Insulin-like growth factor binding protein 2 in bipolar disorder: An expression study in peripheral tissues

TITLE: ***Insulin-like Growth Factor Binding Protein 2 in bipolar disorder: an expression study in peripheral tissues***

RUNNING TITLE: ***IGFBP-2 expression in Bipolar Disorder***

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ABSTRACT

OBJECTIVE: This study aimed to investigate the putative role of Insulin like Growth Factor Binding Protein 2 (IGFBP2) in Bipolar Disorder (BD) pathogenesis and as a peripheral biomarker for the differential diagnosis in mood disorders. IGFBP2 is a member of the family of high-affinity binding proteins (IGFBP1-6) and appears to demonstrate a governing role in Insulin like Growth Factor (IGF) regulation in the central nervous system.

METHODS: IGFBP2protein and mRNA levels were measured respectively in serum of 93 controls, 41BD and 43 Major Depressive Disorder (MDD) patients and in skin fibroblasts from 15 controls, 12 BD and 23 MDD patients.

RESULTS: The results indicated a lower expression of IGFBP2 in both tissues of BD patients, whereas no difference was found in MDD patients compared to controls.

CONCLUSION: Our findings in peripheral tissues are in line with previous results in the brain and support a downregulation of the IGFBP2 expression in BD specific for this disorder, suggesting the potential usefulness of this marker for the differential diagnosis. Further studies in independent and wider cohorts are warranted to confirm the role of IGFBP2 in BD.

KEYWORDS: IGFBP2, Bipolar Disorder, Fibroblasts, Major Depressive Disorder, Serum.

Introduction

Bipolar disorder (BD) is a complex psychiatric illness characterizedby episodes of depression, mania, or mixed state that typically recur and become more frequent across the life span. The aetiology of BD is still not clarified and consistent findings evidenced an impairment in neurodevelopment and neuroplasticity mechanisms that may lead to brain structural alterations (Machado-Vieira et al. 2014).

A number of findings, coming from different experimental settings, indicated alterations of neurotrophins and growth factors levels in BD such as brain-derived neurotrophic factor(BDNF), vascular endothelial growth factor (VEGF), nerve growth factor(NGF) and also insulin like growth factor-1 (IGF-1)(Scola and Andreazza 2015).

With regard to IGF-1, increased serum levels of this factor have been observed in BD patients (Kim et al. 2013; Liu et al. 2014a)and transcriptomic studies on lymphoblastoid cell lines reported that this growth factor may play a role in response to lithium, a mood stabilizer frequently employed as first-line in the treatment of this disorder (Squassina et al. 2013; Milanesi et al. 2015). Furthermore, studies in post-mortem brains of BD patients indicated a reduction of Insulin like Growth factor Binding Protein 2 (IGFBP2)(Bezchlibnyk et al. 2007).

The insulin-like growth factor (IGF) system, comprising 2 growth factors (IGF-1 and IGF-2), 4 receptors and 6 high-affinity binding proteins (IGFBP1 to IGFBP6), is involved in the regulation of

cellular proliferation, differentiation, and apoptosis and accumulating observations reported its involvement in cancer progression, including DNA mutations, cell proliferation and migration, resistance to therapy, and poor prognosis (Yao et al. 2015). In the central nervous system (CNS) the IGF axis plays an important role in neurodevelopment, neuroprotection, neurogenesis and synaptogenesis mechanisms (Chen et al. 2011).

IGFs bind to the IGF receptor 1 (IGF1R) on the cell surface, the IGF receptor type widely expressed in the brain (Pereira et al. 2011). Through this binding the IGF-1signalling cascade activates the PI3K and ras/MAPK pathways, known to regulate neuronal survival and growth during development and functions in adult neurons, as well as to modulate synaptic transmission and plasticity (Machado-Vieira et al. 2009).Although IGF-1 is primarily produced by the liver in response to growth hormone (GH) and affects a wide variety of cell types, its effect on early CNS development and neuronal plasticity suggests that IGF-1 acts as an endocrine, paracrine and autocrine hormone (Yakar et al. 1999).

IGFBPs are produced in a wide variety of cell types including liver, muscle, connective tissue, bone, brain, intestine, ovary, and kidney(Reindl and Sheridan 2012). IGFBPs bind the IGFs with high affinity and transport the growth factors to their site of action or sequester them within tissues, thereby regulating their bioavailability(Leventhal et al. 1999). Thus, differences in expression of IGFBPs may influence the activity of the IGF system. The IGFBP2 is the most abundant IGF binding protein in the brain where it is secreted by neuronal and glial cells(Aizenman and de Vellis 1987) and is the second most abundant in the peripheral blood, next to IGFBP3(Firth and Baxter 2002).

The identification of new diagnostic and assessment biomarkers in psychiatry is needed. In particular, one of the major issues in mood disorders concerns the possibility to differentiate MD from BD, since these pathologies show overlapping symptoms and can be misdiagnosed (Hirschfeld 2013). A correct differential diagnosis of MDD or BD is crucial for an appropriate treatment from the onset and, in turn, for successful treatment outcomes. Identification of biomarkers that can

reflect MDD- and BD-specific pathophysiologic processes, may be used to define early interventions and to develop personalized treatment.

Serum and plasma protein analyses have been performed in several psychiatric disorders to study the pathogenetic mechanisms and to identify biomarkers, easily measurable in vivo and relevant for clinical purposes. However, studies in peripheral blood are limited by the presence of confounding factors such as diet, smoking habits and medications (Kumarasinghe et al. 2012). These limitations might be partially overcome using other tissues such as skin fibroblasts, that recently have been proposed as a suitable model for the investigation of the molecular mechanisms underpinning neurodegenerative and psychiatric disorders (Cattane et al. 2015; Garbett et al. 2015; Kálmán et al. 2016). Indeed, fibroblasts show expression profiles that are in part similar to those of brain tissues and they are relatively easy to obtain from patients through a skin biopsy and can be maintained in culture in a controlled and reproducible environment(Garbett et al. 2015).

Aims of the study

The aim of the present study was to evaluate the protein levels of IGFBP2 in serum and mRNA levels in fibroblast cell cultures from BD patients and matched controls and to compare these findings with those obtained in patients affected by Major Depressive Disorder (MDD) in order to investigate the putative role of this protein in the pathogenesis and its potentiality as biomarker for the differential diagnosis between unipolar and bipolar depression.

Material and methods

Serum study

Subjects forserumIGFBP2 analyses

Participants involved in this study were Caucasians living in Northern Italy. Patients and controls were recruited by the Psychiatry Units of IRCCS Centro S. Giovanni di Dio FBF, Brescia, Italy and

the Department of Neuroscience, University of Torino, Italy. All subjects provided written informed consent form, approved by the local Ethics Committees.

Patients fulfilled the following inclusion criteria: (a) a principal diagnosis of BD type I or II or MDD according to the DSM-IV-TR criteria; (b) at least 18 years of age. The exclusion criteria were: (a) current or previous diagnosis of organic mental disorder, schizophrenia or other psychotic disorder, (b) current alcohol and/or substance-related disorders, (c) current eating disorders, (d) uncontrolled or serious medical condition, (e) pregnancy or postpartum period. All the diagnoses were confirmed by using of the Structured Clinical Interview for DSM Axis I Disorders (SCID-I). For the study on serum 43 MDD and 41 BD patients were recruited. Thirty-five BD patients were in a depressive phase and 5 in mania at the time of enrolment. Severity of the depressive symptoms in MDD and BD patients (in the depressive state) was evaluated using the 17-item Hamilton Rating Scale for Depression (HAMD). The mean HAMD scores in MDD and BD patients were 23.00 ± 3.52 and 23.48 ± 4.08 respectively.

At the moment of blood sampling all 43 MDD patients were drug free from antidepressants and 24 out of them were drug naïve. Thirty-seven BD patients out of 41 were drug free from antidepressants. Eighteen BD patients were in lithium monotherapy, 12 in monotherapy with valproate and 3 were taking both lithium and valproate; one patient was in treatment with lithium and antidepressant and one with valproate, antidepressants and antipsychotics; one patient was taking only antipsychotics, whereas other 3 patients received a combined therapy with antipsychotics plus valproate, lithium and antidepressants, respectively. One patient was in treatment with valproate and antidepressants and one was drug free.

A control group of 93 unrelated volunteers was enrolled. None of these subjects presented a positive personal and familial anamnesis for psychiatric DSM-IV-TR axis I disorders according to the MINI clinical interview(Sheehan et al. 1998).

The socio-demographical and clinical features of patient and control samples are reported in Table

1.

IGFBP-2 protein serum analysis

Venous blood samples for each study participant were drawn in the morning in anticoagulant-free tubes after an overnight fasting. Blood samples were allowed to clot at room temperature for 1 h followed by 1 hr at 4°C. Serum was separated by centrifugation at 1620 x g for 15 min and then stored at −80°C until the time of assay. IGFBP-2 levels were measured by the ELISA method using the human IGFBP-2 Quantikine kit (R&D system, Minneapolis, USA), according to the manufacturer's instructions. The minimum detectable dose of human IGFBP-2 was 0.04 ng/mL, and data were expressed as ng of protein/mL of serum. All samples and standards were measured in duplicate. ELISA plate templates were designed to analyze together samples of different groups in order to control the variability between different assays. The mean inter-assay precision, expressed as the coefficient of variation, was 4.2%.

Fibroblast cell study

Subjects for fibroblast *IGFBP2* mRNA levels analysis

Fifty individuals of Caucasian origin living in Northern Italy, including 15 healthy controls, 23 MDD and 12 BD patients, were enrolled in the study by the Psychiatry Unit of IRCCS Centro S. Giovanni di Dio FBF, Brescia Italy. All the individuals provided written informed consent form, approved by the local Ethics Committee. The inclusion/exclusion criteria were the same described for serum samples; DSM-IV MDD patients with at least moderately severe depression were enrolled in the study. Among the BD patients, 5 were in depressive phase, 6 in mania and 1 patient in mix episode phase. The features of the cohorts are shown in Table 1.

Collection of skin biopsies, establishment of fibroblast cultures and RNA extraction

Specimens for fibroblast cultures were collected by skin punch biopsies ($3mm^2$), taken from the scapular region, under local anesthesia using aseptic conditions and immediately immersed in a saline solution (PBS). All primary cultures were grown in Eagle's Minimum Essential Medium

(MEM; Life Technologies, City, State) supplemented with 10% of fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100µg/ml), non-essential amino acids (1% v/v) and glutamine (1% v/v) under optimal conditions (37° C, 5% CO_2). The medium was changed every 3 days. When fibroblast outgrowth reached confluence, cells were split into larger culture dishes or were frozen using 20% FBS and 10% DMSO. All fibroblasts used for experiments were cultured till the 5th passage in order to minimize any effect from exposure in vivo to possible subject's confounding factors such as diet, smoking or drugs (Akin et al. 2004).

Total RNA was isolated by single-step extraction using TRIzol Reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, (Life Technologies), according with the manufacturer's instructions. Subsequent RNA clean-up was performed using RNeasy mini kit (QIAGEN) to obtain high quality RNA. Purity of each sample was determined by the A260:280 ratio, with acceptable values ranging from 1.8 to 2.2.

***IGFBP2* fibroblast gene expression analysis.**

The *IGFBP2* RNA expression levels were analyzed using Applied Biosystems 7500 real-time PCR system (Life Technologies, Foster City, CA, USA). PCR was carried out using TaqMan Universal PCR Master Mix (Life Technologies). 20 ng of cDNA were used in each real-time PCR reaction in a final volume of 20 µl (TaqMan Gene Expression Assay; Life Technologies).

The expression of *IGFBP2* mRNA (IGFBP2 assay: Hs00167151_m1) in the patient sample groups, in comparison with the control group, was calculated as described by Pfaffel and Colleagues(Pfaffl 2001), using reference genes chosen from a set of housekeeping genes (GAPDH assay: Hs99999905_m1; β-Act assay: Hs99999903_m1; α-Tub assay: Hs00362387_m1; Cyc-1 assay: Hs00357717_m1; ATP5B assay: Hs00969569_m1; B2m assay: Hs99999907_m1). The stability of reference genes was evaluated by geNorm software. The underlying principles and formulas are described in Vandesompele et al. (Vandesompele et al. 2002). Our analysis indicated that the most appropriate reference genes in skin fibroblasts are GAPDH, Cyc-1, and ATP5B. Data were

normalize on the geometric average of these reference genes. Each individual determination was repeated in duplicate.

Statistical analysis

Categorical variables were tested by means of Chi-square test and ANOVA analysis of variance was used to compare quantitative variables and the mean values of IGFBP2 serum concentrations and fibroblasts expression levels between controls and BD/MDD patients. Post hoc tests with Bonferroni correction were applied to analyse significant differences between groups. Pearson correlation analyses were performed to assess associations between the demographic and morphometric variables (BMI, sex and age) and IGFBP2. Data were analysed using the Statistical Package for Social Sciences, Version 17.0 (SPSS Inc).

RESULTS

IGFBP-2 serum levels

Sex distribution was significantly different between patient and control groups ($p<0.01$), while BMI index was different between BD patients and controls ($p= 0.026$). IGFBP-2 serum levels were inversely correlated with BMI in the whole sample ($r= -0.218$ $p=0.007$). IGFBP-2 serum levels in ng/ml ± SD were 173.24 ± 77.95 in BD; 225.82 ± 129,11 in MDD and 232,90 ± 125,48 in controls (Fig.1). ANOVA analysis indicated a significant difference in IGFBP-2 concentrations among the groups ($F=3.83$, $p=0.024$). Post hoc analyses indicated a decrease of the protein levels in BD patients ($p=0.022$) while MDD patients showed serum levels similar to controls ($p=0.124$, $F=3.83$) (Fig.1). Because 36 out of 41 BD patients were in depressive phase and only 5 in maniac phase, we repeated the analysis excluding patients in mania and the IGFBP-2 reduction remained significant ($p=0.037$). The IGFBP-2 decrease in BD patients was confirmed also after adjusting for BMI and

gender differences between groups. As 18 BD patients enrolled in our study for serum analysis were receiving lithium treatment, we compared their IGFBP2 level with those of the 12 patients treated with valproate and we did not observe a statistically significant difference (p=0.937).

IGFBP2 mRNA levels in fibroblasts

The three groups were homogeneous for sex and BMI, but not for age (Table 2). No correlation was found between *IGFBP2* mRNA levels in fibroblasts and age (r=-0.065, p=0.655) or BMI (r=0.039, p=0.82). ANOVA analyses showed an overall p-value of 0.001 (F=7.97) and Bonferroni's multiple comparison post-hoc tests showed decreased expression levels in BD patients compared to controls (FC=-5.39; p=0.001) and MDD patients (p=0.025). No difference was observed comparing MDD patients versus controls (FC=-1.3 ,p=0.334; Fig.2).

As the age was different between BD and control groups, *IGFBP2* mRNA levels analysis was corrected for this variable. Repeating the ANCOVA correction for both age and BMI the p-value remained significant (p=0.002).

Discussion

Our study aimed to contribute to the current understanding of the role of IGF system in mood disorders, by measuring IGFBP2 protein and mRNA levels in serum and biopsied fibroblasts grown in vitro, respectively, from controls, BD and MDD patients.

Our findings show, for the first time, decreased levels of IGFBP2 in serum samles of BD patients compared to controls, whereas no difference was found in serum samples from MDD patients. In serum, about 75–80% of the IGFs binds IGFBP-3 and a smaller percentage (20–25%) is associated with low molecular mass IGFBPs such as IGFBP2, while less than 1% are f in the free (unbound) form (Hasegawa et al. 1996). Clinical studies indicated that, in contrast to IGFBP-1, IGFBP-2 levels in serum are stable and not influenced by postprandial changes (Blum et al. 1993).

Regarding possible effects of psychotropic treatment on patient serum IGFBP2 levels it must be

noted that a dose-dependent relationship between *IGFBP2* mRNA and protein levels and lithium concentration was reported in primary cultures of rat cortical neurons (Bezchlibnyk et al. 2006). Because 18 BD patients enrolled in our study for serum analysis were undergoing lithium treatment, we compared their *IGFBP2* mRNA levels with those of the 12 patients treated with valproate and we did not observe significant differences between the two groups.

Our data in serum are corroborated by the findings in fibroblasts that confirmed a decrease in *IGFBP2* expression specifically in BD patient fibroblasts, but not in MDD patients. This corroboration is important, as confounding factors, such as diet, lifestyles, smoking habit and drug treatment with mood stabilizers may be virtually eliminated in this in vitro model after several cycles of cell division(Hayashi-Takagi et al. 2014). Thus, our observations support again the utility of biopsied fibroblasts as peripheral model for investigating the molecular mechanisms underpinning psychiatric disorders. Furthermore, the decrease of IGFBP2 in both the patients peripheral tissues are in line with previous brain postmortem studies showing a decreased *IGFBP2* mRNA expression in the Prefrontal Cortex (Brodmann's area 9 and 42) of BD patients, whereas no differences it its expression were observed in post-mortem brain tissues between MDD patients and controls (Bezchlibnyk et al. 2007).

The IGF system may be involved in the BD pathogenesis and lithium response (Squassina et al. 2013; Milanesi et al. 2015). Previous studies conducted in BD patients have shown increased serum levels of IGF-1 (Kim et al. 2013; Liu et al. 2014b), and moreover a genome-wide expression profiling microarray study of BD lymphoblastoid cell lines (LCLs) reported that IGF1 was the top gene showing higher expression in LCLs derived from from good versus poor lithium responders (Squassina et al 2013). These observations suggest that IGF-1 may play a crucial role in the pathophysiology of BD, although another study did not find significant alteration of this growth factor in plasma of BD patients at the illness onset (Palomino et al. 2013). Notably, IGFBP-2 is the main IGFs carrier protein in CNS (Collett-Solberg and Cohen 1996), it binds IGF-1 and prevents it

from activating the IGF-1 receptor (IGF-1R) and initiating intracellular signaling cascades, most notably the PI3K/Akt and ERK pathways (Chesik et al.; Aberg et al. 2006) that mediate neuroprotection. Indeed, IGF-1 is able to increase progenitor cell proliferation and new neurons, oligodendrocytes, and blood vessels in the dentate gyrus of the hippocampus. The MAPK signaling pathway is needed for IGF-1–stimulated proliferation in vitro, whereas the PI3K/Akt and MAPK/Erk signaling pathways appear to mediate the antiapoptotic effects of this growth factor (Aberg et al. 2006). The mechanism by which IGFBP-2 executes its function in the regulation of IGF-1 is not clear and data in vitro and in vivo have shown both inhibitory and stimulatory roles of IGFBP-2 on IGF-1 action (Chesik et al.). IGFBPs in general regulate biological activities of IGFs not only by acting as a transport carrier proteins of IGFs, but also by regulating IGFs metabolic clearance, protecting it from degradation by peptidases, and interactions of IGFs with their receptors (Firth and Baxter 2002). These evidences together support the hypothesis that alteration in *IGFBP2* expression might be involved in neurodevelopment abnormalities. Indeed, it has been suggested that IGFBPs may promote the neuroprotective action of IGFs in certain conditions by the binding of extracellular matrix to components of the cellular matrix thereby enhancing IGF bioavailability to cell surface receptors .(Bezchlibnyk et al. 2007)

In addition, many extracellular factor linked to inflammatory responses such as IL-1, GH and TGF-beta are known to be correlated to *IGFBP2* expression. In this context, some observations suggested that IGFBP-2 may be an important factor for the injury/repair processes during the progression of inflammation (Chadelat et al. 1998); moreover IGFBP-1 and IGFBP-2 may also act as proinflammatory factors and are linked to the HIV pathogenic activity (Haugaard et al. 2004) and their CSF concentrations correlate with inflammatory mediators in HIV-positive individuals (Suh et al. 2015). Finally, chronic LPS administration in rats, a model for inflammation, significantly increased circulating IGFBP-3, IGFBP-1 and IGFBP-2 (Soto et al. 1998). It could be that alterations of IGFBP-2 levels are related to immune dysregulation and inflammations that play an important role in the pathogenesis of bipolar disorder (Rege and Hodgkinson 2013).

While our observations suggest that low serum IGFBP-2 levels may serve as a tentative BD biomarker, they do not explain by which mechanism IGFBP-2 is involved in BD pathophysiology: it is possible that IGFBP-2 acts by an IGF-independent pathway or it may be that a compensatory down-regulation of its expression occurs in an attempt to normalize IGF-1 bioavailability to CNS neurons. Moreover, bipolar patients were in treatment at the time of the blood sampling. Thus it is not possible to exclude an effect of the drug treatment on IGFBP2 levels in serum.

Further studies are needed to confirm this finding and to clarify the mechanism by which altered IGFBP-2 expression is implicated in BD.

Declaration of interest.

The authors declare no conflict of interest

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Legend:

IGFBP2, Insulin-like Growth Factor Binding Protein 2; BD, Bipolar Disorder; MDD, Major Depressive Disorder; SE, standard error; RT-qPCR, Quantitative reverse transcription PCR; CTRL, controls.

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Table 1 Clinical and socio-demographical features of individuals involved in serum and fibroblasts analysis

	Serum cohort (n=175)			**Fibroblasts cohort (n=50)**		
	Controls (n=93)	**BD (n=41)**	**MDD (n=43)**	**Controls (n=15)**	**BD (n=12)**	**MDD (n=23)**
Sex (M/F)	52 / 41	14 / 27	8/35	8/7	4/9	6/17
Age	49.56 ± 12.9	46.76 ± 14.5	52.18 ± 12.5	51.3 ± 11.7	41.6± 11.4	52.4 ± 12.8
BMI	24.55 ± 4.2	26.95 ± 4.7	25.59 ± 5.4	25.1± 1.9 **	27.8 ± 4.9 **	25.0 ± 5.9 **

***BMI values were available for 14 HC, 13 MDD and 10 BD*