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Uncovering the metabolic impact of acute psychological stress in young adults

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Stress is associated with the onset of various neurological disorders, such as depression, post-traumatic stress disorder, and anxiety. Although extensively studied, the metabolic changes triggered in response to stress remain unclear. We conducted a descriptive observational study on acute stress responses in university students, combining psychometric, biochemical, and untargeted metabolomic analyses, along with machine learning predictions. In this study, forty participants underwent both relaxation and stress induction through a modified Trier Social Stress Test. Validated psychometric tests confirmed proper induction of both states. Although most biomarkers show significant changes under acute stress state, the machine learning predictive model identified salivary α-amylase and the State-Trait Anxiety Inventory-state (STAI-s) as potential stress markers. Additionally, several metabolic pathways, including steroid hormone biosynthesis, glycerophospholipid metabolism, linoleic acid metabolism, tyrosine metabolism, and aminoacyl-tRNA biosynthesis, presented alterations under acute mental stress. Our findings highlight the impact of acute mental stress on multiple metabolic pathways directly implicated in stress-related disorders. These findings advance the understanding of the adverse effects systematically associated with stress and provide evidence supporting the potential role of salivary α-amylase and STAI-s as stress markers. Yet, they should be regarded as important hypothesis generators. However, further studies are needed for final validation.

Keywords Mental stress reactivity, Metabolic responses, Biomarkers, Untargeted metabolomics, Trier social stress test, Direct infusion mass spectrometry (DI-MS), Machine learning

Abbreviations

ΔAA_{sl}	Difference in salivary α-amylase concentrations between 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 concentration between samples
Cr_{sl}	Salivary cortisol
DHA	Docosahexaenoic acid

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DIMS	Direct infusion mass spectrometry
DOC	11-Deoxycorticosterone
Epi	Epinephrine
ΔFR_{sl}	Difference in salivary flow rate between samples
FR_{sl}	Salivary flow rate
ESI	Electrospray ionization
Glu_{sr}	Serum glucose
HPA	Hypothalamic–pituitary–adrenal
KEGG	Kyoto encyclopedia 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}	Plasma osmolarity
PC	Phosphocholine
PSNS	Parasympathetic nervous system
PPC	Choline-plasmalogen
PPE	Ethanolamine-plasmalogen
Pr_{pl}	Plasma prolactin
PSS	Perceived stress scale
PUFA	Polyunsaturated fatty acids
RS	State after relaxation session
SNS	Sympathetic nervous system
SS	State after stress induction stage
SSC	Symptomatic stress scale
STAI-s/t	State-Trait Anxiety Inventory state and trait tests, respectively
TSST-M	Modified form of the Trier Social Stress Test
VAS	Visual analog scale

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, known as the *homeostatic state*, which is essential for life and well-being[1,2]. However, this optimal balance is constantly challenged by intrinsic and extrinsic adverse forces or *stressors*. While some stressors, such as unexpected events, urgent tasks, traumatic events, and adverse social, economic, and environmental circumstances, often produce psychological effects[3,4], others, such as injuries, noise, or exposure to extreme temperatures, could have physical consequences[1,2,5].

Stressors, when perceived as a threat, lead to a maladaptive stress response or disharmony called *distress* (popularly referred to as bare 'stress'). Stress triggers a complex interplay of physiological and behavioral responses aimed at reestablishing homeostasis, hence improving survival chances[1]. This process involves an intricate network engaging the central nervous system (CNS) and peripheral organs, leading to the activation of the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic nervous system (SNS), followed by the inhibition of the parasympathetic nervous system (PSNS)[1]. If this response is not adequate enough to preserve the balance needed, an inflammatory response is triggered in an attempt to restore the system to its homeostatic state[6]. These biochemical and physiological changes can consequently be used to determine and monitor stress. However, because each individual responds differently according to inherent personality traits along with a myriad of genetic, environmental, and developmental parameters, inter-subject variability is another factor that makes stress diagnosis and monitoring even more challenging[7,8].

Stress is generally classified into three main types: acute, chronic, and negative.

Acute stress triggers a time-limited set of cognitive-behavioral and physiological changes as an immediate response to a stressor[1,2]. Neuropsychologically, acute stress concomitantly enhances alertness and vigilance. Physiologically, intermediate metabolism is adjusted to increase nutrient levels; increased respiratory and heart rates augment oxygen and cardiac output, supporting cardiovascular tone[1]. The resulting nutrient-enriched blood is redistributed to organs directly involved in stress response orchestration (brain, heart, and skeletal muscles). This comes at the expense of a critical but temporary reduction in blood supply to energy-consuming vegetative functions such as digestion, renal and intestinal excretion, reproduction, growth, and immunity[1,7].

Chronic stress involves 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[9].

In turn, negative stress (distress)[10] has 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 function, growth, metabolism, reproduction, and immune competence. Individual performance, behavior, and personality development can be equally affected[7,9].

Nonetheless, stress reactivity depends on (i) the type of stressor, as different stressors activate different metabolic pathways; (ii) the intensity and duration of the stressor, such that the higher the degree of stress is, the lower the specificity of the adaptive response; and (iii) inter-subject variability, considering the manner in which each individual perceives stressors[7].

Psychological stress and distress

Given its influence on human decision-making, psychological stress (negative stress) represents a major public health concern[11–13]. According to the World Health Organization (WHO)[3], the prevalence of social and medical problems associated with mental stress is increasing globally, especially in children, which seriously affects their mental health and well-being. Many factors contribute to the increase in global stress. The COVID-19 pandemic, for example, has become a universal stressor that is involved in a global mental health crisis since it implies enduring, unprecedented, short- and long-term stressful situations that have undermined the mental health of millions of people[12,13]. Nevertheless, especially when chronic, mental stress exacerbates our susceptibility to several diseases, eventually becoming a common cause of morbidity and mortality[11]. Consequently, mental stress has a visible impact on the health system, resulting in elevated healthcare costs, invalidity, or productivity loss. In view of this, finding objective and precise diagnostic methods is currently a pressing need[14,15].

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[16]. 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[5,17,18]. In this sense, 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 still exist[19].

Since the distinctive feature of the stress response is the activation of the SNS and, most importantly, the HPA axis[20,21], the most promising biomarkers point to metabolites released as a result.

Given the multidimensional nature of stress, determining one or only a few reliable biomarkers for diagnosis is unlikely to be a feasible goal. The reported inconsistencies in the literature may be the result of oversimplifying the overall process[22].

To solve this problem, we propose an omics 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[23,24]. It involves systematic identification and quantification of the metabolite profile that characterizes 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, providing a global view of the metabolic changes arising as a result. Metabolites are the intermediate or end products of cellular regulatory pathways, and their levels can be regarded as the ultimate response of biological systems to genetic and environmental changes[25].

In the present study, which was integrated into an ES3-P multidisciplinary project[19,23,26,27] aimed at assessing acute psychological stress, we propose that the main goal is to determine the metabolomic fingerprint of acute psychological stress in a cohort of volunteer university students. This would directly contribute to the discovery of new stress biomarkers and help to unveil the molecular basis of adverse outcomes. As a secondary goal, we will analyze the potential utility of diverse biomarkers proposed in the literature and determine how sex differences operate in the stress response.

Results

Participant characteristics

On the basis of our study design and calculations, the suggested minimum number of subjects for adequate study power was 32 to detect moderate-to-large effects in paired measures (0.4–0.5 standard deviations of the difference). From the initial group recruited, 40 participants qualified. This final group presented a normal body mass index (BMI of 22.4 ± 2.7 kg/m^2) according to WHO guidelines[28] and was composed of young male and female participants in similar proportions (mean age of 22 ± 3.4 years) (Supplementary Table S1). Despite the sex balance, the smaller subgroup size lowers the statistical power. These findings thus remain exploratory, especially given certain risks such as the system's proneness to overfitting.

The perceived stress levels measured before administering the psychometric tests (Supplementary Table S1) were an average of 49.4 units on a scale from 0 to 100, indicating no to mild stress.

In terms of habits, the majority of the participants were nonsmokers (85%), occasional consumers of alcoholic beverages (82.5%), engaged in extracurricular activities (62.5%), practiced 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 respect 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 contraceptive agents, antihistamines, and antiasthmatic medications), which did not hinder the measurement sessions.

Stress evaluation and measurement

Psychometric tests

The State Anxiety Inventory (STAI-s), visual analog scale (VAS), and symptomatic stress scale (SSC) scores significantly increased between the state after the relaxation stage (RS) and the stress induction stage (SS) (Table 1), confirming that the participants became stressed after the modified Trier Social Stress Test (TSST-M) was applied. The Perceived Stress Scale (PSS) and Trait Anxiety Inventory test (STAI-t) results did not

	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

Table 1. Inter-subject median and median absolute deviation (MAD) of stress markers. The variations in psychometric variables and biochemical variables between RS and SS were analyzed via the 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).

significantly differ across 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

Significant increases in the concentrations of the biochemical stress markers ΔAA_{sl} (changes in salivary α-amylase concentration), ΔFR_{sl} (changes in salivary flow rate), plasma copeptin (Cp_{pl}), and plasma prolactin (Pr_{pl}) were detected between sessions. In contrast, the levels of ΔCr_{sl} (changes in salivary cortisol concentration) and the serum glucose concentration (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). Notably, all the variables were within the clinically accepted normal range.

Correlations among the studied variables

Our findings (Fig. 1) revealed a significant positive correlation (r) between VAS score and ΔAA_{sl} ($r = 0.351$, $p < 0.01$) and a significant negative correlation between VAS score 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 was detected for VAS score and PSS score ($r = 0.198$, $p = 0.078$). The correlation between ΔFR_{sl} and ΔAA_{sl} was negative ($r = -0.387$, $p < 0.01$). In contrast, no association was observed between ΔAA_{sl} and ΔCr_{sl}.

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 principal component analysis (PCA) with n = 80 (40 RS and 40 SS) and seven dimensions are shown in Table 2. The first four components presented 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, whereas the second component was positively associated with ΔFR_{sl} and negatively associated with ΔAA_{sl}. The third component had the highest scores for $Cp_{pl,}$ and the fourth component had a strong positive correlation with Pr_{pl}. Together, these components provide information on the different aspects (factors) involved in the response to acute psychological stress. The proposed SRS is expressed as Eq. (1):

$$SRS = \begin{pmatrix} 0.15 * STAI_s + 0.14 * VAS + 0.14 * SSC + 0.12 * AA_{sl} \\ +0.11 * FR_{sl} + 0.19 * Cp + 0.15 * Pr \end{pmatrix} \quad (1)$$

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

Machine learning: decision tree and statistical models

Models created to predict whether an individual is stressed or relaxed provide 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 that ΔFR_{sl} was an additional predictor of acute stress (Fig. 2 and Supplementary Fig. S1). The

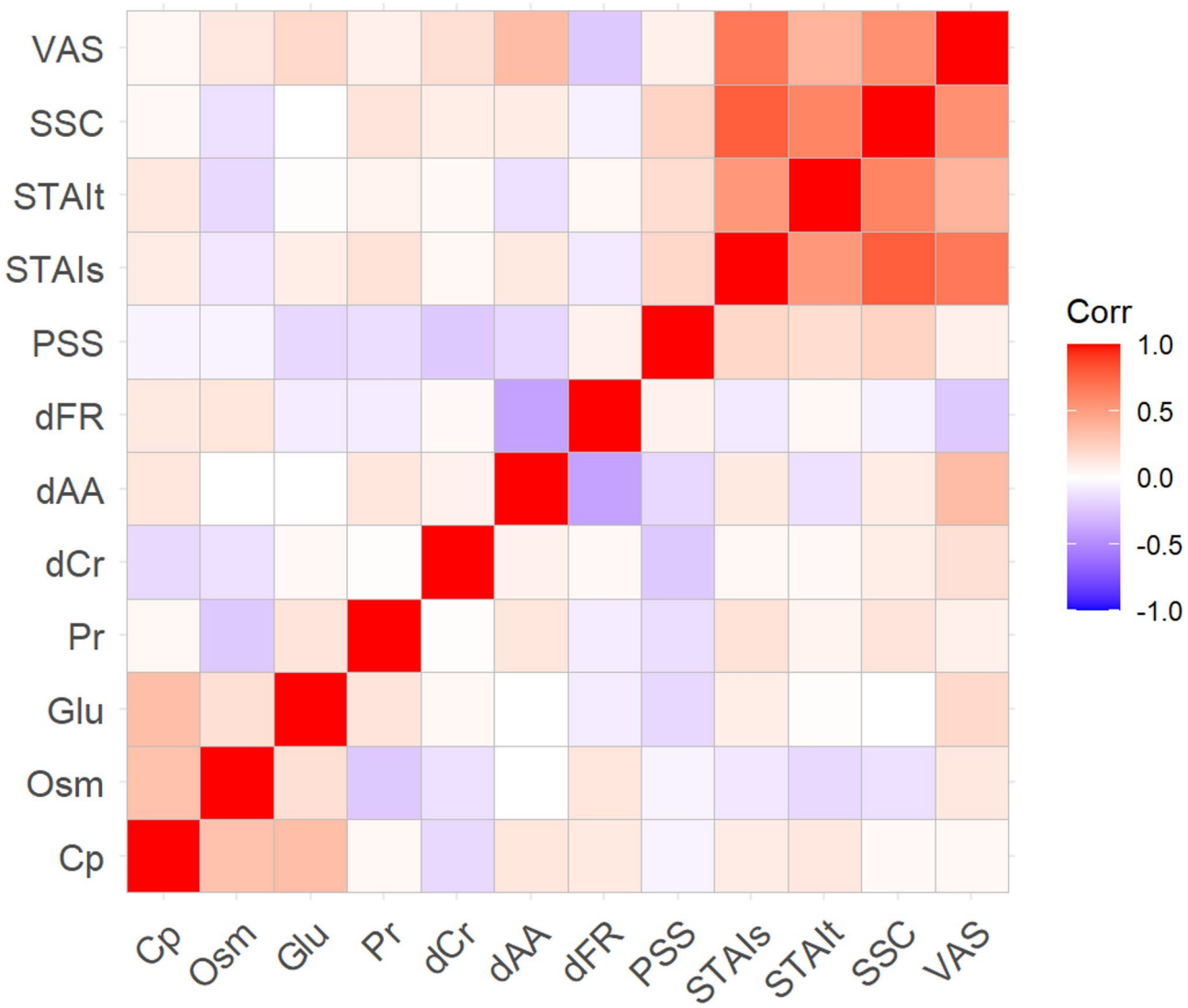


Fig. 1. Spearman rank correlation coefficient matrix heatmap of biochemical and physiological variables (STAI-s, STAI-t, dAA (difference in salivary α-amylase concentrations between stages, ΔAA_{sl}), dCr (difference in salivary cortisol concentrations between stages, ΔCr_{sl}), dFR (difference in salivary flow rate between stages, ΔFR_{sl}), Pr (plasmatic prolactin, Pr_{pl}), Cp (plasmatic copeptin, Cp_{pl}), Glu (serum glucose, Glu_{sr}) and Osm (plasmatic osmolarity, Osm_{pl})) generated via *ggcorplot* in RStudio for Windows. The bar on the right side of the map indicates the color legend of the Spearman correlation coefficients.

predictive accuracy of the decision tree model was 65.21%, whereas the random forest and logistic regression models had accuracies of 73.91% and areas under the receiver operating curves (ROC) of 0.84 and 0.85, respectively.

Metabolomic analyses

The raw direct infusion mass spectrometry (DIMS) profiles revealed approximately 1500 signals in each mode, electrospray ionization in positive mode (ESI (+)) and in negative mode (ESI (−)). After data curation, the remaining features were used for subsequent statistical analysis. Quality control analyses yielded intra-batch Coefficients of variability (CVs) < 7% for the principal metabolites, inter-batch CVs < 10% for the main compounds, and recovery rates of 82–115%, all within internationally accepted ranges, thereby supporting the robustness of our data. PCA plots revealed a clear separation between blood metabolites in RS and SS groups (Fig. 3) for both the ESI (+) and ESI (−) modes, suggesting a clear effect of acute psychological stress on the blood metabolome.

The loading diagram for both modes revealed that the number of potential biomarkers in SS was significantly greater than that in RS (Supplementary Fig. S2). Partial least squares discriminant analysis (PLS-DA) models built with ESI (+) and ESI (−) data provided good clustering of the samples and clearly classified 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. Differentially abundant metabolites with a variable importance in

	PCA Component				Weight (%)
Variables	**1**	**2**	**3**	**4**	
Pr_{pl}	0.2466550	0.00162197	0.57448912	0.776963442*	15
AA_{sl}	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_{sl}	−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

Table 2. Principal component analysis (PCA) summary with eigenvalues, explained variances, and weights of the proposed SRS reference scale. Cum. variance: Cumulative variance; variance expl. : Percentage of variance explained, proportional to the total variance explained by the four components. *variables with the highest weights in each component.

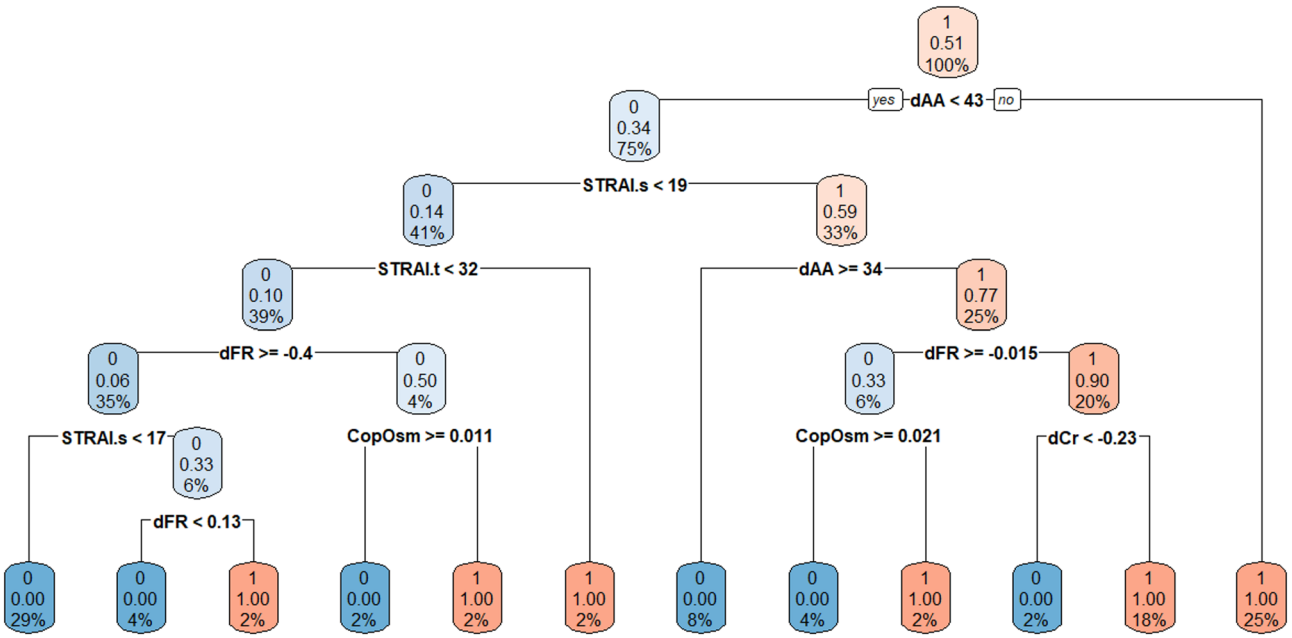


Fig. 2. Decision tree model obtained for stress prediction. Label 0 indicates the relaxed state (RS in blue), label 1 indicates the stress state (SS in red); dAA (difference in salivary α-amylase concentrations between stages, ΔAAsl (U/ml)), dCr (difference in salivary cortisol concentrations between stages, ΔCrsl (ng/ml)), dFR (difference in salivary flow rate between stages, ΔFRsl (ml/min)), CopOsm (plasma Copetin/plasma Osmolarity (mOsm/L)), STRAI.s (STAI-s), and STRAI.t (STAI-t)). The data were generated via RStudio for Windows.

projection (VIP) score > 2[29] and variation coefficients (CV%) below 20% to avoid subjectivity in the selection process, both for RS and SS in ESI (+), are shown in Table 3. Most of the signals obtained in ESI (+) mode presented significantly altered blood levels ($p < 0.05$) of various amino acids and related metabolites (serine, indole, alanine, phenylalanine, valine, histidine, and N-acetyl glutamine), altered sterol and steroid hormone biosynthesis (hydrocortisone, aldosterone, corticosterone, 11-deoxycorticosterone (DOC), progesterone, pregnenolone, cholesterol, 17α-hydroxypregnenolone, 11-deoxycortisol, 17-deoxycortisol, 17β-estradiol, and estrone), and catecholamine neurotransmitters (dopamine, norepinephrine, and epinephrine) (Table 3). The remaining significantly altered metabolites in SS corresponded largely to fatty acids and cellular membrane components (isobutyrate, choline, glycerophosphocholine, and lysophosphatidylcholine (LPC)), sucrose sugars, and muscle-related metabolites (creatine and carnitine). Even so, the most predominant metabolites in RS included tyrosine, tryptophan, and its derivatives (the neurotransmitter serotonin, the neurotoxin quinolinic

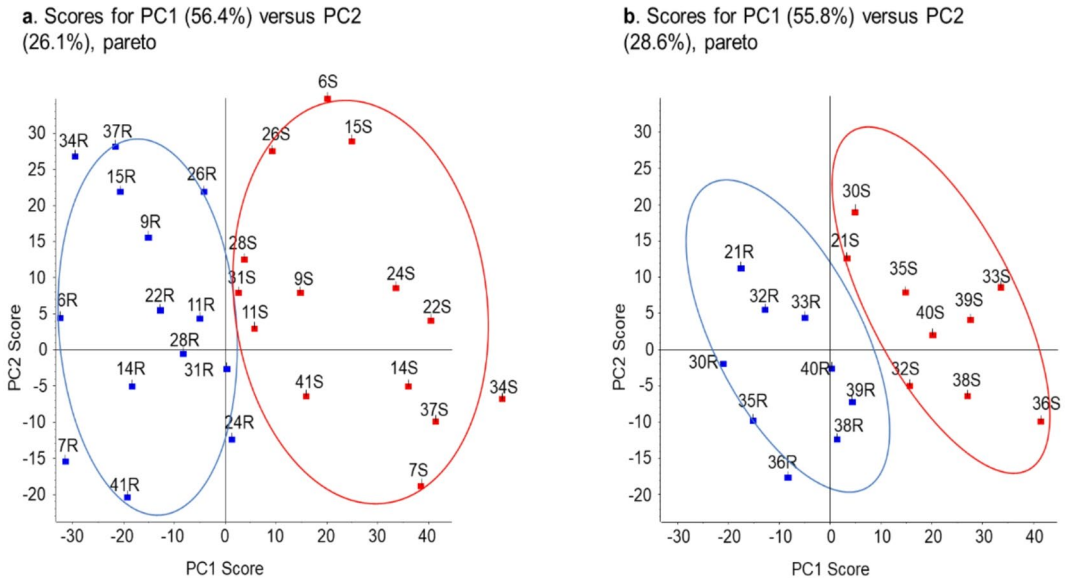


Fig. 3. Score plot of principal component analysis (PCA) of metabolomic data acquired in the ESI (+) (**A**) and ESI (−) (**B**) modes. Each dot represents a blood sample. The samples obtained after the relaxed state (R) are in blue, and those obtained after stress induction (S) are in red.

acid, and the hormone melatonin), derivatives of nitrogenous bases of nucleic acids (hypoxanthine and 2,4-dihydroxypyrimidine), and derivates of the B3 vitamin N-methylnicotinamide (NMN) (Table 3).

Analysis of blood samples in ESI (−) mode revealed 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 in 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 ($p = 4.14e{-}02$) (Fig. 4).

Discussion

In this study, a modified version of the Trier Social Stress Test (TSST-M) was used to induce acute stress in a cohort of 40 university students. We found significant differences between RS and SS in psychometric tests (STAI-s, VAS), SSC, and the biochemical markers AA_{sl}, FR_{sl}, Cp_{pl}, and Pr_{pl} (Table 1). These results confirmed that stress was successfully induced, in agreement with other studies that used the TSST[31,32]. While we anticipated a significant increase in salivary cortisol (Cr_{sl}), no significant difference was found, even though previous studies have shown that cortisol levels typically increase following induced stress[33,34]. This discrepancy could be attributed to the dynamics of cortisol production and saliva detection. Whereas α-amylase is released directly into oral fluid from salivary glands in response to the activation of the SNS, cortisol is first secreted from the adrenal glands into the bloodstream and then passively diffuses into saliva. This process results in a delay of up to 15–20 min before cortisol reaches its peak concentration in saliva in comparison with that of α-amylase[35]. Since saliva sampling was performed after completion of the stress-induction session (25 min after initiation), the 15–20 min peak window for Cr_{sl} may not have been fully captured (Fig. 5). Nevertheless, our metabolomic analysis identified cortisol as a relevant blood biomarker of acute stress, with significant changes in its concentration distinguishing RS from SS (Table 3). Psychometric tests were standardized and administered in a controlled, distraction-free environment, minimizing potential bias.

Concerning sex differences, and within the limitations of this study, significantly higher glucose and copeptin plasma levels were observed in men, in line with findings by Spanakis et al.[31]. This result supports the hypothesis that the response of the HPA axis to acute psychological stress varies by sex, according to previous studies[31,36]. These findings suggest that the risk of suffering from different diseases as a result of stress may vary by sex. However, further research is needed to elucidate such sex-based disparities.

To reduce the multiple dimensions of psychological stress into its main components, we performed a PCA. The top four out of seven components explained 84% of the variance. The first component correlated most strongly with psychometric tests, reflecting variation in the quality of individuals' psychological states produced by the stressors. The second component was associated with SNS activation (involving ΔAA_{sl} and FR_{sl} changes), whereas the third and fourth components were linked to PA axis activation (Table 2). They likely appeared as separate components because Cp_{pl} and Pr_{pl} are secreted from different sources (the posterior and anterior pituitary, respectively). These results highlight the close interaction between the SNS and the HPA axis in eliciting the stress response. By integrating these parameters into the SRS scale[26], we support its utility in quantifying the level of stress perceived by an individual[27]. The scale, however, remains to be validated by additional studies.

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-estradiol[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

Table 3. Differentially abundant metabolites in positive mode (ESI (+)) after relaxation (RS) and stress (SS) stages. 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, considering significant values of $p \leq 0.05$. *Δm*: mass error expressed in ppm. CV: coefficient of variation was 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: Nacetyl glutamine; GPCh: glycerophosphocholine; LPC: lysophosphatidylcholine. [a]previously published in a preliminary report by Lorenzo-Tejedor et al.[23].

The predictive models built via machine learning techniques (decision trees, logistic regression, and random forest classifiers) exhibited a high level of robustness in determining the stress state of the subject (Fig. 2). The risk of model overfitting is acknowledged in the Limitations. Consistently, all our models identified the ΔAA_{sl} and STAI-s as the main predictive biomarkers of acute psychological stress status. These findings support the importance of AA_{sl} as a key biomarker in evaluating stressors that activate the SNS, which is in agreement with previous research reports[37,38]. However, it is important to note that AA_{sl} levels, like all other variables, may be

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

Table 4. Differentially abundant metabolites after stress induction in negative mode (ESI (−)). 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.

influenced by a variety of factors, such as exercise and medication[39]. In the case of the random forest model, FR_{sl} was identified as an additional significant predictor of stress status. Although our models showed high predictive accuracy and the sample size of 40 was sufficient to provide adequate statistical power (Supplementary Fig. S1), the homogeneous cohort reduces the generalizability of our findings to a broader population. Despite these limitations, the present study still provides meaningful insights and a sound basis for future investigations.

With respect to the metabolic signature of acute psychological stress explored here, our results are in line with those of previous studies that documented significant changes in the metabolomic profile in both animal models and humans subjected to different stressors[40–42]. In the PCA plots of the metabolomic data, two clusters were clearly distinguished, indicating that RS and SS samples had markedly different metabolic compositions (Fig. 3). A total of 53 significantly differentially abundant metabolites ($p < 0.05$, VIP > 2) were identified in both the ESI (+) and ESI (−) ion modes. Of these, 9 were predominantly associated with RS, whereas 44 were instead associated with SS. These findings suggest that acute psychological stress generates extensive changes across multiple metabolic pathways involved in an organism's adaptive response. Prolonged stress-induced alterations can have detrimental effects on health. As a result, chronic psychological stress is recognized as a serious risk factor for cardiovascular diseases and metabolic disorders[42].

Notably, one of our most valuable findings was the significant changes in the lipid profile induced by acute mental stress, particularly the substantial increase in fatty acids, polyunsaturated fatty acids (PUFAs), phosphocholines (PCs), plasmalogens (PPCs and PPE), and lysophosphatidylcholines (LPCs) (Table 4). Recent studies have indicated that these lipids and lipid-like molecules play critical roles in cell signaling pathways related to inflammation, immunity, and apoptosis[42,43].

The increases in PPC and PPE levels observed may be attributed to an increased demand for plasmalogens (PPs) in the brain under acute stress conditions to maintain adequate neural function, promote synaptic plasticity, and protect against stress-induced oxidative damage. Several researchers have proposed that PPs, particularly 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[44,45]. However, when stress becomes chronic, this adaptive mechanism is reversed, leading to a decrease in PP levels, which has been associated with degenerative disorders and neurocognitive impairments[42,43].

In addition to the increased PP levels in SS, we also observed elevated levels of LPC. This finding is consistent 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[42]. LPCs are generated through the cleavage of phosphatidylcholine, a major phospholipid in the cell membrane, by phospholipase A_2 (PLA_2), which releases free fatty acids such as arachidonic acid. The observed increase in LPC levels may therefore reflect the body's adaptive complex response to stress, involving PLA_2 activation by mitogen-activated protein (MAP) kinase-related kinases, a family of stress-activated protein kinases[46,47].

The function of LPCs depends on the length and degree of saturation of the fatty acid chain attached to the glycerol moiety[48]. For example, elevated levels of LPC (18:0) and related PPs, PPC (18:0/20:4) and PPC (P18:0/22:6), have been associated with reduced inflammation, lower adiposity, and a decreased risk of

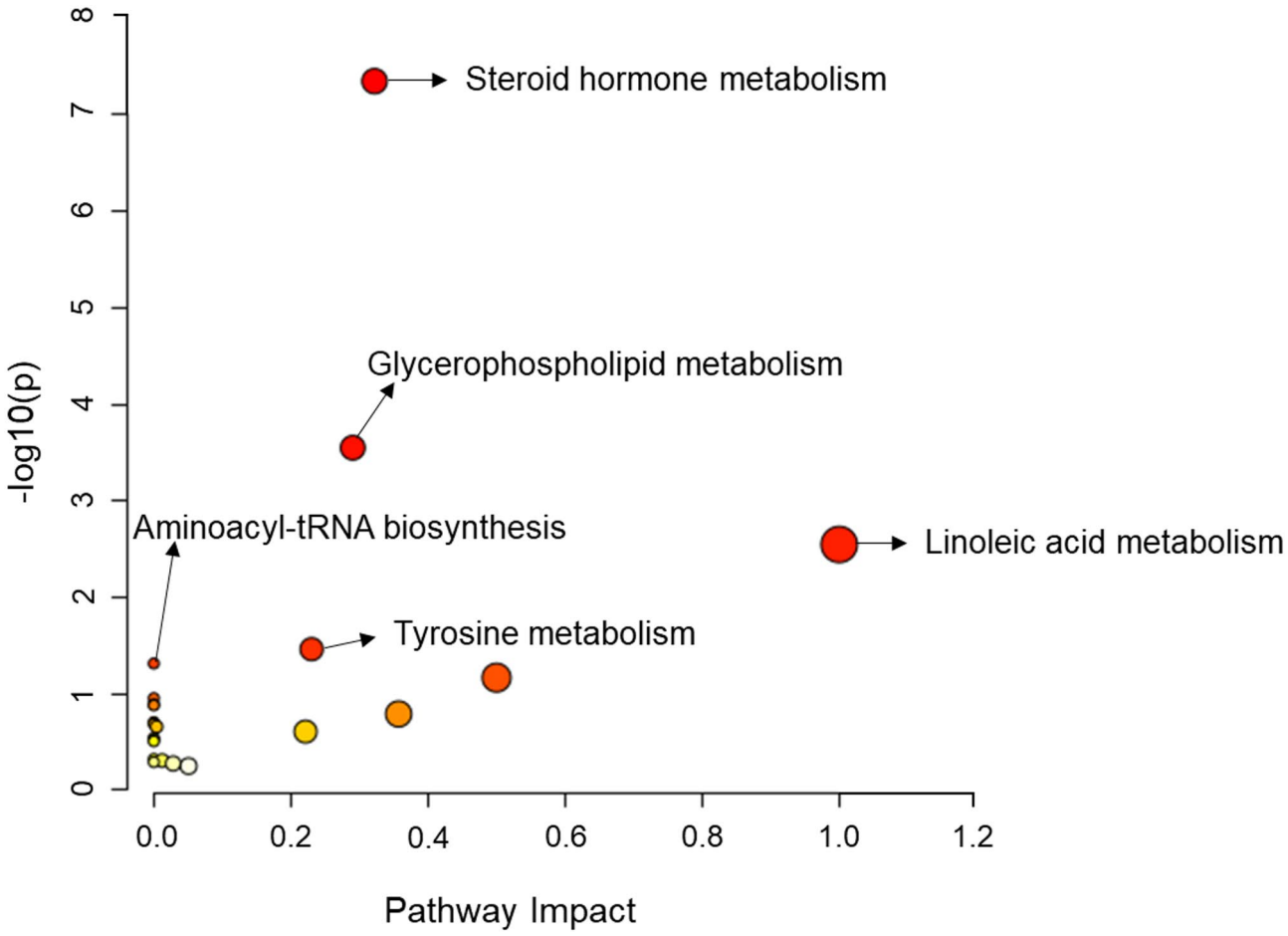


Fig. 4. Metabolic pathways altered after psychological stress induction. Dots represent the affected pathways. Y-axis shows the log-transformed *p* value adjusted for multiple comparisons, whereas X-axis shows the pathway impact. The color indicates significance, ranging from white (not significant) to red (most significant). Dots size reflects the impact score. The figure was generated via *MetaboAnalyst 5.0*[30].

cancer[42,48]. On the other hand, LPCs, such as 18:1 and 20:4 LPCs, exert their biological effects by activating many downstream signaling pathways, including MAP kinase and nuclear factor kappa B (NF-κB) pathways. These pathways promote cell division, chemotaxis, oxidative stress, inflammatory cytokine release, and apoptosis, thereby accelerating the development of atherosclerosis[48]. Additionally, LPC (20:4) is associated with the stress index, and its free fatty acid arachidonic acid (20:4) has been suggested as a marker of depression and stress in humans[42,49].

Another predominant metabolite found under acute stress conditions was linoleic acid (18:2-n6) (Table 4), the most abundant 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[50]. 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, leading to a reduction in its hypocholesterolemic effect[51,52], followed by the consequent accumulation of arachidonic acid. Additionally, LA can undergo nonenzymatic oxidation to produce Oxlams, metabolites that have been shown to promote a strong proinflammatory response in rats[50].

An elevated level of cholesterol in SS, such as that observed here (Table 3), may lead to the generation of a variety of corticosteroids via steroidogenesis. Owing to their lipophilic nature, corticosteroids cannot be presynthesized and stored in adrenal glands but must be rapidly synthesized upon adrenocorticotropic hormone (ACTH) stimulation, which is instead regulated by the HPA axis [53]. Corticosteroids regulate multiple physiological processes, including metabolism, development, homeostasis, cognition, and inflammation[53]. Corticosteroids such as cortisol increase the bioavailability of glucose and the consequent release of energy to the brain[53], as evidenced by the increased levels of carnitine, creatine, and glucogenic amino acids observed in this study, supporting the findings of Singh et al.[40]. Additionally, these amino acids could also serve as substrates for the synthesis of proteins required for the stress response process[54].

Each stressor has a neurochemical signature with distinct central and peripheral mechanisms[55]. In contrast, some studies have demonstrated that the two branches of the sympathoadrenal system (SAS), the adrenal

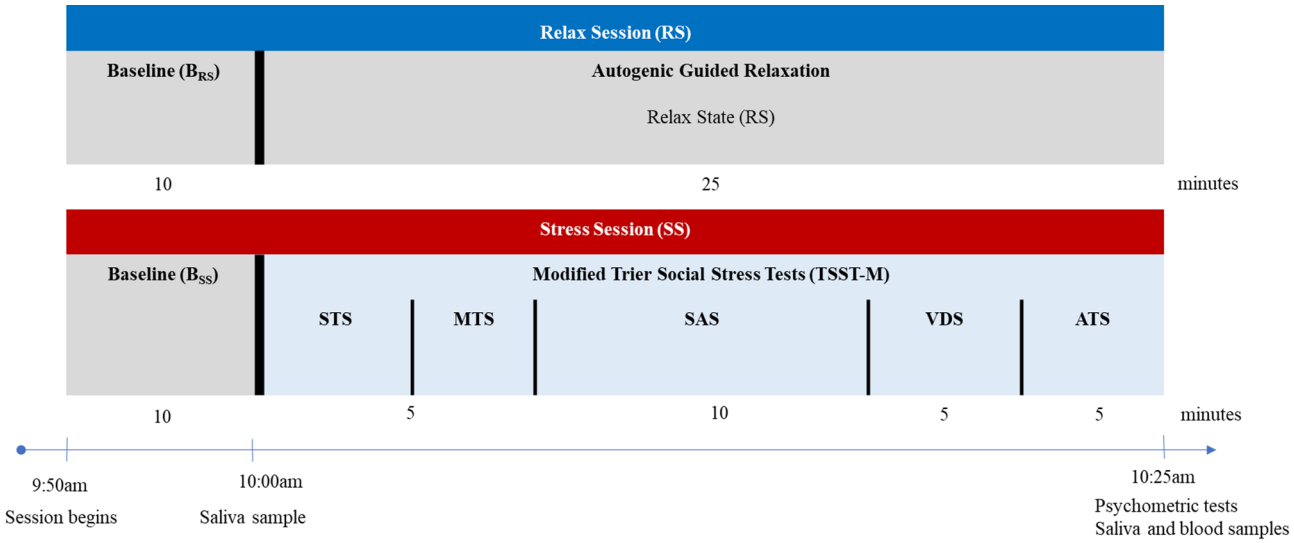


Fig. 5. Schematic representation of the research approach. The stress induction/relaxation protocol steps and sample-collection timeline are shown. **Instructions**: Participants were asked to wake up at least 2 h before the sessions; have a light caffeine-free breakfast; refrain from exercising; take psychotropic substances; and consume alcohol, tobacco, or any other mood-altering substances 24 h before the session. Four saliva samples were collected: two after each baseline stage (B_{RS}, B_{SS}) and two at the end of each session (RS, SS). At the end of each session, blood samples for biochemical and metabolomic analysis and psychometric tests were administered. For TSST-M, a series of stressful tasks—the storytelling stage (STS), memory test stage (MTS), stress anticipation stage (SAS), video display stage (VDS), and arithmetic task stage (ATS)—were administered.

medulla and the sympathetic nerves, can be activated independently by different stressors[55,56]. Nonetheless, our study indicated that acute psychological stress induced by the TSST-M activated both components of the SAS. This stimulates the adrenal medulla system, elevates plasma Epi levels, and activates the sympathetic nervous system, increasing NE and dopamine plasma levels (Table 3).

Epi is known as the hormone that prepares the body for a fight-or-flight response[57]. 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[58]. Dopamine (DA) is a key neurotransmitter that regulates many processes in the CNS, including reward, motivation, and cognition. Importantly, DA can also be produced locally in several peripheral organs, where it has autocrine and paracrine effects influencing many organ functions[59,60] and is released in plasma in response to stress. This response is partly influenced by circulating cortisol levels in the body[61,62]. Moreover, DA regulates critical functions such as metabolic homeostasis, hormone release, sodium balance, blood pressure, renal activity, and gastrointestinal motility. It also modulates inflammatory and immunological processes[59,60]. Prolonged exposure to intense stressors may inhibit DA release and thus disrupt the dopaminergic pathway, leading to psychological disorders such as depression and schizophrenia[63,64].

The elevated levels of cholesterol, steroid hormones, and adrenal catecholamines observed in this study could be explained accordingly by the increase in prolactin, known as *a stress hormone,* along with cortisol. There is substantial evidence supporting the multifaceted role of prolactin in the adrenal response to stress[65]. More specifically, it has been shown to increase the secretion of ACTH, enhance the storage of cholesterol esters, and induce adrenal hypertrophy[65–67]. Under acute stress, prolactin secretion appears to play a crucial and complex role in maintaining metabolic and immune system homeostasis[67–69]. 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[70].

Interestingly, we identified several metabolites that the literature suggests may have protective effects during acute stress. For example, progesterone and pregnenolone (Table 3) are known to suppress HPA activity, thereby reducing stress levels[71,72]. Additionally, caprylic and capric acids (Table 4) have been identified as possessing anti-inflammatory properties, which counteract the inflammatory process often associated with stress[73,74]. Furthermore, 17β-estradiol and estrone (Table 3) have been shown to play neuroprotective roles against stress-related damage[75,76]. 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, the TSST-M was used to induce acute psychological stress and explore multifaceted stress responses through the integration of psychometric assessments, biochemical analyses, and metabolomic profiling. Our findings provide preliminary evidence of sex-related differences in the stress response, particularly in glucose and copeptin plasma levels, further suggesting that stress may affect men and women differently. These observations support the importance of considering sex-sensitive approaches in future stress research.

Our exploratory results also point to the potential utility of the stress reference scale and machine learning prediction models for distinguishing stressed from relaxed states in individuals. Specifically, they present AA_{sl} and STAI-s as promising markers and support the use of direct infusion MS as a minimally invasive method suitable for metabolomic analysis in this context[23].

Within the limits of this study, acute psychological stress appeared to significantly influence several metabolic pathways, reinforcing the possibility of metabolomic profiling as a useful tool for investigating stress-related processes. However, given the relatively small and homogeneous sample, these findings should be regarded as exploratory, requiring validation in larger and more diverse samples to understand the intricate interplay between physiological and psychological domains in acute mental stress responses and clarify the role of the identified altered pathways and biomarkers in stress-related disorders.

Limitations and future directions

This study has several limitations, as mentioned above in the corresponding sections. Most importantly, the relatively small sample size and the focus on a homogeneous group of healthy university students limit the generalizability of the findings. A further limitation is the absence of an independent control cohort, which substantially weakens causal inference and reduces the external validity of our results. Nevertheless, the within-subject repeated-measures design is a key strength of the study, as it allowed each participant to serve as their own control. This minimized inter-individual variability (e.g., genetic, physiological, and lifestyle factors) and increased statistical power with a modest sample size, thereby enabling sensitive detection of dynamic changes in psychological and biochemical stress markers within the same individuals.

Despite these advantages, this design also has limitations. The fixed order of sessions (baseline → relaxation → baseline → stress) raises the possibility of carry-over effects, although the two-week interval between sessions was intended to reduce fatigue and practice influences. Repeated testing may still have introduced learning or adaptation effects, and the lack of follow-up sampling prevents conclusions about the long-term dynamics of the stress response.

Unmeasured variables such as diet, sleep, or hormonal fluctuations, due, for example, to the menstrual cycle phase of female participants or the use of contraceptives, could have influenced the results, specifically variations in prolactin, estrogen, progesterone and other hormone levels. However, the strict focus on acute stress responses over a narrow timeframe minimizes the impact of cyclical hormonal fluctuations. Similarly, since the study evaluated the variation (Δ) between pre- and post-relaxation/stress induction, the prevalence of regular medication use (e.g., antihistamines or bronchodilators) was considered not relevant.

Taken together, our work should be considered preliminary. Within-subject design and a standardized baseline relaxation were used to mitigate short-term hormonal variability; however, menstrual-cycle phase was not stratified and follow-up sampling was not performed. It provides baseline parameters for future research that will be needed to confirm the relationship between the biochemical, metabolic, and psychometric stress measures proposed here. Validation in larger and more heterogeneous samples will increase the generalizability of our findings and further establish the diagnostic and measurement tools introduced.

Materials and methods

Study design

To ascertain the effects of acute psychological stress on biochemical, psychological, and metabolomic variables, we conducted an experimental cross-sectional study in a cohort of university volunteers. Each participant was evaluated under both a relaxed condition and an acute stress condition, allowing individuals to serve as their own control, thereby reducing inter-individual variability and increasing the sensitivity to detect stress-related changes. The study, designed and performed under the ES3-P[19,23,26,27] framework, included two sessions: a 35-min relaxation session (RS) as a control condition, followed by a 35-min stress-induction protocol based on a modified form of the Trier Social Stress Test (TSST-M) previously described by Arza et al.[19], yielding acute psychological stress stage (SS). Protocol details are summarized in Fig. 5.

Sample size calculation

To determine the minimum number of participants required for adequate study power in our within-subject (paired) experimental design, we employed a standard parametric approach for a paired *t* test[77,78]. A two-sided significance level of $\alpha = 0.05$ and a statistical power of $1\text{-}\beta = 0.8$ were assumed. The calculation was performed via Eq. (2):

$$n = \frac{(Z_{1-\alpha/2} + Z_{1-\beta})^2 \cdot \sigma_d^2}{\Delta^2} \tag{2}$$

where Δ is the minimum clinically relevant mean difference and where σ_d is the standard deviation of the within-subject differences (SS-RS). When the standard deviation (σ) of each condition and the correlation between measures (r) are known, then (Eq. (3):

$$\sigma_d = \sigma\sqrt{2\,(1 - r)} \tag{3}$$

Assuming a moderate correlation between measures ($r \approx 0.5$) on the basis of recommendations for paired designs without prior data and $\sigma_d \approx \sigma$, it was estimated that a total of approximately 30 participants would be sufficient to detect significant differences in our continuous variables, with a small allowance for participant withdrawal from the study.

Participants and ethical declaration

Volunteers aged 20–30 years (both sexes) were recruited from the University of Zaragoza. The exclusion criteria included the following: (1) signs of depression or a history of other mental disorders; (2) regular use of psychotropic substances; and (3) pregnancy or breastfeeding at the time of the study (see Supplementary Table S1 for participant details). All participants were informed about the study procedures and provided written informed consent. This documentation is securely archived at the Psychiatric Unit, HCULB, in compliance with the EU's General Data Protection Regulation. The entire study was conducted in accordance with the World Medical Association (WMA) Declaration of Helsinki (2013) and was approved by the Clinical Research Ethics Committee of Aragon (CEICA; protocol number PI14/0044).

Stress induction and relaxation protocols

The sessions were carried out on different days but at the same hour, approximately 10:00 AM, to avoid variations in the circadian rhythm[79]. The RS comprises a baseline (B_{RS}) and relaxation stage (R_{RS}), whereas the SS comprises a baseline stage (B_{SS}) and five distinct stages to induce acute psychological stress[27]. For the RS, 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 following Schultz's method[80]. The SS followed a TSST-M, which is a robust, reliable, and well-documented protocol widely used in stress research[31,32,79,81–83], with slight modifications described in[19] to induce social and cognitive stress. The stress session consisted of storytelling (STS), a memory test (MTS), stress anticipation (SAS), a video display (VDS), and an arithmetic task (ATS) (Fig. 5).

At the end of each session, RS and SS, the participants were required to complete psychometric questionnaires. Saliva samples were collected at the beginning (after the baseline stages, B_{RS} and B_{SS}) and again after RS and SS sessions, with approximately 25 min between collections, corresponding to the duration of each session. These paired samples were used to calculate cortisol (ΔCr) and α-amylase (ΔAA) variation per session. Salivette tubes were used to collect saliva following the manufacturer's recommendations (Sarstedt AG & Co., Nümbrecht, Germany). The samples were immediately preserved on ice and subsequently frozen at − 20 °C until processing, according to the protocol previously described by Garcia Pages et al.[27] in the Endocrinology and Radioimmune Analysis Service of Neurosciences Institute at the Universitat Autònoma de Barcelona (UAB).

Blood and plasma samples were collected only after RS and SS sessions by professional hospital staff. The extraction of blood and plasma for biochemical marker determination was carried out in two tubes: one with the anticoagulant ethylenediaminetetraacetic acid (EDTA) and the other with a gel separator to obtain the serum. Both were preserved on ice until they were centrifuged in the laboratory at 3000 rpm for 10 min. Plasma and serum were then separated into fresh and sterile tubes with the identifying data of the subject, type of session and date. These tubes were kept frozen at − 20 °C until processing at the Core Laboratory of Biochemistry and Molecular Genetics, part of the Biomedical Diagnostic Center (CDB) at Barcelona Clinic Hospital.

Blood samples for metabolomics analysis were taken by pricking the participants' fingers, and approximately 0.3 ml was collected in a sterilized container tube with no chelating agent. These samples were immediately protected from light and stored at − 80 °C until analysis at the Proteomics Core Research Facility of the Aragon Health Sciences Institute (IACS-CIBA). No blood sample underwent any pre-treatment prior to mass spectrometry analysis.

Stress evaluation and measurement: psychometric evaluation

Professionals from the ZARADEMP group, based in the Psychiatric Service (HCU-LB) and the Department of Medicine and Psychiatry (University of Zaragoza), selected the tests, verified the corresponding Spanish versions, and interpreted the results (Data collection notebook published via Zenodo)[84]). Several coordination meetings were held before the start of the study to standardize the administration and interpretation criteria. Before administering the psychometric questionnaires, the participants were asked to indicate their perception of their stress levels (perceived stress) on a scale of 0–100 arbitrary units (Supplementary Table 1). All questionnaires were self-administered and completed in a dedicated quiet room at the Research Psychiatric Department of the University Clinical Hospital. Sessions were monitored to ensure that no interferences occurred during administration other than those required by the study protocol and that there was no time limit for completion.

This team also applied a test designed by themselves on behalf of the ES3 project, 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 and applied in a recent study by Garcia Pages et al.[27]

The validated psychometric tests used were the Spanish versions of the Perceived Stress Scale (PSS)[85], the visual analog scale (VAS), and the State-Trait Anxiety Inventory (STAI)[86]. The PSS is widely used to assess stress levels in young people and adults[87]. It evaluates the degree to which an individual perceives life as unpredictable, uncontrollable, or overloading. The VAS measures subjective stress on a numeric scale ranging from 0 to 100[88]. 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[89,90]. 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)[91].

Measurement of biochemical variables

All of the samples were processed in the same batch to avoid any inter-test variability, achieving an intra-test CV < 5% in all the cases. Serum glucose (Glu_{sr}) was quantified via a glucose oxidase-based enzymatic assay on an ADVIA Chemistry 2400 system (Siemens Healthcare Diagnostics, Erlangen, Germany) at 505/694 nm. Plasma copeptin (Cp_{pl}) was measured with a sandwich enzyme immunoassay kit (Cloud Clone Corp., TX, USA), with a lower limit of detection of 2.9 pg/ml, an intra-assay CV < 10% and an inter-assay CV < 12%. The plasma prolactin

(Pr_{pl}) concentration was determined via an immunometric ELISA kit (Cayman Chemical, MI, USA), which has a minimum detectable concentration of 0.12 ng/ml, an intra-assay CV of 2.8–3.71%, and inter-assay CV of 4.6–5.49%.

Concentrations of salivary cortisol (Cr_{sl}) and salivary α-amylase (AA_{sl}) were quantified via commercial kits from Salimetrics (Salimetrics, State College, PA, United States). Cr_{sl} was measured with a competitive ELISA (catalog #1–3002). The activity of the AA_{sl} enzyme was quantified via a kinetic enzyme assay (#1–1902)[27], which employs a chromogenic substrate, 2-chloro-p-nitrophenol linked to maltotriose. Enzymatic cleavage releases 2-chloro-p-nitrophenol, as measured spectrophotometrically at 405 nm. Changes in salivary cortisol (ΔCr_{sl}), α-amylase (ΔAA_{sl}), and flow rate (ΔFR_{sl}) were calculated as the difference between baseline values (B_{RS} or B_{SS}) and those obtained at the end of the RS or SS stages, respectively.

Stress reference scale

The stress reference scale (SRS) was proposed by Garzon-Rey et al.[26] as a reference standard for measuring acute emotional stress. Significant biochemical and psychometric parameters were used to compute the scale via 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 via Eq. (4):

$$y = \frac{100*(x-Min+\sigma*0.5)}{(Max-Min+\sigma)} \tag{4}$$

where the variable (x) with a standard deviation (σ), minimum (Min), and maximum (Max) values are transformed into a variable (y) ranging from 0–100. Afterwards, principal component 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 via IBM® SPSS® Statistics 25.0 and RStudio for Microsoft Windows, along with the corresponding packages available on the 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 analyzed via the Wilcoxon signed-rank test, a nonparametric statistical test, because the data were not normally distributed after testing for normality via the Lilliefors test. Correlations were computed via Spearman's rank correlation for nonparametric distributions. For all analyses, the significance level was set at $\alpha = 5\%$.

The variables were passed on to create predictive models. Categorical variables were encoded as factors. The grouping RS or SS was considered the response variable for the models, and the other variables were considered 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[92] was used to build a bagging prediction model, and the *random forest* algorithm software package (https://cran.r-project.org/web/packages/randomForest/index.html) was used to obtain the variable relative importance rankings of the variables. We used 70% of the original data as a training set and the remaining data 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 semiquantitative direct-infusion mass spectrometry (DI-MS) untargeted metabolomic study was conducted to characterize biochemical responses to acute psychological stress and as a biomarker development tool. This innovative technique, involving direct injection into the ionization source of the mass spectrometer without prior chromatographic separation with an electrospray ionization (ESI) source, already presents proven advantages and robust results[23,93,94].

Blood samples were collected by pricking the participants' fingers before and after each session (Fig. 5). Approximately 0.5 mL of total blood was collected into an empty and sterilized Eppendorf™ tube. No anticoagulants were used. The samples were immediately protected from light and stored at -80°C until analysis. Sample preparation was carried out as previously described[23].

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, the sample was diluted 1:1000 with MS-grade dichloromethane (Fluka):methanol (ratio of 1:1). The samples to be analyzed were pumped directly into the mass spectrometer.

Measurements were taken in both positive and negative modes via a hybrid triple quadrupole/linear ion trap mass spectrometer 4000 QTRAP LC/MS/MS System (AB Sciex) with an 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 preprocessing were carried out via Analyst® software version 1.5.2 (Build 5704) (Sciex) as previously described[23]. 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-charge ratio (m/z) peaks. To ensure the quality and reliability of the metabolomic data, several analytical quality control parameters were systematically monitored during the DI-MS runs.

Analytical quality control: intra-batch precision was assessed via repeated measurements (n = 5) of a standard reference sample included within each batch[95]. Coefficient of variation (CV) across replicates was < 7% for the major peaks analyzed. Inter-batch precision was evaluated by including a pooled sample composed of aliquots from the study samples as a control in each analytical sequence. The inter-batch CV of the main compounds was < 10%. The recovery rate was assessed by spiking a random subset of samples with internal standards of selected metabolites (amino acids and lipids). Recovery ranged from 82 to 115%. CVs and RRs obtained from the analytical controls were within internationally accepted ranges for untargeted, semiquantitative metabolomics studies[95].

Data normalization, statistical and functional analyses, and compound identification were performed following the protocol previously described by Lorenzo et al.[23].

Enrichment and pathway topology analyses were performed via the corresponding modules of MetaboAnalyst 5.0[30] and categorized with the KEGG pathway *Homo sapiens* database[96,97]. Pathway enrichment analysis allowed the identification of those pathways significantly affected by the stressor and thus improved our understanding of the impact of acute psychological stress on an individual's metabolism.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

G.A.F. and G.G. performed the formal analysis of the psychometric and biochemical data, wrote the computer code and algorithms for the machine learning statistical analysis, and conducted the literature review. They also drafted the original manuscript and prepared the tables. G.A.F. generated Figs. 1 and 4, analyzed sex differences, performed enrichment and pathway topology analyses of the metabolomic data, and contributed to formatting the manuscript according to the journal's style sheet. G.G. and E.M.R. wrote the final sections (Discussion and

Conclusion), thoroughly reviewed the manuscript, and assembled the final version by incorporating minor corrections, rephrasing, and restructuring content to improve conciseness, clarity, fluency, and readability. G.G. also generated Fig. 2; was responsible for conceptualization, validation, and visualization; and oversaw and completed the entire submission process. M.L.T., C.D.L.C., J.A., R.B., and M.B. designed the study. M.L.T. performed the formal analysis for the metabolomic study and generated Fig. 3 and Tables 3 and 4. C.D.L.C. conducted stress and relaxation sessions, administered psychometric tests, coordinated the fieldwork, and compiled the results. E.M.R. contributed to the cognitive component of the manuscript, assisted with the overall review process, and revised the English grammar, terminology, and phrasing throughout the final version. J.L. managed the database registry and contributed to the design of the metabolomics study. R.B. (principal investigator) and J.A. (coinvestigator) supervised project development and managed project funding. M.B. collected and prepared the biological samples for analysis, supervised and coordinated the study, contributed resources, and collaborated on the literature review. All the authors reviewed 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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