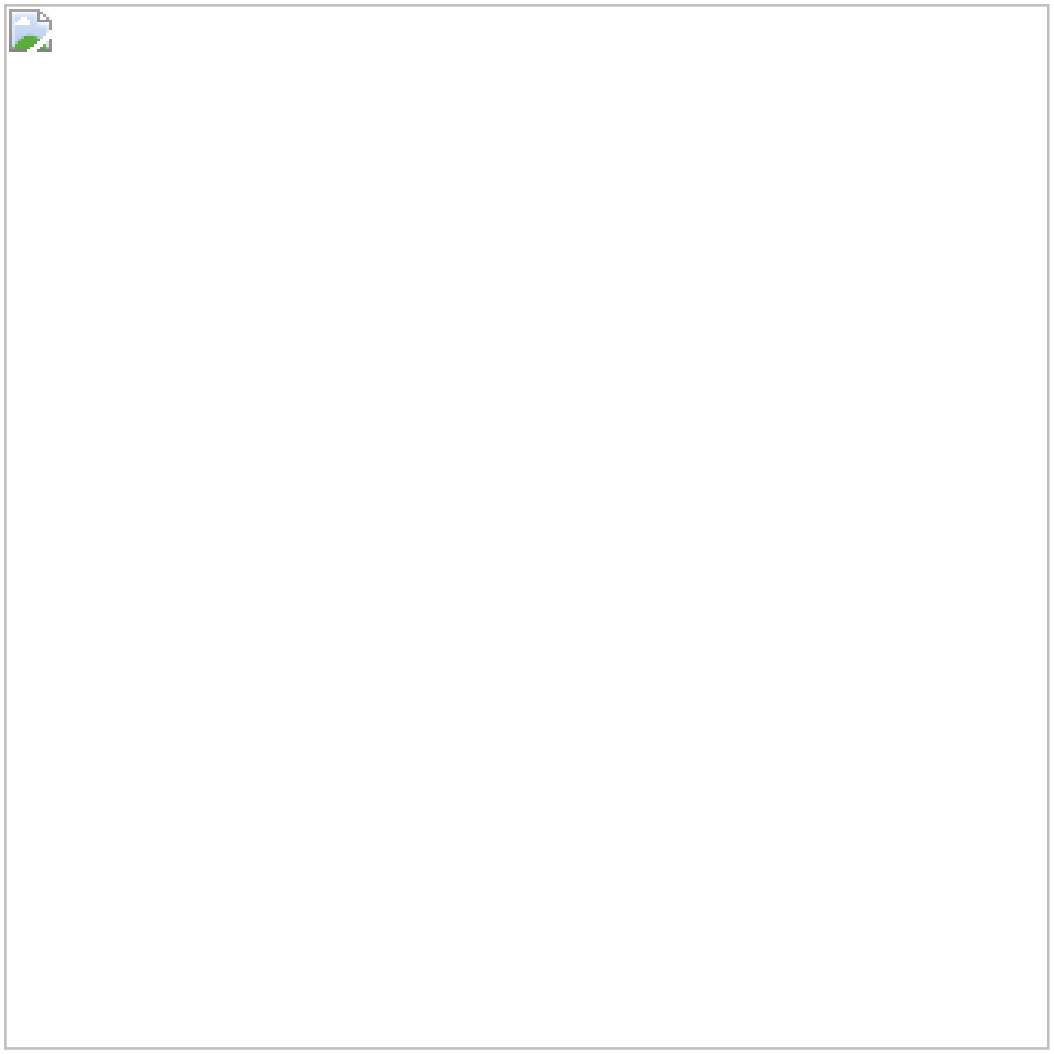

Prenatal stress, anxiety and depression alter transcripts, proteins and pathways associated with immune responses at the maternal-fetal interface†

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Research Article

Prenatal stress, anxiety and depression alter transcripts, proteins and pathways associated with immune responses at the maternal-fetal interface†

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Abstract

During pregnancy, the immune system is modified to allow developmental tolerance of the semi-allogeneic fetus and placenta to term. Pregnant women suffering from stress, anxiety, and depression show dysfunctions of their immune system that may be responsible for fetal and/or newborn disorders, provided that placental gene regulation is compromised. The present study explored the effects of maternal chronic self-perceived stress, anxiety, and depression during pregnancy on the expression of immune-related genes and pathways in term placenta. Pregnancies were clinically monitored with the Beck Anxiety Inventory (BAI) and Edinburgh Postnatal Depression Scale (EPDS). A cutoff threshold for BAI/EPDS of 10 divided patients into two groups: Index group (>10, $n = 11$) and a Control group (<10, $n = 11$), whose placentae were sampled at delivery. The placental samples were subjected to RNA-Sequencing, demonstrating that stress, anxiety, and depression during pregnancy induced a major downregulation of placental transcripts related to immune processes such as T-cell regulation, interleukin and cytokine signaling, or innate immune responses. Expression differences of main immune-related genes, such as *CD46, CD15, CD8α & β ILR7α*, and *CCR4* among others, were found in the Index group ($P < 0.05$). Moreover, the key immune-like pathway involved in humoral and cellular immunity named "Primary immunodeficiency" was significantly downregulated in the Index group compared with Controls. Our results show that mechanisms ruling immune system functions are compromised at the maternal-fetal interface following self-perceived depressive symptoms and anxiety during pregnancy. These findings may help unveil mechanisms ruling the impact of maternal psychiatric symptoms and lead to new prevention/intervention strategies in complicated pregnancies.

Summary Sentence
Mechanisms ruling immune system functions are compromised at the maternal-fetal interface following self-perceived depressive symptoms and anxiety during pregnancy.

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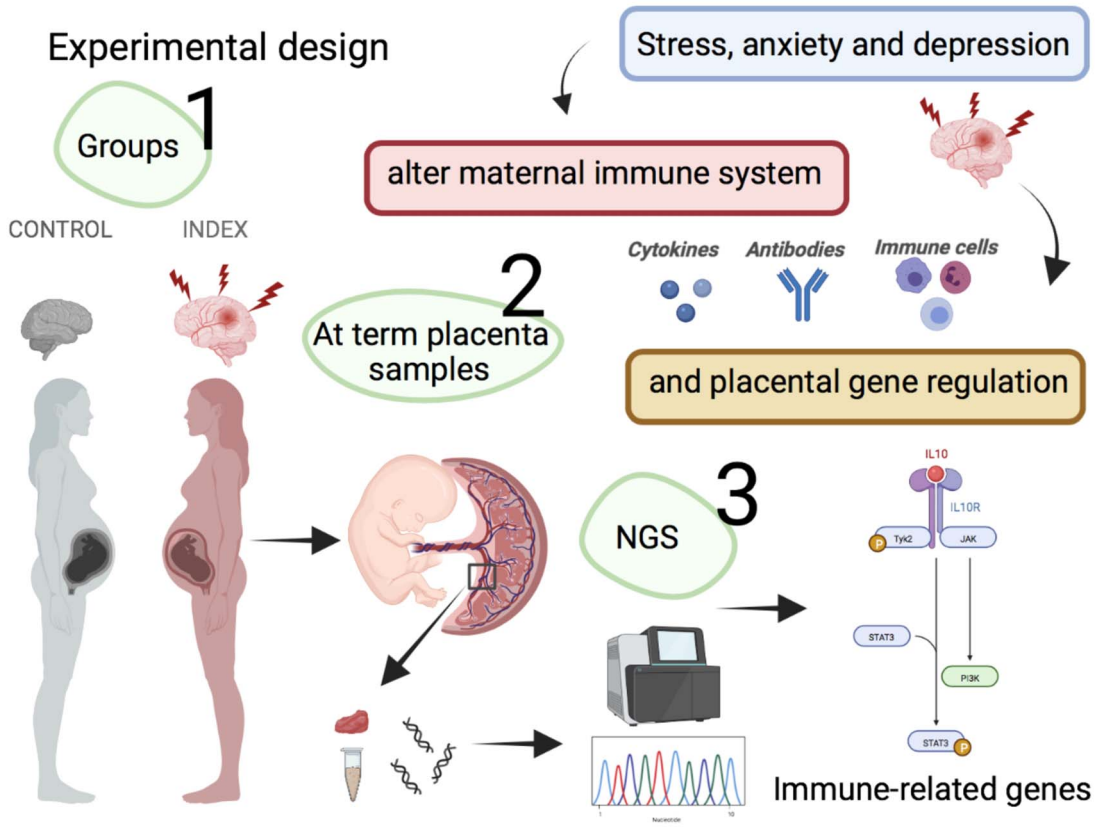
Graphical Abstract



Keywords: antenatal stress, immune system, term-placentae, RNA-Seq, human

Introduction

During pregnancy, placental permeability and immune transfer, including the transfer of maternal antibodies, depend on many factors, including maternal physical and mental condition [1]. For instance, depression during pregnancy (prenatal depression) has been identified as an important clinical risk factor for the transmission of abnormal health and behavior to the offspring [2].

Past and recent studies demonstrated that psychiatric patients exhibit dysregulated immune responses, suggesting that the immune system is strongly associated with psychosomatic illnesses [3]. Evidence of how immunological factors are affected during major depression and stress has been repeatedly presented in the current literature. Whereas patients with major depressive disorder (MDD) reveal increase in both circulating pro-inflammatory cytokines and their respective receptors [4, 5], treatments that reduce or remit depressive symptomology are correlated with normalization of immune signaling levels [6]. For instance, an overrepresentation of IL-6, IL-8, and type I IFN-induced signaling pathways and an increased expression of a wide range of innate immune genes and proteins such as IL-1β, IL-6, TNFα, TLR3, and TLR4 have been found in post-mortem brain samples from depressed suicide victims [7–9]. Furthermore, meta-analyses indicate that the cytokines IL-1β, IL-6, and TNF are consistent biomarkers of inflammation in patients with psychiatric symptoms, with changes in gene expression correlating with depression and even in response to antidepressant treatment [9–11]. These data provide strong evidence implicating the immune system in depression, which may affect the placenta functions and the fetus.

Exposure of the human fetus to high concentrations of biomarkers for inflammation leads to subsequent effects on the HPA axis, affecting behavior, and cognitive function in the offspring [12–15]. These specific prenatal effects suggest a direct biological effect of the in utero environment during depression on the development of the fetal brain which, in turn, could program an abnormal behavior and biological stress responses in the offspring; underlying psychopathology later in life [14].

Women with chronic self-perceived stress, anxiety, and depression during pregnancy had an increased expression of stress-mediating genes in their term placentas [16]. Considering the relation between these psychiatric disorders and immunity [17], it is pertinent to wonder if these women may have a particularly compromised regulation of their immune system in the placenta. Considering the relevance of the placental interface, stress-related maternal dysfunctions could have long-lasting effects on placental gene expression, lead to dysregulation of the fetal immune system, and contribute to some of the neuropsychological components of the newborn health [17]. Studies integrating these complex networks are required to elucidate the immunological and clinical consequences of the maternal programming of the newborn immune system. Comparison of healthy and

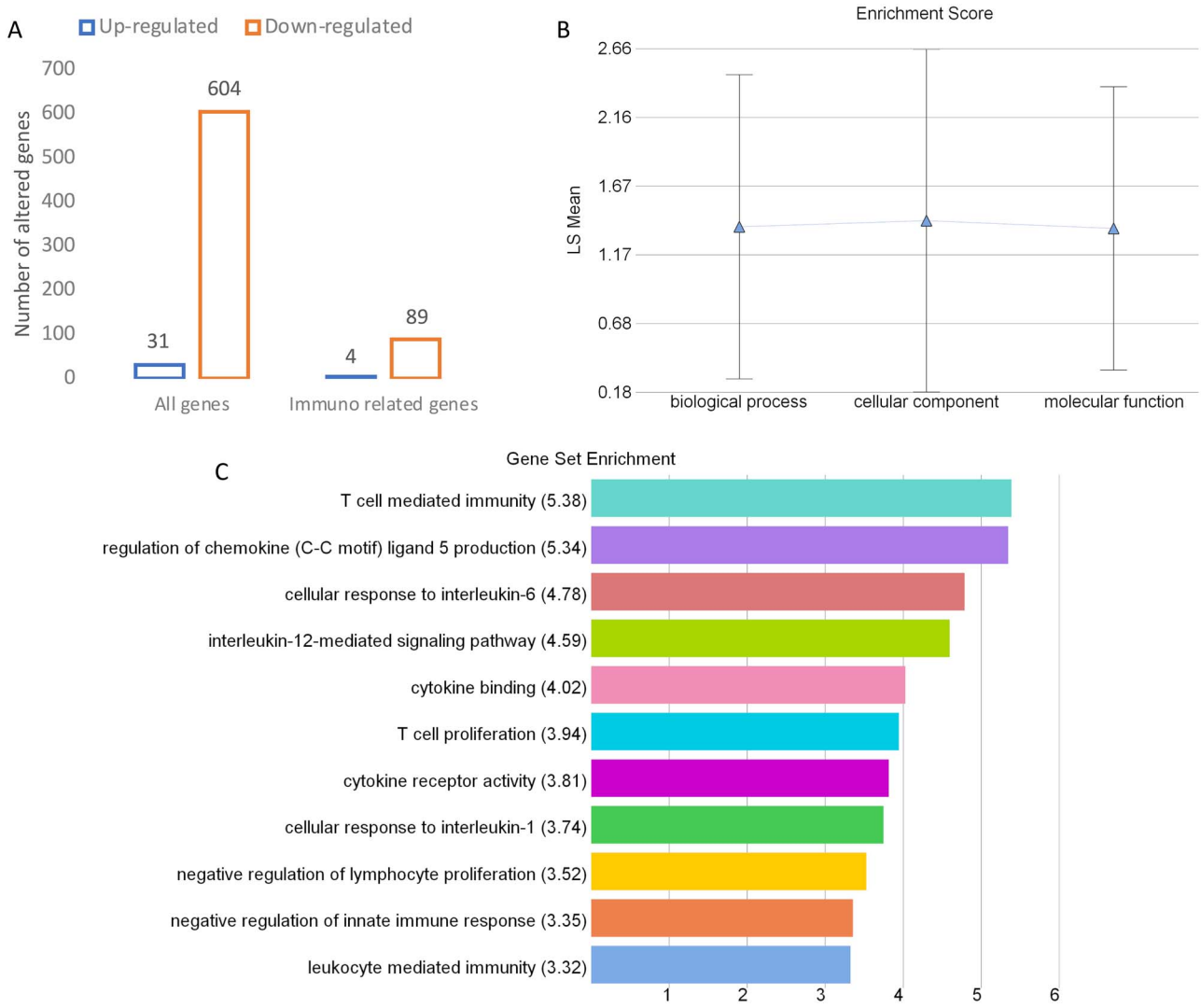


Figure 1. Number of total and immune-related genes (A), the functional categories (B), and the biological processes (C) that are affected in placental samples from women displaying self-perceived stress, anxiety, and depression during pregnancy (Index group, $n = 11$) compared with placental samples from healthy patients (Control group, $n = 11$). The RNA-Seq data were examined using Partek Genomics Suite 7.0 (Partek). Data were normalized using the RMA method. Differently expressed genes between Index and Control groups were established performing a one way-ANOVA setting parameters as a fold change (FC) >1 or <-1 with P-value <0.05.

complicated pregnancies would provide important opportunities to identify key mechanisms influencing neonatal immunity laying a basis for new or improved health interventions targeting the pregnant women or the child.

The present study therefore tested the hypothesis that transcripts and pathways associated with immune responses would have a differential expression in the term placenta of women suffering from chronic self-perceived anxiety and depression during pregnancy, compared with a control population.

Material and methods

Ethical considerations

Patient integrity was ensured by ethical approvals guaranteeing full pre-consent information and full anonymity. Data were handled at the group level only as all data were coded and treated anonymously. This is an established procedure in Swedish clinical investigations. The study was approved by the Regional Ethical Review Board in Linköping (Dnr 2011/499-31, 2013/355-32) and the Swedish Ethical Review Authority (EPM), Uppsala (Dnr 2020-05429).

Experimental design

The primary experimental layout of this study has been published [16, 18]. In brief, data were collected from a total of 390 pregnant women attending an antenatal care clinic in south-eastern Sweden. The women filled out anxiety and depression inventories during pregnancy, which are considered markers of stress. Self-perceived anxiety symptoms were assessed among women at the same time during pregnancy with the Beck Anxiety Inventory (BAI) [19] and depressive symptoms were assessed with the Edinburgh Postnatal Depression Scale (EPDS) [20]. Both inventories are well known, have been validated in Swedish [21, 22] and are frequently used in research settings as well as in clinical settings [23]. A cutoff score of 10 was used as a measure of symptoms of depression and anxiety during pregnancy for both the EPDS and the BAI. By choosing this threshold, the sensitivity for detecting major depression

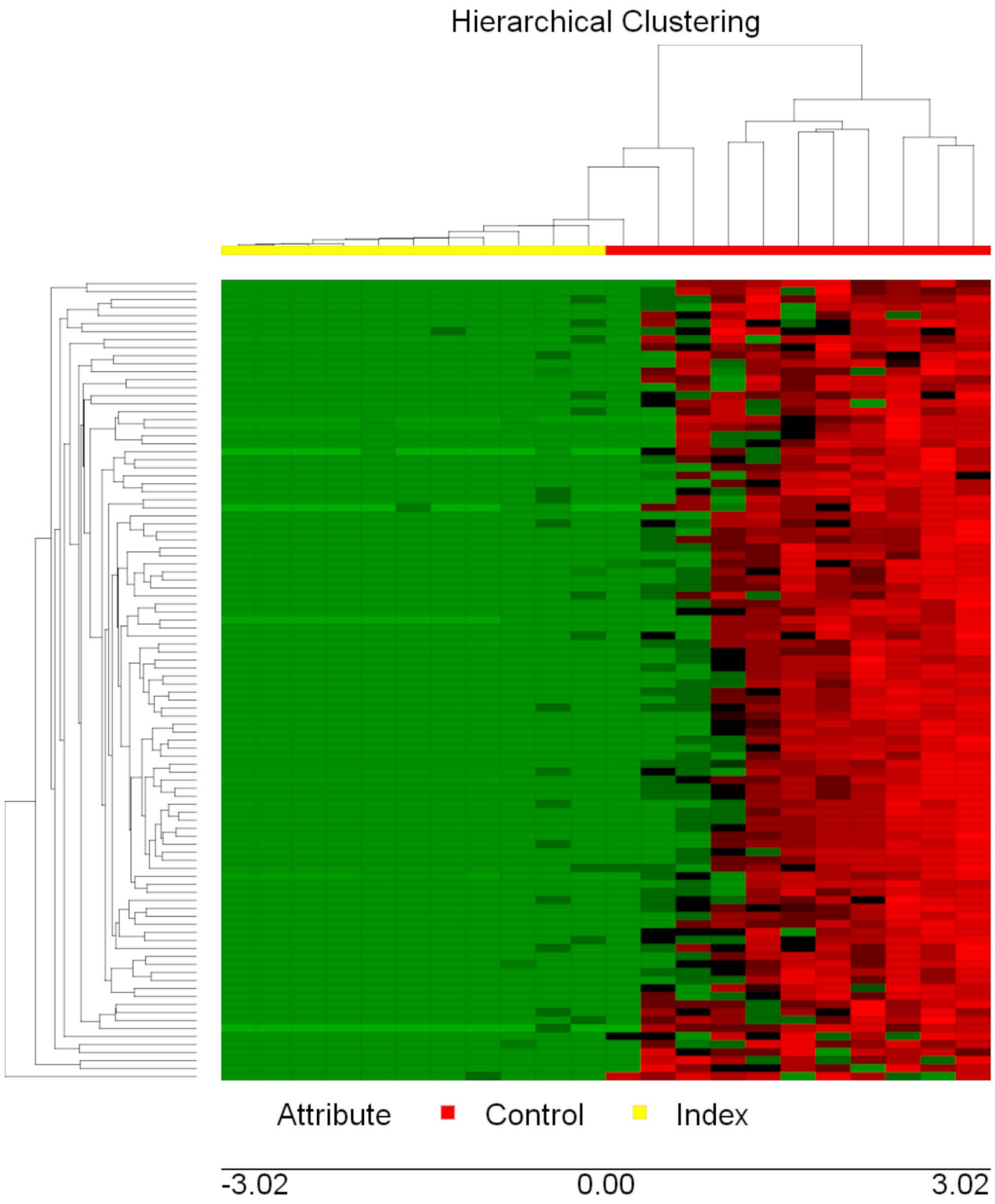


Figure 2. Hierarchical cluster plot of gene expression data of term placenta samples of patients with self-perceived stress, anxiety, and depression (Index group, $n = 11$) and healthy patients (Control group, $n = 11$). Both rows and columns are clustered using correlation distance and average linkage. Colors represent mRNA levels (red: higher, green: lower).

was close to 100% and the specificity was 82% [24]. Women who had pregnancy complications, including preeclampsia and/or preterm delivery, were excluded. A total of 11 women scoring >10 on both EPDS/BAI—indicating symptoms of depression and anxiety—were included in the Index group. A total of 11 controls who scored <10 on both EPDS/BAI were referred as the Control group. After birth, placentas were immediately collected and frozen until transcriptome analyses. Demographic data of patients included in the present study is shown in Table 1.

Table 1. Demographic data of the patients included in this study

	Index n (%)	Control n (%)	*P*-value
University/college degree			1.000*
No	4 (36.4)	5 (45.5)	
Yes	7 (63.6)	6 (54.5)	
Born in Sweden			0.234*
No	0 (0.0)	3 (27.3)	
Yes	11 (100.0)	8 (72.7)	
Married/cohabiting			NA
No	0 (0.0)	0 (0.0)	
Yes	11 (100.0)	11 (100.0)	
Age			0.586*
15–34	10 (90.9)	8 (72.7)	
35-	1 (9.1)	3 (27.3)	
Employed			1.000*
No	0 (0.0)	1 (9.1)	
Yes	11 (100.0)	10 (90.9)	
Offspring's sex			0.4*
Female	5 (45.5)	8 (72.7)	
Male	6 (54.5)	3 (27.3)	
	Mean/SD	Mean/SD	*P*-value
Gestational length, weeks	39.91/1.22	39.54/1.04	0.519**
Birthweight, grams	3650.91/379.37	3700.45/647.45	0.797**

*Fisher's exact test. **Mann–Whitney U-test.

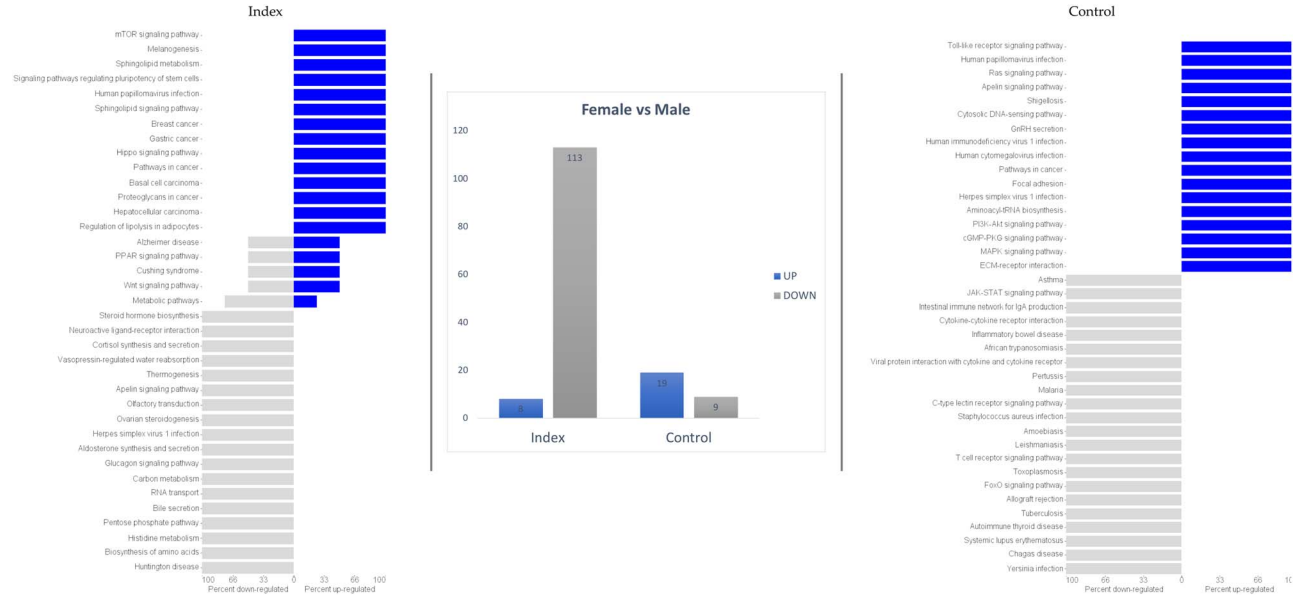


Figure 3. Differential gene expression and GO terms in "female" vs "male" placentas from women displaying self-perceived stress, anxiety, and depression during pregnancy (Index group, $n = 11$) compared with placental samples from healthy patients (Control group, $n = 11$).

Collection and preparation of placenta samples

At-term placentas were placed on ice and samples distant about 5 cm from the confluence of the umbilical cord dissected. This villous parenchyma, approximately 2.5 cm thick, was then perforated to obtain 1 g samples of the fetal (including the chorionic plate), middle, and maternal (including the thin basal plate) regions, snap-frozen, and stored until further analyses.

RNA extraction

Isolation of total RNA was performed from pools of four different placental segments within the same patience using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. Briefly, placental samples were placed in tubes containing 1 mL of Trizol reagent and mechanically disrupted using a TissueLyser II (Qiagen, Hilden, Germany), and were then centrifuged at 12 000 × *g* at 4°C for 10 min. The supernatant was then incubated with bromochloropropane (100 μL/mL homogenized) for 5 min at room temperature (RT). Samples were then centrifuged at 12 000 × g at 4°C for 15 min. The aqueous phases obtained after centrifugation were mixed with isopropanol and RNA precipitation solution (1.2 M NaCl and 0.8 M Na2C6H6O7) and incubated for 10 min at RT. Samples were then centrifuged at 12 000 × *g* at 4°C for 10 min. After discarding the supernatant, 1 mL of 75% ethanol was added to the pellet fraction and centrifuged at 7500 × *g* at 4°C for 5 min. The obtained RNA pellets were air dried for 25 min and mixed with 30 μL of RNase-free water. The total RNA obtained

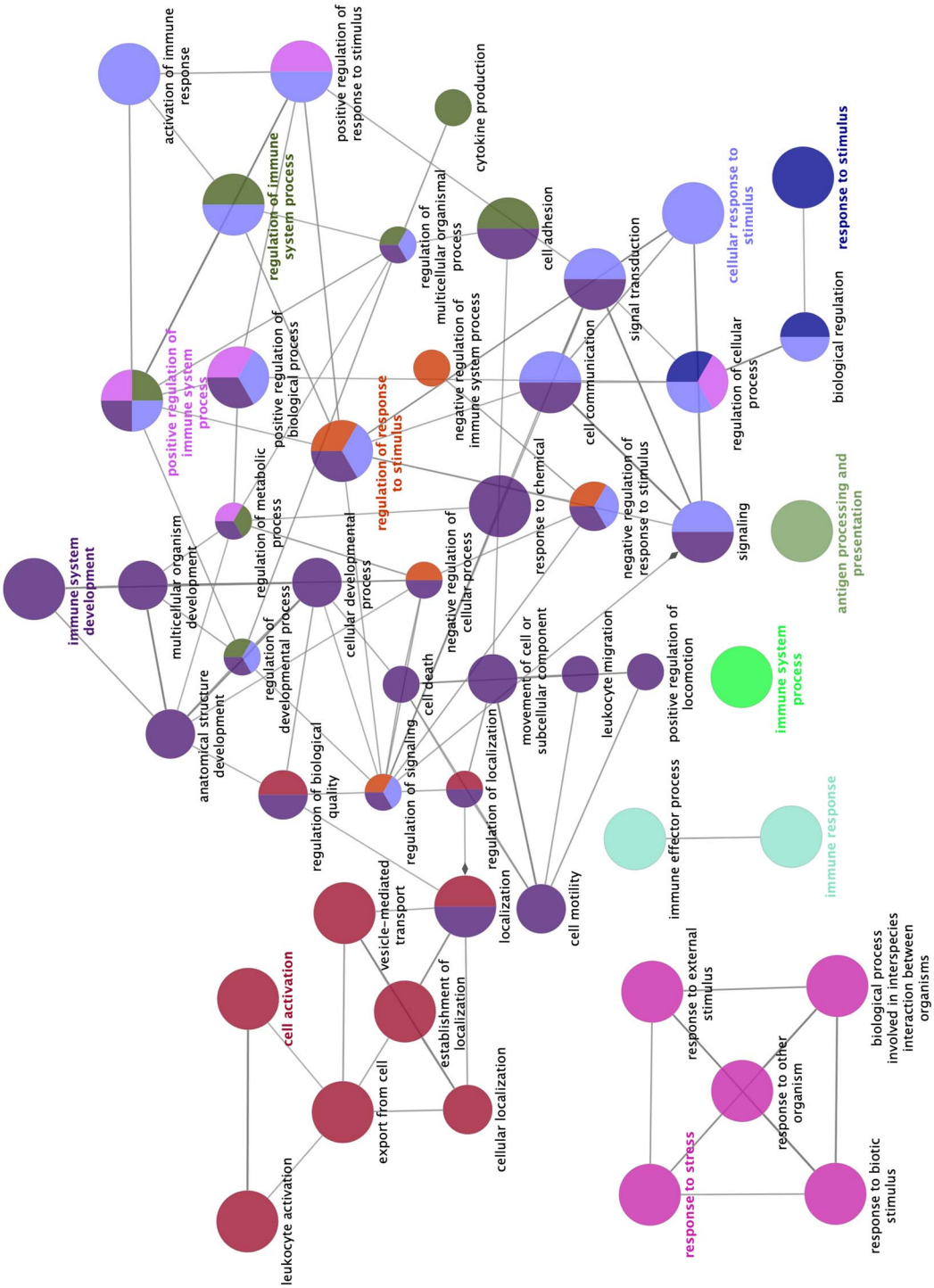


Figure 4. Schematic representation of selected altered transcripts in placentae from women displaying self-perceived stress, anxiety, and depression during pregnancy (Index group, $n = 11$) compared with placental samples from healthy patients (Control group, $n = 11$). Analysis of overrepresented biological functions was performed using the Cytoscape v3.0.0 application ClueGo v2.0.3. The following databases were used: GO subgroups biological process represented as circles. Different colors represent different terms which are functionally grouped based on common genes (kappa score). The size of the circles indicates the level of significance, where the largest circles correspond to the highest significance. The following ClueGo parameters were used: GO tree levels, 1–3 (first level = 0); minimum number of genes, 1; GO term fusion; GO term connection restriction (kappa score), 0.4; GO term grouping.

was quantified using a NanoDrop ND -1000 (Thermo Fisher Scientific, Fremont, CA, USA) and the quality was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), giving 8–10 values for the RNA integrity number (RIN).

Transcriptome analyses

The analyses of altered transcripts were performed using the RNA-Seq technique. The TruSeq stranded mRNA library prep kit (Illumina, San Diego, CA, USA) was used for library preparation, following manufacturer's instructions. Library

sequencing was performed using the NextSeq 500/550 High Output kit v2.5 (150 cycles), with sequencing setup of 2 × 75 bp paired-end, with at least 25 million reads per sample. Quantification and quality of the libraries are done using Qubit DNA Assay kit (Invitrogen) and DNA 1000 kit (Agilent) with an Agilent 2100 Bioanalyzer. Trimmomatics (version 0.36) [25] was used to trim adapter sequence, filter the quality (minimum quality of 15 in 4-bases window) and retain only reads longer than 36 bp. Cleaned reads were aligned with STAR [26] on human genome (GRCh38), using GRCh38.84 annotation to extract gene counts per sample. The raw datasets were deposited at Sequence Read Archive (SRA) with the accession number: PRJNA764856 (https://www.ncbi.nlm.nih.gov/sra/PRJNA764856).

RNA-Seq data analysis

The RNA-Seq data were examined using Partek Genomics Suite 7.0 (Partek). Data were first normalized using Robust Multichip Average RMA method [27]. Differently expressed genes (DEGs) between Index and Control groups were established performing a one way-analysis of variance (ANOVA) setting parameters as a fold change (FC) >1 or <-1 with P-value <0.05. To obtain biological meaning of the significantly modified transcripts, an enrichment analysis was performed. Analysis of altered Gene Ontology (GO) terms and pathways were assessed according to DAVID (database for annotation, visualization, and integrated discover), and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases.

RNA-Seq validation

RNA-Seq results were validated by real time quantitative polymerase chain reaction (RT-qPCR) of nine selected DEGs. The RNA samples used for RT-qPCR assay were the same samples used for RNA-Seq analysis. Primers were commercially synthetized and tested (Bio Rad, Hercules, CA, USA). Total RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). qPCR was performed in 10-μL reactions with 5 μL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, CA, USA), 50 nM for each set of primers, 2 μL of synthetized cDNA and water to a final volume of 10 μL. All reactions were carried out using the Real-Time PCR Detection System (CFX96™; Bio-Rad Laboratories, Inc; CA, USA). The thermal cycling profile was 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. Each sample for each gene was run in duplicate. The gene relative expression levels were quantified using the $2^{-\Delta\Delta ct}$ [28] method and two reference genes (*GAPDH* and *ACTB*) were used for cDNA normalization.

Immunohistochemistry

Placental samples were fixed in 4% paraformaldehyde for 4 h and embedded in paraffin wax. Tissue blocks were cut into 5 μm thick sections and dewaxed with Histoclear (HS-200, National Diagnostics, USA) followed by rehydration through gradients of ethanol (100%, 95%, 90%, 70% and PBS). For antigen retrieval, Tris-EDTA (pH -9.0) buffer was used. Samples were blocked in Hydrogen Peroxide Block (ab64261, Abcam, Cambridge, UK) and Protein block (ab64261, Abcam, Cambridge, UK) and incubated in a humid chamber at 4°C overnight with the primary monoclonal antibodies, namely CD8B (rabbit monoclonal, ABIN6924225, Antibodies-online GmbH, Aachen, Germany at 1:100 dilution) and IL7R (rabbit monoclonal, ab259806, Abcam, Cambridge, UK, dilution 1:100). For protein detection, rabbit specific HRP/DAB (ABC) Detection IHC Kit (ab64261, Abcam, Cambridge, UK) was used, including incubation with biotinylated goat anti-rabbit secondary antibody at RT for 10 min. A washing step (PBS-0.01% Tween20) of 2 × 10 min was performed after each procedure. Primary antibodies were incubated with CD8B & IL7R Blocking peptides (MBS3231207 and MBS8244306, respectively, mybiosources, San Diego, CA, USA) as negative controls. All slides were counterstained (ab245880, Abcam, Cambridge, UK) and mounted in Aqueous Mounting Medium (ab128982, Abcam, Cambridge, UK). Images were obtained with a Nikon E800 microscope connected to software NIS elements (Nikon Instruments Inc. Tokyo, Japan). DAB staining intensity was calculated in a minimum of five areas per section with the Image J Fiji software and differences in DAB staining between groups were statistically compared using Mann–Whitney test.

Results

Chronic self-perceived anxiety and depression during pregnancy modifies the placental transcriptome profile with a strong influence in immune system processes

Following statistical analysis of the data yield by RNA-Sequencing, we found that in the Index group, a total of 635 transcripts were differently expressed when compared with the Control group. Overall, most genes appeared clearly downregulated (604 downregulated and only 31 upregulated). From that general panel we found 225 genes with a fold change <-5 and 28 genes with a fold change >5. After an overall enrichment analysis, there was a conspicuous overrepresentation of biological process associated with immune system process. The most significant findings in this gene set were associated with T cell regulation and interleukin and cytokine production (Figure 1).

To gain insight into similarities among biological samples, differentially expressed genes were subjected to a hierarchical clustering procedure and presented as heatmaps (Figure 2). The heatmap indicates that the selected differential gene set associates the biological samples correctly into two groups, each representing one of the two clinical conditions (Index vs Control).

From the general gene set, we selected 80 significantly altered genes (Table 2) involved in different biological processes and pathways, with potential roles in immune processes, all of them being downregulated in the Index group ($P < 0.05$).

The offspring's sex influences placental gene expression

We further evaluated the impact of offspring sex on gene expression among placentae harvested from Index and Control women at term. This analysis demonstrated different responses on gene expression levels between "female" and "male" placentas with a total of 121 and 28 altered genes in the Index and Control groups, respectively (Figure 3). Eight immune-related genes were dysregulated in "female" placentas compared with "male" placentas in response to

Table 2. Subset of genes associated with immune system processes, differentially expressed in the Index group compared with the Control group ($P < 0.05$)

Gene name	Fold change	*P*-value	Gene name	Fold change	*P*-value
ABL1	−3.23	0.02	*IL17Rα*	−1.87	0.009
ACKR2	−5.12	0.04	*IL7R*	−4.82	0.02
ADAM10	−2.12	0.03	*IMPDH1*	−11.83	0.007
ADGRB1	−2.61	0.02	*IRF7*	−2.34	0.02
AP2A2	−2.54	0.007	*JARID2*	−3.96	0.01
AP3D1	−2.74	0.04	*KAT8*	−7.67	0.03
APEH	−5.37	0.006	*KIF22*	−3.24	0.004
ARHGAP45	−4.22	0.01	*LAX1*	−3.15	0.01
ARHGEF2	−16.28	0.02	*LPCAT1*	−4.13	0.02
ATM	−5.66	0.04	*MKNK2*	−2.44	0.01
BCL11B	−9.73	0.04	*MUC17*	−2.01	0.04
BSG	−2.16	0.04	*MYO10*	−2.02	0.01
BTN2A1	−3.22	0.02	*NFE2L1*	−3.28	0.04
BTN2A2	−3.13	0.007	*NUB1*	−4.07	0.04
BTN2A3	−4.09	0.008	*OPRD1*	−3.78	0.01
C4A	−3.66	0.02	*PCID2*	−3.58	0.02
CACTIN	−3.24	0.01	*PIP5K1C*	−3.79	0.04
CAPZA2	−2.57	0.04	*PMS2P5*	−3.90	0.04
CCR4	−4.33	0.03	*PSMD11*	−5.23	0.04
CD151	−3.65	0.03	*PSMD13*	−1.96	0.02
CD46	−5.13	0.02	*PTPN11*	−6.42	0.03
CD8α	−4.23	0.005	*RAB31*	−1.64	0.01
CD8β	−20.25	0.01	*RAB3A*	−8.81	0.04
CD99L2	−4.31	0.03	*RAB4A*	−3.21	0.01
CERT1	−3.54	0.03	*RC3H1*	−3.79	0.03
CHRNB2	−2.36	0.04	*RPS6*	−1.61	0.04
CHRNB4	−2.84	0.03	*SBNO2*	−2.56	0.03
CLPB	−5.61	0.004	*SCRIB*	−13.78	0.04
CNN2	−2.42	0.03	*SERPINB9*	−2.84	0.002
DAPK3	−3.57	0.01	*SIRPA*	−6.27	0.03
DSP	−1.97	0.04	*SLC16A8*	−24.89	0.04
EDN1	−7.97	0.02	*SLC2A5*	−3.86	0.02
EEF2	−1.97	0.005	*SOD2*	−5.15	0.003
EPPIN	−46.77	0.01	*SOX4*	−2.49	0.01
FAM3α	−4.76	0.02	*SYVN1*	−3.05	0.03
FCGR3α	−5.86	0.01	*TCF3*	−3.28	0.01
FCGR3β	−7.54	0.02	*TEK*	−4.10	0.03
GOLPH3	−1.77	0.02	*TNFRSF13β*	−17.41	0.03
GRAP2	−30.82	0.01	*TRIM34*	−4.14	0.02
ICAM1	−2.74	0.03	*ZBTB7A*	−2.64	0.0005
IL11Rα	−4.11	0.02	*ZDHHC11*	−4.25	0.02
IL12Rβ1	−3.28	0.02			

maternal stress (*CLEC5A*, *VSTM1*, *IGHV23*, *IGKC*, *IGKV2*, *IGKV2D*, *IGLC1*, and *IGLC3*).

Biological meaning of a selected gene subset

The GO biological process analysis revealed several significantly downregulated biological processes associated with immune functions, such as T cell immunity and proliferation and cytokine and interleukin regulation. The details of these analyses are summarized in Table 3. Moreover, to gain insight into the roles of these immune-related genes, this particular set of immune-related genes was subjected to a functional clustering procedure and presented as a network (Figure 4).

One major immune-related pathway dominated among significantly affected pathways

Following pathway enrichment analysis, we found that one of the key pathways ruling mechanisms for cellular and humoral immune regulation named "Primary immunodeficiency" was significantly altered ($P < 0.02$) in placentae from the Index group compared with Controls. Several transcripts belonging to this pathway (*CD8α*, *CD8B*, *IL7R*, and *TNFRSF13B*) were downregulated in the Index group (Figure 5).

Validation of the RNA-Seq data

We selected nine genes to verify the RNA-Seq results by RT-qPCR. These genes presented similar patterns of expression under both methods (Figure 6), proving that RNA-Seq results were reliable.

Chronic self-perceived anxiety and depression during pregnancy alters the protein expression of two candidate immune-related genes

The expression of two candidate proteins (CD8B and IL7R) was assessed by immunohistochemistry to validate the results of gene expression. We confirmed the presence of both proteins within the extra-villous trophoblast of placentas from either studied group (Index and Control groups) (Figure 7Aa–Dd).

Table 3. Most significant differently expressed biological processes ($P < 0.05$) examined with KEGG database in the Index group compared with the Control group

Biological process	Enrichment score	*P*-value	Genes altered (%)	Gene list
T cell mediated immunity	5.38	0.004	16.7	*BTN3A2, BTN3A3, CD46, CD8α*
Negative regulation of chemokine ligan 5	5.34	0.004	50	*MUL1, SIRPA*
Cellular response to interleukine-6	4.78	0.008	20	*ABCC2, ICAM1, SBNO2*
Interleukine-12 signaling	4.59	0.01	10.86	*AIP, BOLA2, CNN2, IL12Rβ1, SOD2*
Cytokine binding	4.02	0.01	6.56	*A2M, ACKR2, BMPR2, CCR4, IL11Rα, IL12Rβ1, LIFR, TGFBR3L, WFIKKN1*
T cell proliferation	3.94	0.01	11.11	*ABL1, CD151, RC3H1, RPS6*
Cytokine receptor activity	3.81	0.02	7.21	*ACKR2, CCR4, IL11Rα, IL12R 1, IL17Rα, IL7R, LIFR*
Cellular response to interleukine-1	3.74	0.02	7.79	*ABCC2, CACTIN, EDN1, ICAM1, RC3H1, SIRPA*
Negative regulation of lymphocyte proliferation	3.52	0.02	7.41	*ATM, GNRH1, GPNMB, RC3H1, SCRIB, TNFRSF13β*
Negative regulation of innate immune response	3.35	0.03	7.93	*A2M, CACTIN, FAM3α, MUL1, SERPINB9*
Leukocyte mediated immunity	3.32	0.03	7.05	*BTN3A2, BTN3A3, CD46, CD8α, IRF7, SERPINB9*

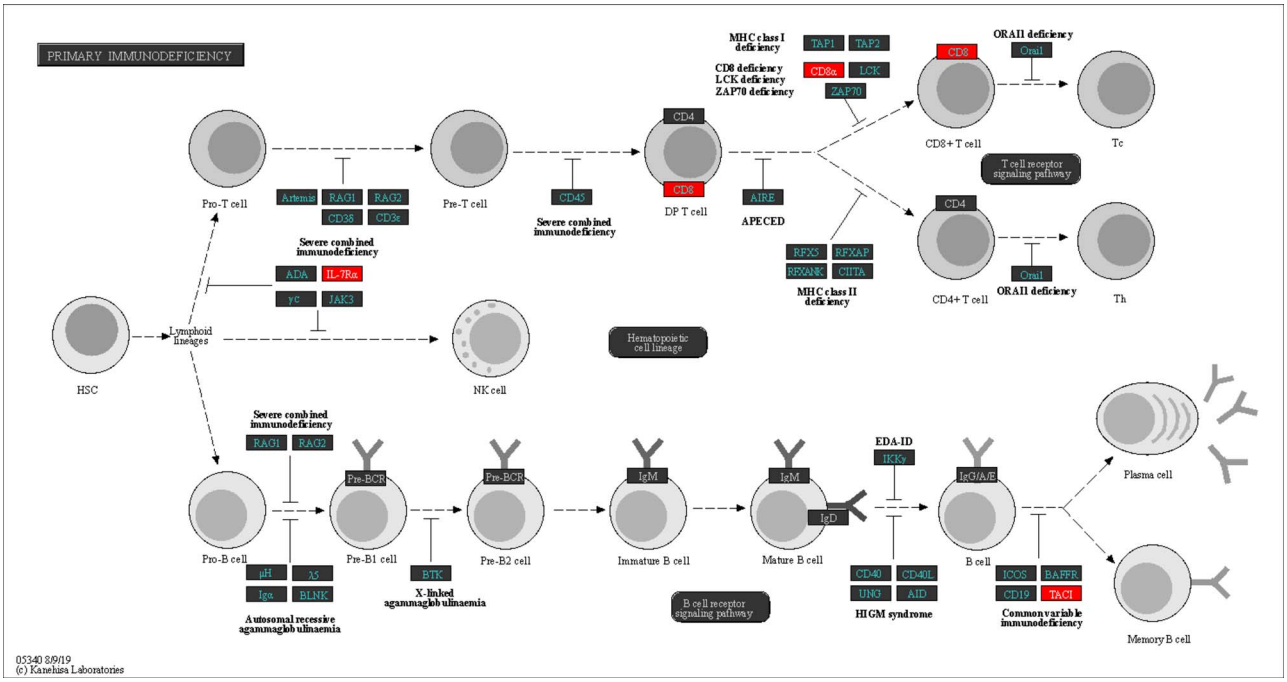


Figure 5. Schematic representation of the "Primary immunodeficiency" pathway (hsa05340). Downregulated genes ($P < 0.05$) in placentae from women displaying self-perceived stress, anxiety, and depression during pregnancy (Index group, $n = 11$) compared with placental samples from healthy patients (Control group, $n = 11$) are red-marked.

Protein expression was calculated by measuring DAB staining intensity in a minimum of five areas per section. Following the line of the results obtained for gene expression, the expression of CD8B and IL7R was significantly downregulated ($P < 0.05$) in placental samples from the Index group compared with the Controls (CD8B: 11.4 ± 0.9 vs 29.9 ± 3.9, respectively and IL7R: 3.9 ± 0.5 vs 39.1 ± 8.7, respectively) (Figure 7E-F). These results suggest the improper protein expression prevailing in placental samples from mothers suffering from stress, anxiety, and depression during pregnancy compared with the Control group.

Discussion

The present study explored the effects of chronic self-perceived stress, anxiety, and depression during pregnancy on the expression of immune-related genes and pathways in term placentae. The results demonstrated the presence of a modified placental transcriptome profile with a strong influence in immune system processes.

Chronic self-perceived anxiety and depression during pregnancy clearly induced a major downregulation of transcripts related to T-cell regulation, interleukins, and cytokine signaling or innate immune response. *CD46*, *CD151*, *and*

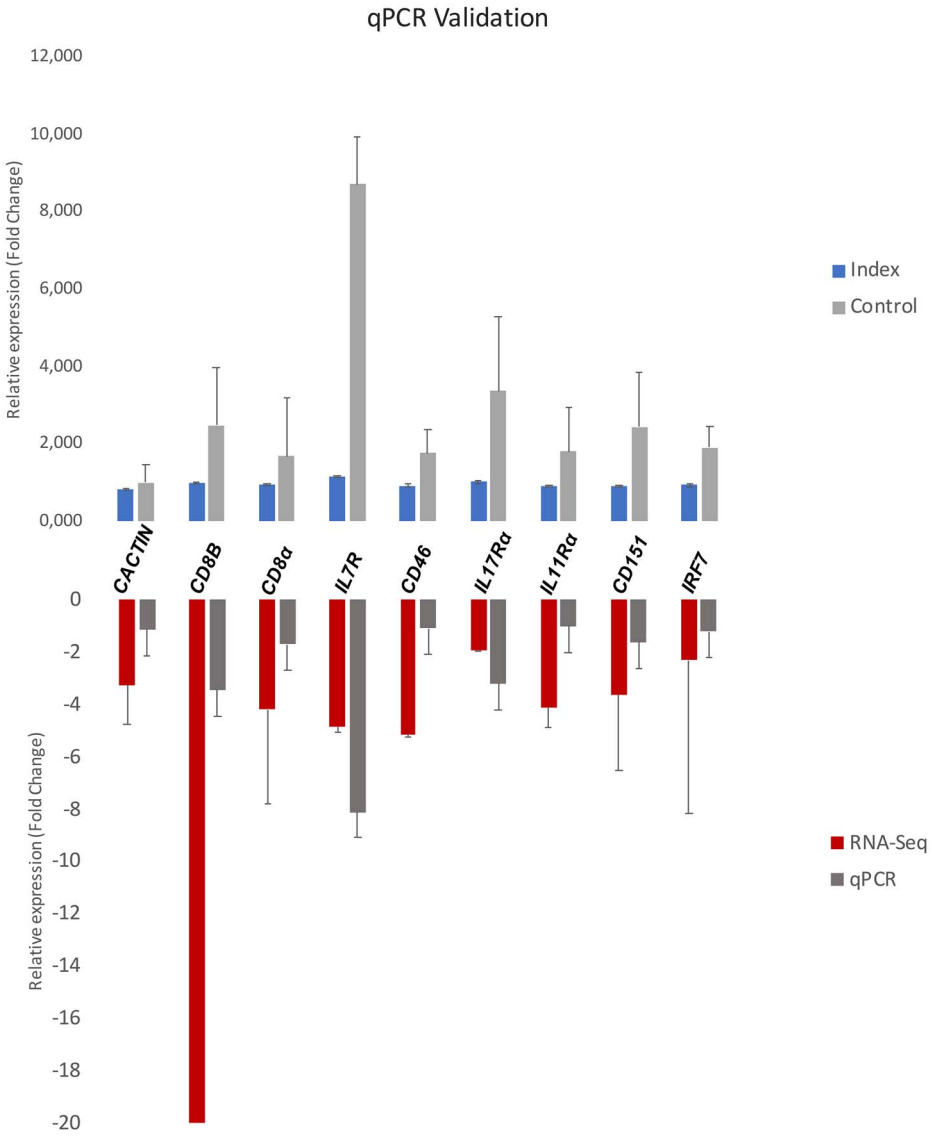


Figure 6. Validation of RNA-Seq results by RT-qPCR of nine differentially expressed genes. The upper graph represents difference of expression of nine immune-related genes between women displaying self-perceived stress, anxiety, and depression during pregnancy (Index group, $n = 11$) compared with placental samples from healthy patients (Control group, $n = 11$). Differences in gene expression between groups were statistically compared using Mann–Whitney test. All genes validated were significantly different ($P < 0.05$) between groups. Values on Y axle represent the relative gene expression (fold change).

CD8α&β genes were downregulated in the Index group (FC: −5.14, −3.65, −4.23, and − 20.25, respectively). *CD46* encodes the membrane cofactor protein which is a regulatory protein that protects host cells from injury by complement [29]. This protein is commonly known as the "pathogen magnet" [30] and is widely recognized for its roles in linking innate and adaptive immune responses [31, 32] and further related to T-cell biology (reviewed in Kemper et al., 2007). The co-engagement of the T-cell receptor and CD46 by $CD4^+$ T cells leads to the induction of interferon-γ (IFN-γ)-secreting effector T-helper type-1 (Th1) cells (reviewed in Kemper et al., 2007 [33]). Moreover, common variable immunodeficiency was found in CD46-deficient patients, a syndrome characterized by hypogammaglobulinemia [34]. CD46-activated T-cells support B-cell activation, evidenced in a CD46-deficient patient whose T-cells were impaired in promoting immunoglobulin G production by B cells [35]. *CD151*, a member of tetraspanins, is an important immunomodulator of Natural Killer (NK) cells [36] and a regulator of antioxidants in placentas. Downregulation of this gene has been recently associated with accused oxidative stress and apoptosis in trophoblast cells [37]. *CD8α*, an adaptive immune gene, has a key role in the "Primary immunodeficiency" pathway, which was downregulated in the Index group.

Primary immunodeficiency disease (PID) is a disorder where components of the immune system are missing or compromised [38]; and likely transferred to the newborn [39]. This pathway involves a group of genes (including *CD8α*, *ILR7α*, and *TAC1*; downregulated in the Index group; FC: −4.23, −4.82, −17.41, respectively) affecting cellular and humoral immunity or nonspecific host defense

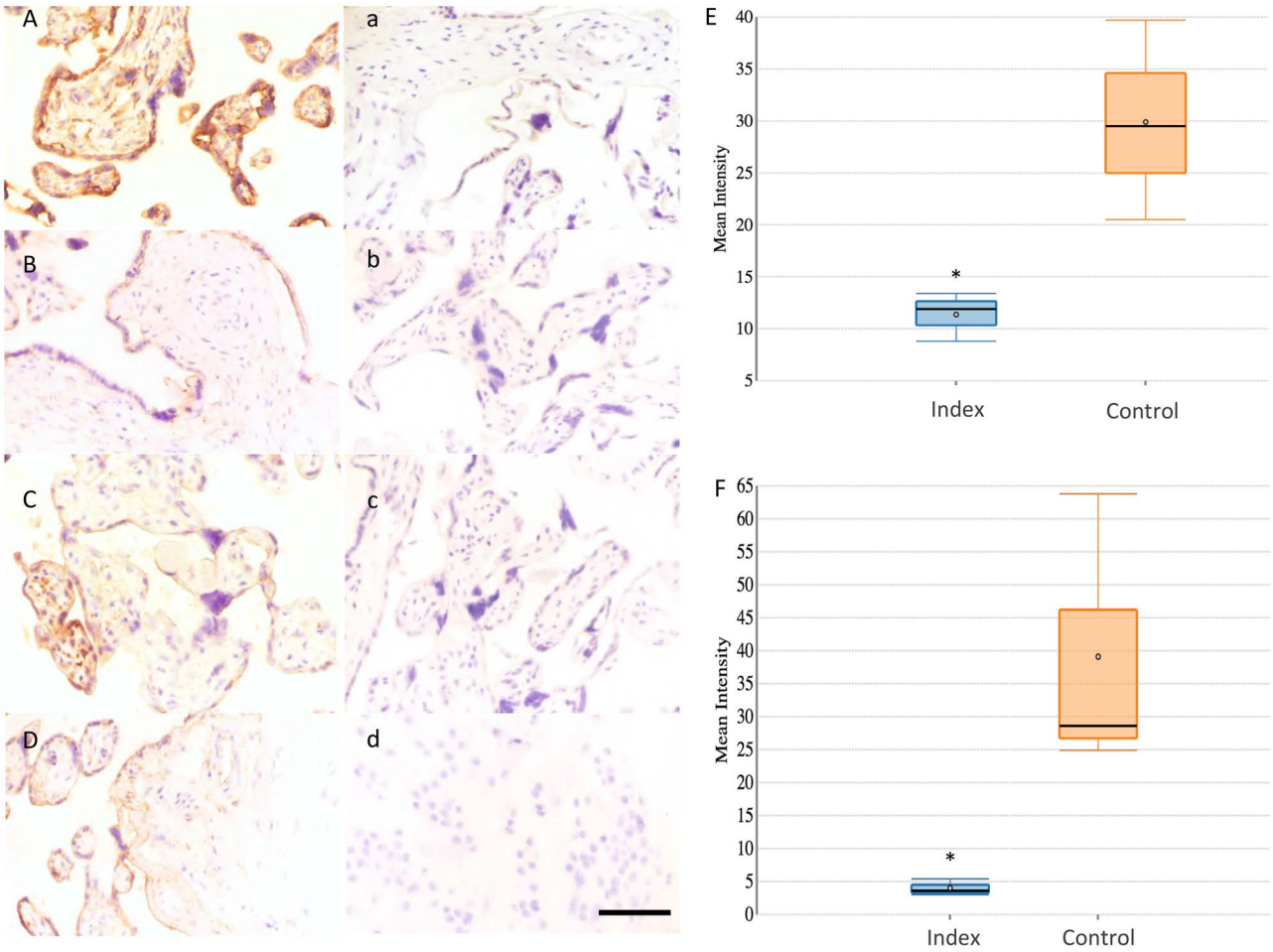


Figure 7. (A–F) Representative immunohistochemical staining for CD8B and IL7R in extra-villous trophoblast of placentas from healthy patients (Control group: A, a- CD8B and C, c- IL7R, $n = 4$) and from patients that were affected by maternal stress during pregnancy (Index group: B,b- CD8B and D,d- IL7R, $n = 4$). A-B images show positive staining of CD8B. a-b images are staining controls of CD8B. C-D images show positive staining of IL7R. c-d images are staining controls of IL7R. Size bar 50 μm. E and F show Whisker-box blots depicting the relative quantification of immunolabeling intensity for CD8B and IL7R proteins using Image J software. The box indicates the interquartile range; the horizontal line in the box depicts the median; whiskers indicate the data range. Differences in DAB staining between groups were statistically compared using Mann–Whitney test. Asterisk indicates that the measured differences were statistically significant ($P < 0.05$).

mechanisms mediated by complement proteins. It would also affect phagocytes and natural killer (NK) cells [40], with responsibility for immune system changes in early life as well as a neuroendocrinological programming of the offspring's brain, which may trigger future neuropsychiatric and/or autoimmune disorders [41]. Such interpretation harmonizes well with the prior findings of high basal levels of inflammatory cytokines and cortisol in blood, associated with reduced GR activity [42] in the offspring of pregnant women with MDD, which makes them highly vulnerable for developing adult affective behaviors. Such postulation is also in line with prior hypothesis that pregnant women suffering from stress and anxiety show dysfunction of circulating inflammatory cytokines, which may be responsible for fetal and/or newborn disorders [43]. Moreover, it is worth noting that the offspring of pregnant women with MDD have high basal levels of inflammatory cytokines and cortisol in blood, associated with reduced GR activity [42], which makes them highly vulnerable for developing adult affective behaviors.

In the present study, the gene encoding the Chemokine receptor *CCR4* was downregulated in the Index group (FC: −4.33). This gene is mainly expressed on näive T-cells [44] and has a key functional role in both cell migration and in cell–cell interaction as well as in the regulation of various immune responses [45]. Lopez et al. (2006) proposed that *CCR4* expression increases in the third trimester of healthy pregnancies implicating T-cell recruitment to inflamed areas, while it reduces in neonates from mothers suffering from atopic immune responses alike the anxious/depressive mothers during pregnancy [46]. This suggests a delayed maturation of cellular immune functions [46] whose impact on the placenta and fetus is unknown and needs further research work.

The receptor for interleukin 7 (*IL7R*), which encodes a protein that plays a critical role during lymphocytes development [47, 48], was repressed in the Index group (FC: −4.82). Dysfunction of *IL7R* has been detected in patients with severe combined immunodeficiency (SCID) leading to chronic inflammatory diseases [49, 50]. Two other interleukin receptors of interest (IL11Rα and IL17Rα) were also decreased in the Index group, which is of interest since it is known that the correct function of these receptors is required for a correct fetoplacental development [51]. In contrast to most interleukin receptors, the expression levels of IL17Rα are functionally significant since high levels of IL17Rα

receptor are required for an effective function [52, 53]. Interestingly, pathological dysfunction of IL-17Rα in pregnant mothers affects fetal brain development and subsequently may contribute to the autism spectrum disorder (ASD)-like behavioral phenotypes in offspring [54].

In summary, these findings suggest that symptoms of chronic anxiety and depression during pregnancy have the potential to suppress immune functions that are physiologically regulated by cytokines, chemokines, and interleukins during pregnancy. Conceivably, these alterations are related to modifications in cellular oxidative stress taking place during the pathogenesis of these disorders since a relation between the accumulation of oxygen radicals and immune dysfunction has been reported before [55]. Indeed, an activation of signaling pathways and alteration of the physiology of inflammatory cytokines and chemokines have been associated with high concentrations of reactive oxygen substances (ROS) in several cell populations [56].

In addition, a downregulation of the two main Toll-like receptors (TLRs) gene regulators (*Cactin*; FC: −3.24 and *IRF7*; FC: −2.34) was observed in the Index group. It is known that TLRs, which are transmembrane proteins, are known to function as primary sensors of the immune system by recognizing specific microbial motifs and inducing proinflammatory genes that promote innate and adaptive immunity [57]. TLRs regulate gene expression through the activation of transcription factors, such as NF-κB and interferon-regulatory factors. Hence, upregulation of these pathways can lead to inflammatory diseases and is a subject to tight control by negative regulators of innate immune signaling. Cactin acts as a negative regulator of TLRs. Overexpression of Cactin suppresses TLR-induced activation of NF-κB and induction of TLR-responsive genes, whereas knockdown of endogenous Cactin enhances TLR-induction of these responses [58]. The downregulation of *Cactin* found in the Index group might be explained by the fact that, under stress conditions, TLRs are needed to counteract the effects of the immune system downregulation and create an environment of immune tolerance toward the fetal allograft.

Conclusions

Taken together, our results demonstrate that an immune dysfunction, evidenced as a differential expression of transcripts and pathways associated with immune responses, was present in the term placenta of women displaying symptoms of chronic self-perceived stress, anxiety, and depression during pregnancy. These findings have potential implications for prevention and intervention strategies. However, more studies are needed to investigate the consequences of these maternal disorders on the immunological condition of the offspring.

Data availability

The data underlying this article are available in Sequence Read Archive (SRA) at (https://www.ncbi.nlm.nih.gov/sra/PRJNA764856), and can be accessed with accession number: PRJNA764856.

Author contributions

Conceptualization and design: IM, AJ, GS, ET and HR-M; analysis: CAM; interpretation of the data: IM, CAM, AJ, GS, ET and HR-M; original draft preparation: CAM and HR-M; review and editing: AJ, GS, ET and HR-M; project administration: IM, AJ, GS and ET; funding acquisition: IM. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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