Mood, immunity and brain connectivity in patients with chronic hepatitis C

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MOOD, IMMUNITY AND BRAIN CONNECTIVITY IN PATIENTS WITH CHRONIC HEPATITIS C

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PhD THESIS

Barcelona, 2019

Programa de Doctorat de Medicina i Recerca Translacional

Departament de Medicina

Facultat de Medicina

Universitat de Barcelona

MOOD, IMMUNITY AND BRAIN CONNECTIVITY IN PATIENTS WITH CHRONIC HEPATITIS C

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A Gabriele, mi hermano

A Camilla, per l'amicizia

Ai miei genitori

E a Valentina.

ACKNLOWLEDGMENTS

El primer agradecimiento va a la Dra. Rocío Martin-Santos. Su constancia, sabiduría, entusiasmo, paciencia (mucha) y humanidad, han sido una preciosa enseñanza, y han permitido que esta tesis viera la luz. Muchas gracias.

Then, I would like to thank Dr. Lucile Capuron, for the time dedicated, for her confidence and disposability, and to give me the opportunity to work in her lab. It has been a great professional experience.

Un agradecimiento especial a la Dra. Blanco-hinojo y al Dr. Pujol, dos científic@s tremendamente brillantes. Ha sido un honor colaborar con vosotr@s.

Agradecer también la Unidad de Hepatitis y de Farmacología del Hospital del Mar/IMIM (Dr. Ricard Solá, Dolors Gimenez y Magi Farré) y la Unidad de Hepatitis del Hospital Clínic (Dra. Zoe Mariño, Dr. Xavier Forns y Concepció Bartres), por la constancia y la preciosa colaboración.

Al "Samba team": a la Dra.Cavero, al Dr.Navinés, a Elfi Egmond y (como outsider) al Dr. García-Rizo por la colaboración y el apoyo moral y hasta psicoterapéutico !!!

A Luis Pintor, Anna Bastidas y Sandra Herranz. Por los buenos momentos pasados en la 9.0.

A la PUR6. Por existir (y sobrevivir !!).

A Salva y al Multisensory Reasearch Group… Where all it has begun!

A Eduard Vieta, por su presencia y escucha incondicionada.

Al Hospital de día de Córsega, mi nueva casa ☺

Y sobretodo, un gracias a tod@s l@s que he cruzado en estos grandiosos años de Hospital Clínic: Co-Rs, R-grandes, R-pequeños, Rotex, becarios, adjunt@s, adjuntill@s, jefes, jefaz@s, enfermer@s, camiller@s... En fin, al Hospital Clínic, casa y escuela de vida.

Per terminare, un grazie speciale alla mia famiglia, allargata, all'osteria "baita mia", e a tutti coloro che mi han sostenuto da lontano e da vicino.

PROLOGUE

This thesis has been developed in the Department of Medicine, University of Barcelona, *Hospital Clinic de Barcelona*, *Institut d'Investigació Biomèdica August Pi i Sunyer* (IDIBAPS), and in the *Centro de Investigación Biomédica en Red de Salud Mental* (CIBERSAM) of Barcelona (Spain).

The studies presented in this thesis have been done in part with grants:

Instituto de Salud Carlos III, grant P110/02206, (Rocio Martin-Santos) and P110/02291 (Ricard Solà), integrated in the *Plan Nacional I+D+I, Ministerio de Economía y Desarrollo*, and co-founded by *Fondo Europeo de Desarrollo Regional* (FEDER-"Una manera de hacer Europa").

The studies presented were supported by *Secretaria d'Universitats I Recerca del Departament d'Economia I Coneixement, Grups consolidats de recerca* (2014_SGR_1431; 2017_SGR_1798) and CIBERSAM.

The thesis includes **2 original scientific papers already published.**

PAPER 1

Title: Systematic review with meta-analysis: neuroimaging in hepatitis C chronic infection.

Authors: **Oriolo G,** Egmond E, Mariño Z, Cavero M, Navines R, Zamarrenho L, Solà R, Pujol J, Bargallo N, Forns X, Martin-Santos R.

Journal: Alimentary Pharmacology & Therapeutics 2018; 47 (9): 1238 – 1252.
DOI: 10.1111/apt.14594

IF: 7,357 (2018); Cites: 4

PAPER 2

Title: Association of chronic inflammation and perceived stress with abnormal functional connectivity in brain areas involved with interoception in hepatitis C patients.

Authors: **Oriolo G**, Blanco-Hinojo L, Navinés R, Mariño Z, Martín-Hernández D, Cavero M, Giménez D, Caso J, Capuron L, Forns X, Pujol J, Sola R, Martin-Santos R.

Journal: Brain, Behavior and Immunity 2019; Epub ahead of print.
DOI: 10.1016/j.bbi.2019.03.008

IF: 6.306 (2018); Cites: 0

Preliminary results of this doctoral thesis have been previously presented in international and national congresses

Simposium XXI Spanish National Congress – CNP, Granada (2018)

"Repercusiones del estrés oxidativo y de la inflamación crónica en el estado de ánimo".

Chair: Dra. Rocío Martín-Santos. Speakers: **Dr. Oriolo**, Dra. Aróstegui, Dra.Jimenez.

Session Dr. Oriolo: *"Relación entre el virus de la hepatitis C con la neuroinflamación y activación de la microglia en áreas cerebrales involucradas en la depresión, neurotransmisión, respuesta endocrina y estrés oxidativo"*.

Presented Poster and Oral Communication

- **Oriolo G,** Egmond E, Cavero M, Mariño Z, Bargalló N, Navinés R, Forns X, Martin-Santos R. *Neuroimaging in hepatitis C chronic infection: a systematic review and meta-analysis.* (5th European Association of Psychosomatic Medicine - EAPM Annual Scientific Conference, Barcelona). 2017

- **Oriolo G,** Navines R, Blanco-Hinojo L, Martin-Hernandez D, Mariño Z, Cavero M, Sola R, Capuron L, Pujol J, Forns X, Martin-Santos R. *Chronic inflammation in hepatitis C patients is associated with increased perceived stress and abnormal connectivity between insula and basal ganglia* (31st European Congress of Neuropsychopharmacology – ECNP, Barcelona). 2018

- **Oriolo G,** Blanco-Hinojo L, Martin-Hernandez D, Mariño Z, Navines R, Sola R, Leza JC, Pujol J, Forns X, Martin-Santos R. *Functional connectivity differences associated to chronic*

hepatitis C: a case-control study (The Lancet Summit: inflammation and immunity in disorders of the brain and mind, Barcelona). 2018

- **Oriolo G,** Navines R, Blanco-Hinojo L, Martin-Hernandez D, Mariño Z, Cavero M, Sola R, Leza JC, Pujol J, Forns X, Martin-Santos R. Aspectos neurobiológicos y clínicos de la conducta de enfermedad mantenida (XXI Spanish National Congress – CNP, Granada). 2018

OTHERS RELATED WORKS

Published Papers

- **Oriolo G**, Huet L, Dexpert S, Beau C, Forestier D, Ladaguenel P, Magne E, Martin-Santos R, Capuron L. *History of Major Depression predicts Neuropsychiatric Symptoms but not Systemic Inflammation in a Cross-sectional Study in Obese Patients*. Brain Behav Immun. 2019; 76:215-222. FI: 6.30

- Machado M, **Oriolo G,** Bortolato B, Kohler C, Maes M, Solmi M, Grande I, Martín-Santos R, Vieta E, Carvalho A. *Biological Mechanism of Depression following treatment with Interferon for chronic hepatitis C: A critical systematic review.* J Affect Disord. 2017; 209:235-245. FI: 3.57

- Udina M, Navinés R, Egmond E, **Oriolo G,** Langorh K, Gimenez D et al. *Glucocorticoid receptors, brain-derived neurotrophic factor, serotonin and dopamine neurotransmission are associated with interferon-induced depression.* Int J Neuropsychopharmacol 2016; 19(4): FI: 4.33

Book Chapters

- **Oriolo G,** Grande I, Martin-Santos R, Vieta E, Carvalho A. *Pathways driving neuroprogression in depression: the role of immune activation.* In BT. Baune editor. Inflammation and immunity in depression. New York: Elsevier; 2018. ISBN: 9780128110737.

Presented Poster and Oral communications

- Egmond E, Navinés R, **Oriolo G,** Mariño Z, Pla A, Bartres C, Cavero M, Subirá S, Forns X, Martin-Santos R. *New antiviral treatments for chronic hepatitis C and health-related quality of life: a systematic review and meta-analysis.*

J Psychosom Res. 2017; 97:147.

- **Oriolo G,** Huet L, Dexpert S, Aubert A, Aouizerate B, Magne E, Beau C, Ledaguenel P, Forestier D, Fuchs D, Martín-Santos R, Capuron L. *History of depression is associated with neuropsychiatric symptoms and augmented inflammatory markers in a cross sectional study on obese patients.*

Eur Neuropsychopharmacol. 2017; 27(4S):S627–S628

- Mariño Z, Egmond E, Pla A, Bartres C, **Oriolo G**, Cavero M, Lens S, Navinès R, Forns X, Martín-Santos R. *Significant incidence of psychiatric disorders despite rapid and positive impact of direct acting antivirals on quality of life.*

J Hepatol 2017; 66:S543-S750

- **Oriolo G,** Egmond E, Navines R, Cavero M, Mariño Z, Forns X, Martin-Santos R. *Prevalencia e incidencia de trastorno depresivo mayor y factores de riesgo asociados en*

pacientes con hepatitis C crónica tratados con antivirales de acción directa (XX Congreso Nacional de Psiquiatría Español, Barcelona). 2017

- Egmond E, **Oriolo G,** Cavero M, Langohr K, Solá R, Navines R, Martín-Santos R. *Substance abuse and quality of life in chronic hepatits C patients receiving antiviral treatment.*

Poster walk session: Selected as an oral communication in the in 24th European Congress of Psychiatry, Madrid.

Eur Psychiat. 2016; 33S:S114-S289.

- Egmond E, **Oriolo G**, Pla A, Bartres C, Cavero M, Mariño Z, Navines R, Forns X, Martín-Santos R. *Do new direct-acting antiviral treatments impact quality of life in chronic hepatitis C patients? Preliminary data.* (18th ISBD Conference, Amsterdam). 2016

- **Oriolo G**, Egmond E, Navines R, Cavero M, Mariño Z, Forns X, Martín-Santos R. *Depression and anxiety disorders incidence during direct acting antiviral treatment in chronic hepatitis C* (18th ISBD Conference, Amsterdam). 2016

- Egmond E, **Oriolo G,** Cavero M, Cañizares S, Navinés R, Solá R, Gimenez D, Martin-Santos R. *Comorbilidad psiquiátrica y calidad de vida en pacientes con hepatitis C crónica y trastorno de dependencia de sustancias a lo largo del tratamiento con interferón-alfa y ribavirina* (XVIII Congreso Nacional de Psiquiatría Español, Santiago de Compostela). 2015

AWARDS AND GRANTS

ECNP Poster Travel Award.: **Oriolo G,** Navines R, Blanco-Hinojo L, Martin-Hernandez D, Mariño Z, Cavero M, Sola R, Capuron L, Pujol J, Forns X, Martin-Santos R. *Chronic inflammation in hepatitis C patients is associated with increased perceived stress and abnormal connectivity between insula and basal ganglia* (31st European Congress of Neuropsychopharmacology – ECNP, Barcelona, 2018).

Grant "Emili-Letang" for one-year project "Longitudinal study of metabolic and inflammatory biomarkers in major depression", provided by Hospital Clínic de Barcelona. 2017-2018

Selection at 10 Best Posters at 5th European Association of Psychosomatic Medicine - EAPM Annual Scientific Conference, Barcelona. (Egmond E, Navinés R, **Oriolo G,** Mariño Z, Pla A, Bartres C, Cavero M, Subirá S, Forns X, Martin-Santos R. *New antiviral treatments for chronic hepatitis C and health-related quality of life: a systematic review and meta-analysis*) 2017

Grant for intership in foreign countries, address to Residents in Psychiatry, announcement 2015-2016, provided by Fundación Española de Psiquiatría y Salud Mental (Spanish Psychiatry and Mental Health Foundation) 2016

Grant for pre-doctoral students to facilitate assistance to an international congress, provided by the Institute of Neuroscience of the Universidad de Barcelona. 2016

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ABBREVIATIONS

Abbreviations

1-MT: 1- Methyl tryptophan

5-HT: Serotonin

AA: Arachidonic acid

ACTH: Adrenocorticotropic hormone

ATP: Adenosine tryphosphate

BDNF: Brain derived growth factor

BH4: Tetrahydrobiopterin

Ca^{2+}: Calcium

CCL-20: Chemokine ligand 20

CHC: Chronic hepatitis C

COX-: Cyclooxygenase-

CRH: Corticotrophin releasing hormone

CRP: C-reactive protein

dACC: Dorsal anterior cingulate cortex

DAMPs: Damaged associated molecular patterns

DGLA: Di-homo-gamma-linolenic acid

DHA: Docosahexaenoic acid

DSM-: Diagnostic and statistical manual of mental disorders-

EPA: Eicosa-pentaenoic acid

fMRI: Functional magnetic resonance imaging

HCV: Hepatitis-C virus

HDRS: Hamilton depression rating scale

HPA: Hypothalamic pituitary adrenal

ICAM-1: Intercellular adhesion molecules-1

IBA-1: Ionized binding calcium adapter-1

Ig-: Immunoglobulin-

IDO: Indoleamine 2,3 dioxygenase

IFN-: Interferon-

IGF: Insulin growth factor

IL-: Interleukin-

iNOS: Inducible nitric oxide synthase

LDL: Low-density lipoprotein

LPS: Lipopolysaccharide

MADRS: Montgomery-Asberg depression rating scale

MAP-Kinase: Mitogen-activated protein kinase

MCP-1: Monocyte chemoattracting protein-1

MDD: Major depressive disorder

NET: Noradrenalin transporter

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NGF: Nerve growth factor

NLRP3: Nod-like receptor pyrin-domain containing 3

NMDA: N-methyl-D-aspartate

O&NS: Oxidative and nitrosative stress

PAMPs: Pathogen associated molecular patterns

PATHOS-D: Pathogen host defence

PET: Positron emission tomography

PGx: Prostaglandin-x

PHQ-9: Physical health questionnaire

PPARγ: Proliferator-activated receptor gamma

rACC: Rostral anterior cingulated cortex

ROS: Reactive oxygen species

RNS: Reactive nitrogen species

SERT: Serotonin transporter

sIL-2R: Interleukin- 2 soluble receptor

sgACC: Subgenual anterior cingulate cortex

TGF- α: Transforming growth factor- α

Th-: Lymphocytes T helper

TLR: Toll like receptor

TNF- α: Tumor necrosis factor- α

TRYCAT: Tryptophan catabolites pathway

TSPO: Translocator protein

VEGF: Vascular endothelial growth factor

ABSTRACT

Abstract

Introduction. Sickness behavior is a highly organized adaptive strategy elicited by inflammation to support the organism's defense against pathogens. It is characterized by changes in behavior, mood and cognition similar to those observed in patients with major depressive disorder. Despite its adaptive function, sickness behavioral changes may become prolonged and dysfunctional when the pathogen stimulus cannot be removed and may contribute to the development of depression in vulnerable patients. The study of the mechanisms linking inflammation to sickness behavior and depression would be crucial for a better understanding of the pathophysiology of depression and to develop new therapeutic approaches.

Hypothesis and Objectives. We hypothesized that patients with a low-degree chronic inflammatory disease such as chronic hepatitis C, compared to healthy controls, would present changes in brain morphology, activity, connectivity and metabolism in areas linked to sickness behavior and depression, and that such alterations would be related to mood symptoms and inflammatory markers. The main objective of this thesis was to elucidate the clinical and neurobiological correlates of a prolonged sickness condition associated with chronic inflammation, such as chronic hepatitis C. We designed two studies. **Study 1:** We reviewed systematically and performed meta-analysis of the neuroimaging evidences in no treated chronic hepatitis C patients. **Study 2:** In a case-control study of no treated chronic hepatitis C patients and healthy controls, we analyzed differences in affective symptoms, cortico-striatal-limbic functional connectivity and serum concentration levels of inflammatory, anti-inflammatory and oxidative stress markers. We studied whether affective

symptoms were correlated with the biological markers and/or with the functional connectivity networks in patients.

Study 1: Methods and Results. *Design:* A systematic review with meta-analysis of neuroimaging research in chronic hepatitis C treatment naïve patients. *Data Sources:* A comprehensive, computerized literature search was conducted in MEDLINE, PsycINFO, and EMBASE from inception up until 1 May 2017 for peer-reviewed studies on structural or functional neuroimaging assessment of no treated patients without cirrhosis or encephalopathy, neuropsychiatric disease or substance use disorder with control group. *Study Selection:* Data were collected with an advanced protocol in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) and the Meta-analysis of Observational Studies in Epidemiology (MOOSE). *Data Extraction:* The primary measures of interest varied according to the neuroimaging technique used and the secondary outcome were the correlation of these measures with clinical variables such as neuropsychiatric symptoms. *Quality assessment:* We used the Newcastle-Ottawa Quality Assessment Scale (NOS). *Data synthesis:* A full review was performed. Meta-analysis was conducted when possible. *Result.* Of 1403 records, 32 full-text articles were assessed for eligibility. The final sample was of 25 studies (magnetic resonance spectroscopy [N=12], perfusion weighted imaging [N=1], positron emission tomography [N=3], single-photon emission computed tomography [N=4], functional connectivity in resting state [N=1], diffusion tensor imaging [N=2] and structural magnetic resonance imaging [N=2]). The whole sample was of 509 patients of mild liver disease, and 491 healthy controls. A meta-analysis of magnetic resonance spectroscopy studies showed increased levels of choline/creatine ratio (mean difference 0.12, 95% confidence interval 0.06-0.18), creatine (0.85, 0.42-1.27) and glutamate plus glutamine (1.67, 0.39-2.96) in basal ganglia and increased levels of

choline/creatine ratio in centrum semiovale white matter (0.13, 0.07-0.19) in chronic hepatitis C patients. These chronic metabolic changes in the brain were similar to those observed in patients with depression. Photon emission tomography studies meta-analyses did not find significant differences in PK11195 binding potential in cortical and subcortical regions of chronic hepatitis C patients compared to controls. Other structural and functional brain abnormalities were also reported by individual studies as marker of neuroinflammation, oxidative stress and neuron-glia or axon-myelin integrity disruption. Central nervous system metabolic changes were mainly correlated with neurocognitive impairment and fatigue symptoms, thought controversial results were observed.

Study 2: Methods and Results. *Design*: A cross-sectional, case-control study of 35 chronic hepatitis C no treated patients, and 30 healthy controls, age and sex matched. Exclusion criteria were decompensated cirrhosis or hepatocarcinoma, any chronic disease or inflammatory condition (e.g., diabetes, asthma, obesity or cancer), auto-immune diseases (e.g., rheumatoid arthritis), any neuropsychiatric and substance use disorder. *Clinical, Biological and Neuroimaging assessment:* After obtaining informed consent, all participants underwent a detailed medical history check, routine laboratory tests and physical examinations to determine whether they met the inclusion and exclusion criteria. All were evaluated for perceived stress (perceived stress scale; PSS), depression (PHQ-9), fatigue and irritability through a visual analog scale (VAS), as well as serum levels of interleukin-6 (IL-6), prostaglandin E2 (PGE_2), 15-deoxy-Δ-12,14-prostaglandin J2 (15d-PGJ_2) and oxidative stress markers. Functional magnetic resonance imaging (1.5T) was performed, measuring resting-state functional connectivity using a region-of-interest (seed)-based approach focusing on the bilateral insula, subgenual anterior cingulate cortex (sgACC) and bilateral putamen. Between-group differences in functional connectivity patterns were assessed with two-

sample t-tests, while the associations between symptoms, inflammatory markers and connectivity patterns were analyzed with multiple regression analyses. *Results:* We observed that chronic hepatitis C patients had higher PSS, PHQ-9 and VAS scores for fatigue and irritability, as well as increased IL-6 levels, PGE_2 concentrations and antioxidant system activation compared to controls. Importantly, increased perceived stress and depressive symptoms were associated with changes in inflammatory marker levels and in functional connectivity between the insula and putamen, areas involved in interoceptive integration, emotional awareness, and orientation of motivational state. Of note, PGE_2 and PSS scores accounted for 46% of the variance in functional connectivity between the anterior insula and putamen.

Conclusions. The present doctoral thesis expanded knowledge about relations between inflammation, neuropsychiatric symptoms and their correlates in brain neuroimaging in chronic hepatitis C patients. The results supported the hypothesis of a direct or indirect involvement of hepatitis C virus in central nervous system disturbances and provide valuable information on the brain areas involved in perceived stress, fatigue and subclinical depressive symptoms during chronic inflammation, highlighting the crucial role of interoception in coordinating prolonged sickness behavior. Using the chronic hepatitis C disease as a model of low-grade inflammation, new neurobiological and neuroanatomical links between sickness behavior and chronic inflammatory conditions have been elucidated. These findings may be crucial in understanding pathophysiological mechanisms related with psychiatric diseases such as depression and open new research perspectives centered on the development of new therapeutic targets.

Resumen

Introducción. La conducta de enfermedad es una estrategia adaptativa y coordinada que tiene la finalidad de defender el organismo en contra de agente patógenos. Está caracterizada por alteraciones inflamatorias junto a cambios en la conducta, el estado de ánimo y la actividad cognitiva, cambios que se parecen a los observados en pacientes con depresión mayor. A pesar de su función adaptativa, los cambios conductuales durante la enfermedad pueden persistir de forma prolongada y volverse disfuncionales, cuando el estímulo patógeno no es eliminado. El estudio de los mecanismos subyacentes que relacionan la inflamación, conducta de enfermedad y depresión podría ser crucial para mejorar el conocimiento de la fisiopatología de la depresión y desarrollar nuevas aproximaciones terapéuticas.

Hipótesis y Objetivos. Hipotetizamos que los pacientes con enfermedades crónicas inflamatorias de bajo grado como la hepatitis C crónica, en comparación con controles sanos, podría presentar cambios cerebrales a nivel estructural, funcional, conectividad y metabolismo en áreas asociadas con la conducta de enfermedad y la depresión, y que dichas alteraciones podrían estar relacionadas con síntomas anímicos y marcadores inflamatorios. El objetivo principal de esta tesis es elucidar los correlatos clínicos y neurobiológicos de la conducta de enfermedad prolongada asociada a patologías inflamatorios crónicos como la hepatitis C crónica. Para ello diseñamos dos estudios. **Estudio 1:** Revisión sistemática y metaanálisis de las evidencias en la literatura de los estudios de neuroimagen en pacientes con hepatitis c crónica no tratada. **Estudio 2**: Estudio caso-control en pacientes con hepatitis C no tratada y controles sanos para analizar las diferencias en la sintomatología afectiva, la conectividad cerebral funcional cortico-estriatal-límbica en estado de reposo y las

concentraciones séricas de marcadores inflamatorios, antiinflamatorios y de estrés oxidativo. También para estudiar la posible correlación entre la sintomatología afectiva con los marcadores biológicos y los cambios en la conectividad funcional en reposo en el grupo de pacientes.

Estudio 1: Métodos y Resultados. *Diseño: R*evisión sistemática con meta-análisis de la investigación sobre neuroimagen en pacientes con hepatitis C crónica no tratados. Los datos de la revisión sistemática fueron recogidos a partir de un protocolo redactado *a priori* basado en las indicaciones de *Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)* y de *Meta-analysis of Observational Studies in Epidemiology (MOOSE)*. *Selección estudios:* Se llevó a cabo una búsqueda comprensiva y computerizada de todos los estudios de neuroimagen publicados y revisados mediante *peer-review* en las bases de datos MEDLINE, PsycINFO y EMBASE hasta el 1 de mayo de 2017, que compararon datos entre pacientes con hepatitis C crónica sin cirrosis o encefalopatía, trastorno neuropsiquiátrico o trastorno por uso de sustancias y grupo control. *Extracción de datos:* La variable primaria de interés dependió de la técnica de neuroimagen utilizada, mientras que las variables secundarias fueron las correlaciones sobre los datos de neuroimagen y las variables clínicas como los síntomas neuropsiquiátricos. *Evaluación de la calidad de los estudios:* Se realizó mediante el *Newcastle-Ottawa Quality Assessment Scale (NOS)*. Siempre que los datos lo permitieron se sintetizó los resultados mediante meta-análisis. *Resultados:* De 1403 estudios encontrados, 32 fueron seleccionados como elegibles. La muestra final comprendió 25 estudios: (espectroscopia, [N=12], imágenes de perfusión ponderadas [N=1], tomografía a emisión de positrones [N=3], tomografía a emisión de fotón único [N=4], conectividad funcional en estado de reposo [N=1], imagen por tensor de difusión [N=2] y resonancia magnética estructural [N=2]). La muestra final comprendió 509 pacientes con hepatitis C

crónica y 491 controles sanos. En los meta-análisis de los estudios de espectroscopia se observaron niveles incrementados de la ratio entre colina y creatina (diferencia de la media: 0.12, 95% intervalo de confianza: 0.06-0.18), de la creatina (0.85, 0.42-1.27) y de glutamato *plus* glutamina (1.67, 0.39-2.96) en los ganglios basales, y niveles incrementados de la ratio entre colina y creatina en la sustancia blanca del centro semiovale (0.13, 0.07-0.19) de pacientes comparados con controles sanos. Las alteraciones metabólicas cerebrales encontradas fueron similares a las observadas en pacientes con depresión. El metaanálisis de los estudios PET no encontraron diferencias significativas entre el potencial de unión de PK11195 en las regiones corticales y subcorticales de los pacientes comparados con controles sanos. En estudios individuales se identificaron otras alteraciones cerebrales estructurales y funcionales, como marcadores de neuroinflamación, de estrés oxidativo y de disrupción de la integridad axo-mielínica y glio-neuronal. Se evidenció que las alteraciones metabólicas en el sistema nervioso central estaban relacionadas a alteraciones neurocognitivas y fatiga, aunque los resultados observados fueran controvertidos.

Estudio 2: Métodos y Resultados. *Diseño:* Estudio, transversal, caso-control, que compara 35 pacientes de ambos sexos, entre 18 y 55 años, con hepatitis C crónica sin tratar y 30 controles sanos emparejados por edad y sexo. *Criterios de exclusión:* Pacientes con cirrosis descompensada o hepatocarcinoma, enfermedades crónicas o condiciones inflamatorias (diabetes, asma, obesidad o tumores), enfermedades autoinmunes (artritis reumatoide) o trastornos neuropsiquiátricos o de uso de sustancias, con controles sanos. *Evaluación clínica, biológica y de neuroimagen:* Tras obtener el consentimiento informado, todos los participantes realizaron una visita médica con anamnesis completa, test de laboratorios rutinarios y un examen físico para revisar los criterios de inclusión y exclusión. Se evaluaron el estrés percibido (escala de estrés percibido; PSS), depresión (cuestionario de salud física-

9; PHQ-9), fatiga e irritabilidad (escala visual analógica; VAS-F y VAS-I), así como las concentraciones séricas de interleuquina-6 (IL-6), prostaglandina E2 (PGE2), 15-deoxy-Δ-12,14-prostaglandina J2 (15d-PGJ2) y marcadores de estrés oxidativo. Se realizó una resonancia magnética cerebral funcional (1.5T) estudiando la conectividad funcional en estado de reposo, mediante la selección *a priori* de regiones de interés (*seed-based approach*): la ínsula bilateral, el cortex cingulado anterior subgenual (sgACC), y el putamen bilateral. Las diferencias entre grupos en los patrones de conectividad funcional fueron analizadas mediante un t-test de dos muestras independientes, mientras que las asociaciones entre síntomas clínicos, marcadores inflamatorios y patrones de conectividad funcional fueron analizadas mediante regresión múltiple. *Resultados:* Los pacientes con hepatitis C crónica presentaban puntuaciones mayores en las escalas de PSS, PHQ-9, VAS-F y VAS-I respeto a los controles sanos; un incremento de las concentraciones séricas de IL-6 y PGE2; y una mayor activación del sistema anti-oxidativo comparado con los controles sanos. El incremento del estrés percibido y los síntomas depresivos estaban asociados a alteraciones de los marcadores inflamatorios y de la conectividad entre ínsula y putamen, áreas involucradas en la integración interoceptiva, la conciencia emocional y la orientación dirigida de un estado motivacional. Por último, los niveles de PGE2 y los valores de PSS eran responsables del 46% de la variación de la conectividad funcional entre ínsula anterior y putamen.

Conclusiones. Esta tesis doctoral incrementa el conocimiento sobre las relaciones entre la inflamación, los síntomas neuropsiquiátricos y sus correlaciones con alteraciones en la neuroimagen cerebral, en los pacientes con hepatitis C crónica. Los resultados observados sostienen la hipótesis que el virus de la hepatitis C esta directa o indirectamente involucrado en alteraciones del sistema nervioso central, y proporciona información importante sobre las

áreas cerebrales involucradas en el estrés percibido, la fatiga y los síntomas depresivos subclínicos durante un estado de inflamación crónica. Además, se resalta el rol crucial de la interocepción en coordinar la conducta de enfermedad prolongada. Utilizando la hepatitis C crónica como modelo de un estado de inflamación de bajo grado, se han podido ilustrar nuevos enlaces neurobiológicos y neuroanatómicos entre la conducta de enfermedad e la inflamación crónica. Los hallazgos de esta tesis podrían ayudar en la comprensión de mecanismos fisiopatológicos relacionados con trastornos psiquiátricos como la depresión, y abre nuevas perspectivas de investigación centradas en el desarrollo de nuevas dianas terapéuticas.

Resum

Introducció. La conducta de malaltia és una estratègia adaptativa i coordinada que té la finalitat de defensar l'organisme enfront d'agent patògens. Està caracteritzada per alteracions inflamatòries juntament amb canvis en la conducta, l'estat d'ànim i l'activitat cognitiva, canvis que s'assemblen als observats en pacients amb depressió major. Malgrat la seva funció adaptativa, els canvis conductuals durant la malaltia poden persistir de forma perllongada i tornar-se disfuncionals, quan l'estímul patogen no és eliminat. L'estudi dels mecanismes subjacents que relacionen la inflamació, conducta de malaltia i depressió podria ser crucial per millorar el coneixement de la fisiopatologia de la depressió i desenvolupar noves aproximacions terapèutiques.

Hipòtesis i Objectius. Hipotetitzem que els pacients amb malalties cròniques inflamatòries de baix grau com l'hepatitis C crònica, en comparació amb controls sans, podrien presentar canvis cerebrals a nivell estructural, funcional, connectivitat i metabolisme en àrees associades amb la conducta de malaltia i la depressió, i que aquestes alteracions podrien estar relacionades amb símptomes anímics i marcadors inflamatoris. L'objectiu principal d'aquesta tesi és esbrinar els correlats clínics i neurobiològics de la conducta de malaltia perllongada associada a patologies inflamatòries cròniques com l'hepatitis C crònica. Per això vam dissenyar dos estudis.

Estudi 1: Revisió sistemàtica i metaanàlisi de les evidències en la literatura dels estudis de neuroimatge en pacients amb hepatitis c crònica no tractada. **Estudi 2:** Estudi cas-control en pacients amb hepatitis C crònica no tractada i controls sans per analitzar les diferències en la simptomatologia afectiva, la connectivitat cerebral funcional cortico-estriatal-límbica en estat de repòs, les concentracions sèriques de marcadors inflamatoris, antiinflamatoris i d'estrès oxidatiu. I la possible correlació entre la simptomatologia afectiva amb els marcadors biològics i els canvis en la connectivitat funcional en repòs en el grup de pacients.

Estudi 1: Mètodes i resultats. Disseny: Revisió sistemàtica amb metaanàlisi de la investigació sobre neuroimatge en pacients amb hepatitis C crònica no tractats. Les dades de la revisió sistemàtica van

ser recollides a partir d'un protocol redactat a priori basat en les indicacions de *Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) i de Meta-analysis of Observational Studies in Epidemiology (MOOSE).* Selecció estudis: Es va dur a terme una recerca comprensiva i computeritzada de tots els estudis de neuroimatge publicats i revisats mitjançant *peer-review* en les bases de dades MEDLINE, PsycINFO i EMBASE fins l'1 de maig de 2017, que van comparar dades entre pacients amb hepatitis C crònica sense cirrosi o encefalopatia, trastorn neuropsiquiàtric o trastorn per ús de substàncies i grup control. *Extracció de dades:* La variable primària d'interès va dependre de la tècnica de neuroimatge utilitzada, mentre que les variables secundàries van ser les correlacions sobre les dades de neuroimatge i les variables clíniques com els símptomes neuropsiquiàtrics. *Avaluació de la qualitat dels estudis:* Es va realitzar mitjançant el *Newcastle-Ottawa Quality Assessment Scale (NOS).* Sempre que les dades ho van permetre es va sintetitzar els resultats mitjançant meta-anàlisi. *Resultats:* De 1403 estudis trobats, 32 van ser seleccionats com a elegibles. La mostra final va comprendre 25 estudis: (espectroscòpia, [N=12], imatges de perfusió ponderades [N=1], tomografia per emissió de positrons [N = 3], tomografia per emissió de fotó únic [N=4], connectivitat funcional en estat de repòs [N=1], imatge per tensor de difusió [N = 2] i ressonància magnètica estructural [N=2]). La mostra final va comprendre 509 pacients amb hepatitis C crònica i 491 controls sans. En les metaanàlisi dels estudis d'espectroscòpia es van observar nivells incrementats de la ràtio entre colina i creatina (diferència de la mitjana: 0.12, 95% interval de confiança: 0,06-0,18), de la creatina (0.85, 0.42-1.27) i de glutamat més glutamina (1.67, 0.39-2.96) en els ganglis basals, i nivells incrementats de la ràtio entre colina i creatina en la substància blanca del centre semioval (0.13, 0,07-0,19) de pacients comparats amb controls sans. Les alteracions metabòliques cerebrals trobades van ser similars a les observades en pacients amb depressió. El metaanàlisi dels estudis PET no van trobar diferències significatives entre el potencial d'unió de PK11195 a les regions corticals i subcorticals dels pacients comparats amb controls sans. En estudis individuals es van identificar altres alteracions cerebrals estructurals i funcionals, com a marcadors de neuroinflamació, d'estrès oxidatiu i de disrupció de la integritat axo-mielínica i glio-neuronal. Es va

evidenciar que les alteracions metabòliques en el sistema nerviós central estaven relacionades amb alteracions neurocognitives i fatiga, encara que els resultats observats fossin controvertits.

Estudi 2: Mètodes i resultats. *Disseny:* Estudi, transversal, cas-control, que compara 35 pacients d'ambdós sexes, entre 18 i 55 anys, amb hepatitis C crònica sense tractar i 30 controls sans aparellats per edat i sexe. *Criteris d'exclusió*: Pacients amb cirrosi descompensada o hepatocarcinoma, malalties cròniques o condicions inflamatòries (diabetis, asma, obesitat o tumors), malalties auto-immunes (artritis reumatoide) o trastorns neuropsiquiàtrics o per ús de substàncies, amb controls sans. *Avaluació clínica, biològica i de neuroimatge*: Després d'obtenir el consentiment informat, tots els participants van realitzar una visita mèdica amb anamnesi complerta, test de laboratoris rutinaris i un examen físic per revisar els criteris d'inclusió i exclusió. Es van avaluar l'estrès percebut (escala d'estrès percebut; PSS), depressió (qüestionari de salut física-9; PHQ-9), fatiga i irritabilitat (escala visual analògica; VAS-F i VAS-I), així com les concentracions sèriques d'interleuquina-6 (IL-6), prostaglandina E2 (PGE_2), 15-deoxy-Δ-12,14-prostaglandina J2 ($15d\text{-}PGJ_2$) i marcadors d'estrès oxidatiu. Es va realitzar una ressonància magnètica cerebral funcional (1.5T) estudiant la connectivitat funcional en estat de repòs, mitjançant la selecció a priori de regions d'interès (*seed-based approach*): l'ínsula bilateral, el còrtex cingular anterior subgenual (sgACC), i el putamen bilateral. Les diferències entre grups en els patrons de connectivitat funcional van ser analitzats mitjançant un t-test per a dues mostres independents, mentre que les associacions entre símptomes clínics, marcadors inflamatoris i patrons de connectivitat funcional van ser analitzades mitjançant regressió múltiple. *Resultats:* Els pacients amb hepatitis C crònica presentaven puntuacions majors en les escales de PSS, PHQ-9, VAS-F i VAS-I respecte als controls sans; un increment de les concentracions sèriques d'IL-6 i PGE_2; i una major activació del sistema anti-oxidatiu comparat amb els controls sans. L'increment de l'estrès percebut i els símptomes depressius estaven associats a alteracions dels marcadors inflamatoris i de la connectivitat entre ínsula i putamen, àrees involucrades en la integració interoceptiva, la consciència emocional i l'orientació dirigida d'un estat

motivacional. Finalment, els nivells de PGE2 i els valors de PSS eren responsables del 46% de la variació de la connectivitat funcional entre ínsula anterior i putamen

Conclusions. Aquesta tesi doctoral incrementa el coneixement sobre les relacions entre la inflamació, els símptomes neuropsiquiàtrics i les seves correlacions amb alteracions en la neuroimatge cerebral, en els pacients amb hepatitis C crònica. Els resultats observats sostenen la hipòtesi que el virus de l'hepatitis C està directament o indirectament involucrat en alteracions del sistema nerviós central, i proporciona informació important sobre les àrees cerebrals involucrades en l'estrès percebut, la fatiga i els símptomes depressius subclínics durant un estat d'inflamació crònica. A més, es ressalta el paper crucial de la interocepció per coordinar la conducta de malaltia perllongada. Utilitzant l'hepatitis C crònica com a model d'un estat d'inflamació de baix grau, s'han pogut il·lustrar nous enllaços neurobiològics i neuroanatòmics entre la conducta de malaltia i la inflamació crònica. Les troballes d'aquesta tesi podrien ajudar en la comprensió de mecanismes fisiopatològics relacionats amb trastorns psiquiàtrics com la depressió, i obre noves perspectives d'investigació centrades en el desenvolupament de noves dianes terapèutiques

INTRODUCTION

1. INTRODUCTION

1.1 Sickness behavior: physical debilitation or adaptive strategy?

Sickness behavior is characterized by changes in behavior, mood and cognition elicited by inflammation (Garcia, Kimeldorf, & Koelling, 1955; Miller & Raison, 2016). The experience of "feeling sick" is common during acute infections or inflammatory trauma (Dantzer, 2001a; Hart, 1988). Clinically, it is featured by a set of neurovegetative symptoms such as fatigue, anorexia, psychomotor retardation and increased sense of pain, as well as augmented irritability, anhedonia, social responsiveness and increased stress sensitivity (Capuron & Miller, 2004; Dantzer et al., 2008; Maes et al., 2012). Soluble mediators such as the pro-inflammatory cytokines Interleukin- (IL-) 1α, IL-1β, IL-6 and Tumor necrosis factor-α (TNF-α) are produced by monocytes/macrophages activated by specific pathogen associated molecular patterns (PAMPs) and are the responsible for the local and systemic inflammatory response against the microbial pathogens. A huge amount of data have demonstrated the direct implication of these cytokines in the induction of sickness behavior (Aubert et al, 1997; Avitsur, Cohen, & Yirmiya, 1997; Dantzer, 2001b; Hart, 1988; Kent et al, 1996; Kent et al, 1992). In animal models, symptoms of sickness behavior can be reliably reproduced by the experimental administration of proinflammatory cytokines, or indirectly by the inoculation of agents able to induce immune response, such as endotoxins or lipopolysaccharide (LPS). For examples, mice and rats injected with IL-1 or microbial LPS have been observed reducing their social exploration and food intake in a time and dose-dependent manner (Kent et al., 1992), as well as altering their sexual behavior (Avitsur et al., 1997). Furthermore, systemic administration of TNF-α consistently suppress feeding (Kent et al., 1996), whereas IL-6 can induce fever response, without inducing behavioral changes (Dantzer, 2001b).

But why we get sick? The interpretation of the sickness behavior as an adaptive behavior was introduced for the first time by Hart in 1988. He argued that the behavioral changes as well as the febrile response related to the inflammation were not signs of physical impairment or debilitation but were part of a highly organized adaptive strategy to support the organism's defence against the pathogen. Hence, cytokines would constitute a system of communication molecules (as well as hormones, neurotransmitters and other peptides), capable of informing the brain about a particular organic state, that is, the occurrence of an infection or an injury. In this line, Dantzer et al. (2001) proposed the sickness behavior as an expression of a central motivational state (Dantzer, 2001b). This concept was elaborated by Bolles and Fanselow (Bolles & Fanselow, 1982), as motivation was defined as a central state that can reorganize perceptions and actions, being flexibility its main feature. Thus, a sick individual should be able to reorganize his behavioral strategies with regard to his needs and capacities, i.e. to accommodate more urgent needs if necessary. Evidence supporting this point of view arrives again from animal experiments (Aubert et al., 1997; Aubert, Kelley, & Dantzer, 1997). In one of these studies (Aubert et al., 1997), administration of LPS to lactating mice at a sickness-producing dose, did not disrupt pup retrieval, thought suppressed the nest building activity. But when the dams and their litters were exposed to 4°C instead of 20°C, also the nest building activity was present, being evident that dams should care for their infants despite sickness. More recently, several theories have tried to explain sickness behavior (and depression) from an evolutionary standpoint (Allen & Badcock, 2006; Anders, Tanaka, & Kinney, 2013; Kinney & Tanaka, 2009; Miller & Raison, 2016; Raison & Miller, 2012; Stieglitz, 2015). In detail, the infection-defence hypothesis of Kinney et al. (2009) sustained that affective and behavioral depressive symptoms have played an adaptive role throughout human history by helping individuals fight or avoiding

infection. The emerging evidence about the bidirectional pathways of communication between the nervous and immune systems provide basis of the relation between depression and immune system (Anders et al., 2013). In the pathogen host defence (PATHOS-D) hypothesis developed by Raison and Miller (2012), the authors show that allelic variants that increase the risk for major depressive disorder intensify host defence mechanisms and innate immune inflammatory responses, enhancing survival in ancestral "highly pathogenic" environment. Conversely, inflammatory activation can increase mortality in modern world. Hence, it is not surprising how depression can be non- adaptive in the social realm, whereas its risk alleles are represented at high prevalence, suggesting an adaptive function (Raison & Miller, 2012). In the Table 1 a list of the main theories with main features is provided.

Table 1. Evolutionary theories of sickness behavior and depressive symptoms

The psychic pain hypothesis (Thornhill & Thornhill, 1990)	Depressed mood provides affective feedback that discourages continued investment in unreachable goals.
The rank theory hypothesis (Gilbert & Erlbaum, 1992)	Depression is a response to loosing status and its adaptive function is to change behavior to promote survival for someone who has been defeated. The function of this depressive adaptation is to prevent the loser from suffering further defeat in a conflict.
The Social Risk Theory Hypothesis (Price, Sloman, Gardner, Gilbert, & Rohde, 1994)	Depression is a signal of submissiveness in response to social failure in order to maintain social acceptance critical for survival and reproduction.
The social navigation hypothesis (Watson & Andrews, 2002)	Depression induces cognitive changes that focus and enhance capacities for the accurate analysis and solution of key social problems, suggesting a social rumination function. The costs associated with anhedonia and psychomotor perturbation can persuade reluctant social partners to provide help suggesting a social motivation function.
The bargaining model hypothesis (Hagen, 2002)	Depressive symptoms serve as distress signals to elicit help during adversity
The infection-defence hypothesis (Kinney & Tanaka, 2009)	Depressed mood stimulates behaviors helping to protect vulnerable individuals and their kin against infectious diseases, to conserve energy and to avoid environmental stressors.
The pathogen-host defence hypothesis (C L Raison & Miller, 2012)	Allelic variants that increase the risk for major depressive disorder enhance host defence mechanisms in general and innate immune inflammatory responses in particular

1.2 Sickness behavior and depression: clinical overlapping syndromes

The affective, cognitive and behavioral features of the sickness behavior are striking similar to the symptoms of depression. Fatigue, anhedonia, psychomotor retardation, reduced interest in the environment and anorexia are all symptoms that form part of the

categorical diagnostic criteria of the Diagnostic and statistical manual of mental disorders-(DSM-) 5 (and its previous editions) for major depressive disorder (American Psychiatric Association. DSM-5 Task Force., 2013). The Table 2 provides the list symptoms of the first criteria.

Table 2. DSM-5 Major Depressive Disorder (MDD) diagnostic criteria A

A – Five (or more) of the following symptoms have been present during the same 2-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure.

NOTE: do not include symptoms that are clearly attributable to another medical condition

1. **Depressed mood** most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad, empty, hopeless) or observation made by others (e.g., appears tearful).

2. Markedly **diminished interest or pleasure** in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation).

3. Significant **weight loss** when not dieting or weight gain (e.g., a change of more than5% of body weight in a month) or decrease or increase in appetite nearly every day.

4. **Insomnia or hypersomnia** nearly every day.

5. **Psychomotor agitation or retardation** nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down).

6. **Fatigue or loss of energy** nearly every day.

7. Feelings of **worthlessness or excessive or inappropriate guilt** (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick).

8. **Diminished ability to think or concentrate**, or indecisiveness, nearly every day (either by subjective account or as observed by others).

9. Recurrent thoughts of death (not just fear of dying), **recurrent suicidal ideation** without a specific plan, or a suicide attempt or a specific plan for committing suicide.

Hence, increased attention was focused on the role of the inflammation in depression. Some experiments on animal models (Frenois et al., 2007; O'Connor et al., 2009; Yirmiya et al., 1999) have been conducted with the aim to disentangle the differences (and analogies) between sickness behavior and depression, and to evidence the role of cytokines in the passage from one condition to another. Some authors observed temporal and phenomenological differences between early onset and late onset LPS-induced symptoms (Dantzer, 2001b). In details, LPS-induced depressive-like behavior occurred much later and lasted much longer (at least 24 hours) than the early events involved in LPS-induced sickness behavior, which symptoms peak should be reached between 2 and 6 hours (Frenois et al., 2007). For this reason, it has been suggested that depressive-like behavior would be mediated by other neurobiological processes that were recruited as a consequence of the increased expression of proinflammatory cytokines. Frenois et al (2007) reported the functional dissociation between the rats' brain structures that underline cytokine-induced sickness behavior and cytokine-induced depressive-like behavior. They found that distinct temporal patterns of brain reactivity in the extended amygdala, hippocampus and hypothalamus were associated with LPS-induced sickness behavior at 6 hours and depressive-like behavior at 24 hours post-LPS respectively (Frenois et al., 2007). In another suggestive experiment, O'Connor et al (2009) show that both LPS-induced sickness behavior and LPS-induced depression-like behavior were blocked by the administration of minocycline, a second-generation tetracycline with potent anti-inflammatory effects. By contrast, the administration of 1-methyl tryptophan (1-MT) does not alter sickness behavior but blocks the LPS-induced depression-like behavior. The 1-MT is a competitive inhibitor of the indoleamine 2,3 dioxygenase (IDO), which activity peaks at 24 hours post LPS-injection and that is considered crucial in the physiopathology of depressive symptoms. These results

implicate IDO as a critical molecular mediator of inflammation-induced depressive-like behavior (O'Connor et al., 2009).

At the end of the 90s, the increased use of recombinant human cytokines IL-2 and interferon- (IFN-) α to treat resistant tumours (mostly melanoma) or chronic hepatitis C (CHC), allow the expansion of new lines of investigation in the field. Many clinicians at the end of the 80s had already observed psychiatric symptoms (mostly depressive symptoms) in patients underwent IL-2 or IFN-α treatment (Denicoff et al., 1987; Renault et al., 1987), but only one decade later immunotherapy became a quasi-experimental model to investigate the physiopathology of cytokine-induced depression (Dantzer et al., 2008). Recent data, based on a comprehensive revision and meta-analysis, had estimated the incidence rate of IFN- α induced depression in CHC patients in 28% (95% CI: 17% to 42%) (Udina et al., 2012). Actually, in previous studies was observed that depressive symptoms developed over a background of neurovegetative and somatic symptoms (that is, the "sickness behavior") in about one third of patients (Capuron, Ravaud, & Dantzer, 2001; Capuron & Miller, 2004). In a compelling study of Capuron et al (2002), it has been observed that in 40 patients with melanoma who underwent IFN-α treatment, a large proportion presented symptoms such as anorexia, fatigue, altered sleep, psychomotor retardation and pain within the first 2 weeks of treatment. On the other hand, symptoms such as sadness, guilt, anhedonia, irritability, anxiety and mild cognitive impairment appeared after 8 and 12 weeks of IFN-α treatment in a smaller proportion of patients, and more specifically in patients who met DSM-IV criteria for major depression. Interestingly, these classes of symptoms were more responsive to paroxetine treatment than the neurovegetative and somatic ones. Hence, the authors suggest the existence of at least two distinct behavioral syndromes: an early-onset neurovegetative syndrome, which could corresponds to a general state of sickness behavior,

and a late-onset mood/cognitive syndrome that could overlap to the first syndrome symptoms and lead to a categorical diagnosis of MDD (Capuron et al., 2002). Many other studies (Castellvi et al., 2009; Krzysztof, Małgorzata, Dorota et al. 2019; Mahajan, Avasthi, Grover et al. 2014; Martin-Santos et al., 2007; Navinés et al., 2009; Sockalingam et al., 2011; Whale et al., 2015) based on dimensional evaluation of depressive symptoms with different psychometric scales such as Hamilton Depression Rating Scale (HDRS), Physical Health Questionnaire for depression (PHQ-9), Montgomery-Asberg depression rating scale (MADRS) support the evidence that somatic complaints, psychomotor retardation and neurovegetative alterations prevail on mood-cognitive and more existential symptoms in the initial phase of inflammatory condition (see Figure 1).

Finally, other important clinical issue is the difference between the courses of sickness behavior and depression, being the former acute, adaptive and oriented to energy preservation. On the other hand, depression is characterized by a waxing and waning course, with sensitization of episodes, neuroprogression and in some case with seasonal variation or manic episodes (Maes et al., 2012).

In synthesis, we have seen that acute inflammatory activation with increased pro-inflammatory cytokines can lead to an adaptive response of the host to the pathogen, called “sickness behavior”, which is characterized by behavioral, cognitive and affective changes. The overlap of clinical features between sickness behavior and depression has induced scientists to study analogies and differences in the phenomenology of the two syndromes, in animal models and in clinical settings. It has been illustrated that somatic and neurovegetative symptoms predominate over mood and cognitive symptoms in depression induced by inflammation. The overlap and the dissimilarities in clinical phenomenology and

in the course of the two clinical pictures, suggest shared and different activation of immune-inflammatory pathways.

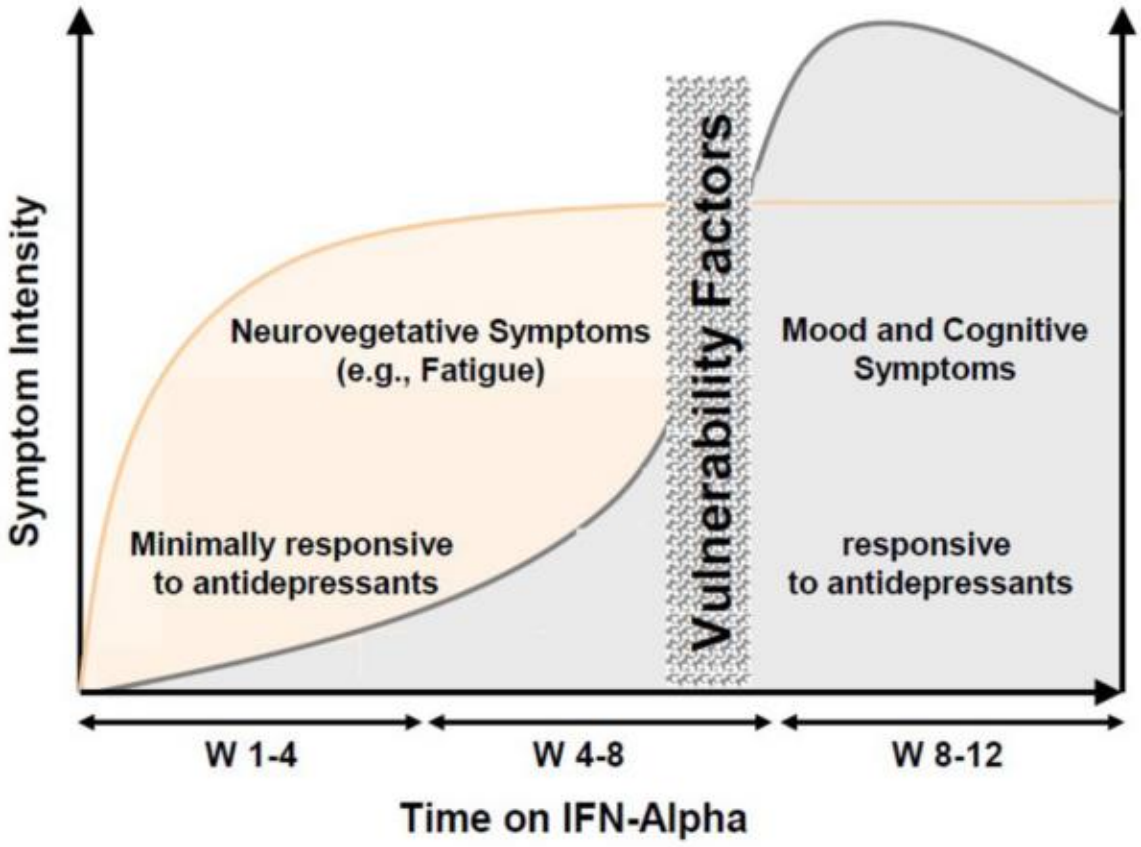

Figure 1. Temporal Evolution of the Neuropsychiatric Symptoms Induced by chronic IFN-α therapy
Interferon (IFN) -α therapy induces two types of behavioral symptoms with differential time course and responsiveness to antidepressants. The neurovegetative symptoms (e.g., fatigue, anergia and psychomotor slowing) develop rapidly (as soon as week 1 [W1]) in almost every individual exposed to cytokines and persist during the duration of IFN-α therapy. These symptoms are minimally responsive to antidepressant treatment. In contrast, the mood and cognitive symptoms (e.g., depressed mood, anxiety, irritability, memory and attentional disturbance) develop in vulnerable patients at later stages of IFN-α therapy (between weeks 8–12) and are highly responsive to antidepressant medication.

1.3 Depression: the role of immune system

Depression is a severe, common and heterogeneous mental disorder, with more than 300 millions of people affected, and it is considered the leading cause of disability worldwide (World Health Organization, 2017), with a lifetime prevalence of more than 15% (Lépine & Briley, 2011). Sufferers are at higher risk of disease and have a higher risk of overall mortality (Cuijpers et al., 2014). More than one third of patients does not adequately respond to a trial with a first-line antidepressant agent (Rush et al., 2006; Stotland, 2012), and a substantial proportion of patients with major depressive disorder (MDD) do not return to premorbid functioning, even though criteria for symptomatic remission are achieved (Stotland, 2012). This condition is related to reductions in quality of life and psychosocial functioning (Alonso et al., 2013), leading to high human capital costs (Cocker et al., 2014). Therefore, a better understanding of pathogenesis and physiopathological mechanisms underlying the disorder, is crucial for the recognition of novel therapeutic targets, and decisive to reduce the global burden of disease (Carvalho et al., 2014). In this line, the relation between depression, inflammation, immunity and oxidative and nitrosative stress (O&NS) is one of the most studied conceptual frameworks. Activated immune-inflammatory pathways in depression were detected in the early 1990s, leading to the formulation of the macrophage theory of depression before (Smith, 1991), and, later on, of the more comprehensive cytokine hypothesis of depression (Dantzer et al., 2008; Maes, 1995).

Actually, multiple studies have shown increased blood levels of pro-inflammatory cytokines in patients with depression. Almost 25 years ago, Maes et al. (1995) observed that depressed patients with melancholic features exhibited higher IL-1β and IL-6 production. Concerning cell mediated immunity response, they found an increase of CD4/CD8 T cell ratios in depressed patients, as well as higher circulation of IL-2 soluble receptors (sIL-2R),

both data indicating T cell activation. The same group show how depression is accompanied by B cell proliferation, as well as increased plasma levels of positive and decreased plasma levels of negative acute phase proteins. Nevertheless, in the last two decades, contrasting results emerged. A recent cumulative meta-analysis showed higher mean levels of IL-6 and CRP in depressed patients compared to non-depressed controls, whereas no consistent association between TNF-α, IL-1β and MDD was observed (Haapakoski et al., 2015). Interestingly, other studies found that CRP levels were increased in acutely depressed patients and normalized after recovery (Goldsmith, Rapaport, & Miller, 2016), and that it was associated with somatic rather than existential symptoms of depression in general population (Jokela et al., 2016; Khandaker, Dantzer, & Jones, 2017). A more recent meta-analysis including 82 studies identified augmented peripheral levels of IL-6, TNF-α, IL-10, the soluble IL-2 receptor, C-C chemokine ligand 2, IL-13, IL-18, IL-12, the IL-1 receptor antagonist, and the soluble TNF-receptor 2 in patients with MDD compared to healthy controls (Kohler et al., 2016). One important consideration that deserves mention is that the majority of these studies were cross-sectional, being impossible disentangle whether increased inflammatory markers are the cause or consequence of depression. In this way, several longitudinal studies have pointed out the role of inflammation as causal risk factor for the development of MDD. IL-6 and CRP levels have been related longitudinally to subsequent development and persistence of depressive symptoms (Zalli et al., 2016).

Other intriguing data were obtained studying mood symptoms and depression in medically ill patients, considering that inflammatory conditions such CHC, obesity, diabetes or coronary disease, show an increased prevalence of mood symptoms compared to the global point prevalence of MDD in the general population, which has been estimated to be 4.7% in a review of 116 prevalence studies (Ferrari et al., 2013; Rosenblat et al., 2014).

Inflammation-associated depression in particular is associated with greater persistence and severity, later age of onset, and reduced motivation (Liu et al., 2017; Zalli et al., 2016). Around 50% of patients with hepatitis-C virus (HCV) infection may develop psychiatric symptoms in absence of cirrhosis or encephalopathy (Adinolfi et al., 2015), being fatigue, depression and cognitive impairment the more frequent (Adinolfi et al., 2015; Poynard et al., 2002; Yarlott, Heald, & Forton, 2017). Actually, up to 80% with CHC can suffer from fatigue (Fletcher et al., 2012) and up to 50% have reported depressive symptoms, anxiety and weakness (Saunders, 2008). Beside some evidence of HCV direct neuroinvasion (Fishman et al., 2008; Wilkinson, Radkowski, & Laskus, 2009) the chronic and systemic activation of the immune system that characterizes CHC, may also account for the pathogenesis of neuropsychiatric symptoms (D'Mello & Swain, 2014; Hartling et al., 2016). Thought a large amount of literature have focused on IFN-α induced depression, little evidence has been also produced regarding systemic inflammation effects (Yarlott et al., 2017). CHC has been related to low grade systemic inflammation which may account for some extra-hepatic manifestations (Rosenthal & Cacoub, 2015), wherein increased proinflammatory cytokines gene-transcription from peripheral blood mononuclear cells has been correlated to depressive symptoms in CHC patients (Pawlowski et al., 2014).

More recently, increased attention was placed on the depressogenic effect of obesity (Capuron, Lasselin, & Castanon, 2016; Jantaratnotai, Mosikanon, Lee et al. 2016; Mac Giollabhui et al., 2019). It has been shown that the incidence of depression in obese individuals was significantly higher than the one measured in the general population (Dawes et al., 2016) and that obesity increased the risk of develop MDD (de Wit et al., 2010). For instance, obesity is considered both a metabolic disorder and an inflammatory condition, being associated with a low-grade systemic inflammation and with enhanced susceptibility

to immune-mediated diseases (Castanon, Lasselin, & Capuron, 2014; Kanneganti & Dixit, 2012; Oriolo et al., 2018).

Moreover, a systematic review by Roy et al. (2012) found that the prevalence rate of depression was more than three-times higher in people with type 1 diabetes (12%, range 5.8-43.3% vs. 3.2%, range 2.7-11.4%) and nearly twice as high in people with type 2 diabetes (19.1%, range 6.5-33% vs. 10.7%, range 3.8-19.4%) compared to those without (Roy & Lloyd, 2012), whereas prevalence rates of depression in patients with coronary heart disease was estimated to be 15 to 28% (Celano & Huffman, 2011). Asthma and arthritis were also inflammatory conditions associated with increased MDD prevalence (Miguel de Díez et al., 2011).

Other line of evidence that associate inflammation and depression derives from the study of stress induction paradigms, which resulted in immune activation along with depressive symptoms. Psychosocial stress is a well-known risk factor for depressive symptoms (Cohen, Janicki-Deverts, & Miller, 2007), and has been observed that can elicit immune activation, beside the classical hypothalamus-pituitary axis (HPA) and sympathetic nervous system activation. In a recent review (Rohleder, 2014), transient increases in systemic inflammation were observed in response to acute psychosocial stress, with larger responses among individuals reporting adverse psychosocial states or conditions such as depression. For example, Miller et al (2004) observed in 72 women exposed to 17 minutes of mock-job interview an increase of shame and anxiety level. This was accompanied by a transient rise in circulating numbers of leukocytes and monocytes, as well as an augmentation of stimulated production of IL-6 and TNF-α. Moreover, the group of women with MDD at the beginning of the study shows greater resistance to molecules that normally terminate the inflammatory cascade, such as glucocorticoids (Miller et al., 2004). A more

recent study illustrate that prolonged restraint stress in animal models increases IL-6, decreases IL-10 and causes persistent depressive-like behavior (Voorhees et al., 2013).

Finally, diet and other environmental triggers such as smoking habit, sleep disturbances, periodontal diseases or lack of Vitamin D have been linked to activation of peripheral low-grade inflammation (Berk et al., 2013). Importantly, diet may also contribute to impairment in gut-brain axis which can influence the core symptoms of neuropsychiatric disorder, such as depression (Kelly et al., 2015; Macedo et al., 2016; Slyepchenko et al., 2017).

Therefore, different lines of investigations pointed out the relation between systemic inflammation, cellular-mediated immunity activation and sensitization of inflammatory and cellular-mediated immune pathways with depression. Some reported data shed a light on how prolonged or out of proportion immune activation could induce the transition from the acute, adaptive response against a pathogen, to a depressive state. But what we know about the impact of cytokines on the neurotransmitter systems and neurocircuits known to be associated with depression?

1.4 Sickness behavior and depression: neurobiological pathways

The pro-inflammatory cytokines IL-1α, IL-1β, IL-6 and TNF-α seem to be crucial in the modulation of the sickness behavior and in the induction of the central nervous system response. Actually, it has been observed that peripheral immunological activation may drive inflammation in central nervous system, interacting with microglia or directly affecting neurons (Haapakoski et al., 2016; Miller et al., 2013), and that immune-to-brain communication is provided by cytokines through different pathways (D'Mello & Swain, 2017). Moreover, cytokines and cellular mediated immunity can interact with virtually every

pathophysiologic domain relevant to depression, including neurotransmitter metabolism, neuroendocrine function, and neural plasticity (Miller, Maletic, & Raison, 2009; Raison, Capuron, & Miller, 2006). In the next paragraphs, an overview of neuroimmune interaction mechanisms and a revision of the principal molecular effects of immune system on neuron-glia interaction, neurotransmission, neuroendocrine and neural plasticity functions relevant to sickness behavior and depression is provided.

1.4.1 Brain-immune communication pathways

The parenchymal tissue of the central nervous system contains a limited immune repertoire, composed mostly by microglia and perivascular macrophages (Marin & Kipnis, 2016). Nevertheless, these structures are not the only responsible of inflammatory activity in the brain. Actually, it has been observed that peripheral inflammatory activation may drive inflammation in central nervous system even when the immune condition is elicited by innocuous stimuli (Miller et al., 2013). Cytokines provide afferent information to the brain about the activity of the immune system, and therefore elicit neuroendocrine responses (Besedovsky & Del Rey, 2011). Works on animal models have illustrating at least five different pathways by which cytokines or active immune cells can cross the blood-brain barrier (Miller et al., 2013). In the so called “humoral route” (1) circulating cytokines pass through leaky regions of the blood-brain barrier, which have fenestrated capillaries located around the circumventricular organs as the area postrema, or the third and fourth ventricle (Breder, Dinarello, & Saper, 1988; Ericsson, Kovács, & Sawchenko, 1994; Komaki, Arimura, & Koves, 1992). A second mechanism of the humoral route (2) is through cytokine specific active transporters (Banks, Kastin, & Durham, 1989). These specific transporters are saturable and dynamics, as they are adapted to or affected by physiological or disease states

(Quan & Banks, 2007). A third mechanism (3) is the activation of macrophage-like cells in circumventricular organs and endothelial cells of brain vessels by circulating cytokines and PAMPs, which results in the production of secondary mediators as prostaglandin E2 (PGE_2) and nitric oxide. PGE_2 can diffuse into the brain and interact with catecholaminergic brainstem nuclei and the paraventricular nucleus of the hypothalamus, inducing hypothalamus-pituitary axis (HPA) alterations and fever (Cao, Matsumura, Yamagata et al., 1997; Dantzer, 2009). Conversely, the "cellular route" (4) consists in the activation of microglia or endothelial cells in central nervous system vasculature that can induce chemokines production and attract immune cells to the brain (D'Mello, Le, & Swain, 2009; Miller & Raison, 2016). Finally, in the "neural pathway" (5) cytokines can bind receptors in afferent nerve fibres (e.g. the vagus nerve), that in turn relay the signal to the nucleus of the solitary tract and from there to different brain regions deputed to the interoceptive awareness and responses (Critchley & Harrison, 2013; D'Mello & Swain, 2017; Luheshi et al., 2000). Actually, cytokines have been related to the upregulation of adhesion molecules such as P-selectin and inter-cellular adhesion molecule 1 (ICAM-1) in the choroid plexus, an epithelial tissue located within the ventricles of the brain that regulate the production of cerebrospinal fluid, providing a permissive environment for immune-cell filtration (Zhang et al., 2013). Evidence of such upregulation become from the observation of increased microglial activity that have been identified in post-mortem and in-vivo human studies (Setiawan, 2015; Torres-Platas et al., 2014). In a post-mortem analysis in dorsal anterior cingulated cortex (dACC) in suicide depressed patients, Torres-Plata et al (2014) observed that the proportion of blood vessels surrounded by a high density of macrophages was more than twice higher in depressed suicides than in controls, and this difference was strongly significant. Consistent with these observations, they also observed that gene expression of

ionized binding calcium adapter -1 (IBA-1) and monocyte chemoattractant protein -1 (MCP-1), chemokines involved in the recruitment of circulating monocytes, was significantly upregulated in depressed suicides, actually demonstrating monocytes traffic to the brain. Setiawan (2015) provide evidence of neuroimmune activation in MDD patients using neuroimaging case-control study with positron emission tomography (PET) and radiolabelled tracer for the translocator protein (TSPO). For an overview of such mechanism, see Figure 2.

Once cytokine signals reach the brain, interaction with glial elements (astrocytes and microglia) and neurons is permitted through the expression of cytokine receptors. Central nervous system cells can also produce cytokines, being the microglia the most active cytokine producing cells (Haroon, Raison, & Miller, 2012; Miller et al., 2009). Thought it remains unclear whether activation of inflammatory pathways in the brain during depression originates primarily in the periphery or from a direct activation of inflammatory responses within the brain (Miller et al., 2009), the experimental and no experimental observations that peripheral inflammatory responses have been associated with mood disorders and sustain the hypothesis that peripheral immune activation spread to the central nervous system, and appear to underpin the different components of sickness behavior.

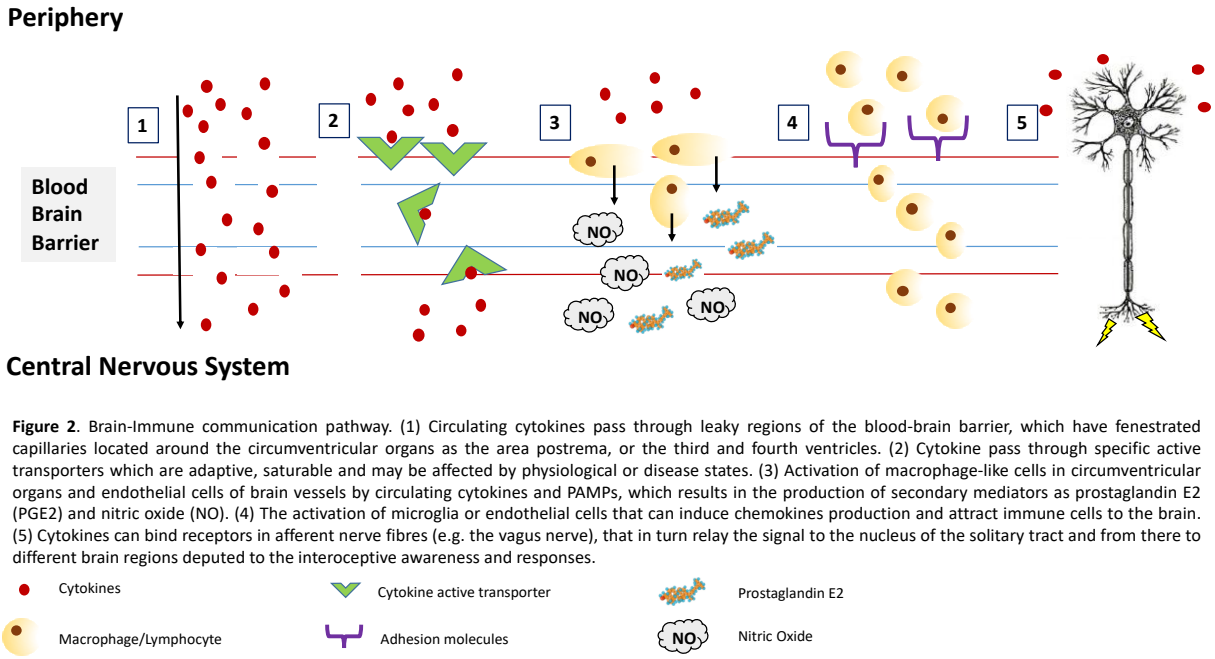

Figure 2. Brain-Immune communication pathway. (1) Circulating cytokines pass through leaky regions of the blood-brain barrier, which have fenestrated capillaries located around the circumventricular organs as the area postrema, or the third and fourth ventricles. (2) Cytokine pass through specific active transporters which are adaptive, saturable and may be affected by physiological or disease states. (3) Activation of macrophage-like cells in circumventricular organs and endothelial cells of brain vessels by circulating cytokines and PAMPs, which results in the production of secondary mediators as prostaglandin E2 (PGE2) and nitric oxide (NO). (4) The activation of microglia or endothelial cells that can induce chemokines production and attract immune cells to the brain. (5) Cytokines can bind receptors in afferent nerve fibres (e.g. the vagus nerve), that in turn relay the signal to the nucleus of the solitary tract and from there to different brain regions deputed to the interoceptive awareness and responses.

1.4.2 Direct mechanisms: the role of microglia and the inflammasome hypothesis

Microglia displays different essential functions in central nervous system, dynamically sustaining neuronal tasks during homeostasis and stress conditions (Wohleb et al., 2016). It has been highlighted how microglia are primed by genetic predisposition in multiple chronic disease states, leading to a stronger response to inflammatory stimulation, thus transforming an adaptive inflammatory activation in the central nervous system to persistent inflammation (Cunningham, 2013). When activated by several stimuli, such as cytokines, hormones or pattern recognition receptors, microglia may contribute to amplify inflammatory signals through the release of pro-inflammatory compounds (i.e. IL-1β, TNF-α). At the same time, microglia can also activate the inducible nitric oxide synthase (iNOS), release reactive oxygen species (ROS) and increase synthesis of tryptophan catabolites pathways (TRYCAT) (Yirmiya, Rimmerman, & Reshef, 2015), which may have a crucial role in the development, maintenance and progression of neuropsychiatric diseases. Indeed, such activation can elicit molecular changes in neurons, leading to neurotoxicity and cellular

death, disruption in neural plasticity or impaired functional brain connectivity and social behavior (Zhan et al., 2014). Importantly, suppression of hippocampal neurogenesis, which is considered an important mechanism underpinning major depression, has been related to activated microglia induced by LPS treatment in rodents (Ekdahl et al., 2003). Moreover, gut microbiota alterations may lead to neural and hormonal dysfunction, through alteration of microglia maturation and function (Cryan & Dinan, 2012; Erny et al., 2015). In humans, abnormal activation of microglia, and increased microglial cell numbers has been observed in depression and in anxiety disorders (Serafini, Amore, & Rihmer, 2015). In the mentioned PET study of Setiawan et al (2015), increased TSPO density was found in prefrontal cortex, insula and anterior cingulated cortex of depressed patients compared to healthy controls, indicating microglia activation. Interestingly, higher TSPO signal correlated with greater severity of depression (Setiawan, 2015). Taken together these findings underline how changes in microglial function elicited by inflammatory stimuli play a significant role in neurodegeneration processes and link several neurobiological pathways in major depressive disorder.

Concerning pro-inflammatory cytokines direct neuronal effects, it has been shown that TNF-α can influence cellular viability, ionic homeostasis, and synaptic plasticity (Park & Bowers, 2010). TNF-α can elicit apoptosis and neuronal damage through the activation of caspase-dependent mechanisms and potentiating of glutamate neurotoxicity (Zou & Crews, 2005). This mechanism can inhibit the long-term potentiation, influencing memory formation (Carvalho et al., 2014; Pickering, Cumiskey, & O'Connor, 2005). Furthermore, in animal models TNF-α over-expression has been shown to antagonize the nerve growth factor (NGF) production in the hippocampus and thereby suppressing hippocampal development (Fiore et al., 2000).

Subchronic elevation of IL-1 has been associated with reduction of the neurotrophins brain derived growth factor (BDNF) and NGF (Song & Wang, 2011) expression in animal models, whereas chronic IL-1 exposure has been shown to disrupt hippocampal neurogenesis (Goshen et al., 2008). Moreover, in animal models this cytokine has been shown to exacerbate neuronal death increasing seizure activity (Patel et al., 2006). This was in line with the neurotrophic theory of depression developed by Duman (1997), based on the observation of decreased neurogenesis and loss of glia in vulnerable cerebral regions such hippocampus in patients with MDD. This was due to decreased neurotrophic support, and was reversible by antidepressant treatment (Duman, 2012; Duman, Heninger, & Nestler, 1997; Duman & Monteggia, 2006).

IL-1β, a pro-inflammatory cytokines activated by the stress-induced inflammasome complex Nod-like receptor pyrin-domain containing 3 (NLRP3) (Walsh, Muruve, & Power, 2014), can alter neuroplasticity enhancing nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activity (Koo et al.,2010). Inflammasomes are a group of protein complexes that recognize PAMPs and damage-associated molecular patterns (DAMPs), and control the production of cytokines (Schroder & Tschopp, 2010). These molecules have been defined as intracellular innate immune sensors and are a key point in the regulation of inflammation (Strowig et al., 2012). The NLRP3 inflammasome is a protein complex expressed in myeloid cells and involved in the regulation of IL-1β and IL-18 activity. The IL-1β is produced in an inactive form (pro-IL-1β) when the Toll-like receptors (TLR) in microglia/macrophages surface are stimulated. The processing and release of IL-1β occurs via inflammasome and the activation of caspase-1 (Martinon, Burns, & Tschopp, 2002). The NLRP3 inflammasome has been considered a bridge between psychosocial stress, systemic diseases and depression (Iwata, Ota, & Duman, 2013). Actually, chronic mild stress activates

inflammasome, whereas DAMPs can be induced in stressors used in animal models of depression, and in turn activates inflammasome (Fleshner, Frank, & Maier, 2017; Miller & Raison, 2016). Furthermore, Iwata et al (2015) observed that acute restraint stress in animal models rapidly increase the active form of the inflammasome and the IL-1β in hippocampus (Iwata et al., 2015). The upregulation of NLRP3 inflammasome and the caspase-1 cleavage can cause resistance to the effects of glucocorticoids (Paugh et al., 2015) which, together with the effects on HPA-axis mediated by the IL-1β, could explain the physiopathology of the stress-induced glucocorticoid resistance, a well-characterized abnormality in patients with depression and linked to increased inflammation (Miller & Raison, 2016). Beside PAMPs and DAMPs, NLRP3 inflammasome can recognize a broad range of danger substances (Schroder & Tschopp, 2010). Indeed, there is evidence in mice and in humans that aberrant inflammasome activation by non-infectious agents, such as hyperglycaemia, fatty acids, cholesterol crystals or amyloid-β may be linked to the pathogenesis of diseases characterized by sterile inflammation, such as diabetes, obesity, cardiovascular disease (Iwata et al., 2015; Strowig et al., 2012). Hence, considering the high prevalence of comorbidity between depression and chronic medical condition, the NLRP3 inflammasome activity can be considered a key biological cross mechanism that link stress, chronic illness and depression to inflammation activity. This can be considered an important future target for therapeutics interventions.

Controversy regarding the effects of IL-6 on the central nervous system emerged in literature, as it was observed both beneficial and detrimental effects of this cytokine in the homeostasis of the brain, with relevance in both physiological and pathological conditions (Spooren et al., 2011). IL-6 has been related to microglial activation (Krady et al., 2008) and calcium (Ca2+) excitotoxicity mediated by the N-methyl-D-aspartate (NMDA) receptor, which

induce neuronal death. Nevertheless, whereas some authors have found that IL-6 enhances NMDA-induced excitotoxicity in cerebellar granule neurons (Conroy et al., 2004), several studies on animals' models evidenced protective properties of IL-6 against excitotoxicity (Wang et al., 2009). Another mechanism by which IL-6 can influence NMDA neurotransmission is through tryptophan catabolites pathway activation (Connor et al., 2008).

Similarly to IL-6, IL-2 induces microglia activation and directly interact with NMDA receptors, altering synaptic plasticity (Shen et al., 2006). Furthermore, IL-2 has been related to chronic myelin damage in multiple sclerosis patients (Mott et al., 2013).

Regarding IFN-α, this cytokine can directly contribute to neurotoxicity and neuronal loss in neurodegenerative, neuroprogressive and neuroimmune diseases (Lambertsen et al., 2004). A recent systematic revision examined biological mechanisms contributing to the onset of depression during IFN-α-based immunotherapy for hepatitis-C virus (Machado et al., 2016). IFN-α is a potent inducer of IL-6 and may lead to immune activation, which may adversely impact BDNF-related neuroprotection. Moreover, IFN-α administration may lead to a reduction in glucocorticoid receptor sensitivity (Felger, Haroon, Woolwine et al., 2016).

The prostaglandins are a group of lipophilic molecules that are synthesised from membrane bound arachidonic acid by the sequential actions of cyclo-oxygenase (COX) 1 and 2, and the respective prostaglandin synthases. They are divided in two groups considering their mechanism of action: conventional types (e.g., PGD_2, PGE_1, and PGE_2) and cyclopentenone-types (e.g., PGJ_2, Δ12-PGJ_2, 15- deoxy-Δ12,14 prostaglandin J2 (15d-PGJ_2), PGA_1, and PGA_2). The conventional type prostaglandins play a major role in the transition and maintenance of chronic inflammation (Leonard, 2018; Narumiya, 2009) by supporting a pro-inflammatory balance through initiating chronic gene dependent expression, inducing

pro-inflammatory cytokines and suppressing Th2 cell differentiation and the anti-inflammatory system (Leonard, 2018). They produce their physiological effects by activating G-protein coupled receptors, prostaglandin-D receptor and EP 1–4 subtypes of the prostaglandin-E receptors. Prostaglandin-E2 (PGE_2) is the prostaglandin subtype that has received the most attention in its ability to mediate central effects in response to circulating cytokines (Lacroix & Rivest, 1998). Thanks to its lipophilic nature, PGE_2 can diffuse into the brain and interact with catecholaminergic brainstem nuclei and the paraventricular nucleus of the hypothalamus, inducing HPA axis alterations and fever (Cao et al., 1997; Dantzer, 2009). Interestingly, PGE_2 receptors are expressed in brain areas implicated in emotional and behavioral control, including the hypothalamus and amygdala (Zhang & Rivest, 1999). It regulates sickness following systemic inflammation, and it is involved in increase body temperature, reduce food intake and influence cognitive functions such as learning and memory (Poon, Ho, Chiu et al., 2015). Actually, inhibition of PGE_2 synthesis in mice reduced sickness behavior induced by direct inoculation of LPS (de Paiva et al., 2010).

The pro-inflammatory activity of conventional prostaglandins is counterbalanced by the anti-inflammatory activity of cyclopentenone-types prostaglandins, as the case of 15d-PGJ_2. This prostaglandin reduces activation of pro-inflammatory cytokines such as IL1 or TNF-α, and it exerts a role of neuroprotection in central nervous system, through the activation of the peroxisome proliferator-activated receptor gamma (PPARγ) (Bell-Parikh et al., 2003; Leonard, 2018).

Other direct mechanism involved the cellular-mediated immunity. The imbalance in the ratio between lymphocyte T-helper 1 (Th1) and T-helper 2 (Th2) due to an excess of CD4+ Th1 cells, has been hypothesized as the basis of the excess of proinflammatory cytokines observed in depression (Slyepchenko, Maes, Machado-Vieira et al., 2016). Th1 are

related to increase proinflammatory activity, leading to activation of macrophages which in turn sustain Th1 propagation (Maes, 2011). CD8+ Th1 cells are another subtype of lymphocytes which drive immune cell proliferation through the release of IL-2, a proinflammatory cytokine which soluble receptor levels are increased in major depressive disorder (Maes et al., 1990). Augmented B cells, stimulated by IL2, have been also observed in MDD (Maes et al., 1992). These observations suggest cellular-mediated immunity activation with a unique lymphocyte profile in depression.

Recently, potential interactions of lymphocytes T-helper 17 (Th17) with mechanisms associated with MDD have been reviewed (Slyepchenko et al., 2016). Cytokine activity, mostly IL-6 and transforming grow-factor-α (TGF-α), induce differentiation of T-helper naïve cells into Th17 cells, which in turn produce several types of cytokines, grouped in the IL-17 family, and the chemokine ligand 20 (CCL-20). Th17 is an independent lineage of T-helper lymphocytes (Dinan & Cryan, 2013), and it has been shown that Th17 can migrate to central nervous system and are important in the bacterial infections clearance and neuroinflammation regulation (Holley & Kielian, 2012). Th17 cells regulate microglia activation and immune response in central nervous system, through release of IL-17 and IFN-α. Such cytokines induce microglia activation and IL-1β production (Murphy et al., 2010). Furthermore, IL-17 and TNF-α together collaborate to oligodendrocyte loss in multiple sclerosis (Paintlia et al., 2011), whereas in microglia, IL-17 production induces release of IL-6 and activation of iNOS and the consequent oxidative and nitrosative stress set off (Meares et al., 2012).

Taken together these findings shed a light on the important role of proinflammatory cytokines and cellular-mediated immunity activation together with central nervous system

microglia interaction, in the major depressive disorders. The Figure 3 provides a general overview of the mentioned direct mechanisms.

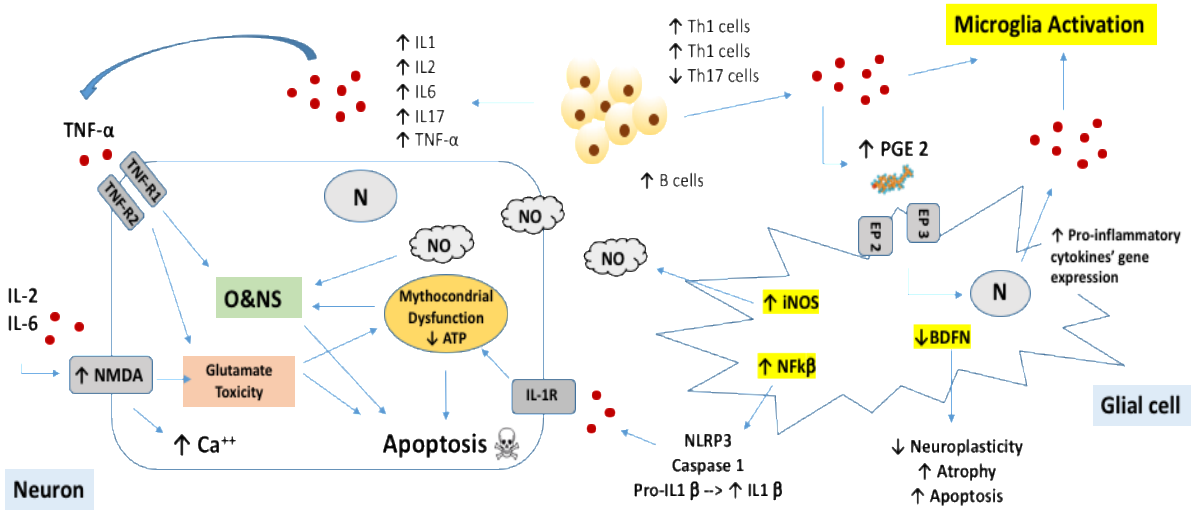

Figure 3. Pro-inflammatory cytokines and cellular mediate immunity activation may drive inflammation in central nervous system, interacting with microglia or directly affecting neurons. These mechanisms may contribute to neuroinflammation, affecting neural plasticity and inducing neuronal apoptosis (see text for details).

Abbreviations: ATP: adenosine triphosphate; BDNF: Brain derived grow factor; IL-: Interleukin; iNOS: inducible nitric oxide synthase; N: nucleus; NLRP3: Nod-like receptor pyrin-domain containing 3; O&NS: oxidative and nitrosative stress; PGE2: Prostaglandin E2; Th: T helper lymphocytes; TNF-α, Tumor-necrosis factor- α; TNF-R, Tumor-necrosis factor receptor.

1.4.3 Pathways Involving Monoaminergic transmission

1.4.3.1 The role of indoleamine 2,3 dioxygenase and the tryptophan catabolism pathway

One familiar mechanism that relates cytokines to depression is the induction of IDO enzyme, a property shown mostly by IL-2, IL-6 and IFN-α (Anderson et al., 2013; Capuron et al., 2001, 2003). The IDO activation leads to tryptophan catabolism into kynurenine, activating the TRYCAT pathway, potentially depleting the availability of serotonin (5-HT) in the brain (Miller et al., 2013). In rodent models, IDO induction by IL-6 has been linked to central and peripheral processes related to depression, somatization and chronic pain (Anderson et al., 2013). Actually, tryptophan is an essential amino acid and a precursor for 5-HT synthesis. More than the 95% of tryptophan is oxidized by hepatic tryptophan-dioxygenase, that can be

induced by cortisol (Maes & Rief, 2012), whereas just a small portion is metabolized by the IDO enzyme. Beside the depletion of 5-HT, that has a well-established role in mood disorder (Rosenblat et al., 2014), activation of TRYCAT pathway may have a role in depressive symptoms (Morris et al., 2015). Indeed, kynurenine can be converted in kynurenic acid or 3-hydroxy-kynurenine and quinolinic acid in astrocytes and microglia, which modulate neurotransmitters (such as 5-HT, dopamine, glutamate and melatonin) production and release (Reus et al., 2015). In general, TRYCATs have been related to impair mitochondrial energy metabolism inhibiting enzymes involved in adenosine triphosphate (ATP) production (Naoi et al., 1987). Disruption in mitochondrial functions and integrity can increase oxidative and nitrosative stress (Maes et al., 2011). Recently, it was observed that heightened TRYCAT activity can disrupt melatoninergic pathway, which was involved in several medical conditions (Morris et al., 2015). Melatonin is an essential anti-inflammatory and antioxidant molecule which modulates mitochondrial functions (Martín et al., 2002). Interestingly, melatonin is produced by gut cells and reduces its permeability avoiding bacteria translocation (Trivedi & Jena, 2013) and gut dysbiosis, which was observed in many medical conditions associated with depression (Slyepchenko et al., 2017). Furthermore, 3-hydroxykynurenine generates free-radicals species contributing to oxidative and nitrosative stress, whereas quinolinic acid is a known agonist at the NMDA receptor, contributing to excessive glutamatergic signalling which may cause destruction of synaptic elements or nerve cells apoptosis (Tavares et al., 2002), a mechanism related to major depression (Müller & Schwarz, 2007). In a study of Raison et al. (2010), positive correlations were found between kynurenine, kynurenic acid, quinolinic acid and proinflammatory and immune variables in the cerebrospinal fluid, with depressive symptoms in patients undergoing IFN-α treatment (Raison et al., 2010). On the other side, other published data show peripheral

decreased of kynurenic acid in depressed patients, postulating a possible neuroprotective role (Maes & Rief, 2012). In this line,, Klein et al (2013) reported kynurenic acid antagonistic effects on NMDA receptors, an effect opposite to the quinolinic acid function, including that an immune-mediated imbalance may contribute to glutamate excitotoxicity (Klein et al., 2013). See Figure 4 for an overview.

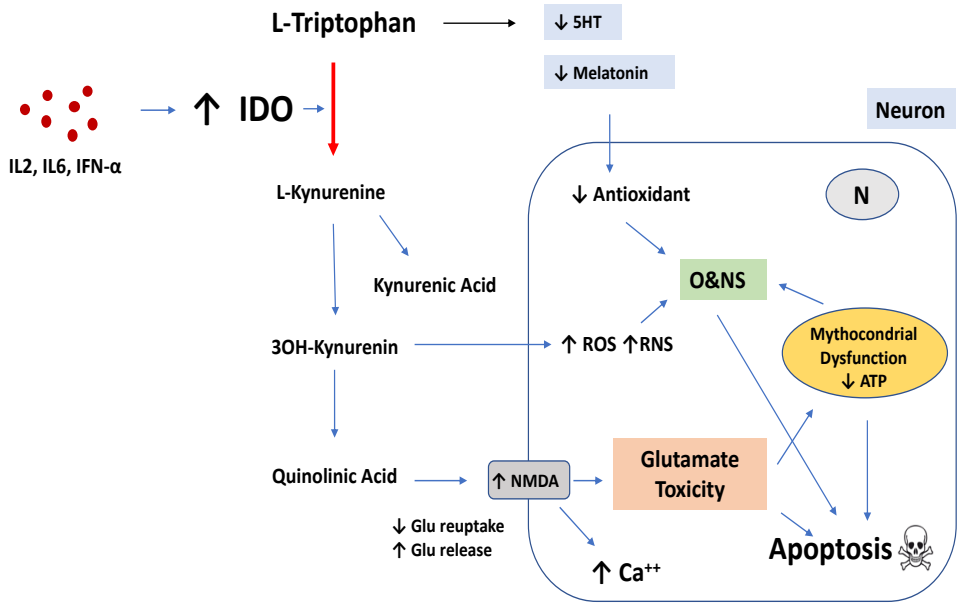

Figure 4. Overview of the tryptophan catabolites (TRYCATs) pathway and their effect on neurons and central nervous system. Cytokines as IL2, IL6 and IFN-α increase activity of IDO enzyme, inducing depletion of 5HT and melatonin, and activating TRYCATs pathway (see text for details).

Abbreviations: 5-HT: Serotonin; ATP: adenosine triphosphate; Glu: Glutamate; IDO: Indoleamine 2,3 dyoxigenase; IFN-α: Interferon- α; IL-: Interleukin; N: nucleus; O&NS: oxidative and nitrosative stress; ROS: reactive oxygen species; RNS: reactive nitric species;

● Cytokine

1.4.3.2 The role of tetrahydrobiopterin activity

Another biological mechanism that may link cytokines with depression is the disruption of tetrahydrobiopterin (BH4), an enzyme cofactor essential for the synthesis of 5-HT, dopamine and norepinephrine, and essential for the conversion of arginine to nitric oxide (Haroon et al., 2012; Neurauter et al., 2008). It has been hypothesized that the induction of iNOS by cytokines during inflammatory response increase BH4 depletion, leading to a reduction of monoamines synthesis, as illustrated in Figure 5 (Capuron & Miller, 2011; Kitagami et al.,

2003). In an interesting study of Felger et al (2013), IFN-α administration was associated with decreased peripheral conversion of phenylalanine to tyrosine, (which ratio is used as an indirect measure of BH4 activity in the brain), that in turn was associated with reduced dopamine in the brain and fatigue (Felger, Li, & Marvar, 2013). One year before, Capuron et al (2012) demonstrated in 14 patients with CHC treated with IFN-α that inflammatory stimuli were associated with decreased basal ganglia activity involving hedonic reward circuitry, which correlated with behavioral alterations, including anhedonia, depression, and fatigue. Moreover, increased uptake and decreased turnover of the radiolabelled dopamine precursor measured by PET, evidenced that inflammatory cytokines can directly affect striatal dopamine function (Capuron & Pagnoni, 2012). The authors pointed out a possible causal mechanism in the disruption of BH4, and consequent reduction of dopamine availability.

1.4.3.3 The role of Mitogen-activated protein kinase pathways

Mitogen-activated protein kinase (MAP-Kinase) pathways, as p38 or extracellular signal-regulated kinases 1 or 2, may increase the expression and function of reuptake transporters for 5-HT, dopamine and noradrenaline (Miller et al., 2009). The MAP-Kinase may be activated by inflammatory cytokines. In vitro models of rat leukaemia cell lines, TNF-α and IL-1 increase expression and activity of the serotonin transporter (SERT) and the reuptake of 5-HT in a dose and time dependent manner (Zhu, Blakely, & Hewlett, 2006). On the same way, inhibition of MAP-Kinase signalling decrease dopamine uptake in a dose and time dependent fashion in human embryonic kidney cell lines (Morón et al., 2003). Finally, even noradrenaline transporter (NET) activity was related to p38 activation (Apparsundaram et al., 2001). Data in humans revealed that increased cerebrospinal fluid levels of IL-6, which

is capable of activating MAP-Kinase, were correlated with depressed mood and decreased cerebrospinal fluid levels of 5-hydroxyindolacetic acid, a 5-HT metabolite (Raison et al., 2010). Finally, MAP-Kinase p38 has been shown to disrupt glucocorticoid receptor translocation from cytoplasm to nucleus, a mechanism probably involved in the breakdown of the HPA axis regulation (Pace, Hu, & Miller, 2007). See Figure 5 for details.

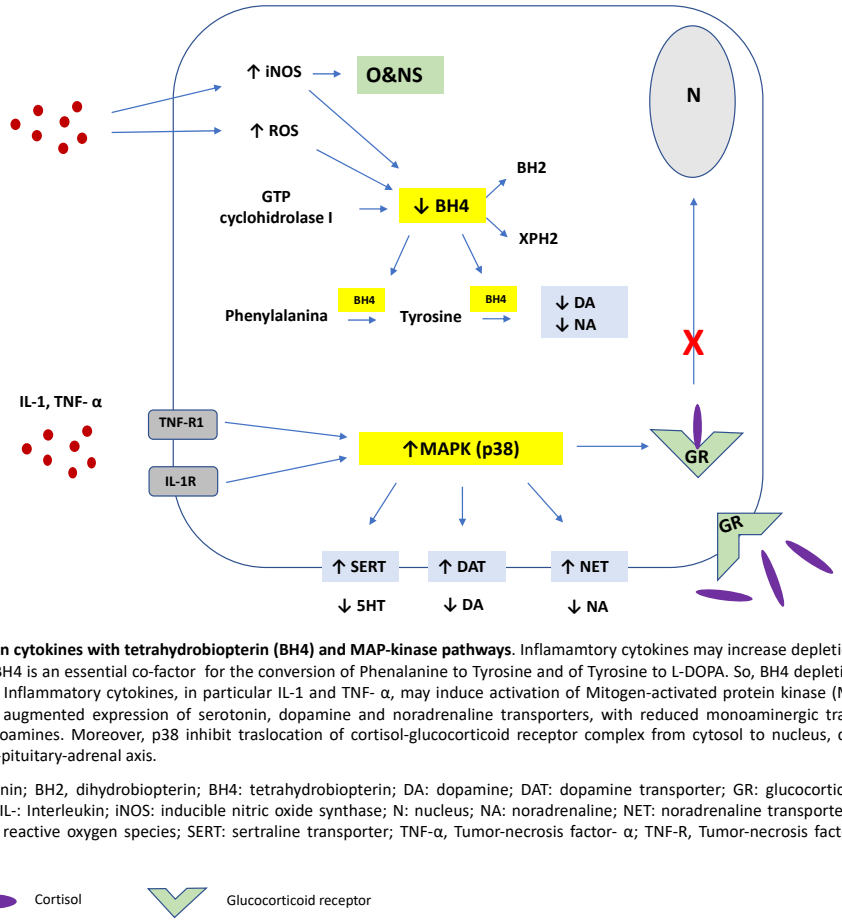

Figure 5. Interaction between cytokines with tetrahydrobiopterin (BH4) and MAP-kinase pathways. Inflamamtory cytokines may increase depletion of BH4, through increase of oxidative stress BH4 is an essential co-factor for the conversion of Phenalanine to Tyrosine and of Tyrosine to L-DOPA. So, BH4 depletion is related to DA and NA synthesis reduction. Inflammatory cytokines, in particular IL-1 and TNF- α, may induce activation of Mitogen-activated protein kinase (MAPK) as p38. This kinase has been related to augmented expression of serotonin, dopamine and noradrenaline transporters, with reduced monoaminergic transmission, due to increased re-uptake of monoamines. Moreover, p38 inhibit traslocation of cortisol-glucocorticoid receptor complex from cytosol to nucleus, contributing to the dysruption of hypothalamus-pituitary-adrenal axis.

Abbreviations: 5-HT: Serotonin; BH2, dihydrobiopterin; BH4: tetrahydrobiopterin; DA: dopamine; DAT: dopamine transporter; GR: glucocorticoid receptor; GTP, guanosine-50-triphosphate; IL-: Interleukin; iNOS: inducible nitric oxide synthase; N: nucleus; NA: noradrenaline; NET: noradrenaline transporter; O&NS: oxidative and nitrosative stress; ROS: reactive oxygen species; SERT: sertraline transporter; TNF-α, Tumor-necrosis factor- α; TNF-R, Tumor-necrosis factor receptor; XPH2, dihydroxanthopterin.

1.4.4 The Hypothalamic-pituitary-adrenal axis

Other than monoaminergic mechanisms have been implicated in cytokine-induced depression physiopathology. Actually, the activation of the HPA axis by pro-inflammatory cytokines, is one of the best elucidated mechanisms that have been related with stress and depressive symptoms (Pariante & Lightman, 2008). Cytokines involved in major depressive

disorder such as IL-1, IL-6, TNF-α and IFN- α stimulate the release of corticotrophin releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and cortisol. One other mechanism by which cytokines may influence the HPA axis is through the decrease in the expression, translocation and downstream effects of glucocorticoid receptors, which may disrupt the axis negative-feedback (Felger et al., 2015). This effect seems to be mediated by the induction of the c-Jun N-terminal kinase (JN-Kinase) and MAPK p38, or through the expression of the inactive β isoform of the glucocorticoid receptors (Dantzer et al., 2008; Pace & Miller, 2009). Hence, the effects of sustained cortisol levels together with a reduction of the negative feedback can lead in turn to immune activation, being part of the same physiopathological process (Pariante & Lightman, 2008). The HPA axis hyperactivity has been repeatedly related to clinical depression, and it is considered one of the most reliable findings in biological psychiatry. A large meta-analysis of 354 studies found that 73% of depressed individuals had elevated cortisol values compared to non-depressed patients (Stetler & Miller, 2011). The pathways by which augmented levels of glucocorticoids induce depression have not been fully elucidated. Maes et al. (2011) observed that cortisol can increase tryptophane-dyoxigenase activity in liver, reducing tryptophan availability for 5-HT synthesis, and boosting the TRYCAT pathway with the consequences already discussed in paragraph 1.5.3.1 (Maes et al. 2011). Furthermore, increased levels of glucocorticoids can cause atrophy in different brain region, such medial prefrontal cortex or hippocampus, contributing to depressive symptoms (Chaouloff & Groc, 2011). See Figure 6.

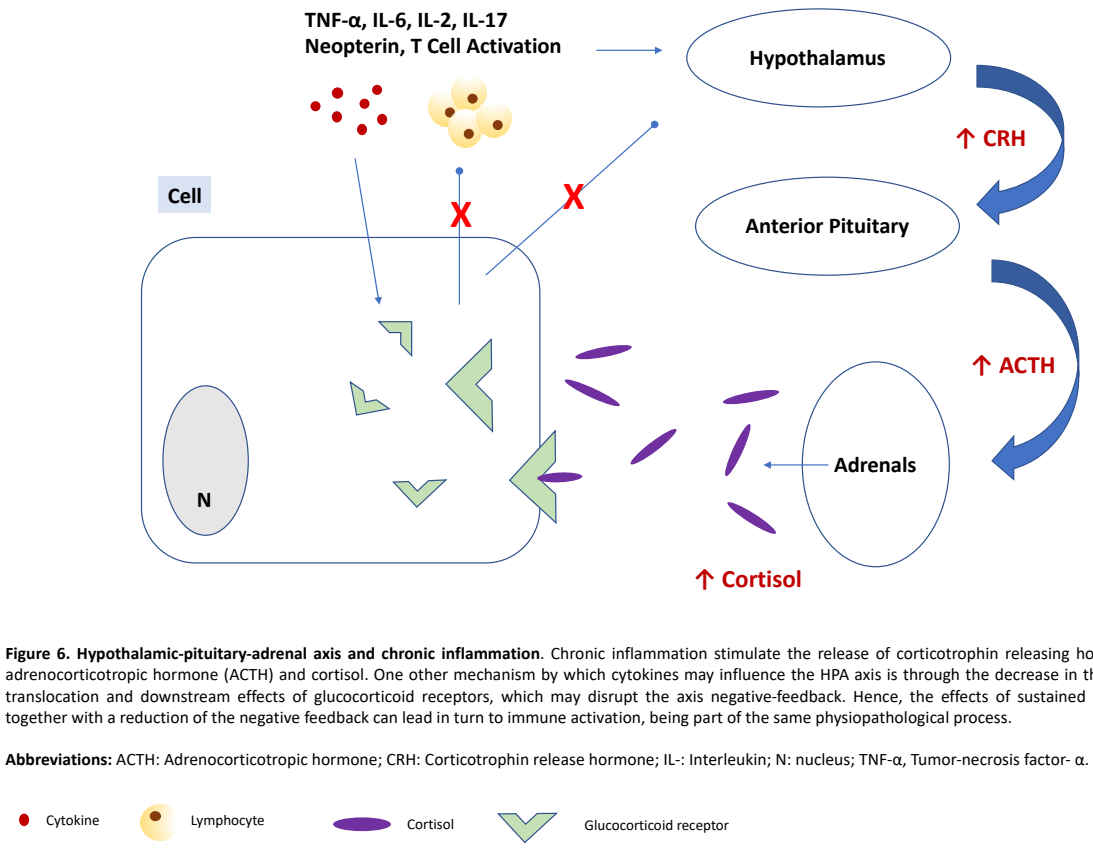

Figure 6. Hypothalamic-pituitary-adrenal axis and chronic inflammation. Chronic inflammation stimulate the release of corticotrophin releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and cortisol. One other mechanism by which cytokines may influence the HPA axis is through the decrease in the expression, translocation and downstream effects of glucocorticoid receptors, which may disrupt the axis negative-feedback. Hence, the effects of sustained cortisol levels together with a reduction of the negative feedback can lead in turn to immune activation, being part of the same physiopathological process.

Abbreviations: ACTH: Adrenocorticotropic hormone; CRH: Corticotrophin release hormone; IL-: Interleukin; N: nucleus; TNF-α, Tumor-necrosis factor- α.

1.4.5 Oxidative and Nitrosative Stress

A balance between endogenous production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and the antioxidant defence systems is essential in order to prevent the oxidative and nitrosative stress (O&NS) (Cobley, Fiorello, & Bailey, 2018). When the reactive oxygen and nitrogen species exceed the elimination capability of antioxidant mechanisms, or because of an increase in their output, either because of a deterioration of the antioxidant defence, then the O&NS ensues. Major depression has been repeatedly associated with O&NS (Palta et al., 2014; Yusuf et al., 2017) and the immune system activation is one of the main cause which drives O&NS in depression (Moylan et al., 2014).

Inflammation as well as mitochondrial processes, can increase the reactive oxygen and nitrogen species production (Maurya et al., 2016; Moylan et al., 2013). This may result in a widespread damage to different biomolecules, such as lipids, proteins, sugars and DNA,

causing impairment in cellular function and rendering them potentially immunogenic (Maes et al., 2011). Actually, the creation of modified neoepitopes due to the O&NS, can induce a secondary autoimmune response and a persistent inflammatory activation, contributing to resistance or recurrence and progression in major depressive disorder (Iseme et al., 2014). For example, the increased protein nitrosilation secondary to the nitrosative stress induces immunoglobulin-M (IgM) response in depression. IgM mediated response can amplify inflammatory reactions, and target essential molecules for cellular physiology, such anchorage molecules, oleic acid or complexes involved in cell signalling pathways, leading to aberration in brain cellular function (Maes et al., 2013).

Another example is the immunoglobulin-G (IgG) activation against oxidized low-density lipoproteins (LDL). Interestingly, depressed patients show increased IgG antibodies against oxidized LDL, compared to normal controls (Maes et al., 2010). Taking into account the pro-atherogenic role of such autoantibodies and their relation with infarction (Anderson & Maes, 2014), it can provide a possible explanation of comorbidity between depression and cardiovascular diseases. Finally, autoimmune activity against 5-HT in major depressive disorder, with consequent apoptosis via activation of pro-caspase 9 (Iseme et al., 2014) has been observed in patients with melancholic depression and it has been associated with number of depressive episodes (Maes et al., 2012).

ROS are reactive species derived from oxygen, as superoxide (a free radical), hydrogen peroxide (a non-radical) or hydroxyl radicals, the most reactive molecule. Nicotinamide-adenine-dinucleotide-phosphate oxidase, xanthine oxidase, cyclooxygenase and lipoxygenase are enzymes which activity can be increased by inflammatory stimuli and are responsible of increased generation of reactive oxygen species. Indeed, these enzymes were in turn associated with depressive symptoms (Cassano et al., 2006; Michel et al., 2010;

Seo et al., 2012). Moreover, in many neurodegenerative disorders it has been demonstrated that oxidative stress, and the subsequent autoimmune response against damaged biomolecules, is one of the major cause of disease progression, reducing neuronal viability, inducing apoptosis or inhibiting mitochondrial respiratory function (Tramutola et al., 2016; Zhao & Zhao, 2013). Concerning oxidative stress and depression, increased level of lipid peroxidation has been found in depressed patients, correlating to the severity of depression (Black, Bot, Scheffer et al., 2015; Dimopoulos, Piperi, Psarra et al., 2008; Lindqvist et al., 2017), whereas DNA oxidative damage in major depressive disorder was related with recurrent depressive episodes (Forlenza & Miller, 2006).

RNS are reactive molecules which derive from nitrogen and are classified in ions and non-ions, as peroxynitrite or nitric oxide respectively. The latter, is synthetized from L-arginine to the NOS. The inducible isoform of such enzyme (iNOS) is induced by cytokines and is responsible of the inflammatory effects of nitric oxide (Maes et al., 2011). This gaseous molecule has regulating effect in neuroendocrine and immune function, and influences 5-HT, dopamine and glutamatergic neurotransmission (Moylan et al., 2013). Increased levels of nitric oxide as well as increased iNOS expression have been found in major depressive disorder patients with recurrent depression (Akpinar et al., 2013; Gałecki et al., 2012). On the other hand, in animal model inhibitors of NOS shown antidepressant properties (Joca & Guimarães, 2006), and fluoxetine has been shown to decrease hippocampal NOS (Zhang et al., 2010).

As mentioned above, the O&NS can be also supported by a decreased antioxidant activity. The antioxidant system is composed by endogenous biomolecules like glutathione or melatonin, as well as enzymatic system such catalase, glutathione peroxidise and superoxide-dismutase, and by exogenous redox modulators, including vitamin C, E, zinc and

coenzyme Q10 (Anderson & Maes, 2014; Yusuf et al., 2017). Inflammation, as well as unhealthy diet, sedentarism and tobacco has been shown to reduce antioxidant activity (Berk et al., 2013). Several lines of evidence indicate a reduction in antioxidant activity in depressed patients. For example, patients with major depressive disorder showed reduced glutathione in prefrontal cortex (Gawryluk et al., 2011). Furthermore, reduced serum levels of different antioxidants, such as coenzyme-Q, superoxide-dismutase and glutathione peroxidise (Morris, Anderson, Berk et al., 2013; Yusuf et al., 2017) have been found in depressed patients. See Figure 7 for details.

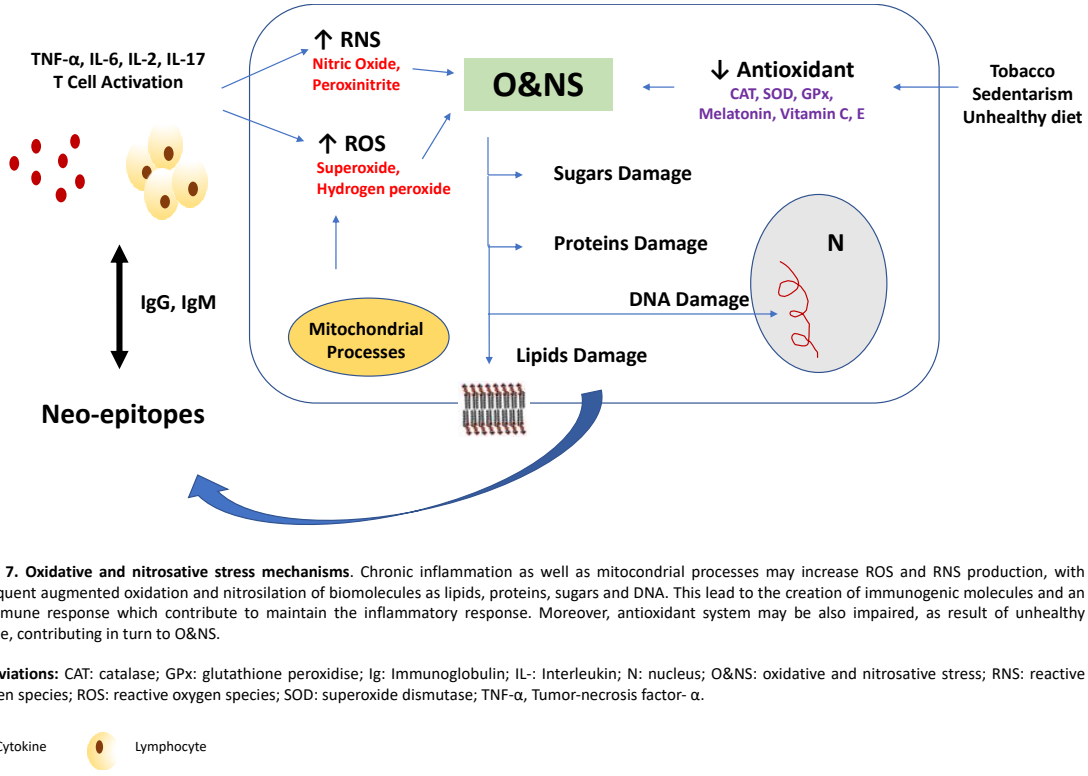

Figure 7. Oxidative and nitrosative stress mechanisms. Chronic inflammation as well as mitocondrial processes may increase ROS and RNS production, with consequent augmented oxidation and nitrosilation of biomolecules as lipids, proteins, sugars and DNA. This lead to the creation of immunogenic molecules and an autoinmune response which contribute to maintain the inflammatory response. Moreover, antioxidant system may be also impaired, as result of unhealthy lifestyle, contributing in turn to O&NS.

Abbreviations: CAT: catalase; GPx: glutathione peroxidise; Ig: Immunoglobulin; IL-: Interleukin; N: nucleus; O&NS: oxidative and nitrosative stress; RNS: reactive nitrogen species; ROS: reactive oxygen species; SOD: superoxide dismutase; TNF-α, Tumor-necrosis factor- α.

1.4.6 Mitochondrial dysfunction

A correct mitochondria functioning is crucial for central nervous system physiology, and alteration in energy production has been related to several neurodegenerative disorders, as Alzheimer disease (Salminen et al., 2015), multiple sclerosis (Gounopoulos et al., 2007) and Parkinson's disease (Trudler et al., 2015). Actually, mitochondrial dysfunction may impair

neurogenesis and cell survival (Volobоueva & Giffard, 2011), and it is a mediator of oxidative and nitrosative stress disruption. Importantly, changes in their size and distribution have been observed in depression (Gardner & Boles, 2011), and a reduction in mitochondrial ATP generation was found in depressed patients compared to controls (Gardner et al., 2003). On the other hand, antidepressant treatment can reverse the mitochondrial impairment observed in a genetic model of depression (Chen et al., 2013). Pro-inflammatory cytokines, such IL-6 or TNF-α, can amplify the mitochondrial dysfunction and contribute to the consequences described on neural integrity (Volobоueva & Giffard, 2011),

1.4.7 Brain-gut interaction and the role of microbiota

It is increasingly recognized that there is a bidirectional communication between the gut and the brain modulated by intestinal gut commensal, called microbiota, and that this can influence the core symptoms of neuropsychiatric disorders, such as major depressive disorder (Kelly et al., 2015). The main mechanisms involved in the communication pathways of the namely “Brain-gut-microbiota axis”, implicate the vagus nerve, the synthesis of neurotransmitters by gut microbes, the production of short chain fatty acids, the influence on HPA axis and immune signalling (Dinan & Cryan, 2016). Actually, balanced interaction between the gut microbiota and the local immune system is crucial in the defence against potentially harmful pathogens (Kamada et al., 2013). In this way, the regulation of nutrient and fluid absorption, beside the prevention from invading damaging substances, is a fundamental function of the intestinal barrier (Johansson, Sjövall, & Hansson, 2013), and microbiota is essential in the development and maintenance of such functions (Shifrin et al., 2012).

Both alterations in microbiota composition, known as “dysbiosis”, and/or alteration in gut permeability, namely “leaky-gut”, may disrupt neuroendocrine or neuroimmune signalling pathways, increasing the vulnerability to stress-related disorder and cooperating to the major depression pathogenesis (Dinan, Borre, & Cryan, 2014; Macedo et al., 2016; Maes et al., 2012). For example, a study of Jiang et al (2015) revealed increased levels of some microbiota species, as *Bacteroidetes, Proteobacteria*, and *Actinobacteria*, and reduction of other species as *Firmicutes* in depressed patients compared to healthy controls (Jiang et al., 2015). Interestingly, the serum level of brain-derived neurotrophic factor (BDNF) differed significantly among the groups. Actually, other studies reported relation between gut microbiota composition with changes in hippocampal BDNF levels of both mRNA and protein (Neufeld et al., 2011), sustaining a possible interaction with stress environment and neurodegeneration mechanisms.

On the other hand, an important study by Maes et al., (2012) demonstrated on a cohort of 112 patients with MDD higher serum levels of IgM and IgA against lipopolysaccharide of several Gram-negative gut commensal, compared to healthy controls (Maes et al., 2012). These findings may be primary explained by an increase permeability of intestinal barrier, with subsequent internal microbiota translocation and immune activation (Maes et al., 2013), Importantly, other studies have shown that diet and stress can be the most important promoters of microbiota imbalance (Berk et al., 2013; Macedo et al., 2016), and that altered gut permeability can activate the TRYCAT synthesis (Dinan & Cryan, 2016).

Taken together these findings highlighted the role of the brain-gut microbiota axis in maintaining brain health, and how an imbalance of such mechanisms may contribute to the activation of immune system and pathways involved in of major depressive disorder and its progressive course.

1.4.8 The neural plasticity and the role of brain derived neurotrophic factor

Immune system activation has been implicated in impaired neuroplasticity, a physiopathological mechanism involved in the evolution of major depressive disorder (Molendijk et al., 2014). The pro-inflammatory cytokines IL-1, IL-6 and TNF-α, in normal condition provide trophic support to neurons and enhance neurogenesis (Bernardino et al., 2008). Nevertheless, evidence from animal studies underline the role of chronic inflammation and stress in decrease expression in neurotrophic factors, such as the BDNF, potentially resulting in reduced hippocampal neurogenesis and increased neuronal atrophy (Sahay & Hen, 2007). For example, in a study of Wu et al (2007), LPS administration in mice decrease BDNF expression and neurogenesis in hippocampus, and it was associated with increased hippocampal concentration of TNF-α and IL-1β (Wu et al., 2007). Also other neurotrophins, such as vascular endothelial growth factor (VEGF) (Carvalho et al., 2015) or insulin growth factor (IGF) (Sievers et al., 2014), have been related to the regulation of neurogenesis in hippocampus (Christian, Song, & Ming, 2014).

In human studies, lower levels of BDNF have been related to severity and recurrent major depressive disorder (Lee et al., 2007), thought this result has not found confirmation (Molendijk et al., 2014). Furthermore, evidence on the role of BDNF in depression derives from data concerning antidepressant treatment. Actually, BDNF levels appear to be higher in treated depressed patients, compared to untreated individuals (Shimizu et al., 2003). In support of these, increase BDNF synthesis in dentate gyrus was correlated with chronic antidepressant treatment in mice (Larsen et al., 2010). Other intriguing results derive from studies on neonatal stress and early trauma. It is widely recognize that early stress exposure as childhood abuse or neglect may predispose to a later onset of major depression or anxiety (Roth & Sweatt, 2011). For example, in a recent cohort study of 473 patients diagnosed of

major depressive disorder, anxiety disorders or somatoform disorder, higher postnatal stress was associated with higher depression severity, and post-natal stress was a predictor factor of depression severity in adult life (Vogt et al., 2016). Furthermore, imaging studies on adults who reported such experiences have identified a number of lasting neural consequences (Roth & Sweatt, 2011). The altered gene expression seems to be responsible of such sensitization, mostly through epigenetic mechanisms involving BDNF genes (Post, 2016). Intense stress experience in the first week of life of rodents can induce methylation of the promoter region of BDNF and thus decrease the BDNF production for the whole animal lifespan (Roth et al., 2009), which is accompanied by depressive-like behavior.

In synthesis, as illustrated in Figure 8, the important role that the BDNF system plays in neural modelling, neuroplasticity and neurogenesis suggests that early stress exposure may increase vulnerability for major depressive disorder in adult life through epigenetic mechanisms, contributing to onset and recurrence of depression.

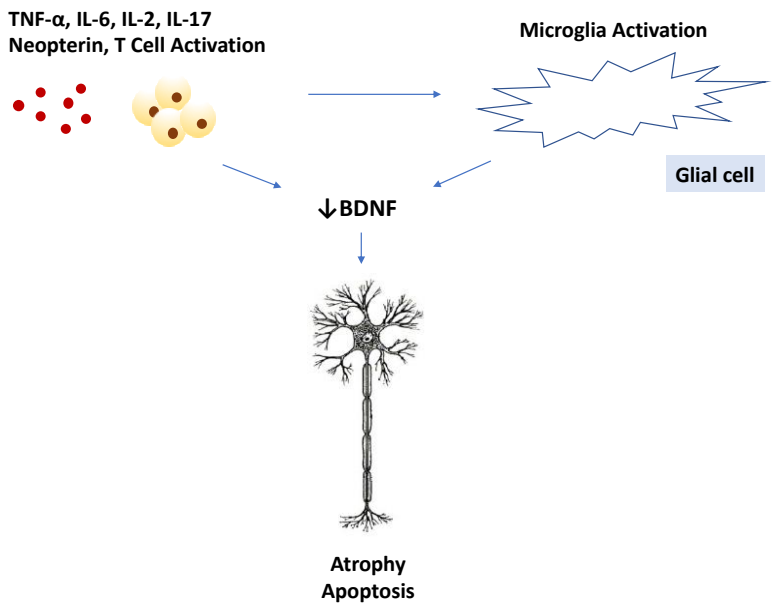

Figure 8. Interaction between chronic inflammation and brain derived grow factor. Chronic inflammation and microglia activation decreased BDNF expression, with deleterious consequences on neuronal fitness. Reduction in BDNF has been related with reduced neurogenesis, impairment neuroplasticity and increase apoptosis .

Abbreviations: BDNF: Brain derived grow factor; IL-: Interleukin; TNF-α, Tumor-necrosis factor- α.

1.4.9 The endocannabinoid system

Finally, thought the evidence is still germinal, it has been observed that the endocannabinoid system plays an important role in immunity modulation, stress response and neurogenesis, and it appears dysregulated in depression (Bhattacharyya, Crippa, Martin-Santos et al., 2009; Campos, Moreira, Gomes et al., 2012; Martin-Santos et al., 2012; Mechoulam & Parker, 2013; Sahu et al., 2019). Endocannabinoids derived from the arachidonic acid, a polyunsaturated fatty acid which oxidation lead to the synthesis of eicosanoids, prostaglandins and leukotrienes (Alhouayek, Masquelier, & Muccioli, 2014). Eicosanoids are molecules involved in inflammatory modulation which can interacts with endocannabinoids signalling, with consequences in the regulation of immune system that could have an influence on the onset and maintenance of depression illness (Boorman et al., 2016).

1.5 The impact of immune system on neurocircuitry

The effects of cytokines on neurotransmitters, neuroendocrine functions and neural plasticity described in the previous paragraphs, augmented the interest in identifying neuroanatomical substrates of such effects. Indeed, neuroimaging studies such as functional magnetic resonance (fMRI) and PET have pointed out the role of cortical and sub-cortical brain structures relevant to the sickness behavior and major depressive disorder, helping to identify those areas sensitive to peripheral inflammation (Harrison, 2017). Most of the studies focus on circuits that have been related with reward, motivation, interoceptive awareness and motor activity, suggesting basal ganglia nuclei as primary target of cytokine effects on brain (Harrison et al., 2009).

A large body of evidence came from experimental paradigms on humans, in which acute inflammation was induced by the administration of endotoxin (e.g. LPS) or typhoid and influenza vaccination (Boyle et al., 2019; Brydon, Harrison, Walker et al., 2008; Eisenberger et al., 2011; Harrison et al., 2009), or from cuasi-experimental paradigms, for example in CHC patients treated with pro-inflammatory cytokine IFN-α (Capuron et al., 2005, 2007). Little evidence regards neuroimaging studies in chronic low-grade inflammation condition, such CHC or obesity patients.

Insula, subgenual anterior cingulate cortex (sgACC), dorsal anterior cingulated cortex (dACC) and basal ganglia, in particular ventral striatum, dorsal striatum and substantia nigra, have been related to individual component of inflammation-related behavioral changes (Harrison, 2017).

The insula cortex is believed to represent and integrate the interoceptive signals, as changes in peripheral inflammation, providing the bases of interoceptive and emotional awareness (Craig, 2009). Indeed, structural and functional changes in posterior, mid or anterior insula, have been associated to subjective feelings of malaise and fatigue following inflammation (Bushara, Grafman, & Hallett, 2001; Farrer et al., 2003; Klein et al., 2007). Moreover, recent studies pointed out a posterior-to-mid-to-anterior pattern of integration of interoceptive information (Craig, 2003). For example, activation of posterior insula is related to objective intensity of heat pain, whereas anterior insula activation is related to subjective pain evaluation (Kong et al., 2006). Importantly, the joint activation of insula and ACC in experiencing interoceptive changes has been reported in several studies, being ACC the probable site for motivational behaviors related to interoceptive awareness (Craig, 2009).

The sgACC has been recognized as crucial in emotional processing and mood regulation (Drevets, Savitz, & Trimble, 2008; Seminowicz et al., 2004; Wu et al., 2016). Impaired connectivity between sgACC to ventral striatum and amygdala was associated with mood symptoms during inflammatory challenge (Harrison et al., 2009). In details, the authors observed an augmentation in circulating IL-6 and significant mood reduction at 3 hours after typhoid injection in 16 healthy participants. Inflammation-associated mood deterioration was correlated in changes in sgACC activity and in functional connectivity during evoked responses to emotional stimuli, which was modulated by peripheral IL-6 (Harrison et al., 2009).

The dACC has been recognized as crucial in conflict monitoring (Carter et al., 1998). CHC patients receiving IFN-α show significant dACC activation in contrast to controls subjects, which correlated with the number of task-related errors. As the authors of the study suggest, the cytokines signalise the need to exert greater mental effort to maintain performance and therefore contribute to cytokine-induced behavioral changes (Capuron et al., 2005). The dACC has been also related to process social pain and threatening stimuli in the social domain, i.e. social rejection (Eisenberger & Lieberman, 2004). In a study of Slavich et al (2010), 31 healthy subjects exposed to a laboratory based social stressor displayed significant increases in soluble receptor for TNF-α (sTNF-αRII), increase that was associated with greater activity in the dACC and anterior insula (Slavich et al., 2010).

Basal ganglia are subcortical structures involved in the integration and coordination of executive functions, reward, emotions and mood, with specific relevance for adaptive shaping and action selection (Bostan & Strick, 2018; Riva, Taddei, & Bulgheroni, 2018). Specifically the ventral part of the striatum is critical for reward processes and motivational state (Schultz et al., 1992). Inflammation has been observed to modulate striatal responses

to reward as well as dopamine release (Borowski et al., 1998; Capuron & Pagnoni, 2012; Schultz et al., 1992). A recent study highlighted the role of inflammation in the re-orientation of motivational state, demonstrating a rapid reduction in ventral striatal encoding of reward prediction error and a significant increase in right insula encoding of punishment prediction error (Harrison et al., 2016). In another study, inoculation of typhoid vaccination in 16 healthy volunteers was associated with increased activation in substantia nigra during a cognitive task, that in turn correlated with increase in IL-6 and psychomotor slowing (Brydon et al., 2008b). Studies in patients with CHC receiving IFN-α evidenced significantly reduced bilateral activation of the ventral striatum during a hedonic reward task, correlating with decreased motivation and increased fatigue (Capuron & Pagnoni, 2012). Recently, Felger et al (2015) observed that decreased connectivity between ventral striatum and ventromedial prefrontal cortex was associated with increased CRP serum levels which correlated with anhedonia (Felger et al., 2015a).

Considering the dorsal part of the striatum, it has been associated with the control of habitual behaviors (Redgrave, Rodriguez, Smith, & Rodriguez-oroz, 2010; Wunderlich, Dayan, & Dolan, 2012) and in conjunction with insula, it is believed to modulate the balance between goal-directed and habitual modes of action control (Hong et al., 2015). In the study of Felger et al. (2015) mentioned above, increased CRP serum levels similarly predicted decreased dorsal striatal to ventromedial prefrontal cortex and presupplementary motor area connectivity, which correlated with decreased motor speed and which was associated with IL-6 and IL-1β increased plasma levels (Felger et al., 2015).

On the counterpart, brain structural and functional alterations in major depressive disorder have been identified in several neuroimaging studies. Alterations in prefrontal-limbic-subcortical areas have been primarily identified, similar to those involved during

acute inflammatory challenge and involved in emotional processing and awareness, (Drevets, Price, & Furey, 2008; Feng et al., 2016; Harrison, 2017; Mulders et al., 2015). For example, increased dorsal and mid insula connectivity to amygdala and orbito-frontal cortex was observed in depressed patients and correlated with symptoms severity (Avery et al., 2014), whereas reduced connectivity between sgACC and insula together with other cortical areas was found in depressed adolescents (Cullen et al., 2009). Considering basal ganglia, increased causal connectivity of the putamen and the caudate on the rostral anterior cingulated cortex (rACC) was observed in treatment-naïve patients during a first depressive episode (Feng et al., 2016). Moreover, in a study of Furman et al (2011), ventral striatum to sgACC connectivity was reduced in depressed patients, whereas increased connectivity between caudate and dorsal prefrontal cortex was positive correlated with severity of the disorder (Furman, Hamilton, & Gotlib, 2011).

Taken together, these data indicate an interaction between immune system activation and brain regions involved in several depressive symptoms, suggesting possible shared physiopathological mechanisms. See Figure 9 for a visual synthesis.

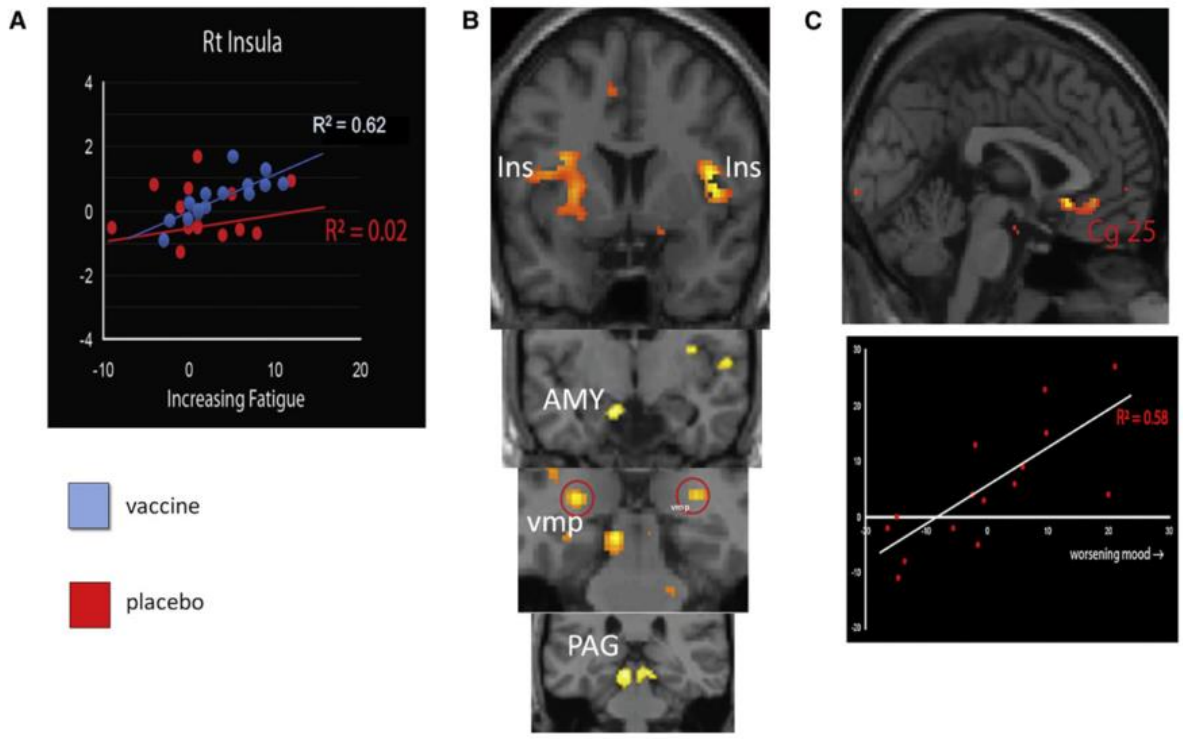

Figure 9. Impact of Peripheral Inflammation on Human Brain Activity, Cognition, and Emotion Group neuroimaging results are shown from fMRI experiments in which participants were scanned during states of (typhoid vaccine) induced peripheral inflammation or after placebo injection. (A) Reactivity of right insula cortex to (Stroop task) stimuli; predicted inflammation (not placebo) induced fatigue over the course of the experiment. (B) Following induced inflammation, brain regions showing greater reactivity map onto known central visceral afferent pathways: dorsal pons and periaqueductal grey matter (PAG); ventromedial pallidum (vmp), amygdala (AMY), and insula. (C) Inflammation-induced enhanced reactivity within subgenual cingulate cortex (cg25) to emotional face stimuli predicts inflammation-induced worsening of mood. Dysfunction of this region is implicated in patho-etiology of clinical depression. This finding (and other changes in functional connectivity) suggests depression hijacks mechanisms for the expression of social withdrawal and motivational blunting in adaptive sickness behaviors.

1.6 Resilience and vulnerability to immunity challenge

Beside the increasing evidence that inflammation increases risk of depression, it is not possible to claim that major depressive disorder is an inflammatory disease. Actually, not all patients with increased inflammation (as chronic ill patients, or the greater proportion of patients treated with IFN-α) develop depression (Lotrich, 2015). On the other hand, it is also true that not all people with MDD show immune activation (Steptoe, Kunz-Ebrecht, & Owen, 2003) or respond to anti-inflammatory treatment (Raison et al., 2013). Two important issues

came out from this concern: first, the inflammation can be considered a trigger of depression when cooperate with other risk factors and, precisely, can be consider a risk factor itself; second, some individuals appear to be resilient to the development of mood symptoms, given the same set of inflammatory conditions (Rosenblat et al., 2014).

Different clinical and biological conditions were associated with increased risk to develop cytokine-induced depressive symptoms, even if a majority set of observations derived from people treated with IFN-α. In a study of Capuron et al (2004), baseline mood and psychosocial characteristics of patients developing IFN-α induced depression were assessed. Patients with depressive symptoms at study endpoint exhibited higher baseline scores of the MADRS in sadness, pessimistic thoughts and sleep disturbances, compared to patients who remained free of depressive symptoms during cytokine therapy. Interestingly, only emotional symptoms and sleep disturbance at baseline, along with low social support, predicted severity of depressive symptoms at the end of the first month of therapy (Capuron al., 2004). A meta-analysis of Udina et al (2012) on 957 patients elucidated how female gender, low education and subthreshold depressive symptoms were associated with IFN-induced depression. Another series of vulnerability factors including early life stress, chronic stress and a personal or family history of depression, may also serve as relevant risk factors for the development of cytokine-induced mood and cognitive symptoms (Capuron & Miller, 2011).

Concerning biological risk factors, emerging evidence induce to consider increased inflammation as a risk factor for the future development of depression or for resistance in treatment response (Kim et al., 2019; Miller & Raison, 2016). By the way, intriguing data were obtained in a recent systematic review of Machado & Oriolo (2016), in which biological mechanisms contributing to the onset of a depression during IFN-α based immunotherapy

for HCV infection were examined. For example, in one selected prospective cohort study of more than 3000 individuals, it was observed how baseline CRP and IL-6 serum levels predicted cognitive symptoms of depression at 12 years follow-up (Gimeno & Kivima, 2009). In this line, a study of Prather et al (2009) underlined how increased of IL-6 plasma levels was strong predictive of major depressive disorder in CHC individuals treated with IFN-α. Other evidence of vulnerability or resilience factors came from studies in aging and/or obesity individuals, in which a state of chronically activated inflammatory signals may contribute to increased vulnerability to depressive symptoms (Capuron & Miller, 2011).

In many of these conditions, depressed patients tend to have higher levels of inflammatory cytokines than patients without major depressive disorder (Lotrich, 2015). Actually, some studies have highlighted the relation between chronic medical condition, depression and immune activation markers. In a study from Musselman et al (2001) plasma concentrations of IL-6 were found to be significantly increased in cancer patients with depression versus healthy comparison subjects and cancer patients without depression (Musselman et al., 2001). In a more recent study of Postal et al (2013), serum TNF-α levels were increased in childhood-onset systemic lupus erythematosus patients who develop depression (Postal et al., 2013). In the same line, Loftis et al (2013) highlighted that somatic symptoms of depression can be significantly exacerbated by IFN-α treatment in HCV infected patients, and may be predicted by higher TNF-α levels and lower 5-HT level at baseline (Loftis et al., 2013). Nevertheless, the relations between chronic diseases and depression seem to be bidirectional (Katon, 2011), as well as patients who show immune activation in the context of depression, can be medically healthy (Capuron et al., 2004; Maes, 1995).

An intriguing novel line of research regard the microbiota, considering that bacterial population stimulate inflammation which can influence the behavior (Lotrich, 2015).

Moreover, HPA axis hyperactivity, lower docosahexaenoic acid (DHA), higher di-homo-gamma-linolenic acid (DGLA) and higher arachidonic acid (AA) / eicosapentaenoic acid (EPA) + DHA ratio have been shown to increase risk to develop IFN-α induced depression (Capuron et al., 2003; Eccles et al., 2012; Lotrich, Sears, & McNamara, 2013; Machado & Oriolo et al., 2016). Genetic variations also appear to be crucial in the interaction with immune to brain signalling to exacerbate risk to major depressive disorder. Genetic variability in the inflammatory cytokines genes can influences inflammatory activity (Lotrich, 2015). For example, in a 2 year prospective study of a community sample of 521 old people, the associations between physical disorders and incident depression were significant in the presence of 2 alleles related to higher proinflammatory cytokine production (TNF-α -850T and IL-8 -251A), and 1 allele related to lower anti-inflammatory cytokine production (IL-4 +33C) (Kim et al., 2013). Furthermore, in a post-mortem study, a gene expression profiling was conducted on brain tissue samples from Brodmann Area 10 in the prefrontal cortex, a region involved in reward-related behavior. Fourteen psychotropic drug-free persons with a history of depression were compared to healthy controls matched for age, gender, and post-mortem interval. Gene set analysis evidenced up-regulation of a variety of pro- and anti-inflammatory cytokines (such IL-1α, IFN-γ and IL-10), suggesting local inflammatory stress in the post-mortem brain tissue samples of patients with history of major depression (Shelton et al., 2011). In the systematic review of Machado & Oriolo mentioned above (2016), the presence of specific functional genetic variations in metabolism of polyunsaturated fatty acids, monoamine neurotransmission, neurotrophic factors and glucocorticoid axis was associated with an increase risk to develop IFN-α induced depression (Machado & Oriolo et al., 2016).

On the other side, factors implicated in resilience to the inflammatory-mood pathway are still poorly understood (Rosenblat et al., 2014). Nevertheless, emerging data on animal models have revealed that T-cells may have a protect role against stress and depression, reducing inflammation and supporting neuronal integrity (Kim et al., 2012; Lewitus, Cohen, & Schwartz, 2008).

1.7 Chronic hepatitis-C: a systematic disease

Hepatitis C virus infection is a major public health problem, with an estimated annual incidence of between 3 and 4 million cases and a worldwide prevalence of 2.8% (World Health Organization, 2017). HCV is a positive single stranded RNA virus that belongs to the Flavivirid family. HCV genome encodes a single large polyprotein which is cleaved co-translationally by viral and cellular proteases to produce three structural proteins (nucleocapsid, E1, and E2), the ion channel protein p7, and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Bartenschlager, Lohmann, & Penin, 2013). Each of these proteins coordinates processes of the HCV life cycle, and confers viral tropism to several cells and tissues, thus, such proteins represent valuable targets for antiviral therapies (Götte & Feld, 2016). Based on genome sequence diversity, HCV has been classified into seven main genotypes and 67 subtypes (Smith et al., 2014).

The major modes of transmission are parenteral via intravenous illicit drug use, blood transfusion or organ transplant, whereas intranasal drug use and unhygienically administered tattoos also confer high risk. Sexual and household contact confer low risk, whereas perinatal transmission is still poorly understood, occurring in 2–8% of infected mothers (Prasad & Honegger, 2013; Wang, D'Souza, & Jacobson, 2016).

HCV infection is usually asymptomatic and often unrecognized by the host, and approximately 20–40% of patients clear the virus spontaneously (Hoofnagle, 2002; Polo &

Laufer, 2017). On the counterpart, between 50% and 80% of infected individuals develop CHC, which may progress to cirrhosis and hepatocellular carcinoma. Chronic infection is indicated by persistent viremia after 12– 24 weeks of infection (Wang et al., 2016), and ethnicity, gender, and age at time of infection have been related to chronicity (Hoofnagle, 2002). Reported rates of progression to cirrhosis have been variable, with a recent large systematic review estimating the prevalence of cirrhosis after 20 years of infection to be about 16% (Thein, Yi, Dore et al., 2008). In those patients with cirrhosis, hepatocellular cancer is the most common complication and the main cause of death (44%) (Sangiovanni et al., 2006). Furthermore, end-stage liver disease due to HCV infection represents the main cause of liver transplantation in the Europe, United States of America and Australia (Polo & Laufer, 2017). Diagnosis can be obtained via serum RNA testing as early as 2 weeks after infection and later by serum antibody (Wang et al., 2016).

Today, because of its extra-hepatic manifestations, CHC is increasingly considered a systemic disease (Medina, García-Buey, & Moreno-Otero, 2004) (See table 3 for details), and this notion is further strengthened by accumulating evidence for HCV entry and replication in all major cellular systems of the body (Adinolfi et al., 2015). In a prospective cohort study performed by Cacoub and co-workers, it was reported that up to 74% of chronic HCV patients suffer from at least one extrahepatic manifestation (Cacoub et al., 1999). In addition, Lee and colleagues demonstrated in a recent work that HCV seropositive patients had increased mortality risks due to extrahepatic manifestations, with a hazard ratio of 1.35 (95%CI: 1.15-1.57) (Lee et al., 2012). Interestingly, it has been estimated that the total annual direct medical cost associated with HCV extrahepatic manifestations was around 2.17 billion euro (€), with a per-HCV-patient cost ranging from €899 to €1647 annually (Cacoub et al., 2018).

Specifically, CHC has been associated with insulin resistance and type-2 diabetes, lymphoproliferative disorders, including mixed cryoglobulinemia and B-cell non-Hodgkin's lymphoma, and with autoimmune diseases, especially Sjögren's syndrome, rheumatoid arthritis and systemic lupus erythematosus (Flores-Chávez, Carrion, Forns et al., 2017; Nagao, Kawasaki, & Sata, 2008). Moreover, it has been shown that HCV may affect the kidney, thyroid, eye, gut, and cardiovascular system (Féray, 2012; Johnson et al., 1993; Zampino et al., 2013). Finally, experimental, virologic, and clinical evidence have demonstrated a close association between HCV infection and neuropsychiatric symptoms and diseases, issue that will be developed in the next paragraph.

Table 3. **Extrahepatic manifestation of Chronic Hepatitis C**

Affected system	**Disease**
Cardiovascular	Coronary artery disease
	Carotid atherosclerosis
	Pericarditis
	Congestive heart failure
Endocrine	Insulin Resistance
	Diabetes type II
Immune	Monoclonal gammopathies
	B-cell lymphoproliferative disease
	Autoantibodies
Dermatologic	Purpura
	Lichen planus
	Psoriasis
	Polyarthritis nodosa
Musculoskeletal	Fatigue
	Arthralgia
	Polyarthritis
Nervous	Cognitive impairment
	Depression
	Multiple mononeuropathy
Renal	Glomerulonephritis
	Membranous nephropathy
	Renal insufficiency
Respiratory	Subclinical alveolitis
	Pulmonary intra-alveolar haemorrhages

1.8 Mood, inflammation and chronic hepatitis-c

Around 50% of infected individuals may develop neurological or psychiatric disorders, which are independent of the severity of the liver disease (Adinolfi et al., 2015). Among psychiatric and somatic symptoms, up to 80% of patients can suffer from fatigue, (Fletcher et al., 2012) and up to 50% present depressive symptoms, anxiety and weakness (Saunders, 2008), causing impairment in social and occupational functioning as well as reduction in quality of life (Adinolfi et al., 2015; Poynard et al., 2002; Yarlott et al., 2017). Moreover, HCV-infected individuals often complain of "brain fog," which specifically includes forgetfulness and difficulty concentrating, in addition to fatigue, malaise and anhedonia (Forton, Taylor-Robinson, & Thomas, 2003). In fact, mild cognitive impairment in CHC patients has been reported in many studies, with slower psychomotor speed, alteration in working memory and deficits in attention and concentration being the most commonly observed features (Forton et al., 2002; Hilsabeck, Hassanein et al., 2003; Hinkin, Castellon, Levine et al., 2008). It has been estimated that around one third of infected patients may show neurocognitive disability, even in the absence of cirrhosis and encephalopathy (Hilsabeck, Perry, & Hassanein, 2002; Kramer et al., 2002; Senzolo et al., 2011). However, such estimation could had been exaggerated, to the extent that no clear and direct attribution to HCV infection can be made, as other factors may contribute to cognitive impairment in such patients.

The high prevalence of neuropsychiatric symptoms reported in CHC is supported by several lines of converging evidence pointing to a neuropathogenic effect of the virus (Senzolo et al., 2011). Several studies suggest that HCV can replicate in monocytes/macrophages, which are able to cross the blood-brain barrier and access to the central nervous system in a process known as "Trojan horse" mechanism (Laskus et al., 2005;

Laskus et al., 2002; Wilkinson et al., 2009). Thus, the brain may serve as an important reservoir for subsequent viral replication, as indicated by the detection of specific HCV-RNA strands in post-mortem brains of CHC patients (Vargas et al., 2002). Furthermore, the evidence of viral quasi-species diversity between the central nervous system and liver, supports the notion of independent viral evolution (Fletcher et al., 2012), rendering the central nervous system a potential source of relapse after anti-viral therapy (Yarlott et al., 2017). Thus, it has been suggested that the infected microglial cells may increase secretion of pro-inflammatory cytokines such as TNF- α or interleukins (Wilkinson, Radkowski, Eschbacher et al., 2010), which have been associated with the physiopathology of depressive and cognitive symptoms (Wohleb et al., 2016).

Beside evidence of HCV direct neuroinvasion, the systemic chronic immune system activation that characterizes CHC may also account for the pathogenesis of neuropsychiatric symptoms (D'Mello & Swain, 2014; Hartling et al., 2016). Thought a large amount of literature have focused on IFN-α induced depression, little evidence has been also produced regarding systemic inflammation effects (Yarlott et al., 2017). CHC has been related to low grade systemic inflammation which may account for some extra-hepatic manifestations (Rosenthal & Cacoub, 2015), wherein increased proinflammatory cytokines gene-transcription from peripheral blood mononuclear cells has been correlated to depressive symptoms in CHC patients (Pawlowski et al., 2014). As mentioned in previous paragraphs, it is well known that pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α, may interact with several neurobiological pathways, interfering with neurotransmission and neurotrophic mechanisms (Furtado & Katzman, 2015). For example, the activation of the IDO enzyme secondary to neuroinflammation and the consequent induction of the TRYCATs pathways have been related to the depletion of serotonin levels and the augmentation of glutamate

neurotoxicity through an increase in quinolinic acid (Morris et al., 2015). In the same way, immune activation in central nervous system has been related to a reduced activity of tetra-hydro biopterin, an enzyme involved in dopamine synthesis and whose disruption has been associated with decreased dopamine levels (Felger et al., 2013). (see paragraph 1.5.3.1,2).

In the last 2 decades, neuroimaging has been used in HCV-infected patients in the search for in vivo evidence of central nervous system alterations (Yarlott et al., 2017). Structural and functional techniques have been used to define anatomical alterations, metabolic or neurotransmission abnormalities, and connectivity disruption in CHC. Nevertheless, the majority of these studies centre on demonstrating the neuropsychiatric effects of therapy with IFN-α, which was the main molecule used to treat CHC (Capuron et al., 2012; Capuron, Neurauter, et al., 2003; Machado et al., 2016) before the development of the new DAAs, and had been associated with MDD, malaise and fatigue (Udina et al., 2012). Few neuroimaging studies have focused on chronic HCV infection in patients without treatment, and even more less studies have selected a healthy control group. Moreover, the attempts to find correlation between neuroimaging findings and cognitive or psychiatric symptoms have yielded inconclusive results (Kharabian Masouleh et al., 2016).

In synthesis, as sickness behavior and depression share clinical phenomenology, inflammatory pathways and brain functional changes, it has been hypothesized that prolonged and dysregulated sickness behavior may contribute to the development of major depressive disorder in vulnerable patients (Capuron & Castanon, 2012; Rosenblat et al., 2014). Nevertheless, there is little information on neurobiological and neuroanatomical links between chronic inflammatory condition and prolonged sickness behavior in subjects without depression or other current psychiatric diagnosis. Several situations in which the

initial noxious stimulus could not be removed and in which sickness behavior may be prolonged and dysfunctional have been identified. This would be the case of chronic infections (i.e. HIV infection, or CHC), auto-immune disorders (i.e. rheumatoid arthritis, inflammatory bowel disease) or chronic inflammatory conditions (i.e. cancer, diabetes, obesity), which have been often related to increased prevalence of major depressive disorder (Liu et al., 2017). A disruption in the mechanisms that regulate inflammation during the sickness behavior (Dantzer et al., 2008), beside a basic higher pro-inflammatory activity, plus independent risk factors for mood disorders may together collaborate in the emergence of major depression. Of course, this has important implications in treatment strategy. In a recent clinical trial that test the monoclonal antibody against TNF- α (infliximab) to treat MDD, a post-hoc analysis revealed that CRP baseline concentration > 5mg/L was the cut-off point at which patients treated with infliximab experience a greater response than placebo, with an effect size similar to that observed with standard antidepressant (Raison et al., 2013).

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

1. HYPOTHESIS

1.1 General hypothesis

We hypothesize that patients with CHC compared to healthy controls will present changes in brain morphology, activity, connectivity and metabolism in areas linked to sickness behavior and depression, and that such alterations will be related to mood symptoms and inflammatory markers.

1.2 Specific Hypothesis

1. CHC patients without decompensated cirrhosis, substance use disorder or other neuropsychiatric disorders, will present neuroimaging alterations compared to healthy controls, which would be correlated with neuropsychiatric symptoms.

1.1. Neuroimaging studies will show differences in brain morphology, activity, connectivity and metabolism, of patients with CHC when compared to healthy controls

1.2. Neuropsychiatric symptoms such as fatigue, depression and cognitive impairment will be associated with neuroimaging changes.

2. Patients with CHC will have a pattern of brain functional connectivity in resting state related to sickness behavior symptoms and inflammatory markers, compared to healthy subjects.

2.1. Patients with no treated CHC will show increased symptoms of depression, irritability, fatigue or perceived stress compared to healthy subjects.

2.2. Patients with no treated CHC will present increased serum levels of pro-inflammatory and decreased levels of anti-inflammatory markers compared to healthy subjects.

2.3. Patients with no treated CHC will present increased serum levels of oxidative markers compared to healthy subjects.

2.4. Patients with no treated CHC will present brain connectivity alterations in areas associated with interoceptive integration and awareness, emotional processing and orientation of motivational state, compared to healthy subjects.

2.5. Affective symptoms, inflammatory activity and oxidative stress presented by patients with no treated CHC will be related to pattern of brain functional connectivity.

2. OBJECTIVES

2.1 Main Objective

The main objective of this thesis was to elucidate the clinical and neurobiological correlates of a prolonged sickness condition associated with chronic inflammation, investigating the effect of CHC on mood, immune system and neuroimaging alterations in those areas implicated in sickness behavior and depression.

2.2 Specific Objectives

1. To conduct a meta-analysis of the current evidence of brain neuroimaging studies in CHC treatment-naïve patients compared to healthy controls, excluding from the analyses those patients with decompensated cirrhosis, substance use disorder or other neuropsychiatric disorders to reduce confounding factors.

1.1. To summarize and to analyse the main structural and functional neuroimaging alterations observed in CHC treatment-naïve patients compared to healthy controls.

1.2. To summarize the correlation between neuroimaging alterations and neuropsychiatric symptoms, such as fatigue, depression and neurocognitive impairment.

2. To study *in vivo* the functional connectivity between cortico-striatal-limbic areas and clinical and neurobiological correlates of a prolonged sickness condition as CHC.

2.1. To compare affective symptoms as depression, anhedonia, fatigue, irritability and perceived stress between no treated CHC patients with healthy controls.

2.2. To compare serum levels of inflammatory and anti-inflammatory markers such as Interleukin-6 (IL-6), prostaglandin-E2 (PGE_2), 15d-prostaglandin-J2 (15d-PGJ_2) between no treated CHC patients with healthy controls.

2.3. To compare serum levels of oxidative stress markers as Malondialdehyde - Thiobarbituric acid reactive substances (MDA-TBARS) and anti-oxidant enzymes as glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) between no treated CHC patients with healthy controls.

2.4 To compare brain connectivity alterations in areas associated with interoceptive integration and awareness, emotional processing and orientation of motivational state, between no treated CHC patients with healthy subjects.

2.5. To study whether affective symptoms are correlated with serum levels of inflammatory, anti-inflammatory or oxidative stress markers and/or with the functional connectivity networks in no treated patients with CHC.

METHODS

3. METHODS

3.1 Study 1

Systematic review and meta-analysis: neuroimaging in hepatitis C chronic infection.

3.1.1 Sample

The first study presented is a systematic review with meta-analysis of neuroimaging research in CHC treatment naïve patients. Database search was conducted from inception up until 1st May 2017 for peer-reviewed studies on structural or functional neuroimaging assessment of CHC patients without cirrhosis, encephalopathy, substance use disorder or other psychiatric disorder, with control group. Other exclusion criteria were non-neuroimaging studies of CHC, participants under 18 years old, patients with human immunodeficiency virus (HIV) co-infection and subjects who had other neurological disorders or a history of stroke or traumatic brain injury. All patients were interviewed by using the Mini International Neuropsychiatric Interview (MINI) (Sheehan et al., 1998), and must have been checked for drug consumption. The final sample comprised 25 studies embracing different techniques, with a whole sample of 509 CHC patients (Table 4).

Table 4. **Design and sample for study 1**

STUDY	**SAMPLE**	**DESIGN**
"Systematic review with meta-analysis: neuroimaging in hepatitis C chronic infection"	25 studies that included 509 no treated CHC patients (without decompensated cirrhosis, encephalopathy, Substance use disorder or any neuropsychiatric disorder) 491 healthy controls	Systematic review with meta-analysis

3.1.2 Data Extraction

The primary measures of interest assessed were the following: for structural imaging data, global and regional volume and thickness; for diffusion tensor imaging (DTI, which measures the three-dimensional anisotropic diffusion of water molecules within tissues) fractional anisotropy, apparent diffusion coefficient and mean diffusivity; for functional imaging data, the primary measures of interest were global and regional activity in the form of cerebral blood flow, blood oxygen level dependent signal or connectivity; for perfusion weighted imaging (PWI) relative cerebral blood volume; for spectroscopy, concentration (absolute value or ratio relative to the creatine reference peak) of cerebral metabolites: [N-acetylaspartate (NAA), N-acetylaspartate-glutamate (NAAG), creatine (Cr), myoinositol (mI), choline (Ch), Phosphoryl-Choline (P-Cho), Glyceryl-phosphoryl-choline (GP-Cho), glutamine (Gln), glutamate (Glu), glutamine plus glutamate (Glx)]; for connectivity, brain activation between areas, assessed using the seed-based and decomposition-based analysis in resting state or under a paradigm; for positron emission tomography (PET), the binding potential of the biochemical marker used or the cerebral metabolic rate of glucose (CMRglc) in case of F-fluoro-deoxy-glucose-PET; and finally, for single photon emission computerized tomography (SPECT), the binding potential of the biochemical marker used. The secondary outcomes were the correlation of these measures with clinical variables (viral genotype, viremia, degree of hepatic fibrosis, depression, fatigue and cognitive domains).

3.1.3 Data Synthesis

The overall methodological quality of the identified articles was tested with the Newcastle - Ottawa Quality Assessment Scale (NOS). A qualitative analysis was conducted for the primary and secondary outcomes, considering the different neuroimaging

techniques, and a meta-analysis was conducted when possible. The mean difference (MD) and 95% confidence interval (CI) were used to compare main outcomes in different brain areas of CHC patients versus healthy controls. The random effect model was selected a priori for all analyses, since heterogeneity was assumed to be present across studies and in order to avoid bias in estimates the parameters of interest. The fixed effect model was subsequently performed as sensitivity test. According to guidelines, we reported only the random effect model. If the overall result differed from the fixed effect model, we reported both results. Tau2, I2 and the chi-squared test statistics were calculated to assess heterogeneity. Statistical analyses were performed using SPSS (version 20.0 for Windows; SPSS, Inc; Chicago, Illinois) and Review Manager (RevMan, Version 5.3, Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014).

3.2 Study 2

Association of chronic inflammation and perceived stress with abnormal functional connectivity in brain areas involved with interoception in hepatitis C patients.

3.2.1 Sample

In the second study, patients were recruited between 2014 and 2016 at the Liver Unit of the Hospital Clinic and from the Hepatology Unit of Parc de Salut Mar, both in Barcelona. Healthy controls were recruited from both personnel hospital and from students of University of Barcelona. The exclusion criteria for the study were as follows: unable to understand the Catalan or Spanish languages, the presence of concomitant liver disease, decompensate cirrhosis or hepatocarcinoma, HIV co-infection, any neurological disease, current drug or alcohol use disorder, any psychiatric disorder diagnosis with a relapse within

a 24-week period before starting treatment, which was ruled out through structured interview using Mini-international neuropsychiatric interview assessment (MINI) (Sheehan et al., 1998) and no metallic prothesis or pacemaker carriers. After obtaining informed consent, all patients were screened for exclusion criteria. Healthy controls were also recruited, matched for sex, age and laterality. Exclusion criteria required that participants were free of any acute or chronic infection (i.e. HCV, HIV) or any chronic disease or inflammatory condition (e.g. diabetes, asthma, obesity, cancer), as well as auto-immune disease (e.g. rheumatoid arthritis). They were also excluded in case of presenting uncontrolled current medical disease, any neurologic disorders or in case of receiving anti-inflammatory treatment (e.g. corticoids, statins, non-steroidal anti-inflammatory drugs). To be included, current psychiatric disease, drug or alcohol use disorder had to be ruled out, through structured interview using MINI assessment which was conducted by senior psychiatrist (see Table 5).

Table 5. **Design and sample for study 2**

STUDY	**SAMPLE**	**DESIGN**
"Association of chronic inflammation and perceived stress with abnormal functional connectivity in brain areas involved with interoception in hepatitis C patients"	35 no treated CHC patients (without decompensated cirrhosis, encephalopathy, Substance use disorder or any neuropsychiatric disorder) 30 healthy controls	Cross-sectional Case-control

3.2.2 Clinical evaluation

All patients were interviewed by using the Mini International Neuropsychiatric Interview (MINI) (Sheehan et al., 1998), in order to assess present or past psychiatric

disorders, as it was an exclusion criteria. In the second study, the Spanish validated version of the physical health questionnaire for depression (PHQ-9) (Diez-Quevedo, Rangil, Sanchez-Planell, Kroenke, & Spitzer, 2001) was used to evaluate subthreshold symptoms of depression. PHQ is a brief instrument to cover a wide range of psychopathology and to diagnose specific disorders, considering that the items correspond to the symptom criteria for each disorder as outlined in the DSM-IV-TR (Kroenke, Spitzer, Williams, & Löwe, 2010). Furthermore, PHQ has been validated across a variety of medical conditions in primary care setting, included CHC patients (R. Navinés et al., 2012; Spitzer, Kroenke, Williams, & Group, 1999). The PHQ-9 has nine items with four response options ("Not at all", "Several days", "More than half the days" and "Nearly every day") rated from 0 to 3. It can be used as a continuous measure with scores ranging from 0 to 27 and cut-points of 5, 10, 15 and 20 representing mild, moderately, moderately severe and severe levels of depressive symptoms, as well as a diagnostic algorithm for MDD if the patients point in ≥ 5 of the 9 symptoms as present "more than half the days" and one the symptoms is depressed mood or anhedonia (Kroenke et al., 2010). If two, three, or four depressive symptoms have been present at least "more than half the days" in the past two weeks, so Other Depressive Disorder (ODD) is diagnosed (R. Navinés et al., 2012). The intensity of fatigue and irritability was assessed through a visual analogue scale (i-VAS-i and VAS-f) (Folstein & Luria, 1973), that is a visual tool in which the patient is asked to place an arrow on a line that reads left to right from, ranging from 0 to 100 mm (0 = no fatigue or no irritability and 100 = severe fatigue imaginable or extreme irritability). VAS is a well-validated scale apt in discriminating illness severity that can be rapidly self-administered (Killgore, 1999). We used irritability and fatigue scores, as they reflect components of sickness behavior and depression that were not exhaustively covered by the PHQ-9. The perceived stress scale (PSS) was applied to

measures the degree to which situations in one's life are appraised as stressful. This scale is easy to score and provides valuable additional information about the relationship between perceived stress and pathology (Sheldon Cohen, 1983). It includes 14 items scored on a 5-point Likert scale (0-4), it is easy to score, and it can be administered in few minutes. The score range from 0 to 56 and there are no cut-offs for the classification for "high, medium or low" stress, as PSS is not diagnostic (Remor, 2006). It has been used in different studies (He, Gao, Li, & Zhao, 2014; Nagano, Nagase, Sudo, & Kubo, 2004; Vere, Streba, Streba, Ionescu, & Sima, 2009) addressing stress in liver diseases, considering that evidence has linked stress to the initiation, course and outcome of liver disease (Vere et al., 2009). See Table 6 for details.

Table 6. **Clinical evaluation scales**

SCALE	PSYCHOPATOLOGY DOMAIN
Mini International Neuropsychiatric Interview (MINI)	Diagnosis of mental health illness, following DSM-IV-TR criteria
Physical health questionnaire for depression (PHQ-9)	Depressive symptoms and anhedonia
Perceived stress scale (PSS)	Symptoms of subjective stress
Visual analogic scale for fatigue (VAS-f)	Intensity of fatigue
Visual analogic scale for irritability (VAS-i)	Intensity of irritability

3.2.3 Biochemical assessment

In the study two, the blood samples of patients and healthy subjects were obtained at the same time of the behavioral assessment for the measurement of serum concentration of inflammatory markers. Collected samples were centrifuged (10 min, 1000g, 4°C) after clotting and sera were stored at -80°C until the assays. Enzyme-Linked Immunosorbent Assay (ELISA) technique with spectrocromography was used to identify and quantify the

immunological (IL-6, PGE_2, $15dPGJ_2$) and oxidative (MDA-TBARS, SOD, CAT, GPx) biomarkers. The spectrophotometry analysis was conducted using the ELISA spectrophotometer Synergy 2 (BioTek®, USA) together with the analysis software Gen5 Data (BioTek®, USA). In Table 7 a summary of the assessed biomarkers is provide.

Table 7. **Biological markers**

Molecule	**Marker**
Inteleukin-6 (IL-6)	Pro-inflammatory marker
Prostaglandin-E2 (PGE_2)	Pro-inflammatory marker
d15-Prostaglandin-J2 ($d15\text{-}PGJ_2$)	Anti-inflammatory marker
Maldonildialdehyde – Thiobarbituric acid reactive substances (MDA-TBARS)	Oxidative stress products
Superoxide dismutase (SOD)	Anti-oxidant enzyme
Glutathione peroxidase (GPx)	Anti-oxidant enzyme
Catalase (CAT)	Anti-oxidant enzyme

3.2.4 Functional Magnetic Resonance Image and Connectivity Analysis

Images were obtained using a 1.5 T Signa Excite system (General Electric, Milwaukee, WI, USA) equipped with an eight-channel phased-array head coil and single-shot echoplanar imaging (EPI) software. Imaging data were processed in a Microsoft Windows platform using Statistical Parametric Mapping software (SPM8; The Wellcome Department of Imaging Neuroscience, London, UK, http://www.fil.ion.ucl.ac.uk/spm/) running on MATLAB (The MathWorks Inc., Natick, MA, USA). Specific procedures to control for the effects of head motion were adopted (Pujol et al., 2014). Resting-state functional connectivity was assessed using

a region-of-interest (seed)-based approach. Selected Regions of interests (ROIs) for both studies were anterior insula, dorsal and ventral putamen and sgACC (see Table 8). More details about methods of image acquisition and processing, functional connectivity analysis and subthreshold criteria are exposed in the method section of the article (see "Papers" section of this thesis).

Table 8. **Region of interests (ROIs)**

ROIs	Coordinates
Anterior Insula (bilaterally)	x = ± 38, y = 25, z = 5
Ventral Putamen (bilaterally)	x = ±20, y = 12, z = -3
Dorsal Putamen (bilaterally)	x = ± 28, y = 1, z = 3
Subgenual anterior cingulate cortex	x = 8, y = 17, z = -9

3.2.5 Statistical analysis

Characteristics of the study sample were summarized using the mean and standard deviation (SD) for continuous variables and percentages for categorical variables. Sociodemographic features of the participants were compared between the groups using two-sample t-tests for continuous variables and chi-square tests for categorical variables. Behavioral assessment scores (PSS, PHQ-9, and VAS scores) were compared between the groups using multivariate analysis, controlling for age, sex and tobacco use, to evaluate possible interactions of these factors. A Shapiro-Wilk test of normality was performed to determine the distribution of the biological marker variables (IL-6, PGE2, 15d-PGJ2, TBARS,

GPx, SOD and CAT). Measurements that had > 3 SD above or below the mean were considered outliers and excluded from the analyses. Biomarker serum levels were log transformed if they were not normally distributed. Univariate analysis for independent samples was conducted (t-test) to compare values between the groups. Multivariate analyses controlling for age, sex and tobacco use were also performed. Finally, a multiple regression analysis was performed to assess the combined contribution of inflammatory markers and behavior to functional connectivity measurements in the patient group. Functional connectivity measurements were included as the dependent variable and the potential predictors were serum PGE2 levels and PSS scores.

Statistical analyses were undertaken with SPSS version 23.0. The tests of significance were two-tailed, with the degree of significance set at $p < 0.05$.

3.2.6 Ethical aspects

The study design was approved by the ethical committee of respective hospitals, in line with ethical principles of Helsinki declaration of 2013. All subjects who participated to the study were appropriately informed and signed the informed consent before the study inclusion.

RESULTS

4. RESULTS

In the next paragraph the main results of the two studies are exposed. The detailed results are described in the papers attached in the Papers section.

4.1 Study 1 *

Systematic review and meta-analysis: neuroimaging in hepatitis C chronic infection.

In this study a systematic review and meta-analysis of neuroimaging research in CHC treatment naive patients, or patients previously treated without sustained viral response, was performed to study structural and functional brain impact of hepatitis C. The final sample comprised 25 studies (see Figure 11 for details).

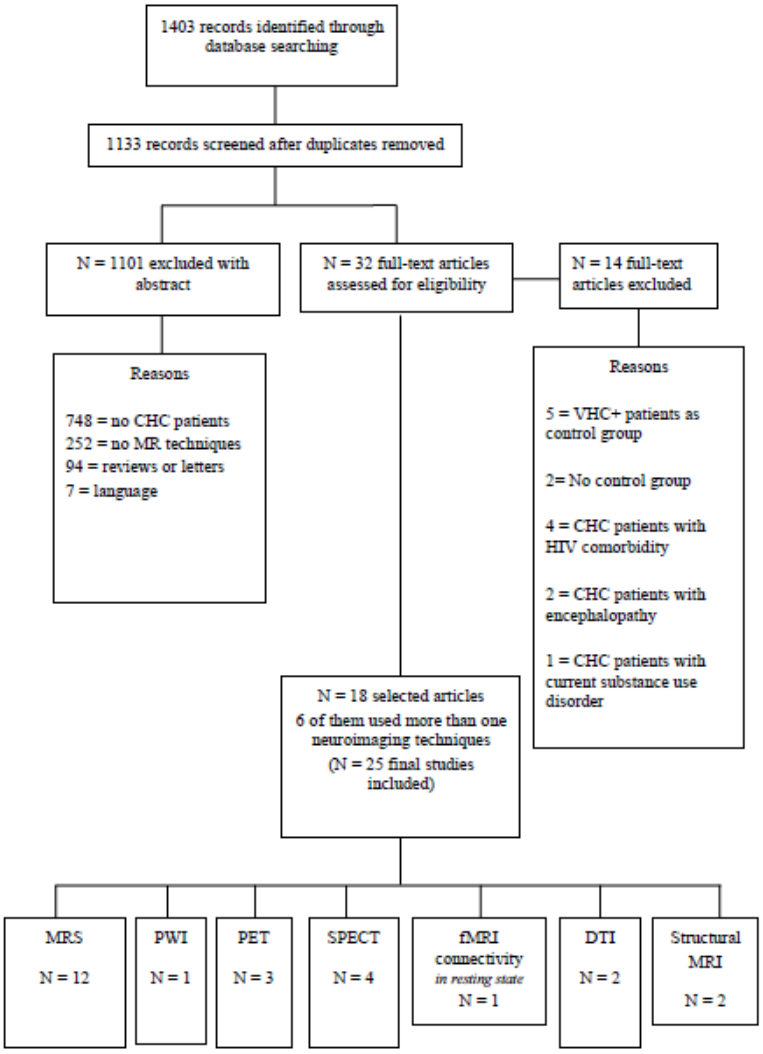

Figure 11. Flowchart showing selection of studies included in systematic review with meta-analysis.

4.1.1 Brain activation, connectivity, metabolism and structural changes

The whole sample was of 509 CHC patients, with an average age of 41.5 years old and mild liver disease. Although most of the studies (12 of 25) used proton magnetic resonance spectroscopy to investigate central nervous system metabolic changes in CHC patients, different brain regions were assessed as well as different metabolites studied, and so it was difficult to summarise the results. Meta-analyses could be performed for 5 metabolites (either peak levels or their ratio with creatine) showing increased levels of choline/ creatine

ratio, creatine and glutamate plus glutamine in basal ganglia and increased levels of choline/creatine ratio in centrum semiovale white matter of CHC patients compared with healthy controls (see Figure 12 for details). Photon emission tomography studies meta-analyses did not find significant differences in PK11195 binding potential in cortical and subcortical regions of CHC patients compared with controls. Other structural or functional neuroimaging abnormalities were found but no meta-analysis could be performed.

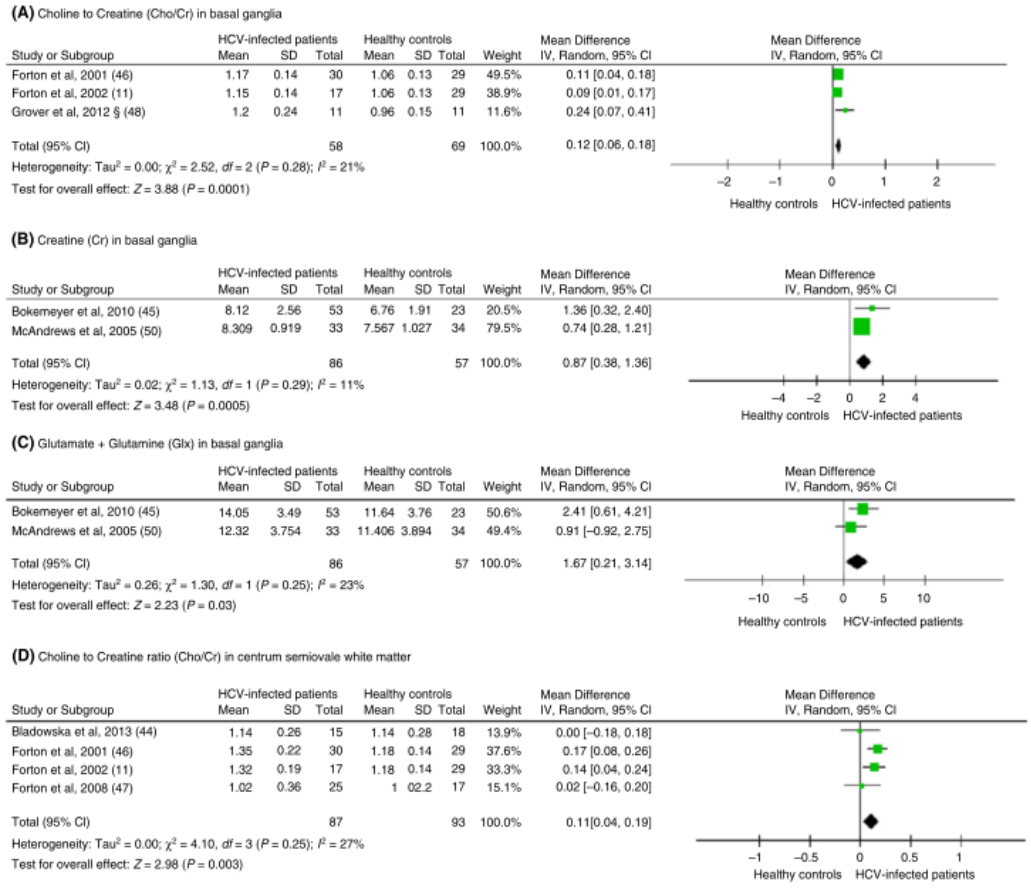

Figure 12. Forest plot of mean difference of significant MRS studies. (A-D) Comparison of metabolite peak levels in basal ganglia and centrum semiovale white matter, between HCV-infected patients and healthy controls. The numbers in parenthesis correspond to the reference list of the published paper. §, §§ indicates 2 different imaging techniques in the same study. Cho, choline; Cr, creatine; Glx, glutamine plus glutamate; HCV, hepatitis C virus; MD, mean difference; MRS, magnetic resonance spectroscopy, SD, standard deviation.

4.1.2 Psychometric and neuropsychological correlations with neuroimaging findings

Five studies identified correlations between cerebral metabolite alterations and cognitive impairment whereas in one study poorer neuropsychological performance was correlated with micros architectural changes in basal ganglia evidenced by the means of diffusion tensor imaging. PET studies identified an association between preserved cognitive function and increased PK11195 binding potential in basal ganglia as well as increased cerebral glucose metabolism in frontal, parietal, and associative cortices with significant cognitive deficits. Three studies reported correlations with high fatigue scores and metabolic alterations in different brain areas with controversial results, while only one reported correlation between high depressive symptoms and metabolic alterations. Fatigue and depression scales scores correlations were analysed in the functional connectivity study and in one PET study.

* After the publication of the results of the study, we performed an actualization of the database search from 1st May 2017 to 31st of March 2019. The aim of this actualization was to confirm that the results we found in the meta-analysis were not changed. We applied the same search criteria and the same inclusion and exclusion criteria (see Method section for details). Three new studies were initially selected (Amin et al., 2018; Kumar, Deep, Gupta, Atam, & Mohindra, 2017; McCready et al., 2018). One study was excluded as part of the sample with CHC had vasculitis (Amin et al., 2018) and another one was excluded as no exclusion of psychiatric disorder was assumed in HCV infected patients (Kumar et al., 2017), as reported in Appendix Section, Supporting information of the Paper 1, Third paragraph, (page 178). A third study was included (McCready et al., 2018), as inclusion and exclusion criteria were accomplished. Data extraction and analysis was conducted as exposed in the Method section. In appendix section, the new data were included in Table 1S and Table 3S of supporting information of Paper 1 (pages 181 and 188). The quality assessment was also reported in Table 2S (page 183).

The study of McReady et al. (2018) included 20 HCV infected patients and 26 healthy control subjects. All participants underwent a fMRI during the execution of a delay discontinuing task (DDT). A whole brain imaging analysis was performed to see whether HCV infected patients exhibit differences in brain activation during the DDT. The task allows the measurement of one's tendency to choose smaller immediate rewards over larger delayed rewards. The study showed that patients with HCV infection exhibit greater impulsive

behavior when presented with difficult choices in the DDT, and less activation in the left lateral occipital gyrus, precuneus and superior frontal gyrus. It was also observed that impulsivity was negatively related to activation in the bilateral medial frontal gyrus, left insula, left precuneus, left inferior parietal lobule, and right temporal occipital gyrus, which are regions important for cognitive control. Moreover, among HCV infected patients, those with more viral load chose immediate rewards more often on hard choices relative to easy choices. In synthesis, HCV is associated with changes in brain activation consistent with reduced cognitive control.

4.2 Study 2

Association of chronic inflammation and perceived stress with abnormal functional connectivity in brain areas involved with interoception in hepatitis C patients.

In this study we observed that CHC patients perceived more subjective stress and presented increased depressive symptoms compared to healthy controls. In line with our hypothesis, increased of proinflammatory markers (i.e. IL-6 and PGE2 serum levels) and decreased of anti-inflammatory markers (i.e. 15d-PGJ2 serum levels) were found in CHC patients, compared to healthy controls. Interestingly, the antioxidant enzymatic system was activated in CHC patients compared to controls, as highlighted by the increased serum levels of SOD and CAT. In line with these results, the serum levels of MDA-TBARS, a final product of lipid peroxidation and an indicator of oxidative stress, were lower in CHC patients than in control subjects, demonstrating the correct functioning of the antioxidant system.

As we hypothesized, PSS scores positively correlated with functional connectivity between the right anterior insula and right putamen, whereas PHQ-9 scores correlated with functional connectivity between most of the seeds and the right anterior insula. Concerning inflammatory markers, we observed that PGE2 serum levels positively correlated with functional connectivity between the right anterior insula and right caudate nucleus and between the right ventral putamen and right putamen/globus pallidus. Conversely, and IL-6

serum levels negatively correlated with functional connectivity between the same brain areas (see Figure 13).

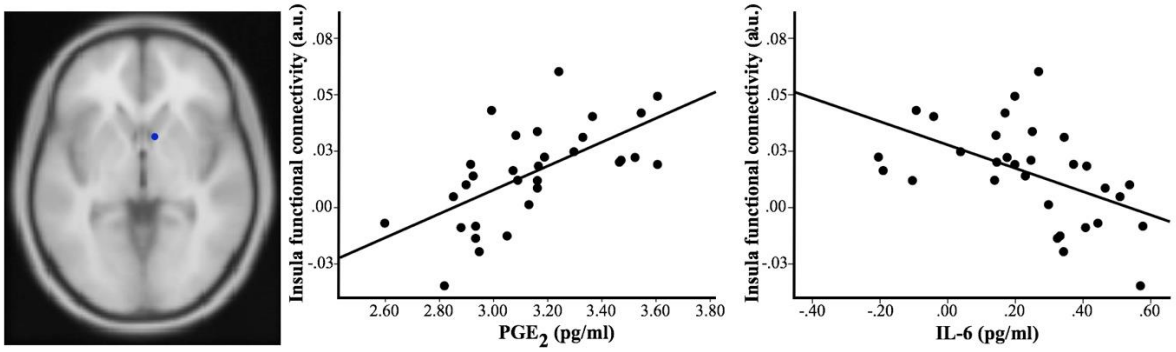

Figure 13. Plots of correlation analysis for the pro-inflammatory cytokines prostaglandin E2 (PGE_2) and interleukin-6 (IL-6) in chronic hepatitis C (CHC) patients. Functional connectivity between the anterior insula (seed map) and the right ventral striatum (caudate nucleus; blue dot in the left panel have the MNI coordinates x = 8, y = 8, z = -2) was positively associated with prostaglandin E2 (PGE_2; R = 0.628, p = 0.00015) and negatively associated with interleukin-6 (IL-6; R = -0.513, p = 0.003).

Overall, results indicated that the inflammatory markers PGE_2 and IL-6 were associated with functional connectivity changes between the insular cortices and structures in the basal ganglia and within the basal ganglia. Increased perceived stress, in turn, was associated with functional connectivity changes in regions that partially overlapped with the changes associated with the inflammatory markers. A multiple regression analysis including measures from both inflammatory markers and clinical outcomes showed that PGE_2 and PSS scores accounted for significant unique variance in the functional connectivity between the anterior insula and putamen (see Figure 14). In a stepwise approach, (1) increased PGE_2 serum levels and (2) increased PSS scores were entered into the equation, accounting for 46% of the variance in functional connectivity measurements (adjusted R square = 0.42).

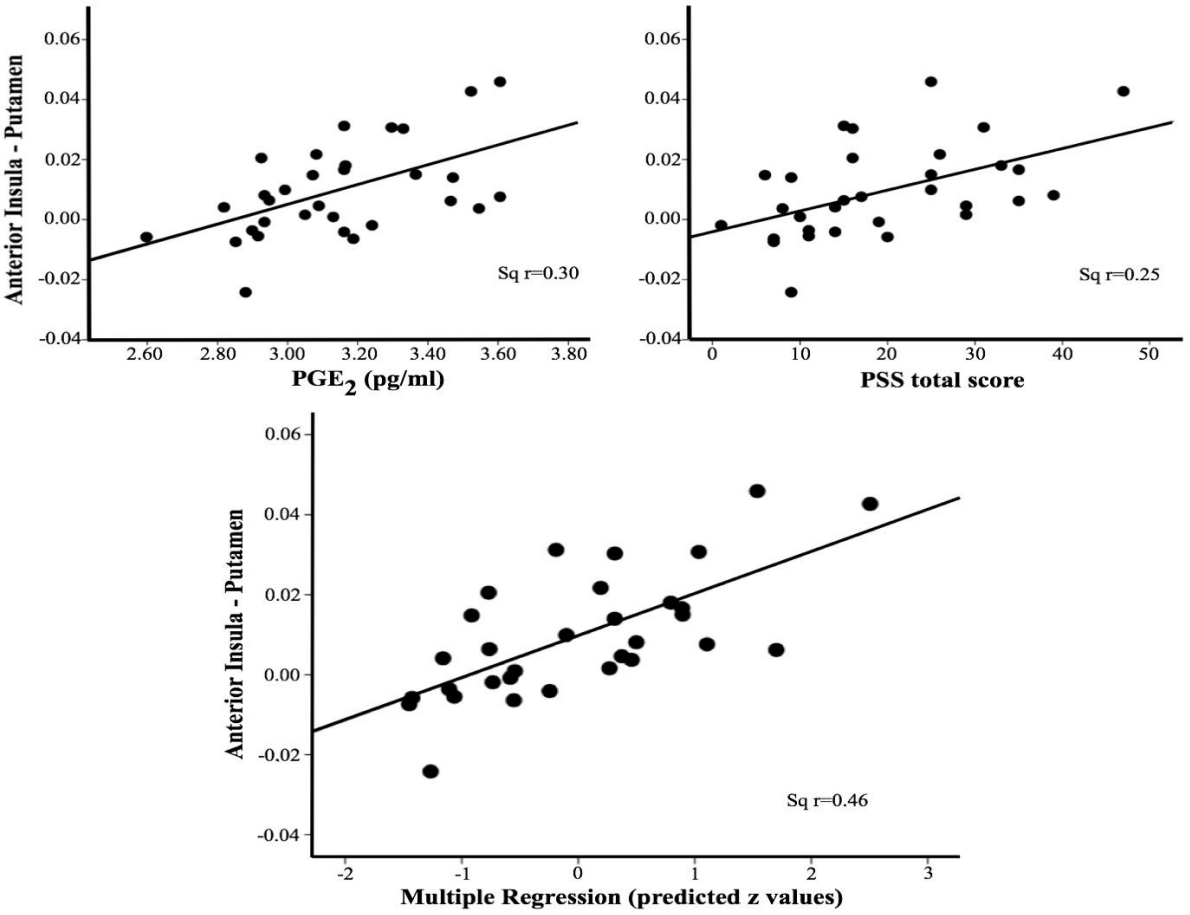

Figure 14. Plots of the correlations between functional connectivity measurements and biological and clinical variables. Functional connectivity values (y axis) indicate the correlation between the right insula (seed region) and the functionally connected region in the right putamen. Top panel: positive correlations for prostaglandin E2 (PGE_2; R = 0.300) and for perceived stress scale (PSS) scores (R = 0.250). Bottom panel: increased PGE_2 serum levels and increased PSS scores were entered into the equation in the multiple regression analysis, accounting for 46% of the variance in functional connectivity measurements (adjusted R = 0.42). All correlations were significant at $p < 0.008$.

DISCUSSION

5. DISCUSSION

The doctoral thesis presented here expands knowledge about relations between inflammation, neuropsychiatric symptoms and their correlates in brain neuroimaging in HCV infected patients. Using the CHC disease as a model of low-grade inflammation, new neurobiological and neuroanatomical links between sickness behavior and chronic inflammatory conditions have been elucidated. These findings are crucial in understanding pathophysiological mechanisms related to psychiatric diseases such as depression, and open new research perspectives centred on the development of new therapeutic targets.

The first study identified the association between HCV and neuroimaging alterations in the absence of severe liver disease or cirrhosis, substance use disorders, or other psychosocial factors. CHC is characterized by a chronic and systemic inflammatory activation (Zampino et al., 2013) which may lead to inflammation in the central nervous system, modulating neurons, astrocytes and microglia activity (Miller et al., 2013). Neuroimmune activation and cytokines may interact with virtually every pathophysiological domain relevant to neuropsychiatric symptoms, including neurotransmitter metabolism, neuroendocrine function, oxidative and nitrosative stress (O&NS) and neural plasticity (Miller et al., 2009; Raison et al., 2006). This provides an explanation for the metabolic, functional and structural neuroimaging alterations associated with CHC which have been highlighted in our systematic revision and meta-analysis. For example, increased levels of the choline/creatine ratio [choline being a marker for cell membrane synthesis and turnover (Bertholdo, Watcharakorn, & Castillo, 2013)] in the centrum semiovale white matter and in basal ganglia of CHC patients, can be considered a marker of neuroinflammation. This

alteration may reflect energy failure, membrane degradation or demyelination (Portella et al., 2011), and it has been observed in patients with other multi-systemic chronic infections such as HIV patients in the presence of macrophage infiltration (Meyerhoff et al., 1999).

Other important findings highlighted the role of microglial activation in CHC patients. We were able to identify significantly increased creatine peak levels in basal ganglia of CHC patients compared to healthy controls in the meta-analysis of proton magnetic resonance spectroscopy studies. Creatine is a marker of the energetic system and intracellular metabolism, and it is considered a glial marker since its concentration is much higher in glial cells and grey matter than in neurons and white matter (Bertholdo et al., 2013). On the other hand, we did not find significant increased levels of the myoinositol absolute peak in basal ganglia, nor increased levels of myoinositol/creatine in centrum semiovale white matter of CHC patients compared to healthy controls, as the meta-analysis was performed, though such results must be interpreted with caution. Due to its osmolar properties, myoinositol participates in the volume regulation of astrocytes, and high myoinositol levels reflect gliosis, astrocytosis and increased cell membrane turnover (Bertholdo et al., 2013; Soares & Law, 2009). Moreover, the meta-analysis of two PET studies (Grover et al., 2012; Pflugrad et al., 2016) did not show significant differences in glial activation in several brain areas of HCV-infected patients. Nevertheless, important observations are highlighted in the single studies. Grover et al. observed increased PK11195 binding potential in caudate nucleus and thalamus of CHC patients versus controls. Increased PK11195 binding potential in caudate nucleus and thalamus were observed in CHC patients versus controls (Grover et al., 2012). Up-regulation in binding potential of PK11195 is a selective marker of activated glia (Banati, 2002) and it has been used as a biomarker of inflammation in Huntington disease or cerebral HIV infection (Hammoud et al., 2005; Tai et al., 2007). On the other hand,

the study of Pflugrad et al. (2016) found increased PK11195 binding potential in caudate, thalamus and putamen only in those CHC patients with attention impairment, compared to controls (Pflugrad et al., 2016).

Other data illustrated the impact of HCV infection on serotoninergic and dopaminergic neurotransmission. The results of two SPECT studies selected in the review showed a reduction in the midbrain serotoninergic and striatal dopaminergic systems in CHC patients who exhibit worse cognitive impairment as well as fatigue and depressive symptoms (Weissenborn et al., 2006). In fact, alterations in serotonin transmission between raphe nucleus and the prefrontal cortex, amygdala and hippocampus have been associated with mood symptoms and sleep disturbances (Pakalnis, Splaingard, Splaingard et al., 2009), whereas dopamine subcortical circuits have been associated with cognitive performance and flexibility, as well as the generation of pleasure (Volkow et al., 2000; Wang & Pereira, 2017). Moreover, data from a randomized clinical trial demonstrated a remission of fatigue symptoms in CHC patients treated with ondansetron, a serotonin receptor 3 antagonist, suggesting an impact of HCV on neurotransmission functions (Piche et al., 2005).

Finally, the meta-analysis showed increased glutamate plus glutamine levels in the basal ganglia of HCV-infected patients, supporting the role of oxidative stress in CHC. The increase of glutamate plus glutamine in the basal ganglia is believed to derive from the increase in glutamate (Bokemeyer et al., 2010; Zahr, Mayer, Rohlfing et al., 2014). Glutamate is an excitatory neurotransmitter, which can participate in the redox cycle (Soares & Law, 2009) and its accumulation in the extracellular space may trigger excessive activation of glutamatergic receptors and lead to excitotoxicity (Murphy-Royal, Dupuis, Groc et al., 2017). These results are in line with certain reports in major depressive patients, which have found

increased glutamatergic transmission that were associated with structural and functional changes in several brain structures as basal ganglia, contributing to oxidative stress (Eyre & Baune, 2012).

Considering correlations between neuropsychiatric evaluations and neuroimaging findings supporting neuroinflammation, glial activation or oxidative stress markers, no clear data were identified, and the absence of a consistent relationship deserve mention. For example, Grover et al. (2012) reported significantly increased myoinositol/creatine ratios in basal ganglia of CHC patients, which correlated with increased error rates in a cognitive task involving attention (Grover et al., 2012), and similar results were found in patients with major depressive disorder (Chen et al., 2009). Conversely, Bokemeyer et al. found a negative correlation between myoinositol levels in basal ganglia and centrum semiovale white matter with fatigue scores, suggesting a beneficial effect of glial activation among HCV-infected patients. In this sense, a neuroprotective role of astrocytes and microglia activation was assumed (Bokemeyer et al., 2010). Moreover, signs of increased functional connectivity in resting state in the right posterior parietal regions to parietal, temporal and occipital cortices observed in HCV patients (Kharabian Masouleh et al., 2016), have been correlated with better cognitive performance, in the domains of attention and episodic memory. Indeed, dorsal parietal regions have been related to top-down attentional domain and to episodic memory (Cabeza, Ciaramelli, Olson et al., 2008). These data suggest that the reorganization of the connectivity in the right posterior parietal lobe may be interpreted as a compensatory mechanism that provides more attentional resources. Finally, the new study included (McCready et al., 2018) showed how HCV was associated with changes in brain activation consistent with reduced cognitive control, as patients with HCV exhibited differences in brain activation during a delay-discontinuing task, a measure of impulsivity.

Taken together, the findings of the first study support the hypothesis of a direct or indirect involvement of HCV in central nervous system disturbances. Cerebral metabolite alterations and functional neuroimaging abnormalities may be considered a marker of neuroinflammation and neuron-glia and axon-myelin integrity disruption, similar to those observed in patients with major depressive disorder (Coloigner et al., 2019; Xu et al., 2016). Nevertheless, few studies included reported association between neuropsychiatric symptoms and neuroimaging alteration, with controversial results. Moreover, a lack of replicated evidence was observed.

The results of the first study prompted the design of the second study, which was focused on the identification of neuropsychiatric and inflammatory impairments in patients with CHC, and their correlation with functional neuroimaging alterations. In line with the hypotheses of this thesis, the results from the second study indicate that increased inflammation, as reflected by increased IL-6 and PGE_2 serum levels, and greater perceived stress and subclinical depressive symptoms in patients with CHC, were associated with abnormal functional connectivity in brain regions associated with interoceptive awareness, psychomotor functions and affective processing.

We observed that patients with CHC perceived more stress and reported greater levels of irritability and fatigue than control subjects, as expected for patients with chronic disease. Moreover, the higher PHQ-9 scores reflected the increased subclinical depressive symptoms in CHC patients, particularly anhedonia and a reduced interest in doing things. This subtle difference was particularly suggestive, as a diagnosis of depression was an exclusion criterion in this study. Symptoms of fatigue, irritability and anhedonia have been widely described as part of sickness behavior (Dantzer, 2009). These may persist in chronic inflammatory conditions without reaching greater clinical relevance and are the most

common complaints of CHC patients (D'Mello & Swain, 2014; Huckans et al., 2014; Yarlott et al., 2017). As expected, CHC patients showed increased serum levels of inflammatory mediators, namely IL-6 and PGE_2, as well as reduced levels of the anti-inflammatory 15d-PGJ_2. These findings, in line with previous studies (Aregay et al., 2018; Senzolo et al., 2011; Shah, Ma, & Scherzer, 2015; Waris & Siddiqui, 2005), demonstrated the increased inflammatory activity in patients with CHC. IL-6 is a highly versatile pro-inflammatory cytokine with pleiotropic effects, which is secreted in response to environmental stress factors, such as infections or obesity (Castanon et al., 2014; Tanaka, Narazaki, & Kishimoto, 2014), and contributes to the development of chronic inflammatory illnesses (Baran et al., 2018). Furthermore, IL-6 is involved in several physiological functions in the central nervous system, such as neuron homeostasis. Thus, its chronic dysregulation may lead to various diseases (Rothaug, Becker-Pauly, & Rose-John, 2016; Spooren et al., 2011). Similarly, PGE_2 has been linked to the transition to and maintenance of chronic inflammation (Leonard, 2018; Narumiya, 2009) by promoting inflammation through inducing the expression of pro-inflammatory cytokines and suppressing Th2 cell differentiation and the anti-inflammatory system (Leonard, 2018). PGE_2 regulates sickness following systemic inflammation and is associated with increased body temperature, reduced food intake and changes in cognitive functions such as learning and memory (Poon et al., 2015). Inhibition of PGE_2 synthesis in mice has been reported to reduce the sickness behavior induced by LPS treatment (de Paiva et al., 2010). In line with these results, our finding of reduced levels of the anti-inflammatory 15d-PGJ_2 in CHC patients was expected, as this prostanoid is known to exert anti-inflammatory effects via its nuclear peroxisome proliferator-activated receptor-γ (PPARγ) (García-Bueno, Madrigal, Pérez-Nievas et al., 2008). This imbalance between cyclooxygenase-produced pro- and anti-inflammatory mediators has been described in

experimental models as well as in patients with psychiatric disorders (García-Álvarez et al., 2018; García-Bueno et al., 2014; Leza et al., 2015).

In addition to the increased inflammation observed in CHC patients, a new and interesting finding was the absence of oxidative stress in these subjects, which was not expected. Actually, in our meta-analysis we found increased glutamate plus glutamine levels in the basal ganglia of HCV-infected patients, supporting the role of oxidative stress in CHC. Generally, it has been demonstrated that chronic inflammation may induce excessive production of ROS and RNS, which can cause nitro-oxidative damage to proteins, lipids or nucleic acids. The resulting O&NS can cause mitochondrial dysfunction, glial activation, neuroinflammation and apoptosis in the central nervous system, and has been associated with several neuropsychiatric conditions (Linqvist, 2017). The brain is particularly vulnerable to oxidative damage due to its high oxygen use and relatively weak antioxidant defences (Ng, Berk, Dean et al., 2008). For example, increased levels of polyunsaturated lipid oxidation, namely MDA-TBARS levels, have been reported in MDD patients (Lopresti, Maker, Hood et al., 2014; Palta et al., 2014), and have been indicated as factors contributing to chronic and recurrent depression, as well as aging (Maurya & Rizvi, 2010). Nevertheless, the intrinsic antioxidant enzymatic system (i.e., SOD, CAT and GPx) may be activated in certain conditions to maintain ROS/RNS concentrations at desirable levels and to prevent O&NS (Sousa et al., 2016). In our second study, increased SOD and CAT activities were reflected by decreased levels of MDA-TBARS in CHC patients. MDA accumulation and no clear disruptions in the antioxidant systems in CHC patients indicate that the antioxidant system in CHC patients in our study was functioning and still able to manage ROS/RNS production. This is noteworthy, as O&NS may be crucial in the development of psychiatric illnesses, which was

an exclusion criterion in our study. Further longitudinal studies are needed to determine whether these changes might be used as status biomarkers in this particular clinical setting.

Our results demonstrated that differences in sub-syndromic clinical symptomatology and inflammation between the groups were reflected by brain functional changes in areas involved in interoceptive awareness, psychomotor functions and affective processing. As emerges from our first study and other literature (Fishman et al., 2008; Laskus et al., 2005), the brain may be a minor replication site for HCV, which can cross the blood brain barrier (BBB) and enter the central nervous system through infected monocytes (Thomas, Török, Forton et al., 1999). HCV can interact with the microglia, inducing its activation by increasing the production of pro-inflammatory mediators. Both studies of this thesis highlighted neuroimaging alterations in areas such as basal ganglia and limbic structures, which are sensitive to peripheral inflammation and associated with symptoms of sickness behavior. Importantly, in the literature similar results were reported for psychiatric conditions. For example, in a recent meta-analysis was observed that GABA and Glx levels were lower in the ACC of MDD patients versus healthy controls (Godfrey, Gardner, Kwon et al., 2018), whereas higher ratios of choline to creatine (Cho/Cr) in the basal ganglia was observed in MDD patients than healthy controls (Yildiz-Yesiloglu & Ankerst, 2006). Other neuroimaging studies showed decreased functional connectivity between the sgACC and the precuneus in patients with MDD compared to healthy controls (Connolly et al., 2013; Ho et al., 2014), whereas increased functional connectivity between ventral putamen and frontal operculum was found in subjects with a high risk of psychosis (Dandash et al., 2014).

Another intriguing result from the second study was the positive correlation between increased PGE_2 serum levels and increased functional connectivity of the insula with the dorsal putamen. The same brain areas were associated with perceived stress, as the insula to

dorsal putamen connectivity positively correlated with PSS scores. Due to its lipid composition, PGE_2 can directly enter the brain parenchyma, spreading through the BBB and mainly interacting with the signalling receptors EP2 and EP3 on neurons to modulate neurotransmission (Furuyashiki & Narumiya, 2011). Interestingly, these receptors are mostly expressed in brain areas implicated in emotional and behavioral control, as PGE_2 has been reported to be involved in the mediation of behavioral response to circulating cytokines (Zhang & Rivest, 1999). By contrast, IL-6 in CHC patients negatively correlated with the functional connectivity between the dorsal and ventral putamen and the caudate nucleus. These same brain areas were associated with subclinical depressive symptoms, as the dorsal and ventral putamen connectivity positively correlated with PHQ-9 scores. Although these results may appear contradictory, animal studies indicate that IL-6 in the brain may contribute to the expression of brain cytokines in response to immune stimuli (Dantzer et al., 2008), resulting in several effects in the central nervous system that can be both detrimental and advantageous. Importantly, the effects of IL-6 partly depend on diverse factors, such as the presence of other cytokines or growth factors in the environment, the brain region involved and the physiological state of the tissue. Moreover, low or high IL-6 concentrations can exert opposite effects (Gadient & Otten, 1997; Spooren et al., 2011), mediating both neuroprotective and neurotoxic microglial responses (Eskes, Honegger, Juillerat-Jeanneret et al., 2002; Krady et al., 2008). It should be noted that PGE_2 and IL-6 are pro-inflammatory mediators that are also produced by the microglia and neurons, their secretion being modulated by the direct neuropathogenic effects of HCV (Wilkinson et al., 2010).

Taken together, our global findings demonstrate how CHC is associated with cerebral metabolite alterations, which may be considered a marker of neuron-glia and axon-myelin integrity disruption, and how changes in peripheral inflammation can influence insula and

basal ganglia connectivity, illustrating how changes in internal bodily states can disturb neural representations, emotional states and executive functions. This is in line with current theories that postulate that emotional feeling states may arise through the perception of bodily signals, given that interoceptive and emotional processes share similar neural substrates (Critchley, 2005; Damasio, 1994; Quadt, Critchley, & Garfinkel, 2018). The insular cortex is believed to represent and integrate interoceptive signals, such as inflammatory markers, providing the basis of interoceptive and emotional awareness, that is, the experiential side of sickness behavior (Craig, 2009). Studies using acute inflammatory challenges (Boyle et al., 2019; Harrison, 2017) have demonstrated that subjective experiences of inflammation-associated symptoms derive from interoceptive signals converging on the insula (Harrison et al., 2009). Indeed, structural and functional changes in the posterior, mid or anterior insula have been associated with subjective feelings of malaise and fatigue following inflammation (Bushara et al., 2001; Farrer et al., 2003; Klein et al., 2007). Moreover, several studies have reported a posterior-to-mid-to-anterior pattern of integration of interoceptive information (Craig, 2003). For example, activation of the posterior insula is linked to the objective intensity of heat pain, whereas anterior insular activation is associated with subjective pain evaluation (Kong et al., 2006). The patients in our second study experienced increased subjective stress, which may be modulated partly by the effects of PGE_2 on the insular cortex. However, results from the multiple regression analyses indicated that increased PGE_2 serum levels and increased PSS scores independently accounted for 46% of the variance in functional connectivity measurements. The basal ganglia consist of subcortical structures involved in the integration and coordination of executive functions, reward, emotions and mood, with a specific relevance for adaptive shaping and action selection (Grace, 2012; Wichmann & De Long, 2013). Specifically, the

dorsal putamen has been associated with the control of habitual behaviors (Redgrave, Rodriguez, Smith et al., 2010; Wunderlich et al., 2012) and is believed to modulate the balance between goal-directed and habitual action control together with the insula (Hong et al., 2015). In the meta-analysis we observed increased choline/creatine ratios, glutamine plus glutamate and creatine levels in the basal ganglia of CHC patients compared to healthy controls, indicating chronic metabolic changes in the basal ganglia induced by CHC. In the second study, disruption of the dorsal putamen-insula interaction correlated with increased subjective perceived stress and reduced interest in doing things in CHC patients, which may be part of the modulation of the goal-directed versus habitual action control. Moreover, several neuroimaging studies have suggested that disrupted connectivity between the basal ganglia and putamen elicits behavioral changes following inflammatory challenges (Brydon et al., 2008; Felger & Miller, 2012). For example, PET studies revealed increased glucose metabolism in the putamen following IFN-alpha administration, which correlated positively with increased fatigue (Capuron et al., 2007; Juengling et al., 2000). Furthermore, an fMRI study revealed increased substantia nigra activity after administering the typhoid vaccine, which correlated with increased IL-6 peripheral blood concentrations and psychomotor retardation (Brydon et al., 2008).

5.1 Limitations

The studies included in this doctoral thesis had several limitations which deserve mention. In the first study, the neuroimaging techniques we selected present considerable heterogeneity. Although most of the studies used proton magnetic resonance spectroscopy to investigate central nervous system metabolic changes in CHC patients, different brain regions were assessed as well as different metabolites studied, and so it was difficult to

summarize the results. Moreover, the lack of reported data in certain cases precluded a quantitative analysis; meta-analyses could be performed for 5 metabolites (either peak levels or their ratio with creatine) in two different brain regions, and only two meta-analyses included more than two studies. With regard to other neuroimaging techniques this systematic review found a lack of replicated evidence of structural and functional brain alterations induced by HCV infection. Another limitation was the fact that all included studies were cross-sectional, which means that is impossible to establish the directions of the association; furthermore, most of them had relatively small samples. A further issue that deserves mention is the controversial results in the relations between neuroimaging alterations and cognitive outcome or severity of psychiatric symptoms. A possible explanation is that the methodological heterogeneity, the several neuroimaging techniques employed, and the wide variety of psychometric scales and neuropsychological test batteries used may limit the comparability between studies. Considering the second study, its cross-sectional design reduced the possibilities of inference. The absence of longitudinal assessment precluded predictive analysis, which could have strengthened the validity of our hypothesis. Moreover, a larger sample size would have increased the statistical power. Another limitation was that we analysed only a few types of inflammatory markers. Analysing other markers such as CRP or TNF-α could have increased the reliability of our results. However, CRP might be a less reliable marker as several reports have found reduced CRP levels in CHC patients that may be due to auto-antibody activities (Sjöwall et al., 2012) or interferences by IL-6 on CRP synthesis (Shah et al., 2015). Finally, we did not know the time elapsed between HCV infection and diagnosis in the patients we studied, making it difficult to ascertain the duration of CHC. In general, the relationship between chronic inflammation and psychiatric symptoms is less easy to identify because patients who have

such medical conditions are examined at different stages of their disease process (Dantzer, 2009). Moreover, it is even more difficult to determine the beginning of the disease in CHC patients as the source of infection is often unknown. This may result in much higher inter-individual variability.

5.2 Future directions

Our results support the hypothesis of a direct or indirect involvement of HCV in central nervous system disturbances and provide valuable information on the brain areas involved in perceived stress, fatigue and subclinical depressive symptoms during chronic inflammation, highlighting the crucial role of interoception in coordinating prolonged sickness behavior. This can offer new research perspectives in the field of hepatology and psychoneuroimmunology.

On one hand, a multimodal neuroimaging approach which embraces structural, functional and metabolic assessment of CHC patients and the inclusion of post-mortem histological study when indicated, may help to identify valid and specific brain abnormalities induced by HCV infection. On the other hand, as the HCV is directly involved in central nervous system disturbances, we can hypothesize that the brain may constitute a potential source of hepatitis C relapse after antiviral therapy. Considering the questionable blood brain barrier penetration ability of the new direct acting antivirals (DAAs) (Cuenca-Lopez, Rivero, & Rivero-Juárez, 2017), such a reservoir should be taken into account in long-term rebound or outcomes. Late viral relapses are extremely rare after successful treatment with DAAs (Banerjee & Reddy, 2016; Sarrazin et al., 2017), but little evidence has been produced to determine the effect of sustained viral response on long-term outcome (Jakobsen et al.,

2017). For this reason, long-term prospective studies of patients with sustained viral response would be helpful in identifying those metabolic and functional changes in central nervous system strictly related with HCV infection, determining long-term outcomes and eventually figuring out markers which may be associated to late viral relapse.

Other suggestive issues and research perspectives derive from the findings that chronic inflammation together with the possible effects of HCV on the central nervous system, may account for the metabolic changes in basal ganglia and disruption in the connectivity between the insula and dorsal putamen, regions that provide cortical representation of the internal state of the body, including changes in peripheral inflammation. In this sense, HCV seems to prime the brain and may account for a chronic sickness condition that is reflected by increased subjective stress perception and/or subclinical depressive symptoms (such as anhedonia), which may derive from aberrant interoceptive processing. This may represent a trigger for psychiatric illnesses in vulnerable patients or be a vulnerability factor itself, inducing a cascade of neurobiological pathways linked to mental disorders. Following these results, one future research direction could be represented by new cross-sectional studies, including a third group of patients with CHC and clinical depression. Such a design may be useful in disentangling differences in neurobiological or neuroimaging impairments between chronic sickness condition and major depression. On one hand, it would permit the recognition of vulnerability factors that may be implicated in the development of major depression in a chronic inflammatory state, such as genetic predisposition, opening perspectives of preventive interventions. On the other hand, the new insights of pathophysiological mechanisms implicated in major depression related to chronic inflammation could provide an impulse for the development of new biological therapeutic targets.

Some other questions arise from the results of this thesis. What happens to the behavioral alterations induced by CHC, after the achievement of a sustained viral response? Will we observe a complete remission of sickness behavior, and will this remission be related to a reduction of inflammatory markers and/or new changes in brain connectivity? And what happens to patients with CHC and major depressive disorder, after sustained viral response? Will we observe a persistence of depressive symptoms, and will these symptoms be associated to specific neurobiological pathways (as oxidative stress) and/or neurophysiological alterations? Which kind of treatment would be the more adequate in these patients? To answer these intriguing questions, studies with longitudinal design are needed, investigating differences in clinical, neurobiological and neuroimaging outcomes between CHC patients with major depression, CHC patients without depression and healthy controls, and among CHC patients before and after hepatitis-C virus eradication. In this line, a new suggestive hypothesis arising from our thesis, is that oxidative and nitrosative stress may represent a crucial neurobiological pathway implicated in the transition between sickness behavior and major depression. The confirmation of such a hypothesis would strengthen therapeutic strategies based on anti-oxidative and nitrosative stress.

CONCLUSIONS

6. CONCLUSIONS

1. The results of the two studies included in this doctoral thesis support the hypothesis that patients with CHC compared to healthy controls present changes in brain morphology, activity, connectivity and metabolism in areas linked to sickness behavior and depression, and that such alterations are related to mood symptoms and inflammatory markers.

2. In the first study, meta-analysis showed that patients with CHC exhibit cerebral metabolite alterations and functional neuroimaging abnormalities, which sustain the hypothesis of HCV involvement in brain disturbances. The alterations were similar to those observed in the literature in patients with major depressive disorder.

3. Central nervous system metabolic changes were mainly correlated with neurocognitive impairment and fatigue symptoms, thought controversial results were observed.

4. In the second study, a case-control study, patients with CHC showed increased symptoms of depression, irritability, fatigue or perceived stress compared to healthy subjects.

5. Patients with CHC presented increased serum levels of pro-inflammatory markers (i.e. PGE_2 and IL-6) and decreased levels of anti-inflammatory markers (i.e. 15-PGJ_2) compared to healthy subjects.

6. Patients with CHC showed a functioning antioxidant system that was still able to manage ROS/RNS production. The absence of MDA accumulation and no clear disruptions in the antioxidant systems is noteworthy, as O&NS may be crucial in the development of psychiatric illnesses, which was an exclusion criterion in our second study.

7. The clinical and inflammatory differences between CHC patients and healthy controls, were reflected by functional connectivity changes in brain areas involved in interoceptive awareness, psychomotor functions and emotional processing.

8. More specifically, patients with CHC experienced increased subjective stress, which may be modulated partly by the effects of PGE_2 on the insular cortex.

9. HCV may account for a chronic sickness condition modulated by inflammatory markers, that is reflected by increased subjective stress perception and/or subclinical depressive symptoms (such as anhedonia), which may derive from aberrant interoceptive processing.

10. In general, chronic inflammation may contribute to brain metabolism changes, which may be considered a marker of neuron-glia and axon-myelin integrity disruption, and specifically may induce prolonged activation of interoceptive pathways. In turn, this prolonged activation may promote long-standing maladaptive neurobiological and behavioral impairments implicated in the pathophysiology of depression.

11. The doctoral thesis presented here expands knowledge about relationships between inflammation, neuropsychiatric symptoms and their correlates in brain neuroimaging in HCV infected patients. Using the CHC disease as a model of low-grade inflammation, new neurobiological and neuroanatomical links between sickness behavior and chronic inflammatory conditions have been elucidated. These findings may be crucial in understanding pathophysiological mechanisms related with psychiatric diseases such as depression, and open new research perspectives centred on the development of new therapeutic targets.

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7. REFERENCES

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PAPERS

Received: 24 September 2017 | First decision: 31 October 2017 | Accepted: 11 February 2018

DOI: 10.1111/apt.14594

WILEY AP&T Alimentary Pharmacology & Therapeutics

Systematic review with meta-analysis: neuroimaging in hepatitis C chronic infection

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Funding information
Fondo de Investigación en Salud. Instituto de Salud Carlos III, Grant/Award Number: FIS: PI10/01827; Fondos FEDER, Grant/Award Number: PI15/00151; Ajuts per el support a grups de recerca (SGR), Grant/Award Number: 2014_SGR_605; Gilead Fellowship Program, Grant/Award Number: GLD17/00273.

Summary

Background: Chronic hepatitis C is considered a systemic disease because of extra-hepatic manifestations. Neuroimaging has been employed in hepatitis C virus-infected patients to find in vivo evidence of central nervous system alterations.

Aims: Systematic review and meta-analysis of neuroimaging research in chronic hepatitis C treatment naive patients, or patients previously treated without sustained viral response, to study structural and functional brain impact of hepatitis C.

Methods: Using PRISMA guidelines a database search was conducted from inception up until 1 May 2017 for peer-reviewed studies on structural or functional neuroimaging assessment of chronic hepatitis C patients without cirrhosis or encephalopathy, with control group. Meta-analyses were performed when possible.

Results: The final sample comprised 25 studies (magnetic resonance spectroscopy [N = 12], perfusion weighted imaging [N = 1], positron emission tomography [N = 3], single-photon emission computed tomography [N = 4], functional connectivity in resting state [N = 1], diffusion tensor imaging [N = 2] and structural magnetic resonance imaging [N = 2]). The whole sample was of 509 chronic hepatitis C patients, with an average age of 41.5 years old and mild liver disease. A meta-analysis of magnetic resonance spectroscopy studies showed increased levels of choline/creatine ratio (mean difference [MD] 0.12, 95% confidence interval [CI] 0.06-0.18), creatine (MD 0.85, 95% CI 0.42-1.27) and glutamate plus glutamine (MD 1.67, 95% CI 0.39-2.96) in basal ganglia and increased levels of choline/creatine ratio in centrum semiovale white matter (MD 0.13, 95% CI 0.07-0.19) in chronic hepatitis C patients compared with healthy controls. Photon emission tomography studies meta-analyses did not find significant differences in PK11195 binding potential in cortical and subcortical regions of chronic hepatitis C patients compared with controls. Correlations were observed between various neuroimaging alterations and neurocognitive impairment, fatigue and depressive symptoms in some studies.

Conclusions: Patients with chronic hepatitis C exhibit cerebral metabolite alterations and structural or functional neuroimaging abnormalities, which sustain the hypothesis of hepatitis C virus involvement in brain disturbances.

As part of AP&T's peer-review process, a technical check of this meta-analysis was performed by Dr Yuan. The Handling Editor for this article was Professor Geoffrey Dusheiko, and it was accepted for publication after full peer-review.

1 | INTRODUCTION

Hepatitis C virus (HCV) infection is a major public health problem, with an estimated annual incidence of between 3 and 4 million cases and a worldwide prevalence of 2.8%. Between 50% and 80% of infected individuals develop chronic hepatitis C (CHC), which may progress to cirrhosis and hepatocellular carcinoma.[1] Today, because of its extra-hepatic manifestations, CHC is considered a systemic disease.[2] As far as the central nervous system is concerned, around 50% of infected individuals may develop neurological or psychiatric disorders, which are independent of the severity of the liver disease.[3] Among psychiatric and somatic symptoms, up to 80% of patients can suffer from fatigue,[4] and up to 50% present depressive symptoms, anxiety and weakness,[5] causing impairment in social and occupational functioning as well as reduction in quality of life.[6,7] Moreover, HCV-infected individuals often complain of "brain fog," which specifically includes forgetfulness and difficulty concentrating, in addition to fatigue, malaise and anhedonia.[8] In fact, mild cognitive impairment in CHC patients has been reported in many studies,[9–11] with slower psychomotor speed, alteration in working memory and deficits in attention and concentration being the most commonly observed features.[9,10] It has been estimated that around one third of infected patients may show neurocognitive disability, even in the absence of cirrhosis and encephalopathy.[12–14] However, such estimation could had been exaggerated, to the extent that no clear and direct attribution to HCV infection can be made, as other factors may contribute to cognitive impairment in such patients. Several reviews[3,15,16] pointed out that confounding factors such as advanced liver disease or history of neuropsychiatric disorder or substance use disorder were not systematically assessed in studies addressing cognitive impairments in CHC patients. Furthermore, most of these studies were cross-sectional, with small samples, and HCV-infected patients were often selected from hospital populations, which may account for selection bias.[16]

The high prevalence of neuropsychiatric symptoms reported in CHC is supported by several lines of converging evidence pointing to a neuropathogenic effect of the virus.[13] Several studies suggest that HCV can replicate in monocytes/macrophages, which are able to cross the blood-brain barrier and access to the central nervous system in a process known as "Trojan horse" mechanism.[17–20] Thus, the brain may serve as an important reservoir for subsequent viral replication, as indicated by the detection of specific HCV-RNA strands in post-mortem brains of CHC patients.[21] Furthermore, the evidence of viral quasi-species diversity between the central nervous system and liver, supports the notion of independent viral evolution,[4] rendering the central nervous system a potential source of relapse after anti-viral therapy.[16] Considering the questionable blood-brain barrier penetration ability of the new direct acting antivirals (DAAs),[22] such reservoir should be taken into account in long-term rebound or outcomes. Late viral relapses are extremely rare after successful treatment with DAAs.[23,24] However, a recent exhaustive systematic review underlined the absence of evidence to determine the effect of sustained viral response on long-term outcome.[25] Regarding neurobiological consequences, it has been suggested that the infected microglial cells may increase secretion of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) or interleukins (IL),[26] which have been associated with the physiopathology of depressive and cognitive symptoms.[27]

Beside some evidence of direct neuroinvasion, the systemic chronic immune system activation that characterises CHC may also account for the pathogenesis of neuropsychiatric symptoms. It is well known that pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α, may interact with several neurobiological pathways, interfering with neurotransmission and neurotrophic mechanisms.[28] For example, the activation of the indoleamine-2,3-dioxygenase enzyme secondary to neuroinflammation and the consequent induction of the tryptophan catabolites pathways have been related to the depletion of serotonin levels and the augmentation of glutamate neurotoxicity through an increase in quinolinic acid.[29] In the same way, immune activation in central nervous system has been related to a reduced activity of tetra-hydro biopterin, an enzyme involved in dopamine synthesis and whose disruption has been associated with decreased dopamine levels.[30]

In the last 2 decades, neuroimaging has been used in HCV-infected patients in the search for in vivo evidence of these central nervous system alterations.[16] Structural and functional techniques have been used to define anatomical alterations, metabolic or neurotransmission abnormalities, and connectivity disruption in CHC. Nevertheless, the majority of these studies centre on demonstrating the neuropsychiatric effects of therapy with interferon-α (IFN-α), which was the main molecule used to treat CHC[31-33] before the development of the new DAAs, and had been associated with major depressive disorder, malaise and fatigue.[34] Few neuroimaging studies have focused on chronic HCV infection in patients without treatment, and even more less those with a healthy control group. Moreover, the samples selected are heterogeneous, especially with regard to the clinical severity of the CHC, the presence of neuropsychiatric comorbidities and the diagnosis of active substance use disorder. Finally, the attempts to find correlation between neuroimaging findings and cognitive or psychiatric symptoms have yielded inconclusive results.[35]

Thus, in order to examine the in vivo evidence of central nervous system alterations in CHC, we performed a systematic review (and meta-analysis if sufficient data were available) of studies investigating HCV chronic-infected patients (treatment naive or previously treated without achieving a sustained viral response), using neuroimaging techniques, and assessed the correlation of these alterations with neuropsychiatric symptoms.

2 | METHODS

Data for this systematic review were collected with an advanced document protocol (see supporting information) in accordance with

the *Preferred Reporting Items for Systematic Reviews and Meta-Analyses* (PRISMA) and with the *Meta-analysis of Observational Studies in Epidemiology* (MOOSE) statements.[36,37] A systematic review includes a literature search, title/abstract screening of eligible references, study selection after full-text review, data extraction and quality assessment of the studies included. All steps in the literature search, identification and selection of studies, data extraction and quality assessment were performed independently by 2 investigators, a psychiatrist and a psychologist (G.O. and E.E.). The interrater agreement κ statistic was 0.82. Disagreements were resolved through discussion, and the opinion of a third senior researcher (R.M.S.) was sought to make the final decision if the consensus was not achieved.

2.1 | Search strategy

A comprehensive, computerised literature search was conducted in MEDLINE, PsycINFO and EMBASE. We searched for the relevant studies published from the earliest available online year until May 2017, using the following key words: "hepatitis C," "chronic hepatitis C," "HCV," "CHC," "structural MRI," "structural magnetic resonance imaging," "functional MRI," "functional magnetic resonance imaging," "fMRI," "connectivity," "MRSI," "H-MRS," "spectroscopy," "DTI," "diffusion tensor imaging," "PWI," "perfusion weighted imaging," "PET," "positron emission tomography" "SPECT", "single-photon emission computerised tomography," mixed with different Boolean operators "AND" and "OR." Additional studies found in the reference lists of the articles identified were also searched. This search strategy was broadened by tracking the citations of the articles included in Google Scholar. The titles and abstracts were examined, and full-text articles of potentially relevant studies were obtained. After this, inclusion and exclusion criteria were applied, and the articles selected were included in the systematic review (see supporting information for references of excluded articles).

2.2 | Study selection

We included only studies published in peer-reviewed journals and written in English, Spanish, Italian and Dutch. To homogenise the selection and to facilitate comparisons, only structural and functional neuroimaging studies with patients with chronic HCV of both genders were included. Additional inclusion criteria were as follows: (1) for cross-sectional designs, studies including treatment naive CHC patients, or patients who had received anti-viral treatment without achieving a sustained viral response, and a healthy control group; (2) for longitudinal design, we selected basal data from studies involving CHC patients treatment naive, or who had received anti-viral treatment without achieving a sustained viral response, with a healthy control group; (3) before the inclusion in the study, patients must have been checked for drug consumption; (4) if a study in the same sample included two or more neuroimaging techniques, these were recorded as separately studies. The exclusion criteria were as follows: (1) non-neuroimaging studies of CHC; (2) participants under 18 years old; (3) patients with human immunodeficiency virus (HIV) co-infection; (4) subjects who had other neurological disorders or a history of stroke or traumatic brain injury; (5) subjects with psychiatric disorders, or alcohol dependence or substance abuse disorders other than nicotine; (6) individuals with advanced cirrhosis or encephalopathy; and (7) grey literature.

2.3 | Data extraction and main outcomes

The variables recorded for each article were as follows: authors, year of publication, socio-demographic features (gender, age, ethnicity, sample size, handedness), hepatitis C virus characteristics (HCV genotype, RNA levels, anaemia, degree of liver fibrosis, anti-viral regimens in treated patients) and exclusion criteria for neurological, psychiatric or drug use-related disorders. History of substance use and confirmation of abstinence from other drugs (if checked by urine test) were also recorded. History of psychiatric disorders and psychopathological variables (eg psychotic or depressive symptoms) were assessed, as were psychometric and neuropsychological evaluations. As regards imaging variables, technique and design, rest/active condition (for functional imaging studies) and type of cognitive task performed during functional imaging were recorded, as well as study features such as blinded design. The primary measures of interest assessed were the following: for structural imaging data, global and regional volume and thickness; for diffusion tensor imaging (DTI, which measures the 3-dimensional anisotropic diffusion of water molecules within tissues) fractional anisotropy, apparent diffusion coefficient and mean diffusivity; for functional imaging data, the primary measures of interest were global and regional activity in the form of cerebral blood flow, blood oxygen level-dependent signal or connectivity; for perfusion weighted imaging (PWI), relative cerebral blood volume; for spectroscopy, concentration (absolute value or ratio relative to the creatine reference peak) of cerebral metabolites: [N-acetylaspartate (NAA), N-acetylaspartate-glutamate (NAAG), creatine (Cr), myoinositol (mI), choline (Ch), phosphoryl-choline (P-Cho), glyceryl-phosphoryl-choline (GP-Cho), glutamine (Gln), glutamate (Glu), glutamine plus glutamate (Glx)]; for connectivity, brain activation between areas, assessed using the seed-based and decomposition-based analysis in resting state or under a paradigm; for positron emission tomography (PET), the binding potential of the biochemical marker used or the cerebral metabolic rate of glucose (CMR_{glc}) in case of F-fluoro-deoxy-glucose-PET; and finally, for single-photon emission computerised tomography (SPECT), the binding potential of the biochemical marker used. The secondary outcomes were the correlation of these measures with clinical variables (viral genotype, viremia, degree of hepatic fibrosis, depression, fatigue and cognitive domains). We recorded the statistically significant results of each outcome variable and noted whether a multiple comparison correction was performed to prevent a bias towards false positives.

2.4 | Quality assessment

The overall methodological quality of the identified articles was tested with the Newcastle-Ottawa Quality Assessment Scale (NOS), a scale recommended by the Cochrane collaboration for quality assessment of observational studies.[38] For this review, we considered the NOS subscale that evaluates case-control studies which consisted in 9 items grouped in 3 domains of selection, comparability and exposure. Scores may vary from 0 to 9, with higher scores indicated better methodological quality.

2.5 | Data synthesis

As stated in paragraph 2.3, the primary outcome varied according to the neuroimaging technique used. A qualitative analysis was conducted for the primary and secondary outcomes of each group. A meta-analysis was conducted for those results obtained from spectroscopy and PET-PK11195 technique. In these cases, the mean difference (MD) and 95% confidence interval (CI) were used to compare the concentration of brain metabolites and the PK11195 binding potential in different brain areas of chronic hepatitis C patients versus healthy controls. The random effect model was selected a priori for all analyses, since heterogeneity was assumed to be present across studies and in order to avoid bias in estimates the parameters of interest. The fixed effect model was subsequently performed as sensitivity test. According to guidelines,[39] we reported only the random effect model. If the overall result differed from the fixed effect model, we reported both results. Tau^2, I^2 and the chi-squared test statistics were calculated to assess heterogeneity. When I^2 was 0% to 30%, heterogeneity was considered unimportant, between 30% and 60% moderate and more than 60% substantial.[40] Publication bias of this group of articles was examined in a funnel plot of MD against its standard error, and the Begg's test was used to test funnel plot's asymmetry. Nevertheless, such analysis was not possible because of the poor number of studies included in the meta-analyses (less than 10).[22,39] Statistical analyses were performed using SPSS (version 20.0 for Windows; SPSS, Inc, Chicago, IL, USA) and Review Manager (RevMan, Version 5.3, Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014).

3 | RESULTS

3.1 | Study selection

Of 1403 records, 270 were removed after screened due to duplication, and 1101 were excluded after title/abstract review because they did not meet the selection criteria a priori. Thirty-two full-text articles were then assessed for eligibility. Of these, 14 articles were excluded for various reasons (see Figure 1 and supporting information). Finally, 18 unique references met the inclusion criteria and were included in this systematic review.[11,35,41-56] Figure 1 provides a PRISMA flowchart of study selection for this systematic review.

3.2 | Characteristics of the included studies

Table S1 summarises the characteristics of each study included. All studies were published, in English, between 2000 and May 2017 and had a cross-sectional, case-control design except one study, which had a longitudinal design.[55] Nevertheless, we extracted data of baseline evaluation, which was cross-sectional between CHC patients and healthy controls. The whole sample comprised 509 CHC patients, with an average age of 41.5 years old and mild liver disease. Few studies reported clinical features of CHC, genotype-1 being the most prevalent HCV genotype (35.8%). In accordance with the exclusion criteria, advanced cirrhotic patients (eg patients with current hepatic encephalopathy) were not present, and a variety of method were used to assess cirrhosis grade.[57-59] Patients with alcohol use disorder were excluded from studies. Nevertheless, only 2 studies report urine check before patients' enrollment,[49,52] whereas 3 studies report the timeframe in which patients were alcohol free, that was 1,[48] 2[49] and 7 years.[50] All studies included a healthy control group, with a whole sample of 491 healthy individuals and an average age of 45.9 years old. Various psychometric evaluations were conducted in the different studies. Depressive symptoms were assessed using the Beck Depression Inventory, the Hospital Anxiety and Depressive Symptoms, the Hamilton Rating Scale for Depression or the symptoms checklist, and Fatigue Severity with the Fatigue Assessment Inventory, the Fatigue Impact Scale, the Fatigue Severity Scale and the Visual Analogue Scale for Fatigue. Various neuropsychological tests were carried out to rate cognitive functions, mostly attention, executive functions and memory. Quality scores vary across studies, three of which had the highest punctuation (see Table S2 in Supporting Information).

3.3 | Brain activation, connectivity, metabolism and structural changes

Twenty-five studies reported in 18 references were identified, which used different neuroimaging techniques [magnetic resonance spectroscopy (N = 12), perfusion weighted images (N = 1), PET (N = 3), SPECT (N = 4), functional connectivity in resting state (N = 1), diffusion tensor imaging (N = 2) and structural magnetic resonance imaging (N = 2)]. Table S3 provides a summary of the main neuroimaging findings of the studies included, presented according to each type of brain imaging technique.

3.4 | Magnetic resonance spectroscopy

Twelve case-control studies were identified using proton magnetic resonance spectroscopy (H-MRS). The single voxel spectroscopy method was the most used; in 2 recent studies,[51,52] a multivoxel technique was applied. The spectral analysis was mainly conducted with the acquisition mode of the point-resolved spectroscopy (PRESS) technique, which guarantees a better spectral quality. The Stimulated Echo Acquisition Mode (STEAM), which permits a more precise volume selection, was applied in 2 studies.[45,54] The most frequently studied metabolites were N-acetylaspartate, myoinositol, choline, glutamate

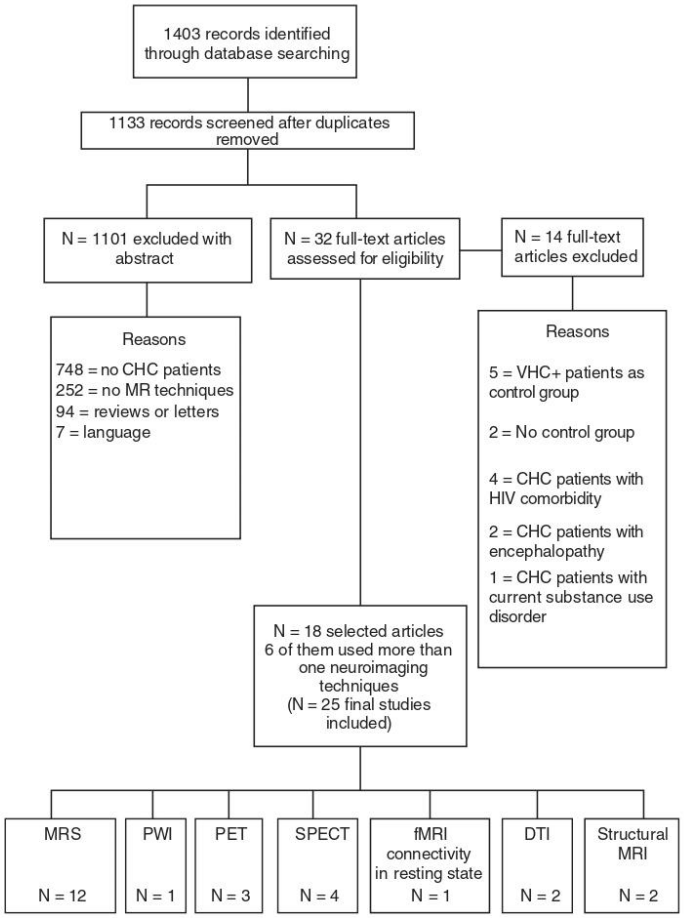

FIGURE 1 Flowchart showing selection of studies included in systematic review

plus glutamine and creatine. In Table 1, a brief synthesis of the location and function of each metabolite is displayed. Metabolite quantification was reported either as a ratio relative to the creatine reference peak, as it is considered the most stable cerebral metabolite or as absolute values. Considerable heterogeneity was observed in the brain regions studied. The basal ganglia and the centrum semiovale white matter were the most studied regions: other brain regions investigated were the posterior cingulate gyrus, frontal cortex, parietal cortex, temporal cortex, occipital cortex, frontal white matter, parietal-occipital white matter and pons. Finally, various psychometric scales were used to assess depressive symptoms or fatigue, and different neuropsychological assessments were conducted across the studies. Meta-analysis was possible in the studies that presented mean with standard deviation data of the metabolite results and considering the same metabolite spectra for the same brain regions. Overall, the meta-analyses results appear to indicate an excess of choline/creatine ratio (MD 0.12, 95% CI 0.06-0.18), glutamine plus glutamate (MD 1.67, 95% CI 0.21-3.14) and creatine (MD 0.87, 95% CI 0.38-1.36) in basal ganglia of patients with CHC, as well as an increased choline/creatine ratio (MD 0.11, 95% CI 0.04-0.19) in the centrum semiovale of HCV-infected patients compared with controls. In Figure 2, the forest plots of significant results are displayed. Sensitivity analyses were performed through fixed effect model. No changes in the significance and direction of results were observed, supporting strength and consistence of the results (for details of sensitivity analyses, see Table S4 in Supporting Information).

3.4.1 | Choline and choline/creatine ratio

Absolute levels of choline were calculated in 2 studies,[45,50] and a meta-analysis was conducted of choline levels in basal ganglia. No significant differences were observed between HCV-infected patients

TABLE 1 Magnetic resonance spectroscopy metabolites: an overview[a]

Metabolite (abbreviation)	Location	Function	Marker
Choline (Cho)	White matter	Nutrient Essential compound of phospholipids and acetylcholine synthesis	Cell membrane synthesis and turnover Myelination Cellular proliferation
Creatine (Cr)	Neurons, astrocytes and oligodendrocytes	Reservoir for energy production Osmolyte	Energetic system and intracellular metabolism Glial activation
Glutamate and Glutamine	Neurons and glia, respectively	Regulation of excitatory neurotransmission	Neuron-astrocyte integrity
Myo-inositol (mI)	Grey matter, glial cells	Storage form of glucose Precursor of the inositol polyphosphate messenger cascade Osmolyte	Glial activation Augmented glial-cell volume
N-Acetyl Aspartate (NAA)	Grey matters, neurons	Energy metabolism Myelin lipid synthesis	Axon-myelin integrity Neuron density and viability

[a]From the following references: 67, 68, 71, 82.

and healthy controls. In the study by Bokemeyer et al,[45] significant increases in choline concentration were also reported in parieto-occipital white matter. No differences were found in occipital grey matter, pons,[45] midline grey matter, frontal grey matter or central white matter.[50] In the meta-analysis of 4 studies, choline/creatine levels were increased in centrum semiovale white matter of CHC patients compared with healthy controls (Figure 2).[11,44,46,47] Same results were obtained in another meta-analysis of 3 studies[11,46,48] as increased choline/creatine levels in basal ganglia of CHC patients versus healthy controls were observed (Figure 2). Considering single studies, no increases in choline/creatine levels were found in midline and frontal grey matter, bilateral temporo-parietal grey matter, fronto-parietal white matter,[44] occipital grey matter,[46,48] posterior cingulate gyrus[41,44] or parieto-occipital white matter.[54]

3.4.2 | Creatine

A meta-analysis of 2 of the studies[45,50] showed significantly increased creatine levels in the basal ganglia of HCV-infected patients compared with healthy controls (Figure 2). As regards single studies, no significant differences were found in occipital grey matter, parietal-occipital white matter, pons,[45] midline grey matter, frontal grey matter, central white matter[50] and frontal grey matter or white matter, parietal grey matter or white matter.[52]

3.4.3 | Glutamate plus glutamine and glutamate plus glutamine/creatine ratio

The meta-analysis of 2 of the studies[45,50] showed increased absolute glutamate plus glutamine levels in basal ganglia of HCV-infected patients compared with healthy controls (Figure 2). Significantly increased glutamate plus glutamine levels were also observed in parieto-occipital white matter,[45] but a meta-analysis was not possible because the data available were insufficient. No significant differences were found in occipital grey matter, parieto-occipital white matter, pons,[45] midline grey matter, frontal grey matter, central white matter[50] and frontal grey matter or white matter, parietal grey matter or white matter.[52] Two studies considered the glutamate plus glutamine/creatine ratio,[47,51] but a meta-analysis could not be conducted due to insufficient data. Nevertheless, no significant differences in glutamate plus glutamine/creatine levels were found between CHC patients and healthy controls in centrum semiovale white matter.

3.4.4 | Myoinositol and myoinositol/creatine ratio

Compared with healthy controls, the meta-analysis conducted for 2 of the studies included[45,50] did not show increased absolute levels of myoinositol in basal ganglia (Figure S1 in Supporting Information) in HCV-infected patients. Nevertheless, sensitivity analysis showed significant differences when the fixed effect model was performed, suggesting caution in the interpretation of such results. I^2 was 46%, suggesting moderate heterogeneity (see Figure S1 and Table S4 in supporting information). In the single studies, increased levels of myoinositol were found in bilateral frontal white matter,[52] but no increase in myoinositol was found in occipital grey matter, parieto-occipital white matter, pons,[45] midline grey matter, frontal grey matter, central white matter[50] and frontal grey matter, parietal grey matter or white matter.[52] A meta-analysis was also conducted in 3 studies which assessed the myoinositol/creatine ratio in centrum semiovale,[44,47,51] but no significant differences were found between CHC subjects and healthy controls. As the previous case, sensitivity analysis with fixed effect model show significant increased myoinositol/creatine ratio in centrum semiovale of CHC patients. I^2 was 78%, suggesting substantial heterogeneity between studies (see Figure S1 and Table S4 in Supporting Information). No significant differences in myoinositol/creatine levels between HCV patients and healthy controls were found in temporo-parietal grey matter, posterior cingulate gyrus,[44] occipital grey matter,[46,48] parieto-occipital white matter or pons.[54]

3.4.5 | N-acetyl aspartate and N-acetyl aspartate to creatine ratio

Two studies analysed the absolute peak value of the N-acetylaspartate metabolite,[50,52] but in different regions, found lower N-acetylaspartate in central white matter in patients than in control group,[50]

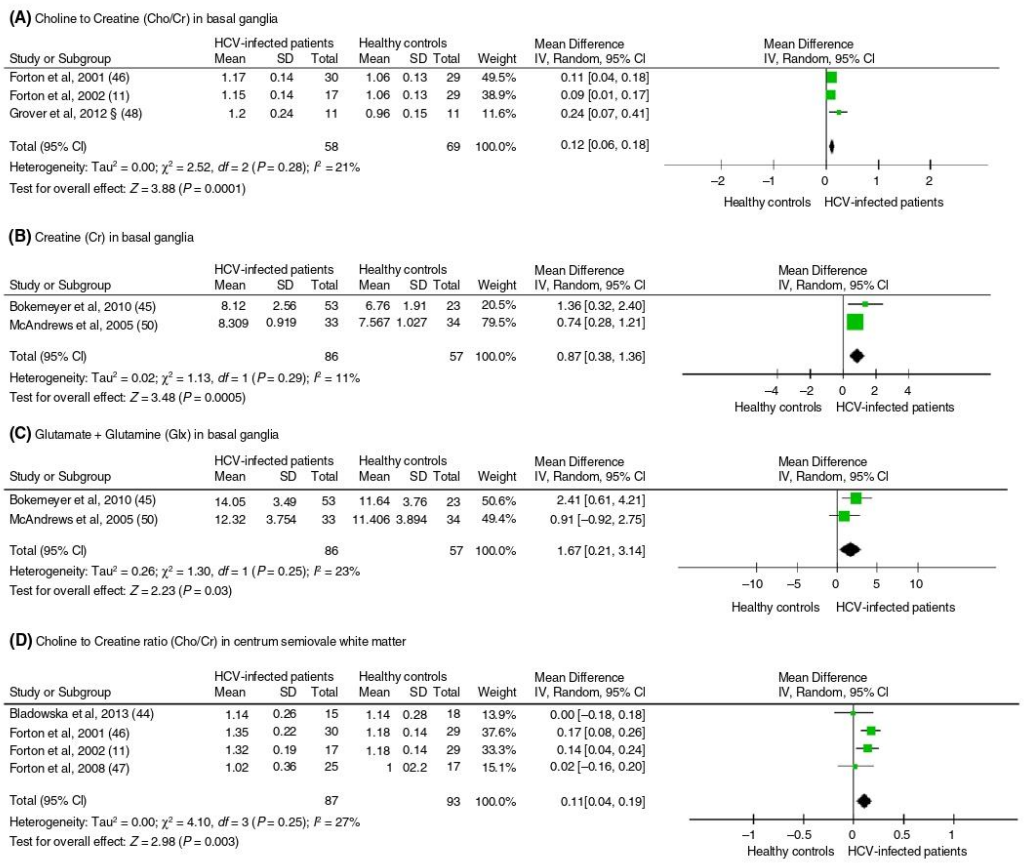

FIGURE 2 Forest plot of mean difference of significant MRS studies. (A-D) Comparison of metabolite peak levels in basal ganglia and centrum semiovale white matter, between HCV-infected patients and healthy controls. The numbers in parenthesis correspond to the reference. §, §§ indicates 2 different imagine techniques in the same study. Cho, choline; Cr, creatine; Glx, glutamine plus glutamate; HCV, hepatitis C virus; MD, mean difference; MRS, magnetic resonance spectroscopy

whereas Thames et al[52] observed reduced N-acetylaspartate in bilateral parietal white matter. No other differences in N-acetylaspartate were reported either in basal ganglia or in frontal grey matter. Seven studies[11,41,44,46-48,54] focused on the N-acetylaspartate/Creatine peak in several and different brain regions, but a meta-analysis could only be conducted for 2 of these studies[44,47] because of heterogeneity and insufficient data. In the meta-analysis, no differences were found between HCV-infected patients and healthy controls in terms of the N-acetylaspartate/creatine ratio in basal ganglia. In the other studies, a reduction of the N-acetylaspartate/creatine ratio was observed in occipital grey matter,[54] in left and right white matter (unspecified)[41] and in frontal and parietal white matter[44] of CHC patients compared with healthy controls. No significant differences were reported in temporo-parietal grey matter, posterior cingulate gyrus,[44] occipital grey matter,[46,48] frontal white matter,[51] parieto-occipital white matter, pons[54] or centrum semiovale white matter.[11,46,47]

Three studies[45,51,52] provided data on the N-acetylaspartate plus N-acetylaspartate-glutamate peak in patients with CHC, but these data were insufficient for the performance of a meta-analysis. Brain metabolite concentrations were quantitatively analysed by using LCmodel software.[60] Bokemeyer et al[45] reported an increased concentration of the N-acetylaspartate plus N-acetylaspartate-glutamate absolute peak in basal ganglia of HCV-infected patients compared with healthy controls, whereas Nagarajan et al[51] found no differences in the N-acetylaspartate plus N-acetylaspartate-glutamate/creatine ratio in frontal white matter. No significant differences were reported in N-acetylaspartate plus N-acetylaspartate-glutamate absolute peak in occipital grey matter, parieto-occipital white matter, pons,[45] frontal and parietal white matter or grey matter.[52]

3.4.6 | Other metabolites

Aspartate, gamma-amino-butyric acid (GABA), glutathione and scyllo-inositol ratios to creatine levels were analysed in one study.[51] Only glutathione to creatine ratio levels were found increased in the frontal white matter of CHC patients compared with healthy controls. As for the other metabolite levels, no significant differences were found in frontal white matter concentrations.

3.5 | Perfusion weighted imaging

Only one study performing perfusion weighted imaging technique in CHC patients was included,[42] which found a reduced regional cerebral blood flow in left temporo-parietal cortex and left frontal cortex in HCV-infected individuals compared with healthy controls. Patients also had an increased regional cerebral blood flow in bilateral basal ganglia compared with healthy controls.

3.6 | Positron emission tomography

Three studies used PET imaging technique to study microglial activation and alterations of cerebral glucose alterations. A meta-analysis was conducted of 2 studies[48,55] using the peripheral benzodiazepine receptor radioligand ^{11}C-(R)-PK11195 (PK11195), a marker of microglial activation, and its binding potential was calculated as main outcome. No significant differences were found in PK11195 binding potential in caudate nucleus, thalamus, putamen, pallidus and in frontal, temporal and occipital cortex of CHC patients compared with controls (see Figure S1 in Supporting Information). Importantly, the study by Grover et al[48] observed increased PK11195 binding potential in caudate nucleus of CHC patients, which correlated with viral load, whereas the subgroup of patients with genotype 1 virus showed increased binding potential in the thalamus compared with healthy controls. Interestingly, the study by Pflugrad et al[55] found significantly increased PK11195 binding potential in caudate, putamen and thalamus regions, only in those patients showing less attention impairment compared with healthy controls.

The study by Heeren et al[56] identified decreased cerebral metabolic rate of glucose in the superior and medial frontal gyri, the anterior cingulated gyrus, the hippocampus and parahippocampal gyrus, and part of the cerebellum in CHC patients compared with healthy controls.

3.7 | Single-photon emission computerised tomography

Two studies from the same group assessed monoaminergic neurotransmission in CHC patients by means of SPECT. Both studies investigated serotonin transporter (SERT) activity in midbrain and hypothalamus and the dopamine transporter (DAT) activity in caudate and putamen. Nevertheless, no meta-analysis could be performed because of insufficient data. Weissenborn et al[53] showed reduced DAT and SERT activity in striatum and hypothalamus/midbrain in CHC patients compared with healthy controls. This study identified that pathological DAT and SERT binding (with reduction above 2SDs) were present in 60% and 50% of patients, respectively. The study by Heeren et al[56] reported similar results, observing reduced DAT and SERT activity in CHC patients compared with healthy controls in striatum and hypothalamus/midbrain, respectively. In addition, DAT availability was correlated positively with cerebral metabolic rate of glucose in the superior and inferior parietal and postcentral gyri, the superior medial and inferior frontal gyri, the superior temporal gyrus and the medial cingulated gyrus (all these gyri bilaterally); with the precentral gyrus, the medial temporal gyrus, the temporal pole (on the left); and with insula, putamen, pallidus precuneus and calcarine cortex bilaterally.

3.8 | Functional magnetic resonance

One study selected assessed functional connectivity in resting state in CHC patients.[35] Resting-state functional magnetic resonance imaging identified higher eigenvector centrality in patients compared with healthy controls in the right postcentral sulcus and superior parietal lobe. The seed at the significant eigenvector connectivity cluster was used for the analysis. CHC patients presented a higher connectivity between the right postcentral sulcus with primary and secondary somatosensory cortex, paracentral lobule, superior temporal gyrus and occipital lobe compared with healthy controls.

3.9 | Diffusion tensor imaging

Diffusion tensor imaging is a neuroimaging technique that provides information on changes at cellular microarchitecture level and is able to detect alterations in normal appearing white matter or grey matter.[61] In our review, we selected 2 studies using diffusion tensor imaging in CHC patients,[43,52] but no meta-analysis could not be performed because the data were heterogeneous and insufficient. Bladowska et al[43] reported lower fractional anisotropy and increased apparent diffusion coefficient in the inferior fronto-occipital fasciculus in CHC patients than in healthy controls. Thames et al[52] observed an increased fractional anisotropy in striatum, which correlated with a poorer overall neuropsychological performance, and with the domain of language fluency. Moreover, CHC patients presented an increased mean diffusion in the fronto-occipital fasciculus and in the external capsule.

3.10 | Structural magnetic resonance imaging

Two recent studies[35,49] evaluated the whole brain grey matter volume, finding no differences between HCV patients and healthy controls. Therefore, no meta-analysis was performed. Hjerrild et al[49] was the only study to evaluate the cortical thickness. Compared with healthy controls, HCV patients showed thinner cerebral cortex in left frontal lobe and left and right occipital lobe. These results did not correlate with fatigue symptoms.

3.11 | Psychometric and neuropsychological evaluations and their correlations with neuroimaging findings

Five studies identified correlations between cerebral metabolite alterations and cognitive impairment,[11,41,47,48,52] whereas in one study, poorer neuropsychological performance was correlated with micros architectural changes in basal ganglia evidenced by the means of diffusion tensor imaging.[52] PET studies identified an association between preserved cognitive function and increased PK11195 binding potential in basal ganglia,[55] as well as increased cerebral glucose metabolism in frontal, parietal and associative cortices with significant cognitive deficits.[56] Three studies reported correlations with high fatigue scores and metabolic alterations in different brain areas,[41,45,52] with controversial results, while only one study reported correlations between high depressive symptoms and metabolic alterations.[41] Fatigue and depression scale scores correlations were analysed in the functional connectivity study[35] and in one PET study.[56] Details of correlations between neuroimaging findings and psychometric or neuropsychological evaluations are summarised in Table S3 of Supporting information.

4 | DISCUSSION

This study confirmed that HCV is associated with chronic metabolic changes in the brain, either in basal ganglia or in centrum semiovale white matter, in the absence of severe liver disease or cirrhosis, substance use disorders, or other psychosocial factors. Moreover, central nervous system metabolic changes were mainly correlated with neurocognitive impairment and fatigue symptoms, though controversial results were observed. Several other structural and functional brain abnormalities were also reported by individual studies, but meta-analyses could not be performed. In the following paragraphs, we discuss the neuroimaging results and their clinical correlates associated with CHC based on the present evidence of central nervous system mechanisms underpinning the HCV chronic infection (Figure 3).

4.1 | Data supporting neuroinflammatory activation in CHC

As mentioned in the introduction, CHC is characterised by a chronic and systemic inflammatory activation,[62] which may lead to inflammation in the central nervous system, interacting with neurons, astrocytes and microglia, even when the immune condition is elicited by innocuous stimuli.[63] A recent study highlighted the role of gut dysbiosis in CHC patients, which is related to endotoxemia and the persistence of systemic inflammation[64] even in patients with sustained viral response. Figure 3 provides an overview of different pathways by which cytokines or active immune cells can cross the blood-brain barrier.[65] Several studies included in our review supported the role of neuroinflammation in CHC patients. Bladowska et al[43] reported an increased myoinositol/creatine ratio in the posterior cingulate gyrus, an area involved in learning and memory processes which deteriorates in dementia syndromes.[66] An increase in myoinositol levels is associated with glial proliferation, or an increase in glial cell size, which may occur in inflammation.[67,68] Moreover, the authors[43] observed a positive correlation between myoinositol/creatine levels and grade of portal inflammation, measured by means of the histology activity index score, indicating that inflammatory processes can play a role in the central nervous system involvement.[13]

Neuroimmune activation and cytokines may interact with virtually every pathophysiological domain relevant to neuropsychiatric symptoms, including neurotransmitter metabolism, neuroendocrine function, oxidative and nitrosative stress and neural plasticity.[69,70] This provides an explanation for the metabolic, functional and structural neuroimaging alterations associated with CHC, which have been highlighted in this review. For example, increased levels of the choline/creatine ratio in the centrum semiovale white matter and in basal ganglia of CHC patients, with choline being a marker for cell membrane synthesis and turnover (Table 1).[71] Augmented choline can be considered a marker of neuroinflammation, reflecting energy failure, membrane degradation or demyelination,[72] and it has been observed in patients with other multi-systemic chronic infections such as HIV patients in the presence of macrophage infiltration.[73] Moreover, blood hypo-perfusion in the frontal and temporo-parietal cortex and hyper-perfusion of basal ganglia of CHC patients was shown by the means of perfusion weighted imaging,[42] and may be considered a marker of neuroinflammation.[74] Disturbances in cerebral perfusion have been related not only to neuroinflammation but also to the cerebrovascular complications and autoimmune syndromes encountered in HCV-infected patients.[75,76] Finally, structural neuroimaging studies revealed reduced cortical thickness in the right and left occipital cortex, as well as in the left frontal lobe of patients with CHC,[49] although these results were not replicated in the study by Kharabian Masouleh et al.[35] Neuropsychiatric disorders involving neuroinflammatory response as multiple sclerosis[77] or major depressive disorder[78] have also been related to reduced cortical thickness or global volume.

4.2 | Data supporting the role of microglial activation in CHC patients

Several studies in our review have highlighted the role of microglial activation in CHC patients, though results are somewhat controversial. The meta-analysis of 2 PET studies[48,55] did not show significant differences in glial activation in several brain areas of HCV-infected patients. Nevertheless, important observations are highlighted in the single studies. Grover et al[48] observed increased PK11195 binding potential in caudate nucleus and thalamus of CHC patients versus controls. Up-regulation in binding potential of PK11195 is a selective marker of activated glia,[79] and it has been used as a biomarker of inflammation in Huntington disease or cerebral HIV infection.[80,81] Interestingly, in the study by Grover et al,[48] HCV viral load was positively correlated with increased PK11195 binding potential,

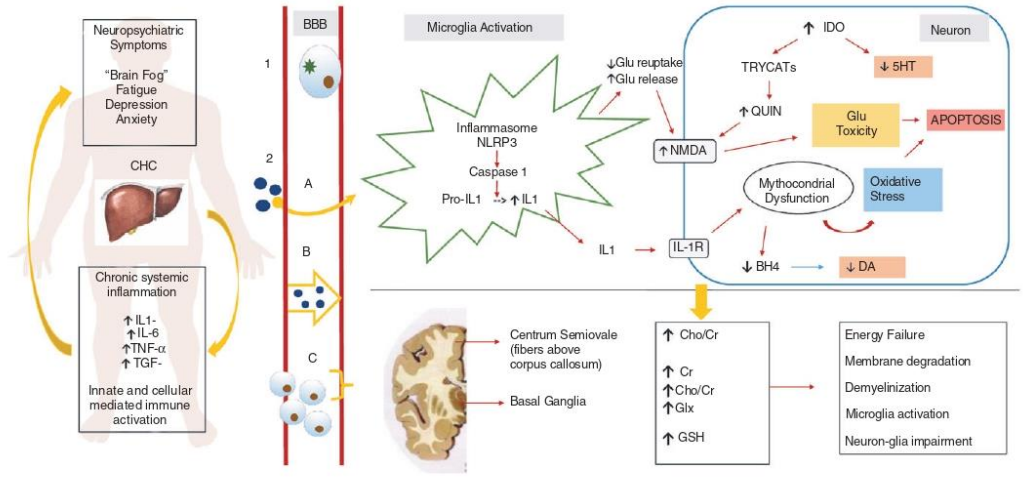

FIGURE 3 Different etiopathogenic and pathophysiological mechanisms have been hypothesised to explain the central nervous system alterations observed with different neuroimaging techniques. Circulating HCV-infected monocytes can access the central nervous system across the blood-brain barrier trafficking (1), as well as pro-inflammatory cytokines and cellular-mediated immune inflammation can communicate with central nervous system crossing the blood-brain barrier through the "neural route" (A), the "humoral route" (B) and the "cellular route" (C). So, both direct virus neuroinvasion and chronic systemic inflammatory activation may contribute to microglia activation and neuroinflammation, which can interact with virtually every pathophysiologic domain relevant to neuropsychiatric symptoms, including neurotransmitter metabolism, oxidative stress and apoptosis. 5HT, serotonin; BBB, blood-brain barrier; BH4, tetra-hydro biopterin; CHC, chronic hepatitis C; CHO, choline; Cr, creatine; DA, dopamine; Glu, glutamate; Glx, glutamate + glutamine; GSH, glutathione; HCV, hepatitis C virus; IDO, indoleamine 2,3 dioxygenase; IL, interleukin; NLRP3, nod-like receptor pyrin-domain containing 3; QUIN, quinolinic acid; TNF-α, tumour necrosis factor-α; TGF-b, transforming growth factor-b; TRYCATs, tryptophan catabolites

suggesting a possible direct implication of the virus. In line with these observations, we identified significant increased creatine peak levels in basal ganglia of CHC patients compared with healthy controls in the meta-analysis of proton magnetic resonance spectroscopy studies. Creatine is a marker of the energetic system and intracellular metabolism, and it is considered a glial marker since its concentration is much higher in glial cells and grey matter than in neurons and white matter.[71]

However, we did not find significant increased levels of the myoinositol absolute peak in basal ganglia, neither increased levels of myoinositol/creatine in centrum semiovale white matter in CHC patients compared with healthy controls, as the meta-analysis was performed, though such results must be interpreted with caution. Due to its osmolar properties, myoinositol participates in the volume regulation of astrocytes, and high myoinositol levels reflect gliosis, astrocytosis and increased cell membrane turnover (Table 1).[71,82]

No clear correlations between neuropsychiatric evaluations and neuroimaging findings of glial activation were identified, and the absence of a consistent relationship deserves mention. For example, Grover et al[48] reported significantly increased myoinositol/creatine ratios in basal ganglia of CHC patients, which correlated with increased error rates in a cognitive task involving attention, and similar results were found in patients with major depressive disorder.[83] Conversely, Bokemeyer et al[45] found a negative correlation between myoinositol levels in basal ganglia and centrum semiovale white matter with fatigue scores, suggesting a beneficial effect of glial activation among HCV-infected patients. In the same line, Pflugrad et al[55] found increased PK11195 binding potential in caudate, thalamus and putamen only in those CHC patients with attention impairment, compared with controls. In this sense, a neuroprotective role of astrocytes and microglia activation was assumed.[45,55] Moreover, signs of increased functional connectivity in resting state in the right posterior parietal regions to parietal, temporal and occipital cortices observed in HCV patients[35] have been correlated with better cognitive performance, in the domains of attention and episodic memory. Indeed, dorsal parietal regions have been related to top-down attentional domain and to episodic memory.[84] Taken together, these data suggest that the reorganisation of the connectivity in the right posterior parietal lobe may be interpreted as a compensatory mechanism that provides more attentional resources.

4.3 | Data related to the impact of HCV infection on monoaminergic neurotransmission

Two SPECT studies were included in the review, which illustrated the impact of HCV infection on serotoninergic and dopaminergic neurotransmission. The results showed a reduction in the midbrain serotoninergic and striatal dopaminergic systems in CHC patients

who exhibit worse cognitive impairment as well as fatigue and depressive symptoms.[53] Actually, alterations in serotonin transmission between raphe nucleus and the prefrontal cortex, amygdala and hippocampus have been associated with mood symptoms and sleep disturbances,[85] whereas dopamine subcortical circuits have been associated with cognitive performance and flexibility, as well as the generation of pleasure.[86,87] Moreover, data from a randomised clinical trial demonstrated a remission of fatigue symptoms in CHC patients treated with ondaserton, a serotonin receptor 3 antagonist, suggesting an impact of HCV on neurotransmission functions.[88]

4.4 | Role of oxidative stress associated with HCV chronic infection

Finally, evidence of oxidative stress derives from the observation of increased glutamate plus glutamine levels in the basal ganglia of HCV-infected patients. Its increase in the basal ganglia is believed to derive from the increase in glutamate.[45,68] Glutamate is an excitatory neurotransmitter, which can participate in the redox cycle,[82] and its accumulation in the extracellular space may trigger excessive activation of glutamatergic receptors and lead to excitotoxicity.[89] These results are in line with certain reports in major depressive patients, which have found increased glutamatergic transmission that were associated with structural and functional changes in several brain structures as basal ganglia, contributing to oxidative stress.[90] Nevertheless, other study observed lower levels of glutamine plus glutamate in the prefrontal regions, amygdala and hippocampus.[91] The reduction of glutamine plus glutamate levels has been associated with impairments in neuron-astrocyte integrity, as glutamine-glutamate cycle in central nervous system, which may be considered as the neurochemical correlation of neuron-glia interaction.[92] Interestingly, Nagarajan et al[51] found increased levels of glutathione in the basal ganglia of HCV-infected patients, which are indicative of oxidative stress. Oxidative stress plays a central role in neuronal toxicity and loss and has been related to neurodegenerative diseases.[93,94] Glutathione is crucial for the neutralisation of reactive oxygen species in the central nervous system,[95] and an increase in its levels can be considered as an early marker of inflammation that precedes neuronal damage. Another mechanism involved is microglia activation, which is responsible for the Nod-like receptor pyrin-domain containing 3 (NLRP-3, known as inflammasome) intracellular activity and the consequent production and release of Interleukin-1β.[65] This cytokine has been associated with the mitochondrial dysfunction, apoptosis and reactive oxygen species increase.[27] Moreover, oxidative and nitrosative stress has been related to neurodegenerative diseases, and it is considered a possible pathway of neuroprogression in major depressive disorder.[96,97]

4.5 | Strengths and limitations of the systematic review

This systematic review has several limitations that may be discussed in advance. The neuroimaging techniques in the selected references present considerable heterogeneity. Although most of the studies (12 of 25) used proton magnetic resonance spectroscopy to investigate central nervous system metabolic changes in CHC patients, different brain regions were assessed as well as different metabolites studied, and so it is difficult to summarise the results. Moreover, the lack of reported data in certain cases precludes a quantitative analysis; meta-analyses could be performed for 5 metabolites (either peak levels or their ratio with creatine) in 2 different brain regions, and only 2 meta-analyses included more than 2 studies. With regard to other neuroimaging techniques, this systematic review found a lack of replicated evidence of structural and functional brain alterations induced by HCV infection. Indeed, the negative and contradictory results emerging from our meta-analysis deserve special mention: for example, no significant increase in the myoinositol absolute peak was observed in the basal ganglia of CHC patients, nor in myoinositol/creatine ratio in the centrum semiovale white matter. A possible explanation for this finding may be the high heterogeneity observed between studies and lack of sensitivity. Moreover, the use of creatine as a referent metabolite, because of its assumed stable concentration, may influence the consistency of the results across studies.[98] It has been shown that creatine levels are higher in grey matter[99] and may change with age,[100] alcohol consumption[101] and over the different stages of HIV-induced dementia.[102] For this reason, the evaluation of absolute metabolite concentration is recommended. Another limitation is the fact that all included studies were cross-sectional, which means that is impossible to establish the directions of the association; furthermore, most of them had relatively small samples. A further issue that deserves mention is the controversial results in the relations between neuroimaging alterations and cognitive outcome or severity of psychiatric symptoms. As explained above, a possible explanation is that the methodological heterogeneity, the several neuroimaging techniques employed, and the wide variety of psychometric scales and neuropsychological test batteries used may limit the comparability between studies. On the other hand, an important strength of the study was the fact that no cirrhotic patients or patients with hepatic encephalopathy were included, as stated in the exclusion criteria, so as to avoid the neuroimaging alterations which have been reported in these patients,[103] and all studies included incorporated a healthy control group. However, only few studies described the HCV genotype, viral load, transaminase levels or degree of fibrosis, a situation that limited the detection of possible associations between clinical features of hepatitis and brain changes. These and other potential confounding factors were not always controlled for. Finally, it is possible that this systematic review failed to identify some other study (i.e. from the grey literature).

5 | CONCLUSIONS

In summary, patients with CHC exhibit cerebral metabolite alterations and structural or functional neuroimaging abnormalities, thus

supporting the hypothesis of a direct or indirect involvement of HCV in central nervous system disturbances. Brain metabolite changes in either direction may be considered a marker of neuroinflammation and neuron-glia and axon-myelin integrity disruption, similar to those observed in patients with major depressive disorder.[92] Both direct virus neuroinvasion and chronic systemic inflammatory activation may contribute to neuroinflammation and microglial activation, which may interact with virtually every pathophysiological domain relevant to neuropsychiatric symptoms, including neurotransmitter metabolism, neuroendocrine function and oxidative stress. These pathways may provide an explanation for the metabolic, functional and structural neuroimaging alterations associated with CHC, which have been highlighted in this review. Nevertheless, a lack of replicated evidence has been observed. A multimodal neuroimaging approach which embraces structural, functional and metabolic assessment of CHC patients and the inclusion of postmortem histological study when indicated may help to identify valid and specific brain abnormalities induced by HCV infection. We consider that prospective studies in larger cohorts are needed to shed further lights on the relation between HCV infection, neuropsychiatric impairment and neuroimaging alterations. Moreover, a recent exhaustive review[104] pointed out the substantial improvement of patient-reported outcomes in clinical trial setting related to the high efficacy of the new DAAs treatments. In the lights of these considerations, the study of patients with sustained viral response would be crucial to identify those metabolic and functional changes in central nervous system strictly related with HCV infection.

ACKNOWLEDGEMENTS

Declaration of personal interests: RMS is grateful to Instituto de Salud Carlos III, Spanish Ministry of Economy and Competiveness, Centro para la Investigación Biomédica en Red de Salud Mental (CIBERSAM); and the Secretaria d'Universitats I Recerca del Departamentd'Economia I Coneixement, Grups consolidats de recerca (2014_SGR_1411). RMS was funded by ISCIII-Subdirección General de Evaluación, grant PI10/01827, CF integrated in Plan Nacional I+D+I and co-founded by Fondo Europeo de Desarrollo Regional (FEDER-"Una manera de hacer Europa"). XF was funded by ISCIII-Subdirección General de Evaluación, grant PI15/00151. CF integrated in Plan Nacional I+D+I and co-funded by Fondo Europeo de Desarrollo Regional (FEDER-"Una manera de hacer Europa") and by Secretaria d'Universitats i Recerca del Departament d'Economia i Coneixement, grant 2014_SGR_605. Other authors have no statements to declare.

Declaration of funding interests: RMS received grant from the Instituto de Salud Carlos III, FIS: PI10/01827, Fondo Europeo de Desarrollo Regional (FEDER): "Una manera de hacer Europa." XF received support by Plan Nacional de I+D+I and co-funded by ISCIII-Subdirección General de Evaluación and Fondo Europeode Desarrollo Regional (FEDER-"Una manera de hacer Europa") (grant PI15/00151) and Secretaria d'Universitats i Recerca del Departament d'Economia i Coneixement (grant 2014_SGR_605). ZM received support by the Gilead Fellowship Program in Hepatitis 2017 (grant GLD17/00273). XF and ZM also received support by the Spanish Health Ministry (Plan Estratégico Nacional contra la hepatitis C).

AUTHORSHIP

Guarantors of the article: G. Oriolo and R. Martin-Santos were the authors who took responsibility for the integrity of the work as a whole, from inception to published article.

Author contributions: G. Oriolo and E. Egmond did all steps in the literature search, study identification and selection, data extraction and quality assessment. G. Oriolo, R. Navinés, Z. Mariño and R. Martin-Santos designed the research study and wrote the paper, and J. Pujol and Z. Mariño contributed to the design and discussion of the study. M. Cavero, L. Zamarrenho, N. Bargallo, R. Solà and X. Forns contributed to the discussion of the results and to the final revision of the systematic review. All authors contributed to and have approved the final manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information will be found online in the supporting information tab for this article.

How to cite this article: Oriolo G, Egmond E, Mariño Z, et al. Systematic review with meta-analysis: neuroimaging in hepatitis C chronic infection. *Aliment Pharmacol Ther*. 2018;00:1–15. https://doi.org/10.1111/apt.14594

Supporting Information Paper 1

List of Supporting Material for the article

I. Protocol

PROTOCOL CHC-NEUROIMAGING REVISION

Aim

The aim of the review was to compile structural and functional neuroimaging studies of the central nervous system (CNS) structural and functional neuroimaging in chronic hepatitis C (CHC) patients, in order to shed lights on brain features and changes during this chronic illness.

Data for this systematic review were collected with an advanced document protocol in accordance with the PRISMA and MOOSE guidelines(Moher, Liberati, Tetzlaff, Altman, & Grp, 2009; Stroup et al., 2000). These protocols provided a quality checklist for reporting systematic reviews based on Newcastle - Ottawa Quality Assessment Scale (NOS)(Stang, 2010).

Search strategy

A comprehensive, computerized literature search was conducted in Medline, PsycINFO and EMBASE databases. We used the following key words: "hepatitis C", "chronic hepatitis C", "HCV", "CHC", "structural MRI", "structural magnetic resonance imaging", "functional MRI", "functional magnetic resonance imaging", "fMRI", "connectivity", "MRSI", "H-MRS", "spectroscopy", "DTI", "diffusion tensor imaging", "PWI", "perfusion weighted imaging", "PET", "positron emission tomography", "SPECT", "single photon emission computed tomography", mixed with different Boolean operators "AND" and "OR". Studies published up to 1st May 2017 written in English, Spanish, Italian and Dutch were searched for. The titles and abstracts were examined, full-text articles of potentially relevant studies were obtained and their reference lists were also examined.

Selection criteria

In order to homogenize the selection and facilitate comparisons, studies were only included if they expressly stated the following inclusion criteria: structural and functional neuroimaging studies of patients of both gender with chronic HCV. Moreover: 1) for cross-sectional designs we selected studies including treatment-naïve CHC patients, or patients who had received antiviral treatment without achieving a sustained viral response (SVR), with a healthy control group, and that comprising at least 5 participants in case of functional imaging studies and spectroscopy or 10 participants in case of structural neuroimaging studies; 2) for longitudinal design, we selected basal data from studies involving CHC patients treatment naïve, or who had received antiviral treatment without achieving a SVR, with a healthy control group; 3) before inclusion in the study, patients must have been checked for drug consumption. The exclusion criteria were: 1) no neuroimaging studies of CHC; 2) participants under 18 years old; 3) individuals with advanced cirrhosis or encephalopathy; 4) patients with HIV co-infection; 5) subjects who had other neurological disorders or a past history of stroke or traumatic brain injury; 6)

subjects with psychiatric disorders, or individuals who met criteria for alcohol dependence or substance abuse disorders (abuse or dependence) other than nicotine; and 7) grey literature.

Publications that reported two forms of different data from the same subjects (e.g. MRI and PET) or a study examining the same subjects with two different cognitive tasks during the functional imaging (e.g. auditory attention and verbal working memory) were considered as two separate studies. Specifically, we selected studies using structural techniques such as structural magnetic resonance (sMRI) and diffusion tensor imaging (DTI). On the other hand, studies using functional techniques such as magnetic resonance spectroscopy (MRS), perfusion weighted imaging (PWI), functional magnetic resonance imaging (fMRI) were selected regardless of the paradigm used during the image acquisition (e.g. emotional recognition), as well as studies using positron emission tomography (PET) and single photon emission computed tomography (SPECT). Connectivity studies were included.

Data extraction

The variables recorded for each article were: authors, year of publication, socio-demographic features (gender, age, ethnicity, sample size, handedness), hepatitis C virus characteristics (HCV genotype, RNA levels, anemia, degree of liver fibrosis, antiviral regimens in treated patients) and exclusion criteria for neurological, psychiatric or drug use-related disorders. History of substance use and confirmation of abstinence from other drugs (if checked by urine test) were also recorded. History of psychiatric disorders and psychopathological variables (e.g. psychotic or depressive symptoms) were assessed, as were psychometric and neuropsychological evaluations. As regards imaging variables, technique and design, rest/active condition (for functional imaging studies) and type of cognitive task performed during functional imaging were recorded, as well as study features such as blinded design. The primary measures of interest assessed were the following: for structural imaging data, global and regional volume and thickness; for diffusion tensor imaging (DTI, which measures the three-dimensional anisotropic diffusion of water molecules within tissues) fractional anisotropy, apparent diffusion coefficient and mean diffusivity; for functional imaging data, the primary measures of interest were global and regional activity in the form of cerebral blood flow, blood oxygen level dependent signal or connectivity; for perfusion weighted imaging (PWI) relative cerebral blood volume; for spectroscopy, concentration (absolute value or ratio relative to the creatine reference peak) of cerebral metabolites: [N-acetylaspartate (NAA), N-acetylaspartate-glutamate (NAAG), creatine (Cr), myoinositol (mI), choline (Ch), Phosphoryl-Choline (P-Cho), Glyceryl-phosphoryl-choline (GP-Cho), glutamine (Gln), glutamate (Glu), glutamine plus glutamate (Glx)]; for connectivity, brain activation between areas, assessed using the seed-based and decomposition-based analysis in resting state or under a paradigm; for positron emission tomography (PET), the binding potential of the biochemical marker used or the cerebral metabolic rate of glucose (CMRglc) in case of F-fluoro-deoxy-glucose-PET; and finally, for single photon emission computerized tomography (SPECT), the binding potential of the biochemical marker used. The secondary outcomes were the correlation of these measures with clinical variables (viral genotype, viremia, degree of hepatic fibrosis, depression, fatigue and cognitive domains). We recorded the

statistically significant results of each outcome variable, and noted whether a multiple comparison correction was performed to prevent a bias toward false positives.

Quality assessment

The overall methodological quality of the identified articles was tested with the Newcastle - Ottawa Quality Assessment Scale (NOS), a scale recommended by the Cochrane collaboration for quality assessment of observational studies34. For this review, we considered the NOS subscale that evaluates case control studies which consisted in nine items grouped in three domains of selection, comparability and exposure. Scores may vary from 0 to 9, with higher scores indicated better methodological quality(Stang, 2010).

II. PRISMA guidelines

Section/Topic	#	Checklist Item	Reported on Page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	**1**
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	**3**
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	**5-7**
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	**8**
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	**8**
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	**9-10**
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	**9**
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	**9**
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	**8-10**
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	**10-11**
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	**10-11**
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	**N/A**
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	**12**
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	**12**
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	**12**
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	**N/A**
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	**13**
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	**13-14**
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome-level assessment (see Item 12).	**N/A**
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group and (b) effect estimates and confidence intervals, ideally with a forest plot.	**15-23**
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	**13-23**
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	**12**
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	**N/A**
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., health care providers, users, and policy makers).	**24-29**
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review level (e.g., incomplete retrieval of identified research, reporting bias).	**29-31**
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	**31-32**
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	**33**

doi:10.1371/journal.pmed.1000100.t001

III. Excluded studies

VHC + control group

Abe K, Wada A, Oshima S, Kono S, Takahashi A, Kanno Y, Imaizumi H, Hayashi M, Okai K,Niwa SI, Yabe H, Ohira H. Reduced frontal activation during verbal fluency task in chronic hepatitis C patients with interferon-based therapy as measured by near-infrared spectroscopy. Hepatol Res. 2016 doi: 10.1111/hepr.12721

Haroon E, Felger JC, Woolwine BJ, Chen X, Parekh S, Spivey JR, Hu XP, Miller AH. Age-related increases in basal ganglia glutamate are associated with TNF, reduced motivation and decreased psychomotor speed during IFN-alpha treatment: Preliminary findings. Brain Behav Immun. 2015; 46:17-22.

Haroon E, Woolwine BJ, Chen X, Pace TW, Parekh S, Spivey JR, Hu XP, Miller AH. IFN-alpha-induced cortical and subcortical glutamate changes assessed by magnetic resonance spectroscopy. Neuropsychopharmacology 2014; 39(7):1777-85.

Capuron L, Pagnoni G, Drake DF, Woolwine BJ, Spivey JR, Crowe RJ, Votaw JR, Goodman MM, Miller AH. Dopaminergic mechanisms of reduced basal ganglia responses to hedonic reward during interferon alfa administration. Arch Gen Psychiatry. 2012; 69(10):1044-53.

Capuron L, Pagnoni G, Demetrashvili M, Woolwine BJ, Nemeroff CB, Berns GS, Miller AH. Anterior cingulate activation and error processing during interferon-alpha treatment. Biol Psychiatry. 2005; 58(3):190-6.

No control group

Taylor MJ, Godlewska B, Near J, Christmas D, Potokar J, Collier J, Klenerman P, Barnes E, Cowen PJ. Effect of interferon-α on cortical glutamate in patients with hepatitis C: a proton magnetic resonance spectroscopy study. Psychol Med. 2014 Mar;44(4):789-95.

Dipasquale O, Cooper EA, Tibble J, Voon V, Baglio F, Baselli G, Cercignani M, Harrison NA.Interferon-α acutely impairs whole-brain functional connectivity network Architecture. A preliminary study. Brain Behav Immun. 2016; 58:31-39.

CHC patients with advanced cirrhosis or encephalopathy

Miese F, Kircheis G, Wittsack HJ, Wenserski F, Hemker J, Mödder U, Häussinger D, Cohnen M. 1H-MR spectroscopy, magnetization transfer, and diffusion-weighted imaging in alcoholic and non alcoholic patients with cirrhosis with hepatic encephalopathy. Am J Neuroradiol. 2006;27(5):1019-26.

Iwasa M, Mifuji-Moroka R, Kuroda M, Moroka H, Fujita N, Kobayashi Y, Adachi Y, Gabazza EC, Matsuda H, Takei Y. Regional reduction in gray and white matter volume in brains of cirrhotic patients: voxel-based analysis of MRI. Metab Brain Dis. 2012; 27(4):551-7.

Co-infected

Vigneswaran S, Rojas JH, Garvey L, Taylor-Robinson S, Winston A. Differences in the variability of cerebral proton magnetic resonance spectroscopy (1H-MRS) measurements within three HIV-infected cohorts. Neuroradiol J. 2015;28(6):545-54.

Garvey LJ, Pavese N, Ramlackhansingh A, Thomson E, Allsop JM, Politis M, Kulasegaram R, Main J, Brooks DJ, Taylor-Robinson SD, Winston A. Acute HCV/HIV coinfection is associated with cognitive dysfunction and cerebral metabolite disturbance, but not increased microglial cell activation. PLoS One. 2012;7(7):e38980.

Gongvatana A, Cohen RA, Correia S, Devlin KN, Miles J, Kang H, Ombao H, Navia B, Laidlaw DH, Tashima KT. Clinical contributors to cerebral white matter integrity in HIV-infected individuals. J Neurovirol. 2011;17(5):477-86.

Winston A, Garvey L, Scotney E, Yerrakalva D, Allsop JM, Thomson EC, Grover VP, Main J, Cox JI, Wylezinska M, Taylor-Robinson SD. Does acute hepatitis C infection affect the central nervous system in HIV-1 infected individuals? J Viral Hepat. 2010;17(6):419-26.

Subatance use disorder

Taylor MJ, Letendre SL, Schweinsburg BC, Alhassoon OM, Brown GG, Gongvatana A, Grant I; HNRC. Hepatitis C virus infection is associated with reduced white matter N-acetylaspartate in abstinent methamphetamine users. J Int Neuropsychol Soc. 2004;10(1):110-3.

*** New Search until 31 of March 2019**

Comorbidity in VHC patients

Amin A, Nawito Z, Zayed HS, Enaba D, Elsayed ND, Alsirafy S, … Abo Elfadl S. Psychiatric and functional neuroimaging abnormalities in chronic hepatitis C virus patients: Is vasculitis a contributing factor? Arab Journal of Gastroenterology 2018; 19(2): 71–75.

VHC+ patients no controlled for psychiatric disorder

Kumar A, Deep A, Gupta RK, Atam V, Mohindra S. Brain Microstructural Correlates of Cognitive Dysfunction in Clinically and Biochemically Normal Hepatitis C Virus Infection. Journal of Clinical and Experimental Hepatology. 2017;7(3): 198–204.

IV.. Supplementary Tables Table 1S. Characteristics of studies included in the review

A.MRS	HCV patients Healthy controls		CHC features			Magnetic Resonance Spectroscopy (MRS)			Neuropsychiatric assessment
Author/year	**N (% fem)**	**Age Mean (SD)**	**HCV genotype Type: N (%)**	**RNA levels IU/mL Mean (range)**	**Fibrosis N: grade Mean/Median**	**Characteristics [Metabolites]**		**ROI**	**Symptoms/Domains Tools**
Bladowska et al., 2013[43,44]	15 (40) 18 (33.3)	42.1 (N/A) 34.6 (N/A)	1:15 (100)	1324553 (58672-4485492)	HAI unspecified 15: ≤2 Mean 0.6	1.5 T SVS PRESS	NAA/Cr, Cho/Cr mI/Cr	Bilateral TPC , Frontal cortex PCG, Frontal and parietal WM Basal ganglia	None
Bokemeyer et al., 2010[45]	53 (73.6) 26 (N/A)	52 (9) 52.5 (12.8)	1: 42 (79) 3: 3 (6) N/A: 8 (15)	N/A	N/A	1.5 T SVS STEAM	NAA+NAAG Glx, Cho, mI, Cr	Parieto-occipital WM Occipital GM, Basal ganglia Pons	HADS, FIS NPS assessment
Forton et al., 2001[46]	30 (53) 29 (48)	44 (N/A) 42 (N/A)	N/A	N/A	N/A	1.5 T SVS PRESS	NAA/Cr, Cho/Cr mI/Cr	Basal ganglia, Centrum semiovale WM, Occipital GM	None
Forton et al., 2002[11 †]	17 (53) 29 (48)	42.1 (N/A) 42 (N/A)	N/A	N/A	N/A	1.5 T SVS PRESS	NAA/Cr, Cho/Cr mI/Cr	Basal ganglia Centrum semiovale WM	BDI, HADS, Fatigue (N/A), NPS assessment
Forton et al., 2008[47]	25 (46) 17 (60)	45.0 (8.3) 45.1 (5.7)	N/A	N/A	ISHAK 25: ≤2 Median 1	1.5 T SVS PRESS	NAA/Cr, Cho/Cr mI/Cr, Glx/Cr	Centrum semiovale WM (Frontal WM)	NPS assessment
Grover et al., 2012[48] (§)[††]	11 (54.5) 11 (54.5)	52.3 (N/A) 52.5 (N/A)	1: 6 (55) 2: 1 (9) 3: 2 (18) 4: 2 (18)	N/A	ISHAK 11: ≤2	1.5 T SVS PRESS	NAA/Cr, Cho/Cr mI/Cr	Basal ganglia Occipital GM	FIS, NPS assessment
McAndrews et al., 2005[50]	33 (N/A) 34 (N/A)	46.1 (9.3) 35.8 (9.3)	N/A	N/A	ISHAK: (0-3) Mean 2.0 (0.8)	N/A SVS PRESS	NAA, Cho, mI Glx , Cr	Putamen, GP, Central WM Midline frontal GM	BDI, FAI, NPS assessment
Nagarajan et al., 2012[51]	14 (N/A) 14 (N/A)	56.2 (N/A) 46.6 (N/A)	N/A	N/A	N/A	3 T L-COSY (2D)	Glu/Cr, Glx/Cr, mI/Cr, Cho /Cr, NAA+NAAG/Cr, GSH/Cr, GABA/Cr, Scy/Cr Asp/Cr, GPCho/Cr	Frontal WM	None
Saadi Allah et al., 2014[41]	20 (45) 20 (40)	40.5 (7.5) 37.8 (7.4)	N/A	49.2 IU/mL	N/A	1.5 T N/A N/A	NAA/Cho , NAA/Cr Cho/Cr	PCG,Centrum semiovale L/R Frontal Lobe ,WM L/R (unspecified)	HDRS, FSS, MMSE
Thames et al, 2015[52] (§)[†††]	29 (N/A) 20 (50)	N/A 54.4 (5.5)	N/A	N/A	N/A	3 T MRSI (2D) PRESS	NAA + NAAG, GPCho + PhCho, mI, Glx, Cr	Basal ganglia Frontal WM, GM Parietal WM,GM	Fatigue (VAS) NPS assessment
Weissenborn et al, 2004[54] (§)[¶]	15 (66.7) 15 (73.3)	54.9 (13.4) 35.8 (9.3)	N/A	11 >5.10^5c/ml 4 <10^5 c/ml	ISHAK 6: ≤2 9: 4	1.5 T SVS STEAM	NAA/Cr, Cho/Cr mI/Cr	Occipital GM, Pons Basal ganglia Parieto-occipital WM	HADS, FIS, NPS assessment
Weissenborn et al, 2004[54] (§§)[¶]	15 (73.3) 15 (73.3)	54.7 (11.9) 35.8 (9.3)	N/A	9 >5.10^5 c/ml 4 <10^5 c/ml 2 N/A	ISHAK 7: ≤2 8: 4	1.5 T SVS STEAM	NAA/Cr, Cho/Cr mI/Cr	Occipital GM,Pons Basal ganglia Parieto-occipital WM	HADS, FIS, NPS assessment

B. PWI	Subjects		CHC features			Perfusion Weighted Images (PWI)		Neuropsychiatric assessment
						Characteristics	ROI	Symptoms/ Tools
Bladowska et al., 2014[42]	14 (45.8) 18 (33.3)	39.5 (N/A) 34.6 (N/A)	1:14 (100)	1324553 (58672-4485492)	HAI (no specified) 15: ≤2 Mean 0.6	1.5 T rCBV	Bilateral TPC, Frontal córtex, PCG, Fronto-parietal WM, Basal ganglia	NPS assessment

C. PET	Subjects		CHC features			Positron Emission Tomography (PET)		Neuropsychiatric assessment
						Marker	ROI	Symptoms /Tools
Grover et al., 2012 (§§)[48]	11 (54.5) 10 (50)	52.3 (N/A) 58 (N/A)	1: 6 (55) 2: 1 (9) 3: 2 (18) 4: 2 (18)	N/A	ISHAK 11: <2	PK11195	Caudate N,Thalamus putamen GP frontal , Parietal temporal Occipital	Fatigue (FIS) NPS assessment
Heeren et al., 2011 (§)[56]	10 (100) 12 (33)	52.6 (2.9) 47 (N/A)	1: 10 (100)	N/A	N/A	F-FDG	Whole Brain	BDI, HADS, FIS SF-36 (QoL), NPS assessment
Pflugrad et al., 2016[55] ¶¶	12 (100) 6 (100)	N/A 51.3 (4.0)	1: 12 (100)	N/A	N/A	PK11195	Amygdala, Caudate, Putamen, putamen, pallidus, Thalamus Pons,GM cerebellum, GM frontal, temporal, occipital cortex	BDI, HADS, FIS SF-36 (QoL), NPS assessment

D. SPECT	Subjects		CHC features			Single Photon Emission Computed TomographySPECT		Neuropsychiatric assessment
						Marker	ROI	Symptoms/Tools
Heeren et al., 2011[56] (§§)	10 (100) 20 (50)	52.6 (2.9) 50 (N/A)	1: 10 (100)	N/A	N/A	DAT	Striatum (caudate & putamen)	BDI, HADS, FIS, SF-36 (QoL), NPS assessment
Heeren et al., 2011[56] (§§§)	10 (100) 16 (55)	52.6 (2.9) 47 (N/A)	1: 10 (100)	N/A	N/A	SERT	Hypothalamus Midbrain	BDI, HADS, FIS, SF-36 (QoL), NPS assessment
Weissenborn et al.,2006[53] (§)	20 (65) 20 (55)	48.8 (5.3) 50.2 (18.9)	1: 18 (90) 3: 2 (10)	N/A	N/A	DAT	Striatum (caudate & putamen)	BDI, HADS, FIS SF-36 (QoL), NPS assessment
Weissenborn et al.2006[53] (§§)	20 (65) 16 (50)	48.8 (5.3) 47.3 (17.2)	1: 18 (90) 3: 2 (10)	N/A	N/A	SERT	Hypothalamus Midbrain	BDI, HADS, FIS,SF-36 (QoL), NPS assessment

E. fMRI	Subjects		CHC features			Functional Magnetic Resonance Imaging (fMRI)	Neuropsychiatric assessment
						Characteristics	Symptoms/Tools
Masouleh et al., 2016[35] (§)‡	9 (100) 10 (100) 23 (100)	55 (3) 58 (6) 57 (4)	1b 19 (100)	4 x 105 12 x 10 5	Fibroscan 5.8 (2.5) kPa 5.7 kPa	fMRI 3 T Resting state Gradient-echo echo-planar image	HDRS, SCL-90, FIS, NPS assessment

***New Study Inserted after actualization until 31th March 2019**

McReady et al., 2018	20 (25) 26 (21)	51 (9) 46 (13)	1 : 9 (45) 2 : 5 (25) 3 : 3 (15) N/A : 3 (15)	6.3 x 105	N/A	fMRI 3 T During DDT** task Gradient-echo echo-planar image	DDT task

**DDT: Delay discontinuing task

F. DTI	Subjects		CHC features			Diffusion Tensor Imaging (DTI)		Neuropsychiatric assessment
						Characteristics	ROI	Symptoms/Tools
Bladowska et al., 2013[44]	15 (40) 18 (33.3)	42.1 (N/A) 34.6 (N/A)	1: 15 (100)	1324553 (58672- 4485492)	HAI 15: ≤2 Mean 0.6	1.5 T FA ADC	Middle cerebellar peduncle L/R, ILF L/R, Inf. FOS L/R, GCC, Splenium corpus callosum, Post. limb internal capsule L/R, SLF L/R, Posterior cingulum L/R	NPS assessment
Thames et al., 2015[52] (§§)††	29 (N/A) 20 (50)	N/A 54.4 (5.5)	N/A	N/A	N/A	3 T FA MD	Insula, Amygdala, Hippocampus, Capsule I/E Corona Radiata, Cingulum, striatum, FOS,Thalamus	VAS(fatigue) NPS assessment

G. MRI	Subjects		CHC features			Structural Magnetic Resonance Imaging (MRI)	Neuropsychiatric assessment
						Characteristics	Symptoms/Tools
Hijerrild et al., 2016[49] ‡‡	43 (58.2) 43 (58.2)	42.5 (10.2) 43.8 (9.7)	1: 19 (44) 2: 3 (7) 3: 20 (47) 4: 1 (2)	N/A	< 13 kPa	3 T GM Cortical thickness	HDRS FSS
Masouleh et al., 2016[35] (§§)‡	9 (100) 10 (100) 23 (100)	55 (3) 58 (6) 57 (4)	1b:19 (100)	4 x 105 12 x 10 5	Fibroscan 5.8±2.5 kPa 5.7 kPa	3 T GM	HDRS, SCL-90 FIS NPS assessment

† Complete data for MRS no available, †† Occipital GM no reported, ††† The results are divided for MRS study and DTI study. No values available for cases that underwent MRS and DTI. No values available for MRS and DTI. ¶ The results on HCV patients are divided in mildly fatigued (FIS average 21.5±12.8) and moderately fatigued (FIS average 79.8±25.6) ($p < .001$). No standard deviation reported. ¶¶ This was the only longitudinal study selected. We report here the data of baseline cross-sectional evaluation. ‡ Sociodemographic data of CHC patients are presented separating SVR and treatment naïve groups. ‡‡ Sociodemographic variables included depressed patients (n=7 in HCV patients, 16%). The analysis was conducted separately. (§), (§§), (§§§). Indicates two different imagine techniques in the same study.

List of abbreviation (alphabetical order): N/A, Not available; SVR, Substained viral response.

Brain areas: FOS, Fronto-occipital fasciculus; GCC, Genu corpus callosum; GM, Gray Matter; GP, Globus pallidus; IFOF, Inferior fronto-occipital fasciculus; ILF, Inferior longitudinal fasciculus; L/R, Left/Right; MCP, Middle cerebellar peduncle; PC, Posterior cingulum; PLIC, Posterior limb of internal capsule; SLF, Superior longitudinal fasciculus.
Brain metabolites: Asp, Aspartate; Cho, Choline; Cr, Creatine; Gln, Glutamine; Glu, Glutamate; Glx, Glutamate + Glutamine; GP-Cho, Glyceryl-phosphoryl-choline; GSH, Glutathione; mI, Myo-Inositol; NAA, N-acetyl-aspartate; NAAG, N-Acetyl-aspartyl-glutamate; PCG, Posterior cingulate gyrus; Ph-Cho, Phosphoryl-choline1; Scy, Scyllo-inositol; SLF, Superior longitudinal fasciculus; SCC, Splenium of the corpus callosum;TPC, Temporal-parietal cortex; WM, White Matter.
Neuroimaging techniques: ADC, Apparent Diffusion Coefficient; D: Dimension; DAT, Dopamine transporter binding; DSC-MR: dynamic susceptibility contrast imaging;; FA, Fractional anisotropy; F-FDG: F-Fluoro-deoxy-glucose; ; L-COSY: localized correlated spectroscopy; MD, Mean Diffusivity ; MRSI: Magnetic resonance spectroscopy imaging (multi-voxel technique); PRESS: pointed-resolved spectroscopy; SERT, Serotonin transporter binding; STEAM: stimulating echo acquisition mode; SVS: Single-Voxel spectroscopy; TE, Echo time; TR, Repetition time
Psychopathological rating scales: *Depression*: BDI, Beck depressive inventory; HADS, Hospital anxiety and depressive scale; HDRS, Hamilton depression rating scale; MADRS, Montgomery-Asberg depressive rating scale; SCL-90: Symptom checklist; *Fatigue:* FAI, Fatigue assessment inventory; FIS, Fatigue impact scale; FSS, Fatigue severity scale; VAS, Visual analog scale; MMSE, Mini-mental state evaluation; NPS, Neuropsychology; *Health perceived quality of life:* SF-36, Short form health survey; QoL, Quality of life.

Table 2S. Quality Checklist for the selected studies based on Newcastle-Ottawa Quality Assessment Scale for case control studies

Reference	Is the case definition adequate?	Representativeness sample	Selection of controls	Definition of controls	Comparability of cases and controls (maximum 2 stars)	Ascertainment exposure	Same method of ascertainment for cases and controls	Non-Response Rate	TOTAL
Bladowska et al., 2013 [44]	*	*	*	*	*	*	*	*	**8**
Bladowska et al., 2013 [43]	*	*	*	*	*	*	*	*	**8**
Bladowska et al., 2014 [42]	*	*	*	*	*	*	*	*	**8**
Bokemeyer et al., 2012 [45]	*	*	*		*	*	*	*	**7**
Forton et al., 2001 [46]	*	*	*		*	*	*	*	**7**
Forton et al., 2002 [11]	*	*	*	*	**	*	*	*	**9**
Forton et al., 2008 [47]	*	*	*	*	**	*	*	*	**9**
Grover er al., 2012 [48]	*	*	*	*	*	*	*	*	**8**
Heeren et al, 2011 [56]	*				*	*	*	*	**5**
Hijerrild et al, 2016 [49]	*	*	*		**	*	*	*	**8**
Masouleh et al, 2016 [35]	*		*		*	*	*	*	**6**
McAndrews et al., 2005 [50]	*	*	*		**	*	*	*	**8**
Nagarajan et al., 2012 [51]	*				*	*	*	*	**5**
Pflugrad et al, 2016 [55]	*	*			**	*	*	*	**7**
Saad Allah et al., 2014 [41]	*	*	*		*	*	*	*	**7**
Thames et al., 2015 [52]	*	*	*	*	**	*	*	*	**9**
Weissenborn et al., 2004 [54]	*	*	*		**	*	*	*	**8**
Weissenborn et al., 2006 [53]	*	*	*		*	*	*	*	**7**
***New Study Inserted after actualization 31th march 2019**									
McCready et al., 2018	*	*	*		**	*	*	*	**8**

Total score may vary from 0 to 9, with higher scores indicated better methodological quality.

Table 3S. Key Findings of the different neuroimaging studies included in the review

A. Magnetic Resonance Spectroscopy (MRS)

Author/Year	MRS results associated to HCV-infected patients vs. Healthy controls		Correlations of [metabolite]/ratio with clinical or biological variable (positive, negative or no significant)		
	↑ [metabolite] /ratio	↓ [metabolite] /ratio	Liver inflammation/fibrosis	Depression/fatigue symptoms	Cognitive domains
Bladowska et al., 2013 [43,44]		[NAA]/[Cr] ratio in frontal WM (p=0.010) [NAA]/[Cr} ratio in parietal WM (p=0.035)	[mI]/[Cr] in PCG with inflammation HAI score (r=0.65, p=0.008) [mI]/[Cr] in PCG with fibrosis HAI score (r=0.54; p=0.03) NS other disease variables		
Bokemeyer et al., 2010 [45]	[Cho] in parieto-occipital WM (p=0.045) and in basal ganglia (p=0.02) [Cr] in basal ganglia (p=0.047) [NAA + NAAG] in basal ganglia (p=0.019) [Glx] in parieto-occipital WM (p=0.001)			[mI] in WM with FIS score (r=-0.495, p=0.001) [mI] in basal ganglia with FIS score (r=-0.592, p=0.012) [Cho] in GM with FIS score (r=-0.464, p=0.02) [Cr] in pons with FIS score (r=-0.374, p=0.04)	NS
Forton et al., 2001 [46]	[Cho]/[Cr] ratio in basal ganglia (p=0.01) [Cho]/[Cr] ratio in centrum semiovale WM (p=0.001)		NS		
Forton et al., 2002 [11]	Cho]/[Cr] ratio in basal ganglia (p=0.04) [Cho]/[Cr] ratio in centrum semiovale WM (p=0.008)			NS	Higher [Cho]/[Cr] ratio in basal ganglia with 2 ≥ cognitive domains vs. those with < 2 domains (p=0.036) and HC (p=0.007) Higher [Cho]/[Cr] ratio in centrum semiovale WM with 2 ≥ cognitive domains vs. HC (p=0.02)

Forton et al., 2008 [47]	[mI]/[Cr] ratio in centrum semiovale WM (p=0.02)		NS		[mI]/[Cr] ratio was associated with prolonged working memory reaction times (r=0.72, p=0.002)
Grover et al., 2012 [48] (§)	[mI]/[Cr] ratio in basal ganglia (p=0.0004) [Cho]/[Cr] ratio in basal ganglia (p=0.01)		NS	NS	[mI]/[Cr] ratio and [Cho]/[Cr] ratio in basal ganglia with number of errors made on line tracing test, a cognitive task of attention (r=0.68, p=0.01 and r=0.72, p=0.01 respectively)
McAndrews et al., 2005 [50]	[Cho] in central WM [t(65) = 2.17; p<0.05] [Cr] in putamen and GP [t(65) = 3.09; p<0.01]	[NAA] in central WM [t(65) = 3.27; P<0.01]	NS	NS	NS
Nagarajan et al., 2012 [51]	[mI]/[Cr] ratio in frontal WM (p=0.029) [GSH] in frontal WM (p=0.003)		NS		
Saad Allah et al., 2014 [41]	[NAA]/[Cr] ratio in L and R WM (p=0.001) [Cho]/[Cr] ratio in L and R WM (p=0.001) [NAA]/[Cho] ratio in L and R centrum semi-ovale (p=0.001)		NS	[Cho]/[Cr] ratio in left WM with FSS score (r=0.523, p=0.046) [NAA]/[Cr] in bilateral WM with HDRS score (r=0.601, p=0.018 right WM and r=0.646, p=0.009 left WM) and with FSS scores (r=0.664, p=0.007 right WM and r=0.681, p=0.005 left WM)	[NAA]/[Cr] in bilateral WM with MMSE (r=-0.797, p<0.01 right WM and r=-0.731, p=0.002 left WM) [Cho]/[Cr] in bilateral WM with MMSE (r=-0.847, p<0.01 right WM and r=-0.833, p<0.01 left WM)
Thames et al., 2015 (§) [52]	[mI] in bilateral frontal WM (p=0.004)	[NAA] in bilateral parietal WM (p=0.02)		[mI] in frontal WM with fatigue scores (r_{29}=0.53, p<0.01)	[mI] in frontal WM with processing speed (r_{29}=-0.43, p=0.02) and verbal/language fluency (r_{29}=-0.41, p=0.007)
Weissenborn et al., 2004 [54] (§)		HCV subgroup (mildly fatigued): [NAA]/[Cr] ratio in occipital GM (p=0.02)	NS	NS	NS

Weissenborn et al., 2004 [54] (§§)		HCV subgroup (moderately fatigued): [NAA]/[Cr] ratio in occipital GM (p=0.04)	NS	NS	NS

B. Perfusion Weighted Images (PWI)

Author/year	PWI results associated to HCV-infected patients vs. Healthy controls		Correlations of [metabolite]/ratio with clinical or biological variables (positive, negative or no significant)		
	↑ rCBV	↓ rCBV	Liver inflammation/fibrosis	Depression/fatigue symptoms	Cognitive domains
Bladowska et al., 2014 [42]	Basal ganglia (Right p=0.0002, Left p<0.0001) Bilateral basal ganglia (after Bonferroni correction p<0.0055)	Bilateral TPC (Right p=0.010, Left p=0.003), Left FC (p=0.005) PCG (P=0.045) Left TPC and left FC (after Bonferroni correction p<0.0055)	NS	NS	NS

C. Positron Emission Tomography (PET)

Author/year	PK11195 binding potential associated to HCV-infected patients vs. Healthy controls		Correlations of PET marker binding potential with clinical or biological Variables (positive, negative or no significant)		
	↑ PK11195 binding potential	↓ PK11195 binding potential	Liver inflammation/fibrosis	Depression/fatigue symptoms	Cognitive domains
Grover et al., 2012 [48] (§§)	PK11195 binding potential in caudate nucleus (p=0.03). In genotype 1: in thalamus was higher than HC (p=0.005)		PK11195 binding potential in caudate with HCV viral load (genotype 1) (r=0.77, p=0.005)	NS	NS
Pflugrad et al., 2016 [55]	NS	NS			PK 11195 binding potential in pons (r= -.570, p <.01), putamen (r= -.498, p <.01), caudate nucleus (r= -.417, p= .03), globus pallidus (r= -.463, p=.02), thalamus (r =-.471, p= .01), amygdala (r= -.515, p=<.01), cerebellum (r= -.402, p=.03) and temporal cortex (r= -.403, p=.03) with

					Attention Tests sum scores (results with PCR+ and PCR- patients mixed)
Author/year	**CMR_{glu} binding potential associated to HCV-infected patients vs. Healthy controls**		**Correlations of PET marker binding potential with clinical or biological Variables (positive, negative or no significant)**		
	↑ Cerebral metabolic rate of glucose	**↓ Cerebral metabolic rate of glucose**	**Liver inflammation/fibrosis**	**Depression/fatigue symptoms**	**Cognitive domains**
Heeren et al, 2011 [56] (§)		Superior and medial frontal gyri (p=.035), the anterior cingulated gyrus, the hippocampus and parahippocampal gyrus (p=.012)		$sCMR_{glu}$ in thalamus, postcentral gyrus, anterior and medial cingulate gyri, the orbital part of the three frontal gyri with the FIS scores $sCMR_{glu}$ in orbitofrontal cortex and cerebellum with BDI scores $sCMR_{glu}$ in caudate, anterior cingulate gyrus, right orbitofrontal gyrus and left postcentral gyrus with HADS-D scores. $sCMR_{glu}$ in parahippocampal gyrus and caudate with HADS-A scores	CMR_{glu} in right superior frontal gyrus, left cerebellum, in caudate and cingulum in Attention Tests results CMR_{glu} in anterior and medial cingulate gyri, central cortex, supplementary motor cortex, inferior forntal gyrus, cerebellum, amygdala, inusla and caudate nucleus, superior temporal gyrus and olfactory cortex with Memory Tests results

D. Single Photon Emission Computed Tomography (SPECT)

Author/year	DAT and SER binding potential associated to HCV-infected patients vs Healthy controls		Correlations of SPET marker binding potency with clinical or biological variables (positive, negative or no significant)		
	↑ DAT/SERT binding potential	↓ DAT/SERT binding potential	Liver inflammation/fibrosis	Depression/fatigue symptoms	Cognitive domains
Weissenborn et al., 2006 [53]		DAT in striatum (p=0.0006) SERT in hypothalamus/midbrain (p<0.0001) DAT and SERT binding (>2SDs) in 60% and 50% of patients respectively.	NS	NS	NS Patients with altered SPECT differed significantly from HC in cognitive assessment.
Heeren et al, 2011 [56] (§§)		DAT in striatum (p=.009) SERT in straitum (p=.006)	NS	NS	NS

E. Functional Magnetic Resonance Imaging (fMRI)

Author/year	Functional connectivity associated to HCV-infected patients vs Healthy controls		Correlations of functional connectivity with clinical or biological variables (positive, negative or no significant)		
	↑ functional connectivity or activation	↓ functional connectivity or activation	Liver inflammation/fibrosis	Depression/fatigue symptoms	Cognitive domains
Masouleh et al., 2016 [35] (§)	EC in right post-central sulcus, which extended to the anterior superior parietal lobule (seed) Connectivity between the seed cluster and primary and secondary somatosensory cortex, paracentral lobule, superior temporal gyrus and occipital lobe.		NS	Adjusted mean EC of different cluster with FIS (Spearman's rank = 0.4, p=0.005) and HDRS (depression) (Spearman's rank = 0.5, p=0.001).	Adjusted mean EC in postcentral cluster with better memory performance (r=0.4, p=0.005) and scores of attention (r= -0.59, p=0.01).

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McCready et al., 2018		Activation in left lateral occipital gyrus, left precuneus and left superior frontal gyrus.	NS	NS	The less activation was negative correlated with hard-easy contrast (impulsvity)

F. Diffusion Tensor Imaging (DTI)

Author/year	FA, ADC, and MD result associated to HVC-infected patients compared to Healthy controls		Correlations of DTI results with clinical or biological variables (positive, negative or no significant)		
	↑ FA, ADC or MD	↓ FA, ADC or MD	Liver inflammation/fibrosis	Depression/fatigue symptoms	Cognitive domains
Bladowska et al., 2013[43]	ADC values in bilateral IFOF (Right p<0.0001, Left p=0.024), and Left ILF (p=0.005). After Bonferroni correction ADC increased was found in Right IFOF	FA in bilateral MCP (Right P=0.005, Left P=0.001), bilateral ILF (Right P=0.003, Left P=0.004), bilateral IFOF (Right P<0.0001, Left P=0.0003), and GCC (p=0.0006). According to Bonferroni correction lower FA were found in Right IFOF.	NS with FA or ADC results		
Thames et al., 2015[52] (§§)	FA in striatum (p<0.005) MD in fronto-occipital fasciculus (p<0.0001) and external capsule (p<0.002)				FA in the striatum with poorer overall NPS performance (r_{29}=-0.45, p=0.01) and with language fluency (r_{29}=-0.41, p=0.02). NS with MD

G. Structural Magnetic Resonance Imaging (MRI)

Author/year	Volume whole brain, GM, WM, and thickness result associated to HVC patients compared to Healthy controls		Correlations of structural results with clinical or biological variables (positive, negative or no significant)		
	↑ Volume or Cortical thickness	↓ Volume or Cortical thickness			
Hijerrild et al., 2016 [49]	No differences in volume of whole brain GM, WM and cerebellum	Thinner cerebral cortex in left frontal lobe and in left and right occipital lobe (p<0.05)	Cortex thickness: NS		Cortex thickness: NS with psychometric assessment.
Masouleh et al., 2016 [35] (§§)	No differences in volume of whole brain	NS in cerebral thickness			

(§), (§§). Indicates two different imagine techniques in the same study.

List of abbreviation (alphabetical order): Cho, Choline; CMR_{glu}: Cerebral metabolism rate of glucose Cr, Creatine; DAT, Dopamine transporter binding; DTI, Diffusion tensor imaging; EC: Eigenvector connectivity; FIS, Fatigue impact scale; FSS, Fatigue severity scale; GCC, Genu corpus callosum; Glx, Glutamate + Glutamine; GM, Gray Matter; GP, Globus pallidus; GSH, Glutathione; HDRS, Hamilton depression rating scale; IFOF, Inferior fronto-occipital fasciculus; ILF, Inferior longitudinal fasciculus; L/R, Left/Right; MCP, Middle cerebellar peduncle; mI, Myo-Inositol; MMSE, Mini-mental state evaluation; NAA, N-acetyl-aspartate; NAAG, N-Acetyl-aspartyl-glutamate; NPS, Neuropsychology; NS: not significant; PCG, Posterior cingulate gyrus; PET, Photon emission tomography; PWI, Perfusion weighted images; $sCMR_{glu}$: scaled cerebral metabolism rate of glucose (obtained dividing CMR_{glu} by global CMR_{glu} voxel by voxel); SERT, Serotonin transporter binding; SPECT, Single photon emission computerized tomography; TPC, Temporal-parietal cortex; WM, White Matter.

Table 4S. Sensitivity test of the different meta-analyses performed. Random effect model was used *a priori* and fixed effect model as sensitivity test. The significant results are highlighted in red.

Magnetic resonance spectroscopy – Basal Ganglia		
Metabolite	**Random effect model MD [CI - 95%]**	**Fixed effect model MD [CI - 95%]**
Choline to Creatine (Cho/Cr)	0.12 [0.06 / 0.18]	0.11 [0.06 / 0.16]
Creatine (Cr)	0.87 [0.38 / 1.36]	0.85 [0.42 / 1.27]
Choline (Cho)	0.20 [-0.17 / 0.56]	0.07 [-0.02 / 0.16]
Myoinositol (mI)	0.42 [-0.36 / 1.20]	0.55 [0.10 / 1.01]
Glutamate + Glutamine (Glx)	1.67 [0.21 / 3.14]	1.67 [0.39 / 2.96]

Magnetic resonance spectroscopy – Centrum Semiovale White Matter		
Metabolite	**Random effect model MD [CI95%]**	**Fixed effect model MD [CI - 95%]**
N-Acetylaspartate to Creatine (NAA/Cr)	-0.10 [-0.39 / 0.20]	-0.12 [-0.24 / 0.01]
Choline to Creatine (Cho/Cr)	0.11 [0.04 / 0.19]	0.12 [0.06 / 0.18]
Myoinositol to Creatine (mI/Cr)	0.10 [-0.06 / 0.25]	0.08 [0.01 / 0.15]

Photon emission tomography – PK11195 Binding Potential		
Region	**Random effect model MD [CI - 95%]**	**Fixed effect model MD [CI - 95%]**
Caudate Nucleus	0.02 [-0.01 / 0.05]	0.02 [0.00 / 0.04]
Thalamus	0.04 [-0.01 / 0.10]	0.04 [-0.01 / 0.09]
Putamen	-0.01 [-0.04 / 0.02]	-0.01 [-0.04 / 0.02]
Pallidum	-0.02 [-0.08 / 0.03]	-0.03 [-0.07 / 0.02]
Frontal Cortex	0.02 [-0.01 / 0.05]	0.02 [-0.01 / 0.05]
Temporal Cortex	-0.01 [-0.03 / 0.02]	-0.01 [-0.03 / 0.02]
Occipital Cortex	0.01 [-0.03 / 0.04]	0.01 [-0.03 / 0.04]

List of abbreviation (alphabetical order):
Cho, Choline; CI: confidence interval; Cr, Creatine; Glx, Glutamate + Glutamine; MD: mean difference; mI, Myo-Inositol; NAA, N-acetyl-aspartate;

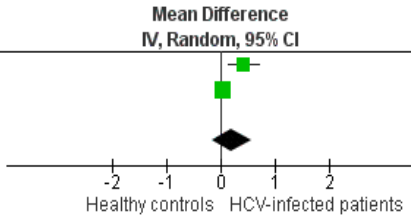
V. Supplementary Figures

Fig.1S Forest plot figures of mean difference of no significant MRS and PET studies.

a. Choline (Cho) in basal ganglia

Study or Subgroup	HCV-infected patients Mean	SD	Total	Healthy controls Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
Bokemeyer et al, 2010 (45)	1.97	0.64	53	1.56	0.53	23	43.8%	0.41 [0.13, 0.69]
McAndrews et al, 2005 (50)	1.664	0.162	33	1.631	0.231	34	56.2%	0.03 [-0.06, 0.13]
Total (95% CI)			**86**			**57**	**100.0%**	**0.20 [-0.17, 0.56]**

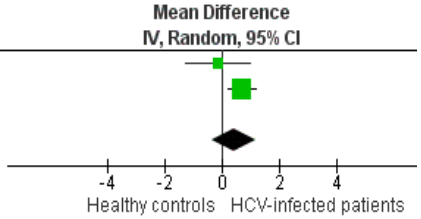
Heterogeneity: Tau² = 0.06; Chi² = 6.37, df = 1 (P = 0.01); I² = 84%
Test for overall effect: Z = 1.06 (P = 0.29)



b. Myoinositol (mI) in basal ganglia

Study or Subgroup	HCV-infected patients Mean	SD	Total	Healthy controls Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
Bokemeyer et al, 2010 (45)	5.12	1.58	53	5.28	2.54	23	32.0%	-0.16 [-1.28, 0.96]
McAndrews et al, 2005 (50)	4.357	1.15	33	3.663	0.912	34	68.0%	0.69 [0.20, 1.19]
Total (95% CI)			**86**			**57**	**100.0%**	**0.42 [-0.36, 1.20]**

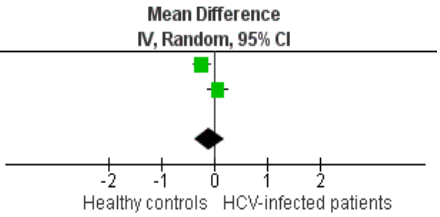
Heterogeneity: Tau² = 0.17; Chi² = 1.86, df = 1 (P = 0.17); I² = 46%
Test for overall effect: Z = 1.06 (P = 0.29)



c. N-Acetylaspartate to Creatine ratio (NAA/Cr) in centrum semiovale

Study or Subgroup	HCV-infected patients Mean	SD	Total	Healthy controls Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
Bladowska et al, 2013 (44)	1.66	0.1	15	1.9	0.33	18	51.7%	-0.24 [-0.40, -0.08]
Forton et al, 2008 (47)	1.92	0.38	25	1.86	0.26	17	48.3%	0.06 [-0.13, 0.25]
Total (95% CI)			**40**			**35**	**100.0%**	**-0.10 [-0.39, 0.20]**

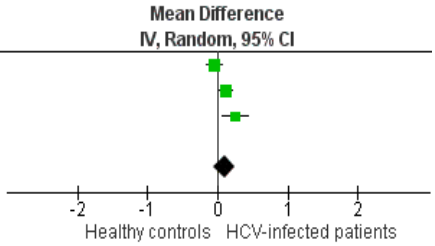
Heterogeneity: Tau² = 0.04; Chi² = 5.46, df = 1 (P = 0.02); I² = 82%
Test for overall effect: Z = 0.63 (P = 0.53)



d. Myoinositol to Creatine ratio (mI/Cr) in centrum semiovale

Study or Subgroup	HCV-infected patients Mean	SD	Total	Healthy controls Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
Bladowska et al, 2013 (44)	0.72	0.08	15	0.77	0.23	18	35.5%	-0.05 [-0.16, 0.06]
Forton et al, 2008 (47)	0.64	0.21	25	0.52	0.1	17	37.6%	0.12 [0.02, 0.22]
Nagarajan et al, 2012 (51)	1.311	0.264	14	1.056	0.237	14	26.9%	0.25 [0.07, 0.44]
Total (95% CI)			**54**			**49**	**100.0%**	**0.10 [-0.06, 0.25]**

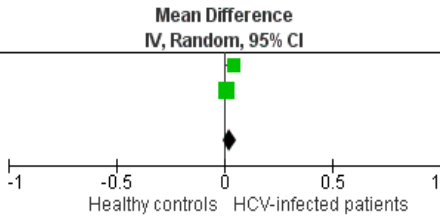
Heterogeneity: Tau² = 0.01; Chi² = 9.10, df = 2 (P = 0.01); I² = 78%
Test for overall effect: Z = 1.21 (P = 0.23)



e. PK11195 Binding Potential in Caudate nucleus

Study or Subgroup	HCV-Infected Patients Mean	SD	Total	Healthy controls Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
Grover et al, 2012 §§ (48)	0.12	0.04	11	0.08	0.03	10	43.0%	0.04 [0.01, 0.07]
Plufgrad et al, 2016 (55)	0.029	0.026	12	0.019	0.018	6	57.0%	0.01 [-0.01, 0.03]
Total (95% CI)			**23**			**16**	**100.0%**	**0.02 [-0.01, 0.05]**

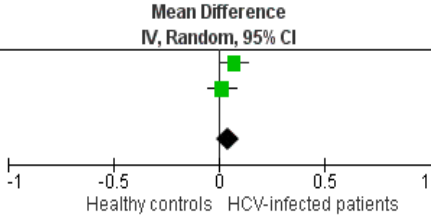
Heterogeneity: Tau² = 0.00; Chi² = 2.60, df = 1 (P = 0.11); I² = 62%
Test for overall effect: Z = 1.54 (P = 0.12)



f. PK11195 Binding Potential in Thalamus

Study or Subgroup	HCV-infected patients Mean	SD	Total	Healthy controls Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
Grover et al, 2012 §§ (48)	0.38	0.09	11	0.31	0.07	10	49.9%	0.07 [0.00, 0.14]
Plufgrad et al, 2016 (55)	0.275	0.075	12	0.262	0.067	6	50.1%	0.01 [-0.06, 0.08]
Total (95% CI)			**23**			**16**	**100.0%**	**0.04 [-0.01, 0.10]**

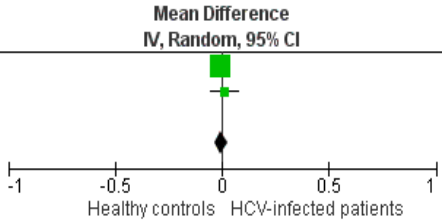
Heterogeneity: Tau² = 0.00; Chi² = 1.33, df = 1 (P = 0.25); I² = 25%
Test for overall effect: Z = 1.45 (P = 0.15)



g. PK11195 Binding Potential in Putamen

Study or Subgroup	HCV-infected patients Mean	SD	Total	Healthy controls Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
Grover et al, 2012 §§ (48)	0.17	0.04	11	0.18	0.04	10	78.1%	-0.01 [-0.04, 0.02]
Plufgrad et al, 2016 (55)	0.189	0.075	12	0.178	0.061	6	21.9%	0.01 [-0.05, 0.08]
Total (95% CI)			**23**			**16**	**100.0%**	**-0.01 [-0.04, 0.02]**

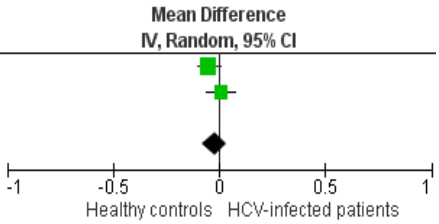
Heterogeneity: Tau² = 0.00; Chi² = 0.32, df = 1 (P = 0.57); I² = 0%
Test for overall effect: Z = 0.35 (P = 0.73)



h. PK11195 Binding Potential in Pallidum

Study or Subgroup	HCV-infected patients Mean	SD	Total	Healthy controls Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
Grover et al, 2012 §§ (48)	0.17	0.07	11	0.22	0.06	10	55.4%	-0.05 [-0.11, 0.01]
Plufgrad et al, 2016 (55)	0.221	0.068	12	0.212	0.069	6	44.6%	0.01 [-0.06, 0.08]
Total (95% CI)			**23**			**16**	**100.0%**	**-0.02 [-0.08, 0.03]**

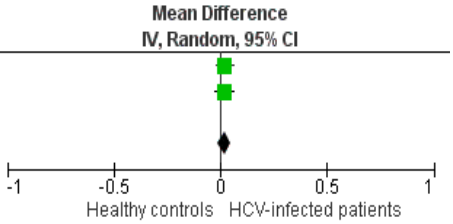
Heterogeneity: Tau² = 0.00; Chi² = 1.75, df = 1 (P = 0.19); I² = 43%
Test for overall effect: Z = 0.81 (P = 0.42)



i. PK11195 Binding Potential in Frontal Cortex

Study or Subgroup	HCV-infected patients Mean	SD	Total	Healthy controls Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
Grover et al, 2012 §§ (48)	0.17	0.06	11	0.15	0.04	10	49.9%	0.02 [-0.02, 0.06]
Plufgrad et al, 2016 (55)	0.152	0.04	12	0.134	0.046	6	50.1%	0.02 [-0.03, 0.06]
Total (95% CI)			**23**			**16**	**100.0%**	**0.02 [-0.01, 0.05]**

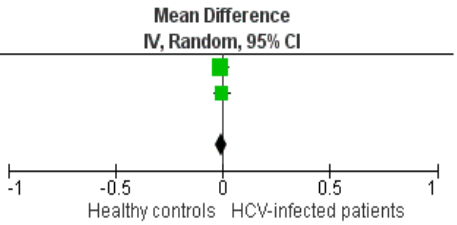
Heterogeneity: Tau² = 0.00; Chi² = 0.00, df = 1 (P = 0.95); I² = 0%
Test for overall effect: Z = 1.22 (P = 0.22)



j. PK11195 Binding Potential in Temporal Cortex

Study or Subgroup	HCV-infected patients Mean	SD	Total	Healthy controls Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
Grover et al, 2012 §§ (48)	0.14	0.05	11	0.15	0.03	10	56.7%	-0.01 [-0.04, 0.02]
Plufgrad et al, 2016 (55)	0.131	0.049	12	0.135	0.036	6	43.3%	-0.00 [-0.04, 0.04]
Total (95% CI)			**23**			**16**	**100.0%**	**-0.01 [-0.03, 0.02]**

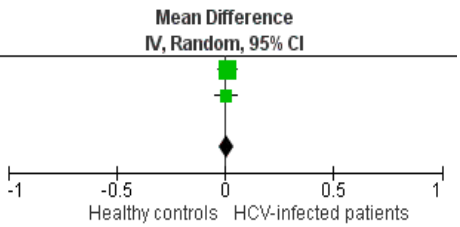
Heterogeneity: Tau² = 0.00; Chi² = 0.05, df = 1 (P = 0.82); I² = 0%
Test for overall effect: Z = 0.55 (P = 0.58)



k. PK11195 Binding Potential in Occipital Cortex

Study or Subgroup	HCV-infected patients Mean	SD	Total	Healthy controls Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
Grover et al, 2012 §§ (48)	0.18	0.06	11	0.17	0.04	10	60.2%	0.01 [-0.03, 0.05]
Plufgrad et al, 2016 (55)	0.155	0.053	12	0.151	0.055	6	39.8%	0.00 [-0.05, 0.06]
Total (95% CI)			**23**			**16**	**100.0%**	**0.01 [-0.03, 0.04]**

Heterogeneity: Tau² = 0.00; Chi² = 0.03, df = 1 (P = 0.86); I² = 0%
Test for overall effect: Z = 0.44 (P = 0.66)



a-d. Comparison of metabolite peak levels in basal ganglia and centrum semiovale white matter, between HCV infected patients and healthy controls. Random model and fixed model as sensitivity test are displayed.

e-k. Comparison of PK11195 binding potential in different brain areas between HCV infected patients and healthy controls. Random model and fixed model as sensitivity test are displayed.

The numbers in parenthesis correspond to the reference. §, §§ indicates two different imagine techniques in the same study.

List of abbreviation (alphabetical order): Cho: Choline; CI: confidence interval; Cr: Creatine; HCV: hepatitis-C virus; mI: Myo-Inositol; NAA, N-acetyl-aspartate; PET: Photon emission tomography; PK11195: peripheral benzodiazepine receptor radioligand ^{11}C-(R)-PK11195; SD: Standard Deviation.

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Brain, Behavior, and Immunity xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Association of chronic inflammation and perceived stress with abnormal functional connectivity in brain areas involved with interoception in hepatitis C patients

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ARTICLE INFO

Keywords:
Basal ganglia
Functional connectivity
Hepatitis C virus
IL-6
Inflammation
Insula
Depression
Perceived stress
Prostaglandin E_2
Sickness behavior
Interoception

ABSTRACT

Background: Sickness behavioral changes elicited by inflammation may become prolonged and dysfunctional in patients with chronic disease, such as chronic hepatitis C (CHC). Neuroimaging studies show that the basal ganglia and insula are sensitive to systemic inflammation.
Aim: To elucidate the clinical and neurobiological aspects of prolonged illnesses in patients with CHC.
Methods: Thirty-five CHC patients not treated with interferon-α or other antiviral therapy, and 30 control subjects matched for age and sex, were evaluated for perceived stress (perceived stress scale; PSS), depression (PHQ-9), fatigue and irritability through a visual analog scale (VAS), as well as serum levels of interleukin-6 (IL-6), prostaglandin E_2 (PGE_2) and oxidative stress markers. Functional MRI was performed, measuring resting-state functional connectivity using a region-of-interest (seed)-based approach focusing on the bilateral insula, subgenual anterior cingulate cortex and bilateral putamen. Between-group differences in functional connectivity patterns were assessed with two-sample t-tests, while the associations between symptoms, inflammatory markers and functional connectivity patterns were analyzed with multiple regression analyses.
Results: CHC patients had higher PSS, PHQ-9 and VAS scores for fatigue and irritability, as well as increased IL-6 levels, PGE_2 concentrations and antioxidant system activation compared to controls. PSS scores positively correlated with functional connectivity between the right anterior insula and right putamen, whereas PHQ-9 scores correlated with functional connectivity between most of the seeds and the right anterior insula. PGE_2 (positively) and IL-6 (negatively) correlated with functional connectivity between the right anterior insula and right caudate nucleus and between the right ventral putamen and right putamen/globus pallidus. PGE_2 and PSS scores accounted for 46% of the variance in functional connectivity between the anterior insula and putamen.
Conclusions: CHC patients exhibited increased perceived stress and depressive symptoms, which were associated with changes in inflammatory marker levels and in functional connectivity between the insula and putamen, areas involved in interoceptive integration, emotional awareness, and orientation of motivational state.

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https://doi.org/10.1016/j.bbi.2019.03.008
Received 21 November 2018; Received in revised form 6 February 2019; Accepted 9 March 2019

Please cite this article as: Giovanni Oriolo, et al., Brain, Behavior, and Immunity, https://doi.org/10.1016/j.bbi.2019.03.008

1. Introduction

Sickness behavior is a highly organized adaptive strategy to support the organism's defense against pathogens, and is characterized by changes in behavior, mood and cognition (Dantzer, 2001a; Garcia et al., 1955; Miller and Raison, 2016; Stieglitz, 2015).

The experience of "feeling sick" is common during acute infections or inflammatory responses to trauma (Hart, 1988; Miller and Raison, 2016). It clinically presents as a set of neurovegetative symptoms such as fatigue, anorexia, psychomotor retardation and increased sensitivity to pain, and is also associated with increased irritability, anhedonia, social responsiveness and increased stress sensitivity (Capuron and Miller, 2004; Dantzer et al., 2008; Maes et al., 2012). Animal and human studies suggest that soluble mediators, such as the pro-inflammatory cytokines interleukin- (IL-) 1, IL-6 and tumor necrosis factor-α (TNF-α), play a direct role in the development of sickness-related behaviors (Aubert et al., 1997; Avitsur et al., 1997; Dantzer, 2001b; Hart, 1988; Kent et al., 1996, 1992). Moreover, it has been observed that peripheral immunological activation may drive inflammation in the central nervous system, involving neurons, astrocytes and the microglia (Dantzer, 2009; Haroon et al., 2012; Miller et al., 2013).

Furthermore, neuroimaging studies have indicated that cortical and sub-cortical brain structures might play a relevant role in sickness behavior, identifying the insula, subgenual anterior cingulate cortex (sgACC) and basal ganglia, particularly the ventral striatum and substantia nigra (Harrison, 2017), as sensitive to peripheral inflammation. These studies involved inducing acute inflammation through the direct inoculation of endotoxins, such as the *Salmonella typhi* vaccine or lipopolysaccharide (LPS), or patients receiving treatment with the pro-inflammatory cytokine interferon- (IFN-) α (Capuron et al., 2012; Eisenberger et al., 2011; Harrison et al., 2009a; Udina et al., 2012). Situations in which the initial noxious stimulus cannot be removed could lead to prolonged and dysfunctional sickness behavior. Examples of this include chronic infections (i.e., human immunodeficiency virus (HIV) or hepatitis C virus (HCV) infections), auto-immune disorders (i.e., rheumatoid arthritis or inflammatory bowel disease) or chronic inflammatory conditions (i.e., cancer, diabetes or obesity), which have been often linked to an increased prevalence of depression (Liu et al., 2017). In this regard, increased perceived stress, fatigue, and irritability, which are also common in depressed patients (Chung et al., 2015; Farabaugh et al., 2004; Fava et al., 2010), have often been observed in chronic inflammatory conditions such as rheumatic diseases (Louati and Berenbaum, 2015), obesity (Capuron et al., 2016), cancer (Bower and Lamkin, 2013), and inflammatory bowel disease (Targownik et al., 2015).

Major depressive disorder (MDD) displays a phenomenological overlap with sickness behavior, and has been consistently associated with increased levels of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) and acute-phase proteins (such as the C-reactive protein) (Haapakoski et al., 2015; Kohler et al., 2016). Moreover, brain structural and functional alterations have been identified in several neuroimaging studies on depression, mainly in the prefrontal-limbic-subcortical areas that are involved in emotional processing and awareness, similar to those involved during acute inflammatory challenges (Drevets et al., 2008; Feng et al., 2016; Harrison, 2017; Mulders et al., 2015; Savitz and Harrison, 2018; Savitz and Drevets, 2009). However, the type of symptoms and illness course differ between MDD and sickness behavior. Typically, MDD is considered a lifetime progressive disease, which differs from the acute and adaptive nature of sickness behavior (Freeman et al., 2017; Oriolo et al., 2018a).

Moreover, depression can involve biological pathways that are different from those associated with acute pro-inflammatory cytokine stimulation, such as cell-mediated immune activation, dysregulated anti-inflammatory mechanisms, neural sensitization to immune responses or auto-immunity processes (Dantzer et al., 2008). Importantly, the activation of oxidative and nitrosative stress (O&NS) pathways, resulting in increased levels of reactive oxygen and nitrogen species (ROS and RNS, respectively) that damage lipids, proteins and DNA, may be crucial in the chronic and progressive course of depression (Liu et al., 2015; Moylan et al., 2013).

Thus, as sickness behavior and depression share clinical phenomenology, inflammatory pathways and brain functional changes, it has been hypothesized that prolonged and dysregulated sickness behavior may contribute to the development of MDD in vulnerable patients (Capuron and Castanon, 2012; Rosenblat et al., 2014). Some studies in chronic hepatitis C (CHC) patients have tried to elucidate the neurobiological and neuroanatomical links between chronic inflammatory conditions and prolonged sickness behavior, excluding subjects with current severe mental illness and considering several ranges of neuropsychiatric symptoms such as depression, anxiety, fatigue or cognition (Aregay et al., 2018; Huckans et al., 2014; Loftis and Hauser, 2008). Depression is the leading cause of disability worldwide (World Health Organization, 2017), affecting > 300 million people, a substantial proportion of whom do not respond adequately to current pharmacological therapies (Rush et al., 2006; Stotland, 2012); therefore, understanding the pathophysiological mechanisms linking inflammation to sickness and MDD seems to be crucial in developing new therapeutic targets (Udina et al., 2015, 2014). Moreover, CHC is a well-known systemic disease with a plethora of extrahepatic manifestations such as chronic kidney disease, mixed cryoglobulinemia, increased rates of insulin resistance, diabetes, and atherosclerosis, increased cardiovascular morbidity and neuropsychiatric symptoms, among others (Grignoli et al., 2015). Accordingly, the purpose of this study was to elucidate the clinical and neurobiological correlates of a prolonged sickness condition associated with chronic inflammation in a case-control study of patients with CHC not treated with IFN-α or others antiviral therapies, and without MDD. We hypothesized that chronic low-grade inflammation secondary to CHC can induce alterations in brain connectivity in areas associated with interoceptive integration and awareness, emotional processing and orientation of motivational state, with such alterations correlating with some aspects of sickness behavior.

2. Materials and methods

2.1. Participants

Fifty-one Caucasian outpatients aged between 18 and 55 years with CHC who were candidates for antiviral treatment, either with the pegylated IFN-α and ribavirin (RBV) combination or with the new direct-acting antivirals (DAA), were recruited between 2014 and 2016 at the Liver Units of two general university hospitals (Hospital Clínic and Hospital del Mar) in Barcelona. None of the patients had previously received anti-viral treatment (pegylated IFN-α or DAA). The exclusion criteria for the study were as follows: not fluent in Spanish language, the presence of other concomitant liver diseases, decompensated cirrhosis or hepatocarcinoma, HIV or HBV co-infection, any chronic disease or inflammatory condition (e.g., diabetes, asthma, obesity (body mass index ≥ 30) or cancer), auto-immune diseases (e.g., rheumatoid arthritis), any lifetime neurological disease or major psychiatric disorders (psychosis or bipolar disorder), any depressive or anxiety disorders until the preceding year, any drug or alcohol use disorder (except tobacco use) up until the preceding year, and the presence of metallic protheses or pacemakers. Patients were also excluded if they presented an uncontrolled medical condition or were receiving any anti-inflammatory treatment (e.g., corticoids, statins, non-steroidal anti-inflammatory drugs or antidepressant/anxiolytic drugs in the last six months). Moreover, 31 control participants without HCV infection, who were matched for age, sex and laterality, were also recruited in the same time period at the Human Pharmacology unit, using the same exclusion criteria outlined above. Controls received a monetary reward

to cover time and travel expenses.

After obtaining informed consent, all the participants underwent a detailed medical history check, routine laboratory tests and physical examinations to determine whether they met the inclusion criteria. Case patients were checked for HCV genotype, source of infection, viral load and grade of liver fibrosis (by means of a liver biopsy, indirect tests of fibrosis, ultrasound examination and/or transient elastography when available). Control participants were checked for anti-HCV antibodies, to ensure the absence of any HCV exposure. All subjects were interviewed by a senior psychiatrist using the Mini-International Neuropsychiatric Interview (MINI) (Sheehan et al., 1998) to assess for current or past psychiatric disorders. Nine patients were excluded due to the presence of current psychiatric disorders. Five patients dropped out as they did not consent to the laboratory and fMRI assessments. Finally, two cases were excluded from the analyses due to excessive head motion during fMRI acquisition. One control subject was excluded due to non-optimal data acquisition. A final sample of 65 participants, 35 patients with CHC and 30 controls, were studied.

Clinical history and sociodemographic variables were collected for all the participants. The institutional review boards approved the study protocol (CEIC of Hospital Clínic and Hospital del Mar), which followed the tenets of the Declaration of Helsinki. All the participants were recruited after providing proper written informed consent.

2.2. Behavioral assessment

The validated Spanish version of Patient Health Questionnaire 9 (PHQ-9) (Diez-Quevedo et al., 2001) was used to evaluate the subthreshold symptoms of depression. PHQ is a brief instrument that covers a wide range of psychopathology and is used to diagnose specific disorders, with the items corresponding to the symptom criteria for each disorder as outlined in the DSM-IV-TR (Kroenke et al., 2010). Furthermore, PHQ has been validated across a variety of medical conditions in primary care settings, including CHC patients (Navinés et al., 2012; Spitzer et al., 1999). PHQ-9 has nine items with four response options ("not at all", "several days", "more than half the days" and "nearly every day") rated from 0 to 3. It can be used as a continuous measure, with scores ranging from 0 to 27 and the cut-off points of 5, 10, 15 and 20 representing mild, moderate, moderately severe and severe levels of depressive symptoms. Patients reporting "more than half the days" for 5 or more of the 9 items of PHQ-9 and presenting a depressed mood or anhedonia were considered to have MDD (Kroenke et al., 2010). Those reporting "more than half the days" in the past two weeks for two, three or four of the items were considerd to have another depressive disorder (Navinés et al., 2012).

The intensity of fatigue and irritability was assessed through a visual analog scale (VAS-f and VAS-i) (Folstein and Luria, 1973) which is a visual tool in which the patient is asked to place an arrow on a line that ranges from 0 to 100 mm from left to right (0 = no fatigue or no irritability and 100 = severe fatigue or extreme irritability). VAS is a well-validated scale that can be used to determine illness severity and can be rapidly self-administered (Killgore, 1999). We used irritability and fatigue scores as they reflect the components of sickness behavior and depression that are not exhaustively covered by PHQ-9.

The perceived stress scale (PSS) is easy to use and provides valuable additional information about the relationship between perceived stress and pathology (Cohen, 1983). It includes 14 items scored on a 5-point Likert scale (0–4) with the total score ranges from 0 to 56, and can be administered in a few minutes (Remor, 2006). It has been used in different studies (He et al., 2014; Nagano et al., 2004; Vere et al., 2009) addressing stress in patients with liver diseases, as stress has been linked to the initiation, course and outcome of liver disease (Vere et al., 2009).

2.3. Biological measurements

Blood samples (10 ml of venous blood) for measuring serum concentrations of inflammatory markers were obtained at the same day as the behavioral assessment was performed, and no > 5 days before the image acquisition. Samples were collected at 09.00 in the morning after overnight fasting and were centrifuged (10 min, 1000*g* at 4 °C) after clotting and sera were stored at −80 °C until analysis.

Enzyme-linked immunosorbent assays (ELISA) were used to identify and quantify the immunological biomarkers. IL-6 was quantified using the Human IL-6 High sensitivity ELISA kit (Diaclone®, Item 950.035.192). No dilution was performed, and the chromatography absorption peak was at 450 nm. The results are shown as pg/ml and the assay had a sensitivity of 0.81 pg/ml and an overall intra-assay coefficient of 4.4% (Cassidy et al., 2002; Mukhopadhyay et al., 2016; Pemberton et al., 2009). The inflammatory prostaglandin PGE_2 was quantified using the PGE_2 ELISA kit - Monoclonal (Cayman® Chemical, Item 514010). Samples were diluted 1:40 in ELISA Buffer and the chromatography absorption peak was at 412 nm. The results are shown as pg/ml and the assay had a detection limit of 15 pg/ml and an overall intra-assay coefficient of 8.8% (Lyons et al., 2014). The anti-inflammatory prostaglandin 15-deoxy-$\Delta^{-12,14}$-prostaglandin J_2 (15d-PGJ_2) was quantified using the 15d-PGJ_2 ELISA kit - Monoclonal (ENZO®, Item ADI-900–023). Samples were diluted 1:4 in ELISA Buffer and the chromatography absorption peak was at 405 nm. The results are shown as pg/ml and the assay had a sensitivity of 36.8 pg/ml and an overall intra-assay coefficient of 6.2% (Wang et al., 2011). Enzymatic colorimetric assays were used to identify and quantify the oxidative stress biomarkers. Superoxide dismutase (SOD) activity was quantified using the DetectX® Colorimetric Activity kit (Arbor Assays, Item K028-H1). Samples were diluted 1:10 in Assay Buffer and the chromatography absorption peak was at 450 nm. The results are shown as units per ml (U/ml), one SOD unit being the amount of enzyme required to inhibit the 50% reduction of superoxide radicals. The sensitivity of the assay was 0.044 U/ml and the overall intra-assay coefficient was 9.6% (MacDowell et al., 2016). Catalase (CAT) activity was quantified using the DetectX® Colorimetric Activity kit (Arbor Assays, Item K033-H1). Samples were diluted 1:20 in Assay Buffer and the chromatography absorption peak was at 560 nm. The results are shown as units per ml (U/ml), one CAT unit being the amount of enzyme required to degrade 1 μM of hydrogen peroxide per minute at 25 °C at a pH of 7.0. Sensitivity was determined as 0.052 U/ml and the overall intra-assay coefficient was 4.1% (Ruiz-Ojeda et al., 2016). Gluthatione peroxidase (GPx) activity was quantified using the Glutathione Peroxidase Assay kit (Cayman Chemical, Item 703102). Samples were diluted 1:4 in Sample Buffer and the chromatography absorption peak was at 340 nm. The results are shown as "activity of GPx" (nmol/min/ml), one GPx unit being the amount of enzyme required to oxidize 1 nM of NADPH into $NADP^+$ per minute at 25 °C. The intra-assay coefficient of variation was 5.7% and the dynamic range 50–344 nmol/min/ml (Ceballos-Picot et al., 1992). As an index of peroxidation of lipid components, malondialdehyde (MDA) levels were quantified without dilution using the TBARS Assay Kit (Cayman Chemical, Item 10009055), which is based on the reaction between thiobarbituric acid (TBA) and MDA. This produces the MDA-TBA complex (which is referred to as thiobarbituric acid reactive substances, TBARS), which presents a chromatography absorption peak at 530–540 nm. The results are shown as μM for the entire sample. The intra-assay coefficient of variation was 5.5% and the dynamic range 0–50 μM (Joshi et al., 2018).

Spectrophotometric analysis was conducted using the ELISA spectrophotometer Synergy 2 (BioTek®, USA) and the Gen5 Data Analysis Software (BioTek®, USA). Statistical analysis was carried out using the statistical software GraphPad Prism 6 (GraphPad Software, Inc., USA).

2.4. Functional magnetic resonance imaging and connectivity analysis

2.4.1. Image acquisition

Resting-state functional magnetic resonance (fMRI) connectivity explores the correlation and integration of brain activity between brain regions regardless of their anatomical connection. The connectivity is assessed by measuring the blood oxygenation level-dependent (BOLD) time series of activations in different brain regions in subjects at resting state (that is, no task is being performed) (Dennis and Thompson, 2014). In our study, images were obtained using a 1.5 T Signa Excite system (General Electric, Milwaukee, WI, USA) equipped with an eight-channel phased-array head coil and single-shot echo planar imaging (EPI) software. The functional sequence consisted of gradient recalled acquisition in the steady state under the following parameters: time of repetition (TR), 2,000 ms; time of echo (TE), 50 ms; pulse angle, 90°; field of view (FOV), 24 cm; 64 × 64 pixel matrix; slice thickness, 4 mm plus an interslice gap of 1.5 mm. Twenty-two interleaved slices were acquired parallel to the anterior-posterior commissure line covering the whole brain. A 6-minute continuous resting-state scan was performed on each participant. Participants were instructed to relax, stay awake and lie still without moving, while keeping their eyes closed throughout. This scan generated 180 whole-brain EPI volumes. The first four (additional) images in each run were discarded to allow magnetization to reach equilibrium.

2.4.2. Image processing

Imaging data were processed in a Microsoft Windows platform using the Statistical Parametric Mapping software (SPM8; Wellcome Department of Imaging Neuroscience, London, UK; http://www.fil.ion.ucl.ac.uk/spm/) running on MATLAB (MathWorks Inc., Natick, MA, USA). Image preprocessing involved motion correction, spatial normalization and smoothing using a Gaussian filter (full-width at half-maximum, 8 mm). Functional images were normalized to the standard SPM EPI template and resliced to a 2-mm isotropic resolution in Montreal Neurological Institute (MNI) space. A high-pass filter set at 128 s was used to remove low-frequency drifts of less than aproximately 0.008 Hz. All image sequences were inspected for potential acquisition and normalization artifacts.

2.4.3. Control of potential head motion effects

To control for the effects of head motion, the following procedures were adopted. Conventional SPM time series alignment to the first image volume was undertaken in each participant and 12 motion-related regressors and estimates of global brain signal fluctuations were included as confounding variables in our first-level (single-subject) analyses. Furthermore, within-subject, censoring-based MRI signal artifact removal (scrubbing) (Power et al., 2014) was used to discard motion-affected volumes. For each participant, interframe motion measurements (head position variations in each brain volume compared to the previous volume) served as an index of data quality to flag volumes of poor quality across the run. At points with interframe motions > 0.2 mm, we discarded the corresponding volume, the volume immediately preceding it and the following two volumes. Finally, potential motion effects were removed using a summary measurement for each participant (mean interframe motion across the fMRI run) as a covariate in the second-level (group) analyses in SPM (Pujol et al., 2014a).

2.4.4. Functional connectivity analysis

Resting-state functional connectivity analysis can be performed in several ways, including seed-based, independent component analysis-based and/or cluster-based methods. In our study, it was assessed using a region-of-interest (seed)-based approach, as detailed previously (Harrison et al., 2013; Pujol et al., 2014b). In this approach, a brain region ("seed") of interest is selected and the time course of activation in that seed is extracted. Brain regions with strong positive correlations with the seed are defined as functionally coupled (Dennis and Thompson, 2014). We based our analysis on brain regions reported to be associated with systemic inflammation and mood changes in previous neuroimaging studies (Felger et al., 2016; Hanken et al., 2014; Labrenz et al., 2016; Seminowicz et al., 2004). Our *a priori* primary region of interest was the insula, which has an important role in interoceptive and emotional awareness, particularly in its anterior part (Craig, 2009). Selected secondary regions representative of our network of interest were the subgenual anterior cingulate cortex (sgACC), considered crucial in emotional processing and mood regulation, and the putamen, which together with the caudate nucleus forms the striatum, the main input structure of the basal ganglia presenting one of the highest metabolic activities in the brain (Wichmann and De Long, 2013). Two maps were obtained using ventral and dorsal striatal seeds to comprehensively assess its functional connectivity, as it is made up of distinct functional subdivisions. Relevantly, activity changes (e.g., glucose metabolism and functional connectivity) in both the insula and the anterior cingulate cortex have been associated with inflammatory markers (Hanken et al., 2014; Hannestad et al., 2012). Thus, a total of four functional connectivity MRI maps were generated using peak coordinates taken from previous studies that were converted into MNI in mm and located bilaterally at the anterior insula ($x = \pm 38$, $y = 25$, $z = 5$), sgACC ($x = 8$, $y = 17$, $z = -9$), and ventral ($x = \pm 20$, $y = 12$, $z = -3$) and dorsal ($x = \pm 28$, $y = 1$, $z = 3$) putamen.

For all the locations, seeds were defined as 3.5-mm radial spheres (sampling approximately 25 voxels) using the MarsBaR region-of-interest toolbox in MNI stereotaxic space (Brett et al., 2002). To generate the seed maps, the signal time course of a selected seed region was calculated as the average signal of the voxels included in the seed at each time point and was used as a regressor to be correlated with the signal time course of every voxel in the brain. The obtained voxel-wise regression coefficients served to build first-level SPM contrast images. This process was performed for each subject and seed separately. To remove potential sources of physiological noise, we derived estimates for white matter, CSF, global brain signal fluctuations and 12 motion-related regressors to be included as confounding ("nuisance") variables alongside the variables of interest in each individual (first-level) SPM analysis.

The resulting first-level contrast images for each participant were then included in second-level (group) random-effects analyses. One-sample t-statistic maps were generated to obtain the functional connectivity maps for each group, while two-sample t-tests were performed to map between-group differences for the contrasts: CHC patients $>$ controls and CHC patients $<$ controls. In addition, whole-brain voxel-wise analyses in SPM were performed to map the correlation between resting-state functional connectivity measurements in our regions of interest and inflammatory markers (i.e., PGE_2, IL-6 and 15d-PGJ_2 as independent regressors) and behavioral outcomes (i.e., PSS, PHQ-9, fatigue and irritability scores as independent regressors) in participants with CHC. To assess the influence of sickness behavior symptoms on the relationship between inflammation and functional connectivity, the correlation maps were re-estimated after covarying for the patients' clinical scores.

Finally, a multiple regression analysis was performed to assess the combined contribution of inflammatory markers and behavior to functional connectivity measurements in the patient group. Functional connectivity measurements were included as the dependent variable and the potential predictors were serum PGE_2 levels and PSS scores.

2.4.5. Thresholding criteria

To control for multiple comparisons within seed-based analyses, results were considered to be significant with clusters above 2.2 ml (277 voxels) at a height threshold of $p < 0.005$, which satisfied the family-wise error (FWE) rate of $P_{FWE} < 0.05$ at the cluster level according to Monte Carlo simulations. Resting-state fMRI data were also adjusted for multiple testing (accounting for seven variables) using Bonferroni

correction (significant cluster size ≥ 3.2 ml).

2.5. Data and statistical analyses

Characteristics of the study sample were summarized using the mean and standard deviation (SD) for continuous variables and percentages for categorical variables. Sociodemographic features of the participants were compared between the groups using two-sample t-tests for continuous variables and chi-square tests for categorical variables. Behavioral assessment scores (PSS, PHQ-9, and VAS scores) were compared between the groups using multivariate analysis, controlling for age, sex and tobacco use, to evaluate possible interactions of these factors.

A Shapiro-Wilk test of normality was performed to determine the distribution of the biological marker variables (IL-6, PGE_2, 15d-PGJ_2, TBARS, GPx, SOD and CAT). Measurements that had > 3 SD above or below the mean were considered outliers and excluded from the analyses. Biomarker serum levels were log transformed if they were not normally distributed. Univariate analysis for independent samples was conducted (*t*-test) to compare values between the groups. Multivariate analyses controlling for age, sex and tobacco use were also performed.

Statistical analyses were undertaken with SPSS version 23.0. The tests of significance were two-tailed, with the degree of significance set at $p < 0.05$.

3. Results

3.1. Characteristics of the study participants

The study sample comprised 35 patients with CHC and 30 control subjects without HCV infection matched for sex, age and laterality. About two-thirds of the participants were male (66.1%) and the mean age of the study sample was 40.2 years (SD = 9.4; range 18–52) (Table 1). Genotype 1 was the most common HCV genotype (80%), whereas the route of HCV infection could not be ascertained in most of the cases (74.3%). The median of HCV RNA (viral load) was $1.5x10^6$ IE/mL (range = 3.0×10^5–6.3×10^6). Only two patients (5.7%) had advanced of fibrosis and compensated cirrhosis (inclusion criteria for antiviral treatment; see Table 1). Furthermore, CHC patients more actively used tobacco than control subjects ($\chi^2 = 5.737$, $p = 0.017$).

In the whole sample, 21.5% of the individuals had a past history of depression/anxiety disorders up until one year before the start of the study. Among the CHC patients, 25.7% had a past history of psychiatric disorders, whereas 16.7% of the control sample had a past history of depression/anxiety. No other differences were found between the groups.

Table 1
Characteristics of the study participants.

	Whole sample N = 65 Mean (SD)/ N (%)	CHC patients N = 35 Mean (SD)/N (%)	Controls N = 30 Mean (SD)/N (%)
Age, Mean (SD)	40.2 (± 9.4)	41.1 (± 9.6)	39.2 (± 9.1)
Female (%)	22 (33.9)	12 (34.3)	10 (33.3)
Marital status	46 (72.1)	26 (74.2)	20 (66.7)
Educational level			
Middle school or less	9 (13.8)	7 (20.0)	2 (6.7)
Professional diploma or undergraduate degree	28 (43.1)	15 (42.9)	13 (43.3)
Graduate degree	28 (43.1)	13 (37.1)	15 (50.0)
Job status			
Student	3 (4.6)	2 (5.7)	1 (3.3)
Active	55 (84.6)	28 (80.0)	27 (90.0)
Unemployed/retired	7 (10.8)	5 (14.3)	2 (6.7)
Depression/Anxiety (> 1 year before)			
Depressive disorders	9 (13.8)	6 (17.1)	3 (10.0)
Anxiety disorders	9 (13.8)	7 (20.0)	2 (6.7)
Family history of psychiatric disorders	27 (41.5)	15 (42.9)	12 (40.0)
Current tobacco use	18 (27.7)	14 (40.0) *	4 (13.3)
HCV genotype			
Gen 1		28 (80.0)	
Gen 2		2 (5.7)	
Gen 3		1 (2.9)	
Gen 4		4 (11.4)	
Source of HCV infection			
Transfusion		3 (8.6)	
Parenteral drug use		3 (8.6)	
Surgery		3 (8.6)	
Unknown		26 (74.3)	
HCV RNA IE/mL median (range)		1.5×10^6 (3.0×10^5-6.3×10^6)	
Compensated cirrhosis		2 (5.7)	

Comparisons were performed between groups of CHC patients and healthy controls. Analyses were performed using two-sample t-tests for continuous variables and chi-square tests for categorical variables.
Abbreviations: CHC, chronic hepatitis C; HVC, hepatitis C virus; SD, standard deviation; SUD, substance use disorder
* $p < 0.05$

3.2. Biological markers

As illustrated in Table 2, CHC patients showed higher serum levels of the pro-inflammatory cytokines IL-6 and PGE_2 compared to control subjects, which was statistically significant ($t = -4.352$, $p < 0.001$ and $t = -4.228$, $p < 0.001$, respectively). Regarding anti-inflammatory markers, significant differences were observed in the 15d-PGJ_2 serum levels ($t = -4.805$, $p < 0.001$), with CHC patients showing lower levels. The antioxidant enzymatic system was activated in CHC patients compared to controls, as highlighted by the increased serum levels of SOD ($t = -2.474$, $p = 0.016$) and CAT ($t = -4.328$, $p < 0.001$). GPx levels were not significantly increased in CHC patients ($t = 0.208$, $p = 0.836$). In line with these results, the serum levels of MDA-TBARS, a final product of lipid peroxidation and an indicator of oxidative stress, were lower in CHC patients than in control subjects ($t = -2.201$, $p = 0.032$), demonstrating the correct functioning of the antioxidant system.

When controlling for age, sex and active tobacco use, the differences in the biological marker serum levels between the groups remained significant after covariance (IL-6: $F = 15.379$, $p < 0.001$; PGE_2: $F = 16.506$, $p < 0.001$; 15d-PGJ_2: $F = 21.779$, $p < 0.001$; MDA-TBARS: $F = 5.764$, $p = 0.020$; SOD: $F = 4.582$, $p = 0.036$; CAT: $F = 18.344$, $p < 0.001$).

3.3. Behavioral assessment: PHQ-9, VAS-f, VAS-i and PSS scores

Total PHQ-9 scores were significantly higher in CHC patients than in healthy controls ($t = -2.914$, $p = 0.005$). As expected, the mean values observed could not be considered clinically relevant (see Table 2). After controlling for age, sex and tobacco use, this difference remained significant ($F = 5.883$, $p = 0.018$). Interestingly, the first item of PHQ-9, which evaluates anhedonia ("little interest or pleasure in doing things"), was also significantly higher in CHC patients ($t = -2.029$, $p = 0.047$). After categorical diagnosis using the PHQ-9 questionnaire, MDD was observed in 1 CHC patient; however, the clinical interview (MINI) was negative for current MDD. The difference with the control group was not statistically significant ($\chi^2 = 3.653$, $p = 0.118$).

Significant differences were observed in both the irritability (VAS-i) and fatigue (VAS-f) scores ($t = -3.484$, $p = 0.001$ and $t = -2.652$, $p = 0.01$, respectively), with the mean scores in CHC patients (Table 2) being in line with those previously reported (Udina et al., 2012). Finally, PSS scores were significantly increased in CHC patients compared to healthy controls ($t = -3.528$, $p = 0.002$). When controlled for age,

Table 2
Clinical outcomes and serum concentrations of biological markers.

Clinical outcomes	CHC patients N = 35 Mean (± SD)	Controls N = 30 Mean (± SD)
PHQ-9		
Total score	3.6 (± 3.9) *	1.4 (± 1.9)
Anhedonia ("Little interest or pleasure in doing things")	0.46 (± 0.7) *	0.17 (± 0.4)
VAS		
Irritability	3.4 (± 2.1) **	1.8 (± 1.5)
Fatigue	3.6 (± 2.2) *	2.2 (± 1.9)
PSS		
Total score	19.5 (± 11.1) **	12.6 (± 5.5)
Pro-inflammatory markers		
IL-6, pg/ml	1.90 (± 0.88) ***	1.15 (± 0.72)
PGE_2, pg/ml	1627.90 (± 995.83)[a] ***	1050.73 (± 1432.67)[b]
Anti-inflammatory markers		
15d-PGJ_2, pg/ml	38904.08 (± 54935.06)[a] ***	232669.17 (± 359036.46)
Oxidative stress markers		
MDA-TBARS, μM	12.70 (± 4.27) *	16.07 (± 6.17)
Anti-oxidant activity markers		
GPx, nmol/min/ml	80.56 (± 13.61)	82.91 (± 19.85)
SOD, U/ml	0.74 (± 0.23) **	0.59 (± 0.20)
CAT, U/ml	52.64 (± 23.60) ***	27.63 (± 16.71)

Comparisons were performed between groups of CHC patients and healthy controls. Analyses were performed using two-sample t-tests for continuous variables, which were log transformed in the case of the serum concentrations of biological markers. Analysis of covariance was further conducted, controlling for age, sex and tobacco use.

Abbreviations: 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; CAT, catalase; CHC, chronic hepatitis C; GPx, gluthatione peroxidase; IL-, interleukin-; MDA-TBARS, malondialdehyde-thiobarbituric acid reactive substances; PGE_2, prostaglandin E_2; PHQ, physical health questionnaire; PSS, perceived stress scale; SD, standard deviation; SOD, superoxide dismutase; VAS, visual analog scale.

*p < 0.05
**p < 0.01
***p < 0.001

[a]Missing data in 1 patient
[b]Missing data in 2 patients
[c]Missing data in 5 patients

sex and tobacco use, differences in the VAS-f, VAS-i and PSS total scores between the groups remained significant (F = 8.374, p = 0.005; F = 4.793, p = 0.032 and F = 6.408, p = 0.014, respectively), highlighting the effects of CHC on psychometric alterations.

3.4. Association between biological markers and clinical outcomes

The association between inflammation and clinical outcome related to sickness behavior was not confirmed, as no significant linear associations were found between the clinical scores and biological markers in CHC patients. Nevertheless, when considering the whole sample, significant positive correlations were observed between PGE_2 serum levels and the PHQ-9 total score (r = 0.298, p = 0.019), and between PGE_2 levels and PSS scores (r = 0.245, p = 0.055), whereas negative correlations were found between 15d-PGJ_2 and the VAS-i score (r = −0.255, p = 0.042) and between 15d-PGJ_2 and the VAS-f score (r = −0.294, p = 0.018). See Table S1 for details.

3.5. Functional connectivity analysis and differences between the groups

Within-group maps. One-sample (group) seed maps corresponded to well-defined functional connectivity networks in both CHC patients and controls. Positive correlations were found between our regions of interest and cortical frontal areas (e.g., insula and operculum, lateral prefrontal cortex, ACC, supplementary motor area) and ventral brain structures. Negative correlations were found with the seed regions mostly involved the medial prefrontal cortex, parietal areas (e.g., angular gyri, precuneus), occipital cortices and the cerebellum. Figures S1 to S4 in the Supplementary Material illustrate the within-group functional connectivity maps. No substantial hemispheric differences were noted for any of the maps.

Between-group differences. A comparison of functional connectivity between groups identified few differences, as illustrated in Fig. 1. Specifically, compared to control subjects in the contrast CHC < controls, patients showed a significant increase in the negative correlation (more anticorrelation) between the left dorsal putamen and left angular gyrus, and between the sgACC and the fusiform gyrus. Conversely, in the contrast CHC > controls, patients showed a significant increase in the positive correlation between the right ventral putamen and left frontal operculum, as well as a reduction in the negative

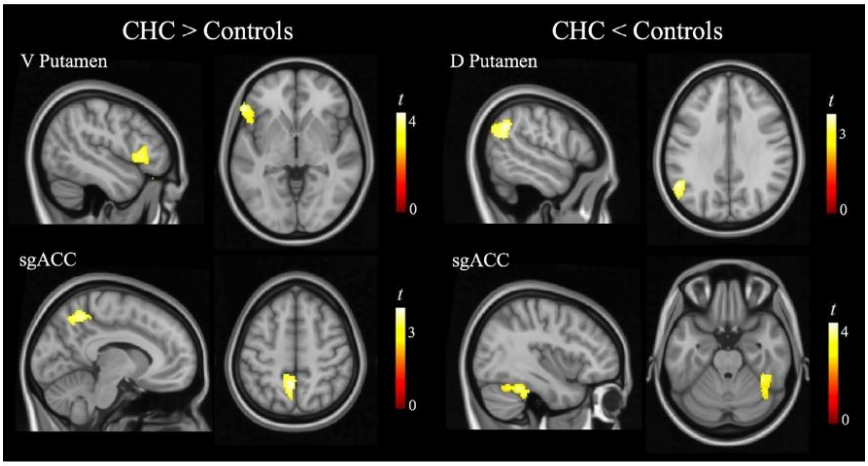

Fig. 1. Between-group differences in functional connectivity. CHC, chronic hepatitis C patients; V, ventral; D, dorsal; ACC, anterior cingulate cortex. The right hemisphere corresponds to the right side of axial and coronal views.

Table 3
Between-group differences in functional connectivity maps.

	CHC patients < Controls					
	Cluster size (ml)	x	y	z	t	Adj. t
Left Dorsal Putamen map						
Angular gyrus	4.3	−54	−56	34	3.8	3.3
Subgenual ACC map						
Fusiform gyrus	2.7	42	−42	−28	4.4	4.4
	CHC patients > Controls					
Right Ventral Putamen map						
Frontal operculum	3.0	−56	32	−6	4.3	4.4
Subgenual ACC map						
Precuneus	2.6	−6	−52	52	3.9	4.0

x y z, coordinates (mm) given in Montreal Neurological Institute (MNI) space. Statistics at cluster-level corrected threshold $P_{FWE} < 0.05$ estimated using Monte Carlo simulations. *Abbreviations:* CHC = chronic hepatitis C; ACC = anterior cingulate cortex. *Adj.t* = Model adjusted for tobacco use.

correlation (lower anticorrelation) between the sgACC and precuneus. No significant differences were observed when controlling for tobacco use, as shown in Table 3.

3.6. Functional connectivity analysis and correlation with biological markers

In CHC patients, serum levels of PGE_2 showed a significant positive correlation with functional connectivity measurements between the right anterior insula and regions in the basal ganglia and related structures (more PGE_2 associated with more connectivity). Positive correlations were also observed with functional connectivity between the right putamen seeds and adjacent regions in the basal ganglia (see Table S2). Fig. 2 illustrates the main findings. No significant positive associations were observed for the left hemisphere seeds.

Regression analysis with IL-6 serum levels showed a significant negative correlation with functional connectivity measurements in the basal ganglia (more IL-6 associated with less connectivity) in both the insula and the dorsal and ventral putamen seed maps only for the right hemisphere (Fig. 3 and Table S3). Of note, functional connectivity between the anterior insula and the right caudate nucleus, as well as between the ventral putamen and the right putamen/globus pallidus (Fig. 3, top and bottom rows), was positively associated with increasing PGE_2 levels and negatively associated with increasing IL-6 levels. Fig. 4 shows the scatter plots illustrating these correlations.

Finally, no significant correlations were found between the serum levels of the anti-inflammatory marker 15d-PGJ_2 and functional connectivity of our regions of interest.

After adjusting for clinical variables, the associations between functional connectivity and the inflammatory markers stayed generally consistent in terms of direction and statistical significance (see Table S2 and S3).

3.7. Functional connectivity analysis and correlation with clinical outcomes

In the whole-brain analysis, the severity of perceived stress positively correlated with functional connectivity between the right anterior insula and the right putamen (more perceived stress being associated with more connectivity). Putamen connectivity maps reciprocally confirmed the specificity of the association, showing a positive correlation of PSS scores with functional connectivity between the dorsal putamen and the left and right insulae (Fig. 5 and Table S4).

Depressive symptoms showed a significant positive correlation with functional connectivity between most of our regions of interest and the insula (higher PHQ-9 score, more connectivity; see Table S5). Fig. 5 illustrates the pattern of correlations and shows the extent to which the identified associations overlap with the results of PSS score analysis. Of note, the largest changes were identified at the anterior insula, predominantly in the right hemisphere. Interestingly, this effect was more specific when only the first item of the PHQ-9 scale, which evaluates anhedonia, was considered. That is, anhedonia scores positively correlated with functional connectivity between the putamen (dorsal and ventral, left and right) and the right anterior insula (see Figure S5).

Fatigue and irritability scores (independently) provided similar results, also demonstrating a significant positive correlation with functional connectivity (higher scores being associated with stronger connectivity) between the dorsal putamen and the left insula in the case of fatigue, and between the dorsal putamen and bilateral insula in the case of irritability scores (see Tables S6, S7).

A similar pattern of correlations was obtained after including inflammatory markers as covariates in the analyses (Tables S4–S7).

3.8. Multiple regression analysis

Overall, results indicated that the inflammatory markers PGE_2 and IL-6 were associated with functional connectivity changes between the insular cortices and structures in the basal ganglia and within the basal ganglia. Symptoms of sickness behavior, in turn, were associated with functional connectivity changes in regions that partially overlapped with the changes associated with the inflammatory markers (e.g., Fig. 2, Fig. 5 and Tables in Supplementary Material). A multiple regression analysis including measures from both inflammatory markers and clinical outcomes showed that PGE_2 and PSS scores accounted for significant unique variance in the functional connectivity between the anterior insula and putamen (Fig. 6). In a stepwise approach, (1) increased PGE_2 serum levels and (2) increased PSS scores were entered into the equation, accounting for 46% of the variance in functional connectivity measurements (adjusted R square = 0.42).

4. Discussion

Results from this study indicate that increased inflammation, as reflected by increased IL-6 and PGE_2 serum levels, and greater perceived stress and subclinical depressive symptoms in patients with CHC are associated with abnormal functional connectivity in brain regions associated with interoceptive awareness (see Figure S6).

We observed that patients with CHC perceived more stress and reported greater levels of irritability and fatigue than control subjects, as expected for patients with chronic disease. Moreover, the PHQ-9 scores reflected the increased subclinical depressive symptoms in CHC patients, particularly anhedonia and a reduced interest in doing things. This subtle difference was particularly suggestive, as a diagnosis of MDD was an exclusion criterion in this study. Symptoms of fatigue, irritability and anhedonia have been widely described as part of sickness behavior (Dantzer, 2009). These may persist in chronic inflammatory conditions without reaching greater clinical relevance and are the most common complaints of CHC patients (D'Mello and Swain, 2014; Huckans et al., 2014; Yarlott et al., 2017).

As expected, CHC patients showed increased serum levels of inflammatory mediators, namely IL-6 and PGE_2, as well as reduced levels of the anti-inflammatory 15d-PGJ_2. These findings, in line with previous studies (Aregay et al., 2018; Senzolo et al., 2011; Shah et al., 2015; Waris and Siddiqui, 2005), demonstrated the increased inflammatory activity in patients with CHC. IL-6 is a highly versatile pro-inflammatory cytokine with pleiotropic effects, which is secreted in response to environmental stress factors, such as infections or obesity (Castanon et al., 2014; Tanaka et al., 2014), and contributes to the development of chronic inflammatory illnesses (Baran et al., 2018). Furthermore, IL-6 is involved in several physiological functions in the central nervous system, such as neuron homeostasis. Thus, its chronic dysregulation may lead to various diseases (Rothaug et al., 2016;

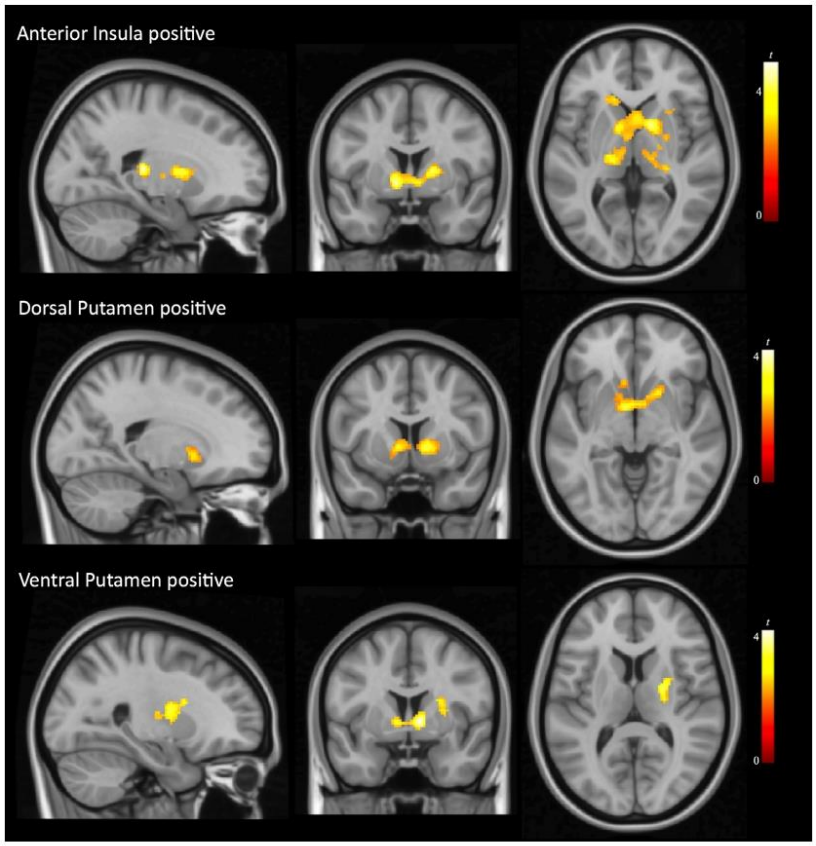

Fig. 2. Representative correlation analysis for the pro-inflammatory marker prostaglandin E_2 (PGE_2) in chronic hepatitis C (CHC) patients. *Top panel:* positive correlation of PGE_2 serum levels with functional connectivity between the anterior insula and the basal ganglia and thalamus. *Middle panel:* positive correlation of PGE_2 serum levels with connectivity between the dorsal putamen and ventral basal ganglia. *Bottom panel:* positive correlation of PGE_2 serum levels with connectivity between the ventral putamen and basal ganglia. The right hemisphere corresponds to the right side of the axial and coronal views.

Spooren et al., 2011). Similarly, PGE_2 has been linked to the transition to and maintenance of chronic inflammation (Leonard, 2018; Narumiya, 2009) by promoting inflammation through inducing the expression of pro-inflammatory cytokines and suppressing Th2 cell differentiation and the anti-inflammatory system (Leonard, 2018). PGE_2 regulates sickness following systemic inflammation and is associated with increased body temperature, reduced food intake and changes in cognitive functions such as learning and memory (Poon et al., 2015). Inhibition of PGE_2 synthesis in mice has been reported to reduce the sickness behavior induced by LPS treatment (de Paiva et al., 2010). In line with these results, our finding of reduced levels of the anti-inflammatory 15d-PGJ_2 in CHC patients was expected, as this prostanoid is known to exert anti-inflammatory effects via its nuclear peroxisome proliferator-activated receptor-γ (PPARγ) (García-Bueno et al., 2008). This imbalance between cyclooxygenase-produced pro- and anti-inflammatory mediators has been described in experimental models as well as in patients with psychiatric disorders (García-Álvarez et al., 2018; García-Bueno et al., 2014; Leza et al., 2015).

In addition to the increased inflammation observed in CHC patients, our results highlighted the absence of oxidative stress in these subjects, which was not expected. It has been demonstrated that chronic inflammation may induce excessive production of ROS and RNS, which can cause nitro-oxidative damage to proteins, lipids or nucleic acids. The resulting O&NS can cause mitochondrial dysfunction, glial activation, neuroinflammation and apoptosis in the central nervous system, and has been associated with several neuropsychiatric conditions (Linqvist, 2017). The brain is particularly vulnerable to oxidative damage due to its high oxygen use and relatively weak antioxidant defences (Ng et al., 2008). For example, increased levels of polyunsaturated lipid oxidation, namely MDA-TBARS levels, have been reported in MDD patients (Lopresti et al., 2014; Palta et al., 2014), chronic and recurrent depression, as well as aging (Maurya and Rizvi, 2010). Nevertheless, the intrinsic antioxidant enzymatic system (i.e., SOD, CAT and GPx) may be activated in certain conditions to maintain ROS/RNS concentrations at desirable levels and to prevent O&NS (Sousa et al., 2016). In our study, increased SOD and CAT activities were reflected by decreased levels of MDA-TBARS in CHC patients. MDA accumulation and no clear disruptions in the antioxidant systems in CHC patients indicate that the antioxidant system in CHC patients in our study was functioning and still able to manage ROS/RNS production. This is noteworthy, as O&NS may be crucial in the development of psychiatric illnesses, which was an exclusion criterion in our study.

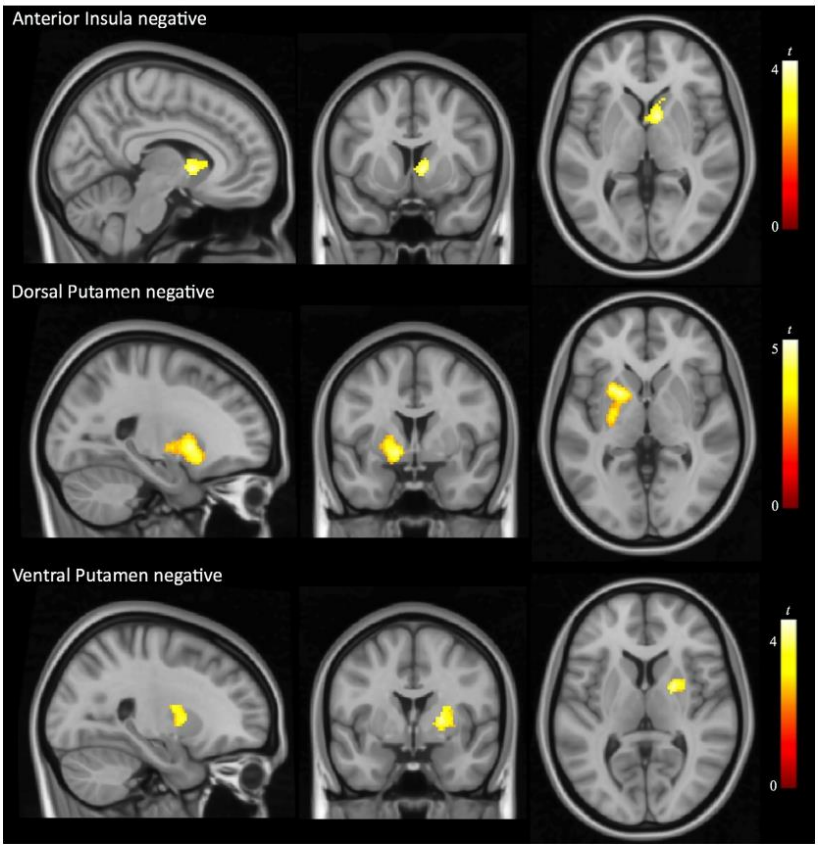

Fig. 3. Representative correlation analysis for the pro-inflammatory cytokine interleukin-6 (IL-6) in chronic hepatitis C (CHC) patients. *Top panel:* negative correlation of IL-6 serum levels with functional connectivity between the anterior insula and the right caudate nucleus. *Middle panel:* negative correlation of IL-6 serum levels with connectivity between the dorsal putamen and the left putamen/globus pallidus. *Bottom panel:* negative correlation of IL-6 serum levels with connectivity between the ventral putamen and the right putamen/globus pallidus. The right hemisphere corresponds to the right side of the axial and coronal views.

Further longitudinal studies are needed to determine whether these changes might be used as status biomarkers in this particular clinical setting.

Our results demonstrated that differences in sub-syndromic clinical symptomatology and inflammation between the groups were reflected by brain functional changes in areas involved in interoceptive

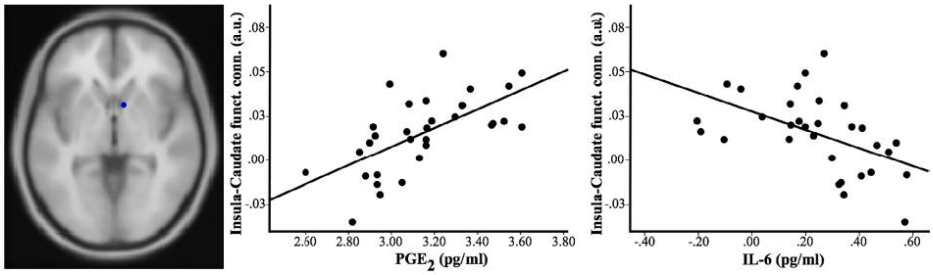

Fig. 4. Plots of correlation analysis for the pro-inflammatory cytokines prostaglandin E_2 (PGE_2) and interleukin-6 (IL-6) in chronic hepatitis C (CHC) patients. Functional connectivity between the anterior insula (seed map) and the right ventral striatum (caudate nucleus; blue dot in the left panel at MNI coordinates x = 8, y = 8, z = -2) was positively associated with prostaglandin E_2 (PGE_2; R = 0.628, p = 0.00015) and negatively associated with interleukin-6 (IL-6; R = -0.513, p = 0.003). See also Figs. 1 and 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

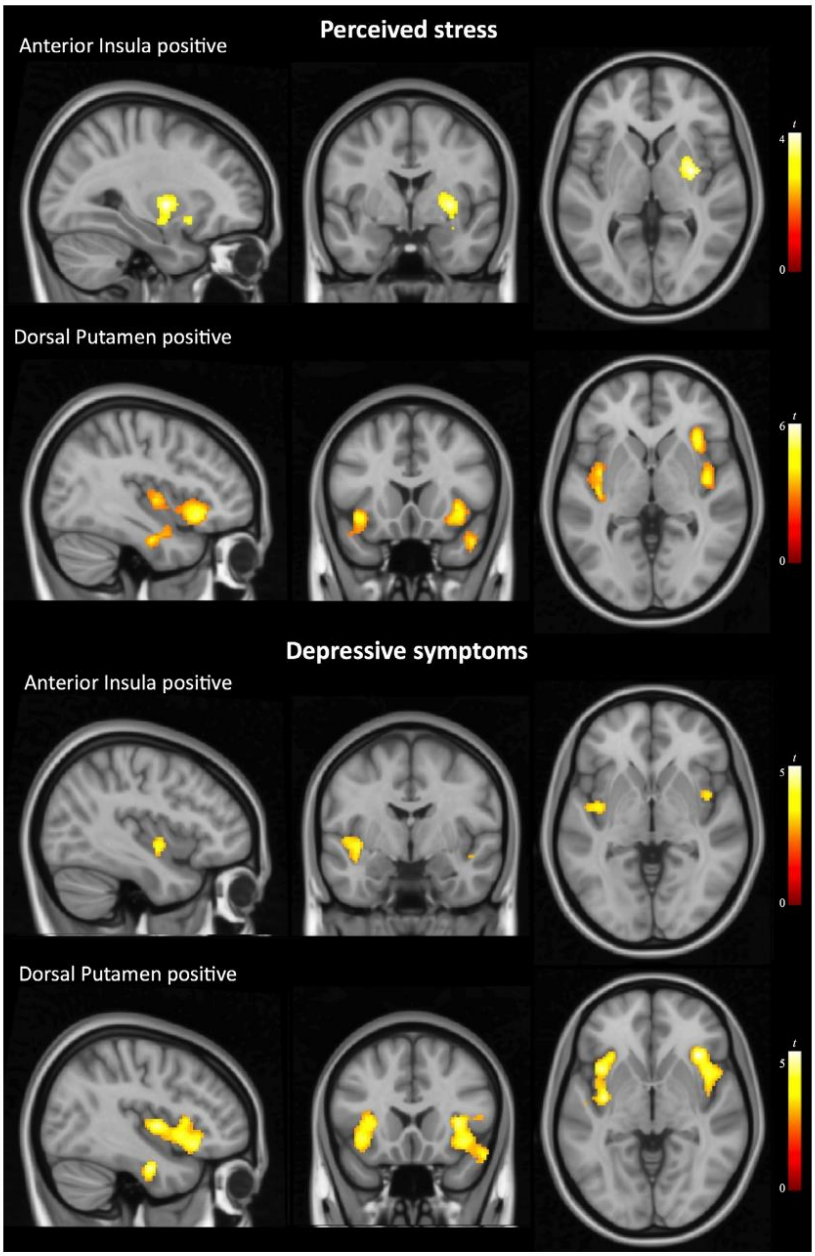

Fig. 5. Representative correlations between functional connectivity measurements and clinical variables. *Top panels:* positive correlations of perceived stress scale (PSS) scores with functional connectivity between the anterior insula and the right putamen and between the dorsal putamen and bilateral insulae. *Bottom panels:* positive correlations of depressive symptoms [measured with Patient Health Questionnaire-9 (PHQ-9) scores] with functional connectivity between both the anterior insula and dorsal putamen and the bilateral insulae. The right hemisphere corresponds to the right side of the axial and coronal views.

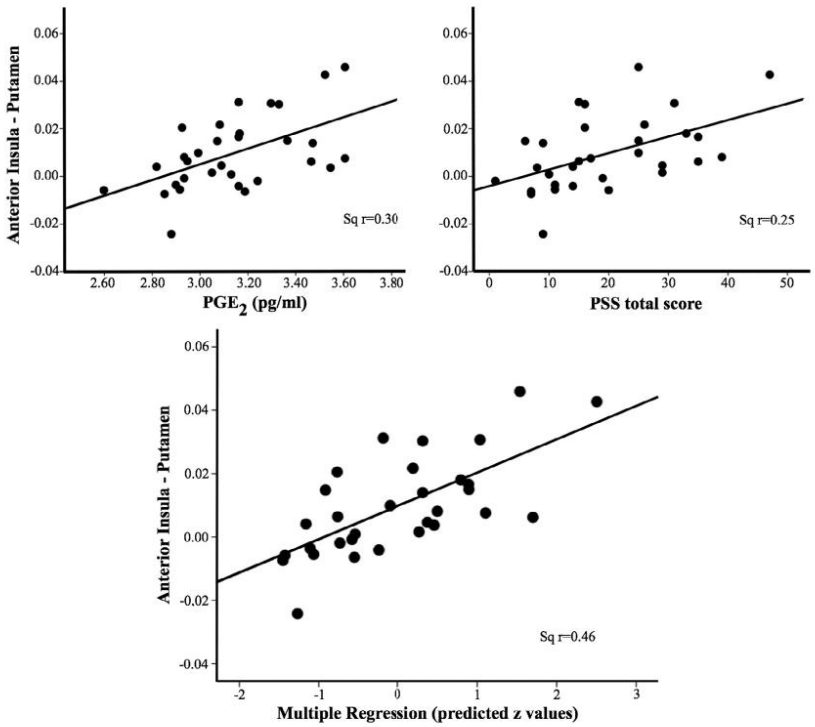

Fig. 6. Plots of the correlations between functional connectivity measurements and biological and clinical variables. Functional connectivity values (y axis) indicate the correlation between the right insula (seed region) and the functionally connected region in the right putamen. *Top panel:* positive correlations for prostaglandin E_2 (PGE_2; R = 0.300) and for perceived stress scale (PSS) scores (R = 0.250). *Bottom panel:* increased PGE_2 serum levels and increased PSS scores were entered into the equation in the multiple regression analysis, accounting for 46% of the variance in functional connectivity measurements (adjusted R = 0.42). All correlations were significant at $p < 0.008$.

awareness, psychomotor functions and affective processing. The chronic inflammatory disruption that characterizes CHC may contribute to the pathogenesis of neuropsychiatric symptoms, as cytokines may interact with several neurobiological pathways involved in psychiatric disorders (Furtado and Katzman, 2015; Oriolo et al., 2018b; Yarlott et al., 2017). Moreover, several studies have postulated that the brain may be a minor replication site for HCV (Fishman et al., 2008; Laskus et al., 2005), which can cross the blood brain barrier (BBB) and enter the central nervous system through infected monocytes (Thomas et al., 1999). HCV can interact with the microglia, inducing its activation by increasing the production of pro-inflammatory mediators. Interestingly, differences in functional connectivity between CHC patients and healthy controls were found in basal ganglia and limbic structures, which are sensitive to peripheral inflammation and associated with symptoms of sickness behavior. Importantly, in the literature similar results were reported for psychiatric conditions. Decreased functional connectivity between the sgACC and the precuneus was also observed in patients with MDD compared to healthy controls (Connolly et al., 2013; Ho et al., 2014), whereas increased functional connectivity between ventral putamen and frontal operculum was found in subjects with a high risk of psychosis (Dandash et al., 2014). Another intriguing result was the positive correlation between increased PGE_2 serum levels and increased functional connectivity of the insula with the dorsal putamen. The same brain areas were associated with perceived stress, as the insula to dorsal putamen connectivity positively correlated with PSS scores. Due to its lipid composition, PGE_2 can directly enter the brain parenchyma, spreading through the BBB and mainly interacting with the signalling receptors EP2 and EP3 on neurons to modulate neurotransmission (Furuyashiki and Narumiya, 2011). Interestingly, these receptors are mostly expressed in brain areas implicated in emotional and behavioral control, as PGE_2 has been reported to be involved in the mediation of behavioral response to circulating cytokines (Zhang and Rivest, 1999). By contrast, IL-6 in CHC patients negatively correlated with the functional connectivity between the dorsal and ventral putamen and the caudate nucleus. These same brain areas were associated with subclinical depressive symptoms, as the dorsal and ventral putamen connectivity positively correlated with PHQ-9 scores. Although these results may appear contradictory, animal studies indicate that IL-6 in the brain may contribute to the expression of brain cytokines in response to immune stimuli (Dantzer et al., 2008), resulting in several effects in the central nervous system that can be both detrimental and advantageous. Importantly, the effects of IL-6 partly depend on diverse factors, such as the presence of other cytokines or growth factors in the environment, the brain region involved and the physiological state of the tissue. Moreover, low or high IL-6 concentrations can exert opposite effects (Gadient and Otten, 1997; Spooren et al., 2011), mediating both neuroprotective and neurotoxic microglial responses (Eskes et al., 2002; Krady et al., 2008). It should be noted that PGE_2 and IL-6 are pro-inflammatory mediators that are also produced by the microglia and neurons, their secretion being

modulated by the direct neuropathogenic effects of HCV (Wilkinson et al., 2010).

Taken together, our findings demonstrate how changes in peripheral inflammation can influence insula and basal ganglia connectivity, illustrating how changes in internal bodily states can disturb neural representations, emotional states and executive functions. This is in line with current theories that postulate that emotional feeling states may arise through the perception of bodily signals, given that interoceptive and emotional processes share similar neural substrates (Critchley, 2005; Damasio, 1994; Quadt et al., 2018). The insular cortex is believed to represent and integrate interoceptive signals, such as inflammatory markers, providing the basis of interoceptive and emotional awareness, that is, the experiential side of sickness behavior (Craig, 2009). Studies using acute inflammatory challenges (Harrison, 2017) have demonstrated that subjective experiences of inflammation-associated symptoms derive from interoceptive signals converging on the insula (Harrison et al., 2009b). Indeed, structural and functional changes in the posterior, mid or anterior insula have been associated with subjective feelings of malaise and fatigue following inflammation (Bushara et al., 2001; Farrer et al., 2003; Klein et al., 2007). Moreover, several studies have reported a posterior-to-mid-to-anterior pattern of integration of interoceptive information (Craig, 2003). For example, activation of the posterior insula is linked to the objective intensity of heat pain, whereas anterior insular activation is associated with subjective pain evaluation (Kong et al., 2006). The patients in our study experienced increased subjective stress, which may be modulated partly by the effects of PGE_2 on the insular cortex. However, results from the multiple regression analyses indicated that increased PGE_2 serum levels and increased PSS scores independently accounted for 46% of the variance in functional connectivity measurements. The basal ganglia consists of subcortical structures involved in the integration and coordination of executive functions, reward, emotions and mood, with a specific relevance for adaptive shaping and action selection (Grace, 2012; Wichmann and De Long, 2013). Specifically, the dorsal putamen has been associated with the control of habitual behaviors (Redgrave et al., 2010; Wunderlich et al., 2012) and is believed to modulate the balance between goal-directed and habitual action control together with the insula (Hong et al., 2015). We observed that disruption of the dorsal putamen-insula interaction correlated with increased subjective perceived stress and reduced interest in doing things in CHC patients, which may be part of the modulation of the goal-directed versus habitual action control. Moreover, several neuroimaging studies have suggested that disrupted connectivity between the basal ganglia and putamen elicits behavioral changes following inflammatory challenges (Brydon et al., 2008; Felger and Miller, 2012). For example, PET studies revealed increased glucose metabolism in the putamen following IFN-alpha administration, which correlated with increased fatigue (Capuron et al., 2007; Juengling et al., 2000). Furthermore, an fMRI study revealed increased substantia nigra activity after administering the typhoid vaccine, which correlated with increased IL-6 peripheral blood concentrations and psychomotor retardation (Brydon et al., 2008). Interestingly, in a recent meta-analysis of neuroimaging and spectroscopic studies on patients with CHC performed by our group (Oriolo et al., 2018b), increased choline/creatine ratios, glutamine plus glutamate and creatine levels were observed in the basal ganglia of CHC patients compared to healthy controls, indicating chronic metabolic changes in the basal ganglia induced by CHC.

Our results provide valuable information on the brain areas involved in perceived stress, fatigue and subclinical depressive symptoms during chronic inflammation, highlighting the crucial role of interoception in coordinating prolonged sickness behavior. Since affective and emotional alterations characterize most psychiatric illnesses (Limanowski and Blankenburg, 2013), they may constitute the link between interoception and mental disorders (Quadt et al., 2018). However, our study had several limitations. First, the cross-sectional design of this study reduced the possibilities of inference. The absence of longitudinal assessment precluded predictive analysis, which could have strengthened the validity of our hypothesis. Moreover, a larger sample size would have increased the statistical power of this study. Second, we analysed only a few types of inflammatory markers. Analysing other markers such as CRP or TNF-α could have increased the reliability of our results. However, CRP might be a less reliable marker as several reports have found reduced CRP levels in CHC patients that may be due to auto-antibody activities (Sjöwall et al., 2012) or interferences by IL-6 on CRP synthesis (Shah et al., 2015). Third, we did not know the time between HCV infection and diagnosis, making it difficult to ascertain the duration of CHC. In general, the relationship between chronic inflammation and psychiatric symptoms is less easy to identify because patients who have such medical conditions are examined at different stages of their disease process (Dantzer, 2009). Moreover, it is even more difficult to determine the beginning of the disease in CHC patients as the source of infection is often unknown. This may result in much higher inter-individual variability. Finally, as mentioned before, HCV may directly affect the central nervous system (Oriolo et al., 2018b; Yarlott et al., 2017), making it difficult to disentangle the effects of chronic inflammation *per se* versus the direct effects of the virus on the brain.

5. Conclusions

Patients with CHC infection exhibited increased perceived stress and subthreshold depressive symptoms, as well as higher levels of inflammatory markers, compared to control subjects. These subtle clinical and inflammatory differences were reflected by functional connectivity changes in brain areas involved in interoceptive awareness, psychomotor functions and emotional processing. Our findings provide evidence that chronic inflammation may induce prolonged activation of interoceptive pathways, which in turn may promote long-standing maladaptive neurobiological and behavioral impairments implicated in the pathophysiology of depression (Miller et al., 2009; Savitz and Harrison, 2018). As we hypothesized, chronic inflammation together with the possible direct effects of HCV on the central nervous system, may account for the disruption in the connectivity between the insula and dorsal putamen, regions that provide cortical representation of internal state of the body, including changes in peripheral inflammation. In this sense, HCV seems to prime the brain and may account for a chronic sickness condition that is reflected by increased subjective stress perception and/or subclinical depressive symptoms (such as anhedonia), which may derive from aberrant interoceptive processing. This may represent a trigger for psychiatric illnesses in vulnerable patients or be a vulnerability factor itself, inducing a cascade of neurobiological pathways linked to mental disorders, such as oxidative and nitrosative stress. However, longitudinal studies are needed to disentangle the intricate interactions between the immune system, HCV neurotropism and the brain. The study of patients before and after a sustained viral response would be crucial in identifying the metabolic and functional changes specifically associated with HCV infection, which may also provide new information on the pathophysiology of neuropsychiatric symptoms and the identification of novel therapeutic targets.

Acknowledgments

RMS is grateful to the Instituto de Salud Carlos III, the Spanish Ministry of Economy and Competiveness, the Centro para la Investigación Biomédica en Red de Salud Mental (CIBERSAM), the Institut d'Investigacions Biomèdica August Pi i Sunyer (IDIBAPS), and the Secretaria d'Universitats i Recerca del Departament d'Economia i Coneixement, Grups consolidats de recerca (2014_SGR_1431 and 2017_SGR_1798).

Funding

This study was supported by the Instituto de Salud Carlos III, FIS: PI10/02206 (RMS) and FIS: PI10/02291 (RS). It was co-funded by ISCIII-Subdirección General de Evaluación and the Fondo Europeo de Desarrollo General (FEDER, "A Way to Build Europe").

Conflicts of interest to declare

RMS, GO, LBH, RN, DMH, MC,DG, JC, LC, and JP: none.

XF received unrestricted grant support from Abbvie and Gilead, and acted as Advisor for Abbvie and Gilead.

ZM acted as advisor for Gilead and received speaker fees from Abbvie, Gilead, Jansen, and MSD.

RS reports receiving consulting fees from Roche Pharma, Bristol Myers Squibb, Gilead Sciences, Novartis, Roche/Genentech, Tibotec and Jansen, lecture fees from Bristol Myers Squibb, Gilead Sciences, Novartis, Roche/Genentech and Jansen, and grant funding from Gilead Sciences, Roche/Genentech and Schering-Plough/Merck.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2019.03.008.

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Supporting Information Paper 2

INDEX

Supplementary Tables

Supplementary Figures

Table S1. Correlations between the clinical scores and biological markers in **A.** Chronic hepatitis-C patients and **B.** the whole sample

	A	**Biological markers**		
		PGE_2*	**IL-6**	**15d-PGJ_2***
Clinical Markers	**PHQ-9**			
	r	.206	-.103	-.021
	p	.243	.556	.908
	PSS			
	r	.266	-.032	.093
	p	.128	.857	.603
	VAS-i			
	r	.113	-.055	-.087
	p	.524	.752	.623
	VAS-f			
	r	.110	.015	-.157
	p	.537	.932	.377

	B	**Biological markers**		
		PGE_2	**IL-6**	**15d-PGJ_2**
Clinical Markers	**PHQ-9**			
	r	**.298**	.144	-.165
	p	**.019**	.251	.191
	PSS			
	r	.245	.150	-.121
	p	.055	.234	.341
	VAS-i			
	r	.148	.109	**-.255**
	p	.252	.389	**.042**
	VAS-f			
	r	.175	.139	**-.294**
	p	.173	.269	**.018**

Bold results are significant. *Abbreviations:* ***15d-PGJ_2*** *=15-deoxy-Δ12,14-prostaglandin J2;* ***IL-6*** *= interleukin-6;* ***PGE_2 =*** *prostaglandin E2;* ***PHQ*** *= Patient Health Questionnaire;* ***PSS*** *= Perceived Stress Scale*

Table S2. Correlations between functional connectivity and serum levels of PGE_2, with and without adjusting for clinical variables.

	*	*CS (ml)*	*x y z*	*t*	*Adj. t PSS*	*Adj. t PHQ*	*Adj. t VASf*	*Adj. t VASi*	*Adj. t HCV-RNA*
Right Anterior Insula map									
Thalamus	+	**5.5[a]**	**20 -26 10**	**5.0**	4.6	-	-	-	4.8
	+	**5.5[a]**	**-18 -22 14**	**4.9**	5.2	5.1	-	4.6	5.2
Globus Pallidus	+	**5.6[b]**	**-10 2 -2**	**4.6**	4.2	4.2	4.0	4.2	4.4
Caudate nucleus	+	**5.6[b]**	**8 8 -2**	**4.4**	4.8	4.5	5.4	4.8	5.0
Putamen	+	**5.6[b]**	**28 -6 6**	**3.2**	-	-	-	-	3.2
Premotor cortex	-	3.1	-40 -8 54	3.9	4.5	4.2	-	-	3.8
Right Dorsal Putamen map									
Caudate n./Globus Pallidus	+	**6.5[c]**	**-8 0 -2**	**5.8**	5.1	5.1	5.4	4.6	5.3
Putamen	+	**6.5[c]**	**18 8 -4**	**4.1**	4.1	4.1	4.3	3.9	4.0
Lingual gyrus	-	**8.7[d]**	**-6 -64 -4**	**5.5**	5.7	5.7	5.5	5.5	5.6
	-	**8.7[d]**	**6 -72 -10**	**4.8**	4.3	4.8	4.3	4.2	5.0
Left Dorsal Putamen map									
Cuneus	-	3.1	-14 -80 34	4.8	5.5	5.1	5.1	5.7	4.7
Right Ventral Putamen map									
Thalamus/Globus Pallidus	+	**4.7[e]**	**8 0 2**	**4.8**	4.5	-	4.4	4.3	4.7
Putamen	+	**4.7[e]**	**24 -6 8**	**3.8**	3.6	-	3.5	3.4	3.7
Prefrontal cortex	-	2.8	-42 18 24	4.7	-	4.7	-	-	4.6
Subgenual ACC map									
Cerebellum	**+**	**4.1**	**22 -72 -42**	**4.6**	4.6	4.3	5.0	4.4	4.5
Supplementary motor area	-	2.2	-12 10 48	7.1	-	-	-	-	6.9
Sensorimotor cortex	**-**	**7.6**	**44 -30 50**	**4.5**	5.4	5.0	5.8	5.1	4.5
	-	**10.5**	**-28 -60 68**	**4.9**	4.4	4.4	4.3	4.6	4.8

x y z, coordinates (mm) given in Montreal Neurological Institute (MNI) space. Statistics at cluster-level corrected threshold $P_{FWE}<0.05$ estimated using Monte Carlo simulations. **Bold** results are significant after Bonferroni correction for multiple comparisons.

* indicates the sign of the correlation.

[a,b,c,d,e] indicate same cluster.

Abbreviations: PGE_2 = prostaglandin 2; CS = cluster size; ACC = anterior cingulate cortex; PSS = Perceived Stress Scale; PHQ = Patient Health Questionnaire; VAS-f = Visual analogue scale (VAS) score for fatigue; VAS-i = VAS score for irritability; HCV-RNA = viral load of hepatitis-C virus. Adjusted models (Adj.) were performed as separate analyses for each covariate.

Table S3. Correlations between functional connectivity and serum levels of pro-inflammatory cytokine IL-6, with and without adjusting for clinical variables.

	*	*CS (ml)*	*x y z*	*t*	*Adj. t PSS*	*Adj. t PHQ*	*Adj. t VASf*	*Adj. t VASi*	*Adj. t HCV-RNA*
Right Anterior Insula map									
Caudate	-	2.4	10 6 2	4.3	4.3	4.4	4.3	4.2	3.7
Right Dorsal Putamen map									
Putamen/Globus Pallidus	-	**7.8**	**-26 10 -2**	**5.7**	5.7	5.9	5.6	5.6	5.4
Right Ventral Putamen map									
Putamen/Globus Pallidus	-	3.1	22 2 4	4.7	4.6	5.0	4.7	4.6	4.7

x y z, coordinates (mm) given in Montreal Neurological Institute (MNI) space. Statistics at cluster-level corrected threshold P_{FWE}<0.05 estimated using Monte Carlo simulations. **Bold** results are significant after Bonferroni correction for multiple comparisons.

*indicates the sign of the correlation.

Abbreviations: IL-6 = interleukin 6. PSS = Perceived Stress Scale; PHQ = Patient Health Questionnaire; VAS-f = Visual analogue scale (VAS) score for fatigue; VAS-i = VAS score for irritability; HCV-RNA = viral load of hepatitis-C virus. Adjusted models (Adj.) were performed as separate analyses for each covariate.

Table S4. Correlations between functional connectivity measurements and PSS total scores, with and without adjusting for biological markers.

	*	*Cluster size (ml)*	*x y z*	*t*	*Adj. t PGE_2*	*Adj. t IL-6*
Right Anterior Insula map						
Putamen	+	**3.6**	**26 0 4**	**5.1**	5.0	4.9
Left Anterior Insula map						
Cerebellum	-	2.3	30 -78 -24	4.0	4.0	-
Right Dorsal Putamen map						
Insula	+	**10.5**	**36 22 -8**	**6.1**	6.2	5.6
	+	**5.7**	**-38 14 -8**	**5.1**	5.1	5.3
Cerebellum	-	**15.3**	**34 -62 -26**	**4.8**	4.3	4.0
Dorsal Prefrontal cortex	-	**4.0**	**-30 12 56**	**4.3**	3.7	5.0
Left Dorsal Putamen map						
Temporal pole	+	**3.4**	**-46 16 -32**	**4.6**	4.3	4.1
Dorsal Prefrontal cortex	-	2.5	-32 14 50	6.5	6.1	7.1
Cerebellum	-	2.2	30 -66 -50	4.7	4.6	5.2
Right Ventral Putamen map						
Lingual gyrus	-	3.0	18 -90 -16	4.0	4.2	4.0
Left Ventral Putamen map						
Temporal pole	+	**4.0**	**52 -8 -24**	**4.9**	4.7	4.2
	+	2.3	-44 8 -28	4.2	3.9	3.5
Cerebellum	-	**3.9**	**30 -62 -50**	**5.2**	4.7	5.3
Precuneus	-	3.0	0 -50 54	4.6	4.4	4.4
Subgenual ACC map						
Frontal pole	-	2.8	28 66 12	3.8	3.5	3.8

x y z, coordinates (mm) given in Montreal Neurological Institute (MNI) space. Statistics at cluster-level corrected threshold $P_{FWE} < 0.05$ estimated using Monte Carlo simulations. **Bold** results are significant after Bonferroni correction for multiple comparisons.

*indicates the sign of the correlation.

Abbreviations: PSS = perceived stress scale; PGE_2 = prostaglandin 2; IL-6 = interleukin 6; ACC = anterior cingulate cortex. Adjusted models (Adj.) were performed as separate analyses for each covariate.

Table S5. Correlations between functional connectivity measurements and PHQ-9 scores, with and without adjusting for biological markers.

	*	*Cluster size (ml)*	*x y z*	*t*	*Adj. t PGE_2*	*Adj. t IL-6*
Right Anterior Insula map						
Supplementary motor area	+	2.5	0 12 50	4.4	4.2	4.0
Left Anterior Insula map						
Insula/Temporal cortex	+	2.9	-48 10 -28	5.1	4.5	6.1
	+	2.2	48 6 -22	4.3	4.3	4.1
Angular gyrus	-	2.9	30 -36 36	4.8	4.2	4.8
Prefrontal cortex	-	2.9	52 22 20	4.6	4.3	4.9
Posterior temporal cortex	-	2.4	54 -56 10	4.3	4.3	3.9
Right Dorsal Putamen map						
Insula	+	**11.7**	**36 24 -4**	**5.2**	4.6	4.8
	+	**7.4**	**-36 -8 -2**	**5.0**	4.8	5.0
Fusiform gyrus	+	2.4	40 -12 -28	4.7	4.2	4.5
Cerebellum	-	**14.2**	**36 -60 -50**	**6.9**	6.3	6.4
Temporal cortex	-	**5.1**	**66 -32 -28**	**4.3**	3.9	4.2
Left Dorsal Putamen map						
Insula	+	**5.3**	**-46 14 -30**	**6.5**	6.0	5.9
	+	**9.7**	**34 18 -2**	**5.1**	4.8	5.9
Cerebellum	-	**6.6**	**44 -58 -54**	**6.7**	6.1	7.1
Posterior temporal cortex	-	**8.9**	**72 -34 -2**	**6.1**	5.8	7.2
	-	**5.3**	**-60 -48 -6**	**5.2**	5.7	5.5
Angular gyrus	-	**5.7**	**-50 -60 52**	**4.8**	4.8	4.7
Right Ventral Putamen map						
Insula	+	**4.5**	**50 10 10**	**4.5**	4.2	4.1
Cerebellum	-	**7.9**	**50 -58 -54**	**5.4**	5.6	5.2
Angular gyrus	-	2.3	-38 -56 18	5.0	4.6	4.9
Precuneus	-	**9.7**	**-8 -66 52**	**4.8**	4.8	4.6
Anterior prefrontal cortex	-	2.3	-16 54 42	4.5	4.1	4.1
Left Ventral Putamen map						
Precuneus	-	**10.0**	**-2 -64 36**	**5.0**	5.1	4.9
Subgenual ACC map						
Cerebellum	-	2.7	-30 -78 -28	4.8	5.2	4.8

x y z, coordinates (mm) given in Montreal Neurological Institute (MNI) space. Statistics at cluster-level corrected threshold $P_{FWE} < 0.05$ estimated using Monte Carlo simulations. **Bold** results are significant after Bonferroni correction for multiple comparisons.

*indicates the sign of the correlation.

Abbreviations: PHQ = Patient Health Questionnaire; PGE_2 = prostaglandin 2; IL-6 = interleukin 6; ACC = anterior cingulate cortex. Adjusted models (Adj.) were performed as separate analyses for each covariate.

Table S6. Correlations between functional connectivity measurements and fatigue scores, with and without adjusting for biological markers.

	*	*Cluster size (ml)*	*x y z*	*t*	*Adj. t PGE_2*	*Adj. t IL-6*
Right Dorsal Putamen map						
Insula	+	2.8	-44 -4 -4	4.6	4.0	4.0
Thalamus	+	2.3	12 -22 16	4.6	3.7	4.8
Visual cortex	-	2.8	24 -102 -16	6.3	5.9	6.1
Left Dorsal Putamen map						
Prefrontal cortex	-	**4.1**	**-52 42 -4**	**4.3**	4.1	4.1
Subgenual ACC map						
Prefrontal cortex	-	2.6	36 46 4	4.5	4.8	4.1
Cerebellum	-	**7.5**	**-6 -70 -48**	**4.7**	4.9	4.6

x y z, coordinates (mm) given in Montreal Neurological Institute (MNI) space. Statistics at cluster-level corrected threshold $P_{FWE} < 0.05$ estimated using Monte Carlo simulations. **Bold** results are significant after Bonferroni correction for multiple comparisons.

*indicates the sign of the correlation.

Abbreviations: PGE_2 = prostaglandin 2; IL-6 = interleukin 6; ACC = anterior cingulate cortex. Adjusted models (Adj.) were performed as separate analyses for each covariate.

Table S7. Correlations between functional connectivity measurements and irritability scores, with and without adjusting for biological markers.

	*	*Cluster size (ml)*	*x y z*	*t*	*Adj. t PGE_2*	*Adj. t IL-6*
Left Anterior Insula map						
Posterior cingulate cortex	-	2.2	14 -40 36	4.3	5.1	4.9
Right Dorsal Putamen map						
Insula	+	2.5	34 -14 10	5.0	3.8	5.5
	+	2.2	-40 4 -6	3.9	3.5	3.8
Temporal cortex	+	3.1	46 -6 -24	4.6	3.8	4.5
Premotor cortex/SMA	-	**8.9**	**2 22 36**	**4.7**	4.3	4.5
Cerebellum	-	**3.9**	**52 -62 -40**	**4.4**	-	-
	-	2.3	-30 -62 -34	4.2	-	-
Left Dorsal Putamen map						
Prefrontal cortex	-	**3.7**	**-28 10 50**	**4.5**	4.0	4.5

x y z, coordinates (mm) given in Montreal Neurological Institute (MNI) space. Statistics at cluster-level corrected threshold $P_{FWE} < 0.05$ estimated using Monte Carlo simulations. **Bold** results are significant after Bonferroni correction for multiple comparisons.

*indicates the sign of the correlation.

Abbreviations: PGE_2 = prostaglandin 2; IL-6 = interleukin 6; SMA = Supplementary motor area. Adjusted models (Adj.) were performed as separate analyses for each covariate.

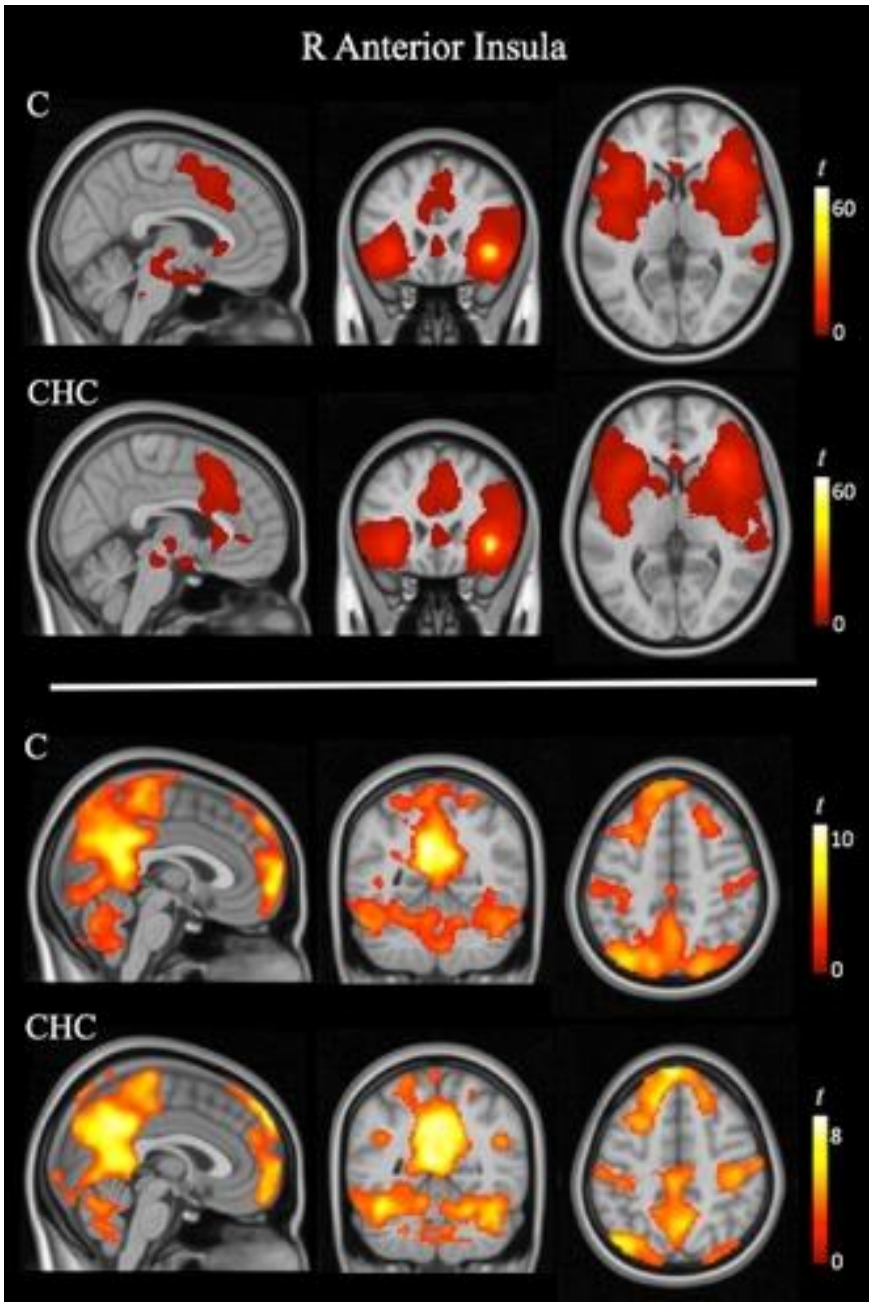

Figure S1. Within-group (one-sample) functional connectivity maps for the anterior insula seed. Positive (top rows) and negative (bottom rows) correlations with the region of interest are shown for control subjects (C) and chronic hepatitis C patients (CHC). R, right hemisphere. Connectivity maps were largely similar for the insula seed in the left hemisphere. The right side of the figure corresponds to the right hemisphere for axial and coronal views.

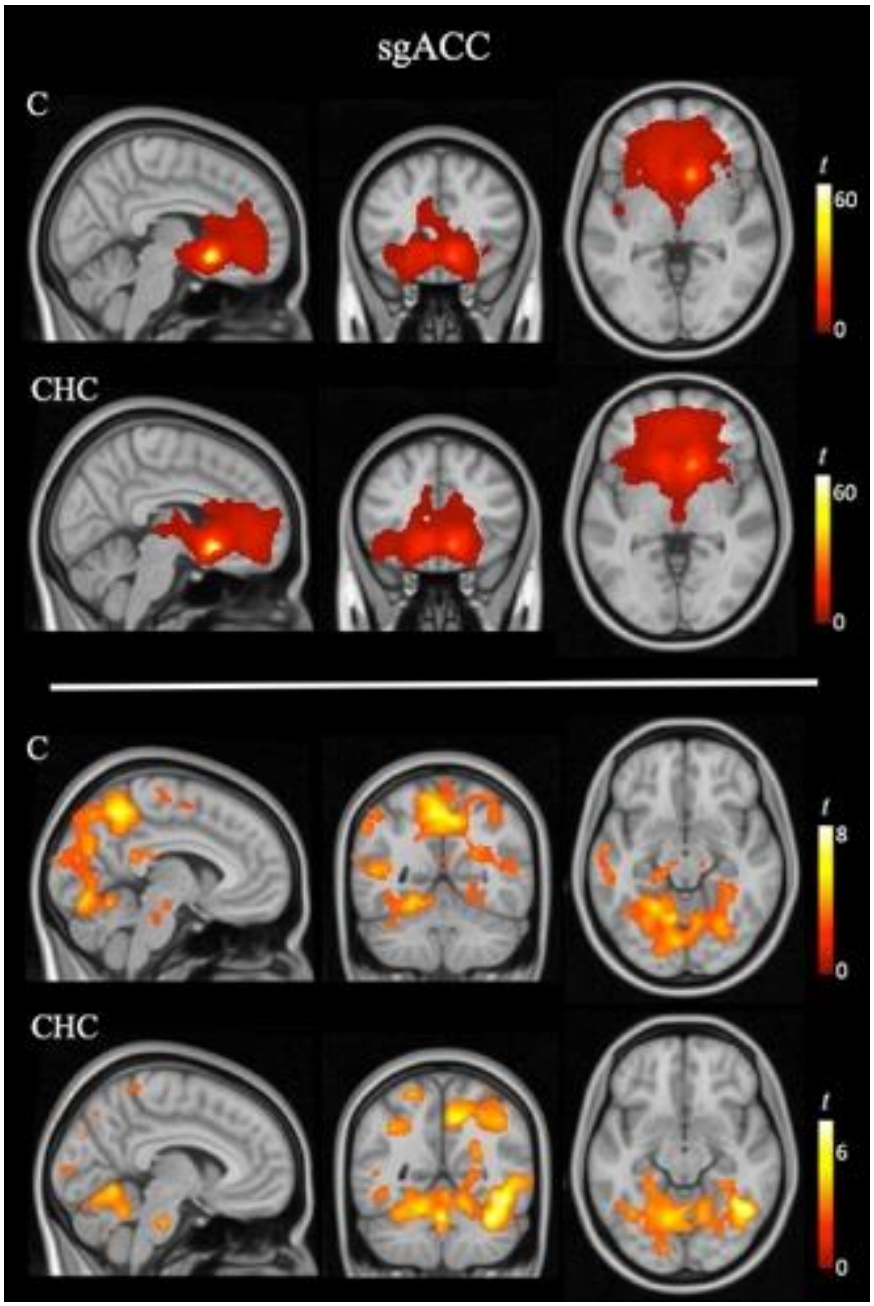

Figure S2. Within-group (one-sample) functional connectivity maps for the subgenual anterior cingulate cortex (sgACC). Positive (top rows) and negative (bottom rows) correlations with the region of interest are shown for control subjects (C) and chronic hepatitis C patients (CHC). The right side of the figure corresponds to the right hemisphere for axial and coronal views.

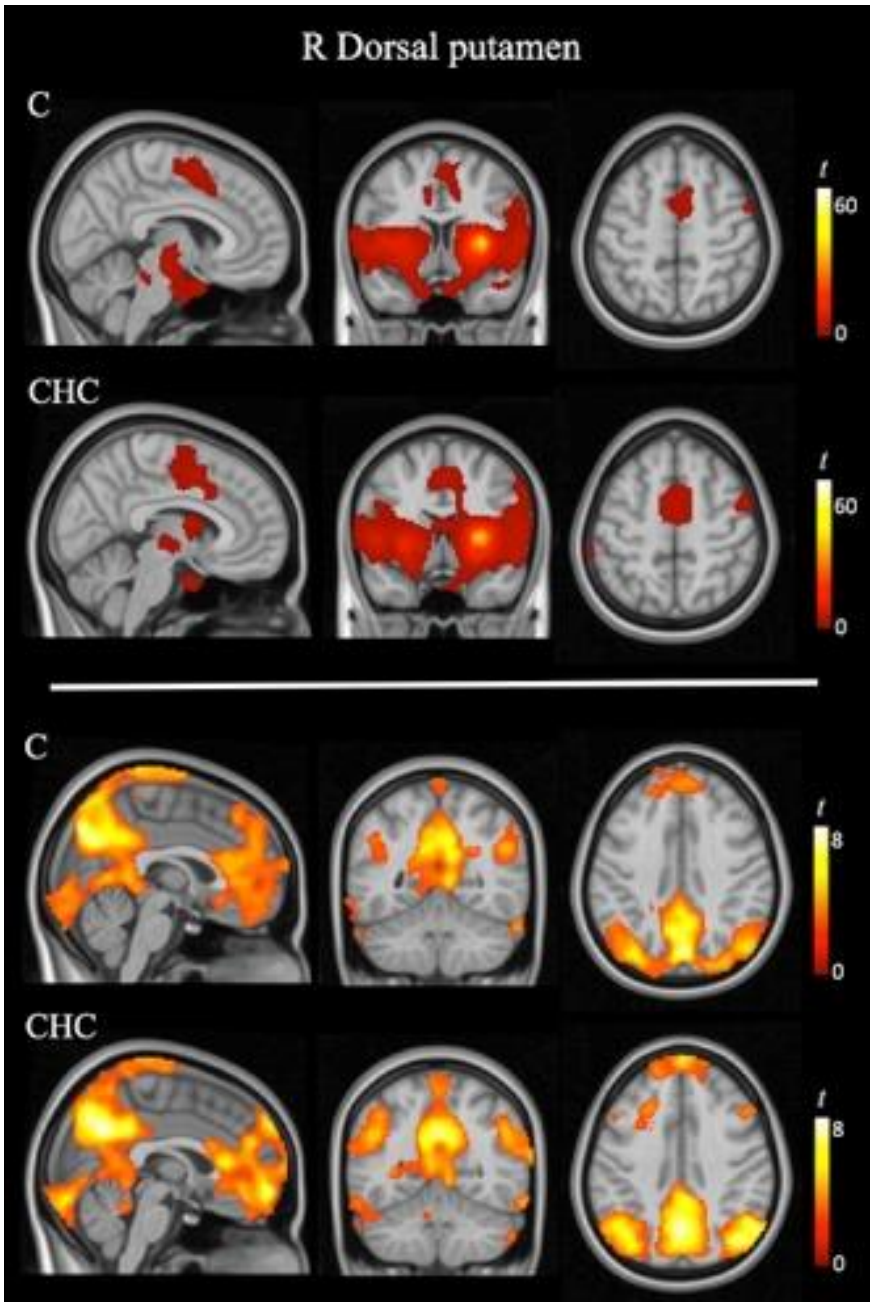

Figure S3. Within-group (one-sample) functional connectivity maps for the dorsal putamen seed. Positive (top rows) and negative (bottom rows) correlations with the region of interest are shown for control subjects (C) and chronic hepatitis C patients (CHC). R, right hemisphere. Connectivity maps were largely similar for the dorsal putamen seed in the left hemisphere. The right side of the figure corresponds to the right hemisphere for axial and coronal views.

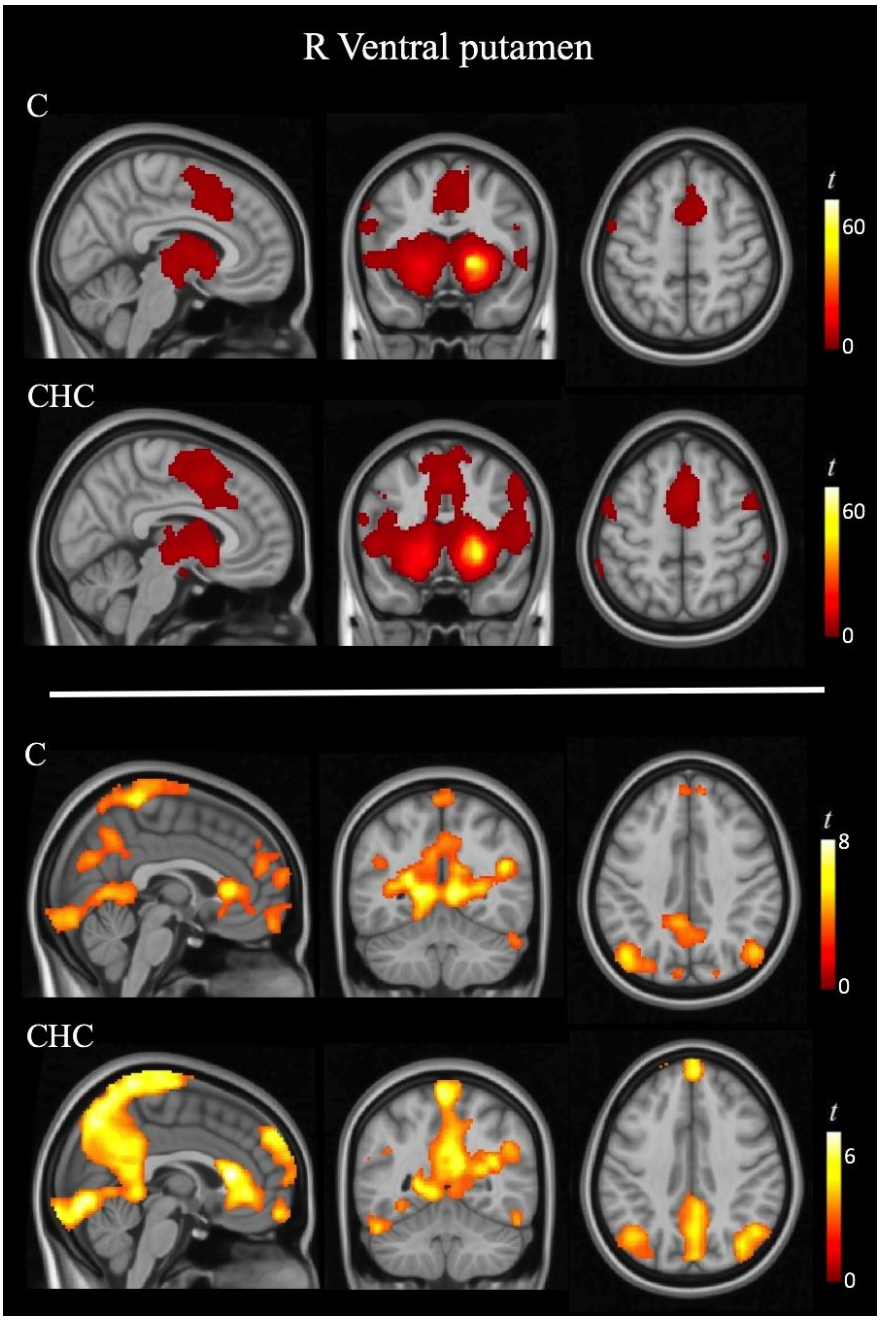

Figure S4. Within-group (one-sample) functional connectivity maps for the ventral putamen seed. Positive (top rows) and negative (bottom rows) correlations with the region of interest are shown for control subjects (C) and chronic hepatitis C patients (CHC). R, right hemisphere. Connectivity maps were largely similar for the ventral putamen seed in the left hemisphere. The right side of the figure corresponds to the right hemisphere for axial and coronal views.

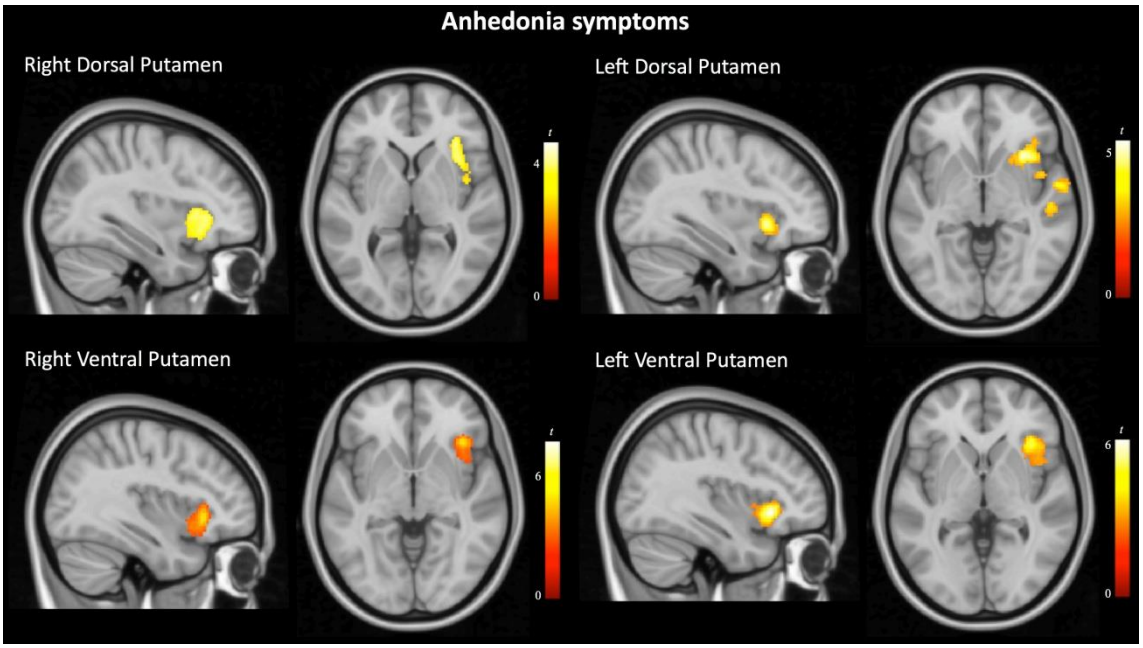

Figure S5. Representative correlations between functional connectivity measurements and anhedonia. *Top panels:* positive correlations of anhedonia score (item 1 of PHQ-9 scale) with functional connectivity between the dorsal putamen (right and left) and right anterior insula. *Bottom panels:* positive correlations of anhedonia score (item 1 of PHQ-9 scale) with functional connectivity between the ventral putamen (right and left) and right anterior insula. The right hemisphere corresponds to the right side of the axial and coronal views.

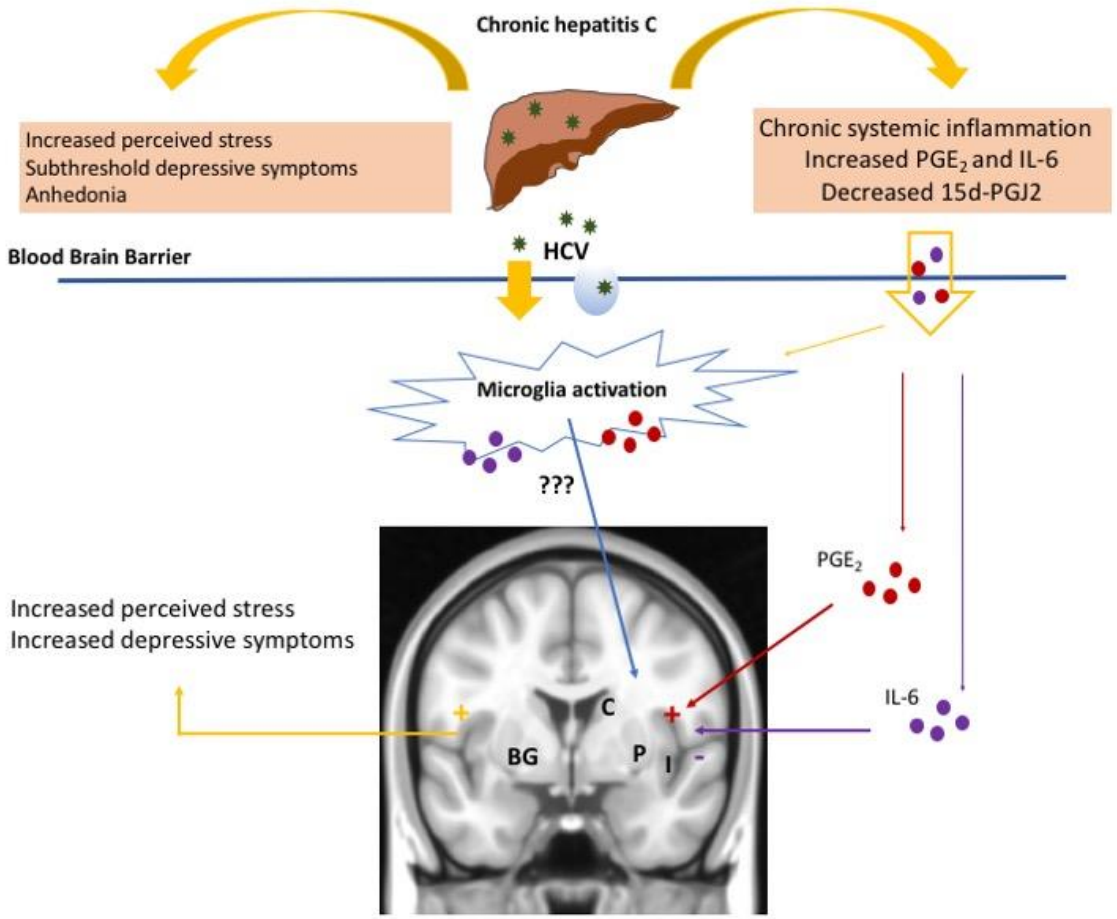

Figure S6. General overview of the interactions between chronic hepatitis C infection, inflammatory markers, neuropsychiatric symptoms and brain connectivity alterations. Chronic hepatitis C infection was associated with increased levels of inflammatory markers, namely IL-6 and PGE2, and with increased perceived stress and subthreshold depressive symptoms. PGE2 and IL-6 influence activity in the brain regions associated with interoceptive awareness and emotional processing, such as the insula and basal ganglia. Moreover, the hepatitis C virus could directly affect the brain, e.g., through activating the microglia, and could be involved in these alterations. Changes in connectivity in the insula and basal ganglia are associated with neuropsychiatric symptoms.

Abbreviations: 15d-PGJ2, 15-deoxy-Δ12,14-prostaglandin-J2; BG, basal ganglia; C, caudate; HCV, hepatitis C virus; I, insula; IL-6, interleukin-6; P, putamen; PGE2, prostaglandin E2.

APPENDIX

I. LICENSES

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II. PSYCHOMETRIC SCALES

Cuestionario de salud del paciente (PHQ-9)

Durante las últimas 2 semanas, ¿con qué frecuencia le han molestado cada uno de los siguientes problemas?

	Nunca	Unos cuantos días	Más de la mitad de los días	Todos o casi todos los días
a) Tener poco interés o disfrutar poco haciendo cosas				
b) Sentirse desanimado, deprimido o sin esperanza				
c) Tener problemas para dormir (coger el sueño o mantenerlo), o tener más sueño de la cuenta				
d) Sentirse cansado o con poca energía				
e) Tener poco apetito o comer demasiado				
f) Sentirse mal consigo mismo – o sentirse fracasado o decepcionado de sí mismo, o pensar que ha decepcionado a los que le rodean				
g) Tener problemas para concentrarse, como por ejemplo para leer el periódico o ver la televisión				
h) Moverse o hablar tan lentamente que los demás lo han notado. O bien lo contrario, estar tan inquieto e intranquilo que ha estado moviéndose de arriba para abajo mucho más de lo habitual				
i) Tener pensamientos sobre que estaría mejor si se muriese o sobre hacerse daño a sí mismo de alguna manera				

Escala de Estrés Percibido - (*PSS*) – versión completa 14 ítems.

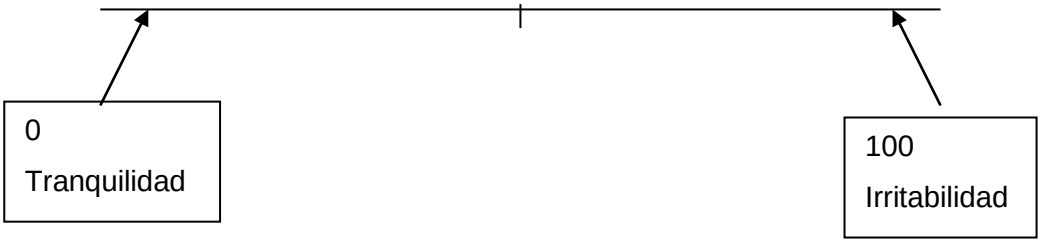
Versión española (2.0) de la *Perceived Stress Scale* (*PSS*) de Cohen, S., Kamarck, T., & Mermelstein, R. (1983) adaptada por el Dr. Eduardo Remor (2001).

Las preguntas en esta escala hacen referencia a sus sentimientos y pensamientos durante el **último mes**. En cada caso, por favor indique con una "X" cómo usted se ha sentido o ha pensado en cada situación.

	Nunca	**Casi nunca**	**De vez en cuando**	**A menudo**	**Muy a menudo**
1. En el **último mes**, ¿con qué frecuencia ha estado afectado por algo que ha ocurrido inesperadamente?	0	1	2	3	4
2. En el **último mes**, ¿con qué frecuencia se ha sentido incapaz de controlar las cosas importantes en su vida?	0	1	2	3	4
3. En el **último mes**, ¿con qué frecuencia se ha sentido nervioso o estresado?	0	1	2	3	4
4. En el **último mes**, ¿con qué frecuencia ha manejado con éxito los pequeños problemas irritantes de la vida?	0	1	2	3	4
5. En el **último mes**, ¿con qué frecuencia ha sentido que ha afrontado efectivamente los cambios importantes que han estado ocurriendo en su vida?	0	1	2	3	4
6. En el **último mes**, ¿con qué frecuencia ha estado seguro sobre su capacidad para manejar sus problemas personales	0	1	2	3	4
7. En el **último mes**, ¿con qué frecuencia ha sentido que las cosas le van bien?	0	1	2	3	4
8. En el **último mes**, ¿con qué frecuencia ha sentido que no podía afrontar todas las cosas que tenía que hacer?	0	1	2	3	4
9. En el **último mes**, ¿con qué frecuencia ha podido controlar las dificultades de su vida?	0	1	2	3	4
10. En el **ultimo mes**, ¿con que frecuencia se ha sentido que tenía todo bajo control?	0	1	2	3	4
11. En el **último mes**, ¿con qué frecuencia ha estado enfadado porque las cosas que le han ocurrido estaban fuera de su control?	0	1	2	3	4
12. En el **último mes**, ¿con qué frecuencia ha pensado sobre las cosas que le quedan por hacer?	0	1	2	3	4
13. En el **último mes**, ¿con qué frecuencia ha podido controlar la forma de pasar el tiempo?	0	1	2	3	4
14. En el **último mes**, ¿con qué frecuencia ha sentido que las dificultades se acumulan tanto que no puede superarlas?	0	1	2	3	4

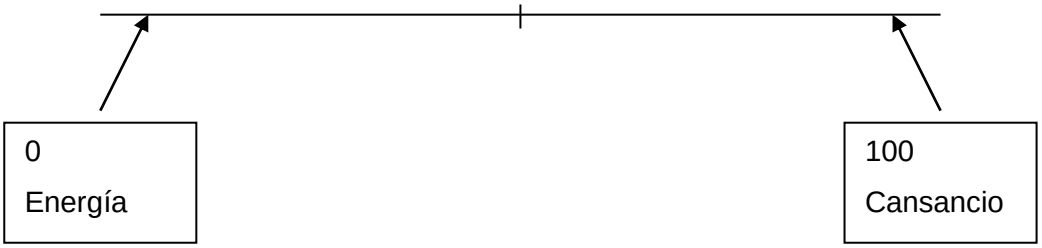
Escala analógico-visual de irritabilidad (VAS-i)

A continuación verá una línea. Representa el grado de irritabilidad o impaciencia que usted ha tenido durante **las dos últimas semanas,** desde el 0, que representa el mayor estado de tranquilidad que pueda imaginarse, hasta el 100, que representa el mayor estado de irritabilidad e impaciencia que pueda imaginarse. Nos gustaría que nos indicara en esta escala, en su opinión, el nivel de irritabilidad que ha tenido en las dos últimas semanas.



Escala analógico-visual de fatiga (VAS-f)

A continuación verá una línea. Representa el grado de cansancio o fatiga que usted ha tenido durante **las dos últimas semanas,** desde el 0, que representa el mayor estado de energía que pueda imaginarse, hasta el 100, que representa el mayor estado de cansancio y fatiga que pueda imaginarse. Nos gustaría que nos indicara en esta escala, en su opinión, el nivel de cansancio o fatiga que ha tenido en las dos últimas semanas.



Consentimiento informado del estudio

"Base neurobiológicas de la depresión inducida por interferon alfa pegilado y ribavirina en la hepatitis C crónica" ESTUDIO PSICOCIT-VHC

Servicio de Psiquiatría/ICN/Hospital Clínic /IDIBAPS/ Sección de Hepatología/HMAR-IMIM/ Barcelona

INFORMACIÓN AL PACIENTE

A. OBJETIVO DEL ESTUDIO

Se está requiriendo su colaboración para participar en esta investigación para estudiar las base neurobiológicas de la depresión inducida por interferon alfa pegilado en la hepatitis C crónica. La hepatitis C crónica está producida por el virus de la hepatitis C y es un importante problema de salud pública que afecta a un 3% de la población mundial. Cerca de un 20% de los pacientes infectados pro el virus pueden desarrollar cirrosis y entre un 1% y un 4% un cancer hepático. Desde hace unos años se dispone de un tratamiento efectivo, el interferón, un medicamento que actua sobre la inmunidad. Por ello es muy importante que las pesonas afectadas puedan llevar a cabo bien el tratamiento. Una de las razones para no seguir bien el tratamiento es la presencia de efectos secundarios del interferón. Alrededor de un 30% de las personas que inician el tratamiento con interferón pueden presentar sintomatología depresiva. Parece ser que el tratamiento con interferón por un lado y la propia infección por otro alteraría el sistema inmunológico que a su vez actúa sobre el cerebro dando lugar a cambios en los neurotransmisores y en la relaciones neuronales entre diferentes áeas cerebrales facilitando que la persona presente síntomas de tristeza, perdida del interés por hacer cosas, pérdida de la capacidad para disfrutar, duerma menos horas, y se sienta mas cansado o irritable.

El objetivo final de nuestro estudio conocer mejor los mecanismos por los cuales se produce esta depresión con el tratamiento y por lo tanto poder predecir que personas con hepatitis C crónica son mas vulnerables de presentar estos efectos secundarios con el tratamiento. Los resultados de este estudio ayudaran a que en el futuro se puedan encontrar tratamientos mas especificos para tratar la depresión y la hepatitis C crónica.

B. EN QUE CONSISTE SU PARTICIPACIÓN

Su participación en este estudio consiste en:

La realización de una entrevista psiquiatrica en la que le preguntaran principalmente por su estado de ánimo ahora y en el pasado. Esta entrrevista tiene una duración aproximada de 15 minutos.Contestar unos test psicológicos sobre como se siente antes de empezar el tratamiento y a lo largo del mismo (a las 4 y 12 semanas de tratamiento). La contestación de estos test le llevará otros 15 minutos.Contestar unos test neuropsicológicos sobre la memoria y capacidad de aprendizaje. Los test se los administrará una neuropsicóloga y la exploración durará cerca de una hora. Algunas de las preguntas las tendrá que contestar de forma muy sencilla en un ordenador.Una prueba de 10 minutos de duración de resonancia magnética funcional. En la que se le pedirá que se extienda en la camilla de la resonancia durante 10 minutos, y esté quieto con los ojos cerrados.Se le solicitará un análisis de sangre de 20 ml de sangre en el mismo centro para estudiar por un lado las citoquinas IL-6 y FNT alfa unas substancias que pueden liberarse con el tratamiento y también para extraer su ADN y poder estudiar variantes genéticas que pueden facilitar que algunas personas tengan mas vulnerabilidad a tener depresión

C. BENEFICIOS Y RIESGOS

El beneficio de este estudio es profundizar en el conocimiento de esta enfermedad que afecta a un número importante de personas de modo que podamos mejorar su diagnóstico, tratamiento y prevención. Estos resultados beneficiarán en el futuro a la población de hombres y mujeres que sufren de hepatitis C y son tratados con interferón. A corto plazo este estudio no supondrá un beneficio directamente para usted. Usted continuará siendo atendido por su equipo médico del Hospital Clínico/Hospital del Mar. Realizaremos una extracción de 10 ml de sangre del brazo. Los riesgos de esta extracción son mínimos o inexistentes, y están limitados a que en algunas personas puede producir un hematoma que dure unos días. La resonancia magnética funcional no se

conoce que tenga ningún riesgo. Sin embargo antes de entrar le pregunatarán si es usted portador de algún marcapasos o lleva alguna protesis metálica ya que en ese caso no se la podrá realizar al tratarse de una mecanismo magnético. Este estudio ha sido aprobado por el Comité Ético de Investigación Clínica de los centros participantes en el estudio.

D. ASPECTOS ÉTICOS

Garantía de participación voluntaria

Su participación en este estudio es totalmente voluntaria y su decisión no afectará en ningún momento la asistencia que está recibiendo en el Hospital Clínico/Hospital del Mar ni la que pueda precisar en el futuro. Además en el caso de que acepte participar en este estudio es Ud libre de abandonarlo sin tener que dar explicaciones cuando lo desee, en cualquier momento del mismo. En el caso de que no quiera participar en el estudio genético ello no impide que usted pueda participar en el estudio general. En el caso poco probable que se le encontrara alguna alteración cerebral durante la realización de la prueba de resonancia, se lo comunicaríamos y nos pondríamos en contacto con su médico de cabecera para poderle orientar de la foma más eficaz respecto a la conducta a seguir.

Confidencialidad

Los investigadores de este centro se responsabilizan de que en todo momento se mantenga la confidencialidad respecto a la identificación del participante, tanto en los datos clínicos, de los datos de neuroimagen como en las muestras de sangre. El nombre y los datos de esta investigación quedarán archivados con un código que será el mismo que aparecerá a lo largo de todo el estudio (datos clínicos y muestras de sangre). Estos procedimientos, que se llaman de anonimización, están sujetos a la Ley Orgánica 15/1999 del 13 de diciembre sobre protección de datos de carácter personal.

¿Qué hacen los investigadores con los datos que recogen?

Los datos se guardan en ficheros de papel o informáticos. Como ya hemos comentado previamente a cada participante se le adjudica un código, de manera que no aparezcan ni su nombre ni su apellido y se mantenga la confidencialidad. Con estos datos se realizarán análisis estadísticos para relacionar los resultados clínicos y de los cuestionarios con los resultados de los análisis genéticos. Finalmente los resultados se publicarán en revistas científicas. Cada vez que los investigadores planteen un nuevo proyecto, este tendrá que ser evaluado por el Comité Ético de Investigación Clínica de los centros que participan en el estudio (Hospital Clínico/Hospital del Mar)

¿Qué hacen los investigadores con la muestra de sangre?

La muestra de sangre se procesa en un laboratorio para separar el plasma de las células. El plasma se guarda congelado para hacer posteriormente análisis bioquímicos relacionados con la depresión, el estrés y la inmunidad. El material se podrá tener almacenado hasta un máximo de 15 años, tras lo cual, el material será destruido según la normativa vigente. Este material podrá ser compartido con otros grupos de investigación de otros centros públicos o centros de investigación privados, españoles o extranjeros, procedimientos que siempre se realizarán bajo las normas de seguridad y confidencialidad necesarias.

En cualquier momento puede Ud solicitar que las muestras genéticas sean eliminadas. Puede Ud realizar cualquier pregunta o duda en relación con el estudio. Los investigadores del estudio están a su disposición para contestarlas. Puede Ud encontrarlos: Dr Ricard Sola, Servicio de Patología Digestiva. Hospital del Mar. Passeig Marítim 25-29. 08003 Barcelona. Dra. Rocío Martín-Santos, Dr. Ricard Navinés, Servicio de Psiquiatría, Hospital Clínico Villarroel 170. 08036 Barcelona

Este protocolo has sido aprobado por el CEIC del Hospital Clínico /Hospital del Mar

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CONSENTIMIENTO INFORMADO POR ESCRITO

Yo (nombre y apellidos) _____

He leído la hoja de información que se me ha entregado

He podido hacer preguntas sobre el estudio.

He recibido suficiente información sobre el estudio.

He hablado con (nombre del investigador) _____

Comprendo que mi participación es voluntaria.

Comprendo que puedo retirarme del estudio: cuando quiera, sin tener que dar explicaciones, sin que esto repercuta en mis cuidados médicos.

De conformidad con lo que establece la LO. 15/1999, de 13 Diciembre, de Protección de Datos de Carácter Personal, declaro haber sido informado:

De la existencia de un fichero o tratamiento de datos de carácter personal, de la finalidad de la recogida de éstos y de los destinatarios de la información.

Del carácter obligatorio o facultativo de mi respuesta a las preguntas que me son planteada.

De las consecuencias de la obtención de los datos o de la negativa a suministrarlo.

De la extracción de una muestra de sangre para la obtención de material inmunológico y genético.

De la identidad y dirección del responsable del tratamiento.

De la identidad y dirección del encargado del tratamiento.

De la posibilidad de solicitar la eliminación de las muestras en cualquier momento.

De la disponibilidad de ejercitar los derechos de acceso, rectificación, cancelación y oposición dirigiéndome por escrito a: Dra. Rocío Martín-Santos /Dr. Navinés, Servicio de Psiquiatría del Instituto de Neurociencias del Hospital Clínico. Villarroel, 150

Y consiento que los datos clínicos referentes a mi enfermedad sean almacenados en un fichero automatizado, cuya información podrá ser manejada exclusivamente para fines científicos.

Presto libremente mi conformidad para participar en el estudio.

(firma del participante y fecha) Barcelona, a ..... de .....200....

(firma del investigador y fecha) Barcelona, a ..... de .....200...

Este protocolo ha sido aprobado por el CEIC del Hospital Clínic