Epigenetic dysregulation in alcohol dependence and borderline personality disorder

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Christof Brückmann
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Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	Jun.-Prof. Dr. Vanessa Nieratschker
2. Berichterstatter:	Prof. Dr. Bernd Wissinger

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I Abbreviations

5hmC	5-hydroxymethylcytosine
AD	Alcohol dependence
AP-1	Activator protein 1
BPD	Borderline personality disorder
CBT	Cognitive behavioral therapy
CGI	CpG island
CpG	Cytosine-phosphate-guanine
CPT	Cell preparation tube
DBT	Dialectical behavior therapy
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DSM	Diagnostic and Statistical Manual of Mental Disorders
EDTA	Ethylenediaminetetraacetic acid
EWAS	Epigenome-wide association study
FACS	Fluorescence-activated cell sorting
FDR	False discovery rate, Benjamini-Hochberg adjusted
GABA	γ-aminobutyric acid
GWAS	Genome-wide association studies
MBD	Methyl-CpG-binding domain
MBT	Mentalization-based therapy
miRNA	microRNA
NK	Natural killer (cells)
NMDA	N-methyl-D-aspartate
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RNAi	RNA interference
SAM	S-Adenosyl Methionine
SNP	Single-nucleotide polymorphism
sRNA	small RNA
TET	Ten-eleven translocation
TFP	Transference-focused psychotherapy
TNF	Tumor necrosis factor

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III Summary

Alcohol dependence and borderline personality disorder are two complex psychiatric disorders. Although studies indicate a high genetic heritability of 40 – 60% for these diseases, the remaining variability still has to be investigated. One major contributor to this “missing heritability” is thought to be explained by epigenetics. Epigenetics includes a number of pathways that ultimately alter gene expression. The most studied mechanism is DNA methylation, which occurs on cytosines in the context of cytosine-guanine dinucleotides. Methylation of DNA can act as steric hindrance and, by recruiting further proteins, promote histone deacetylation and additional *de novo* DNA methylation usually resulting in decreased gene expression.

Here, we examined epigenome-wide T cell DNA methylation in alcohol dependent patients compared to healthy controls, as well as before and after an alcohol treatment. We found that global DNA methylation was decreased in patients compared to controls, but reverted back after the alcohol treatment, leading to the loss of significant differences compared to controls. In addition, we identified unique sets of differentially methylated sites and genes between patients and controls and between patients pre- and posttreatment. From these unique sets, we further identified sites and genes which changed during the alcohol treatment. To verify our epigenome-wide results, we validated our top-ranked hits by pyrosequencing and, additionally, aimed to replicate them in an independent cohort and in whole blood DNA. The fact that we found both *SRPK3* and *HECW2* differentially methylated in T cells and in whole blood supports their potential value as novel blood-based biomarkers for alcohol dependence.

In addition, we investigated DNA methylation of *APBA3* and *MCF2* in borderline personality disorder patients compared to healthy controls as well as before and after a 12-week dialectical behavior therapy. Although we did not detect statistically significant differences in DNA methylation between patients and controls, we found that both genes were higher methylated pre-treatment in patients responding to therapy compared to non-responders. This indicates that *APBA3* and *MCF2* DNA methylation might be potential candidates of novel predictive epigenetic biomarkers for dialectical behavior therapy outcome.

However, further studies are needed to replicate these results in independent cohorts and to decipher the role these genes might play in the respective disease.

IV Zusammenfassung

Zu den komplexen psychiatrischen Erkrankungen zählen unter anderem Alkoholabhängigkeit und die Borderline Persönlichkeitsstörung. Beide Erkrankungen haben eine vergleichsweise hohe genetische Heritabilität von 40 – 60%. Die verbleibende Variabilität kann unter anderem durch die Epigenetik erklärt werden. Epigenetik umfasst mehrere molekulare Mechanismen, darunter die DNA Methylierung. Diese enzymatische Modifikation geschieht an Cytosinen, die in einem Cytosin-Guanin-Dinukleotid präsent sind. Methylierte Cytosine können sowohl als direkte sterische Hinderung von Transkriptionsfaktoren agieren, als auch durch die Rekrutierung weiterer Proteine die Acetlyierung von Histonen und die *de novo* Methylierung der DNA bewirken. Diese Modifikationen führen in der Regel zu einer verminderten Genexpression.

In dieser Arbeit wurde die epigenomweite DNA Methylierung in T-Zellen zwischen alkoholabhängigen Patienten und gesunden Kontrollen sowie zwischen den Patienten vor und nach einer Alkoholentzugstherapie untersucht. Die epigenomweite Methylierung der Patienten war im Vergleich zu den Kontrollen signifikant niedriger, glich sich jedoch nach der Therapie den Werten der Kontrollen an. Des Weiteren wurden etliche signifikant unterschiedlich methylierte Sites und Gene zwischen Patienten und Kontrollen und zwischen Kontrollen vor und nach dem Entzug gefunden. Um die epigenomweiten Ergebnisse zu verifizieren, wurden vier signifikante Sites durch Pyrosequenzierung validiert und in einer weiteren, unabhängigen Kohorte repliziert. Durch die weitere Replizierung in Vollblut könnten die Methylierung von *SRPK3* und *HECW2* künftig potentiell als neue, epigenetische Biomarker für Alkoholabhängigkeit dienen.

Zusätzlich wurde die Methylierung von *APBA3* und *MCF2* in Patienten mit Borderline Persönlichkeitsstörung untersucht. Im Vergleich zwischen Patienten und Kontrollen fanden sich keine signifikanten Unterschiede. Wurden die Patienten jedoch nach der dialektisch-behavioralen Therapie in eine Responder und eine Non-Responder Gruppe eingeteilt, war die Methylierung beider Gene vor der Therapie bei den Respondern signifikant erhöht. Somit könnte die Methylierung von *APBA3* und *MCF2* als prädiktiver Biomarker für den Therapieerfolg der dialektisch-behavioralen Therapie geeignet sein.

Weitere Studien mit größeren und unabhängigen Kohorten werden jedoch benötigt, um diese Ergebnisse zu validieren und replizieren und die Rolle dieser Gene in den jeweiligen Erkrankungen zu untersuchen.

V Publications

Original publications:

1. **Brückmann C**, Di Santo A, Karle KN, Batra A, Nieratschker V. Validation of differential *GDAP1* DNA methylation in alcohol dependence and its potential function as a biomarker for disease severity and therapy outcome. *Epigenetics* 2016; **11**(6)**:** 456-463.
2. **Brückmann C***, Islam SA*, MacIsaac JL, Morin AM, Karle KN, Di Santo A, Wüst R, Lang I, Batra A, Kobor MS*, Nieratschker V*. DNA methylation signatures of chronic alcohol dependence in purified $CD3^+$ T-cells of patients undergoing alcohol treatment. *Scientific Reports* 2017; **7**: 6605
3. Knoblich N, Gundel F, **Brückmann C**, Becker-Sadzio J, Frischholz C, Nieratschker V. DNA methylation of *APBA3* and *MCF2* in borderline personality disorder: potential biomarkers for response to psychotherapy. *In submission*

*contributed equally

Own share:

Paper 1: Validation of differential GDAP1 DNA methylation in alcohol dependence and its potential function as a biomarker for disease severity and therapy outcome.

I designed the study together with VN and performed all laboratory experiments, evaluated the questionnaires and analyzed the data. I recruited patients together with AD and KK. I wrote the first draft of the manuscript and finalized the manuscript with assistance of VN, who was also supervising the study.

Paper 2: DNA methylation signatures of chronic alcohol dependence in purified $CD3^+$ T-cells of patients undergoing alcohol treatment.

VN conceived and designed the study. I organized and coordinated recruitment of study participants. I, KNK, AD, RW, IL and AB took part in recruiting study participants. JLM and AM acquired the 450K data. I performed T cell purification, pyrosequencing experiments, evaluated the questionnaires and analyzed the data. SAI and I analyzed the 450K data and prepared the figures. I and SAI wrote the first draft of the manuscript. I wrote the revision of the manuscript. MSK and VN supervised the work and assisted in finalizing the manuscript.

Paper 3: DNA methylation of APBA3 and MCF2 in borderline personality disorder: potential biomarkers for response to psychotherapy.

VN designed the study. NK, FG, JB and CF recruited patients. I trained NK regarding laboratory work and we performed laboratory experiments and analyzed the data. NK wrote the first draft of the manuscript. I and VN critically reviewed the paper and took part in the writing process.

Further publications:

I was further involved in the following manuscripts which were published during my PhD program:

1. Mladinov M, **Brückmann C**, Nieratschker V, Eschweiler GH. Improvement of Sleep Fragmentation in a Senior Patient with Depression after Short-term Blue-light Treatment: A Case Report. *Submitted to Int Psychogeriatr.* 2017
2. Leehr EJ, Schag K, **Brückmann C**, Plewnia C, Zipfel S, Nieratschker V *et al.* A Putative Association of COMT Val(108/158)Met with Impulsivity in Binge Eating Disorder. *Eur Eat Disord Rev* 2016; **24**(2)**:** 169-173.
3. Nieratschker V, **Brückmann C**, Plewnia C. CACNA1C risk variant affects facial emotion recognition in healthy individuals. *Scientific reports* 2015; **5:** 17349.

Supervision of theses:

The following theses were prepared under my laboratory supervision:

1. Meixner A-S. *Ongoing*. MD thesis, *Universität Tübingen* 2017
2. Gräf Olmos V. Epigenetik und Genexpression ausgewählter Zielgene bei alkoholabhängigen Patienten. Bachelor thesis, *Universität Tübingen* 2017
3. Knoblich N. Epigenetik der Borderline-Persönlichkeits-Störung - Effekte der DBT auf die Methylierungsmuster. MD thesis, *Universität Tübingen* 2017
4. Wallisch A. Epigenetik der Borderline-Persönlichkeitsstörung. Master thesis, *Universität Tübingen* 2016
5. Wiegand AC. Effects of Transcranial Direct Current Stimulation on Cognitive Control and DNA Methylation. Master thesis, *Universität Tübingen* 2016

1 Introduction

1.1 The burden of alcohol dependence

Alcohol dependence (AD) is a very complex and severe disorder that affects a total of 208 million people worldwide, not including a potentially high estimated number of unreported cases. Approximately 3.3 million deaths each year are caused by alcohol abuse [1]. In 2015, a total of approximately 327,000 patients in Germany were enrolled in inpatient treatment programs as a result of their alcohol abuse. Alcohol abuse is not a problem solely adults are confronted with: 22,000 of the above mentioned cases were patients between the age of ten and 19 [2]. Although these numbers are high and mental and behavioral disorders caused by alcohol abuse are, after cardiac insufficiency, the second ranked reason for an inpatient treatment in Germany, the impact of AD has been underestimated in the past and is still underestimated today [3].

To receive a diagnosis of AD, three or more of the seven criteria listed in Table 1 must have been met in the past twelve months according to the *Diagnostic and Statistical Manual of Mental Disorders* DSM-IV [4].

Table 1: Criterions of alcohol dependence according to the DSM-IV [4].

Criterion	Description
Criterion 1	Need for markedly increased amounts of alcohol to achieve intoxication or desired effect; or markedly diminished effect with continued use of the same amount of alcohol
Criterion 2	The characteristic withdrawal syndrome for alcohol; or drinking (or using a closely related substance) to relieve or avoid withdrawal symptoms
Criterion 3	Drinking in larger amounts or over a longer period than intended
Criterion 4	Persistent desire or one or more unsuccessful efforts to cut down or control drinking
Criterion 5	Important social, occupational, or recreational activities given up or reduced because of drinking
Criterion 6	A great deal of time spent in activities necessary to obtain, to use, or to recover from the effects of drinking
Criterion 7	Continued drinking despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to be caused or exacerbated by drinking

To date, the underlying neurobiological mechanisms of AD are only insufficiently understood. Studies indicate a genetic heritability of 40 – 60% while environmental and stochastic effects account for the remaining variability [5, 6]. Regarding genetic factors that contribute to the risk of developing AD, it has been controversially discussed if the age of onset of drinking also is involved in the development of AD [7, 8]. However, it has been shown that with decreasing age at first drink, the risk for AD symptoms increased and that the age of first drink facilitates the expression of genes associated with vulnerability to AD symptoms [7].

AD is considered both a physical and psychological addiction [1, 9]. The physical addiction is experienced as increased tolerance against alcohol and physical symptoms when stopping or reducing alcohol intake, such as nausea, seizures, headache, sweating and the restless leg syndrome, among many others. Primarily, the γ-aminobutyric acid (GABA) and N-methyl-D-aspartate (NMDA) receptors play a key role in mediating various aspects of alcohol dependence syndromes. Specifically, the increased NMDA receptor activity and the decreased mesolimbic dopaminergic function due to chronic alcohol consumption are believed to contribute to the withdrawal symptoms and to the compulsion to drink alcohol [10]. The psychological addiction is presenting itself as intense craving, feelings of anxiety, denial of problems caused by drinking alcohol, depression, and others [11, 12].

Because of the psychological and physiological impact of AD, good treatment options are required. The aim of AD treatments is to alleviate withdrawal symptoms (with drug therapy) and to deal with underlying psychological problems as well as to prevent relapse of the patients through psychotherapy [13-15]. At the Department of Psychiatry and Psychotherapy, Tuebingen, two inpatient alcohol treatment programs are offered, lasting for either three or six weeks. If necessary, clomethiazole is administered at the beginning of either treatment. By acting as positive allosteric modulator of the $GABA_A$ receptor, clomethiazole enhances the effects of the inhibitory neurotransmitter GABA and clomethiazole treatment therefore helps to reduce withdrawal symptoms and avoid delirium tremens [16]. Patients participate in group therapy with a focus on psychoeducational training and attend physiotherapy and occupational therapy with the aim of returning to a normal life-style within a short time period. The six-week program consists of additional individual psychotherapy and a one year post-treatment outpatient therapy to prevent fast relapse. However, in

general, drop-out rates are high and long-lasting success is limited, as shown by high relapse rates and repeated clinical visits of affected patients [11].

1.2 Borderline personality disorder

Borderline personality disorder (BPD) is a complex and severe disorder which is characterized by a high risk of suicide, severe functional impairments and unstable relationships with other people [17, 18]. Prevalence for BPD in the general population is about 0.5 – 5.9% (median 2.8%) and 15 – 25% in clinical settings [19, 20]. BPD patients very often suffer from comorbidities such as depression, anxiety disorders, eating disorders and substance abuse [17].

A number of factors may contribute to the onset of BPD. The estimated heritability of BPD is around 40% [21, 22]. Biological and psychological factors (such as neurobiological dysfunctions or personality traits) may influence the development of the disorder, together with environmental factors such as adverse childhood experiences [17, 23].

The efficacy of several treatment models, including cognitive behavioral therapy (CBT), dialectical behavior therapy (DBT), transference-focused psychotherapy (TFP) and mentalization-based therapy (MBT), have been studied intensively in the past years [24]. DBT, developed by Marsha Linehan [25], focuses on the reduction of suicide risk and behavior antagonizing an inpatient treatment [26] and is exercised as a twelve-week inpatient treatment program at the Clinic of Psychiatry and Psychotherapy in Tuebingen.

1.3 Challenges of current research

One of the conclusions that emerged from the Human Genome Project was the phenomenon of “missing heritability”: Although candidate-gene driven studies showed strong associations of genetic variants with psychiatric diseases, they could not solely account for their heritability [27]. In addition, genome-wide association studies (GWAS) could only replicate a small number of these associations, implicating that there might be a high number of false-positive findings [28]. Vice versa, significant findings in GWAS could also rarely be verified in subsequent studies. This led to two conclusions: First, replication is of paramount importance to prevent false-positive findings. Second, other mechanisms in addition to genetic

heredity are likely to play a role in the development of those complex diseases. In recent years, evidence has emerged that environmental factors play a major role in various psychiatric disorders, among them AD and BPD [29-31], and that epigenetics may account for a significant fraction of the “missing heritability” [32].

Another major challenge of current research is to find disease-specific biomarkers [33]. Biomarkers have a broad range of application from staging diseases to diagnosis to monitoring disease or treatment progress [34]. They also can be used to identify patients more likely to benefit from one or another treatment and, therefore, support the selection of optimal treatment options [35]. These so called predictive or prognostic biomarkers are commonly accepted and used in cancer treatment [36]. Compared to the field of oncology, the use of biomarkers for psychiatric disorders is still very limited. Although a number of biomarkers exist for psychiatric disorders, including Alzheimers’ disease, major depression and AD [37-39], more research is needed to cover a greater range of disorders and to determine the value of specific biomarkers in validation and replication studies.

1.4 Epigenetics

Epigenetics was first defined by Conrad Waddington in 1942 as “the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being” [40] and later revised to the following: “An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence” [41].

Research in epigenetics nowadays comprises mostly the impact of environmental factors on DNA methylation, histone modification and the role of diverse RNA transcripts, such as microRNAs (miRNA) [42], double-stranded interfering RNA (RNAi) and small, regulatory RNAs (sRNA) [43].

Epigenetics had its breakthrough in cancer research, where epigenetic alterations were found to have a vast impact on the etiopathology and could even be one of the major mechanisms driving the development of the disease [44, 45]. A large number of candidate genes were found to be epigenetically altered, mainly leading to reduced expression of DNA repair genes [46, 47]. Epigenetic research has already found its way into modern medicine: For certain types of cancer, treatment with DNA methyltransferase (DNMT) inhibitors, namely Azacitidine and Decitabine [48, 49], can

improve patient outcomes. By acting as cytidine homolog, they indirectly cause a hypomethylating of candidate gene promoters leading to a reactivation of the cellular tumor defense [50, 51]. Since both drugs have a general, nondirectional effect, to date they can only be applied for certain types of cancer and bear considerable side-effects [52, 53]. However, very recently, targeted DNA demethylation has proven possible *in vivo* [54, 55], laying the foundations for future applications in cancer treatment and beyond.

The epigenetic pathway can be divided into three steps (Figure 1): First, a so called "epigenator" functions as the signal for epigenetic changes. Epigenators can be differentiation signals as well as environmental or lifestyle factors, such as temperature, diet, exercise etc. [56]. Second, "epigenetic initiators" such as non-coding RNAs or DNA binding factors transduce the signal onto the DNA in the cell nucleus. In a third step, the actual epigenetic changes are implemented by histone and DNA modifications, which are the "epigenetic maintainers" [41].

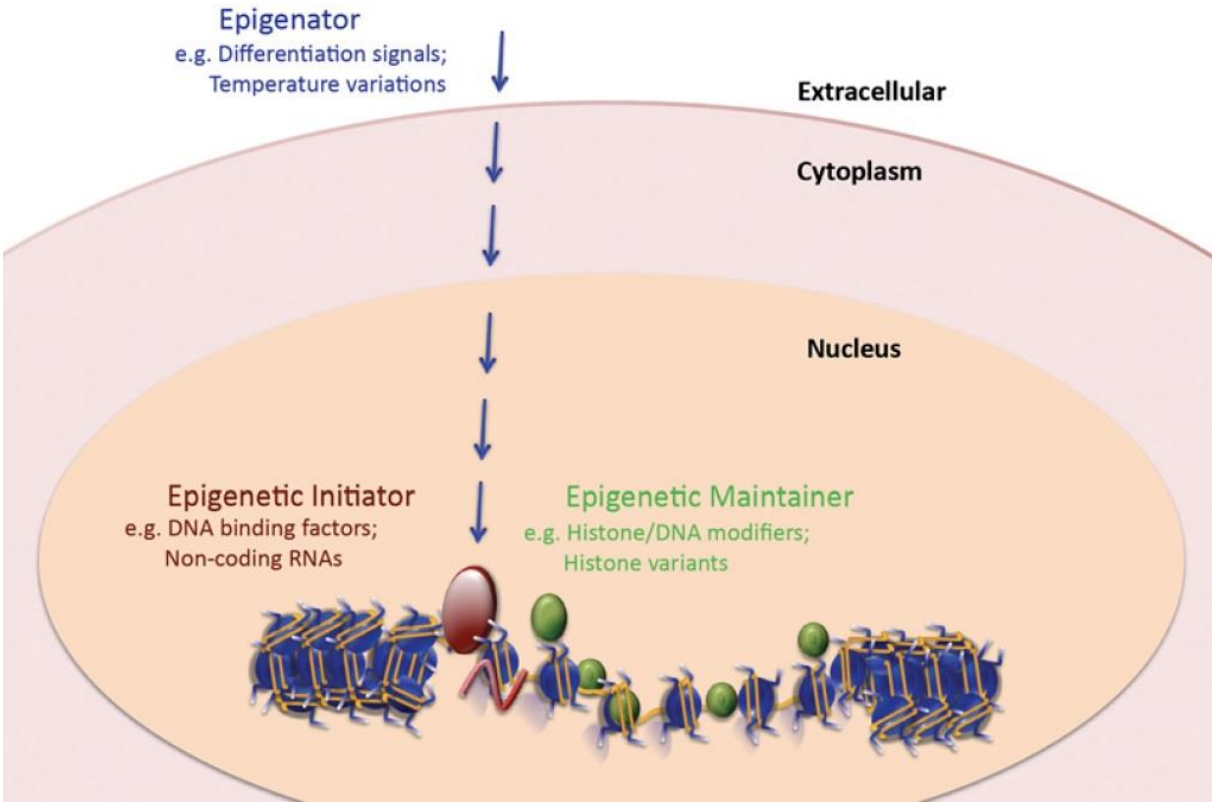

Figure 1: The epigenetic pathway.
Starting with the "epigenator", the signal for inducing epigenetic changes, they are conveyed by the "epigenetic initiator". The actual changes are enforced by histone and DNA modifiers, i.e. DNA methyltransferases ("epigenetic maintainer"). From Berger, S.L. *et al.*, Genes Dev, 2009 [41].

The most common modifications are DNA methylation and histone acetylation, methylation and phosphorylation. Depending on the type of modification, the resulting changes in gene transcription can be very different: In general, DNA methylation and histone deacetylation preserve DNA in a dense, inaccessible state (heterochromatin), which leads to reduced gene transcription. Unmethylated DNA and histone acetylation promote an open, accessible state of the DNA (euchromatin) and lead to increased gene transcription [57-59]. Histone methylation and phosphorylation can lead to either a heterochromatin or euchromatin DNA state, which highly depends on the exact position of the modification [60, 61]. Numerous studies show that DNA methylation and histone modification influence each other [62-64]. However, these general rules do not always apply and recent studies show that the relationship between DNA methylation and gene expression is far more complex than first imagined and involves a number of additional variables [65, 66].
Initially, it was believed that after remodeling in early development, DNA methylation is irreversible [67]. However, by showing that these alterations could change over a lifetime and adapt to different environmental factors, epigenetics became one of the prime research areas in the field [68, 69].

1.4.1 DNA methylation

DNA methylation is one of the best studied epigenetic mechanisms. Hereby, a cytosine is methylated at the fifth position in the pyrimidine ring, forming 5-methylcytosine [70]. This reaction is catalyzed by DNA methyltransferases (DNMTs), which depend on S-Adenosyl methionine (SAM) as methyl group donor [71] (Figure 2).
DNA can be methylated *de novo* or the existing methylation pattern can be maintained, which is a crucial process during cell division [72]. DNA methylation usually only occurs on cytosines in the context of cytosine-phosphate-guanine (CpG) dinucleotides, which are not evenly distributed throughout the genome, but condensed in regions called CpG islands (CGI) [66]. These CGIs are prominently found in gene promoter regions and generally, methylated CGIs are associated with silencing of gene transcription [73]. Non-cytosine DNA methylation, namely adenine methylation, has been reported, but research in this field has just begun and the effects remain unclear to date [74].

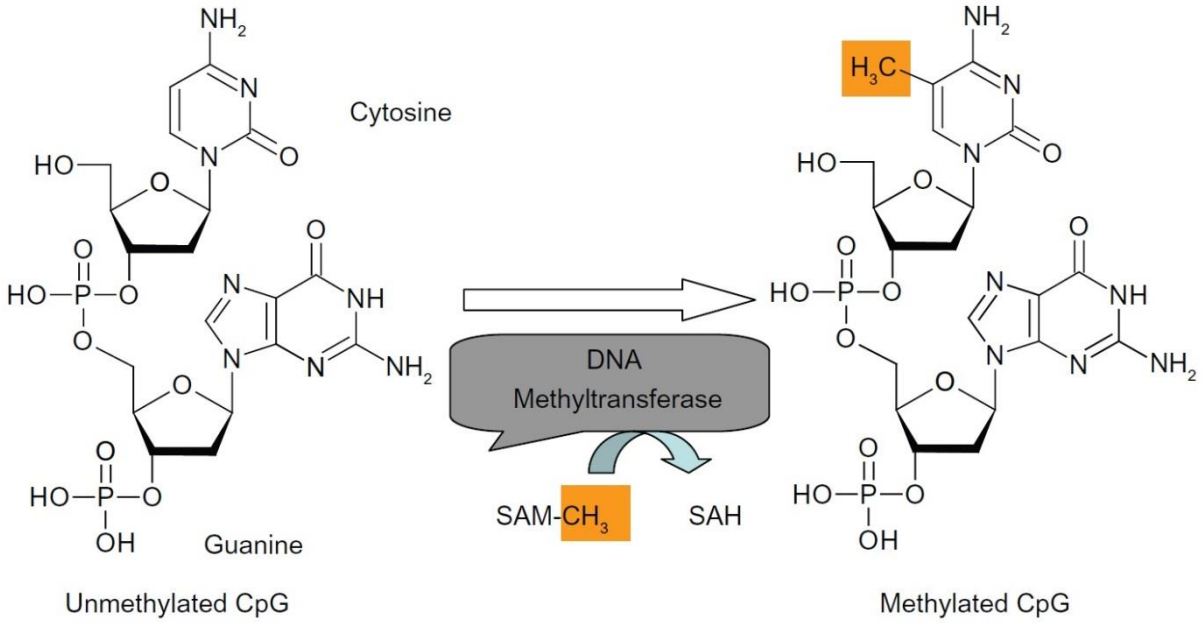

Figure 2: Methylation of cytosine catalyzed by DNA methyltransferases.
A cytosine (top left) in a CpG context can be methylated at the carbon 5 position by DNA methyltransferases to form 5-methylcytosine (top right). DNA methyltransferases require S-Adenosyl methionine as methyl group donor (center). From Ahmed, H., Biomark Cancer, 2010 [71].

Decreased gene transcription through methylated cytosines is mediated by several pathways: First, the addition of a methyl group in transcription factor binding sites can act as steric hindrance [75]. Second, and more importantly, proteins with methyl-CpG-binding domains (MBD) can bind to methylated DNA regions and actively promote a heterochromatin state, modify histones by deacetylation and coordinate *de novo* DNA methylation by recruiting DNMTs [76].

It has been found that the position of the methylated cytosine in respect to the gene location plays an important role. It is widely accepted that DNA methylation in the promoter region of a gene leads to less expression, whereas methylation inside the gene body can lead to enhanced expression of the respective gene [77]. Although methylation in promoter-adjacent regions has proven to be functionally important, further studies are needed to evaluate its role [66].

Since DNA methylation is a reversible process, methyl groups can be removed from cytosines. This process can occur either passively, by missing re-methylating of the DNA during replication by DNMT1, or actively catalyzed by enzymes [78]. Ten-eleven translocation (TET) proteins play a major role in this process: They catalyze the oxidation of 5-methylcytosine, leading to 5-hydroxymethylcytosine (5hmC). Multiple pathways have been shown to contribute to the further demethylation pathway. For example, 5hmC can be directly deaminated enzymatically, forming unmodified

cytosine [79]. It can also be iteratively oxidized by TET enzymes, leading to 5-formylcytosine and 5-carboxylcytosine [80], which in turn can be excised from the DNA [81]. However, only little is known about the regulation of the active demethylation process and how specific loci are demethylated, requiring further investigation.

1.4.2 Factors driving differential DNA methylation

Differential DNA methylation is driven by several factors. The main focus of many studies is to find disease-related differential methylation by comparing a disease-carrying cohort with a healthy cohort.

However, there are several confounding factors which can result in differential DNA methylation and are not always addressed in scientific studies [65, 82]. First, DNA methylation is tissue-specific, meaning that tissue from different origin can be differentially methylated [83]. Second, even within a certain tissue, DNA methylation is still cell type-specific, meaning that variations in cellular composition of samples can confound the analysis of DNA methylation. This is especially and unfortunately true for whole blood, which is commonly used for DNA methylation studies [84].

Third, it is well known that ethnicity [85], sex [86] and age [87] influence DNA methylation. Most obvious differences between males and females include, for example, that only females show hypermethylation of gene promoters on the X chromosome [88]. Also, higher age was found to be correlated with decreased DNA methylation [89]. Further, numerous studies show that nicotine consumption can also have a strong impact on DNA methylation. In fact, the association of smoking and DNA methylation is one of the most studied relations in epigenetic studies and wide-spread changes in the DNA methylation patterns due to smoking have been shown, which were partly reversed after cessation [90]. A study by Besingi and Johansson suggests that differential DNA methylation is not caused by the basic components of tobacco, but from its burnt products, such as arsenic [91].

When conducting DNA methylation studies, these factors should all be taken into account and matched to the best of one's ability to avoid confounding effects. Since high-throughput methods such as Illumina's 450K beadchip array have become popular in recent years, additional caution is necessary to avoid technical variability, such as batch and chip effects.

1.5 DNA methylation and alcohol dependence

Several publications show a major impact of AD on DNA methylation. While candidate-gene driven studies focus on specific sites or genes, epigenome-wide association studies (EWAS) present a hypothesis-free approach to measure a large number of single CpG sites at once, depending on the method and platform used. However, studies so far have found partly conflicting results.

1.5.1 Global DNA methylation studies

Several studies have examined global DNA methylation in AD patients. Within these, three different methodologies were used: I) Endonuclease digestion, II) pyrosequencing of Alu and / or LINE-1 and III) microarray assays following methylated DNA immunoprecipitation. The method most commonly used, pyrosequencing of Alu and / or LINE-1, enables to estimate global DNA methylation by sequencing repetitive elements [92]. LINE-1 (long interspersed nuclear elements) belongs to the family of transposable elements and comprises about 17% of the human genome [93]. Similarly, Alu, which belongs to the group of small interspersed nuclear elements, is the most abundant transposable element with over 1 million copies throughout the genome [94]. Studies using this method have found conflicting results: One study found global DNA hypermethylation in AD patients [95], another one an inverse correlation of higher alcohol consumption resulting in lower global DNA methylation [96]. A third study did not find any effect of AD on global DNA methylation [85]. Earlier studies hypothesized that due to the higher homocysteine levels in AD patients, global DNA methylation patterns should be elevated [97]. However, a study comprising a large cohort did not confirm these findings [98]. Another hypothesis postulates that global DNA hypomethylation in AD patients is attributed to the lack of methionine adenosyl transferase regulation [99, 100]. These controversial results lead to the conclusion that further studies are needed to understand the impact of AD on global DNA methylation.

1.5.2 Candidate-gene driven studies

Taken together, 12 candidate genes have been studied for their associations with AD or alcohol abuse: *ANP*, *AVP*, *GRIN2B*, *HERP*, *MAOA*, *OPRM1*, *Orexin A*, *POMC*, *SLC6A3*, *SLC6A4*, *SNCA* [29] and *GDAP1* as part of this work [101]. The most

prominently studied gene, *SLC6A3*, the solute carrier family 6 member 3 encodes for a dopamine transporter and was analyzed in four different studies. Two studies showed hypermethylation in the promoter region of this gene [102, 103], while another two studies could not detect any differences between controls and AD patients [104, 105]. The methylation status of *GRIN2B* [106], *POMC* [107] and *MAOA* [108] did not show significant changes, but correlated with symptoms of AD such as craving or symptom counts. The majority of the analyzed genes exert functions in neurotransmission.

All the above-mentioned studies have several limitations: A broad variety of different methodologies (i.e. RT-PCR, pyrosequencing, bisulfite sequencing, mass spectrometry etc.) were used, hindering comparability. Additionally, the study material in most cases was peripheral whole blood, which is not optimal due to its multi-cellular composition. Also, not all studies had closely matched patient and control groups. As discussed earlier, this is of paramount importance to gain meaningful results which are not confounded by factors such as age, sex, ethnicity and smoking behavior. Furthermore, not all studies were able to validate or replicate their results. This concludes to the fact that more work and, most of all, closely-matched cohorts are needed to clearly decipher the implication and role of these genes in AD as learning more about these genes may also lead to a better understanding of AD. In addition, a suitable epigenetic biomarker for the disease is missing to date. In clinical settings, biomarkers could facilitate and improve treatment tremendously. However, the mentioned studies were not able to successfully introduce a novel epigenetic biomarker for AD. To circumvent the above-mentioned limitations and to have a hypothesis-free approach, in this work we conducted an EWAS aiming to detect novel differentially methylated sites and regions.

1.5.3 Epigenome-wide association studies

In addition to candidate-gene driven studies, a few EWAS have been conducted to date and have found widespread AD-associated DNAm differences. Differential methylation at 1,710 sites in lymphocytes of alcohol dependent patients [109] as well as 77 differentially methylated regions (DMRs) associated with 62 genes were identified in whole blood samples from discordant monozygotic twin pairs [110]. An additional genome-wide study using postmortem precuneus brain tissue samples

identified a total of 1,046 differentially methylated sites in AD patients compared to healthy controls, of which 432 displayed significant cross-tissue correlation between matched brain and buccal samples [111].

Philibert and colleagues were the first to assess DNA methylation alterations in peripheral blood mononuclear cells (PBMCs) of AD patients before and after participating in a short-term alcohol treatment program [112]. Comparing patients before treatment to healthy controls, they reported differential methylation at 56 sites. Although no significant DNAm differences were observed in patients pre- and post-treatment, 49 of the 56 differential sites reverted to levels similar to controls in patients after treatment [112]. However, this study had a few limitations: Both male and female study participants were included, as well as participants with a different ethnic background. More severely, smoking behavior between patients and controls was not matched. As pointed out earlier, matching patient and control groups is essential, since only a closely matched cohort will reveal results that represent changes specific to the investigated disease.

Although all these previous EWAS identified a multitude of AD-associated differentially methylated sites, they did not account for cell type heterogeneity in their analyses, thereby confounding and limiting the comparability of their findings. Most recently, a study involving 13,317 participants from 13 distinct cohorts reported hundreds of AD-associated differentially methylated sites in DNAm profiles of monocytes and whole blood, which was adjusted for cell composition [113]. This study was the first one to account for cellular heterogeneity in whole blood samples and found a large number of differentially methylated genes and regions, including the γ-Aminobutyric acid-A receptor delta and γ-aminobutyric acid B receptor subunit 1. The limited amount of the studies and their controversial results show the need for more research in this field. Additionally, validation and replication of previous findings is of great importance.

1.6 DNA methylation and borderline personality disorder

Several studies have shown an association between BPD and differential DNA methylation of candidate genes. Using pyrosequencing, a study found differential DNA methylation in *HTR2A*, *NR3C1*, *MAOA*, *MAOB* and soluble *COMT* (*S-COMT*). However, the observed effects were comparatively small, ranging from 0.8% (5-

hydroxytryptamine receptor 2A, *HTR2A*) to 1.8% methylation difference in the glucocorticoid receptor gene *NR3C1* [30]. Another study confirmed the association of BPD with the glucocorticoid receptor gene and found that childhood abuse and its severity positively correlated with *NR3C1* methylation [114]. In addition, differential DNA methylation of the second subunit of the serotonin receptor gene (*HTR3A*) [115], as well as hypermethylation of the brain-derived neurotrophic factor (*BDNF*) gene were found to be associated with BPD [116]. Most recently, an association between BPD and the DNA methylation of *rDNA* and *PRIMA1* was shown [117].

Only one EWAS has been conducted for BPD so far: Comparing a cohort of 24 female BPD patients and 11 healthy controls, Teschler and colleagues found *APBA2*, *APBA3*, *KCNQ1*, *MCF2* and *NINJ2* to be differentially methylated and could validate the findings of *APBA3*, *MCF2* and *NINJ2* by pyrosequencing [31].

As with the previously mentioned findings for AD, a suitable biomarker measuring treatment success has not yet been found. Since BPD treatment is very time intensive, finding a predictive biomarker for treatment outcome would greatly benefit both patients and clinics.

2 Aim of the study

Although the role of DNA methylation and other epigenetic markers in alcohol dependence (AD) and borderline personality disorders (BPD) has been investigated in a number of studies, these had several limitations and many unanswered questions remain. Previous studies did not have closely matched patient and control cohorts. Furthermore, DNA methylation levels reported in previous studies were examined in different tissue and cell types, thereby adding confounding factors and limiting comparability. In addition, changes from pre- to post-treatment were investigated in only one study each for AD [112] and BPD combined with dialectic behavior therapy [116].

The aim of this work is to identify epigenetically altered genes associated with these two disorders. For the study of AD and early recovery, we performed an EWAS of $CD3^+$ T cell DNA methylation of 23 healthy controls compared to 24 alcohol dependent patients on Illumina's HumanMethylation 450K beadchip array platform. We thereby emphasized on matching our groups to the best of our ability to exclude confounding factors. Furthermore, we compared the 24 patients before and after a 3-week alcohol treatment program at the Department of Psychiatry and Psychotherapy in Tuebingen to identify associations related to early recovery of AD. In contrast to the majority of previous studies, we validated our top-ranked hits by pyrosequencing and replicated the top findings from our EWAS analyses in T cell DNA in a second, independent cohort comprising 12 controls and 13 AD patients (replication cohort). To investigate the potential usage as novel biomarkers, we additionally partially validated and replicated top-ranking hits in DNA derived from whole blood.

In BPD, various genes have been found to be differentially methylated. However, their value as predictive epigenetic biomarker has not been assessed so far. Here, we aimed to verify previous results and investigate the predictive role of DNA methylation levels of *APBA3*, *MCF2* and *NINJ2* on therapy outcome in BPD patients. Therefore, we compared 44 BPD patients to 44 controls, who were matched for sex and age. To identify treatment-associated alterations in DNA methylation and the use as novel predictive epigenetic biomarker, we compared patients responding to DBT to non-responders pre- and posttreatment.

3 Results and Discussion

3.1 Study cohorts

3.1.1 Alcohol dependence cohorts

Three cohorts have been used in this work. The EWAS analysis was conducted in the discovery cohort (Table 2) and the EWAS results were validated in the replication cohort (Table 3). Replication of *GDAP1* DNA methylation took place in parts of the above-mentioned cohorts as well as additionally recruited individuals (Table 4). We thereby emphasized on matching as closely as possible for the following aspects: First, we only included male participants in our study. Second, all participants were Caucasian, with all subjects from German descent except one Polish and one Russian participant. Third, since smoking has a vast impact on DNA methylation as mentioned before, we matched the percentage of smokers in each cohort, and within the smokers, we matched the amount of consumed nicotine as closely as possible.

Table 2: Description of the discovery alcohol dependence cohort.
Amount of drinks consumed daily derived from the AUDIT-questionnaire. *P*-values were computed using independent samples *t*-tests. Errors are given as standard deviation (SD).

	Patients	Controls	*P*-value
N	24	23	
Age (years)	47.5 ± 10.1	46.9 ± 10.3	0.8
Smokers (% of total)	19 (79%)	18 (78%)	0.9
Cigarettes smoked daily	15.2 ± 10.7	13.8 ± 12.6	0.7
AUDIT-score	24 ± 6.5	5.9 ± 3.8	4E-15
Days since last drink	1.2 ± 0.6		
Amount of drinks consumed daily in the week before hospital admission	13.7 ± 8.3		
Years of alcohol dependence	10.6 ± 9.4		

Table 3: Description of the replication alcohol dependence cohort.
Amount of drinks consumed daily derived from the AUDIT-questionnaire. *P*-values were computed using independent samples *t*-tests. Errors are given as standard deviation (SD).

	Patients	Controls	*P*-value
N	13	12	
Age (years)	50.9 ± 9.1	45.3 ± 16.2	0.4
Smokers (% of total)	9 (69%)	8 (67%)	0.9
Cigarettes smoked daily	10.5 ± 9.4	8.9 ± 8.0	0.7
AUDIT-score	28.0 ± 4.9	2.8 ± 2.3	3E-14
Days since last drink	0.3 ± 0.4		
Amount of drinks consumed daily in the week before hospital admission	19 ± 11.4		
Years of alcohol dependence	14.6 ± 11.7		

Table 4: Description of the *GDAP1* alcohol dependence cohort.
Amount of drinks consumed daily derived from the AUDIT-questionnaire. *P*-values were computed using independent samples *t*-tests. Errors are given as standard deviation (SD).

	Patients	Controls	*P*-value
N	49	37	
Age (years)	49 ± 10.47	47 ± 12.32	0.3
Smokers (% of total)	38 (79%)	29 (78%)	0.9
Cigarettes smoked daily	20 ± 10.93	16 ± 10.99	0.2
AUDIT-score	25.1 ± 6.1	4.9 ± 3.7	5E-15
Days since last drink	2.9 ± 6.9		
Amount of drinks consumed daily in the week before hospital admission	17 ± 13.1		
Years of alcohol dependence	12.3 ± 9.9		

3.1.2 Psychological effects of the alcohol treatment program

In order to quickly assess the impact of the alcohol treatment program on the psychological wellbeing, patients answered the SCL-90-R questionnaire pre- and posttreatment, reflecting changes in their global distress level (GSI score). Additionally, to investigate the influence of the alcohol treatment program on alcohol craving, we assessed the OCDS score. The OCDS score was calculated as previously described [118]. In the *GDAP1* cohort, calculation was adjusted by dividing

the score by the number of answered items, to allow up to two unanswered items without having an effect on the final score (Table 5 b).

Table 5: Pre- and posttreatment comparison of the discovery and *GDAP1* cohorts.
P-values were computed using paired-samples *t*-tests. Errors are given as standard deviation (SD).
* OCDS score was divided by the number of answered items.

a) Discovery cohort	Patients (T1)	Patients (T2)	*P*-value
N	24	24	
GSI score	0.72 ± 0.45	0.41 ± 0.52	0.036
OCDS score	19.3 ± 6.6	12.0 ± 4.9	3E-05
b) *GDAP1* cohort			
N	49	33	
GSI score	0.78 ± 0.54	0.48 ± 0.49	0.008
OCDS score*	3.93 ± 1.32	2.71 ± 0.96	2E-05

We found that the alcohol treatment program significantly reduced the global distress level of the patients as well as the craving for alcohol (Table 5), indicating that the alcohol treatment has a positive impact on the general well-being of the patients. To date, psychological questionnaires are the means of choice when evaluating treatment outcome. However, this measure is biased by the subjective well-being of the individual subjects and does not reflect an objective measure. Finding a biomarker measuring surrogate endpoints would circumvent this limitation and is a major field of current research.

3.1.3 BPD cohort

The BPD cohort consisted of 44 BPD patients and 44 age- and sex-matched healthy controls (Table 6). This BPD study cohort has been reported as part of a previous thesis [119].

Table 6: Description of the BPD cohort.
P-values were computed using independent samples *t*-tests. Errors are given as standard deviation (SD).

	Patients	Controls	*P*-value
N (male / female)	44 (7 / 37)	44 (7 / 37)	
Age (years)	29.5 ± 8.4	29.7 ± 8.8	
GSI t-score	79.00	44.73	2E-16
BSL23 score	2.42	0.22	5E-15

3.2 Epigenetic dysregulation in alcohol dependence

We performed an EWAS analysis on Illumina's 450K beadchip array. Raw values acquired by Illumina's GenomeStudio were subject to preprocessing and cell type deconvolution, as described in the following paragraph.

3.2.1 Preprocessing

Preprocessing of probes is an essential step to improve the quality of the acquired data. We therefore removed single-nucleotide polymorphism (SNP) probes, probes with missing beta value or bad detection *P*-value and polymorphic (cross-hybridizing) probes (Table 7).

Table 7: Preprocessing of the 450K raw data.

Preprocessing step	Probes	Probe number left
Initial probes		485,577
SNP probes	65	485,512
Missing beta value / bad detection *P*-value	13,903	471,609
Polymorphic probes	19,343	452,266
Final dataset		452,266

After this initial processing, the data was normalized using the Subset-quantile Within Array Normalization (SWAN [120]), normalizing the technical differences between probe types on the 450K beadchip array and ComBat [121], removing batch effects such as chip and position-on-chip effects.

A key rationale of our study was to restrict our DNA methylation analysis to T cells, which are directly influenced by AD, to eliminate cell type heterogeneity and avoiding post-analytical bioinformatic removal of it. Although we purified T cells from our samples with affinity binding of the CD3 receptor via magnetic beads, we additionally checked for residual cell contamination using a bioinformatics approach described in a previous publication [122]. The analysis showed contamination of the purified T cell samples especially with natural killer (NK) cells. We aimed to verify these results experimentally by conducting a fluorescence-activated cell sorting (FACS) analysis. Because the magnetic beads used to purify T cells interfered with the fluorescence measurements, we detached the cells from the beads which led to a decrease in their viability. Further, only 15% of the bead-detached cells showed a positive signal for CD3, if analyzed immediately. However, when incubating the cells for 16 hours after detachment, 90% showed a positive CD3 signal. Additional analysis of the cells by size and granularity confirmed that 92% of the viable cells were T cells. Presumably, the initially measured low CD3 signal was a result of CD3 receptor internalization after binding to the magnetic beads, while the receptor was recycled back to the cell surface after 16 hours of incubation [123].

Since these results were in accordance with the bioinformatic analysis, which predicted an average non-T cell contamination of 5%, we bioinformatically removed contaminating cell types resulting in the final dataset that was used for further analysis. The very low discrepancy of less than 5% between the bioinformatics results and the experimental FACS results makes the data plausible and the residual discrepancy could potentially have been eliminated if viability of the cells after 16 hours had been improved, thus measuring a greater number of cells.

3.2.2 Global DNA methylation

To assess the genome-wide influence of AD on DNA methylation, we calculated global DNA methylation as the average of DNA methylation values across all interrogated sites in each sample. We found that patients had a lower mean global DNA methylation before treatment as compared to controls. However, after the treatment, global DNA methylation values were higher and did not differ significantly from controls any more (Figure 3).

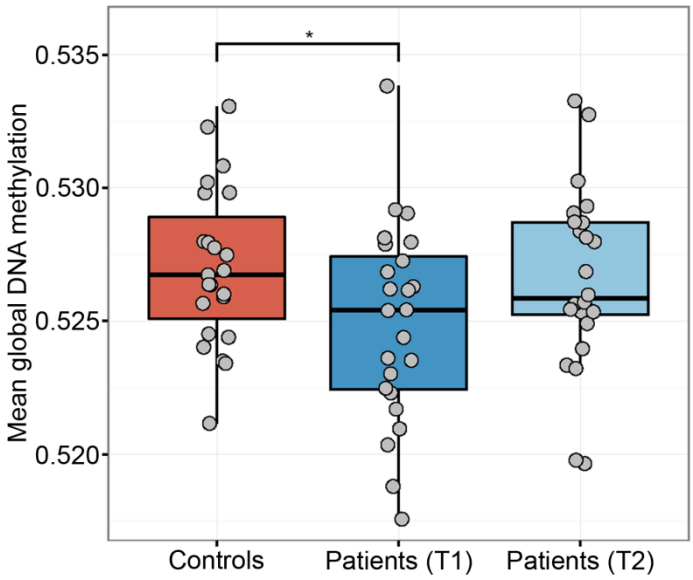

Figure 3: Global DNA methylation levels.
Patients pre-treatment (T1) show significantly lower global DNA methylation compared to controls (*P* = 0.048, Mann-Whitney U test). After the treatment (T2), mean DNA methylation did not differ from the controls any more.

The fact that AD can lead to global DNA methylation changes has been shown previously, however, the results were conflicting. While some studies showed a global hypermethylation [124, 125] others found global hypomethylation [96, 99, 100, 113]. Additionally, the reversal of DNA methylation to control-like levels after treatment was also found by Philibert *et al.* [112].

Initially, global hypermethylation in AD patients was explained by the elevation of homocysteine as a result of alcohol consumption. It was hypothesized that through the higher levels of homocysteine, which is hydrolyzed to methionine and in a further step converted to SAM, more methyl group donors would form and subsequently would stimulate DNA methylation [125]. However, a more recent, extensive study could not find an association between elevated homocysteine levels and global DNA methylation [98]. Contrarily, another hypothesis states that due to alcohol consumption, regulation of methionine adenosyl transferase is impaired and, therefore, global DNA methylation levels decrease [99, 100]. In summary, little is known about the exact relationship between altered homocysteine levels and DNA methylation and, up to now, results often are contradictory [126]. Since we did not measure homocysteine levels or methionine adenosyl transferase activity, we can neither support nor refute a certain theory.

3.2.3 Site-specific analysis results

Two parameters can be adjusted in analyses of high-throughput experiments such as an EWAS. First, *P*-Values have to be adjusted for multiple testing. We adjusted our dataset applying the Benjamini-Hochberg false discovery rate (FDR) method [127]. However, considering the small sample size, we adjusted the significance level to FDR < 0.1, as done in several other studies [128, 129]. With a more stringent FDR, relevant sites could potentially be dismissed in a very early step of the analysis. Second, to compensate for the higher FDR value, we aimed to increase the likelihood of finding biological relevant hits. Therefore, we only analyzed hits with a DNA methylation difference (∆-beta) of more than 5% between groups. Table 8 displays the number of hits for different thresholds of these two parameters in our dataset.

Table 8: Number of significant hits at different FDR and ∆-beta thresholds.

	FDR < 0.2	FDR < 0.1	FDR < 0.05
∆-beta > 0	17,342	1210	47
∆-beta > 0.05	305	59	5
∆-beta > 0.1	17	4	0

First, we compared DNA methylation between controls and patients before treatment, and found 59 CpG sites to be differentially methylated (Table S 1).

Second, to investigate the effect of the alcohol treatment program, we compared DNA methylation between patients pre- and post-treatment and found 48 CpG sites differentially methylated, which were all less methylated before treatment (Table S 2).

Our top-ranked hit *HECW2* from the comparison of controls and patients encodes a HECT-type E3 ubiquitin ligase which is involved in the cellular stress response [130, 131]. This finding is in line with previous evidence for the role of epigenetic regulation of cellular stress response genes in AD, such as *GDAP1*, which was identified in a previous EWAS [112] and replicated in whole blood samples as part of this thesis. Another top hit, *SRPK3*, encodes a serine/arginine protein kinase and plays an important role in the development of the skeletal muscle [132]. However, its homolog *SRPK79D* in *Drosophila melanogaster* plays an important role in the function of synapses [133]. The high amino acid homology between SRPK79D and SRPK3 of

65% might indicate a role of SRPK3 in the nervous system, which has not yet been determined.

Further hits from our analysis have been associated with AD. For example, LIPA, the Lysosomal Acid Lipase A, has been shown to have a lower activity in non-alcoholic fatty liver disease [134]. In addition, TNFSF10 and SKIL are associated with different types of cancer, which is a common comorbidity in AD patients. TNFSF10 is associated with hepatocellular carcinomas [135] and SKIL can act as tumor suppressor [136]. Since the fourth-ranked hit, cg07280807, is in an intergenic area, and the nearest gene (*SMOC1*) is approximately 30,000 base pairs upstream, we additionally performed a motif analysis with JASPAR [137] for this site. The motif analysis revealed putative binding sites for JunB, Fos, FosL1 and Fos:Jun. Fos and Jun can form the activator protein 1 (AP-1) complex, which has been linked to ethanol intake and withdrawal in animal models [138, 139]. Many other hits in the site-specific analysis are sites and genes, which have not been associated with AD before. In addition, we did not find overlapping hits to previous EWAS. However, since these studies have used different cell types for their analyses, different cohorts and different study designs, this was not surprising. Furthermore, for these reasons, EWAS published to date do not show substantial overlapping hits.

It is not surprising that top hits are within the biological processes of cellular stress response (i.e. *HECW2*) or generally related to cancer (i.e. *TNFSF10*, *SKIL*), since both fields play a major role in AD. First, a recent meta-analysis showed that AD can cause cancer at not less than seven sites in the body [140]. Second, cellular stress response is essential to counteract ethanol intake and its toxic effects. In the study by Liu *et al.*, stress response was also one of the top enriched processes [113]. However, gene ontology enrichment analysis of our own study did not show significantly enriched pathways, which is most likely caused by the low number of significant hits.

3.2.4 Regional analysis results

Although it was shown that single CpG sites in promoter regions can impact gene expression, DMRs can control cell-type-specific transcriptional repression of an associated gene more effectively [141]. To identify entire regions spanning several single CpG sites which are differentially methylated, we performed DMRcate

analyses and found 29 DMRs between controls and patients pre-treatment (Table S 3). Interestingly, *SRPK3* was found differentially methylated in both the site-specific and regional analysis, indicating that *SRPK3* is a robust hit in our analyses. Between patients pre- and post-treatment, we did not identify significant DMRs. Presumably, three weeks of treatment were not sufficient to significantly alter the methylation of an entire region.

3.2.5 Post-treatment reversion of differential DNA methylation

Global DNA methylation analysis already showed that mean methylation levels across all sites no longer differed from the controls' levels after alcohol treatment (Figure 3). This was confirmed by the majority of the hits from the site-specific analysis: 7 out of 59 sites showed a complete reversion of DNA methylation to controls' levels. Another 32 sites showed a trend to revert back, although not statistically significant. The DNA methylation of only 20 sites did not change from pre- to posttreatment. This phenomenon of reverting DNA methylation following treatment has been observed previously in the study of Philibert and colleagues. They found that 49 out of 56 significant sites showed reversal of DNA methylation after short-term alcohol treatment [112]. Additionally, other studies showed similar effects after a particular treatment, such as after one month of anti-TNF-α treatment in psoriatic patients [142] or in-vitro treatment of different cancer cell lines with polyphenols [143]. The reversal of DNA methylation and the accompanying change in gene expression could be one key mediator of recovery.

3.2.6 Validation

As reviewed by Mill *et al.* [144], high-throughput experiments such as the 450K beadchip array are prone to false-positive (type I error) results and, therefore, ought to be validated. As described earlier, decreasing costs of high-throughput assays led to massive replication studies of GWAS experiments, often not being able to validate initial candidate-gene driven study findings. In general, validation should be carried out using a different methodology to avoid errors specific to a certain technique. Therefore, we validated our top-ranked hits cg18752527 (*HECW2*), cg07280807 (intergenic), cg16529483 and cg24496423 (both *SRPK3*) by pyrosequencing in the discovery cohort. We found all four sites to be differentially methylated between

patients and controls with a high correlation between the measurements of the different methodologies (Table 9).

3.2.7 Replication

Although we already took several precautions to avoid false positive findings in our EWAS analyses as described before, we additionally replicated our four top-ranked hits, which we successfully validated in the discovery cohort. Therefore, we assessed methylation levels for these sites by pyrosequencing in an independent cohort comprised of 13 AD patients and 12 controls. We found that the intergenic CpG site cg07280807 and cg18752527 (*HECW2*) were also differentially methylated in this cohort (one-sided *t*-test, FDR < 0.05, Table 9).

However, although cg16529483 and cg24496423 in the *SRPK3* gene were higher methylated in patients of our replication cohort, the differences were not significant (Table 9). Although the differences in DNA methylation observed in the replication cohort still had the same direction of change, the extent of methylation differences varied: cg24496423 in the *SRPK3* gene, which had the second biggest difference in the discovery cohort, did not replicate, whereas *HECW2*, whose DNA methylation difference was smaller in the discovery cohort, did. Presumably, the replication cohort with only 25 subjects was too small to achieve statistically robust results.

Table 9: Overview of validation and replication results.
Successful validation or replication is marked with a (+), whereas sites failing to validate or replicate are marked with a (–). * FDR-corrected *P*-Values between patients and controls; Validation, Welch's *t*-test; Replication, one-sided *t*-test.

Site (gene)	Validation (*P*-Value*)	Replication (*P*-Value*)
cg07280807 (intergenic)	+ (1.5E-03)	+ (0.048)
cg18752527 (*HECW2*)	+ (2.7E-06)	+ (0.048)
cg16529483 (*SRPK3*)	+ (1.2E-03)	– (0.183)
cg18752527 (*SRPK3*)	+ (1.6E-03)	– (0.298)

3.2.8 Whole-blood replication

Since one aim of this work was to identify differentially methylated sites that could potentially be used as biomarkers in clinical settings, we aimed to replicate the above-mentioned top-ranked hits in DNA derived from whole blood. In order to serve

as a biomarker, the study material should be easy and cost effective to obtain. This is not applicable for purified T cells, as the purification process is time consuming and cost intensive. Furthermore, T cells can only be purified from freshly drawn blood since freezing the specimen would damage the cells and cause cell death leading to the inability to recover a particular cell type. Finding differentially methylated sites in DNA derived from whole blood would therefore be extremely useful, since immediate processing is not necessary and purification of T cells could be omitted.

We found significant correlations between DNA methylation levels of cg07280807 from T cells and whole blood, both measured by pyrosequencing in the discovery and replication cohort, but we could not replicate the differential methylation between the patient and control group in whole blood. However, as in the EWAS analysis, patients had higher methylation values at this site, though not statistically significant. Potentially, differential DNA methylation of cg07280807 is unique to T cells, and not shared by other blood cells.

Still, we were able to show differential *HECW2* and *SRPK3* DNA methylation in whole blood, implicating their use as potential novel biomarkers for AD. However, since this is the first study to find these two genes associated with AD, other studies have to verify these results. Because biomarkers should be easily and widely applicable, future studies should also investigate *HECW2* and *SRPK3* DNA methylation in different ethnic groups, since we restricted our analyses to Caucasian subjects.

3.2.9 Replication of differential *GDAP1* DNA methylation

GDAP1 was one top-ranked differentially methylated gene in the study by Philibert and colleagues [112]. However, they neither validated nor replicated their findings. Since the study design showed some similarity to ours, i.e. investigating AD and the effects of short-term alcohol treatment, we sought to reproduce this finding in whole blood DNA of our *GDAP1* alcohol dependence cohort, which was comprised of 49 patients and 37 controls. We found that 3 closely spaced CpG sites in the *GDAP1* gene promoter region, including cg23779890, previously reported by Philibert *et al.*, were lower methylated in patients compared to controls. Similarly to the findings of Philibert and to our EWAS analyses, we again found that the DNA methylation differences were reversible by the alcohol treatment: After three weeks of attending

the alcohol treatment program, DNA methylation reverted back to a level where it no longer differed significantly from controls.

However, *GDAP1* did not appear as one of the top hits in our previously described EWAS analysis using DNA isolated from purified T cells. Presumably, the differential methylation of *GDAP1* in whole blood is driven by another cell type than T cells, which could explain why Philibert and colleagues found *GDAP1* differentially methylated in PBMCs and we found it in whole blood, but not in T cells.

3.2.10 *SRPK3* and *GDAP1* gene expression in whole blood

To elucidate the effect of differential *SRPK3* and *GDAP1* DNA methylation on the gene expression of the respective genes, we performed qPCR of whole blood mRNA samples from the cohort used for analyzing *GDAP1* [145]. While the gene expression levels of *GDAP1* did not differ, *SRPK3* showed lowered gene expression in patients pre-treatment by one fourth compared to controls, though the effect was not statistically significant (relative quantification: controls = 1, patients (T1) = 0.75, P = 0.3, Figure 4). Post-treatment, gene expression levels were elevated again and were similar to those of controls (relative quantification: controls = 1, patients (T2) = 1.11, P = 0.8, Figure 4).

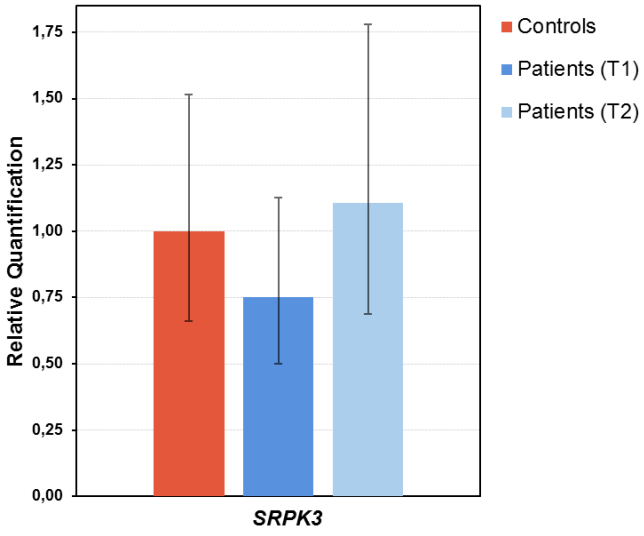

Figure 4: Gene expression of SRPK3.
In comparison to controls, whose expression levels were set to 1, expression levels of patients (T1) were 0.75 (P = 0.3) and patients (T2) were 1.11 (P = 0.8). Error bars depict min-max values. From Gräf Olmos, V. Bachelor thesis, 2017 [145].

GDAP1 DNA methylation was already very low, ranging from 2 to 8%, and the difference between controls and patients was only 1.2%. Therefore, it was not

surprising that we could not detect an altered gene expression. The findings relating *SRPK3* are in accordance to the methylation data, as we expected lower gene expression due to the higher methylation levels in patients pre-treatment compared to healthy controls. Apart from the small effect size, the overall very low expression of SRPK3 we found in whole blood could explain that the results were not statistically significant. However, our finding is consistent with the data from the Expression Atlas by the European Molecular Biology Laboratory [146], where SRPK3 expression in whole blood was below cut-off measured with an Affymetrix microarray [147].

3.2.11 Conclusion & Outlook

In conclusion, we showed differential methylation of various genes associated with AD in disease-relevant T cells. In addition, we found a unique set of genes differentially methylated in patients pre- and posttreatment, implicating their potential role in alcohol abstinence and early recovery. Furthermore, we showed that alcohol dependent patients exhibit global DNA hypomethylation which reverts back after 3 weeks of attending an alcohol treatment program. This data could contribute to the identification of genes playing an important role in AD. To strengthen the results acquired with Illumina's 450K beadchip array, we validated our top-ranked hits and replicated them in an independent cohort.

Our finding of differential methylation of *HECW2* and *SRPK3* in whole blood DNA indicates their potential value as novel blood-based biomarkers for AD. By showing differential *SRPK3* DNA methylation and altered gene expression levels as shown by lower SRPK3 mRNA levels (although not statistically significant), we substantiate the potential use of *SRPK3* as biomarker.

However, the explanatory power of our study is limited by the following factors: First, the cohort size was limited and should be increased in subsequent studies. Second, despite all the advantages, the 450K beadchip array itself has some limitations. Some of them, such as cross-hybridization of certain probes, SNPs within probes and technical variation (batch effects), have been mostly solved using bioinformatic approaches. In addition, the array only covers around 2% of CpG sites in the entire genome [148] and has a great bias for CpG sites within promoter regions, thereby missing potentially important sites, which are not interrogated. At the time of creation, this seemed meaningful, since promoter CpG sites were well studied and their impact

on gene expression was shown in several studies. However, more recent work supports the importance of non-promoter CpG methylation such as in enhancer regions. For this reason, a newer beadchip array (850K, EPIC) was recently developed, adding over 330,000 sites in these regions [149]. Third, we cannot generally rule out that the effects we observed between pre- and posttreatment derived from stochastic temporal variation rather than from alcohol treatment. However, accounting for this in a controlled way is nearly impossible, since controls would have to adjust their entire lifestyle, including physical activity and nutrition, according to the matched patient. In addition, previous studies showed no big influence of temporal variation on the methylation levels interrogated by the 450K beadchip array [150].

Since AD is an addiction, it is meaningful to study effects of AD in brain tissue. However, it is not possible to gain this tissue from living study subjects. The most commonly used approach is to study whole blood DNA methylation as easily obtainable surrogate measure. However, as discussed earlier, methylation profiles between whole blood and brain tissue can vary substantially. Another approach is to indeed use brain tissue of post-mortem samples. Here, study material is drastically limited and, therefore, matching sufficiently sized cohorts is considerably impeded.

Since this is the first study to assess differential methylation levels in T cells from AD patient and we present unique and novel sets of differentially methylated sites and regions, further studies are needed to additionally validate our results. The primary focus should be on the evaluation of *HECW2* and *SRPK3* DNA methylation, since these are the most promising candidates for a novel biomarker. However, future functional studies should also elucidate possible mechanisms of how these genes contribute to the onset or recovery of AD. First implications have been discussed in this work and should be followed up.

3.3 Epigenetic dysregulation in BPD

3.3.1 Comparison of controls and patients

Due to the small number of studies concerning BPD and epigenetics so far, more work is needed to elucidate potential associations. We therefore analyzed methylation levels of *APBA3* and *MCF2* by pyrosequencing in the BPD cohort comprised of 44 patients and 44 controls, seeking to validate and replicate the findings of previous work [31] and, more importantly, to investigate the effect of DBT treatment on DNA methylation. Neither *APBA3,* nor *MCF2* were differentially methylated between controls and patients. To investigate if DBT had an effect on DNA methylation, we additionally compared 24 patients before and after DBT intervention. However, we did not find significant differences in this comparison either. Since we were not able to obtain a PCR product of sufficient quality for further analysis, methylation analysis of the *NINJ2* gene was not possible.

3.3.2 Comparison of responders and non-responders

Although DBT intervention has been investigated in several non-epigenetic studies, a predictive biomarker for treatment outcome does not exist to date. To investigate the predictive power of DNA methylation for treatment success, we divided the patients concluding treatment in responders and non-responders. Usually, this classification is based on the reduction of self-harming tendencies, which is the primary intent of DBT [151]. Unfortunately, we did not retrieve this data for our BPD patient cohort. Therefore, we chose two psychological questionnaires as surrogate measures for response or non-response to DBT: Patients were classified as responders if the GSI t-score calculated from the SCL-90-R questionnaire, which reflects psychological well-being, was lowered by at least 5 points. This reflects a moderate decline of the overall psychological burden. In addition, only if the BSL-23 score, measuring BPD symptoms, was below 2.05 after therapy patients were classified as responders. This score was chosen as it was the average score achieved by patients in earlier studies [152]. However, the mean BSL-23 score in our cohort was considerably higher (2.42), which is why we chose to apply a score of 2.05 as cut-off value. This classification led to a total of 7 responders and 17 non-responders.

Comparing responders and non-responders, we found significant differences in both *APBA3* and *MCF2* DNA methylation. Both genes were hypermethylated in

responders, with mean methylation levels being 13% higher than in non-responders for *MCF2*. In addition, we found an inverse correlation between BPD severity and DNA methylation: Higher DNA methylation pre-treatment resulted in lower GSI t-scores after the treatment. Interestingly, after treatment, methylation values of responders and non-responders did not differ significantly any longer.

To date, except the previous study by Teschler *et al.* [31], neither *APBA3* nor *MCF2* have been associated with BPD. However, associations with other psychiatric disorders such as Alzheimers' disease (*APBA3* [153]), schizophrenia and autism-spectrum disorders (*MCF2* [154]) have been described previously, suggesting a possible, but not yet discovered role of these genes in BPD.

3.3.3 Conclusion & Outlook

Here, we aimed to replicate the differential DNA methylation of *APBA3* and *MCF2* in BPD patients, but found no significant differences compared to controls. Additionally, we did not detect any significant influence of DBT on the DNA methylation of those genes by comparing the patients pre- and post-treatment. These results do not support the findings of the study by Teschler *et al.*, who showed aberrant DNA methylation of both *APBA3* and *MCF2* between their BPD and control cohorts [31]. However, we found *APBA3* and *MCF2* to be significantly hypermethylated at the beginning of treatment in patients responding to DBT compared to non-responders. This supports the hypothesis that DNA methylation of *APBA3* and *MCF2* could serve as an epigenetic biomarker with predictive value for DBT therapy outcome in BPD patients.

Compared to the previous study [31], we were able to almost double the number of patients and quadruple the number of controls, gaining higher statistical power and, therefore, an important reduction in the possibility of false-positive findings. Still, especially considering the size of the responder/non-responder cohort, one main aim should be to increase its size in future studies. Additionally, to facilitate and standardize classification, self-harming behavior should be monitored in upcoming studies, since this is the reference measure of response to DBT.

Although associations with other psychiatric diseases have been shown for both genes, the link to BPD is unknown to date. Subsequent studies have to be conducted

to clearly decipher the role of both *ABPA3* and *MCF2* in BPD. Furthermore, their function as predictive biomarkers for BPD should be validated in a larger cohort.

4 References

1. World Health Organization, *Global status report on alcohol and health*. 2014.
2. Statistisches Bundesamt, *Pressemitteilung Nr. 414.* 2016.
3. Britton, A., D. O'Neill, and S. Bell, *Underestimating the Alcohol Content of a Glass of Wine: The Implications for Estimates of Mortality Risk.* Alcohol Alcohol, 2016. **51**(5): p. 609-14.
4. American Psychiatric Association, *Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision*. American Psychiatric Association. 2000, Washington, DC.
5. Agrawal, A. and M.T. Lynskey, *Are there genetic influences on addiction: evidence from family, adoption and twin studies.* Addiction, 2008. **103**(7): p. 1069-81.
6. Heath, A.C., et al., *Genetic and environmental contributions to alcohol dependence risk in a national twin sample: consistency of findings in women and men.* Psychol Med, 1997. **27**(6): p. 1381-96.
7. Agrawal, A., et al., *Evidence for an interaction between age at first drink and genetic influences on DSM-IV alcohol dependence symptoms.* Alcohol Clin Exp Res, 2009. **33**(12): p. 2047-56.
8. Schwandt, M.L., et al., *Alcohol response and consumption in adolescent rhesus macaques: life history and genetic influences.* Alcohol, 2010. **44**(1): p. 67-80.
9. Skinner, H.A. and B.A. Allen, *Alcohol dependence syndrome: measurement and validation.* J Abnorm Psychol, 1982. **91**(3): p. 199-209.
10. Hoffman, P.L. and B. Tabakoff, *Alcohol dependence: a commentary on mechanisms.* Alcohol Alcohol, 1996. **31**(4): p. 333-40.
11. Becker, H.C., *Alcohol dependence, withdrawal, and relapse.* Alcohol Res Health, 2008. **31**(4): p. 348-61.
12. Cornelius, J.R., et al., *Alcohol and psychiatric comorbidity.* Recent Dev Alcohol, 2003. **16**: p. 361-74.
13. Crowley, P., *Long-term drug treatment of patients with alcohol dependence.* Aust Prescr, 2015. **38**(2): p. 41-3.
14. Jarosz, J., et al., *Naltrexone (50 mg) plus psychotherapy in alcohol-dependent patients: a meta-analysis of randomized controlled trials.* Am J Drug Alcohol Abuse, 2013. **39**(3): p. 144-60.
15. Popova, S., et al., *A literature review of cost-benefit analyses for the treatment of alcohol dependence.* Int J Environ Res Public Health, 2011. **8**(8): p. 3351-64.
16. Bonnet, U., et al., *Comparison of two oral symptom-triggered pharmacological inpatient treatments of acute alcohol withdrawal: clomethiazole vs. clonazepam.* Alcohol Alcohol, 2011. **46**(1): p. 68-73.
17. Leichsenring, F., et al., *Borderline personality disorder.* Lancet, 2011. **377**(9759): p. 74-84.
18. Gunderson, J.G., *Clinical practice. Borderline personality disorder.* N Engl J Med, 2011. **364**(21): p. 2037-42.
19. Zanarini, M.C., et al., *Prevalence of DSM-IV borderline personality disorder in two community samples: 6,330 English 11-year-olds and 34,653 American adults.* J Pers Disord, 2011. **25**(5): p. 607-19.
20. Gunderson, J.G., *Borderline personality disorder: ontogeny of a diagnosis.* Am J Psychiatry, 2009. **166**(5): p. 530-9.

21. Amad, A., et al., *Genetics of borderline personality disorder: systematic review and proposal of an integrative model.* Neurosci Biobehav Rev, 2014. **40**: p. 6-19.
22. Torgersen, S., et al., *A twin study of personality disorders.* Compr Psychiatry, 2000. **41**(6): p. 416-25.
23. Crowell, S.E., T.P. Beauchaine, and M.M. Linehan, *A biosocial developmental model of borderline personality: Elaborating and extending Linehan's theory.* Psychol Bull, 2009. **135**(3): p. 495-510.
24. Dammann, G., et al., *Impact of Interpersonal Problems in Borderline Personality Disorder Inpatients on Treatment Outcome and Psychopathology.* Psychopathology, 2016. **49**(3): p. 172-80.
25. Linehan, M.M., *Dialectical behavior therapy for borderline personality disorder. Theory and method.* Bull Menninger Clin, 1987. **51**(3): p. 261-76.
26. Bohus, M., et al., *Effectiveness of inpatient dialectical behavioral therapy for borderline personality disorder: a controlled trial.* Behav Res Ther, 2004. **42**(5): p. 487-99.
27. Manolio, T.A., et al., *Finding the missing heritability of complex diseases.* Nature, 2009. **461**(7265): p. 747-53.
28. Chabris, C.F., et al., *Most reported genetic associations with general intelligence are probably false positives.* Psychol Sci, 2012. **23**(11): p. 1314-23.
29. Zhang, H. and J. Gelernter, *DNA methylation and alcohol use disorders: Progress and challenges.* Am J Addict, 2016.
30. Dammann, G., et al., *Increased DNA methylation of neuropsychiatric genes occurs in borderline personality disorder.* Epigenetics, 2011. **6**(12): p. 1454-62.
31. Teschler, S., et al., *Aberrant methylation of gene associated CpG sites occurs in borderline personality disorder.* PLoS One, 2013. **8**(12): p. e84180.
32. Trerotola, M., et al., *Epigenetic inheritance and the missing heritability.* Hum Genomics, 2015. **9**: p. 17.
33. Carrigan, P. and T. Krahn, *Impact of Biomarkers on Personalized Medicine.* Handb Exp Pharmacol, 2016. **232**: p. 285-311.
34. Aronson, J.K., *Biomarkers and surrogate endpoints.* Br J Clin Pharmacol, 2005. **59**(5): p. 491-4.
35. Trusheim, M.R., E.R. Berndt, and F.L. Douglas, *Stratified medicine: strategic and economic implications of combining drugs and clinical biomarkers.* Nat Rev Drug Discov, 2007. **6**(4): p. 287-93.
36. Nalejska, E., E. Maczynska, and M.A. Lewandowska, *Prognostic and predictive biomarkers: tools in personalized oncology.* Mol Diagn Ther, 2014. **18**(3): p. 273-84.
37. Craig-Schapiro, R., A.M. Fagan, and D.M. Holtzman, *Biomarkers of Alzheimer's disease.* Neurobiol Dis, 2009. **35**(2): p. 128-40.
38. Olbrich, S., R. van Dinteren, and M. Arns, *Personalized Medicine: Review and Perspectives of Promising Baseline EEG Biomarkers in Major Depressive Disorder and Attention Deficit Hyperactivity Disorder.* Neuropsychobiology, 2015. **72**(3-4): p. 229-40.
39. Neumann, T. and C. Spies, *Use of biomarkers for alcohol use disorders in clinical practice.* Addiction, 2003. **98 Suppl 2**: p. 81-91.
40. Goldberg, A.D., C.D. Allis, and E. Bernstein, *Epigenetics: a landscape takes shape.* Cell, 2007. **128**(4): p. 635-8.
41. Berger, S.L., et al., *An operational definition of epigenetics.* Genes Dev, 2009. **23**(7): p. 781-3.

42. Lim, L.P., et al., *Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs.* Nature, 2005. **433**(7027): p. 769-73.
43. Kowalik, K.M., et al., *The Paf1 complex represses small-RNA-mediated epigenetic gene silencing.* Nature, 2015. **520**(7546): p. 248-52.
44. Jones, P.A. and S.B. Baylin, *The fundamental role of epigenetic events in cancer.* Nat Rev Genet, 2002. **3**(6): p. 415-28.
45. Baylin, S.B. and P.A. Jones, *A decade of exploring the cancer epigenome - biological and translational implications.* Nat Rev Cancer, 2011. **11**(10): p. 726-34.
46. Facista, A., et al., *Deficient expression of DNA repair enzymes in early progression to sporadic colon cancer.* Genome Integr, 2012. **3**(1): p. 3.
47. Agarwal, A., et al., *Role of epigenetic alterations in the pathogenesis of Barrett's esophagus and esophageal adenocarcinoma.* Int J Clin Exp Pathol, 2012. **5**(5): p. 382-96.
48. Kantarjian, H., et al., *Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study.* Cancer, 2006. **106**(8): p. 1794-803.
49. Estey, E.H., *Epigenetics in clinical practice: the examples of azacitidine and decitabine in myelodysplasia and acute myeloid leukemia.* Leukemia, 2013. **27**(9): p. 1803-12.
50. Vendetti, F.P. and C.M. Rudin, *Epigenetic therapy in non-small-cell lung cancer: targeting DNA methyltransferases and histone deacetylases.* Expert Opin Biol Ther, 2013. **13**(9): p. 1273-85.
51. Foulks, J.M., et al., *Epigenetic drug discovery: targeting DNA methyltransferases.* J Biomol Screen, 2012. **17**(1): p. 2-17.
52. Quddus, J., et al., *Treating activated CD4+ T cells with either of two distinct DNA methyltransferase inhibitors, 5-azacytidine or procainamide, is sufficient to cause a lupus-like disease in syngeneic mice.* J Clin Invest, 1993. **92**(1): p. 38-53.
53. Diesch, J., et al., *A clinical-molecular update on azanucleoside-based therapy for the treatment of hematologic cancers.* Clin Epigenetics, 2016. **8**: p. 71.
54. Morita, S., et al., *Targeted DNA demethylation in vivo using dCas9-peptide repeat and scFv-TET1 catalytic domain fusions.* Nat Biotechnol, 2016. **34**(10): p. 1060-1065.
55. Xu, X., et al., *A CRISPR-based approach for targeted DNA demethylation.* Cell Discov, 2016. **2**: p. 16009.
56. Lim, U. and M.A. Song, *Dietary and lifestyle factors of DNA methylation.* Methods Mol Biol, 2012. **863**: p. 359-76.
57. Jenuwein, T. and C.D. Allis, *Translating the histone code.* Science, 2001. **293**(5532): p. 1074-80.
58. Shogren-Knaak, M., et al., *Histone H4-K16 acetylation controls chromatin structure and protein interactions.* Science, 2006. **311**(5762): p. 844-7.
59. Cheung, P. and P. Lau, *Epigenetic regulation by histone methylation and histone variants.* Mol Endocrinol, 2005. **19**(3): p. 563-73.
60. Zhang, Y. and D. Reinberg, *Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails.* Genes Dev, 2001. **15**(18): p. 2343-60.
61. Nowak, S.J. and V.G. Corces, *Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation.* Trends Genet, 2004. **20**(4): p. 214-20.

62. Ooi, S.K., et al., *DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA.* Nature, 2007. **448**(7154): p. 714-7.
63. Zilberman, D., et al., *Histone H2A.Z and DNA methylation are mutually antagonistic chromatin marks.* Nature, 2008. **456**(7218): p. 125-9.
64. Irvine, R.A., I.G. Lin, and C.L. Hsieh, *DNA methylation has a local effect on transcription and histone acetylation.* Mol Cell Biol, 2002. **22**(19): p. 6689-96.
65. Lam, L.L., et al., *Factors underlying variable DNA methylation in a human community cohort.* Proc Natl Acad Sci U S A, 2012. **109 Suppl 2**: p. 17253-60.
66. Jones, P.A., *Functions of DNA methylation: islands, start sites, gene bodies and beyond.* Nat Rev Genet, 2012. **13**(7): p. 484-92.
67. Razin, A. and A.D. Riggs, *DNA methylation and gene function.* Science, 1980. **210**(4470): p. 604-10.
68. Ramchandani, S., et al., *DNA methylation is a reversible biological signal.* Proc Natl Acad Sci U S A, 1999. **96**(11): p. 6107-12.
69. Isles, A.R. and L.S. Wilkinson, *Epigenetics: what is it and why is it important to mental disease?* Br Med Bull, 2008. **85**: p. 35-45.
70. Breiling, A. and F. Lyko, *Epigenetic regulatory functions of DNA modifications: 5-methylcytosine and beyond.* Epigenetics Chromatin, 2015. **8**: p. 24.
71. Ahmed, H., *Promoter Methylation in Prostate Cancer and its Application for the Early Detection of Prostate Cancer Using Serum and Urine Samples.* Biomark Cancer, 2010. **2010**(2): p. 17-33.
72. Schubeler, D., *Function and information content of DNA methylation.* Nature, 2015. **517**(7534): p. 321-6.
73. Egger, G., et al., *Epigenetics in human disease and prospects for epigenetic therapy.* Nature, 2004. **429**(6990): p. 457-63.
74. Wu, T.P., et al., *DNA methylation on N(6)-adenine in mammalian embryonic stem cells.* Nature, 2016. **532**(7599): p. 329-33.
75. Watt, F. and P.L. Molloy, *Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter.* Genes Dev, 1988. **2**(9): p. 1136-43.
76. Du, Q., et al., *Methyl-CpG-binding domain proteins: readers of the epigenome.* Epigenomics, 2015. **7**(6): p. 1051-73.
77. Maunakea, A.K., et al., *Conserved role of intragenic DNA methylation in regulating alternative promoters.* Nature, 2010. **466**(7303): p. 253-7.
78. Wu, H. and Y. Zhang, *Reversing DNA methylation: mechanisms, genomics, and biological functions.* Cell, 2014. **156**(1-2): p. 45-68.
79. Guo, J.U., et al., *Hydroxylation of 5-methylcytosine by TET1 promotes active DNA demethylation in the adult brain.* Cell, 2011. **145**(3): p. 423-34.
80. Ito, S., et al., *Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine.* Science, 2011. **333**(6047): p. 1300-3.
81. He, Y.F., et al., *Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA.* Science, 2011. **333**(6047): p. 1303-7.
82. Barfield, R.T., et al., *Accounting for population stratification in DNA methylation studies.* Genet Epidemiol, 2014. **38**(3): p. 231-41.
83. Christensen, B.C., et al., *Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context.* PLoS Genet, 2009. **5**(8): p. e1000602.
84. Farre, P., et al., *Concordant and discordant DNA methylation signatures of aging in human blood and brain.* Epigenetics Chromatin, 2015. **8**: p. 19.

85. Zhang, F.F., et al., *Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood.* Epigenetics, 2011. **6**(5): p. 623-9.
86. Liu, J., et al., *A study of the influence of sex on genome wide methylation.* PLoS One, 2010. **5**(4): p. e10028.
87. Numata, S., et al., *DNA methylation signatures in development and aging of the human prefrontal cortex.* Am J Hum Genet, 2012. **90**(2): p. 260-72.
88. Hellman, A. and A. Chess, *Gene body-specific methylation on the active X chromosome.* Science, 2007. **315**(5815): p. 1141-3.
89. Heyn, H., et al., *Distinct DNA methylomes of newborns and centenarians.* Proc Natl Acad Sci U S A, 2012. **109**(26): p. 10522-7.
90. Tsaprouni, L.G., et al., *Cigarette smoking reduces DNA methylation levels at multiple genomic loci but the effect is partially reversible upon cessation.* Epigenetics, 2014. **9**(10): p. 1382-96.
91. Besingi, W. and A. Johansson, *Smoke-related DNA methylation changes in the etiology of human disease.* Hum Mol Genet, 2014. **23**(9): p. 2290-7.
92. Yang, A.S., et al., *A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements.* Nucleic Acids Res, 2004. **32**(3): p. e38.
93. Doucet, A.J., et al., *Characterization of LINE-1 ribonucleoprotein particles.* PLoS Genet, 2010. **6**(10).
94. Szmulewicz, M.N., G.E. Novick, and R.J. Herrera, *Effects of Alu insertions on gene function.* Electrophoresis, 1998. **19**(8-9): p. 1260-4.
95. Kim, D.S., et al., *Effect of alcohol consumption on peripheral blood Alu methylation in Korean men.* Biomarkers, 2016. **21**(3): p. 243-8.
96. Zhu, Z.Z., et al., *Predictors of global methylation levels in blood DNA of healthy subjects: a combined analysis.* Int J Epidemiol, 2012. **41**(1): p. 126-39.
97. Bonsch, D., et al., *Homocysteine associated genomic DNA hypermethylation in patients with chronic alcoholism.* J Neural Transm, 2004. **111**(12): p. 1611-6.
98. Semmler, A., et al., *Alcohol abuse and cigarette smoking are associated with global DNA hypermethylation: results from the German Investigation on Neurobiology in Alcoholism (GINA).* Alcohol, 2015. **49**(2): p. 97-101.
99. French, S.W., *Epigenetic events in liver cancer resulting from alcoholic liver disease.* Alcohol Res, 2013. **35**(1): p. 57-67.
100. Varela-Rey, M., et al., *Alcohol, DNA methylation, and cancer.* Alcohol Res, 2013. **35**(1): p. 25-35.
101. Bruckmann, C., et al., *Validation of differential GDAP1 DNA methylation in alcohol dependence and its potential function as a biomarker for disease severity and therapy outcome.* Epigenetics, 2016. **11**(6): p. 456-63.
102. Hillemacher, T., et al., *Promoter specific methylation of the dopamine transporter gene is altered in alcohol dependence and associated with craving.* J Psychiatr Res, 2009. **43**(4): p. 388-92.
103. Wiers, C.E., et al., *Effects of depressive symptoms and peripheral DAT methylation on neural reactivity to alcohol cues in alcoholism.* Transl Psychiatry, 2015. **5**: p. e648.
104. Nieratschker, V., et al., *Epigenetic alteration of the dopamine transporter gene in alcohol-dependent patients is associated with age.* Addict Biol, 2014. **19**(2): p. 305-11.
105. Jasiewicz, A., et al., *DAT1 methylation changes in alcohol-dependent individuals vs. controls.* J Psychiatr Res, 2015. **64**: p. 130-3.

106. Biermann, T., et al., *N-methyl-D-aspartate 2b receptor subtype (NR2B) promoter methylation in patients during alcohol withdrawal.* J Neural Transm (Vienna), 2009. **116**(5): p. 615-22.
107. Muschler, M.A., et al., *DNA methylation of the POMC gene promoter is associated with craving in alcohol dependence.* J Neural Transm (Vienna), 2010. **117**(4): p. 513-9.
108. Philibert, R.A., et al., *MAOA methylation is associated with nicotine and alcohol dependence in women.* Am J Med Genet B Neuropsychiatr Genet, 2008. **147B**(5): p. 565-70.
109. Zhang, R., et al., *Genome-wide DNA methylation analysis in alcohol dependence.* Addict Biol, 2013. **18**(2): p. 392-403.
110. Ruggeri, B., et al., *Association of Protein Phosphatase PPM1G With Alcohol Use Disorder and Brain Activity During Behavioral Control in a Genome-Wide Methylation Analysis.* Am J Psychiatry, 2015. **172**(6): p. 543-52.
111. Hagerty, S.L., et al., *An Exploratory Association Study of Alcohol Use Disorder and DNA Methylation.* Alcohol Clin Exp Res, 2016. **40**(8): p. 1633-40.
112. Philibert, R.A., et al., *A pilot examination of the genome-wide DNA methylation signatures of subjects entering and exiting short-term alcohol dependence treatment programs.* Epigenetics, 2014. **9**(9): p. 1212-9.
113. Liu, C., et al., *A DNA methylation biomarker of alcohol consumption.* Mol Psychiatry, 2016.
114. Perroud, N., et al., *Increased methylation of glucocorticoid receptor gene (NR3C1) in adults with a history of childhood maltreatment: a link with the severity and type of trauma.* Transl Psychiatry, 2011. **1**: p. e59.
115. Perroud, N., et al., *Methylation of Serotonin Receptor 3a in Adhd, Borderline Personality, and Bipolar Disorders: Link with Severity of the Disorders and Childhood Maltreatment.* Depress Anxiety, 2016. **33**(1): p. 45-55.
116. Perroud, N., et al., *Response to psychotherapy in borderline personality disorder and methylation status of the BDNF gene.* Transl Psychiatry, 2013. **3**: p. e207.
117. Teschler, S., et al., *Aberrant DNA Methylation of rDNA and PRIMA1 in Borderline Personality Disorder.* Int J Mol Sci, 2016. **17**(1).
118. Moak, D.H., R.F. Anton, and P.K. Latham, *Further validation of the Obsessive-Compulsive Drinking Scale (OCDS). Relationship to alcoholism severity.* Am J Addict, 1998. **7**(1): p. 14-23.
119. Knoblich, N., *Epigenetik der Borderline-Persönlichkeits-Störung - Effekte der DBT auf die Methylierungsmuster*. 2016, Universität Tübingen.
120. Maksimovic, J., L. Gordon, and A. Oshlack, *SWAN: Subset-quantile within array normalization for illumina infinium HumanMethylation450 BeadChips.* Genome Biol, 2012. **13**(6): p. R44.
121. Johnson, W.E., C. Li, and A. Rabinovic, *Adjusting batch effects in microarray expression data using empirical Bayes methods.* Biostatistics, 2007. **8**(1): p. 118-27.
122. Houseman, E.A., et al., *DNA methylation arrays as surrogate measures of cell mixture distribution.* BMC Bioinformatics, 2012. **13**: p. 86.
123. Valitutti, S., et al., *Degradation of T cell receptor (TCR)-CD3-zeta complexes after antigenic stimulation.* J Exp Med, 1997. **185**(10): p. 1859-64.
124. Bonsch, D., et al., *DNA hypermethylation of the alpha synuclein promoter in patients with alcoholism.* Neuroreport, 2005. **16**(2): p. 167-70.

125. Bonsch, D., et al., *Homocysteine associated genomic DNA hypermethylation in patients with chronic alcoholism.* J Neural Transm (Vienna), 2004. **111**(12): p. 1611-6.
126. Mandaviya, P.R., L. Stolk, and S.G. Heil, *Homocysteine and DNA methylation: a review of animal and human literature.* Mol Genet Metab, 2014. **113**(4): p. 243-52.
127. Benjamini, Y. and Y. Hochberg, *Controlling the false discovery rate: a practical and powerful approach to multiple testing.* Journal of the royal statistical society. Series B (Methodological), 1995: p. 289-300.
128. Nemoda, Z., et al., *Maternal depression is associated with DNA methylation changes in cord blood T lymphocytes and adult hippocampi.* Transl Psychiatry, 2015. **5**: p. e545.
129. Pidsley, R., et al., *Methylomic profiling of human brain tissue supports a neurodevelopmental origin for schizophrenia.* Genome Biol, 2014. **15**(10): p. 483.
130. Miyazaki, K., et al., *A novel HECT-type E3 ubiquitin ligase, NEDL2, stabilizes p73 and enhances its transcriptional activity.* Biochem Biophys Res Commun, 2003. **308**(1): p. 106-13.
131. Yang, A., et al., *p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours.* Nature, 2000. **404**(6773): p. 99-103.
132. Nakagawa, O., et al., *Centronuclear myopathy in mice lacking a novel muscle-specific protein kinase transcriptionally regulated by MEF2.* Genes Dev, 2005. **19**(17): p. 2066-77.
133. Nieratschker, V., et al., *Bruchpilot in ribbon-like axonal agglomerates, behavioral defects, and early death in SRPK79D kinase mutants of Drosophila.* PLoS Genet, 2009. **5**(10): p. e1000700.
134. Hamilton, J., et al., *A new method for the measurement of lysosomal acid lipase in dried blood spots using the inhibitor Lalistat 2.* Clin Chim Acta, 2012. **413**(15-16): p. 1207-10.
135. Nault, J.C., et al., *Recurrent AAV2-related insertional mutagenesis in human hepatocellular carcinomas.* Nat Genet, 2015. **47**(10): p. 1187-93.
136. Jahchan, N.S., G. Ouyang, and K. Luo, *Expression profiles of SnoN in normal and cancerous human tissues support its tumor suppressor role in human cancer.* PLoS One, 2013. **8**(2): p. e55794.
137. Mathelier, A., et al., *JASPAR 2016: a major expansion and update of the open-access database of transcription factor binding profiles.* Nucleic Acids Res, 2016. **44**(D1): p. D110-5.
138. Kuttippurathu, L., et al., *A novel comparative pattern count analysis reveals a chronic ethanol-induced dynamic shift in immediate early NF-kappaB genome-wide promoter binding during liver regeneration.* Mol Biosyst, 2016. **12**(3): p. 1037-56.
139. Bell, R.L., et al., *Gene expression changes in the nucleus accumbens of alcohol-preferring rats following chronic ethanol consumption.* Pharmacol Biochem Behav, 2009. **94**(1): p. 131-47.
140. Connor, J., *Alcohol consumption as a cause of cancer.* Addiction, 2017. **112**(2): p. 222-228.
141. Bock, C., *Analysing and interpreting DNA methylation data.* Nat Rev Genet, 2012. **13**(10): p. 705-19.
142. Roberson, E.D., et al., *A subset of methylated CpG sites differentiate psoriatic from normal skin.* J Invest Dermatol, 2012. **132**(3 Pt 1): p. 583-92.

143. Fang, M., D. Chen, and C.S. Yang, *Dietary polyphenols may affect DNA methylation.* J Nutr, 2007. **137**(1 Suppl): p. 223S-228S.
144. Mill, J. and B.T. Heijmans, *From promises to practical strategies in epigenetic epidemiology.* Nat Rev Genet, 2013. **14**(8): p. 585-94.
145. Gräf Olmos, V., *Epigenetik und Genexpression ausgewählter Zielgene bei alkoholabhängigen Patienten*. 2017, Universität Tübingen.
146. EMBL-EBI. *Expression Atlas*. 2017 [cited 2017; Available from: https://www.ebi.ac.uk/gxa/home.
147. Consortium, G.T., *The Genotype-Tissue Expression (GTEx) project.* Nat Genet, 2013. **45**(6): p. 580-5.
148. Fan, S., et al., *Computationally expanding infinium HumanMethylation450 BeadChip array data to reveal distinct DNA methylation patterns of rheumatoid arthritis.* Bioinformatics, 2016. **32**(12): p. 1773-8.
149. Moran, S., C. Arribas, and M. Esteller, *Validation of a DNA methylation microarray for 850,000 CpG sites of the human genome enriched in enhancer sequences.* Epigenomics, 2016. **8**(3): p. 389-99.
150. Shvetsov, Y.B., et al., *Intraindividual variation and short-term temporal trend in DNA methylation of human blood.* Cancer Epidemiol Biomarkers Prev, 2015. **24**(3): p. 490-7.
151. Linehan, M.M., et al., *Cognitive-behavioral treatment of chronically parasuicidal borderline patients.* Arch Gen Psychiatry, 1991. **48**(12): p. 1060-4.
152. Bohus, M., et al., *The short version of the Borderline Symptom List (BSL-23): development and initial data on psychometric properties.* Psychopathology, 2009. **42**(1): p. 32-9.
153. Csiszar, A., et al., *Synergistic effects of hypertension and aging on cognitive function and hippocampal expression of genes involved in beta-amyloid generation and Alzheimer's disease.* Am J Physiol Heart Circ Physiol, 2013. **305**(8): p. H1120-30.
154. Piton, A., et al., *Systematic resequencing of X-chromosome synaptic genes in autism spectrum disorder and schizophrenia.* Mol Psychiatry, 2011. **16**(8): p. 867-80.

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6 Supplement

6.1 Tables

Table S 1: Site-specific analysis results between controls and patient (T1).
Hits are sorted by FDR-Value.

	Probe ID	Gene	Region	*P*-Value	FDR	Δ-beta	Average beta controls	Average beta patients (T1)
1	cg18752527	HECW2	intragenic	4.30E-07	0.021	0.066	0.342	0.276
2	cg08109624	None	intergenic	8.15E-07	0.023	-0.057	0.760	0.817
3	cg10168086	None	intergenic	1.24E-06	0.026	0.051	0.535	0.484
4	cg07280807	None	intergenic	2.44E-06	0.037	-0.068	0.755	0.822
5	cg12173150	None	intergenic	3.02E-06	0.037	-0.064	0.321	0.385
6	cg01059398	TNFSF10	intragenic	1.07E-05	0.063	0.052	0.261	0.209
7	cg17940902	HLA-DMA	promoter	1.19E-05	0.064	-0.051	0.399	0.450
8	cg22778903	MX2	intragenic	1.34E-05	0.067	-0.051	0.304	0.355
9	cg14612335	SKIL	promoter	1.38E-05	0.067	0.055	0.423	0.368
10	cg11580026	None	intergenic	1.51E-05	0.069	0.051	0.600	0.549
11	cg12284098	MYOM2	intragenic	1.54E-05	0.069	0.056	0.534	0.477
12	cg26091609	CTLA4	intragenic	1.59E-05	0.069	0.060	0.578	0.518
13	cg09768654	SRPK3	promoter	1.65E-05	0.069	-0.092	0.374	0.466
14	cg06851207	PNMAL1	promoter	1.84E-05	0.069	-0.089	0.528	0.617
15	cg14702960	None	intergenic	1.92E-05	0.069	0.052	0.742	0.689
16	cg00449728	MAPRE2	intragenic	2.98E-05	0.070	0.057	0.750	0.693
17	cg22851561	ELMSAN1	intragenic	3.00E-05	0.070	0.052	0.432	0.380
18	cg02536838	ANGPT1	promoter	3.14E-05	0.070	0.075	0.605	0.530
19	cg15841511	None	intergenic	3.42E-05	0.071	-0.059	0.729	0.788
20	cg24392939	CRYBG3	intragenic	3.62E-05	0.072	0.052	0.562	0.510
21	cg12761472	CEP85L	promoter	4.13E-05	0.075	0.055	0.621	0.566
22	cg02652579	SYNGAP1	promoter	4.17E-05	0.076	0.059	0.623	0.563
23	cg22865905	SNORA69	three_plus	4.26E-05	0.076	0.051	0.794	0.743
24	cg27201673	PNMAL1	promoter	5.41E-05	0.078	-0.050	0.213	0.263
25	cg04936619	C17orf75	intragenic	5.88E-05	0.078	0.069	0.314	0.245
26	cg11121969	PCBP3	promoter	6.26E-05	0.078	0.064	0.691	0.627
27	cg00246693	ARHGAP42	promoter	7.10E-05	0.078	-0.053	0.340	0.393
28	cg10399005	None	intergenic	7.11E-05	0.078	-0.057	0.776	0.833
29	cg16529483	SRPK3	promoter	7.18E-05	0.078	-0.105	0.252	0.357
30	cg01220513	SH3KBP1	intragenic	8.08E-05	0.079	0.051	0.506	0.454
31	cg26926002	None	intergenic	8.10E-05	0.079	-0.058	0.719	0.777
32	cg14544087	MIR155HG	intragenic	8.64E-05	0.079	0.063	0.290	0.227
33	cg20893919	TRPC3	intragenic	9.23E-05	0.080	-0.051	0.703	0.754
34	cg18682028	FYCO1	intragenic	9.24E-05	0.080	0.056	0.394	0.338
35	cg04362790	None	intergenic	9.32E-05	0.080	0.052	0.697	0.644
36	cg09060654	LIPA	intragenic	9.51E-05	0.080	-0.079	0.578	0.656

	Probe ID	Gene	Region	*P*-Value	FDR	Δ-beta	Average beta controls	Average beta patients (T1)
37	cg02451774	NBPF8	intragenic	9.98E-05	0.081	-0.053	0.431	0.483
38	cg18723276	USP29	promoter	1.10E-04	0.082	-0.051	0.723	0.774
39	cg13180722	None	intergenic	1.19E-04	0.083	-0.062	0.338	0.401
40	cg12230162	SRPK3	promoter	1.20E-04	0.083	-0.105	0.357	0.463
41	cg18890544	None	intergenic	1.23E-04	0.084	-0.059	0.846	0.905
42	cg24496423	SRPK3	promoter	1.30E-04	0.085	-0.084	0.309	0.393
43	cg02661764	None	intergenic	1.35E-04	0.087	0.059	0.419	0.360
44	cg01400671	None	intergenic	1.40E-04	0.087	0.064	0.409	0.345
45	cg13609457	None	intergenic	1.52E-04	0.090	0.056	0.577	0.521
46	cg25880958	None	intergenic	1.52E-04	0.090	-0.054	0.591	0.645
47	cg18376497	INPP4B	intragenic	1.65E-04	0.092	0.064	0.286	0.223
48	cg13784312	RAPGEF1	intragenic	1.72E-04	0.093	0.051	0.187	0.136
49	cg07135405	MIR1914	three_plus	1.76E-04	0.093	0.146	0.540	0.394
50	cg20475486	None	intergenic	1.80E-04	0.094	-0.058	0.702	0.759
51	cg11858450	CCDC105	intragenic	1.89E-04	0.094	-0.053	0.709	0.762
52	cg05927817	None	intergenic	1.94E-04	0.094	-0.061	0.726	0.787
53	cg00306893	None	intergenic	1.99E-04	0.094	0.062	0.737	0.675
54	cg10365886	TNXB	intragenic	2.08E-04	0.095	-0.105	0.566	0.672
55	cg27503950	None	intergenic	2.19E-04	0.095	-0.063	0.633	0.696
56	cg01089001	GALNT18	intragenic	2.27E-04	0.095	-0.065	0.317	0.382
57	cg12564698	GAL	three_plus	2.30E-04	0.095	0.051	0.312	0.261
58	cg16197188	NRG3	intragenic	2.59E-04	0.100	0.051	0.723	0.672
59	cg04088338	None	intergenic	2.66E-04	0.100	0.052	0.430	0.378

Table S 2: Site-specific analysis results between patients pre- and posttreatment. Hits are sorted by FDR-Value.

	Probe ID	Gene	Region	*P*-Value	FDR	Δ-beta	Average beta patients (T1)	Average beta patients (T2)
1	cg15500907	LAMA4	intragenic	1.01E-06	0.032	-0.056	0.485	0.542
2	cg05266321	CCR2	intragenic	4.63E-06	0.049	-0.061	0.545	0.606
3	cg13279700	C6orf10	intragenic	1.76E-05	0.056	-0.063	0.481	0.544
4	cg14054990	KRTAP19-5	promoter	1.84E-05	0.056	-0.052	0.431	0.482
5	cg21049302	None	intergenic	1.98E-05	0.056	-0.056	0.466	0.522
6	cg17022548	NRG2	intragenic	1.99E-05	0.056	-0.054	0.204	0.258
7	cg22472360	TRIO	intragenic	2.09E-05	0.057	-0.055	0.514	0.569
8	cg07920414	RIMS3	intragenic	2.18E-05	0.057	-0.055	0.438	0.493
9	cg04088338	None	intergenic	2.54E-05	0.059	-0.051	0.378	0.429
10	cg12240358	HOMER2	intragenic	2.68E-05	0.059	-0.057	0.462	0.519
11	cg09712306	AURKA	intragenic	3.48E-05	0.060	-0.058	0.602	0.660
12	cg07939743	None	intergenic	3.50E-05	0.060	-0.052	0.289	0.341

	Probe ID	Gene	Region	*P*-Value	FDR	Δ-beta	Average beta patients (T1)	Average beta patients (T2)
13	cg00803692	CCR5	promoter	3.73E-05	0.062	-0.054	0.370	0.424
14	cg10177030	SNORD12	three_plus	3.85E-05	0.063	-0.053	0.419	0.472
15	cg15439110	None	intergenic	3.93E-05	0.063	-0.080	0.444	0.525
16	cg20385229	SLIRP	intragenic	4.13E-05	0.063	-0.052	0.392	0.444
17	cg02393640	LUZP6	intragenic	5.63E-05	0.067	-0.052	0.390	0.443
18	cg17863551	CD177	promoter	6.27E-05	0.067	-0.059	0.419	0.478
19	cg15279541	None	intergenic	7.14E-05	0.068	-0.051	0.388	0.439
20	cg20171999	RRS1	three_plus	8.93E-05	0.068	-0.070	0.403	0.474
21	cg20559385	None	intergenic	9.43E-05	0.068	-0.052	0.428	0.479
22	cg21429780	MAML3	intragenic	1.01E-04	0.068	-0.052	0.493	0.545
23	cg01482790	HNRNPM	intragenic	1.09E-04	0.068	-0.050	0.289	0.339
24	cg20684197	FGF1	intragenic	1.10E-04	0.068	-0.051	0.395	0.445
25	cg04279139	MANSC4	promoter	1.13E-04	0.069	-0.051	0.410	0.461
26	cg16853860	PSMB9	intragenic	1.16E-04	0.070	-0.060	0.272	0.332
27	cg27062514	CTR9	intragenic	1.33E-04	0.072	-0.064	0.463	0.526
28	cg09931909	MB21D1	intragenic	1.40E-04	0.073	-0.077	0.420	0.497
29	cg13340231	ZNF704	intragenic	1.50E-04	0.075	-0.055	0.528	0.583
30	cg10035831	RPTOR	intragenic	1.60E-04	0.075	-0.057	0.446	0.503
31	cg13927756	MYO10	intragenic	1.62E-04	0.075	-0.056	0.468	0.524
32	cg08749576	None	intergenic	1.67E-04	0.076	-0.058	0.627	0.684
33	cg15484808	RPS18	intragenic	2.20E-04	0.081	-0.054	0.480	0.534
34	cg12802876	None	intergenic	2.46E-04	0.083	-0.059	0.359	0.418
35	cg03548415	None	intergenic	2.93E-04	0.085	-0.051	0.422	0.473
36	cg20547015	PPP1CC	intragenic	3.02E-04	0.086	-0.064	0.453	0.517
37	cg23214895	None	intergenic	3.20E-04	0.088	-0.051	0.569	0.620
38	cg12478092	CCDC116	promoter	3.28E-04	0.088	-0.063	0.510	0.573
39	cg15683542	MIPEP	intragenic	3.41E-04	0.088	-0.053	0.694	0.747
40	cg09514545	MIR525	three_plus	4.04E-04	0.091	-0.060	0.442	0.501
41	cg01789743	NID1	intragenic	4.11E-04	0.091	-0.053	0.499	0.552
42	cg18524114	None	intergenic	4.69E-04	0.093	-0.050	0.339	0.389
43	cg04410448	ZC2HC1B	intragenic	5.12E-04	0.095	-0.051	0.491	0.541
44	cg13714407	RAPGEF1	intragenic	5.23E-04	0.095	-0.059	0.367	0.426
45	cg27367066	None	intergenic	5.63E-04	0.097	-0.054	0.455	0.510
46	cg26837708	YBX1	intragenic	5.85E-04	0.097	-0.058	0.388	0.445
47	cg14817867	PRPSAP2	intragenic	6.07E-04	0.097	-0.052	0.419	0.471
48	cg13598358	PPP1CC	intragenic	6.32E-04	0.098	-0.056	0.362	0.418

Table S 3: Regional analysis between controls and patients (T1).
Hits are sorted by FDR-Value.

	CGI coordinates	Gene(s)	No. of CpG sites	FDR	mean Δ-beta within region
1	chrX:153046175-153047707	SRPK3	10	3.80E-19	-0.059
2	chr1:242220301-242220925	None	5	1.02E-15	-0.054
3	chr6:29648161-29649084	None	21	1.88E-14	0.081
4	chr6:160023581-160024144	None	6	5.47E-12	-0.058
5	chr19:29217858-29218774	None	7	4.20E-06	-0.088
6	chr17:57915665-57918682	TMEM49; MIR21	12	6.97E-06	0.050
7	chr9:124988720-124991047	LHX6	10	1.46E-05	0.070
8	chr12:4916913-4919230	KCNA6	10	3.13E-05	0.051
9	chr10:90985055-90985062	LIPA	2	4.51E-05	-0.076
10	chr7:90895466-90896701	FZD1	5	8.30E-05	-0.063
11	chr7:155150681-155151427	None	5	1.02E-04	0.056
12	chr8:144631768-144631915	None	2	1.36E-04	0.054
13	chr15:66947171-66947617	None	5	2.88E-04	0.086
14	chr19:613111-613818	HCN2	4	3.51E-04	-0.051
15	chr6:95220699-95221182	None	3	5.20E-04	0.057
16	chr1:30240119-30240265	None	2	7.88E-04	-0.053
17	chr8:637468-638330	ERICH1	4	7.88E-04	-0.063
18	chr6:290800-293285	DUSP22	11	4.04E-03	0.089
19	chr9:19378679-19379118	RPS6	2	4.15E-03	0.052
20	chr19:48697722-48698632	None	3	6.54E-03	-0.055
21	chr15:60987894-60987928	RORA	2	6.78E-03	0.090
22	chr6:28664155-28664226	None	3	9.62E-03	-0.053
23	chr8:216578-216788	None	4	1.02E-02	-0.094
24	chr5:179740743-179741120	GFPT2	4	1.85E-02	0.092
25	chr3:73045556-73045686	PPP4R2	2	2.91E-02	-0.086
26	chr2:128168798-128168987	None	2	3.04E-02	0.050
27	chr13:27295928-27296010	None	3	3.54E-02	0.063
28	chr6:32490350-32490444	HLA-DRB5	2	4.17E-02	0.108
29	chr5:118693725-118693764	TNFAIP8	2	4.39E-02	0.059

6.2 Publications

Paper 1

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Validation of differential GDAP1 DNA methylation in alcohol dependence and its potential function as a biomarker for disease severity and therapy outcome.

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RESEARCH PAPER

Validation of differential *GDAP1* DNA methylation in alcohol dependence and its potential function as a biomarker for disease severity and therapy outcome

Christof Brückmann[a], Adriana Di Santo[a], Kathrin Nora Karle[a], Anil Batra[b], and Vanessa Nieratschker[a]

[a]Department of Psychiatry and Psychotherapy, University Hospital of Tuebingen, Tuebingen, Germany; [b]Section for Addiction Medicine and Addiction Research, Department of Psychiatry and Psychotherapy, University Hospital of Tuebingen, Tuebingen, Germany

ABSTRACT

Alcohol dependence is a severe disorder contributing substantially to the global burden of disease. Despite the detrimental consequences of chronic alcohol abuse and dependence, effective prevention strategies as well as treatment options are largely missing to date. Accumulating evidence suggests that gene-environment interactions, including epigenetic mechanisms, play a role in the etiology of alcohol dependence. A recent epigenome-wide study reported widespread alterations of DNA methylation patterns in alcohol dependent patients compared to control individuals. In the present study, we validate and replicate one of the top findings from this previous investigation in an independent cohort: the hypomethylation of *GDAP1* in patients. To our knowledge, this is the first independent replication of an epigenome-wide finding in alcohol dependence. Furthermore, the AUDIT as well as the GSI score were negatively associated with *GDAP1* methylation and we found a trend toward a negative association between *GDAP1* methylation and the years of alcohol dependency, pointing toward a potential role of *GDAP1* hypomethylation as biomarker for disease severity. In addition, we show that the hypomethylation of *GDAP1* in patients reverses during a short-term alcohol treatment program, suggesting that *GDAP1* DNA methylation could also serve as a potential biomarker for treatment outcome. Our data add to the growing body of knowledge on epigenetic effects in alcohol dependence and support *GDAP1* as a novel candidate gene implicated in this disorder. As the role of *GDAP1* in alcohol dependence is unknown, this novel candidate gene should be followed up in future studies.

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Introduction

Causing approximately 3.3 million deaths every year (or 5.9% of all deaths world-wide) and attributing to 5.1% of the global burden of disease, harmful use of alcohol plays a decisive role for health (WHO, 2014). Despite the detrimental consequences of chronic alcohol abuse and dependence, effective preventive strategies and treatment options are still less than optimal.

Genetic and environmental factors modulate susceptibility to chronic alcohol abuse and alcohol dependence. Whereas heritability estimates for alcohol dependence range between 40 and 60%, environmental and stochastic effects account for the remainder of this variability.[1-3] Accumulating evidence suggests that genetic and environmental factors not only act independently of each other but that also their interactions are implicated in the etiology of alcohol dependence.[4-6] Among others, the interaction between genes and environment is mediated by epigenetic mechanisms.[7] The major epigenetic mechanisms involve covalent modifications: DNA methylation and posttranslational histone modifications.[8,9] Both mechanisms are important regulators of gene expression.[10] DNA is methylated at position 5 of the cytosine pyrimidine ring, a reaction catalyzed by DNA methyltransferases (DNMTs). DNA methylation mainly occurs at the cytosine of a CpG dinucleotide. These CpG sites are not evenly distributed throughout the genome but are enriched in regions called CpG islands. CpG islands overlap with the promoter regions of 50 – 60% of human genes and are typically less methylated than CpG sites outside of CpG islands.[11] Methylation of CpG sites is usually correlated with a decrease in gene expression.[12,13]

Initially, DNA methylation was believed to be established during early embryonic development and to remain stable afterwards. However, more recent research hints toward a more complex pattern of transcriptional regulation through DNA methylation and it is now known that DNA methylation is a dynamic mechanism.[14] DNA methylation patterns vary over the lifetime of an organism and allow it to adapt to environmental changes.[15] Various diseases are associated with altered epigenetic regulation and epigenetic mechanisms also play an important role in many neuropsychiatric disorders, [16] such as depression,[17] schizophrenia [18] and addictions [19] including alcohol dependence.

Increased levels of homocysteine have been described in alcohol dependent patients.[20-22] Homocysteine is of importance for DNA methylation as it is metabolized to methionine, which is then transformed into S-adenosyl methionine

CONTACT Vanessa Nieratschker vanessa.nieratschker@med.uni-tuebingen.de Department of Psychiatry and Psychotherapy, University Hospital of Tuebingen, Calwerstrasse 14, 72076 Tuebingen, Germany.
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(SAM), the most important methyl group donor in vertebrates.[23] Consequently, elevated homocysteine levels were associated with DNA hypermethylation in alcohol dependent patients.[24] In contrast to those findings, other studies have reported that alcohol dependent patients lack the regulation of methionine adenosyl transferase resulting in global DNA hypomethylation.[25,26]

Several previous candidate-gene driven studies investigated the interplay between alcohol consumption and DNA methylation. An impact of alcohol intake on the methylation state of various genes, including monoamine oxidase A,[27] dopamine transporter,[28] serotonin transporter,[29] nerve growth factor [30] and, most recently, leptin [31] have been described.

To date, there are only few studies investigating the influence of alcohol consumption on epigenetic mechanisms at an epigenome-wide level. In these studies, a number of genes were found to be significantly differentially methylated epigenome-wide between alcohol dependent patients and control individuals. The epigenetically differentially regulated regions included hyper- as well as hypo-methylated genes in patients.[32-34] The most recent study by Clark et al. identified *CNTN4* as a risk factor for alcohol use by examining the methylation status of approximately 27 million autosomal CpG sites and comparing them to GWAS data.[35] Earlier candidate-gene based studies investigating the influence of therapeutic interventions on DNA methylation reported decreasing homocysteine levels in alcohol dependent patients during alcohol treatment,[20,21,36,37] leading to the hypothesis that DNA methylation levels also decrease during alcohol treatment. However, candidate-gene driven DNA methylation studies conducted thus far have resulted in conflicting findings.[28,30,38]

To date, only one study has investigated the effects of an alcohol treatment on the epigenome using a systematic approach.[32] No gene was epigenome-wide significantly differentially regulated when comparing the patients' methylome at the beginning of the alcohol treatment and after 4 weeks of treatment. However, when comparing patients entering the program and healthy control individuals, 56 genes reached epigenome-wide significance after Bonferroni correction, among them, *GDAP1*. This gene caught our attention, as it was the most significant finding within a promoter region of a characterized gene product. *GDAP1* was significantly hypomethylated in alcohol dependent patients compared to the control group. *GDAP1* is a member of the ganglioside-induced differentiation-associated protein family. Mutations in *GDAP1* have been linked to Charcot-Marie-Tooth disease, a peripheral nerve disorder involving loss of muscle tissue.[39,40] So far, no associations of *GDAP1* with alcohol dependence or other addictions have been reported.

To clarify whether *GDAP1* is indeed a novel epigenetic biomarker for alcohol dependence, we aimed to replicate the DNA methylation status of *GDAP1* in a cohort of 49 alcohol dependent patients entering an alcohol treatment program and 37 healthy control individuals. In addition, we studied *GDAP1* DNA methylation after 3 weeks of participating in an inpatient alcohol treatment program to elucidate whether *GDAP1* DNA methylation could also serve as an epigenetic biomarker of treatment response.

Table 1. Characterization of patients and control individuals. Errors are given as standard deviation (SD). Amount of drinks is the standardized unit originating from the AUDIT questionnaire.

	Control individuals	Patients	*P*-value
Total N	37	49	
Age (years)	47 ± 12.32	49 ± 10.47	0.30
Smokers (% of total)	29 (78%)	38 (79%)	0.93
Cigarettes smoked daily	16 ± 10.99	20 ± 10.93	0.18
Days since last drink		2.9 ± 6.9	
Amount of drinks consumed daily one week before hospital admission		17 ± 13.1	
Years of alcohol dependency		12.3 ± 9.9	

Results

Lower GDAP1 *DNA methylation in patients at the beginning of the alcohol treatment (T1) compared to control individuals*

The demographic characteristics as well as nicotine and alcohol consumption of our cohort is provided in Table 1.

Control individuals and patients did not differ significantly in age (patients: 49 ± 10.47 years, control individuals: 47 ± 12.32 years; $P = 0.3$) or smoking behavior (control individuals: 16 ± 10.99 cigarettes per day, patients: 20 ± 10.93; $P = 0.18$). AUDIT scores differed significantly between control individuals (4.9 ± 3.7; $P = 5.1E-15$) and patients (25.1 ± 6.1) as well as the GSI scores (0.16 ± 0.13 for control individuals, 0.78 ± 0.54 for patients; $P = 1.7E-10$).

For all 3 sites analyzed, DNA methylation levels between control individuals and patients at T1 differed significantly (Table 2 and Fig. 1). For cg23779890 / site 1, the CpG site identified by Philibert et al.,[32] DNA methylation levels were as follows: 7.8 ± 0.2 in control individuals, 6.6 ± 0.3 in patients, $P = 0.001$. For site 2, DNA methylation levels were 4.0 ± 0.1 in control individuals and 3.6 ± 0.1 in patients, $P = 0.015$. For site 3, DNA methylation levels were 2.1 ± 0.1 in control individuals and 1.8 ± 0.1 in patients, $P = 0.012$.

Mean DNA methylation across all 3 sites differed significantly between control individuals and patients (control individuals: 4.6 ± 0.1, patients: 4.0 ± 0.2; $P = 0.001$).

Since the DNA methylation levels of each site were highly correlated with the mean DNA methylation level across all sites (site 1: $r_s = 0.979$, $P = 2.0E-82$; site 2: $r_s = 0.938$, $P = 1.4E-55$; site 3: $r_s = 0.892$, $P = 4.0E-42$), we decided to use the mean DNA methylation levels for further analyses.

First, comparing control individuals and patients at T1, the mean DNA methylation level was significantly negatively associated with the GSI score ($r_s = -0.2066$, $P = 0.016$), and AUDIT Score ($r_s = -0.2041$, $P = 0.009$). Furthermore, a trend toward a negative association between the mean DNA methylation level and the years of dependency ($r_s = 0.266$, $P = 0.08$) was observed. We did not find any association between the DNA methylation levels and the amount of drinks consumed daily in the week before hospital admission ($r_s = -0.1038$, $P = 0.35$).

Alcohol treatment significantly influences GDAP1 *DNA methylation levels in alcohol dependent patients*

After three weeks of alcohol treatment (T2), DNA methylation levels at all 3 sites were increased (Fig. 1, Table 3): 7.3 ± 0.3

Table 2. DNA methylation levels, AUDIT and GSI scores for control individuals vs. patients at T1. DNA Methylation level errors are given as standard error of the mean (SE), questionnaire score errors are given as standard deviation (SD).

	Control individuals	Patients at T1	*P*-value
Site 1 (cg23779890)	7.8 ± 0.2	6.6 ± 0.3	0.001
Site 2	4.0 ± 0.1	3.6 ± 0.1	0.015
Site 3	2.1 ± 0.1	1.9 ± 0.1	0.012
Mean DNA methylation (sites 1 – 3)	4.6 ± 0.1	4.0 ± 0.2	0.001
AUDIT score	4.9 ± 3.7	25.1 ± 6.1	5.1E-15
GSI score	0.16 ± 0.13	0.78 ± 0.54	1.7E-10

(site 1, $P = 0.001$), 3.8 ± 0.1 (site 2, $P = 0.033$) and 2.0 ± 0.1 (site 3, $P = 0.001$). Again, the mean DNA methylation across these sites differed significantly (4.4 ± 0.2, $P = 0.001$) from the values of patients at T1 (4.0 ± 0.2) and were highly correlated with each other (site 1: $r_s = 0.972$, $P = 4.6E\text{-}21$; site 2: $r_s = 0.901$, $P = 9.1E\text{-}13$; site 3: $r_s = 0.877$, $P = 2.2E\text{-}11$). In addition, the GSI score decreased significantly (0.78 ± 0.54 vs. 0.48 ± 0.49, $P = 0.008$, N = 30), as well as the OCDS score (3.93 ± 1.32 vs. 2.71 ± 0.96, $P = 2.1E\text{-}5$, N = 33).

Comparing the mean DNA methylation across all 3 sites between the control individuals and patients at T2, none of the DNA methylation levels differed significantly (site 1: $P = 0.098$; site 2: $P = 0.244$; site 3: 0.377; mean: $P = 0.167$).

The exclusion of 8 patients who had been abstinent for more than 3 d before hospital admission led to a diminishment of the days since the last drink from 2.9 ± 6.9 d to only 1.3 ± 0.8 d. Furthermore, it enhanced the observed effect of differential *GDAP1* methylation between control individuals and patients at T1. At T2, this only had a moderately positive effect (see Table S1).

Discussion

By conducting pyrosequencing of 3 adjacent CpG sites in *GDAP1*, including cg23779890, we were able to replicate the finding of significant differences in DNA methylation between alcohol dependent patients and matched control individuals previously reported by Philibert et al.[32] In addition, we identified significant differences between *GDAP1* DNA methylation levels in patients at the day of hospital admission (T1) and after

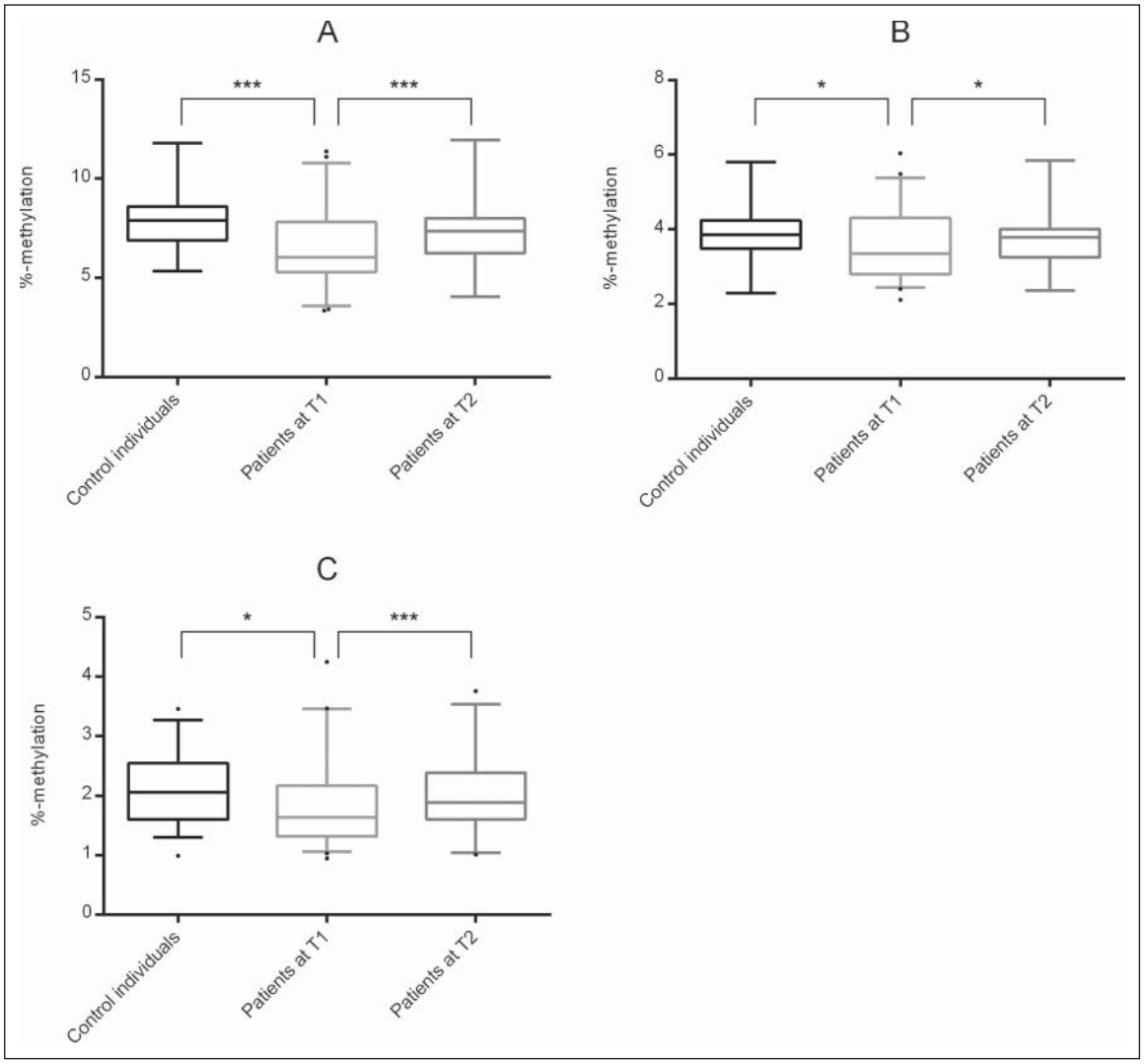

Figure 1. DNA methylation levels at (A) site 1 / cg23779890, (B) site 2 and (C) site 3 for control individuals, patients at T1 and patients at T2. Significant differences are indicated with * ($P \leq 0.05$) and *** ($P \leq 0.001$).

Table 3. DNA methylation levels, OCDS and GSI scores for patients at T1 vs. patients at T2. DNA Methylation level errors are given as standard error of the mean (SE), questionnaire score errors are given as standard deviation (SD).

	Patients at T1	Patients at T2	*P*-value
Site 1 (cg23779890)	6.6 ± 0.3	7.3 ± 0.3	0.001
Site 2	3.6 ± 0.1	3.8 ± 0.1	0.033
Site 3	1.9 ± 0.1	2.0 ± 0.1	0.001
Mean DNA methylation(sites 1 – 3)	4.0 ± 0.2	4.4 ± 0.2	0.001
OCDS score	3.93 ± 1.32	2.71 ± 0.96	2.1E-5
GSI score	0.78 ± 0.54	0.48 ± 0.49	0.008

3 weeks of attending an inpatient alcohol treatment program (T2). Furthermore, the AUDIT score as well as the GSI score at T1 were negatively associated with the DNA methylation levels and we found a trend toward a negative association between the DNA methylation levels and the years of alcohol dependency, but not with the amount of drinks consumed in the week before hospital admission. Our study thus provides additional evidence supporting the hypothesis that *GDAP1* DNA methylation could serve as new biomarker for the severity of alcohol dependence.

In contrast to the hypothesis of increased DNA methylation levels in alcohol dependent patients due to higher levels of homocysteine,[24] our results, as well as the previous results from Philibert et al. [32] show a hypomethylation of the *GDAP1* gene in patients compared to control individuals. This was surprising, but as we did not measure homocysteine levels in our study samples, we can neither support nor contradict a potential correlation between homocysteine levels and DNA methylation of the *GDAP1* gene promoter. Nevertheless, other studies did not find a correlation between homocysteine and global DNA methylation, and some did find a hypothesis-opposing outcome: with higher homocysteine levels in their samples, global DNA methylation was decreased.[41] Other studies speculate that the missing regulation of the methionine adenosyl transferase in alcohol dependent patients results in global DNA hypomethylation.[25,26] A recent study specifically investigating the role of homocysteine in altered DNA methylation in 363 alcohol dependent patients also found no correlation between homocysteine and global DNA methylation.[42] Further studies are therefore necessary to clarify the relationship between homocysteine levels and *GDAP1* DNA methylation.

Furthermore, we observe that increased severity of alcohol dependence in patients, assessed by the AUDIT score, as well as the GSI score, is associated with lower *GDAP1* DNA methylation. However, we did not find a correlation between the amount of alcohol consumed one week before admission to the hospital and *GDAP1* DNA methylation. As the exact amount of alcohol consumed one week before hospital admission does not affect *GDAP1* DNA methylation, but rather the intensity and time span of alcohol dependence, *GDAP1* DNA methylation could serve as an indicator of long-term and severe alcohol dependence rather than for short-term alcohol exposure.

Whereas Philibert et al. did not identify significant differences in *GDAP1* DNA methylation in patients between T1 and T2, our results show an increase in DNA methylation levels in patients at T2, which no longer differed from the levels in control individuals. Our finding therefore supports the hypothesis of DNA methylation as a reversible process and suggests that DNA methylation levels return to their previous state, if the environmental condition underlying the epigenetic alteration—in this case alcohol dependence—is amended. However, to prove this hypothesis, it would be necessary to perform a longitudinal study and compare *GDAP1* DNA methylation in patients before and after the onset of the disease. In our study, we only included patients after disease onset (mean years of dependency: 12.3 ± 9.9 years). After three weeks of attending an inpatient alcohol treatment program, the GSI score as well as the OCDS score, a measure of craving severity, decreased significantly in our patient cohort, suggesting a positive therapy outcome. Questionnaires are the most common means to assess these traits but are not an objective measure as they can be subjectively biased. The reversion of *GDAP1* DNA methylation levels during abstinence could therefore serve as a biological, more objective indicator of a positive therapy outcome.

Although DNA methylation percentages in our study did deviate from the ones reported by Philibert and colleagues,[32] we are able to replicate and validate the effect of alcohol dependence on *GDAP1* DNA methylation. Philibert et al. found the mean DNA methylation level of cg23779890 to be 19.4% in patients and 24.3% in control individuals. We measured DNA methylation levels of 6.6% and 7.8%, respectively. These differences could be explained as follows.

One major difference between these studies is the source of material. Philibert and colleagues used mononuclear cells, whereas we used DNA prepared from whole blood. Whole blood is a heterogeneous mixture of different cell-types and blood composition varies from individual to individual and is depending on numerous factors such as age, sex, and individual health status. This is of importance as DNA methylation patterns are cell-type specific and could therefore explain the differences in DNA methylation levels between our study and the study by Philibert et al.[32]

The use of whole blood could be seen as a limitation of our study. However, we have explicitly chosen to investigate *GDAP1* DNA methylation in whole blood to serve as an epigenetic biomarker for alcohol dependence and as a potential gauge of the therapy efficacy in a clinical setting. To be suitable as a biomarker, the study material needs to be easy and cost effective to obtain. The preparation and use of mononuclear cells for clinical diagnostics is impossible, as it is very time-consuming and labor-intensive in addition to being more expensive than the usage of whole blood.

In addition, as both the Illumina's 450K Chip as well as the pyrosequencing approach have systematic biases the differences could also be explained by the different methods used. As the overall congruency between Illumina's 450K Chip and pyrosequencing data is good, there are however specific sites where a direct translation from β-values originating from the Chip analysis to DNA methylation levels measured by pyrosequencing is difficult.[43] These include, but are not limited to non-specific and cross-hybridizing probes, which represent a combination of multiple loci and therefore can elevate readings of low methylation or diminish readings of high methylation, biasing the results.[44] Another well-known limitation of pyrosequencing is amplification bias. To account for this and to prevent batch effects, the samples were run at least in duplicates and they were assigned to different positions on different plates.

However, the overall effect in DNA methylation changes between patients at T1 and control individuals in both studies is very similar despite being obtained in 2 distinct cohorts using different methods (450K Chip analysis vs. pyrosequencing) as well as different sources of DNA: Philibert et al. found a 4.9% higher DNA methylation in control individuals compared to patients; our data show a 1.2% higher DNA methylation. These data indicate that indeed *GDAP1* DNA methylation levels obtained from whole blood are usable as potential epigenetic biomarkers of alcohol dependence severity. Although the differences in DNA methylation are quite small, the fact that they can be found in different populations, different tissue as well as with different analytical methods suggests that *GDAP1* DNA methylation could serve as biological predictor of alcohol dependence, especially in combination with epigenetic data of other genes of known influence. Unfortunately, we were not able to collect a second sample after 3 weeks from those patients, who did not complete the alcohol treatment. Without having obtained the DNA methylation levels for this group, we can only speculate that *GDAP1* DNA methylation could also serve as a biomarker for treatment outcome. Measuring DNA methylation levels at a second time point from patients, who do not complete the alcohol treatment, should be taken into consideration in future studies.

In contrast to Philibert et al., we used a slightly different matching strategy: The cohort used for this study only consists of Caucasian men, and patients and control individuals were matched for age and smoking behavior. The cohort investigated by Philibert et al. is more heterogeneous, consisting of both sexes and different ethnicities. Furthermore, 27 patients were daily smokers, whereas only one control individual was a daily smoker. This is problematic, because smoking has a major influence on DNA methylation patterns.[45] The authors take this limitation of their study into consideration by comparing the overlap of their 10000 most significant probes to the 910 epigenome-wide significant genes found by Dogan et al., who evaluated the effect of smoking on DNA methylation.[45] Only 22 significant hits were overlapping between the 2 studies, leading Philibert et al. to the conclusion that the effects they are reporting are indeed due to alcohol consumption, and are not biased by differences in smoking behavior. However, this approach is based on the assumption that Dogan et al. were able to identify all genes epigenetically altered by smoking, which is highly unlikely. Furthermore, this strategy does not take into account potential overlapping effects of both smoking and alcohol consumption, which displays a high comorbidity and would therefore have to be further evaluated.

As already mentioned, no associations between the outer mitochondrial membrane protein *GDAP1* and alcohol dependence have been reported thus far. Mutations in *GDAP1* cause Charcot-Marie-Tooth (CMT) disease, a hereditary motor, and sensory neuropathy.[46] The 2 major causes leading to CMT disease are mutations in *PMP22* and *MFN2*, which directly affect the myelin sheath and the axon.[47,48] Mutations in *GDAP1* are associated with decreased mitochondrial fission activity (recessively inherited) or an impairment of mitochondrial fusion (dominantly inherited).[39,40,49] The expression of dominantly inherited mutated forms of *GDAP1* lead to increased production of reactive oxygen species (ROS).[50] Furthermore, wild type *GDAP1* has been reported to protect against oxidative stress.[51] As the production of ROS also is a direct effect of alcohol intake,[52] this could be a potential link explaining *GDAP1* hypomethylation in alcohol dependent patients: DNA hypomethylation should lead to increased expression and consequently increased protein production in alcohol dependent patients. Therefore, *GDAP1* overexpression could counteract and compensate for the increased oxidative stress in alcohol dependence. This hypothesis is supported by the fact that DNA methylation levels rise after 3 weeks of alcohol treatment. In this time period, oxidative stress in alcohol dependent patients should be dramatically reduced. However, as we neither measured *GDAP1* expression, nor GDAP1 protein levels or the levels of ROS, this hypothesis should be followed up in subsequent studies. Further links between CMT and alcohol dependence are provided by recent studies, showing that a triple-therapy with a combination of naltrexone, baclofen, and sorbitol (PXT3003) can improve health of patients suffering from CMT disease.[53,54] While PXT3003 was shown to downregulate PMP22 mRNA expression and improve myelination as well as axonal regeneration,[53] both naltrexone and baclofen are also used (partly off-label) to treat alcohol dependence.[55] Acting as an opioid antagonist (naltrexone) and a GABA-B-receptor agonist (baclofen), respectively, these drugs reduce the rewarding effects of alcohol and inhibit dopaminergic neurotransmission.[56] Whether GDAP1 is also influenced by naltrexone and/or baclofen requires investigation.

The *GDAP1* gene is regulated by the transcription factor YY1.[57] Up to date, there is no evidence linking YY1 to alcohol dependence. However, other putative transcription factor binding sites include the binding sites of EGR1 and ZNF143, among others, as analyzed with JASPAR.[58] For both transcription factors, a potential link to alcohol dependence, such as alcoholic fatty liver disease [59] or the regulation of aldehyde reductase [60] has been previously reported. The lack of functional data is a limitation of our study. Therefore, future studies are needed to better understand the regulation of *GDAP1* as well as its function in the context of alcohol dependence and to investigate the impact of altered DNA methylation on gene expression.

In conclusion, in the present study we were able to validate and replicate the finding of *GDAP1* being significantly hypomethylated in alcohol dependent patients compared to healthy control individuals, which was previously discovered in an epigenome-wide association study.[32] Furthermore, we show that these differences in DNA methylation diminish after 3 weeks of abstinence, leading us to the conclusion that *GDAP1* DNA methylation could serve as a possible epigenetic biomarker for severity of alcohol dependence and potentially for treatment outcome. Our data add to the growing body of knowledge on epigenetic effects in alcohol dependence and support *GDAP1* as a novel candidate gene implicated in alcohol dependence. However, future studies are needed to replicate our finding of epigenetic changes in *GDAP1* during alcohol treatment in independent cohorts, as well as to clarify potential mechanisms of action.

Subjects & methods

This sample was comprised of 49 male patients (mean age 49.14 ± 10.47 years) with a diagnosis of alcohol dependence according to the fourth edition of the Diagnostic and Statistical

Manual of Mental Disorders (DSM-IV) participating in a 3- or 6-week alcohol treatment program at the Clinic for Psychiatry and Psychotherapy, Tuebingen. Subjects with any other addiction except nicotine have been excluded, as well as subjects with any other psychiatric comorbidity necessitating psychiatric medication. Except for 8 patients, the last exposure to alcohol before entering the study had not exceeded 72 h. For the first days of detoxification, clomethiazole was administered if necessary. Population-based male control individuals (n = 37, mean age 47.41 ± 12.32 years) were recruited from the city of Tuebingen, Germany and the surrounding area. Control individuals were matched for age and smoking behavior. Phenotypic information about patients and control individuals was obtained by self-administered questionnaires. The following questionnaires were used in patients: Alcohol consumption was assessed using the AUDIT,[61] alcohol craving using the OCDS [62] and the global distress level (GSI) using the SCL-90-R.[63] The SCL-90-R and OCDS were repeated after 21 d of detoxification (T2). Control individuals were screened for problematic alcohol intake using the AUDIT questionnaire, and control individuals with an AUDIT-Score > 15 were excluded, as a higher value is suggestive for alcohol dependence.[28] The SCL-90-R questionnaire was used in control individuals as well, and in addition, demographic information and health status of both—patients and control individuals—was assessed. All subjects were Caucasian and provided written informed consent prior to participation. The study was approved by the ethics committee of the University of Tuebingen and was conducted in accordance with the Declaration of Helsinki.

Ethylenediaminetetraacetic (EDTA) peripheral venous blood samples were taken from all patients immediately after hospital admission (T1). After 21 d (± 2 d) of treatment (T2), a second EDTA-blood sample was taken from the 33 patients (mean age 48.7 ± 10.92 years) who remained in the program (drop-out rate: 33%). EDTA-blood from control individuals was drawn immediately after study inclusion. Blood samples were instantly frozen and kept at −80°C until further usage. DNA was extracted using the QIAamp DNA Blood Maxi Kit (Qiagen). Genomic DNA (500 ng) was bisulfite converted using the EpiTect Fast Bisulfite Conversion Kit (Qiagen) according to the manufacturer's protocol with the following adjustments: incubation steps at 60°C were prolonged to 15 min (instead of 10 min); converted DNA was eluted in 20 μl instead of 15 μl and stored at −20°C until analysis.

Pyrosequencing was performed as follows: A 166 bp fragment covering the TSS200 region of *GDAP1* and partially overlapping the transcription start site was amplified by PCR from 2 μl bisulfite-treated DNA using the PCR Primer Set from the PyroMark CpG Assay *GDAP1* (PM00035399) and the PyroMark PCR Kit (both Qiagen) according to manufacturer's protocol. The CpG Assay *GDAP1* covers 3 CpG sites located within chromosome 8 (site 1 located at 75,262,523, 95 bp upstream of the TSS; site 2 located at 75,262,532, 86 bp upstream of the TSS; and site 3 located at 75,262,534, 84 bp upstream of the TSS) and includes the CpG site cg23779890 (site 1) which has been previously implicated by Philibert et al.[32] The 3 CpG sites are part of a larger CpG island including 48 CpG sites (chr8:75,262,522-75,263,044).

Cycling conditions were as follows: 95°C for 15 min; 94°C for 30 s, 56°C for 30 s, 72°C for 30 s (45 cycles); 72°C for 10 min. To detect potentially biased amplification of differentially methylated fragments, DNA samples with known methylation levels (0%, 25%, 50%, 75%, and 100%) were included as controls (EpiTect Control DNA, Qiagen) in the amplification and the pyrosequencing reaction.

PCR products and a no template control were visualized on a 2% agarose gel to verify successful amplification and specificity of the products. Processing of the PCR amplicons for the pyrosequencing analysis was performed in accordance with the manufacturer's protocol and PCR products were then pyrosequenced using the PyroMark Q24 system (Qiagen) and the sequencing primer from the PyroMark CpG Assay *GDAP1* (PM00035399). The percentage of methylation at each of the 3 CpG sites analyzed was quantified using the PyroMark Q24 software version 2.0.6 (Qiagen). Pyrosequencing was performed in duplicates. To avoid plate effects, samples from patients and control individuals were mixed on each plate and the samples were randomly assigned to different wells for the 2 sequencing runs. For quality control the coefficient of variance (CV) was calculated. For the 33 samples (18 control individual samples, 14 patient samples at T1 and 1 patient sample at T2) where the CV between 2 runs for any site was ≥ 0.3, a third measurement was obtained. The outlier was eliminated from further analysis, and only the 2 remaining values were used. Using this approach led to a maximum variation of 2.02%. Typically, an intra-sample variation of ≤ 3% is considered reliable.

Statistical analysis was conducted using SPSS version 21.0 (IBM). Each site was examined individually. DNA methylation levels were not normally distributed according to the Shapiro-Wilk test. Hence, non-parametric test methods were applied. Differences in the percentage of DNA methylation between the patient group and the control group were analyzed using the Mann-Whitney U test. For identifying differences in DNA methylation, GSI score, and OCDS score between the 2 time points T1 and T2 of the patients, the Wilcoxon test was used. Correlations between continuous variables were tested using the Spearman correlation test. A significance level of $P \leq 0.05$ was considered significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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References

1. Agrawal A, Lynskey MT. Are there genetic influences on addiction: evidence from family, adoption and twin studies. Addiction 2008; 103:1069-81; PMID:18494843; http://dx.doi.org/10.1111/j.1360-0443.2008.02213.x
2. Heath AC, Bucholz KK, Madden PA, Dinwiddie SH, Slutske WS, Bierut LJ, Statham DJ, Dunne MP, Whitfield JB, Martin NG. Genetic and environmental contributions to alcohol dependence risk in a national twin sample: consistency of findings in women and men. Psychological Med 1997; 27:1381-96; PMID:9403910; http://dx.doi.org/10.1017/S0033291797005643
3. Agrawal A, Lynskey MT, Todorov AA, Schrage AJ, Littlefield AK, Grant JD, Zhu Q, Nelson EC, Madden PA, Bucholz KK, et al. A candidate gene association study of alcohol consumption in young women. Alcohol Clin Exp Res 2011; 35:550-8; PMID:21143251; http://dx.doi.org/10.1111/j.1530-0277.2010.01372.x
4. Laucht M, Treutlein J, Schmid B, Blomeyer D, Becker K, Buchmann AF, Schmidt MH, Esser G, Jennen-Steinmetz C, Rietschel M, et al. Impact of psychosocial adversity on alcohol intake in young adults: moderation by the LL genotype of the serotonin transporter polymorphism. Biological psychiatry 2009; 66:102-9; PMID:19358979; http://dx.doi.org/10.1016/j.biopsych.2009.02.010
5. Nelson EC, Agrawal A, Pergadia ML, Wang JC, Whitfield JB, Saccone FS, Kern J, Grant JD, Schrage AJ, Rice JP, et al. H2 haplotype at chromosome 17q21.31 protects against childhood sexual abuse-associated risk for alcohol consumption and dependence. Addict Biol 2010; 15:1-11; PMID:19878140; http://dx.doi.org/10.1111/j.1369-1600.2009.00181.x
6. Blomeyer D, Treutlein J, Esser G, Schmidt MH, Schumann G, Laucht M. Interaction between CRHR1 gene and stressful life events predicts adolescent heavy alcohol use. Biological psychiatry 2008; 63:146-51; PMID:17597588; http://dx.doi.org/10.1016/j.biopsych.2007.04.026
7. Nieratschker V, Batra A, Fallgatter AJ. Genetics and epigenetics of alcohol dependence. J Mol Psychiatry 2013; 1:11; PMID:25408904; http://dx.doi.org/10.1186/2049-9256-1-11
8. Sutherland JE, Costa M. Epigenetics and the environment. Ann N Y Acad Sci 2003; 983:151-60.
9. Hashimoto H, Vertino PM, Cheng X. Molecular coupling of DNA methylation and histone methylation. Epigenomics 2010; 2:657-69; PMID:21339843; http://dx.doi.org/10.2217/epi.10.44
10. Holliday R. DNA methylation and epigenetic mechanisms. Cell biophysics 1989; 15:15-20; PMID:2476223; http://dx.doi.org/10.1007/BF02991575
11. Wang Y, Leung FC. An evaluation of new criteria for CpG islands in the human genome as gene markers. Bioinformatics 2004; 20:1170-7; PMID:14764558; http://dx.doi.org/10.1093/bioinformatics/bth059
12. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. Nature 2004; 429:457-63; PMID:15164071; http://dx.doi.org/10.1038/nature02625
13. Doerfler W. DNA methylation and gene activity. Ann Rev Biochem 1983; 52:93-124; PMID:6311083; http://dx.doi.org/10.1146/annurev.bi.52.070183.000521
14. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet 2012; 13:484-92; PMID:22641018; http://dx.doi.org/10.1038/nrg3230
15. Feil R, Fraga MF. Epigenetics and the environment: emerging patterns and implications. Nat Rev Genet 2011; 13:97-109; PMID: 22215131; http://dx.doi.org/10.1038/nrg3142
16. Mill J, Heijmans BT. From promises to practical strategies in epigenetic epidemiology. Nat Rev Genet 2013; 14:585-94; PMID: 23817309; http://dx.doi.org/10.1038/nrg3405
17. Januar V, Saffery R, Ryan J. Epigenetics and depressive disorders: a review of current progress and future directions. Int J Epidemiol 2015; 44 (4):1364-1387; PMID:25716985; http://dx.doi.org/10.1093/ije/dyu273
18. Abdolmaleky HM, Smith CL, Zhou JR, Thiagalingam S. Epigenetic alterations of the dopaminergic system in major psychiatric disorders. Methods Mol Biol 2008; 448:187-212; PMID:18370235; http://dx.doi.org/10.1007/978-1-59745-205-2_9
19. Plazas-Mayorca MD, Vrana KE. Proteomic investigation of epigenetics in neuropsychiatric disorders: a missing link between genetics and behavior? J Proteome Res 2011; 10:58-65; PMID:20735116; http://dx.doi.org/10.1021/pr100463y
20. Bleich S, Degner D, Javaheripour K, Kurth C, Kornhuber J. Homocysteine and alcoholism. J Neural Transm Suppl 2000:187-96; PMID:11205139; http://dx.doi.org/10.1007/978-3-7091-6301-6_12
21. Bleich S, Degner D, Wiltfang J, Maler JM, Niedmann P, Cohrs S, Mangholz A, Porzig J, Sprung R, Ruther E, et al. Elevated homocysteine levels in alcohol withdrawal. Alcohol and alcoholism 2000; 35:351-4; PMID:10905999; http://dx.doi.org/10.1093/alcalc/35.4.351
22. Heese P, Linnebank M, Semmler A, Muschler MA, Heberlein A, Frieling H, Stoffel-Wagner B, Kornhuber J, Banger M, Bleich S, et al. Alterations of homocysteine serum levels during alcohol withdrawal are influenced by folate and riboflavin: results from the German Investigation on Neurobiology in Alcoholism (GINA). Alcohol and alcoholism 2012; 47:497-500; PMID:22645037; http://dx.doi.org/10.1093/alcalc/ags058
23. Bleich S, Hillemacher T. Homocysteine, alcoholism and its molecular networks. Pharmacopsychiatry 2009; 42 Suppl 1:S102-9; PMID: 19434547; http://dx.doi.org/10.1055/s-0029-1214396
24. Bonsch D, Lenz B, Reulbach U, Kornhuber J, Bleich S. Homocysteine associated genomic DNA hypermethylation in patients with chronic alcoholism. J Neural Transm 2004; 111:1611-6; PMID:15565495; http://dx.doi.org/10.1007/s00702-004-0232-x
25. French SW. Epigenetic events in liver cancer resulting from alcoholic liver disease. Alcohol Res 2013; 35:57-67; PMID:24313165
26. Varela-Rey M, Woodhoo A, Martinez-Chantar ML, Mato JM, Lu SC. Alcohol, DNA methylation, and cancer. Alcohol Res 2013; 35:25-35; PMID:24313162
27. Philibert RA, Gunter TD, Beach SR, Brody GH, Madan A. MAOA methylation is associated with nicotine and alcohol dependence in women. Am J Med Genet B Neuropsychiatr Genet 2008; 147B:565-70; PMID:18454435; http://dx.doi.org/10.1002/ajmg.b.30778
28. Nieratschker V, Grosshans M, Frank J, Strohmaier J, von der Goltz C, El-Maarri O, Witt SH, Cichon S, Nothen MM, Kiefer F, et al. Epigenetic alteration of the dopamine transporter gene in alcohol-dependent patients is associated with age. Addiction biology 2014; 19:305-11; PMID:22506971; http://dx.doi.org/10.1111/j.1369-1600.2012.00459.x
29. Philibert RA, Sandhu H, Hollenbeck N, Gunter T, Adams W, Madan A. The relationship of 5HTT (SLC6A4) methylation and genotype on mRNA expression and liability to major depression and alcohol dependence in subjects from the Iowa Adoption Studies. Am J Med Genet B Neuropsychiatr Genet 2008; 147B:543-9; PMID:17987668; http://dx.doi.org/10.1002/ajmg.b.30657
30. Heberlein A, Muschler M, Frieling H, Behr M, Eberlein C, Wilhelm J, Groschl M, Kornhuber J, Bleich S, Hillemacher T. Epigenetic down regulation of nerve growth factor during alcohol withdrawal. Addiction biology 2013; 18:508-10; PMID:21392176; http://dx.doi.org/10.1111/j.1369-1600.2010.00307.x
31. Hillemacher T, Weinland C, Lenz B, Kraus T, Heberlein A, Glahn A, Muschler MA, Bleich S, Kornhuber J, Frieling H. DNA methylation of the LEP gene is associated with craving during alcohol withdrawal. Psychoneuroendocrinology 2015; 51:371-7; PMID:25462909; http://dx.doi.org/10.1016/j.psyneuen.2014.10.014
32. Philibert RA, Penaluna B, White T, Shires S, Gunter T, Liesveld J, Erwin C, Hollenbeck N, Osborn T. A pilot examination of the genome-wide DNA methylation signatures of subjects entering and exiting short-term alcohol dependence treatment programs. Epigenetics 2014; 9:1212-9; PMID:25147915; http://dx.doi.org/10.4161/epi.32252
33. Ruggeri B, Nymberg C, Vuoksimaa E, Lourdusamy A, Wong CP, Carvalho FM, Jia T, Cattrell A, Macare C, Banaschewski T, et al. Association of Protein Phosphatase PPM1G With Alcohol Use sDisorder and Brain Activity During Behavioral Control in a Genome-Wide Methylation Analysis. Am J Psychiatry 2015;

172:543-52; PMID:25982659; http://dx.doi.org/10.1176/appi.ajp.2014.14030382
34. Zhang R, Miao Q, Wang C, Zhao R, Li W, Haile CN, Hao W, Zhang XY. Genome-wide DNA methylation analysis in alcohol dependence. Addiction biology 2013; 18:392-403; PMID:23387924; http://dx.doi.org/10.1111/adb.12037
35. Clark SL, Aberg KA, Nerella S, Kumar G, McClay JL, Chen W, Xie LY, Harada A, Shabalin AA, Gao G, et al. Combined Whole Methylome and Genomewide Association Study Implicates CNTN4 in Alcohol Use. Alcohol Clin Exp Res 2015; 39:1396-405; PMID:26146898; http://dx.doi.org/10.1111/acer.12790
36. Hultberg B, Berglund M, Andersson A, Frank A. Elevated plasma homocysteine in alcoholics. Alcohol Clin Exp Res 1993; 17:687-9; PMID:8392819; http://dx.doi.org/10.1111/j.1530-0277.1993.tb00820.x
37. Wedekind D, Neumann K, Falkai P, Malchow B, Engel KR, Jamrozinski K, Havemann-Reinecke U. S100B and homocysteine in the acute alcohol withdrawal syndrome. Eur Arch Psychiatry Clin Neurosci 2011; 261:133-8; PMID:20593192; http://dx.doi.org/10.1007/s00406-010-0121-2
38. Biermann T, Reulbach U, Lenz B, Frieling H, Muschler M, Hillemacher T, Kornhuber J, Bleich S. N-methyl-D-aspartate 2b receptor subtype (NR2B) promoter methylation in patients during alcohol withdrawal. J Neural Transm (Vienna) 2009; 116:615-22; PMID:19350219; http://dx.doi.org/10.1007/s00702-009-0212-2
39. Barneo-Munoz M, Juarez P, Civera-Tregon A, Yndriago L, Pla-Martin D, Zenker J, Cuevas-Martin C, Estela A, Sanchez-Arago M, Forteza-Vila J, et al. Lack of GDAP1 induces neuronal calcium and mitochondrial defects in a knockout mouse model of charcot-marie-tooth neuropathy. PLoS Genet 2015; 11:e1005115; PMID:25860513; http://dx.doi.org/10.1371/journal.pgen.1005115
40. Tazir M, Hamadouche T, Nouioua S, Mathis S, Vallat JM. Hereditary motor and sensory neuropathies or Charcot-Marie-Tooth diseases: an update. J Neurological Sci 2014; 347:14-22; PMID:25454638; http://dx.doi.org/10.1016/j.jns.2014.10.013
41. Yi P, Melnyk S, Pogribna M, Pogribny IP, Hine RJ, James SJ. Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation. J Biol Chem 2000; 275:29318-23; PMID:10884384; http://dx.doi.org/10.1074/jbc.M002725200
42. Semmler A, Heese P, Stoffel-Wagner B, Muschler M, Heberlein A, Bigler L, Prost JC, Frieling H, Kornhuber J, Banger M, et al. Alcohol abuse and cigarette smoking are associated with global DNA hypermethylation: results from the German Investigation on Neurobiology in Alcoholism (GINA). Alcohol 2015; 49:97-101; PMID:25702197; http://dx.doi.org/10.1016/j.alcohol.2015.01.004
43. Roessler J, Ammerpohl O, Gutwein J, Hasemeier B, Anwar SL, Kreipe H, Lehmann U. Quantitative cross-validation and content analysis of the 450k DNA methylation array from Illumina, Inc. BMC Res Notes 2012; 5:210; PMID:22546179; http://dx.doi.org/10.1186/1756-0500-5-210
44. Dedeurwaerder S, Defrance M, Bizet M, Calonne E, Bontempi G, Fuks F. A comprehensive overview of Infinium HumanMethylation450 data processing. Brief Bioinform 2014; 15:929-41; PMID:23990268; http://dx.doi.org/10.1093/bib/bbt054
45. Dogan MV, Shields B, Cutrona C, Gao L, Gibbons FX, Simons R, Monick M, Brody GH, Tan K, Beach SR, et al. The effect of smoking on DNA methylation of peripheral blood mononuclear cells from African American women. BMC Genom 2014; 15:151; PMID:24559495; http://dx.doi.org/10.1186/1471-2164-15-151
46. Krajewski KM, Lewis RA, Fuerst DR, Turansky C, Hinderer SR, Garbern J, Kamholz J, Shy ME. Neurological dysfunction and axonal degeneration in Charcot-Marie-Tooth disease type 1A. Brain 2000; 123 (Pt 7):1516-27; PMID:10869062; http://dx.doi.org/10.1093/brain/123.7.1516
47. Watila MM, Balarabe SA. Molecular and clinical features of inherited neuropathies due to PMP22 duplication. J Neurological Sci 2015; 355:18-24; PMID:26076881; http://dx.doi.org/10.1016/j.jns.2015.05.037
48. Cartoni R, Martinou JC. Role of mitofusin 2 mutations in the physiopathology of Charcot-Marie-Tooth disease type 2A. Exp Neurol 2009; 218:268-73; PMID:19427854; http://dx.doi.org/10.1016/j.expneurol.2009.05.003
49. Kabzinska D, Kotruchow K, Cegielska J, Hausmanowa-Petrusewicz I, Kochanski A. A severe recessive and a mild dominant form of Charcot-Marie-Tooth disease associated with a newly identified Glu222Lys GDAP1 gene mutation. Acta biochimica Polonica 2014; 61:739-44; PMID:25337607
50. Niemann A, Wagner KM, Ruegg M, Suter U. GDAP1 mutations differ in their effects on mitochondrial dynamics and apoptosis depending on the mode of inheritance. Neurobiol Dis 2009; 36:509-20; PMID:19782751; http://dx.doi.org/10.1016/j.nbd.2009.09.011
51. Noack R, Frede S, Albrecht P, Henke N, Pfeiffer A, Knoll K, Dehmel T, Meyer Zu Horste G, Stettner M, Kieseier BC, et al. Charcot-Marie-Tooth disease CMT4A: GDAP1 increases cellular glutathione and the mitochondrial membrane potential. Hum Mol Genet 2012; 21:150-62; PMID:21965300; http://dx.doi.org/10.1093/hmg/ddr450
52. Gonzalez-Reimers E, Santolaria-Fernandez F, Martin-Gonzalez MC, Fernandez-Rodriguez CM, Quintero-Platt G. Alcoholism: a systemic proinflammatory condition. World J Gastroenterol 2014; 20:14660-71; PMID:25356029; http://dx.doi.org/10.3748/wjg.v20.i40.14660
53. Chumakov I, Milet A, Cholet N, Primas G, Boucard A, Pereira Y, Graudens E, Mandel J, Laffaire J, Foucquier J, et al. Polytherapy with a combination of three repurposed drugs (PXT3003) down-regulates Pmp22 over-expression and improves myelination, axonal and functional parameters in models of CMT1A neuropathy. Orphanet J Rare Dis 2014; 9:201; PMID:25491744; http://dx.doi.org/10.1186/s13023-014-0201-x
54. Attarian S, Vallat JM, Magy L, Funalot B, Gonnaud PM, Lacour A, Pereon Y, Dubourg O, Pouget J, Micallef J, et al. An exploratory randomised double-blind and placebo-controlled phase 2 study of a combination of baclofen, naltrexone and sorbitol (PXT3003) in patients with Charcot-Marie-Tooth disease type 1A. Orphanet J Rare Dis 2014; 9:199; PMID:25519680; http://dx.doi.org/10.1186/s13023-014-0199-0
55. Crowley P. Long-term drug treatment of patients with alcohol dependence. Australian Prescriber 2015; 38:41-3; PMID:26648614; http://dx.doi.org/10.18773/austprescr.2015.015
56. Soyka M, Mutschler J. Treatment-refractory substance use disorder: Focus on alcohol, opioids, and cocaine. Prog Neuro-psychopharmacol Biol Psychiatry 2015; PMID:26577297; http://dx.doi.org/10.1016/j.pnpbp.2015.11.003
57. Ratajewski M, Pulaski L. YY1-dependent transcriptional regulation of the human GDAP1 gene. Genomics 2009; 94:407-13; PMID:19720140; http://dx.doi.org/10.1016/j.ygeno.2009.08.014
58. Mathelier A, Fornes O, Arenillas DJ, Chen CY, Denay G, Lee J, Shi W, Shyr C, Tan G, Worsley-Hunt R, et al. JASPAR 2016: a major expansion and update of the open-access database of transcription factor binding profiles. Nucleic Acids Res 2016; 44:D110-5; PMID:26531826; http://dx.doi.org/10.1093/nar/gkv1176
59. Donohue TM, Jr., Osna NA, Trambly CS, Whitaker NP, Thomes PG, Todero SL, Davis JS. Early growth response-1 contributes to steatosis development after acute ethanol administration. Alcoholism Clin Exp Res 2012; 36:759-67; PMID:22141421; http://dx.doi.org/10.1111/j.1530-0277.2011.01681.x
60. Barski OA, Papusha VZ, Kunkel GR, Gabbay KH. Regulation of aldehyde reductase expression by STAF and CHOP. Genomics 2004; 83:119-29; PMID:14667815; http://dx.doi.org/10.1016/S0888-7543(03)00213-1
61. Saunders JB, Aasland OG, Babor TF, de la Fuente JR, Grant M. Development of the Alcohol Use Disorders Identification Test (AUDIT): WHO Collaborative Project on Early Detection of Persons with Harmful Alcohol Consumption–II. Addiction 1993; 88:791-804; PMID:8329970; http://dx.doi.org/10.1111/j.1360-0443.1993.tb02093.x
62. Moak DH, Anton RF, Latham PK. Further validation of the Obsessive-Compulsive Drinking Scale (OCDS). Relationship to alcoholism severity. Am J Addict 1998; 7:14-23; PMID:9522003; http://dx.doi.org/10.1111/j.1521-0391.1998.tb00463.x
63. Mercier C, Brochu S, Girard M, Gravel J, Ouellet R, Pare R. Profiles of alcoholics according to the SCL-90-R: a confirmative study. Int J Addict 1992; 27:1267-82; PMID:1446961; http://dx.doi.org/10.3109/10826089209047349

Table S1: Comparison of p-values before and after the exclusion of eight previously abstinent patients. Values are p-values.

	control individuals vs. patients at T1		patients at T1 vs. patients at T2	
	before exclusion	after exclusion	before exclusion	after exclusion
N	49 patients at T1	41 patients at T1	33 patients at T2	31 patients at T2
Site 1	0.001	2.5E-4	0.001	0.001
Site 2	0.015	0.01	0.033	0.031
Site 3	0.012	0.005	0.001	0.001
Mean DNA methylation (sites 1 - 3)	0.001	0.001	0.001	0.001

Paper 2

Brückmann C*, Islam SA*, MacIsaac JL, Morin AM, Karle KN, Di Santo A, Wüst R, Lang I, Batra A, Kobor MS*, Nieratschker V*.

DNA methylation signatures of chronic alcohol dependence in purified CD3$^+$ T-cells of patients undergoing alcohol treatment.

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DNA methylation signatures of chronic alcohol dependence in purified CD3$^+$ T-cells of patients undergoing alcohol treatment

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Christof Brückmann[1], Sumaiya A. Islam[2], Julia L. MacIsaac[3], Alexander M. Morin[3], Kathrin N. Karle[1], Adriana Di Santo[1], Richard Wüst[1,4], Immanuel Lang[1], Anil Batra[1], Michael S. Kobor[2,3,5,6] & Vanessa Nieratschker[1]

Several studies have shown an association of alcohol dependence with DNA methylation (DNAm), suggesting that environmentally-induced changes on epigenomic variation may play an important role in alcohol dependence. In the present study, we analysed genome-wide DNAm profiles of purified CD3$^+$ T-cells from pre- and post-treatment alcohol dependent patients, as well as closely matched healthy controls. We identified 59 differentially methylated CpG sites comparing patients prior to treatment with healthy controls and were able to confirm 8 of those sites in additional analyses for differentially methylated regions. Comparing patients before and after a 3-week alcohol treatment program we revealed another unique set of 48 differentially methylated CpG sites. Additionally, we found that the mean global DNAm was significantly lower in patients prior to treatment compared to controls, but reverted back to levels similar to controls after treatment. We validated top-ranked hits derived from the epigenome-wide analysis by pyrosequencing and further replicated two of them in an independent cohort and confirmed differential DNAm of *HECW2* and *SRPK3* in whole blood. This study is the first to show widespread DNAm variation in a disease-relevant blood cell type and implicates *HECW2* and *SRPK3* DNAm as promising blood-based candidates to follow up in future studies.

Alcohol dependence (AD) is a severe disorder that has long-lasting detrimental consequences, resulting in considerable health, economic and societal burden. According to the World Health Organization, alcohol related diseases account for approximately 3.3 million deaths per year (WHO, 2014). Although this number is alarmingly high, studies indicate that problematic drinking behaviour still is underestimated[1]. To date, treatment options are limited and the effectiveness of existing alcohol treatment programs is often less than optimal or difficult to assess, warranting a need for improvement.

The pathogenesis of AD is complex and includes genetic as well as non-genetic factors. Evidence is emerging that the interaction between underlying genetic factors and environmental stimuli (gene x environment, GxE) in particular plays a major role in addiction-related disease states[2–4]. Such findings have prompted considerable inquiry into the biological basis of GxE influences, with epigenetic regulation providing one of the most compelling candidate mechanisms for the mediation of GxE effects[5,6].

One of the most frequently studied epigenetic mechanisms is DNA methylation (DNAm), which involves the covalent addition of a methyl group to the 5′ position of a cytosine, primarily in the context of a cytosine-phosphate-guanine (CpG) dinucleotide. CpG dinucleotides are especially prevalent in CpG islands, genomic regions of approximately 1000 base pairs (bp) with a CG content greater than 50%[7]. CpG islands are

[1]Department of Psychiatry and Psychotherapy, University Hospital of Tuebingen, Tuebingen, Germany. [2]Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada. [3]Centre for Molecular Medicine and Therapeutics, BC Children's Hospital, Vancouver, BC, Canada. [4]Department of Neurodegenerative Disease, Hertie-Institute for Clinical Brain Research, Tuebingen, Germany. [5]Human Early Learning Partnership, University of British Columbia, Vancouver, British Columbia, Canada. [6]Canadian Institute for Advanced Research, Toronto, Ontario, Canada. Christof Brückmann and Sumaiya A. Islam contributed equally to this work. Michael S. Kobor and Vanessa Nieratschker jointly supervised this work. Correspondence and requests for materials should be addressed to V.N. (email: vanessa.nieratschker@med.uni-tuebingen.de)

associated with 50–70% of human gene promoters and increased DNAm in these regions is generally correlated with a decreased transcription of the respective gene[8,9]. Furthermore, methylated regions adjacent to CpG islands, called CpG island shores (up to 2 kb in either direction) or shelves (from 2 to 4 kb in either direction), may contribute to and potentiate epigenetic effects on gene expression[10–12]. In recent years, there has been increasing appreciation for the complexity of the relationship between DNAm and gene expression regulation, which tends to be highly dependent on genomic context[9,13]. DNAm profiles of genetic regions can vary substantially between different cell types[14]. It has been shown that after tissue origin, cellular heterogeneity within a tissue is a major driver of DNAm variance, highlighting the need to account for cellular composition in DNAm analyses[15,16].

Several biological factors including age[17], sex[18] and ethnicity[19] also have a profound impact on DNAm patterns. In addition, a number of lifestyle-based environmental exposures, including smoking[20–23] and alcohol consumption[24–36], are associated with variation in DNAm. In particular, DNAm alterations in AD patients have been documented in a number of epigenetic studies in human populations. For example, candidate gene analyses reported differential DNAm of the dopamine[30] and serotonin transporters[32], the nerve growth factor *NGF*[27], leptin[28] and most recently *GDAP1*[25] in AD patients compared to healthy controls. In the context of epigenome-wide association studies (EWAS), previous studies found widespread AD-associated DNAm differences at single sites, differentially methylated regions (DMRs)[26,33–35] and in "bulk" DNAm, representing mean global total levels of DNAm[29,36]. One study assessed DNAm alterations in peripheral blood mononuclear cells (PBMCs) of AD patients participating in a short-term alcohol treatment program compared to healthy controls, and reported differential methylation at 56 CpG sites in patients prior to treatment compared to controls. Although no statistically significant DNAm differences were observed in patients before and after the alcohol treatment program, 49 of the 56 differential sites reverted back in patients post-treatment to levels similar to controls[31]. Together, these previous studies identified a multitude of AD-associated differentially methylated sites, however, they did not account for cell type heterogeneity in their analyses, thereby potentially resulting in associations that are confounded by inter- and intra-individual differences in cellular composition. Most recently, a study involving 13,317 participants from 13 distinct cohorts analysed DNAm profiles in monocytes and whole blood. This analysis, which was adjusted for cell composition, revealed hundreds of AD-associated differentially methylated CpG sites[29].

Although all these previous studies support a potential link between DNAm variation and AD, a number of questions have yet to be explored: I) Are there signatures of AD in a disease-relevant blood cell type? II) Does treatment result in reversion of differential DNAm back to the levels found in controls? III) Importantly, can such AD-associated differential DNAm be replicated in independent cohorts, signifying the robustness of the identified genome-wide hits, and IV) Can the differential DNAm from a purified blood cell type also be detected in whole blood samples, indicating the potential relevance of these associations in other blood cell types?

To address these questions, we assessed genome-wide DNAm profiles of purified $CD3^+$ T-cells of a well-characterized cohort of long-term chronic AD patients participating in a clinical 3-week alcohol treatment program, along with the profiles of healthy controls closely matched for sex, age, ethnicity and smoking behaviour. We restricted our analyses to T-cells due to the known effects of chronic alcohol abuse in modulating the number, activity and relative subtype abundance levels of these immune cells[37]. For example, short-term binge drinkers as well as chronic AD patients exhibit a reduced number of peripheral T-cells[38]. In addition, a shift from $CD4^+$ and $CD8^+$ naïve T-cells towards memory T-cells is observed in AD patients[39]. Furthermore, alcohol consumption influences T-cell activation, leading to elevated numbers of activated $CD8^+$ T-cells, which may contribute to chronic inflammation[37,40]. For these reasons, heightened susceptibility to infections, including tuberculosis, pneumonia and HIV is observed in those patients[37,41]. T-cells have also been used previously in similar epigenetic studies due to their regulatory function in neuroimmune mechanisms[42,43]. Furthermore, by comparing the patients before and after 3 weeks of participating in a clinical alcohol treatment program, we sought to identify differentially methylated sites that may play a potential role in alcohol withdrawal and early recovery. In order to test whether our findings were robust, we validated four of our top-ranked hits by pyrosequencing, replicated the top-ranking hits in an independent second cohort of AD patients and matched controls and additionally confirmed the top-ranking hits in whole blood DNA of our cohort samples.

Results

Study cohorts and DNA methylation array normalization. To identify AD-associated DNAm variation, we utilized a discovery and replication cohort of AD patients and healthy controls, who were closely matched for age, sex and smoking behaviour. Demographic and AD-relevant characteristics as well as AUDIT and GSI scores of both cohorts are provided in Table 1a and b. To measure the effectiveness of the 3-week alcohol treatment program, we compared both GSI and OCDS scores in the discovery cohort at the beginning and after treatment. We found that both values decreased significantly, indicating a reduced alcohol craving and a better overall psychological well-being post-treatment (Table 1c).

In order to assess the association of AD with genome-wide DNAm in our discovery cohort, we measured site-specific DNAm at over 450,000 CpGs using the Illumina 450 K array. To test for potential cellular heterogeneity in the bead-purified $CD3^+$ T-cell samples, we used the Houseman blood deconvolution algorithm to estimate cell-type proportions, observing up to 32% of contaminating non-T-cell DNA in a fraction of our samples, although these proportions were not correlated with group status (Supplementary Figure S1). Regression-based adjustment of 450 K data resulted in the removal of these cell type associations as assessed by PCA (Supplementary Figure S2). The adjusted dataset thereby represented DNAm profiles from T-cells whose inter-individual cell type differences had been normalized to the best of our abilities for subsequent analyses.

Identification of AD-associated differential DNAm. Based on site-specific analyses of the T-cell DNAm array profiles, we identified 59 differentially methylated CpG sites between patients (T1) and controls with DNAm differences (Δ-beta) of at least 5% to increase the likelihood of biological relevance (FDR < 0.1). Of

	a) Discovery study cohort			b) Replication study cohort		
	Controls (N = 23)	Patients (N = 24)	*P*-value	Controls (N = 12)	Patients (N = 13)	*P*-value
age	46.9 ± 10.3	47.5 ± 10.1	0.8	45.3 ± 16.2	50.9 ± 9.1	0.4
active smokers	18 (78%)	19 (79%)	0.9	8 (67%)	9 (69%)	0.9
cigarettes per day	13.8 ± 12.6	15.2 ± 10.7	0.7	8.9 ± 8.0	10.5 ± 9.4	0.7
Years of alcohol dependence		10.6 ± 9.4			14.6 ± 11.7	
Days since last drink before hospital admission		1.2 ± 0.6			0.3 ± 0.4	
Standard drinks consumed each day in the week before hospital admission		13.7 ± 8.3			19 ± 11.4	
AUDIT	5.9 ± 3.8	24 ± 6.5	4E-15	2.8 ± 2.3	28.0 ± 4.9	3E-14
GSI	0.15 ± 0.14	0.72 ± 0.45	6E-07	0.10 ± 0.09	0.11 ± 0.10	0.9
	c) Results after 3-week alcohol treatment in the discovery cohort					
	Patients (T1)		Patients (T2)		*P*-value (paired testing)	
GSI	0.72 ± 0.45		0.41 ± 0.52		0.036	
OCDS	19.3 ± 6.6		12.0 ± 4.9		3E-05	

Table 1. Description of a) the discovery study cohort, b) the replication study cohort and c) results after 3-week alcohol treatment program in the discovery cohort. Errors are given as standard deviation. Abbreviations: AUDIT, alcohol use disorder identification test; GSI, global severity index; OCDS, obsessive compulsive drinking scale.

these 59 hits, 28 sites showed higher methylation, while 31 sites had lower methylation in patients compared to controls. Differences in DNAm ranged from 5 to 14% (Fig. 1a). The top 10 hits, ranked by Benjamini-Hochberg (BH)-adjusted *P*-value significance, are listed in Table 2a. A complete list of all 59 significant hits (FDR < 0.1) is provided in Supplementary Table S1. The top-ranked hit (cg18752527) exhibited a DNAm difference of 6.6% and was located within the intragenic region of the *HECW2* gene.

In addition to single CpG sites, we identified 29 significant DMRs (FDR < 0.01, Δ-beta > 5%) using DMRCate. These DMRs contained 153 CpG sites, of which 8 were also identified as differentially methylated in the site-specific analysis between controls and patients (T1) (Supplementary Table S2). Interestingly, 4 of these overlapping 8 hits were in the *SRPK3* gene region (Fig. 1b).

Treatment-related alterations in T-cell DNAm profiles. To identify differentially methylated sites potentially playing an important role in alcohol withdrawal and early recovery in AD patients, we compared genome-wide T-cell DNAm profiles of patients before (T1) and after 3 weeks (T2) of participation in an alcohol treatment program. Using paired testing in our site-specific analyses, we identified 48 differentially methylated sites between patients (T1) and patients (T2), all of which showed increased methylation at T2 ranging from 5 to 12% difference (FDR < 0.1, Δ-beta > 5%) (Fig. 1c, Supplementary Table S3). The top 10 hits are listed in Table 2b. Utilizing the same threshold as before, we did not observe any DMRs in patients before and after treatment.

Post-treatment reversion of differentially methylated sites. To examine whether AD-associated DNAm is influenced by a 3-week alcohol treatment program, we assessed DNAm levels in patients post-treatment at the 59 sites identified in the analysis comparing controls and patients (T1). After the treatment (T2), the DNAm levels of 7 out of 59 sites reverted back to a level where they no longer significantly differed from controls (Fig. 1d). Based on paired testing, we determined that these 7 sites were indeed differentially methylated between patients (T1) and patients (T2). Moreover, 32 CpG sites showed a trend to revert back, though not significant at an FDR < 0.1. The DNAm levels of the remaining 20 sites did not change from T1 to T2.

Assessment of mean global DNAm differences between groups. Given the unidirectional change in our site-specific analysis of patients before and after treatment, particularly at AD-associated sites which showed post-treatment reversion, we next examined if this trend was related to AD-associated differences in mean global DNAm. Here we defined mean global DNAm as the calculated average of DNAm values across all sites in each sample. We found that although the result was only nominally statistically significant, prior to the alcohol treatment (T1), mean global DNAm was lower in patients compared to controls ($P = 0.048$, Mann-Whitney U test). However, at the end of treatment (T2), global DNAm of the patients approximated the levels seen in controls and no longer differed significantly from controls (Fig. 2a). This finding was consistent with the unidirectional differences, in that all significant sites between patients before and after treatment showed increased methylation at T2 in the site-specific analysis, and supported the observed post-treatment reversion of AD-associated sites. Of note, these differences in mean global DNAm are unlikely to be driven by batch effects or other sources of technical variation due to the fact that all samples were run in a randomized manner on the same set of arrays.

Differences in naïve T-cell subtype abundances between groups. To evaluate if there were differences in underlying T-cell subtypes between the groups, we estimated abundance measures of additional blood

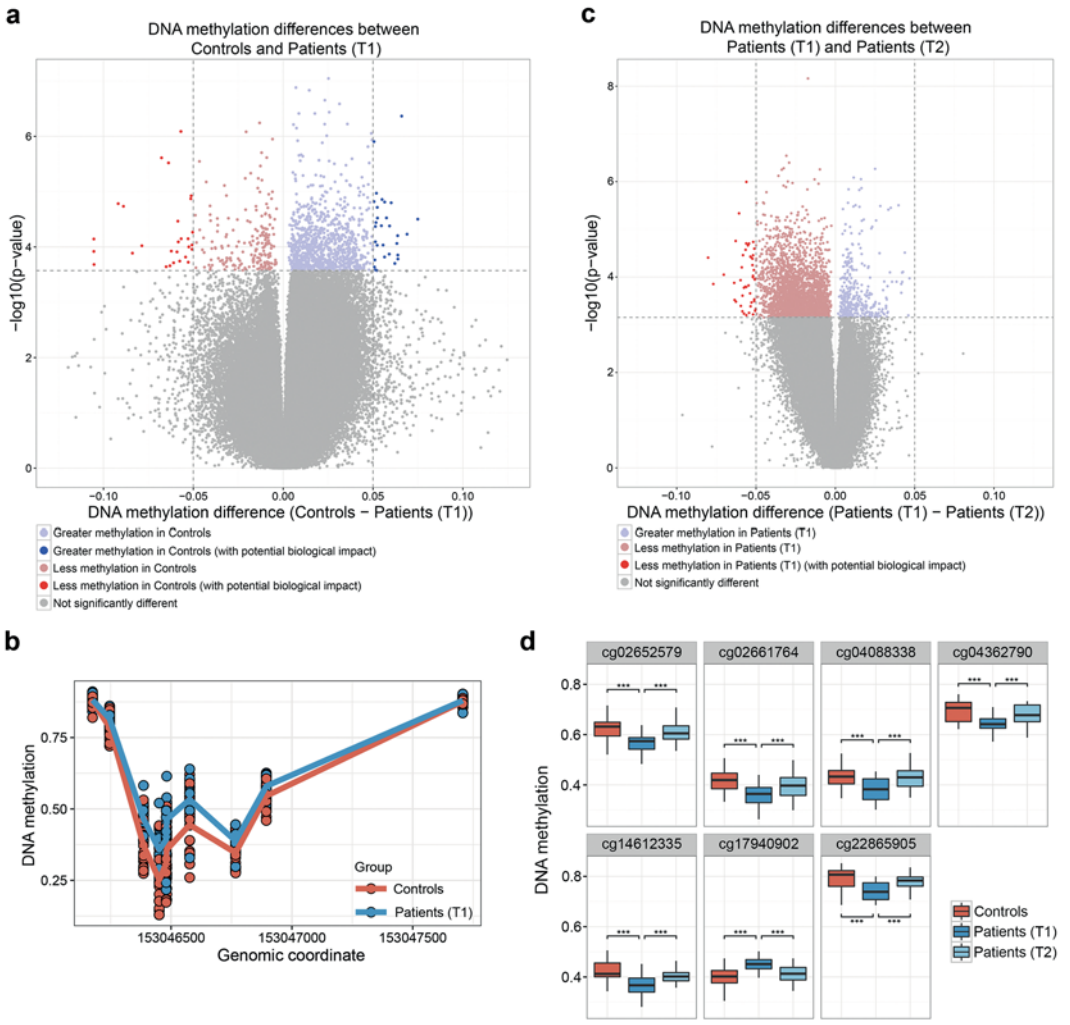

Figure 1. Differential sites and regions identified in the 450 K array analyses. (**a**) Volcano plot depicting differences in DNAm levels between controls and patient (T1) for each probe in the corrected 450 K dataset (indicated on X axis) against FDR (indicated on Y axis, on –log10 scale). Dashed horizontal line denotes FDR threshold of 0.1 while dashed vertical lines denote DNAm difference thresholds of −0.05 and 0.05, respectively. (**b**) Differential DNAm detected by DMRcate in the promoter region of the *SRPK3* gene (chrX:153, 046, 386–153, 046, 482). (**c**) Volcano plot depicting differences in DNAm levels between patients (T1) and patients (T2) as described in panel (a). (**d**) DNAm levels of seven sites which show reversion of DNAm post-treatment. ***Indicate an FDR < 0.001.

cell subsets using an advanced blood analysis option for an epigenetic clock prediction tool[44] on our T-cell 450 K profiles. We observed that the predicted abundance levels of both $CD4^+$ and $CD8^+$ naïve T-cell subsets significantly increased post-treatment in AD patients (FDR < 0.01, Wilcoxon signed rank test) (Fig. 2b). However, the abundance of these naïve T-cell subtypes was not statistically significantly different between controls and patients at either time point.

Validation of AD-associated differential DNA methylation by pyrosequencing. To verify the results from the 450 K dataset, we selected two top-ranking differentially methylated sites between controls and patients (T1) (cg18752527 in the *HECW2* gene and cg07280807 in an intergenic region) for validation using pyrosequencing as an independent readout of DNAm measures. We additionally validated two promoter CpGs of *SRPK3* (cg16529483 and cg24496423) since differential methylation in the *SRPK3* gene region was found to be a robust finding in our DMRcate analyses. We were able to confirm significant differences between controls and patients (T1) at all 4 sites, as shown in Fig. 3a (Student's t-test, FDR < 0.01). Although Bland-Altman plots showed a general bias for lower methylation levels measured by pyrosequencing (Supplementary Figure S3), the correlation in measurements between the two methodologies was highly concordant for all 4 sites (Spearman's correlation $r_s > 0.7$, FDR < 0.001) (Supplementary Figure S3).

Replication of AD-associated differential DNA methylation in an independent cohort. To further test the robustness of our EWAS findings, we analysed the previously mentioned 4 sites in T-cells of an

Probe ID	Gene	Region	Average beta Controls	Average beta Patients (T1)	Δ-beta	*P*-Value	BH-adjusted *P*-value
a) Differentially methylated sites between Controls and Patients (T1)							
cg18752527*	*HECW2*	intragenic	0.342	0.276	0.066	4.30E-07	0.0213
cg08109624		intergenic	0.760	0.817	−0.057	8.15E-07	0.0234
cg10168086		intergenic	0.535	0.484	0.051	1.24E-06	0.0256
cg07280807*		intergenic	0.755	0.822	−0.068	2.44E-06	0.0366
cg12173150		intergenic	0.321	0.385	−0.064	3.02E-06	0.0370
cg01059398	*TNFSF10*	intragenic	0.261	0.209	0.052	1.07E-05	0.0627
cg17940902	*HLA-DMA*	promoter	0.399	0.450	−0.051	1.19E-05	0.0640
cg22778903	*MX2*	intragenic	0.304	0.355	−0.051	1.34E-05	0.0666
cg14612335	*SKIL*	promoter	0.423	0.368	0.055	1.38E-05	0.0666
cg11580026		intergenic	0.600	0.549	0.051	1.51E-05	0.0691
Probe ID	**Gene**	**Region**	**Average beta Patients (T1)**	**Average beta Patients (T2)**	**Δ-beta**	***P*-Value**	**BH-adjusted *P*-value**
b) Differentially methylated sites between Patients (T1) and Patients (T2)							
cg15500907	*LAMA4*	intragenic	0.485	0.542	−0.056	1.01E-06	0.0323
cg05266321	*CCR2*	intragenic	0.545	0.606	−0.061	4.63E-06	0.0487
cg13279700	*C6orf10*	intragenic	0.481	0.544	−0.063	1.76E-05	0.0561
cg14054990	*KRTAP19-5*	promoter	0.431	0.482	−0.052	1.84E-05	0.0565
cg21049302		intergenic	0.466	0.522	−0.056	1.98E-05	0.0565
cg17022548	*NRG2*	intragenic	0.204	0.258	−0.054	1.99E-05	0.0565
cg22472360	*TRIO*	intragenic	0.514	0.569	−0.055	2.09E-05	0.0569
cg07920414	*RIMS3*	intragenic	0.438	0.493	−0.055	2.18E-05	0.0572
cg04088338		intergenic	0.378	0.429	−0.051	2.54E-05	0.0590
cg12240358	*HOMER2*	intragenic	0.462	0.519	−0.057	2.68E-05	0.0590

Table 2. Top 10 differentially methylated sites a) between controls and patients (T1) and b) between patients (T1) and patients (T2). Probe IDs marked with an asterisk were validated by pyrosequencing. Abbreviations: Average beta, mean methylation values (%); Benjamini-Hochberg (BH) adjusted *P*-value.

independent replication cohort by pyrosequencing. The two top-ranking hits, cg07280807 in an intergenic region and cg18752527 in *HECW2*, were differentially methylated in the replication cohort (FDR < 0.05, one-sided t-test) (Fig. 3b). However, the two sites within the *SRPK3* promoter region (cg16529483 and cg24496423) did not replicate in this cohort, likely due to insufficient power with the low sample size in this cohort, but showed a trend in the same direction as in the discovery cohort.

Analysis of differential DNA methylation in whole blood. To identify sites that are not only differentially methylated in T-cells, but also in whole blood DNA, we sought to reproduce our most robust EWAS findings from T-cells in whole blood DNA samples of both our discovery and replication cohorts. Therefore, we analysed DNAm of the 4 previously mentioned sites in whole blood samples by pyrosequencing. We observed differential methylation of cg18752527 in the intragenic region of *HECW2* between controls and patients (T1) in both cohorts (FDR < 0.05, Student's t-test) (Fig. 3c). Furthermore, similar to the findings from T-cells, the two sites within the *SRPK3* promoter region (cg16529483 and cg24496423) were differentially methylated in whole blood samples of the discovery cohort (Fig. 3d), but not of the replication cohort. We found that differential DNAm of cg07280807 did not replicate in whole blood of either cohort. Using a previous 450 K dataset of purified blood cell types[45], we confirmed that the DNAm status of cg18752527 in *HECW2* was highly associated with T-cells, along with NK cells, suggesting that the DNAm differences we measured in whole blood were driven, in part, by T-cells ($P = 7.6E-15$, ANOVA) (Supplementary Figure S4). The DNAm statuses of the two sites in the *SRPK3* promoter were not associated with any specific cell type (Supplementary Figure S4).

Discussion

By analysing genome-wide DNAm profiles of purified $CD3^+$ T-cells using the Illumina 450 K array, we found 59 CpG sites to be differentially methylated in a group of 24 alcohol dependent patients compared to 23 closely matched healthy controls. These site-specific hits showed considerable overlap to detected DMRs, suggesting that the results were not contingent on the analytical approach used. Furthermore, we discovered 48 sites that were differentially methylated between AD patients at the time of hospital admission (T1) and after 3 weeks (T2) of participation in an alcohol treatment program and showed a reversion of some of the AD-associated sites post-treatment. In addition, we were able to validate four of the top-ranking AD-associated hits by pyrosequencing, and replicate two of them in an independent cohort. Finally, we found the top-ranked hits in *HECW2* (cg18752527) and *SRPK3* (cg16529483 and cg24496423) to be differentially methylated in whole blood, signifying the potential relevance of these associations in other blood cell types. To our knowledge, this is the first study to assess and replicate alcohol-associated differential DNAm in purified T-cells and to assess DNAm variation that may be related to early recovery from AD in closely matched human population cohorts.

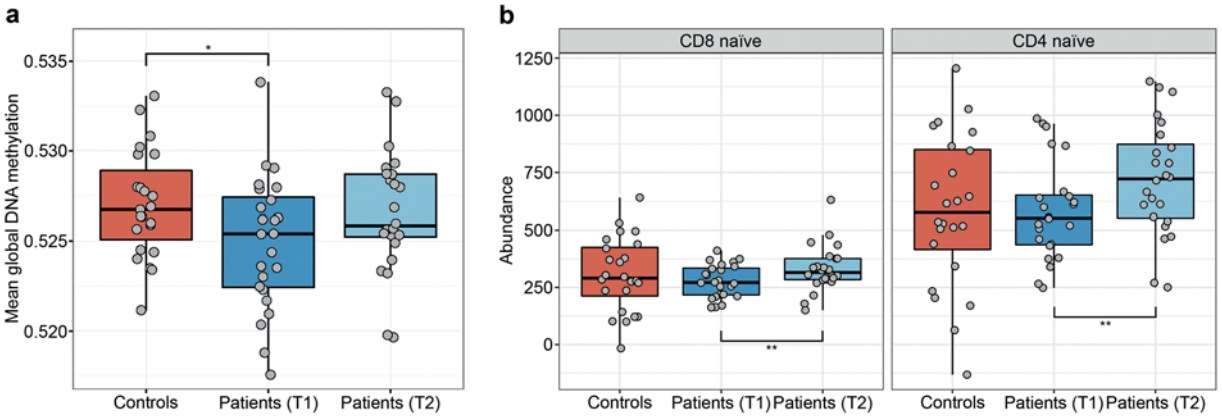

Figure 2. Mean global DNAm differences and naïve T-cell subtype differences between groups. (**a**) Patients (T1) showed significantly decreased mean global DNAm levels compared to controls ($P = 0.048$, Mann-Whitney U test). Differences between controls vs. patients (T2) and patients (T1) vs. patients (T2) were not significant. (**b**) Abundance levels of naïve $CD8^+$ and $CD4^+$ T-cells were predicted using an advanced blood DNA methylation age prediction tool. Both naïve T-cell subtypes significantly increased post-treatment in patients (**Indicates an FDR < 0.01, Wilcoxon signed-rank test) but were not significantly different between controls and patients at either time point.

EWAS pose an excellent hypothesis-free opportunity to identify as yet undiscovered disease-associated genes. Our EWAS findings of AD-associated differential DNAm revealed both site-specific and regional differences between patients before treatment and matched controls in a clinically relevant cell type. The observed bi-directional patterns of changes are consistent with previous evidence of AD-associated differential DNAm[26,29,31,33,35]. However, our findings derived from T-cells did not overlap with previously reported associations of AD with DNAm[26,29,31,33,35]. This can at least in part be explained by the use of heterogeneous biological material (i.e. whole blood, PBMCs), differences in the cohorts used or in the strategies applied to match patients and controls as well as by varying methodologies for DNAm measurement, with reduced or discordant coverage of CpG sites in previous studies[26,31,33,35] compared to the present study. However, our top-ranking hits in *HECW2* and *SRPK3* might contribute to reveal mechanisms that may play a role in AD. HECW2 is a HECT-type E3 ubiquitin ligase involved in the cellular stress response[46,47]. This finding is in line with previous evidence for the role of epigenetic regulation of cellular stress response genes in AD, such as *GDAP1*, which was identified in a previous EWAS[31] and subsequently replicated in whole blood samples derived from an independent cohort[25]. However, *GDAP1* did not come up in this present analysis using DNA isolated from purified T-cells. Presumably, the previously described differential methylation of *GDAP1* in whole blood is driven by another cell type other than T-cells. *SRPK3* encodes a serine/arginine protein kinase and is essential for the development of the skeletal muscle[48]. It was shown that the drosophila homolog *SRPK79D* plays an important role in the function of synapses[49]. Although an association between *SRPK3* and the nervous system in humans has not been described so far, the high homology between SRPK79D and SRPK3 (65%) makes an as yet uncharacterized role in the nervous system possible.

In addition to the assessment of AD-associated differential DNAm in T-cells prior to alcohol treatment, we also examined treatment-related site-specific alterations in DNAm by comparing DNAm profiles in T-cells of patients before (T1) and after a 3-week alcohol treatment (T2). Our findings include numerous sites in which DNAm in patients (T2) reverts back to levels comparable to those observed in controls. More specifically, we showed post-treatment DNAm reversion (at 7 sites) or partial reversion (at 32 sites) back to control levels. These findings confirm the results of a previous pilot study, which also showed reversion of DNAm after a short term alcohol treatment program[31]. Other epigenetic studies in human populations investigating the effect of short-term treatments, including exercise or dietary interventions, on DNAm of relevant tissues have identified similar numbers of site-specific DNAm changes with a comparable magnitude of effect sizes to our findings[50,51].

Based on our assessment of mean global DNAm, measured as averaged methylation across all interrogated CpGs, we found that global DNAm levels were significantly lower in patients prior to the alcohol treatment compared to controls. Following alcohol treatment, the mean global DNAm of patients no longer differed significantly from controls. These results are in accordance with the unidirectionality of our treatment-related hits, with all significant sites exhibiting increased DNAm after treatment, and with our site-specific findings that numerous AD-associated CpGs exhibited post-treatment reversion to levels comparable to controls. The reduction in mean global DNAm observed in AD patients is supported by previous studies, which also demonstrated decreased methylation[29,36]. It has been hypothesized that such alcohol-associated decreases in global DNAm are attributed to the lack of methionine adenosyl transferase regulation in AD patients[14,52]. However, in contrast, earlier studies have postulated that due to the higher homocysteine levels in AD patients, global DNAm patterns should be elevated[53], although such associations have not been confirmed[54]. The lack of consensus in regard to alterations in alcohol-related global DNAm measures highlights the need for further investigation into the biological mechanisms underlying global DNAm patterns in AD patients.

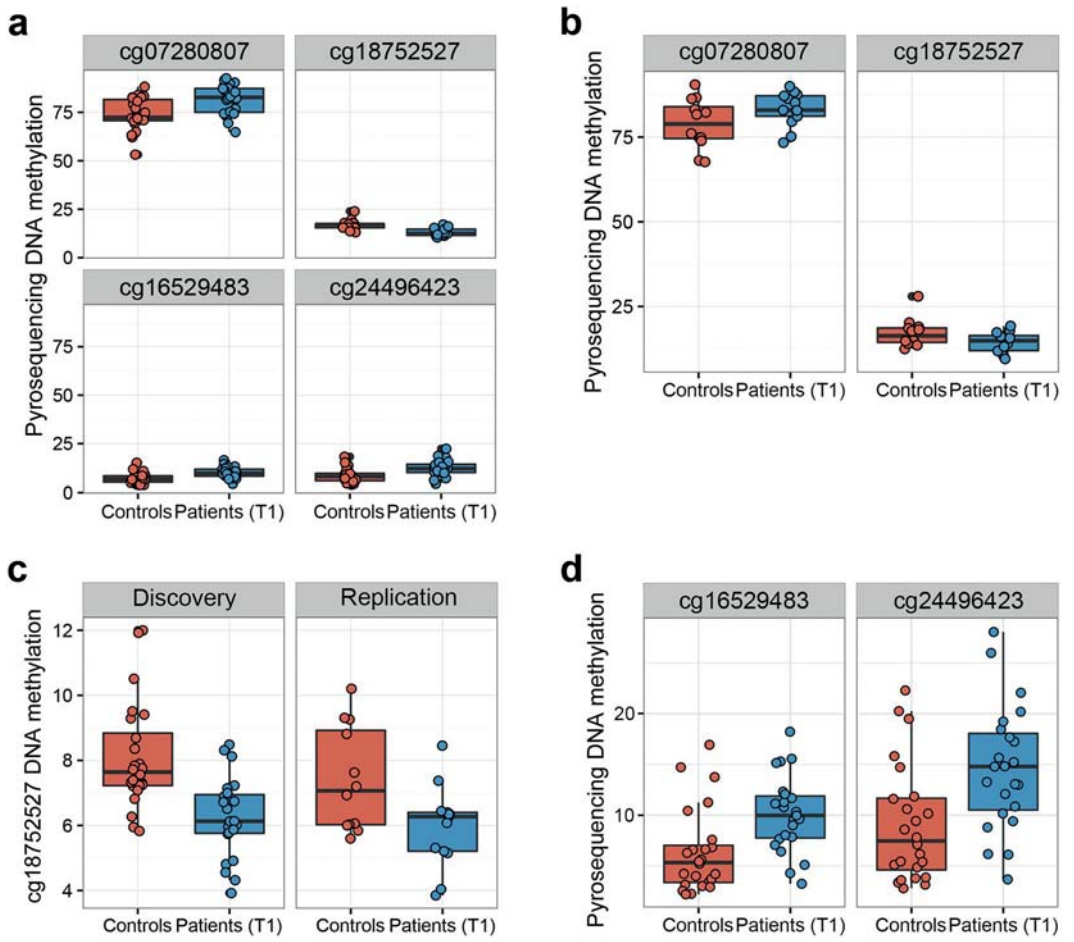

Figure 3. Validation and replication of top-ranking hits by pyrosequencing. (**a**) Boxplots showing differences in DNAm levels of discovery cohort T-cell samples as measured by pyrosequencing (FDR < 0.01, Student's t-test). (**b**) Two top-ranked hits (cg07280807 and cg18752527) were verified as being differentially methylated in T-cell samples of the replication cohort (FDR < 0.05, one-sided t-test). (**c**) Verification of differential methylation of cg18752527 (*HECW2*) in the discovery (left) and the replication cohort (right) in DNA derived from whole blood (FDR < 0.05, two-sided t-test). (**d**) Verification of cg16529483 and cg24496423 (*SRPK3*) differential methylation in the discovery cohort in DNA derived from whole blood (FDR < 0.01, two-sided t-test).

Using bioinformatic predictions from our T-cell DNAm profiles, we observed a significant increase in naive $CD4^+$ and $CD8^+$ T-cell subsets post-treatment, which is consistent with evidence of decreased frequencies of these naïve T-cell subtypes due to chronic AD[37,39] and a resultant restoration of peripheral T-cell numbers following short-term alcohol abstinence[38]. These findings, along with known effects of alcohol dependence on T-cell homeostasis, proliferation and activation[39,55], highlight the importance of understanding alcohol-related effects on T-cell-specific biology, particularly in the context of AD pathophysiology and treatment, of which our study serves as the first to profile such AD-associated changes on the T-cell epigenome.

In order to verify that our results are robust and largely reflective of potential biological variation as opposed to technical variation, we took a number of precautions in our analyses, including I) constraining our hits to sites with DNAm differences greater than 5% between groups in order to increase the likelihood of biological relevance, II) confirming 450 K measures by pyrosequencing and III) validating top-ranked hits by pyrosequencing in an independent replication cohort. Although we observed a general bias between the two methodologies, in which the pyrosequencing measures were lower than 450 K values, there was high concordance of measures between the two methods and we were still able to detect significant differences in DNAm between groups, signifying the strength of our results. Moreover, we were able to confirm three top-ranking hits from purified T-cells in whole blood, further strengthening the robustness of our findings and highlighting their potential importance in AD.

It is important to note that our study had a few inherent limitations. Firstly, using bioinformatic cell type predictions, we detected notable levels (up to 32%) of cellular contamination in our bead-purified T-cell samples. This is consistent with previous work which confirmed the presence of cellular heterogeneity in samples even after purification using cell surface markers[56]. We removed cell heterogeneity using a regression-based method, thereby ensuring inter-individual differences in cell composition were normalized in our dataset prior to DNAm analyses. Secondly, our analyses were limited by a rather small sample size. To work around this limitation, we

utilized a relaxed FDR threshold in the differential methylation modelling to capture more potentially biologically relevant sites and focused on validating and replicating our top-ranked hits to ensure these results were robust. Although we were able to validate the hits within the *SRPK3* promoter by pyrosequencing in T-cell and whole blood samples of the discovery cohort, we could not replicate the differential DNAm of *SRPK3* in our second cohort, unlike our findings for *HECW2*. This probably results from insufficient statistical power due to the low sample size of the replication cohort. We acknowledge that the small samples size analysed in our study could also hinder successful validation of our results in future studies. The phenomenon of non-replication could also be observed in previous transcriptome-wide studies in human populations of AD patients and control individuals, where the overlap between the individual studies was fairly small[57,58]. However, by technically validating and replicating our results in a second cohort, we made an attempt to reduce the risk of false-positive findings to a minimum. Despite these efforts, our results should be verified in a larger cohort spanning different populations to confirm the associations for *HECW2* and *SRPK3*. So far, neither *HECW2* nor *SRPK3* were among top-ranked hits in transcriptome-wide studies. Therefore, functional data is required to investigate the interplay of DNAm, transcription and functioning of these genes related to AD. Thirdly, we cannot rule out that the DNAm differences between the patients before (T1) and after treatment (T2) may be due to stochastic temporal DNAm variation, although previous work in blood has revealed minimal evidence of temporal variation in the majority of 450 K probes across a 9 month period[59]. In addition, differences in DNAm could also be due to direct influences of acute ethanol intoxication, which has been shown to have an effect on transcriptome regulation[57,58]. We tried to circumvent this limitation by only including subjects who had their last drink in a narrow time frame of 1.2 ± 0.6 days. Additionally, the 20 CpG sites which did not change from pre- to post-treatment could potentially be differentially methylated due to chronic alcohol exposure and not due to early withdrawal. To clarify this issue, future longitudinal studies are warranted. Finally, we cannot disregard the potential influence of genetic variation on our differentially methylated CpG sites. However, we attempted to reduce genetic heterogeneity in our cohort by using only Caucasian participants.

In conclusion, we report that AD is associated with lower mean global DNAm and with differential DNAm of specific sites in $CD3^+$ T-cells. Additionally, we were able to identify changes in DNAm related to alcohol treatment in patients. These changes include the reversion of AD-associated DNAm alterations at certain sites to levels comparable to controls. Validation of our top-ranking associations by pyrosequencing and replication of our top-ranked hits in a second independent cohort strongly supports the robustness of our results. Finally, we show that the differential methylation of *HECW2* and *SRPK3* is not only present in T-cells, but also in whole blood, indicating that *HECW2* and *SRPK3* are likely robust findings which should be followed up in future studies.

Methods

Study cohorts. The discovery study cohort was comprised of 24 male AD patients (mean age 47.5 ± 10.1 years) participating in a 3-week in-patient alcohol treatment program at the Clinic for Psychiatry and Psychotherapy in Tuebingen, Germany. AD was diagnosed according to the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV). Twenty-three population based, sex- and age-matched healthy controls (mean age 46.9 ± 10.3 years) were recruited from Tuebingen and the surrounding area. The replication study cohort was comprised of 13 male AD patients (mean age 50.9 ± 9.1 years) and 12 matched healthy controls (mean age 45.3 ± 16.2 years). In addition, the smoking behaviour (measured as cigarettes per day) of both groups was matched. Subjects with a dependence other than nicotine and patients with any psychiatric disorder necessitating psychotropic medication were excluded from the study. All subjects were of Caucasian origin and gave written informed consent after recovering from alcohol intoxication (patients) or prior to participation in the study (controls), which was approved by the ethics committee of the University of Tuebingen and was conducted in accordance with the Declaration of Helsinki.

After recovery from alcohol intoxication and at the time of study inclusion, respectively (time point 1, T1), patients and controls answered a self-administered phenotypic and demographic questionnaire, the Alcohol Use Disorder Identification Test (AUDIT)[60], assessing alcohol consumption, and the Symptom Checklist-90-R (SCL-90-R) questionnaire[61], assessing the global distress level (GSI). Patients also answered the obsessive compulsive drinking scale (OCDS-G) questionnaire, reflecting obsession and compulsivity related to craving and drinking behavior[62]. OCDS-G and SCL-90-R were reassessed after three weeks (± 2 days) of participation in the alcohol treatment program (time point 2, T2). Controls with AUDIT scores >15 were excluded, as a higher value is suggestive for problematic alcohol intake.

At T1 and T2, peripheral venous blood was drawn from patients in Ethylenediaminetetraacetic (EDTA) and Mononuclear Cell Preparation tubes (CPT, both BD, Franklin Lakes, NJ, USA). EDTA and CPT blood samples from the controls were drawn at study inclusion. Samples for whole blood DNA extraction were kept at −80 °C until further usage.

$CD3^+$ T-cell purification and DNA isolation. Immediately after blood draw, PBMCs were first separated via centrifugation of the CPT tubes for 20 min at $1650 \times g$. $CD3^+$ T-cells were then purified from PBMCs following the positive isolation protocol using Dynabeads CD3 (Invitrogen, Carlsbad, CA, USA). The cells were subsequently lysed and DNA was prepared using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to standard protocol.

Bisulfite conversion and Illumina 450 K DNA methylation arrays. T-cell DNA (750 ng) was bisulfite converted using the Zymo Research EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA). DNA yield and purity was assessed using a Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). Samples were subsequently randomized and 160 ng of bisulfite-converted DNA was applied to the Illumina Infinium

HumanMethylation450K (450 K) Beadchip array, as per manufacturer's protocols (Illumina, San Diego, CA, USA).

DNA methylation array data processing, blood cell deconvolution and differential methylation analyses. Raw data from the 450 K array was subjected to quality control, normalization and batch correction. Subsequently, remaining contamination of the purified T-cells was bioinformatically removed from the dataset. After subsetting the corrected data in 3 groups (controls vs. patients (T1); patients (T1) vs. patients (T2); controls vs. patients (T2)), site-specific differential DNAm was assessed by linear regression modelling while differentially methylated region (DMRs) were identified using the DMRcate package as described earlier[63]. A detailed description of all analyses can be found in the supplementary methods. The 450 K data has been made publicly available on the Gene Expression Omnibus database (GSE98876).

Pyrosequencing-based validation and replication in T-cells. 500 ng T-cell DNA was bisulfite-converted using the Epitect Fast Bisulfite Conversion Kit (Qiagen) as described earlier[25]. For amplification of the region of interest, PCR was conducted using the PyroMark PCR Kit (Qiagen) with the following primers: forward (fwd): 5′-GTTATGGTTGGGTTTTTGGG-3′, reverse (rev): 5′-Bio-CCTATCTCCTCAAACAAAAACTAAAAA-3′, sequencing (seq): 5′-AGTTAGGGATTATAGTGTAGTTG-3′ (cg07280807); fwd: 5′-GTGTTTGTGGGAATGTTTTTTATA-3′, rev: 5′-Bio- CACACTACACTTTCATTTTCTATCAA-3′, seq: 5′- TTTTTAGATATATAAATTTTTTTTTT-3′ (cg18752527) and fwd/seq: 5′-GTTATTTATAAAGGAGGGTGAGATTA-3′, rev: 5′-Bio-AACCACTACTCCTATAAAACCCCAC-3′ (cg16529483/cg24496423). A detailed list of PCR primers and programs is provided in Supplementary Table S4. Specificity of the PCR was verified by agarose gel electrophoresis including a negative control. Pyrosequencing was conducted on a PyroMark Q24 according to standard protocol using PyroMark Gold Reagents (both Qiagen). Each sample was measured in triplicates; an intra-sample deviation of $\geq$3% led to the exclusion of the deviating measurement. For each site, measurements of DNA with known methylation levels of 0%, 25%, 50%, 75% and 100% were obtained (Epitect Control DNA, Qiagen). Correlations between the 450 K dataset and pyrosequencing were tested using the Spearman's correlation test.

Pyrosequencing-based validation and replication in whole blood. DNA was prepared from EDTA tubes using QIAamp DNA Blood Maxi Kit (Qiagen) according to manufacturer's protocol. Afterwards, bisulfite conversion and pyrosequencing was carried out as described above.

References

1. Britton, A., O'Neill, D. & Bell, S. Underestimating the Alcohol Content of a Glass of Wine: The Implications for Estimates of Mortality Risk. *Alcohol and alcoholism* **51**, 609–614, doi:10.1093/alcalc/agw027 (2016).
2. Young-Wolff, K. C., Enoch, M. A. & Prescott, C. A. The influence of gene-environment interactions on alcohol consumption and alcohol use disorders: a comprehensive review. *Clin Psychol Rev* **31**, 800–816, doi:10.1016/j.cpr.2011.03.005 (2011).
3. Enoch, M. A. The influence of gene-environment interactions on the development of alcoholism and drug dependence. *Curr Psychiatry Rep* **14**, 150–158, doi:10.1007/s11920-011-0252-9 (2012).
4. Wall, T. L., Luczak, S. E. & Hiller-Sturmhofel, S. Biology, Genetics, and Environment: Underlying Factors Influencing Alcohol Metabolism. *Alcohol research: current reviews* **38**, 59–68 (2016).
5. Meaney, M. J. Epigenetics and the biological definition of gene x environment interactions. *Child development* **81**, 41–79, doi:10.1111/j.1467-8624.2009.01381.x (2010).
6. Boyce, W. T. & Kobor, M. S. Development and the epigenome: the 'synapse' of gene-environment interplay. *Dev Sci* **18**, 1–23, doi:10.1111/desc.12282 (2015).
7. Illingworth, R. S. *et al*. Orphan CpG islands identify numerous conserved promoters in the mammalian genome. *PLoS genetics* **6**, e1001134, doi:10.1371/journal.pgen.1001134 (2010).
8. Choy, M. K. *et al*. Genome-wide conserved consensus transcription factor binding motifs are hyper-methylated. *BMC genomics* **11**, 519, doi:10.1186/1471-2164-11-519 (2010).
9. Jones, P. A. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature reviews. Genetics* **13**, 484–492, doi:10.1038/nrg3230 (2012).
10. Irvine, R. A., Lin, I. G. & Hsieh, C. L. DNA methylation has a local effect on transcription and histone acetylation. *Mol Cell Biol* **22**, 6689–6696 (2002).
11. Sandoval, J. *et al*. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. *Epigenetics* **6**, 692–702 (2011).
12. Edgar, R., Tan, P. P., Portales-Casamar, E. & Pavlidis, P. Meta-analysis of human methylomes reveals stably methylated sequences surrounding CpG islands associated with high gene expression. *Epigenetics Chromatin* **7**, 28, doi:10.1186/1756-8935-7-28 (2014).
13. Lam, L. L. *et al*. Factors underlying variable DNA methylation in a human community cohort. *Proceedings of the National Academy of Sciences of the United States of America* **109**(Suppl 2), 17253–17260, doi:10.1073/pnas.1121249109 (2012).
14. Varela-Rey, M., Woodhoo, A., Martinez-Chantar, M. L., Mato, J. M. & Lu, S. C. Alcohol, DNA methylation, and cancer. *Alcohol research: current reviews* **35**, 25–35 (2013).
15. Farre, P. *et al*. Concordant and discordant DNA methylation signatures of aging in human blood and brain. *Epigenetics Chromatin* **8**, 19, doi:10.1186/s13072-015-0011-y (2015).
16. Jaffe, A. E. & Irizarry, R. A. Accounting for cellular heterogeneity is critical in epigenome-wide association studies. *Genome Biol* **15**, R31, doi:10.1186/gb-2014-15-2-r31 (2014).
17. Chu, M. *et al*. Inferring relative numbers of human leucocyte genome replications. *Br J Haematol* **141**, 862–871, doi:10.1111/j.1365-2141.2008.07142.x (2008).
18. Liu, J., Morgan, M., Hutchison, K. & Calhoun, V. D. A study of the influence of sex on genome wide methylation. *PloS one* **5**, e10028, doi:10.1371/journal.pone.0010028 (2010).
19. Zhang, F. F. *et al*. Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood. *Epigenetics* **6**, 623–629 (2011).
20. Breitling, L. P., Yang, R., Korn, B., Burwinkel, B. & Brenner, H. Tobacco-smoking-related differential DNA methylation: 27K discovery and replication. *Am J Hum Genet* **88**, 450–457, doi:10.1016/j.ajhg.2011.03.003 (2011).

21. Monick, M. M. *et al*. Coordinated changes in AHRR methylation in lymphoblasts and pulmonary macrophages from smokers. American journal of medical genetics. *Part B, Neuropsychiatric genetics: the official publication of the International Society of Psychiatric Genetics* **159B**, 141–151, doi:10.1002/ajmg.b.32021 (2012).
22. Shenker, N. S. *et al*. Epigenome-wide association study in the European Prospective Investigation into Cancer and Nutrition (EPIC-Turin) identifies novel genetic loci associated with smoking. *Human molecular genetics* **22**, 843–851, doi:10.1093/hmg/dds488 (2013).
23. Joubert, B. R. *et al*. 450K epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. *Environ Health Perspect* **120**, 1425–1431, doi:10.1289/ehp.1205412 (2012).
24. Zhang, H. & Gelernter, J. DNA methylation and alcohol use disorders: Progress and challenges. *The American journal on addictions/American Academy of Psychiatrists in Alcoholism and Addictions*. doi:10.1111/ajad.12465 (2016).
25. Bruckmann, C., Di Santo, A., Karle, K. N., Batra, A. & Nieratschker, V. Validation of differential GDAP1 DNA methylation in alcohol dependence and its potential function as a biomarker for disease severity and therapy outcome. *Epigenetics* **11**, 456–463, doi:10.1080/15592294.2016.1179411 (2016).
26. Hagerty, S. L., Bidwell, L. C., Harlaar, N. & Hutchison, K. E. An Exploratory Association Study of Alcohol Use Disorder and DNA Methylation. *Alcoholism, clinical and experimental research* **40**, 1633–1640, doi:10.1111/acer.13138 (2016).
27. Heberlein, A. *et al*. Epigenetic down regulation of nerve growth factor during alcohol withdrawal. *Addiction biology* **18**, 508–510, doi:10.1111/j.1369-1600.2010.00307.x (2013).
28. Hillemacher, T. *et al*. DNA methylation of the LEP gene is associated with craving during alcohol withdrawal. *Psychoneuroendocrinology* **51**, 371–377, doi:10.1016/j.psyneuen.2014.10.014 (2015).
29. Liu, C. *et al*. A DNA methylation biomarker of alcohol consumption. *Mol Psychiatry*, doi:10.1038/mp.2016.192 (2016).
30. Nieratschker, V. *et al*. Epigenetic alteration of the dopamine transporter gene in alcohol-dependent patients is associated with age. *Addiction biology* **19**, 305–311, doi:10.1111/j.1369-1600.2012.00459.x (2014).
31. Philibert, R. A. *et al*. A pilot examination of the genome-wide DNA methylation signatures of subjects entering and exiting short-term alcohol dependence treatment programs. *Epigenetics* **9**, 1212–1219, doi:10.4161/epi.32252 (2014).
32. Philibert, R. A. *et al*. The relationship of 5HTT (SLC6A4) methylation and genotype on mRNA expression and liability to major depression and alcohol dependence in subjects from the Iowa Adoption Studies. *American journal of medical genetics. Part B, Neuropsychiatric genetics: the official publication of the International Society of Psychiatric Genetics* **147B**, 543–549, doi:10.1002/ajmg.b.30657 (2008).
33. Ruggeri, B. *et al*. Association of Protein Phosphatase PPM1G With Alcohol Use Disorder and Brain Activity During Behavioral Control in a Genome-Wide Methylation Analysis. *The American journal of psychiatry* **172**, 543–552, doi:10.1176/appi.ajp.2014.14030382 (2015).
34. Wang, F., Xu, H., Zhao, H., Gelernter, J. & Zhang, H. DNA co-methylation modules in postmortem prefrontal cortex tissues of European Australians with alcohol use disorders. *Sci Rep* **6**, 19430, doi:10.1038/srep19430 (2016).
35. Zhang, R. *et al*. Genome-wide DNA methylation analysis in alcohol dependence. *Addiction biology* **18**, 392–403, doi:10.1111/adb.12037 (2013).
36. Zhu, Z. Z. *et al*. Predictors of global methylation levels in blood DNA of healthy subjects: a combined analysis. *International journal of epidemiology* **41**, 126–139, doi:10.1093/ije/dyq154 (2012).
37. Pasala, S., Barr, T. & Messaoudi, I. Impact of Alcohol Abuse on the Adaptive Immune System. *Alcohol research: current reviews* **37**, 185–197 (2015).
38. Tonnesen, H., Andersen, J. R., Pedersen, A. E. & Kaiser, A. H. Lymphopenia in heavy drinkers–reversibility and relation to the duration of drinking episodes. *Ann Med* **22**, 229–231 (1990).
39. Cook, R. T. *et al*. Fine T-cell subsets in alcoholics as determined by the expression of L-selectin, leukocyte common antigen, and beta-integrin. *Alcoholism, clinical and experimental research* **18**, 71–80 (1994).
40. Cook, R. T. *et al*. Activated CD-8 cells and HLA DR expression in alcoholics without overt liver disease. *J Clin Immunol* **11**, 246–253 (1991).
41. Porretta, E., Happel, K. I., Teng, X. S., Ramsay, A. & Mason, C. M. The impact of alcohol on BCG-induced immunity against Mycobacterium tuberculosis. *Alcoholism, clinical and experimental research* **36**, 310–317, doi:10.1111/j.1530-0277.2011.01624.x (2012).
42. Nemoda, Z. *et al*. Maternal depression is associated with DNA methylation changes in cord blood T lymphocytes and adult hippocampi. *Transl Psychiatry* **5**, e545, doi:10.1038/tp.2015.32 (2015).
43. Guillemin, C. *et al*. DNA methylation signature of childhood chronic physical aggression in T cells of both men and women. *PloS one* **9**, e86822, doi:10.1371/journal.pone.0086822 (2014).
44. Horvath, S. DNA methylation age of human tissues and cell types. *Genome Biol* **14**, R115, doi:10.1186/gb-2013-14-10-r115 (2013).
45. Reinius, L. E. *et al*. Differential DNA methylation in purified human blood cells: implications for cell lineage and studies on disease susceptibility. *PloS one* **7**, e41361, doi:10.1371/journal.pone.0041361 (2012).
46. Miyazaki, K. *et al*. A novel HECT-type E3 ubiquitin ligase, NEDL2, stabilizes p73 and enhances its transcriptional activity. *Biochemical and biophysical research communications* **308**, 106–113 (2003).
47. Yang, A. *et al*. p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature* **404**, 99–103, doi:10.1038/35003607 (2000).
48. Nakagawa, O. *et al*. Centronuclear myopathy in mice lacking a novel muscle-specific protein kinase transcriptionally regulated by MEF2. *Genes & development* **19**, 2066–2077, doi:10.1101/gad.1338705 (2005).
49. Nieratschker, V. *et al*. Bruchpilot in ribbon-like axonal agglomerates, behavioral defects, and early death in SRPK79D kinase mutants of Drosophila. *PLoS genetics* **5**, e1000700, doi:10.1371/journal.pgen.1000700 (2009).
50. Nitert, M. D. *et al*. Impact of an exercise intervention on DNA methylation in skeletal muscle from first-degree relatives of patients with type 2 diabetes. *Diabetes* **61**, 3322–3332, doi:10.2337/db11-1653 (2012).
51. Ronn, T. *et al*. A six months exercise intervention influences the genome-wide DNA methylation pattern in human adipose tissue. *PLoS genetics* **9**, e1003572, doi:10.1371/journal.pgen.1003572 (2013).
52. French, S. W. Epigenetic events in liver cancer resulting from alcoholic liver disease. *Alcohol research: current reviews* **35**, 57–67 (2013).
53. Bonsch, D., Lenz, B., Reulbach, U., Kornhuber, J. & Bleich, S. Homocysteine associated genomic DNA hypermethylation in patients with chronic alcoholism. *Journal of neural transmission* **111**, 1611–1616, doi:10.1007/s00702-004-0232-x (2004).
54. Semmler, A. *et al*. Alcohol abuse and cigarette smoking are associated with global DNA hypermethylation: results from the German Investigation on Neurobiology in Alcoholism (GINA). *Alcohol* **49**, 97–101, doi:10.1016/j.alcohol.2015.01.004 (2015).
55. Cho, B. K., Rao, V. P., Ge, Q., Eisen, H. N. & Chen, J. Homeostasis-stimulated proliferation drives naive T cells to differentiate directly into memory T cells. *The Journal of experimental medicine* **192**, 549–556 (2000).
56. Wijetunga, N. A. *et al*. The meta-epigenomic structure of purified human stem cell populations is defined at cis-regulatory sequences. *Nat Commun* **5**, 5195, doi:10.1038/ncomms6195 (2014).
57. Farris, S. P., Arasappan, D., Hunicke-Smith, S., Harris, R. A. & Mayfield, R. D. Transcriptome organization for chronic alcohol abuse in human brain. *Mol Psychiatry* **20**, 1438–1447, doi:10.1038/mp.2014.159 (2015).
58. Zhang, H. *et al*. Differentially co-expressed genes in postmortem prefrontal cortex of individuals with alcohol use disorders: influence on alcohol metabolism-related pathways. *Hum Genet* **133**, 1383–1394, doi:10.1007/s00439-014-1473-x (2014).

59. Shvetsov, Y. B. *et al*. Intraindividual variation and short-term temporal trend in DNA methylation of human blood. *Cancer Epidemiol Biomarkers Prev* **24**, 490–497, doi:10.1158/1055-9965.EPI-14-0853 (2015).
60. Saunders, J. B., Aasland, O. G., Babor, T. F., de la Fuente, J. R. & Grant, M. Development of the Alcohol Use Disorders Identification Test (AUDIT): WHO Collaborative Project on Early Detection of Persons with Harmful Alcohol Consumption–II. *Addiction* **88**, 791–804 (1993).
61. Mercier, C. *et al*. Profiles of alcoholics according to the SCL-90-R: a confirmative study. *Int J Addict* **27**, 1267–1282 (1992).
62. Moak, D. H., Anton, R. F. & Latham, P. K. Further validation of the Obsessive-Compulsive Drinking Scale (OCDS). Relationship to alcoholism severity. *The American journal on addictions/American Academy of Psychiatrists in Alcoholism and Addictions* **7**, 14–23 (1998).
63. Peters, T. J. *et al*. *De novo* identification of differentially methylated regions in the human genome. *Epigenetics Chromatin* **8**, 6, doi:10.1186/1756-8935-8-6 (2015).

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Author Contributions

V.N. and M.S.K. conceived and designed the study. C.B., K.N.K., A.D., R.W., I.L. and A.B. took part in recruiting study participants. J.L.M. acquired the 450 K data. C.B., S.A.I. and A.M.M. performed and analysed experiments. S.A.I. prepared the figures. C.B. and S.A.I. wrote the first draft of the manuscript. All authors participated in the preparation, modification and revision of the manuscript.

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DNA methylation signatures of chronic alcohol dependence in purified $CD3^+$ T-cells of patients undergoing alcohol treatment

Christof Brückmann, Sumaiya A. Islam, Julia L. MacIsaac, Alexander M. Morin, Kathrin N. Karle, Adriana Di Santo, Richard Wüst, Immanuel Lang, Anil Batra, Michael S. Kobor, and Vanessa Nieratschker

Supplementary Information

Supplementary methods

DNA methylation array data quality control and normalization: Quality control, processing and differential DNAm analysis of 450K array data was performed as previously described.[1,2] Briefly, raw intensity values from the arrays were imported into Illumina GenomeStudio V2011.1 software and subjected to initial quality control checks for array staining, extension and bisulfite conversion followed by color correction and background adjustment using control probes contained on the 450K array. Subsequent processing and analysis were performed in R Version 3.2.1 (http://www.r-project.org). Profiles from 65 probes targeting single nucleotide polymorphisms (SNPs) were used to ensure T1 and T2 samples were indeed matched from the same individual. The 65 SNP probes were also filtered out of the dataset. Additional probe filtering was performed in which poor performing probes including those with detection *P*-values greater than 0.01, probes with missing beta values, and probes for which less than three beads contributed to the signal in any sample were eliminated (a total of 13 903). Recent re-annotation of the Illumina 450K array[3] was used to filter 19 343 probes that are known to be polymorphic at the target CpG. Probes which have nonspecific in silico binding to the sex chromosomes were assessed in a post-hoc analysis following differential DNAm analysis to ensure they did not overlap with identified hits.[3] Together, quality control checks eliminated 33 311 probes, leaving a total of 452 266 probes for further analysis. Following quality control processing, quantile normalization was conducted using the lumi R package[4] after assessment using the quantro package indicated that quantile normalization was appropriate for this dataset.[5] Differences between Type I and Type II probes on the 450K array were normalized using Subset-quantile Within Array Normalization (SWAN).[6] ComBat[7] was then used to remove chip and row effects, while protecting

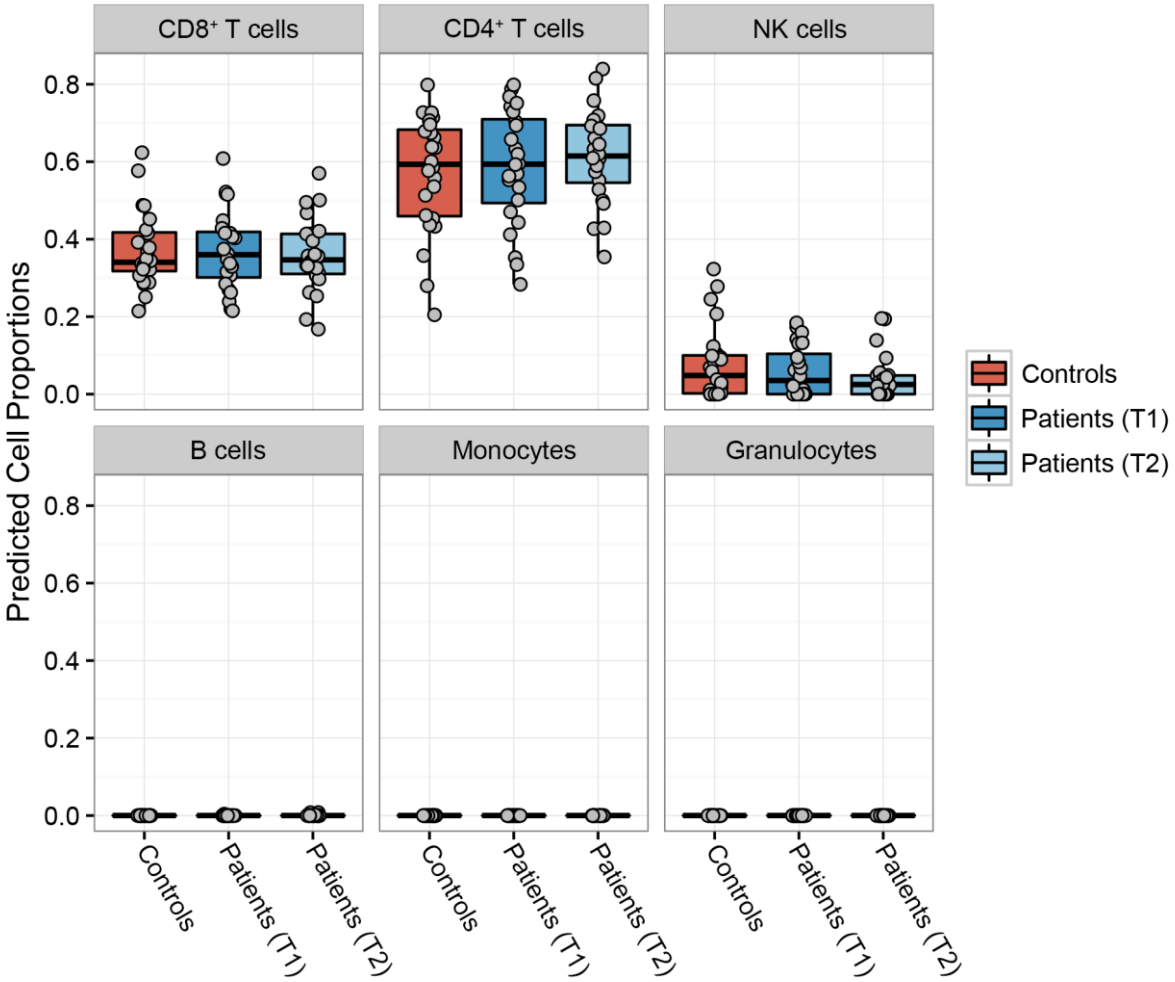
sample group. Removal of technical variation was assessed by principal component analysis (PCA).

Blood cell type deconvolution: To test for potential contamination of bead-purified $CD3^+$ T-cell samples by other blood cell types, a well-established algorithm was used to bioinformatically estimate cell type composition based on underlying reference DNAm profiles.[8,9] In addition, the 450K data was subjected to advanced DNA methylation age analysis in blood using a publicly available DNA methylation age predictor tool in order to obtain predicted abundance measures of additional blood cell types including plasma blasts, $CD8^+CD28^-CD45RA^-$ (memory and effector) T-cells, naïve $CD8^+$ T-cells and naïve $CD4^+$ T-cells.[10] Upon detection of potential non-T-cell contamination in a fraction of samples, we removed this cell-type composition variation by regressing probewise DNAm on estimated cell type proportions, as previously described.[11] The residuals of each regression model were applied to the mean value of each data series to obtain the 'corrected' DNAm data. PCA was subsequently used to check that the presence of the cell type proportions in DNAm variation was minimal in the corrected dataset. PCA was additionally used to check for correlation of other known meta-variables (i.e. sample group, age, daily smoking) with the underlying DNAm patterns of the uncorrected and corrected 450K datasets, respectively. Note that for all PCA analyses, the top-ranking PC (denoted as PC0) was negated as it is not informative of inter-individual variance in the DNAm data.[12]

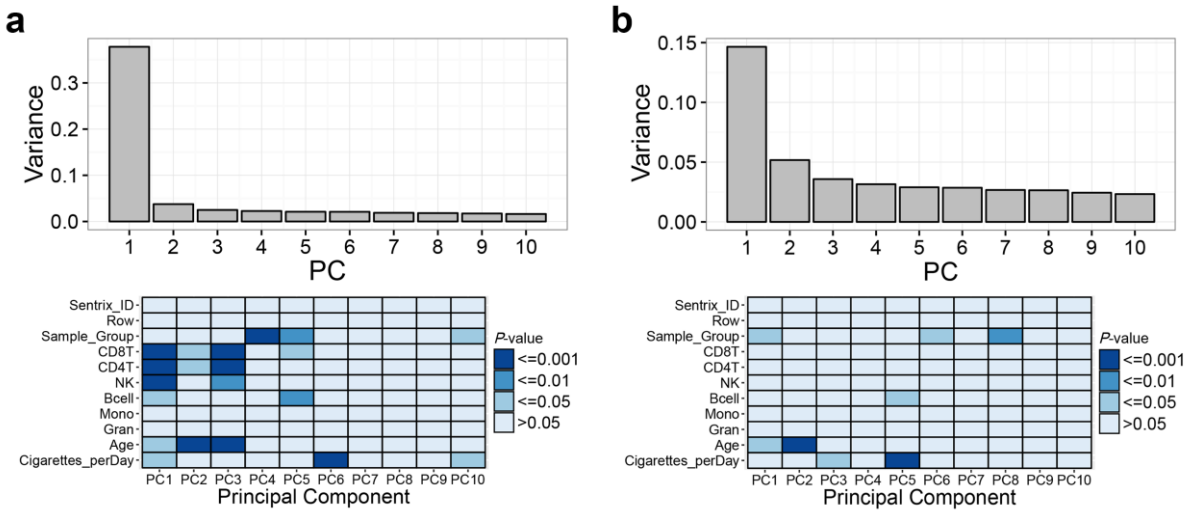
Differential methylation analyses of 450K dataset: The cell-type corrected 450K dataset was subsetted into controls versus patients (T1), patients (T1) versus patients (T2) and controls versus patients (T2) sample sets, respectively, prior to differential DNAm analysis. In the genome-wide analyses, differentially methylated probes were identified using the R limma package's moderated t-statistics with empirical Bayesian variance estimation.[13] Specifically, in the comparison of controls

versus patients (T1), a linear model was fit for each probe's DNAm measures with sample group as the main effect, adjusted for age and smoking levels. In the comparison of patients (T1) and patients (T2) samples, differentially methylated probes were identified using paired testing in linear regression analysis. For both of these comparisons, differentially methylated regions (DMRs) were detected using DMRcate package which uses the moderated t-statistics generated in their respective limma analyses.[14] In the comparison of controls versus patients (T2), we sought to assess which differentially methylated sites between controls and patients (T1) exhibited reversion in the patient (T2) samples such that their DNAm levels were comparable to controls. To address this, we specifically tested the 59 hits identified between controls versus patients (T1) (FDR < 0.1 and DNAm difference > 5%) by fitting individual linear models for each of the 59 probes. For all tests, the resulting *P*-values were adjusted using the Benjamini-Hochberg False Discovery Rate (FDR) method.[15] All statistical analyses were performed on transformed M-values.[16]

Questionnaire evaluation: The AUDIT score is the sum of all 10 items of the questionnaire. The GSI score represents the sum of all the subscales of the SCL-90-R divided by the number of answered items (usually 90). For the OCDS score, the higher value of four item pairs (Items 1 and 2, 7 and 8, 9 and 10, and 12 and 13) was added up with the remaining items, leading to a potential range of 0 to 40. Up to one missing item was allowed and replaced by adding the mean of all other items.



Supplementary Figure S1. Estimations of blood cell proportions in samples based on underlying reference DNAm profiles. Estimates were predicted using the Houseman blood cell deconvolution algorithm. There was no statistically significant association between predicted proportions of any cell type and sample group (Mann-Whitney U test for comparison of controls and patients (T1) or controls and patients (T2); Wilcoxon signed-rank test for comparison of matched patients (T1) and patients (T2) samples).



Supplementary Figure S2. Principal component analyses before and after regression-based adjustment of the 450K data. PCA showing the correlation of known phenotypic and technical variables to the top 10 principal components, each representing an incremental proportion of the variance in the methylation data. a) Top 10 PCs in unadjusted 450K dataset (representing 60% of the DNAm variance) and b) top 10 PCs in the adjusted 450K dataset (representing 45% of the DNAm variance).

Supplementary Table S1. Differentially methylated sites between Controls and Patients (T1)

#	Probe ID	Gene	Region	Average beta Controls	Average beta Patients (T1)	Δ-beta	*P*-Value	BH-adjusted *P*-Value
1	cg18752527	HECW2	intragenic	0.342	0.276	0.066	4.30E-07	0.0213
2	cg08109624	None	intergenic	0.760	0.817	-0.057	8.15E-07	0.0234
3	cg10168086	None	intergenic	0.535	0.484	0.051	1.24E-06	0.0256
4	cg07280807	None	intergenic	0.755	0.822	-0.068	2.44E-06	0.0366
5	cg12173150	None	intergenic	0.321	0.385	-0.064	3.02E-06	0.0370
6	cg01059398	TNFSF10	intragenic	0.261	0.209	0.052	1.07E-05	0.0627
7	cg17940902	HLA-DMA	promoter	0.399	0.450	-0.051	1.19E-05	0.0640
8	cg22778903	MX2	intragenic	0.304	0.355	-0.051	1.34E-05	0.0666
9	cg14612335	SKIL	promoter	0.423	0.368	0.055	1.38E-05	0.0666
10	cg11580026	None	intergenic	0.600	0.549	0.051	1.51E-05	0.0691
11	cg12284098	MYOM2	intragenic	0.534	0.477	0.056	1.54E-05	0.0691
12	cg26091609	CTLA4	intragenic	0.578	0.518	0.060	1.59E-05	0.0691
13	cg09768654	SRPK3	promoter	0.374	0.466	-0.092	1.65E-05	0.0691
14	cg06851207	PNMAL1	promoter	0.528	0.617	-0.089	1.84E-05	0.0691
15	cg14702960	None	intergenic	0.742	0.689	0.052	1.92E-05	0.0691
16	cg00449728	MAPRE2	intragenic	0.750	0.693	0.057	2.98E-05	0.0702
17	cg22851561	ELMSAN1	intragenic	0.432	0.380	0.052	3.00E-05	0.0702
18	cg02536838	ANGPT1	promoter	0.605	0.530	0.075	3.14E-05	0.0702
19	cg15841511	None	intergenic	0.729	0.788	-0.059	3.42E-05	0.0706
20	cg24392939	CRYBG3	intragenic	0.562	0.510	0.052	3.62E-05	0.0725
21	cg12761472	CEP85L	promoter	0.621	0.566	0.055	4.13E-05	0.0754
22	cg02652579	SYNGAP1	promoter	0.623	0.563	0.059	4.17E-05	0.0758
23	cg22865905	SNORA69	three_plus	0.794	0.743	0.051	4.26E-05	0.0764
24	cg27201673	PNMAL1	promoter	0.213	0.263	-0.050	5.41E-05	0.0778
25	cg04936619	C17orf75	intragenic	0.314	0.245	0.069	5.88E-05	0.0778
26	cg11121969	PCBP3	promoter	0.691	0.627	0.064	6.26E-05	0.0778
27	cg00246693	ARHGAP42	Promoter	0.340	0.393	-0.053	7.10E-05	0.0778

#	Probe ID	Gene	Region	Average beta Controls	Average beta Patients (T1)	Δ-beta	*P*-Value	BH-adjusted *P*-Value
28	cg10399005	None	intergenic	0.776	0.833	-0.057	7.11E-05	0.0778
29	cg16529483	SRPK3	promoter	0.252	0.357	-0.105	7.18E-05	0.0780
30	cg01220513	SH3KBP1	intragenic	0.506	0.454	0.051	8.08E-05	0.0791
31	cg26926002	None	intergenic	0.719	0.777	-0.058	8.10E-05	0.0791
32	cg14544087	MIR155HG	intragenic	0.290	0.227	0.063	8.64E-05	0.0791
33	cg20893919	TRPC3	intragenic	0.703	0.754	-0.051	9.23E-05	0.0801
34	cg18682028	FYCO1	intragenic	0.394	0.338	0.056	9.24E-05	0.0801
35	cg04362790	None	intergenic	0.697	0.644	0.052	9.32E-05	0.0801
36	cg09060654	LIPA	intragenic	0.578	0.656	-0.079	9.51E-05	0.0801
37	cg02451774	NBPF8	intragenic	0.431	0.483	-0.053	9.98E-05	0.0806
38	cg18723276	USP29	promoter	0.723	0.774	-0.051	0.0001	0.0819
39	cg13180722	None	intergenic	0.338	0.401	-0.062	0.0001	0.0830
40	cg12230162	SRPK3	promoter	0.357	0.463	-0.105	0.0001	0.0835
41	cg18890544	None	intergenic	0.846	0.905	-0.059	0.0001	0.0839
42	cg24496423	SRPK3	promoter	0.309	0.393	-0.084	0.0001	0.0854
43	cg02661764	None	intergenic	0.419	0.360	0.059	0.0001	0.0867
44	cg01400671	None	intergenic	0.409	0.345	0.064	0.0001	0.0874
45	cg13609457	None	intergenic	0.577	0.521	0.056	0.0002	0.0897
46	cg25880958	None	intergenic	0.591	0.645	-0.054	0.0002	0.0898
47	cg18376497	INPP4B	intragenic	0.286	0.223	0.064	0.0002	0.0919
48	cg13784312	RAPGEF1	intragenic	0.187	0.136	0.051	0.0002	0.0928
49	cg07135405	MIR1914	three_plus	0.540	0.394	0.146	0.0002	0.0928
50	cg20475486	None	intergenic	0.702	0.759	-0.058	0.0002	0.0936
51	cg11858450	CCDC105	intragenic	0.709	0.762	-0.053	0.0002	0.0940
52	cg05927817	None	intergenic	0.726	0.787	-0.061	0.0002	0.0940
53	cg00306893	None	intergenic	0.737	0.675	0.062	0.0002	0.0940
54	cg10365886	TNXB	intragenic	0.566	0.672	-0.105	0.0002	0.0947
55	cg27503950	None	intergenic	0.633	0.696	-0.063	0.0002	0.0952

#	Probe ID	Gene	Region	Average beta Controls	Average beta Patients (T1)	Δ-beta	*P*-Value	BH-adjusted *P*-Value
56	cg01089001	GALNT18	intragenic	0.317	0.382	-0.065	0.0002	0.0953
57	cg12564698	GAL	three_plus	0.312	0.261	0.051	0.0002	0.0953
58	cg16197188	NRG3	intragenic	0.723	0.672	0.051	0.0003	0.0995
59	cg04088338	None	intergenic	0.430	0.378	0.052	0.0003	0.0999

Abbreviations: Average beta, mean methylation values (%); Benjamini-Hochberg (BH) adjusted P-value.

Supplementary Table S2. Top listed hits detected by both site-specific and DMRcate analysis.

Probe ID	Gene	DMR	Position	Average beta Controls	Average beta Patients (T1)	Δ-beta	*P*-Value	FDR
cg16529483	*SRPK3*	chrX:153046175-153047707	153046451	0.252	0.357	-0.105	3.52E-23	5.90E-19
cg24496423	*SRPK3*	chrX:153046175-153047707	153046480	0.309	0.393	-0.084	2.84E-23	4.94E-19
cg12230162	*SRPK3*	chrX:153046175-153047707	153046482	0.357	0.463	-0.105	2.80E-23	4.94E-19
cg09768654	*SRPK3*	chrX:153046175-153047707	153046386	0.374	0.466	-0.092	6.72E-23	1.01E-18
cg18890544		chr1:242220301-242220925	242220538	0.846	0.905	-0.059	1.75E-18	8.88E-15
cg08109624		chr1:242220301-242220925	242220925	0.760	0.817	-0.057	1.69E-19	1.02E-15
cg27503950		chr6:160023581-160024144	160024002	0.633	0.696	-0.063	2.92E-15	6.57E-12
cg09060654	*LIPA*	chr10:90985055-90985062	90985062	0.578	0.656	-0.079	1.96E-07	4.53E-05

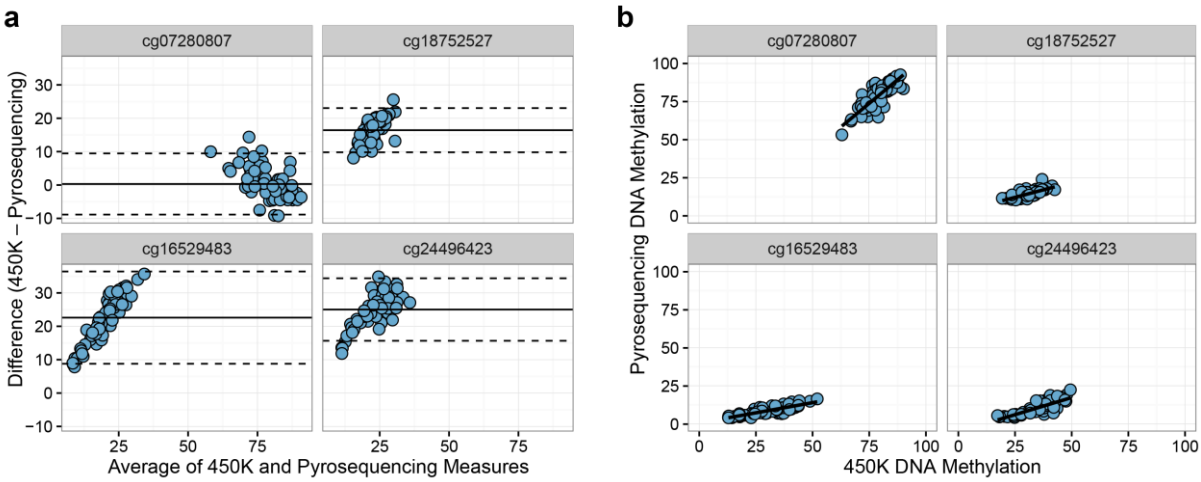
Abbreviations: Average beta, mean methylation values (%); FDR, Benjamini-Hochberg False Discovery Rate; DMR, differentially methylated region.

Supplementary Table S3. Differentially methylated sites between Patients (T1) and Patients (T2)

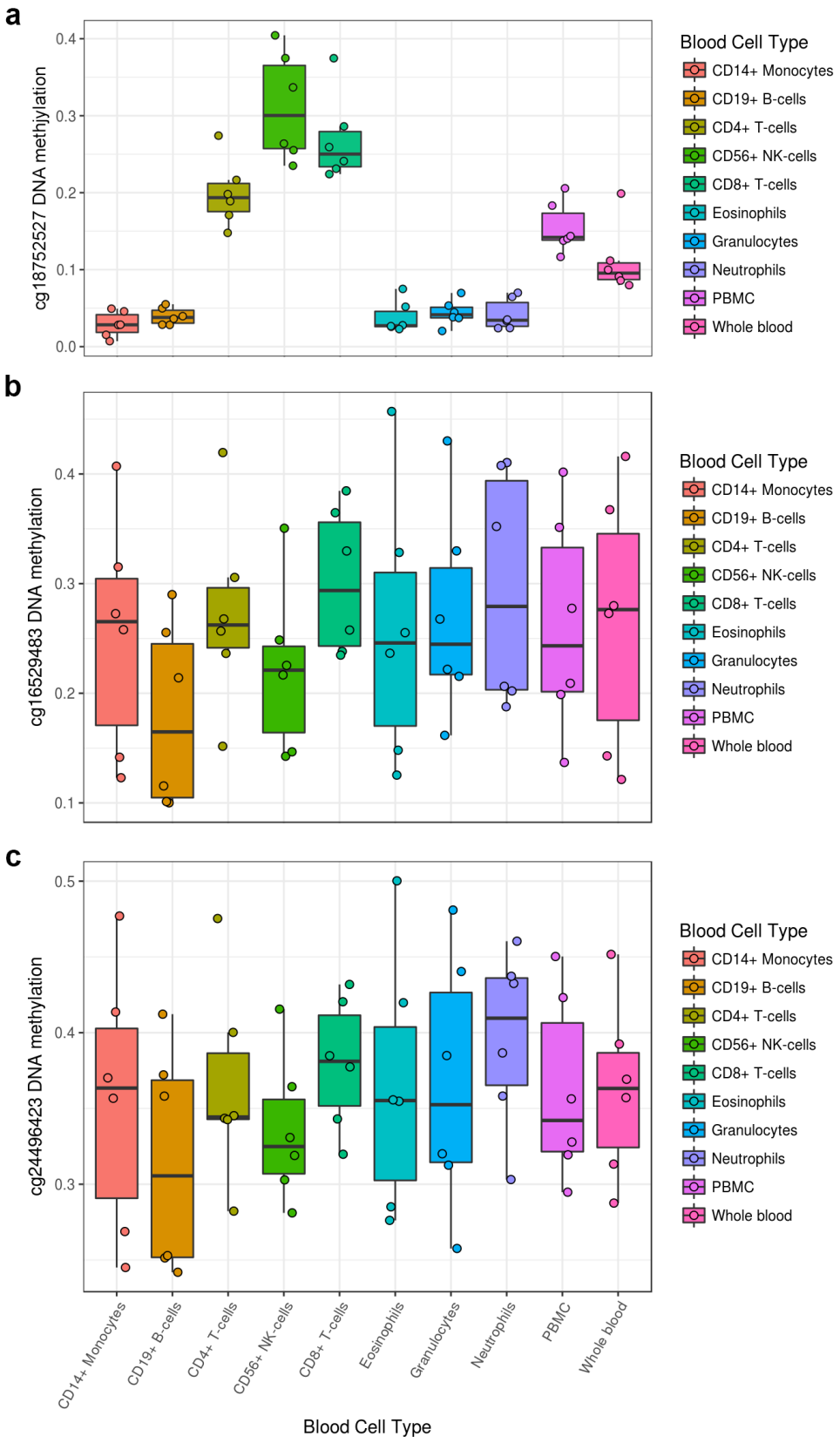
#	Probe ID	Gene	Region	Average beta Patients (T1)	Average beta Patients (T2)	Δ-beta	*P*-Value	BH-adjusted *P*-Value
1	cg15500907	LAMA4	intragenic	0.485	0.542	-0.056	1.01E-06	0.0323
2	cg05266321	CCR2	intragenic	0.545	0.606	-0.061	4.63E-06	0.0487
3	cg13279700	C6orf10	intragenic	0.481	0.544	-0.063	1.76E-05	0.0561
4	cg14054990	KRTAP19-5	promoter	0.431	0.482	-0.052	1.84E-05	0.0565
5	cg21049302	None	intergenic	0.466	0.522	-0.056	1.98E-05	0.0565
6	cg17022548	NRG2	intragenic	0.204	0.258	-0.054	1.99E-05	0.0565
7	cg22472360	TRIO	intragenic	0.514	0.569	-0.055	2.09E-05	0.0569
8	cg07920414	RIMS3	intragenic	0.438	0.493	-0.055	2.18E-05	0.0572
9	cg04088338	None	intergenic	0.378	0.429	-0.051	2.54E-05	0.0590
10	cg12240358	HOMER2	intragenic	0.462	0.519	-0.057	2.68E-05	0.0590
11	cg09712306	AURKA	intragenic	0.602	0.660	-0.058	3.48E-05	0.0605
12	cg07939743	None	intergenic	0.289	0.341	-0.052	3.50E-05	0.0605
13	cg00803692	CCR5	promoter	0.370	0.424	-0.054	3.73E-05	0.0620
14	cg10177030	SNORD12	three_plus	0.419	0.472	-0.053	3.85E-05	0.0627
15	cg15439110	None	intergenic	0.444	0.525	-0.080	3.93E-05	0.0628
16	cg20385229	SLIRP	intragenic	0.392	0.444	-0.052	4.13E-05	0.0628
17	cg02393640	LUZP6	intragenic	0.390	0.443	-0.052	5.63E-05	0.0668
18	cg17863551	CD177	promoter	0.419	0.478	-0.059	6.27E-05	0.0670
19	cg15279541	None	intergenic	0.388	0.439	-0.051	7.14E-05	0.0677
20	cg20171999	RRS1	three_plus	0.403	0.474	-0.070	8.93E-05	0.0680
21	cg20559385	None	intergenic	0.428	0.479	-0.052	9.43E-05	0.0680
22	cg21429780	MAML3	intragenic	0.493	0.545	-0.052	0.0001	0.0680
23	cg01482790	HNRNPM	intragenic	0.289	0.339	-0.050	0.0001	0.0681
24	cg20684197	FGF1	intragenic	0.395	0.445	-0.051	0.0001	0.0684
25	cg04279139	MANSC4	promoter	0.410	0.461	-0.051	0.0001	0.0688
26	cg16853860	PSMB9	intragenic	0.272	0.332	-0.060	0.0001	0.0696
27	cg27062514	CTR9	intragenic	0.463	0.526	-0.064	0.0001	0.0721

#	Probe ID	Gene	Region	Average beta Patients (T1)	Average beta Patients (T2)	Δ-beta	*P*-Value	BH-adjusted *P*-Value
28	cg09931909	MB21D1	intragenic	0.420	0.497	-0.077	0.0001	0.0735
29	cg13340231	ZNF704	intragenic	0.528	0.583	-0.055	0.0002	0.0751
30	cg10035831	RPTOR	intragenic	0.446	0.503	-0.057	0.0002	0.0753
31	cg13927756	MYO10	intragenic	0.468	0.524	-0.056	0.0002	0.0754
32	cg08749576	None	intergenic	0.627	0.684	-0.058	0.0002	0.0761
33	cg15484808	RPS18	intragenic	0.480	0.534	-0.054	0.0002	0.0811
34	cg12802876	None	intergenic	0.359	0.418	-0.059	0.0002	0.0828
35	cg03548415	None	intergenic	0.422	0.473	-0.051	0.0003	0.0853
36	cg20547015	PPP1CC	intragenic	0.453	0.517	-0.064	0.0003	0.0862
37	cg23214895	None	intergenic	0.569	0.620	-0.051	0.0003	0.0878
38	cg12478092	CCDC116	promoter	0.510	0.573	-0.063	0.0003	0.0879
39	cg15683542	MIPEP	intragenic	0.694	0.747	-0.053	0.0003	0.0883
40	cg09514545	MIR525	three_plus	0.442	0.501	-0.060	0.0004	0.0908
41	cg01789743	NID1	intragenic	0.499	0.552	-0.053	0.0004	0.0910
42	cg18524114	None	intergenic	0.339	0.389	-0.050	0.0005	0.0933
43	cg04410448	ZC2HC1B	intragenic	0.491	0.541	-0.051	0.0005	0.0949
44	cg13714407	RAPGEF1	intragenic	0.367	0.426	-0.059	0.0005	0.0953
45	cg27367066	None	intergenic	0.455	0.510	-0.054	0.0006	0.0967
46	cg26837708	YBX1	intragenic	0.388	0.445	-0.058	0.0006	0.0967
47	cg14817867	PRPSAP2	intragenic	0.419	0.471	-0.052	0.0006	0.0971
48	cg13598358	PPP1CC	intragenic	0.362	0.418	-0.056	0.0006	0.0978

Abbreviations: Average beta, mean methylation values (%); Benjamini-Hochberg (BH) adjusted P-value.



Supplementary Figure S3. Correlations between 450K array and pyrosequencing measures. a) Bland-Altman plots for verified CpGs show a slightly biased agreement between 450K dataset and pyrosequencing measures. b) Strong positive correlation between 450K and pyrosequencing measures for cg07280807 (Spearman r_s = 0.85, *P* = 2E-16), cg18752527 (r_s = 0.71, *P* = 3E-12), cg16529483 (r_s = 0.79, *P* = 4E-16) and cg24496423 (r_s = 0.80, *P* = 2E-16).



Supplementary Figure S4. Blood cell type associations of 3 examined CpG sites. a) DNA methylation of cg18752527 in the *HECW2* gene was significantly associated with $CD4^+$ and $CD8^+$ T cells, along with NK cells, as determined by differential DNAm testing using a previous 450K dataset of purified blood cell types[17] (*P* = 7.6E-15, ANOVA). DNA methylation of cg16529483 (b) and cg24496423 (c) in the *SRPK3* gene were not significantly associated with any cell type (*P* > 0.6, ANOVA).

Supplementary Table S4. Primers and PCR programs for validation and replication.

cg07280807 (intergenic) fwd: 5'-GTTATGGTTGGGTTTTTGGG-3' rev: 5'-Bio-CCTATCTCCTCAAACAAAAACTAAAAA-3' seq: 5'-AGTTAGGGATTATAGTGTAGTTG-3' Amplicon length: 156 bp coordinates: chr14:70,317,178-70,317,333 Note: The amplicon contains 3 CpG sites, of which the third is cg07280807	**PCR program:** 95°C – 15 min ---------- 45 cycles: 94°C – 30 sec 50°C – 30 sec 72°C – 30 sec ---------- 72°C – 10 min 4°C – hold
cg18752527 (*HECW2*) fwd: 5'-GTGTTTGTGGGAATGTTTTTTATA-3' rev: 5'-Bio- CACACTACACTTTCATTTTCTATCAA-3' seq: 5'- TTTTTAGATATATAAATTTTTTTTTT-3' Amplicon length: 135 bp coordinates: chr2:197,132,798-197,132,932	**PCR program:** 95°C – 15 min ---------- 45 cycles: 94°C – 30 sec 50°C – 30 sec 72°C – 30 sec ---------- 72°C – 10 min 4°C – hold
cg16529483 / cg24496423 (*SRPK3*) fwd/seq: 5'-GTTATTTATAAAGGAGGGTGAGATTA-3' rev: 5'-Bio-AACCACTACTCCTATAAAACCCCAC-3' Amplicon length: 85 bp coordinates: chrX:153,046,424-153,046,508 Note: The amplicon contains 5 CpG sites, of which the first is cg16529483 and the fourth is cg24496423. Due to CpG sites in the primer binding area, the primers contain 1 (fwd) and 2 (rev) mismatches, which are highlighted underlined.	**PCR program:** 95°C – 15 min ---------- 45 cycles: 94°C – 30 sec 48°C – 30 sec 72°C – 30 sec ---------- 72°C – 10 min 4°C – hold

Abbreviations: fwd, forward primer; rev, reverse primer; seq, sequencing primer; Bio, biotin-modification; bp, basepair.

Supplementary References

1 Esposito, E. A. *et al.* Differential DNA methylation in peripheral blood mononuclear cells in adolescents exposed to significant early but not later childhood adversity. *Dev Psychopathol* **28**, 1385-1399, doi:10.1017/S0954579416000055 (2016).

2 De Souza, R. A. *et al.* DNA methylation profiling in human Huntington's disease brain. *Human molecular genetics* **25**, 2013-2030, doi:10.1093/hmg/ddw076 (2016).

3 Price, M. E. *et al.* Additional annotation enhances potential for biologically-relevant analysis of the Illumina Infinium HumanMethylation450 BeadChip array. *Epigenetics Chromatin* **6**, 4, doi:10.1186/1756-8935-6-4 (2013).

4 Du, P., Kibbe, W. A. & Lin, S. M. lumi: a pipeline for processing Illumina microarray. *Bioinformatics* **24**, 1547-1548, doi:10.1093/bioinformatics/btn224 (2008).

5 Hicks, S. C. & Irizarry, R. A. quantro: a data-driven approach to guide the choice of an appropriate normalization method. *Genome Biol* **16**, 117, doi:10.1186/s13059-015-0679-0 (2015).

6 Maksimovic, J., Gordon, L. & Oshlack, A. SWAN: Subset-quantile within array normalization for illumina infinium HumanMethylation450 BeadChips. *Genome Biol* **13**, R44, doi:10.1186/gb-2012-13-6-r44 (2012).

7 Johnson, W. E., Li, C. & Rabinovic, A. Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics* **8**, 118-127, doi:10.1093/biostatistics/kxj037 (2007).

8 Houseman, E. A. *et al.* DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC Bioinformatics* **13**, 86, doi:10.1186/1471-2105-13-86 (2012).

9 Koestler, D. C. *et al.* Blood-based profiles of DNA methylation predict the underlying distribution of cell types: a validation analysis. *Epigenetics* **8**, 816-826, doi:10.4161/epi.25430 (2013).

10 Horvath, S. DNA methylation age of human tissues and cell types. *Genome Biol* **14**, R115, doi:10.1186/gb-2013-14-10-r115 (2013).

11 Jones, M. J., Islam, S. A., Edgar, R. D. & Kobor, M. S. Adjusting for Cell Type Composition in DNA Methylation Data Using a Regression-Based Approach. *Methods in molecular biology*, doi:10.1007/7651_2015_262 (2015).

12 Lam, L. L. *et al.* Factors underlying variable DNA methylation in a human community cohort. *Proceedings of the National Academy of Sciences of the United States of America* **109 Suppl 2**, 17253-17260, doi:10.1073/pnas.1121249109 (2012).

13 Smyth, G. K. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* **3**, Article3, doi:10.2202/1544-6115.1027 (2004).

14 Peters, T. J. *et al.* De novo identification of differentially methylated regions in the human genome. *Epigenetics Chromatin* **8**, 6, doi:10.1186/1756-8935-8-6 (2015).

15 Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the royal statistical society. Series B (Methodological)*, 289-300 (1995).

16 Du, P. *et al.* Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. *BMC Bioinformatics* **11**, 587, doi:10.1186/1471-2105-11-587 (2010).

17 Reinius, L. E. *et al.* Differential DNA methylation in purified human blood cells: implications for cell lineage and studies on disease susceptibility. *PloS one* **7**, e41361, doi:10.1371/journal.pone.0041361 (2012).

Paper 3

Knoblich N, Gundel F, Brückmann C, Becker-Sadzio J, Frischholz C, Nieratschker V.

DNA methylation of APBA3 and MCF2 in borderline personality disorder: potential biomarkers for response to psychotherapy.

In submission

DNA methylation of *APBA3* and *MCF2* in borderline personality disorder: potential biomarkers for response to psychotherapy

Nora Knoblich[1], Friederike Gundel[1], Christof Brückmann[1], Julia Becker-Sadzio[1], Christian Frischholz[1], Vanessa Nieratschker[1]

[1]Department of Psychiatry and Psychotherapy, University Hospital of Tuebingen, Tuebingen, Germany.

Correspondence to:
Jun.-Prof. Dr. Vanessa Nieratschker
Calwerstr. 14
72070 Tuebingen
Germany
Tel. +49 7071 2985523
Fax. +49 7071 295903
E-Mail: vanessa.nieratschker@med.uni-tuebingen.de

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Abstract

Borderline personality disorder (BPD) is a severe and complex mental disease associated with high suicidal tendencies and hospitalization rates. Accumulating evidence suggests that epigenetic mechanisms are implicated in the etiology of BPD. A recent epigenome-wide study identified several novel genes, which are epigenetically dysregulated in BPD. Those genes include *APBA3*, *MCF2* and *NINJ2*. Psychotherapy such as Dialectical Behavior Therapy (DBT), an established treatment for BPD, provides an excellent setting to investigate environmental influences on epigenetic mechanisms in order to identify biomarkers for disease status and therapy success. However, the effects of DBT on epigenetic regulation have only been researched in one previous study analyzing *BDNF*. In the present study we aimed to investigate the role of DNA methylation of *APBA3*, *MCF2* and *NINJ2* as possible biomarkers for treatment-outcome in BPD, whilst validating the previous findings of differential DNA methylation in a cohort of 44 BPD patients and 44 well matched healthy control individuals.

Unexpectedly, we did not detect significant DNA methylation differences between patients and control individuals. However, we found a high correlation between the methylation status of *APBA3* and *MCF2* and therapy outcome: before DBT treatment, both genes were significantly higher methylated in patients responding to therapy compared to patients that did not respond.

Our study is the first to report results pointing to possible predictive epigenetic biomarkers of DBT outcome in BPD patients. Following replication in independent cohorts our finding could facilitate the development of more personalized therapy concepts for BPD patients by including epigenetic information.

Introduction

With a prevalence of up to 2% in the general population and up to 25% in clinical settings, borderline personality disorder (BPD) is one of the most frequent personality disorders (Gunderson, 2009; Torgersen et al., 2001). A high rate of committed suicides (about 10% of the patients) characterizes the severity of this disease. In addition to the high rate of completed suicides, more than 70% of patients suffering from BPD had at least one suicide attempt in their medical history (Oldham, 2006). BPD is characterized by impairments in emotion and affect regulation, self-perception and interpersonal relationships. The severity of BPD is emphasized by the high rates of relapse after initial successful therapy observed in several short- and long-term studies (Gunderson, 2009; Leichsenring et al., 2011; Zanarini et al., 2006). This renders a sustainable therapy even more essential for a good prognosis.

Dialectical behavior therapy (DBT) established by M. Linehan, is a widely-used psychotherapeutic treatment for BPD, whose efficacy has been shown in many studies (Bohus et al., 2004; Bohus et al., 2000; Linehan, 1993; William D. Barley, 1993). Designed for suicidal patients meeting the BPD criteria, the main aim of the therapy is to reduce suicidal tendencies, including self-harmful behavior, as well as behavior preventing therapy or prolonging inpatient treatment (Bohus et al., 2004; Fleischhaker et al., 2011). Initially designed as outpatient treatment, the DBT was recently modified for inpatient settings, now typically lasting 12 weeks in European psychiatric institutions (Bohus et al., 2004).

The pathomechanism of BPD is not completely understood to date. M. Linehans' model of a biosocial development suggests that BPD is a disorder resulting from biological vulnerability combined with harming environmental influences. A depreciating and emotionally unstable environment during childhood together with genetic vulnerability could result in the disturbances of emotion regulation which is typical for BPD (Crowell et al., 2009; Linehan, 1993). Whereas twin and family studies suggest a heritability of BPD between 35 and 65% (Distel et al., 2009; Torgersen et al., 2000), individual risk genes could not be identified for BPD thus far (Calati et al., 2013; Gunderson, 2009). Over the past years, evidence emerged that epigenetic mechanism play a major role in the mediation of genome-environment-interactions. Alterations in epigenetic regulation have been described for several psychiatric disorders e.g. major depression, schizophrenia and BPD (Januar et al.,

2015; Perroud et al., 2016; Rivollier et al., 2014; Teschler et al., 2016). Epigenetics include posttranslational histone modifications, DNA methylation and the activity of non-coding RNAs (Hashimoto et al., 2010; Nieratschker et al., 2013). DNA methylation is catalyzed by methyltransferases (DNMTs) (Egger et al., 2004; Jones and Takai, 2001), which transfer a methyl group from S-adenosyl-methionine to cytosine creating 5-methyl-cytosine (Sutherland and Costa, 2003). Regulatory DNA methylation mainly occurs at the cytosine of a CpG dinucleotide. Whereas CpG sites are underrepresented throughout the genome, they are enriched in so called CpG islands (Jones and Takai, 2001), areas containing more than 50% of cytosine and guanine (Egger et al., 2004; Sutherland and Costa, 2003). CpG islands overlap with the promoter regions of 50 - 60% of human genes (Nieratschker et al., 2013) and are typically less methylated than CpG sites outside of CpG islands (Wang and Leung, 2004). Hypermethylation of these regions usually inhibits transcription through several mechanisms (Sutherland and Costa, 2003). The epigenome was formerly believed to be stable after the embryonal development (Nieratschker et al., 2013; Razin and Riggs, 1980). However, current studies imply that epigenetic regulation is a more dynamic process that is influenced by environmental factors not only prenatal, but also postnatal (Jones and Takai, 2001; Nieratschker et al., 2014).

Epigenetic research in BPD thus far has mainly focused on candidate genes of other psychiatric disorders e.g. *BDNF, COMT, 5-HTT* and *MAOA* (Dammann et al., 2011; Perroud et al., 2013; Teschler et al., 2013; Teschler et al., 2016). However, Teschler et al. investigated DNA methylation in BDP using a systematic epigenome-wide approach. In this previous study, 259 significantly differentially methylated CpG sites were discovered. The authors selected several of those sites for validation and where able to confirm their findings for three CpG sites located in *APBA3* and one site in *MCF2* and *NINJ2*, respectively (Teschler et al., 2013). An association with BPD has not been described for any of those genes before.

An influence of psychotherapy on DNA methylation levels has been described recently. Roberts et al. found significant alterations in DNA methylation levels of the serotonin transporter gene (*5-HTT*) in the course of anxiety treatment in children (Roberts et al., 2014): Those who responded to cognitive behavior therapy (CBT) showed increased methylation levels at a specific CpG site after treatment, whereas the levels of non-responders decreased significantly. A second study reported similar results for *FKBP5:* Here, a decrease in DNA methylation during therapy was

associated with a strong reduction in symptom severity, whereas an increase in DNA methylation was associated with a weaker response to treatment (Roberts et al., 2015). In addition to the findings in children suffering from anxiety disorder, an epigenetic effect of CBT has also been described in adult anxiety patients: Ziegler et al. detected epigenetic alterations in *MAOA* associated with response to CBT in adult panic disorder patients (Ziegler et al., 2016). Prior to therapy the DNA methylation levels of *MAOA* were significantly reduced in patients compared to healthy controls. While the DNA methylation levels of responders increased and where no longer significantly different from those of control individuals, the DNA methylation levels of non-responders decreased even further during the course of the CBT (Ziegler et al., 2016). A similar correlation has been described for *FKBP5* in the context of PTSD: While therapy responders showed a decrease, non-responders showed an increase in DNA methylation over time (Yehuda et al., 2013). In contrast to those findings, DNA methylation of *GR* was not significantly different in responders and non-responders post-treatment, but the pre-treatment DNA methylation levels predicted treatment response to the extent that responders showed higher levels compared to non-responders (Yehuda et al., 2013). Only one study thus far investigated the epigenetic effects of DBT in BPD: Perroud et al. showed that *BDNF* DNA methylation levels decreased during treatment in therapy responders, but increased in non-responders (Perroud et al., 2013).

The aim of the present study was to investigate differential DNA methylation of *APBA3*, *MCF2* and *NINJ2* (Teschler et al., 2013) as potential epigenetic biomarker for treatment outcome in BPD. In addition, our study was designed to validate the previous findings on differential methylation of those genes and to identify potential influences of the DBT on DNA methylation patterns. Our study included 44 patients and 44 age, as well as gender matched control individuals. In the patient group, DNA methylation analyses were performed pre-treatment and were repeated at the end of the DBT program.

Experimental procedures

Subjects

The sample was comprised of 44 BPD patients (37 females and 7 males, mean age 29.5 ± 8.4 years) hospitalized for a 12-week DBT program and 44 age and gender matched control individuals (37 females and 7 males, mean age 29.7 ± 8.8 years). Patients with a diagnosis of BPD according to the International Personality Disorder Examination (IPDE) and who met at least five diagnostic criteria of the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) were included in the study. Only control individuals with no psychiatric history were accepted in this study.

Phenotypic information about patients and control individuals was obtained by self-administered questionnaires. The following questionnaires were used: SCL90R (Franke, 2002), BSL23 (Wolf et al., 2009) and the childhood trauma questionnaire (CTQ) (Bernstein et al., 2003). All subjects were Caucasian and provided written informed consent prior to participation. The criteria for a BPD diagnosis are identity disturbance (present in 70.5% of our patients), unstable interpersonal relationships (90.9%), chronic feelings of emptiness (86.4%), efforts to avoid real or imagined abandonment (54.6%), affective instability (86.4%), inappropriate anger (77.3%), paranoid or dissociative symptoms (70.5%), impulsivity (86.4%) and self-harmful or suicidal behavior (88.6%). Patients reached an average of 2.42 points in the BSL23, which is considerably higher than the average score described in previous studies (Bohus et al., 2009; Wolf et al., 2009). The average t-score of the GSI was 79 points in patients. Control individuals reached a t-score of 44.7 and a BSL 23 score of 0.22, values indicating the absence of mental / psychological stress.

Therapy responders are usually defined by reduction of self-harmful behavior. Since information about changes in self-harmful behavior through therapy was not available for our cohort, we chose a combination of two parameters that display the overall psychical burden and well-being of the patients for the classification of responders and non-responders, namely the global severity index (GSI) derived from the symptom checklist 90 (SCL90) and the borderline symptom list 23 (BSL23), developed especially for the severity of the borderline personality disorder. A patient was defined as responder if the GSI t-score was reduced by more than 5 points post-

therapy and additionally a score lower than 2.05 for the BSL23 was reached. Applying this classification resulted in 7 responders and 17 non-responders.

The study was approved by the ethics committee of the University of Tuebingen and was conducted in accordance with the Declaration of Helsinki.

DNA methylation analysis

Ethylenediaminetetraacetic (EDTA) peripheral venous blood samples were taken from all patients within the first week of hospital admission (T1). During the last week of the 12-week DBT-program (T2), a second EDTA-blood sample was taken from the 24 patients (mean age 30.75 ± 8.81 years) completing therapy. EDTA-blood from control individuals was drawn immediately after study inclusion. Blood samples were instantly frozen and kept at -80°C until further usage.

DNA extraction was performed using the QIAamp DNA Blood Maxi-Kit (Qiagen, Hilden, Germany). 500 ng of genomic DNA was bisulfite converted using the EpiTect Fast Bisulfite Conversion Kit (Qiagen) according to manufacturer's protocol. PCR amplification and pyrosequencing was performed as described in Teschler et al (Teschler et al., 2013) with slight modifications. Primer (Eurofins Genomics, Ebersberg, Germany) sequences as well as cycling conditions are displayed in Table 1. For *APBA3* a nested PCR was performed. PCR products and a no template control were visualized on an agarose gel to verify successful amplification and specificity of the products.

Table 1: PCR conditions and primers used

Primer	Sequence	PCR-Program	
APBA3BSU1	5´-ATTTTAGTTTGGGTGATAGAGTGAGGTTT	95°C	15min
APBA3BSL1	5´-BIO-CCTATATAAACAATACCCAACTAAAACCTAA	94°C	30s
		49°C	30s
		72°C	30s
		72°C	10min
	(45 cycles, PCR-MasterMix QIAGEN)	8°C	storage

APBA3BSU11	5´-GAGGTTTTGTTTTAAAAAAAATAAATAAATT	95°C	15 min
APBA3BSL1	5´-BIO-CCTATATAAACAATACCCAACTAAAACCTAA	94°C	30s
APBA3BSSeq	5´-TTYGAAAAATAAAAAATTTGAGGTTT	45°C	30s
		72°C	30s
		72°C	10min
	(45 cycles, PCR-MasterMix QIAGEN)	8°C	storage
MCF2BSU1	5'-GGGTAGGAYGAGAGTAAAAAGTATGAGTT	95°C	2min
MCF2BSL1	5'-BIO-CAACTCTCTTCCTAAAAACAAACTTAATAAA	95°C	30s
MCF2BSSeq	5'-TTTATAAAGATTTTTAGTATTTTATTTTAAA	48,5°C	30s
		72°C	30s
		72°C	10min
	(45 cycles, GoTaq-MasterMix Promega)	8°C	storage
NINJ2BSU4	5'-TTTATAYGTGTGTGTAGGTGTATATTTTTTT	95°C	15min
NINJ2BSL2	5'-BIO-AAACAAACTACRTAAACTCCTCCAAA	94°C	30s
NINJ2BSSeq	5'-GTGTGTGTAGGTGTATATTTTTTTTAGAGG	47°C	30s
		72°C	30s
		72°C	10min
	(45 cycles, PCR-MasterMix QIAGEN)	8°C	storage

Processing of the PCR amplicons for the pyrosequencing analysis was performed according to the manufacturer's protocol and PCR products were then pyrosequenced using the PyroMark Q24 system and the PyroMark GoldReagents (Qiagen). To detect potentially biased amplification of differentially methylated fragments, DNA samples with known methylation levels (0%, 25%, 50%, 75% and 100%) were included as controls (EpiTect Control DNA, Qiagen) in the amplification and the pyrosequencing analysis.

The percentage of methylation at each of the three CpG sites analyzed was quantified using the PyroMark Q24 software version 2.0.6 (Qiagen). Pyrosequencing was performed in triplicates. To avoid batch effects, samples from patients and control individuals were mixed on each plate and the samples were randomly

assigned to different wells for each sequencing run. Only runs differing less than 3% were included in the analysis.

Statistical analyses

Statistical analysis was performed using SPSS Statistics Version 21 (IBM, NY, USA). DNA methylation levels of *APBA3* were normally distributed according to the Shapiro-Wilk test. T-tests for either independent or paired samples (for comparing T1 and T2) were applied.

For *MCF2*, DNA methylation levels were not normally distributed according to the Shapiro-Wilk test. Hence, non-parametric test methods were applied. Significance level was set as alpha <0.05.

Results

No difference in *APBA3* and *MCF2* DNA methylation status between patients at the beginning of the DBT program (T1) and control individuals

For *APBA3* two CpG sites were analyzed. The mean DNA methylation value of patients (n = 44, mean age 29.5 ± 8.4 years) was 83.7% for site 1 and 75.2% for site 2, overall DNA methylation was 79.5%. The mean DNA methylation value of control individuals (n = 44, mean age 29.7 ± 8.8 years) was 82.7% (site 1) and 74.5% (site 2), resulting in an overall DNA methylation value of 78.6%. This difference between patients and control individuals was not significant (independent samples t-test; site 1: t = 1.0 p = 0.3; site 2: t = 0.6, p = 0.5; overall DNA methylation: t = 0.8, p = 0.4).

For *MCF2* one CpG site was analyzed. The mean DNA methylation value of this CpG site was 47.2% in patients and 50.2% in control individuals – this difference was not significant (Mann-Whitney U-Test: Z = -1.0, p = 0.3).

Due to technical difficulties, *NINJ2* was not included in further analysis.

No effects of DBT on the DNA methylation status of *APBA3* and *MCF2*

When comparing patients completing the 12 weeks of therapy before and after treatment (n = 24, mean age 30.8 ± 8.8), DBT had no significant influence on the DNA methylation levels of either gene. Mean DNA methylation of *APBA3* before therapy was 78.8%, for site 1, 83.2% and for site 2 74.4%. After completion of therapy DNA methylation was 78.4%, 82.6% and 74.1%, respectively (paired samples t-test; overall: t = 0.5, p = 0.6, site 1: t = 0.7, p = 0.5; site 2: t = 0.3, p = 0.8). For *MCF 2* DNA methylation was 46.9% prior to therapy and 47.9% post-therapy (paired samples t-test; t = -0.010, p = 1.0).

Therapy responders show increased pre-treatment DNA methylation levels (T1)

Comparison of the DNA methylation status of *APBA3* before and after therapy shows that therapy responders display significantly higher overall DNA methylation values prior to therapy than non-responders. The overall methylation value of responders was 81.5%, the value of non-responders 77.7% (independent samples t-test: t = -2.1, p = 0.044, Figure 1).

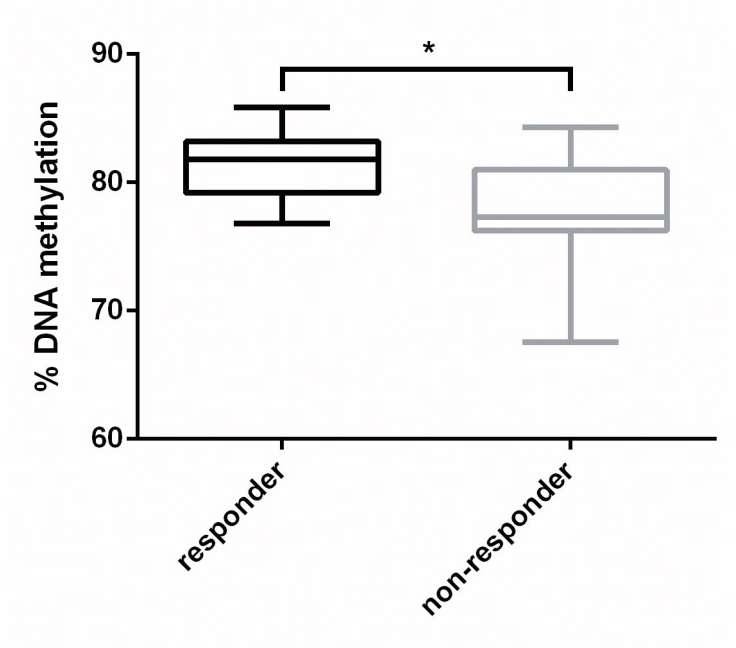

Figure 1: Boxplot comparing overall DNA methylation values for *APBA3* prior to therapy (T1) of non-responders and responders. * $p \leq 0.05$.

For *MCF2* the same relationship between DNA methylation and therapy outcome applied: Patients responding to therapy showed significantly higher DNA methylation prior to treatment (T1). DNA methylation values of responders were 56.2%, non-responders displayed DNA methylation values of 43.3% (Wilcoxon U-test: U = -2.8, p = 0.003; Figure 2).

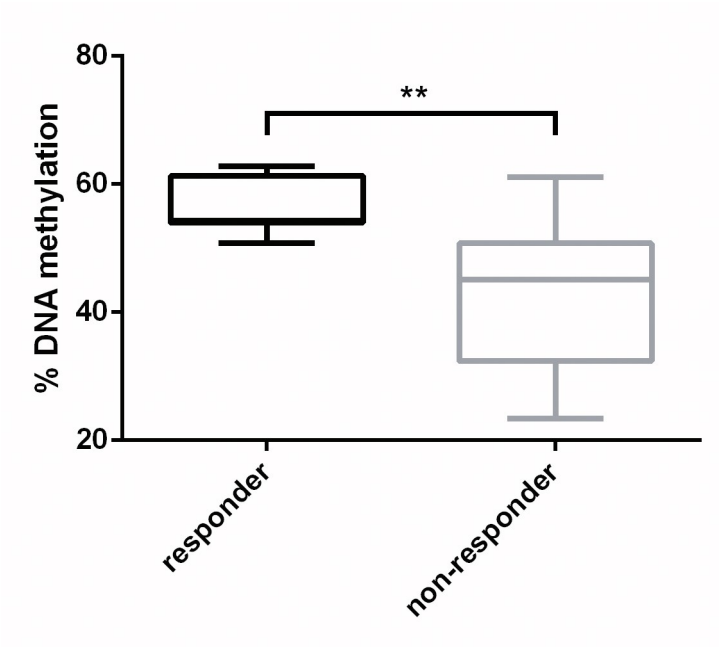

Figure 2: Boxplot comparing DNA methylation values for *MCF2* prior to therapy (T1) of non-responders and responders. ** $p \leq 0.01$.

Moreover, an inverse correlation between the GSI t-score and DNA methylation of *MCF2* before therapy was found: The higher the DNA methylation prior to treatment (T1), the lower the overall GSI t-score post-therapy (T2; Pearson correlation $r = -0.4$, $p = 0.038$).

At T2, no significantly different DNA methylation levels between responding or non-responding patients were detected for either gene. For *APBA3* DNA methylation values were 77.8% for responders and 79.8% for non-responders (independent samples t-test: $t = -1.0$, $p = 0.3$). For *MCF2* DNA methylation values of 48.9% for responders and 46.2% for non-responders were detected (Mann-Whitney U-test: $U = -0.2$, $p = 0.9$).

Discussion

In the present study, we aimed to replicate the differential DNA methylation of *APBA3* and *MCF2* in BPD patients compared to healthy control individuals described in a previous study (Teschler et al., 2013), but found no significant differences between the two groups. Furthermore, we did not detect a significant influence of DBT on the DNA methylation profile of these genes in our overall patient sample. However, *APBA3* and *MCF2* were found to be significantly hypermethylated at the beginning of treatment in individuals responding to DBT compared to non-responders, supporting the hypothesis that DNA methylation of *APBA3* and *MCF2* could serve as an epigenetic biomarker with predictive value for therapy outcome.

Replication of epigenome-wide findings in independent cohorts is generally hampered by the high probability of type 1 errors, also referred to as false-positive results, in high-throughput experiments. This might explain why we were unable to detect DNA methylation differences in the analyzed candidate genes in our cohort although Teschler et al. supported their results by validation of some of their findings – including *APBA3* and *MCF2* – using an independent method, namely pyrosequencing. As one of the major drivers for differential DNA methylation is tissue or cell type (Jaffe and Irizarry, 2014), a second possible explanation for the non-replication is the use of whole blood samples in both studies and the missing correction for blood-cell type composition. Other individual factors influencing DNA methylation are sex, age and health status (Farre et al., 2015; Jaffe and Irizarry, 2014; Liu et al., 2010). To circumvent the issue of confounding factors, we matched our samples of patients and control individuals for age and sex to be able to investigate the influence of the individual's health status (BPD vs. healthy control) on the DNA methylation pattern of the selected genes. Nevertheless, differences in blood-cell type composition between our samples and the samples analyzed by Teschler et al. could have prevented us from confirming their findings.

Unfortunately, we can neither confirm nor refute potential hypermethylation of *NINJ2* as we were not able to obtain pyrosequencing results in sufficient quality for this locus even though the PCR and sequencing reaction was performed following the protocol described previously (Teschler et al., 2013) with slight modifications. This could potentially be explained by differences in the devices used to run the PCR reaction or by differences in primer as well as polymerase quality due to deviating

manufacturing protocols. All our attempts to optimize the analyses protocol and obtain satisfying results failed. This is probably attributed to the rather long target region which spans 172 nucleotides and is therefore difficult to analyse by pyrosequencing.

The two genes successfully analyzed in our study*, APBA3* and *MCF2*, have not been investigated in the context of psychotherapy before. In contrast to our initial hypothesis, we did not detect a significant effect of the psychotherapeutic intervention on the DNA methylation status of those genes when analyzing the overall patient sample. However, after dividing our samples into responders and non-responders, we found both genes to be significantly hypermethylated prior to DBT in therapy responders. This significant difference was no longer detectable post-treatment. Moreover, treatment response for the individual subjects was predicted by the DNA methylation levels of the *MCF2* gene prior to therapy, as the methylation levels at T1 were significantly inversely correlated with the overall psychological burden (GSI t-score) post-treatment (T2).

That we and others (Roberts et al., 2015; Roberts et al., 2014; Yehuda et al., 2013; Ziegler et al., 2016) identified differential DNA methylation in responders compared to non-responders but did not find a general effect of psychotherapeutic treatment in the overall group is not surprising, as one would expect functional changes in neurobiological processes mainly in the group of patients benefiting from the therapeutic intervention. To the best of our knowledge, the only other predictive epigenetic marker for psychotherapeutic treatment outcome described thus far is the glucocorticoid receptor gene (*GR*) in the context of a psychotherapeutic intervention in PTSD (Yehuda et al., 2013). Similar to our findings, an epigenetic effect of the therapeutic intervention itself could not be observed. A recent study demonstrated that HPA axis reactivity is associated with treatment outcome in a psychotherapeutic setting for PTSD (Rauch et al., 2017). As the differential methylation of the *GR* influences the HPA axis this association could explain the differences in response to treatment (Tyrka et al., 2016; Yehuda et al., 2015). Whether the differential DNA methylation of *APBA3* and *MCF2* in treatment responders and non-responders is also associated with neurobiological alterations directly influencing therapy outcome remains unanswered and needs to be addressed in future studies. Only one study published thus far investigated the influence of DBT on DNA methylation levels. The authors demonstrated a decrease in *BDNF* DNA methylation levels in DBT

responders and an increase in non-responders, but this DNA methylation alteration was not of predictive value as methylation prior to therapy was not correlated with treatment outcome (Perroud et al., 2013). In contrast to the findings for *BDNF*, our finding of differential DNA methylation between therapy responders and non-responders prior to DBT suggests that the DNA methylation levels of *APBA3* and *MCF2* could serve as a molecular predictor of therapy outcome in BPD.

The role of *APBA3* and *MCF2* in BPD is unclear. *APBA3* is a member of the amyloid beta precursor protein binding family, interacting with the amyloid precursor protein (APP) whose proteolysis generates beta amyloid (Aβ), the primary component of the amyloid plaques associated with Alzheimers' disease (Csiszar et al., 2013; Sullivan et al., 2014). Moreover, *APBA3* is known to be epigenetically altered in adenoma or gastrointestinal tumors (Kim et al., 2003; Rashid et al., 2001). A link between *MCF2*, a guanine nucleotide exchange factor, and schizophrenia as well as autism-spectrum disorders was reported previously (Piton et al., 2011). *MCF2* is involved in neurite outgrowth (Piton et al., 2011) and alterations in neuronal development associated with genetic or epigenetic alterations could potentially explain the link between this gene and psychiatric diseases. Future functional studies are warranted to clarify the role of *APBA3* and *MCF2* in BPD.

This study is the first to present results pointing towards possible predictive epigenetic biomarkers for therapy response in BPD. However, our study also has a few limitations. First, although we were able to almost double the number of patients compared to a previous study (Teschler et al., 2013) and include four times as many control individuals, our sample size was still relatively small. This is especially true for the group of therapy responders which included only seven individuals, owed to the strict criteria we applied for categorizing therapy responders. However, in previous epigenetic studies of psychotherapeutic interventions the number of therapy responders was comparable to our study (Yehuda et al., 2013; Ziegler et al., 2016). To qualify as therapy responder, the patients were required to achieve a GSI t-score which was reduced by more than 5 points post-therapy and in addition a BSL23 score lower than 2.05 had to be reached. Prior to therapy the patients in our cohort achieved a BSL23 score of 2.42 which is considerably higher than the average score of 2.05 BPD patients reached in several previous studies (Bohus et al., 2009; Wolf et al., 2009). Therefore, a reduction in the BSL score to average levels was considered a therapy success. To confirm our results, replication in larger cohorts is required. A

second limitation is that we were not able to perform a follow-up assessment of our patient group beyond the 12 weeks of inpatient treatment. Without knowing the subsequent health status of that group at a later time point following therapy, we cannot answer the question whether DNA methylation of *APBA3* and *MCF2* prior to therapy could also serve as a biomarker for long-term treatment outcome. Third, our study lacks functional data, as we did not analyze gene expression levels of our candidate genes. However, Teschler et al. showed that DNA methylation levels of *APBA3* correlated with gene expression levels in human cells (Teschler et al., 2013), suggesting that the DNA methylation differences we observed in our sample might also result in differential expression of the respective genes. Nevertheless, functional investigations should be included in future studies to reveal the distinct role of both genes in BPD.

In conclusion, to the best of our knowledge, we report the first findings pointing towards predictive epigenetic biomarkers for a psychotherapeutic treatment outcome in BPD. Our findings contribute to a better understanding of the biological underpinnings of BPD. In addition, the use of predictive biomarkers for therapy response could facilitate the development of more personalized therapy concepts. However, replication in larger cohorts is required to confirm our findings.

Literature

Bernstein, D.P., Stein, J.A., Newcomb, M.D., Walker, E., Pogge, D., Ahluvalia, T., Stokes, J., Handelsman, L., Medrano, M., Desmond, D., Zule, W., 2003. Development and validation of a brief screening version of the Childhood Trauma Questionnaire. Child abuse & neglect 27, 169-190.
Bohus, M., Haaf, B., Simms, T., Limberger, M.F., Schmahl, C., Unckel, C., Lieb, K., Linehan, M.M., 2004. Effectiveness of inpatient dialectical behavioral therapy for borderline personality disorder: a controlled trial. Behaviour research and therapy 42, 487-499.
Bohus, M., Haaf, B., Stiglmayr, C., Pohl, U., Bohme, R., Linehan, M., 2000. Evaluation of inpatient dialectical-behavioral therapy for borderline personality disorder--a prospective study. Behaviour research and therapy 38, 875-887.
Bohus, M., Kleindienst, N., Limberger, M.F., Stieglitz, R.D., Domsalla, M., Chapman, A.L., Steil, R., Philipsen, A., Wolf, M., 2009. The short version of the Borderline Symptom List (BSL-23): development and initial data on psychometric properties. Psychopathology 42, 32-39.
Calati, R., Gressier, F., Balestri, M., Serretti, A., 2013. Genetic modulation of borderline personality disorder: systematic review and meta-analysis. Journal of psychiatric research 47, 1275-1287.
Crowell, S.E., Beauchaine, T.P., Linehan, M.M., 2009. A biosocial developmental model of borderline personality: Elaborating and extending Linehan's theory. Psychol Bull 135, 495-510.
Csiszar, A., Tucsek, Z., Toth, P., Sosnowska, D., Gautam, T., Koller, A., Deak, F., Sonntag, W.E., Ungvari, Z., 2013. Synergistic effects of hypertension and aging on cognitive function and hippocampal expression of genes involved in beta-amyloid generation and Alzheimer's disease. American journal of physiology. Heart and circulatory physiology 305, H1120-1130.
Dammann, G., Teschler, S., Haag, T., Altmuller, F., Tuczek, F., Dammann, R.H., 2011. Increased DNA methylation of neuropsychiatric genes occurs in borderline personality disorder. Epigenetics : official journal of the DNA Methylation Society 6, 1454-1462.
Distel, M.A., Rebollo-Mesa, I., Willemsen, G., Derom, C.A., Trull, T.J., Martin, N.G., Boomsma, D.I., 2009. Familial resemblance of borderline personality disorder features: genetic or cultural transmission? PloS one 4, e5334.
Egger, G., Liang, G., Aparicio, A., Jones, P.A., 2004. Epigenetics in human disease and prospects for epigenetic therapy. Nature 429, 457-463.
Farre, P., Jones, M.J., Meaney, M.J., Emberly, E., Turecki, G., Kobor, M.S., 2015. Concordant and discordant DNA methylation signatures of aging in human blood and brain. Epigenetics & chromatin 8, 19.
Fleischhaker, C., Bohme, R., Sixt, B., Bruck, C., Schneider, C., Schulz, E., 2011. Dialectical Behavioral Therapy for Adolescents (DBT-A): a clinical Trial for Patients with suicidal and self-injurious Behavior and Borderline Symptoms with a one-year Follow-up. Child and adolescent psychiatry and mental health 5, 3.
Franke, G.H., 2002. Symptom-Checkliste von L.R. Derogatis – Deutsche Version (SCL-90-R). Beltz Test, Göttingen.
Gunderson, J.G., 2009. Borderline personality disorder: ontogeny of a diagnosis. The American journal of psychiatry 166, 530-539.
Hashimoto, H., Vertino, P.M., Cheng, X., 2010. Molecular coupling of DNA methylation and histone methylation. Epigenomics 2, 657-669.

Jaffe, A.E., Irizarry, R.A., 2014. Accounting for cellular heterogeneity is critical in epigenome-wide association studies. Genome biology 15, R31.
Januar, V., Saffery, R., Ryan, J., 2015. Epigenetics and depressive disorders: a review of current progress and future directions. International journal of epidemiology 44, 1364-1387.
Jones, P.A., Takai, D., 2001. The role of DNA methylation in mammalian epigenetics. Science 293, 1068-1070.
Kim, S.G., Chan, A.O., Wu, T.T., Issa, J.P., Hamilton, S.R., Rashid, A., 2003. Epigenetic and genetic alterations in duodenal carcinomas are distinct from biliary and ampullary carcinomas. Gastroenterology 124, 1300-1310.
Leichsenring, F., Leibing, E., Kruse, J., New, A.S., Leweke, F., 2011. Borderline personality disorder. Lancet 377, 74-84.
Linehan, M., 1993. Cognitive–behavioral treatment of borderline personality disorder. Guilford Press, New York.
Liu, J., Morgan, M., Hutchison, K., Calhoun, V.D., 2010. A study of the influence of sex on genome wide methylation. PloS one 5, e10028.
Nieratschker, V., Batra, A., Fallgatter, A.J., 2013. Genetics and epigenetics of alcohol dependence. Journal of molecular psychiatry 1, 11.
Nieratschker, V., Massart, R., Gilles, M., Luoni, A., Suderman, M.J., Krumm, B., Meier, S., Witt, S.H., Nothen, M.M., Suomi, S.J., Peus, V., Scharnholz, B., Dukal, H., Hohmeyer, C., Wolf, I.A., Cirulli, F., Gass, P., Sutterlin, M.W., Filsinger, B., Laucht, M., Riva, M.A., Rietschel, M., Deuschle, M., Szyf, M., 2014. MORC1 exhibits cross-species differential methylation in association with early life stress as well as genome-wide association with MDD. Translational psychiatry 4, e429.
Oldham, J.M., 2006. Borderline personality disorder and suicidality. The American journal of psychiatry 163, 20-26.
Perroud, N., Salzmann, A., Prada, P., Nicastro, R., Hoeppli, M.E., Furrer, S., Ardu, S., Krejci, I., Karege, F., Malafosse, A., 2013. Response to psychotherapy in borderline personality disorder and methylation status of the BDNF gene. Translational psychiatry 3, e207.
Perroud, N., Zewdie, S., Stenz, L., Adouan, W., Bavamian, S., Prada, P., Nicastro, R., Hasler, R., Nallet, A., Piguet, C., Paoloni-Giacobino, A., Aubry, J.M., Dayer, A., 2016. Methylation of Serotonin Receptor 3a in Adhd, Borderline Personality, and Bipolar Disorders: Link with Severity of the Disorders and Childhood Maltreatment. Depression and anxiety 33, 45-55.
Piton, A., Gauthier, J., Hamdan, F.F., Lafreniere, R.G., Yang, Y., Henrion, E., Laurent, S., Noreau, A., Thibodeau, P., Karemera, L., Spiegelman, D., Kuku, F., Duguay, J., Destroismaisons, L., Jolivet, P., Cote, M., Lachapelle, K., Diallo, O., Raymond, A., Marineau, C., Champagne, N., Xiong, L., Gaspar, C., Riviere, J.B., Tarabeux, J., Cossette, P., Krebs, M.O., Rapoport, J.L., Addington, A., Delisi, L.E., Mottron, L., Joober, R., Fombonne, E., Drapeau, P., Rouleau, G.A., 2011. Systematic resequencing of X-chromosome synaptic genes in autism spectrum disorder and schizophrenia. Molecular psychiatry 16, 867-880.
Rashid, A., Shen, L., Morris, J.S., Issa, J.P., Hamilton, S.R., 2001. CpG island methylation in colorectal adenomas. The American journal of pathology 159, 1129-1135.
Rauch, S.A., King, A.P., Liberzon, I., Sripada, R.K., 2017. Changes in Salivary Cortisol During Psychotherapy for Posttraumatic Stress Disorder: A Pilot Study in 30 Veterans. The Journal of clinical psychiatry.
Razin, A., Riggs, A.D., 1980. DNA methylation and gene function. Science 210, 604-610.

Rivollier, F., Lotersztajn, L., Chaumette, B., Krebs, M.O., Kebir, O., 2014. [Epigenetics of schizophrenia: a review]. L'Encephale 40, 380-386.
Roberts, S., Keers, R., Lester, K.J., Coleman, J.R., Breen, G., Arendt, K., Blatter-Meunier, J., Cooper, P., Creswell, C., Fjermestad, K., Havik, O.E., Herren, C., Hogendoorn, S.M., Hudson, J.L., Krause, K., Lyneham, H.J., Morris, T., Nauta, M., Rapee, R.M., Rey, Y., Schneider, S., Schneider, S.C., Silverman, W.K., Thastum, M., Thirlwall, K., Waite, P., Eley, T.C., Wong, C.C., 2015. Hpa Axis Related Genes and Response to Psychological Therapies: Genetics and Epigenetics. Depression and anxiety 32, 861-870.
Roberts, S., Lester, K.J., Hudson, J.L., Rapee, R.M., Creswell, C., Cooper, P.J., Thirlwall, K.J., Coleman, J.R., Breen, G., Wong, C.C., Eley, T.C., 2014. Serotonin transporter [corrected] methylation and response to cognitive behaviour therapy in children with anxiety disorders. Translational psychiatry 4, e444.
Sullivan, S.E., Dillon, G.M., Sullivan, J.M., Ho, A., 2014. Mint proteins are required for synaptic activity-dependent amyloid precursor protein (APP) trafficking and amyloid beta generation. The Journal of biological chemistry 289, 15374-15383.
Sutherland, J.E., Costa, M., 2003. Epigenetics and the environment. Annals of the New York Academy of Sciences 983, 151-160.
Teschler, S., Bartkuhn, M., Kunzel, N., Schmidt, C., Kiehl, S., Dammann, G., Dammann, R., 2013. Aberrant methylation of gene associated CpG sites occurs in borderline personality disorder. PloS one 8, e84180.
Teschler, S., Gotthardt, J., Dammann, G., Dammann, R.H., 2016. Aberrant DNA Methylation of rDNA and PRIMA1 in Borderline Personality Disorder. International journal of molecular sciences 17.
Torgersen, S., Kringlen, E., Cramer, V., 2001. The prevalence of personality disorders in a community sample. Archives of general psychiatry 58, 590-596.
Torgersen, S., Lygren, S., Oien, P.A., Skre, I., Onstad, S., Edvardsen, J., Tambs, K., Kringlen, E., 2000. A twin study of personality disorders. Comprehensive psychiatry 41, 416-425.
Tyrka, A.R., Parade, S.H., Welch, E.S., Ridout, K.K., Price, L.H., Marsit, C., Philip, N.S., Carpenter, L.L., 2016. Methylation of the leukocyte glucocorticoid receptor gene promoter in adults: associations with early adversity and depressive, anxiety and substance-use disorders. Translational psychiatry 6, e848.
Wang, Y., Leung, F.C., 2004. An evaluation of new criteria for CpG islands in the human genome as gene markers. Bioinformatics 20, 1170-1177.
William D. Barley, S.E.B., Eric W. Peterson, Amanda S. Hollingsworth, Mara Griva, Susan C. Hickerson, Jane E. Lawson, and Billy J. Bailey, 1993. Development of an Inpatient Cognitive-Behavioral Treatment Program for Borderline Personality Disorder. Journal of personality disorders 7, 232-240.
Wolf, M., Limberger, M.F., Kleindienst, N., Stieglitz, R.D., Domsalla, M., Philipsen, A., Steil, R., Bohus, M., 2009. Short Version of the Borderline Symptom List (BSL-23): Development and Psychometric Evaluation. Psychother Psych Med 59, 321-324.
Yehuda, R., Daskalakis, N.P., Desarnaud, F., Makotkine, I., Lehrner, A.L., Koch, E., Flory, J.D., Buxbaum, J.D., Meaney, M.J., Bierer, L.M., 2013. Epigenetic Biomarkers as Predictors and Correlates of Symptom Improvement Following Psychotherapy in Combat Veterans with PTSD. Frontiers in psychiatry 4, 118.
Yehuda, R., Flory, J.D., Bierer, L.M., Henn-Haase, C., Lehrner, A., Desarnaud, F., Makotkine, I., Daskalakis, N.P., Marmar, C.R., Meaney, M.J., 2015. Lower methylation of glucocorticoid receptor gene promoter 1F in peripheral blood of veterans with posttraumatic stress disorder. Biological psychiatry 77, 356-364.

Zanarini, M.C., Frankenburg, F.R., Hennen, J., Reich, D.B., Silk, K.R., 2006. Prediction of the 10-year course of borderline personality disorder. The American journal of psychiatry 163, 827-832.
Ziegler, C., Richter, J., Mahr, M., Gajewska, A., Schiele, M.A., Gehrmann, A., Schmidt, B., Lesch, K.P., Lang, T., Helbig-Lang, S., Pauli, P., Kircher, T., Reif, A., Rief, W., Vossbeck-Elsebusch, A.N., Arolt, V., Wittchen, H.U., Hamm, A.O., Deckert, J., Domschke, K., 2016. MAOA gene hypomethylation in panic disorder-reversibility of an epigenetic risk pattern by psychotherapy. Translational psychiatry 6, e773.

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Contributors

VN designed the study. NK and CB performed the literature searches, the laboratory work and data analyses. NK wrote the first draft of the manuscript. NK, FG, CF have participated in patient recruitment and clinical interviews. All authors contributed to and have approved the final manuscript.

Disclosure of interest

The authors have no conflicts of interest to report.

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