Thesis

Shared Pathways between Obesity and Bipolar Disorder: Endoplasmic Reticulum Stress, Oxidative Stress and the Circadian Clock -

Genetic and Epigenetic Analysis

submitted by

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Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbständig angefertigt und abgefasst, und jene Personen und Institutionen, die am Zustandekommen der Forschungsdaten beteiligt waren, namentlich genannt habe. Andere als die angegebenen Quellen habe ich nicht verwendet und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen habe ich als solche kenntlich gemacht. Die Arbeit an der Dissertation und daraus entstandener Publikationen wurde gemäß den Regeln der „Good Scientific Practice"durchgeführt.

Graz, am 16.12.2016

Susanne A. Bengesser

Declaration:

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice".

Graz, 16th of December 2016

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Abbreviations

ANK3*… Ankyrin 3; Ankyrin G*

APA… American Psychiatric Association

ARNTL*… Aryl hydrocarbon receptor nuclear translocator like*

ATF6*… Activating transcription factor 6*

BiP… Binding immunoglobulin protein, syn. *GRP78* and *HSPA5*

BD… Bipolar Disorder

CACNA1C*… Calcium voltage-gated channel subunit alpha1 C*

CHOP… Encodes a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors, syn. *GADD153, DDIT*

CLOCK*… Circadian Locomotor Output Cycles Kaput*

CREB*… cAMP responsive element binding protein*

CG… Cytosine and guanosine

CpG islands… Repeats of cytosine and guanine (CG repeats)

CRY*…Cryptochrome* genes

CSNK1ε *… Casein kinase I isoform Epsilon*

CQ… Quantification cycle

DGKH*… Diacylglycerol kinase eta*

DNA... Deoxyribonucleic acid

DSM-IV or 5… Diagnostic and Statistical Manual of Mental Disorders version IV or 5 by the American Psychiatric Association (APA)

eIF2α… Eukaryotic Initiation Factor of Translation

ER…Endoplasmic Reticulum

ER stress… Endoplasmic Reticulum stress

Genes… *Italic written*

GST… Glutathione-S-Transferase

GSK3β*... Glycogen-synthase-kinase-3Beta*

GRP78*... 78 kDa glucose-regulated protein*, syn. *BiP* and *HSPA5*

HSPA5*… Heat shock 70 kDa protein 5*, syn. *GRP78* and *BiP*

ICD-10... International Classification of Diseases-10 (European Classification)

IGF1*… Insulin like growth factor 1*

IRE1α… *Inositol-Requiring Protein-1α*

LMAN2L*... Lectin, mannose binding 2 like*

MDA… Malondialdehyde

MetS… Metabolic Syndrome

MAOA… Monoamine oxidase A

mTOR… Mammalian target of rapamycin

NCAN… *Neurocan*

NGS…Next Generation Sequencing

NPAS2… *Neuronal PAS domain protein 2*

ODZ4... *Odd Oz/ten-m homolog 4*

PER… *Period* gene

PERK… *PKR-like ER kinase*, syn. EIF2AK3 eukaryotic translation initiation factor 2-alpha kinase 3

PKR... *Protein kinase RNA-activated also known as protein kinase R*, syn. Eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2)

PI3K... *Phosphoinositide 3-kinase*

Proteins… Upright written (not *italic*)

qPCR… Quantitative PCR (Polymerase Chain Reaction)

REV-ERBα… syn. *Nuclear receptor subfamily 1 group D member 1*(*NR1D1*)

RNA… Ribonucleic acid

ROS… Reactive oxygen species

RT-PCR… Real Time Polymerase Chain Reaction (quantitative PCR)

SOD… Superoxide dismutase

TAC… Total Antioxidative Capacity

TBARS… Thiobarbituric acid reactive substances

TSS... Transcription start site

UPR… Unfolded Protein Response

XBP1… *X-box binding protein 1*

Zusammenfassung

Hintergrund

Individuen mit bipolarer Erkrankung (BD) leiden überproportional häufig (circa 70%) an Adipositas. In der Literatur wird ein Zusammenhang zwischen Endoplasmatischem Retikulum (ER) Stress, oxidativem Stress und circadianen Rhythmen und der Adipositas diskutiert. Daher wurde die Triade in Synopsis mit der BD und Adipositas molekularbiologisch untersucht.

Methoden

I. Gruppenunterschiede zwischen BD und Kontrollen mittels ANCOVAs,

II. Korrelationen mit Body Mass Index (BMI) oder Unterschiede zwischen Gewichts-Gruppen (normalgewichtig, übergewichtig, adipös) mittels ANCOVAs.

Folgende Methoden wurden in peripherem Blut verwendet:

(1) **ER Stress Quantifizierung** durch Genexpressionsanalyse mittels quantitativer PCR der Gene *BiP* und *CHOP*, sowie semiquantitativer Analyse des *XBP1* Splicing. Hypothesengeleitete Gen Assoziations Analysen von *PERK* rs867529, *ATF6* rs2271013, *LMAN2L* rs9834970 und *LMAN2L* rs6746896 mittels PLINK.

(2) **Oxidativer Stress** wurde durch Detektion der Lipidperoxidationsmarker Malondialdehyd (MDA) und „Thiobarbituric Acid Reactive Substances" (TBARS), sowie den antioxidativen Parametern Glutathion-S-Transferase (GST), Cu/Zn Superoxid Dismutase (SOD) und Totaler antioxidativer Kapazität (TAC) bestimmt.

(3) **Die zirkadiane Uhr** wurde mit hypothesengeleiteten Gen Assoziations Analysen der Uhrengene *CLOCK* und *PER* mit PLINK analysiert. Die Methylierungs-Analyse von *ARNTL* (cg05733463 and PS2 POS1-7) wurde mittels Epitect Kit, PCR und Pyrosequenzierung durchgeführt.

Ergebnisse

Individuen mit BD hatten eine signifikant höhere *BiP* Genexpression im Vergleich zu Kontrollen ($F(1/100)$= 4.449, $p < 0.05$, Partielles Eta^2= 0.043). Die Gesamtsumme der *XBP1* mRNA war in der BD signifikant geringer als in den Kontrollen ($F(1/100)$= 5.265, $p < 0.05$, Partielles Eta^2= 0.050). PatientInnen mit BD hatten signifikant weniger ungesplictes *XBP1* als

Kontrollen (*F(1/100)*= 5.634, $p < 0.05$, Partielles Eta^2= 0.053). Der *LMAN2L* rs6746896 SNP zeigte eine signifikante Assoziation mit der BD im Gen Assoziations Test (X^2= 3.881, $p < 0.05$, OR= 0.65). Marker des ER Stresses unterschieden sich nicht signifikant zwischen den Gewichts-Gruppen; ebenso gab es keine signifikante Korrelation mit dem BMI. Der BMI unterschied sich auch nicht zwischen den Genotyp Gruppen der untersuchten ER Gene.

Der oxidative Stress Marker MDA war signifikant niedriger in der BD im Vergleich zu Kontrollpersonen (*F(1/204)*= 8.485, $p < 0.05$, Partielles Eta^2= 0.040). Die totale antioxidative Kapazität TAC war signifikant niedriger in der BD im Vergleich zu Gesunden (*F(1/189)*= 8.406, $p < 0.05$, Partielles Eta^2= 0.043). Antioxidative Marker korrelierten mit BMI im Gesamt-Sample unabhängig von der BD- TAC korrelierte negativ mit BMI (r= -0.143, $p < 0.05$) und GST korrelierte positiv mit BMI (r= 0.151, $p < 0.05$).

Individuen mit BD zeigten eine signifikant höhere Methylierung von *ARNTL* an cg05733463 im Vergleich zu Kontrollen (*F(1/209)*= 44.500, $p < 0.001$, Partielles Eta^2= 0.176). Die zweite CpG Insel PS2 von *ARNTL* war an PS2 POS1 signifikant niedriger methyliert in der BD (*F(1/128)*= 5.787, $p < 0.05$, Partielles Eta^2= 0.043). Die Methylierung von *ARNTL* unterschied sich nicht zwischen den verschiedenen Gewichts-Klassen. Ebenso gab es keine signifikanten Assoziationen von *PER3* und *CLOCK* mit BD im Gen Assoziations Test. Der BMI unterschied sich nicht zwischen den untersuchten Uhrengen-Genotypen.

Diskussion

Die Resultate dieser Dissertation unterstreichen die enorme Bedeutung der ER Homöostase, des oxidativen Stresses und der gestörten zirkadianen Rhythmen in der Pathogenese der BD. Die ER Stress Genexpressions-Marker *BiP* und *XBP1*, der oxidative Stress Marker MDA, die TAC, sowie die Methylierung des Uhrengens *ARNTL* könnten nach weiterer intensiver Forschung Bio-Marker oder neue „Drug-Targets“ in der BD darstellen.

Summary

Background

Individuals with Bipolar Disorder (BD) suffer disproportionately frequently (about 70%) from overweight and obesity. Literature postulates a relationship between Endoplasmic Reticulum (ER) stress, oxidative stress and circadian rhythms and obesity. Therefore, the triad was studied molecular biologically in synopsis with BD and obesity.

Methods

I. group differences between BD and controls using ANCOVAs and II. correlations with body mass index (BMI) or differences between weight groups (normal weight, overweight, obese) were investigated using ANCOVAs. The following methods were used in peripheral blood:

(1) **ER stress** quantification by gene expression analysis with quantitative PCR of the genes *BiP* and *CHOP*, as well as semiquantitative analysis of *XBP1* splicing. Hypothesis-driven gene association tests of *PERK* rs867529, *ATF6* rs2271013, *LMAN2L* rs9834970 and *LMAN2L* s6746896 in BD and controls.

(2) **Oxidative stress** was determined by detection of the lipid peroxidation markers malondialdehyde (MDA) and Thiobarbituric Acid Reactive Substances (TBARS), as well as the antioxidant parameters glutathione S-transferase (GST), Cu / Zn superoxide dismutase (SOD) and total antioxidant capacity (TAC).

(3) **The molecular circadian clock** was analyzed with hypothesis-driven gene association tests of the *CLOCK* and *PER3* genes with PLINK in BD and controls. Methylation analysis of the clock gene *ARNTL* (g05733463 and PS2 POS1-7) was performed with the Epitect Kit, PCR and pyrosequencing.

Results

Individuals with BD had a significantly higher *BiP* gene expression compared to controls $F(1/100) = 4.449$, $p < 0.05$, partial Eta^2 = 0.043). The total sum of the *XBP1* mRNA was significantly lower in BD than in the controls ($F(1/100) = 5.265$, $p < 0.05$, Partial Eta^2 = 0.050). Similarly, patients with BD had significantly less unspliced *XBP1* than controls ($F(1/100) = 5.634$, $p < 0.05$, Partial Eta^2 = 0.053). The *LMAN2L* rs6746896 SNP showed a significant association with BD in the "basic gene association test" ($X^2 = 3.881$, $p < 0.05$, OR = 0.65). ER stress markers did not differ significantly between the weight

groups and there was no significant correlation between BMI and ER stress parameters. BMI did not differ between the different investigated genotype groups.

The oxidative stress marker MDA was significantly lower in BD compared to controls (*F(1/204)* = 8.485, $p < 0.05$, Partial Eta^2 = 0.040). The total antioxidant capacity TAC was significantly lower in patients with BD compared to controls (*F(1/189)* = 8.406, $p < 0.05$, Partial Eta^2 = 0.043). Antioxidative markers correlated with BMI in the total sample independently of BD - TAC correlated negatively with BMI ($r = -0.143$, $p < 0.05$) and GST correlated positively with BMI ($r = 0.151$, $p < 0.05$).

Individuals with BD showed a significantly higher methylation of *ARNTL* at cg05733463 compared to controls (*F(1/209)* = 44.500, $p < 0.001$, Partial Eta^2 = 0.176). In contrast, the second CpG Island PS2 of *ARNTL* had significantly lower methylation in individuals with BD compared to controls at PS2 POS1 (*F(1/128)* = 5.787, $p < 0.05$, Partial Eta^2 = 0.043). The methylation of *ARNTL* did not differ between the different weight classes. Furthermore, there were no significant associations of *PER3* and *CLOCK* with BD in the gene association test. BMI did not differ between the investigated clock gene genotype groups.

Discussion

The results of this dissertation underline the enormous importance of ER homeostasis, oxidative stress and disturbed circadian rhythms in the pathogenesis of BD. The ER stress gene expression markers *BiP* and *XBP1*, the oxidative stress marker MDA, the TAC, as well as the methylation of the *ARNTL* clock gene could represent bio-markers or new "drug targets" in BD after further intensive research.

1. Introduction

1.1. Bipolar Disorder, Obesity and Shared Pathways

Bipolar Disorder (BD) is a mood disorder that is classified by mood swings between the pole of euphoria and the opposite pole of depression. The lifetime-risk for BD is estimated around 5 % and the average age of onset is located between 25 and 30 years (Dilling H 2010).
Manic episodes are characterized by euphoric mood as a major symptom in the “Diagnostic and Statistical Manual of Mental Disorders” (DSM-IV and DSM-5) published by the American Psychiatric Association (APA) and by the International Classification of Diseases-10 (ICD-10). Manic symptoms endure at least for one week or are that severe that hospitalization is required. In addition to the altered mood, numerous symptoms like inflated self-esteem or grandiosity, decreased need for sleep, flight of ideas, logorrhoea, distractibility, psychomotor agitation, increase in goal-directed activity and excessive involvement in pleasurable activities that have a high potential for painful consequences (e.g. unrestrained buying, foolish business investments or sexual indiscretions) are common in manic episodes. Most important, the symptoms are not caused by substance abuse or medical conditions like hyperthyroidism or other diseases. In contrast to mania, hypomania is characterized by milder manic symptoms and without loss of social inhibitions or harmful psychosocial consequences (Dilling H 2010, Rothenhäusler HB 2007).

Depressive episodes are categorized by depressed mood that persists for at least two weeks (DSM-IV, DSM-5 and ICD-10). In addition to depressed mood, patients suffer from diverse symptoms like anhedonia and reduced energy, further from significant unintentional weight loss or weight gain, insomnia or hypersomnia, agitation or psychomotor retardation, fatigue, loss of energy, feelings of worthlessness or excessive guilt, loss of concentration or indecisiveness. Suicide ideation and suicide attempts might occur in severe depressive states (Dilling H 2010, Rothenhäusler HB 2007).

According to the DSM-IV-criteria, which were used in the current doctoral thesis, different types of the course of BD are known. BD type I comprises at least one manic and one depressive episode, in total at least two affective phases. BD type II includes at least one hypomanic and one depressive episode. Courses of BD with unipolar occurring manias- without depressive episodes are rare (Rothenhäusler HB 2007, Benazzi, Akiskal 2006). Rapid cycling is a special subtype and is characterized by a high frequency of affective episodes, namely at least four episodes per year (Dilling H 2010, Rothenhäusler HB 2007, Rothenhäusler HB 2007, Benazzi, Akiskal 2006, Bengesser S 2013).

Even though the clinical picture of BD has already been described by Hippocrates of Kos and Aretaeus of Cappadocia, the exact pathogenesis of BD is still not clearly elucidated yet (Rothenhäusler HB 2007). Nevertheless, what we know from twin- and adoption studies already for decades is a high heritability (up to 85%) of the disease (Cardno et al. 1999, McGuffin et al. 2003). Concordance rates between monozygotic twins are even as high as 40- 70%. In contrast, the concordance rate lies between 5-10% for dizygotic twins. Children of both parents suffering from BD have an approximate lifetime-risk of 50-65% to fall ill from BD, whereas children with one parent with BD have a risk of 25%. If one first-degree relative suffers from BD, one has the risk of 5-10% to develop BD. In contrast, the estimated risk in the general population is around 1% (Rothenhäusler HB 2007). These facts indicate that genetic predisposition is highly important for the development of the BD, but not to the exclusive cause (Rothenhäusler HB 2007).

Current genetic research using methods like exome sequencing, genome wide association studies (GWAS), hypothesis driven genetic association studies and linkage studies showed that an orchestra of hundreds of gene variants – each alone solely with a very low effect - contribute to genetic vulnerability of BD (Bengesser S 2013). Based on limited space, I want to mention just selected BD susceptibility genes here. The most replicated genome wide significant BD susceptibility genes are *CACNA1C* (*Calcium voltage-gated channel subunit alpha1 C*) and *ANK3* (*Ankyrin 3, Ankyrin G*). Both belong to the ion channel group or the ion channel associated proteins (Cross-Disorder Group of the Psychiatric Genomics Consortium 2013, Zhang et al. 2013, Ferreira et al. 2008, Sklar et al. 2008, Takata et al. 2011, Schulze et al. 2009). Further GWAS top hits include *NCAN* (*Neurocan*), *ODZ4* (*Odd Oz/ten-m homolog 4*), *DGKH* (*Diacylglycerol kinase eta*) and *IGF1* (*Insulin like growth factor 1*). Le-Niculescu and colleagues performed a functional re-analysis of GWAS data and identified beside others the clock gene *ARNTL (Aryl hydrocarbon receptor nuclear translocator-like gene)* as a further possible important BD gene (Bengesser S 2013, Cichon et al. 2011, Pereira et al. 2011, Sklar et al. 2011, Baum et al. 2008, Zeng et al. 2011, Le-Niculescu et al. 2009b). Other top hits of BD GWAS also include one gene encoding for a cargo receptor and ER transmembrane protein responsible for trafficking of glycosylated proteins, namely *LMAN2L (Lectin, mannose binding 2 like)* (Chen et al. 2013).

Even though GWAS are a powerful means to detect predisposing genes without bias by choosing genes, GWAS cannot detect all susceptibility genes by itself. Diverse GWAS consortia are constantly working on increasing sample sizes e.g. the Psychiatric Genomic Consortium (Cross-Disorder Group of the Psychiatric Genomics Consortium et al. 2013, Hou et al. 2016). Cross-disorder designs are additional ways to increase sample sizes (Cross-Disorder Group of the Psychiatric Genomics Consortium et al. 2013). Nevertheless, other approaches are still necessary to paint the whole picture of BD vulnerability. Results of hypothesis driven gene association-, linkage- and gene expression studies are still important for elucidating the missing heritability. Furthermore, new methods like Next Generation Sequencing (NGS) will additionally help to solve the genetic mystery. NGS can help to discover new copy number variations (CNVs) or rare single nucleotide polymorphisms (SNPs) that are not represented on a GWAS chip (Goes et al. 2016).

In addition, we know nowadays that not only the genetic blueprint is important for exhibiting phenotypes, but also the regulation of gene expression. Diverse epigenetic modifications like methylation or acetylation can alter gene expression. Methylation of cytosines at position 5 of the cytosine ring in CG rich regions (CpG islands) in the promoter area leads, according to current scientific knowledge, to silencing of the gene (Barlow, Bartolomei 2014). The effect of methylation at cytosines in exons is more complicated and not clearly elucidated yet (Barlow, Bartolomei 2014, Barlow, Bartolomei 2014, Blattler et al. 2014). Nevertheless, methylation can impact gene expression to a major degree and methylation-analysis can help to reveal the concatenation between genetics, gene-environment interactions and gene expression. Thus, methylation and other epigenetic modifications of BD related genes may contribute to the multifactorial vulnerability-stress-model. Growing evidence suggests that BD is caused on one hand by the genetic vulnerability and on the other hand by several gene-environment-interactions like life-style changes (obesity, hyperglycaemia and metabolic syndrome), biopsychosocial stressors, chronobiological changes (jetlag, night shifts) and negative life events. The combination of polygenic genetic vulnerability, chronic and acute stressors (e.g. jetlag) can finally lead to the outbreak of affective episodes. Diverse pathways may mediate those gene-environment interactions such as oxidative stress, endoplasmic reticulum (ER) stress, chronic inflammation, epigenetic changes or disturbed molecular circadian clock, finally resulting in neurotransmitter imbalances and affective episodes (Rothenhäusler HB 2007, Bengesser S 2013, Abdolmaleky et al. 2011, Ghadirivasfi et al. 2011, Nohesara et al. 2011, Abdolmaleky et al. 2006, Abdolmaleky et al. 2005, Abdolmaleky et al. 2004, Kakiuchi et al. 2007, Kakiuchi et al. 2003).

Of special interest is the fact that obesity is more common in BD than expected by chance (up to 70%). Bipolar individuals have increased levels of abominal obesity compared to the general population. The latter cannot be sufficiently explained by behavioral and/or iatrogenic factors alone (McIntyre et al. 2010b, Fagiolini et al. 2008, Fagiolini et al. 2005). Bipolar patients with obesity show faster illness progression and a worse course of disease (Leboyer et al. 2012). Many previous studies also show that individuals with BD present a higher level of Metabolic Syndrome (MetS) compared to healthy controls. Obesity, overweight, hyperglycaemia and the MetS may influence BD mechanisms in a negative way via the already mentioned biological pathways - ER stress, oxidative stress and circadian rhythms (see Fig. 1) (Bengesser S 2013, Mangge et al. 2014, Reininghaus et al. 2014, Reininghaus et al. 2013, Mangge et al. 2010, Furukawa et al. 2004, Bengesser et al. 2014, Bengesser et al. 2016).

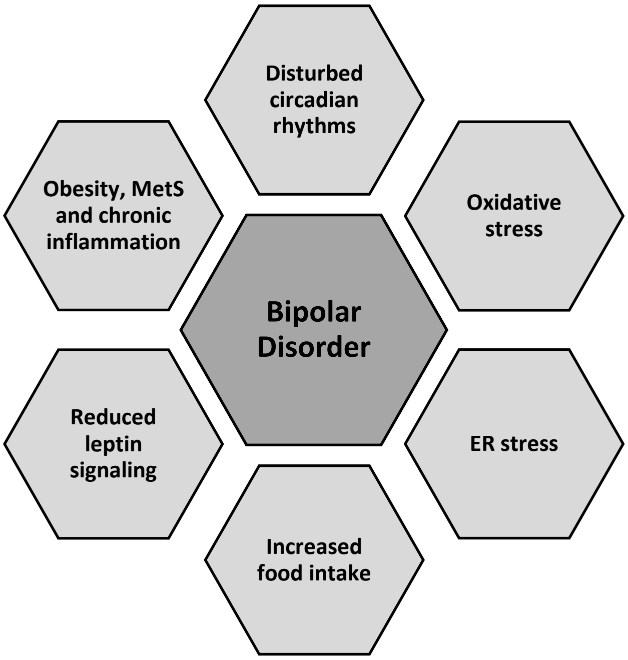


Fig.1. Bipolar Disorder (BD) and metabolic pathways. BD presents more often than by chance comorbid overweight or obesity (McIntyre et al. 2010c, McIntyre et al. 2010a). It is well known, that obesity is leading to chronic inflammation and oxidative stress. Furthermore, obesity induces Endoplasmic Reticulum (ER) stress and disturbs circadian rhythms according to literature. ER stress, oxidative stress and chronic sleep fragmentation are leading to hyperphagia, hypernutrition, obesity, reduced leptin signaling and insulin resistance. **Abbreviations**: MetS… Metabolic syndrome

Obesity, MetS, oxidative stress, chronic inflammation and ER stress are pathophysiologic processes that are closely interlinked with each other via diverse pathways displayed in Fig.1 and 2. Elevated Reactive Oxygen Species (ROS) levels disturb the ER redox state and subsequently inhibit adequate protein folding by interrupting disulfide bond formation in the ER. The increase of unfolded proteins "stresses" the cell resulting in ER stress (Wang, Yang & Zhang 2016). ER stress was also associated with oxidative stress in mice fed with high fat diet (Yuzefovych et al. 2013, Yin et al. 2015). Another concatenation between ER stress and obesity is the relationship between leptin levels and *BiP* (*Binding immunoglobulin protein)* gene expression. The adipokine leptin is produced in white adipose tissue in response to insulin and regulates the food and energy homeostasis. Leptin specifically induces the expression of the "ER stress sensor" *BiP* through the PI3K-mTOR pathway in neuroblastoma cell lines. Leptin is upregulating *BiP* gene expression and is thereby protecting the cell against obesity related ER stress. Although leptin is known as "anti-obesity-hormone", levels of circulating leptin are highly elevated due to the established leptin resistence in obesity (Thon et al. 2014). Obesity is also linked to disturbed circadian rhythms and cellular stress - chronic sleep loss increases cellular stress especially in insulin resistant obese mice (Naidoo 2012, Hakim et al. 2015). Furthermore, fragmented sleep results in increased food intake and reduced leptin signaling in the hypothalamus of mice- a vicious circle (Naidoo 2012, Hakim et al. 2015). Summarizing, there might be shared disease pathways between BD and obesity (see Fig. 1 and 2) (McIntyre et al. 2010c, McIntyre et al. 2010a). Based on this scientific knowledge the following chapters will review the molecular biological concatenation between obesity and BD focussing on three pathways/mechanisms correlated with obesity and BD (see Fig. 2):

1) **ER stress**
2) **Oxidative stress**
3) **Molecular circadian rhythms**

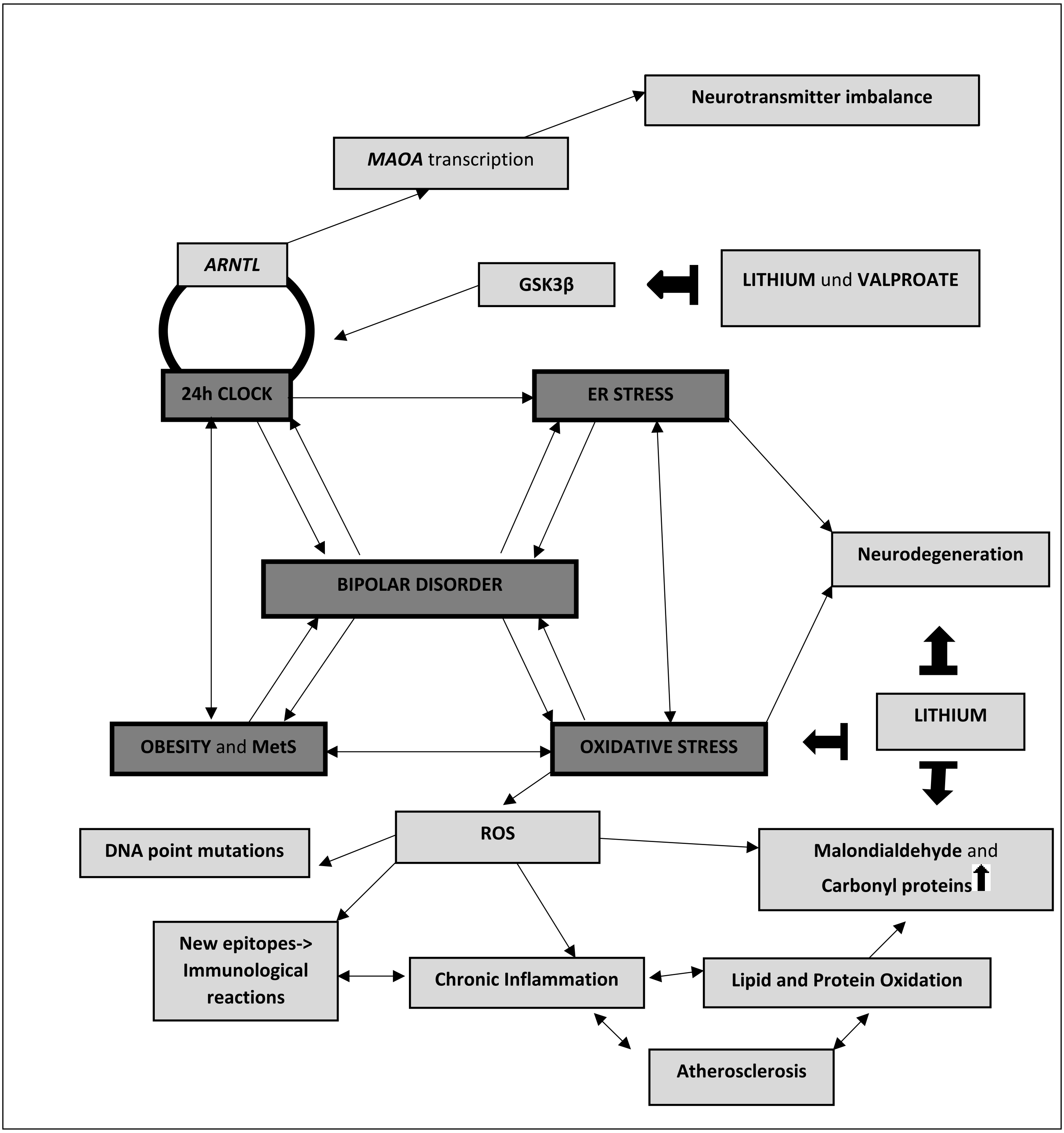


Fig.2. Interplay between Obesity related conditions and Bipolar Disorder. Obesity, Metabolic Syndrome (MetS) and hyperglycaemia lead to increased oxidative stress, chronic inflammation, increased Endoplasmic Reticulum (ER) stress and disturbed circadian rhythms. The clock gene *ARNTL* (*Aryl hydrocarbon receptor nuclear translocator-like gene*) controls the transcription of *MAOA* (*Monoamine Oxidase A*) that may result in neurotransmitter imbalances (Hampp et al. 2008, Hampp, Albrecht 2008). Interrupted circadian rhythms are also associated with different forms of cellular stress (Pluquet, Dejeans & Chevet 2014). Oxidative stress is involved in immunological reactions such as creation of new epitopes, spontaneous DNA mutations, lipid- and protein oxidation and ER stress (Berk et al. 2011). Increased Reactive Oxygen Species (ROS) production in obesity and chronic inflammation causes protein mis- and unfolding resulting in ER stress, which subsequently activates the Unfolded Protein Response (UPR) - this either rescues the cell or activates cell death machinery. **Abbreviations: DNA**... Deoxyribonucleic acid; **GSK3β**… Glycogen synthase kinase-3Beta.

1.2. ER stress and the UPR

The ER is a large membranous network that has diverse important cellular functions like protein synthesis, protein folding and maturation of most secreted and transmembrane proteins. Chaperones assist in folding and modifying proteins correctly. The ER lumen is characterized by a high redox potential, high calcium concentrations and high protein content and provides an ideal milieu for folding and posttranslational modifications of proteins. About one third of newly synthesized proteins are rapidly degraded because of non correct protein folding in the ER "protein folding factory" (Schubert et al. 2000). The accumulation of unfolded and misfolded proteins in the lumen of the ER results in ER stress. Several conditions like calcium depletion, high levels of ROS, folding-defective mutations, viral infections, energy depletion, hypoxia, hyperglycaemia, obesity and the metabolic syndrome were shown to be inducers of ER stress conditions (Bengesser et al. 2016, Volk, Hensel & Kox 1997, Gregersen, Bross 2010).

Accumulation of unfolded and misfolded proteins results in activation of the Unfolded Protein Response (UPR) - a cellular rescue program to overcome states of ER-stress (see Fig. 3). This program either restores the homeostasis of the ER or induces apoptosis- the "suicide of the cell" (Bengesser et al. 2016).

As displayed in Fig. 3, the Mammalian UPR is governed by three major signaling cascades initiated by three ER transmembrane proteins:

1. **IRE1α** (Inositol-Requiring Protein-1α)
2. **PERK** (PKR-like ER kinase)
3. **ATF6** (Activating transcription factor 6).

Under normal physiologic conditions all three ER stress transmembrane receptors (**IRE1α, PERK** and **ATF6**) are occupied by the "ER stress sensor" **BiP** (syn. **GRP78** and **HSPA5**), which binds unfolded proteins. BiP is a chaperone that binds newly synthesized proteins and preserves them in an appropriate state for folding and oligomerization (Bengesser et al. 2016). BiP is a ubiquitously expressed protein, but its synthesis is strikingly induced under ER stress conditions that lead to the accumulation of unfolded proteins in the ER (Bengesser et al. 2016).

ER stress leads to **dissociation** of **BiP** from the mentioned ER stress receptors **IRE1α**, **PERK** and **ATF6**, which activates several downstream signal transduction pathways:

1. **PERK** gets autophosphorylated and dimerizes, which results in activation of the protein. Activated PERK phosphorylates the eukaryotic Initiation Factor of Translation **eIF2α**. Phosphorylated eIF2α leads to inactivation of protein synthesis by blocking the assembly of the ribosomal subunits. PERK induced inhibition of protein synthesis supports the survival of the cell by preventing the ER from obstruction by a devastating amount of nascent protein chains and unfolded proteins. Additionally, phosphorylation of eIF2α leads to expression of *ATF4* (*Activating transcription factor 4*), which activates itself transcription of protective UPR target genes (B'chir et al. 2013).

2. **ATF6** translocates to the Golgi apparatus and gets processed into an active transcription factor. The latter activates transcription of diverse ER stress related genes such as *BiP, CREP/ PPP1R15B* (*Protein Phosphatase 1, Regulatory Subunit 15B*), *CHOP* (*C/EBP homologous protein*) and *XBP1* (*X-box binding protein 1*) (B'chir et al. 2013).

3. **IRE1α** is activated after dissociation of **BiP** and subsequently excises an atypical 26bp intron from the *XBP1* mRNA (see Fig.4). The spliced *XBP1* variant encodes for a transcription factor, which turns on gene expression of many UPR target genes like genes encoding for protective chaperones (van Schadewijk et al. 2012).

1.2.1. The protein folding factory and BD

The importance of ER stress in BD mechanisms is underlined by genetic research. *LMAN2L,* one favourite candidate of a BD GWAS meta-analysis including over 14 000 bipolar cases, encodes for an ER transmembrane protein - a cargo receptor and ER membrane protein, that trafficks glycosylated proteins (Chen et al. 2013). Lim et al. confirmed the positive association between *LMAN2L* rs6746896 and the risk of psychosis in a smaller cross disorder design sample (n= 715 patients with BD and schizophrenia, n= 593 controls). They also found a *LMAN2L*ANK3* gene-gene interaction (Lim et al. 2014).

Only a limited number of ER stress related hypothesis driven gene association studies or gene expression studies in peripheral blood of BD patients and controls are published so far. Nevertheless, there are diverse *in vitro* and *in vivo* studies about ER stress and the UPR in BD, which were summarized in a review article about ER stress and BD that originated from this thesis recently (Bengesser et al. 2016).

For example, Pfaffenseller et al. investigated UPR pathways *in vitro* in lymphocytes obtained from individuals with BD in 2014. Pfaffenseller and colleagues revealed that cell death provoked by the ER stress inducing agent tunicamycin was significantly higher in bipolar patients compared to controls. Tunicamycin induces accumulation of protein chains by preventing the glycosylation of newly synthesized proteins in the ER. Glycosilation of proteins is rather important for quality control and correct trafficking of the proteins. The UPR response to tunicamycin was characterized by Pfaffenseller et al. in BD at protein level. Healthy controls (n= 32) showed an upregulation of UPR related proteins (BiP, eIF2-P and CHOP) in response to ER stress, whereas patients with BD (n= 30) had no adequate response (Pfaffenseller et al. 2014). So et al. (2007) and Hayashi et al. (2009) found similar results at mRNA level after ER stress induction with thapsigargin (inhibitor of the sarco/endoplasmic reticulum Ca^{2+}-ATPase SERCA) and tunicamycin. So et al. found higher *XBP1* and *CHOP* gene expression in healthy controls compared to BD- patients. Thus, the consensus so far is that BD patients may have an impaired response to experimentally induced ER stress (So, Warsh & Li 2007). Hayashi found as well an impaired response to thapsigargin or tunicamycin in BD (n= 59) compared to healthy matched controls (n= 59) on mRNA level with RT-PCR. *XBP1* splicing and *GRP94* gene expression were lower in patients with BD than in controls. *BiP* gene expression did not differ between BD and controls after ER stress induction in lymphoblastoid cells (Hayashi et al. 2009).

CHOP – a transcription factor and another UPR related gene - activates the transcription of numerous proapoptotic factors and is associated with cell death following ER stress (Lakshmanan et al. 2013, Matsumoto et al. 2013). The induction of *CHOP*-expression seems to be impaired in BD after inducing ER stress according to already mentioned *in vitro* studies (Pfaffenseller et al. 2014, So, Warsh & Li 2007, Hayashi et al. 2009). Hypothesis driven gene association studies about *CHOP* are lacking.

In contrast, *XBP1* was quite intensely investigated in the light of BD. Kakiuchi and colleagues published in Nature Genetics in 2003, that the *XBP1* promoter SNP (-116C→G) is associated with BD and leads to altered gene expression of *XBP1* in this mood disorder in lymphoblastoid cells (Kakiuchi et al. 2003). The G allele of the (-116C→G) *XBP1* polymorphism showed reduced *XBP1*-dependent transcription activity compared to the C allele. Furthermore, G genotypes have a worse response to valproate (Kim et al. 2009) and Lithium (Masui et al. 2006). Matigian and his group analyzed global gene expression with whole-genome microarrays in lymphoblastoid cell lines from monozygotic twin pairs discordant for the disease. That gene expression study by Matigian could not replicate Kakiuchi's findings and did not find a dysregulation of *XBP1* and *BiP* (Matigian et al. 2007) in bipolar patients. Also the gene association study by Hou and his group (n= 153 Chinese BD patients, n= 174 healthy controls) could not replicate the accociation between BD and this *XBP1* (-116C→G) SNP (Hou et al. 2004). Summarized, the *XBP1* promoter polymorphism (−116C→G) that is associated with lower gene transcription was associated with BD in a Japanese case-control study by Kakiuchi, but not in European or Chinese studies (Kakiuchi et al. 2003, Hou et al. 2004, Hou et al. 2004, Cichon et al. 2004). So and colleagues investigated the relationship between the *XBP1* (−116C→G) polymorphism and altered gene expression of *XBP1*, but they could not find a correlation between this SNP and attenuated *XBP1* expression (So, Warsh & Li 2007). Based on these inconsistent results, larger replication studies are required, because *XBP1* lies within an interesting region on chromosome 22 (22q12.1). The latter is located close to the highly predisposing region of DiGeorge syndrome, which was highly linked to BD in past linkage studies (Bengesser S 2013).

Based on previous literature, we hypothesized that ER-stress plays a major role in BD and obesity. We furthermore suggest that gene expression of *BiP* and *CHOP* may differ between BD and controls and according to obesity. Moreover, we suggest that *XBP1* splicing differs between the suggested groups. In addition, basic gene association analysis of ER related genes (*LMAN2L, ATF6* and *PERK*) was performed in the course of this thesis.

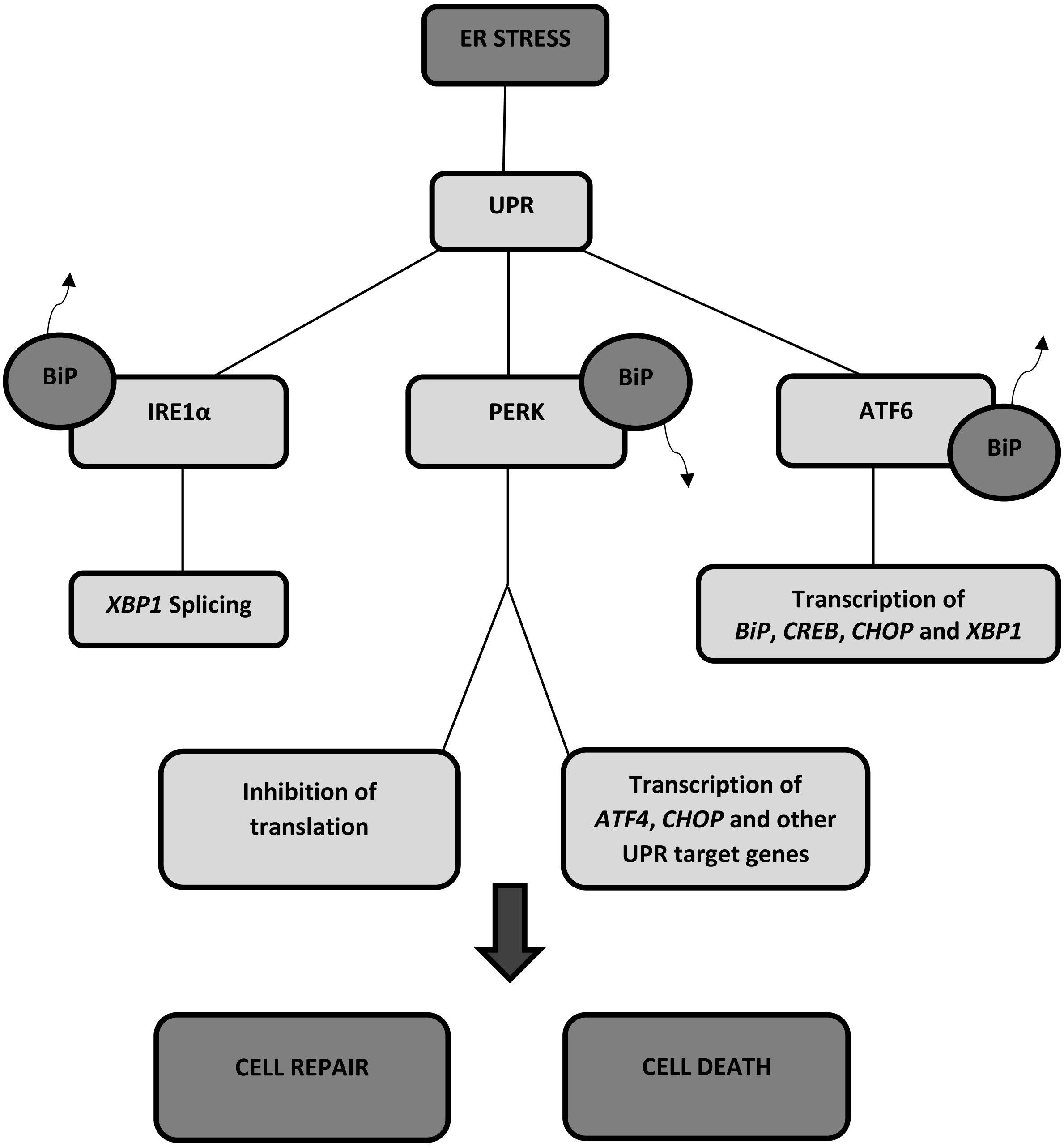


Fig.3. Endoplasmic Reticulum (ER) stress and Unfolded Protein Response (UPR). ER stress results from diverse environmental stressors (e.g. hyperglycaemia, obesity, metabolic syndrome/MetS, radiation or drugs) and is characterized by an accumulation of unfolded and misfolded proteins in the ER. BiP (Binding immunoglobulin protein) is associated with ER transmembrane proteins (IRE1α, PERK and ATF6) and dissociates during ER stress conditions and binds unfolded proteins in the lumen of the ER. Dissociation of BiP activates the three UPR pathways, which results in *XBP1* splicing, inhibition of translation and transcription of *ATF4*, *CHOP*, *CREB*, *XBP1* and other UPR taget genes. **Abbreviations:** ***ATF4***… Activating transcription factor 4; ***ATF6***… Activating transcription factor 6; ***BiP***… Heat shock protein, syn. *HSPA5*, GRP78; ***CHOP***… encodes a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors, syn. ***GADD153***, ***DDIT***; ***CREB***… cAMP responsive element binding protein; ***IRE1α***… Inositol Requiring Enzyme 1 α, splices and activates XBP1; ***PERK***… PKR-like ER kinase, syn. EIF2AK3 eukaryotic translation initiation factor 2-alpha kinase 3; ***XBP1***… X-box binding protein 1;

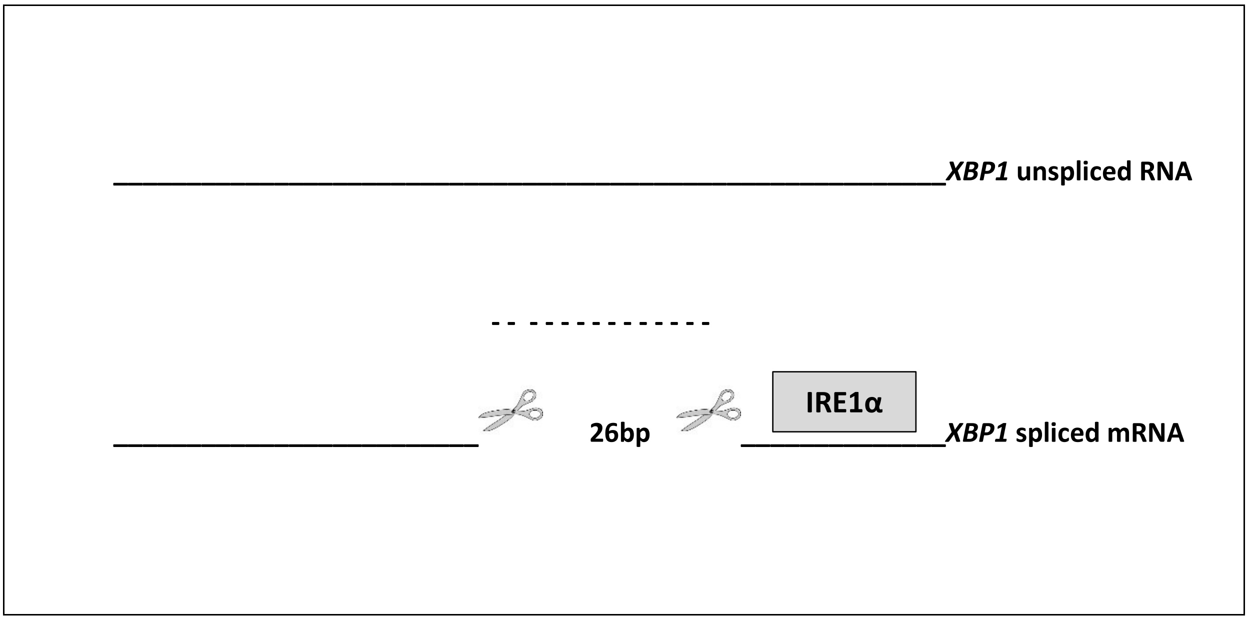


Fig.4. **XBP1 Splicing**. Endoplasmic Reticulum (ER) stress leads to activation of the Unfolded Protein Response (UPR) related enzyme IRE1α (Inositol Requiring Enzyme 1 α). Activated IRE1α excises a 26bp intron from the unspliced XBP1 RNA by means of atypical splicing, which enables translation of the transcription factor *XBP1* and subsequently the expression of further UPR related genes. **Abbreviations**: ***XBP1***… X-box binding protein 1; mRNA… messenger RNA (ribonucleic acid).

1.3. Oxidative Stress

Oxidative stress often results from an imbalance between the generation of Reactive Oxygen Species (ROS) produced by oxygen dependent redox reactions and antioxidative capacity. Under physiological conditions, ROS are generated and eliminated continuously. If the generation of ROS exceeds the antioxidative capacity, or if antioxidative mechanisms are insufficient, ROS levels increase and reach toxic concentrations reffered to as “oxidative stress” (Mangge et al. 2004, 2010)).

Diverse environmental stressors enhance the generation of ROS. Enhanced ROS levels produce damage on lipids, proteins and DNA. ROS-mediated modifications of biomolecules or metabolites such as malondialdehyde (MDA), Thiobarbituric Acid Reactive Substances (TBARS) and carbonylated proteins can be analysed as markers for oxidative stress, because ROS itself are not detectable due to their short half lifes (Mangge et al. 2010, Maes et al. 2013a, Maes et al. 2013b, Mangge et al. 2004). Quantifiable antioxidative markers are GST (Glutathione-S-Transferase), TAC (Total Antioxidative Capacity) and SOD (Superoxide dismutase). GSTs are detoxifying enzymes that catalyze the conjugation of glutathione to xenobiotic substances like drugs etc. SOD, another enzyme that ameliorates the cytotoxicity of ROS, detoxicates the superoxide (O_2^-) radical into ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). The antioxidative marker TAC represents the total amount of antioxidant enzymes and small molecular weight antioxidants (Mangge et al. 2010, Maes et al. 2013a, Maes et al. 2013b, Mangge et al. 2004, Dalvi et al. 2013, Frey et al. 2007, Lenaz et al. 2000, Bengesser et al. 2015, 2016a).

Oxidative stress causes a variety of physiologic dysfunctions as it activates immune-inflammatory pathways that cause increased neurotoxicity and reduced neuroplasticity (Di Dalmazi et al. 2016). ROS damage microstructures and create new epitopes, which can even lead to secondary autoimmune responses to neurotransmitter receptors and neurotransmitters (Maes et al. 2013b). In addition, ROS can induce spontaneous DNA point mutations by deamination of cytosine to uracil (Dutta et al. 2015). Last but not least, oxidative stress is involved in atherosclerosis and other degenerative processes (Berk et al. 2011, Maes et al. 2013a, Maes et al. 2013b, Maes et al. 2011, Moylan et al. 2013).

Oxidative stress belongs, together with ER stress and disturbed circadian rhythms, to the triad of obesity related pathways (Mangge et al. 2004, 2010). There is growing evidence that individuals with BD are disproportionately affected by overweight and obesity and metabolic syndrome when compared to the general population. Cardiovascular diseases, obesity and diabetes are, like in the general population, the most noteworthy reasons of mortality in patients with BD (McIntyre et al. 2010b, Fagiolini et al. 2008, Fagiolini et al. 2008, McIntyre et al. 2010c, Vancampfort et al. 2013, Garcia-Portilla et al. 2009). Evidence from scientific research suggests a disturbed oxidative stress biosignature in BD (Bengesser et al. 2016a, Bengesser et al. 2015). Diverse studies discovered a cross talk between oxidative stress markers (MDA, TBARS and carbonylated proteins) and antioxidative parameters (SOD, GST and TAC) and the pathogenesis of BD (Andreazza et al. 2008, Selek et al. 2008, Gergerlioglu et al. 2007, Kuloglu et al. 2002, Machado-Vieira et al. 2007, Ozcan et al. 2004a, Ranjekar et al. 2003, Savas et al. 2006, Kapczinski et al. 2011b). Furthermore, *in-vitro*-and *in-vivo*-studies of BD revealed that the mood stabilizing agents Lithium and valproate decreased oxidative stress markers like MDA or TBARS and increased antioxidative defense markers like GST (Albayrak et al. 2013, Diniz, Machado-Vieira & Forlenza 2013, Nciri et al. 2013, Khairova et al. 2012, Jornada et al. 2011, Riadh et al. 2011, Bakare et al. 2009). The latter underlines again, that oxidative stress may contribute to disease mechanisms of BD.

Since alterations in oxidative and antioxidative systems are eminent in BD and related to obesity, we analyzed the measurable traces of oxidative stress in our current BIPFAT study cohort. We supposed that the oxidative stress parameters MDA, TBARS and carbonylated proteins are increased in BD. Furthermore, we hypothesized that the measureable antioxidative markers GST, TAC and SOD may be decreased in BD. In addition, we postulated that oxidative markers are increased in obesity and BD.

1.4. The Molecular Clock

Seasonal changes of mood, severe sleeping disorders in mood disorders, antidepressant effects of sleep deprivation and beneficial therapeutic effects of light therapy designate a role of the circadian clock circle in BD disease mechanisms. Clinical experience illustrates that affective episodes are commonly triggered by disturbed circadian rhythms like fragmented or reduced sleep, jetlag and disturbed day structure (Bengesser S 2013).

The circadian rhythms are of high importance for BD because the central 24h clock coordinates many processes in organisms like melatonin or cortisone release, body temperature, sleep wake cycle, eating habits and even neurotransmitter levels. The central pacemaker suprachiasmatic nucleus (SCN) coordinates these circadian processes and peripheral 24h clocks. The central molecular clock in the ventral part of the hypothalamus is directly linked to light/dark cycles by the retinohypothalamic tract (Bengesser S 2013, Abrahamson, Moore 2001). Light applied in the dark synchronizes the circadian clock to function in a 24hour rhythm. Nevertheless, also in complete darkness, without environmental stimuli, the SCN can still maintain almost circadian rhythms (Bengesser S 2013, Porterfield, Mintz 2009).

The molecular clock is regulated by a transcriptional translational feedback loop of more than twenty clock genes (Bengesser S 2013, Shearman et al. 2000). ARNTL plays a major role in the circle as heterodimer with CLOCK and as heterodimer with NPAS2 (Bengesser S 2013, DeBruyne, Weaver & Reppert 2007). Those heterodimers activate gene expression of *PER 1-3*, *CRY 1-2, REV-ERBα* and other clock genes (Langmesser et al. 2008). PER and CRY proteins reach their maximum level in the cytoplasm after 12 hours and are phosphorylated by casein kinases or the serine-threonine kinase GSK3β (glycogen-synthase-kinase-3b) consequently. Phosphorylated PER and CRY enter the nucleus and suppress their own transcription by binding ARNTL-CLOCK heterodimers, which creates a negative feedback loop. The phosphorylation of PER modulates cycle length and is therefore an important mechanism of phase shifts. Of special interest is the fact that the well established and specific mood stabilizers Lithium and valproate inhibit GSK3β and interfere with the molecular clock by this way (Bengesser S 2013, Langmesser et al. 2008).

As mentioned, CLOCK/ARNTL complexes induce gene expression of the clock gene *REV-ERBα*, which inhibits *ARNTL* transcription. *REV-ERBα* is another target of the mood stabilizing Lithium, which degrades *REV-ERBα* gene products leading to increased *ARNTL* gene expression (Bengesser S 2013, Raspe et al. 2002).

One of the most important arms in the clock gene circle in regard of mood is the fact that *ARNTL* initiates the transcription of *MAOA*. According to the model postulated by Hampp et al. in diverse reports, the concatenation of *ARNTL* and *MAOA* gene expression is leading to putative neurotransmitter imbalances (Hampp et al. 2008, Hampp, Albrecht 2008). According to that model, the development of elevated mood goes along with reduced expression of *ARNTL,* respectively depressed mood with high expression of *ARNTL* (Hampp et al. 2008, Hampp, Albrecht 2008).

1.4.1. Clock Genes and Bipolar Disorder

Genetic research underlines the association between BD and the molecular circadian clock. Previous gene association studies are depicted in Table 1. A functional re-analysis of GWAS data with a convergant functional genomics (CFG) approach revealed the association of the clock gene *ARNTL* with BD (Le-Niculescu et al. 2009a). In addition, diverse hypothesis driven gene association studies showed a significant association between individual SNPs of *ARNTL* and BD (Bengesser S 2013, Le-Niculescu et al. 2009a, Mansour et al. 2009, Mansour et al. 2006, Nievergelt et al. 2006). Haplotype analysis discovered an association between BD and *ARNTL* as well as *PER3* haplotypes (Nievergelt et al. 2006). In contrast, some research groups could not replicate these findings (Bengesser S 2013, Kripke et al. 2009, Shi et al. 2008).

NPAS2, encoded on 2q13, is forming a heterodimer with ARNTL and initiates the transcription of other clock genes and *MAOA* (see Fig. 5). Therefore, *NPAS2* plays an important role in the 24h circle and it is not surprising that the *NPAS2* SNPs rs1562313, rs13025524, rs11123857 were significantly associated with BD in hypothesis driven gene association studies (Bengesser S 2013, Mansour et al. 2009, Kripke et al. 2009).

Another essential player in the circadian circle is *REV-ERBα* (*Nuclear Receptor REV-ERBα gene*, syn. *NR1D1*). REV-ERBα inhibits the transcription of *ARNTL* by binding regulatory sites at the promotor of *ARNTL* (Bengesser S 2013, Hampp et al. 2008, Hampp, Albrecht 2008). Diverse gene variants of *REV-ERBα* (e.g. rs2314339) and special haplotypes were significantly associated with BD (Kripke et al. 2009, Severino et al. 2009).

As already mentioned, *PER1-3* are important clock genes, because they regulate the duration of clock cycles. Diverse *PER3* SNPs and *PER* haplotypes were associated with BD in hypothesis driven family-based and case-control association studies (Mansour et al. 2006, Nievergelt et al. 2006). The haplotypes, including rs3789327 and rs2278749, are thought to be central for the resilience against BD (Nievergelt et al. 2006). The *PER3* variable-number-tandem-repeat (VNTR) polymorphism might furthermore influence the age of onset of BD. Homozygocity for this *PER3* VNTR polymorphism was found to lead to BD outbreak at young age (Benedetti et al. 2008). However, Kripke and colleagues could not replicate these findings in their association study (Bengesser S 2013, Kripke et al. 2009).

PER2 SNPs showed modest association with the mood disorder BD (Kripke et al. 2009). *PER2* might be important for mood regulation, as certain mutations of *PER2* subsequently decreased expression of *MAOA* and resulted in increased neurotransmitter levels and elevated mood in mice. Consequently, *PER2* mice mutants showed mania like behavior (Bengesser S 2013, Hampp et al. 2008, Hampp, Albrecht 2008).

CRY1 (encoded on 12q23-q24.1) lies within a locus linked with BD discovered in multiple linkage studies and even in genome wide approaches (Ewald et al. 2002, Glaser et al. 2005, Cassidy et al. 2007, Green et al. 2005). This region on chromosome 12 is associated with Darier's disease, which commonly shows BD as comorbidity (Green et al. 2005).

CRY2 SNPs (chromosomal region: 11p11.2) were not associated with the affective disorder BD in the major amount of genetic studies (Mansour et al. 2006, Nievergelt et al. 2006, Nievergelt et al. 2005). Just one gene association study found a nominal association with BD (Bengesser S 2013, Mansour et al. 2009).

CLOCK (located on 4q12) encodes for a basic-helixloop-helix-PAS transcription factor that activates transcription of several clock genes. Animal testing and gene association studies indicated a role in mood disorders. *CLOCK* mice mutants showed mania like symptoms which could partially be reversed by Lithium (Roybal et al. 2007, Yin et al. 2006). *CLOCK* (e.g. the T3111C SNP rs1801260) seems to play a major role in BD (Bengesser S 2013, Kripke et al. 2009, Benedetti et al. 2003), although other gene association studies showed no significant association (Mansour et al. 2006, Nievergelt et al. 2006, Kishi et al. 2009, Kishi et al. 2009).

The results of hypothesis driven gene association studies and GWAS underline a potential role of clock genes in BD disease mechanisms. Moreover, also knowledge from pharmacological studies point towards involvement of disturbed circadian rhythms in BD. Well established and highly specific mood stabilizing agents like Lithium and valproate influence circadian clock pathways by inhibiting GSK3β and REV-ERBα (Bengesser S 2013, Yin et al. 2006, Jonathan Ryves et al. 2005, McCarthy et al. 2011).

The effect of circadian rhythms on mood is not astounding, as the 24h clock regulates the degradation of tryptamine and catecholamine neurotransmitters indirectly (see Fig.5) (Bengesser S 2013). ARNTL might activate transcription of *MAOA*, which encodes for the neurotransmitter degrading enzyme MAOA/ monoamine oxidase A (Hampp et al. 2008, Hampp, Albrecht 2008). Degradation of serotonin, noradrenaline and dopamine by MAOA diminishs their mood elevating effects (Bengesser S 2013, Hampp et al. 2008, Hampp, Albrecht 2008, Kripke et al. 2009).

Previous scientific reports suggest that reduced *ARNTL* gene expression lower *MAOA* transcription and consequently raise the levels of dopamine, noradrenaline and serotonin (Hampp et al. 2008, Hampp, Albrecht 2008). This is of special interest as neurotransmitter imbalances as one major reason for affective disorders have been discussed for decades. Therefore, I hypothesized that the still not completely elucidated mood swings might be associated with the regulation of *ARNTL* gene expression. It is well known, that methylation of CpG islands (CG rich elements) in close vicinity of a promoter can be an important gene expression regulator (Brenet et al. 2011). Methylation of cytosines in regulatory targets around the promoter leads to silencing of the gene. In contrast, intragenic methylation is usually not associated with silencing of gene expression (Brenet et al. 2011). According to Brenet et al. (2011) dense exonic methylation is far more common than previously recognized or expected statistically. First exons are relatively spared compared to more downstream exons and introns (Brenet et al. 2011).

Thus, according to current knowledge methylation is a means for regulating gene expression. Since gene expression of *ARNTL* may be altered during mood swings of BD, I hypothesized that the generally CG rich clock gene *ARNTL* may be epigenetically modified and that the methylation differs between bipolar patients and controls. Hypermethylation of *ARNTL* could lower gene expression of *MAOA* and may finally results in elevated levels of neurotransmitters serotonin, noradrenaline and dopamine based on reduced degradation.

To my best knowledge, epigenetic analysis of *ARNTL* or other members of the molecular circadian clock have not been performed yet in BD or related mood disorders. Nonetheless, some other epigenetic alterations gained interest in BD research. Promoter methylation of several genes (i.e. *KCNQ3, COMT, HTR1A, HTR2A, SLC6A4*) showed significant methylation alterations in the BD group compared to the control group (Bengesser S 2013, Ghadirivasfi et al. 2011, Nohesara et al. 2011, Abdolmaleky et al. 2006, Carrard et al. 2011, Sugawara et al. 2011).

Table 1. Summary of gene association studies of clock genes in Bipolar Disorder. Abbreviations: *ARNTL*… Aryl hydrocarbon receptor nuclear translocator-like gene, *PER*… period gene, *CRY*… chryptochrome gene, *NR1D1*…nuclear receptor subfamily 1, group D, member 1, syn. *REV-ERBα*, *NPAS2*… neuronal pas domain protein 2, *CSNK1*… Casein kinase I, *CLOCK*… Circadian Locomotor Output Cycles Kaput,

Clock gene	**Chromosomal location**	**Support of association with BD**	**No support of association with BD**
PER1	17p13.1-17p12	Kripke et al. 2009	Mansour et al. 2006 Nievergelt et al. 2006 Shi et al. 2008
PER2	2q37.3	Kripke et al. 2009	Shiino et al. 2003 Mansour et al. 2006 Nievergelt et al. 2006 Shi et al. 2008
PER3	1p36.23	Nievergelt et al. 2006 Mansour et al. 2006 Benedetti et al. 2008 Dmitrzak-Weglarz et al. 2015** Karthikeyan et al. 2014	Shi et al. 2008 Kripke et al. 2009
ARNTL (BMAL1 or MOP3)	11p15	Mansour et al. 2006 Nievergelt et al. 2006 Le-Niculescu et al. 2008 Maciukiewicz et al. 2014 Rybakowski et al. 2014* Dmitrzak-Weglarz et al. 2015**	Shi et al. 2008 Kripke et al. 2009
NPAS2	2q13	Kripke et al. 2009	Nievergelt et al. 2006
CRY1	12q23-q24.1	Ewald et al. 2002 Glaser et al. 2005 Green et al. 2005 Cassidy et al. 2007	Nievergelt et al. 2005 Mansour et al. 2006 Shi et al. 2008
CRY2	11p11.2		Mansour et al. 2006 Nievergelt et al. 2006
CSNK1D	17q25	Kripke et al. 2009	Shi et al. 2008
REV-ERBα (NRIDI)	17q11.2	Kripke et al. 2009 Severino et al. 2009	Shi et al. 2008
CLOCK	4q12	Shi et al. 2008 Kripke et al. 2009	Mansour et al. 2006 Nievergelt et al. 2006 Kishi et al. 2009
TIMELESS	12q12-q13	Mansour et al. 2006	Shi et al. 2008
CSNK1ε	22q13.1	Nievergelt et al. 2006 Shi et al. 2008	

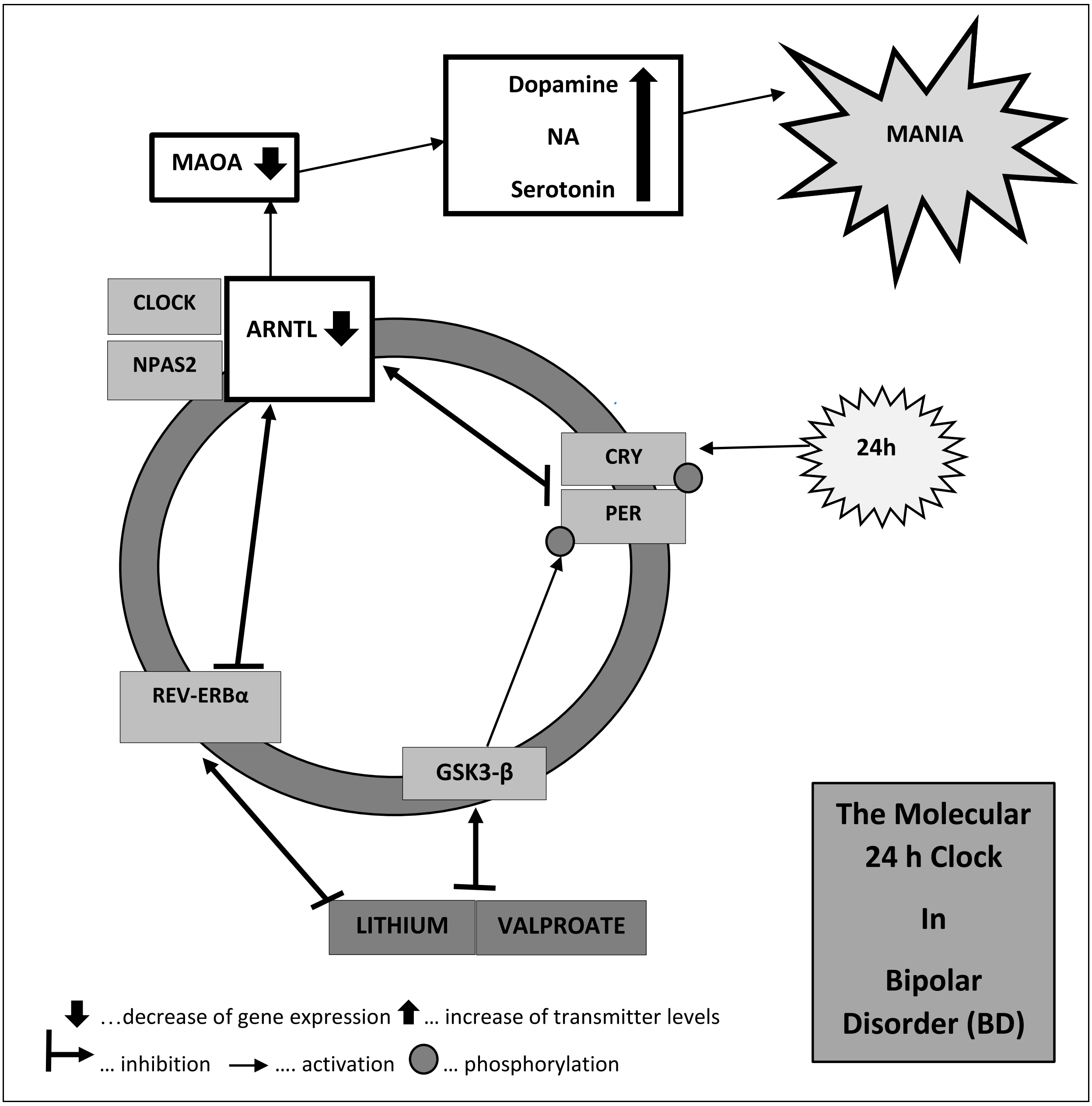


Fig.5. The molecular clock and Bipolar Disorder (BD): Light at dusk or dawn synchronizes the circadian molecular clock in a 24h rhythm. The light is perceived by the retinohypothalamic tract. The light impulse is captured in the suprachiasmatic nucleus (Abrahamson, Moore 2001). ARNTL-CLOCK heterodimers activate transcription of clock genes like *PER and CRY* (Langmesser et al. 2008, Gekakis et al. 1998). Accumulated PER and CRY proteins get phosphorylated by casein kinases or glycogen-synthase-kinase-3Beta (GSK3β), which modulates the length of the cycle (Etchegaray et al. 2009). Lithium and valproate inhibit GSK3β, which can influence cycle length. Phosphorylated PER and CRY enter the nucleus and inhibit their own transcription by binding ARNTL-CLOCK heterodimers. The latter creates a negative feedback loop (Langmesser et al. 2008). In parallel, ARNTL-CLOCK complexes initiate transcription of *REV-ERBα*, which inhibits the transcription of *ARNTL* that represents another feedback regulation (Guillaumond et al. 2005). Most important, NPAS2/ARNTL heterodimers activate transcription of the neurotransmitter-degrading enzyme monoamine oxidase (MAOA) (Hampp et al. 2008). The latter can principally explain mood switches resulting from disturbed circadian rhythms. **Abbreviations**: NA… Noradrenaline, *NPAS2*… Neuronal pas domain protein 2, *ARNTL*… Aryl hydrocarbon receptor nuclear translocator-like gene, *CLOCK*… Circadian Locomotor Output Cycles Kaput, *PER*… period gene, *CRY*… Chryptochrome gene, *NR1D1*… Nuclear receptor subfamily 1, group D, member 1 (syn.*REV-ERBα)*

1.5. Hypotheses and aims

(I) The first major hypothesis aimed at investigating group differences between BD and controls in genetic markers of the triad oxidative stress, ER stress and the molecular circadian clock.

(II) The second hypothesis aimed at analyzing the correlation between the mentioned pathways and overweight/obesity.

The concrete scientific questions included:

- Are ER stress related genes (*BiP* and *CHOP*) differentially expressed between BD and controls?
- Are there differences in *XBP1* splicing between BD and controls?
- Do ER stress related parameters (*BiP*, *CHOP* and *XBP1*) differ according to weigth classes (normal weight, overweight, obesity)?
- Do ER stress related parameters (*BiP*, *CHOP* and *XBP1*) correlate with BMI?
- Are ER related genes (*ATF6* rs2271013, *PERK* rs867529, *LMAN2L* rs9834970, *LMAN2L* rs6746896) associated with BD?
- Are genotypes of ER related genes (*ATF6* rs2271013, *PERK* rs867529, *LMAN2L* rs9834970, *LMAN2L* rs6746896) associated with differing BMI?
- Do (anti)oxidative markers (MDA, TBARS, GST, SOD and TAC) differ between BD and controls?
- Do oxidative (MDA, TBARS) and antioxidative (GST, SOD and TAC) parameters differ between overweight and normal weight?
- Do (anti)oxidative markers correlate with BMI?
- Is methylation of the clock gene *ARNTL* differing between BD and controls?
- Does *ARNTL* methylation correlate with BMI?
- Are clock gene genotypes (*PER3* rs10864315, rs228682, rs228642 and rs10462021 and *CLOCK* rs12649507, rs1801260 and rs534654) differing between BD and controls?
- Are clock genes (*PER3* rs10864315, rs228682, rs228642 and rs10462021 and *CLOCK* rs12649507, rs1801260 and rs534654) associated with differing BMI levels in BD versus controls?

2. Material and Methods

2.1. Description of the BIPFAT and BIPGENETICS study

The data of this doctoral thesis was collected as a part of the Austrian BIPFAT and BIPGEN studies (Reininghaus et al. 2014, Reininghaus et al. 2013). The predominant aim of the BIPFAT study is to identify common underlying pathomechanisms of BD and metabolic disorders. The partially overlapping BIPFAT and BIPGEN (BIPGENETICS) studies have been conducted at the Department of Psychiatry and Psychotherapeutic Medicine of the Medical University of Graz (MUG), Graz, Austria.

Blood was taken between 8.30 a.m. and 9.30 a.m. from overnight fasting study participants. Genetic and epigenetic analyses were performed according the the workflow depicted in the next chapters. Body weight [kg] of patients was assessed and the BMI [kg/m2] was calculated. Weight groups were built: I) three weight groups: normal weight (BMI < 25 kg/m^2) versus overweight (BMI < 25 kg/m^2 and > 30 kg/m^2) versus obese study participants (BMI > 30 kg/m^2). II) only two weight groups if one subgroup contained less than 10 persons: normal weight (BMI<25 kg/m^2) versus overweight/obese study participants (BMI > 25kg/m^2).

Test persons with BD have been recruited over three years at the Department of Psychiatry and Psychotherapeutic Medicine (MUG). Bipolar patients were diagnosed according to the criteria of DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, 4th edition) using the SCID-I (Structured Clinical Interview for DSM-IV Axis I Disorders). Bipolar study participants were former in- or outpatients of the Department of Psychiatry and Psychotherapeutic Medicine. All study participants with BD as well as healthy controls gave written informed consent. The studies had been approved by the local ethics committee at the MUG (Reference numbers: 24-123 ex 11/12 and 23-199 ex 10/11) in compliance with the current revision of the Declaration of Helsinki, ICH guideline for Good Clinical Practice and current regulations. Exclusion criteria of bipolar probands were a history of severe substance abuse and severe personality disorder.

For the healthy control group individuals without a history of severe psychiatric disease and without a severe psychiatric disease in first grade relatives were recruited. Since it was a naturalistic study design, some probands had medical comorbid diseases (e.g. hypothyreosis) and received appropiate treatment. The description of cohorts is given in Table 2-4.

In all BIPFAT and BIPGEN cohort subsets, patients with BD were treated either with a mood stabilizing mono- or combination therapy with Lithium, anticonvulsants (carbamazepine, oxcarbazepine and valproic acid) or atypical antipsychotics (aripiprazole, olanzapine, quetiapine, ziprasidone, risperidone and clozapine) and in some cases with additional antidepressants. For further information about the study design and preliminary results of the BIPFAT study see our previous reports (Reininghaus et al. 2013, Bengesser et al. 2016a, Bengesser et al. 2015, Lackner et al. 2015, Reininghaus et al. 2015).

Table 2. Description of the ER stress gene expression analysis cohort. Gene expression analysis of *BiP* and *CHOP*, as well as unconventional *XBP1* splicing, were performed in BD (n= 54) and controls (n= 54). **Abbreviations**: BD… Bipolar Disorder, BMI… Body Mass Index, M… mean, SD… Standard Deviation

Description of the ER stress gene expression cohort

	Bipolar Disorder	***Controls***	***Differences between Bipolar Disorder and Controls***
Number of study participants	54	54	
Age [years] (M +/- SD)	43 (+/- 14)	39 (+/- 16)	***p*< 0.05**
Age range [years]	18 – 80		
Males (n)	30	17	***p*< 0.05**
Females (n)	24	37	
BMI [kg/m^2] (M +/- SD)	28.32 (+/- 5.04)	25.26 (+/- 4.33)	***p*< 0.05**
Lithium intake in BD	43 %		
Antiepileptics intake in BD	32 %		
Atypical antipsychotics intake in BD	54 %		
Combination therapy	52 %		
Smokers	46 %	35 %	***p*< 0.05**

Table 3. Description of the oxidative stress cohort. Serum markers for oxidative stress (MDA and TBARS) and for the antioxidative defense (GST, SOD and TAC) were analyzed in BD (n= 138) and controls (n= 75). **Abbreviations**: BD… Bipolar Disorder, BMI… Body Mass Index, M… mean, MDA… malondialdehyde, TBARS… TBA-reactive substances, GST… Glutathione-S-transferase, SOD… superoxide dismutase, SD… Standard Deviation, TAC… Total antioxidative capacity.

Description of the oxidative stress cohort

	Bipolar Disorder	*Controls*	*Differences between Bipolar Disorder and Controls*
Number of study participants (n)	138	75	
Age [years] (M +/- SD)	43.8 (+/- 13.4)	38.3 (+/- 15.6)	***p*< 0.05**
Age range [years]	18 – 80		
Males (n)	72	30	***p*< 0.05**
Females (n)	66	45	
BMI [kg/m^2] (M +/- SD)	28.3 (+/- 6.0)	24.7 (+/- 4.8)	***p*< 0.05**
Lithium intake in BD	28 %		
Antiepileptics intake in BD	32 %		
Atypical antipsychotics intake in BD	63 %		
Combination therapy	95%		
Smokers	52 %	27%	***p*< 0.05**

Table 4: Description of the total Epigenetics cohort. **Abbreviations:** BD… Bipolar Disorder, BMI… Body Mass Index, M… mean/ average, SD… Standard Deviation. The Genotyping cohort overlaps with the epigenetics sample and displays a similar distribution of clinical markers.

Description of the Epigenetics cohort

	Bipolar Disorder	*Controls*	*Differences between Bipolar Disorder and Controls*
Number of study participants (n)	159	70	
Age [years] (M +/- SD)	44.27 +/- 13.91	41.23 +/- 15.85	***n.s.***
Age range [years]	18 – 80		
Males	48.77 %	32.67 %	***p*= 0.010**
Females	51.23 %	67.33 %	
BMI [kg/m^2] (M +/- SD)	28.07 (+/- 6.14)	25.08 (+/- 4.73)	***p*< 0.001**
Lithium intake in BD	26 %		
Antiepileptics intake in BD	29 %		
Smokers	50%	32 %	***p*< 0.05**

2.2. ER stress analysis

2.2.1. Overview of ER stress analysis

2.2.1.1. Gene expression study of CHOP and BiP

The workflow is depicted in Fig. 6. Fasting blood was taken from cubital veins of bipolar probands and healthy controls (between 8:30 and 9:30 a.m.) at the Department of Psychiatry and Psychotherapeutic Medicine and collected in Lithium heparin tubes. Then standard "Ficoll-density-gradient-separation" using Histopaque® (Sigma-Aldrich, St.Louis, MO, USA) was performed approximately one hour after blood taking at the Department of Pathophysiology and Immunology (MUG) following the introductions of the supplier. The isolated peripheral blood mononuclear cells (PBMCs) were kept at -20°C till further processing. RNA was isolated from defrosted PBMCs with TRI ReagentRT® (MRCinc, Cincinnati, OH, USA) according to the SOPs of the institute. Total RNA was reverse transcripted to obtain cDNA by means of the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Thermo Fisher, Foster City, CA, USA). Afterwards, gene expression analysis of the ER stress related genes *CHOP*, *BiP* and the reference gene *HPRT1* was performed by quantitative RT-PCR (qPCR).

2.2.1.2. XBP1 Splicing analysis

XBP1 splicing analysis was performed by semiquantitative RT-PCR using the One Step RT-PCR Kit® obtained from Qiagen (Hilden, Germany) and following gel electrophoresis. Image processing software (freeware software "GelAnalyzer") was used to quantify the intensity of *XBP1* and *XBPs* (spliced *XBP1*) cDNA bands in the gels.

The following PCR program was used for amplification of *XBP1*:

a. 30 min at 50°C
b. 15 min at 95°C
c. 30 sec. at 94°C
d. 30 sec.at 60°C
e. 1 min at 72°C:
f. Repetition of point c-e 31 times, à 32 cycles
g. 10 min at 72°C
h. 4° „forever“

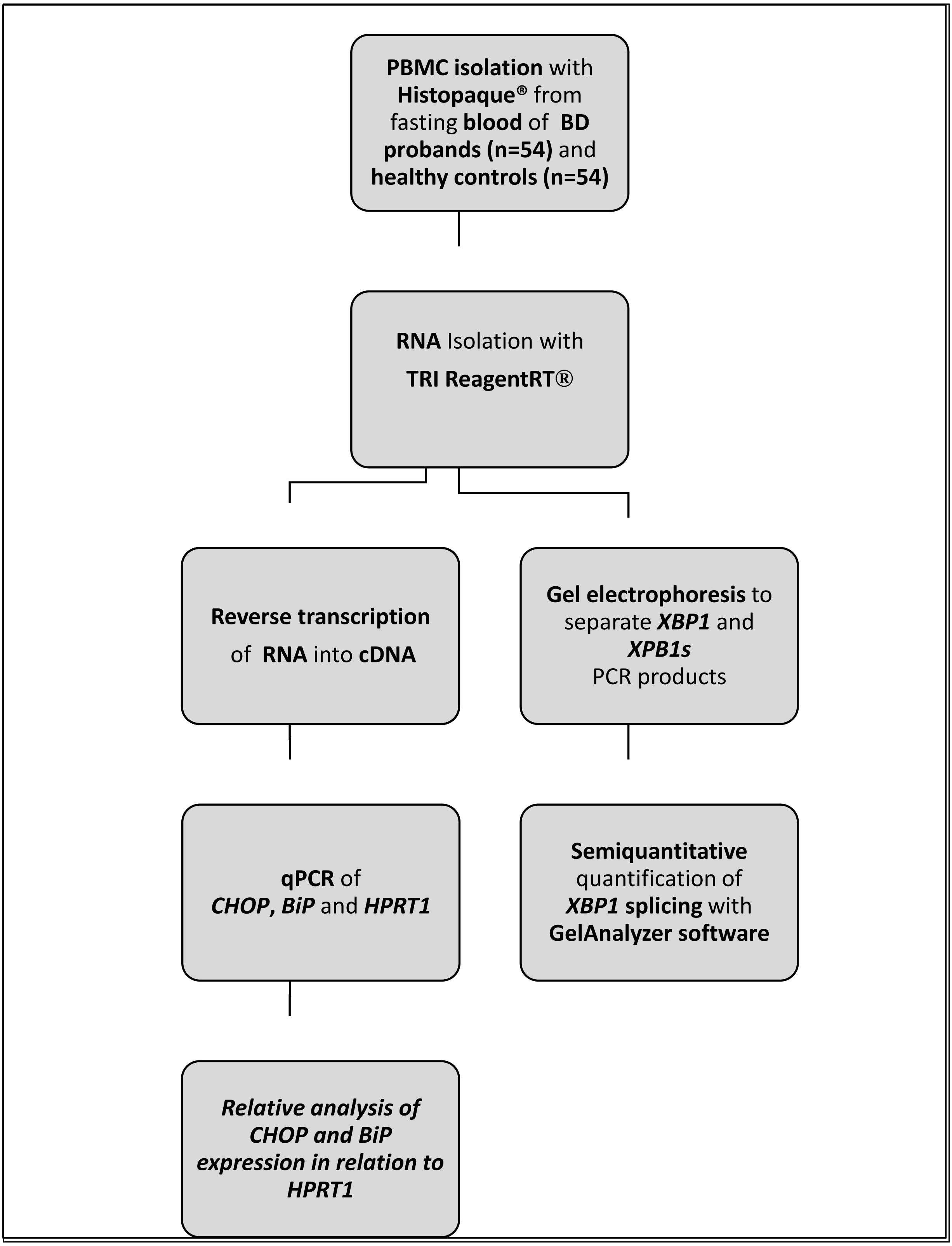


Fig.6. Workflow Endplasmic Reticulum (ER)- Stress. Abbreviations: ***BiP***… Binding immunoglobulin protein syn. *HSPA5*, GRP78; ***CHOP***… encodes a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors, syn. ***GADD153***, ***DDIT***; ***XBP1***… X-box binding protein 1; **XBPs**… spliced XBP1 RNA; **XBP1u**… Unspliced XBP1 RNA, PBMC…peripheral blood mononuclear cells, qPCR…quantitative PCR (polymerase chain reaction), cDNA…complementary DNA, **HPRT1**… constitutively expressed house keeping gene, reference gene

2.2.2. Isolation of Peripheral Mononuclear Cells (PBMCs)

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from Lithium heparin anticoaculated whole blood by means of “Ficoll density centrifugation”. The isolation process was performed in the laminar flow in a sterile environment. Ficoll® is widely distributed but in this present investigation Histopaque®-1077 was used.

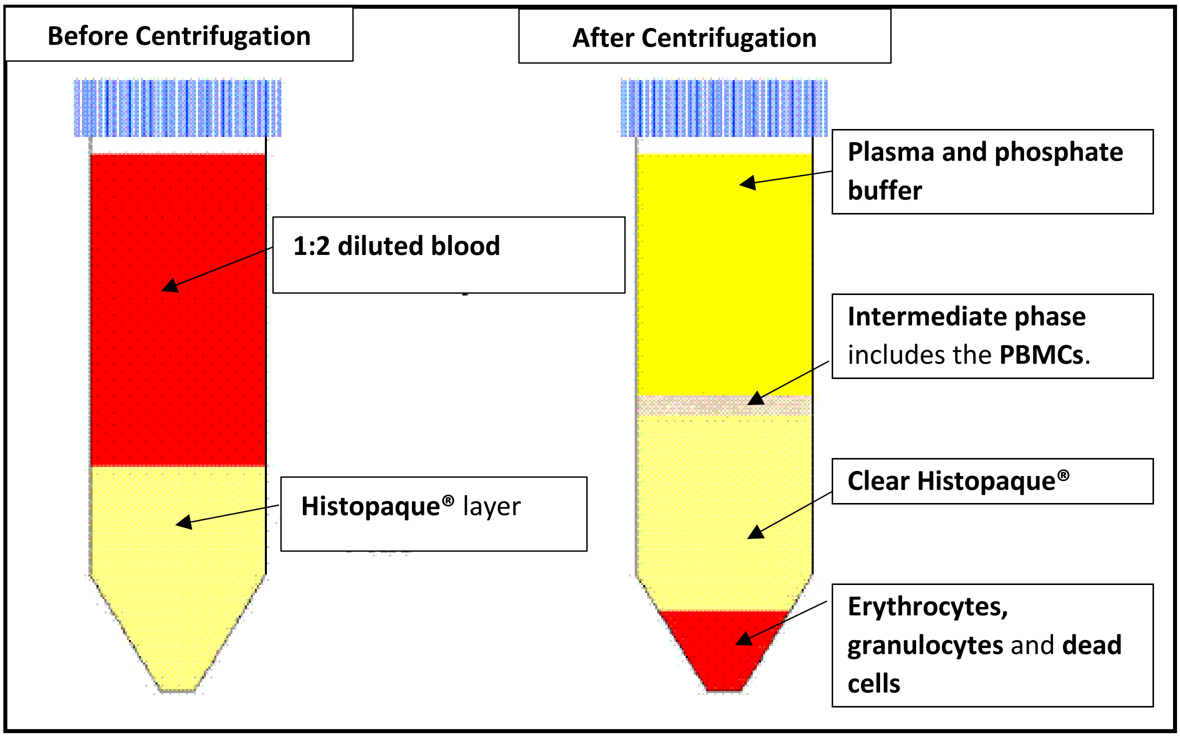


Fig. 8. Histopaque® density-gradient-centrifugation

In summary, 8 ml Lithium heparin whole blood was diluted 1:2 with calcium and magnesium free phosphate buffer (CMF PBS) in a 50ml Sarstedt® tube. Histopaque®-1077 (9 ml) was carefully and slowly overlayed with 16 ml of the 1:2 diluted blood. Centrifugation at 400g for 30 min without break was performed. Then, the interphase containing the mononuclear cells was harvested with a sterile disposable Pasteur pipette and washed twice in CMF PBS at 400 g for 10min with mild break. Afterwards the obtained cell pellet was stored at -20°C.

2.2.3. Isolation of RNA using TRI Reagent RT

RNA isolation from PBMCs was performed with TRI ReagentRT®. The latter is a complete and ready-to-use reagent for the isolation of total RNA or the simultaneous isolation of RNA, DNA and proteins from human or animal samples. The principle is based on the different solubility of RNA, DNA and proteins in a solution with aqueous and organic phases. The pelleted PBMCs were homogenized/ lysed in TRI ReagentRT and the homogenate was separated into the phases by addition of the chaotropic and denaturating reagent Guanidinium thiocyanate. RNA remains entirely in the aqueous phase, DNA stays in the interphase and proteins were kept in the organic phase. RNA was precipitated from the aqueous phase by addition of isopropanol, washed with ethanol and solubilized in nuclease-free water.

For the isolation of RNA 1 ml of TRI ReagentRT was added to each tube containing pelleted cells. Then repetitive pipetting was used for lysis of the pelleted PBMCs. After homogenization 50 µl 4-Bromanisole (BAN) per 1 ml TRI ReagentRT was added to the tubes and tubes were subsequently inverted over 20 times. Then, the suspensions were incubated at room temperature for 5 min. Then the tubes were centrifuged at 12,000 g for 15 min at 4°C. The resulting seperated aqueous phase was transferred to a fresh tube and the RNA was precipitated by adding 500 µl isopropanol per 1 ml TRI ReagentRT. Samples were stored at room temperature for 10 min and centrifuged at 12,000 g for 8 min at 4°C. RNA precipitated and formed a white pellet at the bottom of the tube, which was visible after centrifugation. The supernatant was removed and the pellet was resuspended in 1 ml of 75% EtOH. Subsequently, the tube was centrifugated at 7.500 g for 5 min at 4°C. The supernatant and ethanol were carefully removed and the pellet briefly air-dried under the hood for 3-10 min until the white pellet got more or less transparent. Dependent on the size of the pellet 30 µl –50 µl RNAse free water was added and and the tubes were incubated for 10-15 min at 55-60°C and 300 rpm in a thermo shaker. Then, RNA was immediately placed on ice. RNA concentration was measured by a NanoDrop 1000® spectrophotometer (Thermo Fisher). As a quality check 2 µl of each RNA sample was mixed with 8 µl of nuclease free H_2O and 2 µl 6x loading puffer and loaded on a 1 % agarose gel. RNAs were separated by gel electrophoresis for 40 min at 70V.

2.2.4. Reverse Transcription

RNA was transcribed into cDNA with the **“High Capacity RNA to cDNA Kit”** of Applied Biosystems/Thermo Fisher. Freezed RNA stock solutions (stored at -20°C) were thawed on ice. Then, RNA stock solutions were diluted with nuclease free H_2O to reach a final RNA concentration of 100ng/µl.

10µl of Master Mix (MM) containing RT buffer, dNTP Mix, random primers, reverse transcriptase and nuclease free H_2O were mixed with 1000ng of RNA (total volume 20µl). RT minus controls were used to prove absence of contamination of the used materials.

Thermocycler Program for reverse transcription

The following program was used for reverse transcription in the **THERMAL CYCLER C1000** (Bio-Rad, Hercules, CA, USA):

10 min: 25 °C
120 min: 37 °C
5 min: 85 °C
∞: 4 °C

The resulting cDNA probes of 108 subjects were used for further quantitative PCR (qPCR) analysis of genes of interest and the reference gene *HPRT1* (see next chapter).

2.2.5. Real Time Quantitative PCR (qPCR) of *CHOP, BiP* and *HPRT1*

Real Time Quantitative PCR (qPCR) of the ER stress related genes *CHOP* and *BiP*, as well as the reference gene *HPRT1* was performed with SYBR® Green based techniques. The qPCR was performed for each sample in triplicates.

4µl of 1:10 diluted cDNA (5ng/µl) from PBMCs of probands was pipetted into the wells of a 96 well plate. The complete reaction (15 µl) for qPCR of *CHOP*, *BiP* or *HPRT1* included 7.5 µl iQ™ SYBR® Green Supermix (Bio-Rad), 0.3 µl forward primers, 0.3 µl reverse primers (primer sequences are listed in Table 5) and 2.9 µl nuclease free H_2O.

Table 5. qPCR Primers. **Abbreviations**: *CHOP*… encodes a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors, syn. *GADD153*, *DDIT; BiP*… Binding immunoglobulin protein, syn. *GRP78* and *HSPA5*, *HPRT1*… hypoxanthine phosphoribosyltransferase 1.

	Primer
BiP **forward**	5‘-TGCAGCAGGACATCAAGTTC-3‘
BiP **reverse**	5‘-AATAAGCCTCAGCGGTTTCT-3‘
CHOP **forward**	5’-TGTTTCACCTCCTGGAAATGAAATGAAGAGGA-3’
CHOP **reverse**	5’-TCACCATTCGGTCAATCAGA-3’
HPRT1 **forward**	5’-GACCAGTCAACAGGGGACAT-3’
HPRT1 **reverse**	5’-CTGCATTGTTTTGCCAGTGT-3’

We used in the present study the house keeping gene *HPRT1* as reference gene for calculation of relative gene expression and also as interplate calibrator. After adding the master mix and cDNAs to the PCR-plates, we started the PCR-reaction in a CFX96™ Real-Time PCR Detection System (Bio-Rad). Melting curve analysis was performed to confirm target-specifity. Then, quantification of gene expression was assessed by analysis of the obtained fluorescence curve kinetics of *BiP* or *CHOP* in comparison to the house keeping gene *HPRT1*. First of all the “Cycle Quantification values” (CQ) were analysed. The CQ is equivalent with that amplification cycle, in that the detected fluorescence exceeds the defined threshold (background fluorescence) - indicating amplification of the cDNA of interest. The so called DeltaCQ values were calculated according to the following formula:

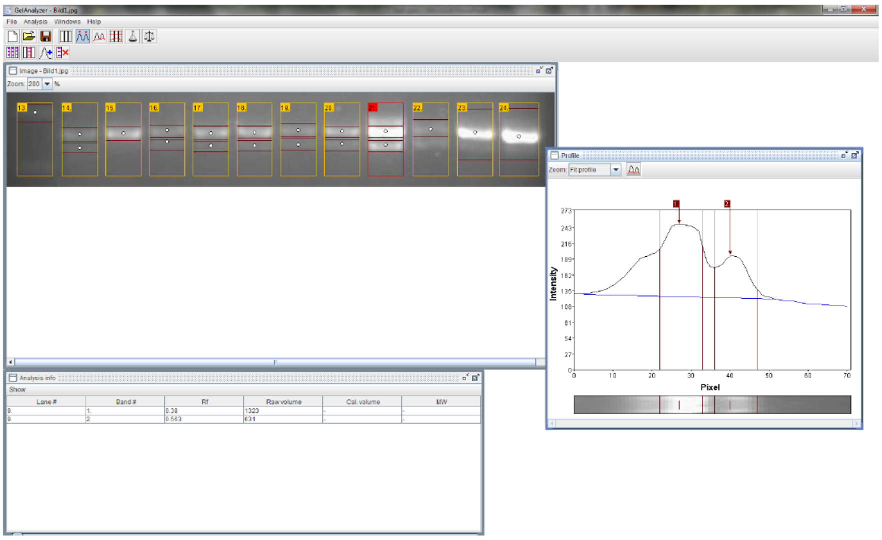
DeltaCQ= CQ (gene of interest) – CQ (reference gene)

Fig.9. Formula for calculating DeltaCQ values. DeltaCQ values represent the normalized mRNA expression levels of genes of interest. **Abbreviations**: CQ… Cycle Quantification values

2.2.6. *XBP1* Splicing-assay

The mRNA of *XBP1* retains an atypical intron, which is cut by the enzyme IRE1α in the course of the UPR (Thorpe, Schwarze 2010). The detection of spliced *XBP1* mRNA (=*XBP1s*) represents a method to detect the activation of an UPR-response at the mRNA level. Splicing was analyzed by RT-PCR in an endpoint analysis of obtained PCR-products using agarose gel electrophoresis. The intensity of the bands of *XPB1* (unspliced form), respectively spliced form of *XBP1* (*XPB1s*) bands, was assessed semiquantitatively by means of the free image processing program GelAnalyzer (LazarSoftware).

The first step of *XBP1* splicing analysis employed the **QIAGEN® One Step RT-PCR Kit** to perform reverse transcription of RNA and quantitative PCR with one single Kit (basically in one tube sequentially). In the first step reverse transcriptase produced cDNA, then the second enzyme (HotStarTaq® DNA polymerase) gets activated by heat-activation after reverse transcription. After amplification, products of *XBP1* were loaded on a 2.3 % agarose gel (2:45h running time, 32 cycles). Bands represented either spliced or unspliced *XBP1* mRNA. The intensity of the bands was quantified with the software GelAnalyzer and measured with [A.U.] respectively [A.U.C.] (Area under the curve) (Fig.10).



Lane #	Band #	Rf	Raw volume	Cal. volume	MW
9.	1.	0.38	1323	-	-
9.	2.	0.563	631	-	-

Fig.10: Semiquantitative analysis of XBP1–splicing using GelAnalyzer software. The figure shows a screenshot of the GelAnalyzer analysis of one XBP1 band on a representative gel. In summary, bands on the gels (Image) are converted to histograms (Profile). The area under the curve of the histogram corresponds to the intensity of the band (= Raw volume [Arbitrary Unit]) as read out of the assay.

2.2.7 Analysis of GWAS data

DNA was extracted from fasting blood samples with the salting out method according to the SOP in the attachment. Bipolar study participants (n= 150) and healthy controls (n= 77) were genotyped with the OmniExpress1.1 BeadChip by Illumina (Munich, Germany) at the „Life & Brain Center"at the University of Bonn (Bonn, Germany) according to the manufacturers' protocols, which are publicly available at:

http://support.illumina.com/array/array_kits/humanomniexpress-24-beadchip-kit/documentation.html

The genotyping was performed with the proven HiScan® or iScan® system (Illumina). Standard quality controls (QC) like SNP calling rates, family error tests, Hardy Weinberg Equilibrium and population stratification analysis were performed by the specialist Urs Heilbronner at the Universiy of Göttingen and Munich (LMU Munich, Germany).

2.2.7.1. Genotype extraction for Genotype X Obesity interaction analysis

ER related SNPs were chosen by literature search via the search engines Pubmed, OMIM and dbSNP. Only those SNPs available on the Omniexpress1.1 chip were extracted hypothesis driven with the software **PLINK** (v1.07, author: Shaun Purcell, URL: http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell et al. 2007).

The following SNPs of ER related genes were analysed in the current sample:

- ***PERK*** rs867529
- ***ATF6*** rs2271013
- ***LMAN2L*** rs9834970 and rs6746896

The general command plink --file data --extract mysnps.txt was used for **SNP extraction** in PLINK.

Three genotype groups (e.g. AA vs. AG vs. GG) were used as independent variables in SPSS analysis to investigate possible differences regarding BMI between ER related genotypes.

2.2.7.2. Gene association study (Case/ control design)

The basic association test was used to analyse the disease trait of BD by comparing allele frequencies between affected cases and controls. Used general commands for the basic gene association test in PLINK: plink --file mydata --assoc

2.3. Oxidative Stress

2.3.1. Description of Oxidative Stress BIPFAT study subset

The description of the analyzed cohort is displayed in Table 3.

2.3.2. Description of Biological Assays for Oxidative Stress Analysis

MDA (malondialdehyde) was investigated by a novel, specific method using GC-MS (gas chromatography-mass spectrometry) provided by Thermo Fisher Scientific (Fremont, CA, USA). The GC-MS method is based on derivatisation of MDA with 2,4-dinitrophenylhydrazine. Second, MDA and other TBA-reactive molecules were measured with an additional method based on TBA-reaction and HPLC/ high-performance liquid chromatography, which is broadly used in other scientific original reports. The use of the second method helps comparing our results to other previously published results. The TBA-reaction followed by HPLC and fluorometric detection was used to detect TBARS (Merck-Hitachi, Stuttgart, Germany). Total Antioxidative Capacity (TAC) was estimated by the TAC ELISA kit from Omnignostica Forschungs GmbH (Vienna, Austria). The antioxidative enzyme GST was determined by the GST-π ELISA Kit (Immundiagnostik, Bensheim, Germany). SOD was investigated with the Serazym® Cu/Zn SOD ELISA (Seramun, Wolzig, Germany). Analysis of carbonylated proteins was done with the Carbonyl Protein ELISA Kit (Immundiagnostik). For further information about the exact oxidative stress study protocol I refer to the original reports based on this thesis (Bengesser et al. 2014, 2016a).

2.4. The Molecular Clock

2.4.1. Methylation analysis of CG rich elements of ARNTL- OVERVIEW

The workflow is depicted in Fig. 11. DNA was isolated from fasting blood at the Institute of Human Genetics, MUG. DNA isolation was performed by the salting out method according to the SOPs in the attachment. To measure methylation status of CG rich regions of *ARNTL* (PS2 POS1-7 and cg05733463) DNA was conversed into bisulfite treated DNA with the Epitect-Kit® by Qiagen. Bisulfite treatment of DNA was performed according to the Epitect-Kit® manual. Afterwards, PCR and pyrosequencing were performed at the University of Bonn, Institute of Neuropathology to distinguish between methylated and unmethylated cytosines. PCR and pyrosequencing were performed according to the SOPs of the institute. The used primers for PCR and pyrosequencing for cg05733463 and PS2 are depicted in Table 6. Methylation itself cannot be measured, but unmethylated cytosines are conversed into uracil by the bisulfite treatment. In contrast, methylated cytosines are not altered by the procedure. Thus, the sequences differ after PCR and pyrosequencing depending on the methylation status and differentiation between methylated and unmethylated Cs is possible. The interpretation of the pyrograms and the calculation of the methylation status in % were performed at the University of Bonn, Institute of Neuropathology.

Table 6.: **Primers for Methylation analysis of *ARNTL*** (Primers for PCR and pyrosequencing) – designed by A.Waha. **Abbreviations**: f… forward, r… reverse, ps… pyrosequencing, BIOT… biotinylated.

Primers for PCR and Pyrosequencing in *ARNTL* Methylation Analysis

Primers for PS2	PS2-f	TAGGGTAGGTAGAGGTGTTGTAGG
	PS2-r	TCACTACCCCCAAAAACAAAACTAT (**BIOT**.)
	PS2-ps	GGTGTTGTAGGAGTTT
Primers for cg05733463	cg05733463-f	GGGAATTGTTTTTTTGGTTGTAGT
	cg05733463-r	CCCACAACACAAAATATTAATCAT (**BIOT**.)
	cg05733463-ps	TGTTTTTTTGGTTGTAGTTTAA

2.4.2 Hypothesis driven gene asscociation study of clock genes and BD

Basic Gene Association analysis was performed as depicted in chapter 2.2.7. The workflow is depicted in Fig.11.

2.4.3. Clock gene Genotypes and BMI

The workflow is depicted in Fig.11. **Hypothesis driven genotype extraction** was performed with **PLINK**. ***PER3*** SNPs (rs10864315, rs228682, rs228642, rs10462021) and ***CLOCK*** SNPs (rs534654, rs1801260 and rs12649507) were extracted. Then, three genotype groups e.g. AA, AG and GG or two groups e.g. AA+ AG and GG were built to compare BMI levels according to genotype groups between BD and controls. **ANCOVAs** with major factors group (BD versus controls) and genotype of the investigated clock gene and **BMI** as dependent variable, as well as age, sex and smoking (yes, no) as covariates were performed to test whether certain genotypes showed significantly differing BMI levels.

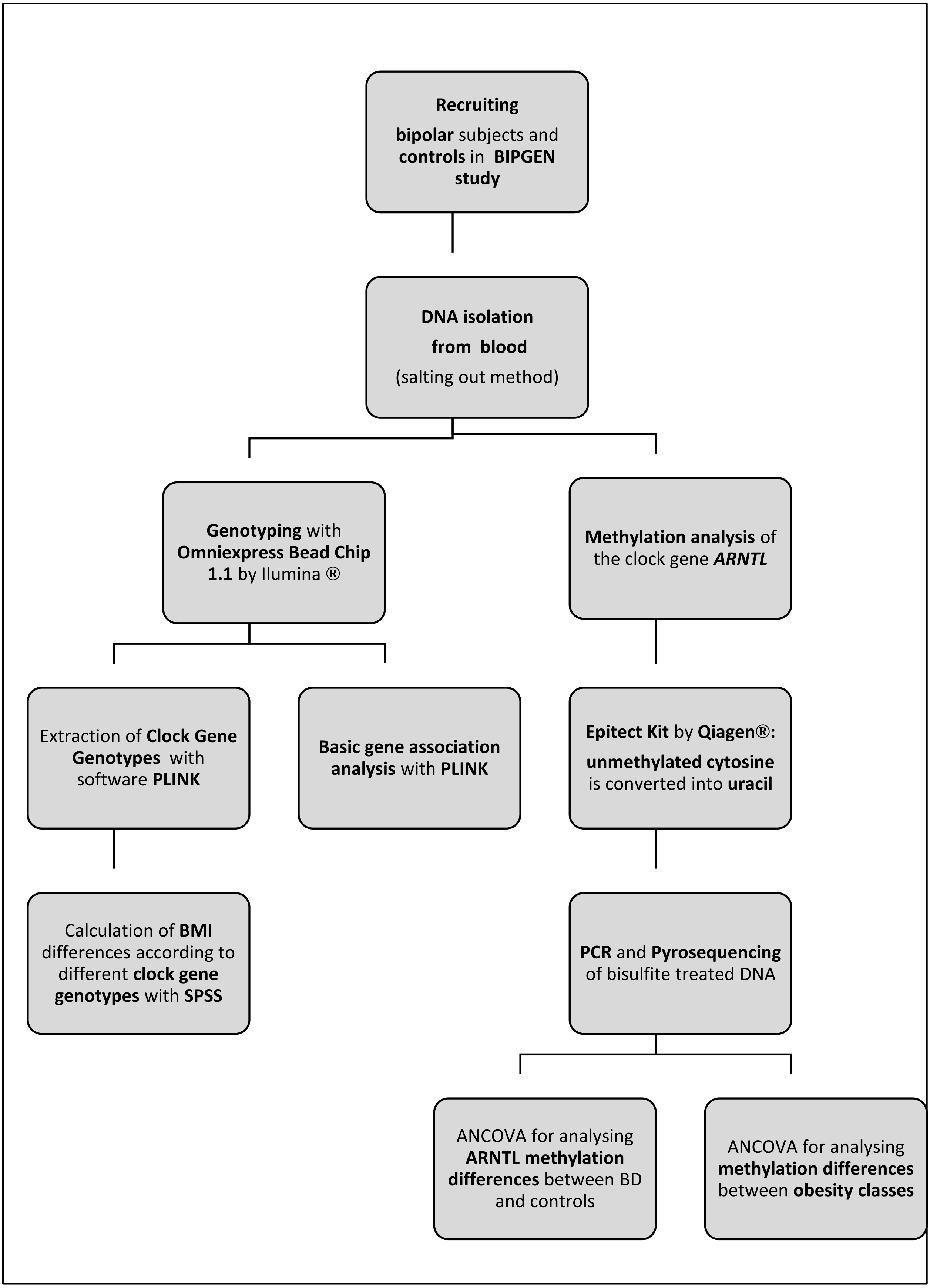


Fig. 11. Workflow CLOCK GENES. Abbreviations: ARNTL… *Aryl hydrocarbon receptor nuclear translocator like*, BD… Bipolar Disorder, BMI…Body Mass Index, BS-DNA… bisulfite treated DNA, PCR…polymerase chain reaction

2.5. Statistical analysis

SPSS version 21.0 (IBM Corp. Armonk, NY) was used for all statistical analyses in the thesis except the basic gene association test, which was performed with the command line program PLINK. Descriptive statistics is depicted with the average of the analyzed marker and the standard deviation in parentheses. In the case of missing normal distribution, the median is shown. Significance levels were set at $p < 0.05$. Prior to testing the hypotheses of the thesis, T-tests and Mann-Whitney-U tests were used to analyse the differences of clinical markers between two groups for the description of the sample (see Table 2-4). The Kolmogorov Smirnov test and Shapiro Wilk test were used to test normality distribution. The Levene's tests were used to analyse the sample heterogeneity.

2.5.1. ER stress Statistics (*BiP, CHOP* and *XBP1*)

2.5.1.1. Group effects of all ER stress markers (*BiP, CHOP and XBP1*)

The following ANCOVA model was used to investigate group differences in *BiP* or *CHOP* gene expression and *XBP1* splicing between BD and controls. The ANCOVA model included group (BD versus controls) as major variable and the ER stress marker (*BiP* or *CHOP* or *XBP1 markers*) as dependent variable. The covariates age, BMI, sex, smoking (yes/no), Lithium (yes/no) and antiepileptics (yes/no) were introduced to correct for their putative confounding effects. The covariates were introduced, because previous literature underlined an association between those covariates and ER stress.

In addition, non parametric tests (Mann-Whitney-U test and Kruskal-Wallis test) were performed in the case of sample heterogeneity (significant Levene test) or missing normal distribution (tested by Kolmogorov Smirnov test).

2.5.1.2. Overweight and obesity effects of all ER stress markers (*BiP, CHOP and XBP1*)

a) To test ER stress gene expression differences between normal weight and overweight/obese individuals, ANCOVAS were performed- group (BD versus controls) and overweight/obesity (yes/no) were used as major factors, ER stress markers were used as dependent factors and age, sex, smoking (yes/no), Lithium (yes/no) and antiepileptics (yes/no) were used as covariates.

b) To test ER stress marker differences between three weight groups (normal weight, overweight and obesity), ANCOVAS were used- group (BD versus controls) and weight classes were used as major factors, gene expression markers were used as dependent factor and the mentioned parameters age, sex, smoking (yes/no), Lithium (yes/no) and antiepileptics (yes/no) as covariates

c) Pearson correlation analysis was used to analyse the putative correlation between ER stress markers and the analysed BMI.

2.5.1.3. Obesity and ER related genotypes

As already mentioned, genotypes were extracted hypothesis driven with PLINK from the preexisting GWAS data set. Then, three genotype groups (e.g. AA, AG and GG) were built. If one genotype group could not achieve the minimum of n= 10 consequently only two groups were built e.g. (AA + AG) and (GG).

ANCOVAs with major factor group (BD versus controls) and genotypes (e.g. AA versus AG versus GG); dependent variable: BMI and covariates: age, sex, Lithium (yes/no) and antiepileptics (yes/no) were used to test differences of BMI between genotypes of ER related genes.

2.5.2. Oxidative Stress Statistics

2.5.2.1. Group Effects- (Anti)oxidative parameters between BD and Controls

Normality tests were performed prior to testing the hypotheses as previously described. To test differences between BD and controls ANCOVA models were used. The ANCOVA models incorporated one (anti)oxidative marker (TBARS or MDA or TAC or SOD or GST) as dependent variable, the group factor (BD vs. controls) as independent variable and age, BMI, sex, smoking (yes/no), atypical antipsychotics (yes/no), Lithium (yes/no) and antiepileptics (yes/no) as covariates.

2.5.2.2. Weight and oxidative stress

a) (Anti)oxidative markers in overweight/obesity versus normal weight:

To test differences in (anti)oxidative markers between normal weight and overweight/ obesity, ANCOVAS were performed- group (BD versus controls) and overweight/ obesity (yes/no) were used as major factors, (anti)oxidative markers were used as dependent factors and age, sex, smoking (yes/no), Lithium (yes/no), antiepileptics (yes/no) and antipsychotics (yes/no) were used as covariates.

b) Pearson correlation between anthropometric measures and (anti)oxidative markers

Pearson correlation analysis was performed for BMI and all (anti)oxidative markers (MDA, TBARS, TAC, GST and SOD) in the total sample independent of group.

2.5.3. Molecular Clock Statistics

2.5.3.1. Methylation differences of ARNTL between BD and controls

SPSS version 22.0 (IBM, 2013) was used for statistical analyses. Normal distribution of the sample used in the ANCOVAs was tested with the Kolmogorov-Smirnov-test, Shapiro-Wilk-test and visually with QQ-plots.

To compare clinical variables between the groups t-test, Mann-Whitney U test, Chi-Square test or correlation analysis were used for the description of the sample/ cohort description (see Table 4).

The major hypothesis that *ARNTL* methylation status differs between BD and healthy controls was tested with analyses of co-variance (ANCOVAs). Methylation status [in %] at one CG site (either cg05733463 or PS2 POS1– POS7) was used as dependent variable and group (BD vs. controls) as major factor. Age, BMI, sex, smoking (yes/no), Lithium intake (yes/no) and anticonvulsant intake (yes/no) were introduced as covariates since they can potentially affect methylation. The covariate anticonvulsants (yes/no) included intake of valproate (VPA) or lamotrigine or carbamazepine. Homogeneity of variance was confirmed using the Levene´s test.

2.5.3.2. Methylation differences according to weight

ANCOVAs were used to investigate obesity effects. The models included overweight/obesity (yes/ no) or weight classes (normal weight, overweight and obesity) and group (BD vs. controls) as major factors, methylation parameters as dependent variables. Age, sex, smoking (yes/no), Lithium intake (yes/no) and anticonvulsant intake (yes/no) were introduced as covariates since they can potentially affect methylation. Pearson’s correlation analysis was used to investigate putative correlations between BMI and methylation markers of *ARNTL*.

2.5.3.3. Gene Association Analysis

Hypothesis driven gene association analysis (Basic association test) was performed with the command line program PLINK, which is statistically based on Chi Square Tests to compare genotype groups between BD and controls.

2.5.3.4. BMI levels in Clock gene Genotypes

ANCOVAs with major factors group (BD versus controls) and genotype of the investigated clock genes (*CLOCK* or *PER3*) and BMI as dependent variable as well as age, sex, Lithium (yes/no) and antiepileptics (yes/no) as covariates were performed to test whether certain genotypes showed significantly differing BMI levels.

3. Results

3.1. ER (Endoplasmic Reticulum) stress

3.1.1. *BiP* gene expression in BD versus Controls

BiP gene expression levels differed significantly ($p < 0.05$) between bipolar patients (n= 54) and healthy controls (n= 54) according to ANCOVA testing and additional non parametric Mann-Whitney-U and Kruskal-Wallis-tests, because data were not normally distributed and a positive Levene test (*F(1/106)*= 12.018, $p < 0.05$) showed sample heterogeneity.

Patients with BD had a mean *BiP* dCQ level of -1.61 +/- 0.51 whereas healthy controls had a mean *BiP* dCQ level of -1.04 +/- 1.10 relativated to expression of the house keeping gene *HPRT1*. The *BiP* dCQ median was -1.28 in healthy controls and -1.64 in BD. The obtained results represent significantly higher *BiP* gene expression in BD than in healthy controls (*F(1/100)*= 4.449, $p < 0.05$, Partial Eta^2= 0.043)- see Fig.12.

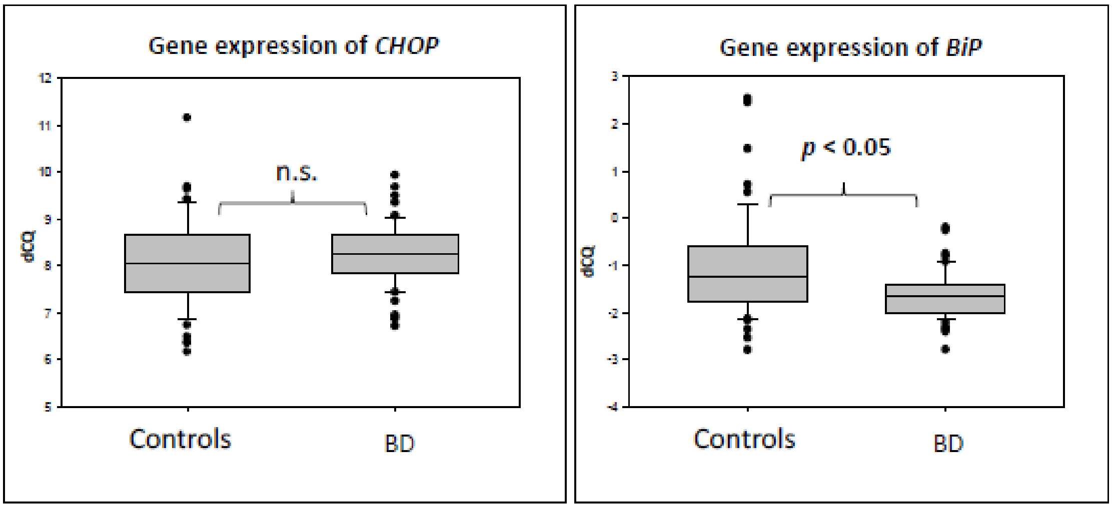


Fig. 12. Gene expression analysis of *CHOP* and *BiP* in Bipolar Disorder (BD) and controls. Quantitative PCR in peripheral blood of individuals with BD (n= 54) and controls (n= 54) of the Unfolded Protein Response genes *BiP (Binding immunoglobulin protein)* and *CHOP.* The latter encodes for a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors, syn. *GADD153, DDIT).* **Abbreviations***: CQ… Cycle Quantification values, dCQ… represent the normalized mRNA expression levels of genes of interest.*

3.1.2. Obesity and *BiP* gene expression

BiP gene expression did not differ significantly between weight classes in the total sample. Furthermore, no weight*BD interactions were found. Additionally, BMI did not correlate with the gene expression marker dCQ *BiP*.

3.1.3. *CHOP* gene expression in BD versus Controls

Relative gene expression of *CHOP* was normally distributed over group (p= 0.087).

There was no significant difference *(F(1/100)*= 1.853, p= 0.176, Partial Eta^2= 0.018) in *CHOP* gene expression between BD (n=54) and controls (n=54)- see Fig. 12. Patients with BD had a mean dCQ *CHOP* level of 8.26+/- 0.66 and healthy controls a mean dCQ level of 8.05 +/- 0.94.

The Levene test was positive (*F(1/106)*= 5.203, $p < 0.05$) and as a consequence non parametric tests were used in addition. Similarly, the Mann-Whitney-U and the Kruskal-Wallis-test showed no significant group difference in *CHOP* gene expression between BD and controls (p= 0.170). No covariate effects were detected. Age, BMI, sex, smoking, Lithium and anticonvulsants intake had no influence on *CHOP* gene expression in the current

ANCOVA. The median of *CHOP* expression [dCQ CHOP] was 8.06 in healthy controls, respectively 8.24 in BD.

3.1.4. Weight and *CHOP* gene expression

CHOP gene expression did not differ significantly between the different weight classes. In addition, no weight*BD interaction was found. No significant correlation between CHOP gene expression and BMI was found. Furthermore, *CHOP* gene expression did not correlate with BMI independent from group.

3.1.5. *XBP1* Splicing

XBP1 splicing in BD versus Controls (group effects)

The total amount of *XBP1* (sum of spliced and unspliced *XBP1*) differed significantly (*F(1/100)*= 5.265, $p < 0.05$, Partial Eta^2= 0.050) between BD (n= 54) and controls (n= 54). Patients with BD had lower expression levels of total *XBP1* than controls (descriptive statistics is depicted in Fig. 13). The band intensity of unspliced *XBP1* differed significantly (*F(1/100)*= 5.634, $p < 0.05$) between BD and controls as well. Patients with BD had lower levels of unspliced *XBP1* than controls.

In contrast, spliced *XBP1*, the ratio unspliced/spliced *XBP1,* the percentage of unspliced and spliced *XBP1* did not differ between study participants with BD and healthy controls.

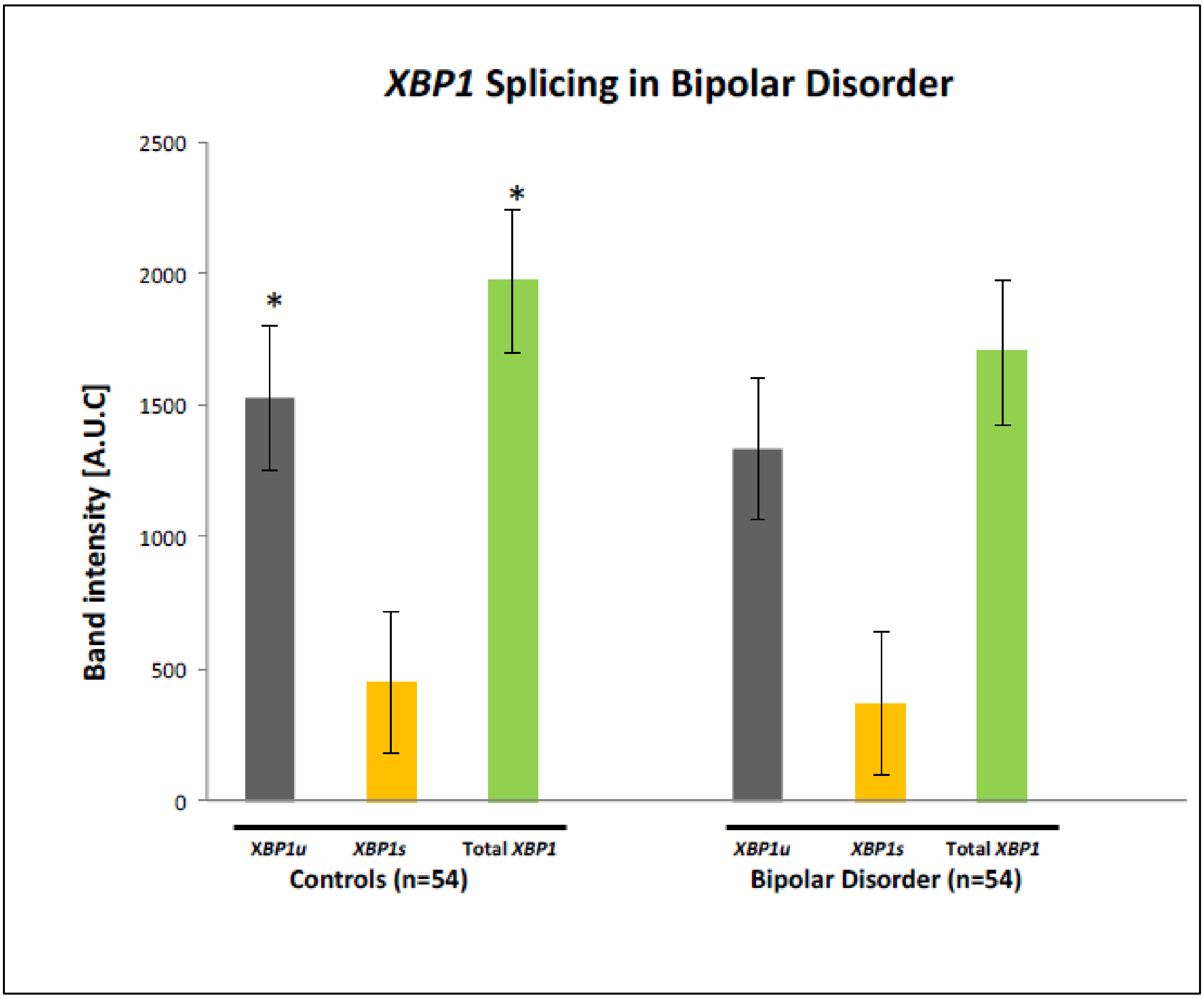


Fig.13. *XBP1* splicing in Bipolar Disorder (BD). Expression, respectively splicing of the transcription factor XBP1 was assessed in lymphocytes by semiquantitative RT-PCR in BD patients in comparison to healthy controls. Expression of unspliced XBP1 (XBP1u) respectively spliced XBP1 (XBP1s) was quantified by densitometry using the software GelAnalyzer. The sum of spliced and unspliced *XBP1* (Total *XBP1*) differed significantly (*) between BD and controls (*F(1/100)*= 5.265, $p < 0.05$, Partial Eta^2= 0.050). Furthermore, unspliced *XBP1* (XBPu) differed significantly (*) between BD and controls as well (*F(1/100)*= 5.634, $p < 0.05$). Spliced XBP1 (XBP1s) did not differ between BD and controls.

Weight and XBP1 splicing

XBP1 values did not differ significantly between two weight classes (normal weight versus overweight/ obesity). Furthermore, *XBP1* parameters did not correlate significantly with BMI.

3.1.6. Hypothesis driven gene association study of ER related genes

ER gene SNPs (***PERK*****,** ***ATF6*** and ***LMAN2L***) were investigated with the basic gene association analysis using the PLINK software (see Table 7). The ***LMAN2L*** rs6746896 SNP was significantly associated with BD in the basic association test including 150 bipolar subjects and 77 controls (X^2= 3.881, $p < 0.05$, OR= 0.6538). The G allele is less common in BD than in controls. The other tested gene variants showed no association with BD.

Table 7. Basic Gene association analysis of ER related genes in BD and controls. Patients with BD (n= 150) and controls (n= 77) were genotyped with the Omniexpress1.1 chip by Illumina. Basic gene association analysis was performed with the free software PLINK. *LMAN2L* rs6746896 was significantly associated with BD. The G allele is less common in BD than in controls. **Abbreviations:** CHR… Chromosome, SNP… SNP ID, BP… Physical position (base-pair), A1… Minor allele name (based on whole sample), F_A… Frequency of this allele in cases, F_U… Frequency of this allele in controls, A2… Major allele name, CHISQ… Basic allelic test chi-square (1df), P… Asymptotic p-value for this test, OR… Estimated odds ratio (for A1, i.e. A2 is reference). *ATF6*... Activating transcription factor 6, PERK… PKR-like ER kinase, syn. EIF2AK3 eukaryotic translation initiation factor 2-alpha kinase 3, *LMAN2L*… Lectin, mannose binding 2 like.

BASIC GENE ASSOCIATION ANALYSIS

CHR	SNP	BP	A1	F_A	F_U	A2	CHISQ	P	OR
1	***ATF6*** **rs2271013**	161753802	G	0.09333	0.06494	A	1.07	0.3009	1.482
2	***PERK*** **rs867529**	88913273	G	0.25	0.2434	C	0.02343	0.8784	1.036
3	***LMAN2L*** **rs9834970**	36856030	A	0.4667	0.5065	G	0.6467	0.4213	0.8526
2	***LMAN2L*** **rs6746896**	97410949	G	0.25	0.3377	A	3.881	**0.04883**	0.6538

3.1.7. BMI levels in ER related Genotypes

BMI did not differ significantly according to the different genotype groups of the ER related genes *ATF6* (rs2271013), *PERK* (rs867529) and *LMAN2L* (rs9834970 and rs6746896) after correction for mood stabilizer intake in bipolar patients (n= 132) and controls (n= 67).

3.2. Oxidative Stress

3.2.1. Group effects (BD group versus control group) and descriptive statistics

Contradicting our expectations, patients with BD had significantly lower levels of the oxidative stress marker MDA compared to controls (*F(1/204)*= 8.485, $p < 0.05$, Partial Eta^2= 0.040). Patients with BD had an average MDA level of 0.72+/- 0.20 µmol/l, whereas healthy controls had a mean MDA level of 0.81+/- 0.26 µmol/l. The Levene test was not significant, representing homogenous sample distribution (*F(1/211)*= 0.005 *p*= 0.946).
The total antioxidative capacity TAC was significantly lower in participants with BD than in controls (*F(1/189)= 8.406*, $p < 0.05$, Partial Eta^2= 0.043). Patients with BD had a mean TAC of 1.17+/- 0.47 mmol/l. In contrast, controls had an average TAC of 1.38 +/- 0.50 mmol/l.

TBARS did not differ between patients with BD and controls (*F(1/205)*= 1.781, *p*= 0.184, Partial Eta^2= 0.009). The Levene test was not significant (*F(1/212)*= 2.638, *p*= 0.106).

The antioxidative enzyme SOD did not differ significantly (*F(1/143)*= 0.260, *p*= 0.611, Partial Eta^2= 0.002) between BD and controls. The other tested antioxidative enzyme GST did not differ between BD and controls either (*F(1/174)*= 0.463, *p*= 0.497, Partial Eta^2= 0.003).

3.2.2. Obesity and Oxidative Stress

(Anti)oxidative markers in overweight/ obese and normal weight

The (anti)oxidative parameters MDA, TBARS, GST, SOD and TAC did not differ significantly between two weight classes (normal weight versus overweight/obese). Furthermore, no weight*group effects were found.

Correlations between (anti)oxidative parameters and BMI

Diverse (anti)oxidative markers correlated with BMI independent from group (BD vs. controls). TAC correlated negatively with BMI (*r*= -0.143, $p < 0.05$) and GST correlated positively with BMI (*r*= 0.151, $p < 0.05$). SOD did not correlate significantly with BMI (*r*= 0.154, *p*= 0.056). MDA (*r*=-0.004, *p*= 0.954) and TBARS (*r*= -0.020, *p*= 0.768) did not correlate with BMI in the total sample independent from BD.

3.3. Clock genes

3.3.1. Hypothesis driven gene association study of Clock genes

The investigated *PER3* SNPs (rs10864315, rs228682, rs228642 and rs10462021) and *CLOCK* SNPs (rs12649507, rs1801260 and rs534654) were not associated with BD in the basic gene association test performed with PLINK (see Table 14).

Table 14. Basic gene association analysis of Bipolar Disorder (n= 150) and controls (n= 77). Abbreviations: CHR… Chromosome, SNP… SNP ID, BP… Physical position (base-pair), A1… Minor allele name (based on whole sample), F_A… Frequency of this allele in cases, F_U… Frequency of this allele in controls, A2… Major allele name, CHISQ…Basic allelic test chi-square (1df), P… Asymptotic p-value for this test, OR… Estimated odds ratio (for A1, i.e. A2 is reference), BD... Bipolar Disorder, CLOCK... Circadian Locomotor Output Cycles Kaput, PER… period genes

BASIC GENE ASSOCIATION ANALYSIS

CHR	SNP	BP	A1	F_A	F_U	A2	CHISQ	P	OR
1	**rs10864315** ***PER3***	7850081	A	0.2833	0.3052	G	0.2358	0.6272	0.9
1	**rs228682** ***PER3***	7856346	G	0.4367	0.4091	A	0.3162	0.5739	1.12
1	**rs228642** ***PER3***	7863293	G	0.4033	0.4481	A	0.8362	0.3605	0.8327
1	**rs10462021** ***PER3***	7897133	G	0.1867	0.1753	A	0.08762	0.7672	1.08
4	**rs12649507** ***CLOCK***	56380484	A	0.3121	0.2792	G	0.5212	0.4703	1.171
4	**rs1801260** ***CLOCK***	56301369	G	0.2867	0.3117	A	0.3062	0.5800	0.8875
4	**rs534654** ***CLOCK***	56290220	A	0.1567	0.2013	G	1.425	0.2326	0.7371

3.3.2. Association of BMI and clock gene genotypes

BMI did not differ according to genotype groups of the clock gene SNPs *PER3* (rs10864315, rs228682, rs228642, rs10462021) and *CLOCK* (rs12649507, rs1801260, rs534654) in bipolar patients (n= 132) and controls (n= 67).

3.3.3. Epigenetics of *ARNTL*- Group Effects

General results and description of the sample

The description of the investigated cohort is given in Table 2. The sample used in the ANCOVAs was normally distributed. In addition, homogeneity of variance was confirmed using Levene´s test. Prior to testing our hypothesis, we found significant associations between methylation of cg05733463 and Lithium intake ($p < 0.01$), anticonvulsants intake ($p < 0.01$) and smoking ($p < 0.05$).

Group effects- Methylation differences of ARNTL between BD and controls

There was a significant difference in methylation status at the CG site cg05733463 of the clock gene *ARNTL* between BD and controls ($F(1/209)= 44.500$, $p < 0.001$, Partial Eta^2= 0.176). Methylation at cg05733463 was significantly higher in study participants with BD than in controls (Fig. 14).

In contrast, methylation status of *ARNTL* was significantly lower at the second CpG island PS2 POS1 between individuals with BD compared to controls ($F(1/128)= 5.787$, $p < 0.05$, Partial Eta^2= 0.043).

There was no significant difference in *ARNTL* methylation status at PS2 POS2 ($F(1/128)= 3.033$, $p= 0.084$, Partial Eta^2= 0.023), PS2 POS3 ($F(1/110)= 0.170$, $p= 0.681$, Partial Eta^2= 0.002), PS2 POS4 ($F(1/59)= 0.025$, $p= 0.876$, Partial Eta^2< 0.001), PS2 POS5 ($F(1/59)= 1.045$, $p= 0.311$, Partial Eta^2= 0.017) and PS2 POS6 ($F(1/48)= 1.036$, $p= 0.314$, Partial Eta^2= 0.021) between BD and controls.

Descriptive statistics of *ARNTL* methylation in BD and controls is presented in Table 15.

Table 15: Descriptive statistics of ARNTL methylation status in Bipolar Disorder and Controls: The mean and standard deviation of *ARNTL* CpG islands (cg05733463 and PS2_POS1-7) methylation [in %] are depicted. Differences in methylation of *ARNTL* over groups (BD vs. controls) were tested with ANCOVAs. The ANCOVA results are marked: $p < 0.05$**, $p < 0.001$***. **Abbreviations:** M…Mean/ average, n… Number of study participants, SD… Standard Deviation, CG...cytosine and guanine repeats, CpG islands...sequence rich in repetitive CG elements.

Methylation [in%] of ARNTL CG sites
in Bipolar Disorder versus healthy controls

Group		cg05733463	PS2 POS1	PS2 POS2	PS2 POS3	PS2 POS4	PS2 POS5	PS2 POS6	PS2 POS7
Bipolar Disorder	**M**	**77.72*****	**1.53****	1.38	2.79	2.21	0.63	1.64	1.67
	SD	5.50	0.44	0.29	1.64	1.17	0.41	0.57	0.81
	N	151	69	69	55	40	31	22	14
Controls	**M**	**70.93*****	**1.78****	1.46	3.21	2.25	0.84	1.98	2.64
	SD	5.70	0.44	0.34	3.61	2.20	0.98	0.85	1.22
	N	66	67	67	63	52	36	34	28

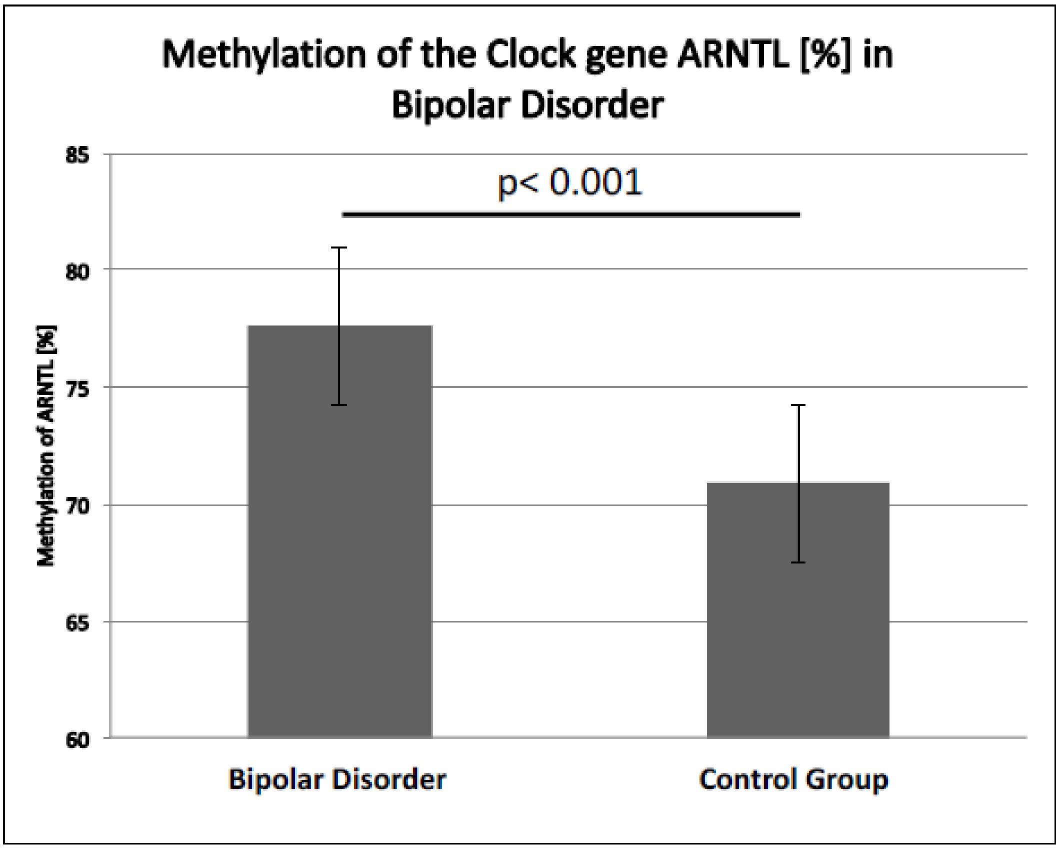


Fig.14: Mean average values of *ARNTL* methylation at the CG site cg05733463 between BD (n= 151) and controls (n= 66). Methylation status [%] at the CG site cg05733463 differed significantly between BD and controls ($F(1/209)$= 44.500, $p < 0.001$, Partial Eta^2= 0.176).

Methylation markers of *ARNTL* in overweight/obese and normal weight

Summarized, no significant differences of *ARNTL* methylation were found between the two weight classes normal weight and overweight/obese study participants in any of the sites, independent from group. Furthermore, no significant weight*group effect was found.

Correlations between methylation parameters of *ARNTL* and BMI

No significant correlations were found between methylation markers of *ARNTL* and BMI.

4. Discussion

This doctoral thesis investigated shared pathways between obesity and BD, because overweight, obesity and other metabolic disorders are more common than by chance in BD (McIntyre et al. 2010a, 2010c). Thus, we hypothesized, that obesity related biological pathways may contribute to the pathogenesis of BD. In this thesis, the results of the parameters of ER stress, oxidative stress and circadian rhythms in BD and obesity are presented.

4.1. ER Stress

In line with our hypothesis that indicators of ER stress differ between BD and controls, we found a magnitude of ER related alterations in our current sample.

The group differences mainly included significant alterations in *BiP* gene expression, total *XBP1* levels and *LMAN2L* genotype distributions. First, there was a significantly higher gene expression of the chaperone and "ER stress sensor" *BiP* in BD compared to controls. Second, *CHOP*-expression and spliced *XBP1* did not differ between BD and controls. Nevertheless, individuals with BD had a significantly lower expression of total *XBP1* as well as lower unspliced *XBP1* levels compared to controls. Third, we found a significant association of the gene variant *LMAN2L* rs6746896 with BD according to the basic gene association analysis performed with PLINK. Fourth, no significant weight*BD interactions were found in the investigated pathways. The markers of ER stress, oxidative stress and the 24h clock did not even differ between weight classes independent of BD.

Gene expression studies of *BiP* in tissue and/or blood cells of bipolar patients in a naturalistic design and in a sufficiently large sample are sparse. Therefore, it is difficult to compare our result of significantly increased *BiP* gene expression in peripheral blood of bipolar study participants with previous literature. Taken that into account, our results broaden the scientific knowledge about *BiP* gene expression in peripheral blood of BD.

Nevertheless, diverse studies investigated *BiP* gene expression *in-vitro* in cultured lymphocytes derived from bipolar patients or in other cell culture models (e.g. cultivation of cerebral rat cells). Even though results obtained with the help of cell culture models are often not completely compareable to *in-vivo* studies, they give some insights in ER stress related mechanisms in BD. Some *in-vitro* studies found impaired reactions to experimentally induced ER stress in cells derived from BD study participants (Pfaffenseller et al. 2014, So, Warsh & Li 2007, Hayashi et al. 2009). So and colleagues analyzed the UPR pathway in B-lymphoblasts of bipolar patients and healthy controls after provoking ER-stress with special agents (e.g. thapsigargin and tunicamycin) (So, Warsh & Li 2007). Basal gene expression of UPR target genes (*BiP*, *CHOP* and *XBP1*) did not differ between BD and controls, but solely a restricted up-regulation of *XBP1* and *CHOP* (not *BiP*) after applying ER stress was found in BD cells compared to controls (So, Warsh & Li 2007). Hayashi and colleagues extended the study design and principally reproduced the results of So et al. in a larger cohort of patients (Hayashi et al. 2009). Thus, patients with BD are not able to cope with experimentally induced ER stress as efficiently as healthy people (So, Warsh & Li 2007, Hayashi et al. 2009).

However, why is *BiP* gene expression increased in BD? Well, there may be diverse putative reasons for elevated *BiP* in BD. According to previous literature, mood stabilizers, obesity, oxidative stress and diverse other conditions interfere with *BiP* gene expression (Thon et al. 2014, Wang, Bown & Young 1999, Bown et al. 2002, Shao et al. 2006).

According to literature, *BiP* gene expression is associated with obesity and related endocrinological conditions (Thon et al. 2014, Bengesser et al. 2016b). Interestingly, increased leptin levels favor increased *BiP* gene expression, which could be one mitigating factor for the association of *BiP* gene expression and obesity (Thon et al. 2014). The increased expression of chaperones could be one rescue mechanism to prevent harm from obesity associated oxidative stress and ER stress. Nevertheless, we could not find a significant difference in *BiP* gene expression according to weight classes between patients and controls in our current sample. A limitation of this finding is that our weight classes were based on differences between normalweight and overweight/ obese individuals, and we did not regard obese individuals as a separate class due to a too small sample size. Further investigations in enlarged sample sizes must reexplore this putative correlation.

The intake of the mood stabilizers Lithium and valproate could principally be another reason for the increased *BiP* gene expression in BD. Hitherto literature shows that mood stabilizers exert positive effects on oxidative stress (Bengesser et al. 2016a) and ER stress (Wang, Bown & Young 1999, Shao et al. 2006, Wada et al. 2005). Already, in the late nineties it was shown for the first time that mood stabilizing drugs are able to modulate the expression of *BiP* in a positive way (Wang, Bown & Young 1999). Chronic treatment with Lithium and valproate stimulates the expression of ER located chaperons *BiP*, *GRP94* and calreticulin in rat brains (Wang, Bown & Young 1999, Shao et al. 2006, Wada et al. 2005). Nevertheless, we have corrected the analyses for mood stabilizer (Lithium and valproate) intake and can conclude that increased *BiP* gene expression may be a trait marker for BD itself. Enhanced *BiP* gene expression might in further consequence reflect an adaption process to mild ER stress in BD leading to stimulation of chaperone expression in order to prevent cell damage.

The second major ER stress related finding concerned the splicing of *XBP1*. Total *XBP1* levels, sum of spliced and unspliced *XBP1* mRNA, and unspliced *XBP1* differed significantly between BD and controls. However, as a limitation of our study, expression of *XBP1* was not normalized to expression of a house keeping gene. In contrast, spliced *XBP1* did not differ between BD and controls in our sample. The latter may be interpreted either as the absence of stimuli activating *XBP1* or as impaired reaction to ER stress in BD, which was previously described in *in-vitro* studies (Pfaffenseller et al. 2014, So, Warsh & Li 2007, Hayashi et al. 2009). Interestingly, reduced *XBP1*-mediated signaling has been associated with impaired insulin receptor signaling and diabetes mellitus in a study by Ozcan and colleagues (Ozcan et al. 2004b). Obesity, hypernutrition and metabolic syndrome, but also energy depletion favors ER stress and influence the rescue mechanisms of the UPR (Fu et al. 2011, Ghosh et al. 2014, Hakim et al. 2014, Khan, Wang 2014, Nakagawa et al. 2014). Interestingly, we could not find any significant association between markers of ER stress (*BiP*, *CHOP* and *XBP1* markers) and weigth in our current investigation.

In contrast to increased *BiP* gene expression, *CHOP* was not differentially expressed in blood samples of study participants with BD compared to controls. This goes in line with previous literature of *in-vitro* studies that point toward impaired up-regulation of gene expression of *CHOP* in reaction to ER stress in BD *in vitro* (Pfaffenseller et al. 2014, So, Warsh & Li 2007, Hayashi et al. 2009).

The third major ER related finding of our study concerned results from hypothesis driven gene association analysis. We replicated in our genotyped BIPGEN sample the association of the *LMAN2L* SNP rs6746896 with BD as described in hitherto literature (Lim et al. 2014). This is remarkable, because *LMAN2L* belongs to the top hits of BD GWAS. *LMAN2L* encodes for a cargo receptor and transmembrane protein in the ER membrane, which is trafficking glycosylated proteins (Wang, Groenendyk & Michalak 2015). The latter may be rather important in synopsis with ER stress, because impaired trafficking of glycosylated proteins could lead to an accumulation of glycoproteins in the lumen of the ER, which could potentially favor ER stress. N-linked glycans that are synthesized by the rough ER are critical for protein maturation and are an important quality control signal in the secretory protein pathway. Impaired glycoprotein quality control, deficient protein glycosylation and wrong protein folding activate the ER stress coping response (Wang, Groenendyk & Michalak 2015). Thus, genetic variations of *LMAN2L* may hypothetically favor ER stress conditions via insufficient transport of glycosylated proteins. Nevertheless, the exact function of the rs6746896 SNP (an A/G single-nucleotide variation on human chromosome 2) and the functional impact of that variant on ER homeostasis is not clearly elucidated yet. Furthermore, the rs6746896 SNP has not yet been associated with ER stress in previous studies (Chen et al. 2013, Lim et al. 2014).

Another interesting question for further studies is the gene expression of *BiP*, *CHOP* and *XBP1* in diverse affective episodes. The current sample was too small to discriminate gene expression and *XBP1* splicing in depression, euthymia and mania. Nevertheless, it is an interesting scientific question for upcoming studies. If ER stress gene expression differed between different affective episodes, it may be useful as state marker. Since the clinical field of Psychiatry has not yet implemented blood-biomarkers for tracking episodes, research must put more effort into finding blood-biomarkers. The latter may improve diagnostics and treatment of mood disorders considerably.

Taken together, the observations of this thesis are consistent with the notion that disturbances in the ER stress response might be involved in BD disease mechanisms.

Furthermore, correlations between all three pathways would have been interesting, but were not possible based on the differing sample sizes. Nevertheless, those scientific questions will be addressed in the future.

4.2. Oxidative Stress

In line with our hypotheses that (anti)oxidative markers differ between BD and controls and in relationship with obesity, we found diverse alterations in the oxidative and antioxidative system between BD and controls. The most prominent findings of our investigation included significantly reduced serum levels of the oxidative stress marker MDA and significantly reduced total antioxidative capacity "TAC" in BD compared to controls (Bengesser et al. 2015). The (anti)oxidative markers GST and TAC correlated with BMI in the total sample independent from group. No specific weight*BD effects were found in the current sample.

The homeostatic imbalance of the oxidative and antioxidative system in BD was postulated by diverse previous studies (Andreazza et al. 2008, Selek et al. 2008, Gergerlioglu et al. 2007, Kuloglu et al. 2002, Machado-Vieira et al. 2007, Ozcan et al. 2004a, Ranjekar et al. 2003, Savas et al. 2006, Kapczinski et al. 2011b). Significant increased levels of the oxidative stress marker TBARS were reported in all affective episodes of BD (Andreazza et al. 2008, Machado-Vieira et al. 2007, Ranjekar et al. 2003, Kapczinski et al. 2011b, Tsai, Huang 2015). MDA levels were increased in BD in rather small studies that included around 30 study participants (Kuloglu et al. 2002, Ozcan et al. 2004a). Nevertheless, study results are conflicting, because some groups could not find any differences in TBARS levels between BD compared to controls (Gubert et al. 2013, Wiener et al. 2013). The latter goes in line with our results. We could not find differences in TBARS levels between BD and controls and MDA, which belongs to the TBA reactive substances, was even significantly decreased in BD compared to controls. The antioxidative marker TAC was also significantly reduced in BD compared to the control group. There is rare previous literature about this sum marker of antioxidants and antioxidative enzymes. Former studies about the antioxidative defense and BD described reduced antioxidants or reduced antioxidative enzymes like SOD (Selek et al. 2008, Raffa et al. 2012, Kapczinski et al. 2011a, Yumru et al. 2009).

(Anti)oxidative markers can be influenced by a variety of biological processes. Mood stabilizers and female sex hormones can lower oxidative stress markers in BD according to previous literature. Women had significantly lower TBARS levels than men in the BIPFAT cohort. Estrogens seem to have an additive protective antioxidative effect similar to the effect of Lithium (Bengesser et al. 2015, 2016a). In our previously published original report about mood stabilizers and oxidative stress we found the lowest MDA and TBARS levels in bipolar patients taking Lithium (Bengesser et al. 2016a). Lithium's antioxidative and neuroprotective effects have been well documented in hitherto literature (Albayrak et al. 2013, Diniz, Machado-Vieira & Forlenza 2013, Nciri et al. 2013, Jornada et al. 2011, Forlenza et al. 2012, Quiroz et al. 2010). Decreased lipid peroxidation under Lithium monotherapy compared to unmedicated BD individuals was described previously (Machado-Vieira et al. 2007, Banerjee et al. 2012, Aliyazicioglu et al. 2007). Those neuroprotective, antioxidative effects were as well replicated in animal- and *in-vitro*- studies (da-Rosa et al. 2012, Macedo et al. 2012, Ghedim et al. 2012).

The group effects concerning MDA and TAC may represent pathogenetic mechanisms of BD itself, because we corrected for putative confounding factors in our ANCOVA designs in the current investigation; covariates included age, BMI, sex, smoking, Lithium and valproate intake. Thus, our observed alterations in the oxidative system seem to be trait markers for BD itself. As mentioned before, research must put more effort into evaluating putative serum biomarkers for tracking the course of the disease, because - in contrast to other medical disciplines - such serum markers are still lacking in the field of Psychiatry. Our follow up study will concentrate on evaluating serum markers especially along the course of BD, which may broaden the knowledge about (anti)oxidative markers in affective episodes during the course of the disease. Previous results by Tsai et al. already pointed towards increased TBARS in mania (Tsai, Huang 2015). Future studies are necessary to further clarify the role of the oxidative stress system in BD and whether oxidative markers are useful biomarkers for tracking the course of BD. Reliable serum biomarkers could improve diagnostics and treatment remarkably.

The second major finding concerned significantly reduced TAC in BD compared to controls. TAC, which is also commonly described as "total reactive antioxidant potential"/TRAP or "total antioxidant status"/TAS, refers to the general ability of the organism to detoxify ROS. TAC is a marker that summarizes diverse players of the "antioxidative defense", antioxidants and antioxidative enzymes, which are required for maintaining the oxidative system in balance. Alterations of TAC in BD were discussed rather sparse in previous literature. Some former studies described reduced antioxidants or reduced antioxidative enzymes (e.g. SOD) in BD (Selek et al. 2008, Raffa et al. 2012, Kapczinski et al. 2011a, Yumru et al. 2009). Kapczinski and colleagues found for instance high oxidative and low antioxidative parameters in BD, which even resembled the results in septic patients (Kapczinski et al. 2011a). Yumru and colleagues reported as well, in concordance to our results, lowered total antioxidant status in patients with BD compared to controls (Yumru et al. 2009).

It is still unclear, whether reduced TAC results from reduced nutritional intake or increased turnover of antioxidants. Literature also claims that sports and nutrition have moderate effects on TAC in healthy individuals (Braakhuis, Hopkins & Lowe 2013). Significantly reduced TAC may underline a possible unfavorable oxidative biosignature in BD, which could be the consequence of altered resiliency and/or excessive occurrence of oxidation and inflammatory processes.

Furthermore, TAC correlated negatively and GST positively with BMI in our cohort independent from group. According to literature, obesity is commonly associated with increased oxidative stress, which could explain increased turnover of TAC (in first line antioxidants). In line with this, reduced total antioxidative status in obesity was described previously in literature (Vehapoglu et al. 2016, Ha et al. 2014, Melissas et al. 2006). Studies about the serum marker GST itself and obesity are rather sparse and must be accomplished in the future. Interestingly, no further significant overweight and obesity effects were found in the current sample.

Summarized, diverse alterations of the oxidative system and the antioxidative defense were found in BD. Nevertheless, no weight*BD effects were found in the oxidative system in the current sample. Future studies must further clarify the role of (anti)oxidative serum markers in BD and obesity.

4.3. Molecular Clock

Based on the previously published positive correlation between *ARNTL* and *MAOA* gene expression (Hampp et al. 2008, Hampp, Albrecht 2008), we hypothesized that epigenetic modification of the clock gene *ARNTL* may differ between patients with BD and controls. We suggested that putative silencing of *ARNTL* via methylation could principally affect gene expression of *MAOA* and in further consequence the degradation of neurotransmitters. The latter could possibly explain to some degree the predisposition to mood swings by altering neurotransmitter levels, which could result from decreased or increased degradation of neurotransmitters by the enzyme MAOA. As a first step to prove this hypothesis we analysed the methylation pattern of the clock gene *ARNTL* in BD and controls in this current study.

The second hypothesis investigated the possible correlation between methylation markers of *ARNTL* and BMI, because obesity is more common than by chance in BD and may influence diverse molecular biological pathways tremendously.

To our very best knowledge, this is the first study examining epigenetic modulation of regulatory CG rich targets of the clock gene *ARNTL* in BD. Methylation of *ARNTL* differed significantly between BD and healthy controls in the current cohort.

Most important, the methylation status of the CG rich area cg05733463 was significantly higher in BD than in controls. In contrast, methylation sites in PS2 showed significantly lower methylation in BD than in controls.

Our results are not surprising, because changes in circadian rhythms have been described for decades in BD. Results from previous BD research point towards an association between BD and the circadian clock. Most gene association studies display an association between *ARNTL* SNPs and BD (Le-Niculescu et al. 2009a, Mansour et al. 2006, 2009, Nievergelt et al. 2006). Results from animal testing underline as well the importance of the molecular circadian clock in BD. Mice with knocked out clock genes present mania like behavior (Coque et al. 2011).

Even though our results are broadening knowledge in the scientific field of circadian rhythms in BD, we must consider that there are some limitations of the study. For instance, it is not clear so far whether the significant difference of methylation status between BD and controls has a significant impact on clinical symptoms of BD. Further analyses are necessary to evaluate the clinical implications of the obtained results.

If the methylation of *ARNTL* contributes to a major clinical effect, then future scientific questions must consider whether the investigated methylated CpG islands might be attractive new drug targets for mood stabilization. One indicator that CpG islands of *ARNTL* may be interesting putative new drug targets is the concatenation between already well established mood stabilizers and the molecular clock. Lithium and valproate interact with the 24h clock by inhibition of Gsk3β and inhibition of REV-Erb-Alpha (Bengesser S 2013, Raspe et al. 2002).

Further limitations include the fact that methylation analysis was performed in DNA of peripheral blood, because CNS biopsies are not possible in living study participants because of ethical reasons. Post mortem brain tissue of BD patients is hard to obtain and the freezing process may alter methylation. Therefore, the investigation in peripheral blood is a necessary compromise. Nevertheless, there is hitherto literature, which underlines that methylation status in peripheral blood mirrors methylation in the brain (van den Oord et al. 2015). In addition, there are advantages to perform the methylation analysis of *ARNTL* in peripheral blood, because analysis is easy and may become a useful state- and trait marker in the future. As mentioned above bio-markers are still lacking in the field of Psychiatry, and therefore effort must be put into further evaluation of putative bio-markers in peripheral blood.

To complete the discussion of our results, we have to note, that methylation of *ARNTL* did not differ between normal weight and overweight/ obesity classes after correction for Lithium and valproate intake. Furthermore, we did not find a correlation between methylation markers of *ARNTL* and BMI.

Summarized, we suggest that methylation of *ARNTL* may contribute to the multifactorial predisposition for mood swings based on the previously published positive correlation between *ARNTL* and *MAOA* gene expression. The latter may possibly explain neurotransmitter imbalances in BD. Nevertheless, the concatenation between methylation of *ARNTL* and serum neurotransmitter levels must be further evaluated in the future.

Conclusions

Diverse lines of evidence point towards a concatenation between BD and obesity related conditions (McIntyre et al. 2010c, McIntyre et al. 2010a). This doctoral thesis investigated a triad of pathways in BD versus controls and in relationship with weight classes:

- 1st ER stress
- 2nd Oxidative stress
- 3rd Circadian rhythms

ER related pathways displayed diverse significant differences between BD and controls. BD differed from controls in regard of *BiP* gene expression, total amount of *XBP1*, unspliced *XBP1* and *LMAN2L* genotype distribution. *BiP* gene expression was significantly higher in BD than in controls. The *LMAN2L* rs6746896 SNP, an A/G single-nucleotide variation on human chromosome 2, was significantly associated with BD. Furthermore, unspliced *XBP1* and the total amount of *XBP1* differed between patients with BD and controls. No significant weight*BD effects were found in the analyses of ER stress in the present cohort.

Oxidative stress markers and markers of the antioxidative defense were altered in the current sample as well. Summarized, the oxidative stress marker MDA and the marker of the total antioxidative capacity TAC were significantly decreased in BD compared to controls. TAC correlated negatively with BMI and GST correlated positively with BMI in the total cohort independent from BD. No other significant overweight and obesity related effects were found in the oxidative stress system in the current sample.

Methylation of the clock gene *ARNTL* differed significantly at the CG rich regions cg05733463 and PS2 POS1 between patients with BD and controls. Study participants with BD had significantly higher methylation at the CG site cg05733463 than controls. In contrast, methylation was significantly lower at PS2 POS1 in individuals with BD compared to controls. Methylation of *ARNTL* did not correlate with BMI in the current sample. Additionally, no weight*BD effects were found.

The results of this thesis highlight diverse alterations in the oxidative stress system, ER stress system and circadian rhythms in BD compared to controls. Contradicting our expectations, no weight*BD effects were found in the three analysed pathways. Further research is necessary to replicate the findings and to investigate the clinical implications. There may be putative new drug targets or potential serum-biomarkers within the triad of investigated pathways.

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Attachment

6.1. Published Original Reports and Reviews originated from this thesis:

1.) Title: „**Peripheral markers of oxidative stress and antioxidative defense in euthymia of bipolar disorder-Gender and obesity effects**“. Authors: **Bengesser** SA, Lackner N, Birner A, Fellendorf FT, Platzer M, Mitteregger A, Unterweger R, Reininghaus B, Mangge H, Wallner-Liebmann SJ, Zelzer S, Fuchs D, McIntyre RS, Kapfhammer HP, Reininghaus EZ. Journal: Journal of Affective Disorders (Top 20% journal). Impact factor (2014): 3.383.

2.) Title: **„Mood Stabilizers, Oxidative Stress and Antioxidative Defense in Euthymia of Bipolar Disorder“**. Authors: **Bengesser** SA, Lackner N, Birner A, Platzer M, Fellendorf FT, Queissner R, Filic K, Reininghaus B, Wallner-Liebmann SJ, Mangge H, Zelzer S, Fuchs D, Kapfhammer HP, McIntyre RS, Reininghaus EZ. Journal: CNS Neurol Disord Drug Targets. Publication date: 2016. PMID: 26996179. Impact factor (2014): 2.628.

3.) Title: **„Endoplasmic Reticulum Stress and Bipolar Disorder - Almost Forgotten Therapeutic Drug Targets in the Unfolded Protein Response Pathway Revisited“.** Authors: **Bengesser** SA, Fuchs R, Lackner N, Birner A, Reininghaus B, Meier-Allard N, Stracke A, Kapfhammer HP, Reininghaus EZ, Wallner-Liebmann S. Journal: CNS Neurol Disord Drug Targets. Publication date: 2016. PMID: 26996177. Impact factor (2014): 2.628.

4.) Title: **„Is the Molecular Clock ticking differently in Bipolar Disorder?-Methylation analysis of the Clock Gene ARNTL“.** Authors: Bengesser SA, Reininghaus EZ, Lackner N, Birner A, Fellendorf FT, Platzer M, Kainzbauer N, Tropper B, Hörmanseder C, Queissner R, Kapfhammer HP, Wallner-Liebmann SJ, Fuchs R , Petek E, Windpassinger C, Schnalzenberger M, Reininghaus B, Evert B, Waha A. Journal: World Journal of Biological Psychiatry (Top 20% journal). Impact factor (2014): 4.183.

6.2. CMF PBS buffer (5 Liter):

-40g NaCl

-1,5g KCl

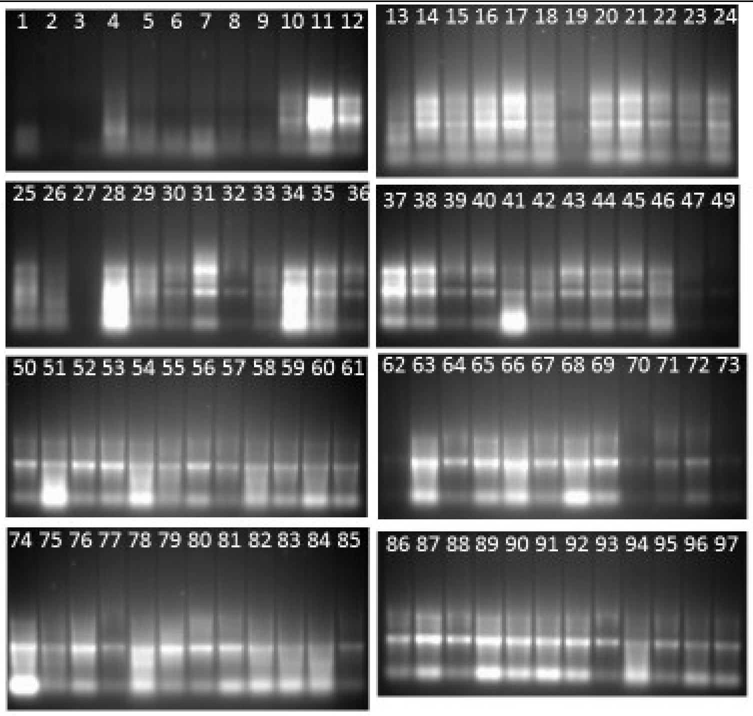
-0,1g KH2PO4

-0,457g Na2HPO4x2H2O

-10g Glucose

6.3. RNA checking gels

RNA checking gels (Gene expression analysis)



1.5. SOPs

Salting out technique (DNA isolation)- SOP from the Human Genetics Department

1 Einleitung

Aus den zellhaltigen Zellen des Blutes wird DNA nach der Aussalzmethode isoliert.

2 Mitgeltende AA bzw. Dokumente und Normen

DNA-Isolierung P – SOP

3 Definitionen / Abkürzungen

DNA ETOH Ethanol HCl Salzsäure KHCO3 Kaliumhydrogencarbonat NaCl Natriumchlorid Na2EDTA Dinatrium-ethylendiamin-tetraacetat NaOH Natriumhydroxid NH4Cl Ammoniumchlorid rpm rounds per minute SDS Sodiumdodecylsulfat SE Sodium EDTA TE Tris EDTA

4 Geräte

Zentrifuge Eppendorf Centrifuge 5810R Rotor: Ausschwingrotor A-4-81, max. Drehzahl 4000 1/min. Kühlschrank Gefrierschrank Brutschrank Pipetten

5 Chemikalien und Lösungen 5.1 Chemikalien

Chemikalie	Firma	Lagerung
Ammoniumchlorid p.A.	Roth	Raumtemperatur
Chloroform p.A.	Roth	Raumtemperatur
Dinatrium-ethylendiamin-tetraacetat p.A.	Roth	Raumtemperatur
Ethanol absolut p.A.	J.T.Baker	Raumtemperatur
Isoamylalkohol p.A.	Roth	Raumtemperatur
Kaliumhydrogencarbonat p.A.	Roth	Raumtemperatur

Natriumchlorid p.A.	Merck	Raumtemperatur
Natriumhydroxid p.A.	Roth	Raumtemperatur
Proteinase K	Amresco	-15 bis -25°C
Roti-Phenol®	Roth	Raumtemperatur
Salzsäure rauchend 37% z.A.	Merck	Raumtemperatur
Sodiumdodecylsulfat ultra pure	Roth	Raumtemperatur
Tris p.A.	Roth	Raumtemperatur

5.2 Lösungen

Lysepuffer: (für 2 Liter)16,58g 2,00g KHCO3

0,074g Na2EDTA autoklavieren

NH4Cl

10x SE: (für 1 Liter): 43,83g NaCl 93,06g Na2EDTA

mit 5M NaOH auf pH=8,0 einstellen autoklavieren

50ml 10x SE 450ml H2O autoklavieren

1x SE:

10x TE: (für 1 Liter) 12,11g Tris 3,72g Na2EDTA

1x TE:

50ml 10x TE 450ml H2O autoklavieren

mit 6M HCl auf pH=8,0 einstellen autoklavieren

20% SDS: (für 1 Liter) sterilfiltrieren

200g Sodium-Dodecyl-Sulfat ultra pure

6M NaCl: (gesättigt, für 1 Liter) 350,64g NaCl autoklavieren

Ethanol 70%: 70ml ETOH abs. 30ml H2O

Ethanol absolut z.A. ProteinaseK: 10mg/ml

1. Tag:

* Blutprobe in ein 50ml Greiner Röhrchen (ohne Stehrand) überführen

* mit 3-5fachem Volumen an Lysepuffer überschichten und schwenken

* für 30 bis max. 60 min. auf Eis stellen bzw. in den Kühlschrank zum Lysieren (Lösung muss klar werden, d.h. die roten Blutkörperchen sind geplatzt)

* zentrifugieren: -- 20 min. -- 1300 rpm

-- bei Raumtemperatur * Überstand verwerfen

* Pellet (Leucocyten) in 20ml Lysepuffer lösen = Waschschritt

* zentrifugieren: -- 20 min. -- 1300 rpm

-- bei Raumtemperatur * Überstand verwerfen

Verdau ansetzen:

* 5ml 1x SE für 10ml an Blut-Ausgangsvolumen (Pellet in 1ml 1xSE resuspendieren und weitere 4ml davon zugeben)

*+ 250 µl 20% SDS * + 25 µl Proteinase K

* Bei 37°C (+/- 2°C) im Brutschrank über Nacht verdauen lassen Dieser Ansatz gilt für 5ml Endvolumen.

3ml Endvolumen 3ml 1x SE + 150 µl 20% SDS

+ 15 µl Proteinase K

2ml Endvolumen 2ml 1x SE + 100 µl 20% SDS

+ 10 µl Proteinase K

2. Tag:

* + 1,4ml 6M NaCl-Lösung zum Verdau dazugeben Dieser Ansatz gilt für 5ml Endvolumen.

3ml Endvolumen + 840 µl NaCl-Lösung 2ml Endvolumen + 560 µl NaCl-Lösung

* am Vortexer kurz mischen

* zentrifugieren: -- 30 min. -- 4000 rpm

-- bei Raumtemperatur Inzwischen Pasteurpipetten mittels Bunsenbrenner biegen (steril, unter der Werkbank)

* Überstand in ein steriles 50ml Greiner Röhrchen schütten (die Zelltrümmer bleiben zurück und werden verworfen)

* Mit 3-5fachem Volumen ETOH absolut überschichten (bezogen auf das Volumen des Überstandes, z.B. 5ml Lösung → auf 25ml im Greiner auffüllen)

* DNA fällt als weißes Knäuel aus Die DNA mit Hilfe der steril gebogenen Pasteurpipette herausnehmen und kurz in 70%igen ETOH (70ml ETOH abs. + 30ml H_2O) tauchen = Waschschritt

* DNA auf der Pasteurpipette an der Luft ca. 10min trocknen lassen (DNA wird farblos)

* In ein steriles Cryo-Tube 1x TE vorlegen (je nach Pelletgröße 500-1000µl) und die DNA durch leichtes bewegen der Pasteurpipette darin lösen Die fertig isolierte DNA wird kurzfristig im Kühlschrank und langfristig im Gefrierschrank gelagert.

7 Besondere Hinweise

Um den Kontakt von potentiell-infektiösen Probenmaterial mit der Augenschleimhaut zu verhindern, ist während der DNA-Isolierung das Tragen einer Schutzbrille erforderlich."

EDTA-Vollblut und DNA nicht direktem Sonnenlicht aussetzen Wichtig: Genaue

Beschriftung der Proben um Verwechslungen auszuschließen!!!

1. Tag Verdau ansetzen:

200 µl DNA 10 µl 20%iges SDS

4 µl Proteinase K

4 µl 0,5M EDTA 200 µl H2O

Diesen Verdau in einem 1,5 ml Eppi machen und über Nacht in den Brutschrank bei 37°C stellen.

2. Tag siehe 2. Tag DNA-Isolierung P - SOP

SOP of Bisulfite Treatment

Part I

1. Ansatz der Reaktion in 200 yl PCR Tubes bei Raumtemperatur
2. DNA Konzentration 1ng - 2yg, max. 20yl, verdünnt in H2O
3. zu der DNA-Verdünnung gibt man 85yl Bisulfite Solution und 35yl Protect Buffer, insgesamt ergibt das ein Volumen von **140yl** im Tube

4. die Bisulfite Conversion erfolgt im Thermocycler:

Denaturation:	95°C	5 Min
Uncubation:	60 °C	10 Min
Denaturation:	95°C	5 Min
Incubation:	60°C	10 Min
Hold:	20°C	

Part II

Aufreinigung der bahandelten DNA

1. Tubes kurz runter zentrifugieren und den kompletten Inhalt (140yl) in 1.5 ml Eppis übertragen
2. 310 yl Buffer BL hinzu geben, kurz vortexen, kurz zentrifugieren

3. 250yl 100% Ethanol dazu, 15 sec vortexen, kurz zentrifugieren
4. den kompletten Inhalt auf eine MinElute DNA spin Säule (werden bei 4°C gelagert) geben und 1 Min bei 14.000 rpm zentrifugieren
5. die Säule in ein neues Collection-Tube geben
6. 500yl Buffer BW auf die Säule geben und 1 Min 14.000 rpm zentrifugieren
7. die Säule in ein neues Collection-Tube
8. 500yl Buffer BD auf die Säule, 15 Min bei RT inkubieren
9. 1 Min 14.000 rpm zentrifugieren, neues Collection Tube
10. 500yl Buffer BW auf die Säule, 1 Min 14.000 rpm, neues collection Tube
11. Wiederholung Schritt 10.
12. 250yl 100% Ethanol hinzu, 1 Min 14.000 rpm zentrifugieren
13. neues Collection Tube, 1 Min 14.000 rpm zentrifugieren
14. die Säule mit offenem Deckel bei 60°C 5 Min trocknen
15. 1.5 ml Eppis mit Proben-Namen beschriften und die zugehörige Säule hinein setzen
16. 15yl Buffer EB auf die Mitte der Säule geben und 1 Min RT inkubieren
17. 1 Min 14.000 rpm zentrifugieren
18. erneut 15yl Buffer EB auf die Mitte der Säule geben, 1 Min RT
19. 1 Min 14.000 rpm zentrifugieren
20. die behandelte DNA bei -20°C lagern

Used SOPs for PCR, bisulfite treatment and Pyrosequencing

Durchführung:

Bestimmung der Konzentration und Qualität der DNA

- die Konz. von 1µl DNA wird photometrisch auf dem Nano Drop ND-1000 Spectrophotometer bei 260nm und 280nm bestimmt

- 1% Agarosegel mit einer 1kb Leiter zum Nachweis möglicher Fragmentierung der DNA 1µl Ladepuffer Sigma Gel Loading Buffer G2526-5ml + 1µl DNA

Bisulfit Behandlung genomischer DNA:

100-500µg DNA werden in 20µl unter Verwendung des EpiTect Bisulfite Kit von Qiagen (Cat-No. 59104) mit Bisulfit modifiziert. Methylierte Cytosin Basen bleiben bei dieser

Behandlung erhalten, alle anderen Cytosin Basen werden zu Uracil umgewandelt, das in der folgenden PCR Reaktion durch Thymidin ersetzt wird. Die Behandlung erfolgt nach den Angaben des Herstellers (Qiagen).

- der Ansatz erfolgt in 200µl Reaktionsgefäßen in der PCR-Maschine: Biometra T1 Thermocycler

PCR:

Das gewünschte DNA Fragment wird mit spezifischen Primern amplifiziert. Hierbei wird der PyroMark PCR Kit (Qiagen Cat No. 978703) nach den Angaben des Herstellers ohne Verwendung der Lösung Q eingesetzt. Die Reaktion muss in der **Pipettierstation** erfolgen um Kontaminationen zu vermeiden. Jede Probe wird in Duplikaten zusammen mit einer Positiv- (methylierte DNA) und einer Negativkontrolle (unmethylierte DNA) und einer Leerkontrolle (H_2O) analysiert. Das PCR Gerät Biometra wird mit dem unten beschriebenen Programm 20 verwendet.

- die Reaktion erfolgt in der Pipettierstation um Kontaminationen zu vermeiden
- jede Probe wird in Duplikaten zusammen mit einer Positiv-und einer Nagativkontrolle und einer Leerkontrolle (H_2O) analysiert.

• Ansatz Mastermix in µl:

Master Mix 2x:	12,5
CoralLoad Concentrate 10x:	2,5
P1 10pmol/µl:	1,5
P2 10pmol/µl:	1,5
Wasser:	4,0

- pro Ansatz werden 22µl Mastermix in eine 96-Well Platte vorgelegt
- nun werden 3µl der BS- DNA dazu gegeben
- mit Mineralöl bedecken und kurz zentrifugiert
- PCR-Maschine: Biometra T1 Thermocycler

- **PCR Programm:** 95°C 15 min

94°C 30 sec

56°C 30 sec 50 X

72°C 30 sec

72°C 10 min

4°C Pause

DNA Fragmentanalyse aus 2% Agarosegel:

Das PCR Produkt wird in einem 2% Agarosegel mit dem PUC19 Längenstandard aufgetrennt (5µL PCR-Produkt) und mit dem UV Geldokumentationssystem sichtbar gemacht. Diese Analyse dient der Kontrolle der PCR Reaktion. Nur Proben, die ein eindeutiges Signal der richtigen Fragmentlänge ohne Nebenbanden zeigen werden weiterverarbeitet. Voraussetzung hierfür ist, dass die Leerkontrolle (Wasser) kein Signal zeigt.

Aufreinigung des biotinylierten DNA-Stranges:

Für die Pyrosequenzierung müssen dNTPs, Primer und der nicht-biotinylierte Strang des PCR Produktes entfernt werden. Hierfür wird die Vacuumstation (Biotage) eingesetzt. In eine Proben Platte wird zur Aufreinigung neben dem verbleibenden PCR Produkt Bindepuffer und Sepharose pipettiert.

Sample-Platte

20µl PCR-Produkt

18,5µl Binding Buffer

1,5 µl Sepharose

Vacuum Pumpe einschalten

1.	HPLC-	5sec
2.	HPLC-H_2O	5sec
3.	Aufsaugen der Proben bzw Sepharose-Beads	10sec
4.	70% Ethanol	5sec
5.	Denaturation-Solution	5sec
6.	Wash-Buffer (1x)	10sec

Vacuum Pumpe ausschalten

Pyrosequenzierung:

Zu dem gereinigten biotinylierten Einzelstrang wird der Sequenzierungsprimer pipettiert und lagert sich für 3 Minuten an. Die Reaktionsplatte wird anschließend in den Pyrosequenzierer (PyroMark Q24, Biotage) gestellt und das spezifische Programm zur Pyrosequenzierung gestartet. Dieses wird auf dem zugehörigen Computer erstellt und per USB Stick auf den PyroMark übertragen. Eine Kontrolle der Bisulfitkonvertierung wird verwendet. Wird hier ein Wert über 1.5 gemessen, ist die Qualität der Bisulfitbehandlung nicht ausreichend und die DNA Probe muss erneut konvertiert werden.

Cartridge vorbereiten

Das Volumen von Enzym- und Substrat-Mix und der Nukleotide ist abhängig von der Anzahl der zu untersuchenden Proben und der zu analysierenden Sequenz (PyroMark Gold Q96 Kit, Qiagen) und wird von der PyroMark Systemsoftware vorgegeben.

• Primer Platte, Annealing	3min bei RT
• **Vacuum Pumpe einschalten**	
• 80°C Denaturation	2 min
• HPLC-H_2O	5 sec
• HPLC-H_2O	5 sec

Vacuum Pumpe ausschalten:

- die Reaktionsplatte wird nun in den Pyrosequenzierer gestellt und das Programm für gestartet

Qualitätssicherung:

1. PCR Produkt korrekter Größe. Leerkontrolle zeigt kein Signal
2. Beide Messungen der Patienten DNA zeigen einheitliche Werte
3. Die in Vorversuchen bestimmten Wichtungsfaktoren für die 4 untersuchten CpG Positionen berücksichtigen altersbedinge Methylierung von normalem Gehirngewebe
4. Die Positiv und Negativkontrollen zeigen eindeutige positive (>40) bzw. negative Metyhlierungswerte (<-15)
5. Die Bisulfitkontrolle zeigt eine mind. 93% Konvertierung
6. Die Signalhöhe der Sequenzierungsreaktion ist mindestens 20%

1.6. Not significant results in detail

To increase the readability of the text, **not** significant results are depicted in this section:

1.6.1. Obesity and *BiP* gene expression

BiP gene expression in overweight versus normal weight study participants

The gene expression of *BiP* [dCQ] was analyzed in 39 persons with normal weight (BMI<25 kg/m^2) and 69 overweight/obese study participants (BMI> 25kg/m^2). *BiP* gene expression did not differ significantly between normal weight and overweight/obese study participants independent of group (*F(1/99)*= 0.813, *p*= 0.369, Partial Eta^2= 0.008). Furthermore, there was no group*overweight effect for dCQ *BiP* in BD versus controls (*F(1/99)*= 0.867, *p*= 0.354, Partial Eta^2= 0.009).

BiP gene expression between weight classes of BD versus controls

A further subclassification of the cohort into normal weight (<25 BMI, n= 40), overweight (BMI 25-30, n= 40) and obesity (>30 BMI, n= 28) did not show differing *BiP* gene expression between the weight groups either (*F(2/98)*= 1.022, *p*= 0.364, Partial Eta^2= 0.020). Furthermore, there was again no *BiP* weight*group effect (*F(2/98)*= 0.896, *p*= 0.412, Partial Eta^2= 0.018).

Correlation of BiP and BMI

In addition, there was no significant correlation between BMI and *BiP* gene expression in the total sample of 108 study participants independent of group (*r*= -0.016, *p*= 0.865).

1.6.2. Weight and *CHOP* gene expression

CHOP gene expression in overweight/ obese versus normal weight study participants

CHOP gene expression did not differ between normal weight and overweight/ obese study participants in a total of 108 tested study participants (*F(1/99)*= 2.687, *p*= 0.104, Partial Eta^2= 0.349). No weight*group effect (*F(1/99)*= 0.560, *p*= 0.456, Partial Eta^2= 0.006) was found.

CHOP gene expression in weight classes of BD versus controls

In addition, *CHOP* gene expression did not differ between three weight classes - normal weight, overweight and obese (*F(1/94)*= 1.368, *p*= 0.245). No weight*group interaction was found either (*F(3/94)*= 0.096, *p*= 0.962, partial Eta^2= 0.003).

Pearson correlation analysis of BMI and CHOP

BMI and *CHOP* gene expression did not correlate with each other independent of BD (*r* = -0.004, *p*= 0.968).

1.6.3. *XBP1* Splicing

XBP1 splicing in BD versus Controls (group effects)

The ratio unspliced/spliced *XBP1* did not differ between BD and controls (*F(1/100)*= 0.773, *p*= 0.382, Partial Eta^2= 0.008). The average of the ratio unspliced/spliced *XBP1* was 4.59 +/- 4.52 in BD and 4.26+/- 2.62 in controls. The percentage of unspliced *XBP1* did not differ between patients and controls (*F(1/100)*= 0.638, *p*= 0.222, Partial Eta^2= 0.002). Similarly, the percentage of spliced *XBP1* did not differ between patients and controls (*F(1/100)*= 0.222, *p*= 0.638, Partial Eta^2= 0.002). 78.51+/- 6.73% of *XBP1* was unspliced in BD, whereas 21.49+/- 6.73% was spliced. In contrast, 76.69+/- 9.58% of *XBP1* mRNA was unspliced in controls and 23.31 +/- 9.58% was spliced.

Weight and XBP1 splicing

All *XBP1* parameters did not differ significantly between the different weight classes. Unspliced *XBP1* did not differ significantly between weight classes in relationship with BD, thus no weight*group effect was found (*F(1/100)*= 3.937, *p*= 0.050, Partial Eta^2= 0.038).

However, there was also no significant difference in unspliced *XBP1* between overweight/ obese and normal weight independent of group (*F(1/100)*= 1.140, *p*= 0.288, Partial Eta^2= 0.011).

Other calculated *XBP1* values did not differ according to overweight/ obesity or normal weight. Spliced *XBP1* did not differ either between overweight/ obese and normal weight participants (n= 39) (*F(1/100)*= 0.206, *p*= 0.651, Partial Eta^2= 0.002). No weight*group effects were found for spliced *XBP1* (*F(1/100)*= 0.055, *p*= 0.815, Partial Eta^2= 0.001).

The total amount of *XBP1* (=sum of unspliced and spliced *XBP1*) also did not differ between overweight/ obese and normal weight study participants (*F(1/100)*= 0.500, *p*= 0.481, Partial Eta^2= 0.005). No weight*group was found for the total amount of *XBP1* (*F(1/100)*= 2.719, *p*= 0.102, Partial Eta^2= 0.026).

The ratio of unspliced/spliced *XBP1* did not differ between overweight/ obese and normal weight (*F(1/100)*= 0.013, *p*= 0.909, Partial Eta^2= 0.019). Similar, no weight*group effect was found (*F(1/100)*= 1.940, *p*= 0.167, Partial Eta^2= 0.019).

The percentage of spliced *XBP1* did not differ between overweight/ obese and normal weight either (*F(1/100)*= 1.298, *p*= 0.257, Partial Eta^2= 0.013). In addition, no weight*group effect was found (*F(1/100)*= 1.839, *p*= 0.178, Partial Eta^2= 0.018).

In line with that, the percentage of unspliced *XBP1* did not differ between overweight/ obese and normal weight participants in regard of group (*F(1/100)*= 0.627, *p*= 0.430, Partial Eta^2= 0.006).

1.6.4. Obesity and Oxidative Stress

(Anti)oxidative markers in overweight/ obese and normal weight

MDA did not differ between overweight/ obese (n= 86) and normal weight (n= 128) study participants independent from group (*F(1/204)*= 0.044, *p*= 0.833, Partial Eta^2= 0.005). Furthermore, there was no group*weight effect concerning the parameter MDA in BD participants and controls (*F(1/204)*= 1.042, *p*= 0.309, Partial Eta^2= 0.005). TBARS did not differ between overweight/ obese and normalweight independent from group in normal weight participants and overweight/ obese participants (*F(1/205)*= 0.100, *p*= 0.753, Partial

Eta2= 0.000). No significant weight*group effect was found (*F(1/205)*= 3.196, *p*= 0.075, Partial Eta^{2}= 0.015).

The GST did not differ between overweight/ obese study participants (BD and controls) and normal weight test persons (*F(1/173)*= 1.376, *p*= 0.242, Partial Eta^{2}= 0.008). No group*weight effects were found (*F(1/173)*= 0.004, *p*= 0.950, Partial Eta^{2}= 0.000).

SOD did not differ between overweight/ obese and normalweight independent from group (*F(1/143)*= 0.000, *p*= 0.990, Partial Eta^{2}= 0.000). Furthermore, SOD did not show any group*weight effects (*F(1/143)*= 0.030, *p*= 0.862, Partial Eta^{2}= 0.000).

The TAC did not differ between overweight/ obese and normal weight independent of BD (*F(1/188)*= 0.221, *p*= 0.639, Partial Eta^{2}= 0.001). No group*weight effect was found for TAC either (*F(1/188)*= 0.353, *p*= 0.553, Partial Eta^{2}= 0.002).

1.6.5. Weight effects and Methylation of *ARNTL*

Methylation at cg05733463 did not differ between normal weight and overweight study participants independent from group (*F(1/210)*= 0.704, *p*= 0.402, Partial Eta^{2}= 0.003). No weight*methylation interaction was found for cg05733463 (*F(1/210)*= 0.006, *p*= 0.936, Partial Eta^{2}= 0.000).

Methylation at PS2 POS1 did not differ between normal weight and overweight/obese study participants independent from BD (*F(1/129)*= 0.587, *p*= 0.445, Partial Eta^{2}= 0.005). No significant weight*group effect was found for BD and controls (*F(1/129)*= 3.019, *p*= 0.085, Partial Eta^{2}= 0.023).

Methylation at PS2 POS2 did not differ between normal weight and overweight/obese study participants independent from group (*F(1/129)*= 0.288, *p*= 0.592, Partial Eta^{2}= 0.002) or in relationship with BD (*F(1/129)*= 0.277, *p*= 0.600, Partial Eta^{2}= 0.002).

Similar, the percentage of methylation did not differ between overweight/obese and normal weight study participants independent from group (*F(1/110)*= 0.187, *p*= 0.666, Partial Eta^{2}= 0.002) or in relationship with BD (*F(1/110)*= 0.1664, *p*= 0.200, Partial Eta^{2}= 0.015) at PS2 POS3.

In line, methylation did not differ between overweight/obese and normal weight study participants (n= 43) independent from group (*F(1/83)*= 0.015, *p*= 0.904, Partial Eta^2= 0.000) or in relationship with BD (*F(1/83)*= 0.014, *p*= 0.906, Partial Eta^2= 0.000) at PS2 POS4.

Methylation at POS5 did not differ between normal weight and overweight/obese study participants independent from group (*F(1/58)*= 0.044, *p*= 0.835, Partial Eta^2= 0.001) or in relationship with BD (*F(1/58)*= 0.273, *p*= 0.604, Partial Eta^2= 0.005).

Methylation did not differ between overweight/ obese and normal weight study participants independent from group (*F(1/47)*= 0.428, *p*= 0.516, Partial Eta^2= 0.009). No significant weight*group interaction was found (*F(1/47)*= 3.563, *p*= 0.065, Partial Eta^2= 0.070) at PS2 POS6.

Correlations between methylation parameters of *ARNTL* and BMI

Methylation at cg05733463 did not correlate with BMI in the total sample of study participants independent from BD (*r*= 0.053, *p*= 0.436).