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FOREWORD

The purpose of this thesis is to evaluate the involvement of Glycogen Synthase Kinase 3 (GSK3) in mood disorders, specifically its role as a marker in differential diagnosis between bipolar and unipolar depression, and as an indicator of the condition's gravity. The topic is dealt with in view of a critical review of the literature and with a clinical-translational contribution carried out on a sample of patients affected by major depressive disorder and bipolar disorder, compared to a control group.

Bipolar disorder and major depressive disorder are among the most common mood disorders, with a total incidence in the general population exceeding 15%. Onset generally occurs during youth, associated with significant morbidity and mortality, so much so it has been included among the first causes of disability in the world population regardless of gender. To date, differential diagnosis between unipolar and bipolar forms is exclusively based on clinical elements, however even in-depth clinical exams may not suffice to reach a correct diagnosis, especially in the initial stages of the condition and during a major depressive episode, with possible negative consequences on treatment and prognosis. Thus acquiring biomarkers in clinical practice would constitute a fundamental step forward to improve diagnostic accuracy, but currently there are no validated molecules to be used in everyday clinical practice.

The first chapter of this thesis is devoted to analyzing the issue of differential diagnosis between unipolar and bipolar depression with the consequences of possible misdiagnosis. Specifically, clinical elements and case history data have been analyzed

which can direct clinicians towards a diagnosis aided by screening scales and the peripheral, genetic and neuroimaging markers currently being researched.

The second chapter analyzes existing literature on the involvement of GSK3 in mood disorders, starting from *in vitro* studies and extending to studies on clinical samples.

The third chapter presents a translational clinical study fruit of a collaboration between the Psychiatry Unit of University Hospital San Luigi Gonzaga and the research group led by Professor Filippo Tempia from the Cavalieri Ottolenghi Institute of Neurosciences of University of Torino.

Patients were recruited from our Psychiatry Unit, all diagnosed with bipolar disorder and major depressive disorder, drug-free for at least 4 weeks, evaluated through a clinical assessment and a blood test aimed at measuring levels of GSK3⟨ and GSK3® in its total and phosphorylated components. A control group was also recruited composed of healthy subjects with no personal or family history of psychiatric disorders.

The study aims at evaluating levels of (total and phosphorylated) GSK3 in polymorphonuclear cells of the peripheral circulation (PBMCs) of patients with bipolar disorder, major depressive disorder and healthy control subjects, in order to study its role as a marker of disease and differential diagnosis; it also aims at assessing whether there is a correlation between levels of GSK3 and the severity of affective symptoms.

The results of the translational case-control study have been critically described and discussed in consideration of the literature available on the subject.

CHAPTER I

Differential diagnosis between bipolar depression and unipolar depression

I.1. Introduction

Bipolar disorder (BD) and major depressive disorder (MDD) are the two most common mood disorders among the general population, with a lifetime prevalence of 4,5% and 16,2% respectively (Kessler et al, 2003: Merikangas et al, 2007). MDD is defined by the presence of one or more major depressive episodes during the patient's life, while bipolar disorder is defined by the presence of at least one lifetime hypomanic episode, regardless of the number of depressive episodes. Thus, although mania and hypomania are pathognomonic and contribute to the definition of a diagnosis, the most common clinical presentation of bipolar disorder is a major depressive episode (MDE). A naturalistic study on a large sample of patients suffering from bipolar disorder has shown how patients spent over half of their time during the 13-year long follow-up period in a depressive episode, while only 10% of the time was taken up by hypo/manic episodes (Judd et al, 2002; Judd et al 2003). MDEs are also the most frequent onset sign; almost half of patients with type I bipolar disorder and up to three quarters of those with type II bipolar disorder will first display depressive symptoms (Goodwin e Jamis, 2010; Tondo et al, 2014).

In light of all this data, when confronted with a major depressive episode it is mandatory to carefully exclude the possibility that it actually is an MDE in the context of bipolar disorder, at onset or where expansive phases have not yet been identified.

But from a phenotypic point of view, both clinical conditions could present in a similar way, so much so that the diagnoses of an MDE in bipolar disorder and of an MDE in major depressive disorder are based on totally identical criteria, in accordance with the DSM-5TR (Tab. 1 and Tab. 2).

Recognizing the episode as belonging to one or the other longitudinal clinical conditions (MDD vs BD) is of fundamental importance because pharmaceutical treatment and

prognosis are different. In the case of an MDE in MDD, a course of antidepressants is advisable lasting at least one year (in the absence of clinical markers suggesting it be maintained, such as a history of at least three depressive episodes during the patient's lifetime or two MDEs separated by an intercritical period shorter than five years), whilst in the case of an MDE in the context of BD, the first choice of therapy would entail mood stabilizers to be maintained long term to cure the acute phase and prevent future relapses without inducing biphasic switching.

Although it is of primary importance, differential diagnosis between bipolar and unipolar depression still is to date a challenge for any clinician, especially at their onset, when cross-reconstruction of illness history investigating possible activation stages may not be possible.

From a realistic clinical point of view, this diagnostic issue may result in an estimated 16-40 % of bipolar depression incorrectly assessed (Angst et al, 2011) with a resulting period of untreated condition (that is, the time between the onset of the condition and the beginning of the first adequate treatment with mood stabilizers) amounting to over six years (Dagani et al, 2017; Di Salvo et al, 2022).

Tab.I.1 MDE diagnostic criteria in Bipolar Disorder (APA, 2022).

Major depressive episode

a. Five (or more) of the following symptoms were concurrently present over a period of two weeks and represent a change compared to the previous level of functioning; at least one of the symptoms consists in 1) depressed mood or 2) loss of interest or pleasure.
   Note: do not include symptoms clearly due to another medical condition
   1. Depressed mood for most of the day, almost every day, as reported by the subject (i.e., feels sad, empty or desperate) or as perceived by others (i.e. complaining).
      Note: In children and adolescents the mood may be irritable.
   2. Significant reduction in interest or pleasure deriving from all or most of the activities carried out during the day, almost every day (as reported by the subject or others).
   3. Significant weight loss not due to dieting, or weight gain (i-e. a 5% change in body weight in one month) or reduction/increase of appetite almost every day.
      Note: in children, the inability to reach a normal ponderal index should be considered.
   4. Insomnia or hypersomnia almost every day.
   5. Agitation or psychomotor retardation almost every day (observable by others, not just subjective feelings of agitation or of being slowed down).
   6. Fatigue or lack of energy almost every day.
   7. Excessive or inappropriate feelings of worthlessness or guilt (which can be delirious), almost every day (not just self-accusation or feeling guilty about being ill).
   8. Reduced ability to think or concentrate or indecision, almost every day (as a subjective impression or observed by others).
   9. Recurring thoughts about death (not just fear of death), recurring suicide ideation without a specific plan, or a suicide attempt, or specific plans to commit suicide.

b. Symptoms cause clinically significant discomfort, or social and work life are compromised, or other important dimensions of life.
c. The episode can not be attributed to the physiological effects of a chemical substance or to another medical condition.

Note: criteria A-C constitute a major depressive episode.

Note: a response to significant loss (e.g. grief, financial breakdown, losses due to natural disasters, serious medical conditions or disability) may include feelings of deep sadness, fixation on loss, insomnia, loss of appetite and weight, noted in criterium A, which may resemble a depressive episode. Although these symptoms may be understandable or considered appropriate to the scale of loss, the presence of a major depressive episode in addition to a normal response to significant loss should be carefully considered. This decision invariably includes the risk of a clinical evaluation based on the individual's history and to the cultural customs connected to expressing unease in the context of loss.

Tab.I.2 Major depressive episode diagnostic criteria in Major Depressive Disorder (APA, 2022).

Major Depressive episode
d. Five (or more) of the following symptoms were concurrently present for a period of two weeks, and represent a change compared to the previous level of functioning; at least one of the symptoms consists in 1) depressed mood or 2) loss of interest or pleasure. *Note*: do not include symptoms obviously caused by another medical condition 10. Depressed mood for most of the day, almost every day, as reported by the subject (i.e., feels sad or empty or desperate) or as observed by others (i.e., appears plaintive). *Note:* In children and adolescents the mood may be irritable. 11. Marked reduction in interest or pleasure for all, or almost all, activities for most of the day, almost every day (as reported by the subject or as observed by others). 12. Significant loss of weight not due to dieting, or weight gain (i.e. a change in body weight over 5% in a month) or reduction or increase of appetite almost every day. *Nota*: in children the inability to reach normal ponderal levels should be taken into account. 13. Insomnia or hypersomnia almost every day. 14. Agitation or psychomotor slowing down almost every day (observed by others, not just subjective feelings of being unsettled or slowed down). 15. Fatigue or lack of energy almost every day. 16. Excessive or inappropriate feelings of worthlessness or guilt (which can be delirious), almost every day (not just self-accusation or feeling guilty about being ill). 17. Reduced ability to think or concentrate or indecision, almost every day (as a subjective impression or observed by others). 18. Recurring thoughts about death (not just fear of death), recurring suicide ideation without a specific plan, or a suicide attempt, or specific plans to commit suicide. e. Symptoms cause clinically significant discomfort, or social and work life are compromised, or other important dimensions of life. f. The episode can not be attributed to the physiological effects of a chemical substance or to another medical condition. *Note:* criteria A-C constitute a major depressive episode. *Note:* a response to significant loss (e.g. grief, financial breakdown, losses due to natural disasters, serious medical conditions or disability) may include feelings of deep sadness, fixation on loss, insomnia, loss of appetite and weight, noted in criterium A, which may resemble a depressive episode. Although these symptoms may be understandable or considered appropriate to the scale of loss, the presence of a major depressive episode in addition to a normal response to significant loss should be carefully considered. This decision invariably includes the risk of a clinical evaluation based on the individual's history and to the cultural customs connected to expressing unease in the context of loss.

There are many reasons for this diagnostic difficulty: first of all, the patient's difficulty in acknowledging the symptoms of a hypomanic episode as pathological, omitting them during in-depth diagnostic interviews if not precisely investigated; the absence of a family member during a phase of diagnostic assessment that may provide information on continuum fractures unacknowledged by the patient; and finally research by the clinician limited to euphoria, ignoring irritability and dysphoric mood changes (Goodwin e Jamis, 2010). In addition, the presence of DSM-5 with a specific marker "with mixed characteristics" for major depressive disorder, could make it harder to immediately spot an MDE with three or four hypomanic symptoms simultaneous to the specter of bipolar disorders.

Finally, as reported above, a considerable percentage of bipolar disorder patients diagnosed with an MDE at onset, based exclusively on DSM-5TR criteria, will thus necessarily be considered as unipolar. Recent studies have in fact estimated that over 20% of patients with an MDE at onset followed for 12-18 months will develop a counter-polar episode with increased risk of a switch during the first year of illness (Ratheesh et al, 2017: Kessing et al, 2017).

I.2. Clinical elements of differential diagnosis

With an occasionally probabilistic approach based on literature, it is possible to identify some intra-episode and treatment-response which, combined with family history information, may function as predictors of bipolarity and bring clinicians to a differential diagnosis of a major depressive episode.

First of all, patients with bipolar disorder are more likely to have a family history featuring bipolar disorder and/or alcohol/drug/suicide related problems (Bowden, 2005; Mitchell et al., 2008; Goodwin e Jamison, 2010; Takeshima and Oka, 2013; Tondo et

al., 2014). But concerning history of illness, BD patients tend to have earlier onset compared to patients with MDD (Zisook et al, 2007; Mitchell et al, 2008), a greater number of lifetime episodes, although of shorter duration (Mitchell et al, 2008: Moreno et al, 2012), an "on/off" type of relapse/remission, a seasonal relapse recurrence (usually in winter), a higher number of suicide attempts during their history and a higher index of comorbidity with other psychiatric conditions such as ADHD and substance abuse disorders (Rihmer e Kiss, 2002; Goldberg and Harrow, 2004; Goodwin e Jamison, 2007).

But regarding the clinical presentation of the depressive episode, patients with bipolar disorder tend to display more varied moods compared to the reduced hedonic tone of MDD patients, and display atypical depressive symptoms such as leaden paralysis, hypersomnia, increased appetite and weight gain, instead of the terminal insomnia and anorexia typical of MDD patients. They also present psychotic symptoms more often and increased cognitive impairment (Borkowska and Rybakowski, 2001; Wolfe et al., 1987; Goodwin and Jamison, 2007; Akiskal et al. 1995, Benazzi 1999, Benazzi 2002a, Benazzi 2002b, Hadjipavlou et al. 2004, Bowden 2005, Mitchell et al. 2008).

Concerning therapy, patients suffering from bipolar depression tend to have a history of resistance/inconstant response to antidepressants, to the point that they can be classified as suffering from treatment-resistant depression (pseudo-resistance), or of mixed symptoms appearing during the course of treatment (Goodwin 2003).

A table quickly summarizing some elements that may be used by clinicians to reach a diagnosis can be found in the guidelines for treating affective disorders used in Australia and New Zealand (Malhi et al., Aust N Z J Psychiatry 2015) (Fig. 1.)

Fig.I.1 Clinical elements that may guide differential diagnosis of an MDE (from: Malhi et al., Aust N Z J Psychiatry 2015).

Features	Bipolar	Unipolar^
Family History	Bipolar disorder (more likely) Alcohol and/or substance use (more likely)	Bipolar disorder (less likely) Alcohol and/or substance use (less likely)
Illness onset	Early onset (approx. 20-25 years)	Later onset (approx. 25-30 years)
Onset/Offset	More often abrupt	More often gradual
Comorbidity	ADHD more often	ADHD less often
Duration of episodes	<6 months	>6 months
Number of Prior Episodes	Multiple prior depressive episodes	Fewer prior episodes
Mood symptoms	Lability of mood/manic symptoms	Depressed mood and low energy
Psychomotor symptoms	Psychomotor retardation	Psychomotor retardation less likely
Sleep disturbances	Hypersomnia and/or increased day time napping	Initial insomnia/reduced sleep
Appetite Changes	Hyperphagia and/or increased weight	Appetite and/or weight loss
Other symptoms	Other 'atypical' depressive symptoms such as hypersomnia, hyperphagia, 'leaden paralysis' Psychotic features and/or pathological guilt	Somatic complaints

However, it should be quite clear that these elements are approximate and must not be taken as criteria on which to base a conclusive differential diagnosis.

I.3. Screening tools

A few scales supporting clinical evaluation may help clinicians to reach a differential diagnosis.

Among the most easily applied tools in clinical practice:

The *Mood Disorder Questionnaire* (MDQ), a short, self-administered questionnaire identifying patients probably suffering from bipolar disorder (Hirschifeld et al, 2000). More than 7 positive answers to the first 13 questions and/or achieving a score equal to "moderate problem" or "serious problem" in the functional impairment section result in a diagnosis of bipolar disorder.

The Hypomania/Mania Symptoms Checklist (HCL-32), another self-administered screening test (Angst et al, 2005) consisting in two introductory questions on the patient's current emotional state and level of energy, followed by 32 multiple choice questions evaluating specific symptoms of hypomania/mania. The score is worked out adding positive answers to closed questions. A score ≥14 is associated with a positive history of hypomanic/manic episodes, therefore leading to a diagnosis of bipolar disorder with a sensitivity of 0.8 and specificity equal to 0.51 (Angst et al, 2005).

The Bipolar Spectrum Diagnostic Scale (Ghaemi et al, 2005) is a screening test divided in two sections: in the first, the patient reads a series of statements describing most of the symptoms of hypomania in simple terms, in the second the patient is asked how much he/she recognizes him/herself in the previous statements and a score between 2 and 6 is assigned to each of 19 statements. The score can vary between 0 and 25 points; a score ≥ 20 means a diagnosis of bipolar disorder is highly probable; a score between 13 and 19 means the diagnosis is moderately probable; scores between 7-12 are associated with a low probability, and scores <6 mean bipolar disorder is very unlikely. A validation study showed how the sensitivity of the scale is the same for the entire bipolar spectrum, reaching 75% for bipolar disorder I and 79% for bipolar disorder II/NAS respectively, with an average sensitivity of 76% and specificity equal to 85%.

Nonetheless, we must bear in mind that all these tests are screening tools and not diagnostic tools, as diagnosis remains exclusively clinical.

I.4. Consequences of misdiagnosis

As described previously, correctly attributing a major depressive episode to one of the two different affective disorders has important therapeutic consequences: in the case of MDD, the use of antidepressants even just for a while, but in the case of BD the use of mood stabilizers usually for life.

Diagnosing a patient suffering from bipolar disorder as unipolar and treating him/her as such can result in potentially disadvantageous prognostic consequences.

First of all, numerous studies conducted in the late Nineties have shown how the use of antidepressants to treat bipolar depression is associated with low clinical efficacy of the treatment of depressive symptoms with an increased rate of depression resilience (actually pseudo-resilience) among this set of patients. In addition, especially in monotherapy, the use of antidepressants seems to be associated with a general deterioration of the condition's development, with an increased number of affective issues, progressive shortening of the inter-episode spells and an increased rate of counter-polar switches or onset of mixed symptoms resulting in increased risk of suicide (Altshuler et al., 1995; Boerlin et al., 1998; Peet, 1994; Wehr et al., 1988).

Most of these studies examined treatment with old school antidepressants, chiefly tricyclic. More recent studies carried out exclusively with selective serotonin reuptake inhibitors seem to perform more effectively during the acute phase (Amsterdam et al, 2016; McGirr et al, 2016) with reduced risk of counter-polar switches (Sidor e MacQueen, 2011), although an increased risk of affective recurrence appears to be confirmed in the long run (MCGirr et al, 2016).

Taken as a whole, these results tell us antidepressants are a possible weapon available to clinicians, but only in selected cases, and above all if used with the awareness that the depressive episode in progress is of a bipolar nature with all the resulting consequences regarding clinical risk and monitoring needs.

I.5. Differential diagnosis biomarkers

In the light of the diagnostic difficulties mentioned above, there is a growing need to identify biomarkers that may help clinicians in differential diagnosis, in addition to acting as a guide in the choice of treatment and staging of disorders.

Despite ongoing efforts, to date the diagnosis of mood disorders is exclusively clinical. A growing number of studies within translational research are trying to contribute to the definition of sets of biomarkers able to define and profile a given psychiatric disorder. However, data related to possible markers, specifically studied for differential diagnosis between bipolar and unipolar depression are scant and often contradictory.

Following below is a brief summary of the markers being studied:

Neuroimaging techniques

Neuroimaging studies have revealed how BD patients appear to display reduced volume of the amygdala and white matter compared to MDD patients, who seem to display a reduction of the anterior cingulate cortex with a predictive power equal to 69% (Redlich et al, 2014). In addition to different volumes in selected brain areas, the two groups appear to show specific activation patterns in response to emotional stimuli: specifically, functional magnetic resonance studies have shown how BD patients display increased activation of the amygdala (Grotegerd et al, 2014) and reduced activation of the anterior cingulate cortex (Burger et al, 2017) compared with patients suffering from unipolar depression.

Genetic markers

It seems a different gene expression profile of the two disorders is also confirmed by genetic studies, especially regarding a few genes involved in inflammation (CCL24, CCR6, NR3C1), although evidence is still limited (Powell et al, 2014; Menezes et al, 2019).

Peripheral biomarkers

Numerous studies have proposed a few molecules as possible peripheral biomarkers for differential diagnosis, such as IGFBP2, VGF and platelet-derived growth factor BB (Milanesi et al, 2017;Chen et al, 2018; Idemoto et al, 2021), all conducted on small clinical samples and waiting for validation on larger samples.

One of the most replicated results is the reduced expression of peripheral levels of brain-derived neurotrophic factor (BDNF) in patients suffering from unipolar disorder (Fernandes et al, 2008; Li et al, 2014; Menezes et al, 2019; Schroeter et al, 2020) compared to MDD patients and healthy subjects, although low levels of protein have also non-specifically been found in other psychiatric disorders (Maina et al, 2010). Although a possible cut-off in peripheral levels of BDNF has been proposed in order to identify BD patients with increased sensitivity and precision (Fernandes et al, 2008), further studies are necessary before it can be validated and applied in everyday clinical practice.

CHAPTER II

Glycogen Synthase Kinase 3 in mood disorders

II.1. Glycogen Synthase Kinase-3(GSK-3)

GSK3 is a Ser/Thr protein kinase originally identified, and consequently named, because of its ability to phosphorylase and deactivate the glycogen synthase enzyme involved in glycogen metabolism. it was later found ubiquitously in all mammal tissues and cellular organelles, and its involvement in regulating and modulating many aspects of neuronal function such as gene expression, neurogenesis, synaptic plasticity and cellular death and survival was discovered, with over 50 target substrates identified (Doble and Woodgett, 2003; Frame and Cohen, 2001; Jope and Johnson, 2004).

There are two isoforms of this protein, GSK3〈 and GSK3®, coded by independent genes but sharing 85% of sequence homology, including kinasic domain which has a sequence homology equal to 97% (Woodgett et al, 1990). Both GSK3〈 and GSK3® are expressed in brain tissue (Yao et al, 2002), with isoform alpha abundant above all in the hippocampus, cerebral cortex, striatum and in the cerebellum's Purkinje cells, while the beta isoform is more universally expressed in all brain areas (Li et al, 2010).

GSK3 is one of the proteins with the most irregular functioning in the human body, for a number of reasons:

1) Some of its substrates need phosphorylase 'priming' by another kinase of four or five residues at c-terminal, before they can be efficiently phosphorylated by GSK3 (Kennelly e Krebs, 1991)
2) unlike many other kinases, GSK3 è constitutionally active in basic conditions;
3) It is inhibited by phosphorylation in serine at the N terminal (Cross et al, 1995; Sutherland et al, 1993), serine-21 for GSK3 alpha and serine-9 for GSK3 beta.

Phosphorylation is mediated by various kinase proteins, including Akt (Cross et al, 1995), kinase protein C (Goode et al, 1992) and kinase protein A (Fang et al, 2000; Li et al, 2000).

II.2. Evidence of GSK3 involvement in mood disorders

The first evidence of GSK3 involvement in bipolar disorder pathogenesis emerged in 1996, when two independent research groups, (Klein e Melton,1996; Stambolic et al, 1996), described an inhibiting effect of lithium as a direct competitor of the molecule through a mechanism mediated by a bond with magnesium.

From this finding, different research groups studied the role of kinase in depth, expanding analysis of lithium's inhibiting role, analyzing the role of other psychiatric drugs, evaluating GSK3 alterations in rat models, in postmortem samples, in genetic studies and finally evaluating molecule alterations in vivo on patients suffering from mood disorders.

Their findings follow below:

II.2.1. Lithium salts and other psychiatric drugs in GSK3 modulation – preclinical studies

Lithium salts

Lithium's direct inhibition on GSK3 through a direct, magnesium-mediated bond, has been confirmed by further studies (Ryves e Harwood, 2001). However, the direct inhibiting effect measured in vitro is rather weak, given that the salt's therapeutic concentration (approx. 1 mM) allows 25-50% of GSK activity to be inhibited, based on physiological concentrations of magnesium (Gurvich e Klein, 2002).

In addition to direct inhibition, lithium increases GSK3 inhibition mediated by phosphorylation serine at the N terminal (Li et al, 2007) in an indirect and effective way already at haematic concentrations within the therapeutic range. Although the mechanism of action is not yet completely clear, increased phosphorylation appears to be due to the inhibition of a phosphatase protein which usually activates GSK3 removing its phosphorylation in serine (Mora et al, 2002; Song et al, 2002). This double inhibiting mechanism, direct and indirect, allows to exceed the inhibition only due to the direct mechanism which would occur too quickly and would be scarcely relevant from a therapeutic point of view (Fig. II.1)

Fig. II.1 Lithium salts and GSK3 regulation (from Jope 2003)

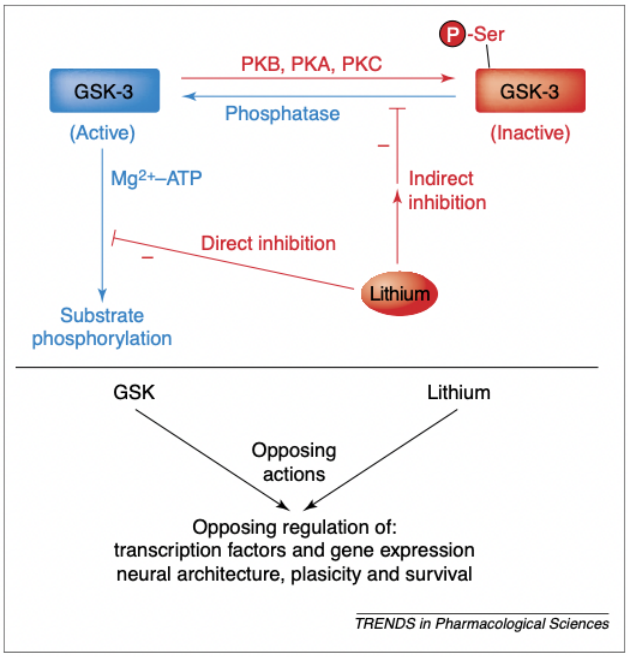

Figura II. 1. Interactions between lithium and GSK-3. GSK3 catalyses the phosphorylation of many substrates in the presence of magnesium. Lithium is a competitive inhibitor of magnesium with a resulting inhibition of kinase (indicated as direct inhibition). In addition, lithium inhibits the action of phosphatases which would cause dephosphorylation (that is, the activation) of GSK3, leaving kinase in its inhibited form (indirect inhibition).
Aspects associated with GSK3 activation are in blue. Signaling pathways associated with its inhibition are in red.

The discovery that lithium acts as a GSK3 inhibitor fueled interest in determining whether other mood stabilizers modulate kinase activity.

Pharmacological studies carried out show how GSK3 inhibition is a mechanism of action shared by many pharmacological classes used in the treatment of bipolar disorder, although the evidence in literature is not always unambiguous.

Table II.1. shows the effects of the main psychiatric drugs used in the treatment of bipolar disorder.

It still is not clear whether GSK3 inhibition is linked to the stabilizing effect of these drugs and if GSK3 can consequently be the target of new treatments.

Table II.1. GSK3 and psychiatric drug treatments

Drug	Effect on GSK3	Experimental treatment
Lithium	↓Activity ↑ pSer	In vitro, in cells, in animals, in human peripheral tissues
Valproate	↓Activity ↑ pSer	In cells, in rats
Carbamazepine	No effect	In cells
Lamotrigine	No effect ↑ pSer	In cells In animals
Fluoxetine	↑ pSer	In animals
Imipramine	↑ pSer	In animals
Clozapine	↑ pSer	In cells, in animals
Olanzapine	↑ pSer	In vitro, in cells, in animals
Risperidone	↑ pSer	In animals
Quetiapine	↑ pSer	In animals
Aripiprazole	↑ pSer	In animals
Haloperidol	↑ pSer	In animals

Valproic acid and other stabilizers

A few studies report that valproic acid directly inhibits the activity of GSK3 (Chen et al, 1999; De Sarno et al, 2002; Roh et al, 2005), although this fact has not been confirmed by other work (Eickholt et al, 2005; Hall et al, 2002; Jin et al, 2005; Phiel et al, 2001). Recent work suggests that GSK3 inhibition via valproic acid could be mediated by the inhibition of the drug of the histone deacetylase (HDAC) (Phiel et al, 2001), the inactivation of which causes increased inhibition of kinase phosphorylation (Aubry et al, 2009; De Sarno et al, 2002; Kozlovsky et al, 2006; Lamarre e Desrosiers, 2008).

Few studies have evaluated the action other mood stabilizers such as carbamazepine or lamotrigine have on GSK3. It seems carbamazepine does not have any direct action on kinase (Aubry et al, 2009; Mai et al, 2002; Ryves et al, 2005), and similarly lamotrigine (Aubry et al, 2009), on which there are conflicting results.

Atypical antipsychotics

Most atypical antipsychotics, including risperidone, olanzapine, clozapine, quetiapine appear to increase GSK3 phosphorylation in serine in rats, causing its inhibition (Alimohamad et al, 2005; Li et al, 2007b; Roh et al, 2007), also at low dosage (Li et al, 2007b).

Haloperidol is the only typical antipsychotic to be reported as having an inhibiting effect on kinase (Alimohamad et al, 2005; Kozlovsky et al, 2006; Roh et al, 2007).

Antidepressants

As well as mood stabilizers and antipsychotics, some antidepressants also appear to promote control on GSK3 through inhibition. Administering fluoxetine and imipramine significantly increased GSK3 phosphorylation in serine in rat brains (Beaulieu et al, 2008b; Li et al, 2004). The inhibiting effect of these antidepressants on GSK3 occurs within a few hours after acute treatment in vivo, suggesting that it could be a response to the rapid increase of brain monoamines caused by these antidepressants, but whether the rapid inhibition of GSK3 is involved in the antidepressants therapeutic action, given the latency in acting, is still an open question.

II.2.2 Behavioural changes in rats with altered GSK3 expression or activity.

Although studies conducted on rat models use different instrumental strategies, they support the idea that GSK3 plays a role in mood disorders.

Mania-like behaviour and GSK3

One of the rat models to mimic manic behaviour in test animals is basal locomotor hyperactivity evaluated in an unknown, open field.

Rats overexpressing GSK3 in a constitutionally active form, showed an increase of motor activity (Prickaerts et al, 2006), exactly like animals with a reduced function of kinase inhibitors (Hikida et al, 2007; Pletnikov et al, 2008). These results are in line with the idea that an increase in GSK3 activity is directly linked to locomotor hyperactivity.

Another model for mania used with rats is hyperactivity induced by amphetamines. The studies of Beaulieu's group (2004) which identify GSK3 as an important modulator of the activation induced by stimulants are particularly interesting. Beaulieu and colleagues show how GSK3 is activated by administering amphetamines in the rats' cortex and striatus (Beaulieu et al, 2004, 2005, 2008a) with consequent hyperactivity. GSK3beta heterozygous knockout rats show diminished motor hyperactivation after being administered amphetamines compared to wild-type rats, basal locomotor hyperactivity being equal (Beaulieu et al, 2004). Other authors have shown how administering GSK3 inhibitors reduces amphetamine-induced hyperactivity, further supporting the conclusion that GSK3 in active form is a mediator of this response (Kozikowski et al, 2007; Kalinichev and Dawson, 2011) and that it is linked to manic-like behaviour.

Depression-like behaviour and GSK3

The theory that GSK3 dysregulation promotes depression-like behaviour in rats has been extensively supported by multiple pharmacological approaches. In the early Nineties studies had already highlighted how administering lithium to rats lowered many

measurements of depression in rat models and increased the effects of SSRIs (Faria and Teixeira, 1993; Nixon et al., 1994; Redrobe and Bourin, 1999). After the discovery in 1996 that lithium is a GSK3 inhibitor (Klein and Melton, 1996; Stambolic et al., 1996), many authors came to the conclusion that the responses to lithium described above were most likely due to GSK3 inhibition.

Together with these pharmacological studies, strategies using molecular modifications have helped to confirm that GSK3 alterations produce depressive behaviour in rats.

Reduced levels of GSK3beta heterozygous knockout rats for isoform beta have shown reduced time of immobility in the forced swim test (FST) and the TST (O'Brien et al, 2004; Beaulieu et al, 2008). Reduced time of immobility in FST and TST has also been shown in rats with a suppressed GSK alpha functioning gene (Kaindanovich-Beilin et al, 2009). All these studies taken as a whole show that reduced expression of both forms of GSK3 reduces vulnerability to depression-like behaviour in rats.

As a whole, preclinical studies have resulted in the hypothesis that a dysregulation of GSK3, mediated by an altered phosphorylation in serine resulting in the protein's aberrant functionality is a feature of mood disorders. Specifically, in depression, the lack of signals which usually keep GSK3 inhibited, such as serotonine or neurotrophins, causes up-regulation of GSK3 activity, able to promote susceptibility to depression. On the contrary, in mania we see excessive dopaminergic transmission which can induce kinase activation through a trickle down mechanism (Fig II.2).

Part of the therapeutic effect of the drugs used for mood disorders thus appears to be mediated by a direct or indirect action on the inhibition of GSK3 kinase.

Fig. II.2 GSK3 involvement in mood disorders (Jope 2011)

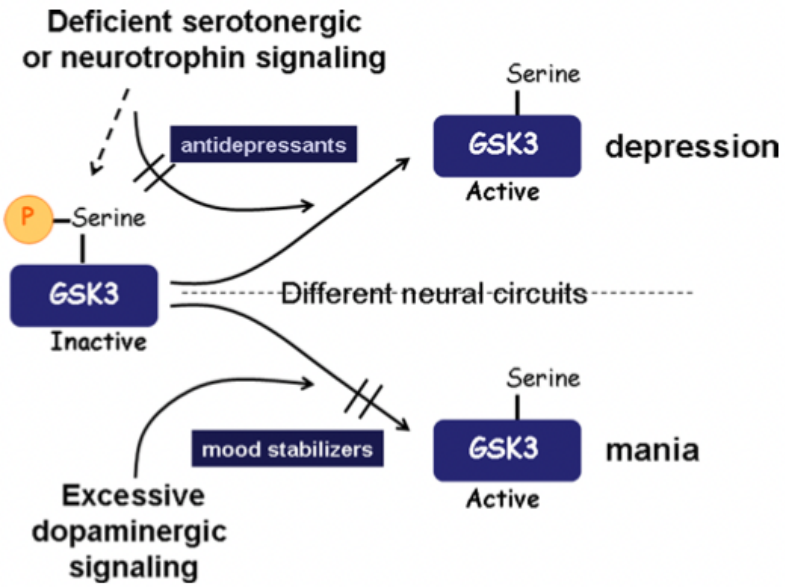

Figura II.2. Diagram illustrating how GSK3 can be dysregulated in mood disorders. In depression, a shortfall of the signals which usually keep kinase inhibited, such as those induced by serotonin and neurotrophins, can cause GSK3 activity up-regulation, which appears to entail increased susceptibility to depression.

Mania on the other hand can entail increased dopaminergic signals which triggers GSK3 activation. Part of the therapeutic action of antidepressants and mood stabilizers could result from direct or indirect kinase inhibition.

II.2.3 Genetic studies

Genetic studies have also contributed to clarify the role of GSK3 in mood disorders.

An Italian research group has in fact shown how a single nucleotide polymorphism (SNP) in the region promoting GSK3beta (-50T/C) is associated to a late onset of bipolar disorder and to improved response to treatment with lithium salts (Benedetti et al, 2004, 2005). Improved response to lithium was also shown in a group of patients suffering from MDD who presented the same genetic polymorphism in the kinase domain (Adli et al, 2007), although this fact has not been confirmed by other studies (Szczepankiewicz et al, 2006).

Other studies have linked the presence of particular genetic GSK3 polymorphisms to the response to lithium and antidepressants (Tsai et al, 2008) and to the volume of grey matter in MDD patients (Inkster et al, 2009). Therefore, GSK3 gene variations seem to

be involved in vulnerability to the condition and in response to treatment, although their impact on GSK3 functionality is not yet clear.

II.2.4. Post-mortem studies

In addition to genetic studies, the study of GSK3 has also been carried out on brain samples from deceased psychiatric patients.

A post mortem study conducted on 40 brain samples from suicidal and non-suicidal subjects revealed an increase of GSK3b activity and a reduction of Akt activity in depressed suicide victims but not in non-depressed ones (Karege et al, 2007). Although two other studies did not report different GSK3 levels in the post-mortem brains of BD patients and healthy subjects (Kozlovsky et al, 2000; Lesort et al, 1999a), these studies did not investigate whether kinase activity was altered. Evaluating GSK3 activity or its level of phosphorylation can be arduous in post-mortem brains, as kinase phosphorylation degrades within a few minutes from death, as shown by animal models (Li et al, 2005). Furthermore, another limit of using post-mortem samples consists in the impossibility of linking possible alterations in the level of the molecule with the stage of the disorder affecting the patients, in addition to the potential influence of other pre mortem drugs on the levels of phosphorylation.

II.2.5. In vivo studies on peripheral tissues

An alternative to studies on brain tissue is the analysis of GSK3 levels in peripheral tissues. However, the literature reports conflicting data when GSK3 levels are analyzed in vivo in the peripheral blood of patients with mood disorders.

Starting from the peripheral tissue used, some studies analyze protein levels in the platelets, while others in peripheral blood mononuclear cells (PBMCs), thus obtaining results which are hard to compare.

Li and colleagues (2007) have evaluated whether lithium treatment in vivo modifies GSK3 levels of expression. The authors evaluated GSK3beta phosphorylation levels analyzing PBMCs from 23 healthy control subjects, 9 BD patients treated with lithium and 13 lithium-free BD patients undergoing treatment with other drugs. The analysis showed how basal phosphorylation levels were lower in healthy control subjects, three times higher in lithium-free BD patients, and peaked (approximately eight times higher compared to control subjects) in patients treated with lithium (Fig.II.3). These results confirm the hypothesis that lithium treatment increases phosphorylation and consequently kinase inhibition, as already suggested by rat models.

Fig. II.3 GSK3® in PBMCs from BD patients and healthy control subjects (from Li et al, 2007)

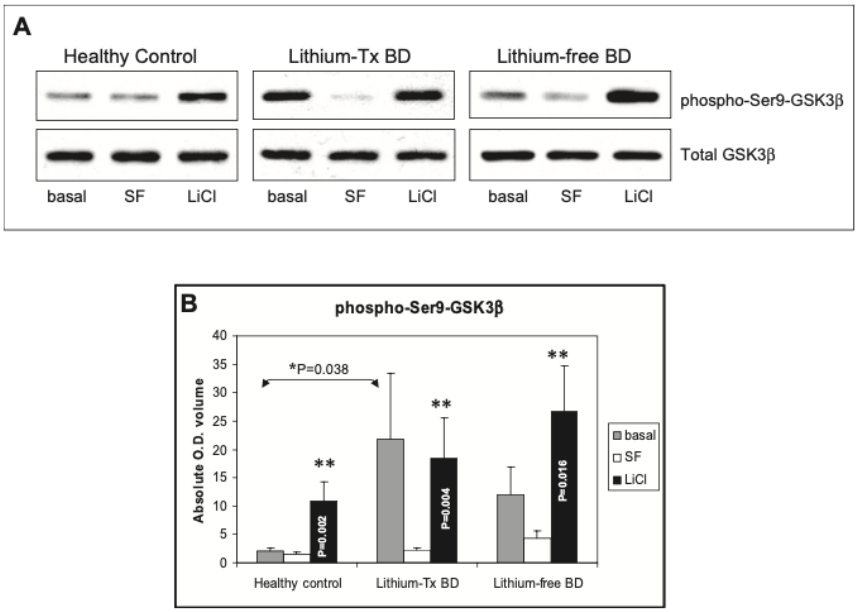

Figura II.3. Phospho-GSK3β in PBMCs from healthy control subjects and BD patients before and after lithium treatment. Cells isolated from healthy controls (n.13), patients treated with lithium (n.9) and lithium-free patients (n.13) were immediately lysed and incubated in ice for two hours (basal), in serum for two hours (SF) or for one hour after administering 20 mmol/L for one hour (LiCl). Immunoblot of total and phosphorylated GSK3® components ***(A)*** *Immunoblot of a healthy control, a BD patient treated with lithium and a lithium-free BD patient* ***(B)****. **p<0.05.*

The same group of authors (Li et al, 2010) later analyzed levels of total and phosphorylated GSK3 in PBMCs from a group of BD type I patients (n:30), admitted to hospital because of a manic episode, and from a group of healthy controls (n: 30), in addition to evaluating GSK3 modifications due to antimanic treatment.

The study showed significantly higher levels of GSK3 alpha and beta in the group of manic patients compared to healthy controls and how improved post-treatment symptoms were accompanied after 8 weeks by an increase of GSK3 phosphorylation levels, the total component being equal. (Fig II.4)

Fig. II.4. The effect of antimanic treatment on GSK3 in PBMCs from BD patients during a manic phase (from Li et al, 2010).

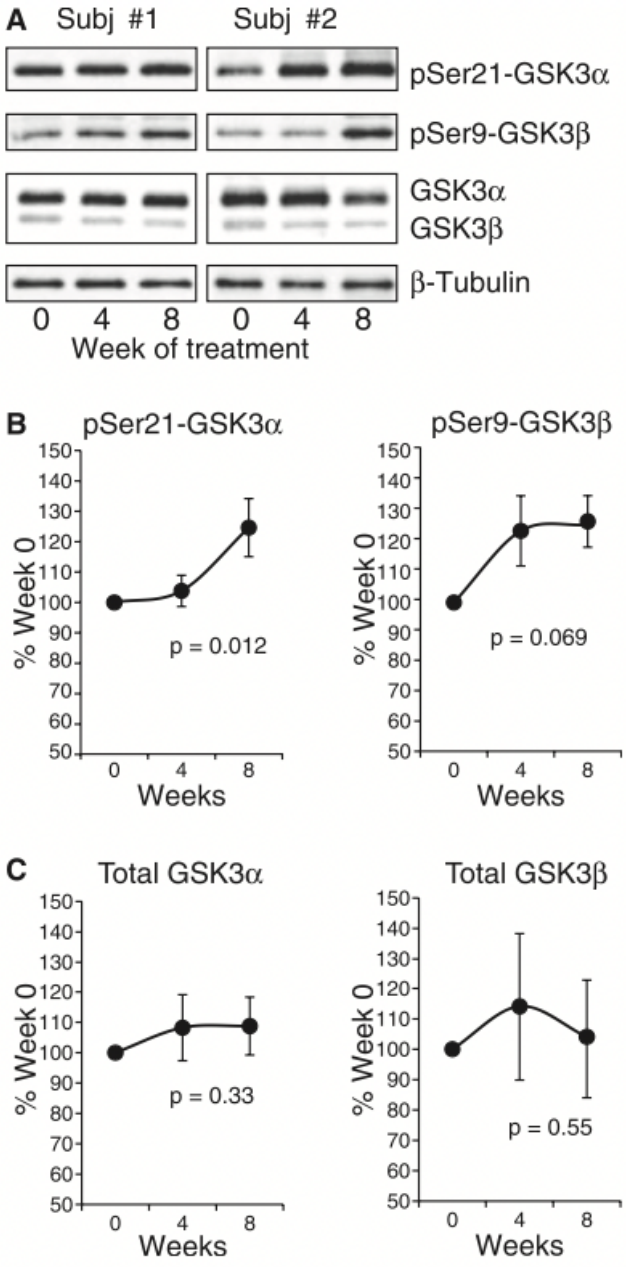

Figura II.4 The effect of antimanic treatment on GSK3 analyzed in PBMCs from BD patients during a manic phase. Immunoblot representing 2 of 47 patients ***(A).*** *Estimate of the total components of the protein* ***(B)*** *and of the phosphorylated components* ***(C)****.*

The same year, Pandey and colleagues (Pandey et al, 2010) analyzed levels of GSK3beta expression in cytosol and in the cellular membranes of platelets obtained from patients suffering from major depressive disorder (MDD; n: 21) and bipolar disorder (BD; n: 21), before and after treatment with antidepressants and stabilizers.

The authors observed how levels of GSK3 beta were significantly lower in the cytosol and in the membrane of platelets from BD patients compared with those from MDD patients. In addition, lithium treatment significantly increased levels of GSK3beta in BD patients compared to pretreatment levels, bringing the protein to a level similar to that found in a group of healthy control subjects (n: 21). Antidepressant treatment did not modify GSK3 levels post-treatment in MDD patients. (Fig.II.5)

Fig. II.5. Effects of psychiatric drug treatment on GSK3 levels (from Pandey et al, 2010).

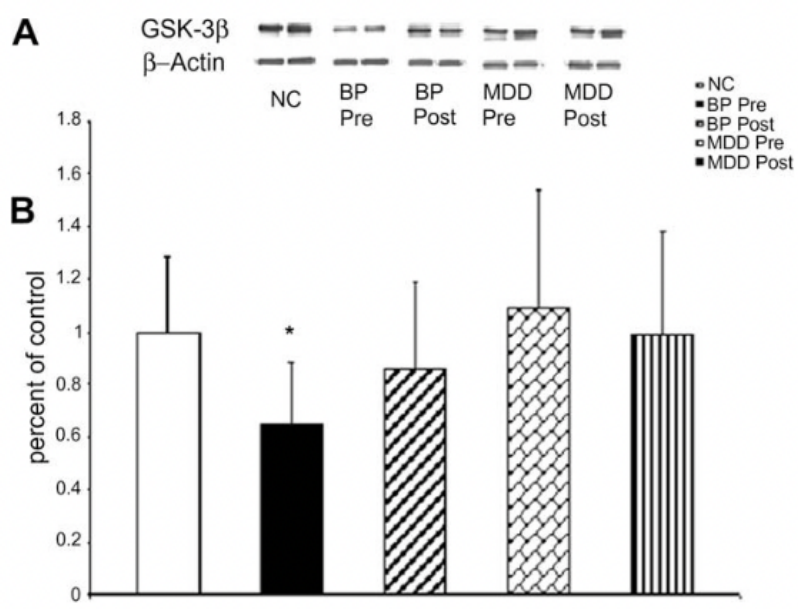

*Figura II.5. Western blot representing GSK3® and ®-actine in the membrane of two healthy control subjects, two BD patients and two MDD patients, before and after 8 weeks of treatment with psychiatric drugs (A). Average GSK3® level in the cellular membrane of platelets obtained from drug-free patients (Pre BD and Pre MDD) and after 8 weeks of treatment (Post BD, Post MDD) and from healthy control subjects. (B) *p<0.001*

Still in 2010, Polter and colleagues (Polter et al, 2010) analyzed levels of GSK3 in PBMCs from 18 patients suffering from bipolar disorder (in every phase of the condition) and 11 healthy controls. They found alpha and beta phosphorylated components were 35% lower in the patients compared to healthy control subjects, with no difference in total components, showing how the reduction of the phosphorylated

component was not due to a reduction of total kinase expression. The authors also analyzed the link between GSK3 phosphorylated components and symptom severity (Fig.II.6)

Fig. II.6. Levels of GSK3 expression (from Polter et al, 2010).

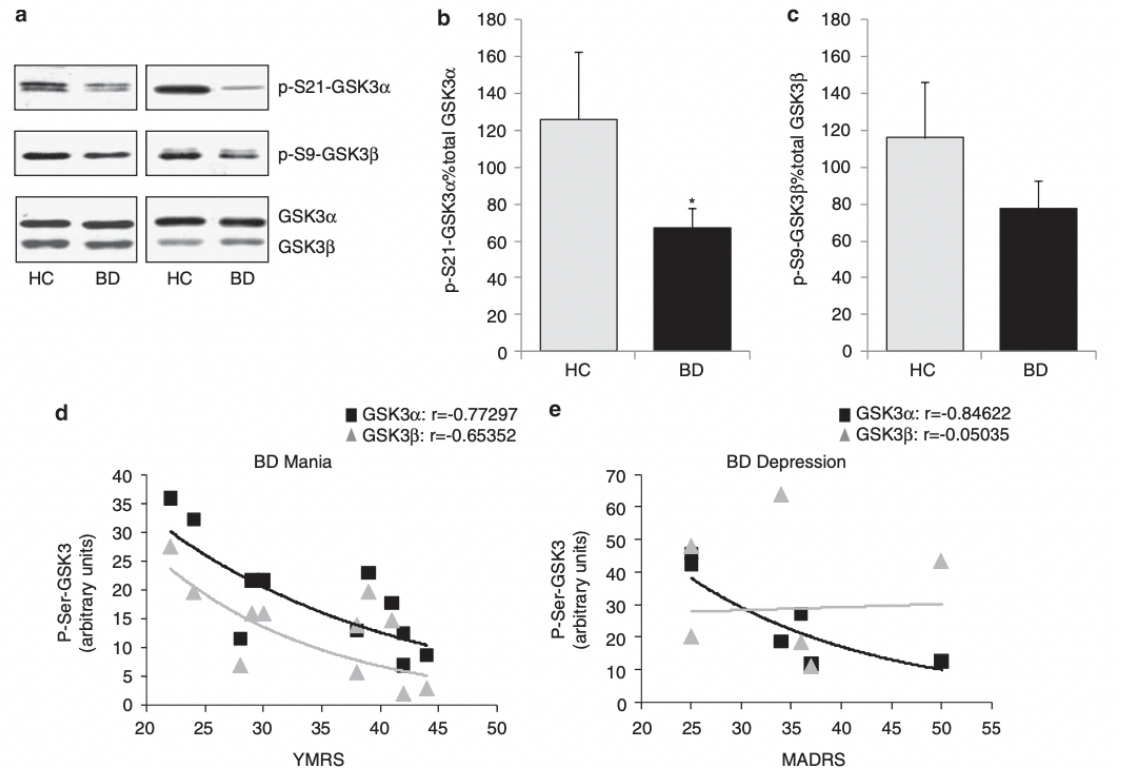

Figura II.6. Immunoblot representing levels of total and phosphorylated GSK3α and GSK3β in PBMCs from healthy controls and BD patients ***(A)****. Estimate of the levels of GSK3* ***(B-C)****. Correlation between pGSK3α and pGSK3β and total YMRS scores in hypo/manic patients* ***(D)****. Correlation between pGSK3α and pGSK3β total MADRS scores in depressed patients.****(E)****.*

Finally, de Sousa and colleagues (2015) analyzed the effect of lithium in vivo treatment on GSK3 over six weeks, analyzing the total and phosphorylated component of the beta isoform in the platelets of 27 patients suffering from bipolar depression. The authors also recruited 22 healthy subjects to be used as control group. No baseline differences in GSK3 expression levels were found between depressed patients and healthy control subjects, nor any correlation between GSK3 levels and symptom severity. After six

weeks of lithium treatment levels of phosphorylated GSK3 beta were found to have significantly increased compared to baseline levels (Fig II.7).

Fig.II. 7. Levels of total and phosphorylated GSK3® in subjects pre and post lithium treatment and healthy control subjects. (from de Sousa et al, 2015).

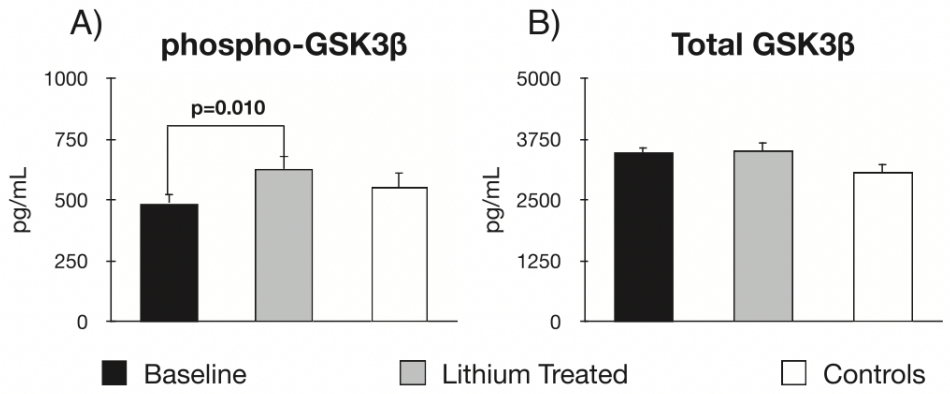

Figure II.7. Levels of phosphorylated (A) and total (B) GSK3 obtained from platelets extracted from 27 patients affected by bipolar depression at baseline and after six weeks of treatment, and 22 healthy control subjects.

As a whole these studies yield conflicting results.

One study did not find any difference between the levels of GSK expression in healthy control subjects and depressed bipolar patients (Polter et al, 2010), a result not confirmed by a concurrent study by Pandey (Pandey et al, 2010) which reported lower levels of GSK3 in depressed bipolar patients compared to control subjects. In manic patients GSK3® expression appears to increase (Li et al, 2010). Similarly, the evaluation of GSK3 activity levels (quantification of the phosphorylated component) at baseline and/or after a course of therapy with psychiatric drugs has yielded uneven results (Polter et al, 2010; Li et al, 2007; de Sousa et al, 2015).

But although partial and conflicting, the results of these in vivo studies confirm the involvement of GSK3 in the pathophysiology of mood disorders and in the mechanisms of action of specific psychiatric drug treatments.

CHAPTER III

Glycogen Synthase Kinase 3 in mood disorders: a biomarker for differential diagnosis between unipolar and bipolar depression?

III.1. Introduction

Mood disorders are a heterogenous group of psychiatric diseases that include bipolar disorders (BD), with both manic and depressive episodes, and depressive (unipolar) disorders. One of the major issues in mood disorders concerns the differential diagnosis between bipolar and unipolar depressive episodes, with crucial implications in treatment choice (mood stabilizers vs antidepressants). Identification of peripheral biomarkers that can reflect specific pathophysiologic processes of bipolar and unipolar depression may promote early interventions with appropriate treatments (Milanesi et al., 2018).

Glycogen synthase kinase-3 (GSK3) is a highly conserved serine-threonine kinase expressed in all mammalian tissues, originally identified as an enzyme involved in glucose metabolism (Embi et al., 1980). Two isoforms, GSK3α and GSK3β, encoded by different genes but sharing 85% sequence homology are known (Woodgett, 1990). Both the isoforms are expressed at similar levels in mouse brain (Yao et al., 2002); instead in the human brain GSK3β seems to be predominantly expressed in all regions, while GSK3α appears to be especially copious in the cerebral cortex, hippocampus, striatum and cerebellum (Li and Jope, 2010). Unlike most other kinases, GSK3 is constitutively active under basal conditions and it is negatively regulated by the phosphorylation of N-terminal serines, serine-21 in GSK3α and serine-9 in GSK3β (Stambolic and Woodgett, 1994). Phosphorylated-GSK3 (pGSK3) remains inhibited while dephosphorylation of the residue results in the activation (disinhibition) of the kinase.

Both GSK3 isoforms are independently (Liang and Chuang, 2006) involved in a multitude of cellular pathways and in several aspects of the neuronal development such as neurogenesis, neural plasticity, cell survival and neurotransmission (Frame and Cohen, 2001; C A Grimes and Jope, 2001; Hur and Zhou, 2010; Rayasam et al., 2009).

Due to its involvement in numerous neural processes, dysregulation of GSK3 has been hypothesized to play a role in the pathophysiology of neuropsychiatric disorders, such as Alzheimer's disease, schizophrenia and mood disorders (Gould and Manji, 2002; Carol A. Grimes and Jope, 2001).

Findings of animal studies corroborate the hypothesis of GSK3 involvement in mood disorders. Modifications of GSK3 in mice are associated with dysregulated behavior: a reduction in GSK3 activity (GSK3β haploinsufficiency and GSK3α deletion) correlates with an antidepressant like behavior (Kaidanovich-Beilin et al., 2009; O'Brien et al., 2004), while GSK3β overexpressing transgenic mice show hyperactivity mimicking mania in humans (Prickaerts et al., 2006). Furthermore, GSK3 is found to be involved in the mechanism of action of mood stabilizers such as lithium, which acts as a direct and indirect inhibitor of GSK3 (Jope, 2003).

However, studies in vivo using peripheral blood cells have shown mixed results. In comparison to healthy controls, no difference was found in GSK3 expression in peripheral blood mononuclear cells (PBMCs) of drug free bipolar depressive patients, while GSK3β seems to be increased in bipolar mania (Li et al., 2010). GSK3β was found decreased in platelets of bipolar patients but not in patients with major depressive disorder (MDD) (Pandey et al., 2010), while, taking into account only depressed bipolar patients, no difference was found in GSK3β total level compared to healthy controls (de Sousa et al., 2015). Similarly, the levels of GSK3 activity (phosphorylated component) have shown conflicting results in peripheral cells of bipolar patients compared to healthy controls (de Sousa et al., 2015; Li et al., 2007; Polter et al., 2010).

Moreover, there is no study evaluating the role of GSK3 as potential biomarker in the differential diagnosis of mood disorders.

The aims of the present study are:

- to evaluate GSK3 levels in PBMCs of patients with BD, with MDD and in healthy subjects, in order to study its role as a marker of disease and for differential diagnosis;
- to evaluate whether there is a correlation between GSK3 levels and severity of mood disorders symptoms

III.2. Material and methods

III.2.1. Study design

This is a translational cross-sectional observational study. The clinical study was conducted in accordance with the Declaration of Helsinki in its most recent version (64th WMA General Assembly, Fortaleza, Brazil, October 2013). The study was reviewed and approved by the local Ethics Committee (approval code:10893/Tit. 02/ Cat. 06; approval date:02/08/2019).

III.2.2. Participants

Before enrolment all participants received information regarding the study rationale, procedures and implications, and were asked written consent. The clinical sample was recruited among patients admitted to the inpatient unit or referred to the outpatient clinic of the Psychiatry Department of the University Hospital San Luigi Gonzaga of Orbassano, Turin. They were patients with mood disorder, BD or MDD, drug-free since at least four weeks and were assessed through a clinical evaluation and a blood test to evaluate GSK3 levels.

In particular, all participants had to fulfill the following inclusion criteria: a) principal diagnosis of MDD or BD according to the DSM-5 criteria; b) drug-free since at least four weeks; c) for participants with MDD, current major depressive episode (MDE); d)

for participants with BD, any disease phase (MDE, hypomanic/manic episode, euthymia); e) at least 18 years of age.

The following exclusion criteria were considered: a) obesity; b) diabetes; c) thyroid function alteration; d) major unstable medical comorbidity.

In parallel, a control group of unrelated healthy subjects was enrolled.

III.2.3. Clinical Assessment

Socio-demographic and clinical characteristics of the study sample were obtained through the administration of a semistructured interview that we developed and used in previous studies.(Rosso et al., 2015) In addition, the following clinical rating scales were administered: the 21-item Hamilton Depression Rating Scale (HAM-D) (Hamilton, 1967), the Hamilton Anxiety Rating Scale (HAM-A) (Hamilton, 1959), the Young Mania Rating Scale (YMRS) (Young et al., 1978), the Clinical Global Impression (CGI) scale (Guy, 1976).

III.2.4, PBMCs isolation and protein extraction

For each subject, 16-18 mL of whole venous blood was collected in EDTA Vacutainers (BD Biosciences, Milan, Italy). Samples were processed within 40' from collection to avoid degradation of phosphorylated proteins (data not shown). PBMCs were isolated by centrifugation over the density gradient medium Lymphoprep™ (Axis-Shield, Oslo, Norway) and using SepMate™-50 tubes (Stem Cell Technologies), according to the manufactures instructions. 8×10^6 PBMCs were stored at -80°C until protein extraction. PBMCs pellets were lysed on ice-cold lysis buffer (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EGTA) plus protease inhibitor cocktail tablet (Roche, 05892970001) and phosphatase inhibitor cocktail tablet (Roche, PHOSS-RO). The homogenate was maintained on ice for 30 min and then centrifuged at 12.000 RPM for 25 min at 4°C.

The supernatant was collected and the protein concentration was measured in triplicate using the Bradford protein assay.

III.2.5. Western blot analysis

Proteins extracted from human PBMCs (30 micrograms) were separated by 4–12% Bis-Tris precast gel (Life Technologies) and transferred onto nitrocellulose membrane using an iBlot™ dry blotting system (Life Technologies). Membranes were than blocked with EveryBlot blocking buffer (Bio-Rad) for 5 min and then incubated overnight at 4°C with primary antibodies to phospho-Ser9-GSK3β (1:1000, Invitrogen, #MA5-14873), phospho-Ser21-GSK3α (1:1000, Invitrogen, #MA5-15021), Phospho-GSK-3α/β (Ser21/9) (1:1000, Cell Signaling Technology, #9331) and total GSK3α/β (1:1000, Invitrogen, #4G-1E). Immunoblots were developed using horseradish peroxidase–conjugated goat antimouse or antirabbit, followed by detection with enhanced chemiluminescence. Protein bands were quantified with a densitometer. An aliquot of the same protein lysate from Jurkat cell lysate (Millipore, #12-303) was included in each immunoblot to adjust the intensity of membrane exposure.

III.2.6. Statistical Analysis

Subjects' characteristics were summarized as means and SD for continuous variables and as frequencies and percentages for categorical variables.

Shapiro-Wilk test was used to confirm normal distribution of data.

Categorical variables were tested by means of chi-square ($\chi 2$) test. Quantitative variables were compared using T-test for independent samples.

Pearson's correlation coefficient method was used to examine the relationship between GSK3 expression and severity of illness in bipolar and major depressive patients. Analyses were conducted using IBM SPSS Statistics 27. Graphs were produced using GraphPad Prism 8.

III.3. Results

A total group of thirty-two drug-free patients with mood disorders were screened: five patients were excluded from the following analyses for incidental conditions, that might interfere with GSK3: thyroid diseases (n=2), benzodiazepine consumption before the blood collection (n=2); one patient was excluded because of consent withdrawal (n=1). The remaining twenty-seven patients were enrolled in the study. Among mood disorder patients, 15 (55.6%) suffered from MDD while 12 (44.4%) from BD. Thirty-two healthy subjects (HC) were enrolled as a control sample.

The socio-demographic and clinical characteristics of the study sample are shown in Table 1.

Table 1. Baseline socio-demographic and clinical characteristics of the study sample.

Parameters	Healthy Controls (N:32)	Bipolar Disorder (N:12)	Major Depressive Disorder (N:15)
Age, years (mean ± SD)	40.7±12.5	38.3±14.5	52.6±10.9
Sex, *n* (%)			
Male	18 (56.3)	6 (50.0)	4 (26.7)
Female	14 (43.8)	6 (50.0)	11 (73.3)
Other medical conditions, *n* (%)			
Yes	3 (9.7)	3 (25.0)	7 (46.7)
No	28 (90.3)	9 (75.0)	8 (53.3)
Metabolic syndrome, *n* (%)			
Yes	0 (0)	1 (8.3)	1 (6.7)
No	29 (100)	11 (91.7)	14 (93.3)
Duration of illness, years (mean ± SD)	-	15.25±13.0	13.13±13.46
Type of BD, *n* (%)	-		
BD Type I		4 (33.3)	-
BD Type II		5 (41.7)	
BD NOS		3 (25.0)	
Current episode, *n* (%)	-		
Eutimic		2 (16.7)	0 (0)
Depressive		7 (58.3)	15 (100)
Hypo/manic		3 (25.0)	0 (0)
HAM-D Score, (mean ± SD)	2.38±1.2	12.8±7.9	18.7±7.1
HAM-A Score, (mean ± SD)	1.97±1.1	9.9±5.3	13±5.4
YMRS Score, (mean ± SD)	0.06±0.2	9.6±11.4	1.9±1.6
CGI-S Score, (mean ± SD)	1±0.0	4.3±2.0	4.1±0.9

Table 1. BD: Bipolar Disorder. HAM-D: 21-item Hamilton Depression Rating Scale; HAM-A: Hamilton Anxiety Rating Scale; YMRS: Young Mania Rating Scale;
CGI-S: Clinical Global Impression Severity

There were no statistically significant differences between patients with mood disorders and controls in terms of age, gender, medical comorbidities and duration of illness. Patients with BD and MDD were comparable for depression severity, assessed by means HAM-D scores.

We found no statistically significant difference in total and phosphorylated GSK3 levels when comparing HC and patients with MDD, while, in patients with BD, the total GSK3β level was significantly lower compared with HC (Table 2).

Table 2. Total and phosphorylated GSK3 levels: Healthy Controls *versus* patients with BD and Healthy Controls *versus* patients with DDM (t-test).

Parameters	Healthy Controls (N:32)	Bipolar Disorder (N:12)	Major Depressive Disorder (N:15)	*p-value HC vs BD*	*p-value HC vs DDM*
Phosphorylated GSK3α levels, (mean ± SD)	5.997±7.591	10.799±11.377	3.676±1.393	0.112	0.265
Phosphorylated GSK3β levels, (mean ± SD)	1.265±1.241	0.926±0.609	1.469±1.217	0.235	0.609
Total GSK3α levels, (mean ± SD)	0.893±0.458	0.754±0.322	1.016±0.513	0.270	0.446
Total GSK3β levels, (mean ± SD)	1.430±0.613	0.909±0.312	1.472±0.708	**0.008**	0.840

Table 2. BD: Bipolar Disorder; DDM: Major Depressive Disorder; in bold: p<0.005

Comparing patients with MDD relative to BD at any clinical phase (depressed, hypomanic/manic/euthymic), no differences were found in total and phosphorylated GSK3α/β (Table 3).

Table 3. GSK3 levels between patients with bipolar disorder and patients with major depressive disorder.

Parameters	Bipolar Disorder - any episode (N:12)	Major Depressive Disorder (N:15)	t	*p-value*
Phosphorylated GSK3α levels, (mean ± SD)	0.615±0.502	0.776±0.929	-,578	0.569
Phosphorylated GSK3β levels, (mean ± SD)	0.354±0.303	0.456±0.536	-,627	0.537
Total GSK3α levels, (mean ± SD)	1.148±1.025	1.302±0.833	-,421	0.678
Total GSK3β levels, (mean ± SD)	0.596±0.567	0.963±0.563	-1.674	0.106

When comparing GSK3 levels only in patients presenting with a current major depressive episode (7 diagnosed with BD and all 15 patients with MDD), total GSK3α and GSK3β were significantly lower in BD than in MDD (Fig. 1; GSK3α: 0.673±0.360 vs 1.302±0.833, p: 0.023; GSK3β: 0.347±0.256 vs 0.963±0.563, p: 0.013). No differences were found in the phosphorylated component of either GSK3 isoforms.

Fig. 1: GSK3 protein levels in PBMCs extracts of currently depressed patients (MDD and BD).

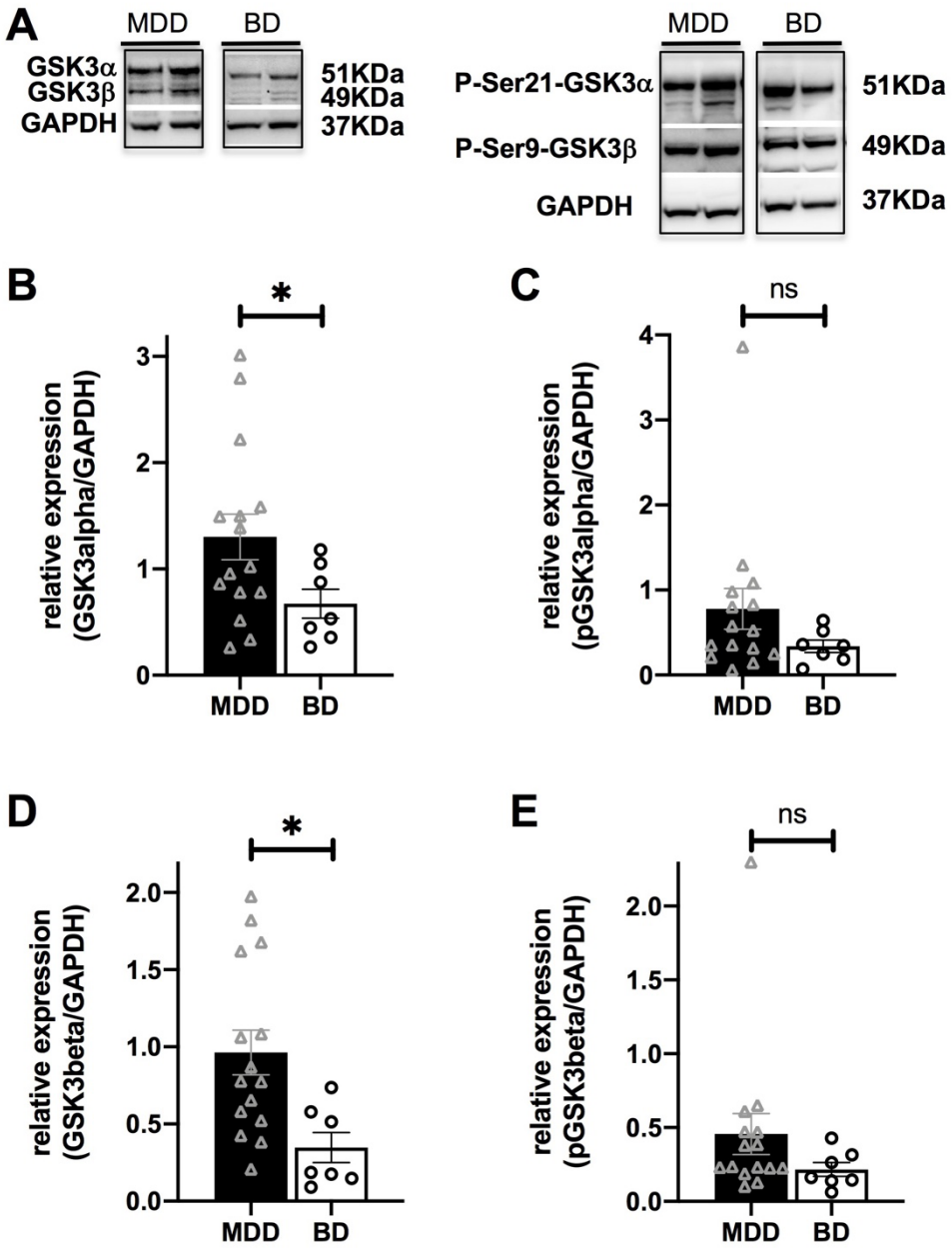

Fig. 1 A. *Representative western blots of phospho-Ser21-GSK3α, phospho-Ser9-GSK3β, and total GSK3α and GSK3β in PBMCs from MDD and BD patients. GAPDH served as loading control.* ***B-E.*** *Densitometric analysis showing significantly lower protein levels of GSK3α and GSK3β in BD (n=7) compared to MDD patients (n=15). The data are expressed as mean ± SEM; * $p < 0.05$ by Student's t-test.*

Regarding the relationship between GSK3 expression and clinical severity, for total GSK3α or GSK3β we did not find any significant correlation with HAM-D, HAM-A, YMRS or CGI-S scores. However, phospho-GSK3β levels in depressive patients (n=22) showed a negative correlation with HAM-D scores (r:-0.441; p: 0.04).

III.4. Discussion

To our knowledge this is the first translational case-control study investigating the levels of both GSK3α and GSK3β in PMBCs of at least 4 weeks drug-free patients with bipolar disorders or major depressive disorder compared to healthy controls. Our main aim was to assess whether GSK3 levels could play a role as a marker of disease and could be useful for differential diagnosis in the early detection of unipolar and bipolar depressive episodes.

Total GSK3β was significantly decreased in PBMCs of patients with BD compared to controls, while no differences were found in the levels of total or phosphorylated GSK3 for patients with MDD. These results corroborate the hypothesis that GSK3 is specifically involved in the pathogenesis of BD, also pointing to it as a possible biomarker. These findings confirm what Pandey and colleagues (Pandey et al., 2010) found by analyzing total GSK3β in platelets of patients with BD and MDD. However, Pandey and colleagues (Pandey et al., 2010) analyzed neither GSK3α nor pGSK3α or β. Similarly, other studies in the literature on PBMCs have focused on only some

aspects: Li and colleagues (Li et al., 2007) evaluated only the beta isoform of GSK3; another study was run only on hypo/manic patients; (Li et al., 2010) while Polter and colleagues (Polter et al., 2010) analyzed only patients with BD. These studies have shown mixed results (Li et al., 2007; Polter et al., 2010), and the patients recruited were either not drug-free (but only lithium-free) or had been drug-free for only a few days, making the results difficult to compare and biased by current drug treatments.

In our clinical sample, serum levels of GSK3α and GSK3β, either total or phosphorylated, were not different between patients with bipolar disorders (all phases) and patients with major depressive disorder. This could be due to higher levels of total GSK3 in hypo/manic episodes compared to bipolar depressive episodes. This is consistent with a previous report of Li (Li et al., 2010). However, considering only the currently depressed subjects among patients with BD, we found levels of total GSK3α and GSK3β to be significantly lower compared to patients with MDD. Since the mean HAM-D scores of patients with bipolar and unipolar depression were similar, this difference cannot be attributed to a difference in the severity of depression. This finding points to total GSK3 as a putative biomarker for differential diagnosis between BD and MDD, helping clinicians in the challenge of differentially identifying acute major depressive episodes in patients with BD versus those with MDD.

The second aim of the present study was to investigate the correlation between GSK3 levels and severity of mood symptoms. We found a correlation between lower levels of phosphorylated-GSK3β and higher HAM-D scores. This is in line with what Polter and colleagues (Polter et al., 2010) previously found for pGSK3α and Montgomery-Asberg Depression Rating Scale analyzing PBMCs of patients with BD. On platelets however, previous studies have found no correlation (de Sousa et al., 2015; Pandey et al., 2010), suggesting that different results may be obtained depending on the cell type analyzed.

Our findings are consistent with the hypothesis of a deregulation of GSK3 activity underlying the pathogenesis of mood disorders, strengthening the concept that a relationship exists between depression and decrease of phosphorylated GSK3β. This suggests that GSK3β might be overactive in patients with depression independently from a reduction of the total component, which seems specific of patients with BD.

III.5. Limitation and strengths

The results of the study should be interpreted in light of some limitations. First of all, GSK3 levels were assessed exclusively in peripheral tissue and further confirmation on brain tissue should be obtained. However, postmortem samples may not be suitable for studying the enzyme since serine-phosphorylation rapidly declines within minutes after death (Li et al., 2005). Therefore, PBMCs proved to be one of the few viable surrogates for studying GSK3, as they share several mechanisms of GSK3 regulation with neurons (Gladkevich et al., 2004; Tsuang et al., 2005). Moreover, the sample size of this study is small and further confirmation in larger samples is needed.

On the other side, our study has some points of strength. Subjects included in our study were enrolled from our emergency department or acute psychiatric unit, making our results applicable to "real-world" patients with mood disorders. Moreover, the inclusion of participants who had been drug-free for at least four weeks without unstable medical conditions ensured that GSK3 levels were not affected by pharmacological treatments and truly represented variations related to disease state. Furthermore, contrary to other studies, we analyzed both GSK3α and GSK3β isoforms increasing the robustness of the results. Finally, to the best of our knowledge this is the first *in vivo* study to assess GSK3 consistently within 40 minutes from venipuncture; in fact, levels of pGSK3 were shown to be unstable in peripheral blood samples, making its measurement unreliable if the extraction occurs later than 40 minutes from sampling.

III.6. Conclusions

A first finding was that GSK3β is decreased in BD patients compared to healthy subjects, supporting the hypothesis that dysregulation in GSK3 might be involved in the pathogenesis and suggesting GSK3 as a biomarker for BD. A second finding regards significantly lower total GSK3 levels in patients in a depressive episode with BD compared to patients with MDD. This finding has important clinical implications as it could be integrated with clinical and other biological information to guide the differential diagnosis of two phenotypically similar clinical conditions (unipolar and bipolar depression). As a third result, the negative correlation found between phosphorylated GSK3β and HAM-D scores suggests that pGSK3β might be a putative marker of disease severity. Studies in larger samples are needed to confirm these results and to find cutoff values of GSK3 levels that maximize the discriminating power of this analysis. In addition, further studies are needed to clarify whether drug treatment and clinical remission may influence GSK3 expression.

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