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A multidisciplinary approach to mental illness: do inflammation, telomere length and microbiota form a loop? A protocol for a cross-sectional study on the complex relationship between inflammation, telomere length, gut microbiota and psychiatric disorders

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Complete List of Authors:	Manchia, Mirko; Dalhousie University, Pharmacology; Universita degli Studi di Cagliari, Medical Science and Public Health Paribello, Pasquale; University of Cagliari, Unit of Clinical Psychiatry, University Hospital Agency of Cagliari Arzedi, Carlo Bocchetta, Alberto Caria, Paola Cocco, Cristina Congiu, Donatella Cossu, Eleonora Dettori, Tinuccia Frau, Daniela Virginia Garzilli, Mario Manca, Elias Meloni, Anna Montis, Maria Antonietta Mura, Andrea Nieddu, Mariella Noli, Barbara Pinna, Federica Pisanu, Claudia Robledo, Renato; University of Cagliari, Department of Biomedical Sciences Severino, Giovanni Sogos, Valeria Chillotti, Caterina Carpiniello, Bernardo; Universita degli Studi di Cagliari, Medical Science and Public Health Del Zompo, Maria Ferri, Gian Luca Vanni, Roberta Squassina, Alessio; University of Cagliari, Biomedical Sciences
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A multidisciplinary approach to mental illness: do inflammation, telomere length and microbiota form a loop? A protocol for a cross-sectional study on the complex relationship between inflammation, telomere length, gut microbiota and psychiatric disorders.

Mirko Manchia[1,2,3], Pasquale Paribello[1,2], Carlo Arzedi[1,2], Alberto Bocchetta[4,5], Paola Caria[6], Cristina Cocco[5], Donatella Congiu[4], Eleonora Cossu[1,2], Tinuccia Dettori[6], Daniela Virginia Frau[6], Mario Garzilli[1,2], Elias Manca[7], Anna Meloni[4], Maria Antonietta Montis[1,2], Andrea Mura[1,2], Mariella Nieddu[6], Barbara Noli[7], Federica Pinna[1,2], Claudia Pisanu[4], Renato Robledo[6], Giovanni Severino[4], Valeria Sogos[8], Caterina Chillotti[4,5], Bernardo Carpiniello[1,2], Maria Del Zompo[4,5], Gian Luca Ferri[7], Roberta Vanni[6], Alessio Squassina[4*]

[1] Unit of Psychiatry, Department of Public Health, Clinical and Molecular Medicine, University of Cagliari, Cagliari, Italy; [2] Unit of Clinical Psychiatry, University Hospital Agency of Cagliari, Cagliari, Italy; [3] Department of Pharmacology, Dalhousie University, Halifax, Nova Scotia, Canada; [4] Department of Biomedical Sciences, Section of Neuroscience and Clinical Pharmacology, University of Cagliari, Italy; [5] Unit of Clinical Pharmacology, University Hospital Agency of Cagliari, Italy; [6] Department of Biomedical Sciences, Unit of Biology and Genetics, University of Cagliari, Italy; [7] Department of Biomedical Sciences, NEF Laboratory, University of Cagliari, Italy; [8]Department of Biomedical Sciences, Section of Cytomorphology, University of Cagliari, Monserrato, Italy.

*Correspondence to:

Alessio Squassina, PhD

Laboratory of Pharmacogenomics, Department of Biomedical Sciences, Section of Neuroscience and Clinical Pharmacology, University of Cagliari, Italy

Cittadella Universitaria di Monserrato

SP8 Sestu-Monserrato, Km 0.700

09042 Cagliari, Italy

E-mail address: squassina@unica.it

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ABSTRACT

Introduction: Severe psychiatric disorders such as bipolar disorder (BD), schizophrenia (SCZ), and major depressive disorder (MDD) are typically associated with a significant reduction in life expectancy compared to the general population. Among the different hypotheses formulated to explain this observation, accelerated aging has been increasingly recognized as the main culprit. At the same time, telomere shortening is becoming widely accepted as a proxy molecular marker of the aging process. With the present study, we aim to fill a gap in the literature by better defining the complex interaction/s between inflammation, age-related co-morbidities, telomere shortening and the gut microbiota in psychiatric disorders.

Methods and analysis: A cross-sectional study is proposed, recruiting 40 patients for each of three different diagnostic categories (BD, MDD, SCZ) treated at the Section of Psychiatry and at the Unit of Clinical Pharmacology of the University Hospital Agency of Cagliari (Italy), compared to 40 age and sex matched non-psychiatric controls (HC). Each group purposely includes a comparable number of individuals suffering, or not, from age-related co-morbidities, to account for the impact of these medical conditions on the biological make-up of recruited patients. The inflammatory state, microbiota composition, and the leukocytes telomere length (LTL) are assessed.

Ethics and dissemination: The present study protocol was approved by the Ethics Committee of the University Hospital Agency of Cagliari (PG/2018/11693, September 5, 2018). The study is conducted in accordance with the principles of good clinical practice, and the Declaration of Helsinki, and in compliance with the relevant Italian national legislation. Written, informed consent is obtained from all participants. Participation to the study is on a voluntary basis only, and patients may withdraw their consent at any point with no disadvantage to their treatment/s.

A psychiatric assessment establishes that the patients' ability to provide their consent is duly retained.

Keywords: biomarker; aging; allostasis; ELISA; sybr green

Article summary

Strengths and limitations of this study

- This is a novel approach proposing a systematic investigation of the complex interplay between inflammation, microbiota, and telomere shortening in modulating the liability for major psychiatric disorders.
- The balanced recruitment of individuals affected and unaffected by age-related co-morbidities among the 4 study groups, allows for a clearer definition of the possible confounding effects of these chronic medical conditions on telomere shortening, inflammatory status, and microbiota composition.
- The cross-sectional design does not allow for a causal inference of any possible finding on the explored psychiatric conditions, as these may simply represent epiphenomena of these disorders.
- The small sample size and the relatively restricted catchment area may limit the generalizability of the results.

Introduction

Bipolar disorder (BD) is a severe and chronic psychiatric disorder characterized by alternating manic and depressive episodes interspersed with periods of well-being. Its prevalence in the general population ranges from 0.8 to 1.2%[1] and is associated with high levels of disability and premature mortality. [2]Indeed, Bipolar disorder patients have a 10 to 20 years reduction in their life expectancy compared to the general population, an epidemiological mark shared with other severe mental disorders, including schizophrenia (SCZ) and major depression (MD). [3] A vast body of data suggests that this excess mortality is accounted for by a higher prevalence of co-morbid chronic somatic disorders compared to individuals without mental illnesses. [4] In fact, age-related illnesses, such as cardiovascular and metabolic disorders, whose pathophysiology carries an inflammatory component, present a significantly higher incidence in psychiatric patients than in the general population. [5,6] This evidence has led to the hypothesis that accelerated aging and inflammation may play a central role in the etiopathogenesis of mental disorders. Biological signatures of these events are represented by telomere shortening (TS) and increased levels of pro-inflammatory markers. Several studies have reported shorter leukocyte telomere length (LTL) as well as increased levels of circulating pro-inflammatory cytokines in BD, SCZ, and MD patients compared to controls. [7–9] These findings strongly support the involvement of altered telomere and inflammatory dynamics in the neurobiological underpinnings of severe psychiatric disorders and suggest that signatures of these processes might be detectable in peripheral tissues. Telomere shortening is a hallmark of cellular aging, and while telomere length reduce with each cell division, the shortening rate is increased by allostatic load and inflammation,[10] thus affecting the ageing process. Consistently, a recent study evaluating mortality in more than 64,000 subjects from the general population, showed that short telomeres in peripheral blood leukocytes were associated with high mortality. [11] So far, findings of shorter telomeres in psychiatric patients have been contradictory,[12] likely due to underpowered study

samples and limitations in methodologies, while the interaction between inflammatory processes, telomeres shortening and psychiatric disorders has been scarcely investigated. There is evidence that pro-inflammatory cytokines are important mediators of the association between depressive and anxiety disorders and LTL. [13] Moreover, the impact of co-morbid age-related disorders has been often overlooked in previous telomere studies in psychiatry. Although current data suggest that TS, inflammation and accelerated aging may play a central role in modulating the liability for severe psychiatric disorders, differences and similarities in their involvement in mental illnesses have yet to be elucidated and therefore need comprehensive investigations.

Alterations of these biological pathways in severe psychiatric disorders might also influence other relevant physiological components. It is known that an estimate of 40,000 bacterial species and 1,800 phyla inhabits our body, paralleling the number of human cells, and approaching a ratio of nearly 1:1 according to recent estimates. [14] As a whole, the microorganisms living in our body are collectively referred to as microbiota, and include bacteria, fungi and protozoa. The microbiota serves multiple physiological functions, although its overall importance has not been fully grasped yet. A state of balance between "good" bacteria and "harmful" bacteria (called "eubiosis") is therefore paramount to maintain the homeostasis of its hosting organism. On the other hand, the perturbation of this balance (called "disbiosis") is related to the development of several pathological conditions. For example, the microbiota works synergistically with the immune system to avoid colonization from pathogenic bacteria and viruses. The microbiota also plays a role in metabolism, participating in the digestion by aiding micronutrients absorption (in particular amino acids and short-chain fatty acids), and part of their further degradation as well. Another significant example is represented by the increased incidence of major cardiovascular events related to high levels of trimethylamine-N-oxide (TMAO). [15] The latter compound derives from the microbiota metabolism of several nutrients (in particular lecithin), and has been

associated with an increased cholesterol accumulation in macrophages. [15] Moreover, broad-spectrum oral antibiotics reduce the TMAO production, and upon suspension of antibiotic therapy, its production increases again, underscoring the direct relationship between TMAO production and the microbiota. An increased production of TMAO might indeed represent an independent risk factor for atherosclerosis. [15]

Although dysbiosis has been clearly associated with numerous ailments, it is not clear whether a causal relationship exists between these two phenomena or, instead, if anomalies in the microbiota composition represent an epiphenomenon of the underlying pathological processes. Similarly, the eventual preemptive or even therapeutic effects deriving from the microbiota manipulation remain unclear. However, there is now consisting evidence that the microbiota, particularly gut microbiota, exerts a modulatory function on the brain, possibly even modulating behavior. [16] Indeed, experimental data in animal models show that CNS neurotransmission can be profoundly disturbed by the absence of a normal gut microbiota [17] and that alterations of eubiosis with induced acute bacterial infection might induce cognitive alterations. [18] In humans, data have correlated alterations of microbiota with the manifestation of autism spectrum disorder (ASD). [19] Interestingly, a recent translational study operated transplants of gut microbiota from human donors with ASD controls into germ-free mice revealing that colonization with ASD microbiota was sufficient to induce hallmark autistic behaviors [20]. Other severe psychiatric disorders, such as SCZ [21] and MDD [22] show substantial microbiota imbalances. Relevant to this study protocol is the evidence that a proportion of this altered gut –brain communication in severe mental disorders, determined by a pathological microbiota, is determined through an immunomodulatory action, which includes a raise in the activity of inflammatory pathways. [23]

Study objectives

The main objective of the present protocol is to clarify the relationship between LTL and inflammation in the etiopathogenesis of BD, SCZ and MDD. Furthermore, we evaluate the impact of the age-related medical co-morbidities on these biological markers. Contextually, we aim to determine the presence of alterations in the gut microbiota composition in individuals affected by SCZ, MDD and BD, as compared with healthy controls (HC). As reported above, several lines of evidence suggest that the microbiota might play a significant role on the brain-gut axis, and microbial derangement may result in the development of several psychiatric conditions. [24]

Specifically, our study tests the following primary and secondary hypotheses:

Primary:

- To determine the differences in LTL among individuals affected by SCZ, MDD, BD and healthy controls;
- To test for the presence of differences in inflammatory markers among patients affected by SCZ, MDD, BD and healthy controls;
- To assess whether microbiota composition differs among individuals affected by SCZ, MDD, BD and healthy controls.

Secondary:

- To clarify the impact of medical co-morbidities related to aging on the differences in LTL and in the number of short telomeres, as well as on inflammatory markers levels and on microbiota;

Methods

Study design

This is a cross-sectional study in the context of which we are performing the recruitment of three cohorts, each comprised of 40 patients affected by SCZ, MDD, and BD diagnosed with the Italian version of the Structured Clinical Interview for DSMIV-TR Axis I Disorders (SCID)[25] . In addition, we are recruiting a sample of 40 HC.

Patient and Public involvement.

There was no involvement of patients in the development of this study protocol. The patients were asked to report their impression on the time required to participate in the study and the burden of the sample procedure as well. An effort is constantly made to facilitate the participation of the eligible individuals, taking into account their indications and minimizing the potential disruption given sampling procedures. Patients were not involved in the development of the recruitment process, but they will be part of the dissemination phase of the study results. Indeed, we plan to describe using lay language the main findings of the study to the participants and their families organizing a local conference.

Recruitment process

The sample is recruited from patients followed-up and treated at the community mental health centre of the Section of Psychiatry of the Department of Medical Science and Public Health, University of Cagliari and University Hospital Agency of Cagliari and the Unit of Clinical Pharmacology, University Hospital Agency, Cagliari, Italy. The recruitment process is performed on the basis of the presence or absence of medical co-morbidities related to aging (e.g. cardiovascular diseases, diabetes mellitus type 2, etc). Approximately half of the subjects for each group are recruited on the basis of the presence of such co-

morbidities. Healthy controls are recruited by word of mouth among hospital staff, their families, and university students.

Inclusion and exclusion criteria

The recruitment process is based on the following inclusion criteria: 1) patients affected by SCZ, BD, MDD according to DSM IV-TR[26] criteria; 2) able to express a consent to participate formulated by signing the consent form; 3) age between 18 and 70 years-old; 4) in euthymic phases for BD and MDD and with at least the six months of stability before recruitment for SCZ. We also apply the following exclusion criteria: 1) presence of acute infections; 2) presence of chronic autoimmune inflammatory conditions (e.g. rheumatoid arthritis, thyroiditis); 3) presence of eating disorders; 4) presence of post-traumatic stress disorder; 5) presence of current substance use disorders; 6) presence of neurological disorders; 7) past traumatic brain injury; 8) presence of severe co-morbidities that may influence molecular testing (such as cancer, HIV infection). The inclusion criteria for healthy controls comprise: 1) the absence of a personal history of mental disorders, 2) the willingness to participate in the study, 3) absence of acute infections; 4) absence of chronic autoimmune inflammatory conditions (e.g. rheumatoid arthritis, thyroiditis); 5) absence of past traumatic brain injury; 6) absence of severe co-morbidities that may influence molecular testing (such as cancer, HIV infection). In addition to the above exclusions criteria for HC and patients, the following are considered in relation to the microbiota study: use of antibiotics in the month preceding the sampling procedure, chronic use of probiotics.

Sample size estimation

Considering the magnitude of the effect size reported in the literature regarding the correlation between LTL and mood disorders (Cohen d=0.67 [27]), our sample has an 85% statistical power to

identify the existing differences between the diagnostic groups (SCZ, BD, MDD) and HC at an $\alpha = 0.05$. Similarly, we assume adequate statistical power for the microbiota analysis as we are recruiting a sample of similar size of that analyzed in the study of Yuan et al. (N=40). [28]

Clinical assessment

Recruited subjects are assessed by trained mental-health professionals (psychiatry residents or senior clinical staff). Clinical information is collected through direct interview of the patient as well as through a systematic assessment of existing medical records. Whenever possible we are collecting collateral information from at least one first degree relative or significant other, after obtaining the consent from the participant. Specific to patients' groups are the following clinical data: age of onset, history of suicide attempt, history of violent attempt, type of onset, number of episodes, including depressive, hypomanic/manic, mixed recurrences (for BD and MDD), family history of psychiatric disorders, presence of past and current substance and/or alcohol use disorder, past and current pharmacological treatments, duration of pharmacological treatment, type of drugs. Treatment resistance for MDD and SCZ patients is defined according to the criteria of Souery et al. [29] and Kane et al. [30], and is based on the clinical course and evaluation of treatment response patterns. In addition, we are collecting, for patients and HC, detailed data on the presence of past and current cigarette smoking status, number of cigarettes per day (categorized according to Fagerström criteria [31]), height, weight, body mass index, cardiovascular and metabolic co-morbidity, presence of physical activity. We are also gathering data on diet, as this is relevant to the analysis of microbiota, including: presence of vegan diet, and type of diet (categorized as Mediterranean, junk food, high meat consumption, high consumption of baked products, high carbohydrates diet, vegetarian, and vegan).

Finally, for healthy controls, the screening study visit includes a clinical interview (1 ½ hrs long). Each subject will be interviewed using the Italian version of the SCID-I/NP [25] to rule out the presence of Axis I psychiatric disorders.

Psychopathological measures

Some psychopathological measures are used to establish the stability of the illness at the moment of recruitment: the Positive and Negative Scale for Schizophrenia (PANSS) [32] and the Clinical Global Impression Scale for Schizophrenia (CGI-SCH)[33], which have both shown to be valid assessment tools for the identification of patients in remission, particularly in routine clinical practice. [34,35] In BD patients we assess the response to mood stabilizers using the "Retrospective Criteria of Long-Term Treatment Response in Research Subjects with Bipolar Disorder" scale. [36]

Blood sampling and assay procedures

Fasting peripheral venous blood samples are taken from all patients within a similar time window (8:00 to 10:00 AM). Blood samples are collected in an euthymic phase (for MDD and BD patients), or at least 6 months after their latest exacerbation of psychopathological symptoms (for SCZ patients). Specimens are divided in 3 aliquots: (1) for measurement of plasma C-reactive protein (high sensitivity CRP assay) and the proinflammatory cytokines TNF-α and IL-6, using enzyme-linked immunosorbent assays (ELISA); (2) for DNA extraction and the subsequent measurement of LTL with qPCR; (3) for measurement of telomere length using Quantitative Fluorescent in situ hybridization (qFISH) on the metaphase chromosomes in lymphocytes. [37]

Fecal microbiota analysis methods

The 16S RNA Microbial Profiling is a test capable of exploiting the recent technological advances of metagenomic in microbiota ecology: it is based on the sequencing of the regions V3 and V4 of the 16S rRNA (the gene responsible for the production of the prokaryote small ribosomal subunit), a gene which represents an important marker widely used in bacterial taxonomy. Starting from biological material (a stool sample) we are isolating bacterial DNA further processed to amplify the 16S gene rRNA. The analysis is completed thereafter by sequencing the 16S rRNA gene pool corresponding to the different microorganisms present in the microbiota, subsequently identified through the use of bioinformatic tools (Metagenomics Illumina Inc. and Kraken APP).

Data analysis plan

The association between LTL or cytokine levels and demographic/clinical variables is tested using parametric or non-parametric tests according to the observed distribution. The presence of statistically significant differences in the LTL and in the cytokine plasma levels among the different study groups (SCZ, BD, MDD, HC) is tested through linear regression. Furthermore, the presence of medical co-morbidities related to aging, and the other demographic and clinical variables significantly associated with the outcome are inserted in the regression model. The analyses will be conducted using R[38] and IBM Statistical Package for Social Science (SPSS®). As for the microbiota analysis, the position, dispersion and shape indexes will be calculated for the quantitative variables, whilst the relative frequency for each class will be calculated for categorical variables. In addition, the correlations between the different analyzed variables will be evaluated by employing the aforementioned univariate and multivariate analysis methods.

Ethics

Ethics, consent and permissions

This study protocol was approved by the Ethics Committee of the University Hospital Agency of Cagliari (PG/2018/11693) on September 5, 2018. The study is conducted in accordance with the principles of good clinical practice, with the Declaration of Helsinki and in compliance with the national legislation. Written, informed consent is being obtained from all participants. Participation to the study is voluntary and patients may be able to withdraw consent at any point with no disadvantage to their treatments. A psychiatric assessment establishes that patients' ability to consent is not compromised by their psychopathological status.

Status of recruitment

Collection of clinical data and biological samples is currently undergoing and at the moment of the preparation of the protocol 87% of the sample has been recruited (104/120). Clinical data have been already cleaned and inputted into a database in preparation of data analysis. The main socio-demographic and clinical characteristics for the BD, MDD, SCZ and HC samples are summarized in Supplementary Table 1.

Discussion

In this study, we sought to explore the relationship between telomere length, inflammation, the microbiota and the impact of their interaction on the risk of developing a severe mental disorder such as SCZ, BD or MDD. In addition, a corollary objective is to establish similarities and differences in how abnormal molecular dynamics among telomeres, inflammation and microbiota manifest in SCZ, BD, MDD. A novel approach in our study is to ascertain the impact of the age-related medical co-morbidities on such molecular dynamics. Previous research has shown that accelerated biological aging is a hallmark of severe psychiatric disorders. [39–41] Molecular signatures of this accelerated decay are currently being identified and point to TS as well as to a series of inflammatory markers. In the case of SCZ, a recent meta-analysis has found that SCZ patients have a highly statistically significant shortening of LTL compared to HC.[42] Further, a recent qualitative synthesis of the literature has found that consisting evidence points to elevated levels of pro-inflammatory cytokines and chemokines in SCZ, including interleukin (IL)- IL-1β, tumor necrosis factor alpha (TNFα), Eotaxin-1, Eotaxin-2, monocyte chemoattractant protein-1, macrophage inflammatory protein-1β, thymus- and activation-regulated chemokine, macrophage-derived chemokine, as well as a decline in the levels of the anti-inflammatory cytokine IL-2. [39] In BD patients there is evidence of a significant LTL shortening compared to HC irrespective of mood-state.[43] Similarly to SCZ, the inflammatory patterns in BD are characterized by increased levels of TNF-α, the soluble tumor necrosis factor receptor type 1 (sTNF-R1) and the soluble inlerleukin-2 receptor (sIL-2R) compared to HC. [44] Of note, there are intriguing findings, although still at the level of experimental pre-clinical data, suggesting that perturbations of the gut microbiota composition and the functional metagenome may be associated with accelerated aging in animal models. [45] In summary, the literature shows that: 1) there is a plausible interaction between

inflammation and TS in modulating the risk of severe psychiatric disorders, and 2) this association might reflect (or be modulated) by specific detrimental alterations of the microbiota. However, available data also tell that: 1) no previous studies have so far examined the role of confounders (mainly the presence of age-related medical co-morbidities) in influencing the dynamics between accelerated biological aging and inflammation in psychiatric disorders, and 2) there is a gap in the knowledge on how microbiota might influence these dynamics in humans.

Our project has been set in this context. We expect to demonstrate that a shortening of LTL and/or a higher number of short telomeres will be present in the diagnostic groups (SCZ, MDD, BD) as compared with HC. We also expect that the pro-inflammatory cytokines levels will be higher among patients and will be negatively correlated with the LTL. Further, we anticipate that patients with medical co-morbidities related to aging will present shorter telomeres and higher pro-inflammatory cytokines levels as compared with patients bereft of such co-morbidities. Finally, we expect to identify specific microbiota clusters associated with the diagnostic groups (SCZ, BD, MDD) and related to specific biological markers of inflammation and accelerated aging.

Clearly, our study findings will need to be interpreted in the context of several limitations. First, due to the cross-sectional design, we will not be able to establish causality (i.e. whether biological markers of aging resulted from an increased inflammatory load or vice versa). Due to the relatively small sample size, our study should be considered as hypothesis generator, capable of clarifying the association strength between the different variables. The resulting data will be of great importance for planning future studies in this area of research.

Notwithstanding the existing limitation deriving from the study design and the small sample size, this project appears unique and original. The role exerted by the interaction between inflammation and telomeres, and the contribution of medical co-morbidities related to aging in

influencing the effect of this interaction, have been hypothesized but never tested in a systematic manner. Moreover, there is no available data linking these biological markers to the microbiota derangements. By using an accurate methodology to better characterize the recruited patients, the synergy between different research groups with renowned and complementary skills allows for a reliable study concerning the three-sided interplay between telomeres, inflammation, and microbiota. Obtaining a deeper understanding of the influence exerted on these systems could help us in developing novel ways of managing these severely disabling conditions.

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Author statement

MM contributed to the design of the study protocol and wrote the first draft of the manuscript. PP co-wrote the first draft of the manuscript. AS designed the study protocol and co-wrote the first draft of the manuscript. CA, AB, PC, CC, DC, EC, TD, DF, MG, EM, AM, MAM, Amur, MN, BN, FP, CP, RR, GS, VS, critically revised the manuscript and contributed to the discussion. CChill, BC, MDZ, GLF, RV, contributed to the design of the study protocol and critically revised the manuscript. We wish to thank all the patients who are participating to this study. We also thank the nurses Ms. Flavia Orrù (Unit of Clinical Pharmacology, University Hospital Agency of Cagliari), and Ms. Anna Greca Floris, Mr. Gianluca Carta, Mr. Sergio Deiana, Ms. Gioia Gabriela Delogu, Mr. Giovanni Dessanai, Ms. Maria Elisabetta Loche, Mr. Giampiero Neri, Ms. Maria Cristina Olmi, Ms. Enrica Pintus, Ms. Loredana Satta (Unit of Clinical Psychiatry of the University Hospital Agency of Cagliari) for helping with the recruitment procedures. CP is supported by Fondazione Umberto Veronesi.

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Conflict of interest

The authors of this paper do not have any competing interest in relation with the content of the present study.

Supplementary Table 1. Main socio-demographic and clinical characteristics of the sample

Variable	Bipolar disorder (N=40)	Schizophrenia (N =36)	Major depressive disorder (N = 19)	Healthy controls (N = 28)	Total (N = 123)
Women, N (%)	23 (57.5)	23 (57.5)	12 (63.2)	11 (39.3)	50 (40.7)
Men, N (%)	17 (42.5)	32 (88.9)	6 (31.6)	17 (60.7)	72 (58.5)
Age (mean ± SD)	51.5±10.7	46.7±12.4	56.1±7.9	42.5±11.6	48.7±11.9
Age of onset (mean ± SD)	25.3±9.4	25.6±7.4	33.8±12.0		27.1±9.7
Paternal age at conception (mean ± SD)	33.8±5.9	33.2±6.5	33.8±4.7	33.4±5.7	33.5±5.8
History of suicide attempt, N (%)	11(27.5)	8 (22.5)	2 (16.7)		22
History of violent attempt, N (%)	4 (10.0)	1 (2.8)	2 (11.1)		7
Vegan diet, N (%)	2 (5.0)	1 (2.9)	0 (0.0)	0 (0.0)	3 (2.5)
Physical activity, N (%)	18 (45.0)	15 (41.7)	5 (27.8)	20 (71.4)	58 (47.5)
Metabolic co-morbidity, N (%)	13 (32.5)	12 (38.7)	5 (27.8)	1 (3.6)	31 (25.4)
Cardiovascular co-morbidity, N (%)	6 (15.0)	5 (13.9)	3 (16.7)	7 (25)	21(17.2)
Current treatment – lithium, N (%)	40 (100)	1 (2.8)	1 (5.6)		
Current treatment – antipsychotics, N (%)	19 (47.5)	36 (100)	4 (22.2)		
Current treatment – general, N (%)	40 (100)	36 (100)	17 (94.4)		

SD: standard deviation

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A multidisciplinary approach to mental illness: do inflammation, telomere length and microbiota form a loop? A protocol for a cross-sectional study on the complex relationship between inflammation, telomere length, gut microbiota and psychiatric disorders

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Complete List of Authors:	Manchia, Mirko; Dalhousie University, Pharmacology; Universita degli Studi di Cagliari, Medical Science and Public Health Paribello, Pasquale; University of Cagliari, Unit of Clinical Psychiatry, University Hospital Agency of Cagliari Arzedi, Carlo; University of Cagliari, Department of Public Health, Clinical and Molecular Medicine Bocchetta, Alberto; University of Cagliari, Biomedical Sciences Caria, Paola; University of Cagliari, Biomedical Sciences Cocco, Cristina; University of Cagliari, Biomedical Sciences Congiu, Donatella; University of Cagliari, Biomedical Sciences Cossu, Eleonora; University of Cagliari, Department of Public Health, Clinical and Molecular Medicine Dettori, Tinuccia; University of Cagliari, Biomedical Sciences Frau, Daniela Virginia; University of Cagliari, Biomedical Sciences Garzilli, Mario; University of Cagliari, Department of Public Health, Clinical and Molecular Medicine Manca, Elias; University of Cagliari, Biomedical Sciences Meloni, Anna; University of Cagliari, Biomedical Sciences Montis, Maria Antonietta; University of Cagliari, Department of Public Health, Clinical and Molecular Medicine Mura, Andrea; University of Cagliari, Department of Public Health, Clinical and Molecular Medicine Nieddu, Mariella; University of Cagliari, Biomedical Sciences Noli, Barbara; University of Cagliari, Biomedical Sciences Pinna, Federica; University of Cagliari Department of Public Health Clinical and Molecular Medicine Pisanu, Claudia; University of Cagliari, Biomedical Sciences Robledo, Renato; University of Cagliari, Department of Biomedical Sciences Severino, Giovanni; University of Cagliari, Biomedical Sciences Sogos, Valeria; University of Cagliari, Biomedical Sciences Chillotti, Caterina; University Hospital Agency of Cagliari, Unit of Clinical Pharmacology Carpiniello, Bernardo; Universita degli Studi di Cagliari, Medical Science and Public Health Del Zompo, Maria; University of Cagliari, Biomedical Sciences Ferri, Gian Luca; University of Cagliari, Biomedical Sciences

	Vanni, Roberta; University of Cagliari, Biomedical Sciences Squassina, Alessio; University of Cagliari, Biomedical Sciences
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A multidisciplinary approach to mental illness: do inflammation, telomere length and microbiota form a loop? A protocol for a cross-sectional study on the complex relationship between inflammation, telomere length, gut microbiota and psychiatric disorders.

Mirko Manchia[1,2,3], Pasquale Paribello[1,2], Carlo Arzedi[1,2], Alberto Bocchetta[4,5], Paola Caria[6], Cristina Cocco[5], Donatella Congiu[4], Eleonora Cossu[1,2], Tinuccia Dettori[6], Daniela Virginia Frau[6], Mario Garzilli[1,2], Elias Manca[7], Anna Meloni[4], Maria Antonietta Montis[1,2], Andrea Mura[1,2], Mariella Nieddu[6], Barbara Noli[7], Federica Pinna[1,2], Claudia Pisanu[4], Renato Robledo[6], Giovanni Severino[4], Valeria Sogos[8], Caterina Chillotti[4,5], Bernardo Carpiniello[1,2], Maria Del Zompo[4,5], Gian Luca Ferri[7], Roberta Vanni[6], Alessio Squassina[4*]

[1] Unit of Psychiatry, Department of Public Health, Clinical and Molecular Medicine, University of Cagliari, Cagliari, Italy; [2] Unit of Clinical Psychiatry, University Hospital Agency of Cagliari, Cagliari, Italy; [3] Department of Pharmacology, Dalhousie University, Halifax, Nova Scotia, Canada; [4] Department of Biomedical Sciences, Section of Neuroscience and Clinical Pharmacology, University of Cagliari, Italy; [5] Unit of Clinical Pharmacology, University Hospital Agency of Cagliari, Italy; [6] Department of Biomedical Sciences, Unit of Biology and Genetics, University of Cagliari, Italy; [7] Department of Biomedical Sciences, NEF Laboratory, University of Cagliari, Italy; [8]Department of Biomedical Sciences, Section of Cytomorphology, University of Cagliari, Monserrato, Italy.

*Correspondence to:

Alessio Squassina, PhD

Laboratory of Pharmacogenomics, Department of Biomedical Sciences, Section of Neuroscience and Clinical Pharmacology, University of Cagliari, Italy

Cittadella Universitaria di Monserrato

SP8 Sestu-Monserrato, Km 0.700

09042 Cagliari, Italy

E-mail address: squassina@unica.it

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ABSTRACT

Introduction: Severe psychiatric disorders are typically associated with a significant reduction in life expectancy compared to the general population. Among the different hypotheses formulated to explain this observation, accelerated aging has been increasingly recognized as the main culprit. At the same time, telomere shortening is becoming widely accepted as a proxy molecular marker of aging. The present study aims to fill a gap in the literature by better defining the complex interaction/s between inflammation, age-related co-morbidities, telomere shortening and gut microbiota in psychiatric disorders.

Methods and analysis: A cross-sectional study is proposed, recruiting 40 patients for each of three different diagnostic categories (bipolar disorder, schizophrenia, major depressive disorder) treated at the Section of Psychiatry and at the Unit of Clinical Pharmacology of the University Hospital Agency of Cagliari (Italy), compared to 40 age and sex matched non-psychiatric controls. Each group includes individuals suffering, or not, from age-related co-morbidities, to account for the impact of these medical conditions on the biological make-up of recruited patients. The inflammatory state, microbiota composition, and the leukocytes telomere length (LTL) are assessed.

Ethics and dissemination: The study protocol was approved by the Ethics Committee of the University Hospital Agency of Cagliari (PG/2018/11693, September 5th, 2018). The study is conducted in accordance with the principles of good clinical practice, and the Declaration of Helsinki, and in compliance with the relevant Italian national legislation. Written, informed consent is obtained from all participants. Participation to the study is on a voluntary basis only. Patients will be part of the dissemination phase of the study results, during which a local conference will be organize and families of patients will also be involved. Moreover, findings will

be published in one or more research papers, and presented at national and international conferences, in posters or oral communications.

Keywords: biomarker; aging; allostasis; ELISA;

Article summary

Strengths and limitations of this study

- This is a novel approach proposing a systematic investigation of the complex interplay between inflammation, microbiota, and telomere shortening in modulating the liability for major psychiatric disorders.
- The balanced recruitment of individuals affected and unaffected by age-related co-morbidities among the 4 study groups, allows for a clearer definition of the possible confounding effects of these chronic medical conditions on telomere shortening, inflammatory status, and microbiota composition.
- The cross-sectional design does not allow for a causal inference of any possible finding on the explored psychiatric conditions, as these may simply represent epiphenomena of these disorders.
- The small sample size and the relatively restricted catchment area may limit the generalizability of the results.

Introduction

Bipolar disorder (BD) is a severe and chronic psychiatric disorder characterized by alternating manic and depressive episodes interspersed with periods of well-being. Its prevalence in the general population ranges from 0.8 to 1.2%[1] and is associated with high levels of disability and premature mortality[2]. Indeed, BD patients have a 10 to 20 years reduction in their life expectancy compared to the general population, an epidemiological mark shared with other severe mental disorders, including schizophrenia (SCZ) and major depressive disorder (MDD). [3] Both disorders are clinically severe, with SCZ characterized by positive symptoms such as delusions and hallucinations, negative symptoms (with avolition and social withdrawal) and significant permanent cognitive impairment[4], and MDD by single or recurrent episodes of low mood and decreased energy often associated with high levels of anxiety and cognitive impairment[5]. A vast body of data suggests that the excess mortality of these severe psychiatric disorders is accounted for by a higher prevalence of co-morbid chronic somatic disorders compared to individuals without mental illnesses[6]. In fact, age-related illnesses, such as cardiovascular and metabolic disorders, whose pathophysiology carries an inflammatory component, present a significantly higher incidence in psychiatric patients than in the general population[7,8]. This evidence has led to the hypothesis that accelerated aging and inflammation may play a central role in the etiopathogenesis of mental disorders. Biological signatures of these events are represented by telomere shortening (TS) and increased levels of pro-inflammatory markers. Several studies have reported shorter leukocyte telomere length (LTL) as well as increased levels of circulating pro-inflammatory cytokines in BD, SCZ, and MDD patients compared to controls [9–11] , suggesting that the interaction and crosstalk between these two biological pathways might play a central role in the neurobiology of psychiatric disorders. Telomere shortening is a hallmark of cellular aging, and while telomere length reduces with each cell division, the shortening rate is increased by allostatic load and inflammation,[12] thus affecting the ageing process. Consistently, a

recent study evaluating mortality in more than 64,000 subjects from the general population, showed that short telomeres in peripheral blood leukocytes were associated with high mortality[13]. So far, findings of shorter telomeres in psychiatric patients have been contradictory,[14] likely due to underpowered study samples and limitations in methodologies, while the interaction between inflammatory processes, telomeres shortening and psychiatric disorders has been scarcely investigated. There is evidence that pro-inflammatory cytokines are important mediators of the association between depressive and anxiety disorders and LTL[15]. Moreover, the impact of co-morbid age-related disorders has been often overlooked in previous telomere studies in psychiatry. Although current data suggest that TS, inflammation and accelerated aging may play a central role in modulating the liability for severe psychiatric disorders, differences and similarities in their involvement in mental illnesses have yet to be elucidated and therefore need comprehensive investigations.

Alterations of these biological pathways in severe psychiatric disorders might also influence other relevant physiological components. It is known that an estimate of 40,000 bacterial species and 1,800 phyla inhabits our body, paralleling the number of human cells, and approaching a ratio of nearly 1:1 according to recent estimates[16]. As a whole, the microorganisms living in our body are collectively referred to as microbiota, and include bacteria, fungi and protozoa. The microbiota serves multiple physiological functions, although its overall importance has not been fully grasped yet. A state of balance between "good" bacteria and "harmful" bacteria (called "eubiosis") is therefore paramount to maintain the homeostasis of its hosting organism. On the other hand, the perturbation of this balance (called "disbiosis") is related to the development of several pathological conditions. For example, the microbiota works synergistically with the immune system to avoid colonization from pathogenic bacteria and viruses. The microbiota also plays a role in metabolism, participating in the digestion by aiding

micronutrients absorption (in particular amino acids and short-chain fatty acids), and part of their further degradation as well.

Although dysbiosis has been clearly associated with numerous ailments, it is not clear whether a causal relationship exists between these two phenomena or, instead, if anomalies in the microbiota composition represent an epiphenomenon of the underlying pathological processes. Similarly, the eventual preemptive or even therapeutic effects deriving from the microbiota manipulation remain unclear. However, there is now consisting evidence that the microbiota, particularly gut microbiota, exerts a modulatory function on the brain, possibly even modulating behavior[17]. Indeed, experimental data in animal models show that CNS neurotransmission can be profoundly disturbed by the absence of a normal gut microbiota [18] and that alterations of eubiosis with induced acute bacterial infection might induce cognitive alterations[19]. In humans, data have correlated alterations of microbiota with the manifestation of autism spectrum disorder (ASD)[20]. Interestingly, a recent translational study operated transplants of gut microbiota from human donors with ASD controls into germ-free mice revealing that colonization with ASD microbiota was sufficient to induce hallmark autistic behaviors [21]. Other severe psychiatric disorders, such as SCZ[22] and MDD[23] show substantial microbiota imbalances. Relevant to this study protocol is the evidence that a proportion of this altered gut –brain communication in severe mental disorders, determined by a pathological microbiota, is determined through an immunomodulatory action, which includes a raise in the activity of inflammatory pathways[24].

Study objectives

The main objective of the present protocol is to clarify the relationship between LTL and inflammation in the etiopathogenesis of BD, SCZ and MDD. Furthermore, we evaluate the impact of the age-related medical co-morbidities on these biological markers. Contextually, we aim to

determine the presence of alterations in the gut microbiota composition in individuals affected by SCZ, MDD and BD, as compared with healthy controls (HC). As reported above, several lines of evidence suggest that the microbiota might play a significant role on the brain-gut axis, and microbial derangement may result in the development of several psychiatric conditions. [25]

Specifically, our study tests the following primary and secondary hypotheses:

Primary:

- To determine the differences in LTL among individuals affected by SCZ, MDD, BD and HC;
- To test for the presence of differences in inflammatory markers among patients affected by SCZ, MDD, BD and HC;
- To assess whether microbiota composition differs among individuals affected by SCZ, MDD, BD and HC.

Secondary:

- To clarify the impact of medical co-morbidities related to aging on the differences in LTL and in the number of short telomeres, as well as on inflammatory markers levels and on microbiota;

Methods

Study design

This is a cross-sectional study in the context of which we are performing the recruitment of three cohorts, each comprised of 40 patients affected by SCZ, MDD, and BD diagnosed with the Italian version of the Structured Clinical Interview for DSMIV-TR Axis I Disorders (SCID)[26] . In addition, we are recruiting a sample of 40 HC.

Patient and Public involvement.

There was no involvement of patients in the development of this study protocol. The patients were asked to report their impression on the time required to participate in the study and the burden of the sample procedure as well. An effort is constantly made to facilitate the participation of the eligible individuals, taking into account their indications and minimizing the potential disruption given sampling procedures. Patients were not involved in the development of the recruitment process, but they will be part of the dissemination phase of the study results.

Recruitment process

The sample is recruited from patients followed-up and treated at the community mental health centre of the Section of Psychiatry of the Department of Medical Science and Public Health, University of Cagliari and University Hospital Agency of Cagliari and the Unit of Clinical Pharmacology, University Hospital Agency, Cagliari, Italy. The recruitment process is performed on the basis of the presence or absence of medical co-morbidities related to aging (e.g. cardiovascular diseases, diabetes mellitus type 2, etc). Approximately half of the subjects for each group are recruited on the basis of the presence of such co-morbidities. Healthy controls are recruited by word of mouth among hospital staff, their families, and

university students. Further, they will undergo a standard medical and laboratory test (including CBC, liver and kidney function) to verify their health status.

Inclusion and exclusion criteria

The recruitment process is based on the following inclusion criteria: 1) patients affected by SCZ, BD, MDD according to DSM IV-TR[27] criteria; 2) able to express a consent to participate formulated by signing the consent form; 3) age between 18 and 70 years-old; 4) in euthymic phases for BD and MDD and with at least the six months of stability before recruitment for SCZ. We also apply the following exclusion criteria: 1) presence of acute infections; 2) presence of chronic autoimmune inflammatory conditions (e.g. rheumatoid arthritis, thyroiditis); 3) presence of eating disorders; 4) presence of post-traumatic stress disorder; 5) presence of current substance use disorders; 6) presence of neurological disorders; 7) past traumatic brain injury; 8) presence of severe co-morbidities that may influence molecular testing (such as cancer, HIV infection). The inclusion criteria for HC comprise: 1) the absence of a personal history of mental disorders, 2) the willingness to participate in the study, 3) absence of acute infections; 4) absence of chronic autoimmune inflammatory conditions (e.g. rheumatoid arthritis, thyroiditis); 5) absence of past traumatic brain injury; 6) absence of severe co-morbidities that may influence molecular testing (such as cancer, HIV infection). In addition to the above exclusions criteria for HC and patients, the following are considered in relation to the microbiota study: use of antibiotics in the three months preceding the sampling procedure, chronic use of probiotics.

Sample size estimation

Considering the magnitude of the effect size reported in the literature regarding the correlation between LTL and mood disorders (Cohen d=0.67 [28]), our sample has an 85% statistical power to

identify the existing differences between the diagnostic groups (SCZ, BD, MDD) and HC at an $\alpha = 0.05$. Even if the microbiota analysis remains mainly exploratory it is important to note that previous research was performed in samples of comparable size both for SCZ[29] and for MDD[30].

Clinical assessment

Recruited subjects are assessed by trained mental-health professionals (psychiatry residents or senior clinical staff). Clinical information is collected through direct interview of the patient as well as through a systematic assessment of existing medical records. Whenever possible we are collecting collateral information from at least one first degree relative or significant other, after obtaining the consent from the participant. Specific to patients' groups are the following clinical data: age of onset, history of suicide attempt, history of violent attempt, type of onset, number of episodes, including depressive, hypomanic/manic, mixed recurrences (for BD and MDD), family history of psychiatric disorders, presence of past and current substance and/or alcohol use disorder, past and current pharmacological treatments, duration of pharmacological treatment, type of drugs. Treatment resistance for MDD and SCZ patients is defined according to the criteria of Souery et al. [31] and Kane et al. [32], and is based on the clinical course and evaluation of treatment response patterns. In addition, we are collecting, for patients and HC, detailed data on the presence of past and current cigarette smoking status, number of cigarettes per day (categorized according to Fagerström criteria [33]), height, weight, body mass index, cardiovascular and metabolic co-morbidity, presence of physical activity. We are also gathering data on diet, as this is relevant to the analysis of microbiota, by asking participants to complete the food frequently questionnaires (FFQ) and 72 hours narrative summary of dietary intake prior to sampling, based on which all subjects will be characterized as belonging to one or more of the following categories: Mediterranean, junk food, high meat consumption, high consumption of baked products, high

carbohydrates diet, vegetarian, and vegan. Finally, for HC, the screening study visit includes a clinical interview (1 ½ hrs long). Each subject will be interviewed using the Italian version of the SCID-I/NP [26] to rule out the presence of Axis I psychiatric disorders.

Psychopathological measures

Some psychopathological measures are used to establish the stability of the illness at the moment of recruitment: the Positive and Negative Scale for Schizophrenia (PANSS) [34] and the Clinical Global Impression Scale for Schizophrenia (CGI-SCH)[35], which have both shown to be valid assessment tools for the identification of patients in remission, particularly in routine clinical practice[36,37]. In BD patients we assess the response to mood stabilizers using the "Retrospective Criteria of Long-Term Treatment Response in Research Subjects with Bipolar Disorder" scale[38].

Blood sampling and assay procedures

Fasting peripheral venous blood samples are taken from all patients within a similar time window (8:00 to 10:00 AM). Blood samples will be collected in an euthymic phase (for MDD and BD patients), or at least 6 months after their latest exacerbation of psychopathological symptoms (for SCZ patients). Specimens will be divided in 3 aliquots: (1) for measurement of plasma C-reactive protein (high sensitivity CRP assay) and the proinflammatory cytokines TNF-α and IL-6, using enzyme-linked immunosorbent assays (ELISA); (2) for DNA extraction and the subsequent measurement of LTL with qPCR, performed according to the method described by Cawthon [39]; (3) for measurement of telomere length using Quantitative Fluorescent in situ hybridization (qFISH) on the metaphase chromosomes in lymphocytes, carried out as described in Cantara et al. [40].

Fecal microbiota analysis methods

The 16S RNA Microbial Profiling is a test capable of exploiting the recent technological advances of metagenomic in microbiota ecology: it is based on the sequencing of the regions V3 and V4 of the 16S rRNA (the gene responsible for the production of the prokaryote small ribosomal subunit), a gene which represents an important marker widely used in bacterial taxonomy. Starting from biological material (a stool sample) we are isolating bacterial DNA using the QIAamp DNA Stool Mini Kit (Qiagen, Milan, Italy). DNA is then employed for amplification of the V3–V4 hypervariable regions of the bacterial 16S ribosomal RNA (rRNA) with Next Generation Sequencing using a MiSeq Instrument (Illumina Inc., San Diego, CA, USA), as previously described[41]. The analysis is completed thereafter by sequencing the 16S rRNA gene pool corresponding to the different microorganisms present in the microbiota, subsequently identified through the use of bioinformatic tools (Metagenomics Illumina Inc. and Kraken APP).

Data analysis plan

The association between LTL or cytokine levels and demographic/clinical variables is tested using parametric or non-parametric tests according to the observed distribution. The presence of statistically significant differences in the LTL and in the cytokine plasma levels among the different study groups (SCZ, BD, MDD, HC) is tested through linear regression. Furthermore, the presence of medical co-morbidities related to aging, and the other demographic and clinical variables significantly associated with the outcome are inserted in the regression model. The analyses will be conducted using R[42] and IBM Statistical Package for Social Science (SPSS®). As for the microbiota analysis, the position, dispersion and shape indexes will be calculated for the

quantitative variables, whilst the relative frequency for each class will be calculated for categorical variables. In addition, the correlations between the different analyzed variables will be evaluated by employing the aforementioned univariate and multivariate analysis methods.

Ethics and dissemination

Ethics, consent and permissions

This study protocol was approved by the Ethics Committee of the University Hospital Agency of Cagliari (PG/2018/11693) on September 5th, 2018. The study is conducted in accordance with the principles of good clinical practice, with the Declaration of Helsinki and in compliance with the national legislation. Written, informed consent is being obtained from all participants. Participation to the study is voluntary and patients may be able to withdraw consent at any point with no disadvantage to their treatments. A psychiatric assessment establishes that patients' ability to consent is not compromised by their psychopathological status.

Dissemination

We plan to describe the main findings of the study to the participants and their families organizing a local conference using lay language. Moreover, we will also organize a public event open to the community where we will explain and discuss the potential impact of the findings of our project on the management of bipolar disorder. Findings will also be published in one or more research papers, and presented at national and international conferences, in posters or oral communications.

Status of recruitment

The enrollment of patients and controls started on January 2019. Collection of clinical data and biological samples is currently undergoing and at the moment of the preparation of the protocol 87% of the sample has been recruited (104/120). Clinical data have been already cleaned and inputted into a database in preparation of data analysis. The main socio-demographic and clinical characteristics for the BD, MDD, SCZ and HC samples are summarized in Supplementary Table 1. The planned end date for the study is February 29th 2020.

Discussion

In this study, we sought to explore the relationship between telomere length, inflammation, the microbiota and the impact of their interaction on the risk of developing a severe mental disorder such as SCZ, BD or MDD. In addition, a corollary objective is to establish similarities and differences in how abnormal molecular dynamics among telomeres, inflammation and microbiota manifest in SCZ, BD, MDD. A novel approach in our study is to ascertain the impact of the age-related medical co-morbidities on such molecular dynamics. Previous research has shown that accelerated biological aging is a hallmark of severe psychiatric disorders[43–45]. Molecular signatures of this accelerated decay are currently being identified and point to TS as well as to a series of inflammatory markers. In the case of SCZ, a recent meta-analysis has found that SCZ patients have a highly statistically significant shortening of LTL compared to HC[46]. Further, a recent qualitative synthesis of the literature has found that consisting evidence points to elevated levels of pro-inflammatory cytokines and chemokines in SCZ, including interleukin (IL)- IL-1β, tumor necrosis factor alpha (TNFα), Eotaxin-1, Eotaxin-2, monocyte chemoattractant protein-1, macrophage inflammatory protein-1β, thymus- and activation-regulated chemokine, macrophage-derived chemokine, as well as a decline in the levels of the anti-inflammatory cytokine IL-2[43]. In BD patients there is evidence of a significant LTL shortening compared to HC irrespective of mood-state[47]. Similarly to SCZ, the inflammatory patterns in BD are characterized by increased levels of TNF-α, the soluble tumor necrosis factor receptor type 1 (sTNF-R1) and the soluble inlerleukin-2 receptor (sIL-2R) compared to HC[48]. Of note, there are intriguing findings, although still at the level of experimental pre-clinical data, suggesting that perturbations of the gut microbiota composition and the functional metagenome may be associated with accelerated aging in animal models[49]. In summary, the literature shows that: 1) there is a plausible interaction between

inflammation and TS in modulating the risk of severe psychiatric disorders, and 2) this association might reflect (or be modulated) by specific detrimental alterations of the microbiota. However, available data also tell that: 1) no previous studies have so far examined the role of confounders (mainly the presence of age-related medical co-morbidities) in influencing the dynamics between accelerated biological aging and inflammation in psychiatric disorders, and 2) there is a gap in the knowledge on how microbiota might influence these dynamics in humans.

Our project has been set in this context. We expect to demonstrate that a shortening of LTL and/or a higher number of short telomeres will be present in the diagnostic groups (SCZ, MDD, BD) as compared with HC. We also expect that the pro-inflammatory cytokines levels will be higher among patients and will be negatively correlated with the LTL. Further, we anticipate that patients with medical co-morbidities related to aging will present shorter telomeres and higher pro-inflammatory cytokines levels as compared with patients bereft of such co-morbidities. Finally, we expect to identify specific microbiota clusters associated with the diagnostic groups (SCZ, BD, MDD) and related to specific biological markers of inflammation and accelerated aging.

Clearly, our study findings will need to be interpreted in the context of several limitations. First, due to the cross-sectional design, we will not be able to establish causality (i.e. whether biological markers of aging resulted from an increased inflammatory load or vice versa). Due to the relatively small sample size, our study should be considered as hypothesis generator, capable of clarifying the association strength between the different variables. The resulting data will be of great importance for planning future studies in this area of research.

Notwithstanding the existing limitation deriving from the study design and the small sample size, this project appears unique and original. The role exerted by the interaction between inflammation and telomeres, and the contribution of medical co-morbidities related to aging in

influencing the effect of this interaction, have been hypothesized but never tested in a systematic manner. Moreover, there is no available data linking these biological markers to the microbiota derangements. By using an accurate methodology to better characterize the recruited patients, the synergy between different research groups with renowned and complementary skills allows for a reliable study concerning the three-sided interplay between telomeres, inflammation, and microbiota. Obtaining a deeper understanding of the influence exerted on these systems could help us in developing novel ways of managing these severely disabling conditions.

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Author statement

MM contributed to the design of the study protocol and wrote the first draft of the manuscript. PP co-wrote the first draft of the manuscript. AS designed the study protocol and co-wrote the first draft of the manuscript. CA, AB, PC, CC, DC, EC, TD, DF, MG, EM, AM, MAM, Amur, MN, BN, FP, CP, RR, GS, VS, critically revised the manuscript and contributed to the discussion. CChill, BC, MDZ, GLF, RV, contributed to the design of the study protocol and critically revised the manuscript.

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Conflict of interest

The authors of this paper do not have any competing interest in relation with the content of the present study.

Supplementary Table 1. Main socio-demographic and clinical characteristics of the sample

Variable	Bipolar disorder (N=40)	Schizophrenia (N =36)	Major depressive disorder (N = 19)	Healthy controls (N = 28)	Total (N = 123)
Women, N (%)	23 (57.5)	23 (57.5)	12 (63.2)	11 (39.3)	50 (40.7)
Men, N (%)	17 (42.5)	32 (88.9)	6 (31.6)	17 (60.7)	72 (58.5)
Age (mean ± SD)	51.5±10.7	46.7±12.4	56.1±7.9	42.5±11.6	48.7±11.9
Age of onset (mean ± SD)	25.3±9.4	25.6±7.4	33.8±12.0		27.1±9.7
Paternal age at conception (mean ± SD)	33.8±5.9	33.2±6.5	33.8±4.7	33.4±5.7	33.5±5.8
History of suicide attempt, N (%)	11(27.5)	8 (22.5)	2 (16.7)		22
History of violent attempt, N (%)	4 (10.0)	1 (2.8)	2 (11.1)		7
Vegan diet, N (%)	2 (5.0)	1 (2.9)	0 (0.0)	0 (0.0)	3 (2.5)
Physical activity, N (%)	18 (45.0)	15 (41.7)	5 (27.8)	20 (71.4)	58 (47.5)
Metabolic co-morbidity, N (%)	13 (32.5)	12 (38.7)	5 (27.8)	1 (3.6)	31 (25.4)
Cardiovascular co-morbidity, N (%)	6 (15.0)	5 (13.9)	3 (16.7)	7 (25)	21(17.2)
Current treatment – lithium, N (%)	40 (100)	1 (2.8)	1 (5.6)		
Current treatment – antipsychotics, N (%)	19 (47.5)	36 (100)	4 (22.2)		
Current treatment – general, N (%)	40 (100)	36 (100)	17 (94.4)		

SD: standard deviation