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***FOXO1*, *A2M* and *TGFB1*: three novel genes predicting depression in gene X environment interactions are identified using cross-species and cross-tissues transcriptomic and miRNomic analyses**

Authors:

Annamaria Cattaneo[1,2], Nadia Cattane[2], Czamara Darina[3], Johan G. Eriksson[4,5,6], Eero Kajantie[4,7,8], Alessia Luoni[9], Chiara Malpighi[2], Anna Suarez[10], Jari Lahti[7,10,11], Valeria Mondelli[1], Paola Dazzan[12], Katri Räikkönen[10], E. B. Binder[3,13] Marco A. Riva[9❖], and Carmine M Pariante[1❖].

[1]Stress, Psychiatry and Immunology Laboratory, Department of Psychological Medicine, Institute of Psychiatry, King's College, London;

[2] Biological Psychiatry Unit, IRCCS Fatebenefratelli S. Giovanni di Dio, Brescia;

[3] Department of Translational Research in Psychiatry, Max-Planck Institute of Psychiatry, Munich, Germany;

[4]National Institute for Health and Welfare, Helsinki, Finland;

[5]Hospital for Children and Adolescents, Helsinki University Hospital and University of Helsinki, Helsinki, Finland;

[6]PEDEGO Research Unit, Oulu University Hospital and University of Oulu, Oulu, Finland;

[7] Folkhälsan Research Centre, Finland; 4National Institute for Health and Welfare, Helsinki, Finland;

[8] Department of General Practice and Primary Health Care, University of Helsinki and Helsinki University Hospital, Helsinki, Finland;

[9] University of Milan, Department of Pharmacological and Biomolecular Sciences, Milan, Italy;

[10] Institute of behavioral sciences, University of Helsinki, Helsinki, Finland;

[11] Helsinki Collegium for Advanced Studies, Helsinki, Finland;

[12] Institute of Psychiatry, Psychology & Neuroscience, King's College London, London, UK;

[13] Department of Psychiatry & Behavioral Sciences, Emory University School of Medicine, Atlanta, GA, USA.

❖ these authors contributed equally

Corresponding Author

Dr. Annamaria Cattaneo, PhD

Department of Psychological Medicine,

Institute of Psychiatry, Psychology & Neuroscience, King's College London

Email: annamaria.cattaneo@kcl.ac.uk

Abstract (max 250 words)

Depression results from the interplay of vulnerability genes with environmental factors, a phenomenon named as 'gene-environment (GxE) interaction'. To date, GxE interaction studies have been limited to hypothesis-based candidate genes, since genome-wide (GWAS)-based GxE interaction studies would require enormous datasets with genetics, environmental and clinical variables. We used a novel, cross-species and cross-tissues "*omics*" approaches to identify genes predicting depression in response to stress in GxE interactions. We integrated the transcriptome and miRNome profiles from the hippocampus of adult rats exposed to prenatal stress (PNS) with transcriptome data obtained from blood mRNA of adult humans exposed to early life trauma, using a stringent statistical analyses pathway. Network analysis of the integrated gene lists identified the Forkhead box protein O1 (*FOXO1*), Alpha-2-Macroglobulin (*A2M*) and Transforming Growth Factor Beta 1 (*TGFB1*) as candidates to be tested for GxE interactions, in two GWAS samples of adults either with a range of childhood traumatic experiences (Grady Study Project, Atlanta, USA) or with separation from parents in childhood only (Helsinki Birth Cohort Study, Finland). Six *FOXO1* SNPs showed significant GxE interactions with emotional abuse in the Grady Study that survived stringent permutation analyses and were all replicated in the Helsinki study. In addition, other SNPs in all the three genes showed significant GxE interactions with emotional, physical and sexual abuse in the Grady Study. We therefore provide a successful 'hypothesis-free' approach for the identification and prioritization of candidate genes for GxE interaction studies that can be investigated in GWAS datasets.

Running title: FOXO1, A2M and TGFB: predictors of early in life stress-associated depression

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Background

Major Depressive Disorder (MDD) has a well-established genetic contribution, although with a modest (30-40%) heritability[1]. However, we have so far failed to identify vulnerability genes for this disorder. Indeed, while genome wide association studies (GWAS) have identified main genetic effects for schizophrenia[2], autism[3] and bipolar disorder[4], even very large GWAS meta-analyses have failed to identify genome-wide significant associations in depression[5, 6]. Only recently, and using enormous datasets from more than 75 thousand individuals with depression and 200 thousand healthy subjects, 15 genome-wide significant loci for MDD have been identified[7]. One of the potential reasons behind this weak genetic effect is the fact that individual genotypic variations may increase the risk of depression only in the presence of exposure to life stressors and other adverse environmental circumstances, a phenomenon named as 'gene-environment (GxE) interaction'[8]. Indeed, environmental factors, and in particular exposure to adversities early in life, have been consistently implicated in the pathophysiology of depression[9]. For example, in a large study conducted by the Center of Disease Control with over 9000 participants, Chapman et al (2004)[10] reported a dose-response relationship between the severity of experienced childhood adversities and the lifetime presence of depressive episodes or chronic depression. However, despite the epidemiological and clinical evidence confirming these effects, there is substantial variability in the outcomes of early life stress, since not all people exposed to early adversities develop depression later in life. GxE interactions may indeed also explain why adverse environmental factors do not increase the risk of depression in each and every individual.

The most established examples of GxE interactions linking stress to depression result from the hypothesis-driven investigation of the serotonin transporter promoter short/long polymorphism (5-HTTLPR) and of a functional single nucleotide polymorphism (SNP) within the FK506-binding protein 51 (FKBP5) gene. Caspi and colleagues were the first to show that individuals with one or two copies of the short allele of the 5-HTTLPR exhibit more depressive symptoms and suicidality in the presence of stressful life events as compared with individuals homozygous for the long allele[11]. Although not all the studies have replicated these findings[12], this GxE interaction has been shown to predict depression

in individuals exposed to childhood maltreatment[13]. Similarly, many studies have shown that a functional SNP within FKBP5 (rs1360780) interacts with childhood abuse to predict a multitude of psychiatric phenotypes in adults, including depression and post-traumatic stress disorder[14-18]. For this latter gene, epigenetic mechanisms leading to different cortisol reactivity to stress seem to explain these effects[19, 20]. These two examples of GxE interactions are built on decades of hypothesis-driven research, and have been instrumental in order to identify not only genetic variants that increase the risk of depression, but also the potential biological and molecular mechanisms underlying this increased risk. However, as extensively discussed elsewhere[8], this hypothesis-driven approach can only discover a fraction of such potential existing genetic variants, and it is at odds with the current strategy of using a hypothesis-free approach to identify genes associated with disorders.

Of course, using GWAS data to test GxE interactions would be a theoretically viable option, but it would require enormous datasets with both exposure (such as life events) and outcome (depression) data. To date, only two genome-wide by environment interaction studies (GWEIS) have been performed in this regard. Dunn and colleagues (2016)[21], using data from the SHARe cohort of the Women's Health Initiative comprising more than 10 thousand African Americans and Hispanics/Latinas, have examined genetic main effects and GxE interactions with stressful life events and social support in the development of depressive symptoms; however, only one interaction signal was genome-wide significant in African Americans (rs4652467 located 14 kb from CEP350), and it was not replicated. The second, subsequent whole genome pilot study, performed in only 320 subjects characterized for recent stressful life events, found no interaction that was genome-wide significant[22].

In the current paper, we propose a different "omics-based" approach to identify candidate genes for GxE interactions studies in GWAS datasets, using cross-species and cross-tissue biological prioritization strategies that limits the number of investigated genes and thus enhances the statistical power to identify significant findings. In particular, we have first analyzed the transcriptome and miRNome data from the hippocampus of adult rats exposed to prenatal stress (PNS), a well-established model of exposure to early life trauma leading to depressive behavior and hypothalamic-pituitary-

adrenal (HPA) axis hyperactivity in adulthood[23], in order to obtain a list of genes that are both modulated by PNS *and* targeted by the miRNAs that are modulated by PNS. We have then integrated the resulting genes list with transcriptome data obtained from blood mRNA of human adults who had suffered from childhood trauma, and analyzed the overlapping genes list using network analysis. Finally, we have tested the top network cluster of genes for GxE interactions in order to examine the effects of early life stress on depressive symptoms in adulthood, in two different clinical samples from the Grady Trauma Project in Atlanta (USA)[24] and the Helsinki Birth Cohort Study (Finland)[25, 26].

MATERIAL AND METHODS

Animal model and clinical samples

Prenatal Stress Model (for transcriptomics and miRNomics analyses): PNS procedure was performed as already published[27-29]; briefly, pregnant dams in the last week of gestation were restrained in a transparent Plexiglas cylinder, under bright light, for 45min, three times a day for one week. Control pregnant females were left undisturbed in their home cages. Male offspring from control and PNS groups were killed at postnatal day (PND) 62 (early adulthood) for whole hippocampal dissection (for further details see **Supplementary Material**).

Subjects exposed to early life trauma (for transcriptomics analysis): We recruited volunteer subjects from the local population living in the area served by the South London and Maudsley NHS Trust, in south-east London. Childhood traumatic events were assessed using the Childhood Experience of Care Abuse Questionnaire (CECA-Q;[30]). We performed transcriptomic analyses in 20 subjects who reported at least one type of abuse (separation and physical abuse= 40%; separation and sexual abuse= 20%; physical abuse only= 20%; physical and sexual abuse=5%; physical and emotional abuse= 5%; emotional abuse only=5%; sexual abuse only=5%), and in 20 subjects matched for age, gender and body mass index (BMI) with no history of early life trauma. Mean age±SD was 27±1.6 and 25±0.9

(df=15; $\aleph^2$ = 0.222, p-value= 0.3), and percentage of females was 45% (7F/13M) and 35% (9F/11M) ($\aleph^2$ = 0.417, p-value= 0.5), respectively, in the subjects with and without childhood trauma. Blood samples were collected by using PaxGene Blood Tubes. After collection, blood samples were kept at room temperature for 2 hours, then at -20°C for 2 days and then at -80°C until their processing. The project was approved by the Local Ethical Committee (for further details see **Supplementary Material**).

Grady Trauma Project (for GxE analysis): In the Grady Trauma Project, recruited in Atlanta (Georgia, USA), detailed trauma interviews were collected in a large sample of 4,791 adults (mean age±SD of 40.1± 13.9) with high rates of current and lifetime PTSD. For this study, we used the Childhood Trauma Questionnaire (CTQ), a 28-item, psychometrically validated inventory assessing self-reported levels of sexual, physical and emotional abuse[31]. The presence of moderate-severe sexual, physical and emotional abuse was coded as described before[32]. Depressive symptoms were measured using the Beck Depression Inventory (BDI)[33]. The institutional review board at Emory University approved the study procedures. All participants provided written informed consent before participating.

Helsinki Birth Cohort Study (for GxE analysis): Between 2001 and 2004, 1,620 individuals (mean age±SD of 61.5±2.9) who were born at Helsinki University Central Hospital between 1934 and 1944, and who were alive and living in Finland in 1971, participated into a clinical examination during which blood samples for DNA were used to obtain GWAS data for both directly genotyped and imputed SNPs. Depressive symptoms were measured using the BDI. Early life emotional stress in this samples was defined as separation from biological parents in 1939-46, during the second world war, when around 80,000 Finnish children were sent to Sweden and Denmark in order to escape the dangers of war; 384 subjects were identified as having been separated in childhood[25,26,34]. The project was approved by the Local Ethical Committee (for further details see **Supplementary Material**).

Biological assessments

mRNA and miRNA isolation from hippocampus of animals and from the blood of subjects: Total RNA, including miRNAs, was isolated from rat brain tissues by using RNeasy mini kit (Qiagen, Italy) and from human blood samples using PaxGene miRNA kit (Qiagen, Italy), according to the manufacturer's instructions. Samples were also treated with DNase, and RNA quantity and quality was assessed by evaluation of the A260/280 and A260/230 ratios using a Nanodrop spectrometer (NanoDrop Technologies).

Whole Genome expression microarray analyses in the hippocampus of animals and in the blood of subjects

Gene expression microarray assays were performed using Rat Gene 2.1st Array Strips (which covers 27,147 coding transcripts) or Human Gene 2.1st Array Strips (which covers 31650 coding transcripts) on GeneAtlas platform (Affymetrix), following the WT Expression Kit protocol described in the Affymetrix GeneChip Expression Analysis Technical Manual, and as we have done before[27, 35] (for further details see **Supplementary Material**). Of note, this is the first report of the rat hippocampal transcriptome following PNS using the novel Rat Gene 2.1st Array Strips, hence we have reported here all the relevant data and pathways analyses.

Real Time validation analyses of transcriptomic findings in the hippocampus of animals and in the blood of subjects

All the genes that were significantly modulated by stress both in the hippocampus of animals and in the blood of subjects (n=22 genes, see Results Section) were validated using Real-Time PCR by using Biorad qPCR Mix (BioRad, Milan) and Taqman Assays on a 96 wells Real Time PCR System (One Step TaqMan Real Time PCR). Each sample was assayed in triplicate and each target gene was normalized to β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in rodents, and to β-2-

microglobulin (B2M) and GAPDH in humans. The Pfaffl Method was used to determine relative target gene expression.

MiRNome expression analysis in the hippocampus of animals

500ng of total RNA (including miRNAs) were processed with the FlashTag Biotin HSR RNA Labeling kit (Affymetrix, Santa Clara, CA, USA) and subsequently hybridized onto the GeneChip miRNA 4.1 Array Strip on a GeneAtlas platform (Affymetrix, Santa Clara, CA, USA), which cover 728 mature rat miRNAs and 490 rat pre-miRNAs annotated in miRBase (online miRNA database, http://www.mirbase.org). Washing/staining and scanning procedures were conducted on the Fluidics station following the manufacturer's instructions.

Genotyping of polymorphisms within the Grady Trauma Study: DNA was extracted from saliva in Oragene collection vials (DNA Genotek, Ottawa, ON, Canada) using the DNAdvance kit (Beckman Coulter Genomics, Danvers, MA, USA), while DNA from blood was extracted using either the E.Z.N.A. Mag-Bind Blood DNA Kit (Omega Bio-Tek, Norcross, GA, USA) or ArchivePure DNA Blood Kit (5 Prime, Gaithersburg, MD, USA). N=4,791 subjects were genotyped on the HumanOmniExpress (6%) and the Omni1-Quad BeadChip (94%) (Illumina Inc), and genotypes were called with Illumina's Genome Studio. The HumanOmniExpress interrogates 730,525 individual SNPs per sample, whereas the Omni1- Quad BeadChip interrogates 1,011,219 individual SNPs (for further details see **Supplementary Material**).

Genotyping of polymorphisms in the Helsinki Birth Cohort Study: DNA was extracted from blood samples using Gentra Kit. Participants were genotyped with the modified Illumina 610k array, at the Wellcome Trust Sanger Institute, Cambridge, UK according to standard protocols[36] (for further details see **Supplementary Material**).

Statistical and Bioinformatics analysis

Transcriptomic and miRNome statistical analysis: raw data were imported and analyzed with the software Partek Genomic Suite 6.6 (Partek, St. Louis, MO, USA). After quality control of the data, Analysis of variance (ANOVA) test was performed to assess the effects of PNS on genes expression or miRNAs expression in rats, and of history of early life adversities on gene expression in humans. A maximum filter of $p<0.05$ (FDR corrected) and a minimum absolute fold change cut-off of 1.4 in animals and of 1.2 in humans was applied to select the lists of significant genes. The miRNAs-mRNAs combining analyses was performed using a specific sub-feature in Partek Genomic Suite 6.6.

Pathway Analyses: List of significant genes were analyzed for pathway analyses by using Ingenuity Pathway Analyses Software (IPA) where, as a background, we used gene lists that we obtained applying the minimum absolute fold change cut-off of 1.4 in animals and of 1.2 in humans and q-value<0.05.

Animal and Human Integration Data: The genes lists deriving from rats (following miRNAs-mRNAs combining analyses) and humans (mRNAs only) were cross-integrated, and the list of common genes was tested for random probability by Hyper-Geometric Distribution test[37] in R.

Network Analyses: the list of common genes from animal and human data were used for network analyses using specific tools in IPA and STRING (http://string-db.org).

GXE Interactions:

Grady Trauma Project: We transformed imputed genotypes to best guessed genotypes using a probability threshold of 90%. To assess gene-environment interaction, we individually tested each SNP within the three genes of interest resulting from the final network analysis (see Results, below: *FOXO1*,

n=156 SNPs; *A2M*, n=151 SNPs; and *TGFB1*, n=26 SNPs) for interactions between childhood abuse (sexual, physical and emotional abuse in childhood) and BDI scores in adulthood, using linear regression analysis in R. Childhood abuse was categorized as present (i.e., at least on type of abuse over the moderate to severe cut-off) or absent. Age, gender and the first two principal components of the identity-by-state (IBS) matrix were used as covariates. We calculated nominal p-values in the interactions with all types of abuse. We then calculated more stringent empirical p-values, using permutation analyses, in the interactions with emotional abuse, in order to select a restricted number of SNPs to be replicated in the Helsinki Birth Cohort Study. Therefore, we randomly permuted genotype status for all tested SNPs in parallel, and determined how often permutation based p-values were lower as compared to original p-values; we ran 10,000 permutations for each phenotype. Additionally, we used the minimal p-value approach as described by Westfall and Young, which compares the minimal permutation based p-value over all SNPs to the original p-value, and hereby corrects for multiple testing. All presented p-values are two-sided.

Helsinki Birth Cohort Study: We tested the SNPs that interacted with emotional abuse in the Grady Project and survived permutation analysis (see Results, below: n=6 SNPs, all in *FOXO1*) for interactions between emotional abuse (whether the individual was separated from parents during WWII, or not) and BDI score in adulthood, using linear regression analysis. Each SNP was tested in a separate model; age at testing, gender, and the first three principal components of the IBS matrix, main effects of the gene and the environment were used as covariates. We made further adjustments for SNP x covariate and separation status x covariate interactions, as suggested by Keller[38]. All presented p-values are one-sided p-values as in this replication sample we wanted to confirm these specific six SNPs.

RESULTS

Transcriptomic changes in the hippocampus of adult male rats following PNS

Our first aim was to identify gene expression changes in the hippocampus of male adult rats (at PND 62) that had been exposed to PNS. We analyzed the entire transcriptome and we found a significant modulation of *916* genes in the comparison with control animals (using 1.4<FC<-1.4, q-value<0.05). We have visualized the most significant genes in a hierarchical clustering in **Figure 1,** and listed the 916 significant genes in **Supplementary Table 1**.

We then performed a pathway analysis on the significantly modulated genes, in order to identify the main biological processes involved. We identified *49* biological processes that are altered by the PNS exposure, including some involved in *neurodevelopment* (Axonal Guidance Signaling, Protein Kinase A Signaling, Glutamate Receptor Signaling, Wnt/β-catenin Signaling, CREB Signaling in Neurons, Synaptic Long Term Depression, Synaptic Long Term Potentiation, Glucocorticoid Receptor Signaling) and *inflammation* (IL-8 Signaling, STAT3 Pathway, CDK5 Signaling, IL-1 Signaling, IL-6 Signaling, BMP signaling pathway and TGF-β Signaling). All the significant pathways are summarized by the pie-chart detailing the relevant functional areas (**Figure 2a**), while the entire list of significant pathways is presented in **Supplementary Table 2**. We conducted the subsequent target prioritisation steps using the full list of 916 differentially regulated genes.

miRNome changes in the hippocampus of PNS rats, and combined analyses with transcriptomic changes

We subsequently investigated whether exposure to PNS causes changes in miRNAs levels, and whether the identified miRNAs target transcripts identified in the transcriptomics analysis. We found that PNS exposure resulted in the modulation of *68* miRNAs (47 of them are mature miRNAs, all detailed in **Table 1**), with sixty-five downregulated and only 3 were up-regulated. As both transcriptomic and miRNome analyses were conducted in the hippocampus of the same animals, we then performed a mRNA-miRNAs combining analysis that allows the identification of a panel of top-hit genes that were both modulated by PNS exposure *and* targeted by the miRNAs that were modulated by PNS (see

Methods). These analyses identified *528* significant genes, presented in **Supplementary Table 3**. The specific pathways analysis shows that these genes are again involved in both *neurodevelopment* (Axonal Guidance, Protein Kinase-A Signaling, Glucocorticoid Receptor Signaling, TGF-beta Signaling) and *inflammation* (STAT3 Pathway, PTEN Signaling, ILK Signaling, IL-8 signaling); the full list of the *42* pathways is presented in **Supplementary Table 4.**

Blood mRNA transcriptomics of early life trauma in humans

Comparing subjects with and without exposure to early life trauma (see Methods for sociodemographic and clinical information), we identified *250* genes that were differentially modulated (FC> 1.2, FDR q-value<0.05) (see **Supplementary Table 5**). These 250 genes are involved in the modulation of *41* significant pathways. Of note, a high number of pathways are related, again, to *neurodevelopment* (Wnt/Ca+ pathway, cAMP signaling, CREB signaling) and *inflammation* (eNOS signaling, chemokine signaling, B cell activation), similar to what we found in the PNS model. All the significant pathways are summarized by the pie-chart detailing the relevant functional areas in **Figure 2b,** and have been listed in **Supplementary Table 6**.

Integrating data from PNS in animals and from early life trauma in humans

In the next step, we integrated the *528 genes* obtained by combined mRNA/miRNA analyses in the hippocampus of rats exposed to PNS with the *250 genes* significantly modulated in the blood of adults exposed to early life trauma. We identified *22* common genes that were present in both lists; according to the hypergeometric distribution test, the probability that these genes are not overlapping due to random probability is $p=1.8X10^{-8}$.

Finally, we validated *all of the 22 genes* by Real Time PCR, both in the hippocampus of animals and in the blood of adults exposed to childhood trauma. In line with the microarray results, 16 genes were modulated in the same direction both in the rat hippocampus and in the human blood, and 6 were

modulated in the opposite direction. Specifically, 15 were up-regulated in both: Alpha-2-Macroglobulin (A2M), AT-Rich Interaction Domain 5B (ARID5B), Arrestin Domain Containing 4 (ARRDC4), EPH Receptor A4 (EPHA4), F-Box Protein 32 (FBXO32), Forkhead box protein O1 (FOXO1), Heat Shock Transcription Factor 2 (HSF2), Isochorismatase Domain Containing 1 (ISOC1), Low Density Lipoprotein Receptor Adaptor Protein 1 (LDLRAP1), Leucine-rich repeat neuronal protein 3 (LRRN3), Myosin ID (MYO1D), Phosphatidylinositol-4-Phosphate 3-Kinase, Catalytic Subunit Type 2 Beta (PIK3C2B), Phosphatidic Acid Phosphatase Type 2A (PPAP2A), Sterile Alpha Motif Domain Containing 12 (SAMD12), and Serine Incorporator 5 (SERINC5); one was down-regulated in both: Transforming Growth Factor, Beta 1 (TGFB1); one was up-regulated in rats and down-regulated in humans: Solute Carrier Family 24 (Sodium/Potassium/Calcium Exchanger) Member 3 (*SLC24A3)*; and 5 were down-regulated in rats and up-regulated in humans: B-Cell CLL/Lymphoma 2 (*BCL2),* B-Cell CLL/Lymphoma 9 *(BCL9),* Lymphoid Enhancer-Binding Factor 1 (*LEF1),* Lin-54 DREAM MuvB Core Complex Component (*LIN54),* and Post-GPI Attachment to Proteins 1 (*PGAP1)*. These genes are presented in **Supplementary Table 7**, where we also present the FCs obtained from the microarray studies and the FCs from Real Time PCR analyses.

Gene network analysis and selection of candidates for GxE interaction:

We then focused on the 16 genes (A2M, ARID5B, ARRDC4, EPHA4, FBXO32, FOXO1, HSF2, ISOC1, LDLRAP1, LRRN3, MYO1D, PIK3C2B, PPAP2A, SAMD12, SERINC5, and TGFB1) that were modulated in the same direction both in animals and in humans, and we applied a gene network analysis to identify possible interactions among these genes. Using String Software and IPA Software, we observed only one cluster of interacting genes, represented by *A2M*, *FOXO1* and *TGFB1* (**Figure 3**). Using a gene enrichment/pathway analyses, we then confirmed that this cluster is involved in cytokines signalling, TGF-ß signalling and glucocorticoid receptor signalling. As we found that *A2M*, *FOXO1* and *TGFB1* form a single individual cluster of genes interacting with each other, we focused on these three genes for the subsequent GxE studies.

Gene x Environment interaction studies

We first used the Grady Trauma Project to test whether *A2M*, *FOXO1* and *TGFB1* were indeed vulnerability genes for depression in the context of exposure to early life stress (GxE interaction), as this sample had a range of severe early life adversities, including sexual abuse, physical abuse and emotional abuse[24]. We focused on all the SNPs located in these three genes (*FOXO1*, n=156 SNPs; *A2M*, n=151 SNPs; and *TGFB1*, n=26 SNPs; see Methods), and tested their interaction between childhood sexual, physical or emotional abuse and depressive symptoms in adulthood (BDI scores), in a total number of 4,791 subjects. Within FOXO1, 6 SNPs showed significant GxE interactions with sexual abuse, 40 SNPs with physical abuse and 40 SNPS with emotional abuse (although with a large overlap); within *A2M*, 7 SNPs showed significant GxE interactions with emotional abuse; and within *TGFB1*, 4 SNPS showed significant GxE interactions with sexual abuse and 1 SNP with emotional abuse (See **Supplementary Table 8**).

We then calculated more stringent p-values using permutation analyses, focusing only on the SNPs that were significant with emotional abuse, in order to select a restricted number of SNPs to be replicated in the Helsinki Birth Cohort Study, where a comparable phenotype of early life emotional stress (parental separation during WWII) was available. In the Grady Project, we found 6 SNPs, all located within the *FOXO1* gene, that survived permutation analyses for the GxE interactions between emotional abuse and adult depression (see **Table 2**); the SNP rs17592371 showed the strongest significance, surviving correction for multiple testing over all SNPs tested (p=0.0006, Westfall-Young's method). All 6 SNPs also showed significant GxE interactions between parental separation and adult depression in the Helsinki Birth Cohort Study (see **Table 2**). The GxE interactions of SNP rs17592371 in both cohorts are represented in **Figure 4**, where we can see that individuals with "at risk" genotypes (CC) developed more depressive symptoms in the presence of early separation as compared to individual with "low risk" genotypes (CT and TT).

Discussion

We provide a novel 'hypothesis-free' approach for the identification and prioritization of candidate genes that can be investigated in GWAS datasets to test GxE interaction studies in depression. In particular, by employing "*omics*" approach in animals (mRNAs and miRNAs) and in humans (mRNAs), and cross-species validation, we have identified one cluster of genes, comprising *FOXO1*, *A2M* and *TGF-β1*, that not only show significant GxE interactions predicting adult depression in the context of early life trauma in our study, but also have been previously involved in the regulation of mechanisms that are well established in stress and depression[39-43]. Indeed, SNPs within *FOXO1, A2M* and *TGF-β1* interacted with different types of childhood trauma phenotypes in predicting adult depression in the Grady Trauma Project; and a stringent list of 6 SNPs within *FOXO1* associated with emotional abuse in the Grady Trauma Project was fully replicated in the Helsinki Birth Cohort Study of individuals exposed to early separation.

A large body of evidence has rapidly accumulated over the past years describing interactive effects of genetic factors and early life stressors in determining the risk of depression[8]. These 'hypothesis-driven' studies have mostly focused on variations in candidate genes acting in neurobiological systems that have been previously implicated in the pathophysiology of major depression. As mentioned in the Introduction, the most consistent examples of this approach are studies examining the 5-HTTLPR polymorphism in the serotonin transporter[13, 44-47] and several functional SNPs within FKBP5[16, 17]. However, numerous studies, and some meta-analyses, have produced discrepant results[44, 48-50]. New gene-environment interactions continue to be reported in psychiatry, including in genes as biologically varied as *BDNF*[51,52], *CD38*[53], *ADCYAP1R1*[54], *DRD2*[55] and *GRIN2B*[56], but always from hypothesis-driven research, which has a high risk of non-replication. Indeed, considering the current emphasis on *omics*-based approach in genetic-research, and especially in the context of a paucity of significant genetic effects for depression in GWAS studies, other statistical and biological prioritization strategies need to be developed to facilitate a systematic discovery of novel gene-environment interactions in genome-wide analyses. To our knowledge, the present study is the first report of a hypothesis-free,

omics-, cross-tissues and cross-species approach to identify candidate genes for GXE interaction analyses. Interestingly, Mamdani and colleagues (2015)[57] recently used a similar approach to detect genes of interest in the context of alcohol dependence, combining data from mRNA and miRNA expression patterns in the brain of 18 patients with alcohol dependence and 18 matched controls; although they did not test for GXE interactions, they identified a network of hub genes that was enriched for genes identified by the Alcohol Dependence Genome Wide Association Study.

Our strongest findings, replicated in both clinical cohorts using stringent statistical criteria, pertain to FOXO1. FOXO1 belongs to the forkhead family of transcription factors, which are characterized by a distinct forkhead domain; it is the main target of insulin signaling, it regulates metabolic homeostasis in response to oxidative stress, and it is modulated by glucocorticoids. It is also an important regulator of cell death acting downstream of Cyclin-dependent kinase 1 (CDK1), AKT Serine/Threonine Kinase 1 (AKT1) and macrophage stimulating 1 (MST1), and indeed several studies have suggested that activation of FOXO1 contributes to glucocorticoid-induced cell death[58]. Importantly, FOXO1 is also involved in the maintenance of human embryonic stem cells pluripotency, a function that is mediated through direct control by FOXO1 of OCT4 and SOX2 gene expression, through occupation and activation of their respective promoters. Interestingly, the rs17592371 SNPs (which not only is replicated in both samples, but also survives the most stringent multiple corrections test) is an intronic SNP, and therefore the mechanisms of its actions remain unclear, although likely affecting gene expression and not the function of the gene product. It is also possible that this SNP can influence the protein conformation or the accessibility to phosphorylation sites; indeed, a number of kinases can phosphorylate and regulate FOXO1 proteins either positively or negatively[59-61], including AKT serine/threonine protein kinase downstream of the PI(3)K (phosphatidylinositol-3-OH kinase) signaling pathway[62, 63] and Serum glucocorticoid kinase 1 (SGK1). In particular, SGK1 phosphorylates FOXO1 at threonine 24, serine 256, and serine 319 residues, leading to subcellular redistribution of FOXO1 from the nucleus to the cytosol, so leading to its inactivation as transcription factor. Importantly, SGK1 is a kinase that is specifically activated by glucocorticoids, and we have previously described that SGK1

mediates the inhibitory effect of glucocorticoids on neurogenesis and that its blood expression levels are elevated in patients with Major Depression as compared with control subjects[27].

The findings that both A2M and TGF-β1 have SNPs with significant GxE interactions in the Grady Trauma Project (they could not be tested in the Helsinki cohort) is also of great interest, even if less statistically robust than the findings in FOXO1, as both genes have been implicated in biological processes relevant to depression. A2M has traditionally been viewed as an inflammatory fluid proteinase scavenger, and recent studies have demonstrated the ability of A2M to bind to a plethora of cytokines, including TGF-β1[64]. TGF-β1, in turn, is a member of TGF-β superfamily, which regulates neuronal survival, neurogenesis, synaptogenesis and gliogenesis[65-68]. Similar to the present study, where we found increased A2M mRNA levels following stress both in the rodent hippocampus and in the human blood, A2M is elevated in clinical samples and in experimental models relevant to depression; for example, higher levels of blood A2M are present in patients with total gastrectomy that develop depression, indicating that A2M elevation may be implicated in depression in the context of a pro-inflammatory status[69]. High levels of A2M have indeed been associated with systemic inflammation[71], and therefore it is possible to speculate that the high levels of A2M described in this and other studies may translate into an increased risk of depression by activating the inflammatory system[35,72,73]. TGF-β1, in contrast, is reduced in conditions related to stress and depression, in the present study as well as in previous studies. For example, *TGF-β1* levels are reduced in the blood of depressed patients[74-76], and we have previously shown that the TGF-β1-SMAD signaling is one of the pathways that is most significantly down-regulated in neurons by *in vitro* exposures to high concentrations of cortisol, which mimics depression *in vitro* and negatively affects neurogenesis[27]. Finally, is of note that several SNPs within A2M and TGF-β1, including those that we have identified through our GxE interaction analyses, are associated with the development of several Complex Diseases and Disorders (see genetic association database from complex diseases and disorders https://geneticassociationdb.nih.gov). Moreover, genetic variants in TGF-β1 have been associated with other brain disorders, such as multiple sclerosis[77] and Alzheimer disease[78, 79].

It is important to emphasize that GxE interactions, while based on the effects of specific changes in DNA sequence, are likely to involve epigenetic molecular mechanisms[80-82]. For example, recent studies have shown that the above-mentioned functional polymorphism in the *FKBP5* gene increases the risk of developing depression and PTSD in adulthood by allele-specific, childhood trauma–dependent DNA demethylation in functional glucocorticoid response elements of *FKBP5,* followed by a long-term dysregulation of the stress hormone system and a global effect on the function of immune cells and of brain areas associated with stress regulation[83]. Besides DNA methylation, miRNAs have also recently emerged as important in the long-term regulation of gene expression associated with stress early in life[84-88]. The ability of miRNAs to selectively and reversibly silence the mRNAs of their target genes[89, 90], together with their involvement in biological processes modulated by stressful life events[91], make miRNAs well-suited to serve as fine regulators of the complex and extensive molecular network involved in stress response; hence, our decision in this paper of merging transcriptomics and miRNomics data in the selection of top-hit genes.

There are limitations in our study that should be mentioned. First of all, we have only been able to test the replication of GxE interactions for exposure to early life emotional stress (emotional abuse in the Grady cohort and early separation from parents in the Helsinki study), as it has been proven very difficult to find a third GWAS cohort where we could confirm also the other findings, because of the difficulty in identifying potential cohorts where there is availability of GWAS data *and* data on exposure to life adversity *and* data on the presence of adult depression. However, we trust that our use of three different clinical populations in our study (the South-East London sample to generate transcriptomics, and the Grady Trauma and the Helsinki Birth Cohort Studies for the GXE analyses), together with the cross-species validation, defend the replicability and generalizability of our findings. Second, although the six *FOXO1* SNPs in the Grady Project associated with emotional abuse were significant after permutation analyses (and multiple testing for rs17592371), and replicated in the Helsinki cohort, the SNPs in *A2M* and *TGF-β1* were only significant using unadjusted analyses. Hence,

we propose that the *FOXO1* SNPs (and especially rs17592371) are our strongest finding, while those in *A2M* and *TGF-β1* require further replication.

In conclusion, our data provide a novel approach for the selection of novel susceptibility genes for GxE interaction in depression, alternative to 'hypothesis-driven research' and resulting in the prioritization of candidate genes starting from a 'hypothesis-free' approach. The validity of our approach is supported by the evidence that indeed we have detected and replicated significant GxE interactions in the three identified genes, FOXO1, A2M and TGF-β1, and also that these genes are all involved in inflammation and glucocorticoid-related signaling, which are known biological vulnerability signaling mechanisms for depression. Our proposed prioritization strategy will limit the number of investigated genes in GWAS-based GxE interaction studies and thus will enhance the statistical power to identify significant findings.

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Legends to Figures and Tables

Figure 1 Hierarchical Clustering of gene expression changes by prenatal stress (PNS) in the hippocampus of rats, in comparison with control animals.

The red bar on the horizontal axis indicates control animals and the orange bar indicates PNS-exposed animals. The blue and the red squares in each group (CTRL or PNS) indicate the modulation of the gene expression, with red squares indicating genes that are up-regulated and blue squares gene that are down regulated.

Figure 2A Pathways pie chart in prenatally stressed animals

The pie chart represents the functional relevance of the significant pathways found modulated in the hippocampus of PNS-exposed adult rats.

Figure 2B Pathways pie chart in adults with a history of childhood trauma

The pie chart represents the functional relevance of the significant pathways found modulated in the blood of adult subjects exposed to childhood trauma.

Figure 3 Network Analyses

Network Analyses of the 16 common genes modulated in the same direction both in the hippocampus of rats exposed to prenatal stress and in the blood of adults exposed to childhood trauma. The blue lines indicate direct interactions between molecules (A2M, FOXO1 and TGFB1). The dot lines indicate the involvement of molecules within pathways.

Table 1

List of miRNAs (both mature and non-mature miRNAs) significantly modulated (all with q-value<0.05) in animals exposed to prenatal stress, compared with control animals.

Table 2

Gene X Environment interactions in the two clinical samples for the six genes identified by permutation analysis to be associated with emotional abuse in the Grady Trauma Project

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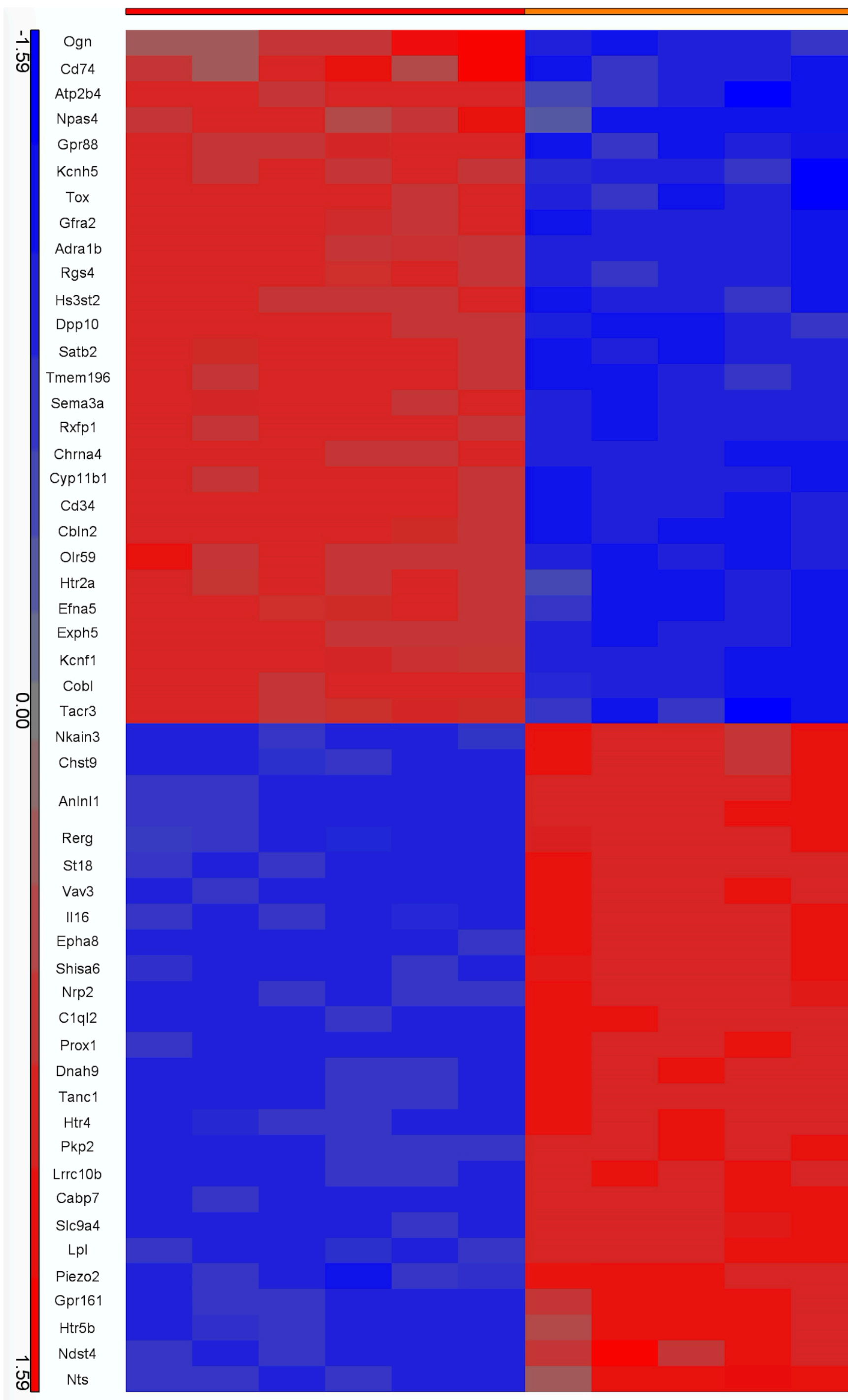
Figure 1

Hierarchical Clustering of gene expression changes by prenatal stress (PNS) in the hippocampus of rats, in comparison with control animals.

The red bar on the horizontal axis indicates control animals and the orange bar indicates PNS-exposed animals. The blue and the red squares in each group (CTRL or PNS) indicate the modulation of the gene expression, with red squares indicating genes that are up-regulated and blue squares gene that are down regulated.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0.00
1.59
Ogn
Cd74
Atp2b4
Npas4
Gpr88
Kcnh5
Tox
Gfra2
Adra1b
Rgs4
Hs3st2
Dpp10
Satb2
Tmem196
Sema3a
Rxfp1
Chrna4
Cyp11b1
Cd34
Cbln2
Olr59
Htr2a
Efna5
Exph5
Kcnf1
Cobl
Tacr3
Nkain3
Chst9
Anln1
Rerg
St18
Vav3
Il16
Epha8
Shisa6
Nrp2
C1ql2
Prox1
Dnah9
Tanc1
Htr4
Pkp2
Lrrc10b
Cabp7
Slc9a4
Lpl
Piezo2
Gpr161
Htr5b
Ndst4
Nts

Figure 2A Pathways pie chart in prenatally stressed animals

The pie chart represents the functional relevance of the significant pathways found modulated in the hippocampus of PNS-exposed adult rats.

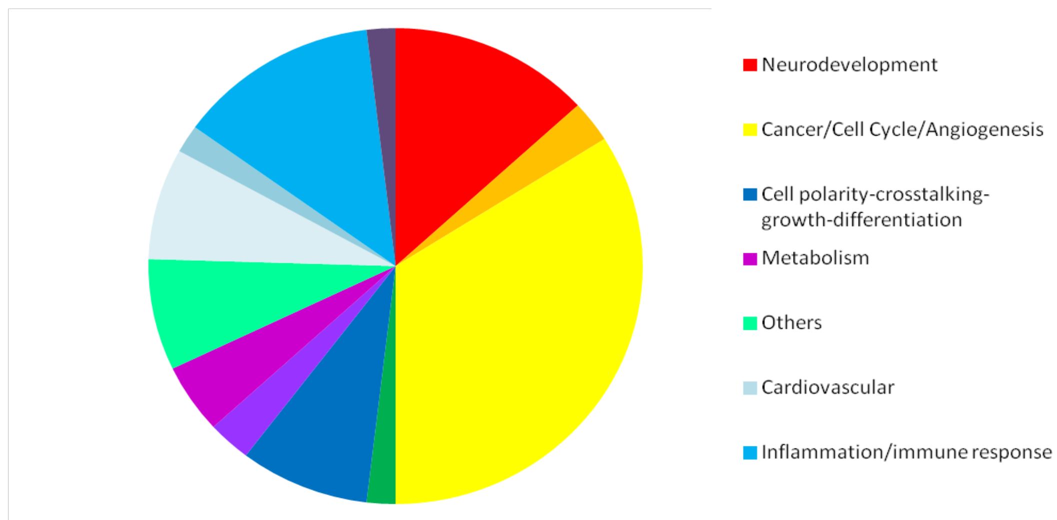
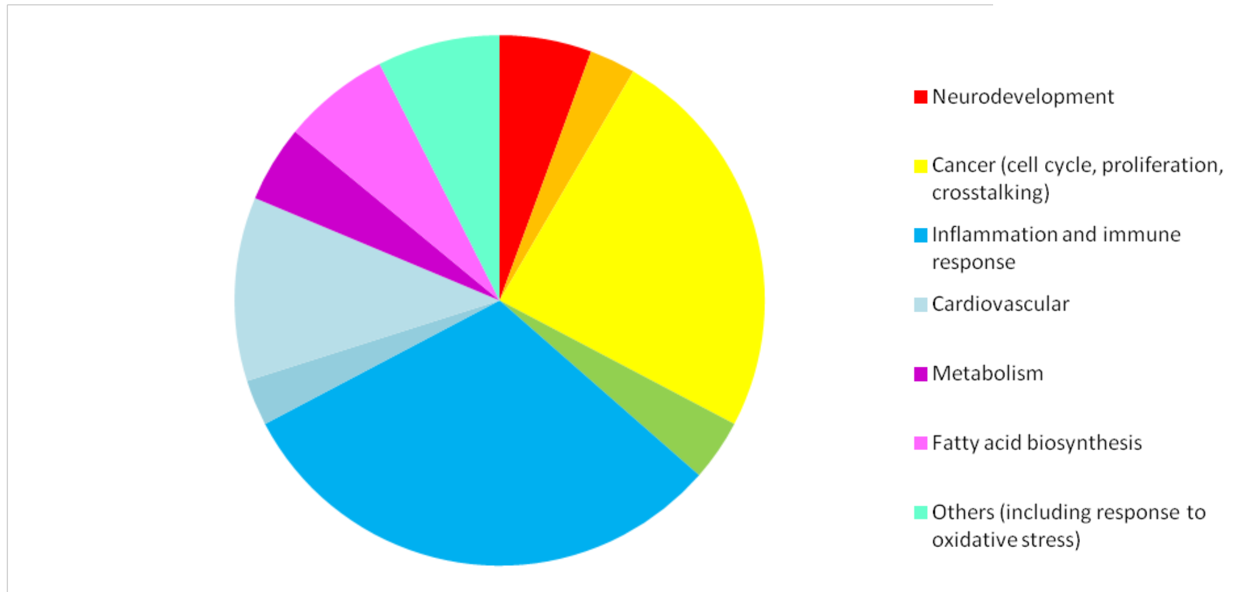


Figure 2B Pathways pie chart in adults with a history of childhood trauma

The pie chart represents the functional relevance of the significant pathways found modulated in the blood of adult subjects exposed to childhood trauma.



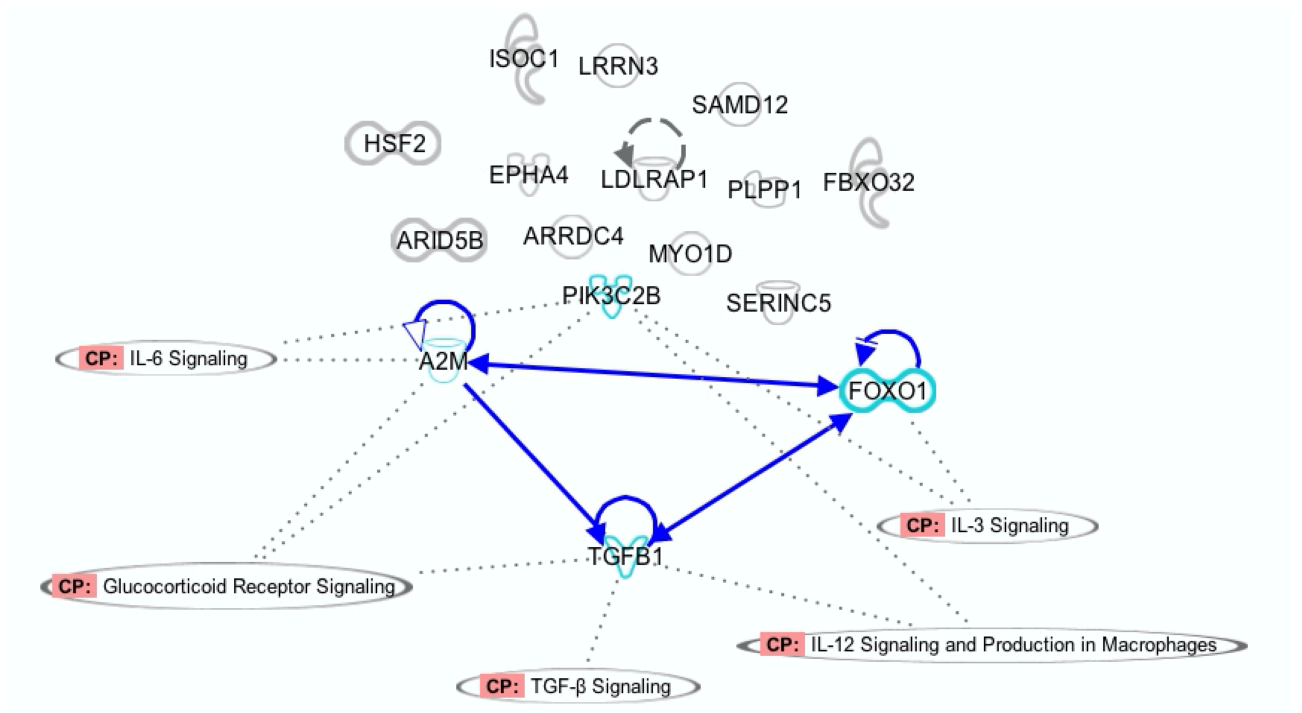


Figure 3 Network Analyses

Network Analyses of the 16 common genes modulated in the same direction both in the hippocampus of rats exposed to prenatal stress and in the blood of adults exposed to childhood trauma. The blue lines indicate direct interactions between molecules (A2M, FOXO1 and TGFB1). The dot lines indicate the involvement of molecules within pathways.

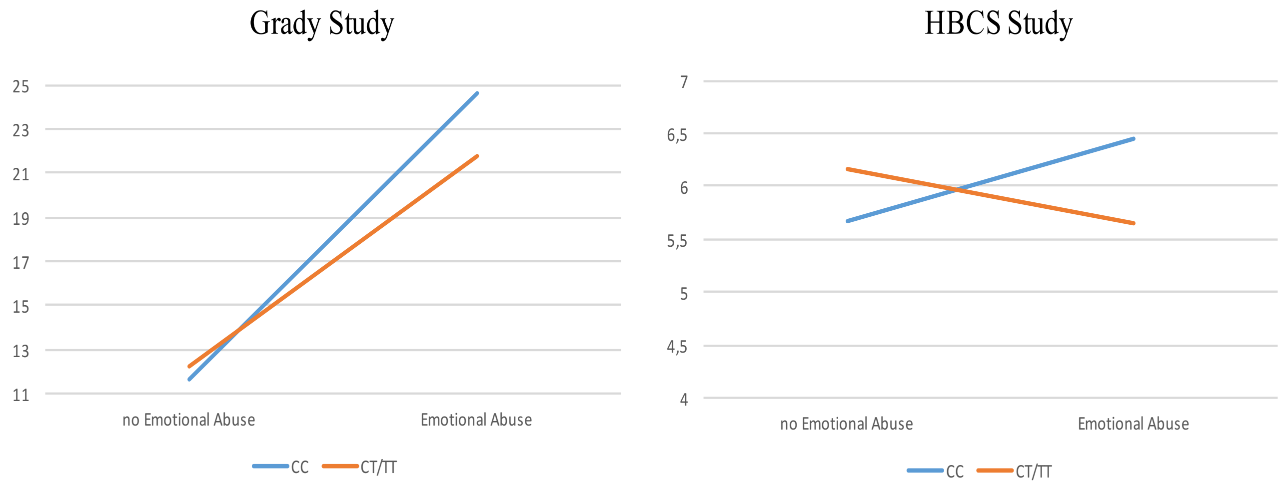


Figure 4

Estimated marginal means of BDI scores for different genotypes of the FOXO1 single nucleotide polymorphism rs17592371 in subjects who were or were not exposed to emotional abuse in the Grady Trauma Project (on the left) and in the Helsinki Birth Study Cohort (on the right). Values are adjusted for gender and age.

Table 1

List of miRNAs (both mature and non-mature miRNAs) significantly modulated (all with q-value<0.05) in animals exposed to prenatal stress, compared with control animals.

	miRNA name	Fold-Change
1	rno-miR-3473	-1,7
2	rno-mir-3593	-1,2
3	rno-miR-339-5p	-1,4
4	rno-mir-1839	-1,2
5	rno-mir-3556b	-1,2
6	rno-miR-133b-5p	-1,3
7	rno-miR-1839-5p	-1,2
8	rno-mir-322	-1,3
9	rno-mir-425	-1,2
10	rno-mir-18a	-1,3
11	rno-mir-3085	-1,3
12	rno-mir-329	-1,2
13	rno-miR-370-3p	-1,4
14	rno-miR-181c-5p	-1,3
15	rno-miR-872-3p	-1,5
16	rno-miR-423-3p	-1,6
17	rno-miR-93-3p	-1,6
18	rno-mir-872	-1,3
19	rno-miR-152-3p	-1,4
20	rno-mir-448	-1,3
21	rno-miR-3068-3p	-1,4
22	rno-mir-532	-1,3
23	rno-mir-290	-1,2

24	rno-miR-106b-3p	-1,4
25	rno-mir-27b	-1,2
26	rno-mir-203a	1,3
27	rno-miR-362-5p	-1,5
28	rno-miR-877	-1,4
29	rno-miR-7a-1-3p	-1,5
30	rno-miR-135b-3p	-1,5
31	rno-miR-6328	-1,2
32	rno-miR-540-3p	-1,4
33	rno-mir-376a	-1,2
34	rno-miR-10b-5p	-1,3
35	rno-miR-425-3p	-1,5
36	rno-miR-330-5p	-1,4
37	rno-miR-6326	-1,3
38	rno-mir-764	-1,3
39	rno-miR-200a-5p	-1,2
40	rno-mir-494	-1,2
41	rno-mir-379	-1,3
42	rno-miR-151-3p	-1,2
43	rno-miR-28-3p	-1,5
44	rno-miR-15b-5p	-1,4
45	rno-miR-324-3p	-1,3
46	rno-miR-871-3p	1,2
47	rno-miR-214-3p	-1,4
48	rno-mir-101b	-1,3
49	rno-miR-325-5p	-1,3
50	rno-miR-339-3p	-1,4

51	rno-mir-196c	-1,4
52	rno-miR-6324	-1,6
53	rno-miR-19b-3p	-1,4
54	rno-miR-20b-5p	-1,6
55	rno-miR-666-3p	-1,5
56	rno-miR-351-5p	-1,2
57	rno-miR-99b-3p	-1,3
58	rno-mir-135a	-1,3
59	rno-miR-139-3p	-1,4
60	rno-miR-342-5p	-1,3
61	rno-miR-376b-3p	-1,3
62	rno-miR-450a-5p	1,3
63	rno-miR-140-5p	-1,6
64	rno-miR-493-3p	-1,5
65	rno-miR-532-5p	-1,3
66	rno-miR-124-5p	-1,3
67	rno-miR-1843-5p	-1,3
68	rno-miR-6215	-1,3

Table 2 Gene X Environment interactions in the two clinical samples for the six genes identified by permutation analysis to be associated with emotional abuse in the Grady Trauma Project

GENE	SNPs FOXO1	Grady Cohort Study		Helsinki Birth Study Cohort	
		β score	p-value	β score	p-value
Foxo1	**rs17592371**	-3,035	<0,0001	-1,780	0,033
Foxo1	**rs2297626**	-2,601	0,0002	-1,808	0,037
Foxo1	**rs17592468**	-2,549	0,0003	-1,763	0,034
Foxo1	**rs28553411**	-2,549	0,0003	-1,763	0,034
Foxo1	**rs7319021**	-2,549	0,0003	-1,763	0,034
Foxo1	**rs12585452**	-2,416	0,0007	-1,796	0,032