Neuroinflammation in first episodes of psychosis

Miquel Bioque Alcázar

Neuroinflammation in first episodes of psychosis

Miquel Bioque Alcázar
PhD Candidate

Dr. Miguel Bernardo Arroyo
PhD Director

Dr. Juan Carlos Leza Cerro
PhD Director

Programa Doctorat Medicina
Departament de Psiquiatria i Psicobiologia Clínica
Facultat de Medicina
Universitat de Barcelona

September 2013

To my family that I adore,

My wife Eva,
my parents Leonor and Bartolomé,
my sisters Sara and Diana,
and my nephew Lucas.

I feel immensely lucky to have you by my side every day.
I love you so much.

This thesis has been developed in the Clinic Schizophrenia Unit of the Hospital Clinic of Barcelona, part of the Clínic Institute of Neuroscience August Pi i Sunyer Biomedical Research Institute (IDIBAPS), University of Barcelona (UB) and the Centro de Investigación Biomédica en Salud Mental (CIBERSAM - G04).

All biochemical determinations (except the genetics) were performed in the Department of Pharmacology, Faculty of Medicine, Complutense University Madrid, CIBERSAM - G12.

The studies here presented were supported by the Instituto de Salud Carlos III (ref. ISCIII 2009-2011: PEPs study PI 080208 - "Phenotype-genotype and environmental interaction. Application of a predictive model in first psychotic episodes. Proyecto PEPs") belonging to the Spanish Ministry of Economy and Competiveness, CIBERSAM (Intramural Projects 2010: Flamm-PEPs study), European Union FEDER, Dirección General de Universidades, Comunidad de Madrid, CANNAB-CM (S2010/BMD-2308), Government of Catalonia, Secretaria d'Universitats i Recerca del Departament d'Economia i Coneixement (2009SGR1295), Esther Koplowitz Center (Barcelona).

ACKNOWLEDGEMENTS

I want to thank my thesis director Miquel Bernardo. Without your help and persistent guidance this thesis would not have been possible. Thank you for trusting me and allow me to be part of this fantastic group.

I would like to express my deepest gratitude to my thesis co-director Juan Carlos Leza, for giving me the opportunity, resources and guidance to carry out such an exciting research. Thank you for your constructive comments and warm encouragement.

Karina McDowell made a huge contribution to conduct the laboratory experiments. Thank you for your tireless work and your patience with me during my unforgettable stays in Madrid.

I owe my deepest gratitude to Borja García-Bueno, for his generous support and nearness. Without your encouragement and guidance this thesis would not have materialized.

I am particularly grateful for the assistance given by Mª Fe Barcones and Javier Santabárbara for the statistical analysis.

I would particularly like to thank Ana Meseguer for her enormously, priceless help in the collection and processing of biological samples.

I would like to offer my special thanks to Bibiana Cabrera for being my traveling companion in the Clínic Schizophrenia Unit, since we landed four years ago.

I am in debt to Clemente Garcia, whose unlimited, unselfish support and friendship have been invaluable during these years.

Special thanks to the members of the research team of the Clínic Schizophrenia Unit and collaborators: Anna Folch, Eduard Parellada, Alicia Durán, Cristina Oliveira, Enric Buisán, Gisela Mezquida, Eduardo Jiménez, Alicia Valiente, Sergi Mas, Patrícia Gassó and Amàlia Lafuente.

TABLE OF CONTENTS

ACRONYMS AND ABBREVIATIONS

15d-PGJ$_2$: Prostaglandin 15-deoxy-PGJ$_2$

2AG: 2-arachidonoylglycerol

AEA: Anandamide

BMI: Body mass index

cAMP: Cyclic AMP

CAN-: Cannabis non-smokers

CAN+: Cannabis smokers

CATIE: Clinical Antipsychotic Trials of Intervention Effectiveness

CB1: Cannabinoid receptor 1

CB2: Cannabinoid receptor 2

C-GAS: Children's Global Assessment Scale

CIBERSAM: Centro de Investigaciones Biomédicas en Red en Salud Mental

CNS: Central nervous system

COMT: Catechol-O-methyl transferase

COX: Cyclooxygenase

COX-2: Cyclooxygenase type 2

CSF: Cerebrospinal fluid

DAGL: Diacylglycerol lipase

DDD: Defined daily dose

DNA: Deoxyribonucleic acid

DSM-IV: Diagnostic and statistical manual, 4th edition.

DUP: Duration of untreated psychosis

ECS: Endocannabinoid system

EDTA: Ethylenediaminetetraacetic acid

EIA: Enzyme immunoassay

ELISA: Enzyme-Linked Immunosorbent Assay

EuropAsi: European Adaptation of a Multidimensional Assessment Instrument for Drug and Alcohol Dependence

FAAH: Fatty acid amide hydrolase (FAAH)

FEP: First episode of psychosis

FIS: Fondo Investigaciones Sanitarias

GAF: Global Assessment of Functioning Scale

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GLU: Glutamate

H_2O_2: Hydrogen peroxide

HCY: Homocysteine

HRP: Horseradish peroxidase

IκBα: Alpha inhibitory complex κB

IFNγ: Interferon gamma

IKKα: Inhibitor of nuclear factor kappa-B kinase subunit alpha

IKKβ: Inhibitor of nuclear factor kappa-B kinase subunit beta

IKKγ: Inhibitor of nuclear factor kappa-B kinase subunit gamma

IL: Interleukin

IL1: Interleukin 1

iNOS: Inducible nitric oxide synthase

IQ: Intelligence quotient

IκB: Inhibitory complex κB

IκB: Inhibitory complex κB

K-SADS-PL: Kiddie-Schedule for Affective Disorders & Schizophrenia, Present & Lifetime Version

MADRS: Montgomery-Asberg Depression Rating Scale

MAGL: Monoacylglycerol lipase

MRI: Magnetic resonance imaging

mRNA: Messenger ribonucleic acid

NAPE: N-acyl phosphatidylethanolamine phospholipase

NAPE-PLD: N-acyl phosphatidylethanolamine-specific phospholipase D

NEDA: N-(1-naphthyl) ethylenediamine dihydrochloride

NFκB: Nuclear transcription factor κB

NGF: Nerve growth factor

NO: Nitric oxide

NO_2^-: Nitrogen dioxide

NSAIDs: Non-steroidal anti-inflammatory drugs

O_2^-: Superoxide radical

OD: Optical density

OH^-: Hydroxyl radical

$ONOO^-$: Peroxinitrite

P: Phosphor

p50: NFκB p50 protein subunit

P65: NFκB p65 protein subunit

PANSS: Positive and Negative Syndrome Scale

PBMC: Peripheral blood mononuclear cells

PEPs: Spanish abbreviation of "Primeros Episodios Psicóticos"

PGDS: Prostaglandin-D synthase

PGE2: Prostaglandin E2

PPAR: Peroxisome proliferator activated receptors

PPARγ: Peroxisome proliferator activated receptors, gamma isoform

PPRE: Peroxisome proliferator response element

SCID: Semi-structured diagnostic interview to establish the diagnosis in adults

SNPs: Single nucleotide polymorphisms

SP-1: Specificity Protein 1

TBA: Thiobarbituric acid

TBARS: Thiobarbituric Acid Reactive Substances (TBARS)

TBS-Tween: Tris Buffered Saline –Tween

TNF: Tumor necrosis factor

TRP channels: Transient receptor potential channels

Ub: Ubiquitin

WB: Western blot

YMRS: Young Mania Rating Scale

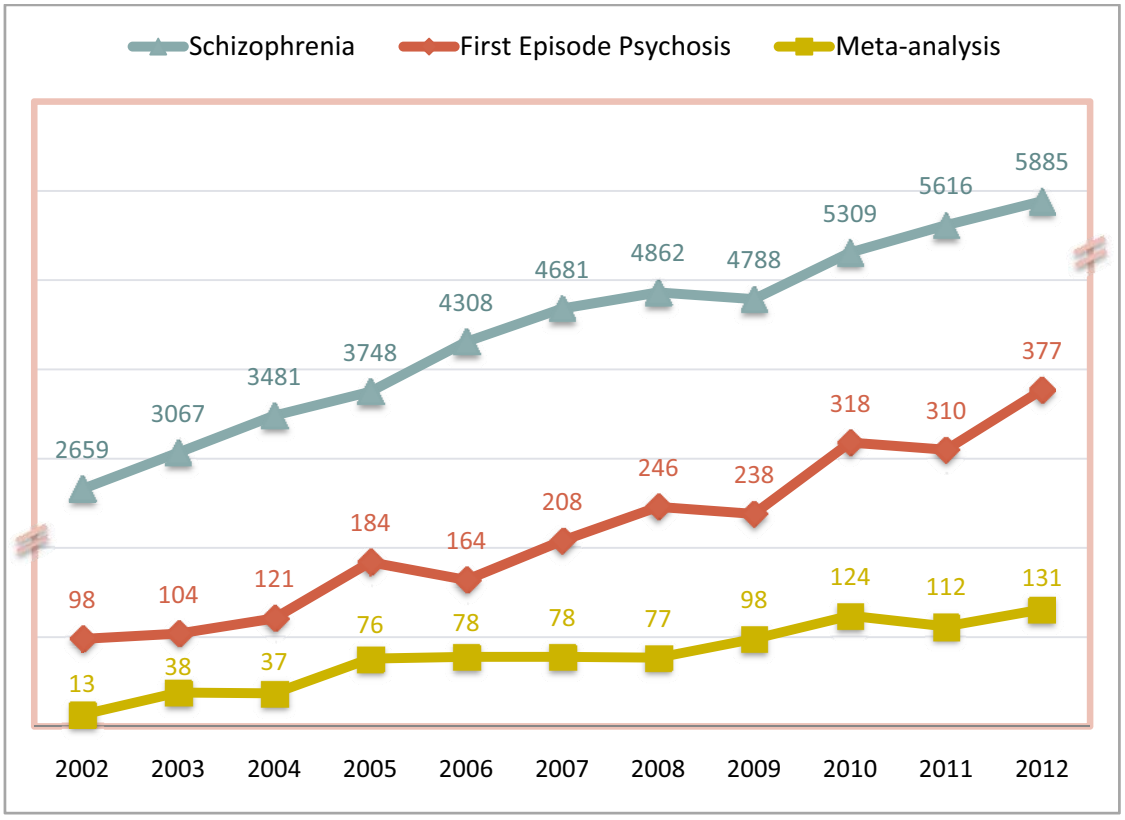
1- INTRODUCTION

Schizophrenia and first-episode psychosis

Psychotic disorders are characterized by the presence of positive (delusions, hallucinations and disorganization), negative (such as apathy or alogia), cognitive and affective symptoms. Among others, disorders that may present episodes with psychotic features are schizophrenia related disorders, affective disorders and psychotic disorders due to drug abuse. Around 3% of the general population suffers a first episode of psychosis (FEP) along their life [1]. The start of a FEP is usually somewhere between 15 and 30 years old, when academic, professional and social skills are in their major expansion [2]. An early onset is associated with a higher genetic load, severe cognitive deterioration, male gender and worse evolution and prognosis [3-5]. The clinical evolution after a FEP uses to be chronic and variable, causing a huge loss in quality of life of patients and their families, and a high cost to society, representing the 10% of the global burden of mental disorders in Europe [6]. Complete remission only occurs in one third of the patients [7]. Up to 80% of the patients relapse during the next five years after a FEP, with a major risk to become resistant to treatment [8].

While the population with chronic schizophrenia has been studied in large, naturalistic studies with real-life patients [9], the FEP population represents a unique opportunity to evaluate the biological, clinical and functional outcomes of psychotic disorders [10]. Conducting longitudinal research in the onset of illness avoids the effect of confounding variables such as the influence of antipsychotic treatment, comorbidity or chronicity [10, 11]. Such variables cause long-term structural changes and may be one reason for the inconsistency of the findings so far [12, 13]. Patients with a first psychotic episode are therefore an excellent group to study the risk factors linked to the development of schizophrenia and other psychotic disorders related to neural stress processes [10]. Establishment of biomarkers as soon as possible after the onset of the disease will enable early disease prevention, and thus improve the prognosis [14]. Indeed, early intervention seems to mitigate progression and improve therapeutic outcomes of the disease [15]. Consequently, the characterization of the FEP population has become a priority area of growing interest for research, with large studies both in the United States and Europe [10-12, 16-18] (figure 1).

Figure 1 – Total number of articles about FEP, schizophrenia and meta-analyses on schizophrenia published in the 2002-2012 decade.

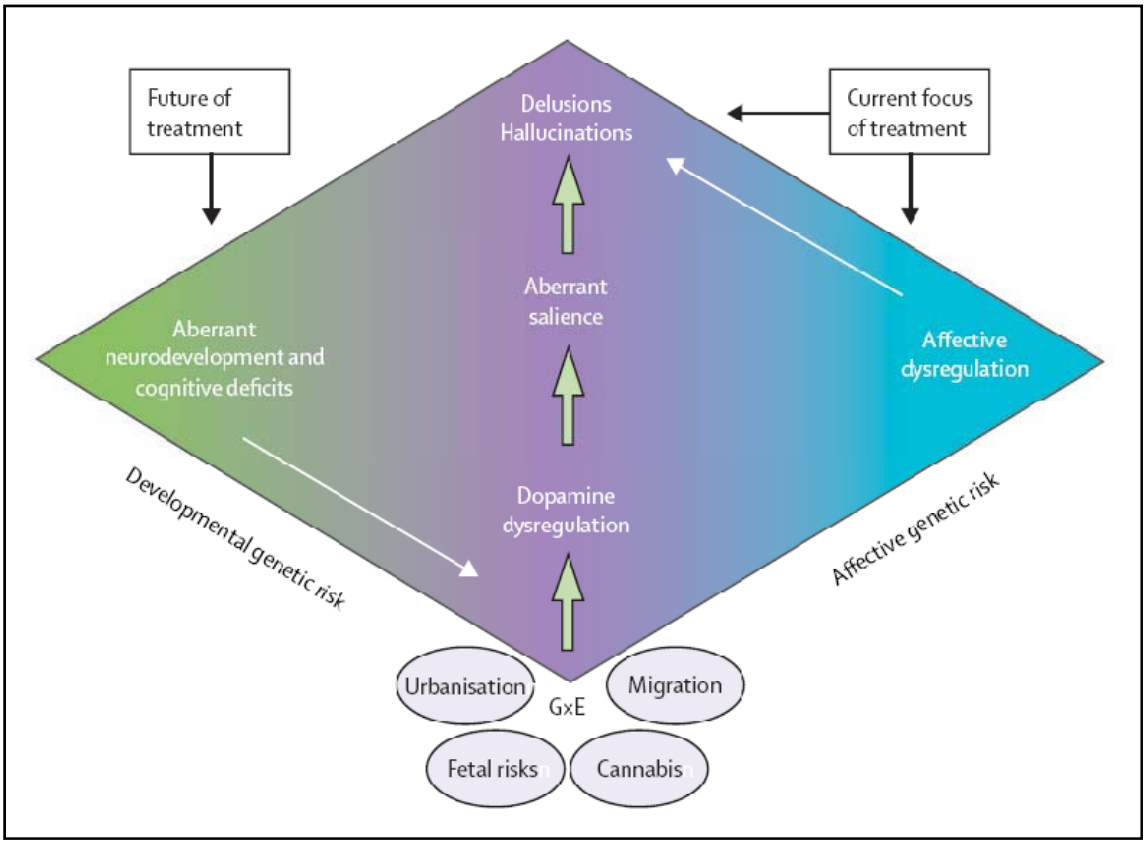


Whilst the enormous scientific efforts to better understand the nature of the disease, we are still far from having ideal effective and safe treatments to offer to our psychotic patients. There is a necessity of a change in the strategy of drug discovery for the treatment of schizophrenia, mainly based in the better comprehension of the pathophysiology [19, 20]. Not surprisingly, less than 50% of patients respond to an initial treatment with antipsychotic medication. Clearly there is a need to open strategies in possible novel therapeutic targets for new drugs [19, 20].

Gene-environment interaction

The research in recent decades demonstrates the heterogeneity of the aetiology of psychotic disorders, where both genetic and environmental factors play a key role [2] (figure 2).

Figure 2: Model of schizophrenia and related psychotic disorders



Adapted from van Os and Kapur [2]

Genetics play a big role in psychotic disorders, as evidenced by the existence of families with multiple affected individuals or monozygotic twin studies [21]. The heritability of the disorder is estimated around 80% [22], with a higher prevalence of pathology at a higher genetic load shared with the affected relative [23]. Common genetic variants tend to have low penetrance and a moderate effect on risk, while rare variants even have a major effect to explain a lower proportion of cases [24]. However, the associations obtained in these studies may be more related to disease mechanisms, evolution and classification than with the risk to suffer it [25]. Moreover, these studies do not evaluate the contribution of the interaction between genes and environment. In fact, environmental factors are not measured in most genetic studies [26-29].

The literature has detected as the main environmental risk conditions for psychosis prenatal stress, high paternal age, malnutrition, infections during pregnancy, perinatal hypoxia, presence of traumatic events, being membership of a minority ethnic groups and cannabis use. Increased risk is associated with the exposure before adulthood, suggesting an interaction with development [30-32]. Following the concept of sensibilization [33], there is evidence that the exposure to certain environmental factors interacting with genetic factors can alter dopaminergic transmission, neuroendocrine and cognitive functioning, patterns of interpersonal interaction and affective processing and may lead to a psychopathology worsening [33-36]. For instance, Caspi and colleagues found that cannabis use in adolescence increases the risk of psychosis in adulthood only to those subjects with specific catechol-O-methyl transferase gene polymorphisms [34]. Despite these studies, research on the impact of the environmental mechanisms such as cannabis in neuroinflammation mechanisms and its relationship with increased vulnerability to diseases such as schizophrenia or affective disorders is in its initial stages [37].

The schizophrenia inflammatory hypothesis

Despite the growing number of published research studies in recent years, the aetiology of psychotic disorders in general, and of schizophrenia in particular, is far from being clarified [20]. Nowadays we are living a reformulation of the classical concept of the psychotic illness [20], being seen as an heterogeneous disorder with a multisystemic impact from the beginning, in addition to its psychiatric expression [38]. Several hypotheses involving the immune system and neuroinflammatory processes – at both peripheral and central nervous system (CNS) - have been proposed as etiological explanations for psychosis [39, 40]. In this context, new research data are appearing in the field of neuroendocrinology, psychoneuroimmunology, genetics or psychopharmacology showing a big overlap between different groups of psychiatric disorders, pointing that different pathological mechanisms in schizophrenia and depression may lead to the same final common pathway of inflammation [41].

In the past fifteen years, a great deal of interest has been focused on immune/inflammatory alterations and the associated oxido/nitrosative consequences associated as key pathophysiological mechanisms involved in schizophrenia. An appreciable body of evidence indicates some immunological dysfunctions in schizophrenia, including immune or inflammatory related genes as risk factors for this disorder [42]. Some studies have shown the association

between schizophrenia risk and autoimmune diseases and severe infections [43]. Recent findings implicated an immune component to schizophrenia risk, with certain genetic variations in the major histocompatibility complex [44, 45].

Most of the evidence supporting the inflammatory hypothesis of schizophrenia referred to elevation of pro-inflammatory cytokine levels, mainly in plasma [46]. Cytokines are important mediators in the regulation between the central nervous system and the immune system [47]. Though recent reviews have demonstrated the role of certain cytokines in the schizophrenia inflammatory hypothesis, it is still soon before thinking proinflammatory cytokines and/or their signalling pathways as a possible novel strategy to treat psychosis [48]. It has also been demonstrated brain microglial activation in post-mortem and positron emission tomography studies, while up-regulated inflammation-related genes in brain tissue have been identified [49-52]. There have also been described increased plasma levels of the inflammatory mediator prostaglandin E2 (PGE_2), the major product of inducible cyclooxygenase (COX-2) and increased COX activity [53, 54]. All these data supported some clinical studies using non-steroidal anti-inflammatory drugs (NSAIDs) in psychotic disorders. Recent meta-analyses reported provisional and limited symptomatic improvement of add-on NSAIDs to antipsychotics in schizophrenia [55].

In contrast, fewer studies have focused on the role of anti-inflammatory signalling pathways in both experimental and clinical settings, with data showing a clear misbalance in some pro/anti-inflammatory mediators in blood of patients with chronic schizophrenia at protein expression level [47, 56]. The stimulation of anti-inflammatory cytokines such as IL-4, IL-10 and IL-17 seems to be a mechanism elicited by several antipsychotics to regulate uncontrolled and potentially deleterious inflammation in schizophrenia [57-59]. In fact, some authors reported an endogenous increase of these molecules in different stages of schizophrenia as an attempt to counteract (or limit) ongoing pro-inflammatory processes [60].

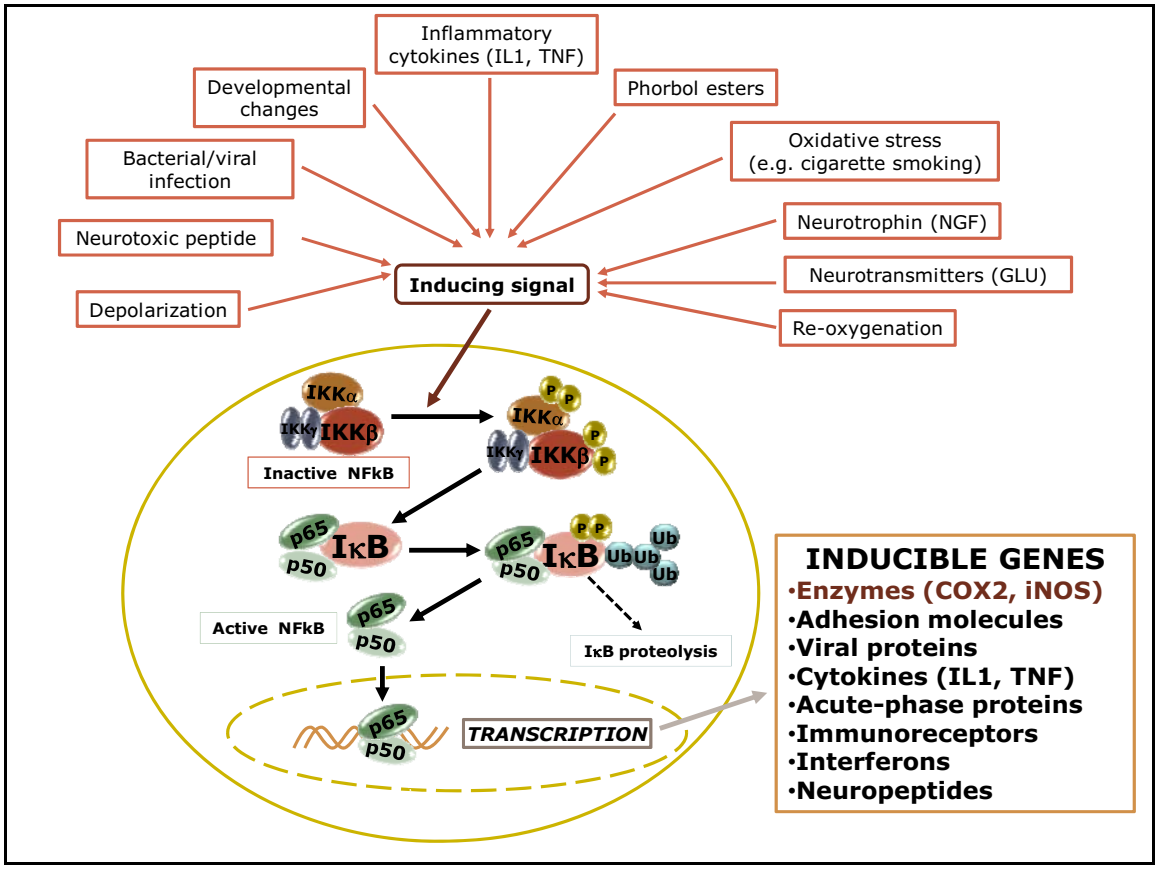
Evidence also suggests an imbalance of immune responses in schizophrenia towards a major humoral response (increased levels of IL 1, 4, 6,10,12 in plasma and CSF of patients), correlated with a worse prognosis [48].

While the inflammatory response is an adaptative mechanism that allows the organism to cope with diverse threatening challenges, under pathological and long lasting conditions the maintenance of this response could become deleterious. The precise regulation of the whole process involves complex endogenous counterbalancing mechanisms that control the effects of potentially deleterious pro-inflammatory mediators. Thus, apart from all data showing inflammatory mechanisms in schizophrenia or psychosis, several studies have focused on the role

of the anti-inflammatory signaling pathways in both experimental and clinical settings with data showing a clear misbalance in some pro/anti-inflammatory mediators in blood of patients with long-lasting schizophrenia at protein expression level [47, 56]. However, there are not data regarding the state of inflammatory mediators and their balance in early phases of the disease, such as after a FEP.

A major pro-inflammatory pathway is the one triggered by the activation of the nuclear transcription factor κB (NFκB) [61] (figure 3). Stimuli of diverse nature start up a series of multi-enzymatic routes that causes the degradation of its inhibitory complex IκB [62-64]. NFκB then translocates to the nucleus where it recognizes specific DNA sequences in the promoter of target genes among which are those that codify for the pro-inflammatory enzymes inducible nitric oxide synthase (iNOS) and the isoform 2 of the enzyme cyclooxygenase-2 (COX-2). The over-activation of these enzymes can produce an accumulation of oxidative and nitrosative mediators (i.e. nitric oxide, peroxinitrite anion, PGE_2), which can cause the depletion of endogenous antioxidant defenses and attack membrane phospholipids causing cell damage in a process known as *oxidative stress* [47].

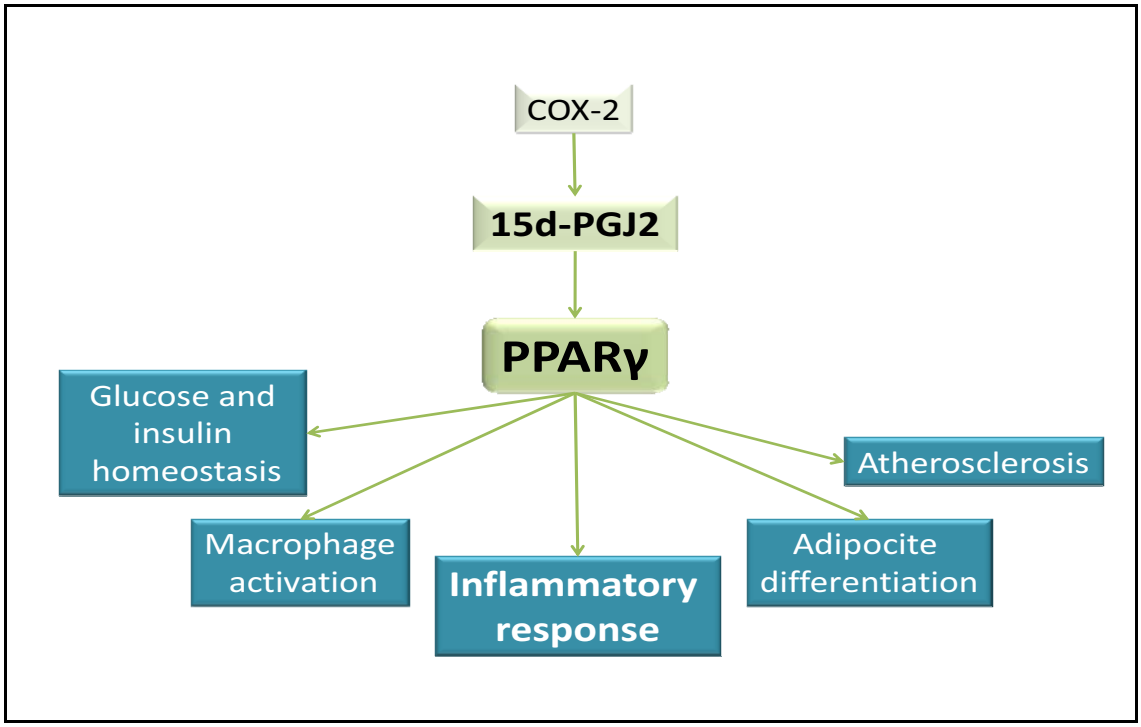
Figure 3: NF-κB activation pathway



GLU: Glutamate; **IKKα**: Inhibitor of nuclear factor kappa-B kinase subunit alpha; **IKKβ**: Inhibitor of nuclear factor kappa-B kinase subunit beta; **IKKγ**: Inhibitor of nuclear factor kappa-B kinase subunit gamma; **IL1**: Interleukin 1; **IκB**: Inhibitory complex κB; **NFκB**: Nuclear transcription factor κB; **NGF**: Nerve growth factor; **P**: Phosphor; **p50**: NFκB p50 protein subunit; **P65**: NFκB p65 protein subunit; **TNF**: Tumor necrosis factor; **Ub**: Ubiquitin

However, in the last few years, some endogenous counterbalancing mechanisms, activated in response to an inflammatory/immune stimulus, have been also described [65]. One of these mechanisms is the activation of peroxisome proliferator activated receptors (PPARs) [66] (figure 4). These nuclear receptors act as ligand-dependent transcription factors, binding to DNA in specific regions and regulating the expression of pro-inflammatory genes [66, 67]. They are expressed in the great majority of brain and peripheral immune cells and recent studies demonstrated that PPARs (mainly their gamma isoform, PPARγ) are master regulators of cerebral physiology and potential therapeutic targets for the treatment of several neuropathological conditions, including those related to neuronal stress exposure [68-70].

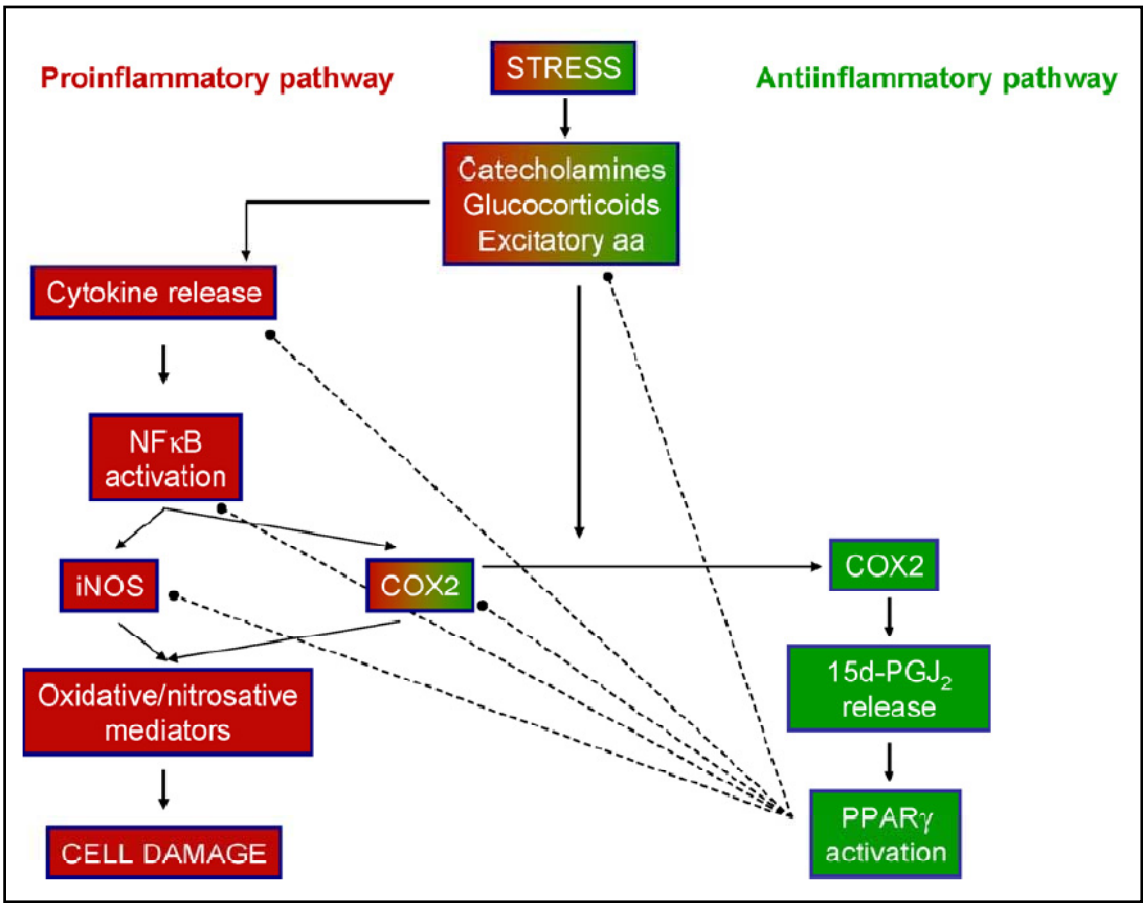
Figure 4: Transcription PPAR pathway



15d-PGJ$_2$: Prostaglandin 15-deoxy-PGJ$_2$; **COX-2**: Cyclooxygenase type 2; **PPARγ**: Peroxisome proliferator activated receptors, gamma isoform

Interestingly, several COX derived products, such as the prostaglandin 15-deoxy-PGJ$_2$ (15d-PGJ$_2$) act as endogenous anti-inflammatory agents by targeting PPARγ [69]. Thus, 15d-PGJ$_2$/PPARγ pathway is involved in the endogenous compensatory mechanism regulating the inflammatory process and that they can also be stimulated pharmacologically, representing not only a potential biomarker but an important new candidate therapeutic target in neurologic/neuropsychiatric diseases with inflammation taking part of their physiopathology (figure 5).

Figure 5: Pro-inflammatory (red) and anti-inflammatory (green) changes induced by stress in the brain



15d-PGJ$_2$: Prostaglandin 15-deoxy-PGJ$_2$; **COX-2**: Cyclooxygenase type 2; **iNOS**: Inducible nitric oxide synthase; **NFκB**: Nuclear transcription factor κB; **PPARγ**: Peroxisome proliferator activated receptors, gamma isoform. Adapted from García-Bueno et al. [47] .

Previous laboratory works using in vivo and in vitro animal models have shown the alteration of the anti-inflammatory compensatory mechanisms in stress and neuroinflammation conditions [47].

The grade and evolution of the inflammatory process, its beneficial/deleterious consequences and the nature of its auto-regulatory mechanisms may vary in the different states of the psychotic illness [71]. The majority of the scientific evidence supporting the idea that inflammatory/immune alterations may play a relevant role in psychotic disorders has been found in established schizophrenia. However, some studies indicate subtle alterations in inflammatory/immune mediators, stress response systems and oxidative/nitrosative stress at the very beginning of the natural course of the disease, as in the FEP [60, 72-74]. In fact, a recent imaging study demonstrates that neuroinflammation is more predominant than axonal degeneration in early stages of schizophrenia [75].

Schizophrenia is associated with impaired oxidative stress parameters, as a consequence of aberrant reduction-oxidation (redox) control [76]. In humans, the most important biomarkers of oxidative stress are the reactive oxygen species hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), and hydroxyl radical (OH^-) and reactive nitrogen species, that include nitric oxide (NO) and peroxinitrite ($ONOO^-$). Hydroxyl radicals, produced from both hydrogen peroxide and nitric oxide, promote apoptosis, DNA damage, protein carbonylation, and lipid peroxidation. Thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA) are important end products of lipid peroxidation [76]. Hyperhomocysteinemia can cause oxidative stress via a number of mechanisms (such as auto-oxidation) to form reactive oxygen species or increased lipid peroxidation [77]. Previous studies indicated that high levels of Homocysteine (Hcy) associate with oxidative stress in schizophrenia, showing a correlation between the increased amount of Hcy and nitrotyrosine in plasma proteins or plasma TBARS, thus being considered as a risk factor for the disease [78, 79].

The endocannabinoid system

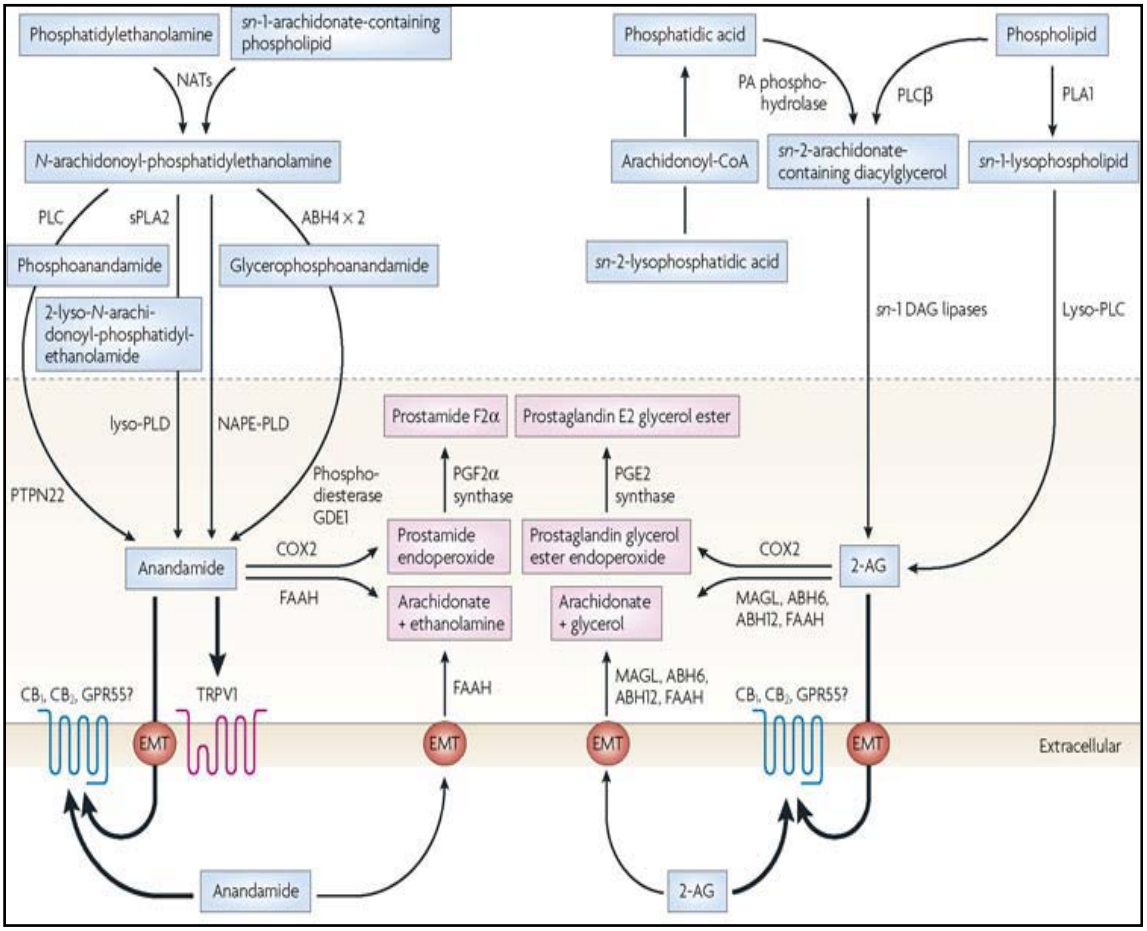
The endocannabinoid system (ECS) is a recently discovered signaling system comprising the arachidonate-based lipids ligands anandamide (AEA) and 2-arachidonoylglycerol (2AG); their cannabinoid G protein-coupled receptors, namely CB1 and CB2, the two main synthesis enzymes N-acyl phosphatidylethanolamine phospholipase (NAPE) and Diacylglycerol lipase (DAGL) and the enzymes fatty acid amide hydrolase (FAAH) and Monoacylglycerol lipase (MAGL) that are responsible for their degradation or reuptake (see figure 6) [80].

The ECS signaling system is a widespread, neuromodulatory system in brain and in the periphery which also modulates metabolic functions and the immune system, having important biological effects [81]. Considerable data support the notion that endocannabinoid signaling has three broad and overlapping functions in mammals: stress recovery, energy balance control and immune regulation [82-84].

Thus, the ECS signaling is activated in a feedback loop by stress and functions to return endocrine, nervous, and behavioral systems to homeostatic balance [82]. It is also triggered by tissue injury [85] and modulates immune and inflammatory responses [84]. So, the ECS has been proposed as a main homeostatic system implicated in the regulation of the complex neuroimmune interactions in diverse neuropathological scenarios [86]. In particular, the ECS is present in stress-responsive neural and peripheral circuits, reducing both

neurodegenerative and inflammatory damage [86, 87], similar to the previously described PPARg/15d-PGJ$_2$ pathway.

Figure 6: The main endocannabinoid system components



Biosynthetic pathways shown in blue, degradative pathways are shown in pink. **ABH4/6/12:** alphabeta-hydrolase 4/6/12; **CB1/2:** cannabinoid receptor 1/2; **COX2:** cyclooxygenase 2; **DAG**: diacylglycerol; **EMT**:'endocannabinoid membrane transporter'; **FAAH**: fatty acid amide hydrolase; **GDE1**: glycerophosphodiester phosphodiesterase 1; **GPR55**: G protein-coupled receptor 55; **MAGL**: monoacylglycerol lipase; **NAPE-PLD**: N-acyl-phosphatidylethanolamine-selective phosphodiesterase; **NATs**: N-acyltransferases; **PA**: phosphatidic acid; **(s)PLA1/2**: (soluble) phospholipase A1/2; **PLC**: phospholipase C; **PLCbeta**: phospholipase Cbeta; **PLD**: phospholipase D; **PTPN22**: protein tyrosine phosphatase, non-receptor type 22; **TRPV1**: transient receptor potential, vanilloid subtype 1 receptor. Adapted from di Marzo [80].

Stress exposure is a major contributing factor to cell death and damage in neurological and neuropsychiatric diseases by three interrelated mechanisms: hypothalamic/pituitary/adrenal axis dysregulation, excitotoxicity, and neuroinflammation. As stated above, stress exposure also activates alternative mechanisms with the aim to correctly resolve this response. In this way, the CB1 upregulation could be a mechanism related to this protective response of the brain [88]. Exposure to stress elicits excitoxicity and neuroinflammation in the brain, contributing to cell death and damage in stress-related neurological and neuropsychiatric diseases such as psychosis [47]. The ECS is present in stress-responsive neural circuits and has been proposed as an endogenous

neuroprotective system activated in some neuropathological scenarios to restore homeostasis [86, 89].

Several studies have related the ECS with psychotic disorders, focusing on CB1 and CB2 receptors. Reduced CB1 expression and activity have been found in different brain areas of patients with schizophrenia [90]. A close relationship has also been reported between a diminished CB2 function (polymorphism Q63R) and an increased susceptibility to schizophrenia, together with other risk factors [91]. Schizophrenia symptom remission has been linked to significant changes in CB2 mRNA transcripts in peripheral blood mononuclear cells (PBMC) [92]. Moreover, deletion of CB2 has been related to schizophrenia-like behaviors in animal models [93]. Therefore, it has been reported that both receptors play a homeostatic role in certain situations and their altered expression has been described in patients with schizophrenia: CB1 mainly in the CNS and CB2 at the peripheral level [81]. Regarding other components of the ECS, cerebrospinal fluid (CSF) AEA levels have been found elevated in subjects with schizophrenia [94, 95]. Finally, it has been recently reported specific alterations in the levels of some endocannabinoids in different brain regions of post-mortem brain tissue form subjects with schizophrenia [96].

Cannabis and psychosis

Exogenous cannabis use is one of the most important and studied enviromental risk factors related to psychosis [31, 32]. Around 25 to 50% of subjects who suffer a FEP use cannabis [97, 98]. Its use in youth increases the risk of developing psychosis, with an estimated odds ratio of 2.10-2.93 [99, 100], decreasing the age of schizophrenia onset [101]. A 15-year follow-up of 50465 Swedish male conscripts reported that those who had tried cannabis by age 18 years were 2.4 times more likely to be diagnosed with schizophrenia than those who had not [102]. However, the cessation of use of cannabis use after a first episode, both in adults and adolescents, is clearly associated with a clinical and functional improvement [103].

The neurobiological mechanisms underlying this increased psychosis susceptibility are poorly understood [104, 105]. Most schizophrenia patients have no history of adolescent cannabis use and the vast majority of young people who use cannabis do not develop psychosis, suggesting the hypothesis that, if cannabis is indeed causal, some individuals may be genetically vulnerable to its effects [34]. Some researchers have suggested that adolescence is a sensitive time period during which the brain may be especially vulnerable to deleterious effects of

marijuana, which may disrupt normal brain maturation and lead to increased schizophrenia risk [106]. This vision has been supported by animal studies [107].

Some studies have found that frequent cannabis exposure may down-regulate AEA signaling in patients with schizophrenia, but not in healthy individuals [108]. It has also been described that FEP who use cannabis present cognitive impairment associated to altered brain structure in particular brain regions rich in CB1 [109, 110].

The effects of both exogenous cannabis and endocannabinoids are mediated by CB1, which is widely expressed in the brain, including dorsolateral prefrontal cortex, hippocampus, posterior cingulated and medial temporal lobe [111, 112]. CB1 is localized to chromosome 6q14–q15, a schizophrenia susceptibility locus [113]. Exogenous cannabinoids could alter endogenous cannabinoid-mediated synaptic plasticity, possibly affecting brain maturation in adolescence [112]. Cannabis may affect neurodevelopmental processes, such as synaptic plasticity, thought to be impaired in schizophrenia [114]. New findings suggest that heavy cannabis use in the context of specific CB1 genotypes (tSNPs rs7766029, rs12720071, and rs9450898) may contribute to greater white matter volume deficits and cognitive impairment, which could in turn increase schizophrenia risk [110].

2- OBJECTIVES AND HYPOTHESIS

Objectives

A. To study the physiological balance between all the elements of the interrelated proinflammatory (NFκB, iNOS, COX2, NO_2^-, TBARS and Homocysteine) and anti-inflammatory pathways (15d-PG_{J2}, PPARγ and the NFκB inhibitory subunit IκBα), from the main single nucleotide polymorphisms (SNPs) to their protein expression level and activity, in plasma and peripheral blood mononuclear cells (PBMC) samples from control and FEP patients, taking advantage of a Spanish multicenter, longitudinal, naturalistic, follow-up study.

- To identify potential risk/protective factors for FEP among these pro-inflammatory and anti-inflammatory pathways.
- To detect if some of these mediators implicated are related to determinate clinical features, as global functioning, symptom severity or diagnosis.

B. To determine, after a period of 6 month of follow-up of the FEP cohort, whether there are changes in some of the components of these pro-inflammatory/anti-inflammatory pathways.

- To identify some of these biomarkers studied as potential risk/protective factors to suffer a FEP and to detect trait or state biomarkers for this condition among them, considering the longitudinal follow-up design.
- To detect if some of these mediators implicated are related to determinate clinical features, as global functioning, symptom severity or diagnosis, at this point of the evolution.

C. To study the expression of the main endocannabinoid system (ECS) components in PBMC samples from healthy controls and FEP patients, taking advantage of a Spanish multicenter, longitudinal, naturalistic, follow-up study.

- To identify some of the ECS components as potential risk/protective factors to suffer a FEP.
- To find possible differences in the ECS status according to prolonged, heavy cannabis use.

Hypothesis

A. Patients with a first episode of psychosis (FEP) will show evident systemic inflammatory conditions compared to matched healthy controls.

- The physiological balance between the interrelated pro/anti-inflammatory pathways will be disregulated in patients with a FEP. Specifically, there will be an increase of the proinflammatory pathway components (NFκB, INOS, COX2, NO_2^-, TBARS and Homocysteine) that lead to an increased oxidative stress and a decrease of the anti-inflammatory pathway components (15d-PGJ_2, PPARγ and the NFκB inhibitory subunit IκBα).
- These pathways will be altered from the main SNPs to their protein expression level and activity in plasma and PBMC samples from FEP patients.
- Some of the mediators implicated in the imbalanced, pro-inflammatory phenotype in FEP patients will be related to determinate clinical features, as global functioning, symptom severity or diagnosis.
- Some of these biomarkers studied will be identified as potential risk/protective factors.
- These results will not be cofounded by gender, age, BMI, smoking, cannabis use, antipsychotic drugs or other potential confounding factors.

B. After a period of 6 month of follow-up there will be changes in some of the components of the disregulated pro-inflammatory/anti-inflammatory pathways described in FEP.

- Some of the mediators implicated in the imbalanced, pro-inflammatory phenotype in FEP patients will be related to determinate clinical features, as global functioning, symptom severity or diagnosis.
- Some of these biomarkers studied will be identified as potential risk/protective factors.
- These results will not be cofounded by gender, age, BMI, smoking, cannabis use, antipsychotic drugs or other potential confounding factors.

C. The endocannabinoid system components studied and their homeostatic role will be disrupted in the peripheral blood mononuclear cells of first-episode of psychosis patients.

- The endocannabinoid status will be modified in those patients who reported prolonged, heavy cannabis use.
- These results will not be cofounded by gender, age, BMI, smoking, cannabis use, antipsychotic drugs or other potential confounding factors.

3- SUBJECTS AND METHODS

Subjects: PEPs & FLAMMPEPs studies

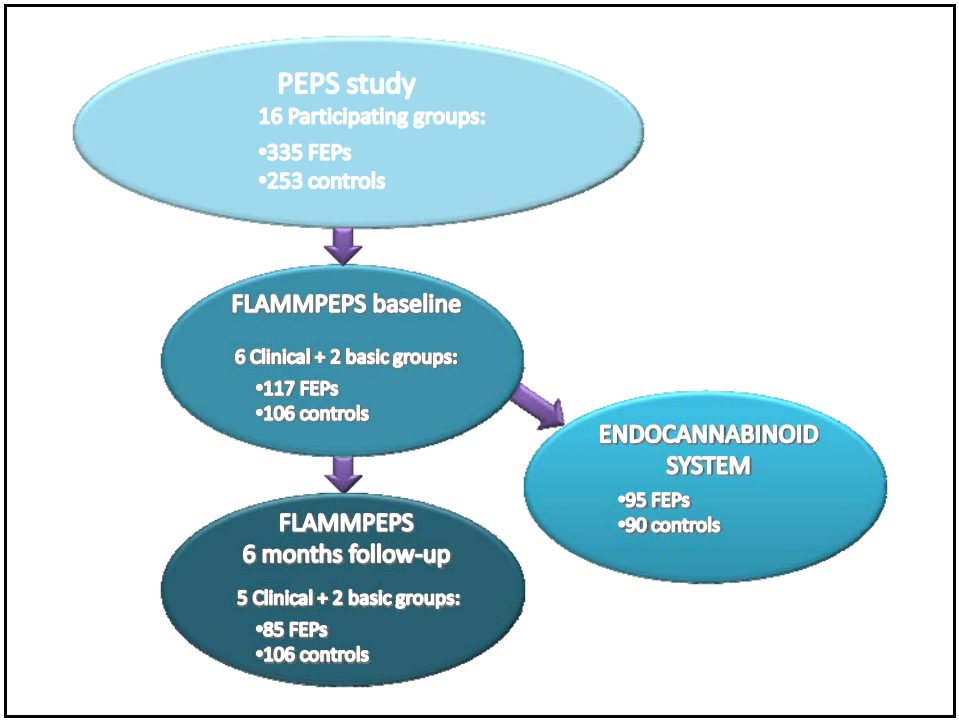
The study population comes from a Spanish multicenter, naturalistic, prospective, longitudinal study called PEPs study, from the Spanish abbreviator of "Phenotype-genotype and environmental interaction. Application of a predictive model in first psychotic episodes." The PEPs study was designed to evaluate clinical, neuropsychological, neuroimaging, biochemical, environmental and pharmacogenetic variables in a sample of more than 300 first-episode psychosis patients matched with healthy controls by age, sex and socio-economic status. Its aim is to assess the clinical characteristics, prognostic factors, diagnostic specificities of findings and pathophysiological changes in the brain during the first 2 years after the psychotic episode through an integrative and translational approach. This project was totally funded by the Spanish public health system by means of the Public National Agency (FIS), during the period 2009-2011. Patients were recruited from sixteen centers located throughout the Spanish territory with experience in performing and assessing diagnoses and in evaluation and treatment with semi-structured interviews and clinical scales from April 2009 to April 2011. All the patients who met the inclusion criteria that were attended at these facilities during the recruitment period were invited to participate in the study. Fourteen of these teams were members of the *Centro de Investigaciones Biomédicas en Red en Salud Mental* (CIBERSAM), the Spanish network of translational research in neuroscientific aspects related to health and mental illness. The other two hospitals were CIBERSAM related centers. The inclusion criteria and the rationale for the assessment approach adopted in the PEPs study, along with an overview of the selected clinical and functional measures were presented in a specific article [10].

Taking advantage of the infrastructure and deployment of human and technical resources of the PEPs project, six of the participant clinical centers and two new basic research teams conducted a sub-study called FLAMMPEPs from *"Inflammatory alterations in schizophrenia: search of biological markers in first psychotic episodes".* Its main objective was to identify possible biochemical pathways leading oxidative/nitrosative and inflammatory status in a subsample of around 100 patients with a FEP and 100 healthy controls. This project was funded by a CIBERSAM grant, and the 8 participants centers were CIBERSAM groups.

Finally, 117 patients with a FEP and 106 healthy controls were included in the FLAMMPEPs baseline visit, which were our population of study. 85 patients were followed during six months to the FLAMMPEPs final visit. From the initial sample, 95

patients with a FEP and 90 controls were included in the endocannabinoid system assay. See figure 7 for details.

Figure 7: The study population



Inclusion/Exclusion criteria

The **inclusion criteria for patients** were:

- Age between 7 and 35 years old at the time of first evaluation.
- Presence of psychotic symptoms of less than a year.
- Speak Spanish correctly.

The **exclusion criteria for patients** were:

- Mental retardation per DSM-IV criteria [115], including not only an intelligence quotient (IQ) below 70 but also impaired functioning.
- History of traumatic head injury with loss of consciousness
- History of organic disease with mental repercussions.

Healthy controls were selected from the same geographic areas following a pair-wise matching. Their **inclusion criteria for controls** were:

- Same gender as patients;
- Similar age (± 10%).

- Similar parental socioeconomic status (±1 level in the Hollingshead-Redlich scale) [116].
- No past o present psychiatric disorder per DSM-IV criteria [117].
- Speak Spanish correctly.
- No history of psychotic disorder among first-degree relatives.

The **exclusion criteria for controls** were the same than for patients.

Having designed a real-life patient, naturalistic study, substance use or having suicidal ideation were not exclusion criteria [10]. Neither the patients nor the controls presented ongoing infections, fever, allergies, other serious medical conditions as cancer or autoimmune, cardiac, pulmonary, chronic infectious diseases, or were receiving immunosuppressive drugs or vaccinations for at least six months prior to inclusion in the study, nor anti-inflammatory or analgesics the two days previous to the extraction of the blood sample.

The study was approved by the ethics committees of all the participating hospitals. The subjects participated after providing a written, informed consent, following the Declaration of Helsinki II. In underage subjects, informed consent was signed by legal guardians. Controls were offered an economic compensation for their participation of around 80€.

Clinical Assessment

Expert clinicians used the Spanish translation of the DSM-IV semi-structured diagnostic interview to establish the diagnosis in adults (SCID) [118, 119], and the Kiddie-Schedule for Affective Disorders & Schizophrenia, Present & Lifetime Version (K-SADS-PL) for subjects less than 18 years old [120].In order to not exclude early-onset psychotic patients, there was a broad age of inclusion allowed [10].

The psychopathological assessment was performed using validated Spanish versions of the Positive and Negative Syndrome Scale (PANSS) [121], the Young Mania Rating Scale (YMRS) [122] and the Montgomery-Asberg Depression Rating Scale (MADRS) [123]. The Global Assessment of Functioning Scale (GAF) and the Children's Global Assessment Scale (C-GAS) were used to measure the global severity of symptoms and the level of functioning [124, 125].

We calculated the potency equivalents to Chlorpromazine of every antipsychotic dosage, following the international consensus [126]. Apart from the interviews with the patient, multiple sources of information (including medical records and interviews with relatives) were used to establish the onset of positive psychotic symptoms (defined as the first week with the PANSS items P1, P3, P5, P6

or G9 scoring four or more). The duration of untreated psychosis (DUP) was defined as the number of days elapsed between this onset and the beginning of the first adequate treatment for psychosis.

Clinical assessment included a complete medical history and physical examination, laboratory tests and body mass index (BMI=weight in kg/height in m^2). Cannabis use was evaluated by a portion of the European Adaptation of a Multidimensional Assessment Instrument for Drug and Alcohol Dependence (EuropAsi) [127]. A systematic recording of drug misuse habits was performed.

Specimen collection and preparation

Venous blood samples (10 mL) were collected by nursery personnel in polypropylene EDTA-containing tubes in the morning (between 8:00 and 10:00) after fasting overnight. All the sample collection and preparation protocols were approved by the technical committee of the Flamm-PEPs study (available in www.cibersam.es). The fresh blood samples were maintained at 4 ºC until preparation after approximately 1 hour.

Blood tubes were centrifuged (641 g x 10 min, 4ºC). The resultant plasma samples were carefully collected and stored at -80ºC until further action was required. The rest of the sample was 1:2 diluted in culture medium (RPMI 1640®, Invitrogen, UK) and a gradient with Ficoll-Paque® (GE Healthcare, Uppsala, Sweden) was used to isolate mononuclear cells by centrifugation (800 g x 40 min, RT). Peripheral Blood Mononuclear Cells (PBMC) layer was carefully aspired and resuspended in RPMI and centrifuged (1116 g x 10 min). The supernatant was removed and the mononuclear cell enriched pellet was manually resuspended in RPMI and stored at -80 ºC until further processing.

For genetic studies, genomic DNA was isolated from 25 µL of the resuspended mononuclear cell enriched pellet using Puregene® (Gentra Systems, Indianapolis, Indiana) in accordance with the manufacturer's protocol. The DNA concentration was determined by means of absorbance (ND1000®, NanoDrop, Wilmington, Delaware).

Biochemical determinations in plasma

Prostaglandin Levels: Plasma levels of COX by-products PGE_2 and 15d-PGJ_2 were measured by enzyme immunoassay (EIA) using reagents in kit form (Prostaglandin E_2 EIA Kit-Monoclonal; Cayman Chemical Europe, Tallinn, Estonia and 15-deoxy-Δ12,14- Prostaglandin J_2 ELISA Kit DRG Diagnostics, Marburg, Germany, respectively)[128].

Nitrites: NO^-_2 the final and stable product of nitric oxide, were measured using the Griess method, where samples are incubated in acidic solution with sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride (NEDA). The nitrites are converted into a pink compound that is measured photometrically at 540nm (Synergy 2, Biotek).

Lipid peroxidation was determined by Thiobarbituric Acid Reactive Substances (TBARS) assay (Cayman Chemical Europe, Tallinn, Estonia), based on the reaction of malondialdehyde (MDA) and thiobarbituric acid (TBA) under high temperature (95 ºC) and acidic conditions. The MDA-TBA adduct formed is measured colorimetrically at 530-540 nm (Synergy 2, Biotek).

Homocysteine were determined using an enzymatic assay (Axis-Shield Diagnostics; Dundee, UK) according to manufacturer's instructions. Measurements were recorded at 340 nm and 37 °C in a Biotek PowerWave HT microplate scanning spectrophotometer (Biotek Instruments, Winooski, VT, U.S.A.). Control plasma samples with normal and abnormal ranges of Hcy were used to calibrate the assay.

Biochemical determinations in PBMC

To carry out all biochemical determinations PBMC samples were first fractionated in cytosolic and nuclear extracts. For preparation of cytosolic and nuclear extracts, a modified procedure based on the Schreiber et al method was used. PBMCs were homogenized in 150 µL buffer [10 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.9); 1 mmol/L EDTA, 1 phenylmethylsulfonyl fluoride, 0.1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL Na-p-tosyl-L-lysine-chloromethyl ketone, 5 mmol/L NaF, 1 mmol/L $NaVO_4$, 0.5 mol/L sucrose, and 10 mmol/L Na_2MoO_4]. After 15 min, Nonidet P-40 (Roche, Mannheim, Germany) was added to reach a concentration level of 1%. The tubes were gently vortexed for 30 sec, and nuclei were collected by centrifugation at 8000g x 5 min. The supernatants were considered to be the cytosolic fraction. The

pellets were resuspended in 50 µL buffer supplemented with 20% glycerol and 0.4mol/L KCl and gently shaken for 30 min at 4ºC. Nuclear protein extracts were obtained by centrifugation at 13.000 g x 5 min, and aliquots of the supernatant were stored at –80 ºC. All steps of the fractionation were carried out at 4ºC. As a control experiment to finely analyze the purity of cytosolic and nuclear extracts, Western blot (WB) assayed against GAPDH (glyceraldehyde-3-phosphate dehydrogenase), SP-1 (Specificity Protein 1) or β-actin were made (in cytosol: 99±1; 19±5; 98±1% of total optical density (OD) signal, respectively; in nuclei: 0; 81±7; 99±1% of total OD signal, respectively).

Determination of pro-inflammatory p65 NFκB subunit and anti-inflammatory PPARγ respective transcriptional activities were carried out in nuclear extracts from PMBC:

Nuclear factor kappa B (NFκB) activity: Activation of NFκB occurs by enzymatic degradation of the bound inhibitory protein (IκBα), allowing movement of the p50/65 subunits from the cytoplasm to the nucleus where they bind to consensus κB sequences in DNA. The presence of p65 subunit in cell nuclei is considered an index of activity. The activity of NFκB was measured in nuclear extracts (obtained as described above) through a commercially available NFκB (p65) Transcription Factor Assay (Cayman Chemicals, MI, USA) following the manufacturer's instructions. Briefly, a specific doublestranded DNA sequence containing the NFκB response element was immobilized to wells of a 96-well plate and nuclear extract was added. NFκB (p65) was detected by addition of a specific primary antibody directed against it and a secondary antibody conjugated to Horseradish peroxidase (HRP) was added to provide a sensitive colorimetric readout at 450 nm. The plate was read in a spectrophotometer (Biotek®, Synergy 2). The OD was normalized using the amount of protein present in the nuclear fraction - OD/mg of protein - and the results are presented as percentage of control.

PPARγ Transcription Factor Assay: PPARγ activity was determined in nuclear extracts from PBMC using ELISA-based kits, which allow the detection and quantification of PPARγ specific transcriptional activity (Cayman Chemical Europe, Tallinn, Estonia). Briefly, nuclear extracts were incubated in a multiwell plate coated with specific peroxisome proliferator response element (PPRE) probes. PPAR bounded to the PPRE probe was detected using a specific antibody against the γ isoform. HRP-labeled secondary antibody was added in and the binding was detected by spectrophotometry at 450nm (Synergy 2, Biotek). Measurement was performed according to the manufacturer's instructions. This assay is specifically for

PPARγ activation and it does not cross-react with other PPAR isoforms such as PPARα or PPARβ/δ.

The protein levels of the inhibitory subunit of NFκB IκBα and the pro-inflammatory enzymes COX2 and iNOS in cytosolic extracts from PBMC were quantified by Western Blot analysis (WB). In addition PPARγ protein expression was quantified in nuclear extracts from PBMC also by WB.

Western Blot Analysis: *A*fter determining and adjusting protein levels using the Bradford method, extracts were mixed with Laemmli sample buffer (Bio-Rad, USA) (SDS 10%, distilled H_2O, 50% glycerol, 1 M Tris HCl, pH 6.8, dithiothreitol and Bromophenol Blue) with b-mercaptoethanol (50 µL/mL Laemmli) and 12.5 µg were loaded into an electrophoresis gel. Once separated on the basis of molecular weight, proteins from the gels were blotted onto a nitrocellulose membrane (Amersham Ibérica, Spain) with a semi-dry transfer system (Bio-Rad) and were incubated with specific antibodies : (1) rabbit polyclonal IκBα in a dilution of 1:1000 in TBS-Tween (sc-371 ; Santa Cruz Biotechnology); (2) COX-2 goat polyclonal antibody in a dilution of 1:1000 in 0.5% skimmed milk in TBS-Tween (sc-1747; Santa Cruz Biotechnology); (3) iNOS rabbit polyclonal antibody in a dilution of 1:750 in 1% skimmed milk in TBS-Tween (ab15323; Abcam, UK); (4) rabbit polyclonal PPARγ in a dilution of 1:1000 in TBS-Tween (sc-7196; Santa Cruz Biotechnology, USA); (5) β-actin mouse monoclonal in a dilution of 1:15000 (Clone AC-15; Sigma, Spain); (6) SP1 rabbit polyclonal antibody in a dilution of 1:2000 (sc-59; Santa Cruz Biotechnology); GAPDH monoclonal antibody at 1:5000 (ab9484; Abcam, UK).

Membranes were incubated with the respective HRP-linked secondary antibodies (1:2000 in TBS-Tween). Blots were imaged using an Odyssey® Fc System (Li-COR Biosciences) and were quantified by densitometry (NIH ImageJ software). In the Western blot analyses carried out in cytosolic extracts, the house keeping gene β-actin was used as loading control (blots shown in the respective figures). In the case of PPARγ, the loading control was the nuclear factor SP1.

Endocannabinoid system components Western Blot Analysis: After determining and adjusting protein levels using the Bradford method (Bradford, 1976), extracts were mixed with Laemmli sample buffer (Bio-Rad, USA) (SDS 10%, distilled H_2O, 50% glycerol, 1 M Tris HCl, pH 6.8, dithiothreitol and Bromophenol Blue) with β-mercaptoethanol (50 µL/mL Laemmli) and 12.5 µg were loaded into an electrophoresis gel. Once separated on the basis of molecular weight, proteins from the gels were blotted onto a nitrocellulose membrane (Amersham Ibérica, Spain) with a semi-dry transfer system (Bio-Rad) and were incubated with specific antibodies: (1) rabbit polyclonal CB2 in a dilution of 1:1000 in TBS-Tween (101550; Cayman Chemical); (2) rabbit polyclonal CB1 in a dilution of 1:750 in TBS-Tween (ab23703; Abcam); (3) rabbit polyclonal NAPE-PLD in a dilution of 1:1000 in TBS-Tween (10306; Cayman Chemical); (4) rabbit polyclonal DAGLα in a dilution of 1:1000 in TBS-Tween (sc-133307; Santa Cruz Biotechnology, USA); (5) rabbit polyclonal FAAH in a dilution of 1:750 in TBS-Tween (101600; Cayman Chemical); (6) rabbit polyclonal MAGL in a dilution of 1:1000 in 5% skimmed milk in TBS-Tween (100035; Cayman Chemical); (7) β-actin mouse monoclonal in a dilution of 1:15000 (Clone AC-15; Sigma, Spain); (8) SP1 rabbit polyclonal antibody in a dilution of 1:2000 (sc-59; Santa Cruz Biotechnology); (9) GAPDH monoclonal antibody at 1:5000 (ab9484; Abcam, UK). Membranes were incubated with the respective HRP-linked secondary antibodies (1:2000 in TBS-Tween). Blots were imaged using an Odyssey® Fc System (Li-COR Biosciences) and were quantified by densitometry (NIH ImageJ software). In the Western blot analyses carried out in cytoplasmatic extracts, the house keeping gene β-actin was used as loading control (blots shown in the respective figures).

Gene Studies*:* A total of 40 SNPs were selected in five candidate gene regions (NFκB, iNOS, COX2, PPARγ, and PGDS: prostaglandin-D synthase, the synthesizing enzyme of PGD_2, from which PGJ_2 derive non enzymatically, covering target loci and upstream and downstream regions) by tagging analysis (as implemented in Haploview 4.1) at an r^2 threshold of 0.8 to capture 98% of the most common HapMap phase II variants based on the CEU panel (minor allele frequency>0.05) (range 91-100% for individual genes). Three SNPs were rejected prior to genotyping for assay rules. The remaining 37 tag SNPs were genotyped by the Mass Array genotyping system (Sequenom Inc., San Diego, USA).

Data collection and statistical analyses

The groups participating in the project needed an effective and friendly tool for the collection and testing of clinical and genetic data of subjects included in the study. The system created, named GRIDSAM, followed the PsyGrid® philosophy [129], defining a Service-Oriented Architecture on which are built several web applications that interact with a central database [10].

Differences between baseline characteristics for patients and controls were assessed using Chi-square, or nonparametric Mann-Whitney U tests, according to the distribution and scales of the variables.

To assess the effect of psychotropic medication we calculated the potency equivalents compared to Chlorpromazine, following the international consensus method described by Gardner *et al. [126]*, and we performed linear regression models for each biomarker.

To calculate the association between FEP and the level of biological markers, we used hierarchical logistic regression models. In order to explore mechanisms explaining the association, we used five models for each biological marker, in which we gradually controlled for potential confounders (age, gender, BMI, cannabis use per month and cotinine levels). Model 1 included the level of biological marker. Model 2 additionally included terms for age and gender. Model 3 additionally included BMI. Model 4 additionally included cannabis use per month and finally, Model 5 additionally included cotinine levels. Only biological markers significantly associated ($p<0.05$) with FEP in model 5 in the previous analyses were selected for the following steps. Logistic regression analyses were again calculated with the same system, and all the biological markers chosen were kept and analyzed together in a new model 1. Model 2 additionally included terms for age and gender. Model 3 additionally included BMI. Model 4 additionally included cannabis use per month. Model 5 additionally included cotinine levels (final model).

Multiple linear regression analysis were used to analyze the change between 6 and 12 months after diagnosis in each biological marker depending on the change in demographic (gender, age, BMI), clinical variables (DUP, GAF), antipsychotic medication (DDD), cannabis, tobacco (cotinine).

Data was managed and analyzed with the IBM SPSS Statistics v.20® [130].

4- RESULTS

Hypothesis A

García-Bueno B, Bioque M, Mac-Dowell KS, Barcones MF, Martínez-Cengotitabengoa M, Pina-Camacho L, Rodríguez-Jiménez R, Sáiz PA, Castro C, Lafuente A, Santabárbara J, González-Pinto A, Parellada M, Rubio G, García-Portilla MP, Micó JA, Bernardo M, Leza JC. Pro-/Anti-inflammatory Dysregulation in Patients With First Episode of Psychosis: Toward an Integrative Inflammatory Hypothesis of Schizophrenia. Schizophr Bull. 2013 Mar 13.

IF (2012): 8.486. Psychatry rank: 7/135 .

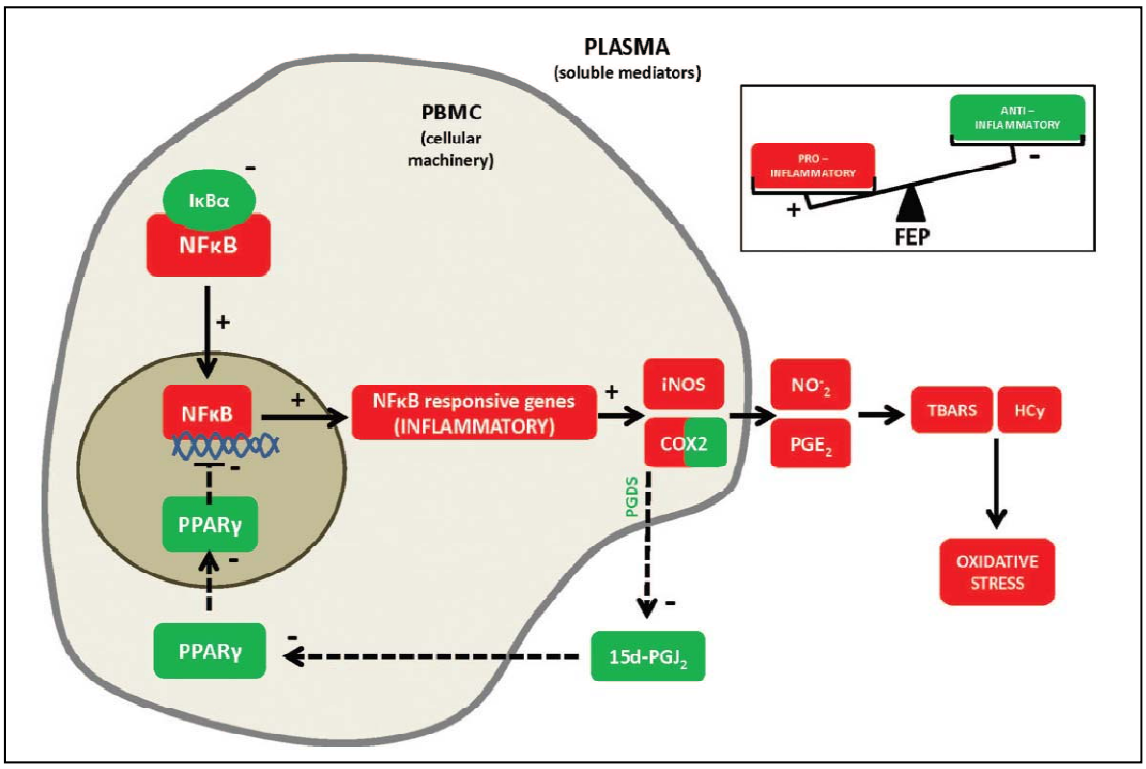
In this study, we found evidence of systemic inflammatory conditions in patients diagnosed of FEP. Specifically we have identified a significant increase in some intracellular components of a main pro-inflammatory pathway, along with a significant decrease in the anti-inflammatory ones (see figure 8).

Multivariate logistic regression analyses conducted identified as the most reliable potential risk factors the expression of inducible isoforms of nitric oxide synthase and cyclooxygenase in PMBC and homocysteine plasma levels, and as potential protection factors the inhibitor of inflammatory transcription factor IκBα and the antiinflammatory prostaglandin 15d-PGJ_2.

Taken as a whole, the results of the study indicate robust phenotypical differences at the cellular machinery level in PBMC of patients with FEP. Although more scientific evidence is needed, the determination of multiple components of pro- and anti-inflammatory cellular pathways including the activity of nuclear receptors have interesting potential as biological markers and potential risk/protective factors for FEP.

Due to its soluble nature, a notable finding in this study is that the antiinflammatory mediator 15d-PGJ_2 might be used as plasmatic biomarker for first episodes of psychosis.

Figure 8: Inflammatory dysregulation in peripheral mononuclear blood cells and plasma from patients with FEP



15d-PGJ$_2$: Prostaglandin 15-deoxy-PGJ$_2$; **COX-2**: Cyclooxygenase type 2; **HCY**: Homocysteine; **IκBα:** Alpha inhibitory complex κB; **iNOS**: Inducible nitric oxide synthase; **NFκB**: Nuclear transcription factor κB; **NO$_2^-$**: Nitrogen dioxide; **PBMC**: Peripheral blood mononuclear cells; **PGE2**: Prostaglandin E2; **PPARγ**: Peroxisome proliferator activated receptors, gamma isoform; **TBARS:** Thiobarbituric Acid Reactive Substances (TBARS)

Hypothesis B

García-Bueno B, Bioque M, Mac-Dowell KS, Barcones MF, Martínez-Cengotitabengoa M, Pina-Camacho L, Sáiz PA, Castro C, Lafuente A, Santabárbara J, González-Pinto A, Parellada M, García-Portilla MP, Micó JA, Bernardo M, Leza JC.
Pro/anti-inflammatory disregulation in incipient psychosis: results from a longitudinal, case-control study with first-episode psychosis.

Submitted.

In this follow-up study we have strengthen the evidence of systemic inflammatory alterations in patients diagnosed of FEP. Previously, with this same cohort of patients, we described phenotypical differences in pro-inflammatory mediators at the cellular machinery level in PBMC, but the resultant soluble elements were not significantly altered.

However, 6 months later the great majority of soluble elements analyzed already appear significantly altered, suggesting the existence of a pro/anti-inflammatory balance more deregulated and potentially more harmful, as can be observed by the lipid peroxidation (TBARS) data found. NO_2^- and TBARS plasma levels and COX-2 expression are the most reliable potential risk factors and the plasmatic levels of 15d-PGJ_2 might be used as protection factor. An interesting correlation exists between antipsychotic dose and the change of PGE_2 (inverse) and 15d-PGJ_2 (direct).

Interestingly, an inverse relationship between global functioning GAF scale and TBARS is also present.

These findings support the existence of a deregulated inflammatory balance in FEP. Pro and anti-inflammatory mediators can be used as state or trait risk/protection biomarkers, respectively.

Hypothesis C

Bioque M, García-Bueno B, Mac-Dowell KS, Meseguer A, Saiz PA, Parellada M, Gonzalez-Pinto A, Rodriguez-Jimenez R, Lobo A, Leza JC, Bernardo B.
Peripheral endocannabinoid system dysregulation in first-episode psychosis.
Neuropsychopharmacology. In press.

IF (2012): 8.678. Psychiatry rank: 6/135.

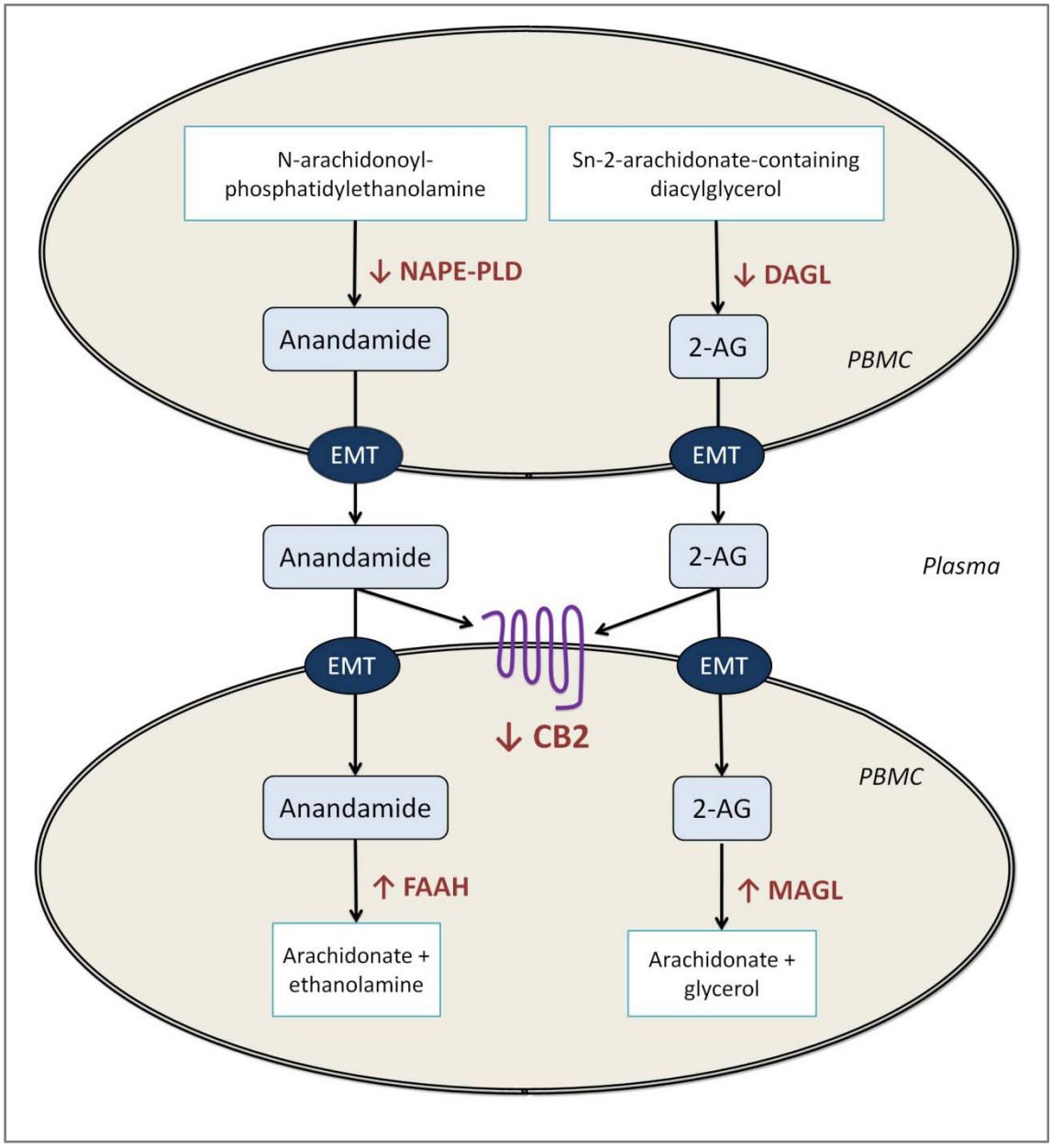
In this study, we found a reduced expression of the CB2 receptor and of the main endocannabinoid synthesis enzymes (NAPE and DAGL) in PBMC of patients with a FEP compared to matched, healthy controls. After controlling for age, gender, body mass index and cannabis use, the group of FEP showed a significantly reduced expression of the endocannabinoid synthesis enzymes (NAPE and DAGL) and an increased expression of the degradative ones (FAAH and MAGL). On the other hand, FEP subjects with history of severe cannabis use showed a larger ECS dysregulation compared to healthy controls.

All together, these results describe, for the first time to our knowledge, a dysregulation of these ECS components in patients who have suffered a FEP (figure 9).

Taking into account that prolonged cannabis use is a risk factor to develop a psychotic disorder, the FEP group was subdivided for further statistical analyses.

The patient subgroup with a history of heavy cannabis use showed a lower CB2 receptor expression, NAPE and DAGL expression in comparison to the control group. No statistically, significant differences were found with the sporadic/non-users subgroup of patients.

Figure 9: Endocannabinoid system dysregulation in peripheral blood mononuclear cells of patients who have suffered a first episode of psychosis.



CB2: cannabinoid receptor 2; **2-AG**: diacylglycerol; **EMT**:'endocannabinoid membrane transporter'; **DAGL**: Diacylglycerol lipase; **FAAH**: fatty acid amide hydrolase; **MAGL**: monoacylglycerol lipase; **NAPE-PLD**: N-acyl-phosphatidylethanolamine-selective phosphodiesterase; **PBMC**: Peripheral blood mononuclear cells

5- ARTICLES

Schizophrenia Bulletin Advance Access published March 13, 2013

Schizophrenia Bulletin
doi:10.1093/schbul/sbt001

Pro-/Anti-inflammatory Dysregulation in Patients With First Episode of Psychosis: Toward an Integrative Inflammatory Hypothesis of Schizophrenia

Borja García-Bueno[†,1], Miquel Bioque[†,2], Karina S. Mac-Dowell[†,1], M. Fe Barcones[†,3], Monica Martínez-Cengotitabengoa[4], Laura Pina-Camacho[5], Roberto Rodríguez-Jiménez[6], Pilar A. Sáiz[7], Carmen Castro[8], Amalia Lafuente[9], Javier Santabárbara[3,10], Ana González-Pinto[4], Mara Parellada[5], Gabriel Rubio[6], M. Paz García-Portilla[7], Juan A. Micó[11], Miguel Bernardo[12], and Juan C. Leza[*,1]

CIBERSAM and: [1]Department of Pharmacology, Faculty of Medicine, Complutense University and Instituto de Investigación Sanitaria-IIS-Hospital 12 de Octubre (i+12), Madrid, Spain; [2]Hospital Clínic, Barcelona, Spain; [3]Hospital Clínico Universitario, Zaragoza, Spain; [4]Hospital Universitario de Alava (sede Santiago) Universidad Nacional de Educación a Distancia, Vitoria, Spain; [5]Child and Adolescent Psychiatry Department, IIS Gregorio Marañón, IISGM, Hospital General Universitario Gregorio Marañón, Madrid, Spain; [6]IIS Hospital 12 de Octubre (i+12), Madrid, Spain; [7]Department of Psychiatry, Faculty of Medicine, University of Oviedo, Oviedo, Spain; [8]Department of Physiology, Faculty of Medicine, University of Cádiz, Cádiz, Spain; [9]Department of Pharmacology, Faculty of Medicine, University of Barcelona, Barcelona, Spain; [10]Department of Preventive Medicine and Public Health, University of Zaragoza, Zaragoza, Spain; [11]Department of Pharmacology, Faculty of Medicine, University of Cádiz, Cádiz, Spain; [12]Hospital Clínic, University of Barcelona, IDIBAPS, Barcelona, Spain

[†]These authors contributed equally to this work.

*To whom correspondence should be addressed; 28040, Madrid, Spain; tel: +34 91 394 1478, fax: +34 91 394 1464, e-mail: jcleza@med.ucm.es

All the authors were from Flamm-PEPs, a Spanish multicentric, collaborative, and translational group inside Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM) aimed to study inflammatory pathways in psychosis both as possible biomarkers and as possible new therapeutical targets.

Background: **Schizophrenia is a chronic syndrome of unknown etiology, predominantly defined by signs of psychosis. The onset of the disorder occurs typically in late adolescence or early adulthood. Efforts to study pathophysiological mechanisms in early stages of the disease are crucial in order to prompt intervention.** ***Methods:*** **Case-control study of first-episode psychotic (FEP) patients and matched controls. We recruited 117 patients during the first year after their FEP according to the DSM-IV criteria and recruited 106 gender-, race-, and age-matched controls between September 2010 and June 2011.** ***Results:*** **Biochemical studies carried out in peripheral mononuclear blood cells (PMBC) and plasma evidence a significant increase in intracellular components of a main proinflammatory pathway, along with a significant decrease in the anti-inflammatory ones. Multivariate logistic regression analyses identified the expression of inducible isoforms of nitric oxide synthase and cyclooxygenase in PMBC and homocysteine plasma levels as the most reliable potential risk factors and the inhibitor of the inflammatory transcription factor NFκB, IκBα, and the anti-inflammatory prostaglandin 15d-PGJ_2 as potential protection factors.** ***Discussion:*** **Taken as a whole, the results of this study indicate robust phenotypical differences at the cellular machinery level in PMBC of patients with FEP. Although more scientific evidence is needed, the determination of multiple components of pro- and anti-inflammatory cellular pathways including the activity of nuclear receptors has interesting potential as biological markers and potential risk/protective factors for FEP. Due to its soluble nature, a notable finding in this study is that the anti-inflammatory mediator 15d-PGJ_2 might be used as plasmatic biomarker for first episodes of psychosis.**

Key words: first-episode psychosis/inflammatory balance/schizophrenia/biomarker

Introduction

Schizophrenia is a chronic syndrome of unknown etiology, predominantly defined by signs of psychosis. The onset of the disorder occurs typically in late adolescence or early adulthood and includes positive, negative, affective and cognitive symptoms.[1] While around 3% of the general population suffers a first episode of psychosis (FEP) along their life, schizophrenia affects approximately 1% of the population worldwide.[2]

After a century of scientific effort to better understand the nature of the disease, we are still far from a clear control of the symptoms, and there is a need to change the strategies followed to understand the pathophysiology of the disorder and the possible therapeutic targets for new drugs.[3,4]

Early intervention seems to mitigate progression and improve therapeutic outcomes of the disease.[5] Establishment of biomarkers as soon as possible after the onset of the disease will enable early disease prevention and thus improve the prognosis.[6] Notably, research in the onset of illness is especially significant because it avoids the effect of confounding variables, such as chronicity. Therefore, in this vein, patients with a FEP are an excellent target to study the risk factors linked to the development of schizophrenia and other psychotic disorders.

Among the different pathophysiological mechanisms involved in schizophrenia and other psychosis, several hypotheses involving inflammatory processes, caused both by external and endogenous factors, have been proposed.[7] An appreciable body of evidence indicates a spectrum of immunological dysfunctions in schizophrenia.[8] This includes genome-wide association study results implicating immune- or inflammatory-related genes as risk factors of this disorder.[9] In addition, recent meta-analyses and reviews have reported favorable effects of add-on classic, nonsteroidal anti-inflammatory drugs to antipsychotics on total, positive and negative symptoms in schizophrenia.[10,11]

A lot of additional data support the inflammatory hypothesis in the pathophysiology of schizophrenia: (*a*) an elevation of proinflammatory cytokines[8]; (*b*) a decrease of anti-inflammatory cytokines and of the interleukin-1 antagonist receptor (IL-1ra)[12,13]; (*c*) anticytokine effect shown by some antipsychotics[14–16]; (*d*) increased plasma levels of the inflammatory mediator prostaglandin E_2 (PGE_2), the major product of inducible cyclooxygenase-2 (COX-2),[17] and increased COX activity[18]; (*e*) microglial activation suggested by postmortem and positron emission tomography studies, at least in subpopulations of individuals with schizophrenia,[19,20] and the identification of inflammation-related genes upregulated in schizophrenic brains.[21,22]

The inflammatory response is an adaptive mechanism that allows the organism to cope with diverse threatening challenges, but under pathological and long-lasting conditions, the maintenance of this response could become deleterious. The precise regulation of the whole process involves complex endogenous counterbalancing mechanisms that control the effects of potentially deleterious proinflammatory mediators. Thus, apart from all data showing inflammatory mechanisms in schizophrenia or psychosis, several studies have focused on the role of anti-inflammatory signaling pathways in both experimental and clinical settings,[23] with data showing a clear misbalance in some proinflammatory/anti-inflammatory mediators in blood of patients with long-lasting schizophrenia at protein expression level.[24] However, there are no data regarding the state of inflammatory mediators and their balance in early phases of the disease, such as after a FEP.

A major proinflammatory pathway is the one triggered by the activation of the nuclear factor κB (NFκB). Stimuli of diverse nature trigger a series of multienzymatic routes that cause the degradation of its inhibitory complex IκB.[25–27] NFκB then translocates to the nucleus where it recognizes specific DNA sequences in the promoter of target genes, among which are those that codify for the proinflammatory enzymes, inducible nitric oxide synthase (iNOS) and the isoform 2 of the enzyme cyclooxygenase-2 (COX-2). The overactivation of these enzymes can produce an accumulation of oxidative and nitrosative mediators (ie, nitric oxide, peroxinitrite anion, and PGE_2), which can cause the depletion of endogenous antioxidant defenses and attack membrane phospholipids causing cell damage in a process known as lipid peroxidation.[23]

However, in the last few years, some endogenous counterbalancing mechanisms, activated in response to an inflammatory/immune stimulus, have been also described.[28] One of these mechanisms is the activation of peroxisome proliferator activated receptors (PPARs).[29] These nuclear receptors act as ligand-dependent transcription factors, binding to DNA in specific regions and regulating the expression of proinflammatory genes.[29,30] They are expressed in the great majority of brain and peripheral immune cells,[31] and recent studies demonstrated that PPARs (mainly their gamma isoform, PPARγ) are master regulators of cerebral physiology and potential therapeutic targets for the treatment of several neuropathological conditions, including stress-related conditions.[23,32] Interestingly, several COX-derived products, such as the prostaglandin 15-deoxy-PGJ_2 (15d-PGJ_2), act as endogenous anti-inflammatory agents by targeting PPARγ.[33] Thus, 15d-PGJ_2/PPARγ pathway is involved in the endogenous compensatory mechanism regulating the inflammatory process. This pathway can also be stimulated pharmacologically, representing not only a potential biomarker but also an important new candidate therapeutic target in neurologic/neuropsychiatric diseases, with inflammation taking part in their physiopathology.

Based on this background, we hypothesized that the physiological balance between these interrelated proinflammatory/anti-inflammatory pathways may be disrupted in FEP (see figure 3). Our purpose is to study in detail all the elements of these pathways from the main single nucleotide polymorphisms (SNPs) to their protein expression level and activity in plasma and Peripheral Blood Mononuclear Cells (PBMC) samples from control and FEP patients, taking advantage of a Spanish multicenter, longitudinal, naturalistic, follow-up study, designed to evaluate clinical, neuropsychological, neuroimaging, biochemical, and genetic variables of a strictly recruited FEP

patient sample between 2010–2011 (PEPs study). Finally, multivariate logistic regression analyses were conducted to identify potential risk/protective factors for FEP.

Methods

See online supplementary material for complete details of each subheading.

Subjects

We recruited 117 patients during the first year after their first episode of psychosis according to the DSM-IV criteria[34] and 106 gender-, race-, and age-matched controls. In adults, diagnosis was established according to DSM-IV criteria (SCID-I and II).[34] For participants under 18 years of age, diagnosis was made using the Kiddie-Schedule for Affective Disorders & Schizophrenia, Present & Lifetime Version (K-SADS-PL).[35] The duration of untreated psychosis (DUP) was defined as the number of days elapsed between the first onset of positive psychotic symptoms (the first week with the Positive and Negative Syndrome Scale [PANSS] items P1, P3, P5, P6, or G9 scoring four or more) and the beginning of the first adequate treatment of psychosis. Full recruitment details are shown in supplementary information (see Methods and online supplementary data 1).

Baseline demographic details of patients involved in the study are detailed in table 1. To ensure diagnosis stability, clinical evaluations were repeated after 6 months of the inclusion of the patients. In order to not exclude early-onset psychotic patients, there was a broad age of inclusion allowed. Inclusion criteria for patients were (*a*) age: 9–35 years at the first evaluation; (*b*) presence of psychotic symptoms of less than 12 months of duration; (*c*) speaking Spanish correctly; and (*d*) having signed the informed consent. Exclusion criteria for patients were (*a*) mental retardation per the DSM-IV criteria, including not only an intelligence quotient below 70 but also impaired functioning; (*b*) history of head trauma with loss of consciousness; and (*c*) organic disease with mental repercussions.

Healthy controls were selected from the same geographic areas. Inclusion criteria were (*a*) same gender as patients; (*b*) similar age (± 10%) as patients; (*c*) similar parental socioeconomic status as patients, measured by the Hollingshead-Redlich scale (±1 level); (*d*) no past or present psychiatric disorder per DSM-IV criteria[34]; (*e*) speaking Spanish correctly; and (*f*) having signed the informed consent. The exclusion criteria for controls were (*a*) mental retardation according to DSM-IV criteria[34] including not only an intelligence quotient below 70, but also impaired functioning; (*b*) history of head trauma with loss of consciousness; (*c*) organic disease with mental repercussions; and (*d*) history of psychotic disorder among first-degree relatives.

Table 1. Baseline Demographic and Clinical Characteristics

Characteristic	Patients (N = 117)	Controls (N = 106)
Demographic characteristics		
Age(y)	23.91 ± 5.83	25.43 ± 6.43
Sex, *n* (%)		
Male	81 (69.2)	70 (66.0)
Female	36 (30.8)	36 (34.0)
Socioeconomic status		
High	22 (18.8)	14 (13.2)
Medium-high	12 (10.3)	17 (16)
Medium	48 (41)	54 (50.9)
Medium-low	27 (23.1)	19 (17.9)
Low	8 (6.8)	2 (1.9)
Ethnic group		
Caucasian	110 (94)	96 (90.6)
Gipsy	1 (0.9)	0 (0)
Maghrebian	1 (0.9)	2 (1.9)
Asian	1 (0.9)	0 (0)
Caribbean	1 (0.9)	0 (0)
Hispanic	3 (2.6)	6 (5.7)
Others	0	2 (1.9)
Psychiatric history		
Duration of untreated psychosis (DUP) in d	98.02 ± 114.38	—
Diagnosis, *n* (%)		
Affective psychosis	21 (17.9)	—
Non-affective psychosis	96 (82.1)	—
Psychopathology score		
PANSS		
Total	53.10 ± 19.50	—
Positive	11.17 ± 6.05	—
Negative	14.32 ± 6.03	—
General	27.62 ± 10.07	—
Young	1.90 ± 4.76	—
Montgomery-Asberg	6.51 ± 6.37	—
Overall functioning score (GAF)	67.20 ± 13.70	—
Baseline antipsychotic medication, *n* (%)		
Risperidone	43 (36.8)	—
Olanzapine	15 (12.8)	—
Aripiprazole	11 (9.4)	—
Paliperidone	9 (7.7)	—
Clozapine	8 (6.8)	—
Quetiapine	7 (6.0)	—
Ziprasidone	2 (1.7)	—
None	22 (18.8)	—
Lithium use, *n* (%)	10 (8.5)	—
Baseline body mass index	**24.92 ± 4.07***	**23.14 ± 3.16**
Baseline Cannabis use, *n* (%)	31 (26.5)	14 (16.0)
Baseline Cannabis per month use	**11.38 ± 34.02***	**1.15 ± 6.36**
Baseline tobacco use, *n* (%)	**56 (57.7)***	**20 (23.0)**
Baseline tobacco per month use	**212.43 ± 248.63***	**45.38 ± 119.32**

Note: Mann-Whitney *U* test, **P*-value < .05. The bold values in the table represent the values reaching statistical significance (*P*-value < .05).

Clinical assessment of patients and controls included a complete medical history and physical examination, laboratory tests, electrocardiogram, weight, height, and

body mass index. The exclusion criteria were ongoing infections, fever, allergies, or the presence of other serious medical conditions (autoimmune, cardiac, pulmonary, endocrine, and chronic infectious diseases and neoplasms). Having designed a real-life patient, naturalistic study, substance use was not an exclusion criterion. Neither the FEP patients nor the healthy control subjects were receiving immunosuppressive drugs or vaccinations for at least 6 months prior to inclusion in the study or anti-inflammatory analgesics the 2 days prior to the extraction of the blood sample.

The study was approved by the Ethics Committee of the 6 participant hospitals. The subjects participated after receiving a full explanation of the study and providing written informed consent in accordance with the Declaration of Helsinki II.

Specimen Collection and Preparation

Venous blood samples (10 ml) were collected between 8:00 and 10:00 h after fasting overnight. All the sample collection and preparation protocols in Flamm-PEPs study are available at www.cibersam.es. Samples were maintained at 4ºC until preparation after approximately 1 h.

Blood tubes were centrifuged (641 g × 10 min, 4ºC). The resultant plasma samples were collected and stored at −80ºC. The rest of the sample was 1:2 diluted in culture medium (Roswell Park Memorial Institute [RPMI] 1640, Invitrogen) and a gradient with Ficoll-Paque (GE Healthcare) was used to isolate mononuclear cells by centrifugation (800 g × 40 min, room temperature [RT]). PBMC layer was aspired and resuspended in RPMI and centrifuged (1116 g × 10 min, RT). The supernatant was removed and the mononuclear cell-enriched pellet was manually resuspended in RPMI and stored at −80ºC.

For genetic studies, genomic DNA was isolated from 25 µl of the resuspended mononuclear cell-enriched pellet using Puregene (Gentra Systems) in accordance with the manufacturer's protocol. The DNA concentration was determined by means of absorbance (ND1000, NanoDrop).

Biochemical determinations in plasma

Prostaglandin Levels. Plasma levels of COX by-products PGE_2 and 15d-PGJ_2 were measured by enzyme immunoassay (EIA) using reagents in PGE_2 EIA Kit-Monoclonal; Cayman Chemical Europe and 15-deoxy-Δ12,14- PGJ_2 Enzyme-linked immunosorbent assay (ELISA) Kit DRG Diagnostics, respectively.

Nitrites. NO^-_2, the final and stable product of nitric oxide, were measured using the Griess method.

Lipid Peroxidation. This was determined by Thiobarbituric Acid Reactive Substances (TBARS) assay (Cayman Chemical Europe), based on the reaction of malondialdehyde and thiobarbituric acid under high temperature (95ºC) and acidic conditions.

Plasma Levels of Homocysteine. Plasma levels of homocysteine (Hcy) were determined using an enzymatic assay (Axis-Shield Diagnostics) according to manufacturer's instructions.

Biochemical Determinations in PBMC

To carry out all biochemical determinations, PBMC samples were first fractionated in cytosolic and nuclear extracts. For preparation of cytosolic and nuclear extracts, a modified procedure based on the Schreiber et al.[36] method was used. Determination of proinflammatory p65 NFκB subunit and anti-inflammatory PPARγ respective transcriptional activities were carried out in nuclear extracts from peripheral mononuclear blood cells (PMBC):

Nuclear Factor Kappa B activity. Activation of Nuclear factor kappa B (NFκB) occurs by enzymatic degradation of the bound inhibitory protein (IκBα), allowing movement of the p50/65 subunits from the cytoplasm to the nucleus where they bind to consensus κB sequences in DNA. The presence of p65 subunit in cell nuclei is considered an index of activity. The activity of NFκB was measured in nuclear extracts (obtained as described above) through a commercially available NFκB (p65) Transcription Factor Assay (Cayman Chemicals) following the manufacturer's instructions.

PPARγ Transcription Factor Assay. PPARγ activity was determined in nuclear extracts from PBMC using ELISA-based kits, which allow the detection and quantification of PPARγ specific transcriptional activity (Cayman Chemical Europe).

Western Blot Analysis. The protein levels of the inhibitory subunit of NFκB, IκBα, and the proinflammatory enzymes COX-2 and iNOS in cytosolic extracts from PBMC were quantified by Western blot (WB) analysis. In addition, PPARγ protein expression was quantified in nuclear extracts from PBMC also by WB analysis. In the WB carried out in cytosolic extracts, the housekeeping genes β-actin and GAPDH were used as loading control (blots shown in the respective figures). In the case of PPARγ, the loading control was the nuclear factor SP1. For clarity, in the figures two WB results are presented, representative of all the samples studied (in each different gel, $n = 3$ per group—control or FEP sample). The insets were the most representative of statistical AU data after densitometric analysis as stated above. All densitometry results are expressed in percent from control.

Gene Studies. A total of 40 SNPs were selected in 5 candidate gene regions (*NFKB*, *NOS2–iNOS*-, *COX-2,*

PPARG, and PGDS: prostaglandin-D synthase, the synthesizing enzyme of PGD_2, from which PGJ_2 is derived non enzymatically, covering target loci and upstream and downstream regions) by tagging analysis (as implemented in Haploview 4.1) at an r^2 threshold of 0.8 to capture 98% of the most common HapMap phase II variants based on the CEU panel (minor allele frequency > 0.05) (range 91%–100% for individual genes). Three SNPs were rejected prior to genotyping for assay rules. The remaining 37 tag SNPs were genotyped by the MassARRAY genotyping system (Sequenom Inc.).

Statistical Analysis

Differences between baseline characteristics for patients and controls were assessed using Chi-square, *t* test, or nonparametric Mann-Whitney *U* tests, according to the distribution and scales of the variables.

To assess the effect of psychotropic medication, linear regression models were performed for each biomarker, and we followed the consensus method described by Gardner et al., 2010[37] to calculate the potency equivalents compared with Chlorpromazine.

To calculate the association between FEP and the level of biological markers, we used hierarchical logistic regression models. In order to explore mechanisms explaining the association, we used 5 models for each biological marker, in which we gradually controlled for potential confounders (age, gender, body mass index [BMI], cannabis use per month, and tobacco use per month). Model 1 included the level of biological marker. Model 2 additionally included terms for age and gender. Model 3 additionally included BMI. Model 4 additionally included cannabis use per month and finally, Model 5 additionally included tobacco use per month. Only biological markers significantly associated ($P < .05$) with FEP in model 4 in the previous analyses were selected for the following steps. Logistic regression analyses were again calculated with the same system, and all the biological markers chosen were kept and analyzed together in a new model 1. Model 2 additionally included terms for age and gender. Model 3 additionally included BMI. Model 4 additionally included cannabis use per month (final model). Model 5 additionally included tobacco use per month (final model).

To estimate the independent contribution of each SNP, genotype frequencies were assessed by means of multivariate methods based on logistic regression analysis and analyzed under codominant, dominant, overdominant, recessive, and additive models.

Results

Demographic and Clinical Features

The demographic and clinical characteristics of the FEP patients and healthy control group are presented in table 1 and online supplementary data 1. Patient and control groups did not differ in gender, age, and race because the case-control match had been designed. The patients and control subjects differed in body mass index (BMI; 24.92 ± 4.07 vs 23.14 ± 3.16, $P < .05$) and in baseline number of cannabis cigarettes smoked per month (11.38 ± 34.02 vs 1.15 ± 6.36, $P < .05$) although no differences were found in the percentage of active cannabis users at the study's baseline.

In addition, there are also differences in baseline tobacco use (56 patients [57.7%] vs 20 controls [23.0%]) and in baseline tobacco cigarettes per month use (212.43 ± 248.63 vs 45.38 ± 119.32).

The clinical characteristics of the sample were similar to other Spanish and European studies with FEP,[38,39] taking into account that subjects younger than 18 years were allowed to participate.

Patients had been diagnosed and treated for 6 months. The PANSS mean total score was 53.10 ± 19.50, and the mean Global Assessment of Functioning (GAF) score was 67.20 ± 13.70, although these patients had been diagnosed and treated for 6 months. The mean age of inclusion was 23.91 ± 5.83 years, while the mean DUP was 98.02 ± 114.38 days. The 82.1% of the patients had been diagnosed of nonaffective psychotic disorders. Only 21 subjects (17.9%) were oriented as affective disorders with psychotic features. Risperidone and olanzapine were the most frequent antipsychotics used. Twenty-two patients had discontinued the antipsychotic treatment at the inclusion time, 13 of which were oriented as affective psychosis and 6 (27.27%) were taking lithium. The clinical variables PANSS, Young, Montgomery-Asberg, GAF, age of inclusion, and DUP were not associated with any of the inflammatory markers studied separately.

However, the lineal regression analysis made to elucidate whether the antipsychotic treatment modify the levels of any of the inflammatory and oxido/nitrosative markers studied showed that only the levels of TBARS are significantly modified ($P = .048$) for the effect of antipsychotic medication. Thus, for each increased unit of chlorpromazine equivalents per day, TBARS levels decrease 0.003 units.

Inflammatory and Oxido/Nitrosative Markers in Control and First-Episode Psychotic Patients

The transcription factor NFκB is a master regulator of the inflammatory and oxido/nitrosative (I&ON) status of a cell. In nuclear extracts from PMBC, the activity of its p65 subunit is increased in samples from FEP patients compared with controls (table 2 and figure 1a). Similarly, the expression of the 2 main enzymatic sources of I&ON soluble mediators, iNOS and COX-2, were significantly higher in FEP group than those in the control subjects (table 2 and figure 1b and c). However, at this particular stage of the disease, the mean levels of the I&ON markers in plasma NO^-_2, PGE_2, TBARS, and Hcy were increased in FEP patients, but they did not reach statistical significance ($P > .05$; table 2 and figure 1d–g, respectively).

Table 2. Biological Markers

Marker	Patients, Total $N = 117$	Controls, Total $N = 106$	Statistics	*df*	*P*-Value
NFκB -act-	**12.79 ± 22.24*** ($n = 53$)	**4.36 ± 3.28** ($n = 35$)	$U = 612.0$	—	**.007**
iNOS -WBc-	**125.36 ± 49.71*** ($n = 91$)	**96.60 ± 40.50** ($n = 88$)	$U = 2585.5$	—	**<.001**
COX2 -WBc-	**145.28 ± 145.50*** ($n = 90$)	**94.58 ± 61.92** ($n = 88$)	$U = 2517.0$	—	**<.001**
NO^-_2 -sol-	14.65 ± 5.90 ($n = 50$)	12.75 ± 4.67 ($n = 61$)	$U = 1270.0$	—	.131
PGE_2 -sol-	524.89 ± 739.41 ($n = 111$)	373.35 ± 264.95 ($n = 104$)	$U = 5522.5$	—	.584
TBARS -sol-	3.49 ± 3.52 ($n = 105$)	2.68 ± 2.69 ($n = 104$)	$U = 4748.5$	—	.109
Hcy -sol-	16.30 ± 17.41 ($n = 71$)	9.87 ± 6.86 ($n = 41$)	$U = 1194.0$	—	.114
IκBα -WBc-	**84.81 ± 47.63*** ($n = 91$)	**103.66 ± 47.13** ($n = 88$)	$U = 2922.0$	—	**.002**
$15dPGJ_2$ -sol-	**571.32 ± 154.63*** ($n = 108$)	**618.87 ± 158.42** ($n = 104$)	$U = 4737.0$	—	**.049**
PPAR -WBn-	**77.64 ± 32.91*** ($n = 14$)	**103.12 ± 27.92** ($n = 16$)	$t = 2.30$	28	**.029**
PPAR -act-	**1.39 ± 0.96*** ($n = 71$)	**1.88 ± 1.45** ($n = 41$)	$U = 2532.5$	—	**.005**

Note: Mean differences (SD) on biomarkers levels between FEP and controls. Two-tailed *t* test was assessed for PPARγ expression because its distribution meets the assumption of normality in the Kolmogorov-Smirnov (with Lilliefors correction) test. For the rest of variables, two-tailed nonparametric Mann-Whitney *U* test was used. **P*-value < .05. The bold values in the table represent the values reaching statistical significance (*P*-value < .05). Analyses carried out in WB: protein expression, determined by western blot in PBMC (WBc: in cytoplasmatic fraction; WBn: in nuclear fraction); sol: plasma levels of soluble compounds; act: activity assay in nuclear extracts. See Methods for details.

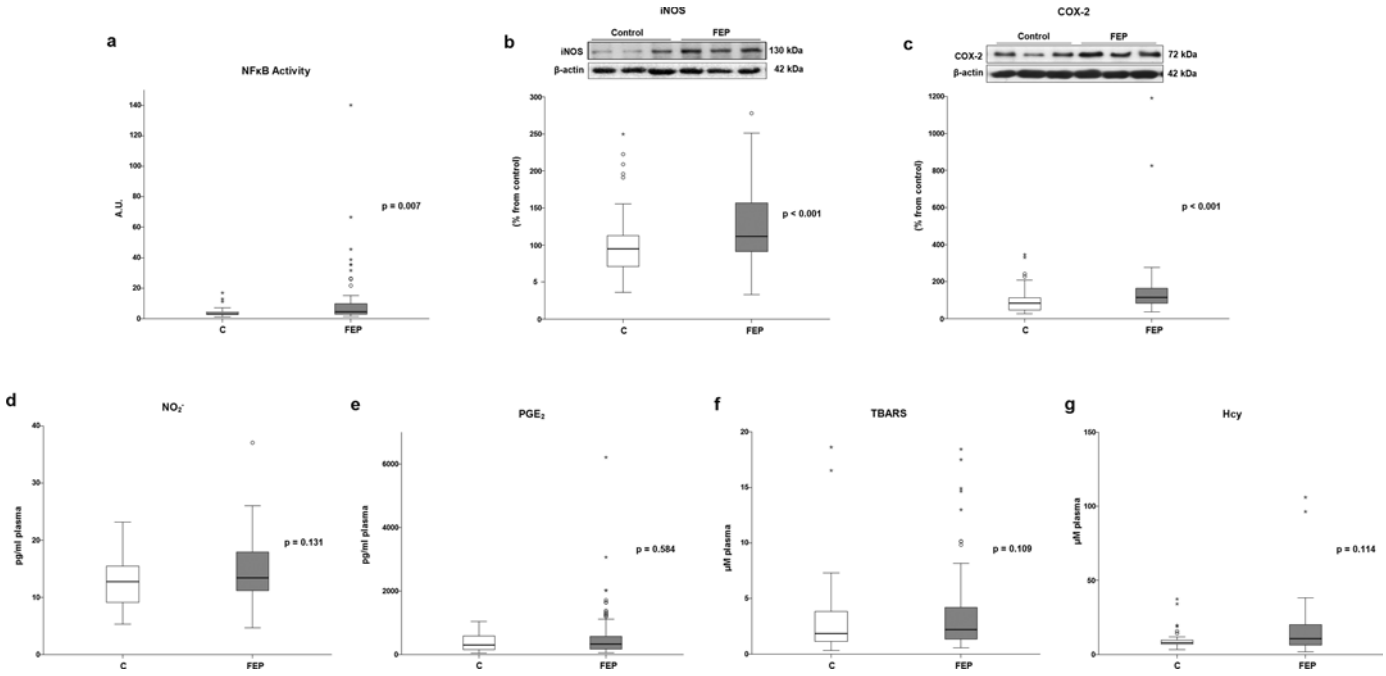


Fig. 1. Mean differences (SD) on biomarkers between FEP and controls (univariate analysis). (a) NFκB activity in PBMC nuclear extracts from FEP patients ($n = 53$) and controls ($n = 35$); (b) Western blot analysis of proinflammatory proteins iNOS (patients $n = 91$, controls, $n = 88$); (c) COX-2 in PBMC cytosolic extracts from FEP patients ($n = 90$) and controls ($n = 88$); and (d) plasma levels of nitrites (NO_2^-; patients $n = 50$, controls, $n = 61$), (e) proinflammatory prostaglandin E_2 (patients $n = 111$, controls, $n = 104$), (f) thiobarbituric acid reactive substances (patients $n = 105$, controls, $n = 104$), and (g) homocysteine from FEP patients ($n = 71$) and controls ($n = 41$). AU, arbitrary units. Two-tailed nonparametric Mann-Whitney *U* test was used. ° represents an atypical value and * an extreme value.

Anti-inflammatory Markers in Control and First-Episode Psychotic Patients

Levels of the NFκB inhibitory subunit IκBα (which maintains NFκB dimers in the cytoplasm of unstimulated cells, blocking its translocation to the nucleus) in cytosolic extracts were decreased in patients compared with those in healthy subjects (table 2 and figure 2a), suggesting the presence of a chronic proinflammatory status in PBMC from patients.

On the contrary, the plasma levels of the anti-inflammatory prostaglandin $15d\text{-}PGJ_2$ were significantly lower in FEP patients than those in the control subjects (table 2 and figure 2b).

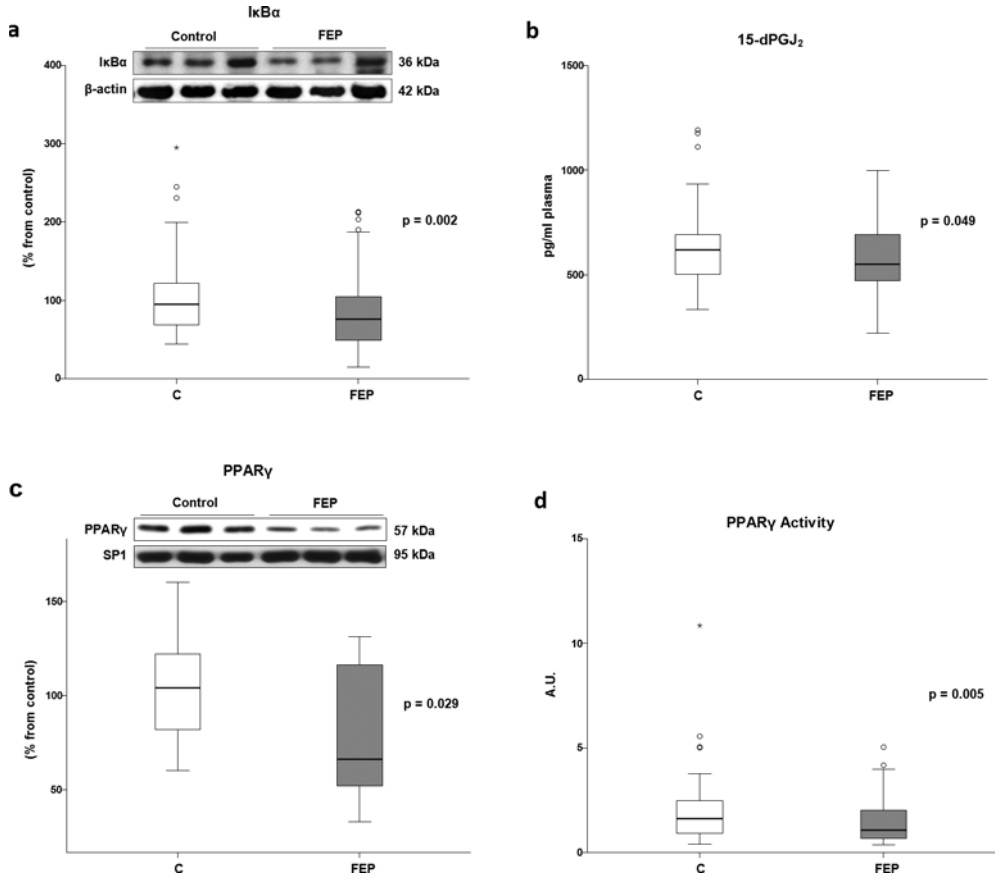


Fig. 2. Mean differences (SD) on (a) Western blot analysis of IκBα in PBMC cytosolic extracts (patients $n = 91$, controls, $n = 88$); (b) plasma levels of anti-inflammatory prostaglandin 15d-PGJ_2 (patients $n = 108$, controls, $n = 104$); (c) Western blot analysis of peroxisome proliferator activated receptor γ (PPARγ; patients $n = 14$, controls, $n = 16$); and (d) transcriptional activity of PPARγ (patients $n = 78$, controls, $n = 87$) in PBMC nuclear extracts. Two-tailed t test was assessed for PPARγ, and for the rest of variables, two-tailed nonparametric Mann-Whitney U test was used. ° represents an atypical value and * an extreme value.

Given that 15d-PGJ_2 acts as an endogenous ligand for PPARγ, which is considered a potent anti-inflammatory transcription factor, we explored the expression (by WB analysis) and transcriptional activity (by studying its ability to bind to its specific DNA response elements) of this receptor in nuclear extracts from PBMC. The WB analysis revealed lower PPARγ expression in patients compared with controls (table 2 and figure 2c). Moreover, the transcriptional activity of PPARγ, analyzed by its ability to bind to its specific DNA response elements, was also reduced in patients (table 2 and figure 2d).

Gene Studies

The results of the association analysis for the 35 SNPs studied in 5 candidate genes (NFκB, iNOS, COX-2, PPARG, and PGDS) are shown in online supplementary data 2. Two polymorphisms presented nominal pointwise, $P < .027$, the nominal P-value expected by chance (rs13348258, NFκB; rs2779248, iNOS). However, none of these showed significant empirical P-values after permutation correction for multiple testing.

Multivariate Analysis

In the final model, only 6 of the 11 biological markers studied were significantly associated with FEP after controlling for all possible confounders (table 3 and online supplementary data 3). The resulting equation was [0.428 × NFκB] + [0.055 × iNOS] + [0.072 × COX-2] + [0.432 × Hcy] + [−0.097 × IκBα] + [−0.069 × 15d-PGJ_2] + [−0.152 × gender (female)] + [0.482 × age] + [1.185 × BMI] + [0.182 × cannabis use per month] + [0.011 × tobacco use per month].

Among the proinflammatory markers, the highest odds ratio (OR) observed was the Hcy (OR = 1.541), meaning that for each unit increased of this biomarker, the risk[40] of FEP increased by 54.1% [$(e^{0.432\times1} - 1) \times 100$] after controlling for remaining biological markers and all possible confounders. Similarly, the results were 53.4% for NFκB (not significant), 5.6% for iNOS, 7.5% for COX-2.

Among the anti-inflammatory markers, the IκBα variable had the lowest OR observed (OR = 0.908), meaning that the association went in the inverse direction of proinflammatory markers, because the risk decreased by 10.2%

Table 3. Multivariate Logistic Regression Analysis

	B	SE	Wald	OR (95 % CI)	*P*-Value
NFκB	0.428	0.234	3.242	1.534 (0.963–2.443)	.072
iNOS	0.055	0.028	4.041	**1.057 (1.001–1.115)**	**.044**
COX2	0.072	0.037	3.889	**1.075 (1.000–1.154)**	**.049**
Hcys	0.432	0.202	4.560	**1.541 (1.036–2.291)**	**.033**
IκBα	−0.097	0.042	5.403	**0.908 (0.837–0.985)**	**.020**
15d-PGJ_2	−0.069	0.032	4.697	**0.933 (0.876–0.993)**	**.030**

Note: Association between FEP and level of biomarker. All the biomarkers were analyzed together and adjusted for age, gender, body mass index, cannabis use per month, and tobacco use per month. The bold values in the table represent the values reaching statistical significance (*P*-value < .05).

$[(e^{0.097\times1} - 1) \times 100]$ for each unit of increased biomarker after controlling for remaining biological markers and all possible confounders. Similarly, the result for 15d-PGJ_2 was 7.1%.

PPARγ protein expression data were excluded of the multivariate analysis due to its small sample size although it was significant ($P < .05$) in its individual regression model controlled for all confounders.

Discussion

In this study, we have found evidence of systemic inflammatory conditions in FEP patients. Specifically we have identified a significant increase in some intracellular components of a main proinflammatory pathway, along with a significant decrease in the anti-inflammatory ones. This is, to our knowledge, the first description of such imbalance in this particular clinical sample. All together, these results describe an imbalanced, proinflammatory phenotype in FEP patients. The multivariate logistic regression analyses conducted allows us to identify Hcy plasma levels as the most reliable potential risk factor, along with iNOS and COX-2, and IκBα and 15d-PGJ_2 as potential protection factors. Due to its soluble nature, a notable finding in this study is that the anti-inflammatory 15d-PGJ_2 might be used as plasmatic biomarker for FEP.

The results of the multivariate analysis applied is of special translational importance because it tries to simulate what actually happens in biological pathways, including both pro- and anti-inflammatory markers in the same statistical model, allowing them to interact even with possible sociodemographic confounders. So in the clinical practice, we could calculate the association between FEP and one of the markers (eg, iNOS) once the influence of other markers (PGE_2, COX-2, Hcy, and 15d-PGJ_2) and confounders (age, sex, BMI, cannabis, and tobacco) have been controlled. The association calculated is quiet stable because this did not change after adjusting for possible confounders. The strength of the association was supported by the stability of OR in the different models calculated.

Taken as a whole, the results of this study indicate phenotypical differences at the cellular machinery level in PBMC of FEP patients, 80% of whom are at the beginning of a multi-episode chronic severe mental illness such as schizophrenia or bipolar disorder.[41] It is worth noting that, contrarily to what occurs in schizophrenia patients, the majority of the proinflammatory soluble elements are not significantly altered although all the parameters follow the same tendency to increase. In addition, the levels of TBARS, a final consequence of cellular damage produced by oxidative stress, follows the same profile. This lack of statistical significance could be explained in base of the heterogeneity of the FEP samples; in fact, only a part of these subjects will develop a full-blown schizophrenia. Longitudinal studies with the same patients will clarify the relevance of the potential oxido/nitrosative cellular damage in FEP subjects, taking into account the increase in lipid hydroperoxides reported in a similar sample.[42]

There are few studies focused on diagnostic tools in FEP; some image studies indicate subtle brain abnormalities[43] and others clinical slight symptoms.[44] In terms of the inflammation process, our results show that while the soluble final products are not significantly modified, their enzymatic sources iNOS and COX-2, both inducible isoforms regulated by the IκBα/NFκB pathway,[45,46] are overexpressed in PMBC. This suggests that FEP patients are at the onset of the inflammatory process.

The great majority of studies reporting inflammatory/immune alterations are described in full-blown schizophrenic patients,[15,17,24,47–50] a situation in which tissue of plasma antioxidant mechanisms are exhausted.[51] Similarly, hyperhomocysteinemia can cause oxidative stress via a number of mechanisms such as auto-oxidation of Hcy to form reactive oxygen species.[52] Previous studies[53] showed a correlation between the increased amount of Hcy and nitrotyrosine in plasma proteins or plasma TBARS,[54] thus being considered as a risk factor for the disease. In this vein, our multivariate statistical approach has identified Hcy levels as a very reliable risk factor for FEP.

There is also a decrease in the counterbalancing pathway mainly controlled by 15d-PGJ_2. Indeed, this mechanism is considered as a possible endogenous regulator of the inflammatory response in neurodegenerative conditions and stress-related diseases.[23] Our group recently described a decrease in this pathway in male, chronic schizophrenic inpatients in acute relapse phase.[24] Now, the data presented here indicate that the changes in 15d-PGJ_2/PPARγ pathway are also present at the very early stages of the disease.

This study suggests an active role for the anti-inflammatory signaling pathway in the pathophysiology of the disorder, which adds support to pharmacological strategies involving the stimulation of the PPARγ activity. Of special interest is the possible use of some thiazolidinediones, potent agonists of PPARγ, used as insulin-sensitizing drugs for the treatment of type 2 diabetes.[55]

Pharmacological activation of PPARγ is a multifaceted therapeutic target due to its anti-inflammatory/antioxidant/antiexcitotoxic/proenergetic profile, reported in some inflammatory-related scenarios (neurological and stress-related diseases).[23,56] Recently, PPARγ activation has been presented as a putative treatment for neurocognitive deficits associated with mood and psychotic syndromes.[57]

In addition to the putative neuroprotective effects of PGD_2/15d-PGJ_2/PPARγ for the negative and cognitive symptomatology treatment of schizophrenia, classical studies already suggested a relevant mechanism to elucidate a specific role for PGD_2 in the management of the positive symptoms. In these studies, PGD_2 stimulated the production of cyclic adenosine monophosphate and thereby exerted functional antagonism of dopamine-D2 receptors.[58] Therefore, PGD_2 and its metabolites could be counteracting the biochemical and behavioral effects of dopamine, and deficient PGD_2/PGJ_2 signaling in the brain could influence dopamine transmission.[59]

After the analysis of the most common SNP variables of pro/anti-inflammatory mediators, the lack of correlation with their studied gene variants could suggest a possible role for epigenetic factors, other less-studied SNPs, or other candidate genes, as well as the need for new methods to detect genetic effects. For example, a recent study used a SNP-based analysis of neuroactive pathways implicates PGE_2 as a novel mediator of antipsychotic treatment response using data from the multiphase, randomized controlled trial Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE).[60]

On the other hand, the absence of findings related to genetic makers and the lack of any clinical correlates of the biomarkers does not yield support to these biomarkers being of etiological relevance. The effects reported could well be reflecting an epiphenomenon related to stress or metabolic complications, which anyway does not diminish their value as putative therapeutic targets. Fortunately, Flamm-PEPs is still an open study, and future longitudinal studies are now conducted with 2 years of follow-up and that will help elucidate these controversial issues and evaluate the utility of this pro-/anti-inflammatory signaling pathway as biological marker for possible second episodes.

Some limitations in this study should be noted: First, we used a single control group of healthy subjects instead of using 2 control groups, 1 from healthy subjects and another including other psychiatric conditions with the aim of controlling and thereby increasing the specificity of our results. In fact, changes in both inflammatory and anti-inflammatory COX-derived pathways occur after acute and chronic stress exposure.[23,30] Second, 81.2% of the FEP patients included in our study were receiving atypical antipsychotic treatment, and there is some evidence on the potential anti-inflammatory effects of antipsychotics,[13–15] most of them at anti-cytokine level. Nevertheless, we have tried to control the possible confounding effect of antipsychotic treatment through a multiple linear regression analysis, and we found only marginal effects on the plasma levels of TBARS. As commented above, increased lipid peroxidation has been found in early-onset first-episode psychosis, but the specific effects of antipsychotic medication were not addressed.[42] Third, a small group (8%) of patients required lithium. Although there are no clear references about lithium and inflammation, the possibility of being a confounding factor was also assessed. However, the result did not modify the association showed in this study. Fourth, the total number of subjects taking part of this study is 117 patients and 106 matched controls, but we could not measure all of the parameters in all the subjects. In general, for the parameters measured in plasma (eg, the 2 prostaglandins) almost all subjects were used, but for the determinations made in the cytosolic/nuclear extracts of PBMC, some methodological limitations existed and the quantity of sample obtained is relatively low. With this limitation in mind, we have tried to get a reasonable number of subjects for each parameter studied to carry out a reliable statistical analysis. No major changes were found between the differences in the whole sample and those in the subsets that were finally analyzed. It is worth noting that PPARγ protein expression data were not chosen and kept together with the other markers selected because of the sample size was small although PPARγ protein was significant in its individual regression model controlled for all confounders. Keeping these data in mind, we cannot discard a role for PPARγ as a potential protective factor in FEP patients. In fact, PPARγ expression and activity are significant in the two-tailed Chi-square tests on categorical data used to identify differences between baseline characteristics for patients and control subjects, both in our study and in schizophrenic inpatients in acute relapse phase.[24]

Despite these limitations, *key strengths of the study deserve mention*: The sample was very homogeneous in the moment in the course of illness and originated from specific areas of 2 major and 3 middle European cities. The diagnostic evaluation was performed with a very comprehensive protocol, and inclusion-exclusion criteria were applied in a strict manner. Finally, another unique feature of the study is that it includes a wide spectrum of biochemical inflammatory markers in both PBMC and plasma samples, allowing in-depth insights and relationships between multiple components of the pro- and anti-inflammatory signaling pathways.

Efforts in describing biological markers for schizophrenia in pathway approaches are claimed by psychiatrists as tools to help early diagnosis and monitor evolution of the disease; this would greatly assist preventive strategies by identifying at-risk individuals who could then be monitored and treated in a way to minimize subsequent morbidity.

In conclusion, and regarding to implications for clinical practice, the importance of early detection and intervention in psychosis has renewed interest in subtle psychopathology beyond positive and negative symptoms and

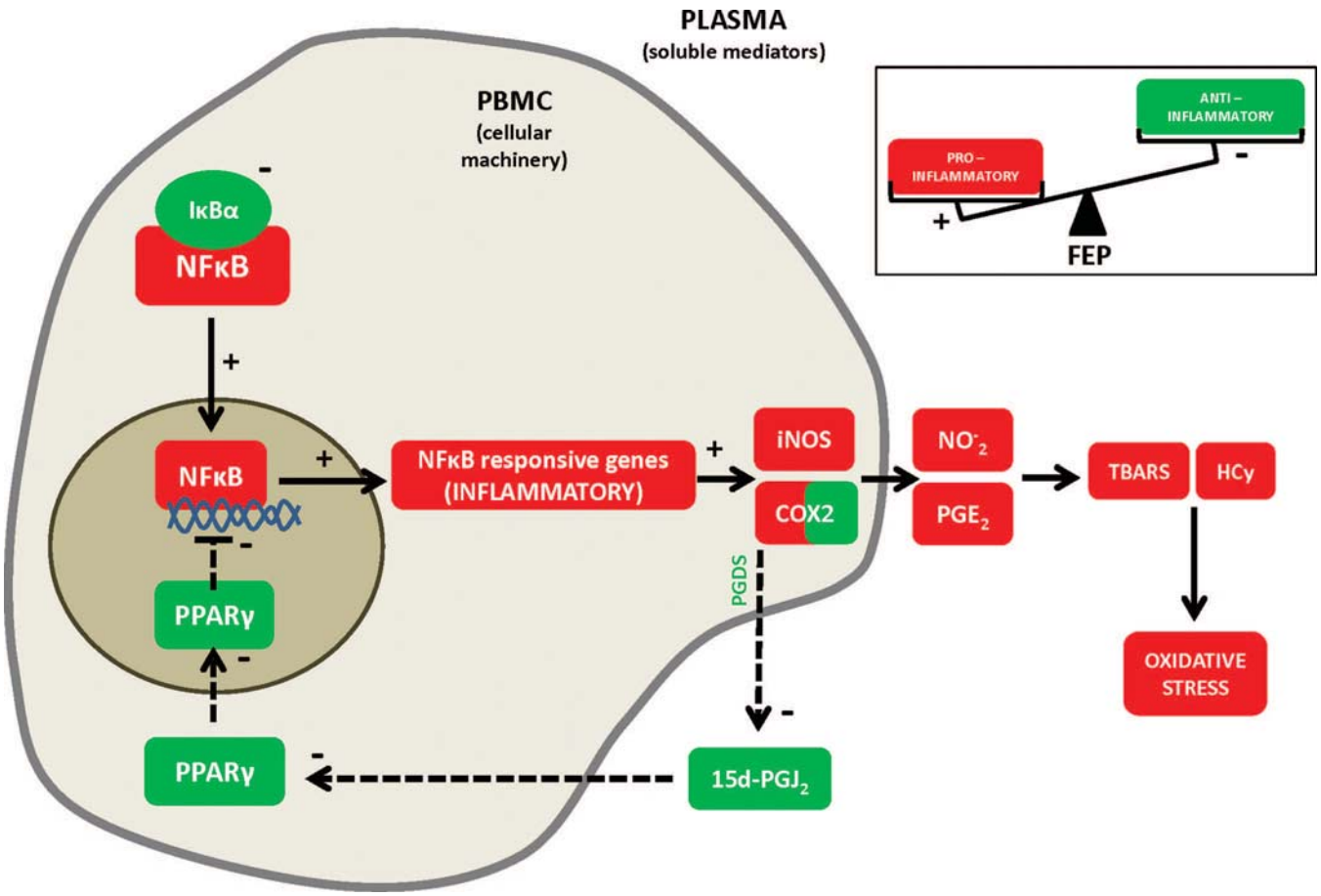


Fig. 3. Inflammatory dysregulation in peripheral mononuclear blood cells and plasma from patients with FEP. Increase in some intracellular components of the main proinflammatory pathway (in red, straight lines) has been also demonstrated: increase in NFκB transcriptional activity and increase in the expression of 2 of the main inflammatory and oxido/nitrosative inducible enzymes, iNOS and COX-2. On the other hand, a decrease in various components of the anti-inflammatory pathway (in green, dotted lines) has been also demonstrated: decrease in the production of 15d-PGJ_2, decrease in PPARγ transcriptional activity and decrease in the expression of the NFκB inhibitory subunit, IκBα. +: activation; −: inhibition.

also in the search for biological markers of the disease. Although more scientific evidence is needed, the determination of multiple components of pro- and anti-inflammatory cellular pathways has interesting potential as biological risk/protective markers for FEP. Their pharmacological modulation can be a promising (see figure 3) therapeutic target to take into account in the future.

Funding

CIBERSAM Intramural Projects 2010 (P02): Flamm-PEPs, Inflammatory alterations in schizophrenia: search of biological markers in first-psychotic episodes. Spanish Ministry of Economy and Competiveness, Instituto de Salud Carlos III, Fondo de Investigaciones Sanitarias (PI 1100325).

Supplementary Material

Supplementary material is available at http:// schizophreniabulletin.oxfordjournals.org.

Acknowledgments

The results presented here conforms a supplementary hypothesis added to the initial hypothesis in the wide PEP study (ISCIII 2009–2011). The authors declare no conflict of interest.

B.G.B. wrote the first version of the article and performed some of the biochemical determinations; M.B. managed and analyzed the clinical data; K.S.M.D. performed biochemical determinations in plasma and in cells and prepared subcellular samples; M.F.B. and J.S. performed the statistical and the first version of the figures; M.M.C., L.P., R.R.J., and P.S. collected the biological samples and the clinical data; C.C. performed the Hcy determination; A.L. performed the gene study and analyzed the data; A.G.P., M.P., G.R., and M.P.G.P. analyzed the clinical data; J.A.M. analyzed the oxidative data; M.B. coordinated PEP study and analyzed the clinical data; J.C.L. coordinated Flamm-PEP study, designed the study, and wrote the article. All of the authors contributed to the final version of the article.

References

1. van Os J, Kapur S. Schizophrenia. *Lancet*. 2009;374:635–645.
2. Perälä J, Suvisaari J, Saarni SI, et al. Lifetime prevalence of psychotic and bipolar I disorders in a general population. *Arch Gen Psychiatry*. 2007;64:19–28.
3. Lewis DA, Gonzalez-Burgos G. Pathophysiologically based treatment interventions in schizophrenia. *Nat Med*. 2006;12:1016–1022.
4. Insel TR. Rethinking schizophrenia. *Nature*. 2010;468:187–193.
5. Kuehn BM. FDA: Gene tests need premarket approval. *JAMA*. 2010;304:145.
6. Tandon R, Nasrallah HA, Keshavan MS. Schizophrenia, "just the facts" 4. Clinical features and conceptualization. *Schizophr Res*. 2009;110:1–23.
7. Lucas SM, Rothwell NJ, Gibson RM. The role of inflammation in CNS injury and disease. *Br J Pharmacol*. 2006;147 Suppl 1:S232–S240.
8. Müller N, Schwarz MJ. A psychoneuroimmunological perspective to Emil Kraepelins dichotomy: schizophrenia and major depression as inflammatory CNS disorders. *Eur Arch Psychiatry Clin Neurosci*. 2008;258 Suppl 2:97–106.
9. Shi J, Levinson DF, Duan J, et al. Common variants on chromosome 6p22.1 are associated with schizophrenia. *Nature*. 2009;460:753–757.
10. Sommer IE, de Witte L, Begemann M, Kahn RS. Nonsteroidal anti-inflammatory drugs in schizophrenia: ready for practice or a good start? A meta-analysis. *J Clin Psychiatry*. 2012;73:414–419.
11. Meyer U, Schwarz MJ, Müller N. Inflammatory processes in schizophrenia: a promising neuroimmunological target for the treatment of negative/cognitive symptoms and beyond. *Pharmacol Ther*. 2011;132:96–110.
12. Arolt V, Rothermundt M, Wandinger KP, Kirchner H. Decreased in vitro production of interferon-gamma and interleukin-2 in whole blood of patients with schizophrenia during treatment. *Mol Psychiatry*. 2000;5:150–158.
13. Maes M, Bosmans E, Ranjan R, et al. Lower plasma CC16, a natural anti-inflammatory protein, and increased plasma interleukin-1 receptor antagonist in schizophrenia: effects of antipsychotic drugs. *Schizophr Res*. 1996;21:39–50.
14. Sugino H, Futamura T, Mitsumoto Y, Maeda K, Marunaka Y. Atypical antipsychotics suppress production of proinflammatory cytokines and up-regulate interleukin-10 in lipopolysaccharide-treated mice. *Prog Neuropsychopharmacol Biol Psychiatry*. 2009;33:303–307.
15. Maes M, Bosmans E, Calabrese J, Smith R, Meltzer HY. Interleukin-2 and interleukin-6 in schizophrenia and mania: effects of neuroleptics and mood stabilizers. *J Psychiatr Res*. 1995;29:141–152.
16. Pollmächer T, Haack M, Schuld A, Kraus T, Hinze-Selch D. Effects of antipsychotic drugs on cytokine networks. *J Psychiatr Res*. 2000;34:369–382.
17. Kaiya H, Uematsu M, Ofuji M, et al. Elevated plasma prostaglandin E2 levels in schizophrenia. *J Neural Transm*. 1989;77:39–46.
18. Das I, Khan NS. Increased arachidonic acid induced platelet chemiluminescence indicates cyclooxygenase overactivity in schizophrenic subjects. *Prostaglandins Leukot Essent Fatty Acids*. 1998;58:165–168.
19. Steiner J, Bielau H, Brisch R, et al. Immunological aspects in the neurobiology of suicide: elevated microglial density in schizophrenia and depression is associated with suicide. *J Psychiatr Res*. 2008;42:151–157.
20. van Berckel BN, Bossong MG, Boellaard R, et al. Microglia activation in recent-onset schizophrenia: a quantitative (*R*)-[11C]PK11195 positron emission tomography study. *Biol Psychiatry*. 2008;64:820–822.
21. Saetre P, Emilsson L, Axelsson E, Kreuger J, Lindholm E, Jazin E. Inflammation-related genes up-regulated in schizophrenia brains. *BMC Psychiatry*. 2007;7:46.
22. Drexhage RC, van der Heul-Nieuwenhuijsen L, Padmos RC, et al. Inflammatory gene expression in monocytes of patients with schizophrenia: overlap and difference with bipolar disorder. A study in naturalistically treated patients. *Int J Neuropsychopharmacol*. 2010;13:1369–1381.
23. García-Bueno B, Caso JR, Leza JC. Stress as a neuroinflammatory condition in brain: damaging and protective mechanisms. *Neurosci Biobehav Rev*. 2008;32:1136–1151.
24. Martínez-Gras I, Pérez-Nievas BG, García-Bueno B, et al. The anti-inflammatory prostaglandin 15d-PGJ2 and its nuclear receptor PPARgamma are decreased in schizophrenia. *Schizophr Res*. 2011;128:15–22.
25. Bierhaus A, Wolf J, Andrassy M, et al. A mechanism converting psychosocial stress into mononuclear cell activation. *Proc Natl Acad Sci USA*. 2003;100:1920–1925.
26. Madrigal JL, Hurtado O, Moro MA, et al. The increase in TNF-alpha levels is implicated in NF-kappaB activation and inducible nitric oxide synthase expression in brain cortex after immobilization stress. *Neuropsychopharmacology*. 2002;26:155–163.
27. Middleton G, Hamanoue M, Enokido Y, et al. Cytokine-induced nuclear factor kappa B activation promotes the survival of developing neurons. *J Cell Biol*. 2000;148:325–332.
28. Galea E, Heneka MT, Dello Russo C, Feinstein DL. Intrinsic regulation of brain inflammatory responses. *Cell Mol Neurobiol*. 2003;23:625–635.
29. Kapadia R, Yi JH, Vemuganti R. Mechanisms of anti-inflammatory and neuroprotective actions of PPAR-gamma agonists. *Front Biosci*. 2008;13:1813–1826.
30. García-Bueno B, Madrigal JL, Lizasoain I, Moro MA, Lorenzo P, Leza JC. Peroxisome proliferator-activated receptor gamma activation decreases neuroinflammation in brain after stress in rats. *Biol Psychiatry*. 2005;57:885–894.
31. Heneka MT, Landreth GE. PPARs in the brain. *Biochim Biophys Acta*. 2007;1771:1031–1045.
32. Feinstein DL. Therapeutic potential of peroxisome proliferator-activated receptor agonists for neurological disease. *Diabetes Technol Ther*. 2003;5:67–73.
33. Forman BM, Chen J, Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. *Proc Natl Acad Sci USA*. 1997;94:4312–4317.
34. First M, Gibbon M, Spitzer RL, Williams JBW, Smith J. *Entrevista clínica estructurada para los trastornos del eje-I del DSM-IV*. Barcelona, Spain: Masson; 1999.
35. Ulloa RE, Ortiz S, Higuera F, et al. [Interrater reliability of the Spanish version of Schedule for Affective Disorders and Schizophrenia for School-Age Children–Present and Lifetime version (K-SADS-PL)]. *Actas Esp Psiquiatr*. 2006;34:36–40.
36. Schreiber E, Matthias P, Müller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res*. 1989;17:6419.

37. Gardner DM, Murphy AL, O'Donnell H, Centorrino F, Baldessarini RJ. International consensus study of antipsychotic dosing. *Am J Psychiatry*. 2010;167:686–693.
38. Castro-Fornieles J, Parellada M, Gonzalez-Pinto A, et al. The child and adolescent first-episode psychosis study (CAFEPS): design and baseline results. *Schizophr Res*. 2007;91: 226–237.
39. Kahn RS, Fleischhacker WW, Boter H, et al. Effectiveness of antipsychotic drugs in first-episode schizophrenia and schizophreniform disorder: an open randomised clinical trial. *Lancet*. 2008;371:1085–1097.
40. Hosmer DW, Lemeshow S. *Applied Logistic Regression*. New York: John Wiley & Sons; 1989.
41. Alvarez-Jiménez M, Parker AG, Hetrick SE, McGorry PD, Gleeson JF. Preventing the second episode: a systematic review and meta-analysis of psychosocial and pharmacological trials in first-episode psychosis. *Schizophr Bull*. 2011;37: 619–630.
42. Micó JA, Rojas-Corrales MO, Gibert-Rahola J, et al. Reduced antioxidant defense in early onset first-episode psychosis: a case-control study. *BMC Psychiatry*. 2011;11:26.
43. Arango C, Rapado-Castro M, Reig S, et al. Progressive brain changes in children and adolescents with first-episode psychosis. *Arch Gen Psychiatry*. 2012;69:16–26.
44. Schultze-Lutter F, Ruhrmann S, Fusar-Poli P, Bechdolf A, Schimmelmann BG, Klosterkötter J. Basic symptoms and the prediction of first-episode psychosis. *Curr Pharm Des*. 2012;18:351–357.
45. Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem*. 1994;269:4705–4708.
46. Newton R, Kuitert LM, Bergmann M, Adcock IM, Barnes PJ. Evidence for involvement of NF-kappaB in the transcriptional control of COX-2 gene expression by IL-1beta. *Biochem Biophys Res Commun*. 1997;237:28–32.
47. Song XQ, Lv LX, Li WQ, Hao YH, Zhao JP. The interaction of nuclear factor-kappa B and cytokines is associated with schizophrenia. *Biol Psychiatry*. 2009;65:481–488.
48. Das I, Khan NS, Puri BK, Sooranna SR, de Belleroche J, Hirsch SR. Elevated platelet calcium mobilization and nitric oxide synthase activity may reflect abnormalities in schizophrenic brain. *Biochem Biophys Res Commun*. 1995;212: 375–380.
49. Yokota O, Terada S, Ishihara T, et al. Neuronal expression of cyclooxygenase-2, a pro-inflammatory protein, in the hippocampus of patients with schizophrenia. *Prog Neuropsychopharmacol Biol Psychiatry*. 2004;28:715–721.
50. Yao JK, Leonard S, Reddy RD. Increased nitric oxide radicals in postmortem brain from patients with schizophrenia. *Schizophr Bull*. 2004;30:923–934.
51. Ghosh N, Ghosh R, Mandal SC. Antioxidant protection: A promising therapeutic intervention in neurodegenerative disease. *Free Radic Res*. 2011;45:888–905.
52. Jones BG, Rose FA, Tudball N. Lipid peroxidation and homocysteine induced toxicity. *Atherosclerosis*. 1994;105:165–170.
53. Brown AS, Bottiglieri T, Schaefer CA, et al. Elevated prenatal homocysteine levels as a risk factor for schizophrenia. *Arch Gen Psychiatry*. 2007;64:31–39.
54. Dietrich-Muszalska A, Malinowska J, Olas B, et al. The oxidative stress may be induced by the elevated homocysteine in schizophrenic patients. *Neurochem Res*. 2012;37:1057–1062.
55. Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J Biol Chem*. 1995;270: 12953–12956.
56. García-Bueno B, Caso JR, Pérez-Nievas BG, Lorenzo P, Leza JC. Effects of peroxisome proliferator-activated receptor gamma agonists on brain glucose and glutamate transporters after stress in rats. *Neuropsychopharmacology*. 2007;32: 1251–1260.
57. McIntyre RS, Soczynska JK, Lewis GF, MacQueen GM, Konarski JZ, Kennedy SH. Managing psychiatric disorders with antidiabetic agents: translational research and treatment opportunities. *Expert Opin Pharmacother*. 2006;7:1305–1321.
58. Ono N, Abiru T, Sugiyama K, Kamiya H. Influences of cyclooxygenase inhibitors on the cataleptic behavior induced by haloperidol in mice. *Prostaglandins Leukot Essent Fatty Acids*. 1992;46:59–63.
59. Condray R, Yao JK. Cognition, dopamine and bioactive lipids in schizophrenia. *Front Biosci (Schol Ed)*. 2011;3:298–330.
60. Adkins DE, Khachane AN, McClay JL, et al. SNP-based analysis of neuroactive ligand-receptor interaction pathways implicates PGE2 as a novel mediator of antipsychotic treatment response: data from the CATIE study. *Schizophr Res*. 2012;135:200–201.

Pro/anti-inflammatory disregulation in incipient psychosis: results from a longitudinal, case-control study with first-episode psychosis

Borja García-Bueno BSci, PhD [a], Miquel Bioque MD [b], Karina S. Mac-Dowell BSci [a], M. Fe Barcones PhD [c], Mónica Martínez-Cengotitabengoa PhD [d], Laura Pina-Camacho MD[e], Roberto Rodríguez-Jiménez MD, PhD [f], Pilar A. Sáiz MD, PhD [g], Carmen Castro PhD[h], Amalia Lafuente PhD[o], Javier Santabárbara PhD[ci], Ana González-Pinto MD, PhD [d], Mara Parellada MD, PhD [e], Gabriel Rubio MD, PhD [f], M. Paz García-Portilla MD, PhD [g], Juan A. Micó MD, PhD [j], Miguel Bernardo MD, PhD [k] and Juan C. Leza MD, PhD [a]

From the FLAMM-PEPs* study, Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM), Spain and:
[a] Dept. of Pharmacology. Faculty of Medicine. Complutense University and Instituto de Investigación Sanitaria -IIS- Hospital 12 de Octubre (i+12). Madrid
[b] Hospital Clínic. Barcelona
[c] Hospital Clínico Universitario. Zaragoza
[d] Hospital Universitario de Alava (sede Santiago) Universidad Nacional de Educación a Distancia. Vitoria
[e] Child and Adolescent Psychiatry Department, IIS Gregorio Marañón, IISGM. Hospital General Universitario Gregorio Marañón. Madrid
[f] IIS Hospital 12 de Octubre (i+12). Madrid
[g] Dept. of Psychiatry. Faculty of Medicine. University of Oviedo. Oviedo
Depts. of [h] Physiology and [j] Pharmacology. Faculty of Medicine. University of Cádiz
[O] Dept of Pharmacology, Faculty of Medicine, University of Barcelona.
[i] Department of Preventive Medicine and Public Health, University of Zaragoza.
[k] Hospital Clínic.University of Barcelona.IDIBAPS. Barcelona.

**FLAMM-PEPs is a Spanish multicentric, collaborative and translational group inside CIBERSAM aimed to study inflammatory pathways in psychosis both as possible biomarkers and as possible new therapeutical targets.*

The first four authors (BGB, MB, KSMD, MFB) contributed equally to this work

Correspondence to: Dr. Juan C. Leza, Departamento de Farmacología, Facultad de Medicina, Universidad Complutense, 28040, Madrid. Spain. jcleza@med.ucm.es

ABSTRACT

Objective: The deregulation of the pro/ antiinflammatory balance has been described in the first psychotic episode (FEP). However, the nature and/or severity of activated inflammatory responses in psychotic disease may critically change as a function of disease progression. To obtain consistent results supporting a role for inflammation in the diagnosis, aetiology and physiopathology of FEP, follow up studies controlling confounding factors and clinical variables are needed.

Method: 117 subjects with a first episode of psychosis (FEP) and 106 matched controls were recruited. From the initial sample, 85 FEP subjects were followed during 6 months. Plasma and Peripheral Blood Mononuclear Cells samples were obtained and the determination of several pro/anti-inflammatory mediators was made. Multivariate logistic regression analyses were used to identify potential risk/protective factors and finally, by means of multiple linear regression models, the change of every biological marker, depending on the changes of explanatory variables during follow-up was analysed.

Results: In this follow-up study we have strengthen the evidence of systemic inflammatory alterations in FEP, because the majority of soluble elements analysed already appear significantly altered, suggesting a more severe dysregulation. NO_2^- and TBARS plasma levels and COX-2 expression are the most reliable potential risk factors and the plasmatic levels of 15d-PGJ_2 might be used as protection factor. An interesting correlation exists between antipsychotic dose and the change of PGE_2 (inverse) and 15d-PGJ_2 (direct). Finally, an inverse relationship between GAF scale and TBARS is also present.

Conclusions: Our findings support the existence of a deregulated inflammatory balance in FEP. Pro and anti-inflammatory mediators can be used as state or trait risk/protection biomarkers, respectively. The direct association between oxidative/bitrosative damage and the GAF scale, and the striking result that one of the targets of antipsychotic therapy could be the restoration of the pro/antiinflammatory balance studied here support the establishment and completion of clinical trials using anti-inflammatory drugs as co-adjuvant strategy to antipsychotics.

INTRODUCTION

Schizophrenia is a chronic form of psychotic illness of unknown etiology, affecting approximately 1% of the population worldwide (van Os and Kapur 2009). The onset of the disorder occurs typically in late adolescence or early adulthood and includes positive, negative, affective and cognitive symptoms (van Os and Kapur 2009). This *first episode of psychosis* (FEP) is suffered by around the 3% of the general population along lifetime.

The clinical evolution after a FEP tends to be chronic and variable, causing a huge loss in quality of life of patients and their families, in their physical health, and a high cost to society (Insel 2010, Olesen, Gustavsson et al. 2011). Up to 80% of the patients relapse during the next five years after a FEP, with a major risk to become resistant to treatment (Alvarez-Jimenez, Parker et al. 2009). Complete remission only occurs in one third of the patients (Huber, Naber et al. 2008).

While the population with chronic schizophrenia has been studied in large, naturalistic studies with real-life patients (Lieberman, Stroup et al. 2005), the FEP population represents a unique opportunity to evaluate the biological, clinical and functional outcomes of psychotic disorders (Bernardo, Bioque et al. 2013). Consequently, the characterization of the FEP population has become a priority area of growing interest for research, with large studies both in the United States and Europe (McEvoy, Lieberman et al. 2007, Bertelsen, Jeppesen et al. 2008, Castro-Fornieles, Parellada et al. 2008, Kahn, Fleischhacker et al. 2008, Bertani, Lasalvia et al. 2011). Conducting longitudinal research in the onset of illness avoids the effect of confounding variables such as the influence of antipsychotic treatment, comorbidity or chronicity (Kahn, Fleischhacker et al. 2008, Bernardo, Bioque et al. 2013). Such variables cause long-term structural changes and may be one reason for the inconsistency of the findings so far (Castro-Fornieles, Parellada et al. 2008, Arango, Rapado-Castro et al. 2012). Patients with a first psychotic episode are therefore an excellent group to study the risk factors linked to the development of schizophrenia and other psychotic disorders related to neural stress processes (Bernardo, Bioque et al. 2013). Moreover, early intervention seems to mitigate progression and improve therapeutic outcomes and prognosis of the disease[5,6].

Despite great efforts involving basic and clinical science, we are still far from a complete understanding of the aetiology and pathophysiology of the disorder. Not surprisingly, less than 50% of patients respond to an initial treatment with antipsychotic medication[3]. Clearly there is a need to open strategies in possible novel therapeutic targets for new drugs [3,4].

In the past fifteen years, a great deal of interest has been focused on immune/inflammatory alterations and the associated oxido/nitrosative consequences associated as key pathophysiological mechanisms involved in schizophrenia and other psychosis[7], and an appreciable body of evidence indicates a spectrum of immunological dysfunctions in schizophrenia[8], including immune or inflammatory related genes as risk factors for this disorder[9]. Most of the evidence supporting the inflammatory hypothesis of schizophrenia referred to elevation of pro-inflammatory cytokine levels (Miller et al, Biol Psychiatr 2011) mainly in plasma, brain microglial activation by post-mortem and positron emission tomography studies[19,20] as well as the identification of up-regulated inflammation-related genes in brain tissue[21,22]. Also, increased plasma levels of prostaglandin E_2 (PGE_2) and cyclooxygenase (COX) activity has been reported in patients[17,18]. All these data supported some clinical studies using non-steroidal anti-inflammatory drugs (NSAIDs). Recent meta-analyses reported provisional evidence about symptomatic favourable effects of add-on NSAIDs to antipsychotics in schizophrenia (Sommer et al., J Clin Psychiatr 2011).

In contrast, fewer studies have focused on the role of anti-inflammatory signaling pathways in both experimental and clinical settings[23], with data showing a clear misbalance in some pro-inflammatory/anti-inflammatory mediators in blood of patients with chronic schizophrenia at protein expression level[24]. The stimulation of anti-inflammatory cytokines such as IL-4, IL-10 and IL-17 seems to be a mechanism elicited by several antipsychotics to regulate uncontrolled and potentially deleterious inflammation in schizophrenia (rev. in Meyer, 2011). In fact, some authors reported an endogenous increase of these molecules in different stages of schizophrenia as an attempt to counteract or limit ongoing pro-inflammatory processes (Borovcanin et al., 2012).

The grade and evolution of the inflammatory process, its beneficial/deleterious consequences and the nature of its auto-regulatory mechanisms may vary in the different states of the psychotic illness (Fineberg and Ellman, 2013). The majority of the scientific evidence supporting the idea that inflammatory/immune alterations may play a relevant role in psychotic disease has been found in established schizophrenia. However, some studies indicates subtle alterations in inflammatory/immune mediators, stress response systems and oxidative/nitrosative stress at the very beginning of the natural course of the disease, as in the FEP (Borovcanin et al., 2012; Herberth et al., 2013; van Venrooij et al., 2012; Martínez-Cengotitabengoa et al., 2012). In fact, a recent imaging study demonstrates that neuroinflammation is more predominant than axonal degeneration in early stages of schizophrenia (Pasternak et al., 2012).

The precise regulation of the whole inflammatory process involves complex endogenous counterbalancing mechanisms that control the effects of potentially deleterious pro-inflammatory mediators. Thus, several studies have focused on the role of anti-inflammatory signalling pathways in both experimental and clinical settings [23], with data showing a clear misbalance in some pro-inflammatory/anti-inflammatory mediators in blood of patients with chronic schizophrenia at protein expression level [24].

Recently, FLAMM-PEPs, a multicentric, collaborative and translational group inside Spanish national network for mental health research (CIBERSAM) aimed to study inflammatory pathways in psychosis both as possible biomarkers and as possible new therapeutical targets, presented the first integrated results about the disbalance of pro- and antiinflammatory components in peripheral blood mononuclear cells (PBMC) in FEP patients (García Bueno et al., 2013), taking advantage of the PEPs project (Spanish research project in FEP) (Bernardo et al. 2013). In particular, the pro-inflammatory pathway studied was the one triggered by the activation of the nuclear factor κB (NFκB), which translocates to the nucleus after release of the bound to its citoplasmatic inhibitory complex IκB[25,26,27]. NFκB then recognizes specific DNA sequences in the promoter of target genes, among which are those that codify for the pro-inflammatory enzymes inducible nitric oxide synthase (iNOS) and the isoform 2 of the enzyme cyclooxygenase-2 (COX-2). The over-activation of these enzymes can produce an accumulation of oxidative and nitrosative mediators (i.e. stable nitric oxide metabolites such as NOx, PGE_2), which can cause the depletion of endogenous antioxidant defences and attack membrane phospholipids causing cell damage in a process known as lipid peroxidation[23]. On the other hand, endogenous counterbalancing mechanisms, activated in response to an inflammatory/immune stimulus, such as peroxisome proliferator activated receptors (PPARs) have been described[29]. These nuclear receptors (mainly their gamma isoform, PPARγ)[29,30] act as ligand-dependent transcription factors, binding to DNA in specific regions and regulating (decreasing) the expression of pro-inflammatory genes. Interestingly, several COX derived products, such as the prostaglandin 15-deoxy-PGJ_2 (15d-PGJ_2) act as endogenous anti-inflammatory agents by targeting PPARγ[33]. Thus, the 15d-PGJ_2/PPARγ pathway is involved in the endogenous compensatory mechanism regulating the inflammatory process. This pathway can also be stimulated pharmacologically, representing not only a potential biomarker but an important new

candidate therapeutic target in those neurologic/neuropsychiatric disorders in whose inflammation may play a role.

In short, the above cited Flamm-PEPs first study (García Bueno et al., 2013), on biochemical analyses in peripheral blood mononuclear cells (PBMC) showed an imbalanced, pro-inflammatory phenotype in FEP patients after 6 months of diagnosis compared to matched, healthy controls. Multivariate logistic regression analyses identified iNOS and COX2 as reliable potential risk factors, along with IκBα and 15d-PGJ_2 as potential protection factors. The disruption of this physiological balance is again re-examined in the present follow up study 12 months after of the inclusion of the patients in order to: (1) analyze the evolution of the physiological balance between inter-related pro-inflammatory/anti-inflammatory pathways described in FEP, (2) identify potential risk/protective factors for FEP by means of multivariate logistic regression analyses, (3) recognize pro/antiinflammatory mediators associated with symptom severity, and finally (4) by means of multiple lineal regression models, to analyze the change of every biological marker after a period of 6 months of longitudinal follow, in correlation to the respective changes of explanatory variables (sociodemographic characteristics, clinical variables and possible confounding factors) during follow-up.

METHODS

Subjects

As described in a previous article, 117 subjects with a first episode of psychosis (FEP) and 106 gender, race and age matched healthy controls were included in the baseline Flamm-PEPs study (García Bueno et al., 2013). From the initial sample, 85 FEP subjects were followed during the following 6 months (maximum 12 months after inclusion) in five Spanish university hospitals.

Baseline inclusion criteria for patients were: 1) Age between 9 and 35 years old; 2) duration of the psychotic symptoms of less than a year; 3) speak Spanish correctly. The exclusion criteria were: 1) mental retardation, including not only an IQ below 70 but also impaired functioning; 2) history of traumatic head injury with loss of consciousness; 3) history of organic disease with mental repercussions.

Having designed a real-life patient, naturalistic study, substance use or having suicidal ideation were not exclusion criteria (Bernardo, Bioque et al. 2013).

Healthy controls were selected from the same geographic areas. Their inclusion criteria were: 1) same gender as patients; 2) similar age (± 10%) 3) similar parental socioeconomic status, allowing ±1 level in the Hollingshead-Redlich scale (Hollingshead and Redlich, 1958); 4) no past or present psychiatric disorder per DSM-IV criteria (American Psychiatric Association 1994); 5) speak Spanish correctly; 6) no history of psychotic disorder among first-degree relatives. The exclusion criteria for controls were the same than for patients.

Neither the patients nor the controls presented ongoing infections, fever, allergies, other serious medical conditions as cancer or autoimmune, cardiac, pulmonary, chronic infectious diseases, or were receiving immunosuppressive drugs or vaccinations for at least six months prior to inclusion in the study or antiinflammatory-analgesics the two days previous to the extraction of the blood sample.

Expert clinicians used the Spanish translation of the DSM-IV semi-structured diagnostic interview to establish the diagnosis in adults (First, Spitzer et al. 1999, First, Spitzer et al. 1999), and the Kiddie-

Schedule for Affective Disorders & Schizophrenia, Present & Lifetime Version (K-SADS-PL) for subjects less than 18 years old (Ulloa, Ortiz et al. 2006).In order to not exclude early-onset psychotic patients, there was a broad age of inclusion allowed (Bernardo et al., 2013).

Apart from the interviews with the patient, multiple sources of information were used to establish the onset of positive psychotic symptoms, including medical records and interviews with relatives. The onset of the FEP was defined as the first week with the PANSS items P1, P3, P5, P6 or G9 scoring four or more[1]. The duration of untreated psychosis (DUP) was defined as the number of days elapsed between this onset and the beginning of the first adequate treatment for psychosis.

Clinical assessment of patients and controls included a complete medical history, physical examination, electrocardiogram and laboratory tests, including complete blood cell count, thyroid, liver and renal function, electrolyte levels, and urinalysis 2. Anthropometric measures were assessed: weight, height and body mass index (BMI=weight in kg/height squared).

The Ethics Committee of the participant hospitals approved the study. Following the Declaration of Helsinki II, all subjects were included after receiving a full explanation of the study and providing written, informed consent.

Specimen collection and preparation

Venous blood samples (10 mL) were collected between 8:00 and 10:00 h after fasting overnight. All the sample collection and preparation protocols in Flamm-PEPs study are available in www.cibersam.es. Samples were maintained at 4 ºC until processing.

Blood tubes were centrifuged (641 g x 10 min, 4ºC). The resultant plasma samples were collected and stored at -80ºC. The rest of the sample was diluted in culture medium (RPMI 1640, Invitrogen, UK) and a gradient with Ficoll-Paque® (GE Healthcare, Uppsala, Sweden) was used to isolate mononuclear cells by centrifugation (800 g x 40 min, RT). Peripheral Blood Mononuclear Cells (PBMC) layer was aspired and resuspended in RPMI and centrifuged (1116 g x 10 min, RT). The supernatant was removed and the mononuclear cell enriched pellet was stored at -80 ºC.

Biochemical determinations in plasma

- *Prostaglandin Levels:* COX by-products PGE_2 and 15d-PGJ_2 plasma levels were measured by enzyme immunoassay (EIA) using reagents in Prostaglandin E_2 EIA Kit-Monoclonal; Cayman Chemical Europe, Tallinn, Estonia and 15-deoxy-Δ12,14- Prostaglandin J_2 ELISA Kit DRG Diagnostics, Marburg, Germany, respectively.

- *Nitrites (NO^-_2),* the final and stable product of nitric oxide, were measured using the Griess method.

- *Lipid peroxidation* was determined by Thiobarbituric Acid Reactive Substances (TBARS) assay (Cayman Chemical Europe), based on the reaction of malondialdehyde (MDA) and thiobarbituric acid (TBA) under high temperature (95 ºC) and acidic conditions.

- *Cotinine levels*: Cotinine is the major degradation product of nicotine metabolism. Measurement of cotinine levels provides a sensitive estimate of tobacco smoke exposure. The levels were determined by enzyme immunoassay using Cotinine EIA Serum kit (Cozart®, Abingdon, UK) following manufacturer's instructions.

Biochemical determinations in PBMC

To carry out all biochemical determinations PBMC samples were first fractionated in cytosolic and nuclear extracts. For preparation of cytosolic and nuclear extracts a modified procedure based on the Schreiber *et al.*[36] method was used. Determination of pro-inflammatory p65 NFκB subunit and antiinflammatory PPARγ respective transcriptional activities were carried out in nuclear extracts from PMBC:

- *Nuclear factor kappa B (NFκB) activity:* Activation of NFκB occurs by enzymatic degradation of the bound inhibitory protein (IκBα), allowing movement of the p50/65 subunits from the cytoplasm to the nucleus where they bind to consensus κB sequences in DNA. The presence of p65 subunit in cell nuclei is considered an index of activity. The activity of NFκB was measured in nuclear extracts through a commercially available NFκB (p65) Transcription Factor Assay (Cayman Chemicals, MI, USA) following the manufacturer's instructions.

-*PPARγ Transcription Factor Assay:* PPARγ activity (PPARγ act) was determined in nuclear extracts from PBMC using ELISA-based kits, which allow the detection and quantification of PPARγ specific transcriptional activity (Cayman Chemical Europe).

-*Western Blot Analysis:* The protein levels of the inhibitory subunit of NFκB IκBα and the pro-inflammatory enzymes COX2 and iNOS were quantified by Western Blot analysis (WB) in cytosolic extracts from PBMC and the protein levels of PPARγ were quantified in nuclear extracts. The house keeping gene β-actin was used as loading control and the nuclear factor SP1 was used like loading control for PPARγ (blots shown in the respective figures). For clarity, in the figures two WB are presented, representative of all the samples studied (in each different gel, n = 3 per group –control or FEP-). The insets were the most representative of statistical AU data after densitometric analysis as stated above. All densitometry results are expressed in % from control.

Statistical analysis

Differences between baseline characteristics for patients and controls were assessed using Chi-square, or nonparametric Mann-Whitney U tests, according to the distribution and scales of the variables.

To assess the effect of psychotropic medication we calculated the potency equivalents compared to Chlorpromazine (following the international consensus method described by Gardner *et al*., 2010[37]) and we performed linear regression models for each biomarker.

To calculate the association between FEP and the level of biological markers, we used hierarchical logistic regression models. In order to explore mechanisms explaining the association, we used five models for each biological marker, in which we gradually controlled for potential confounders (age, gender, BMI, cannabis use per month and cotinine levels). Model 1 included the level of biological marker. Model 2 additionally included terms for age and gender. Model 3 additionally included BMI. Model 4 additionally included cannabis use per month and finally, Model 5 additionally included cotinine levels. Only biological markers significantly associated ($p<0.05$) with FEP in model 5 in the previous analyses were selected for the following steps. Logistic regression analyses were again calculated with the same system, and all the biological markers chosen were kept and analyzed together in a new model 1. Model 2 additionally included terms for age and gender. Model 3 additionally included BMI. Model 4 additionally included cannabis use per month. Model 5 additionally included cotinine levels (final model).

Multiple linear regression analysis were used to analyze the change between 6 and 12 months after diagnosis in each biological marker depending on the change in demographic (gender, age, BMI), clinical variables (DUP, GAF), antipsychotic medication (DDD), cannabis, tobacco (cotinine).

RESULTS

1.- Demographic and clinical features

Table 1 presents the demographic and clinical characteristics of the FEP patients and healthy control group.

22 from the 117 FEP subjects didn't follow the 6 month period of study. Due to these early drop-outs, there were differences in the socioeconomic status between the groups that were not present at baseline. However, patient and control groups did not differ in gender, age and race, as the case-control matching had been designed.

The FEP and control groups differed in BMI (24.65± 5.73 vs. 23.14 ± 3.16, $p<0.05$) and in percentage of active cannabis users (4 patients (5.1%) vs 14 controls (16%), $p<0.05$), although no differences were found in the number of cannabis cigarettes smoked per month. There were also differences in the number of tobacco smokers and in the number of tobacco cigarettes per month use (241.98 vs 45.38, $p<0.05$). Plasma levels of cotinine, the main metabolite of nicotine, appear also higher in patients than in controls (97.27±84.50 *vs* 26.28±49.31 ng/mL).

PANSS mean total score was 40.29 ± 23.67 and the mean Global Assessment of Functioning score (GAF) was 72.08 ± 17.23. The mean age of psychosis onset was 24.37 ± 5.92 years, while the mean duration of untreated psychosis (DUP) was 68.58 ± 77.28 days. The 72.9% of the patients had been diagnosed of non-affective psychotic disorders. Only 17 subjects (20%) were oriented as affective disorders with psychotic features (bipolar, psychotic depression or schizoaffective disorder). Consequently, the Young and the Montgomery-Asberg scales mean scores were low (1.39 and 6.02, respectively).

The mean age of psychosis onset was 24.37 ± 5.92 years, while the mean duration of untreated psychosis (DUP) was 68.58 ± 77.28 days. The 72.9% of the patients had been diagnosed of non-affective psychotic disorders. Only 17 subjects (20%) were oriented as affective disorders with psychotic features, as bipolar disorder, psychotic depression or schizoaffective disorder, so the Young and the Montgomery-Asberg scales mean scores were low (1.39 and 6.02, respectively).

Risperidone and aripiprazole were the most frequent antipsychotics used, while 21 (25.9%) patients had discontinued their antipsychotic treatment in the 6 month follow up period. The defined daily dose of chlorpromazine equivalents was 298.06 ± 303.16 mg/day.

The multiple linear regression analysis made to elucidate whether the antipsychotic treatment modify the levels of any of the inflammatory and oxido/nitrosative markers studied showed that only the levels of PGE_2 and $d15PGJ_2$ are significantly modified for the effect of antipsychotic medication ($p= 0.028$ and 0.006, respectively).

2.- Inflammatory and oxido/nitrosative markers in control and first-episode psychosis patients

The nuclear transcription factor NFκB is a master regulator of the inflammatory and oxido/nitrosative (I&ON) status of a cell. In nuclear extracts from PMBC, its activity is increased in samples from FEP patients when compared with controls (Table 2 and Fig. 1a). Similarly, the expression of the main enzymatic source of I&ON soluble mediators, COX2, was significantly higher in FEP group than those in the control subjects, although the expression of iNOS was not different between groups (Table 2 and Fig. 1b and c). Interestingly, at this particular stage of the disease, the plasmatic levels of the I&ON markers in plasma PGE_2, NO^-_2 and TBARS were increased in FEP patients (Table 2 and Fig. 1d-e, respectively).

3.- Antiinflammatory markers in control and first-episode psychosis patients

Protein expression of the NFκB inhibitory subunit, IκBα (in cytosolic extracts were not decreased in patients compared to healthy subjects (Table 2 and Fig. 2a). However, plasma levels of the anti-inflammatory prostaglandin 15d-PGJ_2 were significantly lower in FEP patients than those in the control subjects (Table 2 and Fig. 2b).

Given that 15d-PGJ_2 acts as an endogenous ligand for PPARγ, which is considered a potent anti-inflammatory transcription factor, we explored the expression (by WB) and transcriptional activity (by studying its ability to bind to its specific DNA response elements) of this receptor in nuclear extracts from PBMC. The WB analysis revealed lower PPARγ expression in patients when compared to controls (Table 2 and Fig. 2c). Moreover, the transcriptional activity of PPARγ, analyzed by its ability to bind to its specific DNA response elements, was also reduced in patients (Table 2 and Fig. 2d).

4.- Multiple logistic regression analysis

In the final model only six of the ten biological markers studied were significantly altered in FEP after controlling for all possible confounders (Table 3 and Supplementary Data 1). The resulting equation was [0.005 x PGE_2] + [0.017 x COX2] + [0.369 x NO^-_2] + [0.182 x TBARS] + [-0.832 x PPARγ act] + [-0.023 x 15d-PGJ_2] + [0.062 x gender (ref.female)] + [-0.194 x age] + [-0.070 x BMI] + [-0.014 x cannabis use per month] + [0.034 x cotinine level]).

Among the pro-inflammatory markers, the highest OR observed was the NO^-_2 (OR=1.447), meaning that for each unit increased of this biomarker, the risk[40] of FEP increased by 44.7% [($e^{-0.369x1}$-1)x100] after controlling for remaining biological markers and all possible confounders (p=0.023)[40]. Similarly, the results were 20% for TBARS (n.s.), 1.7 % for COX2 (p=0.011) and 0.5% for PGE_2 (n.s.).

Among the anti-inflammatory markers, the PPARγ act variable had the lowest OR observed (OR=0.435), meaning that the association went in the inverse direction of pro-inflammatory markers, since the risk decreased by 56.5% [($e^{-0.832x1}$-1)x100] for each unit of increased biomarker after controlling for remaining biological markers and all possible confounders, although it was not significant (p=0.187). Similarly, the result for 15d-PGJ_2 was 2.3% (p=0.001).

PPARγ protein expression data were excluded of the multivariate analysis due to its small sample size, although it was significant (p<0.05) in its individual regression model controlled for all confounders. NFκB expression data were excluded of the multivariate analysis because it was not significant (p=0.175) in its individual regression model controlled for all confounders.

5.- Multiple linear regression analysis.

By means of this analysis, it is possible to predict the change of each biological marker (from baseline to the follow-up point, 12 months) given the set of explanatory variables: gender, age, BMI, cannabis use, plasma cotinine levels, the defined daily dose (DDD) of chlorpromazine equivalents, the DUP and the GAF scale score and the days from diagnostic to the blood sampling for basal values). Four (PGE_2, COX2, NO^-_2 and TBARS) of the six proinflammatory and oxido-nitrosative biological markers studied and two ($15dPGJ_2$ and PPARγ activity) of the four antiinflammatory markers studied were significantly associated with some of the explanatory variables (Table 4 and Supplementary Data 2).

Inflammatory and oxido/nitrosative markers: the increase in plasma levels of PGE_2 from baseline to the follow up appears negatively related with DDD (for each DDD unit increased during these period, PGE_2 will decrease 0.733 units during follow up, once controlling the effect of the increase in the other explanatory variables). Also, PGE_2 will decrease 3.387 units for each increase in one unit of cotinine plasma levels. Finally, PGE_2 levels will increase 0.714 units for each day from diagnostic to the blood sampling for basal values. The PBMC expression of COX2 also appear related with cotinine: it will increase 0.672 units for each unit of cotinine levels increased from basal to follow up.

The increase in nitrite levels, the main stable metabolite of NO and responsible of cellular and tissular oxido/nitrosative damage, appears also related to the explanatory variables chosen: NO^-_2 increase by 7.451 units more in female patients than in male patients, and decrease 0.19 units for each cigarette of cannabis smoked. The increase from basal to follow up TBARS levels in plasma, the final marker of systemic oxido-nitrosative stress appear also related: for each unit of GAF scale increase, TBARS decrease 0.12 units. Finally, for each day from diagnostic to the blood sampling for basal values, TBARS will increase 0.005 units.

Antiinflammatory markers: plasma levels of PGJ_2 from baseline to the follow up appear positively related with DDD (for each DDD unit during these period, PGJ_2 will increase 0.333 units during follow up, once controlling the effect of the increase in the other explanatory variables). Finally, the activity of PPARγ will decrease 0.033 units for each point in GAF score and 0.75 units for each year of age.

DISCUSSION

In this follow-up study we have strengthen the evidence of systemic inflammatory alterations in patients diagnosed of FEP. Previously, with this same cohort of patients, we described phenotypical differences in pro-inflammatory mediators at the cellular machinery level in PBMC, but the resultant soluble elements were not significantly altered (García-Bueno *et al*., 2013). However, 6 months later the great majority of soluble elements analyzed already appear significantly altered, suggesting the existence of a pro/antiinflammatory balance more deregulated and potentially more harmful, as can be observed by the lipid peroxidation (TBARS) data found.

One possible limitation to explain the results reported in our first study would be that the observed changes might be related to stress perception. It is known that a stress component could be one of the factors implicated in the onset of the FEP (rev. in Holtzman et al., 2013). Indeed, psychosocial and/or physical stress could contribute to the observed shift towards enhanced pro- vs. anti-inflammatory signalling (García-Bueno et al 2008). However, past and current complementary results show that the deregulation of the inflammatory balance is permanent (and even worse) in male, chronic schizophrenic inpatients in acute relapse phase (Martínez-Gras et al., 2011) with a history of psychosis of 12.73 yrs.

This evolution could suggest that the inflammatory process found is not only due to acute stress exposure in the beginning of FEP.

The multivariate logistic regression analysis conducted allow us to identify the plasmatic levels of the markers of oxidative/nitrosative damage NO_2^- and TBARS and COX-2 protein expression in PBMC as the most reliable potential risk factors. As occured in our previous study, the plasmatic levels of the antiinflammatory mediator 15d-PGJ_2 might be used as potential protection factor for FEP.

In addition, due to our current longitudinal approach we were able to analyze the significant changes of every biological marker between 6 months after the inclusion of the patients and one year, in correlation to the respective changes of clinical characteristics and confounding factors. Especially remarkable is the result regarding the associations between antipsychotic dose and the change in the plasma levels of PGE_2 (inverse) and 15d-PGJ_2 (direct). Indeed, these results suggest that one of the therapeutic mechanisms of antipsychotic therapy is the restoration of the pro/antiinflammatory balance, disrupted in FEP. It is worth noting that the 25.9% of the FEP patients here recruited were under medication with risperidone. In the last years, it has been shown that this atypical antipsychotic normalizes increased inflammatory mediators (cytokines and prostaglandins) and restores anti-inflammatory pathways in murine models of neuroinflammation elicited by lipopolysaccharide (LPS) (Sugino et al., 2009; MacDowell et al., 2013). Others antipsychotics used, such as olanzapine (7.4%) or clozapine (7.4%), also reduced PGE_2 concentration in rat brain in chronic administration (Cheon et al., 2011; Kim et al., 2012). A recent study using a SNP-based analysis of neuroactive pathways implicated PGE_2 as a mediator of the effects of risperidone, olanzapine and quetiapine (also used by 6.2% of the patients of our study), using data from the CATIE trial[60].

The correlation results between medication with antipsychotics and the restoration of the pro/antiinflammatory balance in FEP represent an important finding supporting the establishment and completion of clinical trials using antiinflamamtory drugs as co-adjuvant strategy to antipsychotics in schizophrenia (Sommer et al., J Clin Psychiatr 2011).

The multiple linear regression analysis results also support the idea that cigarette smoking can activate inflammatory pathways and may represent an important confounding factor. The differential effects of cigarettes consume in COX-2 levels in PBMCs and in the content of its main pro-inflammatory product PGE_2 in plasma should be carefully evaluated. COX-2 is a complex enzyme expressed both in brain and in PBMCs, capable to produce pro and antiinflammatory mediators in different phases of its activity to resolve inflammation, depending of the nature and the level of the stimulus. It is possible that the increase in COX-2 levels observed was related to a correspondent rise in the levels of other antiinflammatory products, such as 15d-PGJ_2, that modulates a massive production of PGE_2. In fact, the levels of 15d-PGJ_2 directly correlate with cotinine levels, although this correlation did not reach statistical significance ($p=0.225$). Indeed, it is well know that nicotine could activate COX-2 and PGE_2 synthesis in brain and other peripheral tissues (De Simone et al., 2005; Toledano et al., 2010; Huang et al., 2011), but on the other hand, other authors have found that a lower dose of nicotine could be antiinflammatory by the inhibition of proinflammatory mediators in human monocytes by suppression of NF-κB transcriptional activity through nicotinic acetylcholine receptor α7 (Yoshikawa et al., 2006). Although there are no studies reporting nicotine effects on 15d-PGJ_2 levels, nicotine can up-regulate PPARγ in dendritic cells and in monocyte/macrophages from healthy smokers (Yanagita et al., 2012; Amoruso et al., 2007). Further research is warranted to elucidate the relationship between both antiinflammatory pathways in physiological and pathological conditions.

Cannabis use is also related to immune alterations and constitutes a possible confounding factor that should be controlled. Supporting this idea, our correlation analysis results show how the cannabinoids

use per month negatively correlates with the levels of the stable metabolites of NO, nitrites in plasma. This specific antioxidant profile of some of the cannabinoids present in the consumed preparations of *Cannabis Sativa*, such as cannabidinol for example, have been demonstrated in different neuropathological scenarios (Ruiz-Valdepeñas et al., 2011; Esposito et al., 2006, 2007). The biological relevance of the correlation here found needs to be explored, for example in a vascular context, where endocannabinoids actions are complex (Randall et al., 2004), and could be altered by exogenous cannabinoid use.

From a clinical point of view, the inverse relationship found here between GAF scale and TBARS plasma levels is particularly relevant to provide scientific evidence to support a direct role for oxidative/nitrosative stress cellular damage in the general symptomatology of FEP and, possibly, in other disorders that course psychotic symptoms. Similarly, in a comparable cohort of patients, it has been reported that cognitive impairment (in learning and memory) is related to oxidative stress in FEP (Martínez-Cengotitabengoa et al., 2012).

As previously said, the longitudinal design let us analyse the temporal evolution of the mild inflammatory response that it is taking place in FEP patients. The comparison of the data obtained in the two selected time-points perfectly illustrates the complexity and the continuous dynamic changes of the inflammatory response in the natural course of incipient psychosis. This complexity is one of the causes why there are so many controversy regarding the specific role of inflammatory mediators in the patho-physiology of the different types of psychotic disorders. Indeed, the nature and/or severity of activated inflammatory responses in schizophrenia may critically change as a function of disease progression (Meyer et al., 2011), and the design and proper use of therapeutical strategies based of antiinflammatory drugs are difficult, producing in the great majority of the cases only relatively modest improvement in clinical symptoms in the early stage of schizophrenia (Sommer et al., 2011).

The results of the multivariate analysis show potential risk/protective factors common both in baseline and follow up visits. This is the case of COX-2 protein levels in PBMC as risk factor and 15d-PGJ_2 plasma levels as putative protection factor. However other plausible biomarkers have lost its validity (i.e. IκBα). These results illustrate how one inflammatory biomarker could be useful in a limited phase of the natural course of the disease (status biomarker), but others are more general for all the phases of the disease (trait biomarker), as has been recently shown for the case of specific cytokines (Miller et al., 2011).

Although nowadays we are seeing a reformulation of the traditional conception of the psychotic illness (Insel et al., 2010), being considered as a heterogeneous disorder with a multisystemic impact from the onset in addition to its psychiatric expression (Kirkpatrick, 2009), one limitation of the data here reported is their systemic nature. Indeed, there is a need to corroborate our findings in brain areas related to schizophrenia to elucidate whether the alterations of these inflammatory risk/protection biomarkers could have etiological relevance and not only utility for diagnosis and monitoring the evolution of the disease. Recent studies compared the same immune biomarkers (cytokines such as IFNγ) in plasma and in post-mortem brain tissue from control and subjects with schizophrenia and found the same alterations in both compartments, validating the concept that schizophrenia can be investigated through studies of systemic biomarkers (Harris et al., 2012). In addition, a recent review suggested a link between peripheral inflammatory/immune processes and MRI detected anomalies in the brain of individuals with schizophrenia (Frodl and Amico, 2013). Furthermore, there are already studies in post-mortem brain tissue from subjects with schizophrenia showing increased levels of some of the pro/antiinflammatory and oxido/nitrosative stress markers studied here (NF-κB, COX-2, TBARS) (Rao et al., 2013; Tang et al., 2012; Wang et al., 2009).

All these findings suggest an active role of this pro/anti-inflammatory signalling pathway in the pathophysiology of the disorder, which support that the possible mechanism/s are not only related to the oxidative/nitrosative cellular damage. Thus, some authors have explored the possibility that abnormalities in prostaglandins and other lipids content in the brain of subjects with schizophrenia could alter synaptic monoaminergic neurotransmission and affect cognition as a result (Orešič et al., 2012). In other approach, some authors have proposed that prostaglandins (PGE_2) could be implicated in the up-regulation of the endogenous glutamate NMDA receptor antagonist kynurenic acid found in the brain of subjects with schizophrenia, which contributes to the pathogenesis of the disease, linking the dopamine hypothesis of schizophrenia together with the idea of a deficiency in glutamatergic function (Erhardt et al., 2007). Regarding the antiinflammatory side of the balance, classical studies showed that PGD_2 stimulated the production of cyclic AMP (cAMP) and thereby exerted functional antagonism of dopamine-D2 receptors[58]. Therefore, PGD_2 and its metabolites could be counteracting the biochemical and behavioural effects of dopamine and deficient PGD_2/PGJ_2 signalling in the brain could influence dopamine transmission[59]. In addition to its antiinflammatory actions, PPARγ may directly regulate glutamatergic neurotransmission at NMDA receptor level (Salehi-Sadaghiani et al., 2012; Almasi-Nasrabadi et al., 2012), which has been involved in positive symptoms of schizophrenia (Stone et al., 2007). PPARγ is a master regulator of glucose metabolism and it has been proposed that alterations in its normal activity could be implicated in some of the metabolic disruptions originated as side effects by chronic antipsychotic medication (rev. in Rolland et al., 2013).

Recently, the pharmacological modulation of PPARγ has been presented as a new treatment for neurocognitive deficits associated with mood and psychotic disorders[57]. However our correlation analyses indicate that PPAR activity present a negative correlation with GAF scale between de two time points considered. It is possible that PPARγ activity increases as an endogenous mechanism of response when the severity of the symptoms is greater, but this possibility needs to be corroborated in more advanced pathological states. Indeed, it is mandatory to re-evaluate the utility of PPARγ as therapeutic target to improve neurocognitive deficits because its chronic exogenous activation could produce metabolic and cardiovascular alterations that masks its neuroprotective actions. In this vein, a recent pilot clinical trial, PPARγ synthetic ligand rosiglitazone failed in the improvement of cognitive deficits in clozapine-treated patients with schizophrenia (Zhenghui et al., 2012).

Some limitations in this study should be noted: Firstly, we used a single control group instead of using two, one from healthy subjects and another including other psychiatric conditions, with the aim to control and increase the specificity of our results. In fact, changes in both inflammatory and anti-inflammatory COX derived pathways occur after acute and chronic stress exposure[23,30]. However, the results here presented need to be confirmed in human samples from other neuropsychiatric disorders, such as depression or bipolar disorder.

Second, 74.1% of the FEP patients included in our study were receiving atypical antipsychotic treatment, and there is some evidence on their potential anti-inflammatory effects[13-15], most of them at anti-cytokine level. Nevertheless, we have tried to control the possible confounding effect of antipsychotic treatment through the multiple linear regression analysis and we found the inverse association between antipsychotic dose and the change in the plasma levels of PGE_2 and the direct association with 15d-PGJ_2 discussed previously.

Third, a small group (8.4%) of patients required lithium. Although there are no clear references about lithium and inflammation, given the broad pharmacological effects of this compound, the possibility of being a confounding factor was also assessed. However, the result did not modify the association showed in this study.

Fourth, the total number of subjects taking part of this study is 117 patients and 106 matched controls but we could not measure all of the parameters in all the subjects from the baseline visit to the 12-month visit. In general, for the parameters measured in plasma (i.e. the two prostaglandins) almost all subjects were used, but for the determinations made in the cytosolic/nuclear extracts of PBMC some methodological limitations existed and the quantity of sample obtained was relatively low. With this limitation in mind we have tried to get a reasonable number of subjects for each parameter studied to carry out a reliable statistical analysis. In addition, we have checked if the sociodemographic characteristics were modified for this reason. No major changes were found between the differences in the whole sample and those in the subsets that were finally analyzed.

It is worth noting that PPARγ protein expression data was not chosen and kept together with the other markers selected because of its small sample size, although it was significant in its individual regression model controlled for all confounders. Keeping this data in mind we cannot discard a role for PPARγ as a potential protective factor in FEP patients. In fact PPARγ expression and activity are significant in the two-tailed Chi-square tests on categorical data used to identify differences between baseline characteristics for patients and control subjects, both in our study and in inpatients subjects with schizophrenia in acute relapse phase[24].

Despite these limitations, we believe that our study has identified vulnerability conditions related to PBMC pro/ anti-inflammatory pathways in FEP in a sample of Spanish patients, which warrant greater attention in future investigations. Furthermore, key strengths of the study deserve mention: the sample of patients was very homogeneous according to the time frame of inclusion, the phase of the illness and originated from specific areas of two major and three middle European cities **(Garcia-Bueno, Bioque et al. 2013)**. The diagnostic evaluation was performed with a very comprehensive protocol and inclusion-exclusion criteria were applied in a strict manner **(Bernardo, Bioque et al. 2013)**. Finally, another unique feature of the study is that it includes a wide spectrum of biochemical inflammatory markers both in plasma samples and peripheral blood mononuclear cells, allowing in-depth insights and relationships between multiple components of the pro- and anti-inflammatory signalling pathways.

Efforts in describing biological markers for schizophrenia in pathway approaches are claimed by psychiatrists as tools to help early diagnosis and monitor evolution of the disease; this would greatly assist preventive strategies by identifying at-risk individuals who should be monitored and treated appropriately to minimize subsequent morbidity.

In conclusion, in this follow-up study we have corroborated the existence of a deregulated systemic pro/anti-inflammatory balance in FEP, which becomes more severe during the initial period of time after the diagnosis of a FEP. The results of the multivariate analysis applied show potential risk/protective factors in both time points studied (COX-2 protein levels in PBMC and 15d-PGJ_2 plasma levels) that can be considered trait markers, and others specific of each one or state biomarkers (IκBα protein levels and NO_2^- and TBARS content in the cytosolic extract of PBMC). The more striking results come from the analysis of the multiple linear regression approach made, which shows how one of the targets of antipsychotic treatment is the restoration of the inflammatory balance. In addition, our results corroborate the need to control cigarette and cannabis use as confounding factors every time that immune/inflammatory alterations are reported in psychotic disorders, and possibly in other psychiatric pathologies too. Finally, from a clinical point of view, the inverse correlation between the final product of oxidative/nitrosative cellular damage TBARS and the GAF scale found is especially relevant to justify the onset and development of antioxidant/anti-inflammatory therapeutical strategies not only for established schizophrenia but in earlier stages of a psychotic disorder.

Author contributions

BGB wrote the first version of the paper and performed some of the biochemical determinations; MBi managed and analysed the clinical data; KSMD performed biochemical determinations in plasma and in cells and prepared sub-cellular samples and the first version of the figures; MFB and JS performed the statistical analyses; MMC, LP, RRJ and PS collected the biological samples and the clinical data; CC performed the Hcy determination; AGP, MP, GR and MPGP analyzed the clinical data; JAM analysed the oxidative data; MBe coordinated PEP study and analysed the clinical data; JCL coordinated Flamm-PEP study, designed the study, wrote the paper. All of the authors contributed to the final version of the paper.

REFERENCES

1. van Os J. & Kapur S. Schizophrenia. Lancet 2009; 374(9690): 635-45.
2. Perälä J, Suvisaari J, Saarni SI, et al. Lifetime prevalence of psychotic and bipolar I disorders in a general population. Arch Gen Psychiatry 2007; 64(1): 19-28.
3. Lewis DA, Gonzalez-Burgos G. Pathophysiologically based treatment interventions in schizophrenia. Nat Med 2006; 12(9): 1016-22.
4. Insel TR. Rethinking schizophrenia. Nature 2010; 468(7321): 187-93.
5. Kuehn BM. Early interventions for schizophrenia aim to improve treatment outcomes. JAMA 2010; 304(2): 139-40,145.
6. Tandon R, Nasrallah HA, Keshavan MS. Schizophrenia, "just the facts" 4. Clinical features and conceptualization. Schizophr Res 2009; 110(1-3): 1-23.
7. Monji et al., 2009…
8. Müller N, Schwarz MJ. A psychoneuroimmunological perspective to Emil Kraepelins dichotomy: schizophrenia and major depression as inflammatory CNS disorders. Eur Arch Psychiatry Clin Neurosci 2008; 258 Suppl 2: 97-106.
9. Shi J, Levinson DF, Duan J, et al. Common variants on chromosome 6p22.1 are associated with schizophrenia. Nature 2009; 460(7256): 753-7.
10. Sommer IE, de Witte L, Begemann M, Kahn RS. Nonsteroidal anti-inflammatory drugs in schizophrenia: ready for practice or a good start? a meta-analysis. J Clin Psychiatry. Dec 13 (2011) [Epub ahead of print].
11. Meyer U, Schwarz MJ, Müller N. Inflammatory processes in schizophrenia: a promising neuroimmunological target for the treatment of negative/cognitive symptoms and beyond. Pharmacol Ther 2011; 132(1): 96-110.
12. Arolt V Rothermundt M, Wandinger KP, Kirchner H. Decreased in vitro production of interferon-gamma and interleukin-2 in whole blood of patients with schizophrenia during treatment. Mol Psychiatry 2000; 5(2): 150-8.
13. Maes M, Bosmans E, Ranjan R, et al. Lower plasma CC16, a natural anti-inflammatory protein, and increased plasma interleukin-1 receptor antagonist in schizophrenia: effects of antipsychotic drugs. Schizophr Res 1996; 21(1): 39-50.
14. Sugino H, Futamura T, Mitsumoto Y et al. Atypical antipsychotics suppress production of proinflammatory cytokines and up-regulate interleukin-10 in lipopolysaccharide-treated mice. Prog Neuropsychopharmacol Biol Psychiatry 2009; 33(2): 303-7.
15. Maes M, Bosmans E, Calabrese J, Smith R, Meltzer HY. Interleukin-2 and interleukin-6 in schizophrenia and mania: effects of neuroleptics and mood stabilizers. J Psychiatr Res. 1995; 29(2): 141-52.
16. Pollmächer T, Haack M, Schuld A, Kraus T, Hinze-Selch D. Effects of antipsychotic drugs on cytokine networks. J Psychiatr Res 2000; 34(6): 369-82.
17. Kaiya H, Uematsu M, Ofuji M et al. Elevated plasma prostaglandin E2 levels in schizophrenia. J Neural Transm. 1989; 77(1): 39-46.

18. Das I, Khan NS. Increased arachidonic acid induced platelet chemiluminescence indicates cyclooxygenase overactivity in schizophrenic subjects. Prostaglandins Leukot Essent Fatty Acids 1998; 58(3): 165-8.
19. Steiner J, Bielau H, Brisch R et al. Immunological aspects in the neurobiology of suicide: elevated microglial density in schizophrenia and depression is associated with suicide." J Psychiatr Res 2008; 42(2): 151-7.
20. van Berckel BN, Bossong MG, Boellaard R et al. Microglia activation in recent-onset schizophrenia: a quantitative (R)-[11C]PK11195 positron emission tomography study. Biol Psychiatry 2008; 64(9): 820-2.
21. Saetre P, Emilsson L, Axelsson E, Kreuger J, Lindholm E, Jazin E. Inflammation-related genes up-regulated in schizophrenia brains. BMC Psychiatry 2007; 7: 46.
22. Drexhage RC, van der Heul-Nieuwenhuijsen L, Padmos RC et al. Inflammatory gene expression in monocytes of patients with schizophrenia: overlap and difference with bipolar disorder. A study in naturalistically treated patients. Int J Neuropsychopharmacol 2010; 13(10): 1369-81.
23. García-Bueno B, Caso JR, Leza JC. Stress as a neuroinflammatory condition in brain: damaging and protective mechanisms. Neurosci Biobehav Rev 2008; 32(6): 1136-51.
24. Martínez-Gras I, Pérez-Nievas BG, García-Bueno B et al. The anti-inflammatory prostaglandin 15d-PGJ2 and its nuclear receptor PPARgamma are decreased in schizophrenia. Schizophr Res 2011; 128(1-3): 15-22.
25. Bierhaus A, Wolf J, Andrassy M, et al. A mechanism converting psychosocial stress into mononuclear cell activation. Proc. Natl. Acad. Sci. U.S.A. 2003; 100(4):1920–1925.
26. Madrigal JL, Hurtado O, Moro MA, et al. The increase in TNF-alpha levels is involved in NF-kappaB activation and inducible nitric oxide synthase expression in brain cortex after immobilization stress. Neuropsychopharmacology 2002; 26(2):155-63.
27. Middleton, G., Hamanoue, M., Enokido, Y, et al. Cytokine-induced nuclear factor kappa B activation promotes the survival of developing neurons. J. Cell Biol 2000; 148 (2): 325–332.
28. Galea E, Heneka MT, Dello Russo C, et al. 2003. Intrinsic regulation of brain inflammatory responses. Cell. Mol. Neurobiol. 2003; 23 (4–5): 625–635.
29. Kapadia R, Yi JH, Vemuganti R. Mechanisms of anti-inflammatory and neuroprotective actions of PPAR-gamma agonists. Front. Biosci. 2008; 13: 1813–1826.
30. García-Bueno B., Madrigal JL, Lizasoain I, et al. Peroxisome proliferator-activated receptor gamma activation decreases neuroinflammation in brain after stress in rats. Biol. Psychiatry. 2005; 57(8): 885–894.
31. Heneka MT, Landreth GE. PPARs in the brain. Biochim. Biophys. Acta 2007; 1771: 1031–1045.
32. Feinstein DL. Therapeutic potential of peroxisome proliferator-activated receptor agonists for neurological disease. Diabetes Technol. Ther. 2003; 5(1): 67–73.
33. Forman BM, Chen J, Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc. Natl. Acad. Sci. U.S.A. 1997; 94(9): 4312–4317.
34. First M, Gibbon M, Spitzer RL, Williams JBW, Smith J. Entrevista clínica estructurada para los trastornos del eje-I del DSM-IV. Barcelona, Masson 1999.
35. Ulloa RE, Ortiz S, Higuera F et al. Interrater reliability of the Spanish version of Schedule for Affective Disorders and Schizophrenia for School-Age Children--Present and Lifetime version (K-SADS-PL)]. Actas Esp Psiquiatr 2006; 34(1): 36-40.
36. Schreiber E, Matthias P, Müller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res 1989; 17: 6419.
37. Gardner DM, Murphy AL, O'Donnell H, et al. International consensus study of antipsychotic dosing. Am J Psychiatry 2010; 167(6):686-93.
38. Castro-Fornieles J, Parellada M, Gonzalez-Pinto Aet al. The child and adolescent first-episode psychosis study (CAFEPS): design and baseline results. Schizophr Res 2007; 91(1-3): 226-37.
39. Kahn RS, Fleischhacker WW, Boter H et al. Effectiveness of antipsychotic drugs in first-episode schizophrenia and schizophreniform disorder: an open randomised clinical trial. Lancet 2008; 371(9618): 1085-97.
40. Hosmer DW, Lemeshow S. Applied logistic regression. New York, NY: John Wiley & Sons 1989.
41. Alvarez-Jimenez M, Parker AG, Hetrick SE, et al.. Preventing the Second Episode: A Systematic Review and Meta-analysis of Psychosocial and Pharmacological Trials in First-Episode psychosis. Schizophr Bull 2011; 37(3):619-30.

42. Micó JA, Rojas-Corrales MO, Gibert-Rahola J et al. Reduced antioxidant defense in early onset first-episode psychosis: a case-control study. BMC Psychiatry 2011; 11: 26.
43. Arango C, Rapado-Castro M, Reig S et al. Progressive brain changes in children and adolescents with first-episode psychosis. Arch Gen Psychiatry 2012; 69(1): 16-26.
44. Schultze-Lutter F, Ruhrmann S, Fusar-Poli P et al. Basic Symptoms and the Prediction of First-Episode Psychosis. Curr Pharm Des. 2012 Jan 10. [Epub ahead of print].
45. Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J Biol Chem 1994; 269(7): 4705-8.
46. Newton R, Kuitert LM, Bergmann M., et al. Evidence for involvement of NF-kappaB in the transcriptional control of COX-2 gene expression by IL-1beta. Biochem Biophys Res Commun 1997; 237(1): 28-32.
47. Song XQ, Lv LX, Li WQ, et al. The interaction of nuclear factor-kappa B and cytokines is associated with schizophrenia. Biol Psychiatry 2009; 65(6): 481-488.
48. Das I, Khan NS, Puri BK et al. Elevated platelet calcium mobilization and nitric oxide synthase activity may reflect abnormalities in schizophrenic brain. Biochem Biophys Res Commun 1995; (2): 375-80.
49. Yokota O, Terada S, Ishihara T et al. Neuronal expression of cyclooxygenase-2, a pro-inflammatory protein, in the hippocampus of patients with schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry 2004; 28(4): 715-21.
50. Yao JK, Leonard S, Reddy RD. Increased nitric oxide radicals in postmortem brain from patients with schizophrenia. Schizophr Bull 2004; 30(4): 923-34.
51. Ghosh N, Ghosh R, Mandal SC. Antioxidant protection: A promising therapeutic intervention in neurodegenerative disease. Free Radic Res 2011; 45(8): 888-905.
52. Jones BG, Rose FA, Tudball N. Lipid peroxidation and homocysteine induced toxicity. Atherosclerosis 1994; 105(2): 165-70.
53. Brown AS, Bottiglieri T, Schaefer CA et al. Elevated prenatal homocysteine levels as a risk factor for schizophrenia. Arch Gen Psychiatry 2007; 64(1): 31-9.
54. Dietrich-Muszalska A, Malinowska J, Olas B et al. The Oxidative Stress May be Induced by the Elevated Homocysteine in Schizophrenic Patients. Neurochem Res. 2012 Jan 24. [Epub ahead of print].
55. Lehmann JM, Moore LB, Smith-Oliver TA, et al.. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). J Biol Chem 1995; 270(22): 12953-6.
56. García-Bueno B, Caso JR, Pérez-Nievas BG, et al. Effects of peroxisome proliferator-activated receptor gamma agonists on brain glucose and glutamate transporters after stress in rats. Neuropsychopharmacology 2007; 32(6): 1251-60.
57. McIntyre RS, Soczynska JK, Lewis GF, et al. Managing psychiatric disorders with antidiabetic agents: translational research and treatment opportunities. Expert Opin Pharmacother 2006; 7(10):1305-21.
58. Ono N, Abiru T, Sugiyama K, Kamiya H. Influences of cyclooxygenase inhibitors on the cataleptic behavior induced by haloperidol in mice. Prostaglandins Leukotrienes Essent Fatty Acids 1992; 46: 59–63.
59. Condray R., Yao JK. Cognition, dopamine and bioactive lipids in schizophrenia. Front Biosci (Schol Ed) 2011; 3: 298-330.
60. Adkins DE, Khachane AN, McClay JL et al. SNP-based analysis of neuroactive ligand-receptor interaction pathways implicates PGE2 as a novel mediator of antipsychotic treatment response: Data from the CATIE study. Schizophr Res 2012; 135(1-3): 200-1.
Alvarez-Jimenez, M., A. G. Parker, S. E. Hetrick, P. D. McGorry and J. F. Gleeson (2009). "Preventing the second episode: a systematic review and meta-analysis of psychosocial and pharmacological trials in first-episode psychosis." Schizophr Bull **37**(3): 619-630.
Arango, C., M. Rapado-Castro, S. Reig, J. Castro-Fornieles, A. Gonzalez-Pinto, S. Otero, I. Baeza, C. Moreno, M. Graell, J. Janssen, M. Parellada, D. Moreno, N. Bargallo and M. Desco (2012). "Progressive brain changes in children and adolescents with first-episode psychosis." Arch Gen Psychiatry **69**(1): 16-26.
Bernardo, M., M. Bioque, M. Parellada, J. Saiz Ruiz, M. J. Cuesta, A. Llerena, J. Sanjuan, J. Castro-Fornieles, C. Arango and B. Cabrera (2013). "Assessing clinical and functional outcomes in a gene-environment interaction study in first episode of psychosis (PEPs)." Rev Psiquiatr Salud Ment **6**(1): 4-16.
Bertani, M., A. Lasalvia, C. Bonetto, S. Tosato, D. Cristofalo, S. Bissoli, K. De Santi, R. Mazzoncini, L. Lazzarotto, M. Santi, A. Sale, D. Scalabrin, M. Abate, M. Tansella and M. Ruggeri (2011). "The influence

of gender on clinical and social characteristics of patients at psychosis onset: a report from the Psychosis Incident Cohort Outcome Study (PICOS)." Psychol Med: 1-12.
Bertelsen, M., P. Jeppesen, L. Petersen, A. Thorup, J. Ohlenschlaeger, P. le Quach, T. O. Christensen, G. Krarup, P. Jorgensen and M. Nordentoft (2008). "Five-year follow-up of a randomized multicenter trial of intensive early intervention vs standard treatment for patients with a first episode of psychotic illness: the OPUS trial." Arch Gen Psychiatry **65**(7): 762-771.
Castro-Fornieles, J., M. Parellada, C. A. Soutullo, I. Baeza, A. Gonzalez-Pinto, M. Graell, B. Paya, D. Moreno, E. de la Serna and C. Arango (2008). "Antipsychotic treatment in child and adolescent first-episode psychosis: a longitudinal naturalistic approach." J Child Adolesc Psychopharmacol **18**(4): 327-336.
First, M., R. Spitzer, M. Gibbon and J. Williams, Eds. (1999). Entrevista clínica estructurada para los trastornos del eje-I del DSM-IV. Barcelona, Masson.
First, M., R. Spitzer, M. Gibbon and J. Williams, Eds. (1999). SCID-II: guía del usuario para la entrevista clínica estructurada para los trastornos de la personalidad. Barcelona, Masson.
Garcia-Bueno, B., M. Bioque, K. S. Mac-Dowell, M. F. Barcones, M. Martinez-Cengotitabengoa, L. Pina-Camacho, R. Rodriguez-Jimenez, P. A. Saiz, C. Castro, A. Lafuente, J. Santabarbara, A. Gonzalez-Pinto, M. Parellada, G. Rubio, M. P. Garcia-Portilla, J. A. Mico, M. Bernardo and J. C. Leza (2013). "Pro-/Anti-inflammatory Dysregulation in Patients With First Episode of Psychosis: Toward an Integrative Inflammatory Hypothesis of Schizophrenia." Schizophr Bull.
Huber, C. G., D. Naber and M. Lambert (2008). "Incomplete remission and treatment resistance in first-episode psychosis: definition, prevalence and predictors." Expert Opin Pharmacother **9**(12): 2027-2038.
Insel, T. R. (2010). "Rethinking schizophrenia." Nature **468**(7321): 187-193.
Kahn, R. S., W. W. Fleischhacker, H. Boter, M. Davidson, Y. Vergouwe, I. P. Keet, M. D. Gheorghe, J. K. Rybakowski, S. Galderisi, J. Libiger, M. Hummer, S. Dollfus, J. J. Lopez-Ibor, L. G. Hranov, W. Gaebel, J. Peuskens, N. Lindefors, A. Riecher-Rossler and D. E. Grobbee (2008). "Effectiveness of antipsychotic drugs in first-episode schizophrenia and schizophreniform disorder: an open randomised clinical trial." Lancet **371**(9618): 1085-1097.
Lieberman, J. A., T. S. Stroup, J. P. McEvoy, M. S. Swartz, R. A. Rosenheck, D. O. Perkins, R. S. Keefe, S. M. Davis, C. E. Davis, B. D. Lebowitz, J. Severe and J. K. Hsiao (2005). "Effectiveness of antipsychotic drugs in patients with chronic schizophrenia." N Engl J Med **353**(12): 1209-1223.
McEvoy, J. P., J. A. Lieberman, D. O. Perkins, R. M. Hamer, H. Gu, A. Lazarus, D. Sweitzer, C. Olexy, P. Weiden and S. D. Strakowski (2007). "Efficacy and tolerability of olanzapine, quetiapine, and risperidone in the treatment of early psychosis: a randomized, double-blind 52-week comparison." Am J Psychiatry **164**(7): 1050-1060.
Olesen, J., A. Gustavsson, M. Svensson, H. U. Wittchen and B. Jonsson (2011). "The economic cost of brain disorders in Europe." Eur J Neurol **19**(1): 155-162.
Ulloa, R. E., S. Ortiz, F. Higuera, I. Nogales, A. Fresan, R. Apiquian, J. Cortes, B. Arechavaleta, C. Foulliux, P. Martinez, L. Hernandez, E. Dominguez and F. de la Pena (2006). "[Interrater reliability of the Spanish version of Schedule for Affective Disorders and Schizophrenia for School-Age Children--Present and Lifetime version (K-SADS-PL)]." Actas Esp Psiquiatr **34**(1): 36-40.
van Os, J. and S. Kapur (2009). "Schizophrenia." Lancet **374**(9690): 635-645.

Table 1.- Demographic and Clinical Characteristics.

Characteristic	Patients (N=85)	Controls (N=106)
Demographic characteristics		
Age – years	25.21 ± 6.03	25.18 ± 6.79
Sex – no. (%)		
Male	60 (70.6)	70 (66.0)
Female	25 (29.4)	36 (34.0)
Socioeconomic status		
High	16 (18.8)	14 (13.2)
Medium-High	7 (8.2)	17 (16.0)
Medium	**30 (35.3)***	54 (50.9)
Medium-Low	24 (28.2)	19 (17.9)
Low	**8 (9.4)***	2 (1.9)
Ethnic Group		
Caucasian	79 (92.9)	96 (90.6)
Gipsy	1 (1.2)	0 (0)
Maghrebian	1 (1.2)	2 (1.9)
Asian	1 (1.2)	0 (0)
Caribbean	1 (1.2)	0 (0)
Hispanic	2 (2.4)	6 (5.7)
Other	0 (0)	2 (1.9)
Age of psychosis onset	24.37 ± 5.92	-
Duration of Untreated Psychosis (DUP) - days	68.58 ± 77.28	-
Duration of illness (DOI) - days	690.33 ± 320.89	
Diagnosis – no. (%)		
Affective Psychosis	17 (20.0)	-
Non-affective Psychosis	62 (72.9)	-
Drugs Psychosis	6 (7.1)	
Psychopathology score		
PANSS		
Total	40.29 ± 23.67	-
Positive	8.01 ± 5.03	-
Negative	11.76 ± 8.15	-
General	20.52 ± 11.99	-
YOUNG	1.39 ± 3.57	-
Montgomery-Asberg	6.02 ± 6.94	
Overall functioning score (GAF)	72.08 ± 17.23	-
Baseline Antipsychotic medication – no. (%)		
Risperidone	21 (25.9)	-
Olanzapine	6 (7.4)	-
Aripiprazole	15 (18.5)	-
Paliperidone	5 (6.2)	-
Clozapine	6 (7.4)	-
Quetiapine	5 (6.2)	-
Ziprasidone	2 (2.5)	-

None	21 (25.9)	-
Defined Daily Dose of chlorpromazine equivalents – mg	298.06 ± 303.16	-
Lithium use – no. (%)	7 (8.4)	-
Body Mass Index	**24.65 ± 5.73 ***	23.14 ± 3.16
Cannabis use - no. (%)	**4 (5.1) ***	14 (16.0)
Cannabis use per month – no. cigarettes	1.09 ± 6.37	1.15 ± 6.36
Tobacco use – no. (%) **Tobacco use per month – no. cigarettes**	**54 (65.1)*** **241.98 (254.11)***	25 (29.4) 45.38 (119.31)
Cotinine – ng/mL	**97.27 ± 84.50***	26.28 ± 49.31

*** p – value < 0.05**

Table 2 Biological markers

Characteristic	Patients (N=73)	Controls (N=106)	statistics	df	p value
INOS	107.92 ± 54.89	96.60 ± 40.50	U=2796.0	-	0.320
COX2	235.34 ± 176.51	94.58 ± 61.92	U=1230.0	-	**<0.001**
NFkB	5.75±2.87	4.36±3.28	U=709.0	-	**<0.001**
PGE2	664.41 ± 569.50	373.35 ± 264.95	U=2309.5	-	**<0.001**
NO2	17.97 ± 7.28	12.75 ± 4.67	U=1337.0	-	**<0.001**
TBARS	4.48 ± 1.78	2.68 ± 2.69	U=1509.0	-	**<0.001**
iKBa	104.22 ± 84.78	103.66 ± 47.13	U=2627.0	-	0.113
d15PGJ2	418.69 ± 155.34	618.87 ± 158.42	U=1101.0	-	**<0.001**
PPARg	56.25 ± 50.87	103.12 ± 27.92	U=143.0	-	**<0.001**
PPARgAct	1.14 ± 0.57	1.88 ± 1.45	U=2055.0	-	**<0.001**

Table 3. Multivariate logistic regression analysis

	B	SE	Wald	OR	IC 95%	p value
PGE2	0.005	0.002	3.721	1.005	1.000-1.009	0.054
COX2	0.017	0.007	6.509	1.017	1.004-1.031	**0.011**
NO2	0.369	0.163	5.137	1.447	1.051-1.991	**0.023**
TBARS	0.182	0.162	1.269	1.200	0.874-1.649	0.260
PPARyAct	-0.832	0.631	1.741	0.435	0.126-1.498	0.187
d15PGJ2	-0.023	0.007	10.099	0.977	0.963-0.991	**0.001**

Table 4. Multiple linear regression analysis

	PGE_2	COX2	NO^-_2	TBARS	PGJ_2	PPARg act.
Gender	-45.635	-37.395	**7.451**	1.793	-31.095	-0.179
Age	-5.010	-0.462	-0.042	0.108	-11.284	**0.075**
Body Mass Index	-1.898	5.596	-0.038	-0.217	2.463	-0.141
Antipsychotic DDD	**-0.733**	0.148	0.006	-0.001	**0.333**	0.000
DUP	1.020	0.187	0.019	-0.013	-0.025	0.003
DOI	**0.714**	-0.047	0.001	**0.005**	-0.057	0.000
GAF	-3.401	2.490	0.122	**-0.120**	0.783	**-0.033**
Cannabis use per month	-0.619	-1.296	**-0.190**	0.015	0.994	-0.003
Cotinine plasma levels	**-3.387**	**0.672**	0.001	0.001	0.503	0.002

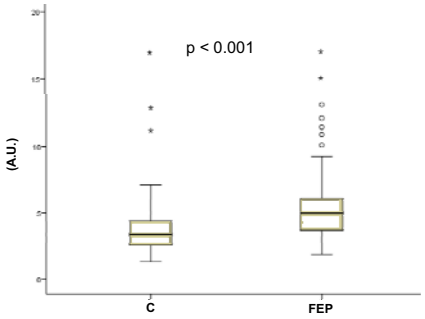
Changes of each biological marker (from baseline to the follow-up point) depending on demographic and clinical variables. The bold values in the table represents the values reaching statistical significance (p-value<0.05) BMI: Body Mass Index, DDD: defined daily dose of antipsychotic chlorpromazine equivalents; DUP: Duration of Untreated Psychosis, DOI: Duration of illness (number of days between the first-episode psychosis onset and the study follow up visit), GAF: Global Assessment of Functioning scale.

Figure legends:

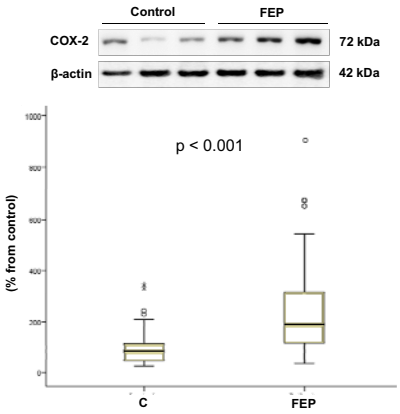
Fig. 1. Mean differences (SD) on proinflammatory biomarkers between FEP and controls (univariate analysis). (a) NFκB activity in PBMC nuclear extracts from FEP patients (*n* = 85) and controls (*n* = 35); (b) Western blot analysis of proinflammatory proteins COX-2 (patients *n* = 85, controls, *n* = 88); (c) iNOS in PBMC cytosolic extracts from FEP patients (*n* = 85) and controls (*n* = 88); and (d) proinflammatory prostaglandin E2 (patients *n* = 85, controls, *n* = 104); (e) plasma levels of nitrites (NO2−; patients *n* = 85, controls, *n* = 61); (f) thiobarbituric acid reactive substances (patients *n* = 85, controls, *n* = 104). AU: arbitrary units. Two-tailed nonparametric Mann-Whitney *U* test was used. ° represents an atypical value and * an extreme value.

Fig. 2. Mean differences (SD) on anti-inflammatory biomarkers. (a) Western blot analysis of IκBα in PBMC cytosolic extracts (patients *n* = 85, controls, *n* = 88); (b)plasma levels of anti-inflammatory prostaglandin 15d-PGJ_2 (patients *n* = 85, controls, *n* = 104); (c) Western blot analysis of peroxisome proliferator activated receptor γ (PPARγ; patients *n* = 85, controls, *n* = 16); and (d) transcriptional activity of PPARγ (patients *n* = 85, controls, *n* = 87) in PBMC nuclear extracts. Two-tailed *t* test was assessed for PPARγ protein levels, and for the rest of variables, two-tailed nonparametric Mann-Whitney *U* test was used. ° represents an atypical value and * an extreme value.

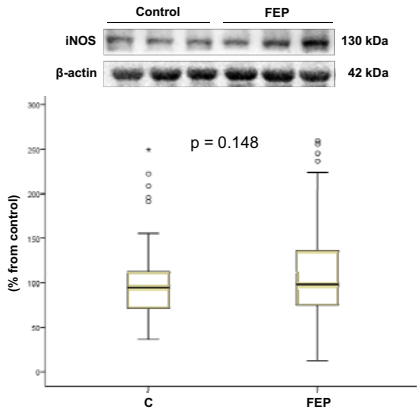
NFκB



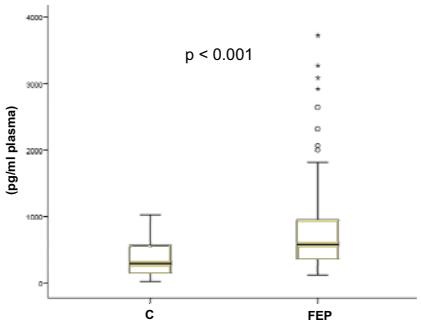
COX-2



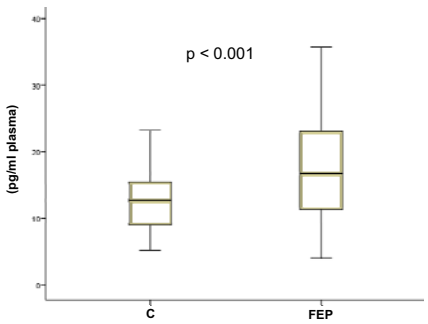
iNOS



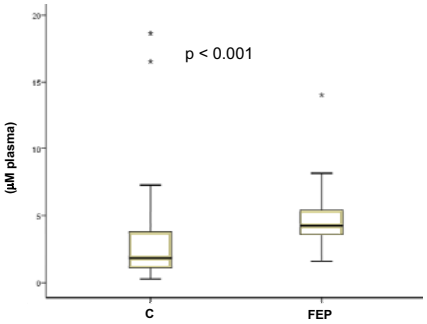
PGE_2

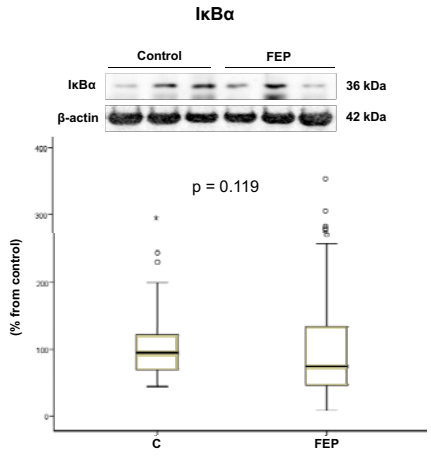


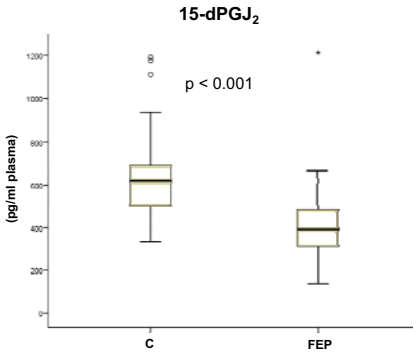
NO_2^-

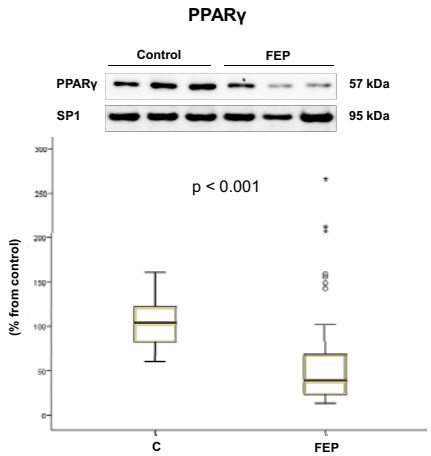


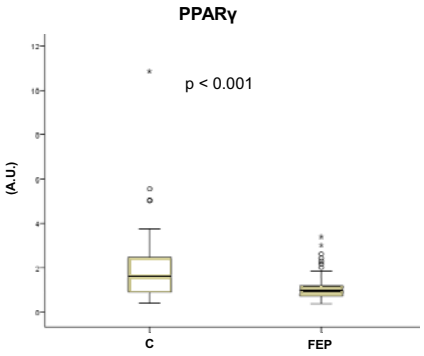
TBARS




IκBα
Control
FEP
IκBα
36 kDa
β-actin
42 kDa
p = 0.119
(% from control)
C
FEP


15-dPGJ2
p < 0.001
(pg/ml plasma)
C
FEP


PPARγ
Control
FEP
PPARγ
57 kDa
SP1
95 kDa
p < 0.001
(% from control)
C
FEP


PPARγ
p < 0.001
(A.U.)
C
FEP

Neuropsychopharmacology (2013), 1–10

www.neuropsychopharmacology.org

Peripheral Endocannabinoid System Dysregulation in First-Episode Psychosis

Miquel Bioque*[,1], Borja García-Bueno[2,3], Karina S MacDowell[2,3], Ana Meseguer[1], Pilar A Saiz[4], Mara Parellada[5], Ana Gonzalez-Pinto[6], Roberto Rodriguez-Jimenez[3], Antonio Lobo[7], Juan C Leza[2,3], Miguel Bernardo*[,1,8] and From the FLAMM-PEPs study—Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM)[9]

[1]Schizophrenia Clinic Unit, Neuroscience Institute, Hospital Clínic de Barcelona, Barcelona, Spain; [2]Department of Pharmacology, Faculty of Medicine, Universidad Complutense de Madrid, Madrid, Spain; [3]Instituto de Investigación, Hospital 12 de Octubre (i + 12), Madrid, Spain; [4]Department of Psychiatry, Faculty of Medicine, University of Oviedo, Oviedo, Spain; [5]Child and Adolescent Psychiatry Department, IIS Gregorio Marañón, Hospital General Universitario Gregorio Marañón, Madrid, Spain; [6]Hospital Universitario de Álava (sede Santiago), Universidad Nacional de Educación a Distancia, Vitoria, Spain; [7]Department of Psychiatry. Hospital Clínico Universitario and University of Zaragoza, Zaragoza, Spain; [8]Department of Psychiatry and Clinical Psycobiology, University of Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain

Several hypotheses involving alterations of the immune system have been proposed among etiological explanations for psychotic disorders. The endocannabinoid system (ECS) has a homeostatic role as an endogenous neuroprotective and anti-inflammatory system. Alterations of this system have been associated with psychosis. Cannabis use is a robust risk factor for these disorders that could alter the ECS signalling. In this study, 95 patients with a first episode of psychosis (FEP) and 90 healthy controls were recruited. Protein expression of cannabinoid receptor 2 (CB2), the protein levels of the main endocannabinoid synthesizing enzymes *N*-acyl phosphatidylethanolamine phospholipase (NAPE) and diacylglycerol lipase (DAGL), and of degradation enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) were determined by western blot analysis in peripheral blood mononuclear cells (PBMCs). Patients with a FEP showed a decreased expression of CB2 and of both endocannabinoids synthesizing enzymes (NAPE and DAGL) in comparison to healthy controls. After controlling for age, gender, body mass index, and cannabis use, NAPE and DAGL expression remained significantly decreased, whereas FAAH and MAGL expression were increased. On the other hand, FEP subjects with history of severe cannabis use showed a larger ECS dysregulation compared with healthy controls. These results indicate an ECS dysregulation in PBMC of FEP patients. The alteration of the ECS presented at the initial phases of psychosis could be contributing to the pathophysiology of the disease and constitutes a possible biomarker of psychotic disorders and an interesting pharmacological target to take into account for therapeutic purposes.

Neuropsychopharmacology advance online publication, 31 July 2013; doi:10.1038/npp.2013.165

Keywords: biomarker; cannabis; endocannabinoid system; first episode of psychosis; schizophrenia

INTRODUCTION

Nowadays, we are living a reformulation of the classical concept of the psychotic illness (Insel, 2010), being seen as an heterogeneous disorder with a multisystemic impact from the beginning, in addition to its psychiatric expression (Kirkpatrick, 2009). Despite the growing number of published research studies in recent years, the etiology of psychotic disorders is far from being clarified (Bernardo and Bioque, 2010). The exposure to certain environmental factors interacting with genetic factors can alter dopaminergic transmission, neuroendocrine, and cognitive functioning, patterns of interpersonal interaction and affective processing and may lead to a psychopathology worsening (Caspi *et al*, 2005).

Among other pathophysiological alterations, several hypotheses involving the immune system—at both peripheral and central nervous system (CNS)—have been proposed as etiological explanations for psychosis (Garcia-Bueno *et al*, 2013; Garcia-Rizo *et al*, 2012; Meyer *et al*, 2011).

*Correspondence: Professor Miguel Bernardo or Miquel Bioque, Unitat d'Esquizofrènia Clínic, Neuroscience Institute, Hospital Clínic de Barcelona, CIBERSAM (G04), C. Villarroel 170. Esc 12. Pta 0, 08036 Barcelona, Spain, Tel: +34 93 227 54 00 Ext. 3142, Fax: +34 932275548, E-mail: bernardo@clinic.ub.es or mbioque@clinic.ub.es

[9]FLAMM-PEPs is a multicentric, collaborative and translational group inside CIBERSAM aimed to study inflammatory pathways in psychosis both as possible biomarkers and as possible new therapeutic targets, incorporated in the PEPs study, a Spanish research project in first episodes of psychosis.

Received 28 March 2013; revised 27 May 2013; accepted 25 June 2013; accepted article preview online 4 July 2013

The endocannabinoid system (ECS) has been proposed as a main homeostatic system implicated in the regulation of the complex neuroimmune interactions in diverse neuropathological scenarios (Wolf *et al*, 2008). In particular, the ECS is present in stress-responsive neural and peripheral circuits, reducing both neurodegenerative and inflammatory damage (Centonze *et al*, 2008; Wolf *et al*, 2008).

In short, the ECS refers to the arachidonate-based lipids anandamide (AEA) and 2-arachidonoylglycerol (2AG); their cannabinoid G protein-coupled receptors, namely CB1 and CB2, the two main synthesis enzymes *N*-acyl phosphatidylethanolamine phospholipase (NAPE) and diacylglycerol lipase (DAGL), and the enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) are responsible for their degradation or reuptake.

Several studies have related the ECS with psychotic disorders (particularly in schizophrenia), focusing on CB1/CB2 receptors. Reduced CB1 expression and activity have been found in different brain areas of patients with schizophrenia (Eggan *et al*, 2008). A close relationship has also been reported between a diminished CB2 function (polymorphism Q63R) and an increased susceptibility to schizophrenia, together with other risk factors (Ishiguro *et al*, 2010). Schizophrenia symptom remission has been linked to significant changes in CB2 mRNA transcripts in peripheral blood mononuclear cells (PBMC; De Marchi *et al*, 2003). Moreover, deletion of CB2 has been related to schizophrenia-like behaviors in animal models (Ortega-Alvaro *et al*, 2011). Therefore, it has been reported that both receptors have a homeostatic role in certain situations and their altered expression has been described in patients with schizophrenia: CB1 mainly in the CNS and CB2 at the peripheral level (Hillard *et al*, 2012). Regarding other components of the ECS, cerebrospinal fluid (CSF) AEA levels have been found elevated in subjects with schizophrenia (Giuffrida *et al*, 2004; Leweke *et al*, 1999).

On the other hand, cannabis is one of the most important and studied enviromental risk factors related to psychosis (Torrey *et al*, 2012). Around 25–50% of subjects who suffer a first episode of psychosis (FEP) present with cannabis use (Koskinen *et al*, 2009; Volkow, 2009). Its use in youth increases the risk of developing psychosis, with an estimated odds ratio of 2.10–2.93 (Henquet *et al*, 2005; Moore *et al*, 2007), decreasing the age of schizophrenia onset (Sugranyes *et al*, 2009). The neurobiological mechanisms underlying this increased psychosis susceptibility are poorly understood (D'Souza *et al*, 2009; Gage *et al*, 2013). However, some studies have found that frequent cannabis exposure may downregulate AEA signalling in patients with schizophrenia, but not in healthy individuals (Leweke *et al*, 2007). It has also been described that FEP patients who use cannabis present cognitive impairment associated to altered brain structure in particular areas rich in CB1 (Bangalore *et al*, 2008; Ho *et al*, 2011).

Based on these data, we hypothesized that the ECS may be disrupted in the FEP. We aimed to study the expression of the main ECS components in PBMC samples from healthy controls and FEP patients, taking advantage of a Spanish multicenter, longitudinal, naturalistic, follow-up study (PEPs study, from the Spanish abbreviation of *Primeros Episodios Psicóticos*; Bernardo *et al*, 2013). Multiple logistic regression analyses were conducted to identify potential risk/protective factors for suffering a FEP among the ECS components studied. Finally, further statistical analyses were conducted to find possible differences in the ECS status according to prolonged, heavy cannabis use.

SUBJECTS AND METHODS

Subjects

Six Spanish university hospitals recruited 95 FEP patients and 90 matched controls from September 2010 to April 2011 (see Supplementary Data 1 for details). The inclusion criteria were described and discussed in previous articles (Bernardo *et al*, 2013; Garcia-Bueno *et al*, 2013). Inclusion criteria for patients were: (1) age between 9 and 35 years; (2) duration of the psychotic symptoms of less than a year; (3) speak Spanish correctly. The exclusion criteria were: (1) mental retardation, including not only an IQ below 70 but also impaired functioning; (2) history of traumatic head injury with loss of consciousness; (3) history of organic disease with mental repercussions. Healthy controls were selected from the same geographic areas following a pairwise matching. Their inclusion criteria were: (1) same gender as patients; (2) similar age ($\pm 10\%$); (3) similar parental socioeconomic status (± 1 level in the Hollingshead–Redlich scale; Hollingshead and Redlich, 1958); (4) no past or present psychiatric disorder per DSM-IV criteria (American Psychiatric Association, 1994); (5) speak Spanish correctly; (6) no history of psychotic disorder among first-degree relatives. The exclusion criteria for controls were the same than for patients.

The study was approved by the ethics committees of all the participating hospitals. The subjects participated after providing a written, informed consent. In underage subjects, informed consent was signed by legal guardians.

Clinical Assessment

The clinical assessment in the PEPs study was detailed described in a previous article (Bernardo *et al*, 2013). Briefly, the diagnosis was established by the semi-structured diagnostic interviews according to DSM-IV criteria (SCID; First *et al*, 1999). The psychopathological assessment was performed using validated Spanish versions of the Positive and Negative Syndrome Scale (PANSS; Kay *et al*, 1987), the Young Mania Rating Scale (Young *et al*, 1978), and the Montgomery-Asberg Depression Rating Scale (MADRS; Montgomery and Asberg, 1979). The Global Assessment of Functioning Scale (GAF) and the Children's Global Assessment Scale were used to measure the global severity of symptoms and the level of functioning (Endicott *et al*, 1976; Shaffer *et al*, 1983). We calculated the potency equivalents to Chlorpromazine of every antipsychotic dosage, following the international consensus (Gardner *et al*, 2010). Apart from the interviews with the patient, multiple sources of information (including medical records and interviews with relatives) were used to establish the onset of positive psychotic symptoms (defined as the first week with the PANSS items P1, P3, P5, P6, or G9 scoring four or more). The duration of untreated psychosis was defined as the

number of days elapsed between this onset and the beginning of the first adequate treatment for psychosis.

Clinical assessment included a complete medical history and physical examination, laboratory tests, and body mass index (BMI = weight in kg/height in m^2). Cannabis use was evaluated by a portion of the European Adaptation of a Multidimensional Assessment Instrument for Drug and Alcohol Dependence (EuropAsi; Kokkevi and Hartgers, 1995). A systematic recording of drug misuse habits was performed.

Biochemical Determinations in PBMC

Blood sample collection, preparation, and cytosolic extraction conditions were described in a previous article (Garcia-Bueno *et al*, 2013).

Preparation of cytosolic extracts. PBMCs were homogenized in 150 µl buffer (10 mmol/l N-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.9); 1 mmol/l EDTA, 1 phenylmethylsulfonyl fluoride, 0.1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml Na-p-tosyl-L-lysine-chloromethyl ketone, 5 mmol/l NaF, 1 mmol/l $NaVO_4$, 0.5 mol/l sucrose, and 10 mmol/l Na_2MoO_4). After 15 min, Nonidet P-40 (Roche, Mannheim, Germany) was added to reach a concentration level of 1%. The tubes were vortexed for 30 s, and nuclei were collected by centrifugation at 8000 *g* for 5 min. The supernatants were considered to be the cytosolic fraction, being stored at −80 °C. All steps of the fractionation were carried out at 4 °C. As an analysis of their purity, cytosolic extracts were assayed by western blot (WB) analysis against GAPDH, SP-1, or β-actin (in cytosol: 99 ± 1; 19 ± 5; 98 ± 1% of total optical density signal, respectively).

WB analysis. After determining and adjusting protein levels using the Bradford method, extracts were mixed with Laemmli sample buffer (Bio-Rad, USA; SDS 10%, distilled H_2O, 50% glycerol, 1 M Tris HCl, pH 6.8, dithiothreitol and bromophenol blue) with β-mercaptoethanol (50 µl/ml Laemmli) and 12.5 µg were loaded into an electrophoresis gel. Once separated on the basis of molecular weight, proteins from the gels were blotted onto a nitrocellulose membrane (Amersham Ibérica, Spain) with a semi-dry transfer system (Bio-Rad) and were incubated with specific antibodies: (1) rabbit polyclonal CB2 in a dilution of 1 : 1000 in TBS-Tween (101550; Cayman Chemical); (2) rabbit polyclonal CB1 in a dilution of 1 : 750 in TBS-Tween (ab23703; Abcam); (3) rabbit polyclonal NAPE-PLD in a dilution of 1 : 1000 in TBS-Tween (10306; Cayman Chemical); (4) rabbit polyclonal DAGLα in a dilution of 1 : 1000 in TBS-Tween (sc-133307; Santa Cruz Biotechnology, USA); (5) rabbit polyclonal FAAH in a dilution of 1 : 750 in TBS-Tween (101600; Cayman Chemical); (6) rabbit polyclonal MAGL in a dilution of 1 : 1000 in 5% skimmed milk in TBS-Tween (100035; Cayman Chemical); (7) β-actin mouse monoclonal in a dilution of 1 : 15 000 (Clone AC-15; Sigma, Spain); (8) SP1 rabbit polyclonal antibody in a dilution of 1 : 2000 (sc-59; Santa Cruz Biotechnology); (9) GAPDH monoclonal antibody at 1 : 5000 (ab9484; Abcam, UK). Membranes were incubated with the respective HRP-linked secondary antibodies (1 : 2000 in TBS-Tween). Blots were imaged using an Odyssey Fc System (Li-COR Biosciences) and were quantified by densitometry (NIH ImageJ software).

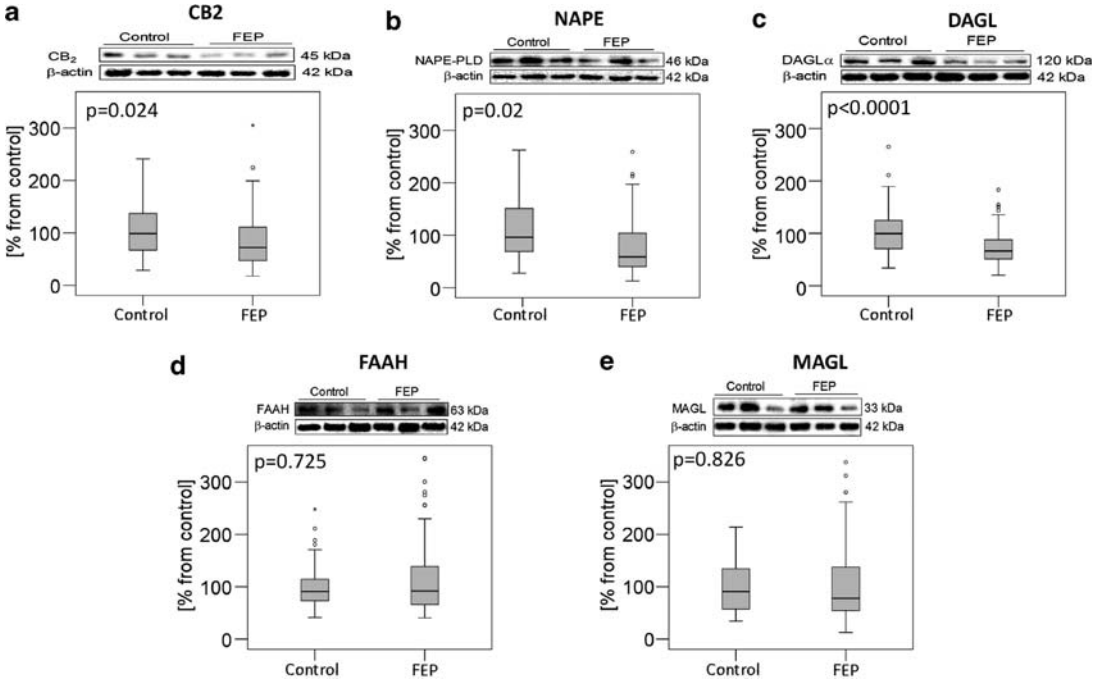


Figure 1 Western blot analysis of ECS components in PBMC cytosolic extracts from FEP patients and controls. WB analysis (insets) of proteins of interest (upper) and loading control (lower) and densitometric analysis (% from control). Mean differences (SD) on ECS markers between FEP and controls (univariate analysis). Western blot analysis in PBMC cytosolic extracts from FEP patients and controls of (a) CB2 receptor, (b) NAPE, (c) DAGLα, (d) FAAH, and (e) MAGL. Two-tailed *t*-test was assessed for CB2, NAPE, and DAGL, and for the rest of variables, two-tailed nonparametric Mann–Whitney *U*-test was used. °Represents an atypical value and * an extreme value.

In the WB analyses carried out in cytoplasmatic extracts, the house keeping gene β-actin was used as loading control (blots shown in the respective figures). In the Figure 1, two WBs are presented, representing all the samples studied (in each different gel, $n=3$ per group –control or FEP). The insets were the most representative of statistical AU data after densitometric analysis as stated above. All densitometry results are expressed in percentage from control.

Statistical Analysis

Differences between control and patient features were assessed using two-tailed χ^2-tests (on categorical data) and *t*-tests (on variables with approximately normal distributions, as ECS components were). A multiple linear regression analysis was performed to assess the effect of psychotropic medication and clinical variables. *T*-tests were used to differentiate the ECS component expression in the FEP group according to the diagnosis (affective *vs* non-affective psychosis), gender, and active cannabis users. Bivariate analyses were used to find differences according to age, clinical scales scores, chlorpromazine's equivalent antipsychotic dosage, BMI, and number of cannabis cigarettes smoked per month.

To calculate the association between FEP and each ECS marker, we used hierarchical logistic regression models with FEP/control status as the dependent variable, and we controlled for in a step-wise manner for potential confounders. Given that the expression of the ECS can be modified by aging (Bilkei-Gorzo, 2012), can be different between genders (Battista *et al*, 2012) and it is related to sustained food intake (Di Marzo *et al*, 2001), analyses were controlled for age, gender, and BMI. Model 1 included the expression of every ECS marker. Model 2 additionally included age and gender. Model 3 additionally included BMI. Model 4 additionally included cannabis use per month. Only those markers with a significant association ($p<0.05$) with the FEP group in the previous analyses were selected for the following steps. Logistic regression analyses were again calculated with the same system, and all the ECS components chosen were kept and analyzed together in a new Model 1, Model 2 (adding age and gender), Model 3 (adding BMI), and Model 4 (adding cannabis use per month—final model).

Data were managed and analyzed with the IBM SPSS Statistics v.20 (IBM Corp, 2011).

RESULTS

Demographic, Clinical, and Cannabis Use Features

Demographic, clinical, and cannabis use information are presented in Table 1 and Supplementary Data 1. Patient and control groups did not differ in gender, age, ethnic group, and socioeconomic status.

The antipsychotic mean dose was of 357.33 mg/day of chlorpromazine equivalents. According to the linear regression analysis, none of the ECS component expression was modified by the use of antipsychotic medication or lithium. We did not find any significant difference between those

Table 1 Demographic, Clinical, and Cannabis use Features

Characteristic	Controls ($N=90$)	Patients ($N=95$)
Demographic characteristics		
Age (years)	25.30 ± 6.41	23.59 ± 5.60
Gender, no. (%)		
Male	62 (68.9)	67 (70.5)
Female	28 (31.1)	28 (29.5)
Socioeconomic status, no. (%)		
High	14 (15.56)	19 (20)
Medium–High	14 (15.56)	10 (10.53)
Medium	45 (50)	37 (38.95)
Medium–Low	15 (16.67)	23 (24.21)
Low	2 (2.22)	6 (6.32)
Ethnic group, no. (%)		
Caucasian	83 (92.22)	90 (94.74)
Gipsy	0	1 (1.05)
Maghrebian	0	1 (1.05)
Asian	0	1 (1.05)
Caribbean	0	0
Hispanic	5 (5.56)	2 (2.11)
Others	2 (2.22)	0
Body mass index	23.30 ± 3.16	24.93 ± 5.33 [a]
Cannabis, active users, no. (%)	8 (10.53)	16 (16.84)
Cannabis, age of use (years)	16.54 ± 2.02	16.04 ± 2.84
Cannabis, use per month—cigarettes	1.33 ± 6.83	12.67 ± 35.76 [a]
Cannabis, time of use (years)	0.51 (2.36)	2.72 ± 3.84 [a]
Cannabis, lifetime contact, no. (%)	20 (26.32)	52 (54.74) [a]
Cannabis, lifetime abuse/dependence, no. (%)	5 (6.58)	46 (48.42) [a]
Cannabis, unknown use habits, no. (%)	14 (15.55)	0 (0)
Diagnosis, no. (%)		
Affective psychosis	—	16 (16.8)
Non-affective psychosis	—	79 (83.2)
Psychopathology score		
PANSS		
Total	—	53.75 ± 18.303
Positive	—	11.13 ± 5.693
Negative	—	14.85 ± 6.154
General	—	27.77 ± 8.856
Young Mania Rating Scale	—	1.67 ± 4.457
Montgomery-Asberg Depression Rating Scale		6.58 ± 6.718
Overall functioning score (GAF)	—	66.39 ± 13.153
Antipsychotic medication, no. (%)		
Risperidone	—	36 (37.9)
Olanzapine	—	11 (11.5)
Aripiprazole	—	9 (9.5)
Paliperidone	—	7 (7.4)
Clozapine	—	7 (7.4)
Quetiapine	—	5 (5.3)
Ziprasidone	—	2 (2.1)
None[b]	—	18 (18.9)
Lithium use, no. (%)	—	9 (9.47)

[a]*T*-test, *p* value <0.05. Results are based on two-sided tests assuming equal variances with significance level 0.05. For each significant pair, the key of the smaller category appears under the category with larger mean. Tests are adjusted for all pairwise comparisons within a row of each innermost subtable using the Bonferroni correction.

[b]Includes both never treated patients and those who had stopped the antipsychotic treatment.

Table 2 Differences in the ECS Markers Expression in FEP and Controls Group (Univariate Model)

ECS marker	Controls ($N=90$)	Patients ($N=95$)	Univariate model		Multivariable model				
			Statistics	*P*-value	*B*	SE	Wald	OR (95% CI)	*P*-value
CB2	101.77 ± 45.23	86.02 ± 48.87	$t=2.27$	**0.024**	−0.007	0.004	2.347	0.993 (0.985–1.002)	0.126
NAPE	102.84 ± 59.64	77 ± 51.05	$t=3.17$	**0.02**	−0.012	0.004	7.824	0.988 (0.980–0.996)	**0.005**
DAGL	104.10 ± 41.86	72.87 ± 32.36	$t=5.69$	**<0.0001**	−0.032	0.007	20.041	0.969 (0.955–0.982)	**<0.001**
FAAH	97.41 ± 36.33	113.47 ± 67.69	$U=4147$	0.725	0.015	0.004	11.333	1.015 (1.006–1.024)	**0.001**
MAGL	99.17 ± 48.75	105.59 ± 71.16	$U=4355$	0.826	0.010	0.004	5.521	1.10 (0.002–1.019)	**0.019**

Association of the ECS components analyzed together and adjusted for age, gender, body mass index, and cannabis use per month (Multiple Logistic Regression Analysis). Bold values indicate significant results ($P<0.05$).

FEP subjects treated with antipsychotic and those who were not taking antipsychotic treatment ($n=18$).

Sixteen patients (16.8%) were oriented as affective disorders (unipolar major depression or bipolar disorder) with psychotic features.

It was determined that 46 out of the 95 FEP patients met criteria of cannabis abuse or dependence (48.4%, FEP CAN+), whereas 49 did not (51.6%, FEP CAN−). These subgroups did not statistically differ in the clinical evaluation, the duration of untreated psychosis, the age of onset, and the BMI (Supplementary Data 2).

ECS in Control and FEP Groups

The WB analysis revealed a significant lower expression of the CB2 receptor and of the two main enzymatic sources of endocannabinoids, NAPE and DAGL, in PBMC from FEP group than in the control subjects (Table 2 and Figure 1a–c, respectively). The expression of the two main degradation enzymatic pathways, FAAH and MAGL, trend to be increased in FEP patients but they did not reach statistical significance in the univariate analysis (Table 2 and Figure 1d and e, respectively). After controlling for possible confounders (age, gender, BMI, and number of cannabis cigarettes per month), four of the five components of the ECS studied were significantly associated with FEP. Although univariate analysis showed CB2 differences between groups, these differences were no longer statistically significant after covarying for BMI, cannabis use per month, and the rest of the ECS components.

Among the ECS synthesis enzymes, DAGL had the lowest OR observed (OR = 0.969), meaning that for each decrease in one unit of DAGL expression the probability for suffering a FEP increased in a 3.1% $[(e^{0.084\times 1}-1)\times 100]$ (Table 2 and Supplementary Data 3).

When analyzing the ECS degradation enzymes, the highest OR observed was for the FAAH (OR = 1.015), meaning that for each unit of this biomarker, the risk of a FEP increased by 1.5% $[(e^{0.489\times 1}-1)\times 100]$ after controlling for remaining ECS components and all possible studied confounders. Similarly, the results were 1% for MAGL.

Unfortunately, in our experimental conditions it was not possible to reliably detect CB1 receptor expression in PBMC in the great majority of samples.

ECS in Different Clinical Subgroups

The mean expression of the FAAH of the FEP subjects was significantly higher in men than in women (124.36 *vs* 87.42, $p=0.014$). We found a negative correlation between the FAAH expression and total PANSS scores ($p=0.019$). FAAH expression also showed a negative correlation with the MADRS scale ($p=0.032$).

According to the diagnosis, patients were classified as non-affective or affective psychosis. There were no differences in the ECS components among these groups. These groups were different in the clinical scale scores and the antipsychotic mean dose, as expected.

Age did not correlate with any of the ECS components studied. The group of patients younger than 18 years old was not different in any of ECS components studied in comparison to the older patients.

Differences between FEP Cannabis Heavy Users/Non-Heavy Users and Controls

Considering the potential confounding effect of prolonged cannabis use on the peripheral ECS expression, the FEP group was divided in two subgroups according to prolonged use of cannabis, defined as those subjects that had fulfilled DSM-IV criteria for dependence or abuse (at least during 12 consecutive months) throughout their lives. These two groups (FEP CAN+ *vs* FEP CAN−) were compared with the healthy control group ($n=71$), excluding from these analyses those volunteers who had presented heavy cannabis use in the past ($n=5$) and those without complete information of cannabis use ($n=14$).

One-way ANOVA revealed statistically significant differences between the three diagnostic groups (FEP CAN+, FEP CAN− and control) on CB2 ($F=3.86$, $p=0.023$), NAPE ($F=5.47$, $p=0.005$), and DAGL ($F=18.35$, $p<0.001$, Figures 2 and 3). Bonferroni *post hoc* analysis indicated that FEP CAN+ patients had a significant lower expression of CB2 compared with controls (76.72 ± 41.93 *vs* 100.65 ± 5.1 AU, $p=0.036$; Figure 2a), lower NAPE expression (71.64 ± 48.20 *vs* 105.75 ± 65.05 AU, $p=0.006$; Figure 2b), and lower DAGL expression (69.29 ± 25.97 *vs* 108.01 ± 43.21 AU, $p<0.001$; Figure 2c). In this analysis, we found no statistically significant differences between the three groups in the FAAH and MAGL expression. Interestingly, there were no differences between FEP CAN+ and CAN− groups in the expression of any of the ECS elements studied.

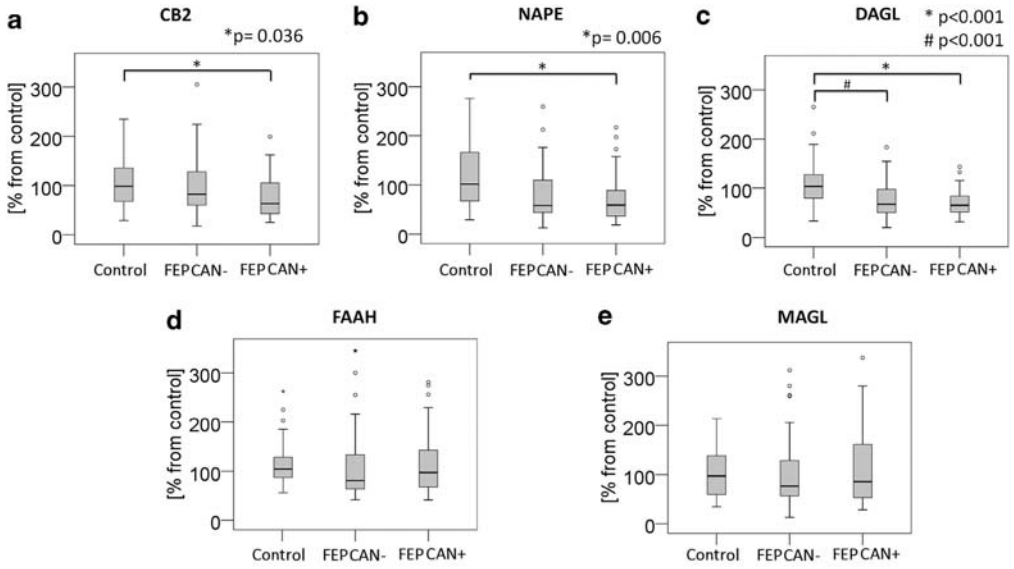


Figure 2 Western blot analysis of the ECS components in PBMC cytosolic extracts from FEP patients (divided according to prolonged cannabis users/non-users) and controls. Densitometric analysis (% from control). Mean differences (SD) on ECS markers between FEP cannabis non-users (FEP CAN −), FEP cannabis users (FEP CAN +), and controls (univariate analysis). Western blot analysis in PBMC cytosolic extracts from FEP patients and controls of (a) CB2 receptor, (b) NAPE, (c) DAGLα, (d) FAAH, and (e) MAGL. Two-tailed *t*-test was assessed for CB2, NAPE, and DAGL, and for the rest of variables, two-tailed nonparametric Mann–Whitney *U*-test was used. ° represents an atypical value and * an extreme value.

These results did not change significantly using a semi-quantitative index for a better characterization of the cannabis use habit (considering its level and duration) nor by analyzing the ECS results according to the early onset of the cannabis use disorder.

DISCUSSION

In this study, we found a reduced expression of the CB2 receptor and of the main endocannabinoid synthesis enzymes in PBMC of patients with a FEP compared with matched healthy controls. After controlling for possible confounders, the group of FEP showed a significantly reduced expression of the endocannabinoid synthesis enzymes and an increased expression of the degradative ones. All together, these results describe, for the first time to our knowledge, a dysregulation of these ECS components in patients who have suffered a FEP (Figure 3). Taking into account that prolonged cannabis use is a risk factor to develop a psychotic disorder (Moore *et al*, 2007; Torrey *et al*, 2012), the FEP group was subdivided for further statistical analyses. The patient subgroup with a history of heavy cannabis use showed a lower CB2 receptor expression, NAPE and DAGL expression in comparison to the control group. No statistically significant differences were found with the sporadic/non-users subgroup of patients.

Data reported so far indicate a dysregulation in the ECS (both in terms of ligands and receptors) in patients with schizophrenia and in animal models of psychosis (Ortega-Alvaro *et al*, 2011; Zamberletti *et al*, 2012). Our results agree with the described relationship between a diminished CB2 function (polymorphism Q63R) and an increased susceptibility to schizophrenia (Ishiguro *et al*, 2010), although from our data we cannot ensure whether its reduced expression is previous or concomitant to the psychotic episode. The ECS has been implicated as a neuroprotective system activated in certain neurodegenerative and neuroinflammatory damage (Wolf *et al*, 2008; Zoppi *et al*, 2011). The synthesis of endocannabinoids could be a defense mechanism adopted by the brain in a psychotic state (Giuffrida *et al*, 2004; Giuffrida and Seillier, 2012). A lower expression of CB2 in the group of FEP might indicate a loss of this protector system.

Leweke *et al* (1999) found elevated AEA levels in the CSF of patients with schizophrenia. Later studies also described elevated CSF AEA levels in antipsychotic-naive first-episode paranoid schizophrenia subjects and in prodromal states of psychosis, with no changes in serum levels (Giuffrida *et al*, 2004; Koethe *et al*, 2009). At peripheral level, De Marchi *et al* (2003) found elevated AEA levels in blood from a small sample of patients with acute schizophrenia. Clinical remission was accompanied by a significant drop in the AEA levels and in the mRNA transcripts for CB2 and FAAH, suggesting that during the acute phase of schizophrenia the ECS signalling might be altered, not only in the CNS but also at systemic level (De Marchi *et al*, 2003).

In addition to schizophrenia, several studies in neuroinflammatory diseases (multiple sclerosis, Huntington's, and Parkinson's diseases) have described ECS alterations in the CNS as well as in PBMC (Centonze *et al*, 2008; Hillard *et al*, 2012). Non-neuronal cell populations have an active and contributory role in the pathogenesis of these neurodegenerative disorders (Perry *et al*, 2007), and signs of neuroinflammation can be detected preceding neuronal loss (McGeer *et al*, 1988). In these CNS pathologies, it can be expected that endocannabinoid concentrations in the circulation and brain are in equilibrium (Hillard *et al*, 2012), PBMC being a mirror of the CNS endocannabinoid status (Centonze *et al*, 2008). There is also evidence that AEA and 2AG concentrations are increased at the peripheral circulation when the immune system is activated during

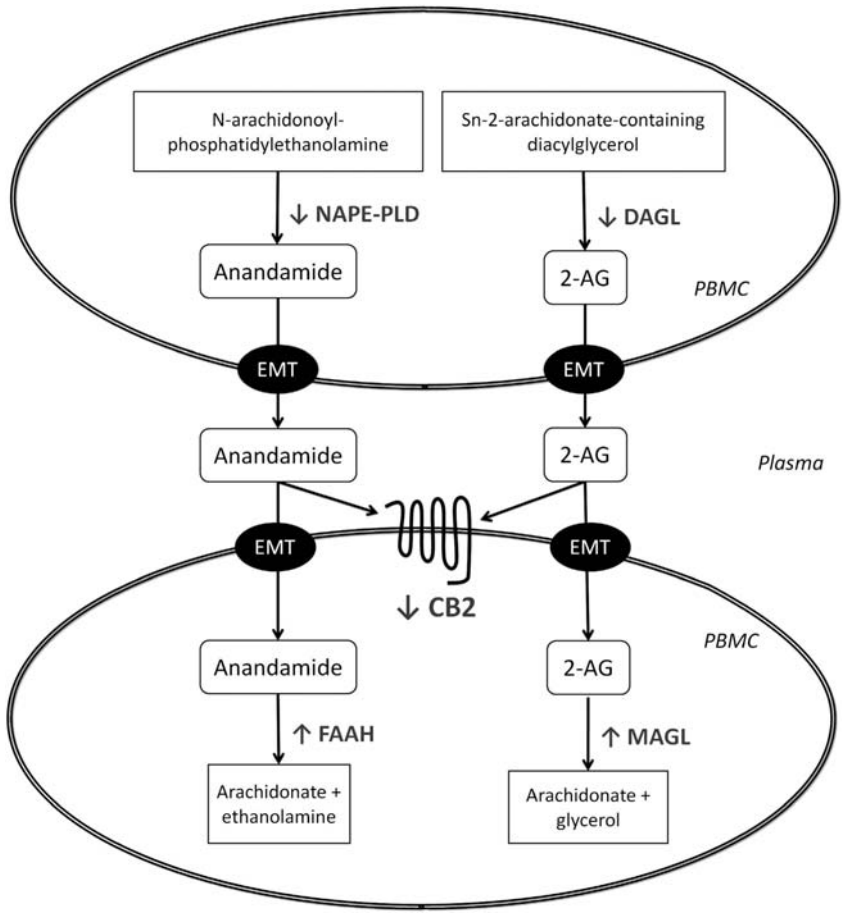


Figure 3 Endocannabinoid system dysregulation in peripheral blood mononuclear cells of patients who have suffered a first episode of psychosis. PBMC, peripheral blood mononuclear cells; NAPE-PLD, *N*-acyl phosphatidylethanolamine phospholipase; DAGL, diacylglycerol lipase; 2-AG, 2-arachidonoylglycerol; EMT, endocannabinoid membrane transporter; CB2, cannabinoid receptor 2; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase. In gray: ECS components studied; ↑: higher expression than healthy controls; ↓: lower expression than healthy controls.

inflammation, infection, or injury (Hillard *et al*, 2012). Finally, a recent review suggests a strong association between inflammatory processes and magnetic resonance imaging anomalies in the brain of subjects with schizophrenia, Alzheimer's disease, or major depressive disorder (Frodl and Amico, 2013).

Some of our findings suggest the value of the determination of peripheral ECS components to obtain potential biomarkers for FEP (Hillard *et al*, 2012). The identification of accessible blood biological markers for psychotic disorders is one of the main needs for both patients and psychiatrists for early diagnosis and evolution monitoring (Schwarz *et al*, 2012), maybe being the best option to approach to their cerebral expression (Rollins *et al*, 2010). Taking into account the results of our multivariable analysis, a low expression of DAGL and NAPE or a high expression of FAAH and NAPE would be associated to a highest risk of suffering a FEP. We will be in good position to establish the predictive value of these findings, as this cohort is being followed during a 2-year period (Bernardo *et al*, 2013).

FAAH expression was higher in men compared with women. It was expected to find differences in FAAH expression between genders, as its activity is regulated by sexual hormones (mainly progesterone and estrogens; Battista *et al*, 2012; Lazzarin *et al*, 2004). Knowing that males have a higher lifetime risk of developing schizophrenia (with a male–female relative risk of about 1.4 (Aleman *et al*, 2003; McGrath *et al*, 2004)), the different FAAH expression found in this study could be one of the factors involved in these risk differences between genders.

We also found a negative correlation between FAAH expression and PANSS and MADRS score. It seems that more severe, acute patients (with higher PANSS scores) would have higher AEA levels (and lower FAAH expression), suggesting that AEA elevation in acute psychosis may reflect a compensatory adaptation to the disease state (Giuffrida *et al*, 2004). In our sample, the mean PANSS score was 53.75 (Table 1), pointing to the fact that the majority of the patients were under remission. This could be the reason why FAAH expression is higher in the FEP group. All together, these findings highlight the variations that the ECS could present depending on the state of the psychotic disorder, severity, or presence of depressive symptoms.

The pharmacological manipulation of the ECS may be a novel therapeutic target for the treatment of psychotic disorders. A recent study has shown that cannabidiol moderately inhibits the degradation of AEA, reducing psychotic symptoms of schizophrenia (Leweke *et al*, 2012). Pharmacological blockade of AEA degradation attenuates induced psychotic-like behaviors in animal

models (Beltramo *et al*, 2000; Seillier *et al*, 2010). The classic focus on CB1 and CB2 has shown the complexity and versatility of the hypothetical ECS role in psychotic disorders (Giuffrida and Seillier, 2012). A recent clinical trial with the CB1 receptor antagonist/inverse agonist rimonabant for improving neurocognition in schizophrenia did not reported clear positive results (Boggs *et al*, 2012). There are various clinical trials recruiting subjects with schizophrenia to test the utility of cannabidiol (ClinicalTrials.gov identifiers: NCT00588731, NCT00309413, and NCT00916201) and AVE1625 (a potent, selective CB1 antagonist. NCT00439634.)

When controlling for other variables, the association between CB2 and the case–control group stopped being significant, and the association between group and FAAH and MAGL became significant (Table 2). These changes appeared when controlling each ECS component between each other, whereas age, gender, BMI, and active cannabis use did not change much these results (Supplementary Data 3). Thus, these changes were caused by the interaction of the ECS between themselves, which seems logical being part of the same biological system.

Some limitations in this study should be noted. First, CB1 receptor expression was undetectable in almost all samples of PBMC. Although both receptors have a role to restore homeostasis mechanisms, CB1 receptors perform their function mainly in the CNS, whereas CB2 receptors do it mainly at the peripheral level. Some authors have reported CB1 expression in human PBMCs, but its levels are much lower than CB2 and considerable work is still needed to define its relevance and regulatory mechanism/s (Klein *et al*, 2003). Second, 81.1% of the patients were receiving antipsychotic treatment. In order to control this possible confounding effect, a multiple linear regression analysis (with each ECS element as dependant variable) was carried out with the chlorpromazine equivalents dosing (Gardner *et al*, 2010). No effect on the level of any of the ECS components was found. In addition, 9% of patients were taking lithium and, given its broad pharmacological effects, the possibility of being a confounding factor was also taken into account. This association did not modify the results either. Third, 16.8% of FEP subjects were diagnosed of affective disorders with psychotic features. This subgroup showed no statistically significant differences in the ECS status compared with non-affective psychosis group. Fourth, cannabis use may have a confounding role when determining the peripheral expression of the different components of ECS. Repeated cannabis use in adolescence produces tolerance to cannabinoid-mediated effects, including brain cannabinoid receptors desensitization and downregulation (Lazenka *et al*, 2012). The altered expression of CB1 and CB2 in cannabis smokers has also been described in PBMC (Nong *et al*, 2002). In our study, we found no significant differences between FEP CAN+ *vs* FEP CAN− subgroups. Bigger subgroups could have shown larger statistically significant differences. Some peripheral endocannabinoids (AEA and oleoylethanolamide) levels are reduced in substance abusers without schizophrenia in comparison to non-abusing schizophrenia subjects (Desfosses *et al*, 2012). Along with other drug use disorders, cannabis use should be an important issue to manage in future research.

It is worth mentioning as strength of our study that the diagnostic evaluation was performed with a very comprehensive protocol, with strict inclusion–exclusion criteria. This naturalistic design makes the sample much closer to the 'real life' FEP population. Owing to the heterogeneity of schizophrenia as a clinical entity, the FEP subgroup is of great interest because it avoids the effect of confounding variables, such as prolonged antipsychotic treatment or chronicity (Bernardo *et al*, 2013). Another key feature of this study is that the age of inclusion is wider than in other previous works, including 23 subjects under 18-year old. Apart from this feature, clinical characteristics of the sample were similar to other studies with FEP in our context (Castro-Fornieles *et al*, 2008; Kahn *et al*, 2008). In addition, complex statistical analyses were conducted to limit biases in the results described.

Thus, future clinical investigations should describe the ECS status in medication-free samples and explore the therapeutic potentials of different ECS targets such as the degrading enzymes studied here, TRP channels, PPAR receptors, and cannabinoid membrane transporters (Giuffrida and Seillier, 2012). MAGL activity could be involved in the regulation of cognitive function (Chanda *et al*, 2010). Studies in high-risk populations will allow determining whether the described alterations in the ECS are present before the psychotic episode starts. This knowledge will have relevant implications to understand the physiopathology of psychosis and also for possible therapeutic implications.

In conclusion, this study has identified that the ECS, which under normal conditions is involved in restoring the homeostatic balance after neural stress, inflammation, or cell damage, appears deregulated in PBMC of patients who had suffered a FEP. Continuous cannabis use could accentuate the malfunction of this endogenous protective system. Some of the peripheral components of the ECS could be used as biomarkers of the disorder. The ECS pharmacological modulation is a promising therapeutic target. Such findings warrant greater attention in future investigations and in the translational significance of these data.

FUNDING AND DISCLOSURE

Study was supported by the Spanish Ministry of Economy and Competiveness, Instituto de Salud Carlos III (ref. ISCIII 2009-2011: PEPs study PI 080208), CIBERSAM (Intramural Projects 2010: Flamm-PEPs study), European Union FEDER, Dirección General de Universidades, Comunidad de Madrid, CANNAB-CM (S2010/BMD-2308), Government of Catalonia, Secretaria d'Universitats i Recerca del Departament d'Economia i Coneixement (2009SGR1295), Esther Koplowitz Center (Barcelona), I+D Madrid (ref. S2010/BMD-2308 CANNAB to JCL). The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank M Fe Barcones (Hospital Clínico Universitario, Zaragoza) and Javier Santabárbara (Department of Preventive Medicine and Public Health, University of Zaragoza) for their methodological support.

Author Contributions

MB wrote the first version of the paper and the figures, performed the statistical analyses, managed and analyzed the clinical data and performed some biochemical determinations; KSM-D and BG-B performed biochemical determinations in plasma and cells; the rest of authors collected the biological samples and the clinical data and analyzed the clinical data; JCL coordinated Flamm-PEPs study; MBer coordinated PEPs study. All of the authors contributed to the final version of the paper.

REFERENCES

Aleman A, Kahn RS, Selten JP (2003). Sex differences in the risk of schizophrenia: evidence from meta-analysis. *Arch Gen Psychiatry* **60**: 565–571.

American Psychiatric Association (1994). *DSM-IV: Diagnostic and Statistical Manual of Mental Disorders*. 4th edn. American Psychiatric Association: Washington, DC.

Bangalore SS, Prasad KM, Montrose DM, Goradia DD, Diwadkar VA, Keshavan MS (2008). Cannabis use and brain structural alterations in first episode schizophrenia–a region of interest, voxel based morphometric study. *Schizophr Res* **99**: 1–6.

Battista N, Meccariello R, Cobellis G, Fasano S, Di Tommaso M, Pirazzi V *et al* (2012). The role of endocannabinoids in gonadal function and fertility along the evolutionary axis. *Mol Cell Endocrinol* **355**: 1–14.

Beltramo M, de Fonseca FR, Navarro M, Calignano A, Gorriti MA, Grammatikopoulos G *et al* (2000). Reversal of dopamine D(2) receptor responses by an anandamide transport inhibitor. *J Neurosci* **20**: 3401–3407.

Bernardo M, Bioque M (2010). Schizophrenia: from neurobiology to nosology of mental disorders. *Actas Esp Psiquiatr* **38**(Suppl 3): 15–17.

Bernardo M, Bioque M, Parellada M, Saiz Ruiz J, Cuesta MJ, Llerena A *et al* (2013). Assessing clinical and functional outcomes in a gene-environment interaction study in first episode of psychosis (PEPs). *Rev Psiquiatr Salud Ment* **6**: 4–16.

Bilkei-Gorzo A (2012). The endocannabinoid system in normal and pathological brain ageing. *Philos Trans R Soc Lond B Biol Sci* **367**: 3326–3341.

Boggs DL, Kelly DL, McMahon RP, Gold JM, Gorelick DA, Linthicum J *et al* (2012). Rimonabant for neurocognition in schizophrenia: a 16-week double blind randomized placebo controlled trial. *Schizophr Res* **134**: 207–210.

Caspi A, Moffitt TE, Cannon M, McClay J, Murray R, Harrington H *et al* (2005). Moderation of the effect of adolescent-onset cannabis use on adult psychosis by a functional polymorphism in the catechol-O-methyltransferase gene: longitudinal evidence of a gene X environment interaction. *Biol Psychiatry* **57**: 1117–1127.

Castro-Fornieles J, Parellada M, Soutullo CA, Baeza I, Gonzalez-Pinto A, Graell M *et al* (2008). Antipsychotic treatment in child and adolescent first-episode psychosis: a longitudinal naturalistic approach. *J Child Adolesc Psychopharmacol* **18**: 327–336.

Centonze D, Battistini L, Maccarrone M (2008). The endocannabinoid system in peripheral lymphocytes as a mirror of neuroinflammatory diseases. *Curr Pharm Des* **14**: 2370–2442.

Chanda PK, Gao Y, Mark L, Btesh J, Strassle BW, Lu P *et al* (2010). Monoacylglycerol lipase activity is a critical modulator of the tone and integrity of the endocannabinoid system. *Mol Pharmacol* **78**: 996–1003.

D'Souza DC, Sewell RA, Ranganathan M (2009). Cannabis and psychosis/schizophrenia: human studies. *Eur Arch Psychiatry Clin Neurosci* **259**: 413–431.

De Marchi N, De Petrocellis L, Orlando P, Daniele F, Fezza F, Di Marzo V (2003). Endocannabinoid signalling in the blood of patients with schizophrenia. *Lipids Health Dis* **2**: 5.

Desfosses J, Stip E, Bentaleb LA, Lipp O, Chiasson JP, Furtos A *et al* (2012). Plasma endocannabinoid alterations in individuals with substance use disorder are dependent on the "Mirror Effect" of schizophrenia. *Front Psychiatry* **3**: 85.

Di Marzo V, Goparaju SK, Wang L, Liu J, Batkai S, Jarai Z *et al* (2001). Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature* **410**: 822–825.

Eggan SM, Hashimoto T, Lewis DA (2008). Reduced cortical cannabinoid 1 receptor messenger RNA and protein expression in schizophrenia. *Arch Gen Psychiatry* **65**: 772–784.

Endicott J, Spitzer RL, Fleiss JL, Cohen J (1976). The global assessment scale. A procedure for measuring overall severity of psychiatric disturbance. *Arch Gen Psychiatry* **33**: 766–771.

First MSpitzer RGibbon MWilliams J (eds) (1999). *Entrevista Clínica Estructurada Para los Trastornos del eje-I del DSM-IV*. Masson: Barcelona.

Frodl T, Amico F (2013). Is there an association between peripheral immune markers and structural/functional neuroimaging findings? *Prog Neuropsychopharmacol Biol Psychiatry* (http://dx.doi.org/10.1016/j.pnpbp.2012.12.013).

Gage SH, Zammit S, Hickman M (2013). Stronger evidence is needed before accepting that cannabis plays an important role in the aetiology of schizophrenia in the population. *F1000 Med Rep* **5**: 2.

Garcia-Bueno B, Bioque M, Mac-Dowell KS, Barcones MF, Martinez-Cengotitabengoa M, Pina-Camacho L *et al* (2013). Pro-/anti-inflammatory dysregulation in patients with first episode of psychosis: toward an integrative inflammatory hypothesis of schizophrenia. *Schizophr Bull* (e-pub ahead of print 13 March 2013).

Garcia-Rizo C, Fernandez-Egea E, Oliveira C, Justicia A, Bernardo M, Kirkpatrick B (2012). Inflammatory markers in antipsychotic-naive patients with nonaffective psychosis and deficit vs. nondeficit features. *Psychiatry Res* **198**: 212–215.

Gardner DM, Murphy AL, O'Donnell H, Centorrino F, Baldessarini RJ (2010). International consensus study of antipsychotic dosing. *Am J Psychiatry* **167**: 686–693.

Giuffrida A, Leweke FM, Gerth CW, Schreiber D, Koethe D, Faulhaber J *et al* (2004). Cerebrospinal anandamide levels are elevated in acute schizophrenia and are inversely correlated with psychotic symptoms. *Neuropsychopharmacology* **29**: 2108–2114.

Giuffrida A, Seillier A (2012). New insights on endocannabinoid transmission in psychomotor disorders. *Prog Neuropsychopharmacol Biol Psychiatry* **38**: 51–58.

Henquet C, Murray R, Linszen D, van Os J (2005). The environment and schizophrenia: the role of cannabis use. *Schizophr Bull* **31**: 608–612.

Hillard CJ, Weinlander KM, Stuhr KL (2012). Contributions of endocannabinoid signaling to psychiatric disorders in humans: genetic and biochemical evidence. *Neuroscience* **204**: 207–229.

Ho BC, Wassink TH, Ziebell S, Andreasen NC (2011). Cannabinoid receptor 1 gene polymorphisms and marijuana misuse interactions on white matter and cognitive deficits in schizophrenia. *Schizophr Res* **128**: 66–75.

Hollingshead ABRedlich FC (eds) (1958). *Social Class and Mental Illness*. Wiley: New York.

IBM Corp (2011). *IBM SPSS Statistics for Windows*. IBM Corp.: Armonk, NY.

Insel TR (2010). Rethinking schizophrenia. *Nature* **468**: 187–193.

Ishiguro H, Horiuchi Y, Ishikawa M, Koga M, Imai K, Suzuki Y *et al* (2010). Brain cannabinoid CB2 receptor in schizophrenia. *Biol Psychiatry* **67**: 974–982.

Kahn RS, Fleischhacker WW, Boter H, Davidson M, Vergouwe Y, Keet IP *et al* (2008). Effectiveness of antipsychotic drugs in first-episode schizophrenia and schizophreniform disorder: an open randomised clinical trial. *Lancet* **371**: 1085–1097.

Kay SR, Fiszbein A, Opler LA (1987). The positive and negative syndrome scale (PANSS) for schizophrenia. *Schizophr Bull* **13**: 261–276.

Kirkpatrick B (2009). The Concept of Schizophrenia. *Rev Psiquiatr Salud Ment* **2**: 105–107.

Klein TW, Newton C, Larsen K, Lu L, Perkins I, Nong L *et al* (2003). The cannabinoid system and immune modulation. *J Leukoc Biol* **74**: 486–496.

Koethe D, Giuffrida A, Schreiber D, Hellmich M, Schultze-Lutter F, Ruhrmann S *et al* (2009). Anandamide elevation in cerebrospinal fluid in initial prodromal states of psychosis. *Br J Psychiatry* **194**: 371–372.

Kokkevi A, Hartgers C (1995). EuropASI: European adaptation of a multidimensional assessment instrument for drug and alcohol dependence. *Eur Addict Res* **1**: 208–210.

Koskinen J, Lohonen J, Koponen H, Isohanni M, Miettunen J (2009). Rate of cannabis use disorders in clinical samples of patients with schizophrenia: a meta-analysis. *Schizophr Bull* **36**: 1115–1130.

Lazenka MF, Selley DE, Sim-Selley LJ (2012). Brain regional differences in CB1 receptor adaptation and regulation of transcription. *Life Sci* **92**: 446–452.

Lazzarin N, Valensise H, Bari M, Ubaldi F, Battista N, Finazzi-Agro A *et al* (2004). Fluctuations of fatty acid amide hydrolase and anandamide levels during the human ovulatory cycle. *Gynecol Endocrinol* **18**: 212–218.

Leweke FM, Giuffrida A, Koethe D, Schreiber D, Nolden BM, Kranaster L *et al* (2007). Anandamide levels in cerebrospinal fluid of first-episode schizophrenic patients: impact of cannabis use. *Schizophr Res* **94**: 29–36.

Leweke FM, Giuffrida A, Wurster U, Emrich HM, Piomelli D (1999). Elevated endogenous cannabinoids in schizophrenia. *Neuroreport* **10**: 1665–1669.

Leweke FM, Piomelli D, Pahlisch F, Muhl D, Gerth CW, Hoyer C *et al* (2012). Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia. *Transl Psychiatry* **2**: e94.

McGeer PL, Itagaki S, Akiyama H, McGeer EG (1988). Rate of cell death in parkinsonism indicates active neuropathological process. *Ann Neurol* **24**: 574–576.

McGrath J, Saha S, Welham J, El Saadi O, MacCauley C, Chant D (2004). A systematic review of the incidence of schizophrenia: the distribution of rates and the influence of sex, urbanicity, migrant status and methodology. *BMC Med* **2**: 13.

Meyer U, Schwarz MJ, Muller N (2011). Inflammatory processes in schizophrenia: a promising neuroimmunological target for the treatment of negative/cognitive symptoms and beyond. *Pharmacol Ther* **132**: 96–110.

Montgomery SA, Asberg M (1979). A new depression scale designed to be sensitive to change. *Br J Psychiatry* **134**: 382–389.

Moore TH, Zammit S, Lingford-Hughes A, Barnes TR, Jones PB, Burke M *et al* (2007). Cannabis use and risk of psychotic or affective mental health outcomes: a systematic review. *Lancet* **370**: 319–328.

Nong L, Newton C, Cheng Q, Friedman H, Roth MD, Klein TW (2002). Altered cannabinoid receptor mRNA expression in peripheral blood mononuclear cells from marijuana smokers. *J Neuroimmunol* **127**: 169–176.

Ortega-Alvaro A, Aracil-Fernandez A, Garcia-Gutierrez MS, Navarrete F, Manzanares J (2011). Deletion of CB2 cannabinoid receptor induces schizophrenia-related behaviors in mice. *Neuropsychopharmacology* **36**: 1489–1504.

Perry VH, Cunningham C, Holmes C (2007). Systemic infections and inflammation affect chronic neurodegeneration. *Nat Rev Immunol* **7**: 161–167.

Rollins B, Martin MV, Morgan L, Vawter MP (2010). Analysis of whole genome biomarker expression in blood and brain. *Am J Med Genet B Neuropsychiatr Genet* **153B**: 919–936.

Schwarz E, Guest PC, Rahmoune H, Harris LW, Wang L, Leweke FM *et al* (2012). Identification of a biological signature for schizophrenia in serum. *Mol Psychiatry* **17**: 494–502.

Seillier A, Advani T, Cassano T, Hensler JG, Giuffrida A (2010). Inhibition of fatty-acid amide hydrolase and CB1 receptor antagonism differentially affect behavioural responses in normal and PCP-treated rats. *Int J Neuropsychopharmacol* **13**: 373–386.

Shaffer D, Gould MS, Brasic J, Ambrosini P, Fisher P, Bird H *et al* (1983). A children's global assessment scale (CGAS). *Arch Gen Psychiatry* **40**: 1228–1231.

Sugranyes G, Flamarique I, Parellada E, Baeza I, Goti J, Fernandez-Egea E *et al* (2009). Cannabis use and age of diagnosis of schizophrenia. *Eur Psychiatry* **24**: 282–286.

Torrey EF, Bartko JJ, Yolken RH (2012). Toxoplasma gondii and other risk factors for schizophrenia: an update. *Schizophr Bull* **38**: 642–647.

Volkow ND (2009). Substance use disorders in schizophrenia–clinical implications of comorbidity. *Schizophr Bull* **35**: 469–472.

Wolf SA, Tauber S, Ullrich O (2008). CNS immune surveillance and neuroinflammation: endocannabinoids keep control. *Curr Pharm Des* **14**: 2266–2278.

Young RC, Biggs JT, Ziegler VE, Meyer DA (1978). A rating scale for mania: reliability, validity and sensitivity. *Br J Psychiatry* **133**: 429–435.

Zamberletti E, Rubino T, Parolaro D (2012). The endocannabinoid system and schizophrenia: integration of evidence. *Curr Pharm Des* **18**: 4980–4990.

Zoppi S, Perez Nievas BG, Madrigal JL, Manzanares J, Leza JC, Garcia-Bueno B (2011). Regulatory role of cannabinoid receptor 1 in stress-induced excitotoxicity and neuroinflammation. *Neuropsychopharmacology* **36**: 805–818.

Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)

6- DISCUSSION

In the first study, we found evidence of systemic inflammatory conditions in patients diagnosed of FEP. Specifically we identified a significant increase in some intracellular components of a main pro-inflammatory pathway, along with a significant decrease in the anti-inflammatory ones. These results describe an imbalanced pro-inflammatory phenotype in FEP patients. The multivariate logistic regression analyses conducted allows us to identify Hcy plasma levels, along with iNOs and COX2, as the most reliable potential risk factors, and IκBα and 15d-PGJ2 as potential protection factors.

In the 6 months follow-up study we have strengthen this evidence of systemic inflammatory alterations in patients diagnosed of FEP. In the baseline study we described phenotypical differences in pro-inflammatory mediators at the cellular machinery level in PBMC, but the resultant soluble elements were not significantly altered. However, 6 months later the great majority of soluble elements analyzed already appear significantly altered, suggesting the existence of a more deregulated pro/anti-inflammatory balance (and potentially more harmful) as can be observed by the lipid peroxidation (TBARS) data found. In this follow-up study, the multivariate logistic regression analysis conducted allowed us to identify the plasmatic levels of the markers of oxidative/nitrosative damage NO_2^- and TBARS and COX-2 protein expression in PBMC as the most reliable potential risk factors.

These results indicate phenotypical differences at the cellular machinery level in PBMC of patients in an initial clinical setting of the disorder (the first episode), in which around the 80% of the patients may be at the beginning of a multi-episode chronic severe mental illness such as schizophrenia or bipolar disorder [131].

The continued search for quantifiable biological markers is particularly useful for the widely known difficulties in the diagnosis of psychiatric diseases [132]. Efforts in describing biological markers for schizophrenia in pathway approaches are claimed by psychiatrists as a tool for early diagnosis and also for monitoring the evolution of the disease; this would greatly assist preventive strategies by identifying at-risk individuals who could then be monitored and treated in a manner with a view to minimizing subsequent morbidity. In this vein, the results of the multivariate analysis applied in these studies are of special translational importance because it tries to simulate what actually happens in biological pathways, including both pro and anti-inflammatory markers in the same statistical model and allowing

them to interact even with possible socio-demographic and clinical confounders. So in the clinical practice we would be able to calculate the association between FEP and one of the markers (for example iNOS), once the influence of other markers (PGE_2, COX2, Hcy and 15d-PGJ2) and confounders (age, sex, BMI, cannabis and tobacco use per month) have been controlled. The association calculated is quiet stable as this did not change after adjusting for possible confounders. The strength of the association was supported by the stability of OR in the different models calculated, because changes in OR were minor and occurred in upper direction only at decimal places. Due to its soluble nature, a notable finding in both studies is that the anti-inflammatory mediator 15d-PGJ2 might be used as plasmatic biomarker for FEP.

There are few studies focused on diagnostic tools in FEP. Some neuroimaging studies indicate subtle brain abnormalities [13], and others clinical subtle symptoms [133]. In terms of inflammation our results coincide with these findings: while the soluble final products are not significantly modified, their enzymatic sources iNOS and COX2, both inducible isoforms regulated by the IκBα/NFκB pathway are over-expressed in PMBC [134, 135]. Thus, our results suggest that FEP patients are at the onset of the inflammatory process. It has to be taken into account that the great majority of studies reporting inflammatory/immune alterations in this pro/anti-inflammatory pathway are described in full-blown, chronic patients with schizophrenia [53, 56, 58, 136-139]. In this subpopulation tissue o plasma antioxidant mechanisms might be exhausted [140].

Similarly, hyperhomocysteinemia can cause oxidative stress via a number of mechanisms (such as auto-oxidation) to form reactive oxygen species or increased lipid peroxidation [77]. Previous studies indicated that high levels of Hcy associate with oxidative stress in schizophrenia, showing a correlation between the increased amount of Hcy and nitrotyrosine in plasma proteins or plasma TBARS, thus being considered as a risk factor for the disease [78, 79]. In this vein, our multivariate statistical approach has identified Hcy levels as a very reliable risk factor for FEP.

On the other hand, we found also a decrease in the anti-inflammatory counterbalancing pathway, mainly controlled by 15d-PGJ_2. Indeed, this mechanism is considered as a possible endogenous regulator of the inflammatory response in neurodegenerative conditions and stress-related diseases [47]. It has been recently described a decrease in this anti-inflammatory pathway in chronic schizophrenic inpatients in acute relapse phase [56]. Now, the data presented in our studies indicate that the changes in 15d-PGJ_2/PPARγ pathway are also present at the very early stages of the disease.

These results suggest an active role for the anti-inflammatory signaling pathway in the pathophysiology of the disorder, which opens up the possibility of using pharmacological strategies involving the stimulation of the PPARγ activity. Of special interest is the possible use of some thiazolidinediones, which are potent agonists of PPARγ, widely used as insulin-sensitizing drugs for the treatment of type 2 diabetes [141]. The pharmacological activation of PPARγ can be considered as a multi-faceted therapeutic target due to its anti-inflammatory/antioxidant/anti-excitotoxic/pro-energetic profile, reported in some inflammatory-related scenarios (neurological and stress-related diseases) [47, 142]. The pharmacological modulation of PPARγ has been presented as a putative treatment for neurocognitive deficits associated with mood and psychotic syndromes [143].

Due to the longitudinal design, we were able to analyze the significant changes of every biological marker between 6 months after the inclusion of the patients in correlation to the respective changes of clinical characteristics and confounding factors. Especially remarkable is the result regarding the associations between antipsychotic dose and the change in the plasma levels of PGE_2 (inverse) and 15d-PGJ_2 (direct), suggesting that one of the therapeutic mechanisms of antipsychotic therapy is the restoration of the pro/anti-inflammatory balance, disrupted in FEP. It is worth noting that the around 26% of the FEP patients recruited were being treated with risperidone. In the last years, it has been shown that risperidone normalizes increased inflammatory mediators (cytokines and prostaglandins) and restores anti-inflammatory pathways in murine models of neuroinflammation [57, 144]. Chronic administration of others antipsychotics, such as olanzapine (7.4%) or clozapine (7.4%), also reduced PGE_2 concentration in rat brain [145]. A recent study using a SNP-based analysis of neuroactive pathways implicated PGE_2 as a mediator of the effects of risperidone, olanzapine and quetiapine (also used by 6.2% of the patients of our study) [146]. The correlation results between medication with antipsychotics and the restoration of the pro/anti-inflammatory balance in FEP represent an important finding supporting the establishment and completion of clinical trials using anti-inflammatory drugs as co-adjuvant strategy to antipsychotics in schizophrenia [55].

From a clinical point of view, the inverse relationship found here between GAF scale and TBARS plasma levels is particularly relevant to provide scientific evidence to support a direct role for oxidative/nitrosative stress cellular damage in the general symptomatology of FEP and, possibly, in other disorders that course psychotic symptoms. Similarly, in a comparable cohort of patients, it has been reported that oxidative stress is related to cognitive impairment (in learning and memory) in FEP [147].

All these findings suggest an active role of this pro/anti-inflammatory signaling pathway in the pathophysiology of the disorder, which support that the possible mechanism/s are not only related to the oxidative/nitrosative cellular damage. Thus, some authors have explored the possibility that abnormalities in prostaglandins and other lipids content in the brain of subjects with schizophrenia could alter synaptic monoaminergic neurotransmission and affect cognition as a result [148]. In other approach, some authors have proposed that prostaglandins (PGE_2) could be implicated in the up-regulation of the endogenous glutamate NMDA receptor antagonist kynurenic acid found in the brain of subjects with schizophrenia, which contributes to the pathogenesis of the disease, linking the dopamine hypothesis of schizophrenia together with the idea of a deficiency in glutamatergic function [149]. Regarding the anti-inflammatory side of the balance, classical studies showed that PGD_2 stimulated the production of cyclic AMP (cAMP) and thereby exerted functional antagonism of dopamine-D2 receptors [150]. Therefore, PGD_2 and its metabolites could be counteracting the biochemical and behavioral effects of dopamine and deficient PGD_2/PGJ_2 signaling in the brain could influence dopamine transmission [151].

In addition to its anti-inflammatory actions, PPARγ may directly regulate glutamatergic neurotransmission at NMDA receptor level [152, 153], which has been involved in positive symptoms of schizophrenia [154]. PPARγ is a master regulator of glucose metabolism and it has been proposed that alterations in its normal activity could be implicated in some of the metabolic disruptions originated as side effects by chronic antipsychotic medication [155]. Recently, the pharmacological modulation of PPARγ has been presented as a new treatment for neurocognitive deficits associated with mood and psychotic disorders. However our correlation analyses indicate that PPAR activity present a negative correlation with GAF scale between the two time points considered. It is possible that PPARγ activity increases as an endogenous mechanism of response when the severity of the symptoms is greater, but this possibility needs to be corroborated in more advanced pathological states. Indeed, it is mandatory to re-evaluate the utility of PPARγ as therapeutic target to improve neurocognitive deficits because its chronic exogenous activation could produce metabolic and cardiovascular alterations that masks its neuroprotective actions. In this vein, a recent pilot clinical trial, PPARγ synthetic ligand rosiglitazone failed in the improvement of cognitive deficits in clozapine-treated patients with schizophrenia [156].

After the analysis of the most common SNP variables of pro/anti-inflammatory mediators, the lack of correlation with their studied gene variants could suggest a possible role for epigenetic factors, other less studied SNPs or other

candidate genes, as well as the necessity of new methods to detect genetic effects. For example, a recent study used a SNP-based analysis of neuroactive pathways implicates PGE_2 as a novel mediator of antipsychotic treatment response using data from the multiphase, randomized controlled trial CATIE [146]. On the other hand, the absence of findings related to genetic makers and the lack of any clinical correlates of the biomarkers here studied raises the issue of whether the effects reported could be reflecting an epiphenomenon related to stress or metabolic complications, rather than a mechanism contributing to symptomatic expression of the illness.

Regarding the ECS study, we found a reduced expression of the CB2 receptor and of the main endocannabinoid synthesis enzymes in PBMC of patients with a FEP compared to matched, healthy controls. After controlling for possible confounders, the group of FEP showed a significantly reduced expression of the ECS synthesis enzymes and an increased expression of the degradative ones. All together, these results describe, for the first time to our knowledge, a dysregulation of these ECS components in patients who have suffered a FEP. Taking into account that prolonged cannabis use is a risk factor to develop a psychotic disorder [31, 99] the FEP group was subdivided for further statistical analyses. The patient subgroup with a history of heavy cannabis use showed a lower CB2 receptor expression, NAPE and DAGL expression in comparison to the control group. No statistically significant differences were found with the sporadic/non-users subgroup of patients.

Data reported so far indicate a dysregulation in the ECS (both in terms of ligands and receptors) in patients with schizophrenia and in animal models of psychosis [93, 157]. Our results agree with the described relationship between a diminished CB2 function (polymorphism Q63R) and an increased susceptibility to schizophrenia [91], although from our data we cannot ensure whether its reduced expression is previous or concomitant to the psychotic episode. The ECS has been implicated as a neuroprotective system activated in certain neurodegenerative and neuroinflammatory damage [86, 88]. The synthesis of endocannabinoids could be a defense mechanism adopted by the brain in a psychotic state [95, 158]. A lower expression of CB2 in the group of FEP might indicate a loss of this protector system.

Leweke et al. found CSF elevated AEA levels in patients with schizophrenia [94]. Later studies described elevated CSF AEA levels in antipsychotic-naive first-episode paranoid schizophrenia subjects and in prodromal states of psychosis, with no changes in serum levels [95, 159]. At peripheral level, Di Marchi et al. found elevated AEA levels in blood from a small sample of patients with acute schizophrenia [92]. Clinical remission was accompanied by a significant drop in the

AEA levels and the mRNA transcripts for CB2 and FAAH, suggesting that during the acute phase of schizophrenia the ECS signaling might be altered not only in the CNS but also in the blood [92]. Finally, it has been recently reported specific alterations in the levels of some endocannabinoids in different brain regions of post-mortem brain tissue form subjects with schizophrenia [96].

In addition to schizophrenia, several studies in neuroinflammatory diseases (multiple sclerosis, Huntington's and Parkinson's diseases) have described ECS alterations in the CNS as well as in PBMC [81, 87]. Non-neuronal cell populations play an active and contributory role in the pathogenesis of these neurodegenerative disorders [160], and signs of neuroinflammation can be detected preceding neuronal loss [161]. In these CNS pathologies, it can be expected that endocannabinoid concentrations in the circulation and brain are in equilibrium [81], being PBMC a mirror of the CNS endocannabinoid status [87]. There is also evidence that AEA and 2-AG concentrations are increased at the peripheral circulation when the immune system is activated during inflammation, infection or injury [81]. Finally, a recent review suggest a strong association between inflammatory processes and magnetic resonance imaging anomalies in the brain of subjects with schizophrenia, Alzheimer’s disease or major depressive disorder [162].

Some of our findings suggest the value of the determination of peripheral ECS components to obtain potential biomarkers for FEP [81]. As previously said, the identification of accessible blood biological markers for psychotic disorders is one of the main needs for both patients and psychiatrists for early diagnosis and evolution monitoring [132, 163], maybe being the best option to approach to their cerebral expression [164]. Taking into account the results of our multivariable analysis, a low expression of DAGL and NAPE or a high expression of FAAH and MAGL would be associated to a highest risk of suffering a FEP.

FAAH expression was higher in men compared to women. It was expected to find differences in FAAH expression between genders, as its activity is regulated by sexual hormones (mainly progesterone and estrogens) [165, 166]. Knowing that males have a higher lifetime risk of developing schizophrenia (with a male–female relative risk of about 1.4 [167, 168]), the different FAAH expression found in this study could be one of the factors involved in these risk differences between genders.

We also found a negative correlation between FAAH expression and PANSS and MADRS score. It seems that more severe, acute patients (with higher PANSS scores) would have higher AEA levels (and lower FAAH expression), suggesting that anandamide elevation in acute psychosis may reflect a compensatory adaptation to

the disease state [95]. In our sample, the mean PANSS score was 53.75, pointing to the fact that the majority of the patients were under remission. This could be the reason why FAAH expression is higher in the FEP group. All together, these findings highlight the variations that the ECS could present depending on the state of the psychotic disorder, severity or presence of depressive symptoms.

The pharmacological manipulation of the ECS may be a novel therapeutic target for the treatment of psychotic disorders. A recent study has shown that cannabidiol moderately inhibits the degradation of AEA, reducing psychotic symptoms of schizophrenia [169]. Pharmacological blockade of AEA degradation attenuates induced psychotic-like behaviors in animal models [170, 171]. The classic focus on CB1 and CB2 has shown the complexity and versatility of the hypothetical ECS role in psychotic disorders [158]. A recent clinical trial with the CB1 receptor antagonist/inverse agonist rimonabant for improving neurocognition in schizophrenia didn't reported clear positive results [172]. There are various clinical trials recruiting subjects with schizophrenia to test the utility of cannabidiol (ClinicalTrials.gov identifiers: NCT00588731, NCT00309413 and NCT00916201) and AVE1625 (a potent, selective CB1 antagonist. NCT00439634.)

Thus, future clinical investigations should describe the ECS status in medication-free samples and explore the therapeutic potentials of different ECS targets such as the degrading enzymes studied here, TRP channels, PPAR receptors and cannabinoid membrane transporters [158]. MAGL activity could be involved in the regulation of cognitive function [173]. Studies in high risk populations will allow determining if the described alterations in the ECS are present before the psychotic episode starts. This knowledge will have relevant implications to understand the physiopathology of psychosis and also for possible therapeutic implications.

It is worth mentioning as strength of these studies is that the diagnostic evaluation was performed with a very comprehensive protocol, with strict inclusion-exclusion criteria. This naturalistic design makes the sample much closer to the "real life" FEP population. Due to the heterogeneity of schizophrenia as a clinical entity, the FEP subgroup is of great interest because it avoids the effect of confounding variables, such as prolonged antipsychotic treatment or chronicity [10]. Another key feature of this study is that the age of inclusion is wider than in other previous works, including 23 subjects under 18 years old. Apart from this feature, clinical characteristics of the sample were similar to other studies with FEP in our context [11, 12]. In addition, complex statistical analyzes were conducted to limit biases in the results described.

7- LIMITATIONS

- We used a single control group of healthy subjects instead of using two control groups, one from healthy subjects and another including other psychiatric conditions, with the aim of controlling and thereby increasing our results specificity. So, the results here presented need to be confirmed in human samples from other neuropsychiatric disorders, such as depression or bipolar disorder.

- A possible limitation to explain the results reported in the first study would be that the observed changes might be related to stress perception. It is known that a stress component could be one of the factors implicated in the onset of the FEP [174]. Indeed, psychosocial and/or physical stress could contribute to the observed shift towards enhanced pro/anti-inflammatory signalling [47], showing the importance to asses stress biomarkers or tests. However, past and current complementary results show that the deregulation of the inflammatory balance is permanent (and even worse) in male inpatients in acute relapse phase with a history of schizophrenia of more than 10 years [56]. This evolution could suggest that the inflammatory process found is not only due to acute stress exposure in the beginning of FEP.

- Another limitation of our results is the systemic nature of the data. Although nowadays we are seeing a reformulation of the traditional conception of the psychotic illness [20], being considered as a heterogeneous disorder with a multisystemic impact from the onset in addition to its psychiatric expression [38], there is a need to corroborate our findings in brain areas related to schizophrenia. This could be useful to elucidate whether the alterations of these inflammatory risk/protection biomarkers could have etiological relevance and not only utility for diagnosis and monitoring the evolution of the disease. Recent studies compared the same immune biomarkers (cytokines such as IFNγ) in plasma and in post-mortem brain tissue from control and subjects with schizophrenia and found the same alterations in both compartments, validating the idea that schizophrenia can be investigated through studies of systemic biomarkers [175]. This model has been also supported by a recent review that suggested a link between peripheral inflammatory/immune processes and MRI detected anomalies in the brain of individuals with schizophrenia [162]. Furthermore, there are already studies in post-mortem brain tissue from

subjects with schizophrenia showing increased levels of some of the pro/anti-inflammatory and oxido/nitrosative stress markers studied here (NF-κB, COX-2, TBARS) [176, 177].

Similarly happens to the endocannabinoid system. Several studies in neuroinflammatory diseases (multiple sclerosis, Huntington's and Parkinson's diseases) have described ECS alterations in the CNS as well as in PBMC [81, 87], and signs of neuroinflammation can be detected preceding neuronal loss [161]. In these CNS pathologies, it can be expected that endocannabinoid concentrations in the circulation and brain are in equilibrium [81], being PBMC a mirror of the CNS endocannabinoid status [87].

- 81% of the FEP patients included in the study (74% at the 6 month follow up visit) were receiving atypical antipsychotic treatment. There is some evidence on the potential anti-inflammatory effects of antipsychotics [57, 58, 178], most of them at anti-cytokine level. Nevertheless, we have tried to control the possible confounding effect of antipsychotic treatment through a multiple linear regression analysis. We found no effects over the endocannabinoids system and only marginal effects on the plasma levels of TBARS in baseline visit ad inverse association between antipsychotic dose and the change in the plasma levels of PGE_2 and the direct association with 15d-PGJ_2. Increased lipid peroxidation has been found in early onset FEP psychosis, but the specific effects of antipsychotic medication were not addressed [179]. Thus, future clinical investigations should explore these biomarkers in medication-free samples [158].

- Similarly, a small group (8%) of patients required lithium. Although there are not clear references about lithium and inflammation, given the broad pharmacological effects of this compound, the possibility of being a confounding factor was also assessed. However, the result did not modify the association showed in this study.

- 16.8% of FEP subjects were diagnosed of affective disorders with psychotic features. This subgroup showed no statistically significant differences in any of the biomarkers compared to non-affective psychosis group.

- We could not measure all of the parameters in all the subjects (117 patients and 106 matched controls) taking part of this study. In general, for the parameters measured in plasma (i.e. the two prostaglandins) almost all subjects were used, but for the determinations made in the cytosolic/nuclear extracts of PBMC some

methodological limitations existed and the quantity of sample obtained is relatively low. With this limitation in mind we have tried to get a reasonable number of subjects for each parameter studied to carry out a reliable statistical analysis. In addition, we have checked if the sociodemographic characteristics were modified for this reason. No major changes were found between the differences in the whole sample and those in the subsets that were finally analyzed.

- PPARγ protein expression data was not chosen and kept together with the other markers selected because of its small sample size, although it was significant in its individual regression model controlled for all confounders. Keeping this data in mind we cannot discard a role for PPARγ as potential protective factor in FEP patients. In fact PPARγ expression and activity are significant in the two-tailed Chi-square tests on categorical data used to identify differences between baseline characteristics for patients and controls subjects, both in our study and in schizophrenia inpatients in acute relapse phase [56].

- Cannabis use is also related to immune alterations and constitutes a possible confounding factor that had to be controlled. Supporting this idea, our correlation analysis in the follow-up study results show how the cannabinoids use per month negatively correlates with the levels of the stable metabolites of NO, nitrites in plasma. This specific antioxidant profile of some of the cannabinoids present in the consumed preparations of *Cannabis Sativa*, such as cannabidiol, has been demonstrated in different neuropathological scenarios [180-182]. The biological relevance of the correlation here found needs to be explored, for example in a vascular context, where endocannabinoids actions are complex [183] and could be altered by exogenous cannabinoid use [184]. Moreover, cannabis use may play a confounding role when determining the peripheral expression of the different components of ECS. Repeated cannabis use in adolescence produces tolerance to cannabinoid-mediated effects, including brain cannabinoid receptors desensitization and downregulation [185]. The altered expression of CB1 and CB2 in cannabis smokers has also been described in PBMC [186]. In our study, we found no significant differences between FEP CAN+ vs. FEP CAN – subgroups. Bigger subgroups could have shown larger, statistically significant differences. Some peripheral endocannabinoids (AEA and oleoylethanolamide) levels are reduced in substance abusers without schizophrenia in comparison to non-abusing

schizophrenia subjects [187]. Along with other drug use disorders, cannabis use should be an important issue to manage in future research.

- The multiple linear regression analysis results of the follow-up study also support the idea that cigarette smoking can activate inflammatory pathways and may represent an important confounding factor. The differential effects of cigarettes consume in COX-2 levels in PBMCs and in the content of its main pro-inflammatory product PGE_2 in plasma should be carefully evaluated. COX-2 is a complex enzyme expressed both in brain and in PBMCs, capable to produce pro and anti-inflammatory mediators in different phases of its activity to resolve inflammation, depending of the nature and the level of the stimulus. It is possible that the increase in COX-2 levels observed was related to a correspondent rise in the levels of other anti-inflammatory products, such as 15d-PGJ_2, that modulates a massive production of PGE_2. In fact, the levels of 15d-PGJ_2 directly correlate with cotinine levels, although this correlation did not reach statistical significance ($p=0.225$). Indeed, it is well know that nicotine could activate COX-2 and PGE_2 synthesis in brain and other peripheral tissues[188, 189], but on the other hand, other authors have found that a lower dose of nicotine could be anti-inflammatory by the inhibition of proinflammatory mediators in human monocytes by suppression of NF-κB transcriptional activity through nicotinic acetylcholine receptor α7 [190]. Although there are no studies reporting nicotine effects on 15d-PGJ_2 levels, nicotine can up-regulate PPARγ in dendritic cells and in monocyte/macrophages from healthy smokers [191, 192]. Further research is warranted to elucidate the relationship between both anti-inflammatory pathways in physiological and pathological conditions.

- CB1 receptor expression was undetectable in PBMC. Although both receptors play a role to restore homeostasis mechanisms, CB1 receptors perform their function mainly in the CNS, while CB2 receptors do it mainly at the peripheral level [158].

8- CONCLUSIONS

- Systemic inflammatory conditions have been evidenced in patients diagnosed of FEP. Specifically, a significant increase in some intracellular components of a main pro-inflammatory pathway, together with a significant decrease in the anti-inflammatory ones have been identified. These results describe an imbalanced pro-inflammatory phenotype in FEP patients.

- Although more scientific evidence is needed, the determinations of multiple components of pro- and anti-inflammatory cellular pathways, including the activity of nuclear receptors, have interesting potential as biological markers and potential risk/protective factors for FEP.

- With the follow-up study, the existence of a deregulated systemic pro/anti-inflammatory balance in FEP, which becomes more severe during the initial period of time after the diagnosis of a FEP, has been corroborated. The results of the multivariate analysis applied show potential risk/protective factors in both time points studied (COX-2 protein levels in PBMC and 15d-PGJ2 plasma levels) that can be considered trait markers, and others specific of each one or state biomarkers (IκBα protein levels and NO_2^- and TBARS content in the cytosolic extract of PBMC).

- From a clinical point of view, the inverse correlation between the final product of oxidative/nitrosative cellular damage TBARS and the GAF scale found is especially relevant to justify the onset and development of antioxidant/anti-inflammatory therapeutic strategies not only for established schizophrenia but in earlier stages of a psychotic disorder.

- The multiple linear regression approach has shown how one of the targets of antipsychotic treatment is the restoration of the inflammatory balance.

- The endocannabinoid system (ECS), which under normal conditions is involved in restoring the homeostatic balance after neural stress, inflammation or cell damage, appears deregulated in PBMC of patients who had suffered a FEP.

- Continuous cannabis use could accentuate the malfunction of this endogenous protective system.

- Some of the peripheral components of the ECS could be used as biomarkers of the disorder.

- The pharmacological modulation of these of pro- and anti-inflammatory cellular pathways and of the ECS can be promising therapeutic targets to take into account in the future.

- It is necessary to control cigarette and cannabis use as confounding factors every time that immune/inflammatory and endocannabinoid system components alterations are reported in psychotic disorders, and possibly in other psychiatric pathologies too.

- Despite the limitations referred, this study has identified vulnerability conditions related to peripheral pro/anti-inflammatory pathways and of the ECS components in a very well characterized sample of FEP, very close to the "real-life" population of patients. Thus, the sample of patients was very homogeneous according to the time frame of inclusion, the phase of the illness and origin; the diagnostic evaluation was performed with a very comprehensive protocol and inclusion-exclusion criteria were applied in a strict manner; and these studies include a wide spectrum of biochemical inflammatory and endocannabinoid markers (both in plasma samples and peripheral blood mononuclear cells) allowing in-depth insights and relationships between multiple components, from the genes to the intracellular machinery (cytoplasmatic and nuclear), enzymes, proteins and soluble mediators and ending with the clinical features, following a translational design.

9- REFERENCES

1. Perala, J., et al., *Lifetime prevalence of psychotic and bipolar I disorders in a general population.* Arch Gen Psychiatry, 2007. **64**(1): p. 19-28.
2. van Os, J. and S. Kapur, *Schizophrenia.* Lancet, 2009. **374**(9690): p. 635-45.
3. Gordon, C.T., et al., *Childhood-onset schizophrenia: an NIMH study in progress.* Schizophr Bull, 1994. **20**(4): p. 697-712.
4. Edwards, J., et al., *Prolonged recovery in first-episode psychosis.* Br J Psychiatry Suppl, 1998. **172**(33): p. 107-16.
5. Linszen, D., P. Dingemans, and M. Lenior, *Early intervention and a five year follow up in young adults with a short duration of untreated psychosis: ethical implications.* Schizophr Res, 2001. **51**(1): p. 55-61.
6. Olesen, J., et al., *The economic cost of brain disorders in Europe.* Eur J Neurol, 2011. **19**(1): p. 155-162.
7. Huber, C.G., D. Naber, and M. Lambert, *Incomplete remission and treatment resistance in first-episode psychosis: definition, prevalence and predictors.* Expert Opin Pharmacother, 2008. **9**(12): p. 2027-38.
8. Alvarez-Jimenez, M., et al., *Preventing the Second Episode: A Systematic Review and Meta-analysis of Psychosocial and Pharmacological Trials in First-Episode psychosis.* Schizophr Bull, 2009.
9. Lieberman, J.A., et al., *Effectiveness of antipsychotic drugs in patients with chronic schizophrenia.* N Engl J Med, 2005. **353**(12): p. 1209-23.
10. Bernardo, M., et al., *Assessing clinical and functional outcomes in a gene-environment interaction study in first episode of psychosis (PEPs).* Rev Psiquiatr Salud Ment, 2013. **6**(1): p. 4-16.
11. Kahn, R.S., et al., *Effectiveness of antipsychotic drugs in first-episode schizophrenia and schizophreniform disorder: an open randomised clinical trial.* Lancet, 2008. **371**(9618): p. 1085-97.
12. Castro-Fornieles, J., et al., *Antipsychotic treatment in child and adolescent first-episode psychosis: a longitudinal naturalistic approach.* J Child Adolesc Psychopharmacol, 2008. **18**(4): p. 327-36.
13. Arango, C., et al., *Progressive brain changes in children and adolescents with first-episode psychosis.* Arch Gen Psychiatry, 2012. **69**(1): p. 16-26.
14. Tandon, R., H.A. Nasrallah, and M.S. Keshavan, *Schizophrenia, "just the facts" 4. Clinical features and conceptualization.* Schizophr Res, 2009. **110**(1-3): p. 1-23.
15. Kuehn, B.M., *Early interventions for schizophrenia aim to improve treatment outcomes.* JAMA, 2010. **304**(2): p. 139-40, 145.
16. McEvoy, J.P., et al., *Efficacy and tolerability of olanzapine, quetiapine, and risperidone in the treatment of early psychosis: a randomized, double-blind 52-week comparison.* Am J Psychiatry, 2007. **164**(7): p. 1050-60.
17. Bertelsen, M., et al., *Five-year follow-up of a randomized multicenter trial of intensive early intervention vs standard treatment for patients with a first episode of psychotic illness: the OPUS trial.* Arch Gen Psychiatry, 2008. **65**(7): p. 762-71.
18. Bertani, M., et al., *The influence of gender on clinical and social characteristics of patients at psychosis onset: a report from the Psychosis Incident Cohort Outcome Study (PICOS).* Psychol Med, 2011: p. 1-12.

19. Lewis, D.A. and G. Gonzalez-Burgos, *Pathophysiologically based treatment interventions in schizophrenia.* Nat Med, 2006. **12**(9): p. 1016-22.
20. Insel, T.R., *Rethinking schizophrenia.* Nature, 2010. **468**(7321): p. 187-93.
21. Waddington, J.L., et al., *Functional genomics and schizophrenia: endophenotypes and mutant models.* Psychiatr Clin North Am, 2007. **30**(3): p. 365-99.
22. Harrison, P.J. and M.J. Owen, *Genes for schizophrenia? Recent findings and their pathophysiological implications.* Lancet, 2003. **361**(9355): p. 417-9.
23. Sham, P.C., et al., *Age at onset, sex, and familial psychiatric morbidity in schizophrenia. Camberwell Collaborative Psychosis Study.* Br J Psychiatry, 1994. **165**(4): p. 466-73.
24. Owen, M.J., N. Craddock, and M.C. O'Donovan, *Suggestion of roles for both common and rare risk variants in genome-wide studies of schizophrenia.* Arch Gen Psychiatry, 2010. **67**(7): p. 667-73.
25. Allen, A.J., et al., *Endophenotypes in schizophrenia: a selective review.* Schizophr Res, 2009. **109**(1-3): p. 24-37.
26. van Os, J. and P. McGuffin, *Can the social environment cause schizophrenia?* Br J Psychiatry, 2003. **182**: p. 291-2.
27. van Os, J., et al., *Do urbanicity and familial liability coparticipate in causing psychosis?* Am J Psychiatry, 2003. **160**(3): p. 477-82.
28. van Os, J. and P. Delespaul, *Psychosis research at Maastricht University, The Netherlands.* Br J Psychiatry, 2003. **183**: p. 559-60.
29. Sanders, A.R., et al., *No significant association of 14 candidate genes with schizophrenia in a large European ancestry sample: implications for psychiatric genetics.* Am J Psychiatry, 2008. **165**(4): p. 497-506.
30. Cannon, M. and M.C. Clarke, *Risk for schizophrenia--broadening the concepts, pushing back the boundaries.* Schizophr Res, 2005. **79**(1): p. 5-13.
31. Torrey, E.F., J.J. Bartko, and R.H. Yolken, *Toxoplasma gondii and other risk factors for schizophrenia: an update.* Schizophr Bull, 2012. **38**(3): p. 642-7.
32. Matheson, S.L., et al., *A systematic meta-review grading the evidence for non-genetic risk factors and putative antecedents of schizophrenia.* Schizophr Res, 2011. **133**(1-3): p. 133-42.
33. Collip, D., I. Myin-Germeys, and J. Van Os, *Does the concept of "sensitization" provide a plausible mechanism for the putative link between the environment and schizophrenia?* Schizophr Bull, 2008. **34**(2): p. 220-5.
34. Caspi, A., et al., *Moderation of the effect of adolescent-onset cannabis use on adult psychosis by a functional polymorphism in the catechol-O-methyltransferase gene: longitudinal evidence of a gene X environment interaction.* Biol Psychiatry, 2005. **57**(10): p. 1117-27.
35. Rutter, M., *How the environment affects mental health.* Br J Psychiatry, 2005. **186**: p. 4-6.
36. Lataster, T., et al., *Childhood victimisation and developmental expression of non-clinical delusional ideation and hallucinatory experiences: victimisation and non-clinical psychotic experiences.* Soc Psychiatry Psychiatr Epidemiol, 2006. **41**(6): p. 423-8.
37. Leboyer M, M.-L.A., Stefanis N, Linszen D, Kahn R, van Os J, Wiersma D, Rutten BPF, Arango C, Jones P, Kapur S, Lewis S, Murray R, Owen MJ, *Schizophrenia aetiology: do gene-environment interactions hold the key?* Schizophr Res, 2008. **102**(1-3): p. 21-6.

38. Fernandez-Egea, E., et al., *Metabolic profile of antipsychotic-naive individuals with non-affective psychosis.* Br J Psychiatry, 2009. **194**(5): p. 434-8.
39. Meyer, U., M.J. Schwarz, and N. Muller, *Inflammatory processes in schizophrenia: a promising neuroimmunological target for the treatment of negative/cognitive symptoms and beyond.* Pharmacol Ther, 2011. **132**(1): p. 96-110.
40. Garcia-Rizo, C., et al., *Inflammatory markers in antipsychotic-naive patients with nonaffective psychosis and deficit vs. nondeficit features.* Psychiatry Res, 2012. **198**(2): p. 212-5.
41. Muller, N. and M.J. Schwarz, *A psychoneuroimmunological perspective to Emil Kraepelins dichotomy: schizophrenia and major depression as inflammatory CNS disorders.* Eur Arch Psychiatry Clin Neurosci, 2008. **258 Suppl 2**: p. 97-106.
42. Shi, J., et al., *Common variants on chromosome 6p22.1 are associated with schizophrenia.* Nature, 2009. **460**(7256): p. 753-7.
43. Benros, M.E., et al., *Autoimmune diseases and severe infections as risk factors for schizophrenia: a 30-year population-based register study.* Am J Psychiatry, 2011. **168**(12): p. 1303-10.
44. Stefansson, H., et al., *Common variants conferring risk of schizophrenia.* Nature, 2009. **460**(7256): p. 744-7.
45. Ripke, S., et al., *Genome-wide association analysis identifies 13 new risk loci for schizophrenia.* Nat Genet, 2013.
46. Miller, B.J., et al., *Meta-analysis of cytokine alterations in schizophrenia: clinical status and antipsychotic effects.* Biol Psychiatry, 2011. **70**(7): p. 663-71.
47. Garcia-Bueno, B., J.R. Caso, and J.C. Leza, *Stress as a neuroinflammatory condition in brain: damaging and protective mechanisms.* Neurosci Biobehav Rev, 2008. **32**(6): p. 1136-51.
48. Potvin, S., et al., *Inflammatory cytokine alterations in schizophrenia: a systematic quantitative review.* Biol Psychiatry, 2008. **63**(8): p. 801-8.
49. Steiner, J., et al., *Immunological aspects in the neurobiology of suicide: elevated microglial density in schizophrenia and depression is associated with suicide.* J Psychiatr Res, 2008. **42**(2): p. 151-7.
50. Saetre, P., et al., *Inflammation-related genes up-regulated in schizophrenia brains.* BMC Psychiatry, 2007. **7**: p. 46.
51. van Berckel, B.N., et al., *Microglia activation in recent-onset schizophrenia: a quantitative (R)-[11C]PK11195 positron emission tomography study.* Biol Psychiatry, 2008. **64**(9): p. 820-2.
52. Drexhage, R.C., et al., *Inflammatory gene expression in monocytes of patients with schizophrenia: overlap and difference with bipolar disorder. A study in naturalistically treated patients.* Int J Neuropsychopharmacol, 2010. **13**(10): p. 1369-81.
53. Kaiya, H., et al., *Elevated plasma prostaglandin E2 levels in schizophrenia.* J Neural Transm, 1989. **77**(1): p. 39-46.
54. Das, I. and N.S. Khan, *Increased arachidonic acid induced platelet chemiluminescence indicates cyclooxygenase overactivity in schizophrenic subjects.* Prostaglandins Leukot Essent Fatty Acids, 1998. **58**(3): p. 165-8.
55. Sommer, I.E., et al., *Nonsteroidal anti-inflammatory drugs in schizophrenia: ready for practice or a good start? A meta-analysis.* J Clin Psychiatry, 2011. **73**(4): p. 414-9.

56. Martinez-Gras, I., et al., *The anti-inflammatory prostaglandin 15d-PGJ2 and its nuclear receptor PPARgamma are decreased in schizophrenia.* Schizophr Res, 2011. **128**(1-3): p. 15-22.
57. Sugino, H., et al., *Atypical antipsychotics suppress production of proinflammatory cytokines and up-regulate interleukin-10 in lipopolysaccharide-treated mice.* Prog Neuropsychopharmacol Biol Psychiatry, 2009. **33**(2): p. 303-7.
58. Maes, M., et al., *Interleukin-2 and interleukin-6 in schizophrenia and mania: effects of neuroleptics and mood stabilizers.* J Psychiatr Res, 1995. **29**(2): p. 141-52.
59. Meyer, U., *Anti-inflammatory signaling in schizophrenia.* Brain Behav Immun, 2011. **25**(8): p. 1507-18.
60. Borovcanin, M., et al., *Elevated serum level of type-2 cytokine and low IL-17 in first episode psychosis and schizophrenia in relapse.* J Psychiatr Res, 2012. **46**(11): p. 1421-6.
61. Perkins, N.D., *Integrating cell-signalling pathways with NF-kappaB and IKK function.* Nat Rev Mol Cell Biol, 2007. **8**(1): p. 49-62.
62. Bierhaus, A., et al., *A mechanism converting psychosocial stress into mononuclear cell activation.* Proc Natl Acad Sci U S A, 2003. **100**(4): p. 1920-5.
63. Madrigal, J.L., et al., *The increase in TNF-alpha levels is implicated in NF-kappaB activation and inducible nitric oxide synthase expression in brain cortex after immobilization stress.* Neuropsychopharmacology, 2002. **26**(2): p. 155-63.
64. Middleton, G., et al., *Cytokine-induced nuclear factor kappa B activation promotes the survival of developing neurons.* J Cell Biol, 2000. **148**(2): p. 325-32.
65. Galea, E., et al., *Intrinsic regulation of brain inflammatory responses.* Cell Mol Neurobiol, 2003. **23**(4-5): p. 625-35.
66. Kapadia, R., J.H. Yi, and R. Vemuganti, *Mechanisms of anti-inflammatory and neuroprotective actions of PPAR-gamma agonists.* Front Biosci, 2008. **13**: p. 1813-26.
67. Garcia-Bueno, B., et al., *Peroxisome proliferator-activated receptor gamma activation decreases neuroinflammation in brain after stress in rats.* Biol Psychiatry, 2005. **57**(8): p. 885-94.
68. Feinstein, D.L., *Therapeutic potential of peroxisome proliferator-activated receptor agonists for neurological disease.* Diabetes Technol Ther, 2003. **5**(1): p. 67-73.
69. Forman, B.M., J. Chen, and R.M. Evans, *Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta.* Proc Natl Acad Sci U S A, 1997. **94**(9): p. 4312-7.
70. Heneka, M.T. and G.E. Landreth, *PPARs in the brain.* Biochim Biophys Acta, 2007. **1771**(8): p. 1031-45.
71. Fineberg, A.M. and L.M. Ellman, *Inflammatory cytokines and neurological and neurocognitive alterations in the course of schizophrenia.* Biol Psychiatry, 2013. **73**(10): p. 951-66.
72. Herberth, M., et al., *Identification of a molecular profile associated with immune status in first onset schizophrenia patients.* Clin Schizophr Relat Psychoses, 2013: p. 1-14.

73. van Venrooij, J.A., et al., *Impaired neuroendocrine and immune response to acute stress in medication-naive patients with a first episode of psychosis.* Schizophr Bull, 2010. **38**(2): p. 272-9.
74. Fraguas, D., et al., *Decreased glutathione levels predict loss of brain volume in children and adolescents with first-episode psychosis in a two-year longitudinal study.* Schizophr Res, 2012. **137**(1-3): p. 58-65.
75. Pasternak, O., et al., *Excessive extracellular volume reveals a neurodegenerative pattern in schizophrenia onset.* J Neurosci, 2012. **32**(48): p. 17365-72.
76. Flatow, J., P. Buckley, and B.J. Miller, *Meta-analysis of oxidative stress in schizophrenia.* Biol Psychiatry, 2013. **74**(6): p. 400-9.
77. Jones, B.G., F.A. Rose, and N. Tudball, *Lipid peroxidation and homocysteine induced toxicity.* Atherosclerosis, 1994. **105**(2): p. 165-70.
78. Brown, A.S., et al., *Elevated prenatal homocysteine levels as a risk factor for schizophrenia.* Arch Gen Psychiatry, 2007. **64**(1): p. 31-9.
79. Dietrich-Muszalska, A., et al., *The oxidative stress may be induced by the elevated homocysteine in schizophrenic patients.* Neurochem Res, 2012. **37**(5): p. 1057-62.
80. Di Marzo, V., *Targeting the endocannabinoid system: to enhance or reduce?* Nat Rev Drug Discov, 2008. **7**(5): p. 438-55.
81. Hillard, C.J., K.M. Weinlander, and K.L. Stuhr, *Contributions of endocannabinoid signaling to psychiatric disorders in humans: genetic and biochemical evidence.* Neuroscience, 2012. **204**: p. 207-29.
82. Hill, M.N., et al., *Endogenous cannabinoid signaling is essential for stress adaptation.* Proc Natl Acad Sci U S A, 2010. **107**(20): p. 9406-11.
83. Di Marzo, V., A. Ligresti, and L. Cristino, *The endocannabinoid system as a link between homoeostatic and hedonic pathways involved in energy balance regulation.* Int J Obes (Lond), 2009. **33 Suppl 2**: p. S18-24.
84. Klein, T.W. and G.A. Cabral, *Cannabinoid-induced immune suppression and modulation of antigen-presenting cells.* J Neuroimmune Pharmacol, 2006. **1**(1): p. 50-64.
85. Pacher, P. and R. Mechoulam, *Is lipid signaling through cannabinoid 2 receptors part of a protective system?* Prog Lipid Res, 2011. **50**(2): p. 193-211.
86. Wolf, S.A., S. Tauber, and O. Ullrich, *CNS immune surveillance and neuroinflammation: endocannabinoids keep control.* Curr Pharm Des, 2008. **14**(23): p. 2266-78.
87. Centonze, D., L. Battistini, and M. Maccarrone, *The endocannabinoid system in peripheral lymphocytes as a mirror of neuroinflammatory diseases.* Curr Pharm Des, 2008. **14**(23): p. 2370-42.
88. Zoppi, S., et al., *Regulatory role of cannabinoid receptor 1 in stress-induced excitotoxicity and neuroinflammation.* Neuropsychopharmacology, 2011. **36**(4): p. 805-18.
89. Mechoulam, R. and E. Shohami, *Endocannabinoids and traumatic brain injury.* Mol Neurobiol, 2007. **36**(1): p. 68-74.
90. Eggan, S.M., T. Hashimoto, and D.A. Lewis, *Reduced cortical cannabinoid 1 receptor messenger RNA and protein expression in schizophrenia.* Arch Gen Psychiatry, 2008. **65**(7): p. 772-84.
91. Ishiguro, H., et al., *Brain cannabinoid CB2 receptor in schizophrenia.* Biol Psychiatry, 2010. **67**(10): p. 974-82.
92. De Marchi, N., et al., *Endocannabinoid signalling in the blood of patients with schizophrenia.* Lipids Health Dis, 2003. **2**: p. 5.

93. Ortega-Alvaro, A., et al., *Deletion of CB2 cannabinoid receptor induces schizophrenia-related behaviors in mice.* Neuropsychopharmacology, 2011. **36**(7): p. 1489-504.
94. Leweke, F.M., et al., *Elevated endogenous cannabinoids in schizophrenia.* Neuroreport, 1999. **10**(8): p. 1665-9.
95. Giuffrida, A., et al., *Cerebrospinal anandamide levels are elevated in acute schizophrenia and are inversely correlated with psychotic symptoms.* Neuropsychopharmacology, 2004. **29**(11): p. 2108-14.
96. Muguruza, C., et al., *Quantification of endocannabinoids in postmortem brain of schizophrenic subjects.* Schizophr Res, 2013.
97. Volkow, N.D., *Substance use disorders in schizophrenia--clinical implications of comorbidity.* Schizophr Bull, 2009. **35**(3): p. 469-72.
98. Koskinen, J., et al., *Rate of Cannabis Use Disorders in Clinical Samples of Patients With Schizophrenia: A Meta-analysis.* Schizophr Bull, 2009.
99. Moore, T.H., et al., *Cannabis use and risk of psychotic or affective mental health outcomes: a systematic review.* Lancet, 2007. **370**(9584): p. 319-28.
100. Henquet, C., et al., *The environment and schizophrenia: the role of cannabis use.* Schizophr Bull, 2005. **31**(3): p. 608-12.
101. Sugranyes, G., et al., *Cannabis use and age of diagnosis of schizophrenia.* Eur Psychiatry, 2009.
102. Andreasson, S., et al., *Cannabis and schizophrenia. A longitudinal study of Swedish conscripts.* Lancet, 1987. **2**(8574): p. 1483-6.
103. Gonzalez-Pinto, A., et al., *Cannabis and First-Episode Psychosis: Different Long-term Outcomes Depending on Continued or Discontinued Use.* Schizophr Bull, 2009.
104. D'Souza, D.C., R.A. Sewell, and M. Ranganathan, *Cannabis and psychosis/schizophrenia: human studies.* Eur Arch Psychiatry Clin Neurosci, 2009. **259**(7): p. 413-31.
105. Gage, S.H., S. Zammit, and M. Hickman, *Stronger evidence is needed before accepting that cannabis plays an important role in the aetiology of schizophrenia in the population.* F1000 Med Rep, 2013. **5**: p. 2.
106. Murray, R.M., et al., *Cannabis, the mind and society: the hash realities.* Nat Rev Neurosci, 2007. **8**(11): p. 885-95.
107. O'Shea, M., I.S. McGregor, and P.E. Mallet, *Repeated cannabinoid exposure during perinatal, adolescent or early adult ages produces similar longlasting deficits in object recognition and reduced social interaction in rats.* J Psychopharmacol, 2006. **20**(5): p. 611-21.
108. Leweke, F.M., et al., *Anandamide levels in cerebrospinal fluid of first-episode schizophrenic patients: impact of cannabis use.* Schizophr Res, 2007. **94**(1-3): p. 29-36.
109. Bangalore, S.S., et al., *Cannabis use and brain structural alterations in first episode schizophrenia--a region of interest, voxel based morphometric study.* Schizophr Res, 2008. **99**(1-3): p. 1-6.
110. Ho, B.C., et al., *Cannabinoid receptor 1 gene polymorphisms and marijuana misuse interactions on white matter and cognitive deficits in schizophrenia.* Schizophr Res, 2011.
111. Pazos, M.R., et al., *Functional neuroanatomy of the endocannabinoid system.* Pharmacol Biochem Behav, 2005. **81**(2): p. 239-47.

112. Robbe, D., G. Alonso, and O.J. Manzoni, *Exogenous and endogenous cannabinoids control synaptic transmission in mice nucleus accumbens.* Ann N Y Acad Sci, 2003. **1003**: p. 212-25.
113. Kohn, Y. and B. Lerer, *Excitement and confusion on chromosome 6q: the challenges of neuropsychiatric genetics in microcosm.* Mol Psychiatry, 2005. **10**(12): p. 1062-73.
114. Keshavan, M.S., S. Anderson, and J.W. Pettegrew, *Is schizophrenia due to excessive synaptic pruning in the prefrontal cortex? The Feinberg hypothesis revisited.* J Psychiatr Res, 1994. **28**(3): p. 239-65.
115. American Psychiatric Association (Washington), *DSM-IV: diagnostic and statistical manual of mental disorders*. 4th ed. 1994, Washington, DC: American Psychiatric Association.
116. Hollingshead, A.B. and F.C. Redlich, eds. *Social Class and Mental Illness*. 1958, Wiley: New York.
117. American Psychiatric Association, *DSM-IV: diagnostic and statistical manual of mental disorders*. 4th ed. 1994, Washington, DC: American Psychiatric Association.
118. First, M., et al., eds. *Entrevista clínica estructurada para los trastornos del eje-I del DSM-IV*. 1999, Masson: Barcelona.
119. First, M., et al., eds. *SCID-II: guía del usuario para la entrevista clínica estructurada para los trastornos de la personalidad*. 1999, Masson: Barcelona.
120. Ulloa, R.E., et al., *[Interrater reliability of the Spanish version of Schedule for Affective Disorders and Schizophrenia for School-Age Children--Present and Lifetime version (K-SADS-PL)].* Actas Esp Psiquiatr, 2006. **34**(1): p. 36-40.
121. Kay, S.R., A. Fiszbein, and L.A. Opler, *The positive and negative syndrome scale (PANSS) for schizophrenia.* Schizophr Bull, 1987. **13**(2): p. 261-76.
122. Young, R.C., et al., *A rating scale for mania: reliability, validity and sensitivity.* Br J Psychiatry, 1978. **133**: p. 429-35.
123. Montgomery, S.A. and M. Asberg, *A new depression scale designed to be sensitive to change.* Br J Psychiatry, 1979. **134**: p. 382-9.
124. Endicott, J., et al., *The global assessment scale. A procedure for measuring overall severity of psychiatric disturbance.* Arch Gen Psychiatry, 1976. **33**(6): p. 766-71.
125. Shaffer, D., et al., *A children's global assessment scale (CGAS).* Arch Gen Psychiatry, 1983. **40**(11): p. 1228-31.
126. Gardner, D.M., et al., *International consensus study of antipsychotic dosing.* Am J Psychiatry, 2010. **167**(6): p. 686-93.
127. Kokkevi, A. and C. Hartgers, *EuropASI: European Adaptation of a Multidimensional Assessment Instrument for Drug and Alcohol Dependence.* Eur Addict Res, 1995. **1**(4): p. 208-210.
128. Schreiber, E., et al., *Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells.* Nucleic Acids Res, 1989. **17**(15): p. 6419.
129. Department of Health and the Medical Research Council. *Psygrid*. Available from: http://www.psygrid.org.
130. IBM Corp, *IBM SPSS Statistics for Windows*. 2011, IBM Corp
Armonk, NY.
131. Alvarez-Jimenez, M., et al., *Preventing the second episode: a systematic review and meta-analysis of psychosocial and pharmacological trials in first-episode psychosis.* Schizophr Bull, 2011. **37**(3): p. 619-30.

132. Kapur, S., A.G. Phillips, and T.R. Insel, *Why has it taken so long for biological psychiatry to develop clinical tests and what to do about it?* Mol Psychiatry, 2012. **17**(12): p. 1174-9.
133. Schultze-Lutter, F., et al., *Basic symptoms and the prediction of first-episode psychosis.* Curr Pharm Des, 2012. **18**(4): p. 351-7.
134. Xie, Q.W., Y. Kashiwabara, and C. Nathan, *Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase.* J Biol Chem, 1994. **269**(7): p. 4705-8.
135. Newton, R., et al., *Evidence for involvement of NF-kappaB in the transcriptional control of COX-2 gene expression by IL-1beta.* Biochem Biophys Res Commun, 1997. **237**(1): p. 28-32.
136. Song, X.Q., et al., *The interaction of nuclear factor-kappa B and cytokines is associated with schizophrenia.* Biol Psychiatry, 2009. **65**(6): p. 481-8.
137. Das, I., et al., *Elevated platelet calcium mobilization and nitric oxide synthase activity may reflect abnormalities in schizophrenic brain.* Biochem Biophys Res Commun, 1995. **212**(2): p. 375-80.
138. Yokota, O., et al., *Neuronal expression of cyclooxygenase-2, a pro-inflammatory protein, in the hippocampus of patients with schizophrenia.* Prog Neuropsychopharmacol Biol Psychiatry, 2004. **28**(4): p. 715-21.
139. Yao, J.K., S. Leonard, and R.D. Reddy, *Increased nitric oxide radicals in postmortem brain from patients with schizophrenia.* Schizophr Bull, 2004. **30**(4): p. 923-34.
140. Ghosh, N., R. Ghosh, and S.C. Mandal, *Antioxidant protection: A promising therapeutic intervention in neurodegenerative disease.* Free Radic Res, 2011. **45**(8): p. 888-905.
141. Lehmann, J.M., et al., *An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma).* J Biol Chem, 1995. **270**(22): p. 12953-6.
142. Garcia-Bueno, B., et al., *Effects of peroxisome proliferator-activated receptor gamma agonists on brain glucose and glutamate transporters after stress in rats.* Neuropsychopharmacology, 2007. **32**(6): p. 1251-60.
143. McIntyre, R.S., et al., *Managing psychiatric disorders with antidiabetic agents: translational research and treatment opportunities.* Expert Opin Pharmacother, 2006. **7**(10): p. 1305-21.
144. MacDowell, K.S., et al., *Risperidone normalizes increased inflammatory parameters and restores anti-inflammatory pathways in a model of neuroinflammation.* Int J Neuropsychopharmacol, 2013. **16**(1): p. 121-35.
145. Cheon, Y., et al., *Chronic olanzapine treatment decreases arachidonic acid turnover and prostaglandin E(2) concentration in rat brain.* J Neurochem, 2011. **119**(2): p. 364-76.
146. Adkins, D.E., et al., *SNP-based analysis of neuroactive ligand-receptor interaction pathways implicates PGE2 as a novel mediator of antipsychotic treatment response: data from the CATIE study.* Schizophr Res, 2012. **135**(1-3): p. 200-1.
147. Martinez-Cengotitabengoa, M., et al., *Cognitive impairment is related to oxidative stress and chemokine levels in first psychotic episodes.* Schizophr Res, 2012. **137**(1-3): p. 66-72.
148. Oresic, M., et al., *Phospholipids and insulin resistance in psychosis: a lipidomics study of twin pairs discordant for schizophrenia.* Genome Med, 2012. **4**(1): p. 1.

149. Erhardt, S., et al., *The kynurenic acid hypothesis of schizophrenia.* Physiol Behav, 2007. **92**(1-2): p. 203-9.
150. Ono, N., et al., *Influences of cyclooxygenase inhibitors on the cataleptic behavior induced by haloperidol in mice.* Prostaglandins Leukot Essent Fatty Acids, 1992. **46**(1): p. 59-63.
151. Condray, R. and J.K. Yao, *Cognition, dopamine and bioactive lipids in schizophrenia.* Front Biosci (Schol Ed), 2011. **3**: p. 298-330.
152. Salehi-Sadaghiani, M., et al., *NMDA receptor involvement in antidepressant-like effect of pioglitazone in the forced swimming test in mice.* Psychopharmacology (Berl), 2012. **223**(3): p. 345-55.
153. Almasi-Nasrabadi, M., et al., *Involvement of NMDA receptors in the beneficial effects of pioglitazone on scopolamine-induced memory impairment in mice.* Behav Brain Res, 2012. **231**(1): p. 138-45.
154. Stone, J.M. and L.S. Pilowsky, *Novel targets for drugs in schizophrenia.* CNS Neurol Disord Drug Targets, 2007. **6**(4): p. 265-72.
155. Rolland, B., et al., *Therapeutic Prospects of PPARs in Psychiatric Disorders: A Comprehensive Review.* Curr Drug Targets, 2013. **14**(7): p. 724-32.
156. Yi, Z., et al., *Rosiglitazone and cognitive function in clozapine-treated patients with schizophrenia: a pilot study.* Psychiatry Res, 2012. **200**(2-3): p. 79-82.
157. Zamberletti, E., T. Rubino, and D. Parolaro, *The endocannabinoid system and schizophrenia: integration of evidence.* Curr Pharm Des, 2012. **18**(32): p. 4980-90.
158. Giuffrida, A. and A. Seillier, *New insights on endocannabinoid transmission in psychomotor disorders.* Prog Neuropsychopharmacol Biol Psychiatry, 2012.
159. Koethe, D., et al., *Anandamide elevation in cerebrospinal fluid in initial prodromal states of psychosis.* Br J Psychiatry, 2009. **194**(4): p. 371-2.
160. Perry, V.H., C. Cunningham, and C. Holmes, *Systemic infections and inflammation affect chronic neurodegeneration.* Nat Rev Immunol, 2007. **7**(2): p. 161-7.
161. McGeer, P.L., et al., *Rate of cell death in parkinsonism indicates active neuropathological process.* Ann Neurol, 1988. **24**(4): p. 574-6.
162. Frodl, T. and F. Amico, *Is there an association between peripheral immune markers and structural/functional neuroimaging findings?* Prog Neuropsychopharmacol Biol Psychiatry, 2013.
163. Schwarz, E., et al., *Identification of a biological signature for schizophrenia in serum.* Mol Psychiatry, 2012. **17**(5): p. 494-502.
164. Rollins, B., et al., *Analysis of whole genome biomarker expression in blood and brain.* Am J Med Genet B Neuropsychiatr Genet, 2010. **153B**(4): p. 919-36.
165. Battista, N., et al., *The role of endocannabinoids in gonadal function and fertility along the evolutionary axis.* Mol Cell Endocrinol, 2012. **355**(1): p. 1-14.
166. Lazzarin, N., et al., *Fluctuations of fatty acid amide hydrolase and anandamide levels during the human ovulatory cycle.* Gynecol Endocrinol, 2004. **18**(4): p. 212-8.
167. Aleman, A., R.S. Kahn, and J.P. Selten, *Sex differences in the risk of schizophrenia: evidence from meta-analysis.* Arch Gen Psychiatry, 2003. **60**(6): p. 565-71.
168. McGrath, J., et al., *A systematic review of the incidence of schizophrenia: the distribution of rates and the influence of sex, urbanicity, migrant status and methodology.* BMC Med, 2004. **2**: p. 13.

169. Leweke, F.M., et al., *Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia.* Transl Psychiatry, 2012. **2**: p. e94.
170. Beltramo, M., et al., *Reversal of dopamine D(2) receptor responses by an anandamide transport inhibitor.* J Neurosci, 2000. **20**(9): p. 3401-7.
171. Seillier, A., et al., *Inhibition of fatty-acid amide hydrolase and CB1 receptor antagonism differentially affect behavioural responses in normal and PCP-treated rats.* Int J Neuropsychopharmacol, 2010. **13**(3): p. 373-86.
172. Boggs, D.L., et al., *Rimonabant for neurocognition in schizophrenia: a 16-week double blind randomized placebo controlled trial.* Schizophr Res, 2012. **134**(2-3): p. 207-10.
173. Chanda, P.K., et al., *Monoacylglycerol lipase activity is a critical modulator of the tone and integrity of the endocannabinoid system.* Mol Pharmacol, 2010. **78**(6): p. 996-1003.
174. Holtzman, C.W., et al., *Stress and neurodevelopmental processes in the emergence of psychosis.* Neuroscience, 2013.
175. Harris, L.W., et al., *Comparison of peripheral and central schizophrenia biomarker profiles.* PLoS One, 2012. **7**(10): p. e46368.
176. Rao, J.S., et al., *Increased neuroinflammatory and arachidonic acid cascade markers, and reduced synaptic proteins, in the postmortem frontal cortex from schizophrenia patients.* Schizophr Res, 2013. **147**(1): p. 24-31.
177. Tang, J., et al., *Decrease in temporal gyrus gray matter volume in first-episode, early onset schizophrenia: an MRI study.* PLoS One, 2012. **7**(7): p. e40247.
178. Maes, M., et al., *Lower plasma CC16, a natural anti-inflammatory protein, and increased plasma interleukin-1 receptor antagonist in schizophrenia: effects of antipsychotic drugs.* Schizophr Res, 1996. **21**(1): p. 39-50.
179. Mico, J.A., et al., *Reduced antioxidant defense in early onset first-episode psychosis: a case-control study.* BMC Psychiatry, 2011. **11**: p. 26.
180. Ruiz-Valdepenas, L., et al., *Cannabidiol reduces lipopolysaccharide-induced vascular changes and inflammation in the mouse brain: an intravital microscopy study.* J Neuroinflammation, 2011. **8**(1): p. 5.
181. Esposito, G., et al., *Cannabidiol inhibits inducible nitric oxide synthase protein expression and nitric oxide production in beta-amyloid stimulated PC12 neurons through p38 MAP kinase and NF-kappaB involvement.* Neurosci Lett, 2006. **399**(1-2): p. 91-5.
182. Esposito, G., et al., *Cannabidiol in vivo blunts beta-amyloid induced neuroinflammation by suppressing IL-1beta and iNOS expression.* Br J Pharmacol, 2007. **151**(8): p. 1272-9.
183. Randall, M.D., D.A. Kendall, and S. O'Sullivan, *The complexities of the cardiovascular actions of cannabinoids.* Br J Pharmacol, 2004. **142**(1): p. 20-6.
184. Pacher, P. and S. Steffens, *The emerging role of the endocannabinoid system in cardiovascular disease.* Semin Immunopathol, 2009. **31**(1): p. 63-77.
185. Lazenka, M.F., D.E. Selley, and L.J. Sim-Selley, *Brain regional differences in CB1 receptor adaptation and regulation of transcription.* Life Sci, 2012.
186. Nong, L., et al., *Altered cannabinoid receptor mRNA expression in peripheral blood mononuclear cells from marijuana smokers.* J Neuroimmunol, 2002. **127**(1-2): p. 169-76.
187. Desfosses, J., et al., *Plasma Endocannabinoid Alterations in Individuals with Substance Use Disorder are Dependent on the "Mirror Effect" of Schizophrenia.* Front Psychiatry, 2012. **3**: p. 85.

188. De Simone, R., et al., *Activation of alpha7 nicotinic acetylcholine receptor by nicotine selectively up-regulates cyclooxygenase-2 and prostaglandin E2 in rat microglial cultures.* J Neuroinflammation, 2005. **2**(1): p. 4.
189. Toledano, A., M.I. Alvarez, and A. Toledano-Diaz, *Diversity and variability of the effects of nicotine on different cortical regions of the brain - therapeutic and toxicological implications.* Cent Nerv Syst Agents Med Chem, 2010. **10**(3): p. 180-206.
190. Yoshikawa, H., et al., *Nicotine inhibits the production of proinflammatory mediators in human monocytes by suppression of I-kappaB phosphorylation and nuclear factor-kappaB transcriptional activity through nicotinic acetylcholine receptor alpha7.* Clin Exp Immunol, 2006. **146**(1): p. 116-23.
191. Yanagita, M., et al., *Nicotine modulates the immunological function of dendritic cells through peroxisome proliferator-activated receptor-gamma upregulation.* Cell Immunol, 2012. **274**(1-2): p. 26-33.
192. Amoruso, A., et al., *Quantification of PPAR-gamma protein in monocyte/macrophages from healthy smokers and non-smokers: a possible direct effect of nicotine.* Life Sci, 2007. **81**(11): p. 906-15.