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Inflammation and depression: A study protocol to dissect pathogenetic mechanisms in the onset, comorbidity and treatment response

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ABSTRACT

About one third of patients suffering from Major Depressive Disorder (MDD) do not respond to any antidepressant medications and 75% experience relapses and general health deterioration. Importantly, inflammation can contribute to such negative outcomes, as well as to cause depression in patients who have been exposed to adverse childhood experiences and/or to viral infections, including COVID-19. Depressed patients also have an increased risk for developing comorbidities, such as cardio-metabolic dysfunctions, where inflammatory alterations, again, play a role in connecting MDD and these comorbid conditions.

Here, we present our study protocol funded by the Italian Ministry of Health in the context of the PNRR call (M6/C2_CALL 2022; Project code: PNRR-MAD-2022-12375859). The project aims to clarify the role of inflammation: i) in the onset of depression in association with environmental factors; ii) in the mechanisms associated with treatment response/resistance; iii) in depression and its comorbidity. To reach all these aims, we will perform biochemical, transcriptomic, genetic variants analyses on inflammatory/immune genes, pharmacokinetics and machine learning techniques, taking advantage of different human cohorts (adolescent depressed patients exposed to childhood trauma; adult depressed patients; treatment resistant depression patients; both prevalent and incident depression cases identified within a large population cohort). Moreover, we will use *in vitro* models (primary cultures of astrocytes, neurons and microglia) treated with pro-inflammatory or stressful challenges and preventive compounds to clarify the underlying mechanisms.

This 2-years project will increase the knowledge on the role of inflammation in the prevention and treatment of MDD and in comorbid disorders, and it will also provide experimental evidence for the development of novel targets and tools for innovative personalized intervention strategies.

1. Introduction

Major depressive disorder (MDD) is one of the most common and debilitating public health problems with a cross-national lifetime risk of 15–18% (Bromet et al., 2011), and accounts for 4.4% of the disease burden worldwide (Murray et al., 2012). MDD is often recurrent;

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50–80% of patients have at least two episodes, typically with increasing severity and frequency. The crucial STAR*D study revealed that one third of MDD patients had not achieved remission after four consecutive antidepressant trials and developed a chronic treatment course (Rush et al., 2006). While the symptoms of MDD can be severe and, at its worst, depression is a life-threatening condition, pharmacological treatment resistance to antidepressant therapy is one of the most challenging situations in the clinical management of affective disorders. Indeed, 75% of patients experience relapses and general health deterioration (Radua et al., 2017; Stegenga et al., 2012).

Inflammation has been widely implicated in the pathophysiology of depression and a dysregulation of the innate and adaptive immune system has been implicated in unfavourable prognosis, including a lack of drugs response (Benedetti et al., 2021b; Beurel et al., 2020; Cattaneo et al., 2013; Osimo et al., 2020). Indeed, depressed patients show increased blood levels of several inflammatory mediators, not only observed in single studies (Cattaneo et al., 2016), but also confirmed in extended meta-analyses (Islam et al., 2023; Zhang et al., 2023). For instance, proinflammatory interleukin (IL) IL-6 and Tumor Necrosis Factor (TNF)-α were significantly increased in depressed patients as compared to controls (Dowlati et al., 2010; Goldsmith et al., 2016; Haapakoski et al., 2015; Kohler et al., 2017; Valkanova et al., 2013). In line with this, higher levels of proinflammatory cytokines were observed also in patients with 'treatment resistant depression' (Cattaneo et al., 2020). Indeed, resistant depression is associated with a dysregulation of the immune system with an imbalance between the pro- and the anti-inflammatory cytokines. Increased levels of TNF-α, IL-6 and C-reactive protein (CRP) levels, in particular, were associated with a lack of treatment response in MDD patients (Carvalho et al., 2013; Haroon et al., 2017; Strawbridge et al., 2015), whereas a meta-analysis (Strawbridge et al., 2015) found that TNF-α levels may decrease in responsive patients to pharmacological treatments as compared to non-responsive ones.

The development of depression is also associated with environmental factors, including inflammatory triggers, such as viral infections and adverse childhood experiences (ACEs), with persistent low-grade inflammation as the core deregulated biological pathway. For instance, over 30% of COVID-19 survivors had clinical onset of depression after the virus clearance, with symptoms that can persist for several months (Benedetti et al., 2021a). Interestingly, among the biomarkers of post-COVID-19 depression, Lorkiewicz and Waszkiewicz (2001) found increased peripheral blood levels of IL-6, soluble interleukin 6 receptor (sIL-6R), IL-1β, TNF-α, interferon gamma (IFN-γ), IL-10, IL-2, soluble interleukin 2 receptor (sIL-2R), CRP, Monocyte Chemoattractant Protein-1 (MCP-1), serum amyloid a (SAA1) and metabolites of the kynurenine pathway (Lorkiewicz and Waszkiewicz, 2021). Similarly, the results of a recent meta-analytic structural equation model showed that complex interactions of psychoneuroimmunological and metabolic factors underlie the association between ACEs and adulthood depression (Zagaria et al., 2024), as CRP and composite inflammation levels significantly mediated the association between ACEs and adult depression.

People with depression and concurring inflammation may represent a high-risk group for cardiometabolic disorders. Co-morbid depression with cardiovascular diseases (CVD) (Anda et al., 1993), type 2 diabetes mellitus (T2DM) (Roy and Lloyd, 2012) and obesity (Luppino et al., 2010) is well established, and there is evidence for genetic overlap between depression, inflammation and obesity (Milaneschi et al., 2017). It has been proposed that "immuno-metabolic depression" may represent a particular 'sub-type' of depression characterised by a distinct symptom profile (e.g., increased appetite and weight gain), with increased inflammatory and cardiometabolic markers (Lamers et al., 2018; Penninx et al., 2013; Simmons et al., 2020). Therefore, alterations in inflammation may represent the underlying substrate connecting depression with other cardio-metabolic conditions and are promising novel targets for intervention (Milaneschi et al., 2020).

Although inflammation has been widely implicated in different aspects of MDD, it still remains unclear: i) where inflammation comes from; ii) how inflammatory system and the presence of a pro-inflammatory status can influence antidepressant efficacy, and iii) whether and how specific inflammatory patterns can map patients with MDD and cardio-metabolic comorbidity.

On these bases, we here describe, as a study protocol, our project funded by the Italian Ministry of Health in the context of the PNRR call (M6/C2_CALL 2022; Project code: PNRR-MAD-2022-12375859). By using a multidisciplinary approach, cutting-edge bioinformatic methodologies and large Italian multicenter clinical cohorts, the project aims to investigate the biological and environmental mechanisms leading to a pro-inflammatory status and contributing to MDD and its comorbidity. It also aims to identify specific inflammatory signatures associated with treatment response/resistance and to investigate, for the first time, the possible influence of inflammation on the pharmacokinetics of antidepressant drugs. By using preclinical *in vitro* models, our project will also demonstrate the causality between inflammatory or stressful insults and specific neuronal morphological and functional outcomes.

In the following paragraphs, we have reported details on the aims of the project, methodology, and statistical/bioinformatic analyses to enhance transparency of research, reduce publication bias and prevent selective publication and selective reporting of research outcomes, as well as to support reproducibility of results for future research studies.

Overall aims of the Project: By taking advantage of large human cohort samples, the following project aims at investigating the contribution of inflammation: i) in the onset of depression in association with environmental factors, ii) in the mechanisms associated with treatment response and iii) in depression and its comorbidity.

2. Methods

In this project, a stratification approach will be applied by using variables related to peripheral inflammation and exposure to environmental factors, as well as clustering analyses to map specific inflammatory patterns with clinical, biological or medical outcomes, by focusing on patients who have been exposed to childhood trauma or COVID-19, who do not respond to antidepressants, or on those that develop comorbidities.

The flow chart representing the study design is reported in Fig. 1.

2.1. Description of human cohorts

2.1.1. COVID-19 cohort

This cohort includes 912 patients (62.39% male, mean age and standard deviation 57.14 ± 12.83), survivors of the COVID-19 infection, of which 515 recruited during the first wave (from 04/06/20 to 11/23/20), and 397 recruited from the second wave onwards (from 10/21/20 to 11/28/22). They were admitted to IRCCS Ospedale San Raffaele with SARS-CoV-2 infection confirmed by clinical, radiological findings and positive RT-PCR test. Patients were recruited and evaluated 1 month, 3 months and 6 months after the resolution of the infection, reaching 808, 345 and 312 subjects respectively. At follow-ups, 31% of the 402 adults who survived COVID-19 fell within the psychopathological ranges for depression (42% anxiety, 28% PTSD and 40% insomnia). In particular, 81/226 patients (35.8%) showed clinically relevant symptoms after 12 weeks post-infection in at least one psychopathological dimension, according to validated self-report questionnaires (28% for depression, 22% for PTSD, and 30% for anxiety), indicating a potential long-COVID syndrome (Mazza et al., 2021).

Patients were evaluated through a battery of neuropsychological tests to detect subtle neurocognitive profiles, that consist of various questionnaires, whose compilation was preceded by the anamnestic collection. Participants belonging to the COVID-19 follow-up, aged 18–70 years, were also offered to undergo magnetic resonance imaging (MRI) performed on a Philips 3.0 T scanner at the High Field Magnetic

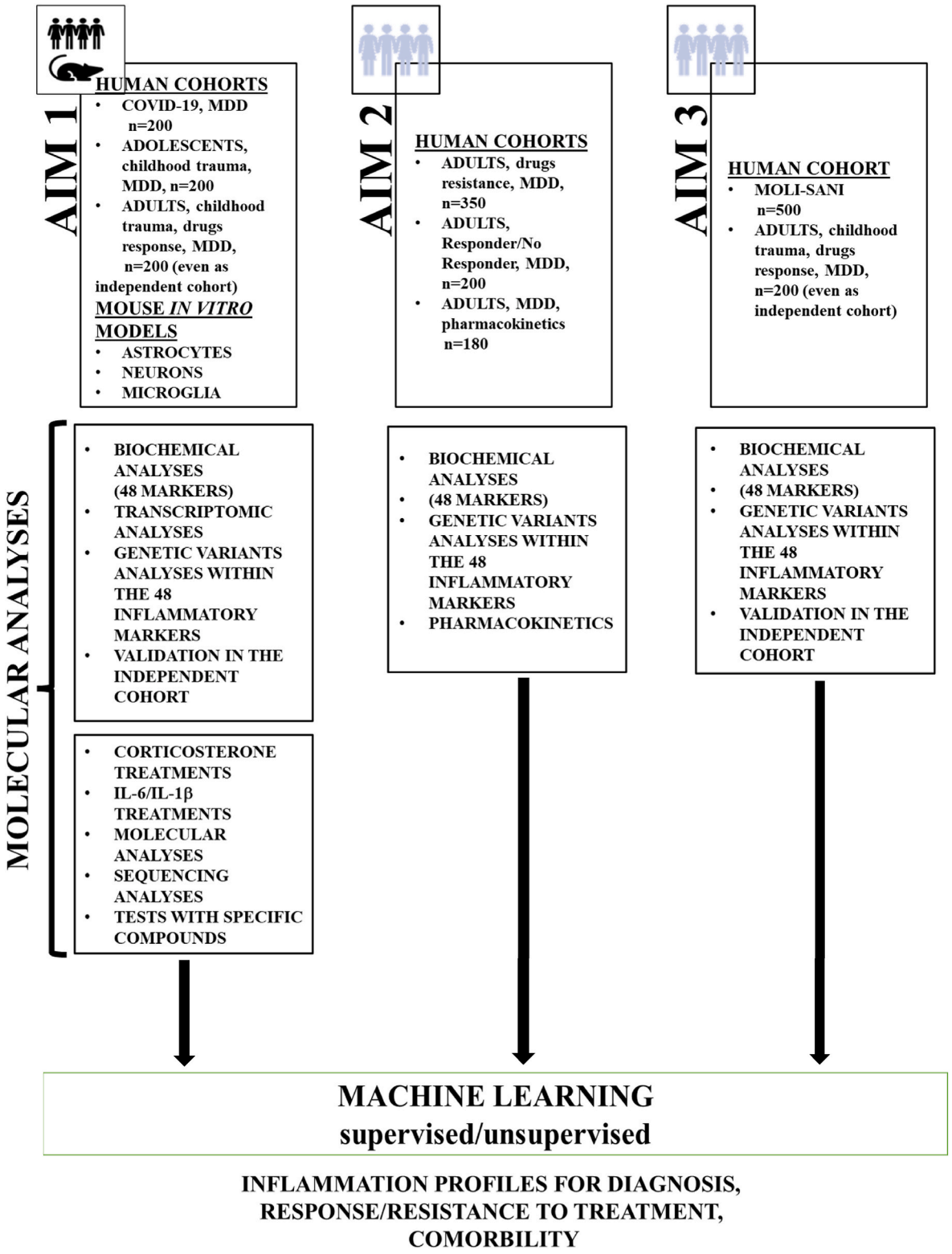

Fig. 1. Flow chart of the project.

Resonance Excellence Center (CERMAC), IRCCS Ospedale San Raffaele, Milan, Italy. MRI (Benedetti et al., 2021a), available for a subsample, has been performed on a 3T Ingenia CX, Philips, The Netherlands with a 32-channel sensitivity encoding SENSE head coil. The images acquired were T1, T2, DWI, and Resting state. Moreover, these participants were asked to undergo a blood sample each for the three follow-up periods. From these stored samples, the quantification of cytokines, chemokines and other immuno-inflammatory factors was carried out using the Bio-Plex Pro Human Cytokine Screening Panel (48 markers).

Patients had to meet the following inclusion criteria: (a) diagnosis of COVID-19 infection, detected by clinical and radiological findings suggesting COVID-19 pneumonia and confirmed by RT-PCR test or clinical examination; (b) older than 18 years of age; (c) signing the informed consent; (d) primary school diploma (as a minimum requirement for

understanding the questions in the tests); (e) Italian native speakers (or bilingualism). Patients had also to meet the following exclusion criteria: (a) subjects with intellectual disability or neurological pathologies.

2.1.2. Adult depressed patients' cohort

It includes 200 adult inpatients with detailed medical and clinical assessment (childhood trauma; Hamilton Depression Rating Scale, HDRS; Beck Depression Inventory, BDI; Brief Assessment of Cognition in Schizophrenia, BACS), including treatment response. Blood samples and MRI data 3.0 T (Poletti et al., 2019; Vai et al., 2020) are available. Blood inflammatory markers levels from the panel Bio-Plex ProTM Human Cytokine Screening Panel (48 markers) and genotypes (Illumina PsychArray BeadChip) are already generated.

2.1.3. Adolescent depressed patients' cohort

It includes 450 adolescents at increased risk for mental disorders because of childhood trauma history, who were clinically assessed (HDRS, BDI, and BACS) at the age of 18 years or more. Blood samples were collected and out of these 450, 200 agreed to take part in neuroimaging study (MRI scan 3.0 T) and completed the 3 years of clinical follow up. Blood samples are available.

2.1.4. Treatment resistant depression cohort

It includes 350 resistant (failure to respond to two or more adequate trials with two or more different classes of antidepressant drugs and to an adequate trial with a tricyclic drug (Maffioletti et al., 2021),) depressed patients with a diagnosis performed according to DSM-5, and with HAMD-(HDRS)-17 score >14. Diagnoses were confirmed using the Structured Clinical Interview for DMS-5 Disorders-SCID-5-CV and SCID-5-PD. Blood samples are available and genotyping (GWAS) data have been generated.

2.1.5. MOLI-SANI cohort

The Moli-sani study is a longitudinal population cohort of adult Italians residing in the Molise region (recruitment 2005–2010; N = 24,325; ≥35 years; 48.11% men). Exclusion criteria were pregnancy, disturbances in understanding/willing processes, ongoing poly traumas or coma. The Moli-sani study was approved by the Ethical Committee of the Catholic University of Rome, and all the participants provided a written informed consent. Upon recruitment, a wealth of clinical, sociodemographic, and lifestyle information was collected from all participants, as well as blood and urine samples, which are currently stored in the Neuromed Biobanking Centre. DNA, plasma, serum, RNA and buffy coat (and more than 40 blood markers) are available, and several inflammatory markers have been generated within the cohort. Since basal recruitment, participants have been followed up both through active and through passive follow-up. This makes it possible to identify both prevalent and incident cases of depression and their cardiometabolic comorbidities, through psychometric tests administered and linkage with Electronic Health Records (EHRs) databases like the Italian National mortality (ReNCaM) registry, the Molise regional registry of hospital discharge records (HDRs) and the regional drug prescription registry, using fiscal code of each participant as unique identifier. Specifically, prevalent depression cases will be identified as presence of depressive symptoms at baseline (PHQ-2 and PHQ-9) and/or having drugs prescriptions for the treatment of depression (including antidepressants, benzodiazepines and their derivatives, mood stabilizers, antipsychotic and sedative-hypnotics drugs), before recruitment date. The latter criterion will be used to identify also incident depression cases in participants free of the disease upon recruitment, in a period ranging from 2006 through 2018, as well as to identify incident T2D and CVD cases in the same follow-up period identified through interpolation of drug prescriptions, main and secondary diagnosis in hospital discharge records and mortality registries.

Main cardiometabolic comorbidities through December 31st, 2018 will be defined as primary fatal and nonfatal incident cases of Coronary Heart Disease (or CHD, namely unstable angina, myocardial infarction, coronary revascularization and sudden death for unspecified cardiac event) and cerebrovascular disease (Ischemic and Hemorrhagic strokes) that occurred in the cohort during follow-up will be ascertained through linkage of the study cohort to the hospital discharge files and to the ReNCaM and death certificates (ISTAT form), by using the International Classification of Diseases, ninth revision (ICD-9). For CHD, ICD 9 codes 410–414 and/or reperfusion procedure (ICD-9 codes 36.0–36.9) and for cerebrovascular disease, ICD9 codes 430–432, 434, 436–438 or procedure codes for carotid revascularization (ICD 9 code 38.12) are considered.

Incident type 2 diabetes (T2D) fatal/non-fatal cases will be ascertained by individual-level record linkage to Molise regional registers of hospital discharge records (HDRs), deaths (ReNCaM register) or drug prescription records. For the HDRs register, primary and secondary diagnoses for hospitalizations are coded using the International Classification of Diseases, version 9 (ICD-9), and codes ICD-9250.X0 and 250.X2 (type 1 excluded) were used, from 2005 until December 31, 2018. Cause-specific mortality will be assessed through the Italian mortality register, validated by Italian death certificates (ISTAT form), and coded according to the ICD-9. In the ReNCaM register, incident cases of T2D are identified when ICD-9 code 250 is reported as the underlying or secondary cause of death. The regional drug prescription register provides information on the type of drug prescribed during the follow-up, and coded according to the Anatomical Therapeutic Chemical (ATC) classification. For each Moli-sani participant, a data download of all prescribed pharmacological therapies is performed by using the Farmastat platform (Statistics and Analysis Pharmaceutical Assistance), a complete web tool available to Italian ASL (Azienda sanitaria locale) to gather information, monitor and evaluate pharmaceutical assistance.

2.2. Description of preclinical in vitro models

2.2.1. Primary astrocytes cultures

Primary astrocytes will be obtained from P0-P4 C57BL6J pups. Hippocampi will be dissected in Hanks' balanced salt solution (HBSS), incubated with 200 U of Trypsin (15 min, 37 °C) and mechanically dissociated in culture media (Dulbecco's modified Eagle's medium, 100 U/ml penicillin, and 100 μg/ml streptomycin, 10% heat-inactivated fetal bovine serum). Cells will be centrifuged (7 min, 1500 rpm, RT) and the pellet resuspended in culture medium and seeded onto T25-flasks pre-coated with poly-d-lysine. After 10 days, to produce astrocyte cultures free of microglia, oligodendrocytes and neurons, flasks will be shaken at 250 rpm for 12 hours (37 °C). After that, cells will be split and seeded onto IBIDI chamber slides imaging analysis or dish for biochemical and molecular analysis which will be pre-coated with poly-d-lysine.

2.2.2. Primary neurons cultures

Primary neuronal cultures will be isolated from C57BL6J and Thy1-YFP pups in the P0-P2 window. After brain dissection, hippocampi will be incubated with papain (30 min, 34 °C), trypsin inhibitor (45 min, RT), and mechanically dissociated. Neurons will be plated onto IBIDI chamber slides for imaging analysis or dish for biochemical and molecular analysis, which will be pre-coated with poly-L-lysine. Thy1-YFP mice express spectral variants of green fluorescent protein (GFP, yellow-YFP) at high levels in neurons. Expression is strong from a mid-gestational stage into adulthood.

2.2.3. Primary microglia cultures

Primary microglia cultures will be isolated from C57BL6J or CX3CR-1GFP pups in the P0–P4 time window through whole brain dissection. Dissociated cells will be seeded as mixed glial culture. Microglia will grow on top of a confluent astrocyte layer.

After four weeks, the mixed glial cultures will be shaken on a rotary shaker (250 rpm) at 37 °C for 1 hour to collect microglial cells that will

be used for molecular analyses.

Microglia cells derived from CX3CR-1GFP knock-in/knock-out mice have an enhanced green fluorescent protein (EGFP) sequence replacing the first 390 bp of the coding exon (exon 2) of the chemokine (C-X3-C motif) receptor 1 (Cx3cr1) gene. The EGFP, but not the endogenous gene, is expressed in monocytes, dendritic cells, NK cells, and brain microglia - mimicking endogenous gene expression.

2.3. Biological investigations

2.3.1. Biochemical analyses

A panel of 48 blood inflammatory blood markers (Pro™ Human Cytokine Screening Panel) will be analysed in the blood samples from the following cohorts: i) the 18 years old MDD adolescents; ii) the treatment resistant MDD adults; iii) the Moli-sani. The results of this panel are already available in the COVID-19 cohort, as well as in the cohort of adult MDD patients.

The panel includes the following markers: FGF basic, IL-10, IL-2, MIP-1α, Eotaxin, IL-12 (p70), IL-4, MIP-1β, G-CSF, IL-13, IL-5, PDGF-BB, GM-CSF, IL-15, IL-6, RANTES, IFN-γ, IL-17A, IL-7, TNF-α, IL-1β, IP-10, IL-8, VEGF, IL -1ra, MCP-1 (MCAF), IL-9, CTACK, IL-1α, MIG, GRO-α, MIF, IL-2Rα, β-NGF, HGF, TRAIL, IL-3, SCF, IFN-α2, IL-18, IL-12 (p40), SCGF-β, LIF, M-CSF, IL-16, SDF-1α, MCP-3, and TNF-β.

EDTA tubes were used for plasma preparation. Plasma samples were immediately centrifuged and stored at −80 °C until the time of the assay.

For OSR cohorts, including MDD and COVID-19 patients, Bio-Plex Pro™ Human Cytokine 48-Plex (BIO-RAD) were used to detect plasma concentrations of immune analytes, through the bead-based Luminex system according to xMAP technology (Luminex 200™ system, Merck Millipore). Whole blood was collected into commercially available anticoagulant-treated tubes (K3_EDTA tubes) and centrifugated to separate plasma from blood cells (red cells and buffy coat, which encompasses platelets and white cells). The blood samples were collected in fasting condition for MDD patients, no fasting for COVID-19 cohort.

2.3.2. Transcriptomic analyses

Transcriptomic analyses will be performed on an Illumina Systems starting from RNA samples of the 18 years old MDD adolescents, of the adult MDD cohort in a replication study, and, finally, in the *in vitro* models following cell treatments with stressful and inflammatory stimuli.

2.3.3. Genetic variants analysis within the 48 inflammatory markers

Genetic variants (to be generated with a Custom panel or already generated with Infinium PsychArray 24 BeadChip (Illumina)) within the 48 inflammatory and immune-related genes will be analysed in the cohorts of adult depressed patients, COVID-19 patients and the treatment resistant MDD adults; while candidate rare and common variants will be tested for the replication in the Moli-sani cohort, where feasible.

2.3.4. Pharmacokinetics analyses

A subgroup of 180 depressed patients with detailed information on pharmacological treatments and response will be extrapolated from the treatment resistant MDD adult cohort, and we will measure the serum/plasma concentrations of antidepressants and their active metabolites by High Performance Liquid Chromatography (HPLC).

The following antidepressants will be measured: fluoxetine (and active metabolite norfluoxetine), fluvoxamine, paroxetine, sertraline, citalopram, escitalopram, venlafaxine (and active metabolite desvenlafaxine), duloxetine, mirtazapine, and vortioxetine.

2.3.5. Mouse cell culture treatments

Cell cultures will be challenged with corticosterone. 3 days after the microglia plating, co-cultures will be treated with IL-6 and IL-1β. Finally, tests with specific compounds will be performed. To set up an *in vitro* model of stress induced by corticosterone, we will study its effect on astrocytes, neurons and microglia in a dose-response manner by testing different concentrations (100 nM −10 μM – 25 μM – 50 μM – 75 μM – 100 μM) for 24 and 48h hours. We will investigate its effects on these cell cultures by detecting both cell vitality (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT assay) and cell death (lactate dehydrogenase, LDH assay) in the same dishes at 24 and 48h. Once identified, the selected dose able to induce stress responses without causing cell death will be used to perform the treatment with pro-inflammatory mediators (e.g. *IL-6 and IL1β*) as well as with protective compounds. Then, protein and gene expression analyses will be performed by using Western blotting, Real Time PCR and RNAseq.

2.4. Statistical and bioinformatics analyses

Clinical/medical, biological and MRI data will be harmonised to reduce the impact of centers batch effects, including ComBat algorithm and visualization checks for neuroimaging data to avoid inappropriate removal of meaningful subpopulation effects (Goh et al., 2017; Pounis et al., 2016).

2.4.1. Gene variants

Frequencies will be compared to reference databases (1000 Genomes Project; Exome Sequencing Project; Exome Aggregation Consortium), with variants prioritized for absence from or with a minor allele frequency MAF<0.1% and predicted to affect protein function or structure (missense, nonsense, frameshift, splice site, start codon, indel). Bioinformatic analyses will use PolyPhen-2, SIFT, CADD-score.

2.4.2. Transcriptomic analyses

Transcript-level raw counts will be quantified against the human transcriptome (e.g. Salmon 1.4.0), comparing differential expression between groups (e.g. DESeq2, edgeR). Genes differentially expressed at a log2 fold change cutoff and FDR<0.01 will be entered in Ingenuity Pathway Analyses Software.

2.4.3. Biomarkers

Inflammatory markers will be analysed using parametric and/or non-parametric multivariate statistics. Peak areas of pharmacokinetics analytes will be normalized to internal standards, validating methods for linearity, detection limit, precision, recovery of antidepressant drugs and metabolites from serum/plasma (Greiner et al., 2007; Mandrioli et al., 2006; Romiguieres et al., 2002).

2.4.4. Neuroimaging

Available features include: T1 images for grey matter cortical and subcortical volumes (Computational Anatomy Toolbox CAT12 (Vai et al., 2020) for SPM12); Diffusion tensor imaging for white matter fractional anisotropy, axial, radial and mean diffusivity (tract-based spatial statistics analysis in FSL); resting state T2* images, pre-processed with HALFpipe.

2.4.5. Unsupervised clustering of participants

Machine learning techniques will be used to cluster depressed participants including COVID-19 patients, identified both through clinical diagnosis and through linkage with EHRs within the Moli-sani cohort, with a data-driven approach to uncover subtypes of depressed patients based on MRI features, inflammatory markers, stress exposure, comorbidities and clinically-meaningful outcomes based on data available in each cohort. To evaluate the clustering solution, we will employ stability-based relative clustering validation methods that seek to identify the best clustering solution as the one that best generalize to unseen data through supervised learning (Landi et al., 2021). Clustering solutions' will be evaluated through the normalized stability (i.e., misclassification error) and most commonly used internal validation measures (elbow method, silhouette-based approach) (Lange et al., 2004; Walther, 2005). We will compare performance of clustering

algorithms (e.g. k-means, hierarchical clustering, Hierarchical Density-Based Spatial Clustering of Applications with Noise) and select the best performing algorithm (i.e., minimum normalized stability). Principal component analyses analysis will identify components if irrelevant features would lead to biased clustering solutions. Clusters will be compared through multivariate classification analysis (e.g., MANOVA, Canonical Correlation Analysis [CCA], Partial Least Squared [PLS]).

2.4.6. Supervised machine learning classification

Several algorithms will be employed: support vector machine, multiple kernel learning (Pattern Recognition for Neuroimaging Toolbox), and elastic net penalized regression (Poletti et al., 2021; Vai et al., 2020). K-Fold nested cross-validation will optimize hyper-parameters and assess the out-of-sample classification performance, to avoid overfitting. Algorithms will be trained to predict cluster label defined in unsupervised analyses, onset of depression during 6 months after COVID-19 infection, treatment response, and cardiometabolic comorbidities. Performances of the models will be compared through balanced accuracy, F1 score, class accuracies, predictive values, and AUC. Analyses will be performed independently in each cohort. Results will be compared to identify shared or unique involved factors.

2.4.7. Survival analyses of incident depression and comorbidity risk

Within the Moli-sani cohort, Cox Proportional Hazard models - incrementally adjusted for sociodemographic, polygenic and lifestyle variables - will be applied to determine whether circulating inflammation predisposes to an increased risk of depression and its main cardiometabolic comorbidities, over ~12 years follow-up. Inflammation will be investigated through both single markers like high sensitivity C-reactive protein levels and through composite markers like the INFLA-score, which is also based on cellular and haemostatic components of the inflammatory process (Pounis et al., 2016). Should we identify significant associations with both depression and comorbidities, we will perform a mediation analysis to estimate the proportion of total effect of INFLA-score on comorbidity risk explained by depression. Moderation effects by gender will also be investigated in this relationship, through interaction and stratified analyses. Survival Random Forest will also be used to predict incident comorbidities, based on the same exposures and covariates used in Cox PH regression. This will imply variable selection and normalization, proper hyperparameter tuning through k-fold cross-validation, training and testing of the optimized model (70:30 ratio), with Permutation Feature Importance and SHapley Additive exPlanations (SHAP) values analysis to establish the most influential features and the direction of their influence.

2.4.8. Correlation analyses

We will perform bioinformatic analyses where machine learning approaches will be applied to: i) correlate the inflammatory/immune status, including immune-genetics, with brain morphological features and depressive symptoms; ii) correlate the inflammatory/immune status, including immune-genetics, with pharmacokinetics and treatment response and resistance; iii) correlate the inflammatory/immune status, including immune-genetics, with comorbidities and depressive symptoms.

We will also apply unsupervised clustering algorithms to identify cluster of depressed patients with specific inflammatory/immune system features that are able to map specific clinical conditions, including the response or resistance to treatment or the development of comorbidities.

3. Aims and hypotheses

Our project aims to address three major objectives related to the role of inflammation: i) in the onset of depression in association with environmental factors; ii) in the mechanisms associated with treatment response; iii) in depression and comorbidity; by exploiting cutting-edge bioinformatic and machine learning methodologies and large Italian multicenter cohorts, described in details above.

3.1. Aim 1

In our first aim, we will explore the possible role of environmental factors such as i.e. ACEs and COVID-19 infection as possible triggers of the development of depression via activation of the inflammatory response. We will first identify the most sensitive panel of inflammatory mediators able to detect a low-grade inflammatory status in association with depression and we will test these biomarkers in a wide sample of people recruited from the general population, namely COVID-19, >18 years old MDD adolescents, MDD adults' cohorts. Then, we will apply unsupervised clustering algorithms and algebraic topology tools on these variables to identify possible homogeneous subgroups of people characterized for specific inflammatory and environmental factors (stress and inflammatory insults). We will also explore the clinical relevance of the obtained associations and stratification, exploring whether the identified findings can be replicated in an independent deeply phenotyped clinical sample of patients with a confirmed diagnosis of major depressive episodes (MDD adults cohort used as independent cohort to replication results). Finally, the identified subgroups of MDD patients will be deeply characterized for other already collected clinical and biological variables, including measures of disease severity (e.g. recurrency, duration and symptom severity), neuropsychology, genetics and multimodal neuroimaging features obtained with MRI. Finally, to better unveil the mechanisms linking environmental insults (e.g. stress and inflammatory triggers) with the pathogenesis of depression, we will use preclinical *in vitro* models that offer the advantage to manipulate systems and networks to test causality and also to test the protective effect of specific compounds.

3.2. Aim 2

In the second aim, we will investigate the mechanisms through which inflammation can drive treatment response. In particular, we will focus the attention on already available cohorts of MDD patients that have been clinically followed-up over time and have been characterized for treatment response/resistance (treatment resistant cohort and MDD adult cohort responders/no responders). We will investigate whether low grade inflammation predisposes to the incident risk of treatment resistant depression, and genetic variants within the panel of inflammatory related genes will be analysed. Moreover, to explore the impact of inflammation on treatment response/resistance, serum/plasma samples of depressed patients, treated with antidepressants and characterized for response to pharmacological treatment, will be quantified. In particular, concentrations of antidepressants and their active metabolites will be measured, and they will be correlated with clinical outcomes, the inflammatory status and other variables influencing drug response. Supervised machine learning algorithms (eg. support vector machine, elastic net penalized regressions) will be then used to predict treatment response, individually in each identified subpopulation. These techniques allow to define a predictive function by accounting for possible relationships among markers, therefore dealing with multicollinearity, and eliminating redundant variables operating an embedded feature selection.

3.3. Aim 3

In the third aim, we will investigate the complex relationship among depression, circulating inflammation and the incident risk for comorbidities, which remains largely unclear, especially concerning the direction of effects and potential mediation roles. To this end, by assessing already available cohorts of MDD patients (Moli-sani study), we will: i) validate incident cardiovascular and diabetes cases; ii) test potential

associations between inflammation with these risks; and finally, iii) test the potential mediation of incident depression inferred from drug treatment in the association between circulating inflammation at baseline and later cardio-metabolic risk. Interactions with gender will also be tested and possibly deepened through gender-stratified analyses, to identify potential modifications of the effect in this association. Through unsupervised cluster analyses, we will identify, in the available cohort, specific inflammatory profiles among patients and test whether these are associated with i) specific comorbidity groups (e.g. depression and CVD, depression and diabetes) and ii) latent factors tagging somatic and affective depressive symptoms.

We hypothesise that a comprehensive inflammatory and environmental characterization of depressive symptoms dimensions is essential to detect specific disease signatures related to relevant health outcomes. We also hypothesise that defining the mechanisms associated with treatment response or treatment resistance as well as unveiling the biological and environmental mechanisms that link the pro-inflammatory status to depression comorbidity allow to deliver diagnostic and computational tools and biomarkers to predict treatment response and comorbidities onset. Finally, we hypothesise that, by using a preclinical *in vitro* animal model, we will uncover novel causative immune-inflammatory mechanisms driving depression and identify new targets for interventions. All this could lead to the development of new therapeutic targets, through a personalized medicine approach, with implications in reducing the personal and social burden of depression.

4. Discussion

The PNRR-MAD-2022-12375859 is a multimodal 2-years project that will perform biochemical, transcriptomic, genetic variants, pharmacokinetic and bioinformatics analyses including machine learning tools taking advantage of different human cohorts, and of a murine *in vitro* model, with the main aim to define the impact of inflammatory dysregulations on depressive symptomatology, its comorbidities and response/resistance to antidepressants. By using data from well characterized clinical cohorts and population study, we will integrate peripheral inflammatory profiles with immune-genetics to identify biological clusters mapping patients with specific clinical and medical outcomes, treatment response/resistance and the presence of comorbidities. For the first time, we will test the influence of inflammatory/immune status, including immune-genetics, on treatment response via influencing antidepressants metabolisms and the related active metabolites. Our preclinical experiments will uncover novel causative immune-inflammatory mechanisms driving depression and will identify new targets for interventions. We will not only increase knowledge in the field, but also deliver diagnostic and computational tools and biomarkers to predict depression development, treatment response and comorbidities onset.

The results obtained from this study will support future work and could lead to the identification of: 1) the role of the immune system in the development of depressive symptoms in those individuals who were exposed to COVID-19 infection; 2) the role of the immune system in the development of depressive symptoms in those adolescents who were exposed to childhood trauma; 3) clusters of patients characterized for specific inflammatory/immune status, including immune-genetics, defined by specific environmental factors and clinical phenotype; 4) an inflammatory/immune status, including immune-genetics, associated with brain features and mapping patients with depressive symptoms; 5) the molecular mechanisms associated with exposure to inflammatory or stressful challenges by using *in vitro* neuronal models; 6) potential compounds with preventive properties that may moderate the negative effects of environmental triggers; 7) molecular mechanisms through which inflammatory/immune status, including immune-genetics, can drive treatment response/resistance; 8) specific pattern of inflammatory/immune clusters, that can influence treatment response via pharmacokinetics of antidepressants; 9) definition of inflammatory and environmental predictors, including immune-genetics, estimation of machine learning predictive accuracy, of treatment response/resistance; 10) inflammatory and environmental predictors, including predictive accuracy, of incident depression and cardiometabolic comorbidities in each identified subpopulation; 11) inflammatory pathways/markers linking depression, inflammation status, including immune-genetics, and the incident risk for comorbidities; 12) potential chronic inflammation subtypes within depression associated with cardiometabolic comorbidities.

By focusing on patients who do not respond to antidepressants, or on those that develop comorbidities, this study will benefit a large number of depressed patients. We will deliver immediate clinical benefit: if we demonstrate the way inflammation can trigger treatment resistance and the onset of medical comorbidities, we will be then able to develop biomarkers that can predict such conditions, but also to identify novel targets to improve symptomatology, long-term prognosis, and prevent the onset of cardio and metabolic comorbidities. Overall, this will reduce the economic, personal and social burden associated with depression and cardio-metabolic comorbid disorders.

5. Ethics approval and dissemination

The protocol (version 2.0) was approved by the local ethical committee, IRCCS Centro San Giovanni di Dio – Fatebenefratelli, Brescia (rif. Parere 16–2023) and then by Comitato Etico Territoriale Lombardia 1. Any modification to the study objectives, study design, participant population, sample size, study procedures, or significant administrative aspects will require an amendment to the protocol.

The results from this project will be publicly presented to scientific researchers and health care professionals through peer-reviewed journals and scientific conference presentations. The authorship requirements will adhere to the scientific journal guidelines. We will transmit and disclose findings, when appropriate, to the general population using media coverage, such as newspaper articles and television interviews.

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Declaration of competing interest

none.

Data availability

No data was used for the research described in the article.

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