Huntington disease and other polyglutamine diseases : using CAG repeat variations to explain missing heritability

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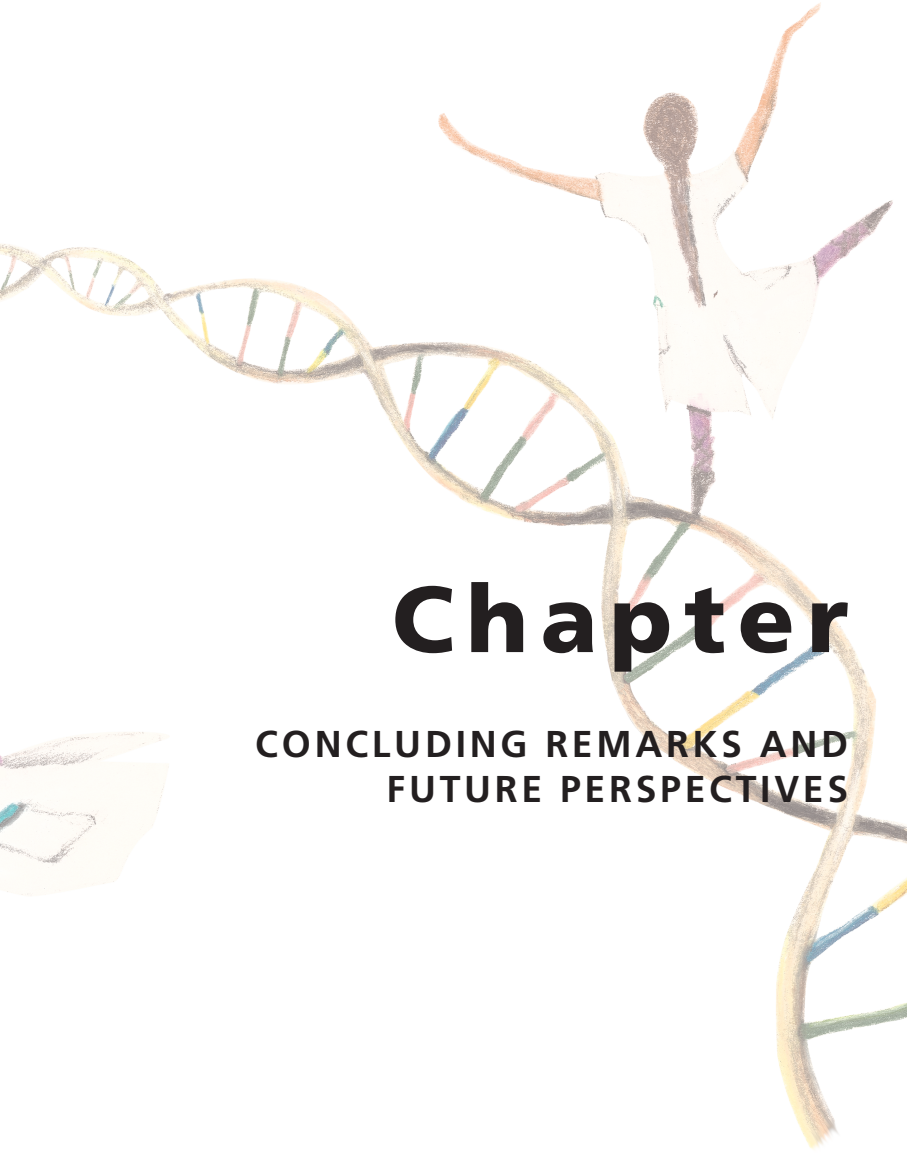

Chapter 7

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

SUMMARY

In this thesis, we provide ample evidence in support of the notion that cytosine-adenine-guanine (CAG) repeat variations within the 'normal' range in polyglutamine disease-associated genes (PDAGs) can affect various aspects of disease. We found associations between CAG repeat polymorphisms in PDAGs and the age of onset in Huntington disease (HD), cognitive function, the risk of lifetime depression and body mass index (BMI) in patients and participants included in various large cohorts. Finally, we found a relatively large prevalence of intermediate and pathological PDAG alleles in the general population.

Age of onset in Huntington disease

We found that the age of onset in HD was associated with CAG repeat variations in *ATXN3*, *CACNA1A* and *AR*, and differences in bioenergetic status of HD patients. Specifically, larger CAG repeat numbers in the longer *ATXN3* allele were associated with a later onset of HD symptoms independent of the CAG repeat number in both *HTT* alleles. Furthermore, the age of onset was affected by the interaction between the sequence length in the mutated *HTT* allele and the CAG repeat number in the longer *CACNA1A* and *AR* alleles. In addition, we found that the adenosine triphosphate (ATP) concentration and several indices of mitochondrial respiration were lower in the fibroblasts of patients with an earlier age of onset, independent of CAG repeat number, sex, disease duration and age. These findings support previous reports that biological interactions between PDAGs can influence the age of onset in polyglutamine diseases[1,2]. Our results also emphasize the major importance of bioenergetics in HD pathology. Together, these results suggest new pathophysiological mechanisms through which the timing of symptom onset is affected and can perhaps be modified. To gain more insight into these mechanisms, future research should focus on the effect of 'normal' CAG repeat variations within *ATXN3*, *CACNA1A* and *AR* on cellular outcomes associated with HD progression and symptom onset, including for instance *HTT* aggregation or striatal neuronal cell death. Likewise, the cause of the difference in bioenergetic status between HD patients with an earlier and later age of onset should be further investigated. We may discover that the mitochondrial DNA of patients with a worse bioenergetic status and an earlier age of onset is more vulnerable to lesions.[3] The resulting increased understanding of how the age of onset in HD is affected and could be modified, would greatly benefit patients for instance by developing therapeutic molecules that could perhaps improve the resistance of mitochondrial DNA to lesions and thereby delaying the onset of symptoms.

Cognitive function

In Alzheimer disease (AD) patients, we found that memory and atrophy of the medial temporal lobes were associated with the CAG repeat number in *ATXN1* and *AR*, respectively. 'Normal' cognitive ageing was associated with the CAG repeat number in six of the nine PDAGs: *ATXN2*, *CACNA1A*, *ATXN7*, *TBP*, *HTT* and *AR*. In both AD and

'normal' cognitive aging, differences in cognitive function and the size of various brain volumes were associated with CAG repeat polymorphisms in *AR*. Throughout evolution, larger CAG repeat numbers in *AR* were associated with organisms having a more complex central nervous system.[4-6] In contrast, larger CAG repeat numbers in *AR* have also been associated with a faster decline in cognitive function in older men.[7] These findings suggest a complex association between cognitive function and CAG repeat variations in *AR*, which is reflected in our findings. The molecular mechanism through which CAG repeat polymorphisms in *AR* affect cognitive function is still unknown. Research has found that larger CAG repeat numbers in *AR* are associated with a decreased sensitivity of the androgen receptor and that decreased levels of androgen are associated with the accumulation of β-amyloid (Aβ).[8-10] Perhaps the decreased sensitivity of the androgen receptor allows for the creation of a more complex central nervous system during development. However, as people get older and evolutionary selection is no longer relevant, this decreased sensitivity might also lead to the accumulation of β-amyloid in the same way decreased levels of androgen do, eventually causing AD. Of course, proving such hypotheses warrants more detailed investigation in future experiments.

The risk of lifetime depression

In this thesis, we reported that the risk of lifetime depression was associated with variations in CAG repeat number within *ATXN7*, *TBP* and *HTT*. Larger CAG repeat numbers in both alleles of *ATXN7* and *TBP* were associated with a higher risk of lifetime depression, whereas the longer *HTT* allele had a positive curvilinear association. Depression is a common characteristic of polyglutamine disorders. Consequently, the positive association between CAG repeat number and depression susceptibility is not surprising. However, the causal mechanism of this association has not yet been established. Various studies have linked decreased levels of several neurotrophic factors, including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) to depression and upregulation of these factors was associated with responses to treatment with antidepressants.[6] Interestingly, expression of BDNF is regulated by the Huntington protein.[7] In addition, HD patients suffer from reduced function and expression of striatal BDNF.[8] However, how 'normal' variations in the length of the CAG repeat sequence might affect in this regulation, is still unknown. To better understand how the repeat polymorphisms in *ATXN7*, *TBP* and *HTT* affect depression susceptibility, future research could focus on assessing the levels of different neurotrophic factors in models expressing these PDAGs with different sequence lengths within the 'normal' range.

Body mass index

BMI was associated with the CAG repeat number in seven of the nine PDAGs: *ATXN1*, *ATXN2*, *ATXN3*, *CACNA1A*, *ATXN7*, *TBP* and *AR*. The proteins encoded by these PDAGs

are primarily expressed in the central nervous system. In particular, neuronal circuits in the hypothalamus and brain stem sense and integrate many local and peripheral cues to regulate the appropriate neuroendocrine, autonomic and behavioural responses to preserve a systemic energy balance.[9] Supporting the link between the central nervous system and metabolic disturbances, is the fact that BMI has been associated with dementia, memory and depression.[10-12] In addition, an inverse association was identified between BMI and the CAG repeat number in HD and spinocerebellar ataxia type 3 (SCA 3) patients.[13-16] Future research investigating the effect of CAG repeat polymorphisms in the PDAGs on the neural circuits involved in the regulation of metabolism could lead to a better understanding of the found associations. Studies could for instance study the effect of CAG repeat variations on leptin resistance.[17] Furthermore, investigating through what metabolic pathways (i.e. appetite, basal metabolic rate, glucose tolerance, insulin insensitivity and physical activity) the association between BMI and the CAG repeat number in PDAGs is mediated, would allow us to gain more insight into our findings.

Intermediate and pathological polyglutamine disease-associated alleles

For this thesis, we determined the CAG sequence length in both alleles of more than 14 000 participants from five large cohorts. These participants were devoid of an established polyglutamine disease diagnosis, allowing us to determine the prevalence of intermediate and pathological PDAG alleles within the general population. We found a relatively high prevalence of these alleles among the genotyped participants.This finding suggests that a higher proportion of the population may be at risk of developing a polyglutamine disease or that certain individuals carry specific unknown traits protecting them from the development of polyglutamine disease symptoms. Investigating these individuals and their potential protective traits could prove beneficial in understanding the pathophysiology of polyglutamine diseases and in the development of polyglutamine disease treatments.

REPETITIVE DNA VARIATIONS IN THE HUMAN GENOME

CAG repeats are not the only type of repetitive DNA in the human genome. Over half of the human genome is estimated to consist of repetitive elements, which together form the repeatome (**Figure 1**).[18] Repetitive DNA sequences can be divided into satellite DNA, tandem repeats, copy number variants (CNVs) and interspersed repetitive DNA. Satellite DNA consists of large arrays of tandemly repeated DNA (5-100 bp), is highly repetitive, constitutes about 10% of the human genome and is found primarily in centromeres and telomeres.[19] Tandem repeats include microsatellites and minisatellites.[20] Microsatellites consists of 1-6 repeated bp motifs, making trinucleotide CAG repeats a type of microsatellites. Minisatellites consists of more than 6 repeated motifs. Collectively, tandem repeats constitute around 3% of the human genome.[21] CNVs comprise about

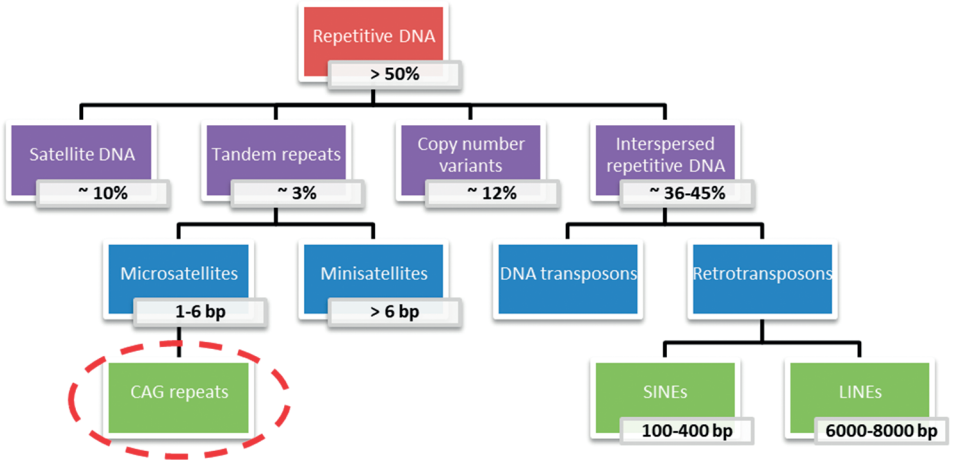

Figure 1. Overview of different repetitive DNA types. More than 50% of the human genome consists of repetitive elements. These repetitive DNA sequences can be divided into satellite DNA (~10%), tandem repeats (~3%), copy number variants (~12%) and interspersed repetitive DNA (~36-45%). Cytosine-adenine-guanine (CAG) repeats constitute only a small portion of the entire repeatome. bp=base pair motifs. CAG=cytosine-adenine-guanine. SINEs=short interspersed elements. LINEs=long interspersed elements.

12% of the human genome and involve variations in copy numbers of chromosomal segments larger than 1 kb.[22-24] In contrast to tandem repeats and CNVs, repeat sequences in interspersed repetitive DNA are not adjacent, but consist of degenerate copies of transposable elements spread throughout the genome.[25] Transposition of these elements can occur via reverse transcription of an RNA intermediate, resulting in retrotransposons that can either contain short (100-400 bp) interspersed elements (SINEs) or long (6-8 kb) interspersed elements (LINEs). Transposition can also occur through excision and reintegration of DNA itself, called DNA transposition. In total, interspersed repetitive DNA sequences constitute about 36-45% of the human genome.[26,27]

Considering the enormous potential genetic variation of repetitive sequences of DNA in the human genome and the many polygenic disorders found associated with CAG repeat polymorphisms, an obvious subsequent step would be to investigate the association of polygenic disorders with these additional repetitive DNA variations. Different studies have indeed presented associations between different complex genetic disorders and repetitive DNA variations other than the CAG repeat polymorphisms in PDAGs. For instance, hexanucleotide GGGGCC repeat expansions in *C9ORF72* were associated with the risk of amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD) and Alzheimer disease.[28-31] Furthermore, variations in CNVs have been associated with autism and schizophrenia.[32,33] Schizophrenia was also associated with variations of microsatellites in the genes *BDNF*, *GCLC*, *JARID2*, *NOS1* and *NUBL*.[34-38] The risk of depression and

anxiety was affected by minisatellite variations within *5-HTT* and *MAOA*.[39-42] Repeat polymorphisms within *5-HTT* have additionally been associated with bipolar disorder and behavioural and psychological symptoms of Alzheimer disease.[43-46] Moreover, the risk of bipolar disorder was associated with tandem repeat variations in *BDNF* and *Per3*.[47,48] Stroke has been associated with repeat polymorphisms in *IL1RN* and *GPIbα*.[49-51] Attention deficit hyperactivity disorder (ADHD) was associated with minisatellite variations in *DAT1* and *DRD4*,[52,53] and the risk of Alzheimer disease was associated with a dinucleotide repeat polymorphisms in the *NOS1* gene.[54] In addition, repeat polymorphisms have been associated with variations in certain traits and endophenotypes, including human cognitive function, affective states and behaviours.[55-62] However, these studies frequently contained small sample sizes and were underpowered. Consequently, well-powered validations in large independent cohorts as well as meta-analyses are required to confirm these findings.[63,64]

POTENTIAL THERAPEUTIC MECHANISMS TO ALTER REPEAT SEQUENCES

As more evidence accumulates regarding the association between repetitive DNA and polygenic disorders, targeting these sequences in order to treat or prevent diseases, becomes more relevant. The investigation in the treatment of tandem repeat disorders, such as Huntington disease, has resulted in several techniques which could be applied to achieve such a therapy (**Table 1**).[64]

First, the affected allele could be altered or silenced by targeting DNA directly through zinc finger proteins or via clustered regularly interspaced short palindromic repeat (CRISPR)-associated Cas9 nuclease.[65] Zinc fingers are naturally occurring structural motifs that efficiently bind specific DNA sequences and can be generated synthetically. Various functional domains are coupled to these zinc fingers, including repression domains, activation domains or endonucleases. Zinc finger peptides fused to a repression or activation domain are engineered to selectively switch genes off or on. Zinc fingers coupled to an endonuclease introduce double strand breaks at specific DNA sites. Subsequently, a donor DNA strand can be incorporated at this location via homologous recombination, changing the gene permanently. However, this process is not decidedly precise or predictable.[66] CRISPR-Cas9 therapeutic strategies can also be employed to inactivate or alter affected alleles. The Cas9 nuclease combined with a specific single guide RNA produces a construct that causes double strand breaks with high precision at a chosen site. The double strand break is repaired via two mechanisms. In absence of a homologous DNA template, non-homologous end joining occurs, which is an error-prone process that introduces small insertions or deletions. When a synthetic DNA template is present, this template is integrated via homologous recombination, which enables the introduction of any desired base-pair changes.[67] A disadvantage of zinc finger proteins and CRISPR-Cas9

Table 1. Overview of gene silencing or altering mechanisms to target repetitive DNA sequences.

Compound	Target	Effect	Advantages	Disadvantages
ZFP	DNA	transcriptional repression transcriptional activation genome editing	single administration to provide long-term treatment; ameliorates all pathogenic pathways	permanent; risk of inflammatory response; not precise or predictable
CRISPR-Cas9	DNA	genome editing	permanent removal of genetic cause; highly specific and targeted; ameliorates all pathogenic pathways	permanent; risk of inflammatory response
ASO	pre-mRNA	pre-mRNA degradation splicing modulation	more target sequence options; diffuses well through the CNS	several administrations necessary
RNAi	mRNA	mRNA degradation	more straightforward targeting; lifelong treatment from a single dose	enhanced delivery methods or viral vectors necessary for compound delivery

ZFP=zinc finger protein. CRISPR-Cas9= clustered regularly interspaced short palindromic repeat (CRISPR)-associated Cas9 nuclease. ASO=antisense oligonucleotide. RNAi=RNA interference.

methods as therapeutic agents is that the production of non-native proteins could trigger inflammatory responses. However, targeting DNA directly, permanently alters the gene and ameliorates all aspects of the respective disorder due to that gene.[68]

In addition to DNA, RNA molecules can be therapeutically targeted. RNA is not protected by repair mechanisms and has a unique secondary or tertiary structure, making targeting these molecules more straightforward. mRNA levels can be reduced by using antisense oligonucleotides (ASOs) or RNA interference (RNAi) compounds. Both ASOs and RNAi compounds are nucleotide-based molecules that selectively bind to mRNA through Watson-Crick complementarity. ASOs are synthetic single-stranded DNA molecules that bind pre-mRNA for degradation by RNase H in the nucleus or to modulate its splicing. By acting on pre-mRNA, ASOs can target exons as well as introns, allowing for more target sequence options.[69] RNAi compounds include double stranded RNA-based molecules, such as short interfering RNA, short hairpin RNA and microRNA. These molecules bind to mature spliced cytosolic mRNA, which is subsequently targeted for degradation by argonaute 2, the RNase enzyme within the RNA-induced silencing complex.[70]

Overall challenges in the development of the above-mentioned treatments include distinguishing the target alleles from the healthy alleles, distinguishing the target repeat

sequence form similar or identical sequences in other genes, and adequately dispensing the drug to the correct tissues. Unique single nucleotide polymorphisms (SNPs) that flank the repeat sequence of interest could be used to distinguish target sequences.[71,72] However, this method poses its own problems. The fact that the SNP sequence would need to be included in the recognition site of the therapeutic molecule, limits the selection options for target motifs. Moreover, the SNP must be linked to the target allele and not the 'healthy' allele with absolute certainty, and the prevalence of the SNP in the affected population greatly determines its feasibility.[68] Furthermore, in order to assure correct delivery of the developed compound to the target organs and tissues, different administration methods should be carefully evaluated. Systemic administration for instance, could result in a higher risk of unwanted off-target effects.[73,74] In addition, the blood brain barrier could prevent the therapeutic agents from reaching target neurological structures. Interestingly, the single-stranded DNA of ASOs diffuses quite well in the central nervous system and is efficiently absorbed by neurons with a corresponding reduction in mRNA and protein levels.[75] For this reason administration of ASOs to the brain via an injection into the cerebral spinal fluid is an accepted delivery option. In contrast, the double stranded RNA of RNAi has low diffusion and cellular uptake in the central nervous system. Enhanced delivery methods or the use of viral vectors, such as the adeno-associated virus (AAV) are required to administer these compounds to the brain parenchyma. However, limited tissue distribution and the immunogenicity of AAVs in the human brain pose challenging issues.[76-81] Although highly promising, these synthetic molecules warrant considerably more research before these agents can be incorporated as the standard therapy for tandem repeat diseases and perhaps future treatment of different polygenic disorders.

CONCLUSION

In this thesis we demonstrated that investigating the pathogenesis of rare diseases, such as polyglutamine diseases, can result in novel insights into more prevalent, but genetically complex disorders. In addition, we provided support for the role of repetitive DNA polymorphisms in elucidating the 'missing heritability' of polygenic disorders and emphasized the necessity to include these variations in future genetic research. Although treatment of tandem repeat disorders via gene silencing or altering methods remains complex, once available these methods could be employed to treat or even prevent the more common diseases in which repetitive DNA sequences are implicated.

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