The effect of chronic alcoholism on epigenetic patterns regulating gene expression and neurodegeneration in the human brain

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Akademisk licentiatavhandling

Som för avläggande av medicine licentiatexamen vid Karoliska Institutet offentligen försvaras på engelska språket i Magnus Huss föreläsningssal, Magnus Huss M4:00. Karolinska Universitetssjukhuset Solna, fredagen den 2a februari 2007 klockan 9.30

STOCKHOLM 2007
The greatest friend of Truth is time, her greatest enemy is Prejudice, and her constant companion Humility.

Charles Caleb Colton
(1780 - 1832)
ABSTRACT

Chronic alcoholism causes about 3.2% of all deaths worldwide. It has an association to about 60 different diseases and injuries with enormous social and economical impact. Despite these facts, very few pharmacological treatments exist and we can still not explain the mechanisms behind addiction, craving and cognitive impairments seen in the abuser. Alcohol targets most areas of the brain and produces an overall decline in many basic functions. Cognitive functions, such as memory, decision making and problem solving is often impaired after years of heavy drinking. The frontal lobe region is strongly connected to cognitive functions. It regulates the magnitude of response to an environmental stimulus and determines that based on former experiences and balancing positive and negative consequences in uncertain outcomes. This higher-order function is what separates humans from primates.

Alcohol abuse can harm the brain in three ways: 1) Acetaldehyde, metabolite of ethanol, is directly toxic to neurons; 2) alcohol challenges glutamate receptors by blocking the binding of the excitatory neurotransmitter glutamate, therefore increasing the numbers of receptors which causes indirect neurotoxicity during alcohol withdrawal; and 3) dietary deficiencies due to lower food intake and deteriorated intestine resorption directly causes altered nucleic acid synthesis (DNA) and methyl group substrates to DNA and proteins.

A decrease of methyl group donation to DNA and proteins will affect the epigenetics within the genome. Methyl-groups attached to the DNA and the histone proteins regulate gene activity. Dynamic regulation of the epigenetic marking is the way the environment can impact on the activity of our genes and consequently the proteins and all functions in the organism.

The aims of this thesis were to evaluate DNA methylation and gene activity changes in the brain of chronic alcoholics. This resulted in optimization of a method to measure global DNA methylation, and establishing the activity of common housekeeping genes as well as selected genes regulating neuronal cell viability, using real-time PCR. We developed the LUMA (LUminometric Methylation Assay), based on restriction enzyme cleavage with HpaII and MspI, cleaving DNA in CpGs, dependently and independently of cytosine methylation, respectively. Detection of nucleotide incorporation and hence level of methylation was performed using Pyrosequencing platform. By using a high-throughput real-time PCR, Low Density Arrays (LDA), we evaluated 16 endogenous control genes and found brain region specific genes for optimal normalization in mRNA quantification assays. The brain regions analyzed, prefrontal and motor cortices had different gene expression stability patterns. We further identified three reference genes which were significantly downregulated in motor cortex of alcoholics compared to controls, and one gene upregulated in the prefrontal cortex. This shows the importance of careful endogenous control validation prior to quantification experiments.

Finally, we show preliminary data of downregulation of genes which activity is related to cell survival, myelination, cell cycle, DNA repair and signaling in the prefrontal cortex, while the motor cortex remained less affected in alcoholics. This suggests a higher vulnerability of alcohol abuse to the frontal cortex region, mainly in the myelin genes that were mostly downregulated. The results from the thesis serve as a good platform to further study gene specific epigenetic changes in chronic alcoholics.
LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by their roman numbers (I, II)


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# ABBREVIATIONS

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<tr>
<td>18S</td>
<td>18s ribosomal RNA</td>
</tr>
<tr>
<td>ACTC</td>
<td>beta-actin</td>
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<tr>
<td>B2M</td>
<td>Beta-2-microglobulin</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin ImmunoPrecipitation</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-phosphate-Guanosine</td>
</tr>
<tr>
<td>CpNpG</td>
<td>Cytosine-phos-Any-of-the-four-nucleotides-phos-Guanosine</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variance</td>
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<tr>
<td>DTI</td>
<td>Diffusion Tensor Imaging</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA Methyl Transferase</td>
</tr>
<tr>
<td>FA</td>
<td>Fractional Anisotropy</td>
</tr>
<tr>
<td>FN1</td>
<td>Fibronectin1</td>
</tr>
<tr>
<td>GAPD</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>GT</td>
<td>Gambling Task</td>
</tr>
<tr>
<td>GUSB</td>
<td>Beta-Glucuronidase</td>
</tr>
<tr>
<td>H3-K9-Ac</td>
<td>Acetylation of Histone H3 at Lys9</td>
</tr>
<tr>
<td>HMBS</td>
<td>Hydroxymethylbilane synthase</td>
</tr>
<tr>
<td>HPRT1</td>
<td>Hypoxanthine phosphoribosyl transferase</td>
</tr>
<tr>
<td>IPO8</td>
<td>Importin 8</td>
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<tr>
<td>IT</td>
<td>Incompatibility Task</td>
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<tr>
<td>LUMA</td>
<td>Luminometric Methylation Assay</td>
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<tr>
<td>MBP</td>
<td>Myelin Basic Protein</td>
</tr>
<tr>
<td>MDS</td>
<td>Mesocorticolimbic Dopaminergic System</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NA</td>
<td>Nucleus Accumbens</td>
</tr>
<tr>
<td>NF</td>
<td>Normalization Factor</td>
</tr>
<tr>
<td>NGFR</td>
<td>Nerve Growth Factor Receptor</td>
</tr>
<tr>
<td>OA</td>
<td>Object Alternation</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PGK1</td>
<td>Phosphoglycerate kinase</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
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<tr>
<td>PFC</td>
<td>Prefrontal Cortex</td>
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<tr>
<td>PLP1</td>
<td>Peripheral Lipid Protein1</td>
</tr>
<tr>
<td>Abbreviation</td>
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<tr>
<td>PMI</td>
<td>Post Mortem Interval</td>
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<tr>
<td>POLR2A</td>
<td>RNApolymerase II</td>
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<tr>
<td>PPIA</td>
<td>Peptidylprolyl isomerase A</td>
</tr>
<tr>
<td>RPLP0</td>
<td>Ribosomal large P0</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single Photon Emission Computed Tomography</td>
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<tr>
<td>TBP</td>
<td>TATA binding protein</td>
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<tr>
<td>TF</td>
<td>Transferrin</td>
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<tr>
<td>TFRC</td>
<td>Transferrin receptor</td>
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<tr>
<td>UBC</td>
<td>Ubiquitin C</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
</tr>
<tr>
<td>WE</td>
<td>Wernicke encephalopathy</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>YWHAZ</td>
<td>Tyrosin-monooxygenase/tryptophan-monooxygenaseactivation protein</td>
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PREFACE

Alcoholism is a hidden disease, filled with shame and guilt, despite the sad truth that alcoholism for many countries is a national disease. It is estimated that approximately 10% of the world’s adult population are alcohol abusers and that every fourth man in the USA have at least once during life, been addicted to alcohol. This estimate can probably be transferred to many other countries. Alcoholism of course also affects women; however the frequency of men abusing alcohol is much greater. Alcoholism is also an increasing problem in younger people shown from the World Health Organization reports on alcohol abuse, especially accelerating problems seen in middle Europe where alcohol for centuries has been a natural part of the culture.

Alcoholism is not only the disease of the alcoholic. It is also the disease of the relative. This is because the denial, the guilt and the consequences alcohol abuse has is transferred to the closest environment. World Health Organization claims that 76.3 million people worldwide have diagnosable alcohol use disorders. A brief calculation that every individual has at least 4 close relatives would mean that 381.5 million people worldwide are suffering from alcoholism, physically or psychologically.

Despite all this, we still don’t know the true mechanisms leading to the disease: craving, relapse and addiction, or the mechanisms underlying the alcohol-related dementia symptoms. One reason for this, I believe, is the attitude towards this type of diseases. Sadly enough, some people wouldn’t even call it a disease. By changing attitudes, life quality for many people could increase, not only for the alcoholic himself.

The general perception is, still today, that alcoholism is a consequence of “bad” self discipline, a matter of character. This is true also for other addicted behaviors, such as food addiction, gambling addiction, power addiction, training addiction, control addiction to name just a few. Because addiction IS a behavior, just like any other behavior you have. The truth is that addiction is increasing and new types of addiction are constantly being identified. I believe that every person is “addicted” to something. It is a reward, which is one of the strongest drifts in life. In science, the term reward is not only referring to getting kicks and euphoria, it also means getting a pleasurable feeling within you. This happens when you eat food, have personal relations or sleep. It is a feeling of wellbeing and can therefore drive you into certain behavior when you suffer from for example anxiety or boredom, states of non wellbeing, but they are likewise natural psychological states. All people have different ways of finding wellbeing, it
might be reading a book or running the marathon. It both creates a feeling of flow and internal pleasure. This is a type of addiction, however less harmful to your body.

Why is then harmful addiction increasing? Is this a replacement for something mentally or physically that is disappearing throughout evolution? As a person, one of the things that hurt me the most are prejudices I hear from people around me. Believe me when I say I’ve been hearing a few of those when I’m telling people I’m drug researcher. I guess that is one of the reasons why my personal choice of career fell into life science. Because then, you can explore things, you can find knowledge and thereby have the opportunity to influence not only medical care but general perceptions and consequently life quality. I hope that I can, through this work and future research, contribute to some of this by identifying and explaining persistent changes in the brain after alcohol abuse.

Decades of research have been able to estimate that human diseases and behaviors, not only addiction, is a result of 40% inheritance residing in our genes, and 60% environmental factors. Environmental factors means for example what you eat and drink, exercise, how much stress you put yourself into and how your relations have been to others during childhood and adult life. Drug addiction is a combination of learnt behavior and weakened ability of the brain to sustain environmental stimulus due to persistent physical changes. Drugs produce, most likely, life long changes in the brain, soon after the addicted behavior initializes; changes that also accumulate by continuous drug intake. Persistent changes make you vulnerable to relapse any time throughout life. This brain changing process can even happen without the usage of drugs. Self-producing endorphins can generate the same pleasurable feeling. This happens in gambling addicts or for monks during meditation. The same reward system in your brain is activated. What is it then that differentiates an addict from a non-addict if we all have the addicted behavior inside us? Of course the genetic factor, some people are at risk more than others. But also, the power of harm the “drug” has to the brain. Toxic compounds like alcohol or amphetamine produce faster changes in the brain, which soon puts you in an addicted situation with persistent changes in the brain.

Then, it is no longer a matter of character.
INTRODUCTION

Epidemiological aspect of alcoholism
World Health Organization (WHO) has in their Global Status Report on Alcohol (2004) estimated that 76.3 million people worldwide had diagnosable alcohol use disorders [1]. Overall there is a causal relationship between alcohol consumption and more than 60 types of disease and injury, and alcohol is estimated to cause about 20-30% of oesophageal cancer, liver cancer, cirrhosis of the liver, homicide, epileptic seizures, and motor vehicle accidents worldwide. Alcohol consumption is the leading risk factor for disease burden in low mortality developing countries and the third largest risk factor in developed countries. Alcohol causes 1.8 million deaths (3.2% of total) worldwide and in Europe alone, alcohol consumption was responsible for over 55 000 deaths among young people aged 15-29 years in 1999 [2].

Analysis of alcohol consumption over a period of almost 40 years reveal that the European Region (EUR), the African Region (AFR) and the Region of the Americas (AMR) all reached their highest consumption about the same time, in the early 1980s, although the level of consumption is obviously much higher in the European Region (EUR) than in the other regions. The two regions showing recent and continuing increases in consumption are the South-East Asian Region (SEAR) and the Western Pacific Region (WPR), which falls within the explanatory model that many developing countries are increasing their alcohol consumption with an increasing level of economic development. The European Region (EUR) and the Regions of the Americas (AMR) are clearly the parts of the world consuming most alcohol in the world when recorded adult per capita consumption reached above 10 and 6 liters of pure alcohol per year respectively (Figure 1).
Today, alcohol abuse is considered one of the largest national diseases in industrialized countries, not only in the perspective of personal health problems but also in relation to the huge social and economic impacts it has to the society. Therefore, requirements for research in the field of alcohol consumption are evident.

**Addiction in the brain**

The brain is severely affected by repeated alcohol consumption. However, there are several stages of addiction and consequently different levels of changes to consider. First, there is a distinction between duration of alcohol consumption, on a morphological and molecular level as well as on a behavioral level. There is a clear difference between the stages: 1) acute drug effects, 2) transition from recreational use to patterns of use characteristic of addiction, i.e. uncontrollable drinking and 3) end-stage addiction. End-stage addiction is characterized by an overwhelming desire to obtain alcohol, a diminished ability to control drug seeking, and reduced pleasure from biological rewards [3]. Second, there are also several phases of post-intoxication of alcohol consumption as of any other drug including acute withdrawal, protracted withdrawal, and abstinence, to consider when discussing alterations detected in the alcoholic brain.

Therefore, the behavioral changes seen in an alcoholic person can vary from impulsive craving and drug seeking behavior to minor alterations in memory function developing to more severe impairments in memory, decision making and problem solving. Alcoholism can even develop to Wernicke encephalopathy (WE)
or finally Korsakoff’s psychosis with symptoms of severe dementia, nystagmus, conjugate gaze palsies, ataxia and altered mental status [4]. Although WE is a consequence of thiamine deficiency and not alcohol per se, it is a common outcome of heavy long term alcohol abuse. It is of crucial importance to define the stage of addiction and the mental and physical condition of an alcoholic individual and connect the neurobehavioral alterations to its brain circuit, in order to be able to connect that to the morphological and molecular changes detected.

Brain morphological abnormalities of alcoholism

**In vivo morphology**

It is well established that long term heavy alcohol consumption produces pathological alterations in the brain [5-9]. Several techniques have been used to evaluate alcohol related brain damage: *in vivo* MRI (Magnetic Resonance Imaging), CT (Computed Tomography), PET (Positon Emission Tomography), SPECT (Single Photon Emission Computed Tomography), post mortem MRI and neuropathology. There are also several newer modifications of older techniques such as Magnetic Resonance Diffusion Tensor Imaging (DTI) [10-13], a technique to visualize white matter bundles and microstructure. Another is the supratentorial white matter Fractional Anisotropy (FA) which is a DTI measure of intravoxel orientational coherence of tissue [14], and there are also stereological methods [15]. The results from different studies performed on alcohol related brain morphology vary, partly due to different subject inclusion criteria and method used. *In vivo* MRI studies often report similar extent of gray matter and subjacent white matter volume deficits in the cortex [16-19], but also a significant reduction in white matter volumes has been seen in adolescents, young adults and adult alcohol use disorders using MRI and DTI [13, 20]. The same study provided data showing significant correlation between prefrontal cortex volume and measures of alcohol consumption. Recently, data using FA detected a widespread distribution of white matter deficits in fluid diffusion in both left and right hemisphere and in midsagittal sections in both men and women recovering from alcohol intoxication, although significantly greater in men than in women [14].
Figure 2. Images displayed in the coronal orientation from MRI and DTI studies of a 61-year-old healthy man (upper images) and a 60-year-old alcoholic man (lower images). The MRI reveals a thinner corpus callosum displaced upward by enlarged ventricles and, on the DTI, less well delineated white matter tracts in the alcoholic man compared with the healthy man [13].

Post mortem neuropathology
Post mortem neuropathological studies are often reporting greater white matter loss than grey matter loss [21, 22]. However, they are in agreement in that the greatest cortical loss occurs in the frontal lobes [23-25]. Although frontal cortex might be mostly affected by alcoholism other brain regions have also been reported to be affected. The corpus callosum becomes thin [26], central pontine myelinolysis probably occurs in nutritionally based alcoholism [27-30], and there has also been reports on a reduced grey matter density within the amygdala [31]. In recent years several lines of evidence have also pointed out that cerebellar and cerebellothalamocortical systems are morphologically affected [20, 32-35]. However, the cerebellar dysfunction might be a consequence of nutritional deficiency like the thiamin deficiency of Wernicke’s encephalopathy and not by alcohol toxicity per se [34]. Furthermore, morphological studies have also shown changes in structures important for the reward pathway of alcoholism such as caudate and putamen which shows enduring reduced volume in alcoholics and even nucleus accumbens which show reduction following recent alcohol exposure [36].

Several studies have been trying to evaluate the reason for a white matter reduction in alcoholic brain tissue. There have been documented changes in the thickness of the myelin lamellae in an experimental model [37], disruption of
white matter microstructural integrity [38, 39], disruption of axonal integrity [23], disruption of white matter microstructure by accumulation of intracellular and extracellular fluid widespread in both hemispheres, in the corpus callosum and in the centrum semiovale [14, 40]. However, there are also evidence supporting the hypothesis that chronic alcoholics do not suffer from degeneration of axons in the white matter of the cerebral hemispheres [15].

Cognitional changes

**General dysfunction**
Over the past few decades, a vast number of rigorous studies of chronic alcoholics have identified selective cognitive, sensory, and motor deficits that persist even after period of detoxification. After an average of 4 weeks of sobriety, alcoholics have been shown to exhibit impairments in problem solving [41-44], short-term memory [45-48], visuospatial abilities [35, 49-51], executive functions [35, 52, 53] perceptual and motor skills [54], balance [35, 55] and olfaction [56, 57]. The substantial amounts of studies reveal a decline in many basic physiological and psychological functions which point to a general neuronal dysfunction in most areas of the alcoholic brain, although frontal cortex is overrepresented. As seen in previous paragraph this is also true when researchers have been studying structural alterations of different brain regions.

**Prefrontal cortex functions**
Frontal cortex is not only responsible for the cognitive control, it is also part of the Mesocorticolimbic Dopaminergic System (MDS). This system arises in the Ventral Tegmental Area (VTA) and projects to the Nucleus Accumbens (NA), the amygdala, the septum and the Prefrontal Cortex (PFC). Hence, PFC is a major part of the reward pathway in positive reinforcing effects of biological rewards such as food, drink and sex, but also to non-biological rewards like drugs of abuse [58-61]. This makes PFC important in other types of cognition, regulated by the reward system. Apart from PFC, the amygdala and the NA are believed to be the major regions regulating activation of behavior. Amygdala has been shown to be involved in fear-motivated behaviors, while the NA was identified from a connection with reward-motivated behaviors. The prefrontal cortex is believed not to judge if a stimulus is negative or positive but to control the overall motivational salience and determine the intensity of behavioral response [62, 63].

**Frontal cortex psychology**
Frontal cortex is a large area of the brain and control different cognitive functions. Earlier studies have been able to prove a rather global dysfunction of the frontal
cortex of alcoholics. However, since more knowledge develop about the different prefrontal functions, more recent studies have tried to specify which prefrontal brain regions that might be more vulnerable to alcohol abuse. Behavioral studies point to a greater vulnerability of the ventromedial and subcortical areas rather than the lateral prefrontal cortex in alcoholics [42, 64-68]. Ventromedial prefrontal cortex has been connected to emotions, organization of personality and appropriate social behavior. The medial region controls the ability to shift between motor responses based on stimuli concerning internal states or goals. Lateral prefrontal cortex on the other hand is controlling word fluency, working memory, planning and decision making based on conditional responses to external information [64]. Alcoholics have been show to exhibit problems in shifting between motor responses (Object Alteration, OA and Incompatibility Task, IT) and decision making (Gambling Task, GT), in tasks connected to the ventromedial region and subcortical regions [64, 69-71]. Functional brain imaging has shown that GT activates these structures by decision making [31, 72]. The GT task test weather a person is able to give up short term rewards for perhaps better long term rewards in uncertain situations [73]. Unchanged results in performance of word fluency task and in the shift between stimuli task [64] and in working memory task [65-68] indicated no major pathological changes in the dorsolateral and superior medial prefrontal cortex. Anatomically, an intact lateral prefrontal cortex is essential for proper word fluency and shift between stimuli learning [72, 74]. Moreover, PET and SPECT studies have suggested a diminished perfusion of the ventromedial prefrontal cortex [75-79].

**Predictors of alcohol brain pathology**

Today it is still not established how alcohol damages the neuronal complexity and consequently the physiological and psychological functions controlled by several brain regions. Findings from behavioral, structural and neuropathological studies have not been able to explain the reason for the neuronal dysfunction and the specific behavioral impairments associated with heavy alcohol drinking, and have not contributed to the development of neuroprotective pharmacotherapy. It has been surprisingly difficult to identify predictors of cognitive performance in alcoholics based on regular consumption variables [80-86]. Mainly, there are three hypotheses for the neuronal dysfunction seen in alcoholics. First, there is a direct effect on the neuronal populations by the ethanol neurotoxicity. Its cleavage product, acetaldehyde is known to be toxic to neurons. Second, indirect excitotoxicity during withdrawal occur when there is a change in the glutamatergic and gamma-aminobutyric acid-ergic neurotransmission due to
glutamate receptor antagonism of ethanol, which leads to increased neuronal sensitivity to glutamate [87-90]. Third, there are various forms of alcohol-related nutritional deficiencies that are well known to influence brain functions [91-93]. In either way, it is plausible that chronic alcohol consumption affects specialized molecular systems, such as regulatory expression proteins, signaling cascade proteins, synaptic transmission and protein trafficking molecules that control neuronal death and viability in the brain. This could eventually result in cognitive impairment.

Gene expression changes in the alcoholic brain

**Frontal cortex gene activity**
The activity of specialized molecular systems is regulated by gene transcription activity, protein modifications and protein degradation. Only recently have cDNA microarray analyses been performed in order to find changes in gene expression patterns among alcoholic abusers [94-100]. Most of them have restricted the analysis to the prefrontal cortex due to limited tissue availability of other regions and identified many functional gene groups to be mainly downregulated in alcoholics. Only one study used linear amplification protocols and was able to identify alcohol-responsive genes in reward pathways: ventral tegmental area and nucleus accumbens [94]. Several of the studies identified downregulation or inconsistent expression of myelin gene expression as a target for alcohol in the PFC [94, 95, 97, 99]. Oxidative stress as indicated by increase in genes associated with DNA repair and heat-shock proteins was detected by many of the studies [94, 97, 100]. This might also be reflected by the decrease in mitochondrial genes associated with electron transport or energy production [94, 95]. Signal transduction genes was another group shown to have altered expression in many of the studies. However, the identity and specific pathway description is often missing and conclusions regarding this group of genes are difficult to draw. Immediate early genes (e.g. transcription factors) have been reported to be induced in rats treated with alcohol [101-104], however there is inconsistent data on humans. Two studies report alcohol-sensitive transcription factor genes [94, 100], whereas three studies did not specify immediate early gene groups to be altered [94, 95, 97, 99, 100].

**Gene activity in other brain regions of alcoholics**
Other regions that have been transcriptionally analyzed in comparison to the highly affected frontal cortex are the motor, the temporal cortex and the mesocorticolimbic system. Also here, results are slightly inconsistent. In two
studies changes of gene transcription in motor cortex fell into the same functional
groups as changes seen in prefrontal cortex [95, 97] and identified a greater
number of genes changed in the motor cortex [95] but also a higher number of
genes down regulated frontal cortex [95, 97]. Furthermore, both studies found
changes in myelin-related genes in the motor cortex. Another study also found
motor cortex changes in most functional groups altered in PFC: signaling-related
genes, mitochondrial genes and transcription factors except that no differential
expression was detected in stress-response genes or myelination genes [98]. The
ubiquitination system and proteasome-related genes have also been reported in
two studies to be altered in the temporal lobe [98] in both motor and prefrontal
cortex [95]. The only study that has been studying transcriptional activity within
the mesocorticolimbic dopaminergic system has been able to identify region
specific expression changes. Of known alcohol-responsive genes based on
sequence alignments to publicity accessible sequence data (out of 19 000 probe
sets): 125 in the VTA, 68 in the PFC and 51 in the nucleus accumbens were
differentially expressed, and none of these genes was common to all three
regions [94]. Moreover, less than 4 % were common for any two. When divided
into functional groups the NA and the VTA exhibited a similar pattern in gene
groups affected by chronic alcoholism and was well separated from expressional
changes in PFC. Characteristic findings in the VTA and NA were alterations in
neuronal plasticity and neurotransmission and was limited to the PFC. Also cell
signaling was a major finding in the VTA. Changes seen in PFC on the other hand
were mainly in energy production, oxidative stress and DNA damage which were
not significant features in the VTA or NA. Surprisingly, no region in this study had
major changes in myelin related genes.

In conclusion, a variety of gene expression data may indicate significant
differences in selection criteria, cut offs, and important variables such as tissue
source and therefore variations in mode of death, tissue pH and post mortem
interval. Moreover, working with substance abuse individuals create problems in
collecting objective data on onset of drinking, amount of intake and stage of
intoxication, withdrawal or abstinence states.

Epigenetics
In terminological terms, epi refers to “around” or “surrounding”. Hence,
epigenetics is what surrounds the DNA and controls it in one way or another.
Since the DNA sequence is simply the blueprint of the body’s production and
synthesis of proteins another level of regulation must exist to in a static or
dynamic way regulate the genes and consequently the proteins [105-107]. This surrounding layer of information is deciding if and to what extent a gene should be active depending on cell type, external stimuli, environmental factors and time in life. Biologically, this is established as a chemical attachment modifying either the cytosine base of the DNA by a methyl group (CH₃); or methyl (CH₃), -phospho (PO₄) -or acetyl (COCH₃) groups attached to the proteins directly interacting with the DNA; the histones (Fig 4). These “marking” of the genome respond in a dynamic way to external stimuli. The histone modifications is suggested as a “histone code” [108]. Under normal conditions the epigenetic marking systems are able to maintain stable for the cell’s life time and even through cell divisions, i.e. an inheritance of the epigenetic state. Although long term region wide silencing or activation occur, also dynamic states of gene regulation must exist to provide for quick transcriptional responses to external stimuli. However, during pathological conditions either silencing or dynamic regulation of epigenetic marks may be disturbed followed by an abnormal gene activity.

**Figure 4.** The basics of epigenetics. The core histone proteins H2A, H2B, H3 and H4 assemble to form one octameric nucleosome core particle by wrapping 146 base pairs of DNA. This wrapping continues throughout the genome. Chemical modifications of the histone tails; methylation (CH₃), phosphorylation (PO₄) and acetylation (COCH₃), and methylation (CH₃) of cytosine residues in the DNA compromise the epigenetic marking of the genome, regulating gene activity.

**DNA methylation and alcoholism**

**Predictors of altered DNA methylation**

As stated above, there are three major predictors of alcohol damage to the neuronal systems in the brain: direct toxicity by acetaldehyde, indirect toxicity by sensitized glutamate transmission or nutritional deficiencies. These external factors may participate in forcing a “reprogramming” of epigenetic patterns thereby affecting gene regulation. Acetaldehyde, the first oxidation product of ethanol, inhibits enzymes that adds methyl groups to DNA and it is possible that
this may be one of the mechanisms behind the fetal alcohol syndrome [109], since lowering of DNA methylation may greatly affect regulation of several genes important for normal development [107]. Long term alcohol exposure may affect gene regulation also in the adult brain by similar mechanisms.

There are also dietary consequences of long term and heavy alcohol consumption. These include severe effects on folate metabolism since chronic alcohol consumption often is followed by thiamine and folate deficiency, due to both lowered intake of healthy food and to a deteriorated nutrition uptake in the intestine. This can induce DNA hypomethylation, as well as change the normal histone methylation pattern, since folate acts as a methyl donor (via S-adenosylmethionine) for both DNA methyltransferase [98, 110] and histone methyltransferase [111].

**Alcohol effects of DNA and histone methylation**

The previously described alcohol-induced alterations could lead to DNA- and histone disturbed methylation. Numerous studies have reported epigenetic changes in alcoholic tissue, mainly in blood cells and liver. Bönsch and colleagues have found that the elevated homocysteine concentration in alcoholics is associated with global DNA hypermethylation [112] in peripheral mononuclear cells, decreases in mRNA expression of DNA methyltransferases DNMT3a and DNMT3b [113], and DNA hypermethylation of the alpha synuclein promoter [114], a gene linked to alcohol preference and craving [115-117]. Moreover, they have also found direct regulation of another gene suggested to be important for neuronal Ca\(^{2+}\) signaling: HERP (homocysteine-induced endoplasmic reticulum protein). Decreased HERP mRNA levels correlated with increased homocysteine levels [118] and increased promoter methylation together with an association to AARE (amino acid response element) and CREB (cyclic AMP response element-binding protein) binding sites within the HERP promoter *in vitro* [119].

Decreased DNA methylation with a concomitant decrease in DNA methyl transferase activity after ethanol exposure of pregnant rats has been reported in fetal tissues [109]. There is also a report of genomic DNA hypomethylation in the rat colonic mucosa and rat liver tissue after chronic alcohol ingestion [120, 121]. Apparently, both global DNA hyper- and hypomethylation have been detected as a consequence of alcohol abuse, however only hypermethylation of promoters investigated have been detected, independently of DNMT expression. This raises the question if this reflects method validity and/or actual targeted gene specific epigenetic regulation, independently of the global status of the genome. In support to this are several studies in cancer research showing increased expression of DNMTs in hepatocellular carcinoma [122] and gastrointestinal
cancer but this increased expression is associated with both hypomethylation and hypermethylation of DNA [123-125].

Only a few studies have documented neuronal epigenetic changes caused by ethanol. One study shows that prenatal exposure to ethanol reduces Glial Fibrillary Acidic Protein (GFAP) immunoreactivity and its mRNA levels in both astrocytes in primary culture and in brains from pups from alcohol-fed mothers [126]. The fetal brains were also hypermethylated in the GFAP gene. GFAP is a cytoskeletal protein important for the morphogenesis in Central Nervous System (CNS). Another study detected a hypomethylation of the NMDA receptor NR2B promoter and a decrease of expression of NR2B in adult cortex and cultured fetal cortical neurons after chronic ethanol treatment, however not after acute ethanol treatment [127, 128]. To date, there is one publication on histone methylation, H3-K9-Me, after acute alcohol intragastric administration in rat. Results showed little (insignificant) changes in all 14 tissues analyzed [129].

**Nutritional effects of DNA methylation**

Several studies have correlated a nutrition deficiency to DNA methylation changes. One study has detected demethylated brain and liver tissue after restricted folate (choline-diet) in rats [130]. Moreover, another study detected promoter hypermethylation of APC, p14 (ARF), p16 (INK4A), hMLH1, O6-MGMT, and RASSF1A in colorectal cancer (CRC). For each of the tested genes, the prevalence of promoter hypermethylation was higher in CRCs derived from patients with low folate/high alcohol intake when compared with CRCs from patients with high folate/low alcohol intake [121, 131].

**Histone acetylation and alcoholism**

Several studies have also investigated histone modifications after alcohol ingestion, mostly in liver tissue after short-term exposure. In *in vitro* studies, ethanol caused a dose- and time- [132] dependent selective acetylation of histone H3 at Lys9 (H3-K9-Ac). Other H3 lysine residues i.e. Lys14, Lys18 and Lys23 are not acetylated under these conditions, which is supported by an *in vivo* study of acute ethanol exposure [129]. The same study showed that intragastric administration of ethanol increases the level of acetylated H3-Lys9 in a tissue specific manner as it is noted in liver, lung and spleen but not in tissues from the brain, heart, kidney, muscle, vessels, stomach and intestine. Furthermore, in hepatocytes exposed to ethanol, Chromatin ImmunoPrecipitation (ChIP) assays demonstrated an association of the acetylated H3-K9 with the alcohol dehydrogenase I (ADH 1) gene in the nuclear chromatin [133]. These data argue
that ethanol-elicited epigenetic changes cause an increased association between acetylated H3 and specific genes, a process which favors transcription [133]. Another study has shown that the chromatin in brains of rats chronically fed with ethanol show increased molar ellipticity in neuronal, astrocyte and oligodendroglial cells by circular dichroism spectrophotometry, which directly indicates relaxed state of chromatin facilitating transcription and replication [134]. Thus it appears that ethanol can modulate histone/chromatin to influence transcriptional activation and rat brain epigenetic changes.
AIMS OF THE THESIS

The present thesis was aimed to study epigenetic and gene transcription vulnerability in the brain of chronic alcoholics. For this purpose methods analyzing epigenetic patterns and gene activity was applied.

Two main aims were defined:

- To develop and optimize a method to study the global DNA methylation pattern in prefrontal and motor cortex of human chronic alcoholics.
- To validate endogenous control gene transcription activity and select the most stable control genes in prefrontal and motor cortex of alcoholics, in order to ensure optimal normalization in gene expression assays.
- To investigate expression activity of genes regulating neuronal cell survival, myelination, cell cycle, DNA repair and signaling, using high-throughput screening real-time PCR (Low Density Arrays).
METHODOLOGICAL OVERVIEW

This section will give a brief description of the materials and techniques used in my thesis. Detailed information is provided in paper I or II in the thesis or in original papers referred to.

Table 1: Tissue, cell line and techniques used in the present thesis

<table>
<thead>
<tr>
<th>Tissue/cells</th>
<th>Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paper I</td>
<td>Lambda DNA, HCT116 cells, MEF-p53-/- cells</td>
</tr>
<tr>
<td>Paper II</td>
<td>Human prefrontal and motor cortex</td>
</tr>
</tbody>
</table>

The table summarizes the tissue or cell lines used in the present thesis and the methods applied in each paper. A general description of the material and methods is found in the following section. A detailed description is found in each paper.

Human subjects

The subject tissues of the present thesis were obtained from the New South Wales Tissue Resource Centre (TRC), University of Sydney, Australia (http://www.pathology.usyd.edu.au/trc.htm). There, Professor Clive Harper has established a human brain bank specifically focusing on alcohol cases, with or without the Wernicke-Korsakoff syndrome, which can provide researchers with fresh, frozen or paraffin embedded brain sections carefully divided into the specific brain regions [135]. Stringent selection criteria are used for all cases obtained from TRC:

- Alcohol cases meet criteria for Diagnostic and Statistical Manual for Mental Disorders, 4th edition, (American Psychiatric Association)
- Do not have a history of multi drug abuse
- No prolonged agonal life support
- No history of cerebral infarction, head injury, or neurodegenerative disease (e.g. Alzheimer’s disease).
- No cases used in this study were diagnosed as Wernicke-Korsakoff syndrome
- Age greater than 18 years
- No developmental disorder
- No history of other psychiatric or neurological disorder
- Negative screen for AIDS and Hepatitis B/C
- Brain on gross examination has no obvious abnormalities
The alcohol consumption was > 80 g per day during the majority of their adult lives, except in one case with a daily alcohol consumption of 60 g. Control cases are matched to alcoholic cases by sex, age, race and Post Mortem Interval (PMI), and had alcohol consumption less than 10 g per day, except in one control case who consumed 35 g of alcohol per day (see Table 2). Control subjects were patients with no neurological abnormalities or neuropathological disorders (stroke, Alzheimer’s disease etc). All subjects, both alcoholics and controls in the present thesis, were male Caucasians. Regions used for analysis included Superior frontal gyros, Brodmann area 9 (Figure 3) and motor cortex, Brodmann area 4. All tissues were obtained after written consent of the next of kin for each subject. The study was approved by the local ethical committee of the Karolinska Institutet, dnr: 04-849/4.

**Table 2.** Demographic data on human subjects used for analyses

<table>
<thead>
<tr>
<th>Group</th>
<th>Controls</th>
<th>Alcoholics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Age, year</td>
<td>58.80 ± 14.5 (34-82)</td>
<td>58.87 ± 14.0 (34-81)</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>Male</td>
</tr>
<tr>
<td>PMI, hours</td>
<td>26.9 ± 16.4 (6.5-50)</td>
<td>31.8 ± 15.1 (8.5-60)</td>
</tr>
<tr>
<td>pH</td>
<td>6.46 ± 0.29 (5.88 – 6.9)</td>
<td>6.47 ± 0.27 (5.66 – 6.78)</td>
</tr>
<tr>
<td>Ethanol (blood)</td>
<td>n = 0</td>
<td>n = 2</td>
</tr>
</tbody>
</table>

Values presented as mean ± SD and range is shown in parentheses. Abbreviations: PMI, post-mortem interval.
Materials used for LUMA development
To validate the LUMA method in the present thesis four systems were used for the evaluation: 1) pBlueScript plasmid, 2) lambda DNA, 3) human colon carcinoma cell lines: wild type HCT116 and HCT116 derived cell lines with knockouts for DNA methyltransferase genes DNMT1 (MT1KO), DNMT3b (3bKO), or both (DKO) kindly provided by Dr Bert Vogelstein at Johns Hopkins University School of Medicine, USA) and 4) MEF-p53-/- cells kindly provided by Dr. Howard Cedar at Hebrew University medical School in Israel. Please see original paper in this thesis for detailed description of method procedure.

DNA methylation detection in the genome
The analysis of DNA methylation, global or regional, can produce an estimate of level of general or specific gene activity in the genome. 5-methylcytosine (5mC) in normal human tissue DNAs constitutes 0.75–1% of all nucleotide bases and approximately 3–4% of all cytosines are methylated in normal human DNA [136], preferentially in CpG sequences. CpG dinucleotides are not randomly distributed throughout the vast human genome. There are CpG-rich regions, known as CpG islands, which are usually unmethylated in all normal tissues and frequently span the 5' end region of genes. Computer analysis of the human genome has revealed
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the presence of about 29,000 CpG islands [137] and has shown that about 60% of the human genes have associated CpG islands, most of which remain non-methylated during development in most tissues [138]. Several methods are developed to analyze global DNA methylation [139]. However, many of them are work intensive and sometimes require radioactive molecules. In this thesis my colleagues and me have developed a new method, LUMA (LUminometric Methylation Assay) that has the advantages of being easy to scale up for high throughput application in clinical studies, it is quantitative and rapid (completed within half a day) and it contains an internal control for DNA input amount. The LUMA method is based on the quantification of DNA restriction digestion, using the Pyrosequencing™ platform in a polymerase extension assay with stepwise addition of dNTPs for specific detection of restriction enzyme cuts. Following successful extension of a dNTP to the DNA, inorganic pyrophosphate (PPI) is released and converted to ATP by ATP-sulfurylase and adenosine-5´-phosphosulfate. Luciferin is subsequently converted to oxyluciferin by luciferase and ATP to produce a proportional amount of visible light which is detected by a Charge Couple Device (CCD) camera [140]. The LUMA method was evaluated by usage and comparison to a well established method to measure global DNA methylation where genomic 5-methylcytosine levels are determined by capillary electrophoresis. In this method, genomic DNA is converted to 2´deoxynucleoside-3´-phosphatase by enzymatic hydrolysis and fluorescently labeled by Bodipy FL EDA and analyzed by a P/ACE MDQ LIF system.

High throughput Real-Time PCR
Real-time PCR was used in the study in order to quantify mRNA and thus gene transcription activity. To be able to analyze many genes and many samples, a high throughput real-time PCR developed by Applied Biosystems (Applied Biosystems, ABI, Foster City, CA) was applied. TaqMan® Low Density Endogenous Control Panel was used to investigate optimal genes for internal control purposes in alcoholic brain tissue. The method uses a 384-well micro fluidic card containing 16 human TaqMan® Gene Expression Assays (see the respective paper for detailed description of genes etc). This enables loading of 8 samples per plate for a triplicate well repeat for each gene. The platform that ABI is using is based on oligonucleotide probe specificity for a 30 bp sequence within the target gene. The probe is constructed with a fluorescent reporter dye (e.g. FAM™) bound to the 5´end and a quencher on the 3´end. While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer through space.
However, if the target sequence is present, the probe anneals between primer sites and is cleaved by the 5´nuclease activity of AmpliTaq Gold polymerase during extension. This separates the reporter from the quencher dye, yielding an increase in the reporter dye signal which is proportional to the starting amount of template, i.e. the cDNA (mRNA converted to cDNA by reverse transcriptase synthesis) amount. The 384-well micro fluidic card is preloaded with probe and primers for each specific gene. With the addition of cDNA and a mastermix, the plate is ready to run in an amplification program for analysis.

Figure 4. Principle of real-time PCR using oligonucelotide probe targeting specific genes (1). In the 5´end of the probe is a quencher dye located quenching the fluorescent activity from the reporter dye located on the 3´end of the probe. When the reporter is separated from the quencher by cleavage of DNA polymerase during amplification (2,3) it emits fluorescent light (4).

Evaluation of gene expression stability using geNORM
In the present thesis, the evaluation of commonly used housekeeping genes was performed by applying a frequently used and well accepted mathematical program: geNORM Visual Basic application for Microsoft Excel [141]. This method relies on the principle that the expression ratio of two ideal internal control genes is identical in all samples, regardless of the experimental condition or cell type. This means that the most expression-stable gene within a sample population is determined by the lowest variation in expression between that gene in comparison with the remaining genes for all samples. The geNORM program thereafter determines the number of genes required for a proper normalization, as calculated and set as a cut-off by the authors. Of those selected genes a Normalization Factor, NF, is calculated as the geometric mean of the included genes. By using the geNORM mathematical program it is possible to circumvent the most problematic step in real-time PCR analysis, namely to control the
precise cDNA amount input between samples which is required when evaluating housekeeping gene expression, since no normalization is possible.

**Statistical analysis**

In the present thesis statistical analysis was performed as presented in the second paper “Validation of endogenous controls for quantitative gene expression analysis: application on brain cortices of human chronic alcoholics”. General linear stepwise regression analysis (multiple regression) was performed to evaluate group differences in gene expression and identify covariates (age, brain pH and PMI). Other factors such as toxicology (alcohol, psychoactive drugs and other presumptive medication) were also evaluated. If no covariance was detected from the multiple regression, Student’s t-test was applied for group difference analysis for normally distributed gene values (equal or unequal variances). Mann Whitney U-test was employed for non normally distributed gene values, and analyzed for covariate influence using Spearman Rank correlation. Normal distribution was estimated by Shapiro-Wilk’s W test of normality. In addition to this analysis methodological variation was estimated by calculations of the Coefficient of Variance (CV) of Ct values within the six observations for each sample. Statistical evaluations were carried out using the Statistica software package. P-values are considered significant when $p< 0.05$. No advanced statistics was required in paper I.
RESULTS OVERVIEW

In this section I will present the results obtained from the two papers included in this thesis. This will be an overview presentation and I refer to each paper for a detailed description of results and figures. In addition, preliminary results from gene expression screening in the alcoholic brain are presented.

Paper I: LUMA development
In paper I “LUMA (LUminometric Methylation Assay)- A high throughput method to the analysis of genomic DNA methylation” a new platform was developed to analyze global DNA methylation. The LUMA method has the advantages of being easy to scale up for large sets of samples, for example in the clinic. It is time efficient making it easy to analyze up to 100 samples within half a day. It is very quantitative with the requirement of only 0.5 µg per sample, and it includes an internal control for exact DNA amount input. The evaluation of the LUMA assay included 4 main principles: 1) Linearity and sensitivity of the method using methylated plasmid and lambda DNA serial dilutions: 2) Biological applicability tested in human colon carcinoma cell lines with knocked-out DNA methyltransferases: 3) Experimental manipulation of DNA methylation and method sensitivity tested by treating murine embryonic fibroblasts with DNA methyltransferase inhibitors: and 4) LUMA validation using a second method for DNA methylation: capillary electrophoresis.

Principle of LUMA
The LUMA method includes three steps: 1) Genomic DNA digestion with methylation sensitive (HpaII) and insensitive (MspI) restriction enzymes, 2) DNA polymerase extension assay using Pyrosequencing technology incorporating nucleotides in cleavage overhangs, and 3) Peak-height analysis and HpaII/MspI ratio calculations.

Every sample is incubated in two reaction mixtures; one including the DNA methylation sensitive enzyme HpaII and the internal control restriction enzyme EcoRI. The other reaction mixture includes the isoschizomer enzyme MspI, insensitive to DNA methylation (i.e. cleaves the DNA independently of CpG methylation) and the internal control EcoRI enzyme. The HpaII and MspI isoschizomeric enzymes both cleave the sequence CCGG within the genome and leave 5´-CG overhangs. The EcoRI enzyme cleaves a sequence unrelated to CpG methylation: GGAATT and leave 5´-AATT overhangs. Both reaction mixtures are incubated for 4 hours before nucleotide extension is performed using the
pyrosequencing instrument PSQ96™MA. After complete DNA digestion dNTPs are added in four sequential steps: 1) dATPαS, 2) dGTP + dCTP, 3) dTTP, and 4) dGTP + dCTP. In step 1 and 3, EcoRI cleaved overhangs are filled with nucleotides A and T respectively, and in step 2, HpaII and MspI cleaved overhangs respectively, are filled with C plus G. Step 4 is used as an internal control for completion of step 2, where all overhangs should have been nucleotide filled in and no peak is therefore expected in this step.

**Linearity of the LUMA**

pBlueScript plasmid in serial dilutions ranging from 125 to 2000 ng was used to evaluate the sensitivity of LUMA. In this range there was a consistent linearity between both C and A peaks to the amount of plasmid, displaying close to identical HpaII/MspI ratios. This indicates a high sensitivity of the method in the range from 125 ng to 2000 ng. If this linearity can be extrapolated to lower and higher amount of plasmid requires further testing, however this range clearly covers the often used and available amount of DNA.

The sensitivity of LUMA was further tested by in vitro methylated lambda DNA using SssI methylase. Fully methylated and unmethylated lambda DNA was mixed in different proportions to produce mixtures of 0, 25, 50, 75 and 100% methylation. Analysis showed linearity in HpaII/MspI ratios to level of methylation, with a reverse correlation (R² = 0.98). When plotting HpaII/EcoRI ratio to HpaII/MspI ratio, there was a linear correlation (R² = 0.99) indicating that HpaII/EcoRI can also be used to estimate CpG methylation levels.

**Biological applicability of LUMA**

Human colon carcinoma cell lines with knocked out of DNA methyltransferase genes, DNMT1 and DNMT3b, were used to evaluate LUMA in a biological system. Results show that the DNMT1 KO cell line and the DNMT1 and DNMT3b double knockout cell line were hypomethylated (HpaII/MspI ratios 0.42 and 0.91 respectively) while the DNMT3 KO cell line and wild type cell line had the same level of methylation (HpaII/MspI ratios 0.30 and 0.27 respectively). Our results are consistent with the data originally reported for these cell lines using standard HPLC method [142]. Furthermore, when reanalyzed using a second method for validation; capillary electrophoresis, the results were in good agreement with the LUMA results. Moreover, the HpaII/EcoRI ratio alone correlated strongly with the (HpaII/EcoRI)/(MspI/EcoRI) ratio, indicating that the measurement of methylation were in relation the same between the two ratios. This shows that the LUMA is applicable in biological systems and that HpaII/EcoRI ratios can replace the HpaII/MspI ratio for analysis of CpG genomic methylation.
Experimental manipulation using 5-Azacytidine treatment
The murine embryonic fibroblast cell line MEF-p53-/- was demethylated using the compound 5-Azacytidine (5-AzaC). The cells were treated for three days in two different concentrations, 2 and 5 µM. This revealed a dose dependent hypomethylation of the cells ($R^2 = 0.96$) further indicating the sensitivity of the LUMA method as a tool for clinical evaluations of tumor stage, epigenetic therapies etc.

Paper II: Validation of endogenous controls
In paper II “Validation of endogenous controls for quantitative gene expression analysis: Application on brain cortices of human chronic alcoholics”, we have analyzed a panel of 16 commonly used housekeeping genes to evaluate best the normalization gene in quantitative experiments. Three parameters were tested in order to evaluate the genes: 1) gene expression stability was analyzed within the alcohol and control sample population using geNORM Microsoft excel visual application program: 2) gene expression differences was evaluated between alcohol and control group using statistical calculations like stepwise linear regression models or Mann-Whitney U-test: and 3) methodological reproducibility was evaluated by calculating $\Delta Ct$ between repeated measures.

Gene expression stability measured by geNORM
By applying geNORM we could evaluate the expression of the 16 endogenous control genes without prior normalization. The geNORM program allows validation of expression stability based on pure Ct values (threshold values for detectable fluorescence based on number of starting copies of cDNA). The geNORM tool produces two important results: 1) rank of stability from the least expression stable gene to the two most expression stable genes: and 2) evaluation of the number of genes required for accurate normalization, since often not only one gene is enough. We found that the genes that differ the least in expression stability within the alcohol and control subjects were brain region specific. In the motor cortex region, importin 8 (IPO8) and RNA polymerase II (POLR2A) were determined to represent the most stable genes. In frontal cortex the beta-actin (ACTB) and ribosomal large P0 (RPLP0) genes were found to be the most stable in mRNA expression in the alcohol and control samples. Furthermore, the pattern of gene expression stability was similar between the two regions since the rank between the genes was similar. geNORM also evaluated that the two most stable genes were sufficient for normalization factor calculation since the $V2/3$ value were below the cut off of 0.15 in both motor and frontal cortex. Therefore, it was possible to obtain an accurate way of normalization using a normalization factor...
(geometric mean) calculated from IPO8 and POLR2A for motor cortex and ACTB and RPLP0 for frontal cortex in chronic alcoholics and controls.

**Expression differences between alcoholics and controls**
When comparing the alcohol group with the control group in gene expression for all the 16 endogenous control genes we found significant increase in three genes in motor cortex of alcoholics compared to controls. The hypoxanthine phosphoribosyl transferase (HPRT1) gene was increased by 1.68-fold in relative expression (mean±SD: 6.20±10.07 vs. 10.41±10.63, stepwise linear regression F2,13 = 6.16, p = 0.04), the phosphoglycerate kinase (PGK1) gene was increased by 1.6-fold in relative expression (mean±SD: 2.15±0.76 vs. 3.44±0.8, Student’s t-test p = 0.01), and the peptidylprolyl isomerase A (PPIA) gene was increased by 1.5-fold in relative expression (mean±SD: 1.64±0.51 vs. 2.48±0.52, Student’s t-test, p = 0.007). There was also a significant decrease with 1.3-fold in relative expression in beta-2-microglobulin (B2M) in frontal cortex of alcoholics (mean±SD: 1.47±0.39 vs. 1.92±0.35, Student’s t-test p = 0.03). These data indicate that HPRT1, PGK1 and PPIA in motor cortex and B2M in frontal cortex are not suitable housekeeping genes for normalization when analyzing chronic alcoholics and control subjects.

**Method reproducibility**
As a third parameter the methodological reproducibility was measured between the endogenous control genes. The reproducibility was evaluated based on variation in Ct values in repeated measures. The ΔCt was calculated from the six repeated measures for each gene and sample, and the mean ΔCt for all genes was compared. Five genes had a ΔCt greater than 0.6 in both motor and frontal cortex: Beta-glucoronidase (GUSB), hydroxymethylbilane synthase (HMBS), phosphoglycerate kinase (PGK1), TATA binding protein (TBP) and tyrosin-monoxygenase activation protein (YWHAZ). A ΔCt of 0.6 corresponds to a 1.5-fold difference in cDNA/RNA amount (2^{0.6} = 1.52) which we considered not acceptable in repeated measures. This low reproducibility was shown to correlate with the average Ct values for that gene. The greater the Ct value the higher ΔCt. This seems likely since a high Ct value reflect a lower level of expression which requires a higher sensitivity of the real-time PCR instrument. Therefore, GUSB, HMBS, PGK1, TBP and YWHAZ are not recommended to use as reference genes in quantitative studies in motor and frontal, cortex of alcoholics.
III: Preliminary results in gene activity

We have analyzed and obtained gene transcription results in 36 preselected genes using Low Density Arrays described in the method section (Applied Biosystems, ABI, Foster City, CA). The preliminary result will be presented in this section. A manuscript reporting these data is in preparation.

The 36 genes were selected based on function in selected gene groups relevant for neuronal survival and viability. They include: cell survival/apoptosis, myelination, DNA repair, cell cycle, NF-κB and signaling/inflammation (Table 3).

Table 3. Selected genes analyzed in real-time PCR.

<table>
<thead>
<tr>
<th>Cell death/survi</th>
<th>Myelination</th>
<th>DNA repair</th>
<th>Cell cycle</th>
<th>NF-κB</th>
<th>Signaling</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAX</td>
<td>MBP</td>
<td>MRE11A</td>
<td>Cyclin-D1</td>
<td>p65</td>
<td>CAMK2A</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>PLP1</td>
<td>RAD50</td>
<td>p21- (CDKN1A)</td>
<td>Rel B</td>
<td>NGFR</td>
</tr>
<tr>
<td>XIAP- (BIRC2)</td>
<td>Fibronectin-1 (FN1)</td>
<td>Nibrin (NBN)</td>
<td>p27- (CDKN1B)</td>
<td>IKKB- (IKBKB)</td>
<td>NOS1</td>
</tr>
<tr>
<td>BCL-XL- (BCL-2L1)</td>
<td>Tranferrin- (TF)</td>
<td></td>
<td>E2F1</td>
<td>CREBBP</td>
<td></td>
</tr>
<tr>
<td>BID</td>
<td>GFAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDNF-r- (NTRK2)</td>
<td>NCAM1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFLAR</td>
<td>TNFRSF1A</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PDCD8</td>
<td>TNFRSF1B</td>
<td></td>
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<tr>
<td>DFFA</td>
<td></td>
<td></td>
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<tr>
<td>DFFB</td>
<td></td>
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</tbody>
</table>

Genes marked with bold are significantly downregulated in frontal cortex of alcoholics compared to controls (see text for mean and p-values). Only NOS1 was also significantly decreased in motor cortex of alcoholics compared to control.

The result from the low density arrays show that 15 genes out of 36 analyzed are significantly downregulated (marked in bold in table 3) in prefrontal cortex of chronic alcoholics when normalized to normalization factor calculated from Beta-actin (ACTB) and ribosomal large P0 (RPLP0) expression. No upregulation was observed within frontal cortex. Genes downregulated were:

- XIAP (Median (range) controls vs. alcoholics: 2.04 (1.00-6.32) vs., 1.48 (1.18-1.90), Mann-Whitney U-test p = 0.003);
- Bcl-2L1 (Median (range) controls vs. alcoholics: 2.89 (1.00-6.96) vs. 2.46 (1.79-3.41), Mann-Whitney U-test p = 0.045);
- Bid (Mean±SD: 4.47±1.79 vs. 3.19±0.88, Stepwise linear regression p = 0.023, covariate Age, no Age x group interaction);
• PDCD8 Mean±SD: 2.16±0.68 vs. 1.43±0.26, Student’s t-test p = 0.0004;
• MBP (Mean±SD: 5.45±3.08 vs. 2.96±2.14, Student’s t-test p = 0.0042);
  PLP1 (Mean±SD: 7.30±5.46 vs. 2.89±1.36, stepwise linear regression p = 0.0018, covariate PMI, no PMI x group interaction);
• Fibronectin (FN1) (Mean±SD: 2.64±1.43 vs. 1.76±0.72, stepwise linear regression p = 0.034, covariate pH, no pH x group interaction);
• Transferrin (Mean±SD: 10.51±7.48 vs. 3.93±2.48, stepwise linear regression p = 0.0005, covariate PMI, no PMI x group interaction);
• Cyclin D1 (Mean±SD: 3.56±2.04 vs. 2.21±0.79, Student’s t-test p = 0.0057); p27 (Mean±SD: 2.13±0.98 vs. 1.48±0.25, Student’s t-test p = 0.016);
• E2F1 (Mean±SD: 2.83±0.96 vs. 2.11±0.73, Student’s t-test p = 0.026);
• PCNA (Mean±SD: 1.96±0.47 vs. 1.33±0.23, Student’s t-test p = 0.00004);
• p65 (Mean±SD: 2.59±0.97 vs. 1.84±0.74, stepwise linear regression p = 0.003, covariate pH, no pH x group interaction);
• NGFR (Mean±SD: 5.87±6.09 vs. 2.59±1.86, Student’s t-test p = 0.018);
• NOS1 (Mean±SD: 2.43±1.00 vs. 1.73±0.44, stepwise linear regression p = 0.0094, covariate Age, no Age x group interaction).

Motor cortex was analyzed in parallel and revealed only one gene, NOS1, downregulated (Mean±SD controls vs. alcoholics: 4.91±1.34 vs. 3.79±1.79, Student’s t-test p = 0.044) when normalized to 18s and RPLP0 (based on results obtained from endogenous control validation experiments, see paper I: suggested reference genes from the paper were IPO8 and POLR2A, however 18s and RPLP0 were also considered “stable” and therefore for work convenience selected in the experiments). There was also a tendency of increase in alcoholics compared to controls in two DNA repair genes:
• MRE11A (Median (range) controls vs. alcoholics: 3.43 (2.06-5.01) vs. 3.80 (1.80-4.70), Mann-Whitney U-test p = 0.081);
• RAD50 (Median (range) controls vs. alcoholics: 2.68 (1.86-5.92) vs. 3.51 (1.00-5.16), Mann-Whitney U-test p = 0.074);
• a tendency of decrease in MBP (Median (range) controls vs. alcoholics: 2.42 (1.02-9.21) vs. 2.00 (1.00-3.79), Mann-Whitney U-test p = 0.089) and Cyclin D1 (Mean±SD controls vs. alcoholics: 2.37±0.56 vs. 1.98±0.82, Student’s t-test p = 0.076).
These data suggest that the major gene activity changes occur in the frontal cortex of chronic alcoholics in the specific groups of genes analyzed, with minor changes in the motor cortex. The decrease in expression is greatest in myelin genes. For example PLP1 (Peripheral Lipid Protein) is downregulated by 2.5-fold, transferrin by 2.67-fold, MBP (Myelin Basic Protein) by 1.84-fold and fibronectin by 1.5-fold. NGFR (Nerve Growth Factor Receptor, p75NR) is also decreased by 2.27-fold. Moreover, the DNA repair system might be more active in the motor cortex of alcoholics than in the frontal cortex as indicated by the trend of increased expression in 2 out of 3 genes analyzed in this gene group.
DISCUSSION

The development of the LUMA method (paper I) was performed in order to easily analyze global DNA methylation in large sets of samples in a sensitive and efficient way. It is based on DNA cleavage with well known restriction enzymes cutting either dependently (HpaII) or independently (MspI) of CmCGG sites in the genome. This will generate 5´-CG overhangs which are filled with dCTP and dGTP nucleotides during luminometric polymerase extension assay using Pyrosequencing. The novelty of the LUMA is firstly the inclusion of an internal control to normalize to DNA amount in the reaction and secondly the accurate way of quantitating the amount of digestion. The EcoRI restriction enzyme serves the first function, by cutting in CpG unrelated sequences, namely to 5´-GAATTC-3´, leaving 5´-AATT overhangs which are filled by dATPαS and dTTP nucleotides by the same polymerase extension assay, but in different sequential steps. The level of methylation in the genome is therefore calculated as the (HpaII/EcoRI)/(MspI/EcoRI) ratio. In paper I, we further concluded that this ratio can be replaced by the HpaII/EcoRI ratio alone by showing a strong correlation between these two ratios. This will enable even lower amounts of DNA in the assay. It will also improve the method even further since HpaII and MspI cutting can be analyzed separately which will provide a tool for plant analysis reported to be rich in CpNpG methylation, affecting also MspI cutting [143]. We also showed that the assay is reproducible over a 10-fold difference in DNA quantity (125-2000 ng) using plasmid DNA. This is highly advantageous since tissue availability is often limited in clinical and human analysis. The specificity and sensitivity of the method was further evaluated by using both “nonbiological” as well as biologically manipulated systems. For instance, lambda DNA was experimentally methylated and mixed in various methylated an unmethylated proportions, which generated a strong inverse correlation between DNA methylation and HpaII/MspI ratio. Dnmt1 and Dnmt3b knockout cell lines were used to measure the sensitivity of LUMA by comparing to other global DNA methylation methods used for the same cell lines [142]. Moreover, 5-azacytidine treatment of embryonic fibroblasts in vitro proved the LUMA to be dose dependent and highly reproducible. LUMA was applied to the analysis of global DNA methylation in human alcoholic frontal and motor cortices. The results suggest a trend of hypomethylation in the frontal cortex of chronic alcoholics, although not significant (data not shown), but not in the motor cortex. Hence, the almost significant p-values prompt for further
samples and other brain regions to be studied. These LUMA results on human chronic alcoholics are not presented in the present thesis.

Following the development of LUMA and global DNA measurements our next step was to analyze gene specific epigenetic modifications. However, before this can be done, the activity of genes important for neuronal survival and viability should be screened in order to establish which genes are affected by chronic alcoholism. Also, different reference genes for the screening of target genes had to be evaluated in order to find the most appropriate gene for normalization in the real-time PCR experiments. Selection of an endogenous control that is unstable or different in expression between alcoholics and controls will skew the normalized data and make it impossible to produce correct results. Hence, in paper II we report the most accurate way of normalization within human prefrontal and motor cortices when quantifying gene transcription. We report brain region specific endogenous control genes for optimal normalization. In motor cortex, importin 8 (IPO8) and RNA polymerase II (POLR2A) were the two most expression stable genes in alcoholics and controls. In frontal cortex, corresponding reference genes were β-actin (ACTB) and ribosomal large P0 (RPLP0). Using the two most stable genes for calculation of the normalization factor is sufficient if the pairwise variation for V2/3 is below cutoff of 0.15, according to the researchers who developed the geNORM program [141]. The pairwise variation for motor and frontal cortex was 0.10 and 0.11, respectively. Therefore, using IPO8 and POLR2A for motor cortex, and ACTB and RPLP0 for frontal cortex, will generate the most accurate normalization without the requirements for more reference genes. However, it is important to note that the general rank of expression stability between the 16 genes was similar between the two regions, and that the rank does not reveal the level of “stability” per se, only the relation between one another. Out of the five most stable genes in both motor and frontal cortex, three of them were represented in both regions (IPO8, POLR2A and RPLP0, Fig. 1A and B in paper II). Noteworthy is also that GAPD, a commonly used reference gene, was ranked as the 3rd and 4th least stable gene in motor and frontal cortex respectively (Fig 1A and B in paper II), which further indicates the requirements for endogenous control validation prior to the quantification experiments. The requirement for endogenous control validation was emphasized when gene expression were compared between alcoholics and controls. This revealed three genes downregulated in the motor cortex of alcoholics; PGK1, HPRT1 and PPIA, and one gene, B2M, upregulated in frontal cortex. Moreover, five genes did not fulfill the requirements for reproducibility when calculating the ΔCt values
between the six repeated measures. The GUSB, HMBS, PGK1, TBP and YWHAZ genes all had \( \Delta C_t \) values greater than 0.6. Translated into DNA amount this corresponds to a 1.5-fold difference between measurements. This was most likely due to low expression since they represented the genes with the highest Ct values, which limited the sensitivity in the real-time PCR detection. Finally, we recalculated the level of expression in all reference genes between alcoholic and controls, normalizing to the most commonly used reference genes: 18S, ACTB and GAPD separately. By this, we could show slightly different results in the genes reported different between groups. It was no longer the same genes differentially expressed between groups: PGK1, HPRT1, PPIA and B2B. There were either other genes added or old fell off (see discussion paragraph in paper II for details). This further proves the necessity of validation for potential reference genes.

In the result section in the thesis present preliminary data is presented on the activity of preselected genes important for cell survival and viability using low density array (LDA) real-time PCR and normalization according to the evaluation performed in paper I. As described in the previous paragraph, selected genes for normalization factor calculation was ACTB and RPLP0 for frontal cortex and 18S and RPLP0 for motor cortex. The ACTB and the RPLP0 were the most stable genes for frontal cortex presented in the paper, whereas IPO8 and POLR2A were the most stable for motor cortex. However, the selected reference genes in the motor cortex, 18S and RPLP0, were selected for several reasons: 1) they were the 4th and the 5th most stable genes (Fig 1A and B in paper II), 2) 18S were included in the LDA plates by default, and 3) the number of genes/samples were limited in the plate. We consider this as a good and accurate way of normalization.

The results from the preselected gene analysis revealed a significant reduction in gene activity in 15 out of 36 genes analyzed in the frontal cortex of alcoholics, in the gene groups of cell survival/death, myelination, cell cycle and signaling/inflammation, while no change in DNA repair gene group was evident (Table 3, result section). In comparison, there was only one gene, NOS1, with a significant gene activity reduction in the motor cortex. The major reduction in gene activity was seen in the myelin genes; PLP1, transferrin, MBP and fibronectin with a decrease of 2.5-fold; 2.67-fold; 1.84-fold; and a 1.5-fold respectively. NGFR (nerve growth factor receptor, p75NR) was also decreased by a 2.27-fold. These findings are supported by others who have reported myelin transcription changes in chronic alcoholics in microarray studies [94, 95, 97, 98],

[40]
and may indicate an important explanation for neuronal and cognitive dysfunction in alcoholics. It further suggests a higher vulnerability to alcohol abuse in the frontal cortex than in the motor cortex. Of the myelin genes, MBP, fibronectin and transferrin all have CpG islands in the 10 Kb upstream promoters when performing prediction analysis using MethPrimer tool on the web (http://www.urogene.org/methprimer). Also NGFR predicted to have CpG islands close to the transcription start site in the promoter. Therefore, future analysis should be focused on promoter specific epigenetic changes: DNA methylation as well as histone modifications in these genes.

As discussed in the introduction of my thesis it is important to have careful and detailed information on investigated alcohol subjects, i.e. to know in what addiction and post-intoxication states the subjects are in. It is also essential to analyze the region of interest for a specific cognitive or reward function in order to explain molecular and biochemical changes. In this study we have used cortex tissue from 15 alcoholics (see Table 2) who have consumed about 100 g of alcohol per day for at least 20 years. Most of them were abstinent drinkers since only two of them had detectable levels of alcohol in the blood by the time of death. Hence, most likely they were going through either: acute withdrawal, protracted withdrawal or abstinence when death occurred and tissues were collected. This, of course, brings uncertainty into the interpretation of data since the exact state of post-intoxication is not known. We used prefrontal cortex, Brodmann area 9, to study what is believed to reflect cognition related alterations. However, as I mentioned in the introduction, prefrontal cortex is also highly involved in the reward pathway via the mesocorticolimbic system and the changes detected in this study may also reflect changes in the reward pathway, not only in cognition, as suggested. Brodmann area 9 is a part of the prefrontal cortex that is spanning both dorsomedial and dorsolateral frontal cortex (Fig. 3A). In this study, we have used superior frontal gyrus sections, located in the dorsomedial frontal cortex. As mentioned in the introduction, recent studies have suggested the medial part of the prefrontal cortex to be more vulnerable to alcohol, in particular ventromedial prefrontal cortex [64]. The lateral part is thought to be less affected. In conclusion, studying dorsomedial Brodmann area 9 is important since this region has been suggested to be more vulnerable to alcohol abuse from a cognitional perspective.
GENERAL CONCLUSIONS

The aim of my thesis was to 1) optimize an analytic tool for global DNA methylation and apply it to genomic analysis of the brain cortex of alcoholics, 2) evaluate the expression of 16 endogenous control genes in prefrontal and motor cortex of alcoholics in order to establish the most accurate way of normalization in gene transcription assays, and 3) analyze the activity of preselected genes by high-throughput real-time PCR. Results from this was expected to generate a general picture of epigenetic and transcriptional changes occurring in the alcoholic brain, serving as a possible explanation for the molecular changes leading to a cognitive dysfunction. It was further expected to guide our alcohol research into more specific epigenetic analysis of gene promoter changes and histone modifications.

The thesis include papers I and II, and preliminary results that indeed have given us good knowledge and tools to start evaluating epigenetic and transcriptional regulation in the human alcoholic brain cortex. In paper I, we developed a highly sensitive and high-throughput technique to analyze global DNA methylation from genomic DNA. In paper II, we evaluated the expression of several endogenous controls and established the most accurate way of normalization in gene expression studies when working with chronic alcoholic prefrontal and motor cortices. In this paper we also identified genes not suitable as reference genes. Usage of such genes could even skew your results due to significant expression differences between alcohol and control groups, or too low methodological reproducibility due to low gene expression. Finally, we have presented interesting preliminary data showing that among 36 selected genes regulating neuronal cell survival, myelination, cell cycle, DNA repair, signaling and inflammation, 15 genes are significantly downregulated in prefrontal cortex of alcoholics, while in the motor cortex only one appears downregulated. This indicates a vulnerability of the frontal cortex to alcohol abuse and the data can serve as a platform for further study. In particular, specific epigenetic mechanisms regulating the transcriptional alteration will be investigated. These studies may contribute to the understanding of alcoholism related cognitive impairments.
CHRONIC ALCOHOLISM AND EPIGENETICS

SUMMARY IN SWEDISH

Alkoholism är en bakomliggande orsak till ungefär 3,2% av samtliga dödsfall i världen. Det finns en koppling till runt 60 olika sjukdomar eller skador såsom cancer i esosophagus och i levern, levercirrhos, epileptiska anfall eller bilolyckor. Uppskattningsvis ca 10% av världens vuxna befolkning missbrukar alkohol, kanske ännu fler, mörkertalet är stort. Alkoholism är därmed en folksjukdom eftersom minst 1% av befolkningen är drabbad.

Trots detta är kunskapen liten om de bakomliggande orsakerna till varför vissa individer utvecklar missbruk, mekanismerna bakom återfall eller de demenssymptom som uppvisas hos många alkoholister, s.k kognitiva förändringar. Många års forskning har visat på morfologiska förändringar av hjärnan. Flera studier har påvisat en minskning av framförallt hjärnans frontallob samt en förlust av vit substans. Hjärnans frontallob är viktig för just de kognitiva förmågorna, t.ex. minnet, problemlösning och beslutsfattande. Det är frontalloben som är högre utvecklat och fattar beslut om hur mycket man ska reagera på ett stimuli utifrån en avvägning av gamla erfarenheter, samt fördelar och nackdelar med ett sådant beslut i en situation som kan te sig osäker.


I andra delen av studien utvärderade vi aktiviteten av 16 olika endogena kontrollgener, s.k “housekeeping genes” i frontal och motor cortex hos alkoholister. Detta gjordes för att fortsättningsvis kunna kvantifiera geners aktivitet på ett så korrekt sätt som möjligt, eftersom endogena kontrollgener används för att normalisera relativa data. Detta genomfördes med hjälp av real-tids PCR och ett excel baserat program, geNORM, vilket kan beräkna uttrycksstabiliteten hos flertalet gener mellan alla individer. Vi fann att för varje hjärnregion var det olika gener som föreföll mest stabila inom vår population. Inom frontal cortex var β-actin (ACTB) samt large ribosomal P0 (RPLP0) mest stabil och i motor cortex var importin8 (IPO8) samt RNA polymerase II (POLR2A) de mest stabila. Tillämpning på detta skulle innebära att för att utföra den mest korrepta normaliseringen i kvantifieringen av genaktivitet så bör dessa olika kontrollgener användas specifikt inom varje hjärnregion. Vi kunde utöver detta visa att 4 utav de 16 analyserade generna var signifikant skilda i uttryck mellan alkoholister och kontroller, tre i motor cortex (phosphoglycerate kinase (PGK1), peptidylprolyl isomerase A (PPIA) samt hypoxanthine phosphoribosyl
transferase (HPRT1)), och en i frontal cortex (beta-2-micoglobulin, B2M). Dessa är alltså inte lämpliga som kontrollgener eftersom en normalisering skulle generera felaktiga data. Vi fann dessutom 5 gener som troligen på grund av för lågt genuttryck inte var reproducerbara och heller inte lämpliga som kontrollgener vid kvantitativa mRNA analyser.

I den tredje delen av avhandlingen presenteras preliminära data på aktivitet hos gener som styr celldöd, myelinering, cell cykel, DNA reparation och cell signalering. Vi kunde visa att i frontal cortex sker en större genaktivitetsförändring än i motor cortex i de utvalda gengrupporna, då 15 av de 36 analyserade generna har en lägre aktivitet i frontal cortex, medan endast en gen har lägre aktivitet i motor cortex. Inga gener påvisade en signifikant ökad aktivitet hos alkoholist. Framförallt var det myelingener som var minskade, med en upp till nästan 3 gångers minskad aktivitet (uttryck).

Resultaten från denna avhandling har skapat en bra grund för att fortsättningsvis kunna studera de genspecifika epigenetiska förändringar inom de gengrupper vi har analyserat. Vi har nu verktyg och resultat för att studera promoterrelaterade förändringar av DNA metylering och histonmodifieringar.
Jag skulle vilja uttrycka en stor tacksamhet till min huvudhandledare Professor Tomas Ekström, och mina bihandledare Professor Yasmin Hurd och PhD Georgy för att ni har låtit mig ta del av ett mycket intressant forskningsprojekt och väglett mig genom forskningsvärlden.

Ett stort tack till AFA försäkringar och Systembolagets Råd för Alkoholforskning (SRA) för möjligheten att bedriva alkoholforskning.

Ett stort tack till, Clive Harper, Terese Garrick och Donna Sheedy i Sydney, Australien för först och främst tillhandahållandet av hjärnmaterial och utförlig information om materialet. Tack även för ett mycket trevligt samarbete och tacksam hjälp till artiklarna.

Tack även till kollegor i USA: Adron Harris, Igor Ponomarev och Dayne Mayfield vid University of Texas i Austin för ett fint samarbete.


Tack till alla andra på CMM som gjort vardagen rolig.
Tack Delphi Post för allt smått och gått av det nödvändiga.

Tack till alla mina andra vänner utanför den vetenskapliga världen som ger mig balans och det andra viktiga i livet: Maria Bergsland, Ylva Stangdell, Ulrika Sporrek, Sofia Svensson, Johanna Lövborg, Emma Persson, Petra Lindberg, Karin Staflin, Maria Ansin, Karin Larsson, Maria Fjellgren, Magnus Jonnerby, Mikaela Berglund, Mikael Kullberg, Michael Lundell, Sofia Braag, Markus Dagnell, Jesper van Dongen, Valtteri Virta.

Tack till min mentor Mauro D’Amato och min personliga karriärcoach Annika Arnberg.

Stort Tack till mitt karriär -vänskaps nätverk: Anna Ökvist, Sara Bruce, Charlotta Dagnell, Sandra Sheerwood, Aurelija Klimavicitue.


Tack alla för rolig skidåkning och nyttiga neurosciencekonferenser i svenska fjällen: Jenny Häggkvist, Olle Andersson, Mikael Nygård, Maurice Perrinjaquet, Johan Öckinger, Mattias Gunnarsson, Erik Södersten, Anna Sandebring, Susanne Akterin, Camilla Hansson, Line Lundfald, Alison Canty och många fler.

Mest av allt, Tack till min familj: min mamma Anita, min syster Johanna, min pappa Ulf och Ewa-Lena, Simon och Janne för all obeskrivlig stöttning och tilltro till min förmåga när jag själv har tvivlat.
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