

Persistent low-grade inflammation, SARS-CoV-2 infection and mental health deterioration signs impact on systemic levels of aminoacid neurotransmitter precursors

Supplementary Material

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Supplementary Methods

Software

Proteome and metabolome data were analyzed with R version 4.2.0. General data transformation tasks were accomplished with the *tidyverse* package bundle (1), *rlang* (2) and the development package *trafo* (<https://github.com/PiotrTymoszek/trafo>). Statistical data testing was done with the packages *rstatix* (3), *ggpubr* (4) along with the development package *ExDA* (<https://github.com/PiotrTymoszek/ExDA>). In linear modeling, base R functions, the development packages *lmqc* (<https://github.com/PiotrTymoszek/lmqc>) and *caretExtra* (<https://github.com/PiotrTymoszek/caretExtra>), and the package *caret* (5) were utilized.

Results were visualized with tools provided by the packages *ggplot2* (6), *cowplot* (7) and *ExDA*. Manuscript and supplementary tables were created with *flextable* (8). Supplementary Material file was written in the *rmarkdown* environment (9) and rendered with the *knitr* (10) and *bookdown* (11) packages.

INCOV cohort data import and transformation

Proteome and metabolome data in form of normalized, age- and sex-adjusted, log₂-transformed serum levels as well as clinical information (sex, SARS-CoV-2 infection status, COVID-19 severity, timepoint, post-COVID-19 syndrome status and particular symptoms) for the INCOV cohort were extracted from supplementary tables accompanying the report by Su and colleagues (12).

Characteristic of the INCOV cohorts is presented in **Table 2**. Numbers of available INCOV cohort samples and the sampling timepoints are shown in **Supplementary Table S1**.

Variable distribution and transformation

Distribution normality and variance homogeneity of normalized cytokine and metabolite serum levels was assessed by Shapiro-Wilk and Levene test, respectively. The distribution testing revealed substantial deviations from normality for multiple study parameters. For this reason statistical hypothesis testing in the INCOV dataset was done with non-parametric tests. For STIGMA cohort metabolite and inflammation marker levels (KYN, KYN/TRP ratio, PHE, TYR and PHE/TYR ratio, NEO, CRP, IL6) used in analysis of correlation with age, comparison between participants stratified by SARS-CoV-2 infection status, anxiety/depression signs or gender as well as for linear modeling, logarithm and

square root transformations was used, which improved both normality and variance homogeneity.

Statistical hypothesis testing

Comparison of normalized serum cytokine and metabolite levels between uninfected controls and COVID-19 individuals from the INCOV cohort at consecutive timepoints after symptom onset was done with Kruskal-Wallis test. Differences between SARS-CoV-2-negative controls and consecutive timepoints of COVID-19 were investigated with Mann-Whitney post-hoc U test corrected for multiple testing with Benjamini-Hochberg method (13).

Correlation of serum cytokine and metabolite levels in uninfected controls and COVID-19 individuals at consecutive timepoints in the INCOV and STIGMA datasets was analyzed by Spearman test.

Comparison of STIGMA dataset metabolite and inflammatory marker levels between participants stratified by SARS-CoV-2 infection status, anxiety/depression signs or gender was done with two-tailed T test. Correlation of STIGMA dataset metabolite levels with age was assessed by Pearson test.

Multi-parameter linear regression with backwards elimination

Effects of the representative inflammation marker (NEO), SARS-CoV-2 infection status, depression/anxiety signs, age and gender on systemic levels of amino acid neurotransmitter precursor and their decay products (TRP, KYN, KYN/TRP ratio, PHE, TYR and PHE/TYR ratio) was assessed by multi-parameter linear regression with backwards elimination.

Full models including the complete set of explanatory variables listed above were constructed (function `make_lm()`, package *lmqc*) and optimized by Akaike information criterion (AIC) driven backwards elimination of non-significant terms (method `step()`, package *lmqc*). Normality and homogeneity of distribution of the model residuals was checked by Shapiro-Wilk and Levene test, respectively (method `summary(type = 'assumptions')`, package *lmqc*) and visually inspected in standard diagnostic plots of model residuals (residuals vs fitted, quantile-quantile plots, method `plot()` called for the model objects). Fit stats (R^2 and root mean squared error [RMSE]) were retrieved from the model objects by calling `summary(type = 'fit')` (package *lmqc*). Validity of the optimized models was determined by likelihood-ratio test (LRT) versus the respective null models (method `anova()`). Reproducibility of the optimized multi-parameter models was investigated by repeated cross-validation (50 repeats, 10 folds, function `train(method = 'lm')`, package *caret*) and by comparison of the RMSE and R^2 statistics obtained with in the training dataset and in cross-validation (method `summary()`, package *caretExtra*). As

presented in **Supplementary Figure S5A**, similar values of error fit in the training and cross-validation data suggest good reproducibility of the optimized models and lack of over-parameterization.

Data and code availability

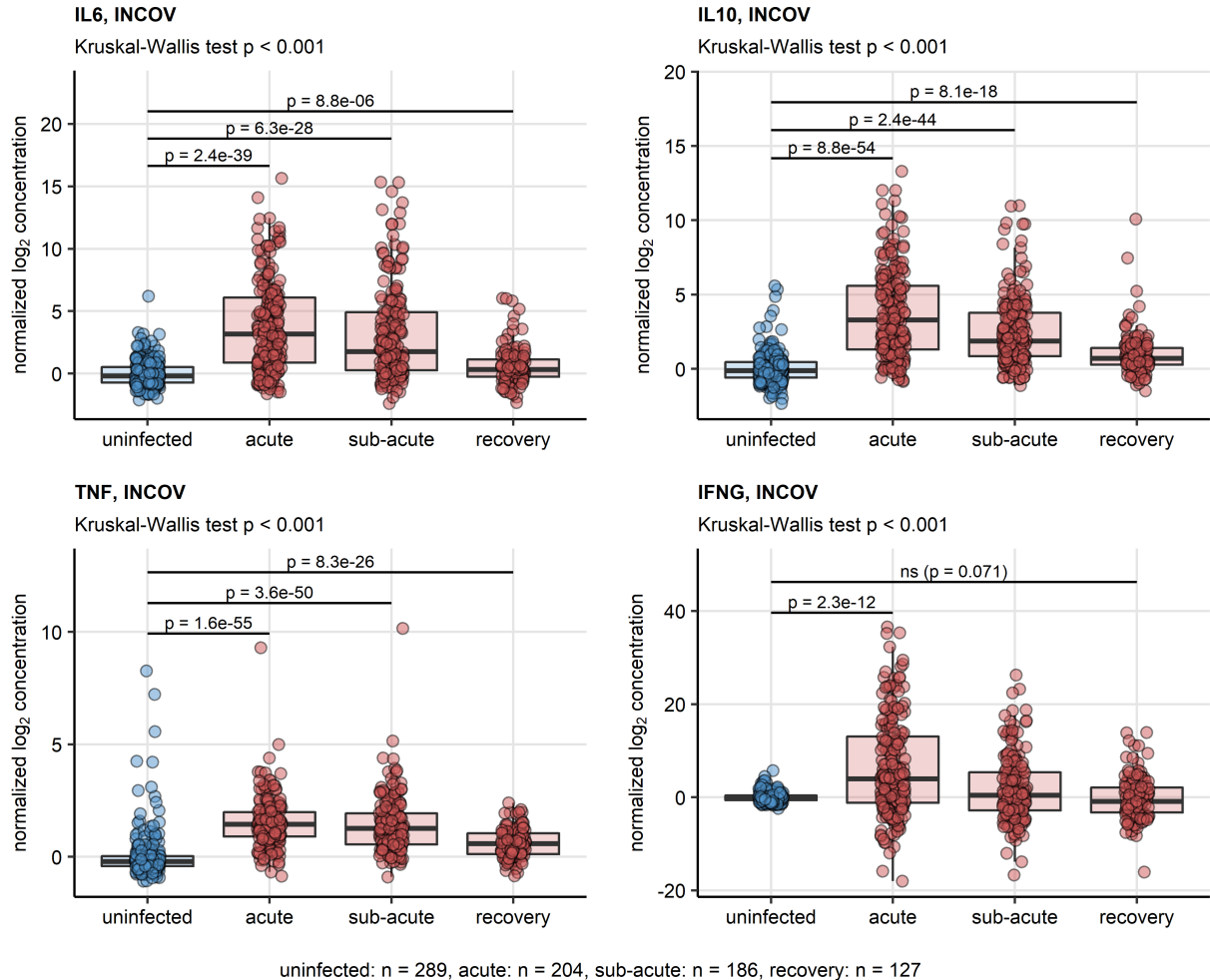
Anonymized local cohort data will be made available upon request to the corresponding author. The INCOV cohort data are publicly available. The study pipeline code is available at https://github.com/PiotrTymoszuk/stigma_validation.

Supplementary Tables

Supplementary Table S1: Number of available samples and sampling timepoints in the INCOV cohort.

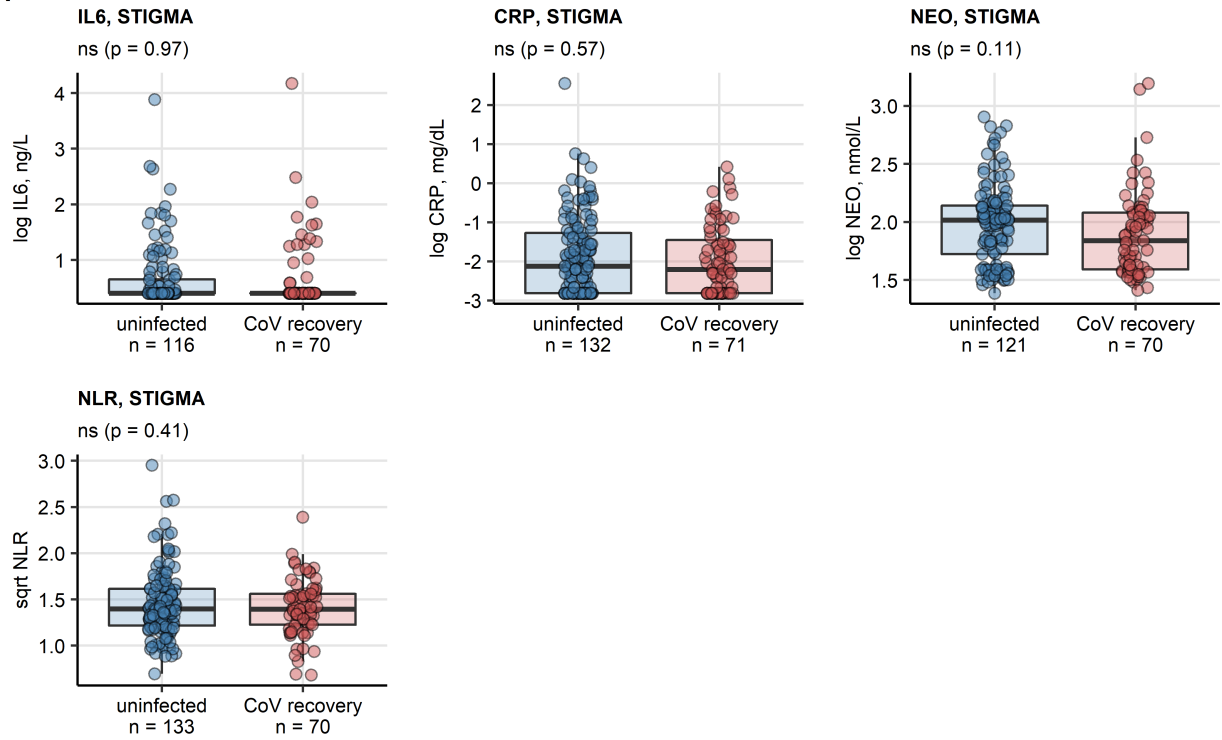
| Time point | Days post infection ^a | Sample number |
|---|----------------------------------|---------------|
| healthy | | 440 |
| acute | 11 [7.9 - 16] | 205 |
| sub-acute | 17 [12 - 23] | 187 |
| recovery | 64 [53 - 90] | 127 |
| ^a Median with interquartile range, days after symptom onset. | | |

Supplementary Figures

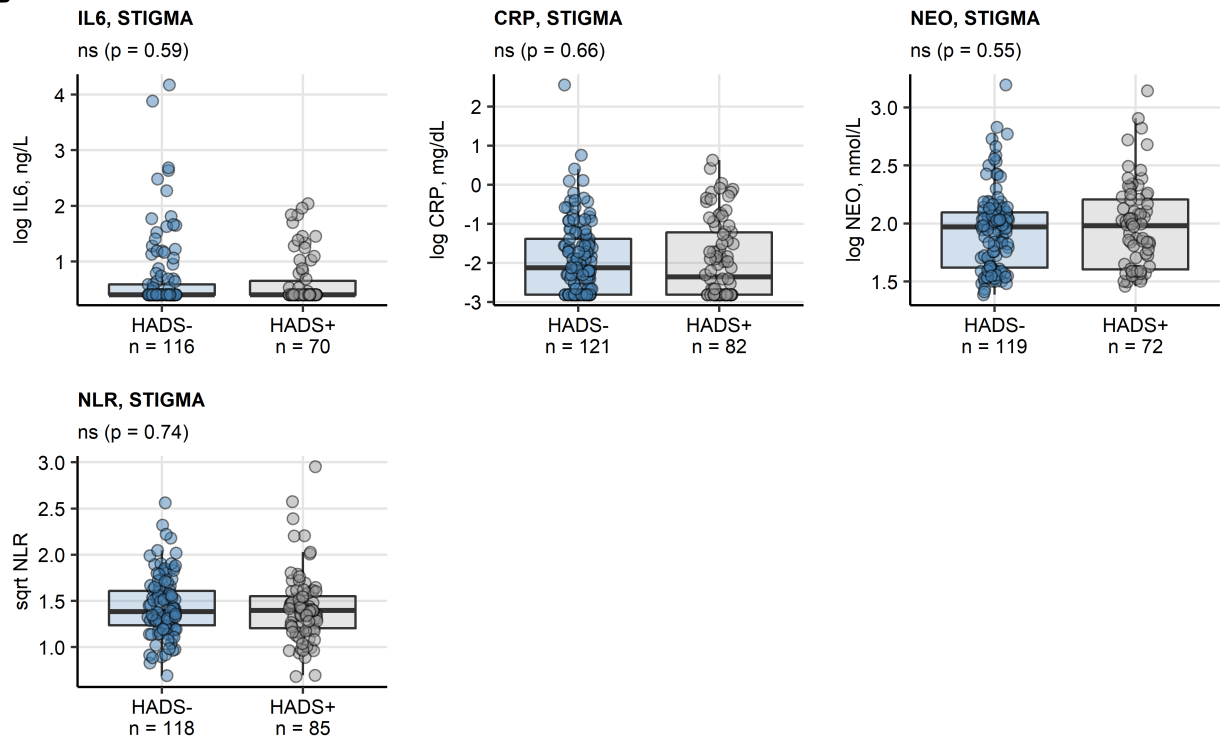


Supplementary Figure S1. Serum levels of inflammatory cytokines in course of COVID-19 and recovery. Serum levels of interleukin 6 (IL6), interleukin 10 (IL10), tumor necrosis factor alpha (TNF) and interferon-gamma (IFN) in serum of uninfected controls and COVID-19 individuals during acute, sub-acute and recovery phase of the disease in the INCOV cohort. Statistical significance was determined by Kruskal-Wallis test with Benjamini-Hochberg-corrected Mann-Whitney U test. Normalized serum level concentrations are presented in box plots. Points represent single samples. The Kruskal-Wallis test results are indicated in the plot captions. Results of the post-hoc tests are indicated in the plots. Numbers of complete observations are displayed under the plots.

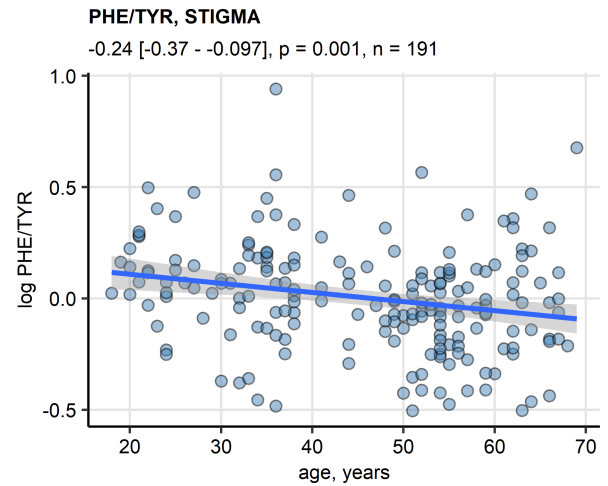
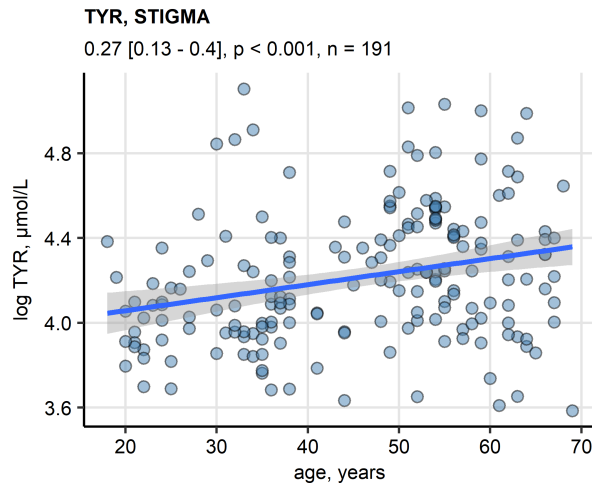
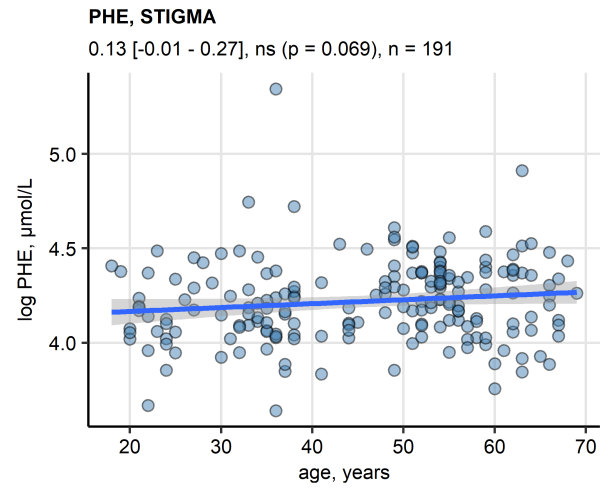
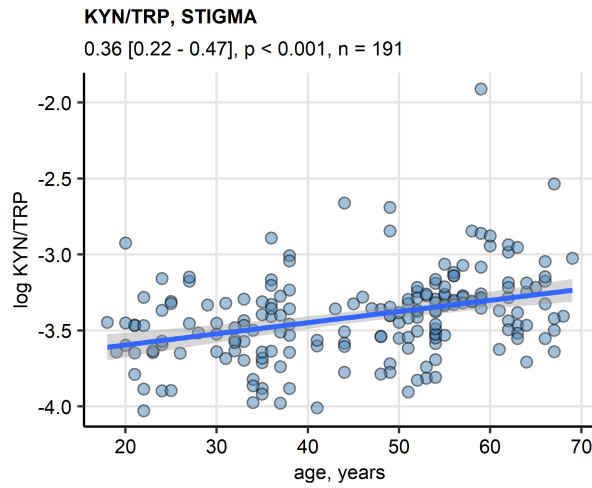
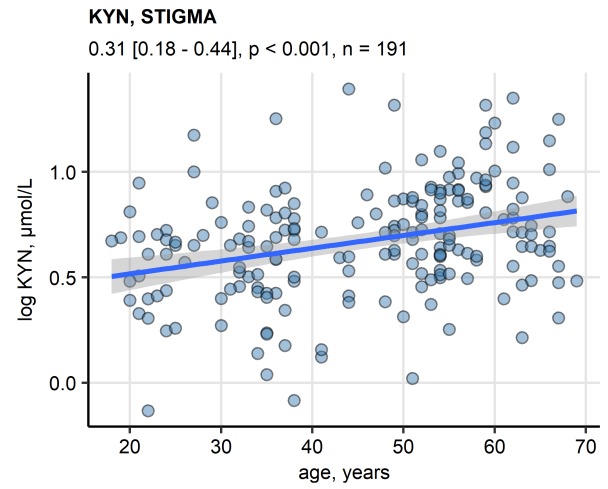
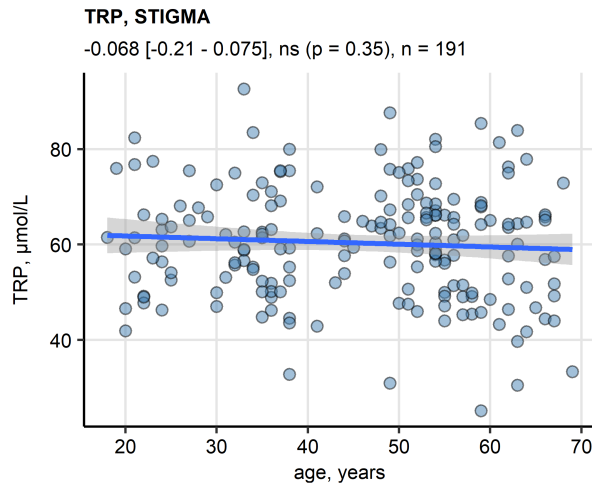
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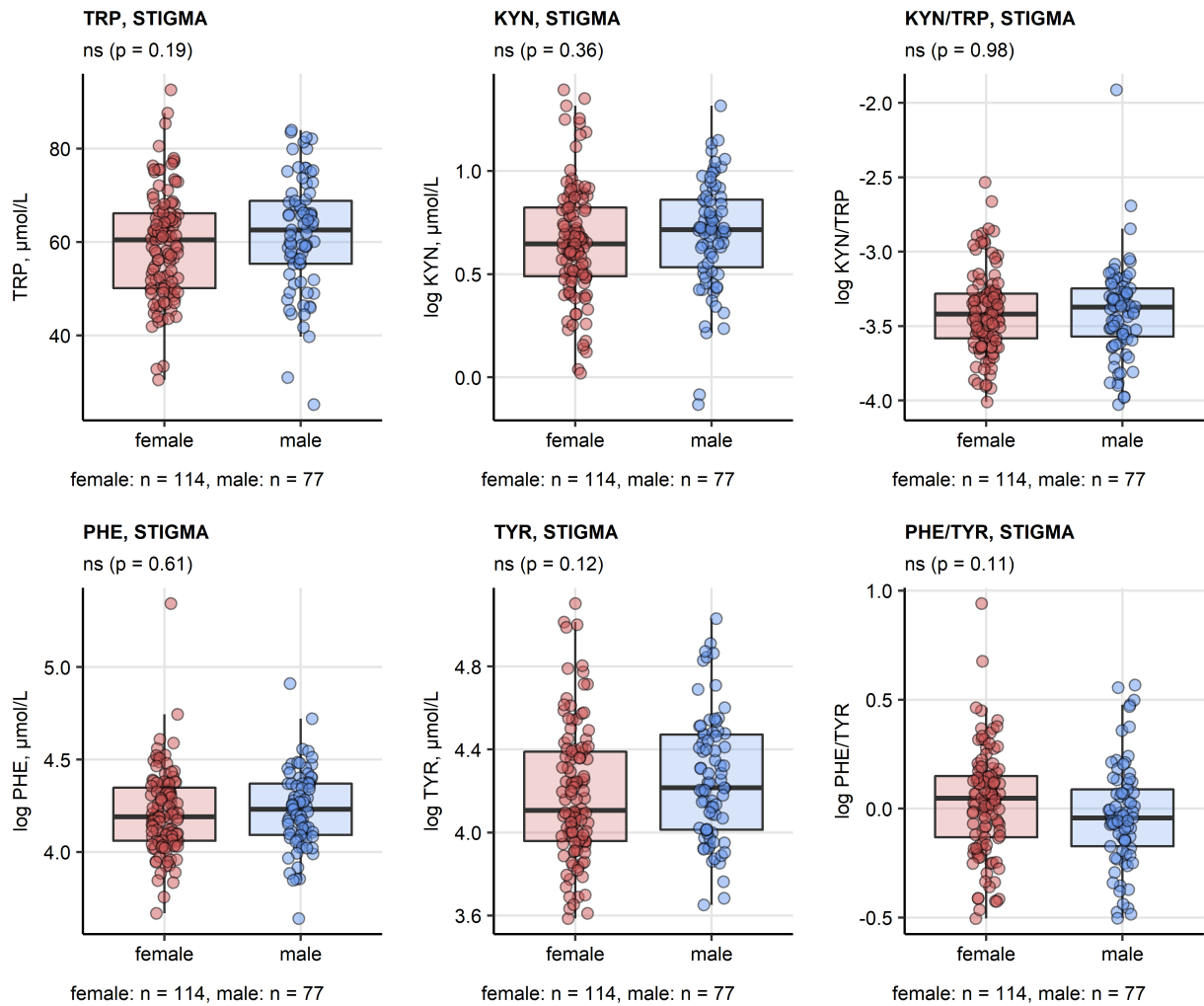
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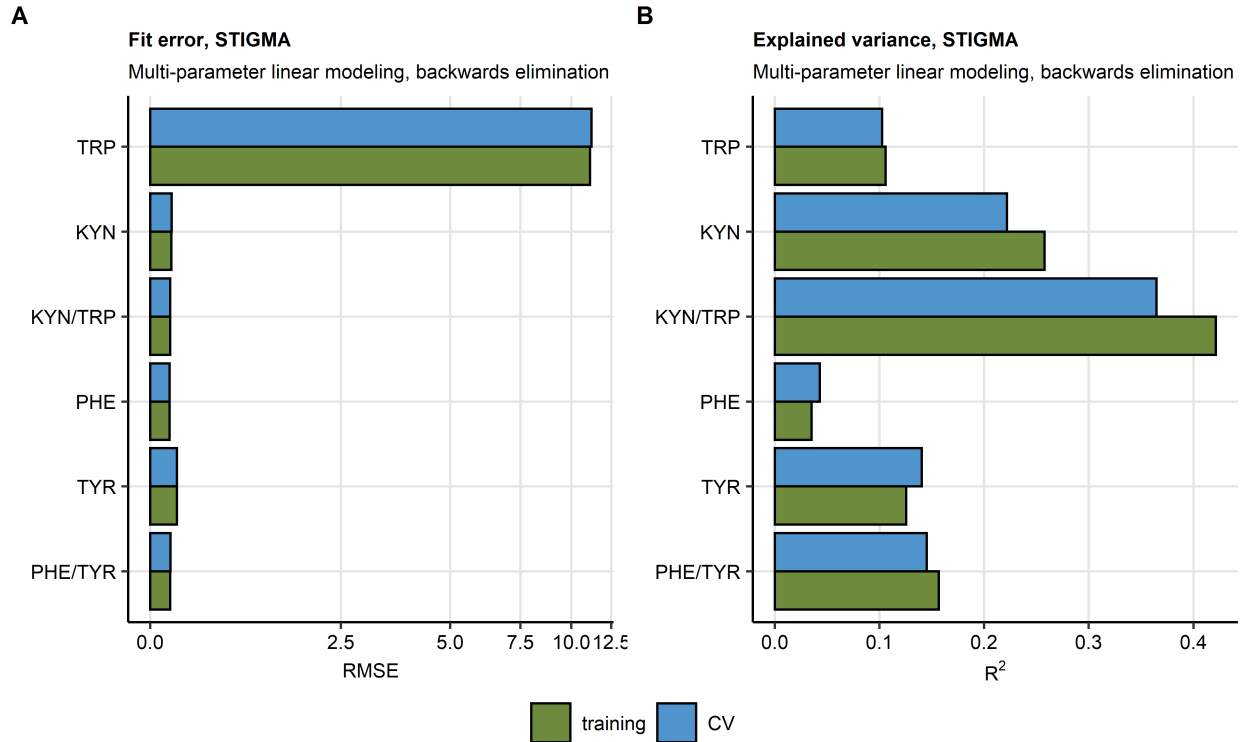
Supplementary Figure S2. Levels of inflammatory markers in STIGMA cohort participants stratified by COVID-19 status and depression/anxiety signs. *Serum levels of interleukin 6 (IL6), C-reactive protein (CRP), neopterin (NEO) and neutrophil-leukocyte ratio (NLR) in the STIGMA study participants stratified by SARS-CoV-2 infections status (A) and presence of depression/anxiety signs defined as HADS \geq 8 (B). Statistical significance was determined by two-tailed T test. Serum levels are shown in box plots. Points represent single observations. Significance p values are displayed in the plot captions, numbers of complete observations are indicated in the plot axes.*



Supplementary Figure S3. Correlation of aminoacid neurotransmitter precursors and their decay products with age. *Correlation of tryptophan (TRP), kynurenine (KYN), kynurenine/tryptophan ratio (KYN/TRP), phenylalanine (PHE), tyrosine (TYR) and phenylalanine/tyrosine ratio (PHE/TRP) with age in the STIGMA cohort was investigated by Pearson test. Points represent single observations, blue lines with gray ribbons depict fitted linear trends with 95% confidence intervals. Values of correlation coefficients with 95% confidence intervals, significance and numbers of complete observations are indicated in the plot captions.*



Supplementary Figure S4. Levels of aminoacid neurotransmitter precursors and their decay products in females and males. Serum concentrations of tryptophan (TRP), kynurenine (KYN), kynurenine/tryptophan ratio (KYN/TRP), phenylalanine (PHE), tyrosine (TYR) and phenylalanine/tyrosine ratio (PHE/TRP) in female and male participants of the STIGMA study. Statistical significance was assessed by two-tailed T test. Serum levels are shown in box plots. Points represent single observations. Significance p values are displayed in the plot captions. Numbers of complete observation are indicated under the plots.



Supplementary Figure S5. Root mean square error and R-squared statistics for multi-parameter linear models of aminoacid neurotransmitter precursors and their decay products in the STIGMA cohort. Multi-parameter linear regression models of serum levels of tryptophan (TRP), kynurenine (KYN), kynurenine/tryptophan ratio (KYN/TRP), phenylalanine (PHE), tyrosine (TYR) and phenylalanine/tyrosine ratio (PHE/TRP) in the STIGMA models were optimized by backwards elimination and their reproducibility was tested by repeated cross-validation (CV, 50 repeats, 10 folds). Values of root mean square error (RMSE, A) and R^2 (B) in the training data set and CV are plotted. Note pseudo-logarithmic scale in (A).

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