ABERRANT GLOBAL DNA METHYLATION IN NEURODEGENERATION: ALS AND TRINUCLEOTIDE REPEAT DISORDERS

by

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ABERRANT GLOBAL DNA METHYLATION IN NEURODEGENERATION: ALS AND TRINUCLEOTIDE REPEAT DISORDERS

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To being inspired…

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ABSTRACT

ABERRANT GLOBAL DNA METHYLATION IN NEURODEGENERATION: ALS AND TRINUCLEOTIDE REPEAT DISORDERS

Amyotrophic lateral sclerosis (ALS) is a late onset neurodegenerative disease of the motor neurons, leading to death within two-three years of onset. Extensive studies have thus far helped identify many genetic causes for this devastating disease, but despite such efforts, more than 80% of ALS still remains unexplained. ALS is generally considered to be a polygenic disease, and many different factors may be involved in its pathogenesis, including a combination of rare mutations and environmental factors which could ultimately lead to epigenetic modifications. Here, we investigated ALS from an epigenetic perspective, focusing on 5-methylcytosine (5-mC), a well-characterized epigenetic modification, aiming to conclude disputes between different studies reporting inconsistent results for global 5-mC levels detected in blood samples of sporadic ALS (sALS) patients. The study was further extended to different subtypes of ALS, including familial ALS (fALS), *C9orf72* expansion carrier ALS (C9orf72+ ALS) and *ATXN2* intermediate expansion ALS along with spinocerebellar ataxia types 1 and 2 (SCA1 and SCA2), Huntington's disease, Friedreich's ataxia and myotonic dystrophy type 1. In order to analyze the global 5-mC levels, in DNA isolated from blood, an enzyme-linked immunosorbent assay (ELISA) kit was selected upon testing of commercially available kits. The results showed that increased global 5-mC levels are not exclusive to sALS ($p < 0.001$ [$F(1, 214) = 11.993$, $p = 0.000645$]) and that this can also be observed in different subtypes of fALS. Interestingly, SCA1 ($p < 0.01$ [$F(1, 32) = 8.778$), $p = 0.00571$]) and SCA2 ($p < 0.01$ [$F(1, 56) = 10.784$, $p = 0.001768$]) patients also showed increased levels of global 5-mC when compared to age- and sex-matched healthy controls. Additionally, direct bisulfite sequencing was utilized to investigate the *C9orf72* promoter in C9orf72+ ALS patients and healthy controls. Promoter hypermethylation was observed in patients and was moderately correlated ($r_s = 0.3902$, $p < 0.05$) with the global levels of 5-mC. We also tested several commercial kits for the quantification of 5-hydroxymethylcytosine and could not find a suitable kit for its detection in blood.

ÖZET

NÖRODEJENERASYONDA ANORMAL GLOBAL DNA METİLASYONU: ALS VE TRİNÜKLEOTİD TEKRAR HASTALIKLARI

Geç başlangıçlı bir motor nöron hastalığı olan amiyotrofik lateral skleroz (ALS), iki-üç yıl içinde ölümle sonuçlanır. Bugüne kadar yapılan kapsamlı araştırmalar bu hastalığa yol açan pek çok genetik faktörü ortaya çıkardıysa da, olguların %80'inden fazlasının genetiği hala açıklanamamaktadır. ALS patogenezinde birçok faktör etkendir, buna nadir mutasyonlar ve çevresel faktörler de dahildir. ALS tüm bu faktörlerin epigenetik modifikasyonlara da neden olduğu poligenik bir hastalık hatta sendrom olarak düşünülmektedir. Bu çalışma kapsamında, iyi tanımlanmış 5-metilsitozin (5-mC) değişimlerine odaklanarak ALS'nin epigenetik yönünü inceledik. Aynı zamanda literatürdeki sporadik ALS (sALS) hastalarının kan örneklerindeki global 5-mC düzeylerini ölçen çalışmalardaki tutarsızlıkları çözmeyi amaçladık. Çalışma ALS'nin alt tiplerinin, ailesel ALS (fALS), *C9orf72* pozitif ALS (C9orf72+ ALS) ve *ATXN2* nedenli ALS'nin, yanı sıra spinoserebellar ataksi 1 ve 2 (SCA1 ve SCA2), Huntington, Friedreich ataksisi ve myotonik distrofi hastalarını da incelemek üzere genişletildi. Kandaki global 5-mC düzeylerini tanımlamak üzere kullanılacak kite piyasadaki enzim immunotest (ELISA) yöntemi ile çalışan çeşitli kitlerin denenmesi sonucu karar verildi. Elde edilen sonuçlar kanda global 5-mC seviyelerindeki artışın sadece sALS'ye özgü olmadığını ($p < 0.001$ [$F(1, 214) = 11.993$, $p = 0.000645$]), ve benzer artışların tüm ALS alt tiplerinde de var olduğunu gösterdi. SCA1 ($p < 0.01$ [$F(1, 32) = 8.778$), $p = 0.00571$]) ve SCA2 ($p < 0.01$ [$F(1, 56) = 10.784$, $p = 0.001768$]) hastaları da, yaş- ve cinsiyet-uyumlu kontrollerle karşılaştırıldığında, kandaki global 5-mC seviyelerinde artış görüldü. Ayrıca, *C9orf72* tekrar artışı mutasyonu taşıyıcılarında bu genin promotor bölgesindeki metilasyonu incelemek amacıyla bisülfit dizilemesi yöntemi kullanıldı. Bu hastalarda gözlemlenen promotor hipermetilasyonu global 5-mC seviyeleri ile istatistiksel olarak orta derecede korelasyon içinde bulundu ($r_s = 0.3902$, $p < 0.05$). Bu çalışmalara ilaveten, piyasadaki 5-hidroksimetilsitozin (5-hmC) ölçüm kitleri de denendi, fakat hiç birinden tutarlı bir sonuç alınamadı.

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LIST OF SYMBOLS

min	Minute
ml	Milliliter
mM	Milimolar
ng	Nanogram
nm	Nanometer
sec	Second
µl	Microliter
µM	Micromolar
%	Percent
*	Asterisk
/	Divided by
<	Smaller than
=	Equals to
>	Bigger than

LIST OF ACRONYMS/ABBREVIATIONS

3'	3 prime
5'	5 prime
5-caC	5-Carboxylcytosine
5-fC	5-Formylcytosine
5-hmC	5-Hydroxymethylcytosine
5-mC	5-Methylcytosine
A	Adenine
AD	Alzheimer's disease
ADCA	Autosomal dominant cerebellar ataxia
ALS	Amyotrophic lateral sclerosis
ALS2	Alsin
ALSoD	Amyotrophic lateral sclerosis online genetics database
ANG	Angiogenin
ANOVA	Analysis of variance
AO	Age of onset
Ap-1	Activator protein 1
ARHGEF28	Rho guanine nucleotide exchange factor 28
ATXN	Ataxin
ATXN2int	Intermediate ATXN2 expansion
BER	Base excision repair
BHMG1	Basic helix-loop-helix and HMG-box containing 1
BSP	Bisulfite primer
BST	Bisulfite
C	Cytosine
C9orf72	Chromosome 9 open reading frame 72
Ca^{2+}	Calcium ion
CAG	Cytosine adenine guanine (DNA repeat unit)
CELF	CUGBP Elav-like family member 1
CHCHD10	Coiled-Coil-Helix-Coiled-Coil-Helix Domain Containing 10

CHGB	Chromogranin B
ChIP-seq	Chromatin immunoprecipitation sequencing
CHMP2B	Charged Multivesicular Body Protein 2B
CNS	Central nervous system
CO_2	Carbon dioxide
CpG	5'-C-phosphate-G-3'
CRYM	Crystallin Mu
CV	Coefficient of variation
DAO	D-Amino-Acid Oxidase
dAtx2	Drosophila homolog of the SCA2 gene
DCTN1	Dynactin Subunit 1
ddH2O	Double-distilled water
DM	Myotonic dystrophy
DMPK	Dystrophia Myotonica Protein Kinase
DMWD	Dystrophia Myotonica, WD Repeat Containing
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxynucleotide triphosphate
DRP	Dipeptide repeat protein
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMP1	Epithelial Membrane Protein 1
ERBB4	Erb-B2 Receptor Tyrosine Kinase 4
fALS	Familial amyotrophic lateral sclerosis
FIG4	FIG4 Phosphoinositide 5-Phosphatase
FRDA	Friedreich ataxia
FTD	Frontotemporal dementia
FUS	Fused in Sarcoma/Translocated in Sarcoma
FXN	Frataxin
G	Guanine
GBM	Glioblastoma multiforme
GLE1	RNA Export Mediator

Gln	Glutamine
H_2O	Water
H3K9	Histone 3 Lysine 9
HD	Huntington's disease
HDAK	Histone deacetylases
HNRNPs	Heterogeneous nuclear ribonucleoproteins
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase
HTT	Huntingtin
LINE-1	Long Interspersed Nuclear Elements
MATR3	Matrin-3
MBNL	Muscleblind-like
MECP2	Methyl-CpG Binding Protein 2 Gene
MeCP2	Methyl-CpG Binding Protein 2 Gene Protein
MeDIP-seq	Methylated DNA immunoprecipitation sequencing
MS	Microsoft
MTR	5-Methyltetrahydrofolate-Homocysteine Methyltransferase
N	NH2-terminus
NDAL	Neurodegeneration research laboratory
NDD	Neurodegenerative disorders
NEFH	Neurofilament
Nes	Nestin
NGS	Next generation sequencing
No.	Number
O_2	Oxygen
OPTN	Optineurin
p	Probably value
Pax6	Paired box protein Pax 6
PCR	Polymerase chain reaction
PD	Parkinson's disease
PFN1	Profilin 1
PNS	Peripheral nervous system

PPP2R2B	Protein Phosphatase 2
PSP	Progressive Supranuclear Palsy
Q	Glutamine
RAN	Repeat-associated non-ATG
RNA	Ribonucleic acid
RSPH6A	Radial Spoke Head 6 Homolog A
SAH	S-Adenosyl-L-homocysteine
sALS	Sporadic amyotrophic lateral sclerosis
SAM	S-Adenosyl methionine
SC	Sample Collection
SCA	Spinocerebellar ataxia
SD	Standard deviation
SETX	Senataxin
SG	Stress granule
Sig.	Significant
SIX5	SIX Homeobox 5
SMA	Spinal muscular atrophy
SNP	Single nucleotide polymorphism
SOD1	Superoxide dismutase 1
Sox2	SRY-Box 2
SPG11	Spastic Paraplegia 11
SPSS	Statistical analysis software
SQSTM1	Sequestosome 1
T	Thymine
TAF15	TATA-Box Binding Protein Associated Factor 15
TARDBP	TAR DNA Binding Protein
TDP-43	TAR DNA-binding protein 43
TET	Ten-eleven translocation
TGD	Thymine DNA glycosylase
TND	Trinucleotide disorders
TUBA4A	Tubulin Alpha 4a
UBQLN2	Ubiquilin 2

UPD	Uniparental disomy
UTR	Untranslated region
UV	Ultra violet
VAPB	VAMP-Associated Protein B And C
VCP	Valosin Containing Protein
WES	Whole exome sequencing
WGS	Whole genome sequencing
WHO	World Health Organization
α-KG	α-ketoglutarate

1. INTRODUCTION

With the advents of modern day high-throughput technologies, delivering ever more elaborate tools and means of deciphering the complex nature of the living world, biological sciences have experienced a massive surge in the amount of data produced (Cook *et al*., 2016). This has in turn led to a considerably large number of novel discoveries, helping mankind gain a more comprehensive understanding of the mechanisms that govern the order within biological systems. In the post-genomic era, epigenetics (first coined by Waddington in 1942), leading to changes in the phenotype without any change in the genotype (Dupont *et al*., 2009; Waddington, 1968), has also gained special attention due to its extensive role in the regulation of many different mechanisms within the cell.

Epigenetic modifications add an extra level of regulatory control upon the genome, the ultimate governing factor within every living entity. Such modifications and regulatory mechanisms within the cell can influence the transcription of both coding and noncoding RNA, and range from DNA methylation to various histone modifications, nucleosomal positioning and also modifications at the RNA level (Allis and Jenuwein, 2016). This is crucial for the ultimate identity of any cell as it is determined by the tightly controlled gene expression patterns via transcription factors that depend on both genetic and epigenetic cues, such as regulatory sequences, chromatin structure and nucleosomal positioning (Cantone and Fisher, 2013).

During early developmental stages, epigenetic patterns have been shown to undergo drastic changes such as global elimination and restoration (Suelves *et al*., 2016), but on the contrary they are found to be quite stable during adulthood. These marks and modifications are crucial for stable gene expression, and thus cellular fate determination and maintenance (Cantone and Fisher, 2013). Whether it be the aberrant positional or chemical modifications in each epigenetic mark, or mutations in the regulatory machinery responsible for the maintenance of the epigenome, the resulting changes at the transcriptional level have been linked to many different diseases (Zoghbi and Beaudet, 2016).

Recent years have seen a significant increase in the number of epigenetic studies being conducted both at the cellular and clinical levels, mainly due to the rapid advancements in various microarray and sequencing technologies (Sarda and Hannenhalli, 2014), such as chromatin immunoprecipitation sequencing (ChIP-seq) and methylated DNA immunoprecipitation sequencing (MeDIP-seq). Such technologies have allowed researchers to investigate the many known epigenetic marks and modifications at very high resolutions. Nevertheless, these technologies as with others, are certainly not flawless, and their error-prone nature demands sound hypotheses prior to study design and data analysis in order to prevent any further inconsistencies that may arise.

1.1. Neurodegenerative Disorders

Neurodegenerative disorders (NDDs) are a large group of neurological diseases caused by the death and atrophy of one or more subtypes of neurons, both in the central and the peripheral nervous system (CNS and PNS, respectively) (Figure 1.1). Such large scale loss of specific neuronal populations are especially problematic as neurons lack regenerative features, such as cellular reproduction and regeneration, which are needed to rebuild the lost neuronal networks and restore their normal function, leading to permanent and irreversible damage. Considering these facts, the primary focus in the search for a cure for NDDs has been on strategies to stop or slow the death of neurons as regenerative medicine has proven to be especially difficult due to the nature of the nervous systems

The most common NDDs worldwide are Alzheimer's and Parkinson's diseases (AD and PD, respectively), followed by amyotrophic lateral sclerosis (ALS) (Özoğuz *et al*., 2015; Renton *et al*., 2014). These diseases are highly heterogeneous and can be both sporadic and hereditary with different modes of inheritance and varying penetrance. Due to the progressive nature of most NDDs, patients usually experience worsening symptoms which can adversely affect their quality of life, and eventually lead to death in the case of loss of vital functions, such as cognition, respiration and heart rate. Most NDDs have no effective cure and currently treatments offered to patients mostly aim to alleviate the symptoms of these complex diseases.

According to the latest reports from the world health organization (WHO), it is predicted that by the year 2040, with advances in the cure of other fatal diseases, such as cancers, and with higher life expectancy leading to an ever-aging population in the developed world, neurodegenerative diseases that affect cognition, such as AD, and others that affect motor function, such as PD and ALS, will become the second most common cause of death after cardiovascular diseases (Gammon, 2014). This prediction is mainly based on the fact that aging is identified as the primary risk factor in neurodegenerative diseases (Lin and Beal, 2006).

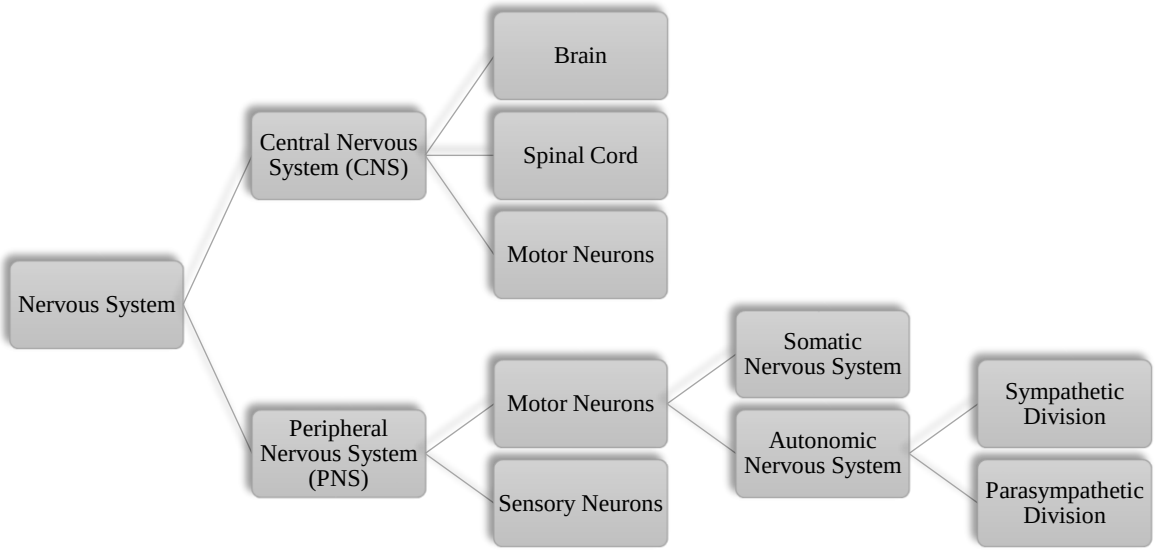

Figure 1.1. Classification of the nervous system.

NDDs have many complex and overlapping phenotypes making their diagnoses quite difficult (Armstrong *et al*., 2005). This has called for the design of different classification methods such as characterizing NDDs by the most predominant site of lesion, whether it be the cerebral cortex, basal ganglia, brain stem, cerebellum or the spinal cord (Figure 1.2). Moreover, one can also further classify these disease with respect to their clinical features and symptoms. Recent advances in high-throughput molecular and genetic diagnosis techniques have also provided physicians with very effective means of diagnosing and classifying various NDDs, with respect to the biomarkers and mutations found within disease-associated genes (Agrawal and Biswas, 2015).

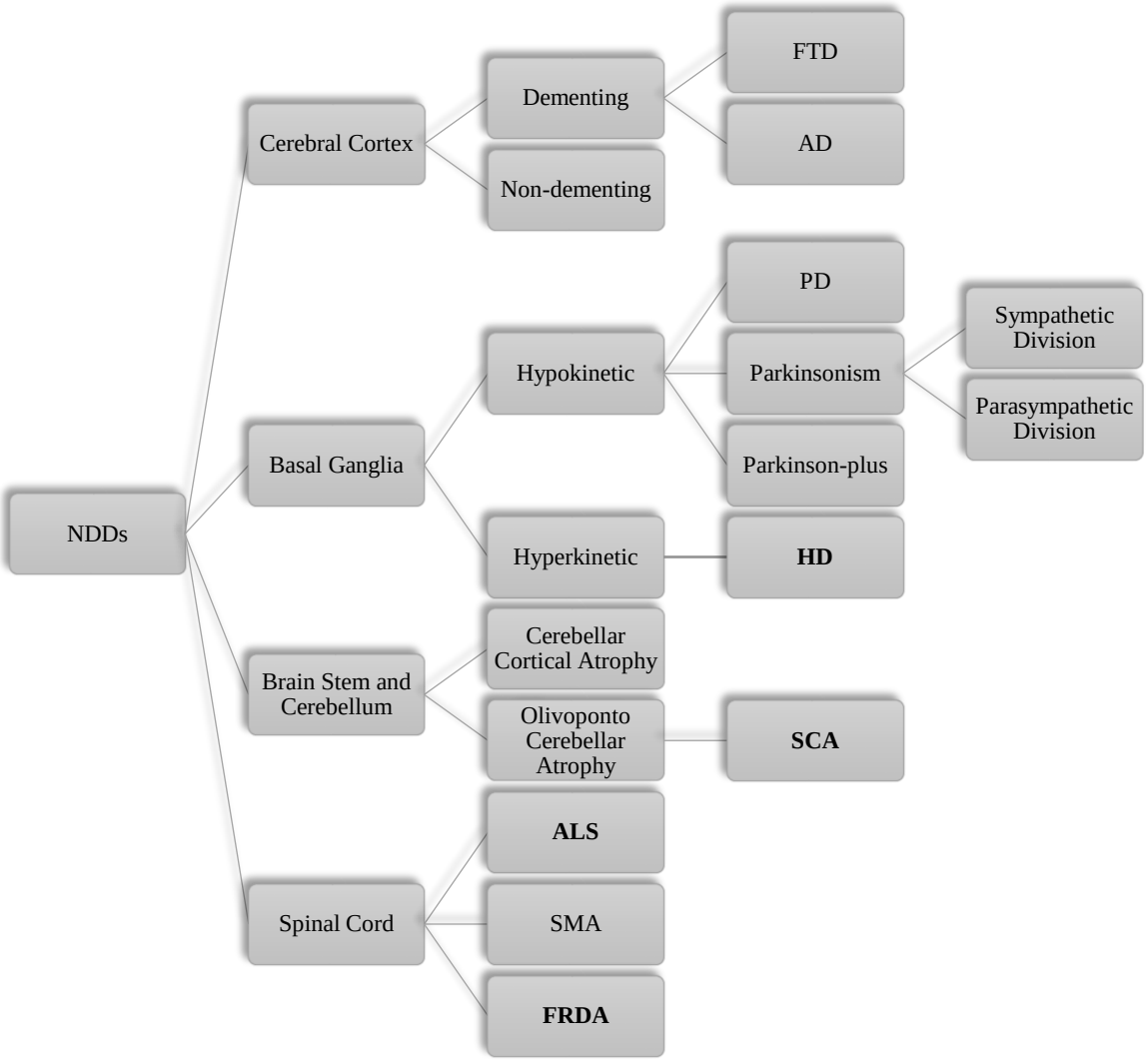

Figure 1.2. Anatomical classification of NDDs (Przedborski *et al*., 2003). The NDDs investigated in the framework of this thesis are shown in bold.

Although large scale sequencing technologies, such as next generation sequencing (NGS), now allow for cheap analysis of entire genomes as opposed to previously utilized techniques, such as Sanger sequencing, its inability to adequately detect repeat expansion mutations make NGS not suitable for the molecular diagnosis of every NDD. This is especially important as up to 30 different NDDs are associated with repeat expansions. Repeat expansion mutations vary in size, and each disease is associated with its own disease-causing threshold (La Spada and Taylor, 2010). The size of the repeating unit, its position within the gene, as well as the bases it contains, are also variable between different diseases (Figure 1.3).

In general, most repeat expansion mutations are inherited in a Mendelian fashion and may be prone to further expansions, and even rarely to contractions, as they are passed down to the next generation. In most cases, it is believed that the presence of interrupting sequences within such repetitive stretches protects them from further expansion, and the loss of these interrupting units can interfere with the cell's DNA damage repair mechanisms, leading to even larger expansions. This phenomenon, often denoted as anticipation, usually leads to an earlier age of onset, faster progression and adverse prognosis, and will eventually become deleterious after a certain number of generations (McMurray, 2010; Mirkin, 2007; Rüb *et al*., 2013).

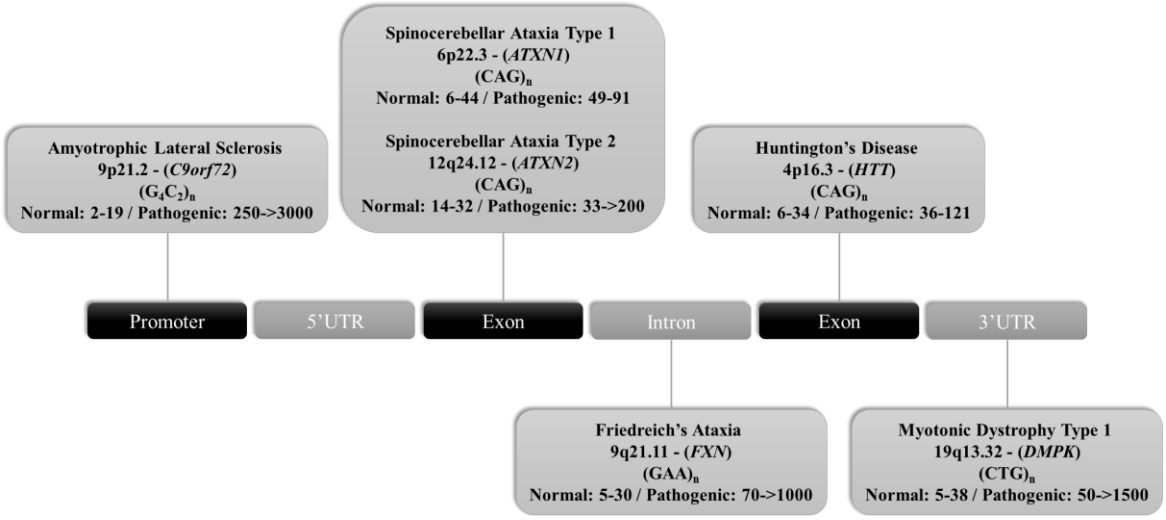

Figure 1.3. Some known repeat expansions in various disorders. The localization and the sizes of the repeats are quite variable among different diseases (Richards *et al*., 2013). The diseases investigated in the framework of this thesis are shown in bold.

1.2. DNA Methylation and Neurodegeneration

The last decade has seen an extraordinary pace in the discovery of many genetic causes to various human diseases, and many more novel discoveries are anticipated to be made with the advance of both sequencing techniques and computational approaches for data mining and analysis of such large scale high-throughput data (Brown and Meloche, 2016). Such advances in sequencing technologies have not only elucidated the genetic bases of different

diseases, but have also been helpful in studying the transcriptome (Lee-Liu *et al*., 2012; Mutz *et al*., 2012) and the epigenome (Sarda and Hannenhalli, 2014), as well. These approaches have helped show how other non-genetic factors could be responsible for various phenotypes (Cooper *et al*., 2013), and to explain why many studies, despite their size and power, have failed to link genetic causes and disease effectively. This has, in turn, led many to believe that a majority of diseases with no known genetic factor may, in fact, be due to other disease-causing factors, such as epigenetic modifications which can both be inherited or gained from the environment (Allan, 2015; Feil and Fraga, 2012).

Epigenetic diseases in principle could be caused by direct changes and modifications of epigenetic marks at the molecular level, such as changes in DNA methylation patterns that could have drastic effects most commonly on imprinted gene regulation, or via indirect mechanisms, such as genetic mutations within epigenetic regulators (Brookes and Shi, 2014). Keeping in mind that genetic mutations often lead to dysfunctional proteins or complete loss-of-function, whereas epigenetic modifications lead to misregulations in gene expression, it is interesting that both phenomena could ultimately lead to similar phenotypes. Observing such events adds to the complexity of regulation within living organisms, and at the same time further improves our understanding of the machinery behind these mechanisms. Whether it be inherited or *de novo*, genetic, epigenetic or environmental, every factor which has an effect on the phenotype can provide a mechanistic insight into the cell. Environmental cues, including diet and lifestyle, along with toxins which can trigger epigenetic modifications, such as changes in DNA methylation patterns, could possibly affect the maintenance and regulation of different epigenetic marks, and provide exciting new areas of research which may prove to be promising for developing novel strategies for the intervention and treatment of various diseases.

Maybe the first and most convincing evidence for the role of the environment and epigenetics in the onset and progression of complex diseases has been monozygotic twin studies where one of the twins has been reported to be discordant for a specific phenotype, such as a disease. This has been the main driver observation for the importance of epigenetics in disease, and many twin studies have taken place, in which monozygotic twins discordant for a phenotype have been studied at many different levels in order to identify the potential

mechanisms responsible for such varied phenotypes (Bell and Spector, 2011; Kim *et al*., 2015). This has been the case both for monozygotic twins, both of whom carry an identified disease-causing mutation and yet present discordant phenotypes, and those where again only one twin presents a sporadic complex disease with no known disease-causing factor (Castillo-Fernandez *et al*., 2014).

Furthermore, complex inheritance patterns, such as varying disease phenotypes despite identical mutations based on the gender of the disease-causing allele carrier parent, have also suggested that factors independent of genetic variations can be responsible in disease onset and progression. An example of this has been shown where specific regions of the genome have variations in functions depending on the origin of the allele; paternal or maternal (Cooper *et al*., 2013). The expression of either the paternal or the maternal allele could be lost completely in the case of uniparental disomy (UPD) as the individual inherits both homologous chromosomes from the same parent (Figure 1.4). In this case, aberrant DNA methylation patterns are believed to lead to several different developmental and neurological disorders (Hannula-Jouppi *et al*., 2014; Soellner *et al*., 2016).

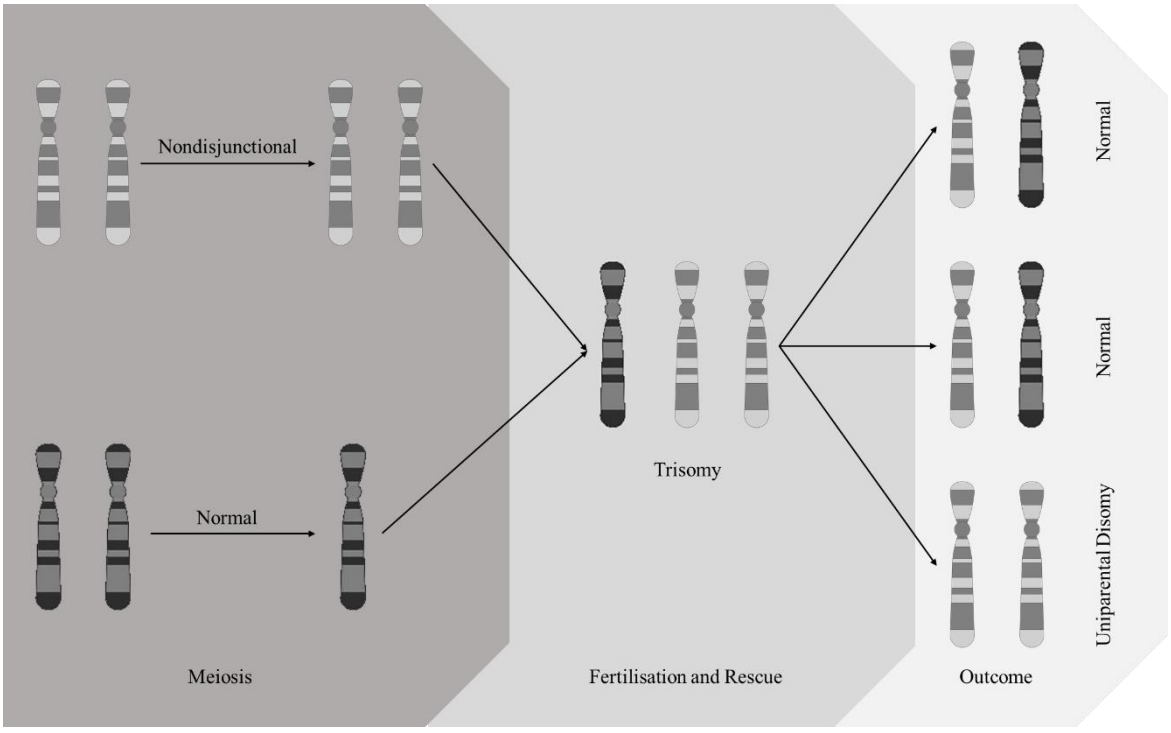

Figure 1.4. Uniparental disomy, where two copies of the same chromosome are inherited from the same parent.

Environmental factors, such as lifestyle choices including diet and exercise, along with others such as exposure to toxins and stress have also been suggested to contribute to disease onset and progression through various epigenetic modifications (Cannon and Greenamyre, 2011; Feil and Fraga, 2012). These modifications, as mentioned before, can be heritable and may also be reversible, which could potentially be exploited if the exact mechanisms of both their role in disease and regulatory pathways were known (Dolinoy and Jirtle, 2008). This is especially exciting as other disease causing factors such as genetic mutations are far more difficult to reverse and may have many adverse effects. Many genetic factors and mutations have been shown to cause NDDs, but there still exists a vast majority of sporadic forms for which no disease-causing factor has thus far been detected (Tsuji, 2010). These diseases are believed to have multifactorial causes, meaning that the disease onset and progression do not only depend on genetic factors, but also on different environmental factors (Marques *et al*., 2011) (Figure 1.5).

Figure 1.5. The importance of genetics and the environment in Mendelian and complex diseases (Drong *et al*., 2012).

One should also keep in mind that the epigenome is much more dynamic than the genome. This is evident in the great diversity that it shows within different tissue types and also during the course of life, whereas the genome is almost completely identical throughout the body and life (Benayoun *et al*., 2015; Suelves *et al*., 2016). Exploiting this feature of epigenetic regulation of gene expression may also prove to be of great value in future efforts to utilize such regulatory pathways for the modification and control of different biological processes in specific populations of cells and tissues.

During the last decade, many different diseases have been studied from an epigenetic perspective (Zoghbi and Beaudet, 2016) and some have even moved on towards clinical trials where different approaches are utilized to modify epigenetic marks in hopes of alleviating different disease (Manal *et al*., 2016; Schapira and Arrowsmith, 2016). NDDs,

being complex in their nature, have so far remained very puzzling and this has led to their analysis from very different perspectives, and epigenetics is no exception (Qureshi and Mehler, 2011). Complex, extensive and multi-layered studies promise to provide very comprehensive and valuable large datasets, and lay the foundations upon which different therapeutic approaches could be based.

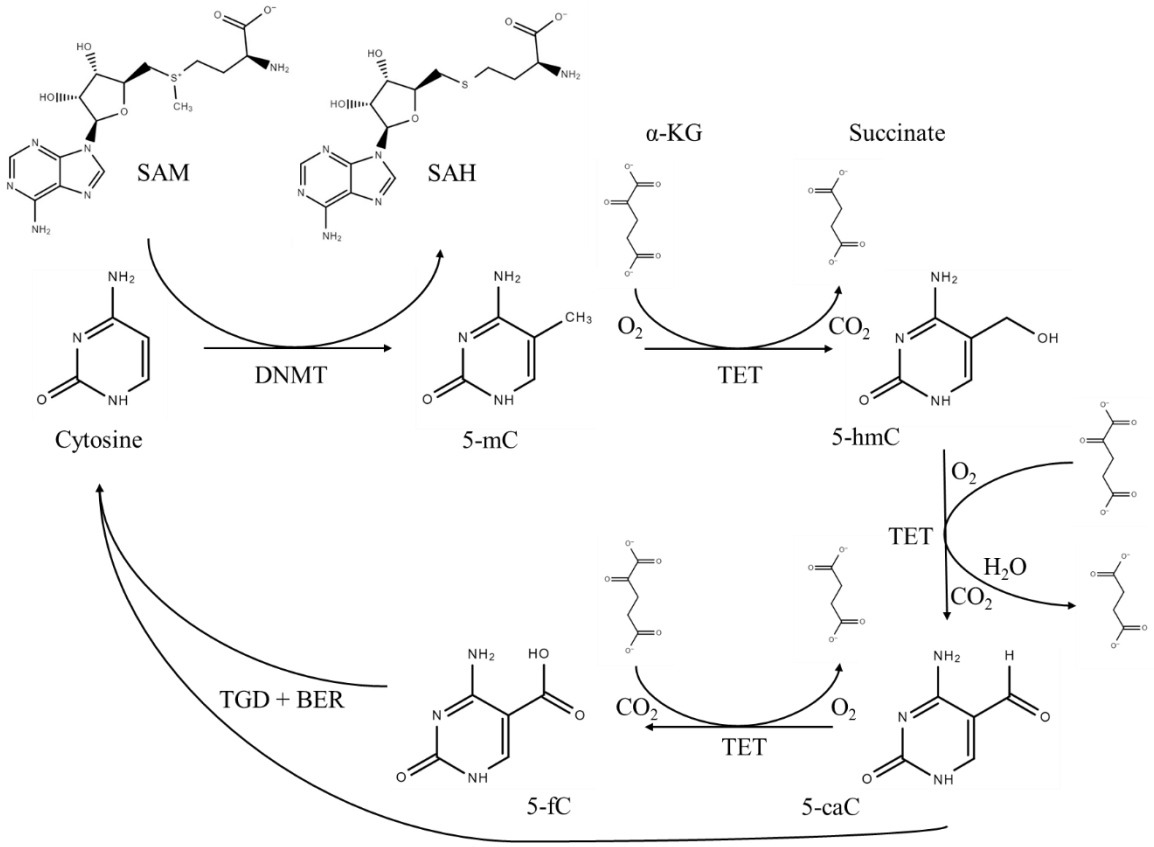

Figure 1.6. Methylation and demethylation of cytosine.

DNA methylation may be the most thoroughly studied and well-characterized epigenetic modification to date and is carried out and maintained by DNA methyltransferases (DNMT) (Uysal *et al*., 2015). All known DNA methyltransferases use S-adenosylmethionine (SAM) as a methyl donor (Figure 1.6). The DNMT family has five members and thus far three active DNMTs have been identified in mammals; DNMT1, DNMT3a and DNMT3b. DNA methylation is maintained in each cell division through the activity of DNMT1 on the hemi-methylated DNA of the daughter cells (Figure 1.7).

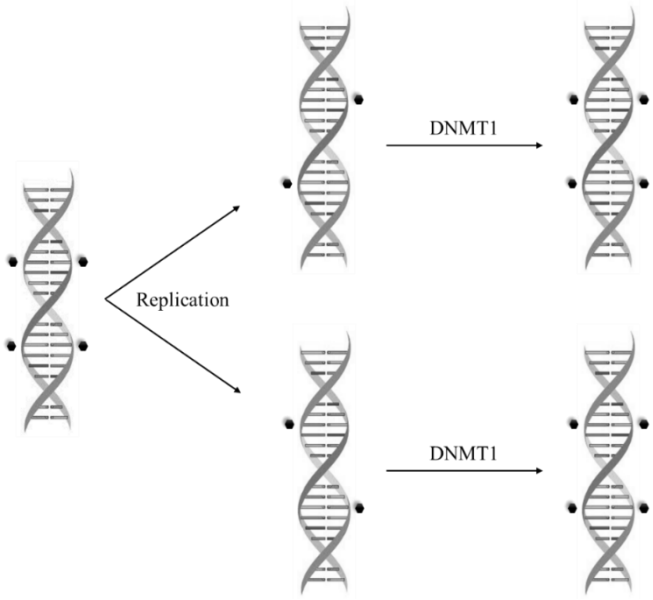

Figure 1.7. Maintenance of DNA methylation during replication.

Promoter DNA methylation is generally a repressive mark and leads to gene silencing (Figure 1.8). This gene-regulatory mechanism depends on DNMTs that transfer methyl groups to the carbon-5 of the cytosine residues, converting them to 5-methylcytosine (5-mC). Gene-regulatory regions and mainly the CpG islands (5′-CpG-3′) of gene promoters are 5-mC free (Figure 1.9). These so called CpG islands are common in promoter regions of genes, and are defined as regions of about 200 base pairs in length, with a CG content of about 50%, where CpGs density is considerably higher than the expected density within the organism under study. In contrast to promoter 5-mC, the presence of 5-mC in intragenic regions (the gene body) can be correlated positively with gene expression.

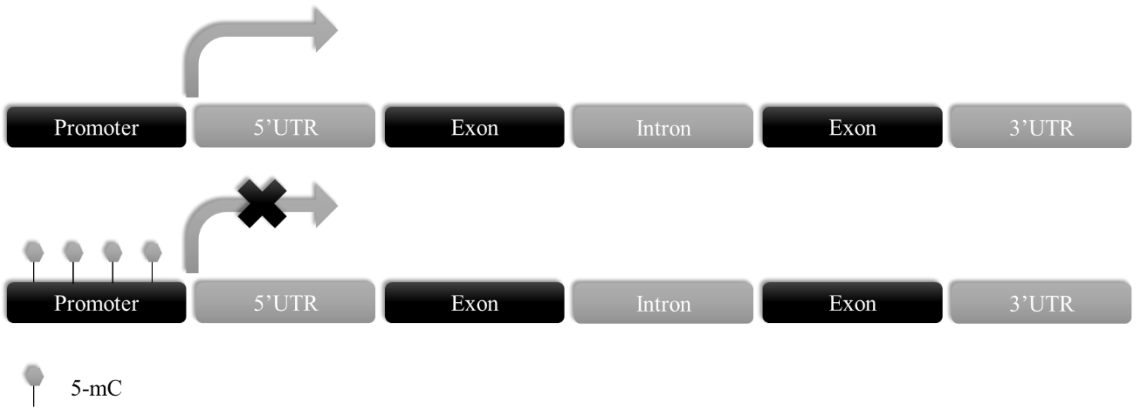

Figure 1.8. The effect of non-methylated and methylated promoters on transcription.

CpG islands have been shown to be evolutionarily conserved, and are thought to arise due to the many deamination events of the methylated cytosines throughout the course of evolution. While deamination of cytosine produces uracil which is reversible via proofreading mechanisms; the deamination of 5-mC leads to the formation of thymine. Thus, the methylation of cytosine may cause spontaneous mutations as CG methylation sites are hotspots for most C to T and G to A transition mutations. This phenomenon has led to the formation of CpG islands across the genome at important regulatory regions such as some promoters (Figure 1.9). DNA methylation is especially important for the suppression of endogenous retroviral genes and other harmful stretches of DNA. One the other hand, it has also been directly correlated with aging as the global decrease observed in the course of ageing is thought to be responsible for the destabilization of the genome and cause organismal dysfunction over time. Furthermore, DNA methylation can act synergistically with chromatin modifications via histone deacetylation to cause transcriptional repression. Methyl-CpG binding domain family of proteins can be recruited to methylated CpGs and cause transcriptional-repression via histone modifications and nucleosome remodeling (Nan *et al*., 1998; H. H. Ng *et al*., 1999; Wade *et al*., 1999).

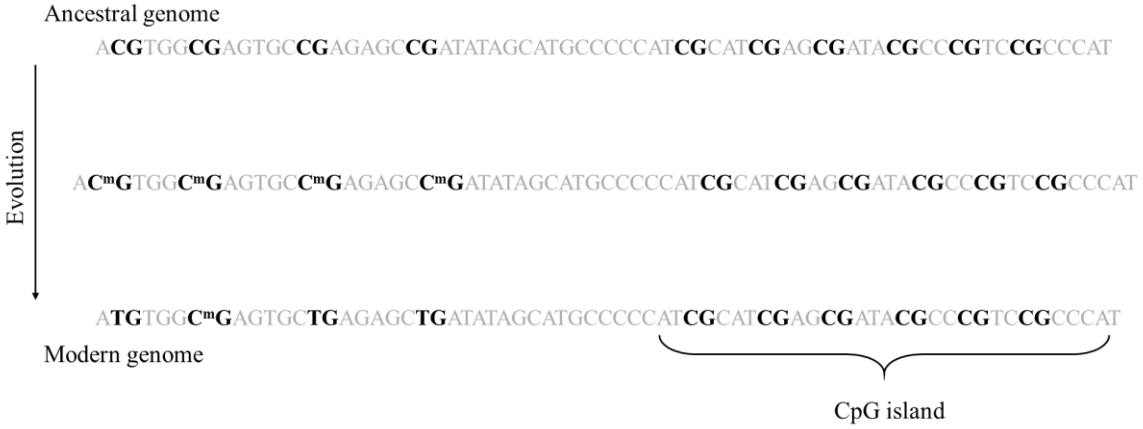

Figure 1.9. The emergence of CpG islands throughout the course of evolution.

Other important regulators of the DNA methylation pathway are the ten-eleven translocation methylcytosine dioxygenase 1 (TET1) enzymes (Rasmussen and Helin, 2016). This group of enzymes are the oxidases responsible for the conversion of 5-mC to 5-hydroxymethlcytosine (5-hmC), the first step in DNA demethylation, and are thought to be crucial in the mechanisms that allow for the regulation of epigenetic on and off switches

within the promoter regions (Figure 1.6). Substantial amounts of 5-hmC are found in certain tissues, such as the brain. The physiological role and significance of 5-hmC, which has been shown to be more prevalent on the 5′ regions of genes, are yet to be entirely elucidated. However, current data suggests that these epigenetic modifications oppose the role of 5-mC within the promoter region by hindering the binding of methyl-CpG binding protein 2 (MECP2) (Ragione *et al*., 2016) and other DNA binding proteins that may cause transcriptional silencing. Modifications, such as 5-hmC, 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) (Figure 1.7) are currently also under intensive study, since they are believed to be especially important in tissue-specific regulation of gene expression which has already been shown to be the case for 5-hmC with its enrichment in mice and human brain samples (Kriaucionis and Heintz, 2009; Madrid and Alisch, 2016). With the advance of both biochemical methods and high-throughput technologies, such as next generation sequencing, studying such differential modifications of cytosine and also other bases within the genome are becoming more feasible (Plongthongkum *et al*., 2014). This is, without doubt, going to be the first step in understanding and characterizing complex epigenetic mechanisms and the nature of their regulatory machinery within biological systems. Models such as different disease states which have been previously characterized on a global state could provide the basis for such higher resolution studies.

With ageing being the main risk factor for many NDDs and recent studies reporting a significant correlation between ageing and DNA methylation (Hernandez *et al*., 2011; Weidner *et al*., 2014), the scientific community has turned its attention towards the possible role of epigenetic factors in NDD pathogenesis (Klein and De Jager, 2016). These studies have focused on the possible role of epigenetic factors, such as DNA methylation and chromatin remodeling, both as sporadic and inherited modifications leading to the onset and progression of disease, similar to genetic mutations (Slatkin, 2009). The main themes in such studies have been aberrant gene promoter methylation, using high-throughput microarray and sequencing technologies along with the quantification of important metabolites such as folate, vitamin B12, S-adenosylhomocysteine (SAH) and SAM (Marques *et al*., 2011). Other more complex epigenetic modifications such as post-translational histone modifications and non-coding RNAs have also been under investigation in various NDDs, but to a much lesser degree (Lee *et al*., 2013).

1.3. Amyotrophic Lateral Sclerosis

ALS is a devastating and rapidly progressive, adult-onset disease of the motor neurons, where the selective and continuous loss of these neurons in the brain and spinal cord lead to muscular atrophy and eventually the loss of all voluntary movement (Morgan and Orrell, 2016) (Figure 1.10). Like many other NDDs, ALS has both familial and sporadic forms with about 10% of all ALS patients being classified as familial ALS (fALS) and the remaining 90% being sporadic (sALS) (Renton *et al*., 2014). Although considered to be a rare disease, it is the third ranking NDD in frequency after AD and PD, ranking first and second, respectively (Özoğuz *et al*., 2015; Renton *et al*., 2014).

Diagnosis of ALS is a lengthy and hard process, mainly due to subtle symptoms at early stages of the disease, which could easily be overlooked or related to other medical conditions. The median time for the diagnosis of ALS patients in the USA could be anything between 1.25 and 2.5 years, depending on the site of onset, whether it be limb or bulbar, and its speed of progression (Williams *et al*., 2013). This is extremely important as the CNS and PNS lack the regenerative features that are needed to replace any lost neurons and thus diagnosing and stopping the disease at earlier stages would be crucial for more effective treatments. This is further emphasized as most of the current treatments that have been shown to be beneficial for mice models of ALS have been administrated prior to disease onset.

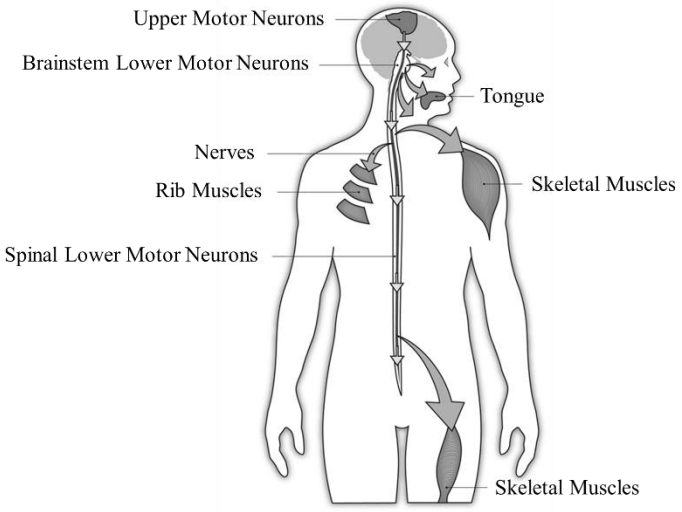

Figure 1.10. Main neurons and muscles affected in ALS.

The loss of voluntary movement is gradual in ALS and starts with patients experiencing weakness and fatigue. The prognosis of the disease is not good and many patients die mainly of respiratory failure 2-3 years after disease onset. This is also correlated with the age of onset which show great variability in ALS (Figure 1.11) and interestingly earlier ages of onset show longer survival. Currently, the only medication available for ALS is benzothiazole riluzole, which is reported to increase life expectancy by about a mere three months (Miller *et al*., 2012).

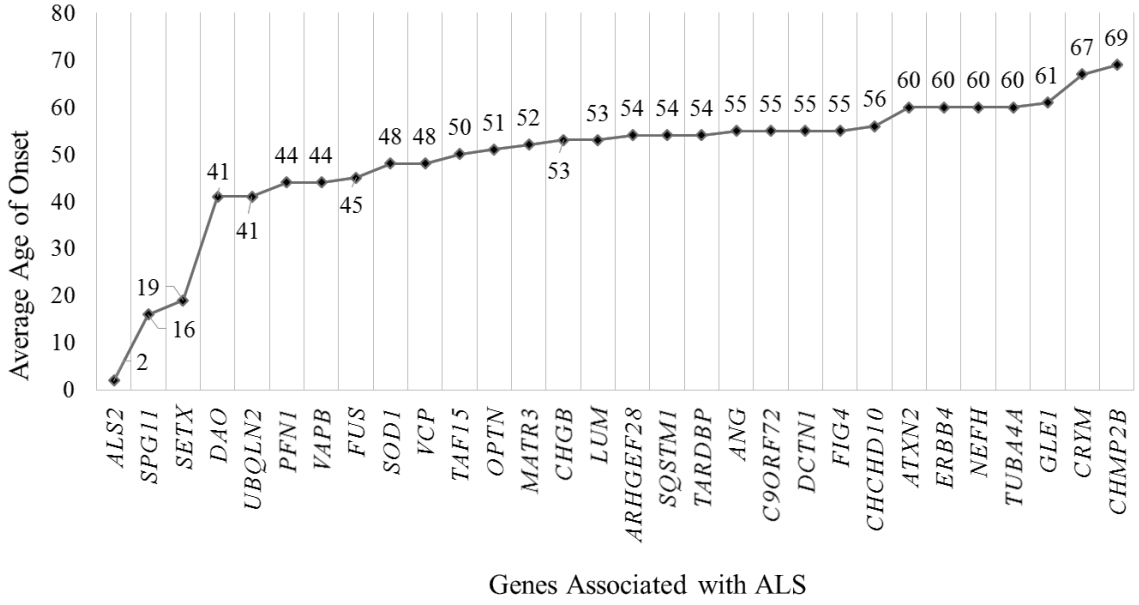

Figure 1.11. Data from ALSoD which is the main online genetics database for ALS (Abel *et al*., 2013). The graph contains data from 684 fALS and 237 sALS gathered from different parts of the world.

The past decade, especially with the development of microarray and NGS technologies which have allowed for large scale association and sequencing studies, the search for novel disease-causing genes and mutations have increased both in size and number. Such studies have utilized a series of different intricate strategies, ranging from linkage analysis in familial cases to whole exome and whole genome sequencing (WES and WGS, respectively), which has led to an exponential rate of disease-causing gene discoveries (Renton *et al*., 2014) (Figure 1.12).

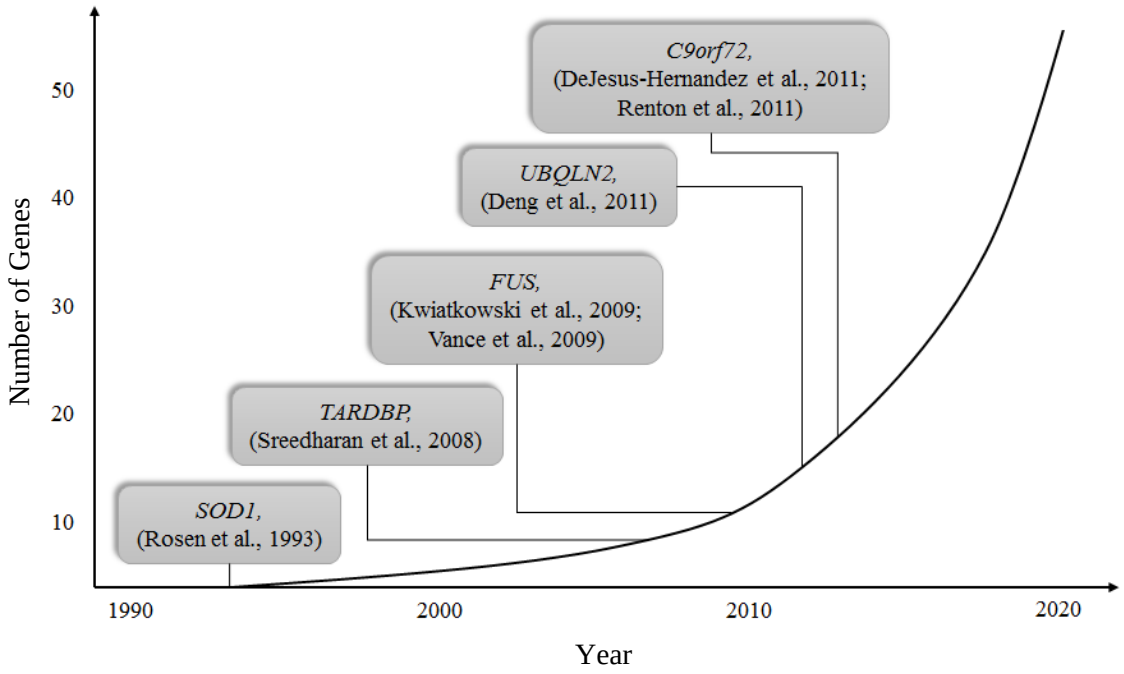

Figure 1.12. The exponential rate of ALS-related gene discoveries (Renton *et al*., 2014).

Even with the technological advances of recent years and large scale international efforts, more than 80% of ALS cases (fALS and sALS together) still remain unexplained (He *et al*., 2015; Kenna *et al*., 2016; van Rheenen *et al*., 2016) leading to a wide-spread belief within the ALS research community that ALS may predominantly be a polygenic disease. Thus, the combination of several SNPs, lifestyle and environmental factors that may act through epigenetic modifications may cumulatively be responsible for disease onset and progression. Such hypotheses are further strengthened by observations such as monozygotic twins which have been found to be discordant for ALS (Meltz Steinberg *et al*., 2015; Xi, Yunusova, *et al*., 2014).

Currently, within our Turkish cohort using both conventional techniques such as Sanger sequencing and also more modern techniques such as whole exome and genome sequencing, we are able to identify disease-causing mutations in only about 42% of familial cases (Özoğuz *et al*., 2015) (Figure 1.13); this number is slightly higher in Europe and North America at about 65% (Renton *et al*., 2014). This is to some extent an indicator of the vast genetic heterogeneity in Turkey.

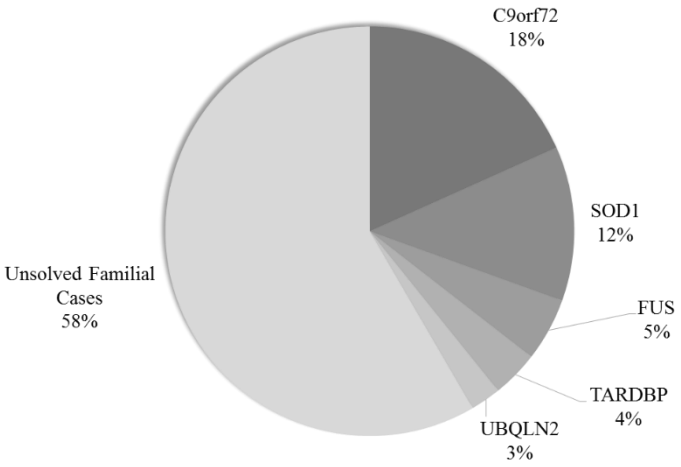

Figure 1.13. Disease-causing mutations in Turkish fALS patients (Özoğuz *et al*., 2015).

The most common ALS-associated genes within our cohort are *C9orf72* (DeJesus-Hernandez *et al*., 2011; Renton *et al*., 2011), *SOD1* (Rosen *et al*., 1993), *FUS* (Kwiatkowski *et al*., 2009; Vance *et al*., 2009), *TARDBP* (Sreedharan *et al*., 2008) and *UBQLN2* (Deng *et al*., 2011). In sporadic patients, with no other affected family members (sALS), the number of cases with identified disease-causing mutations are roughly about 4% (Özoğuz *et al*., 2015) (Figure 1.14) as compared to 11% in the Caucasian populations of Europe and North America (Renton *et al*., 2014).

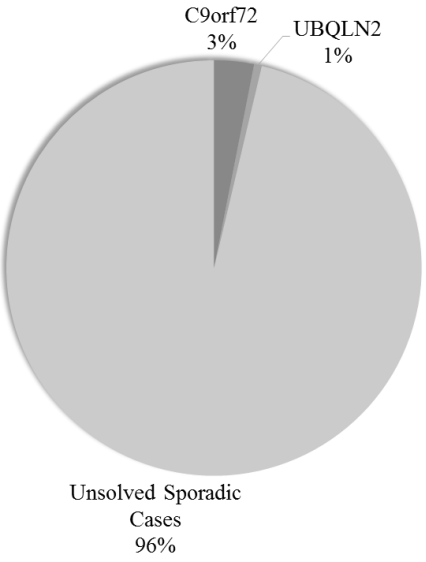

Figure 1.14. Disease-causing mutations in Turkish sALS patients (Özoğuz *et al*., 2015).

Ever since the discovery of the dynamic hexanucleotide $(GGGGCC)_n$ microsatellite repeat expansion in the *C9orf72* gene, it has been widely accepted as the most common cause of ALS. The repeat expansion is in the core promotor of the *C9orf72* gene and lays upstream of the coding region (Ilse Gijselinck *et al*., 2012). Interestingly, the *C9orf72* expansion mutation has also been shown to cause frontotemporal dementia (FTD), making it the first molecular link between FTD and ALS (Özoğuz *et al*., 2015; Rohrer *et al*., 2015; Smith *et al*., 2013).

RNA toxicity is a central theme in many repeat expansion mutations. In the case of *C9orf72*, the expanded RNA molecules are believed to give rise to G-quadruplex toxic RNA structures (Zhou *et al*., 2015). The G-quadroplex structures can give rise to elaborate assemblies that ultimately come together to form intranuclear toxic RNA foci (Reddy *et al*., 2013) (Figure 1.15). Other toxic mechanisms suggested for this mutation include the accumulation of toxic dipeptides due to repeat-associated non-ATG (RAN) translation (Peter O Bauer, 2016) and impaired regulation of endosomal trafficking and autophagy (Farg *et al*., 2014).

DNA methylation has been investigated in NDDs such as ALS both on the global and the gene/promoter level. Maybe the most extensively studied epigenetic modification in ALS is the promoter hypermethylation of the *C9orf72* gene. This is mainly due to the recently identified hexa-nucleotide repeat $(GGGGCC)_n$ expansion mutation in the *C9orf72* gene, whose exact function within the cell is yet to be clearly defined (DeJesus-Hernandez *et al*., 2011; Renton *et al*., 2011). This mutation is currently the most common defect in many different ALS (Özoğuz *et al*., 2015) and frontotemporal dementia (FTD) cohorts worldwide (Smith *et al*., 2013). Furthermore, it has also been linked to AD (Kohli *et al*., 2013) and has become an intensely studied gene in several NDDs. It is believed that the hypermethylation of the promoter region of *C9orf72* reduces the toxic build-up of pathologies observed in post mortem samples from ALS patients (Cooper-Knock *et al*., 2014). Even though neurotoxic pathologies (gain-of-function) are reduced in such a scenario, the disease is suggested to continue now to progress due to the loss-of-function as a result of transcriptional repression, caused by the hypermethylation (Ilse Gijselinck *et al*., 2015).

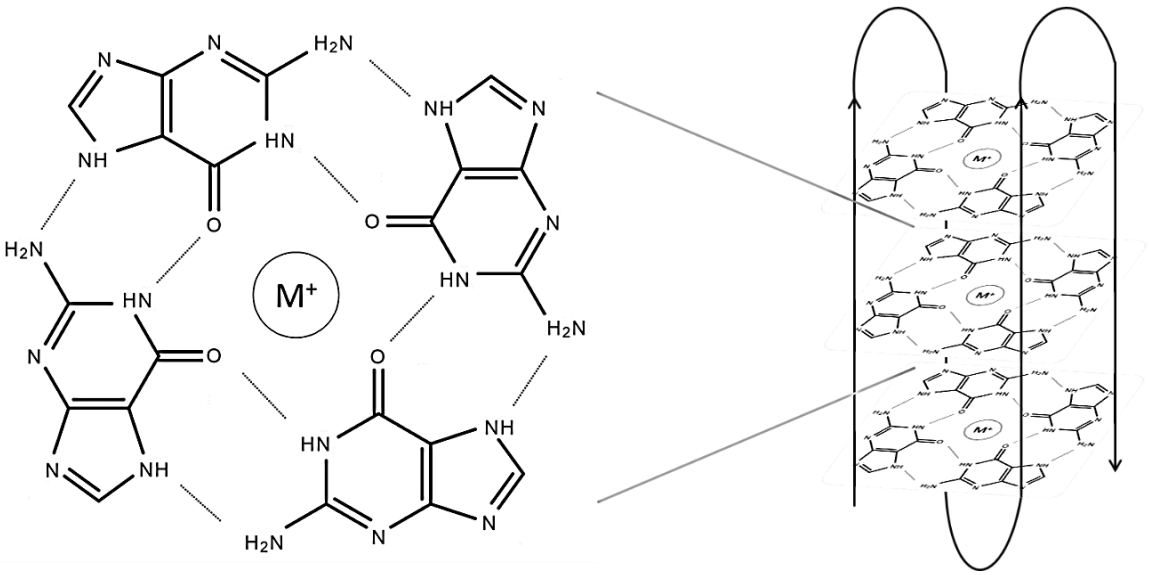

Figure 1.15. G-quadruplex toxic RNA structures can form intranuclear RNA foci.

Higher resolution epigenome-wide studies coupled with data at the transcriptomic level in post-mortem spinal cord samples of sALS patients have also been conducted on a limited number of sALS patient samples and have helped gain some information related to cell death and immune response in neurodegeneration. They also suggest that an increase in global levels of 5-mC is observable in spinal cord samples of sALS patients, but not in the blood (Figueroa-Romero *et al*., 2012). On the other hand, other more recent holistic studies conducted on DNA, isolated from the blood, have reported an increase in global DNA methylation levels in both early- and late-onset ALS patients (Tremolizzo *et al*., 2014). Some other studies have also reported abnormalities in the regulatory machinery responsible for the maintenance of DNA methylation (Martin and Wong, 2013). The epigenetic basis of ALS has so far not been investigated in great detail, and such observations suggest that further in-depth analyses may help elucidate the epigenetic factors that may contribute to the pathogenesis of ALS.

1.4. Trinucleotide Repeat Disorders

Trinucleotide repeat disorders (TND) are the largest group of repeat expansion mutations and are caused by the instability and aberrant expansion of normally stable endogenous tandem repeats within different regions of disease-associated genes (Figure 1.3).

Many different neurological diseases including an array of different NDDs, such as SCA1, SCA2, HD and FRDA, along with DM1, have been linked to trinucleotide repeat expansions. Out of these diseases, SCA1, SCA2 and HD along with several other neurodegenerative diseases share the common CAG-repeat unit which codes for glutamine (Gln, Q) and are referred to as poly-glutamine (poly-Q) diseases. The CAG-repeat, as mentioned before, can be present in different regions of the gene and is not always translated. Such non-coding expansions are thought to contribute to disease pathogenesis by disrupting regulatory and RNA-mediated mechanisms (Nalavade *et al*., 2013). The instability and expansion of trinucleotide repeat regions are thought to arise due to errors in DNA replication, recombination and mismatch repair mechanisms (Mohan *et al*., 2014). Such mutations usually have a high penetrance and can sometimes also affect other organs, which is often the case for FRDA and DM1 patients. Maybe the most interesting aspect of TNDs is how they interfere with neuronal mechanisms in a way that each different TND affects a specific subset of neurons, commonly referred to as selective vulnerability.

1.4.1. Spinocerebellar Ataxia Types 1 and 2

SCA1 and SCA2 belong to the larger group of progressive cerebellar atrophies and are autosomal dominant in their inheritance pattern. They are often referred to as autosomal dominant cerebellar ataxia (ADCA) and are characterized by uncoordinated movement of the limbs, gait ataxia and dysarthria. Unstable CAG-repeat expansions in *ATXN1* (SCA1) and *ATXN2* (SCA2) are known to be the genetic factors behind these diseases and together with SCA3, SCA6 and SCA7, they are the most common subtypes of ADCA, with current therapeutic approaches focusing mainly on symptom management due to a lack of potent treatments. As mentioned before, both diseases share the poly-Q domain expansion as the main pathological cause of disease. Such poly-Q domain expansion-associated diseases share quite a few overlapping features, such as neuronal loss and atrophy in the cerebellum along with other extra-cerebellar tissues at advanced stages of the disease. Purkinje cells and fastigial nuclei of the cerebellum, Betz neurons of the primary motor cortex of the cerebellum, substantia nigra within the midbrain and the superior olive in the pons are all commonly affected regions in poly-Q SCAs such as SCA1 and SCA2 (Figure 1.16).

Toxic RNA foci are thought to be the main cause of cellular dysfunction at the molecular level for SCA1 and SCA2, along with protein mis-folding leading to aggregation and differential subcellular localization. Such events ultimately lead to the disruption of many other downstream mechanisms, such as altered protein degradation pathways due to the aggregation of expanded proteins, altered Ca^{2+} homeostasis, transcriptional dysregulation and mitochondrial dysfunction which is especially interesting as several mitochondrial mutations have also been directly linked to a number of other NDDs, including ataxias (Gorman *et al*., 2014; Jobling *et al*., 2015; Park *et al*., 2014), PD (Lin *et al*., 2012) and HD (Cha *et al*., 2015).

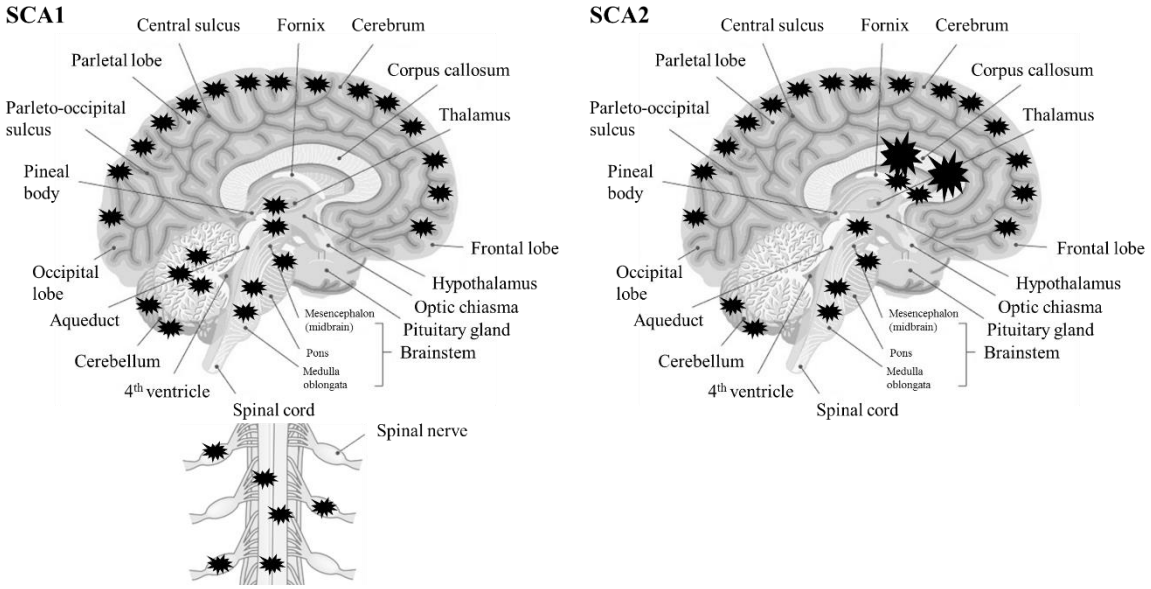

Figure 1.16. The main areas of the CNS affected in SCA1 and SCA2 (Taroni and DiDonato, 2004).

SCA1 is a rapidly progressive adult-onset disease which usually manifests in the 4th decade (Subramony and Ashizawa, 2014). It accounts for about 6% of all ADCAs (Geschwind *et al*., 1997) and the number of repeats shows a significant correlation with the age of onset which is a common phenomenon in repeat expansion mutations, known as anticipation (Rüb *et al*., 2013; Subramony and Ashizawa, 2014). The disease-causing threshold for SCA1 is 39-40 repeats and molecular diagnosis of the disease is crucial for an accurate diagnosis as the clinical manifestation of the disease is not very specific due to the many overlapping phenotypes with other SCAs.

The role of epigenetic factors in SCA1 is yet to be investigated and very few studies have suggested an epigenetic factor in this NDD (Kumaran *et al*., 2014), but on the other hand abnormal histone acetylation and chromatin remodeling have been suggested to be important in other SCAs, calling for more attention to possible epigenetic mechanisms in such diseases (Kazantsev and Thompson, 2008).

SCA2 accounts for 13% of all ADCAs (Geschwind *et al*., 1997) and the disease-causing threshold in the poly-Q tract of the *ATXN2* gene for SCA2 is 32-34 CAG units. SCA2 has a generally slower progression rate when compared to SCA1 and interestingly, repeats between 27 and 33 (intermediate length expansions) have been linked to other diseases. These include an increased risk for ALS (ATXN2int ALS) (Elden *et al*., 2010) and progressive supranuclear palsy (PSP) (Ross *et al*., 2011). Moreover, slightly longer expansions of about 32 and higher have also been linked to L-dopa-responsive Parkinsonism (Charles *et al*., 2007). A more recent study (van Blitterswijk *et al*., 2014), has also suggested that the *ATXN2* intermediate repeat expansion may act as a disease modifier in C9orf72+ ALS patients, making them more prone to develop ALS, rather than FTD which further emphasizes the importance of *ATXN2* in ALS pathology. Similar to SCA1, there is no effective treatment available for SCA2 and the average disease duration is reported to be around 10 years, but rarely patients can live up to 50 years.

Epigenetic studies of the *ATXN2* locus have suggested that promoter hypermethylation could be correlated with CAG-repeat expansions and could also modify the age of onset (Laffita-Mesa *et al*., 2012). Milder disease phenotypes have also been reported in the case of *ATXN2* promoter hypermethylation, which can be explained by the reduction in the expression of the mutant ataxin-2 and thus reduced toxicity (P O Bauer *et al*., 2004). Other than intra-familial differences for the SCA2 phenotype in repeat-carrier patients, reports of monozygotic twins who were discordant for motor symptoms, ocular movement and age at onset for SCA2 indicate an important role for the environment and epigenetics in modifying different aspects of the disease (Anderson *et al*., 2002). As with other rare NDDs, epigenetic studies of SCA2 are limited and further investigations may open new doors towards a better understanding of these devastating diseases.

1.4.2. Huntington's Disease

HD is a member of the TND family of NDDs that occurs due to abnormal CAG-repeat expansions, inherited in an autosomal dominant manner. The poly-Q containing gene responsible for HD is *HTT* (MacDonald, 1993), which is an ubiquitously expressed cytoplasmic protein found throughout the brain. The protein product of this gene harbors the poly-Q tract in its N-terminus (exon 1) and the disease-causing threshold is known to be 40 repeats, with repeats between 36 and 39 demonstrating incomplete penetrance (Rubinsztein *et al*., 1996). The expanded protein has been shown to have aberrant interactions with other proteins and is suggested to disrupt normal cellular functions causing neuronal vulnerability and stress, eventually leading to neuronal death and atrophy (Beal and Ferrante, 2004). As with many other TNDs, longer repeats are associated with anticipation of an earlier disease onset with more severe symptoms in the subsequent generations. HD is associated with symptoms such as weakness, involuntary movements termed chorea and psychiatric symptoms such as emotional distress along with dementia. With no potent treatment available today (Kazantsev and Hersch, 2007) and generally fast progression, the disease leads to death within 10 years with rare cases of survival up to 20 years.

The huntingtin protein (HTT) may be one of the most thoroughly studied proteins in NDD research due to its many important functions within the cell, and interestingly it has also been shown to have a significant impact on many epigenetic marks, ranging from DNA methylation to various histone modifications (Lee *et al*., 2013; Thomas, 2015). Such modifications are thought to cause dysregulations at the transcriptomic level which would certainly be important in disease pathogenesis and progression (Glajch and Sadri-Vakili, 2015). Aberrant DNA methylation has been shown in both HD patients and transgenic HD mice models, especially in the promoter regions of the *Nes, Ap-1, Pax6* and *Sox2* genes which are known to be key elements and regulators of neurogenesis (C. W. Ng *et al*., 2013; Wood, 2013). Moreover, non-coding RNAs, such as microRNAs have also been linked to disease pathogenesis, the mechanisms of which are currently under study and yet not clear (Lee *et al*., 2013). Chromatin level histone modifications, such as differential acetylation and methylation have also been reported in HD. Further evidence for the involvement of epigenetic factors in HD pathogenesis come from monozygotic twins, discordant for

phenotypic features, such as age-of-onset, motor and behavioral symptoms and cognitive differences (Ketelaar *et al*., 2012).

1.4.3. Friedreich's Ataxia

FRDA is a progressive neurodegenerative TND caused by a homozygous GAA-repeat expansion in the *frataxin* gene (exon1) (Campuzano *et al*., 1996) and is the most common form of recessive hereditary ataxias (Muthuswamy and Agarwal, 2015). The disease-causing repeat expansion causes partial silencing and thus frataxin deficiency (Yandim *et al*., 2013), and patients may also rarely have a compound heterozygous genotype in the form of a GAA expansion along with a point mutation (Cossée *et al*., 1999). The disease-causing repeat threshold for FRDA is 66 within the *FXN* gene which can go up to 1,600 (Labuda *et al*., 2000). Longer repeats are associated with younger age of onset along with other disease-associated phenotypes, such as cardiomyopathy, diabetes mellitus and scoliosis (Parkinson *et al*., 2013). As reduced levels of frataxin are correlated with disease onset and progression, it is not surprising to observe that the protein is expressed in a tissue-specific manner (Jiralerspong *et al*., 1997) and some tissues, including the heart, liver and skeletal muscles along with the cerebellum and spinal cord, are mostly affected. Symptomatic treatments are currently the only from of treatment available and include surgical procedures to treat and slow the progression of scoliosis and heart-related symptoms. Drugs such as Idebenone (Kearney *et al*., 2012) have also been introduced, but have had a controversial reception as they are thought to have very little benefit.

When compared to other NDDs and especially TNDs, FRDA is one of the more thoroughly studied diseases with respect to its epigenetics. Aberrant DNA methylation, both upstream and downstream (Evans-Galea *et al*., 2012) and especially in the first intron of the *FXN* gene has been observed in patient samples and correlated with the expansion size and age of onset (Castaldo *et al*., 2008). Other studies have also reported modifications on the chromatin level, including decreased histone H3K9 acetylation and increased H3K9 methylation (Al-Mahdawi *et al*., 2008). Recently, in line with such observations, nicotinamide, a class III histone deacetylase (HDAC) inhibitor, has been put to clinical trials with the aim of increasing the levels of frataxin (Libri *et al*., 2014).

1.4.4. Myotonic Dystrophy Type 1

DM1 is an autosomal dominant TND and is the most common adult onset muscular dystrophy associated with a non-coding CTG repeat expansion in the 3' region of the *DMPK* gene (Brook *et al*., 1992; Fu *et al*., 1992). Patients are reported to have 50 to 4000 repeats, while healthy individuals have 5 to 37 repeats (Meola and Cardani, 2015). The disease can present at any age, but is more commonly seen in the 3rd or 4th decade, showing a significant anticipation in the subsequent generations. As with most other non-coding repeat expansion diseases, such as C9orf72+ ALS and myotonic dystrophy type 2 (DM2), mutant RNAs have been shown to aggregate and form nuclear foci leading to a toxic gain-of-function at the RNA level (Mankodi *et al*., 2000). It has been shown that the mutant RNAs can fold and form stable stem-loop structures which interfere with normal functions of several RNA binding protein families, including *CELF* and *MBNL*, leading to the impairment of their respective signaling pathways (Chau and Kalsotra, 2015). Particularly severe forms of the disease are also associated with implications in the heart (Lund *et al*., 2014), eyes (Ashizawa *et al*., 1992) and gastrointestinal tract (Bellini *et al*., 2006) along with other symptoms such as insulin resistance (Peric *et al*., 2013).

The CTG repeat expansion in DM1 is located within a large CpG island of about 3.5 kb in length (Boucher *et al*., 1995). This region is known to be a gene-rich locus, and the expansion has been shown to lead to epigenetic modifications, such as aberrant DNA methylation both upstream and downstream of the *DMPK* gene. The fact that the CTG repeat locus acts as the downstream promoter of *SIX5*, important in organogenesis, and that it can also influence the transcription of *DMWD*, another myotonic dystrophy-associated gene, along with *BHMG1* and *RSPH6A*, further highlights the importance of DNA methylation in DM1 (Frisch *et al*., 2001). Interestingly, such epigenetic modifications have been suggested to occur in a tissue-specific manner, which can help explain how the expansion mutation can affect some tissues more severely than others (Buckley *et al*., 2016).

2. PURPOSE

Epigenetics has been an emerging field of science within the last decade as different studies have been able to show the importance of epigenetic marks and modifications on the complex machinery governing the inner workings of the cell. Having considered this and the huge impact that such mechanisms could have on human disease, whether it be monogenic or complex, such as the case for ALS, this thesis focuses on one of the most studied epigenetic modifications, namely 5-mC, on a global level. ALS as a complex neurodegenerative disorder along with monogenic trinucleotide repeat-associated diseases were investigated in the hope of unraveling the yet very new field of epigenetics in neurodegeneration. This is the first epigenetic study performed on Turkish ALS patient samples and the largest of its kind worldwide in terms of the number of patient samples (299) and healthy controls (145) analyzed. To the best of our knowledge, this thesis is also the first study, investigating the global levels of 5-mC in 5 additional diseases including SCA1, SCA2, HD, FRDA and DM1.

This thesis aims to:

- Determine if the global 5-mC levels change due to neurodegeneration or during the course of a neurodegenerative disease.
- Conclude controversial disputes regarding aberrant global 5-mC in ALS patients.
- Help elucidate some of the epigenetic aspects of NDDs, especially ALS. The ultimate goal is to pave the ways to the development of early molecular biomarkers for these fatal diseases.

3. SUBJECTS AND MATERIALS

3.1. General Laboratory Equipment and Chemicals

The general laboratory equipment and chemicals used during the experiments conducted within the framework of this thesis are listed in Tables 3.1 and 3.2, respectively.

Table 3.1. General laboratory equipment.

Equipment	**Brand**	**Model**	**Catalog No.**
96-well PCR Plate	Axygen	PCR96FLTC	-
Autoclave	Astell	Front Loading Autoclave	ASB260BT
DNA Extraction System	Roche Diagnostics GmbH	MagNA Pure Compact	3731146001
Incubator	Müve	Incubator EN 120	-
Micro-centrifuge	Beckman Coulter	Microfuge 16	-
Micro-centrifuge Tubes	Axygen	1.5 ml Boil-Proof Microtubes	-
	Axygen	0.5 ml Thin Wall Flat Cap PCR Tubes	-
Micro-pipettes	Rainin	Pipet-Lite, 2 µl, 10 µl, 20 µl, 200 µl, 1000 µl	-
	Thermo Scientific Finnpipette F2	30-300 µl	MH13289
Micro-pipette Tips	Axygen	Universal Fit Tips, 10 µl, 200 µl, 1000 µl	-
Parafilm	Parafilm	PM-996	EW-06720
Pipet Controller	Pipet Boy	accu-jet	-
Plate Reader	Molecular Devices	VersaMax ELISA Microplate Reader	-
	Thermo Electron	Fluoroskan Ascent FL	-
Refrigerator	Arçelik	2021D (-20° C)	-

Table 3.1. General laboratory equipment (cont.).

Equipment	Brand	Model	Catalog No.
Refrigerator	Hettich	HT5786-A (-80° C)	-
Shakers	Heidolph	Duomax 1030	-
Spectrophotometer	Thermo Scientific	NanoDrop 2000c UV-Vis Spectrophotometer	-
Sterile tubes	Greiner Bio-One	Cellstar Tubes, 15 ml	-
Thermal Cycler	Thermo Scientific	Arktik Cycler	-
Vortex	Fisons	WhirliMixer	-
Water purification system	Sartorius	Arium R 611VF	-
Wipes	Kimberly-Clark	Kim Precision Wipes	5511

Table 3.2. General laboratory chemicals used.

Chemical	Brand	Catalog No.
Eau Bi-Distillee	Galen İlaç Sanayii	40503012
Ethanol	Sigma-Aldrich	32205

3.2. Patients and Healthy Controls

Patients diagnosed with various NDDs are referred to NDAL, as the reference laboratory for NDD research in Turkey, by collaborating expert neurologists across the country. Blood samples from patients and affected family members are collected along with unaffected relatives and spouses, with no direct kinship as neurological healthy controls. All blood samples are collected with written consent into EDTA containing tubes and stored at 4° C.

Details regarding the cohort studied within the framework of this thesis, including patients and healthy controls, can be found in Table 3.3 which lists information regarding the mean age of onset (AO) and mean age at sample collection (SC) along with their standard deviations (SD). The number of males and females and the total number of samples are also listed in Table 3.3.

Table 3.3. Clinical and phenotypical details of the cohort studied in the framework of this thesis.

	Mean AO ± SD	Mean Age at SC ± SD	No. Male	No. Female	Total No. Samples
sALS	51.49 ± 12.96	51.95 ± 13.08	104	99	203
fALS	42.12 ± 17.87	42.93 ± 12.72	27	16	43
C9orf72+ ALS	56.44 ± 10.43	57.17 ± 8.74	22	18	40
ATXN2int ALS	55.38 ± 15.65	56.69 ± 15.41	7	6	13
SCA1	33.62 ± 9.54	36.21 ± 10.34	9	8	17
SCA2	28.06 ± 12.23	38.29 ± 16.43	19	10	29
HD	38.67 ± 13.33	46.19 ± 16.79	13	8	21
DM1	26 ± 12.99	35.6 ± 13.48	11	14	25
FRDA	17.13 ± 6.28	25.45 ± 10.47	19	12	31
Healthy Control	-	46.06 ± 16.61	78	67	145

3.3. Equipment and Solutions for DNA Isolation

MagNA Pure Compact Nucleic Acid Isolation Kit I and the MagNA Pure Compact Instrument were used for DNA extraction from blood samples. Concentration and quality of the isolated DNA samples were measured with NanoDrop 2000c UV-Vis Spectrophotometer.

Information regarding the brand and the catalog numbers of the MagNA Pure Compact Instrument and NanoDrop 2000c UV-Vis Spectrophotometer are listed in Table 3.1. The MagNA Pure Compact Nucleic Acid Isolation Kit I was purchased from Roche Diagnostics GmnH (Catalog No. 3730964001).

3.4. Equipment, Kits and Solutions for ELISA

The different kits tested for the selection of the best ELISA-based assays for the detection of 5-mC and 5-hmC are Tables 3.4 and 3.5, respectively. These include four different commercially available kits tested for the detection of 5-mC, along with four different commercially available kits tested for the detection of 5-hmC in DNA isolated from blood.

Detection of the colorimetric signals for three 5-mC and 5-hmC kits was performed using VersaMax ELISA Microplate Reader. The fluorometric signal detection for the other two kits, 5-mC and 5-hmC, were performed using Thermo Electron Fluoroskan Ascent FL.

Table 3.4. Commercially available ELISA-based kits for the detection of 5-mC.

Company	**Kit**	**Short Name**	**Catalog No.**
Abcam	Methylated DNA Quantification Kit (Fluorometric)	Abcam 5-mC	ab117129
Enzo	5-Methylcytosine DNA ELISA Kit	Enzo 5-mC	ADI-900-224
Epigentek	MethylFlash Methylated DNA Quantification Kit (Colorimetric)	Epigentek 5-mC	P-1034
Zymo	5-mC DNA ELISA Kit	Zymo 5-mC	D5325

Table 3.5. Commercially available ELISA-based kits for the detection of 5-hmC.

Company	**Kit**	**Short Name**	**Catalog No.**
Abcam	Hydroxymethylated DNA Quantification Kit (Fluorometric)	Abcam 5-hmC	ab117131
Enzo	5-Hydroxymethylcytosine DNA ELISA Kit	Enzo 5-hmC	ADI-900-225
Epigentek	MethylFlash Hydroxymethylated DNA Quantification Kit (Colorimetric)	Epigentek 5-hmC	P-1036
Zymo	Quest 5-hmC DNA ELISA Kit	Zymo 5-hmC	D5425

The contents in terms of the supplied equipment, controls, antibodies and buffers are listed in Tables 3.6 and 3.7 for 5-mC and 5-hmC detection kits, respectively.

Table 3.6. Contents of various ELISA-based 5-mC detection kits.

Colorimetric			**Fluorometric**
Zymo	**Enzo**	**Epigentek**	**Abcam**
Equipment			
96-well plate	Microtiter Plate	8-Well Assay Strips	8-Well Assay Strips
Controls			
Negative Control (100 ng/µl)	Negative Control (100 ng/µl)	Negative Control (20 µg/ml)	Negative Control (20 µg/ml)
Positive Control (100 ng/µl)	Positive Control (100 ng/µl)	Positive Control (20 µg/ml)	Positive Control (20 µg/ml)
Antibodies			
Anti-5-m-cytosine Primary Antibody (1 µg/µl)	Primary Antibody (1 ng/µl)	Capture Antibody (1000 µg/ml)	Capture Antibody (1000 µg/ml)

Table 3.6. Contents of various ELISA-based 5-mC detection kits (cont.).

Secondary Antibody (1 µg/µl)	Antibody-conjugate (1 µg/µl)	Detection Antibody (400 µg/ml)	Detection Antibody (400 µg/ml)
Buffer & Solutions			
5-mC Coating Buffer	Coating Buffer	10X Wash Buffer	10X Wash Buffer
5-mC ELISA Buffer	ELISA Buffer	Binding Solution	Binding Solution
HRP Developer	HRP Developer	Enhancer Solution	Enhancer Solution
-	-	Developer Solution	Fluoro-developer
-	-	Stop Solution	Fluoro-dilutor
-	-	-	Fluoro-enhancer

Table 3.7. Contents of various ELISA-based 5-hmC detection kits.

Colorimetric			**Fluorometric**
Zymo	**Enzo**	**Epigentek**	**Abcam**
Equipment			
96-well ELISA Plate	Microtiter Plate	8-Well Assay Strips	8-Well Assay Strips
Controls			
Control DNA Set (5 Controls)	Control DNA Set (5 Controls)	Negative Control I (20 µg/ml)	Negative Control I (20 µg/ml)
-	-	Negative Control II (20 µg/ml)	Negative Control II (20 µg/ml)
-	-	Positive Control (20 µg/ml)	Positive Control (20 µg/ml)
Antibodies			
Anti-5-hm-cytosine Primary Antibody (1 mg/ml)	Primary Antibody (1 mg/ml)	Capture Antibody (1000 µg/ml)	Capture Antibody (1000 µg/ml)
Polyclonal Antibody (1 mg/ml)	Antibody-conjugate (1 mg/ml)	Detection Antibody (400 µg/ml)	Detection Antibody (400 µg/mL)
Buffer & Solutions			
Coating Buffer	Coating Buffer	10X Wash Buffer	10X Wash Buffer
10X ELISA Buffer	ELISA Buffer	Binding Solution	Binding Solution
HRP Developer	HRP Developer	Enhancer Solution	Enhancer Solution
-	-	Developer Solution	Fluoro-developer
-	-	Stop Solution	Fluoro-dilutor
-	-	-	Fluoro-enhancer

3.5. Equipment, Kits and Solutions for Direct Bisulfite Sequencing

For the bisulfite conversion of C9orf72+ ALS patient DNA samples and healthy controls, EZ DNA Methylation Kit was utilized according to manufacturer's instructions, using Arktik™ Thermal Cycler from Thermo Scientific. Kit contents are listed in Table 3.8.

Table 3.8. Content of the bisulfite conversion kit used prior to direct sequencing.

EZ DNA Methylation Kit (D5002, Zymo Research)
CT Conversion Reagent
M-Dilution Buffer
M-Binding Buffer
M-Wash Buffer
M-Desulphonation Buffer
M-Elution Buffer
Zymo-Spin™ IC Columns
Collection Tubes

Following the bisulfite conversion of the DNA, the 26 CpG island containing region of the *C9orf72* gene promoter was amplified using Hot Start Taq DNA polymerase, together with compatible master mix components in both patients, age- and sex-matched healthy controls and standard control samples. Information regarding the content of the master mix and standard controls are listed in Table 3.9. The sequence of the primers used for the amplification of the region of interest are listed in Table 3.10.

Table 3.9. Solutions and chemicals used in the amplification of the CpG islands in the *C9orf72* promoter.

Hot Start Taq DNA Polymerase (M0495, New England Biolabs)
Standard Taq Reaction Buffer Pack (B9014S, New England Biolabs)
$MgCl_2$ Solution, 25 mM
dNTP Solution Mix (N0447S, New England Biolabs)
dATP, dCTP, dGTP and dTTP (10 mM each)
Human Methylated & Non-methylated DNA Set (D5014, Zymo)
Human HCT116 DKO Non-methylated DNA, 5 µg / 20 µl
Human HCT116 DKO Methylated DNA, 5 µg / 20 µl
DAPK1 Primers, 20 µl

Table 3.10. Primers used in the amplification of the CpG islands in the *C9orf72* promoter.

1st PCR	
BSP_1F	5'-TTT ATT AGG GTT TGT AGT GGA GTT TT-3'
BSP_1R	5'-AAA TCT TTT CTT ATT CAC CCT CAA C-3'
2nd PCR	
BSP_2F	5'-TAT TAG GGT TTG TAG TGG AGT TTT-3'
BSP_2R	5'-CCA CAC CTA CTC TTA CTA AAC CC-3'

3.6. Software, Online Tools and Databases

Software, online tools and databases used for various tasks, such as viewing documents, sequences, statistical analysis, quantification of both colorimetric and fluorometric signals, DNA quantification, calculations and writing during this thesis are listed in Table 3.11.

Table 3.11. Software, tools and databases used.

Software/Tool/Database	**Source**
CLC Main Workbench v5.7	CLC bio
GraphPad Prism v6.0	GraphPad Software Inc.
Mendeley Desktop	Mendeley Ltd.
MS Office	Microsoft
NanoDrop 2000 / 2000c Software	Thermo Scientific
SPSS Statistics Software v.22	IBM
UniProt	http://www.uniprot.org/
SoftMax Pro v5	Molecular Devices
Ascent™ Software	Thermo Scientific

4. METHODS

The workflow of this thesis, including sample collection and selection, is summarized in Figure 4.1 and shaded gray.

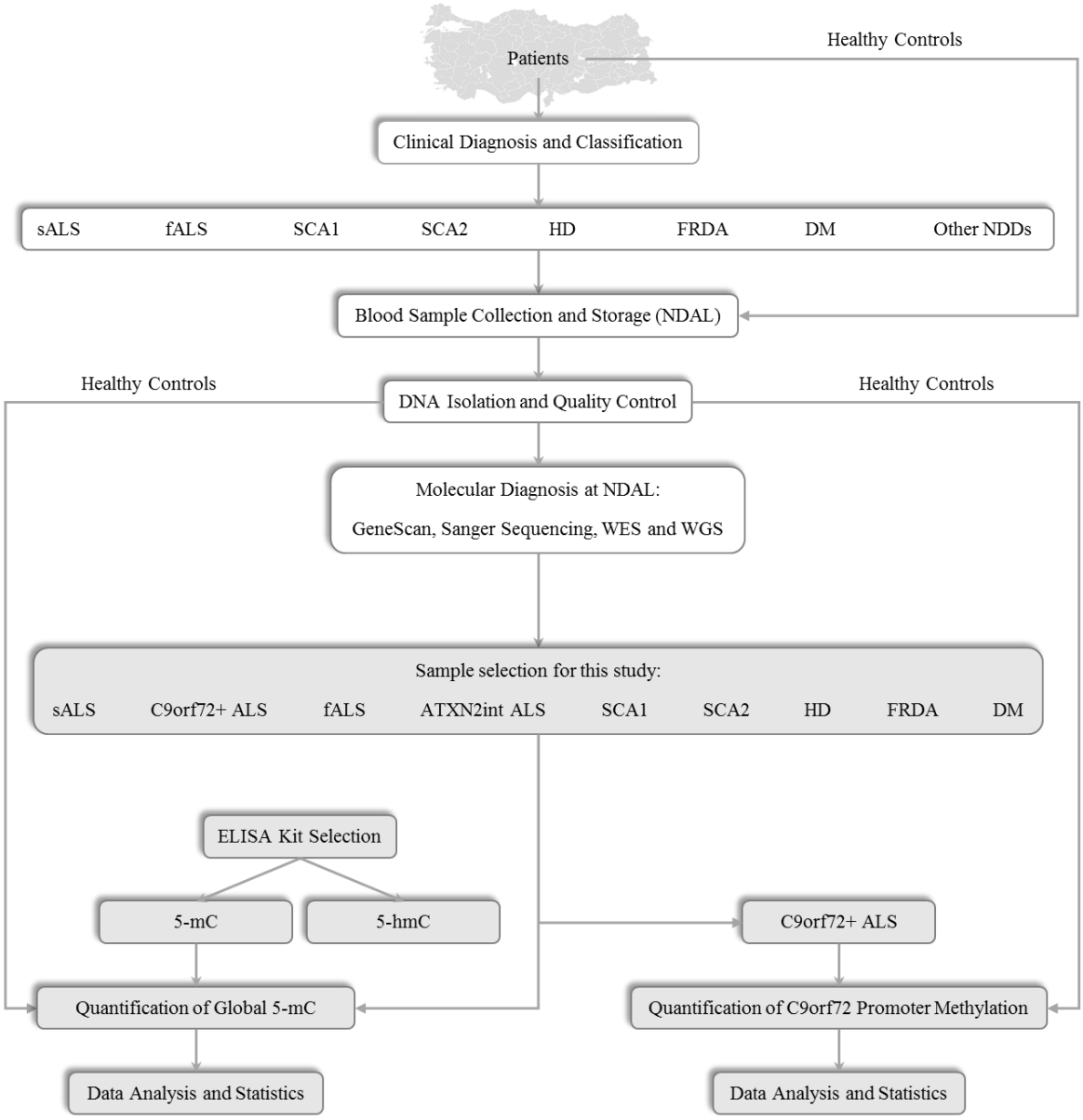

Figure 4.1. The general workflow of this study.

4.1. Blood Sample Collection and Storage

The blood samples of both, patients and healthy controls, were collected into EDTA containing tubes and stored at NDAL.

4.1.1. Patient Samples

Patients were referred to NDAL by collaborating neurologists from across Turkey, along with special forms containing their clinical data. Peripheral blood samples were collected into EDTA containing tubes for DNA extraction with informed written consent and approval of the Ethics Committee of Boğaziçi University. The blood samples were stored at 4° C until later use for DNA extraction.

Various different molecular diagnosis strategies, such as GeneScan for repeat expansion detection and direct sequencing technologies (Sanger sequencing), whole exome sequencing (WES) and whole genome sequencing (WGS), were used in order to further classify the patients into distinct groups, which was crucial for the selection of samples to be included in this thesis (Figure 4.1)

4.1.2. Healthy Control Samples

Blood samples from healthy Turkish individuals were obtained either from unaffected family members (e.g. spouses with no direct kinship: a total of 47 individuals) or from Haydarpasa State Hospital Haematology Department. All blood samples were collected with informed written consent and approval of the Ethics Committee of Boğaziçi University. The average ages at blood collection for patient and healthy controls, are listed in Table 3.3.

4.2. DNA Isolation and Quality Control

The extraction of the DNA from 1,000 µl blood samples of our patient and healthy control cohorts was performed using MagNA Pure Compact and the MagNA Pure Compact Nucleic Acid Isolation Kit I according to the user guide provided by the manufacturer. The

concentration and quality of the extracted DNA were assessed using 1 µl of the sample and NanoDrop 2000c UV-Vis Spectrophotometer. DNA stocks were stored at -20° C.

4.3. ELISA Kit Selection

The kit selection strategy for global 5-mC and 5-hmC detection in DNA, isolated from blood are explained in the following sections and summarized in Figure 4.3 and Figure 4.4. The two main aspects considered for the selection of the kits were the intra-plate variability and the consistency of results between the different kits utilizing similar working principles. The general working principle of each different kit is shown in Figure 4.2. The Epigentek and abcam 5-mC and 5-hmC kits use a direct ELISA setup, the Enzo and Zymo 5-mC kits use an indirect ELISA setup, while the Enzo and Zymo 5-hmC kits use a sandwich ELISA setup (Table 5.1 and 5.2).

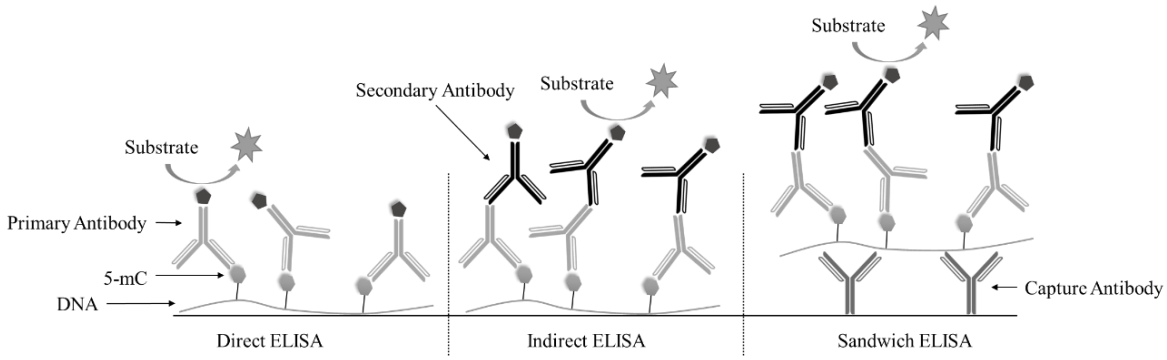

Figure 4.2. The three different ELISA setups utilized in the kits tested in the framework of this thesis.

4.3.1. Global 5-mC Detection Kit Selection

In order to find the most sensitive and precise DNA ELISA-based detection kit for the quantification of 5-mC, four commercially available kits (Table 3.4) were tested using a set of 22 samples comprising of both, patient and healthy control samples (common sample set) (Figure 4.3) according to each manufacturer's guidelines. Non-methylated and fully-methylated standard control samples provided by the kits were mixed in different ratios as

indicated in the user manuals in order to generate a standard curve used to quantify the unknown samples. The standard control samples were processed in parallel with the common sample set in each plate. Three hundred and fifty ng of patient or healthy control DNA was diluted to 20 ng/µl aliquots in separate tubes and five µl of these dilutions along with the standard control samples were loaded in triplicates into 96-well plates.

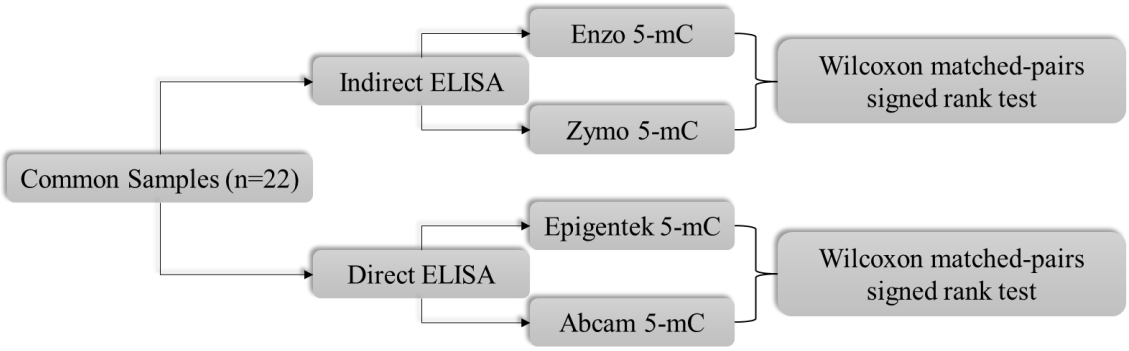

Figure 4.3. The 5-mC ELISA kit selection strategy.

After the protocol for each kit was completed, in other words, once the development solutions were added to the wells, colorimetric absorbance values were measured at three different wavelengths (405 nm, 430 nm and 450 nm) at three different time points (15 min, 35 min and 55 min). The signal detection values for the reading with the best standard curve were exported to MS Excel files via SoftMax Pro v5. For the fluorometric kit, 590 nm emission values were recorded following an excitation at 530 nm immediately after the completion of the protocol. The recorded values were exported to MS Excel files via Ascent™ Software. The intra-plate consistency was analyzed by looking at the SD and coefficient of variation (CV) between the triplicate readings within each of the plates using MS Excel and the consistency of each ELISA setup was assessed by applying the Wilcoxon matched-pair signed rank test using the GraphPad Prism® 6 software (Figure 4.3).

4.3.2. Global 5-hmC Detection Kit Selection

The same approach as in the 5-mC kit selection was also employed in choosing the most sensitive and precise 5-hmC detection kit. Four commercially available kits (Table 3.5) were tested using 24 samples within the common sample set of patient and healthy control

samples, together with the provided controls in each plate, as indicated in the user guides. Similar to the 5-mC detection, DNA dilutions were prepared, and a total of 100 ng of DNA was loaded on 96-well plates in triplicates, and prior to the strategy shown in Figure 4.4, the intra-plate consistency was also analyzed by looking at the SD and CV between the triplicate readings within each of the plates using MS Excel.

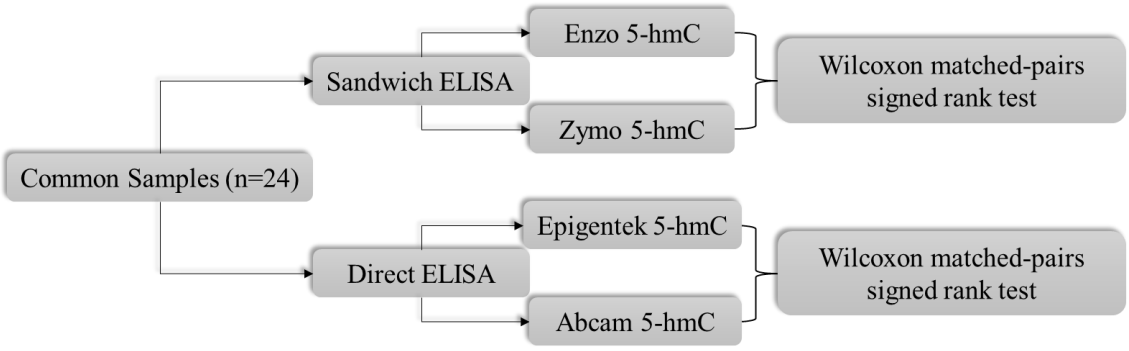

Figure 4.4. The 5-mC ELISA kit selection strategy.

The measurement of colorimetric (at 405 nm, 430 nm, and 450 nm; after 15 min, 35 min, and 55 min) and fluorometric absorbance (excitation at 530 nm, emission at 590 nm) were performed exactly as described before.

4.4. Global Quantification of 5-mC Levels

The experimental and the data analysis stages of global 5-mC percentage detection in different patient groups and healthy controls using the Enzo 5-mC kit are explained in the following sections.

4.4.1. Detection of 5-mC in Patients and Healthy Controls

Detection of the 5-mC levels in different patient groups and healthy controls was performed as described above for global 5-mC kit selection with minor changes. A total of 100 ng patient and healthy control DNA samples were prepared as described previously, and were loaded on 96-well plates in triplicates. Non- and fully-methylated controls were mixed in such a way that the final 5-mC percentages would be between 0 and 10%, rather than 0

and 100% as suggested in the user guide provided by the manufacturer. This was done to confine the standard curve to a smaller interval to increase the accuracy and precision within this region and make it more suitable for human DNA samples considering the average global 5-mC percentages expected.

After the addition of the HRP developer solution, colorimetric absorbance was measured at 405 nm, 430 nm and 450 nm after 15, 35 and 55 minutes. The detected values with the best standard curve were exported to MS Excel files via SoftMax Pro v5.

4.4.2. Data Analysis and Statistics

The calculations needed for quantifying 5-mC levels were carried out according to manufacturer's instructions. The absorbance of the standard samples was used to calculate the slope and the y-intercept of the standard curve using the SLOPE and INTERCEPT functions of MS Excel. Later, the equation below (Equation 4.1), derived from the logarithmic second-order regression equation for the standard curve, was used to determine the 5-mC % for patient and healthy control samples.

$$5mC\ \% = e^{\left\{\frac{absorbance\ -\ y-intercept}{slope}\right\}} \tag{4.1}$$

Quade's rank analysis of covariance (Quade, 2012) was used to compare the global 5-mC levels in sALS, C9orf72+ ALS, SCA1, SCA2, HD, FA and DM1 patients against age- and sex-matched healthy controls using IBM® SPSS® Statistics (Version 22) software. Considering the clinical information available for the patient and healthy control samples and the possible role of different environmental factors in altering global levels of 5mC, Quade's rank analysis of covariance was performed with smoking (Armon, 2009; de Jong *et al*., 2012; Wang *et al*., 2011), alcohol consumption (de Jong *et al*., 2012; E Yu *et al*., 2016) and physical activity (Huisman *et al*., 2013; Mattsson *et al*., 2012; Zhang *et al*., 2011) as covariates, global levels of 5-mC as the dependent variable and the phenotype as the grouping factor. This allows for correcting any biases towards variations that might arise due to these factors. Apart from these covariates, the plate number was also considered as a covariate to account for any inter-plate variation that may have an effect on the results. This

was checked by performing a paired one-way analysis of variance (ANOVA), using GraphPad Prism® 6 software on the standard control samples present on each plate. Such statistical tests are extremely important as each experiment is performed on different days, and variations due to experimental errors could drastically change the results.

For patient samples which lacked information regarding the covariates mentioned, a Mann-Whitney U test was performed in comparison to age- and sex-matched healthy control samples using GraphPad Prism® 6 software.

The significant results for each statistical test are displayed as p-values and asterisk within box plots, graphs and tables (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

4.5. Quantification of *C9orf72* Promoter Methylation and Correlation with Global DNA Methylation

In order to determine the region-specific methylation pattern at the CpG islands, located in the *C9orf72* gene promoter, bisulfite conversion and Sanger sequencing techniques were used, as explained in the following sections.

4.5.1. Direct Bisulfite Sequencing

For the bisulfite conversion reaction, the EZ DNA Methylation Kit from Zymo Research was used according to the manufacturer's instructions. Four hundred ng of C9orf72+ ALS patient samples, age- and sex-matched healthy control samples and commercially available known non- (0%) and fully-methylated (100%) control human DNA samples (as negative and positive controls, respectively) were used for this experiment.

Following the bisulfite conversion of unmethylated cytosines (C) into thymines (T), 30 ng of the DNA yield from this reaction was further utilized in the targeted amplification of the CpG island within the *C9orf72* gene promoter. A nested PCR methodology with two consecutive reactions was utilized in the amplification of this region; 30 ng of the converted

DNA was used as template for the first PCR, and 1/10 of this initial reaction was used as template in the following nested-PCR. Contents of the master mixes for both reactions are shown in Tables 4.1 and 4.2.

Table 4.1. Content of the first PCR mix for *C9orf72* promoter amplification.

Reagent	**Volume (µl)**	**[Final]**
Template / Control DNA	Variable	0.6 ng/µl
Standard Taq Reaction Buffer (10X)	5	1X
dNTPs (10 mM each)	1	200 µM
Forward Primer (BSP_1F, 10 µM)	1	0.2 µM
Reverse Primer (BSP_1R, 10 µM)	1	0.2 µM
Hot Start Taq DNA Polymerase	0.25	
ddH20	to 50 µl	
Total volume	50 µl	

Table 4.2. Content of the nested-PCR mix for *C9orf72* promoter amplification.

Reagent	**Volume (µl)**	**[Final]**
Template / Control DNA	5	
Standard Taq Reaction Buffer (10X)	5	1X
dNTPs (10 mM each)	1	200 µM
Forward Primer (BSP_2F, 10 µM)	1	0.2 µM
Reverse Primer (BSP_2R, 10 µM)	1	0.2 µM
Hot Start Taq DNA Polymerase	0.25	
ddH20	to 50 µl	
Total volume	50 µl	

The PCR cycling conditions for both of the PCR reactions performed after the bisulfite conversion and prior to sequencing are shown in Tables 4.3 and 4.4 for the first and second PCR reactions, respectively.

Table 4.3. The conditions for the first PCR reaction, for the amplification of the *C9orf72* promoter.

Initial Denaturation	95º C	4 min	
Denaturation	95º C	4 min	10 cycles
Annealing	68º C *	30 sec	
Extension	72º C	3 min	

Table 4.3. The conditions for the first PCR reaction, for the amplification of the *C9orf72* promoter (cont.).

Denaturation	95° C	4 min	30 cycles
Annealing	58° C	30 sec	
Extension	72° C	3 min	
Final Extension	72° C	7 min	
*** 1° C decrease at each cycle (68° C → 58° C)**			

Table 4.4. Nested-PCR conditions for the *C9orf72* promoter amplification.

Initial Denaturation	95° C	4 min	
Denaturation	95° C	4 min	10 cycles
Annealing	67° C*	30 sec	
Extension	72° C	3 min	
Denaturation	95° C	4 min	20 cycles
Annealing	57° C	30 sec	
Extension	72° C	3 min	
Final Extension	72° C	7 min	
***1° C decrease at each cycle (67 °C → 57 °C)**			

After the amplification of *C9orf72* promoter CpG island region, all the patient samples together with standard controls were subjected to Sanger sequencing at Istituto Auxologico Italiano Sequencing Core Facility. Results were evaluated using the CLC Main Workbench Software. At the positions of expected Cytosines (C); presence of a single Thymine (T) peak was considered as an indicative of a "non-methylated" state. In the case of a double C/T peaks at these positions, the results were grouped into three; higher T peaks were considered "low", equal peaks were considered "intermediate", and higher C peaks were considered to depict "high" methylation states at these positions.

4.5.2. BST-PCR Data Analysis and Correlation Statistics

Four categories were defined to assess the methylation levels qualitatively at each of the 26 CpG sites in the *C9orf72* promoter, as mentioned in the previous section. The percentage of the promoter methylation was later calculated by assigning each of these states

with a score (high: 3, intermediate: 2, low: 1 and unmethylated: 0). The total score was later used to calculate the promoter methylation percentage using the equation below (Equation 4.2).

$$promoter\ methylation\ \% = \left(\frac{total\ score}{26 \times 3}\right) \times 100 \quad (4.2)$$

In order to investigate a possible correlation between the *C9orf72* promoter methylation and the global methylation in C9orf72+ ALS patients, Spearman correlation statistics was applied using GraphPad Prism® 6 software.

5. RESULTS

In the following sections the results obtained from extensive testing of commercially available 5-mC and 5-hmC DNA ELISA kits, and the global 5-mC levels for the NDAL NDD cohort are presented. The global 5-mC levels are reported for 422 patient samples and 145 healthy controls using the Enzo 5-mC kit chosen due to its higher precision and accuracy (Table 5.1). Figure 5.1 highlights (shaded grey) the patients and disease groups which showed elevated levels of global 5-mC when compared to age- and sex-matched healthy controls. Finally, results for 34 C9orf72+ ALS patients regarding their *C9orf72* promoter methylation using direct bisulfite sequencing and its correlation with global 5-mC levels are also reported.

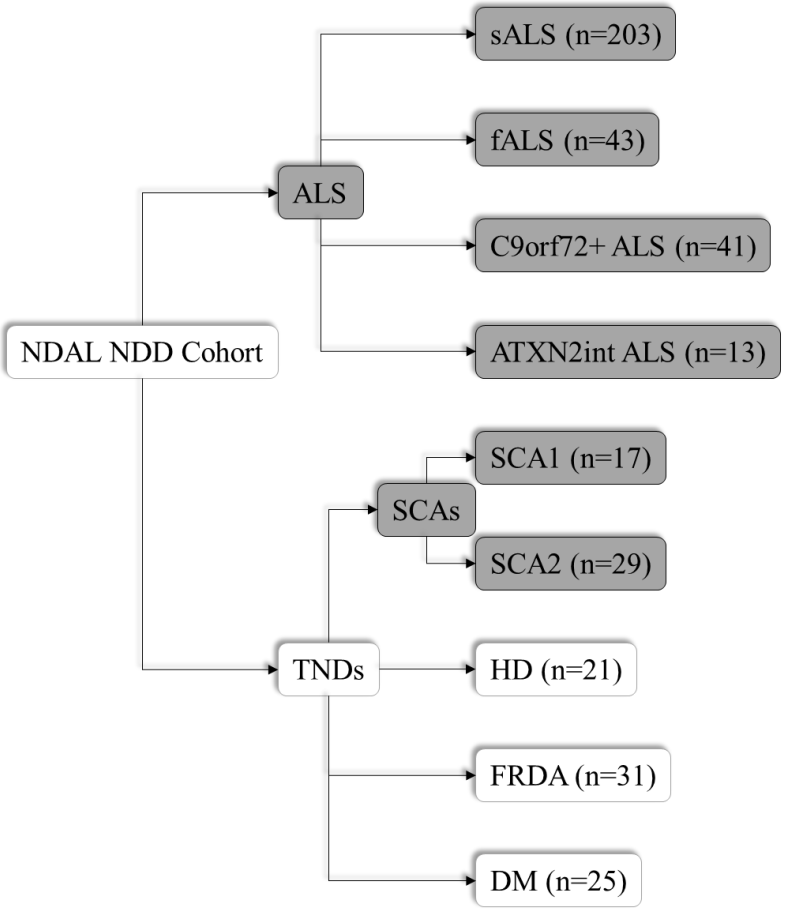

Figure 5.1. The NDD cohort investigated in the framework of this thesis.

5.1. 5-mC and 5-hmC ELISA Kit Selection

In order to select the most reliable commercially available ELISA kit for the quantification of both 5-mC and 5-hmC, a series of tests were conducted on four different 5-mC and four different 5-hmC kits. These tests were conducted using 22 and 24 samples within a common sample set, meaning that identical samples were used across all the plates, for the 5-mC and 5-hmC kits, respectively. These tests aimed to determine both the intra-plate variability and the variability between the kits which utilized a similar working principle with respect to their ELISA setup. This helped ensure more consistent and reproducible results which is of great importance in such high-throughput which are sensitive to even very small errors.

5.1.1. Comparison of 5-mC ELISA Kits

The four 5-mC DNA ELISA kits tested in the framework of this thesis could be classified into two groups with respect to their working principle, whether it be direct or indirect (Figure 4.2 and Figure 4.3). First, in order to determine if each kit had consistent intra-plate readings, the common sample set (n=22) was processed in a triplicate manner in each plate, and the SD and CV were calculated for each of the samples within the common sample set, along with the standard samples provided with each kit. The average SD and CV for each 5-mC kit is reported in Table 5.1 and the kit with the lowest SD and CV, chosen for this thesis (Enzo 5-mC), is shaded in grey. Secondly, in order to analyze the variability between the kits with the same working principle, whether it be direct or indirect, the results for 22 samples within the common sample set were compared by applying the Wilcoxon matched-pairs signed rank test (Figure 4.3). The indirect ELISA system utilized by the Enzo and Zymo 5-mC kits were found to successfully replicate the results for the samples within the common sample set ($Z = 61$, $p > 0.1$). On the other hand, the direct ELISA system utilized by the two other 5-mC kits from Epigentek and Abcam did not give consistent results and both showed a significant difference for the samples within the common sample set ($Z = 237$, $p < 0.0001$). This, along with higher SD and CV values calculated for these two kits, reduces their confidence and reliability.

Table 5.1. The working principle, SD and CV of the intra-plate triplicate readings for the 5-mC ELISA kits. The most consistent kit has been shaded gray as it showed the lowest SD and CV for the triplicate intra-plate readings.

Kit / Manufacturer	Working Principle	Average SD for Triplicate Readings	Average CV for Triplicate Readings
Enzo 5mC	Indirect (colorimetric)	0.003	0.0177
Zymo 5mC	Indirect (colorimetric)	0.003	0.04703
Epigentek 5mC	Direct (colorimetric)	0.052	0.10162
Abcam 5mC	Direct (fluorometric)	0.737	0.0695

5.1.2. Comparison of 5-hmC ELISA Kits

The four 5-hmC ELISA kits tested in the framework of this thesis could be classified into two groups with respect to their working principle; sandwich and direct (Figure 4.2 and Figure 4.4). The common sample set (n=24) was used in order to test these kits exactly as described for the 5-mC DNA ELISA kits (Table 5.2). After testing all four kits, none was found to demonstrate reliable SD and CV due to the high variability in the intra-plate triplicate readings. Also the fact that the kits had a limited range of detection and that all the quantifications were at the lower limit of the kits, led to a concern regarding the performance and the sensitivity of the kit and thus none was chosen for further assays for the quantification of 5-hmC within the NDD cohort. Nevertheless, to check whether such inconsistent intra-plate readings were to be acceptable and if they could somehow be tolerated, the inter-plate variability between the kits that utilized the same working principle were also analyzed. To do so, similar to the approach used for the 5-mC kits, the results for the samples within the common sample set were compared. The sandwich ELISA systems utilized by the Enzo and Zymo 5-hmC kits were found to give highly inconsistent results for the 24 samples within the common sample set ($Z = 300$, $p < 0.0001$), and this was also the case for the direct ELISA system utilized by the two other 5-hmC kits from Epigentek and Abcam ($Z = 300$, $p < 0.0001$). Such high variability in the results produced make these kits unsuitable for the quantification of 5-hmC in DNA isolated from blood.

Table 5.2. The working principle, SD and CV of the intra-plate triplicate readings for the 5-hmC ELISA kits. None of the kits tested gave consistent results for the triplicate intra-plate reading.

Kit / Manufacturer	Working Principle	Average SD for Triplicate Readings	Average CV for Triplicate Readings
Enzo 5-hmC	Sandwich (colorimetric)	0.004	0.21995
Zymo 5-hmC	Sandwich (colorimetric)	0.020	0.057
Epigentek 5-hmC	Direct (colorimetric)	0.007	0.12629
Abcam 5-hmC	Direct (fluorometric)	0.077	0.08861

5.2. 5-mC Levels in Patient and Healthy Control Samples

The global 5-mC levels reported in this thesis have been generated using the Enzo 5-mC kit on 26 different plates. Figure 5.1 shows the mean and the standard error margin for the standard controls (n=5) used across all 26 plates. The results indicate that there is no significant difference at the $p > 0.1$ [$F(1.225, 4.898) = 0.1411$, $p = 0.7714$] between the 26 different plates used in this study considering the results obtained from each plate for the 5 standard controls provided with each kit. This is crucial for reliable and reproducible results for such high-throughput studies.

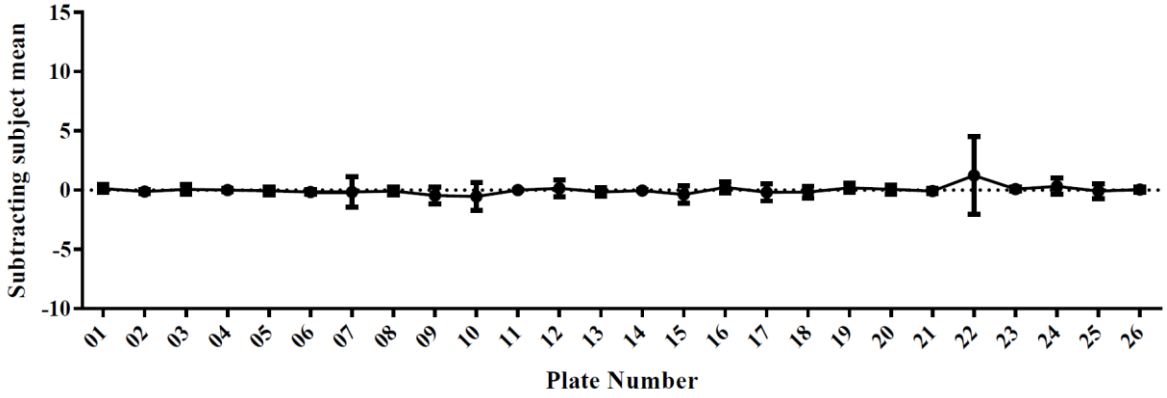

Figure 5.2. The mean and standard error margins for the standard controls used across the different ELISA plates.

5.2.1. Global 5-mC Levels in ALS and Healthy Controls

The global 5-mC levels in ALS patients were compared to age- and sex-matched healthy controls using two strategies: (i) without controlling for external factors for samples lacking supplementary clinical information, such as lifestyle choices which may have an effect on the global 5-mC levels, using the Mann-Whitney U test (Figure 5.3A and Table 5.3), and (ii) using Quade's rank analysis of covariance and by considering external factors such as smoking, alcohol consumption and physical activity for samples with such information available (Figure 5.3B and Table 5.4).

These analyses show that all subtypes of ALS exhibit increased levels of global 5-mC when compared to age- and sex-matched healthy controls both in the presence and absence of the three external factors mentioned. Figure 5.3 illustrates the global 5-mC levels in all different subtypes of ALS along with the corresponding age- and sex-matched healthy controls investigated within the framework of this thesis. Each box plot in Figure 5.3 shows the minimum and the maximum levels of 5-mC observed in each group, along with the median, 25th and 75th percentile. Table 5.3 and Table 5.4 list detailed information regarding the statistical tests conducted for the global 5-mC results obtained.

Table 5.3. Comparison of the global levels of 5-mC in different subtypes of ALS patients lacking information regarding smoking, alcohol consumption and physical activity.

Disease	Significant Increase	Patient Median	Control Median	Mann-Whitney U	p-value
sALS (n=95)	Yes	18.54	15.27	2644	< 0.0001
C9orf72+ ALS (n=20)	Yes	24.67	14.21	50	< 0.0001
fALS (n=43)	Yes	21.8	14.85	347.5	< 0.0001
ATXN2int ALS (n=13)	No	16.8	15.49	78	> 0.1

Table 5.4. Comparison of the global levels of 5-mC in different subtypes of ALS patient cohorts when compared to age- and sex-matched healthy controls, correcting for smoking, alcohol consumption and physical activity.

Disease	Significant Increase	p-value
sALS	Yes	$p < 0.001$ [$F(1, 214) = 12.096$, $p = 0.000645$]
C9orf72+ ALS	Yes	$p < 0.001$ [$F(1, 40) = 16.539$, $p = 0.000217$]

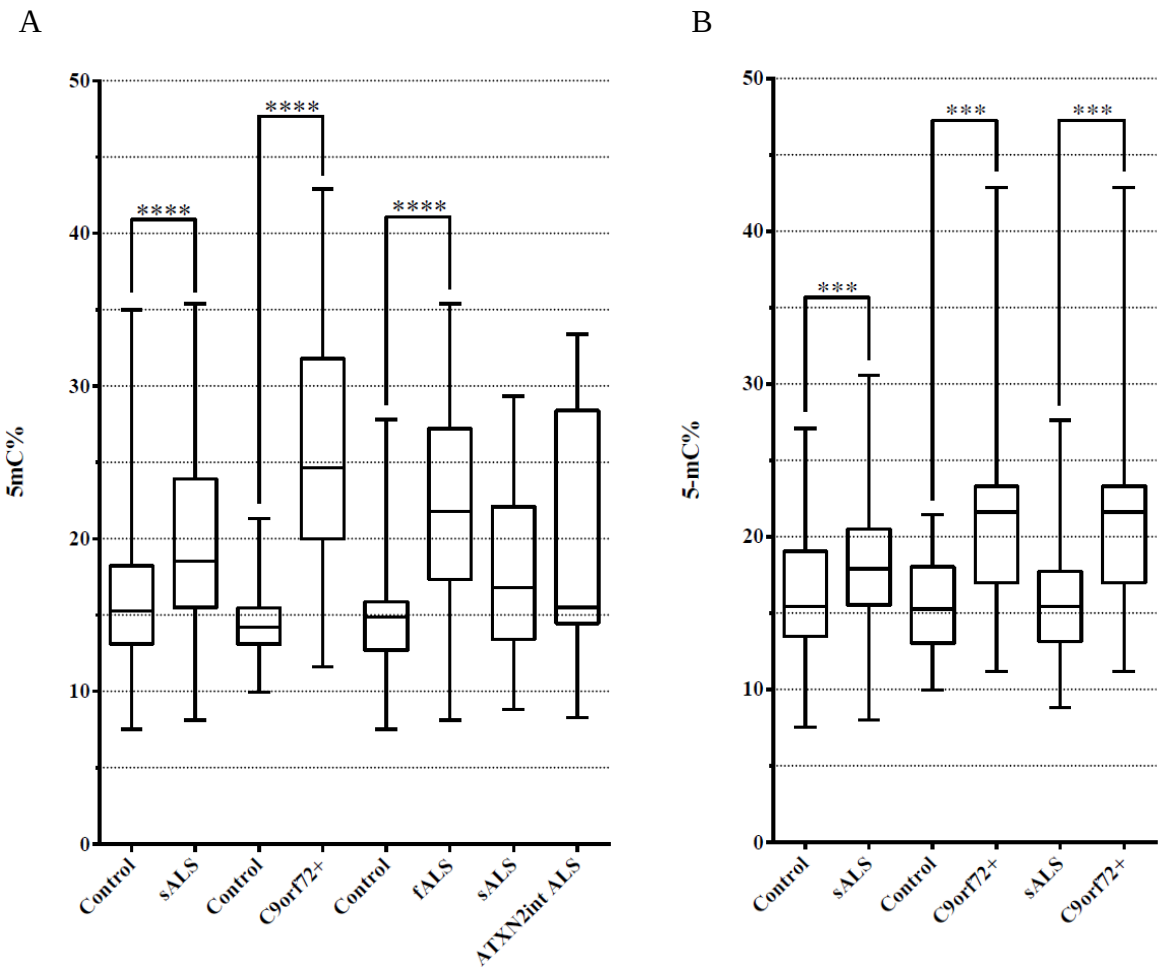

Figure 5.3. Box plots for the global levels of 5-mC in patients and age- and sex-matched healthy controls. A) Mann-Whitney U test (no covariates) and B) Quade's rank analysis of covariance (including covariates). Significant changes are marked with asterisk.

C9orf72+ ALS patients were also compared to age- and sex-matched sALS patients while controlling for age of onset, disease duration at sample collection, smoking, alcohol consumption and physical activity, and a significant increase in the global 5-mC levels was observed at the $p < 0.001$ [$F(1, 40) = 12.962$, $p = 0.000867$] (Figure 5.3B).

Furthermore, a limited number of ATXN2int ALS (n=13) patients were also compared to sALS patients using the Mann-Whitney U test, since information regarding external factors that could be taken into consideration were not available. No significant increase could be observed for the global levels of 5-mC in ATXN2int ALS vs. sALS (Figure 5.3A and Table 5.3).

5.2.2. *C9orf72* Promoter Methylation and its Correlation with Global 5-mC Levels

The promoter region of the *C9orf72* gene, subjected to bisulfite conversion and sequencing was quantified for the 26 CpG islands, and the methylation percentage of the promoter was calculated for 34 C9orf72+ ALS patient samples. Figure 5.4A shows the moderate correlation (r_s = 0.3902, $p < 0.05$) between the absolute global 5-mC levels and the *C9orf72* promoter methylation calculated using Spearman's correlation analysis in C9orf72+ ALS patients, while Figure 5.4B shows the moderate correlation (r_s = 0.3466, $p < 0.05$) between the increase in the global 5-mC levels, with respect to age- and sex-matched healthy controls, and the *C9orf72* promoter methylation in the same cohort.

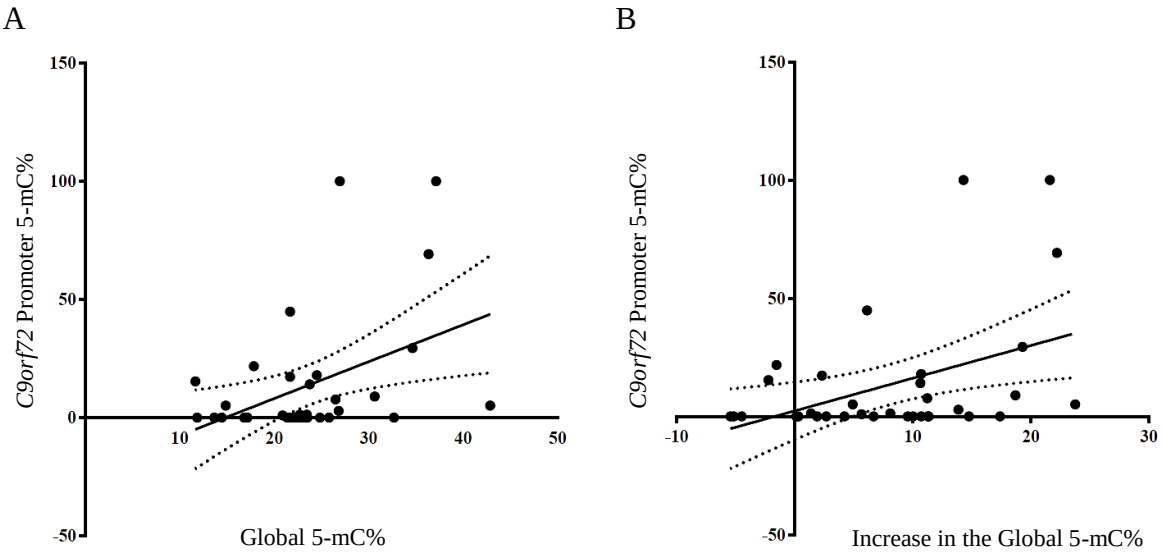

Figure 5.4. Correlation of the global 5-mC levels and promoter methylation in C9orf72+ ALS patients.

5.2.3. Global 5-mC Levels in Trinucleotide Repeat Disorders

Similar to ALS, the TNDs investigated in this thesis have been compared with age- and sex-matched healthy controls while controlling for smoking, alcohol consumption and physical activity using Quade's rank analysis of covariance (Figure 5.5 and Table 5.5). Each box plot shows the minimum and the maximum levels of 5-mC observed in each group, along with the median, 25th and 75th percentile. Table 5.5 lists detailed information regarding the statistical tests conducted for the global 5-mC results obtained for this cohort.

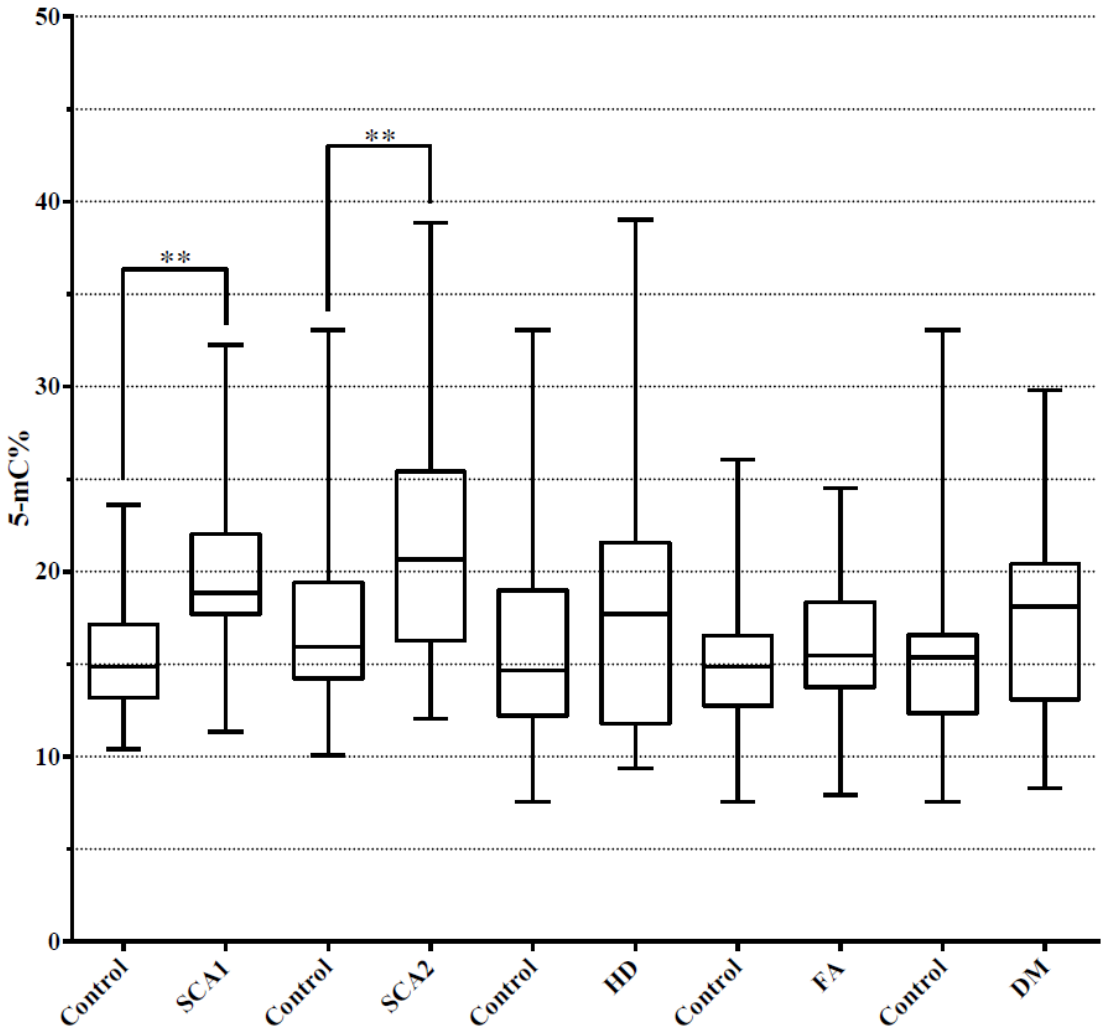

Figure 5.5. Box plots for the global 5-mC levels in different groups of patients (SCA1, SCA2, HD, FA, and DM1) along with age- and sex-matched healthy controls. Significant changes are marked with asterisk.

Table 5.5. Results of the comparison of 5-mC levels in TNDs with respect to age- and sex-matched healthy controls, with smoking, physical activity and alcohol consumption as covariates.

Disease	Significant Increase	p-value
SCA1	Yes	$p < 0.01$ [$F(1, 32) = 8.778$, $p = 0.00571$]
SCA2	Yes	$p < 0.01$ [$F(1, 56) = 10.784$, $p = 0.001768$]
HD	No	$p > 0.1$ [$F(1, 40) = 2.495$, $p = 0.122095$]
FRDA	No	$p > 0.1$ [$F(1, 60) = 0.803$, $p = 0.373799$]
DM1	No	$p > 0.1$ [$F(1, 48) = 0.400$, $p = 0.530261$]

5.3. Effects of External Factors on Global 5-mC Levels

Many different external factors which might have an effect on global 5-mC levels, such as smoking, physical activity, alcohol consumption, and other disease-related factors, including age of onset and disease duration have been taken into consideration within the framework of this thesis. In order to assess the impact of these factors, Quade's rank analysis of covariance was carried out for each covariate within a suitable cohort and by including other factors. The results of these analyses are compiled in Table 5.6. Interestingly, alcohol consumption was found to be a statistically significant modifier of the global 5-mC levels in healthy controls at the $p < 0.001$ [$F(2, 138) = 8.981$, $p = 0.000215$].

Table 5.6. The effect of different external factors on the global levels of 5-mC.

	Cohort	Groups	Controlling Covariates	Sig.	Statistics
Age of Onset	sALS	< 40 , 41 – 60, 61 <	Blood Age, disease duration, sex, smoking, alcohol, physical activity, site of onset, consanguinity, sALS/fALS	No	$p > 0.1$ [$F(2, 122) = 0.123$, $p = 0.885$]
Disease Duration	sALS	0 – 5, 6 – 10, 11 – 20, 21 <	AO, blood age, sex, smoking, alcohol, physical activity, site of onset, consanguinity, sALS/fALS	No	$p > 0.1$ [$F(3, 122) = 0.176$, $p = 0.913$]
Alcohol Consumption	sALS	Yes, No, Quit	Blood age, disease duration, sex, smoking, physical activity	No	$p > 0.1$ [$F(2, 133) = 1.182$, $p = 0.310$]
Smoking	sALS	Yes, No, Quit	Blood age, disease duration, sex, alcohol, physical activity	No	$p > 0.1$ [$F(2, 133) = 1.189$, $p = 0.308$]
Physical Activity	sALS	Yes, No, Quit	Blood age, disease duration, sex, alcohol, smoking	No	$p > 0.1$ [$F(2, 133) = 1.168$, $p = 0.314$]
Age at Blood Collection	Healthy	< 40, 41 – 60, 61 <	Sex, smoking, alcohol, physical activity	No	$p > 0.1$ [$F(2, 138) = 0.666$, $p = 0.666$]
Alcohol Consumption	Healthy	Yes, No, Quit	Sex, smoking, age at blood collection, physical activity	Yes	$p < 0.001$ [$F(2, 138) = 8.981$, $p = 0.000215$]
Smoking	Healthy	Yes, No, Quit	Sex, alcohol consumption, Age at blood collection, physical activity	No	$p > 0.1$ [$F(2, 138) = 1.421$, $p = 0.245$]
Physical Activity	Healthy	Yes, No, Quit	Sex, smoking, age at blood collection, alcohol consumption	No	$p > 0.1$ [$F(2, 138) = 1.862$, $p = 0.159$]

6. DISCUSSION

The results presented in this thesis indicate a significant elevation in global 5-mC levels in sALS patients when compared to age- and sex-matched healthy controls. Such findings are in line with previous studies which have reported an increase in the global levels of 5-mC, independently of age of onset, in DNA isolated from blood and spinal cord samples of sALS patients (Figueroa-Romero *et al*., 2012; Tremolizzo *et al*., 2014). This work also takes a step further and concludes disputes between Figueroa-Romero *et al*., 2012 and Tremolizzo *et al*., 2014, regarding the detection of elevated global 5-mC levels in blood, by screening a large cohort of sALS patients using a high-throughput ELISA-based 5-mC assay. More importantly, elevated levels of global 5-mC are also reported for the first time in different subtypes of ALS, including fALS and C9orf72+ ALS, along with two other NDDs, SCA1 and SCA2.

6.1. Epigenetics in Understanding NDDs

NDDs are a group of highly heterogeneous and complex disorders with many overlapping features and phenotypes, making their diagnosis and treatment extremely difficult, especially due to the lack of competent biomarkers and potent treatments. The past decade has seen an unprecedented increase in our knowledge regarding the pathogenic nature of several NDDs at the cellular and molecular levels: various impaired cellular mechanisms have been shown to trigger disease onset and progression. Such efforts have helped scientists gain a general overview of the pathogenic mechanisms responsible for neurodegeneration and will certainly fuel future studies in hopes of elucidating the exact causes of different NDDs, paving the ways for potent therapeutic approaches that aim to cure the disease rather than simply treating the devastating symptoms. Aging is the number one risk factor for many NDDs and thus, extensive research is of extreme importance in the upcoming years as the ever aging societies of today's world come to the brink of huge ways of different NDDs which will undoubtedly impose a great burden both on governments, society and mankind in general. Considering the complexity of ALS, the third most common NDD after AD and PD (Özoğuz *et al*., 2015; Renton *et al*., 2014), and the fact that more

than 80% of patients diagnosed with ALS have no clear known cause for the disease (He *et al*., 2015; Kenna *et al*., 2016; van Rheenen *et al*., 2016), the need for new approaches and perspectives in attempts to decipher the yet undiscovered underlying factors governing the pathogenesis of this fatal disease is evident. As for other monogenic NDDs, such as SCA1, SCA2, HD and FRDA along with DM1, the largely variable phenotypes and the lack of potent treatments point to a need for more extensive and comprehensive studies that take a closer look at such phenomena from different angles, in hope of new ideas and strategies that could be utilized to tackle such incurable diseases.

Epigenetics, being a relatively new field in biological sciences, has just started to gain momentum in helping to understand some of the previously baffling aspects of various different diseases and conditions. The post-genomic era has admittedly led scientists to believe that the complexity of the spatiotemporal fine-tuning of cellular mechanisms via the regulation of gene expression cannot be explained solely by studying the genome, and that many other factors can effect these mechanisms at other regulatory levels. Each such modifying factor can in principle play a substantial role in explaining the phenotypic differences between individuals. This is largely apparent in the phenotypic differences seen in monogenic disorders and maybe even more so in monozygotic twins, who are discordant for certain phenotypes and diseases. The complexity and diversity of epigenetic factors and modifications, and the error-prone nature of many of the current technologies available for conducting such studies, motivated this work to take a global look at one of the most well-known and extensively studied epigenetic modifications, namely 5-mC. This approach promises to be a good starting point in dissecting the role of epigenetic marks and modifications in the pathogenesis of complex disorders, as it provides a top-down approach prior to higher resolution gene-specific and genome-wide studies. Furthermore, it has already been shown that global variations in DNA methylation can be indicative of gene-specific promoter 5-mC levels (Ohka *et al*., 2011).

Our findings call for further high-resolution studies with the additional analysis of different tissue types and presymptomatic patients using suitable assays. This should be followed by more in-depth studies at the molecular level in order to understand the epigenetic dynamics of these diseases. The correlation of the promoter hypermethylation of

the *C9orf72* gene with the global levels of 5-mC also call for the analysis of genes and maybe also the entire genome. This will help clarify the importance of such correlations and maybe even point toward other mechanism that may be implicated in the pathogenies of ALS.

6.2. Global DNA Methylation in NDDs

Previous studies on global 5-mC levels in ALS patients have had many shortcomings, such as small, limited cohorts and biases towards one or more disease-specific phenotypes. This led us to investigate global 5-mC levels in blood within a Turkish ALS cohort. Here, these limitations have been overcome by screening a large and heterogonous cohort of ALS patients which were classified into different subtypes using molecular screening techniques ranging from conventional methods such as GeneScan and Sanger sequencing to NGS including WES and WGS. External and environmental factors, such as smoking, alcohol consumption and physical activity that have been previously suggested to have an effect on global 5-mC levels were also taken into consideration to limit the possible effects that they may have on the results. Other than ALS, we were also keen to find out if the global 5-mC levels also changed in other neurodegenerative diseases. In order to do so we chose to investigate SCA1, SCA2, HD and FRDA, along with DM1, for which to the best of our knowledge, there has not been any global studies of DNA methylation.

In order to investigate the global levels of 5-mC and also 5-hmC, which is a relatively newly identified cytosine modification, commercially available DNA ELISA kits were extensively tested prior to use. This was mainly due to their ease of use, low cost and acceptable precision when compared to the gold standard that are HPLC-based assays (Knothe *et al*., 2016). Two different ELISA setups from four manufacturers were tested for the detection of each of the cytosine modifications, namely 5-mC and 5-hmC, in order to select the best available high-throughput DNA ELISA system for their quantification. The results obtained from the kit selection procedure prove this step to be important prior to high-throughput studies, such as the one conducted within the framework of this thesis. The results obtained here regarding the kit comparison clearly indicate that both the manufacturer and the ELISA setup along with other factors such as kit storage, handling, delivery conditions and standards of local distributers, are all important aspects that could affect the

final results. These factors were even more evident when testing the Zymo 5-hmC kit as we could only get readings at our third try, having later realized that the previous failures were due to improper antibody storage.

Interestingly, the kit selection procedure also points to a possible reason for the inconsistencies between Tremolizzo *et al*., 2014 and Figueroa-Romero *et al*., 2012 regarding the detection of elevated 5-mC levels in DNA isolated from blood. Figueroa-Romero *et al*., 2012, reported to use the Epigentek 5-mC kit and observed no elevated levels of global 5-mC in blood samples of a small cohort of only 11 sALS patients. When compared to the other kits, the direct ELISA system utilized by the Epigentek 5-mC kit and also the Abcam 5-mC kit which utilizes a similar ELISA setup, failed to produce consistent results. Although one can argue that these kits use different detection methods (colorimetric vs. fluorometric), at least some correlation in their results was expected. Furthermore, the correlation of the results obtained within the framework of this thesis and the study of Tremolizzo *et al*., 2014, which used an HPLC-based assay that is widely accepted as the gold standard for the quantification of 5-mC (Knothe *et al*., 2016), adds more confidence to this approach when compared to the direct ELISA system used by Figueroa-Romero *et al*., 2012. All such findings underline the importance of vigorous testing regimes both on experimental approaches developed in-house and those acquired from third-party companies. The results of the global 5-mC assays in this thesis clearly indicate that the increase in the global 5-mC levels is not exclusive to the CNS in ALS, as was previously reported by Figueroa-Romero *et al*., 2012, and that it presents itself also in blood. Similar to the approach for finding the best available kit for the quantification of 5-mC, four different DNA ELISA kits for the quantification of 5-hmC were tested. The results indicated that none of the kits was able to effectively quantify the very low levels of 5-hmC in blood, and suggest that it should not be utilized for the quantification of 5-hmC in blood, practiced by Figueroa-Romero *et al*., 2012.

6.3. The Epigenetic Link between ALS, SCA1 and SCA2

The results of the 5-mC DNA ELISA assays conducted in ALS and a series of other diseases show that elevated global 5-mC is not only observable in all subtypes of ALS, but also seen in SCA1 and SCA2, but not in HD, FRDA or DM1. This is especially interesting

as the causative genes for both SCA1 and SCA2 are the most relevant to ALS pathology among the NDDs studied here, and also because they share a few phenotypic features, shown in Table 6.1. Further information regarding the diseases examined in the framework of this thesis, such as the affected regions and subsequent lesions, mean age of onset, life expectancy and prevalence can be found in Table 6.1 and Table 6.2 (Bidichandani and Delatycki, 2014; Bird, 2015; Kanehisa *et al*., 2014; Kinsley and Siddique, 2015; Orsini *et al*., 2015; Özoğuz *et al*., 2015; Pagon *et al*., 2015; Parkinson *et al*., 2013; Subramony and Ashizawa, 2014; Theadom *et al*., 2014; Warby *et al*., 2014; Whaley *et al*., 2011)

Table 6.1. General features of the diseases which show elevated global 5-mC levels. The affected regions common for ALS, SCA1 and SCA2 are underlined.

	ALS	**SCA1**	**SCA2**
Affected region	motor cortex (frontal lobe), brain stem, spinal cord	cerebellum, dentate nucleus, brain stem	cerebellum, brain stem, frontal lobe, temporal lobe
Microscopic lesion	hyaline inclusions	neuronal inclusions	neuronal inclusions
Mean age of onset	4th or 6th decade	3rd or 4th decade	3rd or 4th decade
Life expectancy	2-5 years	10-30 years	10-15 years
Prevalence	4-6 in 100,000	1-2 in 100,000	1-2 in 100,000

Table 6.2. General features of the diseases which do not show elevated global 5-mC levels.

	HD	**FRDA**	**DM1**
Affected region	caudate nucleus, globus pallidus, thalamus, hippocampus	spinal cord, peripheral nerves	skeletal and smooth muscle, eyes, heart, testicles, endocrine system, central nervous system
Microscopic lesion	neuronal intranuclear inclusions	-	-
Mean age of onset	4th to 5th decade	2nd to 3rd decade	3rd to 4th decade
Life expectancy	10-20 years	30-40 years	40-50 years
Prevalence	2-3 in 100,000	1-5 in 100,000	5 in 100,000

Ataxin-1, causing SCA1 when mutated, is ubiquitously expressed throughout the body and is mainly localized within the nucleus. It has been suggested to be involved in regulating various aspects of transcription and RNA processing (Irwin *et al*., 2005). Feedback mechanisms involved in the RNA processing pathway can lead to various epigenetic modifications (Holoch and Moazed, 2015), and considering the importance of DNA

methylation in nucleosomal positioning and chromatin remodeling through the activity of MECP2 (Nan *et al*., 1998; H. H. Ng *et al*., 1999), the increased levels of global 5-mC may be the direct result of expanded ataxin-1. Furthermore, ataxin-1 has been shown to physically interact with ataxin-2, expansion mutations of which cause SCA2, despite their distinct subcellular localizations. The mutant form of ataxin-1 also induces intranuclear accumulation of ataxin-2, possibly triggering further stress on the cell. Moreover, in a *Drosophila* model of SCA1, the wild-type *Drosophila* ataxin-2 homolog (*dAtx2)* has been shown to suppress and modify the toxicity of the expanded ataxin-1 (Al-Ramahi *et al*., 2007).

Ataxin-2 has been found to be associated with translating polysomes, and is known to interact with various mRNAs, thus controlling their translation (Satterfield and Pallanck, 2006). Such highly important roles within the cell explain how the expansion mutation within this protein could lead to cellular stress in the form of RNA foci, protein aggregates, altered stress granule (SG) dynamics and proteasomal degradation (Magaña *et al*., 2013; Rüb *et al*., 2013). Disruptions in these pathways, especially in those related to the regulation of protein expression, may explain how expansion mutations within this protein could have an effect on global 5-mC levels during stress conditions. Intermediate length expansion in the polyQ domain of *ATXN2* has previously been linked to ALS as a major risk factor (Elden *et al*., 2010; Lahut *et al*., 2012) and ataxin-2 is known to localize to SGs together with several ALS-associated proteins, namely heterogeneous nuclear ribonucleoproteins (hnRNPs) TDP-43 (Arai *et al*., 2006) and FUS/TLS (Mackenzie *et al*., 2011). All these factors are involved in the assembly and disassembly of the SGs during and after cellular stress conditions (Bosco *et al*., 2010; Dormann *et al*., 2010; Liu-Yesucevitz *et al*., 2010). Ataxin-2 is known to interact with TDP-43 in an RNA-dependent manner, and ataxin-2/TDP-43 complexes were shown to be mislocalized in the spinal cord samples of ALS patients. The intermediate expansions in *ATXN2* are also found to be further enriched in C9orf72+ ALS patients, and may act as a disease modifier making C9orf72+ patients more prone to ALS than to FTD (van Blitterswijk *et al*., 2014). This may be due to reduced SG dissolving rates in the presence of an intermediate-length expansion in ataxin-2, leading to a stronger binding affinity to TDP-43 and FUS, thus preventing their nuclear translocation (Elden *et al*., 2010; Farg *et al*., 2013; Nihei *et al*., 2012). Moreover, intermediate length ataxin-2 expansions when combined with pathological mutations in TDP-43 and FUS, lead to post-translational modifications, such as

hyperphosphorylation and cleavage (Hart *et al*., 2012) along with Golgi fragmentation and activation of the caspase pathway (Farg *et al*., 2013), respectively. These findings are especially interesting as TDP-43 and FUS are also associated with FTD (Ling *et al*., 2013), and differentiating factors such as intermediate length expansions in ataxin-2 could help outline the exact mechanisms leading to each disease. Further links between ataxin-2 and other ALS-associated genes have also been shown as in the case of Profilin-1 (Figley *et al*., 2014).

The findings presented here along with some similarities shown in Table 6.1 can be indicative of a similar or partially overlapping pathological mechanism for ALS, SCA1 and SCA2. Such overlapping mechanisms may include an epigenetic feedback system where by a global increase in 5-mC levels and overall transcriptional repression, the cell tries to reduce stress caused by toxic RNA foci or toxic protein aggregates, such as in the case of C9orf72+ ALS and SCA1 and SCA2, respectively. This scenario may be further plausible when local promoter methylation is not sufficient in reducing cellular stress or when reduced transcription as a result of promoter hypermethylation (DeJesus-Hernandez *et al*., 2011; Laffita-Mesa *et al*., 2012; C. W. Ng *et al*., 2013; Renton *et al*., 2011; Wood, 2013) leads to loss-of-function as seen in some repeat expansion disorders such as C9orf72+ ALS (Belzil *et al*., 2013; Russ *et al*., 2015; Xi *et al*., 2013). Other possible scenarios include impairment in the proteasomal degradation pathway due to toxic protein aggregates, which in-turn will activate the unfolded protein response and reduce transcriptional levels which may be achieved via epigenetic modifications such as DNA methylation. At the end stage, when the cell is no longer capable of handling the various forms of stress mentioned, the autophagy response is activated which is known to signal global transcriptional repression, and this too can cause changes in global 5-mC levels.

6.4. *C9orf72* Promoter Hypermethylation and its Correlation with Global 5-mC

Apart from global DNA methylation studies, the promoter region of the *C9orf72* gene has been under intensive study in order to investigate the possible modifying effects of promoter methylation in C9orf72+ ALS patients (Belzil *et al*., 2014; Russ *et al*., 2015; Xi, Rainero, *et al*., 2014; Xi *et al*., 2013). Hypermethylation of the *C9orf72* gene promoter has

been shown to be quite frequent in C9orf72+ ALS patients, and it has been suggested that larger hexanucleotide expansions are correlated with promoter hypermethylation, supporting a loss-of-function hypothesis for the disease (I Gijselinck *et al*., 2015). This idea, although plausible, cannot entirely explain the pathogenic nature of the *C9orf72* expansion as other reports have shown convincing evidence supporting a toxic gain-of-function for the expanded *C9orf72* in the form of dipeptide repeat proteins (DRPs) (Yamakawa *et al*., 2015) which arise due to RAN translation (Cleary and Ranum, 2014), and also due to repeat-containing RNA-induced toxicity as a result of RNA foci (DeJesus-Hernandez *et al*., 2011). A more holistic study conducted on differentially methylated promoter regions in a genome-wide manner using a limited number of post mortem brain and spinal cord samples of sALS patients has also helped to gain a general idea about some of the most important pathways related to cell death and immune response (Figueroa-Romero *et al*., 2012). Other important cellular pathways related to DNA methylation and the regulatory machinery which takes part in the maintenance of DNA methylation have also been studied, and dysregulations in these mechanisms have been reported in ALS (Martin and Wong, 2013). Others have also reported that increased levels of DNA methylation are lethal for motor neurons (Chestnut *et al*., 2011). All such studies and findings pose DNA methylation as a strong candidate for further research in search of the missing links between the many different mechanisms that are suggested to take part in NDDs and also for clinical purposes such as the development of competent biomarkers and potent novel treatments.

Interestingly, C9orf72+ ALS patients also show an increase in their global 5-mC levels when compared to age- and sex-matched sALS patients, besides age- and sex-matched healthy controls. This, along with the hypermethylation of the *C9orf72* promoter, motivated an investigation into whether these phenomena are correlated. This hypothesis has been previously shown to be true in the case of glioblastoma multiforme (GBM) using LINE-1 promoter hypermethylation as a marker of increased global 5-mC, presenting global DNA methylation as a biomarker for gliomas (Ohka *et al*., 2011). Indeed, a moderate correlation between global 5-mC and *C9orf72* promoter hypermethylation was observed similar to GBMs. Other ALS-related gene promoters could be analyzed to see whether a similar hypermethylation is observed in those genes or any other gene that could possibly be related to ALS via altered DNA methylation patterns within their promoter regions. This could

potentially lead to the development of potent biomarkers for ALS as in the case of GBMs. Considering the lengthy diagnostic procedures and a lack of biomarkers for ALS, not only does this seriously affect the quality of life in patients, leaving them uninformed and confused about their condition, but it is also a great burden on the health care systems across the world. Thus, an effective and quick diagnosis strategy and the development of potent biomarkers will not only help patients lead a higher quality of life and reduce the costs for the health care systems, but maybe even more importantly, keeping in mind the lack of a regenerative mechanism in neurons, this will be crucial for possible preventive treatment strategies that could be utilized for this perplexing disease (Benatar *et al*., 2015).

6.5. Can DNA Methylation be used as a Biomarker for NDDs?

The global increase in 5-mC levels reported in this thesis are relatively small when compared to the study of Tremolizzo *et al*., 2014 which made use of HPLC-based 5-mC assays. This suggests that when compared to the ELISA-based assays used here, their approach may be better suited for clinical purposes. Furthermore, the increase in the global 5-mC levels is reported to be independent of disease-specific traits such as age of onset and disease duration, which could be used to monitor disease progression, making it an unsuitable candidate as a biomarker indicative of different stages of the disease or as a predictor of disease prognosis. Nevertheless, studies on pre-symptomatic mutation carrier NDD patients are needed to check whether such increases in global 5-mC are also observed prior to disease manifestation and if so, investigate the power of these measurements in predicting disease onset. Including other covariates, like body mass index (Mattsson *et al*., 2012) or medications such as statins (Seelen *et al*., 2014) or Riluzole, may increase the power of these studies as they may have a more profound effect on DNA methylation; thus they should be considered in future studies. Apart from these, other enzymes like L-methionine S-adenosylmethionine (MAT) that regulate SAM, the methyl donor for DNA methylation by DNMTs, should also be studied closely in order to unravel their mechanism in increasing DNA methylation in ALS, SCA1 and SCA2 patients. This is especially important as previous studies have shown that SAM supplementation can be beneficial in delaying age of onset in transgenic (SOD1-G93A) mouse models of ALS (Suchy *et al*., 2010). Moreover, histone deacetylase inhibitors (HDIs) such as sodium phenylbutyrate (Ryu *et al*., 2005), especially

when combined with riluzole (Del Signore *et al*., 2009), along with others such as trichostatin A (Yoo and Ko, 2011), have also been shown to slow disease progression in ALS patients and models, respectively.

6.6. The Effect of External Factors on Global 5-mC

In our statistical analyses, we used several different covariates including smoking, physical activity and alcohol consumption, and checked to see if these covariates had a significant effect on the global 5-mC levels in patients when compared to age- and sex-matched healthy controls. Additional covariates such as disease duration upon sample collection and site of onset were also used when comparing different patients groups and when checking for a significant effect of each of these covariates on the global 5-mC levels within each group (Table 5.6). Although none of the covariates mentioned above were found to have a significant effect on the global levels of 5-mC in sALS patients, interestingly alcohol consumption was significantly correlated with reduced levels of global 5-mC in our healthy control cohort and several reports on alcohol consumption and ALS have suggested that alcohol may act as a protective factor as it has been associated with a reduced risk of ALS (de Jong *et al*., 2012; E Yu *et al*., 2016; Ji *et al*., 2015). Alcohol consumption has been shown to change the global and local patterns of DNA methylation by both directly affecting the expression and activity of DNMTs and by reducing folate levels and inhibition of important components of the one-carbon metabolism, causing a decrease in the availability of SAM. Such events have already been associated with disease, as in the case of carcinogenesis where polymorphisms in the key components of the one-carbon metabolism have been suggested to cause alcohol-related carcinogenesis. The primary and highly toxic metabolite of ethanol, acetaldehyde, may be the most predominant component regarding the effect of alcohol on DNA methylation and oxidative stress (Seitz and Stickel, 2007). Reduced levels of global 5-mC that lead to extensive chromosomal instability via the loss of methylation within repetitive regions of the genome such as endoparasitic sequences, have been observed in cancer cells, which are normally extensively methylated. Unlike the loss of global methylation, promoter sequences are known to become hypermethylated within cancer cells causing transcriptional silencing of many different genes. Ethanol is also suggested to interfere with retinoid metabolism and to decrease the levels of vitamin A and

retinoic acid. This is especially important as retinoic acids are crucial for cellular proliferation and differentiation, as they take part in the transcriptional regulation of these events via nuclear retinoic acid receptors (Seitz and Stickel, 2007). There have also been disputes on whether carotenoid consumption (provitamin A), which can subsequently be converted to retinol, may be associated with ALS (Fitzgerald *et al*., 2013; Michal Freedman *et al*., 2013). Furthermore, alcohol can also interfere with DNA methylation via the reduction of methionine synthase (MS) activity, encoded by the *MTR* gene, and result in an increase in betaine homocysteine methyltransferase (BHMT) levels, which compensates for the lack of MS. However, prolonged exposure to alcohol does eventually lead to decreased levels of SAM as the compensatory BHMT pathway cannot be maintained for extended periods of time (Barak *et al*., 1996). Oxidative stress is also a central theme in alcohol metabolism (Seitz and Stickel, 2007) and is also known to have a profound role in ALS (Talbot, 2014). It too, can further lower the availability of SAM by inactivating methionine adenosyltransferase I and II (MAT I and II) (Mato *et al*., 2008). Such findings further highlight the importance of global levels of 5-mC in ALS and NDDs in general and encourage more comprehensive studies on alcohol and changes in global 5-mC.

6.7. Is Blood a Suitable Tissue for Epigenetic Studies in NDDs?

Due to the relative ease of access and convenience, blood is the number one tissue for many studies. However, the fact that it represents a dynamic and mixed population of white blood cells may add some bias to studies conducted using blood (Houseman *et al*., 2015). Having considered these factors, one might suggest that the increase in global 5-mC levels may in fact reflect the role of the immune system in the pathogenesis of ALS (Henkel *et al*., 2013), SCA1 and SCA2. This has also been shown by epigenome-wide studies conducted on sALS spinal cord samples (Figueroa-Romero *et al*., 2012), which suggest that epigenetic changes induce differential expression of immune-related genes. However, the fact that such an increase is only seen in ALS, SCA1 and SCA2 and not in other neurodegenerative diseases such as HD and FRDA, along with DM1, makes us doubt that this phenomenon could simply be classified as a consequence of the disease. This is further emphasized by the effects of alcohol on the DNA methylation pathways and also global 5-mC, and reports on its possible neuroprotective role.

7. CONCLUSION

Having considered the enormous impact that epigenetic changes could have on neurodegenerative disorders (NDDs), this thesis aimed at investigating NDDs from an epigenetic perspective and focuses mainly on the global levels of 5-mC in blood samples of several NDDs including complex diseases such as ALS and monogenic diseases such SCA1, SCA2, HD and FRDA, along with DM1. This thesis represents the largest study of its kind in ALS and a first for the others. Elevated levels of global 5-mC are reported for ALS, SCA1 and SCA2 when compared to age- and sex-matched healthy controls. Furthermore, an increased elevation in global 5-mC levels are reported for C9orf72+ ALS patients which has also been found to be correlated with *C9orf72* promoter hypermethylation.

The role of DNA methylation in the development and progression of several different diseases, especially cancers, have shown that this epigenetic modification could play a central role in disease onset and progression. Many different external factors can alter such epigenetic marks and alcohol has so far presented itself as the most potent modifier of the DNA methylation pathway during adulthood. Furthermore, alcohol has been suggested to reduce the risk of ALS and this was again underlined here as alcohol was shown to significantly lower the global levels of 5-mC in the healthy control cohort.

The results and findings presented in this thesis clearly indicate aberrant modifications in DNA methylation in some neurodegenerative diseases and call for further high-resolution studies, preferably with the additional analysis of different tissue types and presymptomatic patients. This should be followed by more in-depth studies at the molecular level using adequate models in order to understand the epigenetic dynamics of these diseases and to clarify the causality chain in hopes of unraveling yet another part of the puzzle that is neurodegeneration.

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