NK) cells, monocytes, and b-cells.

meQTL Replication Sample

The data from the meQTL replication analysis described in Footnote #3 in the main text comes from an on-going study involving 133 veterans who screened positive for PTSD over the telephone (PTSD was subsequently assessed as part of the study but as the study is on-going, these data are not yet available for analysis). The sample was comprised of 77% men with a mean age of 55.28 years (SD: 30.04). We received the first set of genotypes and DNAm data from this study (more samples will be sent out for analysis), which were processed using the same methods as that described above. We examined the bivariate correlation between the rs9527025 and cg00129557 to look for evidence of replication of the meQTL reported in this study and found a near identical correlation coefficient (within .01) in the second sample as that reported in the main text.

Confounder Analyses

We conducted follow-up analyses examining potential confounds of the effects of the KL-VS SNP investigated in this study (rs9527025) and rs9527025 X PTSD severity on methylation at cg00129557 in the cross-sectional sample. We retained the model as in the primary analysis but also included self-reported cigarette use (yes/no, as assessed with the

Fagerström Test for Nicotine Dependence; FTND; Heatherton et al., 1991; 26.9% of the sample reported cigarette use), current major depressive and alcohol use disorders (present in 25.6% and 6.5% of the sample, respectively, as assessed with the Structured Clinical Interview for DSM-IV Disorders; SCID; First et al., 1997; Spitzer et al., 1998), total number of lifetime traumatic brain injuries (TBIs; *M* = 1.78, *SD* = 2.26), and body mass index (*M* = 28.01, *SD* = 4.25) as main effects and also included their interaction with rs9527025 in the third step of the model. We found that none of these variables were directly associated with methylation at this locus (smallest $p = .19$). The main effect of the SNP remained significant in the second step of the model ($p < .001$). When examining the interaction terms in the third step of the model, there was a significant interaction between rs9527025 and current cigarette use ($p = .017$), but it did not account for the interaction between rs9527025 and PTSD severity ($p = .001$), which remained significant. Thus, the data suggest that the significant interaction between the SNP and PTSD is not due to a third variable effect with respect to BMI, alcohol-use diagnoses, major depression, TBIs, or cigarette use.

We also examined time between assessments as a potential confound in our longitudinal analysis (that depicted in Figure 3), by including time between assessments as a covariate of T2 CRP in a follow-up model. Time between assessments was not associated with T2 CRP ($p = .98$) while the indirect effect of rs9527025 X PTSD severity on T2 CRP via methylation at cg00129557 remained significant (standardized $\beta = -.13$, $p = .036$).

Interaction and longitudinal models for cg02706658

Our analyses revealed a corrected-significant main effect of rs9527025 on cg02706658 in the cross-sectional data (Table S1), although weaker than the association observed between rs9527025 and the other 5'UTR *KL* site (cg00129557). Therefore, we also tested rs9527025 X PTSD severity interaction as a predictor of methylation at cg02706658 in the cross-sectional data and this methylation locus as a potential predictor of CRP in the longitudinal data to see if

results were consistent with those observed for cg00129557. A similar pattern of results would suggest a broader *KL* 5'UTR methylation effect.

The PTSD severity by rs9527025 interaction was not a significant predictor of methylation at cg02706658 in the cross-sectional dataset (p = .12). In the longitudinal cohort, the main effect of rs9527025 (dominant coding) on methylation at this locus was evident (B = -.98, standardized β = -.77, p < .001). In addition, there was a rs9527025 X PTSD severity effect on cg02706658 in the longitudinal dataset, which had not been significant in the larger cross-sectional dataset (B = .007, standardized β = .34, p = .033, Figure S2). In turn, methylation at this locus was negatively associated with residualized change in CRP at T2 (B = -.12, standardized β = -.19, p = .030; Figure S2). However, the indirect effect of the interaction term on residualized change in CRP via methylation at cg02706658 was not significant (p = .15).

Models predicting mean methylation across cg02706658 and cg00129557

We tested if combining (averaging) methylation values at cg02706658 and cg00129557 would provide more information than examining each locus alone. Given their proximity to each other and their correlation, associations with mean methylation across the two loci might indicate a general effect across the DNase hypersensitivity region. Cross-sectional results were nearly identical to that reported for cg00129557 in Table 4 of the main text. Specifically, rs9527025 was associated with mean methylation across the two CpG loci at B = -.60, SE = .053, p < .001 and the rs9527025 X PTSD severity term was associated with mean methylation values across the two loci at B = .004, SE = .002, p = .034. The longitudinal model also did not yield different results when we analyzed mean methylation at the two loci relative to focusing on cg00129557 alone. Specifically, mean methylation values were still strongly predicted by rs9527025 (dominant coding; B = - 1.09, SE = .18, standardized β = -.92, p < .001), and by the rs9527025 X PTSD severity term (B = .01, SE = .003, standardized β = .49, p = .002). CRP at T2 was associated with mean methylation values (B = -.20, SE = .06, standardized β = -.31, p = .001). The indirect effect of rs9527025 X PTSD severity on CRP at T2 via mean methylation

across the two CpG sites was significant (B = -.002, SE = .001, standardized β -.15, p = .031). Thus, it did not appear that combining these loci provided any more information than did focusing on the strongest associated loci (cg00129557), as we did in the main text of the manuscript.

Additional Models

Though we have previously evaluated associations between PTSD, *KL* genotypes, and advanced epigenetic age (Wolf et al., 2019), we did not include epigenetic age in this study due to concerns that associations between *KL* DNAm loci and epigenetic age might simply reflect the concurrent covariation of DNAm across the epigenome. However, to be thorough, and in response to a helpful reviewer critique, we did analyze associations between the primary *KL* DNAm locus examined in this study (cg00129557) and Horvath-defined epigenetic age relative to chronological age. To do so, we first residualized cg00129557 on the KL-VS SNP rs9527025 (given that this was an meQTL). We also residualized Horvath epigenetic age estimates (see description in Wolf et al., 2019) on chronological age to form DNAm age residuals (a dimension ranging from slowed to advanced epigenetic age relative to chronological age). The residualized cg00129557 variable was then used to predict Horvath-defined epigenetic age residuals, controlling for estimated white blood cell type proportions, sex, and the top 3 ancestry PCs within the white non-Hispanic sample (*n* = 307). We found that methylation at cg00129557 (residualized for rs9527025) was associated with slowed epigenetic age (B = -.90, standardized β = -.15, *p* = .024), which is consistent with the direction of effect for this locus in association with (reduced) CRP, as described in the main text of the paper. However, when we tested a similar model in the longitudinal data to see if methylation at cg00129557 at T1 (residualized for rs9527025) predicted T2 Horvath-defined epigenetic age residuals, controlling for T1 Horvath epigenetic age residuals, ancestry, and sex in the white non-Hispanic longitudinal sample (*n* = 115), we found no evidence for an association between T1 methylation at SNP-residualized cg00129557 and T2 epigenetic age residuals (B = .08, standardized β = .02, *p* = .84). It is not

clear how best to interpret this pattern of results across the cross-sectional and longitudinal data, and as noted above, we had concerns that the cross-sectional results might simply reflect the covariation of DNAm across the epigenome. Thus, we have elected not to include these results in the main text of the manuscript, but include them here for reader edification.

Table S1

Complete results from models predicting KL DNA methylation at 10 Loci

	cg00129557					cg02706658					cg19154940			
Variable	*β*	SE	*p*	*p*-corr		*β*	SE	*p*	*p*-corr		*β*	SE	*p*	*p*-corr
Step 1: Covariates														
Age	-.01	.01	.054	NA		-.01	.01	.319	NA		.01	.004	.170	NA
Sex	-.21	.16	.198	NA		-.25	.14	.075	NA		.05	.12	.651	NA
CD8-T	3.24	1.20	**.007**	NA		2.13	1.05	**.044**	NA		-.39	.87	.651	NA
CD4-T	4.47	.91	**1.63E-06**	NA		1.29	.80	.105	NA		-.40	.66	.542	NA
NK	6.28	1.40	**1.00E-05**	NA		5.09	1.22	**3.93E-05**	NA		-.54	1.01	.592	NA
B cells	3.44	1.91	.072	NA		3.73	1.67	**.026**	NA		-.34	1.38	.806	NA
Mono	-1.44	1.72	.404	NA		-.20	1.50	.896	NA		1.21	1.25	.333	NA
PC1	-.90	.79	.257	NA		-.37	.69	.591	NA		.14	.57	.805	NA
PC2	.69	.79	.382	NA		-.22	.68	.746	NA		.61	.57	.287	NA
PC3	-.94	.77	.221	NA		-1.07	.67	.112	NA		.63	.56	.259	NA
PTSD sev	-.001	.001	.642	NA		-.002	.001	.206	NA		-.001	.001	.385	NA
Step 2: SNP[a]														
rs9527025	-.65	.07	**1.29E-20**	**< .0001**		-.55	.06	**1.30E-19**	**< .0001**		.12	.05	**.027**	.538
rs398655	.12	.06	**.028**	.54		.10	.05	**.036**	.6325		-.08	.04	**.035**	.620
rs9315202	-.05	.07	.460	1		-.01	.06	.933	1		.06	.05	.191	1

	cg20672059					cg17806623					cg23584087			
Variable	*β*	SE	*p*	*p*-corr		*β*	SE	*p*	*p*-corr		*β*	SE	*p*	*p*-corr
Step 1: Covariates														
Age	-.002	.002	.252	NA		.003	.002	.188	NA		-.004	.004	.384	NA
Sex	.03	.05	.601	NA		.002	.06	.973	NA		-.21	.13	.114	NA
CD8-T	-.18	.40	.658	NA		-.04	.47	.936	NA		-5.30	.97	**9.73E-08**	NA
CD4-T	-.71	.30	**.019**	NA		.34	.36	.346	NA		-2.54	.74	**6.29E-04**	NA
NK	-.70	.46	.132	NA		.78	.55	.156	NA		-6.80	1.13	**4.96E-09**	NA
B cells	1.62	.63	**.011**	NA		.58	.75	.443	NA		-2.78	1.54	.072	NA
Mono	-.70	.57	.222	NA		.48	.67	.474	NA		-1.23	1.39	.377	NA
PC1	-.07	.26	.786	NA		-.01	.31	.972	NA		-.015	.64	.981	NA
PC2	.50	.26	.053	NA		-.08	.31	.806	NA		1.54	.63	**.015**	NA
PC3	.25	.25	.334	NA		-.07	.30	.817	NA		-.13	.62	.834	NA
PTSD sev	-.0004	.0004	.316	NA		-.00001	.001	.982	NA		-.002	.001	**.029**	NA
Step 2: SNP[a]														
rs9527025	.02	.03	.476	1		-.02	.03	.616	1		.06	.06	.328	1
rs398655	-.02	.02	.376	1		.05	.02	**.014**	.323		-.02	.05	.59	1
rs9315202	.01	.02	.758	1		-.01	.03	.721	1		-.05	.05	.387	1

	cg09886946					cg26325430					cg18056695			
Variable	*β*	SE	*p*	*p*-corr		*β*	SE	*p*	*p*-corr		*β*	SE	*p*	*p*-corr
Step 1: Covariates														
Age	.01	.003	**.019**	NA		-.001	.004	.762	NA		.012	.003	**.0001**	NA
Sex	.04	.09	.626	NA		-.16	.13	.199	NA		.16	.09	.864	NA
CD8-T	.32	.67	.634	NA		2.7	.94	**.004**	NA		4.51	.68	**1.53E-10**	NA
CD4-T	-.06	.51	.914	NA		2.21	.71	**.002**	NA		2.08	.52	**7.05E-05**	NA
NK	.06	.78	.935	NA		4.64	1.09	**2.67E-05**	NA		5.45	.79	**3.23E-11**	NA
B cells	1.44	1.07	.178	NA		1.67	1.49	.262	NA		.20	1.08	.856	NA
Mono	-.12	.96	.900	NA		-2.35	1.34	.079	NA		1.49	.97	.126	NA
PC1	-.19	.44	.673	NA		-.34	.61	.576	NA		-.81	.45	.071	NA
PC2	.54	.44	.223	NA		-1.4	.61	**.025**	NA		-.14	.44	.746	NA
PC3	-.2	.43	.647	NA		-.12	.60	.845	NA		.24	.43	.581	NA
PTSD sev	.001	.001	.073	NA		.0003	.001	.776	NA		-4.5E-06	.001	.995	NA
Step 2: SNP[a]														
rs9527025	.01	.04	.898	1		-.04	.06	.461	1		.032	.043	.449	1
rs398655	.08	.03	**.010**	.24		.06	.04	.139	.98		.07	.03	**.019**	.40
rs9315202	-.08	.04	**.025**	.05		-.02	.05	.776	1		-.04	.04	.333	1

	cg25223823			
Variable	*β*	SE	*p*	*p*-corr
Step 1: Covariates				
Age	-.01	.003	**.047**	NA
Sex	-.04	.08	.631	NA
CD8-T	-3.78	.57	**1.23E-10**	NA
CD4-T	-2.17	.43	**7.41E-07**	NA
NK	-4.90	.66	**1.02E-12**	NA
B cells	-1.81	.90	**.046**	NA
Mono	-1.09	.81	.178	NA
PC1	.05	.37	.900	NA
PC2	.13	.37	.733	NA
PC3	.02	.36	.953	NA
PTSD sev	-.001	.001	.119	NA
Step 2: SNP[a]				
rs9527025	.07	.04	.056	.79
rs398655	-.04	.03	.18	.99
rs9315202	-.05	.03	.096	.93

Note. Beta values are unstandardized. p-corr = multiple testing corrected p-value; NK = natural killer; mono = monocytes; PC = principal component; PTSD sev = posttraumatic stress disorder severity; IX = interaction.
[a]The SNPs were evaluated in separate models but are listed here together for the sake of simplicity.

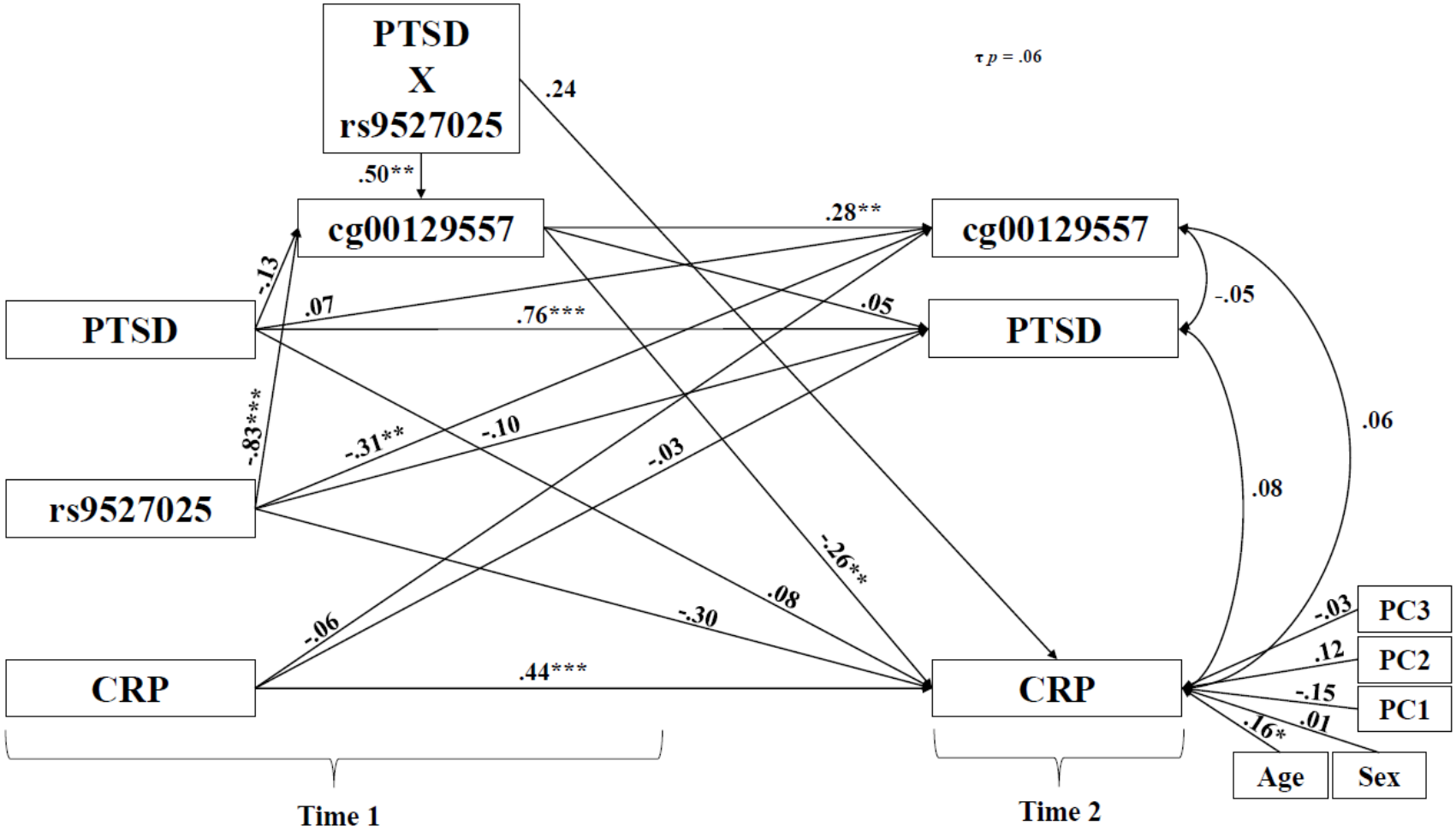
Table S2

Fit of Longitudinal Path Models

Model	χ^2	df	RMSEA	SRMR	CFI	TLI
cg00129557						
CRP	6.76	6	.034	.050	.986	.956
Follow-up CRP 1	6.11	6	.013	.048	.998	.994
Follow-up CRP 2	10.11	11	0.00	.042	1.00	1.03
Follow-up CRP 3	14.14	18	0.00	.045	1.00	1.05
cg02706658	6.24	6	.019	.045	.996	.987

Note. The CRP model refers to the primary longitudinal result as shown in Figure 2 (with dominant genotype coding). Follow-up CRP 1 refers to the model referenced in the main text in which rs9527025 was coded additively. Follow-up CRP 2 refers to the model referenced in the main text in which estimated white blood cells were included as additional covariates to the CRP model. Follow-up CRP 3 refers to the model referenced in the main text in which additional T2 variables were included in the model (as in Figures S1). None of the χ^2 values were statistically significant, consistent with good model fit. Df = degrees of freedom; RMSEA = root measure square error of approximation; SRMR = standardized root mean square residual; CFI = confirmatory fit index; TLI = Tucker-Lewis index; CRP = C-reactive protein.

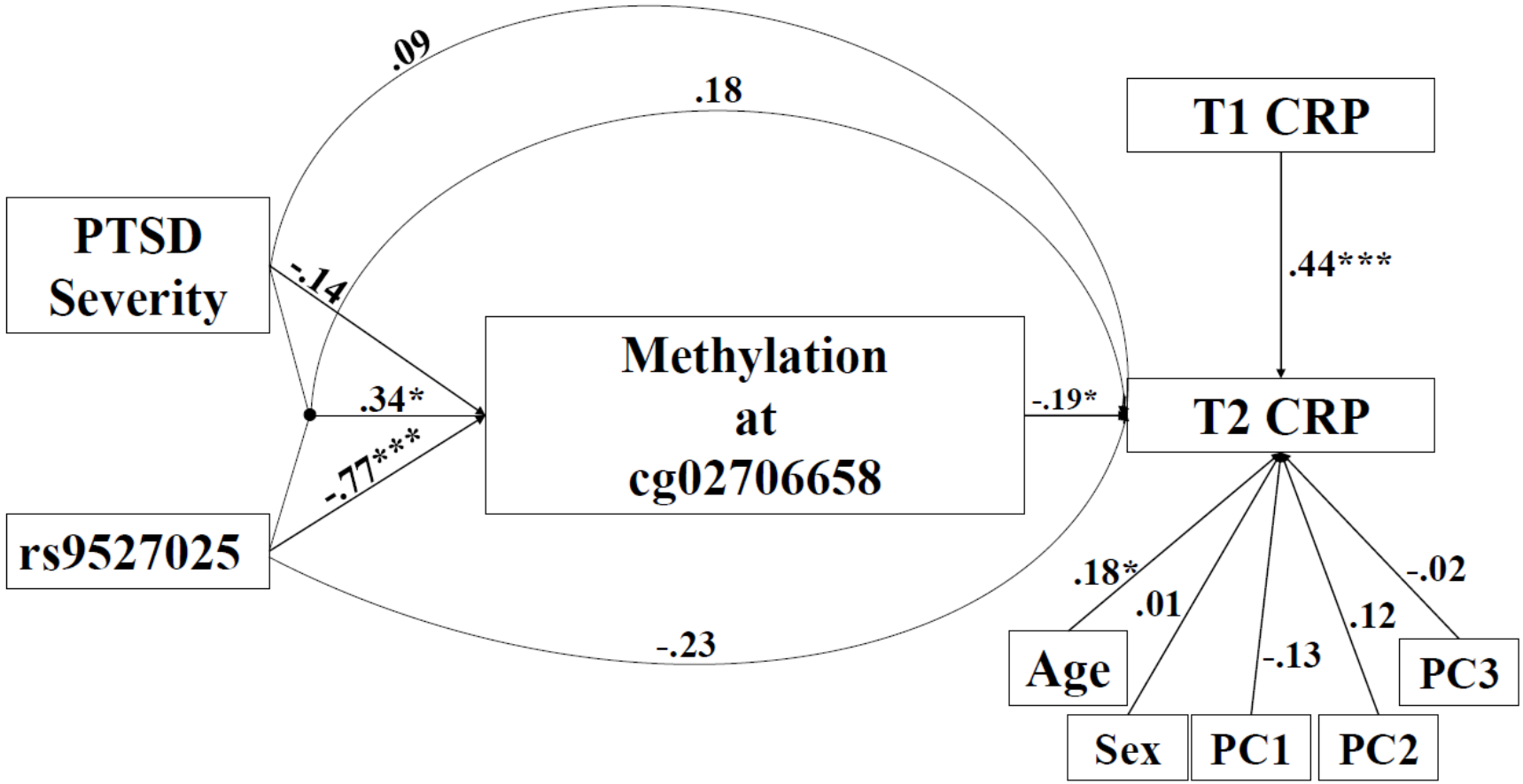
Figure S1



The figure shows the path model for the KL-VS SNP (dominant coding) and cg00129557 with additional Time 2 variables included (i.e., building on the primary model shown in the main text; *n* = 111).

*p < .05. **p < .01. ***p < .001.

Figure S2



The figure shows the path model for the KL-VS SNP (dominant coding) and cg02706658 (n = 111).

*p < .05. **p < .01. ***p < .001.

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