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Endometrial cortisol level and its relationship with psychological stress, molecular tissue changes, and clinical outcomes in infertile women

Diana Marti-Garcia[1,5], Patricia Sebastian-Leon[1,5], Pilar Dolz del Castellar[2], Almudena Devesa-Peiro[1], María del Carmen Vidal[2], Laura Caracena[2], Francisco José Sanz[1], Rebeca Esteve-Moreno[1,3], Estrella Dura-Ferrandis[4] & Patricia Diaz-Gimeno[1]✉

Despite fertility treatments are a stressful journey, there remains a lack of studies assessing how stress is affecting endometrial function in infertility. This prospective cohort study, including 84 endometrial biopsies, aims to elucidate the effect of stress, measured by different approaches, in the endometrial function in In Vitro Fertilization treatments. This population was molecularly characterized by measuring endometrial cortisol levels and endometrial transcriptomic profiles, and psychological aspects measured by the State-Trait Anxiety Inventory test. A relationship between endometrial cortisol levels and psychological stress punctuation was found. Psychologically stressed patients had increased endometrial cortisol levels (5.4 ng/g vs. 3.45 ng/g; $p = 0.05$, in the limit of significance) and cortisol levels correlated with psychological test punctuations (cor = 0.97, $p < 0.05$). From the clinical point of view, patients with cortisol levels ≥ 13.9 ng/g had a 32% relative higher risk of not becoming pregnant ($p = 0.003$). Molecular evidences showed, increased cortisol levels were significantly associated with changes in 182 genes in endometrium ($p < 0.001$) and psychological stress scores were significantly associated with changes in 12 genes involved in key functions for embryo implantation and development ($p < 0.001$). Psychological evaluation could serve as a less-invasive screening tool to identify at-risk infertility patients and implement preventive psychological interventions in the clinical setting.

Keywords State-Trait Anxiety Inventory (STAI), Cortisol, Stress biomarkers, Endometrial microenvironment, Endometrial factor in infertility

About 40% of infertile women face mental health issues, including anxiety, depression, and stress[1–3], with assisted reproductive treatments further adding significant physical and emotional stress[4,5]. Although some studies report that stress is linked to lower pregnancy rates and higher miscarriage rates[6–9], others have not identified such associations[10–12]. Aside from this controversy, there remains a lack of studies directly assessing the link between stress and endometrial function in infertility. Understanding the effect of stress on in vitro fertilization (IVF) patients and its contribution to infertility is important to determine how patients could benefit from personalized psychological support, potentially decreasing drop-outs and increasing IVF treatment success rates.

Various psychological interventions have been developed for infertile women—while some studies report these interventions improve treatment outcomes[13–16], others do not[17,18]. These differential results may be

[1]IVIRMA Global Research Alliance, IVI Foundation, Instituto de Investigación Sanitaria La Fe (IIS La Fe), Av. Fernando Abril Martorell 106, Torre A, Planta 1ª, Valencia 46026, Spain. [2]IVIRMA Global Research Alliance, IVI RMA Valencia, Plaza de la Policía Local nº 3, Valencia 46015, Spain. [3]Department of Paediatrics, Obstetrics, and Gynaecology, University of Valencia, Av. Blasco Ibáñez 15, Valencia 46010, Spain. [4]Instituto de Investigación en Políticas de Bienestar Social -Polibienestar, University of Valencia, C/del Serpis, 29, Algirós, Valencia 46022, Spain. [5]These authors jointly supervised this work: Diana Marti-Garcia and Patricia Sebastian-Leon. ✉email: patricia.diaz@ivirma.com; patricia_diaz@iislafe.es

due to heterogeneity among studies in terms of intervention programs and psychological questionnaires to assess various psychological aspects (e.g., stress, anxiety, depression)[19]. Further, studies have not assessed the impact of psychological interventions directly on endometrial function in infertility. Thus, there remains a lack of standardization in psychological evaluation and treatment of patients undergoing assisted reproductive treatments.

Stress, defined as disrupted homeostasis due to real or perceived threats, activates the hypothalamic–pituitary–adrenal (HPA) axis, increasing cortisol production and its dissemination throughout the body[20,21]. Cortisol helps manage the stress response[22] and thus is a reference stress biomarker. Yet prolonged cortisol exposure can disrupt the HPA axis and affect critical reproductive functions such as hormone production[23–25] as well as follicle maturation, reducing the number of retrieved oocytes[26]. However, the effect of cortisol on the endometrial factor in infertility is still unknown.

The endometrium is a key player in the reproductive process, especially in the window of implantation[27–29] when precise steroid hormonal signaling is required[30]. Several authors have reported that blood and endometrial steroid levels are not correlated[31–33], so local endometrial hormonal production (i.e., intracrinology)[34] could be essential for implantation[35] and early pregnancy. This underscores the need to evaluate steroid levels directly in the endometrial microenvironment.

Our recent research on endometrial function in infertility shows that high endometrial cortisol levels impair endometrial progression[35]. Because one of the main factors that disturbs cortisol levels is stress, in the present study we examined whether stress, assessed using the State-Trait Anxiety Inventory (STAI) questionnaire, was associated with endometrial cortisol levels, gene expression, and reproductive success.

Materials and methods

Ethics statement

This study was performed at the IVI Foundation/IIS La Fe and IVI Valencia in 2019–2024. All participants provided written informed consent. The study protocol was approved by the Institutional Review Board of the Instituto Valenciano de Infertilidad (1805-FIVI-033-PD). This study was adhered to the fundamental principles set forth in the Declaration of Helsinki, the Council of Europe Convention on Human Rights and Biomedicine, and the UNESCO Universal Declaration on the Human Genome and Human Rights. It will also comply with the requirements established in Spanish legislation in the fields of biomedical research, personal data protection, and bioethics.

Study population

This study prospectively included infertile women undergoing IVF, defining infertility as the inability to achieve a clinical pregnancy after at least 12 months of regular unprotected sexual intercourse. Inclusion criteria included women < 50 years old with good-quality embryos who were recommended to undergo an endometrial evaluation cycle including collecting an endometrial biopsy. We excluded patients: with uncorrected uterine pathologies (fibroids, polyps, or congenital Müllerian abnormalities) or hydrosalpinx; with systemic, inflammatory, autoimmune, and/or renal diseases or significant endocrine and/or metabolic disorders; and receiving concomitant medication that could interfere with the study objectives, such as insulin, androgens, corticosteroids, or psychiatric medications (e.g., antidepressants).

Endometrial preparation, biopsy collection, and clinical follow-up

Biopsies were collected either in a natural cycle or hormonal replacement therapy (HRT) cycle. For the HRT cycle, endometrium was prepared by externally adding estradiol and progesterone, as previously described[35]. Endometrial biopsies were scheduled on the fifth day from the start of progesterone administration. For the natural cycle, either administration of human chorionic gonadotrophin (hCG) or luteinizing hormone peak determination was considered a reference for ovulation, and biopsies were scheduled seven days later.

Biopsies were collected from uterine fundus under sterile conditions using a Cornier pipelle cannula (CCD Laboratories, Paris, France) and stored at − 80 °C.

A complete follow-up (i.e., from embryo transfer until live birth, if applicable) of the first single-embryo transfer in the next cycle after biopsy collection was performed to evaluate reproductive success, including consulting electronic health records. Since no patients in our population experienced a clinical or biochemical miscarriage during embryo transfer, we classified patients as pregnant when a healthy live birth was confirmed or not pregnant when presenting a negative serum beta-hCG result (≤ 10 IU/I) 14–16 days after embryo transfer.

Endometrial cortisol concentration

Sample preparation was performed as detailed in our previous work[35]. Endometrial cortisol concentration was measured by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) using an Acquity UPLC system equipped with Acquity UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm) (Waters Corporation, Wilmslow, UK), with water and acetonitrile with 0.1% formic acid as the mobile phases. Samples were measured on a Waters Xevo TQ-S mass spectrometer with electrospray ionization source working in positive-ion and multiple-reaction monitoring mode.

Endometrial gene expression

Total RNA was extracted from endometrial biopsies using the miRNeasy Mini Kit (Qiagen, Madrid, Spain) according to the manufacturer's instructions. Quality of extracted RNA was evaluated using a Nanodrop One spectrophotometer (Thermo Fisher Scientific, Valencia, Spain) and TapeStation 4200 system (Agilent, Valencia, Spain). Only high-quality samples with A260/A280 ratio = ~ 2, A260/A230 ratio = 1.8–2.2, RNA Integrity Number ≥ 3, and ≥ 70% of fragments with > 200 nucleotides were included in subsequent analyses.

For RNA-sequencing (RNA-seq), RNA libraries were generated according to the AmpliSeq for Illumina Transcriptome Human Gene Expression Panel commercial protocol[36,37] in a NextSeq500/550 sequencer, using a paired-end design with 150 cycles and 10 M reads per sample. Raw transcriptomic data were evaluated using FastQC[38,39]. Reads from the RNA-seq library were aligned against a reference sequence using Spliced Transcripts Alignment Reference[40] and quantified using featureCounts[41] to exclude low-quality counts ($Q < 30$). Genes with low expression were filtered by low counts per million ($CPM < 1.5$) using the EdgeR R-package (v.3.32.1)[42]. Once filtered, remaining raw counts were normalized using voom (limma R-package v.3.46.0)[43]. Principal variance component analysis (PVCA R-package)[44] was used to assess possible technical, clinical, and demographic batch effects that could affect the transcriptomic behavior of samples. The limma R-package was used to remove unwanted effects using linear models.

To evaluate clusters of genes that varied expression with increased endometrial cortisol concentrations or STAI results, dose–response analysis was performed using maSigPro R-package (v.1.62.0)[45,46], considering genes with $p < 0.001$ as significant. The identified significant genes were functionally characterized using the Kyoto Encyclopedia of Genes and Genomes human pathways (release 99.0, August 1, 2021)[47] and Gene Ontology (version July 2, 2021; employing only experimental annotations)[48] databases.

Psychological characterization

Anxiety was assessed using the Spanish version of STAI[49]. All patients completed the questionnaire on the same day of biopsy collection or some days later in the same cycle. STAI is a validated questionnaire widely used in general and clinical populations. The questionnaire comprises two scales that evaluate two different dimensions of anxiety: state anxiety (STAI-S), a transitory condition triggered by a situational stressor (e.g., fertility journey); and trait anxiety (STAI-T), a stable personality trait referring to individual differences in the tendency to be anxious about the present and consider situations as life-threatening.

Participants were classified into two groups based on STAI results: those who scored >60th percentile were considered stressed, while those who scored ≤60th percentile were considered not stressed. STAI-S and STAI-T scales were analyzed independently, so a participant could be classified as having state anxiety but not trait anxiety, and vice versa.

In vitro effects of cortisol in immortalized human endometrial stromal cells (HESCs)

A commercialized cell line of immortalized HESCs was cultured in 6-well plates with Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) containing 10% (v/v) fetal bovine serum and supplemented with 0.2% fungizone and 0.2% penicillin/streptomycin until they reach confluence. Once confluent, cells were treated with 0.5 µM or 10 µM cortisol (hydrocortisone, #H0888, Sigma-Aldrich, St. Louis, MO, USA) or vehicle control for 24 h. Total RNA was extracted using the Qiagen miRNeasy Mini Kit according to the manufacturer's instructions. Complementary DNA was synthesized using the PrimeScript Reagent Kit (Perfect Real Time, Takara, Shiga, Japan) on a T3000 thermocycler (Biometra, Dublin, Ireland). Samples were sequenced using the AmpliSeq for Illumina® Transcriptome Human Gene Expression Panel in a NextSeq 500/550 system. Expression of selected genes was evaluated with quantitative real-time PCR (RT-qPCR) using specific primers (Invitrogen, Waltham, MA, USA) (Supplementary Table 1). Reactions were carried out with five technical replicates on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using Power-Up SYBR Green (Thermo Fisher Scientific, Waltham, MA, USA). B-actin was used as a housekeeping gene, water was used as the negative control, and Universal Human RNA (Agilent, Valencia, Spain) was used as the positive control. Relative mRNA expression was calculated using the $2^{-\Delta\Delta}Ct$ method[50].

Statistical analysis

R statistical software version 4.0.5[51] was used for all statistical analyses. Statistical significance was set at $p \leq 0.05$. Plots were generated using ggplot2 R-package[52].

For analysis of demographic and clinical variables, the normality of numerical variables was assessed using Shapiro-Wilk test. Non-parametric variables were analyzed using Wilcoxon and Kruskal-Wallis tests. Parametric variables were analyzed using t-test and ANOVA. For categorical variables, differences in proportions were evaluated using Fisher's exact test.

Cortisol levels in the IVF population were stratified according to boxplot distributions, using interquartile ranges to define categories. Barnard's test (Barnard R-package v.1.8)[53], which considers minority and extreme groups corresponding to patients with greater dysregulation in the endometrial microenvironment, was used to compare endometrial cortisol levels with reproductive outcomes and psychological classifications. Relative risk of not becoming pregnant when endometrial cortisol levels were high was calculated as a ratio comparing the probability of an event occurring in an exposed compared to non-exposed group. Correlation between endometrial cortisol levels and the proportion of stressed patients as assessed by STAI was evaluated using Pearson's correlation test.

Results

Demographic characteristics

From 91 initially assessed patients, our final sample included 84 IVF patients (Fig. 1) with a mean age of 39.10 years, body mass index (BMI) of 22.87 kg/m^2, and endometrial thickness of 8.71 mm. Following biopsy collection, 88.1% of patients ($n = 74$) underwent an embryo transfer, so there was an 11.9% ($n = 10$) treatment discontinuation rate. Among these 10 patients, six did so due to the absence of viable embryos for transfer and chose not to pursue an additional cycle. The remaining four patients dropped out the treatment despite having embryos available.

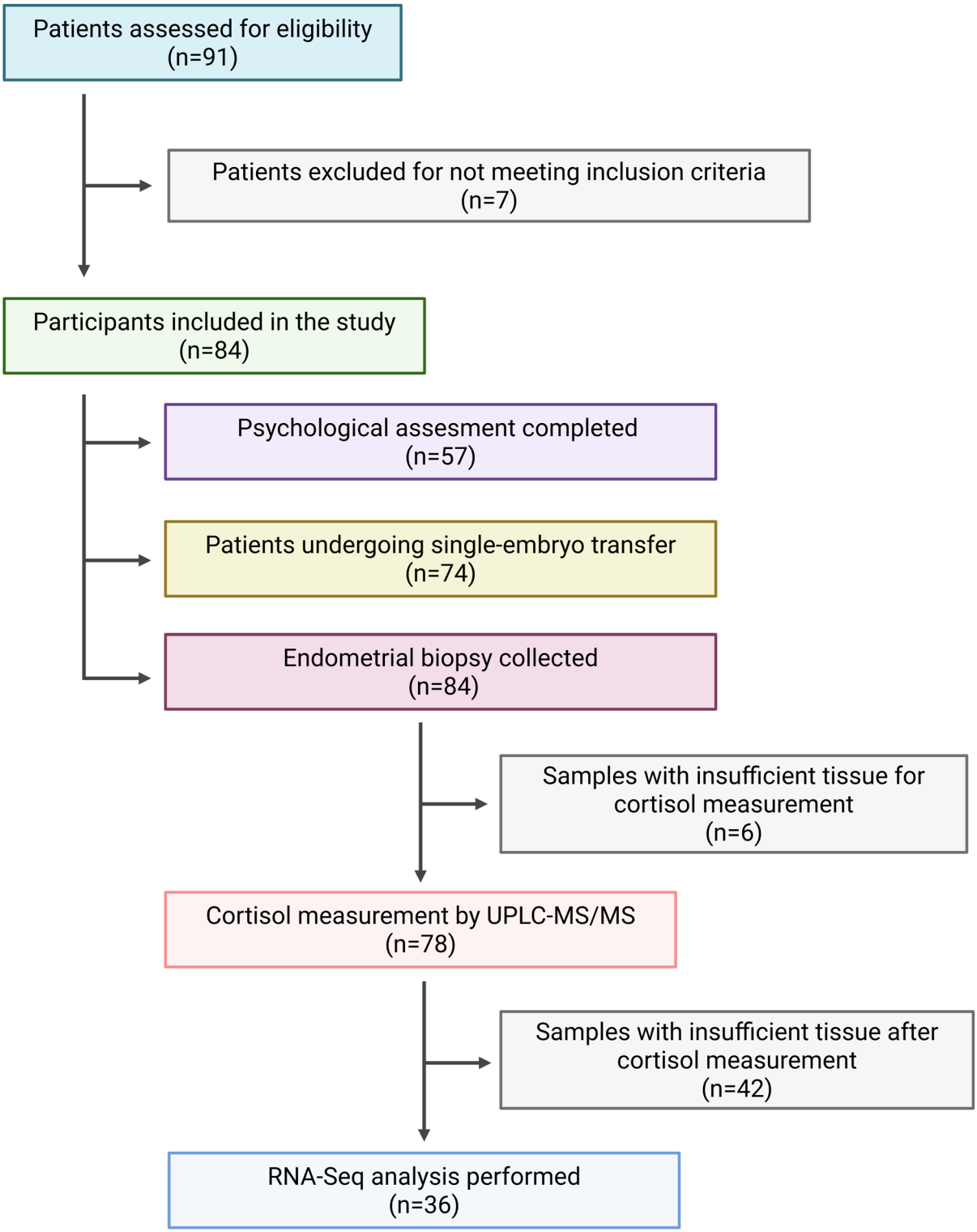


Fig. 1. Flow diagram of patient inclusion, psychological assessment and endometrial samples available for each analysis. From 91 initially assessed patients, seven patients did not meet the inclusion criteria. Our final sample included 84 IVF patients which underwent an endometrial biopsy collection. Following biopsy collection, 74 patients underwent an embryo transfer, and 57 completed the psychological assessment. Six out of the 84 endometrial samples collected had to be discarded due to insufficient tissue quantity for cortisol measurement; hence, the endometrial cortisol measurement by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was performed in 78 samples. After the cortisol measurement, 42 samples had insufficient tissue for subsequent analyses. For that reason, the RNA-seq analyses of endometrial gene expression evaluation was performed in 36 samples. Graphic created with Biorender.com.

Six out of 84 endometrial tissue samples were discarded due to insufficient tissue quantity for cortisol measurement by UPLC-MS/MS. Therefore, endometrial cortisol concentration information was only available for 78 patients (Fig. 1). Mean cortisol concentration in the endometrial microenvironment was 5.89 ng/g. In terms of endometrial preparation, 71.79% of patients ($n = 56$) underwent an HRT cycle, and 28.21% of patients ($n = 22$) underwent a natural cycle. No significant differences were observed in median cortisol concentration between patients undergoing a natural cycle (5.19 ng/g [1.78–30.90 ng/g]) or HRT cycle (3.72 ng/g [0.80–26.70 ng/g]) ($p = 0.163$). Therefore, all subsequent analyses considered patients undergoing both protocols together.

Figure 1 summarizes the study flow including patients and samples available for each analysis.

Psychological stress is correlated with endometrial cortisol levels

From the 78 patients with cortisol measurements, 57 (73.1%) completed the STAI questionnaire (Fig. 1). Nearly half of the patients (40.4%, $n = 23$) were stressed according to STAI-S, 19.3% ($n = 11$) were stressed according to STAI-T, and 12.3% ($n = 7$) were stressed considering both dimensions. Therefore, 59.7% of patients in our population were stressed in at least one STAI dimension.

Patients who were stressed according to STAI-S had significantly increased endometrial cortisol levels (5.40 ng/g [1.10–26.70 ng/g]) compared to STAI-S non-stressed patients (3.45 ng/g [0.80–30.90 ng/g]) ($p = 0.05$, in the limit of significance). We further parsed the data using different cutoff points based on endometrial cortisol concentration. We found that 80% of patients with endometrial cortisol concentrations ≤ 2.5 ng/g (representing quartile 1; $n = 12$) were not stressed according to STAI-S, while 80% of patients with concentrations > 12.6 ng/g (representing relative maximum; $n = 4$) were stressed according to STAI-S—thus showing a proportional significant difference associating cortisol levels with STAI-S scores ($p = 0.038$) (Fig. 2A, Supplementary Table 2). When analyzing the overall trend in the increase of the proportion of stressed patients in relation to cortisol levels, we observed that as cortisol levels increased, the proportion of patients classified as stressed according to STAI-S increased linearly (cor = 0.977, $p = 0.023$) (Fig. 2B). A similar trend was observed when examining the proportion of stressed patients according to STAI-T as cortisol levels increased (cor = 0.965, $p = 0.035$) (Fig. 2C). This relation between endometrial cortisol and psychological stress punctuations is consistent with that obtained using other analytical approaches in this work (Fig. 2A) and further support the relationship between psychological anxiety and endometrial cortisol levels. However, non-significant results were observed for STAI-T when comparing median cortisol levels (3 ng/g vs. 4.7 ng/g of cortisol, $p = 0.944$) or proportions (all $p > 0.05$, Supplementary Table 2).

To identify potential bias, comparison of demographic and clinical variables between psychological stressed and non-stressed patients demonstrated homogeneity in our population (Supplementary Table 3).

High endometrial cortisol levels potentially impair pregnancy

Following biopsy collection, 69 of 78 patients underwent a single-embryo transfer, resulting in 62.31% ($n = 43$) of patients becoming pregnant and 37.69% ($n = 26$) of patients not becoming pregnant. We observed that 80% of patients with cortisol concentrations ≥ 13.9 ng/g ($n = 4$) did not become pregnant ($p = 0.029$) (Fig. 2D, Supplementary Table 4). This resulted in a 32% relative higher risk of not becoming pregnant when endometrial cortisol levels were ≥ 13.9 ng/g compared to when levels were < 13.9 ng/g ($p = 0.0028$). Yet we did not observe a direct relationship between psychological stress and reproductive outcomes (Supplementary Table 3). Our assessment of bias revealed no significant differences in demographic and clinical variables between pregnant and non-pregnant patients (*all* p-values > 0.05) (Supplementary Table 5).

Increased endometrial cortisol levels and STAI-T scores are associated with gene expression and endometrial function

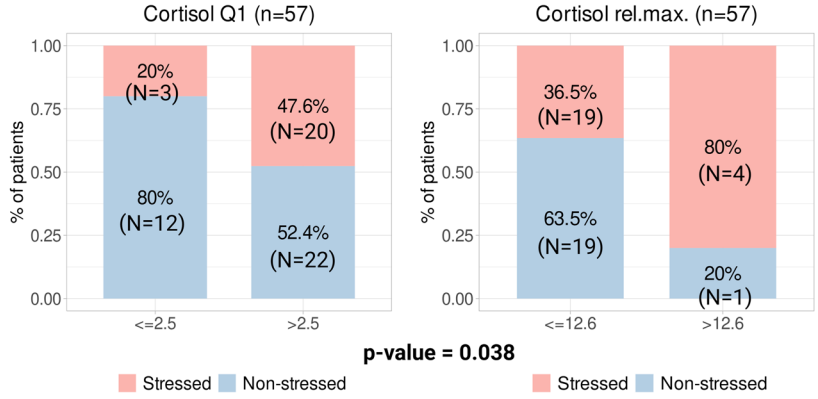
From the initial 78 endometrial samples, only 36 were available for RNA-seq analysis (Fig. 1). We identified 182 genes with changes in expression significantly associated with increased cortisol concentrations ($p < 0.001$) (Supplementary Table 6): 87 genes with increased expression, and 95 genes with decreased expression (Fig. 3A). These genes were mainly involved in 197 functions, with the most represented functions including transcription regulation, signaling, protein processing, and metabolism (Fig. 3B). Other notable functions involved in embryo implantation, although less well-represented, included immune response, apoptosis, and adhesion (Fig. 3B).

While we did not identify altered gene expression with increased STAI-S scores, 12 genes were significantly affected by increased STAI-T scores ($p < 0.001$) (Supplementary Table 7). This included one group of seven genes with decreased expression that mediated functions such as signaling and cell cycle and transcription regulation, and another group of five genes with increased expression that were mainly involved in inflammation, coagulation, immune system, and cell adhesion (Fig. 3C).

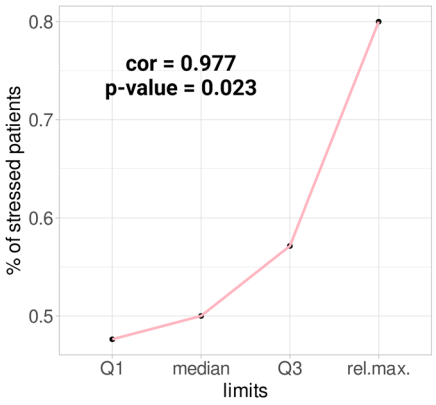
Cortisol disrupts gene expression in cultured HESCs

We selected six genes with high R^2 values, low p-values in dose–response analysis, and high endometrial expression levels to assess their expression in a cell culture model: three genes with decreased expression in response to increased cortisol levels – glycogen synthase kinase 3 alpha (*GSK3A*), regulation of nuclear pre-mRNA domain containing 2 (*RPRD2*), and SET domain containing 2, histone lysine methyl transferase (*SETD2*); and three genes with increased expression in response to increased cortisol levels – hemoglobin subunit beta (*HBB*), nicastrin (*NCSTN*), and small nucleolar RNA, H/ACA box 1 (*SNORA1*). Treating HESCs with 0.5 or 10 µM cortisol significantly decreased expression of *GSK3A* ($p = 7.308e^{-6}$, log2FC = −1.71 (70%) and $p = 5.180e^{-8}$, log2FC = −1.80 (71%)), *RPRD2* ($p = 9.494e^{-9}$, log2FC = −1.85 (72%) and $p = 4.928e^{-9}$, log2FC = −2.01 (75%)), and *SETD2* ($p = 2.710e^{-6}$, log2FC = −1.60 (67%) and $p = 2.423e^{-6}$, log2FC = −1.65 (68%)) in HESCs compared to vehicle control (Fig. 4A). Similarly, both cortisol concentrations significantly decreased expression of *HBB* ($p = 4.471e^{-4}$, log2FC = −4.04 (94%) and $p = 0.002$, log2FC = −2.14 (77%)) and *NCSTN* ($p = 1.489e^{-7}$, log2FC

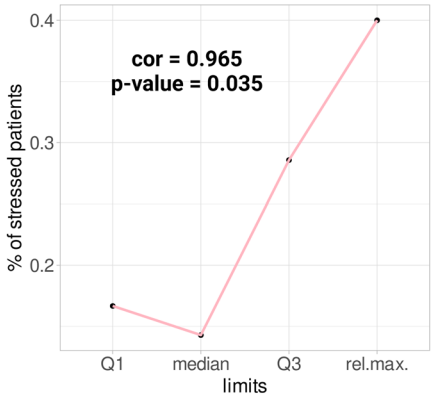
A) Proportions of state-stressed and non-stressed patients according to different cutoff points for endometrial cortisol levels



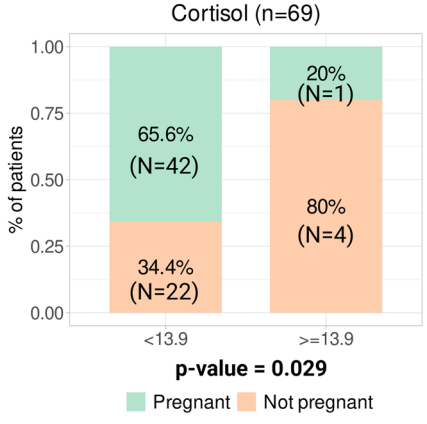
B) Tendency graph and correlation according to STAI-S scores



C) Tendency graph and correlation according to STAI-T scores



D) Proportions of pregnant and non-pregnant patients when endometrial cortisol levels are <13.9 ng/g or ≥13.9 ng/g



= −2.40 (81%) and $p = 3.214e^{-6}$, log2FC = −1.73 (70%)) in HESCs compared to vehicle control (Fig. 4B). Conversely, *SNORA1* expression was significantly increased in cells treated with 0.5 µM cortisol ($p = 0.001$, log2FC = 1.67 (218%)) (Fig. 4B).

Discussion

This study evaluated, for the first time, the relationship among endometrial cortisol levels as a measure of molecular stress, anxiety as a measure of psychological stress, associated molecular tissue changes, and reproductive outcomes, thus integrating multiple perspectives of stress for endometrial factor in infertility. Psychological stress measured by STAI was significantly correlated with endometrial cortisol levels, suggesting an association similar to what others have reported for circulating cortisol levels[21].

◀**Fig. 2**. Endometrial cortisol concentrations are associated with stress and reproductive outcomes in IVF patients. (**A**) Proportions of state-stressed and non-stressed patients according to different cutoff points for endometrial cortisol levels [first quartile (Q1) or relative maximum (rel.max.)]. Bar graph shows significant Barnard's test results comparing endometrial cortisol levels and STAI-S scores (stressed, > 60; non-stressed, ≤ 60). **(B)** Tendency graph and correlation of the proportion of state-stressed patients at each endometrial cortisol cutoff point. Graph shows the proportion of stressed patients according to STAI-S scores for various cortisol cutoff points: Q1 (first quartile), median, Q3 (third quartile), and relative maximum (rel.max.). (**C**) Tendency graph and correlation of the proportion of trait-stressed patients at each endometrial cortisol cutoff point. Graph shows the proportion of stressed patients according to STAI-T scores for various cortisol cutoff points: Q1 (first quartile), median, Q3 (third quartile) and relative maximum (rel.max.). **(D)** Proportions of pregnant and non-pregnant patients when endometrial cortisol concentrations are < 13.9 ng/g or ≥ 13.9 ng/g. Graphic created with Biorender.com.

We previously found that increased endometrial cortisol levels are significantly associated with impaired endometrial progression but found no association with pregnancy[35]. However, the present study shows that endometrial cortisol levels ≥ 13.9 ng/g significantly reduced the probability of pregnancy. Discrepancies in the results between our studies may be due to differences in patient classification in terms of reproductive outcomes—in the current cohort no embryo losses occurred after the first embryo transfer, whereas in the previous study[35] biochemical and clinical miscarriages were included in the non-pregnancy group, making this group more heterogeneous and thus potentially limiting significant findings. Cortisol has been extensively evaluated in reproduction due to its direct association with stress. While some studies report a relationship between serum/hair cortisol levels and reduced pregnancy rates[54,55], others do not[56,57]. Given the previously described lack of correlation between serum and endometrial steroids levels[31–33], measuring cortisol levels directly in the endometrium might better reflect how this could affect implantation and early pregnancy. Yet studies of cortisol in the endometrial microenvironment are scarce[58,59], with only one study evaluating reproductive outcomes and reporting no association between endometrial cortisol levels and pregnancy in natural cycles[59]. This difference could be explained by varied population characteristics such as age and sample size as well as controlling embryo factors and cycle type for endometrial preparation. Therefore, ours is the first study to suggest an association between endometrial cortisol levels and pregnancy outcomes.

Numerous studies report that patients consider infertility and its associated treatments as one of the most stressful experiences of their lives[60], and that stress levels tend to increase after initiating treatment[4,61]. The reported prevalence of stress among IVF patients is 25%–65%, in contrast to the 4%–12% global incidence in the general population, highlighting the psychological burden associated with assisted reproductive treatments[62,63]. In our study, 58.7% of patients were classified as stressed according to at least one STAI dimension. Thus, a high percentage of IVF patients could benefit from personalized psychological support to reduce their stress levels while undergoing this highly demanding psychological process.

One of the main reasons for IVF treatment discontinuation and drop-out is psychological[64]. Early identification of patients at-risk of treatment discontinuation due to psychological factors would enable preventive measures to reduce discontinuation rates and increase success rates in fertility treatments. Patients desire better psychosocial care in assisted reproduction clinics, particularly for those experiencing stress or those with previous failed treatments[65,66], highlighting the importance of more comprehensive mental health-centered approaches in fertility.

We found an association between endometrial cortisol levels and reproductive outcomes. However, psychological stress was not directly associated with IVF pregnancy outcomes in our cohort. We found that increased endometrial cortisol levels were significantly associated with expression of 182 genes with key functions in embryo implantation and early pregnancy. Therefore, our findings suggest that the stress effects may be indirectly mediated through local endometrial cortisol regulation, although the exact mechanism remains to be elucidated. Among the main functions in which these 182 genes were involved we found transcription regulation, which is crucial for endometrial receptivity, embryo implantation and embryo-maternal crosstalk[67–69]. These genes were also involved in other important functions for implantation and early embryo development, such as apoptosis[70] or immune response[71], among others. In fact, in accordance with our results, some researchers have reported that cortisol treatment affects the ability of endometrial cells to migrate, differentiate[72] and proliferate[73,74]. However, other functions proposed here have not yet been directly validated. Therefore, our study lays the groundwork for understanding the relationship between stress, cortisol, and endometrial function, and can serve as a starting point for future research to provide further evidence for this relationship and elucidating the underlying mechanisms.

We found that stress as assessed by STAI-T was associated with altered endometrial expression of 12 genes, yet we found no association with STAI-S. Although both STAI-S and STAI-T scores correlated with increased endometrial cortisol levels, with a stronger association for STAI-S, gene expression changes were only observed in relation to STAI-T. This could be because the long-term effect of experiencing anxiety as a stable personality trait persists over time and thus may exert a lasting impact on endometrial transcriptional profiles that is easier to detect.

More than half of downregulated genes were pseudogenes or regulatory RNA-related genes, such as long non-coding RNAs (lncRNAs). Various studies have highlighted the relevance of lncRNAs in implantation and early pregnancy[75–79], with alterations in recurrent implantation failure[80], recurrent pregnancy loss[81], and endometriosis. Other downregulated genes—such as *INCA1*, which mediates maternal–fetal communication[82], and *TGIF1*, which mediates endometrial receptivity acquisition[83]—are reported as associated with stress for

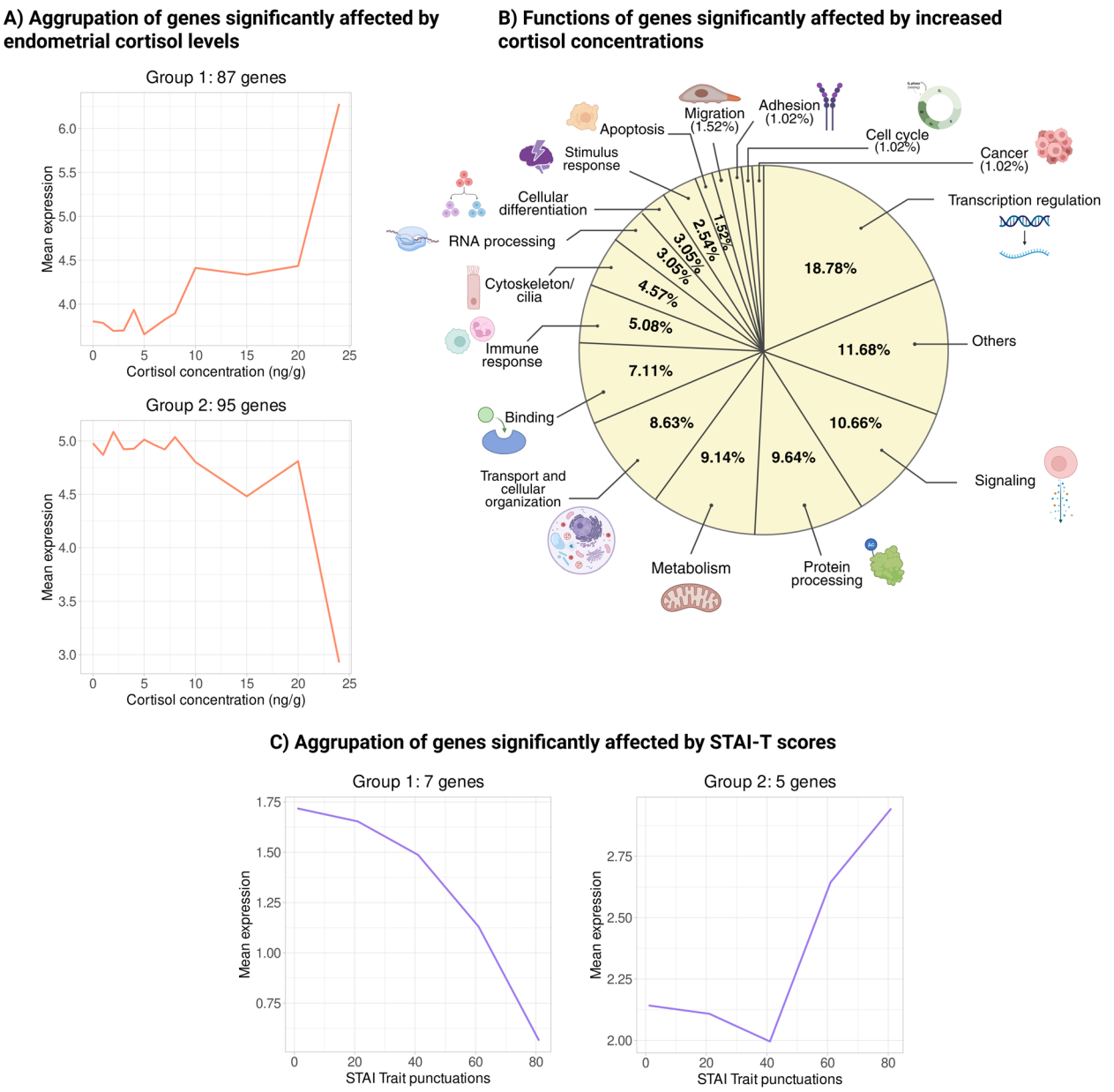


Fig. 3. Increased endometrial cortisol levels and stress affect gene expression and key functions for embryo implantation. (**A**) Aggrupation of the 182 genes with significant changes in expression in response to increased endometrial cortisol concentrations. Genes are grouped by direction of expression change: 87 genes with increased expression (top), and 95 genes with decreased expression (bottom). **(B)** Functions of genes significantly affected by increased cortisol concentrations. Pie chart represents grouped functions involving significant genes and their proportion within the total 197 functions. **(C)** Aggrupation of 12 genes with significant changes in expression in response to increased STAI-T scores. Genes are grouped by direction of expression change: 7 genes with decreased expression (left), and 5 genes with increased expression (right). Graphic created with Biorender.com.

the first time in our study. Conversely, the five upregulated genes we identified with increased stress levels were associated with inflammation (*SAAL1*), immune system (*CORO1A*, *WAS*), coagulation (*SERPINE1*), and adhesion (*RPSA*), critical functions for embryo implantation and early pregnancy maintenance[71,84–86]. Altogether, these dysregulated functions may contribute to reduced pregnancy rates in patients with increased stress and endometrial cortisol levels.

In vitro analysis showed that genes whose expression decreased with increasing endometrial cortisol in the RNA-seq analysis (*GSK3A*, *RPRD2*, and *SETD2*) exhibited the same trend in cultured stromal cells, with both cortisol concentrations inducing this effect. For genes showing the opposite trend in RNA-seq (*HBB*,

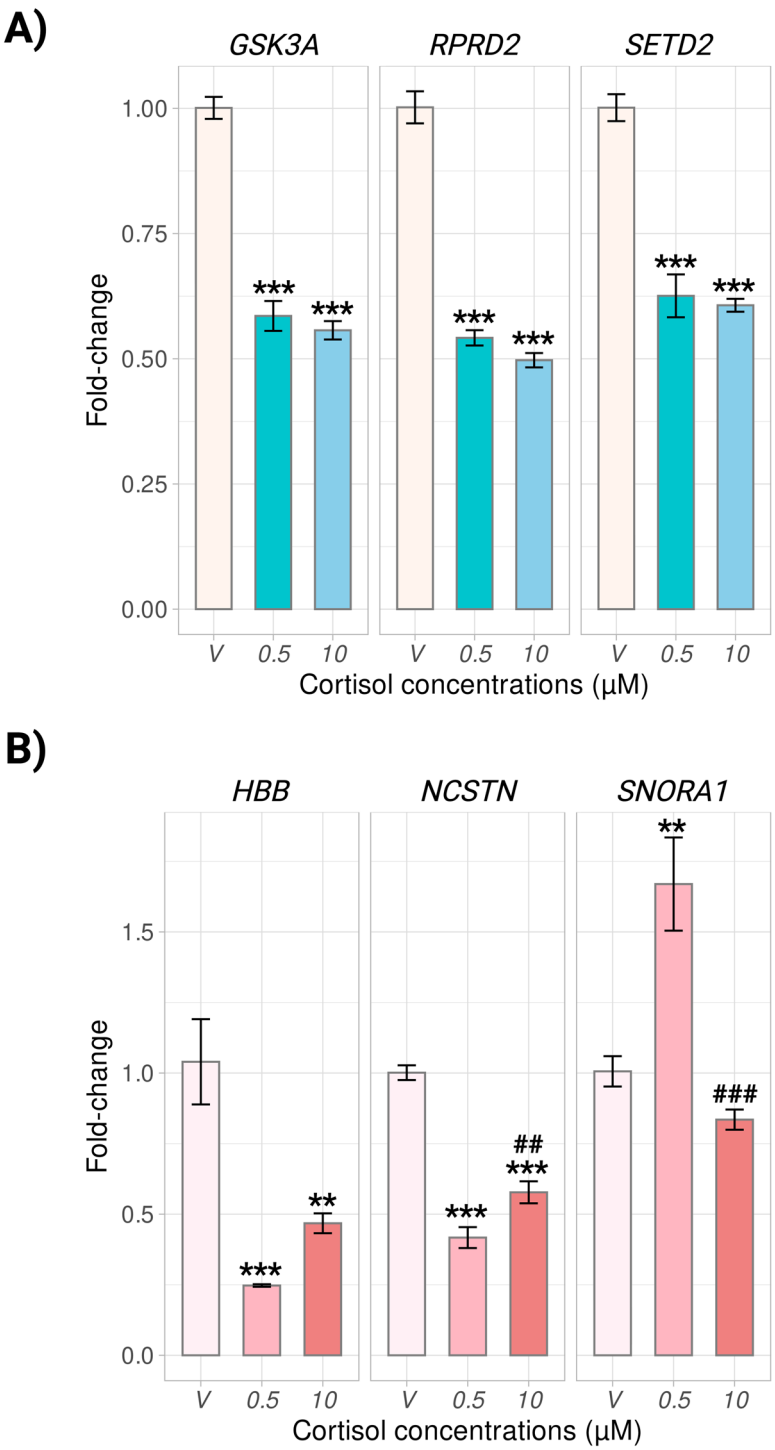


Fig. 4. Cortisol significantly affects expression of (A) ***GSK3A***, ***RPRD2***, and ***SETD2*** and (B) ***HBB***, ***NCSTN***, and ***SNORA1*** in cultured endometrial stromal cells. Relative expression (fold-change) of each gene in response to treatment with vehicle control (V) or cortisol (0.5 μM or 10 μM). $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, comparison to vehicle. $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$, comparison between cortisol concentrations.

NCSTN, and *SNORA1*), only SNORA1 maintained increased expression in vitro. Despite these models being fundamentally different—RNA-seq of endometrial biopsies versus cortisol-treated stromal cells—, expecting some variation in results, our study demonstrate that cortisol affects the expression of key endometrial genes in both systems. Specifically, the in vitro model showed a 94% reduction in *HBB* expression, a gene involved in

preimplantation embryonic development[87], and a 218% increase in *SNORA1* expression, a potential marker of endometrial receptivity and reproductive success[88], highlighting the relevance of the findings.

Despite these promising results, this study is limited by its cohort size due to the difficulty in obtaining endometrial biopsies and the multiple variables included in this large data analysis. Identified non-significant relationships and p-values in the limit of significance could be due to low statistical power. In this same line, the reported correlations, although strong, are based on few data points and may not be generalizable, but they support other findings of this study. Future studies with larger populations are recommended to reach stronger conclusions. Furthermore, while the functional genomics analysis points to certain biological functions, specific experimental validations would be required to further investigate the underlying molecular mechanisms. Finally, although the study population was homogeneous and all clinical variables were carefully controlled, other factors such as lifestyle and coping strategies still may have influenced endometrial cortisol concentrations and the likelihood of pregnancy. Nonetheless, due to lack of differences in HRT versus natural cycles in endometrial preparation, results of our study can be extrapolated to all IVF populations regardless of cycle type.

In conclusion, high endometrial cortisol levels were associated with molecular alterations affecting embryo implantation and development, resulting in 32% higher relative risk of not becoming pregnant. Therefore, despite its invasive nature, direct measurement of endometrial cortisol levels remains a more accurate marker of reproductive outcomes. However, STAI could serve as a less-invasive screening tool to identify patients with higher risk of endometrial failure. These findings underscore the need for a combination of more objective approaches such as molecular measurement of stress with standardized psychological evaluation to identify patients who could benefit from personalized psychological interventions, specifically those with altered endometrial cortisol levels. Future prospective studies are needed to evaluate the effectiveness of personalized therapies to reduce psychological stress and endometrial cortisol levels, potentially improving reproductive outcomes for IVF patients.

Data availability

Some of the findings reported in this scientific article were presented at the 39th Annual Meeting of European Society of Human Reproduction and Embryology held in Copenhagen, Denmark, June 25–28, 2023; and at the 40th Annual Meeting of European Society of Human Reproduction and Embryology held in Amsterdam, Netherlands, July 8–10, 2024. Data including STAI results and metabolic concentrations will be made available to the editors of the journal for review, upon request, should there be any queries. The RNA-seq dataset generated and analysed during the current study are available in the Gene expression omnibus (GEO) repository, [GSE310309].

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Author contributions

D.M.-G.: Investigation (participant recruitment, clinical follow-up, sample pre-processing, RNA extraction, sequencing), formal analysis, validation (cell culture and RT-qPCR), visualization, writing – original draft. P.S.-L.: Conceptualization, formal analysis, writing – review & editing. P.D.: Conceptualization, investigation (psychological evaluation, participant recruitment). A.D.-P.: Conceptualization, investigation (participant recruitment). M.C.V.: Conceptualization, investigation (participant recruitment, biopsy collection) and funding acquisition. L.C.: Investigation (participant recruitment). F.J.S.: Validation (cell culture). R.E.-M.: Validation (cell culture). E.D.-F.: Conceptualization, methodology, supervision. P.D.-G.: Conceptualization, methodology, formal analysis, writing – original draft, writing – review & editing, supervision, project administration, funding acquisition. All authors read and approved the final version of the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to P.D.-G.

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