New insights into stress metabolomics. Looking for new diagnostic biomarkers.

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ABSTRACT

Background: Stress is associated with the onset of several neurological disorders such as depression, post-traumatic stress disorder (PTSD), and anxiety. Even though extensive research on stress has been performed, the specific metabolic changes that occur in response to acute psychological stress remain, to date, unclear.

Aims: The goal of the present study was to evaluate currently proposed biomarkers of stress and investigate its adverse effects of acute psychological stress on the human body.

Methods: The study involved relaxation and stress state induction in 40 participants using autogenic training and a modified Trier Social Stress Test (TSST-M), respectively. To confirm the positive achievement of these states, psychometric questionnaires were administered after each session. Saliva and blood were equally sampled for biochemical and untargeted metabolomics analyses.

Results: Our findings revealed that although most biomarkers assessed suffered changes under induced acute mental stress state, the predictive model that we obtained from machine learning clearly identified salivary α-amylase and State-Trait Anxiety Inventory-state (STAI-s) as prominent markers in defining stress state in an individual. Relatedly, we found that several metabolites involved in the biosynthesis pathway of steroid hormone, the glycerophospholipid metabolism, the linoleic acid metabolism, the tyrosine metabolism, and the aminoacyl-tRNA biosynthesis were also affected by acute psychological stress. Such alterations allow us to understand several adverse effects typically noted in stress states.

Conclusion: Taken together, our results demonstrate that psychological stress has a considerable influence on multiple metabolic pathways directly implicated in stress-related disorders.

Keywords: “Stress, Psychological”; “Biomarkers”; “Metabolomics”; “Mass Spectrometry”; “Metabolic Pathway”; “Machine Learning”.

ABBREVIATIONS:

ΔAA_{sl} (difference in α-amylase concentrations between the second and first samples), AA_{sl} (Salivary α-amylase), ACTH (Adrenocorticotropic hormone), B_{RS} (Baseline relaxation session), B_{SS} (Baseline stress session), CNS (Central Nervous System), Cp_{pl} (Plasma copeptin), ΔCr_{sl} (difference in salivary cortisol between the second and first samples),Cr_{sl} (Salivary cortisol), DHA (docosahexaenoic acid), DIMS (Direct Infusion Mass Spectrometry), DOC (11-deoxycorticosterone), Epi (epinephrine), ΔFR_{sl} (difference in salivary flow rate between the second and first samples),FR_{sl} (Salivary flow rate), ESI (Electrospray Ionisation), Glu_{sr} (Serum glucose), HPA (Hypothalamic-Pituitary-Adrenal), KEGG (Kyoto Encyclopaedia of Genes and Genomes), LA (Linoleic acid), LC-MS (Liquid Chromatography - Mass Spectrometry), LPC (Lyso-phosphatidylcholine), MAPK (mitogen-activated protein kinase), NAG (N-acetyl glutamine), NE (norepinephrine), NF-κB (nuclear factor kappa B), Osm_{pl} (Osmolarity from plasma samples), PC (Phosphocholines), PSNS (Parasympathetic Nervous System), PPC (Choline-plasmalogens), PPE (Ethanolamine-plasmalogens), Pr_{pl} (Plasma prolactin), PSS (Perceived Stress Scale), SNS (Sympathetic Nervous System), SS (Stress session), SSC (Symptomatic stress scale), STAI-s/t (State-Trait Anxiety Inventory state and trait tests, respectively), RS (Relaxation session), TSST-M (Modified form of the Trier Social Stress Test), VAS (Visual Analogue Scale)

INTRODUCTION

Stress

Physiological systems in the body are inherently programmed following rigorous fine-tuning of regulated variables. These variables must be kept within an acceptable dynamic range, that is, the homeostatic state, which is essential for life and well-being (1–3).

However, this optimal balance is constantly challenged by intrinsic and extrinsic adverse forces or *stressors*. Stressors can be psychological, such as unexpected events, urgent tasks, traumatic events, and adverse social, economic, and environmental circumstances (4,5). Otherwise, they can be physical, such as injuries, noise, or exposure to extreme temperatures (1–3,6,7).

Stressors lead to a state of disharmony called *stress*, triggering a complex repertoire of physiological and behavioural responses to re-establish threatened homeostasis to improve chances of survival (6). This adaptive response implicates an intricate network involving stress systems in the Central Nervous System (CNS) and peripheral organs. This leads to activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis and Sympathetic Nervous System (SNS), as well as inhibition of the Parasympathetic Nervous System (PSNS) (1). If the stress response does not suffice to preserve homeostasis, an inflammatory response is induced in an attempt to restore the system to its homeostatic state (8). Crucially, such specific biochemical and physiological changes can be used to determine and monitor stress. However, the response varies for each individual according to personality traits and a myriad of genetic, environmental, and developmental parameters, making stress diagnosis or monitoring challenging (9–11).

Stress can be acute, chronic or negative. Acute stress presents a set of time-limited cognitive-behavioural and physiological changes as an immediate response to a stressor (1–3,12).

Physiological adaptation in this case involves the redirection of nutrients to organs crucial to stress response, such as the brain, heart, and skeletal muscles (1). Cardiovascular tone, heart and respiratory rates, and intermediate metabolism (gluconeogenesis and lipolysis) are increased, while energy-consuming functions, such as digestion, renal and intestinal excretion, reproduction, growth, and immunity, are critically but temporarily reduced. The neuro-psychological adaptation includes heightened alertness and vigilance. Nonetheless, the type of response is dependent on (i) the type of stressor, such that different stressors activate different pathways; (ii) the intensity of the stressor, such that the higher the degree of stress, the lower specificity of the adaptive response (iii) the intra-subject sensibility to stressors (10).

Negative stress (distress) (13) occurs when the response to stress is inadequate. These changes yield detrimental effects on several psychological and physiological functions, such as altered cognitive and affective capacities, mental processing, and sleep-arousal cycle disorders along with simultaneous inhibition of vegetative functions, such as feeding and reproduction. It can also affect gastrointestinal and cardiovascular functions, growth, metabolism, reproduction, and immune competence. Individual performance, behaviour, and personality development can be equally affected (10,14,15).

Chronic stress involves, by contrast, a constant stress stimulus. This can consequently lead to a stage where the body can no longer achieve homeostatic balance, and the individual can no longer deal with the stressors (14,16).

Psychological Stress and Distress

Given its influence on human decision-making, psychological stress (negative stress) represents a major public health concern (7,17–20). According to the World Health Organization (WHO) (4), the prevalence of social and medical problems associated with

mental stress is globally increasing also in children, seriously affecting their mental health and well-being. There are many factors contributing to global stress increase. The COVID-19 pandemic, for instance, became a universal stressor centrally involved in a global mental health crisis, since it implied enduring unprecedented short and long-term stressful situations that undermined the mental health of millions (18–20). In any event, and especially when chronic, mental stress exacerbates our susceptibility to several diseases eventually becoming common causes of morbidity and mortality (17). Consequently, mental stress has a visible impact on the Health System, resulting in elevated costs in means of healthcare, invalidity or productivity loss. In view of this, finding objective and precise diagnosis methods is nowadays a pressing question to be resolved (21,22).

Stress Diagnosis

To date, stress diagnosis and estimation remain complex and clouded, carrying considerable chances of uncertainty. Current standard diagnostic methods build on validated psychometric questionnaires tracking stress-induced changes in cognitive and behavioral abilities (23). Although they are considered highly reliable methods, the interpretation of the questions by the patients and/or the results by the specialist is still highly subjective, thus leading to various biases that can compromise the diagnosis itself (7,24,25). In this sense, and despite many efforts, an objective and reliable method for stress diagnosis has not yet been developed. While different biomarkers have been proposed for acute psychological stress determination in the literature, important disparities in the results (26) still exist.

Since the distinctive feature of stress response is the activation of SNS and, most importantly, the HPA axis, (27,28), the most promising biomarkers point to metabolites released as a result.

Given the multidimensional nature of stress, we submit that determining one or only a few reliable biomarkers for diagnosis is unlikely to be a feasible goal. Reported inconsistencies in the literature may probably be the result of oversimplifying the overall process (29).

To solve this, we propose an omic analysis aiming to identify a significant set of empirically relevant biomarkers, which would result in a more effective approach. In this proposal, metabolomics is presented as the most appropriate strategy (30,31). It involves the systematic identification and quantification of metabolites profile that characterize the phenotype of an organism in a specific situation. Moreover, metabolomics allows the simultaneous determination of the altered set of metabolites in response to stress process, providing a global vision of the metabolic changes arising as a result. Metabolites are the intermediate or the end-products of cellular regulatory pathways, and their levels can be regarded as the ultimate response of biological systems to genetic and environmental changes (32).

Integrated into a multidisciplinary project aimed at assessing acute psychological stress, we develop a proposal where a main goal is to determine the metabolomic fingerprint of acute psychological stress. This would directly contribute to the discovery of new stress biomarkers and help to unveil the molecular basis of its adverse outcomes. As a secondary goal, we will analyse the potential utility of diverse biomarkers proposed in the literature and determine how gender differences operate in stress response.

MATERIALS AND METHOD.

Study design.

A quasi-experimental pre-post study without a control group was employed to ascertain the effects of acute psychological stress on biochemical, psychological, and metabolomic variables in a group of healthy volunteers. The study was designed and performed under the framework of the "ES3 project" (26,30,33–35). It included two sessions, a 35-minute

Relaxation (RS) (control condition) and another 35-minute Stress induction (SS), on the same participants. Acute psychological stress state was induced using a modified form of the Trier Social Stress Test (TSST-M), previously described by Arza *et al*. (26).

Participants.

Young and healthy volunteer students between the ages of 20 and 30 years (both sexes) from the University of Zaragoza were recruited for this study. The exclusion criteria were as follows: (1) signs of depression and pre-existing history of other mental disorders; (2) regular use of psychotropic substance(s); and (3) pregnancy or breastfeeding at the time of the study. The demographic data of the participants are presented in Supplementary Table 1. Participants were duly informed about the details of the study, and they gave informed consent. Participants were instructed to wake up at least 2 hours before the sessions, have a light breakfast without caffeine or tea, and refrain from exercising or consuming any psychotropic substance, drinking alcohol, or smoking 24 hours before the session day. This study was conducted in accordance with the guidelines established in the Declaration of Helsinki of 2013 by the World Medical Association (WMA) (36) and approved by the Clinical Research Ethics Committee of Aragon (CEICA; protocol number PI14/0044).

Stress Induction Protocol /The Relaxation and Stress Sessions.

The sessions were carried out on different days, but at the same hour, around 10:00 AM, to avoid variations in the circadian rhythm (37). The relaxation session (RS) comprised a baseline (B_{RS}) and relax stage (R_{RS}), whereas the stress session (SS) comprised a baseline stage (B_{SS}) and five distinct stages to induce acute psychological stress (26).

For the relaxation session, the subjects were seated in a comfortable position in a dimly lit room and were exposed to audio recording and guided relaxation to induce autogenic relaxation in accordance with Schultz's method (38). The stress sessions followed a TSST-M,

which is a robust, reliable, and well-documented protocol widely used in stress research (39–44), with slight modifications, as described in (26). The stress session consisted of storytelling (STS), memory test (MTS), stress anticipation (SAS), video display (VDS), and arithmetic task (ATS) (26) (Figure 1).

The participants were required to complete psychometric questionnaires at the end of each session, RS and SS. Saliva samples were collected at the end of the baseline stages (B_{RS} and B_{SS}) and again after RS and SS, whereas blood and plasma samples were only collected after RS and SS.

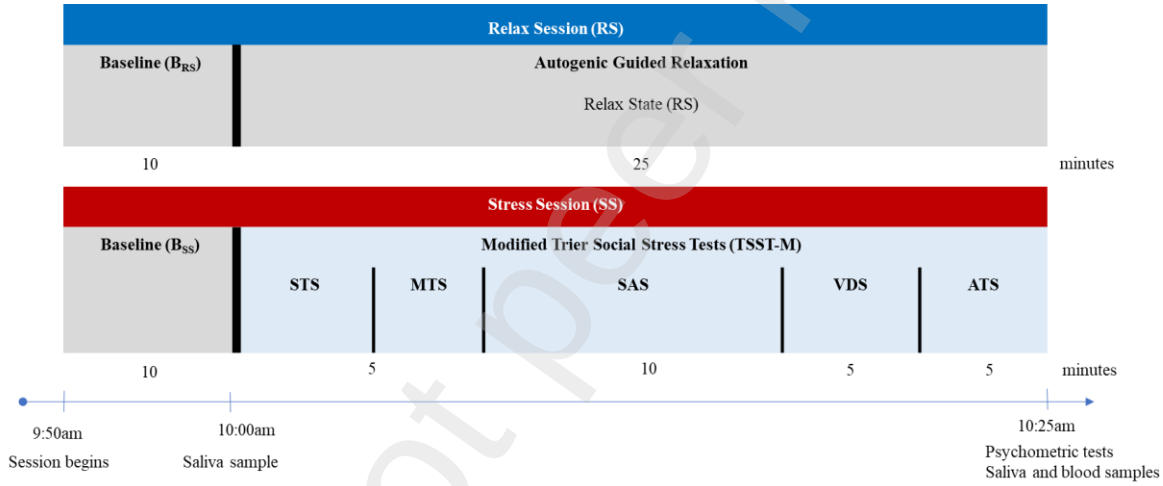

Figure 1. Schematic representation of the research approach. Details of stress induction/relaxation protocol showing the time of sample collection. Therefore, four saliva samples were collected: two after each baseline stage (B_{RS}, B_{SS}) and the other two at the end of each session (RS, SS). Blood samples were drawn, and psychometric tests were administered at the end of each session. The TSST-M involved a series of stressful tasks including story telling stage (STS), memory test stage (MTS), stress anticipation stage (SAS), video display stage (VDS), and arithmetic task stage (ATS).

Stress Evaluation and Measurement: Psychometric Evaluation.

Before administering psychometric questionnaires, participants were asked to indicate their perception of their stress levels (Perceived Stress) on a scale of 0 – 100 arbitrary units.

The professionals of the ZARADEMP group from the Psychiatry Service (HCU-LB) and Department of Medicine and Psychiatry (University of Zaragoza) selected the tests, verified the corresponding Spanish versions, administered the tests to the subjects, and subsequently interpreted the results. This team also applied a test designed by themselves on behalf of the ES3 Project (35), 'the Symptomatic Stress Scale" (SSC). The SSC scale is a Likert-type scale that consists of 20 questions that evaluate the subjective effect of the stressor on the subject from somatic and psycho-cognitive points of view. This scale was validated by Garzón-Rey (45) and applied in a recent study by Garcia Pages *et al*. (46)

The validated psychometric tests used were the Perceived Stress Scale (PSS), Visual Analogue Scale (VAS), and State-Trait Anxiety Inventory tests (STAI). The PSS, originally developed by Cohen *et al*. (47), is widely used to assess stress levels in young people and adults. It evaluates the degree to which an individual perceives life as unpredictable, uncontrollable, or overloading. The Spanish version of this scale, developed by Remor (48) has demonstrated adequate reliability, validity, and sensitivity. The VAS is a valid and reliable technique for measuring subjective stress on a numeric scale ranging from 0 to 100 (49). This test highlights the differences in stress levels between groups and determines the connection between the VAS stress assessment and the evaluation of various related concepts (50,51). Finally, two STAI questionnaires were used: one to measure the trait or general tendency to increase anxiety in stressful situations (STAI-t), and another to evaluate the state of the subject in a specific situation (STAI-s) (52). The Spanish adaptation of this scale frequently used in clinical practice was developed by Guillén and Buela (53).

Measurement of Biochemical Variables.

Biological samples for analysis were collected by professionals from HCU-LB and stored in sterile, airtight compartments at adequate temperature until analysis. Biochemical markers determined were glucose (Glu_{sr}) from serum samples; prolactin (Pr_{pl}), copeptin (Cp_{pl}), and

osmolality (Osm_{pl}) from plasma samples; and salivary cortisol (Cr_{sl}), salivary flow rate (FR_{sl}), and α-amylase (AA_{sl}) in saliva samples. All samples were processed using the same tests to avoid inter-test variability, thereby achieving intra-test variation coefficients < 5% in all cases. Salivette tubes were used to collect saliva, following the manufacturer's recommendations (Sarstedt AG & Co., Nümbrecht, Germany). Subsequently, samples were immediately preserved on ice and later kept frozen at −20°C until processing, according to the protocol previously described by Garcia Pages et al. (46). Concentrations of Cr_{sl} and AA_{sl} were measured in the endocrinology and radioimmune analysis service of Neurosciences Institute at the Universitat Autònoma de Barcelona (UAB) using fully validated immunoassay and kinetics enzyme assay kits from Salimetrics/USA respectively (46). The changes in salivary cortisol (ΔCr_{sl}), α-amylase (ΔAA_{sl}), and flow rate (ΔFR_{sl}) in response to the applied stress stimulus were calculated.

The extracted blood was partitioned into two tubes: one with EDTA anticoagulant and the other with a clot accelerator and gel serum separator. Both samples were preserved on ice and later centrifuged at 3000 rpm for 10 min. Plasma and serum were kept frozen at −20°C until processing at the Biomedical Diagnostics Centre at the Hospital Clinic of Barcelona. Quantification of Glu_{sr}, Pr_{pl}, $Cp_{pl,}$ and Osm_{pl} was performed using molecular absorption and immunoassay spectrometry techniques.

Stress Reference Scale

The stress reference scale *(SRS)* was proposed by Garzon-Rey *et al*. (33) as a reference standard for measuring acute emotional stress. Significant biochemical and psychometric parameters were used to compute the scale using a multivariate approach as described previously. To assign weights to the different variables, their mean scores were first normalized by rescaling to a 0-100 range of arbitrary units using the following equation:

$$y = \frac{100 * (x - Min + \sigma * 0.5)}{(Max - Min + \sigma)}$$

where the variable (*x*) with a standard deviation (*σ)*, minimum (*Min*), and maximum *(Max)* values are transformed into a variable (*y)* ranging from 0 to100. Afterwards, the principal components analysis (PCA) was performed to assign the corresponding weights to each variable. Only features with eigenvalues greater than 0.8, which explained 84% of the total variance, were selected to build the scale.

Statistical Analyses.

Statistical analyses were performed using IBM® SPSS® Statistics 25.0 and RStudio (54) for Microsoft Windows, along with its corresponding packages available on CRAN or Bioconductor repositories.

The states of the volunteers at the end of each session, RS and SS, were considered to be the lower and higher ranges of the stress state. The variations in psychometric, biochemical, and SRS variables between RS and SS were analysed using the Wilcoxon signed-rank test, a non-parametric statistical test, because the data were not normally distributed after testing for normality using the Lilliefors test. Correlations were computed using Spearman's rank correlation for non-parametric distributions. For all analyses, the significance level was set at α=5%.

Variables were passed on to create predicting models. Categorical variables were encoded as factors. The grouping RS or SS was considered as the response variable for the models, and the other variables as predictors of the state of the group. The study employed the Recursive PARTitioning (*rpart)* algorithm based on *CART* (classification and regression tree) to build decision tree models (https://cran.r-project.org/web/packages/rpart/rpart.pdf). The *adabag* package (55) was used to build a bagging predicting model and the *Random-Forest* algorithm software package (https://cran.r-project.org/web/packages/randomForest/index.html) to obtain

the variable relative importance rankings of variables. We used 70% of the original data as a training set and the remaining as a testing set to assess the model afterwards. The Gini Index was used to split nodes and pruning was performed to avoid overfitting the model. A multivariate logistic regression model was constructed and compared with the decision tree, bagging, and random forest models.

Metabolomic Sample Processing and Data Analysis.

A semi-quantitative direct-infusion mass spectrometry (DIMS) untargeted metabolomic study was conducted to characterize biochemical responses to acute psychological stress and as a biomarker development tool. Direct injection into the ionization source of the mass spectrometer without prior chromatographic separation is an innovative technique used with electrospray ionization (ESI) source that presents many advantages and has proven to be robust (30,56–58).

Blood samples were collected by pricking participants' fingers. Approximately 0.5ml of total blood was collected into an empty and sterilized Eppendorf^{TM} tube. No anticoagulants were used. Samples were immediately protected from light and stored at -80°C until analysis. Sample preparation was carried out as previously described (30).

For positive mode MS detection, immediately before analysis, each sample was diluted 1:1000 with a protonating agent solution of LC-MS grade methanol with 0.1% formic acid (Fluka) at 99% purity. For negative mode detection, dilution 1:1000 of the sample was made with MS grade dichloromethane (Fluka): methanol (ratio 1:1). Samples to be analysed were pumped directly into the mass spectrometer.

Measurements were taken in both positive and negative modes using a hybrid triple quadrupole/linear ion trap mass spectrometer 4000 QTRAP LC/MS/MS System (AB Sciex) with electrospray ionization (ESI) source interface for high-sensitivity, full-scan MS, MS/MS, and MS^3 spectra with high selectivity from true triple quadrupole precursor ion (PI) and

neutral loss (NL) scans. Data acquisition and pre-processing were carried out using Analyst® software version 1.5.2 (Build 5704) (Sciex) as previously described (30). A scan range of 50 - 1,200 m/z was used. The mass accuracy and resolution were 5 ppm and 20,000 ppm, respectively. The instrument settings were as follows: ion spray voltage, 5,000 V; curtain gas, 20 AU; GS1 and GS2, 50 and 30 psi, respectively; probe temperature, 550°C and run time 10.0 min. For MS/MS analysis, collision-induced dissociation (CID) mode was used and was set to 30% to 50% normalized collision energy (CE) for selected mass-to-charge ratio (m/z) peaks.

Data normalization, statistical and functional analyses, and compound identification were performed following the protocol previously described by Lorenzo *et al*. (30).

Enrichment and pathway topology analyses were performed using the corresponding modules of MetaboAnalyst 5.0 (59) and categorized with the Kegg pathway *Homo sapiens* database (60). Pathway enrichment analysis allowed for the identification of those pathways significantly affected by the stressor, and thus, to better understand the impact of acute psychological stress on an individual's metabolism.

RESULTS

Participant Characteristics.

Forty-one healthy young participants were enrolled in this study. However, one participant opted out, resulting in a final sample size of 40. The group constituted a socio-demographic homogeneous data sample (Supplementary Table 1), including both young males and females in similar proportions (mean age of 22 ± 3.4 years), and a normal Body Mass Index (BMI of 22.4 ± 2.7 kg/m^2) according to guidelines established by the WHO (61).

The perceived stress levels measured prior to administering psychometric tests (Supplementary Table 1A) showed an average of 49.4 units on a scale from 0 to 100, indicating no to mild stress.

Based on habits (Supplementary Table 1B), majority of the subjects were non-smokers (85%), occasional consumers of alcoholic beverages (82.5%), and engaged in extracurricular activities (62.5%), mainly practised sports regularly, learned foreign languages or engaged in other types of artistic activities. Approximately half of the participants (45%) reported regular coffee consumption. In terms of their social background, most participants lived in urban areas (77.5%), were single (72.5%), and lived with their families (72.5%). With regard to health status, the vast majority of participants did not suffer from chronic diseases (95%) or take medications (75%). However, a small percentage (5%) had chronic diseases such as allergies, migraines, or intestinal reflux, and only 25% were on prescribed medications (mainly contraceptives, antihistamines, and antiasthmatics), which did not hinder the measurement sessions.

Stress Evaluation and Measurement.

Psychometric tests

Scores for STAI-s, VAS, and SSC showed statistically significant increases between RS and SS (Table 1), thus confirming that the participants had become stressed after applying the TSST-M test. The PSS and STAI-t tests did not show significant variation between the states. This reflects coherence in the evaluation since these questionnaires indicate one's predisposition (trait) to respond to stressful situations, but do not evaluate the subject's current state.

Biochemical variables

Statistically significant increases in the biochemical stress markers ΔAA_{sl}, ΔFR_{sl}, Cp_{pl}, and Pr_{pl} were observed between sessions. In contrast, the levels of ΔCr_{sl} and Glu_{sr} did not change significantly after the stressor was applied (Table 1).

Sex-based disparities were observed in Cp_{pl} and Glu_{sr}, with comparatively lower levels in females (Table 1). It is worth mentioning that all variables were within the clinically accepted normal range.

Table 1. Inter-subject median and median absolute deviation (MAD) of stress markers.

	All		Female		Male	
Stress markers	Relax session	Stress session	Relax session	Stress session	Relax session	Stress session
Psychometric variables						
PSS (0-40)	21.0 ± 2.2	20.0 ± 3.0	21.67 ± 1.5	21.5 ± 3.7	21.5 ± 3.7	19.5 ± 3.7
STAI-s (0-80)	15.5 ± 6.7	23.0 ± 8.9**	16.0 ± 8.9	24.0 ± 8.2	14.0 ± 4.5	20.0 ± 8.2
STAI-t (0-60)	20.5 ± 9.6	19.5 ± 8.9	24.0 ± 12.6	21.5 ± 12.6	18.5 ± 8.2	18.5 ± 3.7
SSC (0-80)	17.5 ±10.4	27.5 ± 18.5**	19.0 ± 12.6	32.5 ± 15.6	17.0 ± 9.64	23.0 ± 18.5
VAS (0-100)	30.0 ± 18.5	50.0 ± 29.7**	35.0 ± 22.2	50.0 ± 29.7	30.0 ± 25.9	50.0 ± 29.7
Biochemical Parameters						
Cp_{pl} (pmol/L)[a]	5.9 ± 2.6	6.2 ± 2.9*	3.7 ± 1.6	3.6 ± 1.8	7.0 ± 3.6	8.5 ± 4.2
Osm_{pl} (mOsm/L)	303.0 ± 3.0	304.0 ± 4.0	303.0 ± 5.9	299.0 ± 2.9	304.0 ± 2.9	306.0 ± 5.2
Pr_{pl} (ng/ml)	7.7 ± 1.7	8.3 ± 2.1*	7.9 ± 2.5	8.9 ± 2.7	7.1 ± 2.1	7.6 ± 2.8
ΔCr_{sl} (ng/ml)	-0.06 ±0.03	-0.04 ±0.03	-0.03 ± 0.04	-0.03 ± 0.04	-0.06 ± 0.03	-0.06 ± 0.04
ΔAA_{sl} (U/ml)	2.2 ± 18.2	45.3 ± 28.2**	-2.2 ± 44.8	64.4 ± 35.3	2.3 ± 26.7	31.8 ± 22.8
Glu_{sr} (ng/ml)[a]	91.0 ± 3.0	88.0 ± 5.0	89.0 ± 5.9	86.0 ± 5.9	91.0 ± 4.4	88.5 ± 5.9
ΔFR_{sl} (ml/min)	-0.1 ± 0.4	-0.1 ± 0.2*	-0.05 ± 0.5	-0.1 ± 0.2	-0.05 ± 0.4	-0.1 ± 0.1

The variations in psychometric variables and biochemical variables between RS and SS were analysed using Wilcoxon Signed-Rank Test at a significance level of α=5%. Marked features show significant differences between sessions; *p-values <0.05, **p-values <0.001. [a]: statistically significant differences between sexes (p-value < 0.05).

Correlations among studied variables

Our findings (Figure 2 and Supplementary Table 2) indicated a significant positive correlation (r) between VAS and ΔAA_{sl} ($r = 0.351$, $p< 0.01$) and a significant negative correlation (r) between VAS and ΔFR_{sl} ($r = -0.277$, $p< 0.01$). In addition, a positive association was observed among all psychometric variables, whereas a much less significant association (r) for VAS and PSS ($r = 0.198$, $p=0.078$). The correlation (r) between ΔFR_{sl} and ΔAA_{sl} was negative ($r = -0.387$, $p< 0.01$). In contrast, no association (r) was observed between ΔAA_{sl} and ΔCr_{sl}.

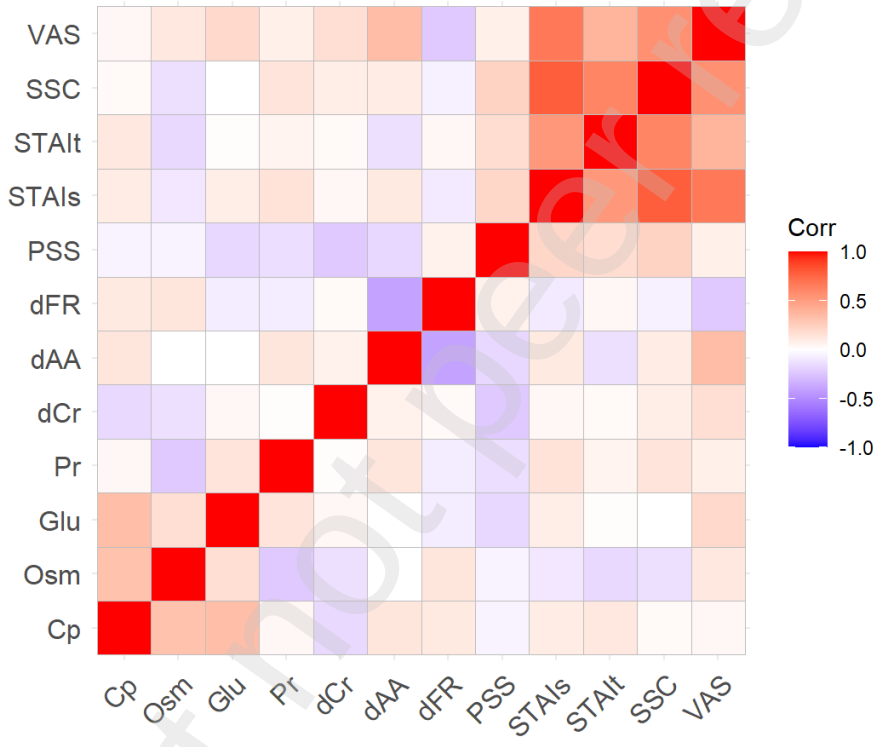

Figure 2. Spearman rank correlation coefficient matrix heatmap of biochemical and physiological variables (STAIs = STAI-s, STAIt = STAI-t, dAA= ΔAA_{sl}, dCr = $\Delta Cr_{sl,}$ dFR = ΔFR_{sl}, Pr = Pr_{pl}, Cp = Cp_{pl}, Glu = Glu_{sr} and Osm = Osm_{pl}) generated using *ggcorplot* in RStudio for windows. The bar on the left side of the map indicates the colour legend of the Spearman correlation coefficients.

Stress Reference Scale (SRS)

To build the SRS, psychometric and biochemical variables that were statistically significant in differentiating RS and SS states were included. The results of the PCA with n=80 (40 RS and 40 SS) and seven dimensions are shown in Table 2. The first four components exhibited eigenvalues greater than 0.7 and explained 84% of the total variance. The loading vectors (correlation coefficient scores) of each component allowed for the interpretation of the type of information collected by each component (Table 2). Thus, the first component mainly collected information corresponding to the psychometric tests, while the second component was positively associated with ΔFR_{sl} and negatively with ΔAA_{sl}. The third component had the highest scores for Cp_{pl} and the fourth had a strong positive correlation with Pr_{pl}. Together, these components provide information on the different aspects (factors) involved in responses to acute psychological stress. The proposed SRS is expressed as:

$$SRS = (0.15 * STAI_s + 0.14 * VAS + 0.14 * SSC + 0.12 * AA_{sl} + 0.11 * FR_{sl} + 0.19 * Cp + 0.15 * Pr)$$

Our findings indicated that SRS scores were significantly higher in SS than in RS (Supplementary Table 3) (p =1.299e-05). In addition, no significant sex-based variation was observed in SRS scores.

Table 2. Principal Components Analysis (PCA) summary with eigenvalues, explained variances and weights of the proposed SRS reference scale.

	PCA Component				Weight (%)
Variables	1	2	3	4	
Pr_{pl}	0.2466550	0.00162197	0.57448912	0.776963442*	15
AA_s	0.4094267	-0.74777448*	0.22566106	-0.143047078	12
STAI-s	0.8509134*	0.38870408	-0.10066183	0.005090755	15
SSC	0.8341677*	0.30798238	-0.04963621	-0.004881756	14
VAS	0.8367070*	-0.01633558	-0.19598637	-0.094137137	14
FR_s	-0.3964135	0.71681296*	0.20191654	-0.086553101	11
Cp_{pl}	0.1713332	0.09291078	0.79956896*	-0.518754316	19
Eigen value	2.5349358	1.3278334	1.1120487	0.9096437	
Variance (%)	36.213368	18.969049	15.886410	12.994910	
Cum. variance (%)	36.21337	55.18242	71.06883	84.06374	
Variance expl. (%)	43	23	19	15	100

Cum. variance: Cumulative variance; Variance expl.: Percentage of variance explained, proportional to the total variance explained by the four components. *variables with highest weights in each component.

Machine Learning: Decision tree and Statistical models

Models created to predict whether an individual is stressed or relaxed provided similar results, indicating their robustness. Decision tree, bagging decision tree, and logistic regression models revealed that the most important variables for the prediction of acute psychological stress were ΔAA_{sl} and STAI-s, whereas the random forest models indicated ΔFR_{sl} as an additional predictor of acute stress (Figure 3 and Supplementary Figure 1 and 2). The predictive accuracy of the decision tree model was 65.21%, while the random forest and logistic regression models had accuracies of 73.91% and an area under the receiver operating curves (ROC) of 0.84 and 0.85, respectively.

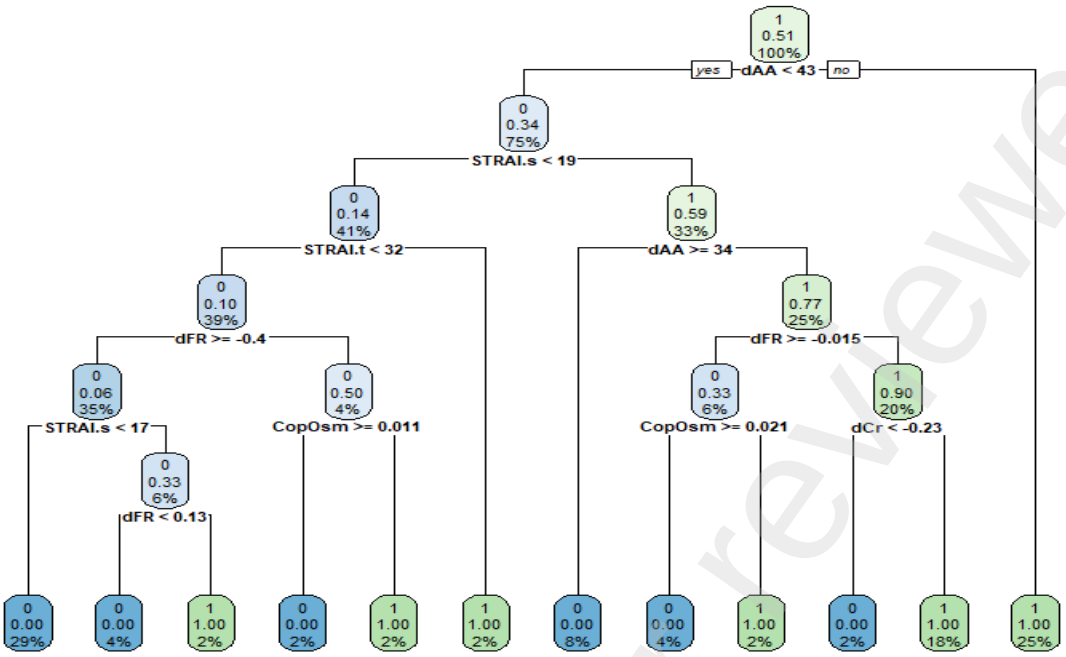

Figure 3. Decision tree model obtained for stress prediction; dAA=ΔAAsl, dFR=ΔFRsl, dCr=ΔCr_{sl}, CopOsm=Copetin/Osmolarity, STRAI.s= STAI-s, STRAI.t= STAI-t

Metabolomics Analyses.

Raw DIMS profiles showed approximately 1500 signals for each mode (ESI (+) and ESI (-)). After data curation, features that remained were passed on for subsequent statistical analysis.

PCA plots revealed a clear separation between blood metabolites for RS and SS (Figure 4) for both ESI (+) and ESI (-), suggesting a clear influence of acute psychological stress on the blood metabolome. The loading diagram for both modes showed that the number of potential biomarkers in SS was significantly larger than that in RS (Supplementary Figure 3). PLS-DA models built with ESI (+) and ESI (-) data provided good clustering of the samples and displayed a clear classification of each state. For ESI (+) mode, the model provided good explained variance (R^2) and predictive variance (Q^2) parameters with values of 0.8 and 0.259, respectively. Differential metabolites, those with a Variable Importance in Projection (VIP)

score > 2 (62), and variation coefficients (CV%) below 20% for the metabolites identified to avoid subjectivity in the selection process, identified for both RS and SS in ESI (+), are shown in Table 3. Most of the signals obtained in ESI (+) mode showed significantly ($p < 0.05$) altered blood levels of many amino acids and related metabolites (serine, indole, alanine, phenylalanine, valine, histidine, N-acetyl glutamine), altered sterols and steroid hormone biosynthesis (hydrocortisone, aldosterone, corticosterone, 11-deoxycorticosterone (DOC), progesterone, pregnenolone, cholesterol, 17α-hydroxypregnenolone, 11-deoxycortisol, 17-deoxycortisol, 17β-oestradiol, oestrone), and catecholamine neurotransmitters (dopamine, norepinephrine, epinephrine). The remaining significantly altered metabolites in SS corresponded largely to fatty acids and cellular membrane components (isobutyrate, choline, glycerophosphocholine, lyso-phosphatidylcholine (LPC)), sucrose sugar, and changes in muscle-related metabolites (creatine and carnitine). Nonetheless, the most predominant metabolites in RS included tyrosine, tryptophan, and its derivatives (the neurotransmitter serotonin, the neurotoxin quinolinic acid, and the hormone melatonin), derivatives of nitrogenous bases of nucleic acids (hypoxanthine and 2,4-dihydroxypyrimidine), and derivate of B3 vitamin N-methylnicotinamide (NMN).

Analysis of blood samples in ESI (-) mode showed a comparable R^2 of 0.84, but a comparatively lower Q^2 of 0.04. The significant signals obtained in this mode were identified as fatty acids and phospholipids (Table 4), suggesting that stress leads to a substantial alteration of the lipid profile.

Subsequent pathway analysis revealed many metabolic pathways that were significantly altered by acute mental stress. These included steroid hormone biosynthesis ($p = 1.09E-07$), glycerophospholipid metabolism ($p = 4.03E-04$), linoleic acid metabolism ($p = 3.27E-03$), aminoacyl-tRNA biosynthesis ($p = 1.09E-02$), and tyrosine metabolism ($p = 4.14E-02$) (Supplementary Figure 4).

Table 3. Significantly differential metabolites determined using positive ion mode (ESI (+)) after relax session (RS) and after stress induction (SS).

Predominant metabolites in SS	Formula	*m / z* [M+H]$^+$	Δm (ppm)	*p*-value	CV (%)	VIP
Hydrocortisone[a]	$C_{21}H_{30}O_5$	363.4653	-7.3	$1.8 \cdot 10^{-2}$	6.2	2.18
Aldosterone[a]	$C_{21}H_{28}O_5$	361.4485	1.8	$2.6 \cdot 10^{-3}$	7.6	2.09
Corticosterone[a]	$C_{21}H_{30}O_4$	347.2245	6.6	$2.9 \cdot 10^{-2}$	5.3	2.05
DOC[a]	$C_{21}H_{30}O_3$	331.2253	-6.0	$3.1 \cdot 10^{-4}$	6.5	2.10
Progesterone (P4)[a]	$C_{21}H_{30}O_2$	315.2314	-3.2	$4.1 \cdot 10^{-2}$	9.7	2.68
Pregnenolone (P5)[a]	$C_{21}H_{32}O_2$	317.2498	5.7	$4.0 \cdot 10^{-2}$	7.8	2.09
Cholesterol[a]	$C_{27}H_{46}O$	387.3598	-7.2	$5.1 \cdot 10^{-3}$	4.4	2.01
17-OHP[a]	$C_{21}H_{32}O_3$	333.2403	-7.8	$1.1 \cdot 10^{-3}$	6.3	2.62
11-deoxycortisol[a]	$C_{21}H_{30}O_4$	347.2257	10.1	$2.1 \cdot 10^{-2}$	7.3	2.09
17-deoxycortisol[a]	$C_{21}H_{30}O_4$	347.2257	10.1	$2.1 \cdot 10^{-2}$	7.3	2.09
17β-oestradiol[a]	$C_{18}H_{24}O_2$	273.1878	8.8	$1.7 \cdot 10^{-2}$	11.2	2.36
Oestrone (E1)[a]	$C_{18}H_{22}O_2$	271.1706	2.9	$8.0 \cdot 10^{-3}$	12.3	3.01
Sucrose	$C_{12}H_{22}O_{11}$	342.29648	2.05	$4.4 \cdot 10^{-2}$	2.9	2.71
Serine[a]	$C_3H_7NO_3$	106.0514	9.4	$3.2 \cdot 10^{-3}$	7.2	2.41
Indole[a]	C_8H_7N	118.0670	11.8	$2.9 \cdot 10^{-2}$	5.8	2.34
Alanine	$C_3H_7NO_2$	89.09318	8.32	$6.1 \cdot 10^{-3}$	3.1	2.53
Phenylalanine[a]	$C_9H_{11}NO_2$	166.0858	-6.0	$1.7 \cdot 10^{-2}$	5.1	2.42
Dopamine[a]	$C_8H_{11}NO_2$	154.0857	-7.1	$9.4 \cdot 10^{-3}$	5.3	2.37
Isobutyrate	$C_4H_7O_2$	87.0971	-3.21	$2.63 \cdot 10^{-2}$	4.2	2.57
Norepinephrine[a]	$C_8H_{11}NO_3$	170.0826	5.3	$2.4 \cdot 10^{-2}$	5.8	2.27
Epinephrine[a]	$C_9H_{13}NO_3$	184.0959	-7.6	$8.1 \cdot 10^{-3}$	6.0	2.35
Choline[a]	$C_5H_{13}NO$	103.1628	-15.0	$3.4 \cdot 10^{-2}$	8.2	2.81
Valine[a]	$C_5H_{11}NO_2$	117.1463	-7.5	$1.5 \cdot 10^{-3}$	6.4	2.31
Creatine[a]	$C_4H_9N_3O_2$	131.1331	-17.1	$4.1 \cdot 10^{-2}$	10.0	2.03
Histidine[a]	$C_6H_9N_3O_2$	155.1545	-12.2	$1.7 \cdot 10^{-3}$	5.4	2.07

Carnitine[a]	$C_7H_{15}NO_3$	161.1989	-11.8	$3.5 \cdot 10^{-3}$	9.4	2.24
NAG[a]	$C_7H_{12}N_2O_4$	188.1811	-10.8	$1.8 \cdot 10^{-2}$	7.9	2.13
GPCh[a]	$C_8H_{20}NO_6P$	257.2212	-9.5	$2.5 \cdot 10^{-2}$	9.8	2.19
LPC (18:1)[a]	$C_{26}H_{52}NO_7P$	521.6673	12.6	$1.4 \cdot 10^{-3}$	8.5	2.28
LPC (18:0)[a]	$C_{26}H_{54}NO_7P$	523.6832	-11.2	$3.1 \cdot 10^{-3}$	6.4	2.11
Predominant metabolites in RS	**Formula**	***m / z* [M+H]$^+$**	**Δm (ppm)**	***p*-value**	**CV (%)**	**VIP**
L-Tryptophan[a]	$C_{11}H_{12}N_2O_2$	205.0967	-4.9	$4.10 \cdot 10^{-3}$	5. 3	2.56
Serotonin[a]	$C_{10}H_{12}N_2O$	177.1039	6.8	$1.9 \cdot 10^{-2}$	5.6	2.18
Melatonin[a]	$C_{13}H_{16}N_2O_2$	233.1270	-8.6	$3.0 \cdot 10^{-2}$	6.8	2.41
Tyrosine	$C_9H_{11}N_1O_3$	181.1885	-2.15	$5.2 \cdot 10^{-2}$	4.1	2.75
Aminoethanol	C_2H_7NO	61.0831	3.40	$3.15 \cdot 10^{-3}$	3.9	2.05
Hypoxanthine	$C_5H_4N_4O$	136.1115	2.95	$5.27 \cdot 10^{-3}$	2.7	2.98
Quinolinic acid	$C_7H_5NO_4$	167.1189	-3.04	$25.0 \cdot 10^{-2}$	3.2	2.43
2, 4- dihydroxypyrimidine	$C_4H_6N_2O$	98.1032	-5.53	$7.35 \cdot 10^{-3}$	5.0	2.12
N-Methylnicotinamide	$C_7H_8N_2O$	136.1512	-3.95	$2.90 \cdot 10^{-2}$	4.5	2.31

MS/MS (Tandem Mass Spectrometry) data, and elucidation of fragmentation patterns for each m/z which confirms unequivocal structural and chemical characterization in all the cases; *p*-value was calculated by T-test analysis for each of the m/z / intensity relations and considering significant values of $p \leq 0.05$; *Δm* is the mass error expressed in ppm; CV: coefficient of variation were considered values <20 % to obtain a method with good reproducibility; VIP: variable importance in projection was set up at a minimum value of 2 to ensure selection of predominant m/z in each group. DOC: 11-deoxycorticosterone; 17-OHP: 17α-hydroxypregnenolone; NAG: N-acetyl glutamine; GPCh: glycerophosphocholine; LPC: lysophosphatidylcholine. [a]: previously published in a preliminary report by Lorenzo-Tejedor *et al* (30)

Table 4. Significantly differential metabolites identified after stress induction using negative mode (ESI (-))

Predominant metabolites in SS	**Formula**	**MS/MS product ions *m/z***	**Δm (ppm)**	***p*-value**	**CV (%)**	**VIP**
Caprylic acid	$C_8H_{16}O_2$	143.10 (−H+)	-6.1	$3.7 \cdot 10^{-2}$	5.2	2.01
Capric acid	$C_{10}H_{20}O_2$	171.10 (−H+)	-9.8	$3.3 \cdot 10^{-3}$	9.2	2.45
Linoleic acid	$C_{18}H_{32}O_2$	279.20 (−H+)	-5.7	$2.3 \cdot 10^{-2}$	2.4	2.80
DHA	$C_{22}H_{32}O_2$	327.20 (−H+)	3.2	$5.0 \cdot 10^{-4}$	7.0	2.32
LPC (20:5)	$C_{28}H_{48}NO_7P$	359.26, 184.07, 104.10, 86.09	-4.3	$3.1 \cdot 10^{-2}$	10.0	2.45
PPE (16:0/22:6)	$C_{43}H_{74}NO_7P$	746.50 (−H+), 327.23, 196.07	-7.6	$2.6 \cdot 10^{-2}$	6.5	2.96
PPE (18:1/20:4)	$C_{43}H_{76}NO_7P$	748.50 (−H+), 303.30, 196.10	5.2	$5.4 \cdot 10^{-3}$	8.1	2.06
PPE (18:0/20:4)	$C_{43}H_{78}NO_7P$	750.50 (−H+), 303.20, 196.10	-9.2	$2.2 \cdot 10^{-3}$	3.4	2.32
PPE (18:0/22:6)	$C_{45}H_{78}NO_7P$	774.50 (−H+), 327.20, 196.10	8.5	$1.9 \cdot 10^{-2}$	9.7	2.47
PC (16:0/20:5)	$C_{44}H_{78}NO_8P$	313.20, 359.30, 184.10, 104.10, 86.0	-6.1	$2.0 \cdot 10^{-2}$	4.3	2.65
PPC (16:0/22:6)	$C_{46}H_{80}NO_7P$	387.20, 184.0, 104.10, 86.0	-8.5	$2.2 \cdot 10^{-2}$	6.2	2.15
PPC (18:1/22:6)	$C_{48}H_{82}NO_7P$	385.20, 184.0, 104.10, 86.0	5.5	$6.3 \cdot 10^{-3}$	7.9	2.98
PC (18:1/20:4)	$C_{46}H_{82}NO_8P$	339.20, 361.0, 184.0, 104.10,86.0	-6.8	$2.6 \cdot 10^{-2}$	5.8	2.50
PC (18:0/22:6)	$C_{48}H_{84}NO_8P$	341.0, 38.0, 184.0, 104.10, 86.0	11.4	$3.0 \cdot 10^{-2}$	11.5	2.21

MS/MS (Tandem Mass Spectrometry) data, and elucidation of fragmentation patterns for each m/z which confirms unequivocal structural and chemical characterization in all the cases; *p*-value was calculated by T-test

analysis for each of the m/z / intensity relations and considering significant values of $p \leq 0.05$; Δm is the mass error expressed in ppm; CV: coefficient of variation were considered values <20 % to obtain a method with good reproducibility; VIP: variable importance in projection was set up at a minimum value of 2 to ensure selection of predominant m/z in each group. DHA: docosahexaenoic acid; LPC: lyso-phosphatidylcholine; PPE: ethanolamine-plasmalogen; PC: phosphocholine; PPC: choline-plasmalogen.

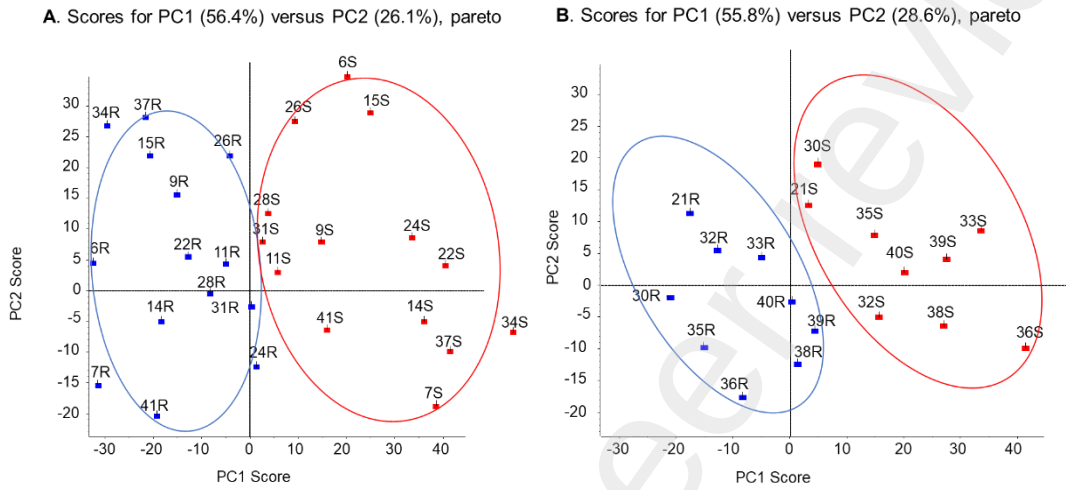

Figure 4. Score plot of principal component analysis (PCA) on metabolomic data acquired in ESI (+) (A) and in ESI (-) (B) modes. Each dot represents a blood sample. Samples obtained after relax state (R) are in blue and the ones obtained after stress induction (S) are in red.

DISCUSSION

In this study, a modified form of the Trier Social Stress Test (TSST-M) was used to induce acute stress. We found significant differences between RS and SS in psychometric tests (STAI-s, VAS), SSC, and in biochemical markers like AA_{sl}, FR_{sl}, Cp_{pl} and Pr_{pl}. These results confirmed that stress was successfully induced, in agreement with other studies that used the TSST (63,64). While we had anticipated a significant increase in salivary cortisol, no significant difference was eventually found even though previous studies have shown that cortisol levels typically rise following induced stress (65,66). This discrepancy could be attributed to the dynamics of cortisol release and detection in saliva. Whereas α-amylase

(AA) is released directly into the oral fluid in response to the activation of the HPA axis, cortisol is instead first released from the adrenal glands into the bloodstream; only then, it passively diffuses into saliva. This process results in a delay of up to 15-20 minutes before cortisol reaches its peak concentration in saliva in comparison with AA (67). Since saliva sample collection in our study was conducted immediately after stress induction, the peak of cortisol may not have been captured. Setting aside this limitation, our metabolomic analysis identified cortisol as a relevant blood biomarker of acute stress, with significant changes in its concentration distinguishing RS from SS.

Concerning sex differences, we observed significantly higher glucose and copeptin levels in men, in line with recent findings by Spanakis *et al.* (63). This result supports the hypothesis that the HPA axis response to acute psychological stress varies by sex according to previous studies (63,68). This indicates that the risk of suffering different diseases as a result of stress may vary between men and women.

Given these results, a PCA analysis was performed in order to reduce the multiple dimensions of the psychological stress state into its main components. From the seven components identified, the top four ones explain 84% of the variance. The first principal component correlates most strongly with psychometric tests, reflecting the variation in the quality of individuals' psychological state produced by the stressor. Whereas the second principal component is related to SNS activation (involving ΔAA_{sl} and FR_{sl} changes), the third and fourth principal components (Cp_{pl} and Pr_{pl}, respectively) related to the HPA axis activation, emerge as separate factors probably because each is secreted by different sources (the posterior and anterior pituitary, respectively). These results highlight the close interaction between the SNS and HPA axis in eliciting stress response. By integrating these significant factors into the SRS scale (33) we could check its utility in quantifying the level of stress

perceived by an individual (46) (Supplementary Table 4). Still, this has to be validated by additional studies.

The predictive models we built using machine learning technique (decision trees, logistic regression and random forest classifiers) exhibited high level of robustness in determining the stress state of an individual. Consistently, all models identified ΔAA_{sl} and STAI-s as main predictive biomarkers of acute psychological stress status. Such a result support the importance of AA_{sl} as a key biomarker in evaluating stressors that activate the SNS, in agreement with previous research reports (69–71). Nonetheless, it is important to note that AA_{sl} levels, like all other variables, may be influenced by a variety of factors such as exercise and medication (72). In the case of the random forest model, FR_{sl} was identified as an additional significant predictor of stress status.

We are aware that, even though our models showed high predictive accuracy indicating their potential reliability for stress monitoring, the small sample size (n=40) in this study limited the statistical power of our analyses, reducing the generalizability of our findings to a broader population. Nonetheless, our findings provide sound bases for further studies.

In this study, we also explored the metabolic signature of acute psychological stress. Our results are in line with previous research that has documented significant changes in the metabolomic profile in both animal models and humans subject to different stressors (73–75). In PCA plots of the metabolomic data two clusters are clearly distinguished, thus indicating that RS and SS samples had remarkably differential metabolic compositions. A total of 53 significantly differential metabolites ($p<0.05$, VIP>2) were identified from both ESI (+) and ESI (-) ion models. Of these, 9 were predominantly associated with RS, while 44 were predominantly associated with SS. These findings showed that acute psychological stress yields extensive changes across multiple metabolic pathways involved in the organism's adaptative response. It is well established that prolonged stress-induced alterations can have

detrimental effects on health. Consequently, chronic psychological stress is recognized as a serious risk factor for cardiovascular diseases and metabolic disorders (75).

Precisely, one of our most striking findings concerns the significant changes in the lipid profile induced by acute mental stress, notably the substantial increase in fatty acids, polyunsaturated fatty acids (PUFAs), phosphocholines (PCs), plasmalogens (PPCs and PPEs), and lysophosphatidylcholines (LPCs). Recent studies on this topic indicate that these lipids and lipid-like molecules play critical roles in cell signaling pathways related to inflammation, immunity and apoptosis (75,76).

The increases in plasmalogens (PPC and PPE) levels observed in our research may be attributed to the increase in the brain's demand for PPs under acute stress conditions to keep an adequate neural function, endorse synaptic plasticity, and protect against stress-induced oxidative damage. Some authors proposed that PPs, crucially those containing omega-3 fatty acids such as LPC (20:5), PPE 16:0/22:6, PPE 18:0/22:6 and docosahexaenoic acid (DHA), as observed in our study, may reduce HPA axis activation in response to acute physiological stress, thereby protecting the brain from subsequent cellular damage (77,78). When stress becomes chronic, this adaptative mechanism leads instead to a decline in PP levels, which is associated with several degenerative disorders and neurocognitive impairments (75,76).

In addition to the increased PP levels in SS, we also observed elevated levels of LPCs. This finding is in line with previous studies suggesting that LPCs containing medium-chain saturated fatty acids may serve as potential biomarkers not only for stress but also for adiposity and inflammation (75). LPCs are generated through the cleavage of phosphatidylcholine, a major phospholipid in cell membrane, by phospholipase A_2 (PLA_2), which produces free fatty acids, including arachidonic acid. The observed rise in LPC levels may reflect the body's complex response to the induced stress adaptation, involving the

activation of PLA_2 by mitogen-activated protein (MAP) kinase-related kinase, a family of stress-activated protein kinases (79,80).

The function of LPCs depend on the length and degree of saturation of the fatty acid chain attached to the glycerol moiety (81). For instance, elevated levels of LPC (18:0) and related plasmalogens, PPC (18:0/20:4) and PPC (P18:0/22:6), have been associated with reduced inflammation, lower adiposity, and a decrease risk of cancer (75,81). On the other hand, LPCs like 18:1 and 20:4 exert their biological roles by activating many downstream signaling pathways, including mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB). These pathways promote cell division, chemotaxis, oxidative stress, inflammatory cytokine release and apoptosis, thereby accelerating the development of atherosclerosis (81). Additionally, LPC (20:4) has been associated with stress index, and its free fatty acid, the arachidonic acid (20:4), has been suggested as a marker of depression and stress in human (75,82).

Another predominant metabolite found under acute stress conditions was linoleic acid (18:2-n6), the most abundant polyunsaturated fatty acid (PUFA) in human nutrition. Linoleic acid (LA), is an essential n-6 PUFA and a precursor to arachidonic acid. While normal levels of LA are crucial for neurological and cognitive development and overall health, elevated levels of LA have been linked to inflammation and metabolic diseases (83). Our data indicate that its metabolic pathway was among the most significantly affected. One such alteration involves the inhibition of the enzymes responsible for catalyzing LA epoxidation, which leads to a reduction in its hypocholesterolemic effect (84,85), followed by the consequent accumulation of arachidonic acid. Additionally, LA can undergo non-enzymatic oxidation to produce Oxlams, metabolites that have been shown to promote a strong pro-inflammatory response in rats (83).

An elevated level of cholesterol in SS like the one observed here may lead to the generation of a variety of corticosteroids via steroidogenesis. Due to the lipophilic nature of corticosteroids, they cannot be pre-synthesized and stored in adrenal glands but have to be rapidly synthesized upon Adrenocorticotropic hormone (ACTH) stimulation, which is instead regulated by the HPA axis (86). Corticosteroids regulate multiple physiologic processes, including metabolism, development, homeostasis, metabolism, cognition and inflammation (86). Cortisol in turn increases the bioavailability of glucose and the consequent release of energy to the brain (98), as evidenced by the increased levels of carnitine, creatine, and glucogenic amino acids observed in this study, corroborating findings by Singh et al. (73). Additionally, these amino acids could also serve as substrate for the synthesis of protein required for the stress response process (99).

Each stressor has a neurochemical signature with distinct central and peripheral mechanisms (87). Some studies have demonstrated that the two branches of the sympathoadrenal system (SAS), the adrenal medulla and the sympathetic nerves, can be activated independently by different stressors (87,88) however, this affirmation remains controversial and poorly understood (89). In our research, we observed that acute psychological stress induced by TSST_M activated both components of SAS. It stimulates the adrenal medulla system elevating plasma Epi levels, and activates the sympathoneural system increasing NE and dopamine plasma levels.

EPI is known as the hormone preparing the body for a fight-or-flight response (90). NE, which is the main sympathetic neurotransmitter in circulatory regulation, is also a central neurotransmitter thought to be involved in alertness, memory of distressing events, nociception, and anxiety (89). Dopamine (DA) is a key neurotransmitter that regulates many processes in the CNS, including reward, motion, and cognition. Importantly, DA can also be produced locally in several peripheral organs, where it has autocrine and paracrine effects

influencing many organ functions (91,92) and is released in plasma in response to stress. This response is partly influenced by circulating cortisol levels in the body (93,94). DA moreover regulates critical functions such as metabolic homeostasis, hormone release, sodium balance, blood pressure, renal activity, gastrointestinal motility. It also modulates inflammatory and immunological processes (91,92). A prolonged exposure to intense stressors inhibits the release of DA and disrupts the dopaminergic pathway, leading to psychological disorders such as depression and schizophrenia (95,96).

The elevated levels of cholesterol, corticosteroids, steroid hormones, and adrenal catecholamines observed in this study could be accordingly explained by the increase in prolactin, which is known as *the stress hormone*.

There is substantial evidence supporting prolactin's multifaceted role in the adrenal response to stress (97). More specifically, it has been shown to increase the secretion of ACTH enhance the storage of cholesterol esters, and induce adrenal hypertrophy (97–99). Under acute stress, prolactin secretion appears to play a crucial and complex role in maintaining metabolic and immune system homeostasis (99–101). Therefore, while Pr may induce a protective proinflammatory state during acute stress, chronic exposure to prolactin can by contrast lead to habituation and potentially contribute to the development of cardiovascular pathologies (102).

Interestingly, we identified several metabolites that the literature suggest may have protective effects during acute stress. For instance, progesterone and pregnenolone are known to suppress HPA activity, thereby reducing stress levels (103,104). Additionally, DHA, caprylic and capric acid have been identified by possessing anti-inflammatory properties, which counteract the inflammatory process often associated with stress (105,106). Furthermore, 17β-oestradiol and oestrone have been shown to play a neuroprotective role against stress-related

damage (107,108). Collectively, these metabolites contribute to the body's adaptive response aimed at restoring homeostasis and mitigating the adverse effects of stress.

CONCLUSIONS

In this study a modified version of the Trier Social Stress Test (TSST-M) is applied in order to induce acute psychological stress. Under this state, we could explore the multifaceted effects of stress response by using psychometric assessments, biochemical analyses, and metabolomic profiling. Our findings further mark significant sex differences in stress response, particularly in glucose and copeptin levels, indicating that stress impacts men and women differently. This highlights the necessity for gender-sensitive approaches in stress research, especially given their implications for disease risk assessment.

Our study also demonstrates the utility of Stress Reference Scale (SRS) in stress quantification and the importance of machine learning predictive models to distinguish distinct stressed vs relaxed states in individuals. Specifically, our predictive models identified salivary α-amylase (AA_{sl}) and STAI-s prominent stress markers.

Despite its limitations, an important strength of our study was the validation of a direct infusion MS method that is minimally invasive, requiring only a finger prick and a drop of blood for metabolomics analysis (30). The results further indicate that acute psychological stress significantly affects bodily systems, triggering relevant metabolic alterations.

These findings help to understand the intricate interplay between physiological and psychological domains in acute mental stress responses. Taken together, our findings contribute to a complex and careful study of stress by integrating tools and advanced analytical methods for acute psychological stress diagnosis and understanding the mechanisms involved in this type of stress response and related disorders. Future research may benefit from longitudinal studies to elucidate the relationship between proposed

biochemical, metabolic and psychometric stress measures, and biomarker validation in stress diagnosis and measurement.

BIASES AND LIMITATIONS

This study presents several limitations. For this study we used a relatively small sample size narrowed down to young, healthy university students, hence diminishing its relative homogeneity with regard to general demographic data. As a result, the generalizability of these findings to a broader population is limited, and should be interpreted in the context of the study's limitations. Furthermore, we did not account for the menstrual cycle phase of the female participants, nor did we consider their use of contraceptives, as these could affect prolactin and other hormone levels such as oestrone and progesterone. Additionally, we did not consider the use of antiallergic and/or bronchodilators (e.g., salbutamol) affecting steroid hormone levels (e.g., cortisol).

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

This work is part of a multidisciplinary project formed with the objective of studying different aspects of the genesis of stress and its adverse effects on health. G. A. Frempong: Investigation, Data Curation, Software, Formal Analysis, Writing - Original Draft & Editing, Visualization. G. Goni: Conceptualization, Software, Formal Analysis, Validation, Writing - Original Draft, Review & Editing, Visualization. M. Lorenzo-Tejedor: Methodology, Investigation, Data Curation, Formal Analysis. C. De la Cámara: Methodology, Investigation, Formal Analysis. J. Lázaro: Methodology, Investigation. J. Aguiló: Investigation, Writing - Review. E. M. Rasia: Writing - Original Draft, Review & Editing. R. Bailon: Conceptualization, Project administration, Resources, Funding acquisition, Writing - Review. M. L. Bernal: Conceptualization, Supervision, Resources, Writing - Review.

DECLARATION OF CONFLICTS OF INTEREST

All authors have read the journal's policy on the disclosure of potential conflicts of interest and have no conflicts of interest to declare.

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