# CCL11 AND GDF11 LEVELS IN DRUG-NAIVE YOUNG ADULTS WITH BIPOLAR DISORDER

# CCL11 AND GDF11 LEVELS IN DRUG-NAIVE YOUNG ADULTS WITH BIPOLAR DISORDER

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## Descriptive Note

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## Abstract

Bipolar disorder (BD) is a chronic and often progressive illness that has a significant impact on quality of life and functioning. Pharmacological treatments are not effective for all patients, emphasizing the need to better understand the pathophysiology of the disorder. It is well known that patients with BD present with increased levels of inflammatory markers during mood episodes and often exhibit chronic low grade inflammation, implicating the immune system in the etiology of the disorder. Furthermore, patients with BD show deficits in neurotrophic factors suggesting that alterations in neurogenesis may precipitate clinical features. Recent evidence indicates that accelerated aging processes may underlie the pathophysiological changes observed in BD, implicating biomarkers related to aging. The chemokine C-C motif chemokine 11 (CCL11) and the cytokine growth differentiation factor 11 (GDF11) have been identified as proteins that increase and decrease with age, respectively. As such, this thesis presents research examining serum levels of these proteins in drug-naive young adults with BD and a matched healthy control group. We analyzed serum levels of CCL11 and GDF11 using enzyme linked immunosorbent assay (ELISA). Our results indicate that serum levels of CCL11 and GDF11 do not differ between the BD group and the healthy control group, however CCL11 levels were elevated in males and in individuals with tobacco abuse/dependence when considering the entire sample. Our results suggest that serum levels of these proteins do not differ between drug-naive young adults with BD and healthy controls, but that alterations may be due to demographic and lifestyle factors. Small sample size and low power should be considered when interpreting these results.

## Acknowledgements

I would like to thank Dr. Kapczinski for his unwavering support, continual guidance and encouragement throughout my graduate studies. I owe a tremendous thank you to our post-doctoral fellows Dr. Wollenhaupt de Aguiar, Dr. de Azevedo Cardoso and Dr. Pfaffenseller for their constant support, counsel and friendship throughout my time as a graduate student. I would like to especially thank Dr. Wollenhaupt de Aguiar for her mentorship, countless revisions and continued dedication to my success. I would like to thank my committee members, Dr. Frey and Dr. Fahnestock for their encouragement and contributions towards this project. I also thank Dr. Minuzzi for his support and statistical expertise. It has been an honour to work with all of you.

I would also like to acknowledge my family for their constant encouragement and support, and for always believing in me and reminding me that I could succeed during the hardest of times. To my father, for always being my biggest cheerleader, to my mother for uplifting me during the more difficult days, and to my 'little' brother, who served as my role model through his dedication and perseverance towards his own work. I would like to thank my two older sisters for their love and support, and my niece and nephew for always bringing a smile to my face.

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**List of Abbreviations**

| | |
|---|---|
| ALK | Activin receptor-like kinase |
| BD | Bipolar disorder |
| BDNF | Brain-derived neurotrophic factor |
| BMP | Bone morphogenic protein |
| CCL11 | C-C motif chemokine 11 |
| CCR3 | C-C chemokine receptor 3 |
| CRP | C-reactive protein |
| DG | Dentate gyrus |
| GDF | Growth differentiation factor |
| GDF11 | Growth differentiation factor 11 |
| GDNF | Glial cell-derived neurotrophic factor |
| IFN- γ | Interferon gamma |
| IL | Interleukin |
| MDD | Major depressive disorder |
| mtDNA | Mitochondrial DNA |
| NSC | Neural stem cells |

| | |
|---|---|
| SEZ | Subependymal zone |
| SGZ | Subgranular zone |
| SVZ | Subventricular zone |
| TGF- β | Transforming growth factor-beta |
| Th2 | T Helper Cell Type 2 |
| TNF- α | Tumor necrosis factor alpha |

## Declaration of Academic Achievement

This thesis consists of 4 chapters: Chapter 1 provides an overview of bipolar disorder, accelerated aging, inflammation, CCL11 and GDF11; Chapter 2 is a manuscript reporting the analysis of CCL11 which has been submitted to the *Journal of Affective Disorders*; Chapter 3 reports the analysis of GDF11 and discusses potential avenues for further investigation; Chapter 4 discusses the overall results and future directions of this research. Clinical data collection and blood draw took place between the years 2012-2014 as part of the second wave of a large cohort study located in the city of Pelotas in southern Brazil, by students and faculty members from the Department of Health and Behavior, Universidade Católica de Pelotas (UCPel). The original study was conceived and designed by Dr. Karen Jansen, Dr. Luciano Dias de Mattos Souza, and Dr. Ricardo Azevedo da Silva. Participant recruitment was conducted by psychologists, who were graduate students from the Department of Health and Behaviour (UCPel). Participants for our nested study were selected by Dr. Kapczinski, Dr. Wollenhaupt-Aguiar, Dr. de Azevedo Cardoso and I. Dr. Kapczinski, Dr. Wollenhaupt-Aguiar and I conceived and designed the present study involving the analysis of collected blood and participant data. I performed the ELISA with the assistance of Dr. Wollenhaupt-Aguiar and Dr. Pfaffenseller, and analyzed and interpreted the results with the assistance of Dr. Wollenhaupt-Aguiar and Dr. de Azevedo Cardoso.

# CHAPTER ONE

## General Introduction

BD has a profound impact on functioning and quality of life in patients and is a leading cause of disability worldwide. Some patients present with a progressive course of illness, emphasizing the need to understand molecular aspects of the disorder close to illness onset. The underlying neurobiology of BD is not entirely understood, and there are many theories to explain its complex pathophysiology. Over the past decade it has been suggested that an accelerated aging process may underlie some of the biological changes observed. The aim of this thesis is to investigate age-related proteins in drug-naive young adults with BD, to elucidate their involvement in the biology of BD. The inflammatory pro-aging factor CCL11 increases with age and is thought to cause decreases in neurogenesis. As patients with BD exhibit chronic low-grade inflammation and effective pharmacological treatments for BD enhance neurogenesis, CCL11 levels may be increased in this population. The potential role of CCL11 in BD is explored in Chapter 2. Contrarily, GDF11 has been shown to decrease with age and has been reported to increase neurogenesis and angiogenesis. As factors affecting these processes are altered in patients with BD, GDF11 levels may also differ in individuals with this disorder. The potential

role of GDF11 in BD is explored in Chapter 3. This thesis reports our analysis of CCL11 and GDF11 levels in drug-naive young adults with BD.

**Bipolar Disorder**

Bipolar disorder (BD) is a severe chronic illness in which recurrent episodes of (hypo)mania and depression alternate with periods of remission (Tondo et al., 2017). BD affects 2.4% of the general population (Merikangas et al., 2011), having great impact on quality of life, functioning and overall health from a young age (Ratnasingham et al., 2013). Manic and hypomanic episodes are each characterized by a distinct period of abnormal euphoria and/or irritability, coupled with a persistent increase in activity or energy, and often intense goal-directed behaviour (Parker et al., 2018). They differ in their duration, as a hypomanic episode is defined as lasting for a maximum of four days while a manic episode is characterized as lasting at least seven days unless hospitalization is required (Parker et al., 2018). Manic episodes often include psychotic features, while hypomanic episodes do not (Parker et al., 2018). Criteria for a diagnosis of BD type II are a depressive episode (characterized by persistent low mood, loss of interest in activities and sleep disturbances) lasting at least two weeks, and a hypomanic episode (Parker et al., 2018). A diagnosis of BD type I merits a full manic episode but does not require a depressive episode (Parker et al., 2018).

The pathophysiology of BD is not entirely understood, and many theories have emerged to explain its complex neurobiology, such as the allostatic load hypothesis. In 2007, Post et al. suggested that unfavourable outcomes in patients with BD could be due to a failure of compensatory mechanisms that would usually minimize the impact of mood states on central

nervous system function (Post et., al 2007). This model was further expanded by Kapczinski et al., (2008) to the allostatic load hypothesis of BD. It is suggested that over time, the stress of activating homeostatic mechanisms after mood episodes to regain normal function eventually exceeds the ability to maintain homeostasis (Kapczinski et al., 2008; George et al., 2008). Allostatic load has been referred to as the 'wear and tear' on the brain and body from continued overactivity that is involved in the adaptation to environmental challenges, such as mood episodes (McEwen 2004). It is theorized that the chronic overactivity of these mechanisms leads to a persistent state of physiological dysregulation, which can be detected through markers of inflammation and oxidative stress (Berk et al., 2011; Kapczinski et al., 2008). As such, this overactivity leaves individuals with BD at increased vulnerability to stress and physiological dysregulation, increasing the likelihood of further mood episodes. BD follows a progressive course in about 50% of cases (López-Villarreal et al., 2019), in which an increasing number of mood episodes is associated with increasing (i) the risk of recurrence of episodes, (ii) the length of episodes, (iii) the severity of episodes, and (iv) the risk of long-term cognitive deficits (Kessing & Anderson, 2017). Other studies have shown that multiple mood episodes are associated with poorer response to treatment and increased functional impairment (Rosa et al., 2010).

The onset of BD generally occurs during adolescence and young adulthood (Joslyn et al., 2016), however many studies investigating its pathophysiology have not focused exclusively on this population. As BD can be a progressive illness, it is important to understand its underlying biology around the time of illness onset. An improved understanding of BD at its early stages could facilitate the development of novel early intervention strategies, and ultimately improve

patient outcomes by halting illness progression.

**Accelerated Aging in Bipolar Disorder**

In recent years, it has been suggested that the pathophysiology of BD may be associated with an accelerated aging process (Rizzo et al., 2014; Fries et al., 2020). While chronological age is determined by calendar units, biological age is the result of accumulated damage to various cells and tissues in the body over the lifespan (Rose et al., 2012). The neurobiology of aging and BD bear similarities, broadly including the progressive deterioration of overall homeostatic brain mechanisms, accompanied by cognitive decline (Budni et al., 2015).

It is hypothesized that environmental factors interact with multiple biological systems to induce accelerated aging in patients with BD (Fries et al., 2020). The biological changes that occur during aging are frequently referred to as 'senescence', a concept used to explain the limited capacity of cells to replicate (Rizzo et al., 2014). Shortened telomeres are considered to be a reasonable indication of cellular senescence and have been thoroughly investigated as a marker of aging in BD (Fries et al., 2020). Many studies have shown increased telomere shortening in patients with BD relative to healthy controls, indicating a potential accelerated aging process (Barbe-Tuana et al., 2016; Lima et al., 2015; Elvsashagen et al., 2011; Simon et al., 2006). Yet it is worth noting that there have been conflicting results on this matter (Colpo et al., 2015). Nonetheless, studies have shown that medications used to treat BD (particularly lithium) are protective against the effects of aging. For example, in a study exploring telomere length in the context of lithium treatment found that BD type I lithium responders had longer telomere lengths than non-responders (Martinsson et al., 2013). It was also found that treatment

with lithium led to a decrease in DNA methylation, which has also been associated with aging (Fries et al., 2017). Many studies have also shown that other factors including oxidative stress, DNA methylation, inflammation, genetics and mtDNA copy number are altered in patients with BD compared to age-matched controls (Fries et al., 2020). It is hypothesized that several stimuli including chronic stress, mood episodes and lifestyle habits interact with biological systems in our bodies and cause alterations in these parameters (Figure 1).

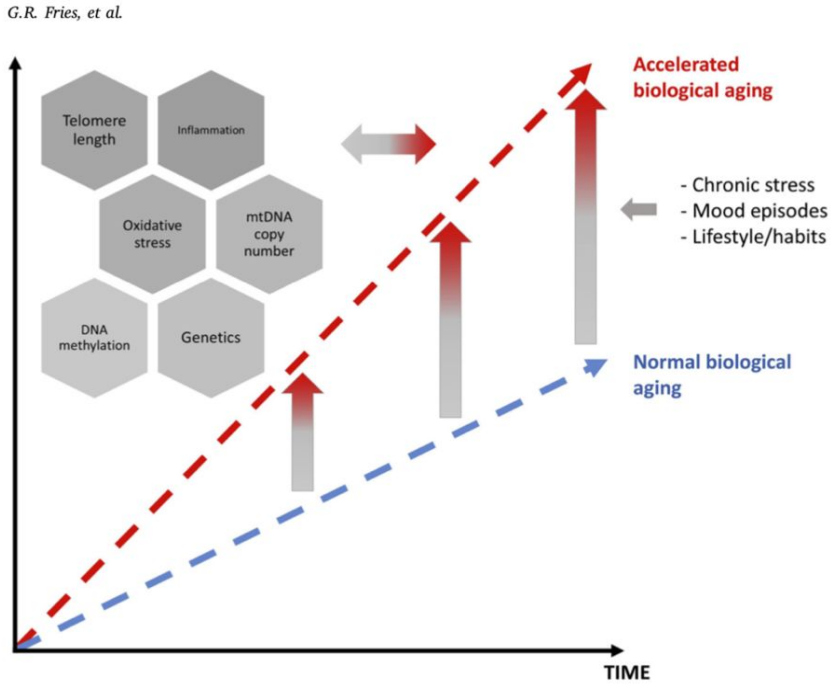


Figure 1: Environmental factors such as lifetime chronic stress exposure, mood episodes, and lifestyle factors and habits (such as diet, smoking, exercise and others) may contribute to acceleration of the normal aging process and promote a premature aging phenotype. Such premature aging processes can be detected through biomarkers such as telomere length, inflammatory molecules, oxidative stress, mtDNA copy number, DNA methylation, and genetic markers, which may also interact with environmental factors to further foster biological aging

processes. The interaction of these biological mechanisms and environmental factors is believed to contribute to the premature aging in patients and ultimately speed up the onset of age-related clinical signs and symptoms. (Fries et al., 2020).

*Clinical evidence for accelerated aging*

The clinical evidence supporting accelerated aging processes in BD is worthy of attention. Studies have shown that patients with BD present with earlier onset of cognitive decline, executive function and functional impairment, suggesting that they are likely to experience the cognitive effects of aging earlier than matched controls (Seelye et al., 2019; Lewandowski et al., 2014; Cacilhas et al., 2009). There is a higher prevalence and earlier age of onset in age-related medical conditions such as cardiovascular disease, hypertension, metabolic imbalances, autoimmune disorders and cancer, which account for a decreased life span of up to 12 years (Rizzo et al., 2014; Kessing et al., 2015). Additionally, changes in brain structure commonly associated with aging such as loss of grey matter in the hippocampus and the cerebellum, decreased volume of prefrontal cortex and ventricular enlargement have been found in patients with BD compared to age matched controls (Roda et al., 2015).

There is growing evidence to suggest that individuals with BD undergo an accelerated aging process. For example, alterations in brain structure manifest earlier in individuals with BD than in age-matched controls (Mohite et al., 2020). However, there are still factors that remain relatively unexplored in this context in BD, particularly in younger populations. While it is known that biological changes present themselves in general adult populations with BD, less is known about the effects of BD on the physiology of younger adults specifically. Particularly,

age-related factors have scarcely been explored in younger populations with BD.

It is interesting to note the continued evolution in the understanding of the pathophysiology of bipolar disorders. In 1992, a renowned researcher in the field of BD, Dr. Robert Post, published a theory that was not confirmed later on, but it helped in the development of the neuroprogression hypothesis. In Dr. Post's hypothesis, it is suggested that BD would have a progression similar to what is seen in patients with epilepsy, for instance, where a sufficient number of repetitions of full-blown seizures will eventually result in the appearance of spontaneous epilepsy. Post (1992) postulated that life stressors may leave long-term vulnerabilities, which could lower the threshold of stress exposure required for triggering a mood episode. Of note, Post (1992) developed a parallel of the kindling model, used in epilepsy, and mood disorders. Later on Post and Kalivas (2013) updated this theory, making it clearer that it was not kindling the main biological underpinning of mood disorders. Rather, Post and Kalivas (2013) postulated that the long-term persistence of the sensitization effects could be an important underlying factor linking mood disorders to trauma and cocaine abuse. The fact that cocaine use is a predisposing factor to conversion to BD was recently confirmed by de Azevedo Cardoso et al., 2020.

**Inflammation in aging and Bipolar Disorder**

Inflammation is a physiological defence process that repairs tissues in response to endogenous or exogenous aggressions (Michaud et al., 2013). The immune system involves many cell types and is present in nearly all tissues of the body. While acute inflammatory responses are an integral part of the body's defence system, they can become pathogenic when

activated in excess (Goldstein et al., 1992). When acute inflammatory responses persist, additional defence components are mobilized to create long-term unresolved immune responses known as chronic inflammation (Chung et al., 2019). Importantly, this low-grade chronic inflammation is associated with changes in cell death signalling pathways including caspase pathways (Opdenbosch & Lamkanfi 2019), and the TNF-α pathway (Annibaldi et al., 2018). During aging, the immune response becomes dysregulated, leading to a chronic systemic inflammatory environment (Chung et al., 2019). It is reported that this wide-spread dysregulation during aging is due to the infiltration of excess macrophages into multiple body tissues including tissues of the liver, heart, kidney, adipose and brain - ultimately propagating inflammation all over the body (van Deursen 2014). Modifications of the immune system associated with aging are known as immunosenescence, which can be broken down into the dysregulation of pro-inflammatory mediators, cytokines, and chemokines (Ventura et al., 2017). Inflammatory mediators are released by immune cells and serve as messengers to promote inflammatory responses (Abdulkhaleq et al., 2018). These messengers include vasoactive amines and peptides, eicosanoids, acute-phase proteins and cytokines (Abdulkhaleq et al., 2018). Cytokines serve various functions such as the activation of neutrophils, proliferation of β cells, synthesis of acute phase proteins, and increasing vascular permeability (Muneer 2015), while chemokines are a subset of cytokines, and work to control cell migration and residence of immune cells (Palomino & Marti 2015). Cumulatively, the activation of these mechanisms results in wide-spread apoptosis resulting in tissue damage. As aging can be defined as physiological deterioration over time, it can be inferred that this decline is due to the disruption in cell death signalling pathways caused by inflammatory processes.

It is well known that immunological abnormalities are involved in the pathophysiology of BD. Chronic stress experienced during mood episodes can lead to microglial activation, causing the release of pro-inflammatory cytokines and the recruitment of peripheral immune cells to the brain, creating an inflammatory environment (Naaldijk et al., 2016). This inflammatory environment leads to widespread cell death and neuronal dysfunction, ultimately impacting overall brain function. Disturbances in immune function have also been highly associated with the severity and recurrence of mood episodes and illness progression (Tatay-Manteiga et al., 2017; Kauer-Sant'Anna et al., 2009). As such, mood episodes have been described as pro-inflammatory states (Rosenblat et al., 2017; Modabbernia et al., 2013). Previous studies have reported increased peripheral levels of cytokines such as IL-2, IL-4, IL-6, IL-1 β and TNF-α in patients with BD during manic episodes, and IL-6 and TNF-α during depressive episodes, in comparison to euthymic patients and healthy controls (Rosenblat et al., 2017; Modabbernia et al., 2013; Brietze et al., 2009; Kim et al., 2007). Additionally, a known marker of inflammation, CRP, has also been associated with the severity of manic episodes (Goldstein et al., 2011). It was also reported that CRP levels were elevated in treatment-naive BD Type 1 patients in manic episodes compared to controls, and decreased following pharmacological treatment (Uyanik et al., 2015).

Effective pharmacotherapies for BD target inflammation. Commonly used therapeutic drugs for BD include lithium and valproic acid, which have been shown to decrease peripheral markers of inflammation (Basselin et al., 2010; Kim et al., 2007; Boufidou et al., 2004). Treatment with lithium in patients with BD was shown to decrease IL-6 production (Knijff et al., 2007), and patients with BD under chronic lithium treatment had significantly lower numbers of

IL-2, IL-6, IL-10 and IFN- γ secreting cells compared to healthy controls (Boufidou et al., 2004). Additionally, a study showed that TNF-α levels were elevated in patients with poor lithium response compared to those with good response (Guloksuz et al., 2012). Anti-inflammatory pharmacotherapies have also been investigated as potential treatments for bipolar depression including nonsteroidal anti-inflammatory drugs, omega-3 polyunsaturated fatty acids, N-acetylcysteine and pioglitazone (Rosenblat et al., 2016). In this meta-analysis, the overall effect of adjunctive anti-inflammatory agents on depressive symptoms was moderate and statistically significant (Rosenblat et al., 2016), which highlights the relevance of further understanding the role of inflammation in the pathophysiology of BD.

**Proteins of Interest**

*CCL11*

As a chemokine, CCL11 induces the recruitment of leukocytes to inflammatory sites, driving inflammation (Charo et al., 2006). CCL11 is also known as eotaxin-1 and is a member of the eotaxin family that activates the G protein-coupled receptor CCR3 present on eosinophils, Th2 lymphocytes, basophils and mast cells (Teixeira et al., 1997; Kitaura et al., 1996; Teixeira et al., 2018). The activation of CCR3 induces eosinophils in particular, stimulating chemotaxis, and driving allergic responses which promote inflammation (Matthews et al., 1998). CCL11 also binds to CCR3 receptors on Th2 lymphocytes which in turn secrete inflammatory cytokines, further propagating the inflammatory response (Ohshima et al., 2002).

In the periphery CCL11 is secreted by epithelial cells, fibroblasts and smooth muscle cells, while in the central nervous system it is secreted by astrocytes, microglia and choroid

plexus epithelial cells under inflammatory stimuli (Teixeira et al., 2018; Huber et al., 2016). CCL11 also crosses the blood-brain barrier, allowing penetration into the brain from peripheral sources (Erickson et al., 2014). In the central nervous system, multiple cells including microglia, astrocytes, oligodendrocytes and choroid plexus epithelial cells express CCR3 receptors (Huber et al., 2016) (Figure 2).

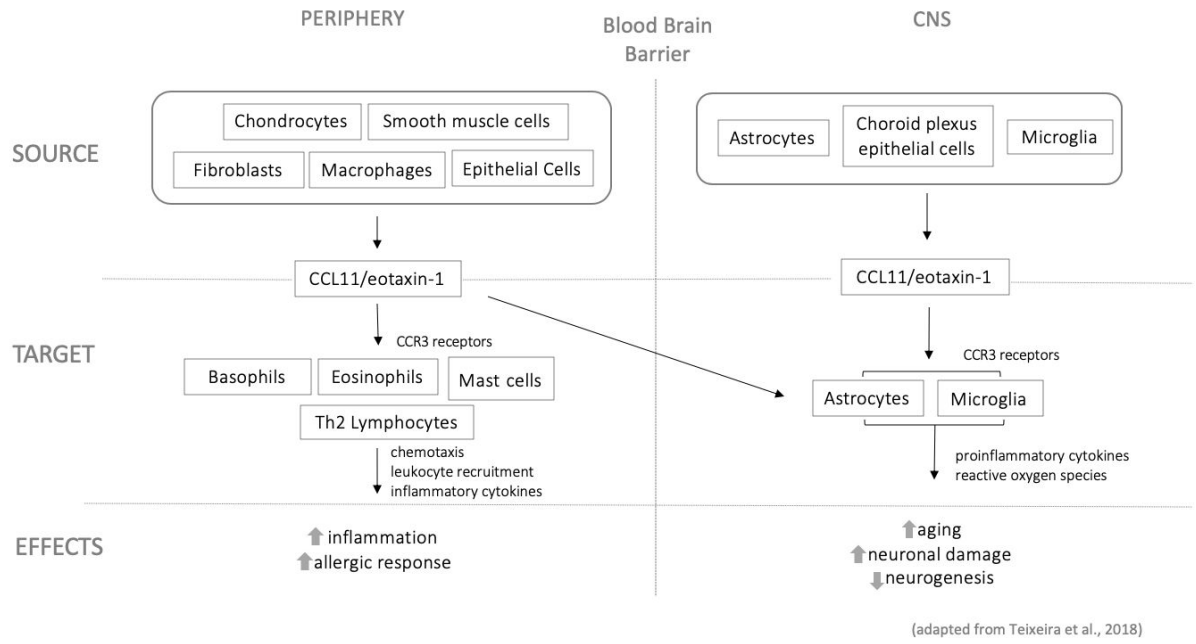


**Figure 2:** Schematic overview of the effects of CCL11/eotaxin-1 in adult subjects. The main sources, targets and effects of CCL11 are shown in the periphery and in the CNS. In the periphery, CCL11 secretion leads to the activation of inflammatory pathways and allergic responses, while in the CNS CCL11 secretion results in aging, neuronal damage and decreased neurogenesis. CCL11 from the periphery can cross the blood-brain barrier.

An overabundance of CCL11 may promote inflammation that is greater than necessary, perhaps contributing to the prevalence of chronic, low-grade inflammation found in psychiatric disorders such as BD (Teixeira et al., 2018).

CCL11 has also been investigated in models of aging. It is known that aging results in a progressive decline in neurogenesis and adult neural progenitor cells, with coinciding impairments in cognitive function (Villeda et al., 2011). Systemic levels of CCL11 increase with age (Hoefer et al., 2017), suggesting a role of CCL11 in this precipitous decline. This relationship was investigated in a heterochronic parabiosis model where it was demonstrated that the plasma of aging mice (containing higher levels of CCL11) decreased synaptic plasticity and impaired contextual fear conditioning as well as spatial learning and memory in young mice (Villeda et al., 2011). Increasing peripheral levels of CCL11 in young mice also decreased neurogenesis and impaired learning and memory (Villeda et al., 2011). These findings suggest a role of CCL11 in age-related cognitive decline. CCL11 was also found to be negatively associated with cognitive performance in a sample of adult humans who lived in a rural setting (Butcher et al., 2018). As there is evidence to suggest that CCL11 increases with age, levels may be altered in patients with BD due to the proposed accelerated aging process (Hoefer et al., 2017; Rizzo et al., 2014).

Chemokines were first investigated in psychiatry in patients with schizophrenia, where increased levels of CCL11 in relation to other chemokines were reported (Teixeira et al., 2008). Following this breakthrough, these findings were extended to hypothesize the role of CCL11 as a biomarker for other psychiatric illnesses. Increased levels of the inflammatory cytokine CCL11 have been reported in euthymic late-stage patients with BD compared to age-matched controls,

as well as across mood states and in patients with BD Type I (Panizzutti et al., 2015; Magalhaes et al., 2014; Barbosa et al., 2013). Apart from the study conducted by Magalhaes et al., these studies focused on either late-stage patients or middle-aged adults. Serum levels of CCL11 were elevated in medicated young adults with BD (Magalhaes et al., 2014), however, CCL11 levels have not been assessed in a drug-naive young adult population. In a different sample, Magalhães et al. 2014, found that CCL11 levels were elevated in medicated young adults. Then, to further refine the interpretation of these findings, we set forth to investigate whether drug-naive young adults with BD would also present increased CCL11 levels. There is evidence that medications used to treat BD may change peripheral levels of inflammation (Rosenblat & McIntyre 2016). Thus, it is important to know whether these changes also take place among drug-naive subjects. Additionally, there is evidence to suggest that levels of CCL11 increase with age (Hoefer et al., 2017). As patients with BD may undergo accelerated aging, it is predicted that levels would be increased among young adults with BD.

*GDF11*

Adult neurogenesis occurs in neurogenic niches, primarily in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus (Alvarez et al., 2004; Gage et al., 2000). Cues within the neurogenic niche are believed to drive the synthesis of new neurons, contributing to cognitive processes such as learning and memory through their integration into the neurocircuitry of the brain (Deng et al., 2010; Saxe et al., 2006). The neurogenic niche is concentrated around blood vessels, permitting communication with the systemic environment **(**Shen et al., 2004). It is known that age-related cognitive decline and

neurogenesis are associated with reduced blood flow and number of Neural Stem Cells (NSC)s (Katsimpardi et al., 2014). As such, diminished neurogenesis during aging may be modulated by factors circulating in the blood. Thus, increasing blood flow to the neurovascular niche has the potential to attenuate these processes. In recent years, GDF11 has been identified as a potential agent responsible for this modulation. Contrary to CCL11, GDF11 is prevalent in young blood, and levels decrease with age (Poggioli et al., 2016).

In humans, GDF11 is expressed in the majority of organs and tissues (Uhlen et al., 2015). The highest level of expression takes place in the hippocampus, while the lowest is in the liver (Uhlen et al., 2015). In the periphery, GDF11 is primarily secreted by lymphocytes, macrophages, endothelial cells and fibroblasts (Uhlen et al., 2015), while in the nervous system it is secreted by neurons, astrocytes and ependymal cells (Rochette et al., 2019). It plays a prominent role in the development of many tissues and processes including embryonic development, the spinal cord, retinal development and pancreatic development. It also plays a key role in skeletal formation (Jamaiyar et al., 2017). GDF11 is part of the transforming growth factor-beta (TGF-β) superfamily of proteins. TGF-β superfamily members include TGF-βs, bone morphogenic proteins BMPs), growth and differentiation factors (GDFs), and activins (He et al., 2014; Krieglstein et al., 2011; Attisano et al., 2002). The cell-surface receptor for the TGF-β family signal cascade is a complex of transmembrane receptors containing an intracellular kinase domain that phosphorylates serine and threonine residues (Attisano et al., 2002). GDF11 exerts its function by interacting with type IIA and IIB activin receptors and binding with activin-like receptor kinase (ALK) ALK4, ALK5 and ALK7 (Ozek et al. 2018; Jamaiyar et al., 2017), inducing the phosphorylation of SMAD2/3 (He et al. 2014). It acts predominantly through ALK4

and ALK5 receptors (Jamaiyar et al., 2017). Notably, genetic activation of the ALK5 receptor improved cognition, neurogenesis, neuronal activity, and synaptic plasticity in the hippocampus of old mice (He et al. 2014). SMAD2/3 further activates co-SMAD4, which has a resulting influence on gene expression. This signalling cascade is thought to up-regulate pro-angiogenic and pro-neurogenic factors through a positive influence on the cerebral vasculature (Schafer et al., 2019; He et al., 2014; Krieglstein et al., 2011; Attisano et al., 2002) (Figure 3).

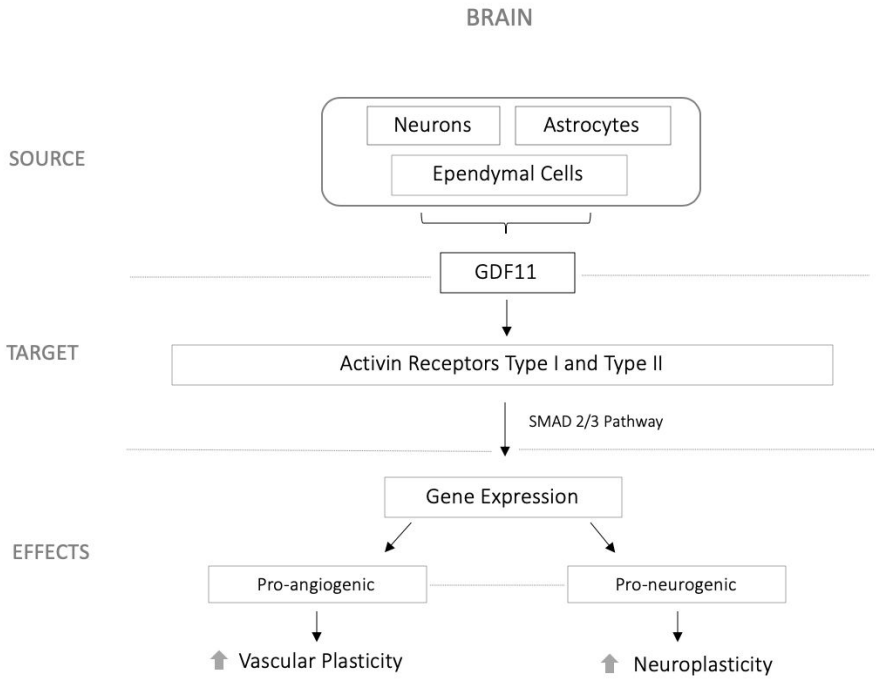


Figure 3: Schematic overview of the sources, targets and effects of GDF11 in the brain. GDF11 is secreted from neurons, astrocytes and ependymal cells and acts on activin receptors (activin type I: activin-like receptor kinase (ALK) 4, ALK5, ALK7, and activin type II: ActRIIA, ActRIIB), influencing gene expression leading to pro-angiogenic and pro-neurogenic processes.

For example, it was found that systemic injection of GDF11 induced vascular remodelling in aged mice (Katsimpardi et al., 2015). There is also evidence to suggest that GDF11 acts directly on endothelial cells, as in vitro treatment with GDF11 caused an increase in endothelial proliferation (Katsimpardi et al., 2014).

It was reported that in a parabiosis model, the exposure of an aged animal to young blood can counteract and reverse the effects of aging at the molecular, structural, functional, and cognitive levels (Villeda et al., 2011). The study conducted by Ozek et al. further investigated the role of GDF11 specifically in a heterochronic parabiosis model. The results showed that systemic GDF11 treatment enhanced neurogenesis, improved vasculature, and increased neuronal activity markers in the hippocampus of old mice (Ozek et al., 2014). Also, a study using injected GFD11 intraperitoneally was able to reduce markers of oxidative stress, including levels of advanced glycosylation end products, protein oxidation and lipid peroxidation in rodents (Loffredo et al., 2013). In addition, a recent study showed that GDF11 can restore age-related deficits in both neurogenesis and cerebral vasculature in the aged brain (Katsimpardi et al., 2014 & 2015).

Although research pertaining to GDF11 and aging continues to emerge, it has yet to be investigated in the context of BD or any other psychiatric disorder. Due to the proposed accelerated aging processes in BD, it is possible that GDF11 levels may be altered in patients with BD. It can be hypothesized that patients with BD would have lower levels of GDF11 compared to controls.

**Aims and Hypotheses**

Premature aging in BD is indicated by alterations in inflammatory markers as well as clinical factors including early cognitive deficits, structural brain changes and the early onset of age-related medical conditions. While this evidence supports accelerated aging in BD, there are still factors that remain relatively unexplored, particularly in young populations. The majority of studies pertaining to accelerated aging in BD have focused on older populations, however it remains unknown if accelerated aging is prominent at an earlier age. The aforementioned proteins CCL11 and GDF11 have been explored as factors related to aging, however they have seldom been explored in the context of BD, especially in a young population. Additionally, these proteins have not been explored in a drug-naive population.

The objective of this thesis was to examine whether there are differences in CCL11 and GDF11 levels between drug-naive young adults with BD and healthy controls. As CCL11 is considered a pro-aging factor, we hypothesized that serum levels would be higher in young adults with BD compared to controls. This is reported in Chapter 2 in the form of a manuscript submitted to the *Journal of Affective Disorders*, titled "CCL11 in drug-naive bipolar patients: the role of sex and smoking status". Conversely, as GDF11 levels decrease with age, we hypothesized that serum levels would be lower in our sample compared to the control group, considering the proposed accelerated aging in BD. This study is presented in Chapter 3.

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## CHAPTER TWO

## CCL11 levels in drug-naive bipolar patients: the role of sex and smoking status

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**Abstract**

**Background:** It has been reported that patients with bipolar disorder (BD) present with changes in peripheral levels of the inflammatory cytokine CCL11, particularly at late stages. The aim of this study is to evaluate CCL11 levels in a population-based sample of drug-naive young adults.

**Methods:** This is a cross-sectional study nested within a large population-based study. 29 drug-naive young adults with BD and 29 controls selected from this cohort were matched for sex, age, and years of education. The diagnosis of psychiatric disorders was performed using the Mini International Neuropsychiatric Interview PLUS. Serum levels of CCL11 were measured using sandwich ELISA. Independent samples T-test was used to assess differences in CCL11 levels between groups. Multivariate linear regression analyses were used to evaluate the effect of independent factors on CCL11 levels.

**Results:** There were no demographic differences between individuals with BD and controls. No significant differences were found in CCL11 levels between groups. The final multivariate model showed that the variables that remained independently associated with higher CCL11 levels were male sex (B:41.41 [CI95%: 15.66 - 67.15], p=0.002) and tobacco abuse/dependence (B: 22.71 [CI95%: 1.28 - 44.15], p=0.038).

**Conclusions:** The present study suggests that there is a possible influence of sex and tobacco abuse/dependence on CCL11 levels, as male sex and smoking status were associated with higher CCL11 levels in our sample. It also suggests that peripheral levels of CCL11 may not be involved in the pathophysiology of BD at early stages of the disorder.

**Keywords:** bipolar disorder; eotaxin-1; CCL11; biomarkers; sex; smoking

## Introduction

Bipolar disorder (BD) is a chronic psychiatric disorder characterized by episodes of (hypo)mania and depression interspersed with periods of remission (Tondo et al., 2017), which affects about 2.4% of the population worldwide (Merikangas et al., 2011). BD has a substantial impact on quality of life (Jansen et al., 2013) and functioning (Jansen et al., 2012) even among youth. The underlying neurobiology of BD remains unclear, and current pharmacological treatments are often insufficient to maintain remission and sustain quality of life (Dimitrakopoulos et al., 2015). Importantly, studies have shown that a subset of patients with BD follow a progressive course of illness (Gama et al., 2013). An increasing number of mood episodes is associated with an increased risk of episode recurrence, severity of episodes, and long- term cognitive deficits, emphasizing the need for intervention during early stages of the disorder (Kessing & Anderson 2017). In this context, further understanding the pathophysiology of early-stage BD is paramount to improving treatment strategies and ultimately patient outcomes.

Changes in immunological markers have been associated with the severity and recurrence of mood episodes as well as illness progression (Berk et al., 2011). Mood episodes have been referred to as pro-inflammatory states based on reports of increased peripheral levels of pro-inflammatory cytokines such as IL-6 and TNF-α compared to controls (Benedetti et al., 2020). Among these cytokines is CCL11, or eotaxin-1, which is associated with allergic and

inflammatory responses (Amerio et al., 2005), and more recently it has been implicated in the aging process and psychiatric disorders (Teixeira et al., 2018).

Increased levels of CCL11 have been correlated with telomere length, brain structure and cognitive functioning in schizophrenia (Czepielewski et al., 2018; Sirivichayakul et al., 2019). Additionally, recent studies showed that CCL11 levels are associated with accelerated aging (Panizzutti et al., 2015) and brain volumetric changes in BD (Mohite et al., 2020). An imbalance of CCL11 levels may over stimulate the synthesis of inflammatory markers, serving as a possible contributor to the chronic low-grade inflammation observed in BD (Teixeira et al., 2018). Increases in CCL11 levels have been reported across mood states in BD (Magalhaes et al., 2014), in late-stage patients with BD (Panizzutti et al., 2015) and in patients with BD type 1 (Barbosa et al., 2013). Altogether, the aforementioned evidence suggests that CCL11 levels might be involved in an accelerated aging process in BD. However, CCL11 has not been investigated in drug-naive individuals or in early-stage population-based samples, which are not as much biased by multiple interventions as clinical samples usually are. Thus, the aim of this study is to assess the factors associated with CCL11 levels in a community sample of drug-naive young adults with BD and matched controls.

## Methods

### Study design and participants

This is a cross-sectional study with a matched sample of drug-naive young adults, nested within a large population-based study. The recruitment was conducted in the city of Pelotas located in southern Brazil. (Full details of the original study have been published by Jansen et al.,

2011, and Moreira et al., 2016.) For this nested study, we selected drug-naive subjects with BD (n=29) from the second wave of the population-based study which took place between the years 2012 and 2014. The inclusion criteria for the BD group were: (1) being diagnosed with BD and (2) self-reported no lifetime psychiatric medication use (drug-naive status). A healthy control group was also recruited (n=29) from the second wave of the population-based study. The inclusion criteria for the healthy control group were: (1) do not have a diagnosis of mood disorders (Major Depressive Disorder or BD), (2) do not have a diagnosis of anxiety disorder (panic disorder, agoraphobia, social phobia, specific phobia, or Generalized Anxiety Disorder), (3) do not have a diagnosis of Obsessive Compulsive Disorder, (4) do not have a diagnosis of Posttraumatic Stress Disorder, (4) do not have a diagnosis of Attention Deficit Hyperactivity Disorder, (5) do not present as a current suicide risk, (6) do not have lifetime psychotic symptoms, (7) do not present with current abuse/dependence of illicit drugs, and (7) self-reported no lifetime psychiatric medication use. Participants were matched by sex, age, and years of education. The age range for participants in this selected sample was 23-30 years old.

**Clinical assessments**

The diagnosis of psychiatric disorders was performed using the Mini International Neuropsychiatric Interview – PLUS (MINI-PLUS) (Sheehan et al., 1998). The Montgomery–Åsberg Depression Rating Scale (MADRS) (Davidson et al., 1986) and the Young Mania Rating Scale (YMRS) (Vilela et al., 2005) were used to assess depressive and manic symptoms, respectively. All assessments were administered by masters and Ph.D. level trained psychologists. The Alcohol, Smoking, and Substance Involvement Screening Test (ASSIST) was

used to evaluate tobacco and other substances abuse/dependence (Ferraz et al., 2004). The cut-off for abuse or dependence was 4.

The study was approved by the Research Ethics Committee of the Universidade Católica de Pelotas (UCPel), under the protocol number 2008/118, and all subjects provided their written informed consent before inclusion in the study.

**CCL11 Assay**

Ten milliliters of blood was withdrawn from each subject by venipuncture into an anticoagulant-free vacuum tube after the interview. Serum was separated within 2 hours by centrifugation at 4000xg for 15 minutes and was kept frozen at -80 °C until biochemical analysis.

CCL11 serum levels were measured using a commercial sandwich ELISA kit according to the manufacturer's instructions (Invitrogen – Catalogue # KAC2231, Massachusetts, USA), and results are expressed in pg/mL. Briefly, 50 uL incubation buffer was added to the wells, followed by the addition of 50 uL of sample or standards to the appropriate wells, and the wells for chromogen blanks were kept empty. The standard curve of CCL11 ranged from 0 pg/mL to 500 pg/mL. After an overnight incubation at 4°C, a biotinylated anti-CCL11 solution (biotin conjugate) was added to each well except for the chromogen blanks, which control for background signal. The plates were then incubated for 2 hours at room temperature, followed by four washes with a wash buffer. Streptavidin peroxidase at the working dilution was added to each well except for the chromogen blanks. Plates were then incubated for 30 minutes at room temperature. Finally, plates were washed again and incubated with the Stabilized Chromogen

(substrate solution) in all wells for 30 minutes, followed by the stop solution. CCL11 levels were determined by absorbance at 450 nm in a microplate reader (Epoch™ 2 Microplate Spectrophotometer, BioTek, Vermont, USA), and the background signal obtained from the chromogen blank was subtracted. The assay was run one time, with a total of 77 wells analyzed (8 standards in duplicate, 58 samples in simplicate and chromogen blanks in triplicate).

**Statistical Analysis**

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 26.0. Independent Samples *t*-test and Pearson correlation were used for parametric variables, and Spearman correlation and Mann Whitney U test were used for non-parametric variables. Fischer's Exact Test was used for qualitative variables. The quantitative variables were expressed as mean ± standard deviation or median and interquartile range (25th–75th percentiles) according to the samples' distribution. Multivariate linear regression models were performed to evaluate the effect of independent factors on the CCL11 levels. The initial model included variables associated with CCL11 levels at $p<0.20$ in the bivariate analysis. BD diagnosis was defined to be included *a priori* and kept in the model due to its importance in the literature. A final model was reached using a manual stepwise removal of each non-statistically significant variable (except BD, which was included *a priori*). Statistical significance was set at $p < 0.05$.

## Results

The sample was composed of 58 drug-naive individuals: 29 with BD and 29 healthy controls. The sample was 79.3% female, with a mean age of 25.81±1.97, and the mean years of education was 10.31±2.66. The sample was successfully matched and there were no significant differences between groups for these characteristics as described in Table 1a.

**Table 1.** Clinical and sociodemographic characteristics between the groups.

| | **Healthy Controls n= 29** | **Bipolar Disorder n=29** | ***p-value*** |
|---|---|---|---|
| **Age (years)** | 25.90±1.90 | 25.72±2.07 | 0.742[a] |
| **Sex (F)** | 79.3% (23) | 79.3% (23) | 1.000[b] |
| **Years of education** | 10.76±2.36 | 9.86±2.91 | 0.203[a] |
| **MADRS (score)** | 0.00 (0.00; 1.00) | 10.00 (2.00; 16.00) | <0.001[c] |
| **YMRS (score)** | n/a | 4.00 (2.00; 7.50) | |
| **Tobacco abuse/dependence** | 34.5% (10) | 55.2% (16) | 0.186[b] |

**Legend:** Age, years of education: data is shown as mean ± standard deviation. Data is presented % (N) for categorical variables. MADRS score: data is shown as median and interquartile range (25th and 75th percentiles). MADRS: Montgomery–Åsberg Depression Rating Scale; YMRS: Young Mania Rating Scale; F: female.

[a] = Independent Samples t-Test, [b] = Fisher's Exact Test, [c] = Mann-Whitney U test.

From our bivariate analysis, we were able to determine that higher CCL11 serum levels were associated with male sex (p=0.003) and showed a positive trend with tobacco abuse/dependence (p=0.073) in our sample, regardless of diagnosis. There was no significant association between CCL11 levels and age (p=0.096), years of education (p=0.913) or MADRS

score (0.497). In addition, there was no significant difference between the BD group and the control group in serum levels of CCL11 (p = 0.957) (Table 2a).

**Table 2a.** Clinical and sociodemographic characteristics associated with CCL11 levels.

| | **CCL11 Levels** | ***p-value*** |
|---|---|---|
| **Sex** | | 0.003[a] |
| Male | 121.47±44.09 | |
| Female | 80.97±39.53 | |
| **Age (years)** | r= 0.221 | 0.096[b] |
| **Years of Education** | r= - 0.015 | 0.913[b] |
| **MADRS (score)** | r= - 0.007 | 0.959[c] |
| **Tobacco abuse/dependence** | | |
| No | 80.16±38.15 | 0.073[a] |
| Yes | 100.66±47.40 | |
| **Diagnosis (BD)** | | 0.957[a] |
| Healthy Controls | 89.66±41.82 | |
| Bipolar Disorder | 89.04±45.65 | |

**Legend:** Sex, diagnosis and tobacco abuse: data is shown as mean ± standard deviation. Continuous independent variables were presented through the correlation coefficient (r). MADRS: Montgomery–Åsberg Depression Rating Scale.

[a] = Student t-test, [b] = Pearson correlation, [c] = Spearman correlation

The final linear regression model ($R^2$ = 0.211) showed that the variables that remained independently associated with higher CCL11 levels were male sex (B: 41.41 [CI95%: 15.66 - 67.15], p=0.002) and tobacco abuse/dependence (B: 22.71 [CI95%: 1.28 - 44.15], p=0.038). The BD diagnosis remained not significantly associated with CCL11 levels (B: -5.32 [CI95%: -26.63 - 15.99], p=0.619) (Table 3a).

**Table 3a.** Linear regression model of the factors associated with CCL11 levels.

| | **B (CI 95%)** | ***p-value*** |
|---|---|---|
| **Sex** | | |
| Female | Reference | |
| Male | 41.41 (15.66 - 67.15) | 0.002 |
| **Tobacco abuse/dependence** | | |
| No | Reference | |
| Yes | 22.71 (1.28 - 44.15) | 0.038 |
| **Group** | | |
| Healthy controls | Reference | |
| Bipolar disorder | -5.32 (-26.63 - 15.99) | 0.619 |

**Legend:** Linear regression model ($R^2 = 0.211$) including the variables male sex, tobacco use and diagnosis. Data is shown as the linear regression coefficient (B) and confidence intervals (CI).

## Discussion

The present study showed no changes in CCL11 serum levels among drug-naive young adults with BD. Of note, tobacco abuse/dependence and male sex showed association with higher CCL11 levels in our sample in a linear regression model. These results support the findings of Magalhaes et al. which also found that males had higher CCL11 levels than females (Magalhaes et al., 2014). Our results support the findings of Brietze et al., who found no differences in serum levels of CCL11 between individuals with BD and healthy controls (Brietze et al., 2009). Additionally, in the field of MDD, a systematic review and meta-analysis with over 400 participants did not identify significant differences in CCL11 levels between individuals with depression and control subjects (Leighton et al., 2018).

Our results differ from the findings of Barbosa et al., as they found increased levels of CCL11 across mood states in patients with BD type I (Barbosa et al., 2013). However, it is worth mentioning that the mean age of participants in that study was 49.79 years ± 11.84, while the participants in our study were young adults. Also, the majority of our sample was diagnosed with BD type II (we had only one BD type I). Possibly our findings are not aligned with the previous literature (Barbosa et al. 2013) because BD type I is more severe than BD type II, and CCL11 may be altered in more severe cases. An additional study found differences in CCL11 levels between the BD group and a healthy control group, however, this difference was only pronounced in late-stage patients (Panizzutti et al., 2015). As BD can be a progressive disorder, it is possible that alterations in CCL11 levels might be a marker for late stage BD. The findings of these two reports are in line with our current understanding of BD trajectories, as it is thought that peripheral levels of inflammation change with illness progression (Passos et al., 2016).

In line with our findings, Targowski et al. (2005) showed that CCL11 levels were significantly higher in healthy adult males than females in a study examining the effects of age and sex on CCL11 levels (Targowski et al., 2005). The study conducted by Targowski et al, also found that CCL11 levels were significantly lower in younger individuals. Another study examining alcohol use disorder in patients with mood disorders also found that CCL11 levels were elevated in males compared to females (Garcīa-Marchena et al., 2017). It can be presumed that sex hormones may play a role in this distinction. Estradiol and progesterone play a role in the production, release, and action of cytokines involved in various immune functions, including allergic responses (Piccinni et al., 1995). Among these cytokines is interleukin-4 (IL-4), which influences CCL11 expression (Mohizuki et al., 1998). As such, it can be suggested that hormonal

differences between males and females may influence CCL11 production. There is evidence that childhood trauma influences biological pathways including those of the immune system (Aas et al., 2016), suggesting that childhood trauma may influence immune-related markers such as CCL11. Childhood trauma is a risk factor for BD (Bortolato et al., 2017) and affects females disproportionately to males (Piccinelli et al, 2000), which may contribute to the sex differences observed in CCL11 levels.

We also found that CCL11 levels were elevated in individuals with tobacco abuse/dependence as compared to individuals without tobacco abuse/dependence. There is evidence to suggest that lifestyle factors such as smoking have effects on CCL11 levels. Cigarette smoking has been associated with changes in immune and inflammatory markers, including CCL11 (Shiels et al., 2014). Indeed, previous work reported that levels of CCL11 were significantly increased in smokers compared to non-smokers (Shiels et al., 2014). It stands to reason that CCL11 levels would be altered in smokers due to the well-established relationship between smoking, asthma and allergies (Strzelak et al., 2018). It seems that exposure to tobacco smoke can change immune responses by altering the functions of a variety of immune cells and aggravating allergic inflammation, and exposure to cigarette smoke can promote the release of a proinflammatory cytokine (interleukin (IL)-17A), which is implicated in the pathogenesis of asthma (Strzelak et al., 2018). Magalhaes et al., 2014, included smoking status as a factor in their analysis, and they also found that smoking status predicted CCL11 levels in their crude analysis, which did not survive in the adjusted analysis. As individuals with BD are 2-3 times more likely to consume tobacco than the general population (Mederios et al., 2018), smoking status may be a confounding variable in studies investigating CCL11 levels in BD.

This study has limitations and strengths that should be considered when interpreting our results. A potential limitation of our study is the small sample size. The small number of controls and individuals with BD may be a factor that prevented us from finding statistical differences between groups, as the number of comparisons in a small sample risks a type 2 error. Additionally, males were underrepresented in our sample. As a cross-sectional study, the data collected cannot be used to determine causality. However, it is important to highlight that one strong advantage of our study is the population-based sample, which is much less biased by multiple interventions than clinical studies. Our sample was also drug-naive, which is advantageous considering that pharmacology studies showed that mood stabilizers, such as lithium, would have an impact on the immune system with both anti-inflammatory (e.g., suppression of cyclooxygenase-2 expression, inhibition of IL-1β and TNF-α production, and enhancement of IL-2 and IL-10 synthesis) and pro-inflammatory effects (e.g., induction of IL-4, IL-6 and other pro-inflammatory cytokines synthesis) (Rosenblat and McIntyre, 2017). In this sense, studying drug-naive participants allows us to better understand the role of inflammatory markers in the pathophysiology of BD. In conclusion, in the present study we investigated serum levels of CCL11 in a community sample of drug-naive young adults with BD, matched to a healthy control group. Previous research showed that CCL11 levels differ in late-stage patients with BD (Panizzutti et al., 2015; Barbosa et al., 2013), so we sought to investigate if levels also differ in drug-naive young adults from a population-based sample. We found that CCL11 levels did not differ between individuals with BD and healthy controls, but that CCL11 levels were predicted by sex and smoking status. These results suggest that CCL11 levels may be regulated by demographic and lifestyle factors such as smoking. Changes in immune markers have been

associated with illness progression (Berk et al., 2011). Therefore, levels of CCL11 might not differ in BD until later stages of illness. Due to the progressive nature of BD (Berk et al., 2011), it is crucial to better understand the pathophysiology at early stages of illness. Further research is warranted with larger sample sizes to determine the potential role of CCL11 in early stages of BD.

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# CHAPTER THREE

## Introduction

BD is a chronic psychiatric condition marked by recurrent mood episodes (Tondo et al., 2017). Studies have shown that a subset of patients experience a more severe and progressive course of the disorder with low treatment response (López-Villarreal et al., 2019), emphasizing the need to better understand the pathophysiology of this illness. Further understanding these mechanisms would provide information for developing early intervention strategies and more specific pharmacological treatments. In recent years it has been suggested that individuals with BD may undergo an accelerated aging process (Rizzo et al., 2014, Fries et al., 2020), implicating aging factors such as GDF11.

There are similarities between the pathophysiologies of aging and of BD, including alterations in brain structure, changes in white matter hyperintensities, immunosenescence, oxidative stress imbalance and cellular senescence (Rizzo et al., 2014). Notably, multiple studies have demonstrated that individuals with BD present with early telomere shortening, which is a known indicator of cellular aging (Simon et al., 2006). It is also common for patients with BD to experience an early onset of age-related medical conditions including cardiovascular disease,

hypertension, obesity, metabolic syndrome and Type 2 diabetes, accounting for a decreased life span of up to 12 years (Kessing et al., 2015).

Studies have shown a potential association between altered adult neurogenesis and mood disorders, including BD (Ruan et al., 2014**;** Hayashi et al., 2018). It has been reported that mood stabilizers used to treat BD enhance the self-renewal of NSCs (Higashi et al., 2008). For example, the administration of mood stabilizers in adult mice was able to activate neurogenesis in the subependymal zone (SEZ) and the dentate gyrus (DG) (Boku et al., 2009; Chen et al., 2000; Higashi et al., 2008). As treatments are able to enhance neurogenesis in patients in accordance with treatment response, it can be inferred that alterations in neurogenesis may be driving some of the clinical features of BD, implicating a deficit of neurogenesis in the pathophysiology of the disorder. Patients with BD also show deficits in neurotrophic factors that promote cell survival and neurogenesis such as brain-derived neurotrophic factor (BDNF) (Munkholm et al., 2016), and glial cell-derived neurotrophic factor (GDNF) (Barbosa et al., 2011), further implying that the pathophysiology of BD involves deficits in neurogenesis and neuroplasticity. As such, it can be inferred that other factors affecting neurogenesis and neuroplasticity may also be involved in the pathophysiology of BD.

The cytokine GDF11 has recently been investigated as an anti-aging factor, however, it has yet to be examined in BD or any other psychiatric illness. GDF11 is part of the TGF-β superfamily of proteins, which influence gene expression through the activation of SMAD2/3 signalling pathways (Krieglstein et al., 2011). Through this gene expression, it is thought to induce pro-angiogenic and pro-neurogenic factors (Ozek et al., 2018). GDF11 has been explored as an anti-aging factor in several animal models. Reports of parabiosis models suggest that when

infused into an older animal, young blood is able to counteract and reverse the effects of aging at the molecular, structural, functional, and cognitive levels (Villeda et al., 2011). In additional studies, systemic treatment with GDF11 was able to enhance neurogenesis, improve vasculature, increase neuronal activity markers in the hippocampus of old mice (Ozek et al., 2014) and reduce markers of oxidative stress (Loffredo et al., 2013). Another study also showed that GDF11 was able to restore age-related deficits in both cerebral vasculature and neurogenesis in the aged brain (Katsimpardi et al., 2014 & 2015). As there is significant evidence to suggest that the pathophysiology of BD may involve deficits in neurogenesis and neuroplasticity, it can be hypothesized that deficits in factors which attenuate these processes, such as GDF11, may be involved in the pathophysiology of BD.

## Methods

### Study design and participants

This is a cross-sectional study with a matched sample of drug-naive young adults, nested within a large population-based study. The recruitment was conducted in the city of Pelotas located in southern Brazil. (Full details of the original study have been published by Jansen et al., 2011, and Moreira et al., 2016.) For this nested study, we selected drug-naive subjects with BD (n=29) from the second wave of the population-based study which took place between the years 2012 and 2014. The inclusion criteria for the BD group were: (1) being diagnosed with BD and (2) self-reported no lifetime psychiatric medication use (drug-naive status). A healthy control group was also recruited (n=29). The inclusion criteria for the healthy control group were: (1) do not have a diagnosis of mood disorders (Major Depressive Disorder or BD), (2) do not have a

diagnosis of anxiety disorder (panic disorder, agoraphobia, social phobia, specific phobia, or Generalized Anxiety Disorder), (3) do not have a diagnosis of Obsessive Compulsive Disorder, (4) do not have a diagnosis of Posttraumatic Stress Disorder, (4) do not have a diagnosis of Attention Deficit Hyperactivity Disorder, (5) do not present as a current suicide risk, (6), do not have lifetime psychotic symptoms, (7) do not present with current abuse/dependence of illicit drugs, and (7) self-reported no lifetime psychiatric medication use. Participants were matched by sex, age, and years of education. The age range for participants in this selected sample was 23-30 years old.

**Clinical assessments**

The diagnosis of psychiatric disorders was performed using the Mini International Neuropsychiatric Interview – PLUS (MINI-PLUS) (Sheehan et al., 1998). The Montgomery–Åsberg Depression Rating Scale (MADRS) (Davidson et al., 1986) and the Young Mania Rating Scale (YMRS) (Vilela et al., 2005) were used to assess depressive and manic symptoms, respectively. All assessments were administered by masters and Ph.D. level trained psychologists.

The study was approved by the Research Ethics Committee of the Universidade Católica de Pelotas (UCPel), under the protocol number 2008/118, and all subjects provided their written informed consent before inclusion in the study.

**GDF11 Assay**

GDF11 serum levels were measured using a commercial sandwich ELISA kit according to the manufacturer's instructions (R&D Systems - Catalogue # DY1958-05, Minnesota, USA). As a control for the specificity of the antibodies in the GDF11 ELISA kit, we used recombinant myostatin (MST) (Peprotech, USA), at the same concentration as the highest GDF11 standard (2 ng/ml) as well as at the middle value of the GDF11 standard curve (0.5 ng/ml). This assay was run in duplicate, and MST was not detected at any concentration, confirming the specificity of this assay for GDF11. To ensure the sensitivity of the kit, we used recombinant human GDF11 (Raybiotech, USA) at three different concentrations (2.560 ng/mL 0.410 ng/mL and 0.164 ng/mL).

Plate preparation: The mouse anti-human GDF11 capture antibody was diluted to 4.00 ug/mL with PBS and was used to coat the 96-well microplate (100 uL/well). The plate was then sealed and incubated overnight at room temperature. Each well was aspirated and washed with 300 uL of Wash Buffer (0.05% Tween 20 in PBS, pH 7.2-7.4) three times. Plates were blocked using 300 uL per well of Reagent Diluent (1% BSA in PBS, pH 7.2-7.4, 0.2 um filtered) and incubated at room temperature for one hour. Each well was then aspirated and washed three times with Wash Buffer.

Assay Procedure: 100 uL of samples or standards in Reagent Diluent were added to each well. The standard curve of GDF11 ranged from 0 to 500 pg/ml. The plate was covered with an adhesive strip and incubated overnight at 4$^{o}$C. Aspiration/washing was repeated. 100 uL of the

biotinylated mouse anti-human GDF11 detection antibody (400 ng/mL) was added to each well, covered with an adhesive strip and incubated for 2 hours at room temperature. Aspiration/washing was repeated. 100 uL of Streptavidin-HRP (40-fold dilution) was added to each well followed by a 20-minute incubation at room temperature (direct light avoided). Aspiration/washing was repeated. 100 uL of Substrate Solution (hydrogen peroxide + Tetramethylbenzidine) was added to each well, prior to a 20-minute incubation at room temperature (direct light avoided). 50 uL of Stop Solution (2N sulfuric acid) was added to each well, and wells were tapped gently to ensure proper mixing. GDF11 levels were determined by absorbance at 450 nm with correction at 540 nm in a microplate reader (Epoch™ 2 Microplate Spectrophotometer, BioTek, Vermont, USA). We ran one independent experiment, and ran the samples and standards in duplicate.

**Statistical Analysis**

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 26.0. Independent Samples *t*-test was used to assess age and years of education between groups (BD or control), while Fischer's exact test was used to assess sex differences between groups. Mann Whitney U test was used to assess differences in GDF11 levels and MADRS scores between groups.

## Results

Out of the 58 samples tested, 27 had readings that were within the standard curve, and 31 were below the standard curve. 13 samples from patients with BD and 14 samples from the

healthy control group were within the curve. We were able to determine that our sample was still successfully matched by sex, age and years of education when considering these samples only (Table 1b).

**Table 1b. Clinical and demographic characteristics**

| | **Healthy controls n=14** | **Bipolar Disorder n=13** | ***p-value*** |
|---|---|---|---|
| **Age (years)** | 25.64±1.95 | 25.31±1.49 | 0.742[a] |
| **Sex (F)** | 85.7% (12) | 76.9% (10) | 0.648[b] |
| **Years of education** | 10.21±2.15 | 9.23±2.65 | 0.299[a] |
| **MADRS (score)** | 0.00 (0.00; 2.00) | 10.00 (2.00; 16.00) | 0.002[c] |
| **YMRS (score)** | n/a | 4.00 (2.00; 7.50) | |

Age, years of education: data are shown as mean ± standard deviation. Data are presented as % (N) for sex. MADRS and YMRS score: data are shown as median and interquartile range (25$^{th}$ and 75$^{th}$ percentiles). MADRS: Montgomery–Åsberg Depression Rating Scale; YMRS: Young Mania Rating Scale; F: female;

[a] = Independent Samples t-Test, [b] = Fisher's Exact Test, [c] = Mann-Whitney U test.

There were no significant differences in serum levels of GDF11 between individuals with BD and controls (p=0.577) (Figure 4). The statistical power and effect size were both very low at 2.65% and D=0.0581, respectively.

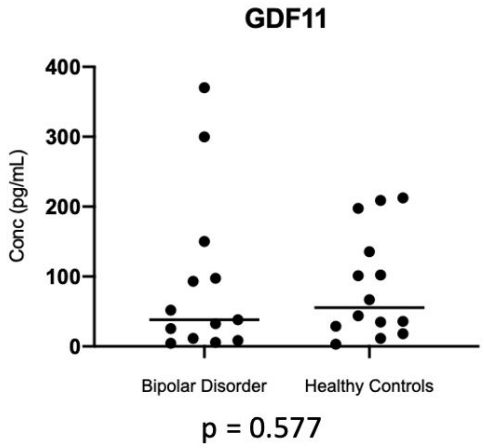


Figure 4: Box plot representing serum levels of GDF11 in individuals with bipolar disorder and healthy controls.

## Discussion

The present analysis showed no significant differences in serum levels of GDF11 between drug-naive young adults with BD and matched healthy controls. Our sample size was lowered considerably due to a lack of readings in the assay, which is also reflected in the low statistical power and effect size. Although we were able to determine that our assay had high specificity for GDF11 through testing with MST, it was not sensitive enough to detect GDF11 within all of the samples. As such, the null results must be interpreted with caution.

After reviewing general causes for low sensitivity (including improper storage of ELISA kit, not enough target, inactive detection reagent, incorrect plate reader settings, not enough substrate, incompatible sample type, interfering buffers or sample ingredients, mixing or

substituting reagents from different kits or assay format not sensitive enough (Abcam 2018)), it appears that future work assessing human GDF11 levels in serum in our study may require a more sensitive assay format. Increasing the incubation time or temperature may also increase the sensitivity of this analysis, however, it is worth noting that the samples were incubated overnight. Additionally, diluting the recombinant protein further to create more points on the standard curve could allow detection at lower concentrations. Considering time constraints and quantity of the sample, in the proposed thesis we were not able to refine our procedures by using a different sample type, increasing sensitivity through a different type of assay.

For future studies, it would be of value to utilize different detection methods, different methodologies, and potentially a different type of sample. It is important to note that a common challenge faced when studying GDF11 is antibody specificity, since many also recognize MST (GDF8) due to their structural homology. As the lack of sensitivity may be due to the assay format (absorbance/colorimetric), using fluorescence may provide better detection, as it is known to be more sensitive. There is also the possibility in the future of using other techniques including flow cytometry, Luminex assay or liquid chromatography/mass spectrometry, as previously demonstrated by Schafer and colleagues (2016). To the best of our knowledge, there are no kits or antibodies available to evaluate GDF11 levels using the above-mentioned techniques (flow cytometry and Luminex). Luminex suppliers have the option of custom development, which is a potential avenue to explore in the future.

There is also evidence suggesting that GDF11 levels are highly concentrated in platelets (Bueno et al., 2016). As we analyzed serum in our study, it is worth considering that assessing platelet-enriched plasma in future studies instead of serum may lead to greater detection.

To the best of our knowledge, this is the first study to analyze GDF11 levels in a psychiatric population. Further studies are needed to better understand the potential role of GDF11 in the physiopathology of BD, considering different methods for the detection of this analyte and different types of samples.

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# CHAPTER FOUR

## General Discussion

In the present study, we investigated if age-related proteins were altered in individuals with BD, as it is hypothesized that patients undergo an accelerated aging process (Rizzo et al., 2014; Fries et al., 2020). These proteins included GDF11, which decreases with age (Poggioli et al., 2016), and CCL11, which increases with age (Hoefer et al., 2017). This study assessed a community sample of drug-naive young adults with BD matched by sex, age, and years of education with a healthy control group. We hypothesized that the individuals with BD would have lower serum levels of GDF11 compared to controls, and that individuals with BD would have higher serum levels of CCL11 compared to controls.

Serum levels of GDF11 and CCL11 were measured using ELISA. After our analyses, we found that there were no statistically significant differences in CCL11 or GDF11 levels between the BD group and the control group. However, when considering the entire sample, males and individuals with tobacco abuse/dependence had higher levels of CCL11 compared to females and individuals without tobacco abuse/dependence.

Our results suggest that changes in CCL11 levels may not be due to BD, but to lifestyle and demographic factors. These findings are in accordance with previous literature, as Targowski

et al., (2005), Garcia-Marchena et al., (2017), and Magalhaes et al., (2014) also found that CCL11 levels were significantly higher in males compared to females. As previously mentioned, it is possible that sex hormones play a role in this distinction, as estradiol and progesterone play a role in the production, release, and action of cytokines that are involved in allergic responses (Piccinni et al., 1995). CCL11 is involved in allergic responses, as it activates eosinophils which induce allergic reactions, particularly in the airways (Eng et al., 2016).

We also found that CCL11 levels were elevated in individuals with tobacco abuse/dependence compared to individuals without tobacco abuse/dependence, suggesting that this lifestyle factor has an influence on CCL11 levels. This finding is consistent with pre-existing literature, as it has been reported that CCL11 levels are affected by smoking (Krisiukeniene et al., 2009; Magalhaes et al., 2014). However, in the study conducted by Krisiukeniene et al., CCL11 levels were measured in sputum, not serum. Also, in the study conducted by Magalhaes et al., the association between smoking status and CCL11 levels did not remain after adjusting for confounders, including clinical illness, mood state, substance use disorder, alcohol use disorder, use of psychiatric medication, self-reported medical illness, sex and social class (Magalhaes et al., 2014).

Given the evidence of accelerated aging in BD (Fries et al., 2020), it is interesting that we did not see differences between groups regarding serum levels of these proteins. However, it was previously reported that there were no differences in CCL11 levels between early-stage patients with BD and healthy controls (Panizzutti et al., 2015). Other studies that reported increased levels of CCL11 compared to controls were conducted on late-stage patients or older adults

(Panizzutti et al., 2015; Barbosa et al., 2013). Given that BD may follow a progressive course, it is possible that changes in these markers may become apparent only in these cases.

**Limitations**

Our study had several limitations. Firstly, as mentioned, we had a low sample size resulting in a low statistical power (1.638% and 2.651% for the comparison between BD and controls regarding CCL11 and GDF11, respectively). Although we selected our participants from a much larger sample from a cross-sectional study, our drug-naive criteria limited us on the number of participants we could include. Our sample consisted of 29 participants in each group. We were only able to obtain readings for approximately half the samples in our GDF11 assay, reducing the total sample size to 27 (13 BD and 14 controls) for this protein. Given the small sample size(s) and lack of readings, we had very low statistical power. Our statistical powers were 1.638% and 2.651% for the comparison between BD and controls regarding CCL11 and GDF11 respectively. Therefore, it is possible that we may have been unable to detect a true effect. As we only had a small amount of serum to use, a significant limitation of our study was that we were unable to run additional analysis with troubleshooting.

Another limitation of our study, that is commonly associated with large cohort studies, is that we had missing clinical data for some participants. For example, only a small portion of participants were able to report their age of illness onset or the number of past depressive, hypomanic or manic episodes. As we were missing this information for many participants, we were unable to determine if participants met the specific criteria for 'early stage' BD in accordance with staging models (Kapczinski et al., 2009). However, because it is a community

sample of young adults, and they were drug-naive, it is probable that they were in an early stage. Additionally, 28 out of our 29 participants with BD had BD type II while only one had BD type I, which may explain why we did not see differences in CCL11 and GDF11 levels between groups. We were unable to select participants with BD type I due to our drug naive criteria. Recruiting drug-naive individuals with BD type I is challenging, as typically patients presenting with a more severe course of BD are medicated.

**Strengths**

Our study has several strengths, including primarily that our sample was drug-naive and was a population-based sample. A drug-naive sample is rare and is of value, as most samples are complicated by varying pharmacological treatments among participants. As pharmacological treatments may affect inflammation and the presence of inflammatory markers (Basselin et al., 2010; Kim et al., 2007; Boufidou et al., 2004, Rosenblat and McIntyre, 2017), the lack of pharmacological intervention in our sample allows a more accurate biological representation of the disorder than many other studies. Additionally, our sample was community-based, which is advantageous given that this type of sample provides a more accurate representation of the general population compared to recruitment from an outpatient program.

Another strength of our study was that we investigated young adults specifically, rather than across the lifespan as most studies do. Investigating a particular age group allows a more specific insight into the biology of the disorder at distinct ages. The mean age of our sample was 25.90±1.90 for the control group and 25.72±2.07 for the BD group, which is relatively close to the average age of onset which was reported as 22.7 years in a sample of 441 patients (Benazzi et

al., 2008). As BD shows a progressive course in a subset of patients (López-Villarreal et al., 2019), it is valuable to observe biological and clinical factors at a young age close to the age of illness onset to work towards targeted intervention. Having a better understanding of the disorder in its early stages may provide insight into developing unique treatments tailored towards this etiological period.

**Future Directions**

The main shortcomings of our study were the lack of sensitivity in the GDF11 assay and the small sample size. Working with a larger sample size in the future would yield a much higher power and decrease the likelihood of a Type 2 error. Other lab methods may also be utilized to detect GDF11 levels in serum, including single molecule array (SIMOA). As the samples in our study showed relatively low concentrations of GDF11, in future studies it could prove valuable to add extra standard points by diluting the recombinant protein further. As we only ran one test, running multiple analyses to assess CCL11 levels in future studies would yield greater consistency and test-retest reliability, allowing a truer reflection of levels within the samples. In future studies, it would be appropriate to leave out the primary antibody as a negative control for the secondary antibody in the CCL11 assay, and to leave out the sample as a negative control for the primary antibody in the GDF11 assay.

Until this study, GDF11 levels had not been investigated in BD or in any other psychiatric disorder. Due to the lack of readings from our analysis, it would be worth repeating our experiment to further assess GDF11 levels in a young population with BD. However, it would also be interesting to assess GDF11 levels in an older and/or late-stage population. As BD

can be a progressive disorder, it is possible that changes in GDF11 levels may not be present until the disorder reaches later stages. Neuroprogression in BD results in pronounced deficits in functioning and cognition, particularly in late stages of the disorder (Passos et al., 2016). It has been reported in animal models that GDF11 is able to reverse the effects of aging at the molecular, structural, functional and cognitive levels (Villeda et al., 2011). Therefore, levels could be altered in late-stage patients with BD who may have undergone accelerated aging with similar deficits in functioning and cognition.

**Conclusions**

The main objective of this thesis was to examine CCL11 and GDF11 levels in drug-naive young adults with BD. The results presented in this thesis suggest that at a young age, drug-naive individuals with BD do not show changes in serum levels of CCL11 and GDF11 compared to controls. As it has been reported that levels of CCL11 are increased in late-stage and older individuals with BD, it is possible that these changes do not occur until past young-adulthood. However, more studies are needed to confirm this hypothesis. Due to the role that these factors play in aging, further elucidating their role in BD would contribute towards the increasing body of work investigating accelerated aging in BD. Future studies should aim to investigate levels of these proteins in individuals with BD in larger sample sizes in young, in early and late stage patients and in an older population to further understand their relevance in this disorder. These findings contribute towards the search for novel biomarkers in BD that may contribute towards identifying effective targeted intervention strategies and earlier diagnosis.

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