UNIVERSITY OF COPENHAGEN
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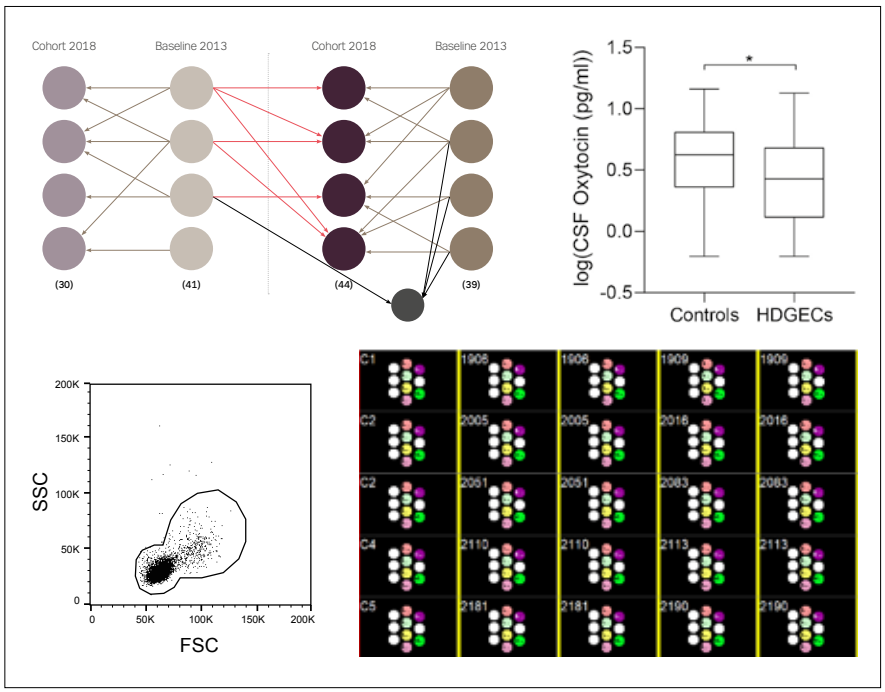
Huntington's Disease

Endophenotypes and biomarkers



PhD thesis

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Endophenotypes and biomarkers

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Preface and acknowledgements

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Manuscripts

This Ph.D. thesis is a synopsis and based on three manuscripts:

I. **Hellem MNN**, Hendel RK, Vinther-Jensen T, Larsen IU, Nielsen TT, Hjermind LE, Budtz-Jørgensen E, Vogel A, Nielsen JE. Endophenotypical drift in Huntington's disease: a 5-year follow-up study. Orphanet J Rare Dis. 2021 Aug 3;16(1):340. doi: 10.1186/s13023-021-01967-2. PMID: 34344392.

II. von Essen MR, **Hellem MNN**, Vinther-Jensen T, Ammitzbøll C, Hansen RH, Hjermind LE, Nielsen TT, Nielsen JE, Sellebjerg F. Early Intrathecal T Helper 17.1 Cell Activity in Huntington Disease. Ann Neurol. 2020 Feb;87(2):246-255. doi: 10.1002/ana.25647. Epub 2019 Nov 27. PMID: 31725947.

III. **Hellem MNN***, Cheong RY*, Tonetto S*, Vinther-Jensen T, Hendel RK, Larsen IU, Nielsen TT, Hjermind LE, Vogel A, Budtz-Jørgensen E, Petersén Å* and Nielsen JE*. Decreased CSF Oxytocin relates to measures of social cognitive impairment in Huntington's Disease patients. Neurology, Submitted January 2022.

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Related publications:

Hellem MNN, Vinther-Jensen T, Anderberg L, Budtz-Jørgensen E, Hjermind LE, Larsen VA, Nielsen JE, Law I. Hybrid 2-[18F] FDG PET/MRI in premanifest Huntington's disease gene-expansion carriers: The significance of partial volume correction. PLoS One. 2021 Jun 11;16(6):e0252683. doi: 10.1371/journal.pone.0252683. PMID: 34115782; PMCID: PMC8195345.

Hendel RK, **Hellem MNN**, Hjermind LE, Nielsen JE, and Vogel A. Intellectual Curiosity and Action Initiation are Subtypes of Apathy Affected in Huntington's Disease Gene Expansion Carriers. Cognitive and Behavioral Neurology, accepted May 2021.

Summary

Huntington's disease (HD) is an autosomal dominantly inherited neurodegenerative disease caused by a CAG repeat expansion in the gene encoding huntingtin. The disease is characterized by progressive motor, psychiatric and cognitive symptoms. This triad of symptoms represent the phenotype of HD; However, the symptoms vary within the individual HD gene expansion carriers (HDGECs) and different endophenotypes are observed. Despite immense research, the exact pathogenesis is still not clear and there is no cure.

In 2013 a cohort of HDGECs and HD gene expansion negative family controls were recruited and characterized according to motor, psychiatric, and cognitive symptoms. During my Ph.D. study, we completed a follow-up on these participants while recruiting new participants also. We aimed to describe the development of the symptoms, the drift between endophenotypes, investigate whether neuroinflammatory activity was measurable in the cerebrospinal fluid (CSF) and if the levels of plasma and CSF oxytocin could be correlated to the cognitive and psychiatric symptoms of HD.

The participants were examined and evaluated by a physician and a neuropsychologist; blood, cerebrospinal fluid and saliva samples were collected, and the participants were categorized according to their endophenotypical presentation.

We included 114 HDGECs, 45 premanifest and 69 motor manifest, and 36 HD gene expansion negative family controls.

In the first study we reexamined the HDGECs from Cohort 2013 and described the drift of the endophenotypes. We found that the drift was unpredictable and only an increasing Disease Burden score (CAP score) increased the risk of developing cognitive impairment and motor symptoms. We did, however, find that while the motor and cognitive symptoms were stationary, the psychiatric symptoms were potentially resolvable. The probability of improving on the psychiatric symptoms were reduced with increasing age.

In the second study we analyzed the CSF and cells of the CSF from HDGECs for signs of early neuroinflammation. Measurements were compared to the HD gene expansion negative family controls, inflammatory controls represented by participants with primary progressive multiple sclerosis and, patients with non-inflammatory neurological disease. CSF was analyzed by

electrochemiluminescence and the cells by flow cytometry. We found a cytokine profile compatible with early proinflammatory lymphocyte activity intrathecally in the HDGECs and an increased amount of T-helper 17.1 cells in the premanifest HDGECs compared to motor manifest HDGECs, controls and patients with non-inflammatory neurological disease. This increased frequency of T-helper 17.1 cells correlated negatively with Disease Burden score, motor score and the level of neurofilament light chain implying an early involvement of the T-helper 17.1 cells.

The third study compared the levels of plasma and CSF oxytocin in HDGECs and controls. We also investigated potential differences in oxytocin levels within the endophenotypes of the HDGECs. We measured the oxytocin levels in all first-time lumbar punctures and the corresponding plasma samples, using radioimmunoassays. We found significantly lower levels of oxytocin in the CSF of the motor manifest HDGECs compared to controls. Within the HDGECs we found the participants with cognitive impairments to have significantly lower levels of oxytocin in the CSF. We also found associations between performance on tests of social cognition and the levels of oxytocin.

In conclusion, we illustrate the unpredictability of the endophenotypical drift in HD and that different modifiers may regulate the development of symptoms and emphasize the importance of considering individual therapy. We suggest replication of both our neuroinflammatory findings and our oxytocin study and suggest potential treatment trials with oxytocin and anti-VLA4 to target social cognitive dysfunction and inflammation respectively. We further challenge the utility of the classical classification of HDGECs according to motor symptoms.

Resumé (Summary in Danish)

Huntingtons sygdom (HS) er en autosomal dominant arvelig sygdom, der skyldes en forlængelse af et CAG repeat i genet, som koder for proteinet huntingtin. Sygdommen er karakteriseret ved progredierende motoriske, psykiatriske og kognitive symptomer. Denne triade af symptomer repræsenterer fænotypen for HS, men symptomerne hos den enkelte HS-anlægsbærer kan dog variere og medføre forskellige endofænotyper. På trods af intensiv forskning er den eksakte patogenese og en helbredende behandling stadig ikke fundet.

Som en del af et tidligere Ph.D.-projekt i 2013, blev en kohorte af HS-anlægsbærere og HS-anlægsbærer-negative familie-kontroller rekrutteret og karakteriseret i forhold til tilstedeværelsen af motoriske, psykiatriske og kognitive symptomer. I løbet af mit Ph.D.-projekt er der gennemført en opfølgning af denne kohorte og sideløbende rekrutteret nye deltagere. Målet var at beskrive udviklingen af symptomer, ændringer i endofænotyperne, undersøge om det var muligt at måle tegn på inflammation i spinalvæsken, samt hvorvidt niveauet af oxytocin i plasma og spinalvæske kunne korreleres til de kognitive og psykiatriske symptomer ved HS.

Vi rekrutterede 114 HS-anlægsbærere, 45 præmanifeste, 69 motor manifeste og 36 HS-anlægsbærer-negative kontroller.

Det første studie beskriver opfølgningen på HS-anlægsbærerne fra 2013 og bevægelserne i deres endofænotyper. Bevægelserne viste sig at være uforudsigelige, og kun en stigende Disease Burden score øgede risikoen for at udvikle kognitiv dysfunktion og motor symptomer. Vi fandt derudover, at de psykiatriske symptomer var dynamiske, mens de kognitive og motoriske symptomer var permanente. Sandsynligheden for at bedres i de psykiatriske symptomer viste sig at mindskes ved stigende alder.

I det andet studie undersøgte vi spinalvæske-markører og cellerne i spinalvæsken fra HS-anlægsbærerne for tegn på tidlig neuroinflammation. Vi sammenlignede resultaterne med familie-kontroller, inflammatoriske kontroller, dvs. deltagere med primær progressiv multipel sklerose, og patienter med non-inflammatorisk neurologisk sygdom. Spinalvæsken blev undersøgt ved elektrokemiluminescens og cellerne ved flowcytometri. Vi fandt en cytokinprofil forenelig med en tidlig proinflammatorisk intratekal lymfocyt aktivering hos HS-anlægsbærerne og en øget frekvens af T-hjælper 17.1 celler hos de præmanifeste HS-anlægsbærere sammenlignet med familie kontroller og patienter med non-inflammatorisk neurologisk sygdom.

Denne øgede frekvens af T-hjælper 17.1 celler korrelerede negativt med Disease Burden score, motor symptomer og målinger af neurofilament light chain protein, hvilket tyder på en tidlig involvering af T-hjælper 17.1 cellerne.

I det tredje studie, sammenlignede vi niveauet af oxytocin i plasma og spinalvæske hos HS-anlægsbærere og familie-kontroller. Vi undersøgte også om der var forskelle inden for de forskellige HS endofænotyper. Vi målte niveauet i alle første-gangs lumbalpunkturer og de tilhørende plasmaprøver ved brug af radioimmunoassay. Vi fandt et signifikant lavere niveau af oxytocin hos de motor manifeste HS-anlægsbærere sammenlignet med familie-kontrollerne og inden for gruppen af HS-anlægsbærere fandt vi at de med kognitive symptomer havde et signifikant lavere niveau af oxytocin end de uden kognitive problemer. Niveauet af oxytocin associerede til scoren på de socialkognitive tests.

Med disse studier har vi beskrevet de individuelt uforudsigelige bevægelser af endofænotyperne ved HS og vist at der kan ligge forskellige modificerende årsager til grund for udviklingen af symptomerne samt understreget vigtigheden af at overveje individuelle behandlingstiltag ved HS. På baggrund af resultaterne anbefaler vi, at der gennemføres replikationsstudier af både inflammationsstudiet og oxytocin-studiet og evt. efterfølgende behandlingsforsøg med oxytocin og anti-VLA4 målrettet henholdsvis den socialkognitive dysfunktion og inflammationen. Derudover udfordrer vi den traditionelle klassifikation af HS-anlægsbærere i forhold til tilstedeværelsen af motoriske symptomer.

Abbreviations

AAO	Age at onset
ASO	Antisense Oligonucleotide
BDNF	Brain Derived Neurotrophic Factor
CAG	Cysteine-adenine-guanine
CAP	CAG age product
CNS	Central nervous system
CSF	Cerebrospinal fluid
EHt	Emotion Hexagon test
HAM -17	Hamilton depression scale
HD	Huntington's disease
HDGECs	Huntington's Disease gene expansion carriers
HTT	Huntingtin gene
HTT	Huntingtin protein
Htt	Huntingtin protein in mice
IL	Interleukin
mHTT	Mutant Huntingtin
MMSE	Mini Mental State Examination
MoCA	Montreal Cognitive Assessment
NIND	Non inflammatory neurological disease
PBA-s	Problem Based Assessment short
PPMS	Primary progressive multiple sclerosis
RIA	Radioimmunoassay
RME	Reading the Mind in the Eyes
SCL-90-r	Symptom Checklist 90 revised
SDMT	Symbol digit modality test
TASIT	The Awareness of Social Inference Test
TFC	Total functional capacity
TMS	Total motor score
UHDRS	Unified Huntington's Disease Rating Scale
VLA4	Very late antigen-4

Introduction

Huntington's Disease

Huntington's disease (HD) is an autosomal dominantly inherited, neurodegenerative disease. It is caused by a cysteine-adenine-guanine (CAG) repeat expansion in exon 1 of the gene encoding huntingtin *HTT* on chromosome 4. It leads to a triad of symptoms consisting of motor, cognitive, and psychiatric symptoms [1].

The disease has been described as early as 1842 by Charles Oscar Waters and by George Huntington in 1872. In "The Medical and Surgical Reporter" he described the characteristic choreatic movements, its hereditary nature and the tendency to insanity and suicide [2]. Eventually, the disease was named after him. Despite the long-term knowledge of the disease, it was not until 1993 that the mutation in the *HTT* gene was identified [1]. Despite intensive research, there is still no cure or disease modifying treatment.

The prevalence of HD is 5-10 per 100,000 in the Caucasian population [3,4]. In the Danish HD registry, presumably the oldest registry of HD, 14,245 individuals from 445 families were registered in 2015; of these individuals 1926 were motor manifest HD gene expansion Carriers (HDGECs) and the remaining individuals had a 50% risk or less of developing HD. The registry resulted in a prevalence of 5-8:100,000 [5].

Pathogenesis

The CAG repeat expansion in *HTT* leads to the formation of an excessively long polyglutamine tract (PolyQ) in the huntingtin protein (HTT) resulting in mutant huntingtin (mHTT). HTT is ubiquitously expressed in humans, with the highest concentration being in the central nervous system (CNS) [6]. There are no greater regional differences in HTT expression within the CNS [7] but HD results in an early specific deterioration of the medium spiny neurons of the striatum (caudate nucleus and putamen) and cortical thinning [4,8,9]; however, what makes these neurons more vulnerable to the effects of mHTT is still unclear and both neuronal dysfunction and neuronal death have been observed.

The function of wild type HTT and mHTT is not fully understood. It is known that Htt is critical for embryonic development as complete inactivation in mice causes embryonic death before the formation of the nervous system [4]. HTT has been shown to have an antiapoptotic function, a

role in regulation of brain derived neurotrophic factor (BDNF) production, axonal and vesicle transport, and in control of synaptic activity [10].

Mutant HTT is found both in the nucleus of the cell and in the cytoplasm, but with higher concentration in the latter [7]. It is prone to misfold and aggregate [11]. Mutant HTT aggregates have been located in the striatum, cerebral cortex, cerebellum and spinal cord. While some studies suggest that the aggregation is part of a protective cellular response [11,12], other evidence points to a predominantly toxic gain-of-function role for the mutated HTT [12]. Several mechanisms suspected of causing neurodegeneration in HD have been identified. These include cytoskeletal disruption, protein mishandling, altered mitochondrial dynamics, and excitotoxicity [7]. No single mechanism causing the initial striatal neuronal death has been identified, but rather a pathological cascade of events triggered by mHTT.

As HD most often debuts in midlife and considering the importance of Htt during embryonal development, mHtt must fulfill the essential roles of wild type Htt, otherwise we would have expected the disease to have manifested itself earlier in life.

Genetics

Six to 35 CAG repeats are normal and do not cause HD. A CAG repeat causing HD contains 40 repeats or more, while a CAG repeat between 36 and 39 entails reduced penetrance (**Table 1**) and a 40% chance of being asymptomatic at the age of 65 [13].

The intermediate alleles (27-35 CAG repeats) are considered instable and at risk of increasing in length from one generation to the next (a process called anticipation), especially if passed on by a male [3,14]. Juvenile HD, with onset before the age of 20, caused by a CAG repeat over 50, is a result of such anticipation, and therefore predominantly observed by paternal transmission [15].

Up to ten percent of all HD cases are considered de novo mutations in some populations and are suspected of being the result of anticipation of the intermediate alleles [16]. It has even been suggested that people in the high-normal range are actually carriers of a potential pathogenic expansion, but that age of onset is beyond the normal lifespan [17].

Table 1.

Number of CAG repeats:	
6-26	Normal alleles
27-35	Intermediate alleles
36-39	Incomplete penetrance range
40 or more	Complete penetrance range

The age at onset (AAO) of HD, motor symptoms, has been registered between 2-80 years of age but HD typically debuts between the age of 35 and 45 [14] and the average disease duration is 15 – 20 years. The most critical determinant of AAO is the number of CAG repeats, which accounts for 50-70 % of the overall variance in AAO [15]. There is an inverse correlation between the length of the CAG repeat and the AAO. The CAG repeat length is also a rate-determining factor for the motor impairment, cognition and daily function [18,19]. The correlation between cognitive symptoms and CAG repeat length is comparable to the correlation between CAG repeat length and motor onset, while there is no correlation between CAG repeat length and psychiatric symptoms [19]. The unaccounted 30-50% of variance are attributed genetic, environmental, and stochastic effects. Some studies have found approximately half of the AAO residual to be heritable/genetic, and the other half environmental [20–22]. In the normal allele, the length of the CAG repeat has been shown to affect AAO, and an increase in the size of the normal allele seemed to mitigate the disease in the HDGECs with longer alleles (47-83 repeats) [20]. In 2012, a large study including 4078 HDGECs however found that the length of the normal CAG repeat had no effect on the AAO [15,23].

In an attempt to estimate the Disease Burden of HD, Penney *et al.* in 1997 proposed a function of CAG repeat length and age: Age x (CAG – 35.5); age being the current age, CAG the repeat length of the mutated allele, and 35.5 being the largest CAG repeat with no expected pathology [24]. This formula was based on findings of a linear correlation between the CAG repeat number and the degree of atrophy in the striatum. The formula has since been known as the CAG age product (CAP) score, however with adjustment, but still in the form Age x (CAG – L), L being a constant. It has been used both as an index of cumulative toxicity of HTT, an entry criterion to studies, and a way to distinguish which HDGECs were far from or close to predicted onset [19]. Several groups have tried to estimate the constant L. An overview of the different equations and

constants can be seen in **Table 2**. Some studies have even included an additional constant (S) to the equation. S leading to a CAP score of a fixed approximate value [19].

Table 2. **The different CAG Age Product (CAP) scores**

CAP score = Age x (CAG – L)				
Study (Author)	L			CAP [a]
Penney [24]	35.5			220
PREDICT (Zhang et al) [25]	33.66			293.6
CAP_S score = (Age x (CAG – L))/S				
Study (Author)	L	S	Score at estimated 50% risk for debut in 5 years	CAP_S[a]
PREDICT (Zhang et al) [25]	33.66	432.3326	1	0.68
CAP_S score = 100 x Age x (CAG – L)/S				
Study (Author)	L	S	Approximate score at expected age of onset	CAP_S[a]
TRACK (Ross et al) [19]	30	627	100	70.18

CAP: CAG age product, L: scaling constant, S: normalization constant, [a] Example: HD gene expansion carrier, 40 years of age and CAG repeat length of 41.

Clinical Features

The diagnosis HD is established when a person carrying the gene for HD develops an otherwise unexplained extrapyramidal movement disorder, for example, chorea, dystonia, bradykinesia or rigidity [19]. It is, however, acknowledged that both cognitive and psychiatric symptoms may precede the motor symptoms which are often not of the greatest concern for the HDGECs [7,26].

The European Huntington's Disease Network's REGISTRY registered the phenotype and HD genotype in a group of 615 European HDGECs. The first symptom of disease was motor symptoms in 48 % of cases, 19.6 % had psychiatric symptoms at onset, 8.4 % cognitive, and 13.2 % mixed [27]. Similarly, in a cohort of 960 HDGECs, Marder *et al.* retrospectively found 56 % to have had motor debut, 22 % psychiatric, 14 % cognitive and 9 % mixed [28]. Vinther-Jensen et al. found 25.5 % of the premanifest HDGECs to have psychiatric symptoms, 9.8 % to have cognitive symptoms, and 3.9 % to have both [29].

The first motor symptom is typically chorea. These involuntary dance-like movements often begin in the distal extremities as suppressible fleeting movements that, over time, become more prominent involving larger and more proximal muscles. Clinical examination also detects saccadic eye movement abnormalities, ataxia and impairment of voluntary movements. Progression of HD leads to bradykinesia and rigidity in later stages, features also seen at juvenile onset [7,19]. Assessment of motor symptoms is conventionally performed using the Unified Huntington's Disease Rating Scale (UHDRS) Total motor score [30]. The rating of the motor symptoms is a 31items list ranging from 0 to 124, with increasing score for increasing symptoms.

The cognitive symptoms in HD reflect the early striatal degeneration and disruption to striatocortical circuitry. The earliest changes are psychomotor slowing and impaired emotion recognition. The most prominent impairments are in the domains of executive functions, psychomotor performance, memory, and the ability to process emotions [31,32]. This can greatly affect everyday functions [19] and the cognitive changes have along with behavioral changes been found to associate with functional decline and be predictive of institutionalization [26]. For the everyday function of the HDGEC the identification and acknowledgement of this functional impairment is of great importance.

The term dementia covers the impairment of the cognitive functions beyond what we might expect from ageing resulting in deficits in occupational and social functioning [33]. The criteria for dementia have generally been based on dementia associated with Alzheimer's disease and therefore efforts are made trying to characterize and evolve diagnostic criteria for dementia in HD. In HD dementia is subcortical, the memory loss is not necessarily significant but cognitive impairment is characterized by attention deficits, cognitive slowing, impaired planning, problem solving and construction and visuoperceptual deficits [34], and it was suggested to include impairment in at least two domains of cognition in the diagnosis of dementia in HD [34]. Mild cognitive impairment (MCI) in HD was suggested to be defined as an objective loss in one cognitive domain without loss of functional independence [35]. In Alzheimer's disease MCI is regarded as a stage between normal cognition and dementia, and individuals with MCI has been found to progress faster to dementia than individuals without MCI [36]. The reported prevalence of dementia in HD is highly dependent on the applied diagnostic criteria. Studies found the prevalence of mild cognitive impairment (MCI) in the premanifest HDGEC to be 40% [36] while the prevalence of MCI at onset of motor symptoms was 84% and the prevalence of dementia 5% [35], whereas frequencies of cognitive domain impairments are rarely described

(**Table 3)**. The cognitive impairments may be found up to 15 years before motor onset [26]. Cognitive impairments can be assessed by several neuropsychological tests, but some of the most sensitive tests to evaluate progression are Symbol Digit Modalities Test (SDMT) and Stroop Word Reading Test [19].

Table 3. Frequencies of impaired domains in Huntington's disease

	Premanifest [36] (N=575)	Motor manifest [35] At motor onset (N=19)	> 5 years after motor onset (N=108)
Executive function (%)		47.4	80.6
Processing speed (%)	9.1	47.4	71.3
Language (%)		36.8	63.0
Memory (%)	7.5	42.1	63.0

(Percentage of cognitive domain impairments)

The major psychiatric symptoms observed in HDGECs are depression, anxiety, irritability, and apathy. Personality changes, aggressiveness, disinhibition, perseverations, and psychosis are also frequent manifestations [37]. Frequencies vary in the different studies, most likely due to different assessment methods (**Table 4**). Psychiatric symptoms have been registered up to 10 years before motor onset and the ratings have been found negatively associated with daily functioning [38]. The psychiatric status is most often assessed with Problem Behaviours Assessment short (PBA-s), Hamilton depression scale (HAM-17), and Symptom Checklist 90 revised (SCL-90-R).

Table 4. Frequencies of psychiatric symptoms in Huntington's disease

Psychiatric symptom	Review by Paoli et al. [39]
Depression	9-63%
Anxiety	33-61%
Irritability and aggression	35-73%
Obsessions-compulsions	7-50%
Psychosis	3-11%
Apathy	52-76%

The assessment of disease onset, progression, and registration of treatment response is conventionally performed using the UHDRS. The UHDRS was developed as a clinical rating scale to assess four domains of clinical performance and capacity in HD: motor function, cognitive function, behavioral abnormalities, and functional capacity. The scale includes a Diagnostic Confidence Level Criteria where the clinician evaluates whether the motor symptoms are sufficient, persistent, and with 99% confidence caused by HD [40]. This evaluation is affected by interrater reliability. In general, the determination of disease onset is difficult with often various symptoms and abnormalities developed over several years [40]. The earliest symptoms are often non-motor and they can be even harder to quantify. Large longitudinal studies like PREDICT-HD and TRACK-HD have observed and described the natural disease course and potential biomarkers for HD. These studies find the pace of finger tapping, Symbol Digit Modalities Test (SDMT), Stroop word score, and atrophy of the striatum to be predictors and reliable outcome measures [19,41,42].

In addition to the prominent clinical features described above, HDGECs also suffer from metabolic disturbances. Some of the most common features are weight loss, skeletal muscle wasting, altered function of the peripheral inflammatory cells, sleep disturbances, and autonomic dysfunction [6,43].

The phenotype of HD is characterized by the presence of individually varying clinical expression of involuntary movements, cognitive and psychiatric symptoms. An endophenotype is a biological trait that reliably and quantitatively reflect the function of a biological system. An endophenotype is partly heritable and therefore more closely related to the cause of the disease than the broad clinical phenotype[44,45]. Here, we define the endophenotypes of HD as the three symptom domains that characterize the disease; motor symptoms, psychiatric symptoms, and cognitive impairment which we apply to both the premanifest and the motor manifest HDGECs. Further, there are clearly sub-endophenotypes within each symptom domain. The individual endophenotype can be variable both within the same CAG repeat length and within families. Familial aggregation of, for example, a certain symptom has been found to reflect genetic modifiers [7], while other studies have found even monozygotic twins to differ in endophenotypes [46]. The precise manifestations and their timing seem clearly modifiable, and contributions are attributed to environmental factors, genetic variation, and difference in epigenetic patterns [47,48]. These endophenotypes and their biological basis may be potential treatment targets.

Neuroinflammation

Inflammation is the response of the tissue when exposed to pathogens. The inflammatory mechanisms work to repair, regenerate, and remove damaged tissue or infectious agents. The inflammatory response of the CNS leads to a fast activation of microglia that function similarly to macrophages of the periphery. The microglia undergo phagocytosis, antigen presentation, rapid proliferation, and cytotoxic secretion. They release chemokines to attract more microglia and secrete inflammatory factors to promote further microglia proliferation. When the microglia are activated, they can present antigens to T-cells that have passed the compromised blood brain barrier [49].

In HD, microglia and to a certain degree also astrocytes have been found to play an important role in neuroinflammation [50]. Up to 15 years prior to predicted AOO, microglial activation has been detected by PET imaging [51], and accumulation of reactive microglia in the striatum and cortex of HDGECs has been found [52,53]. The amount of activated microglia has been found to be correlated to the degree of neuronal loss [54]. This early activation of microglia could indicate that the inflammatory process is initiated from the CNS and not by the peripheral immune system. Activation of microglial cells leads to the release of several inflammatory mediators, including tumor necrosis factor-α (TNF- α), interleukin-1 (IL-1), IL-6, IL-12, and nitric oxide. Initially, this response has a protective effect, but an excessive exposure to the proinflammatory mediators is harmful [50,55]. The microglial activation leads to the release of chemokines that will allow for the peripheral T-cells to aggregate to and penetrate the blood brain barrier. If the T-cells then recognize the healthy tissue or register damage, potentially caused by microglia, they can release additional cytokines and chemokines that can cause further damage.

Whether the immune activation is indirectly caused by neuronal cells in degeneration or directly by promotion of cell-autonomous immune activation by the mHTT within the immune cells has not been determined [56].

Investigation of the cerebrospinal fluid (CSF) of HDGECs described upregulation of IL-6, IL-8, and TNF-α. But whether the neuroinflammation in HD is a reactive process or a primary process is still unclear [57].

The function of the peripheral immune system has also been found altered in HD, and several cytokines produced by the innate immune cells are elevated in the blood [58]; the levels of these have been associated with cognitive dysfunction and disease stage [59].

Biomarkers

A biomarker is a marker that can be objectively measured and evaluated as an indicator for a pathogenic process [19]. It can be diagnostic, it can give a more precise time of onset, it can be prognostic and indicate the individual disease progression, it may be more precise in determining disease stages, and it may assess a therapeutic response [60]. In HD, it is possible to identify gene expansion carriers, but biomarkers are crucial to determine disease onset more precisely, assess disease progression, and assess a potential treatment response.

Structural imaging is a robust biomarker of HD [19]. Studies like PREDICT-HD and TRACK-HD have found MRI to show striatal changes more than 15 years before estimated motor onset [19,41]. In HDGECs, less than 10 years from predicted motor onset, SDMT appears to be one of the most sensitive markers for cognitive change, while finger-tapping speed and finger-tapping interval are reliable outcome measures, and apathy ratings correlate best with psychiatric change [19,41,61].

Biomarkers are not necessary for diagnosing HD, and biomarkers should ideally be non-invasive and acceptable for the patient [62]. Therefore, the incentive to look for wet biomarkers especially in CSF have been limited. However, blood, urine, and saliva have been investigated thoroughly for immune markers, metabolic markers, and endocrine markers [62]. The most promising biomarkers at the moment are neurofilament light chain (NfL) measured in blood and mHTT and NfL in CSF. Both biomarkers increase in amount with increasing disease [40,62].In general, the findings need validation and independent replication preferably in larger cohorts.

Oxytocin

Oxytocin is a neuropeptide predominantly produced in the paraventricular and supraoptic nucleus of the hypothalamus. Neurons from these nuclei project to the posterior pituitary where oxytocin is released into the periphery [63]. Oxytocin plays a role in reproduction, childbirth and social bonding [64]. It has also been described to influence social cognition i.e. social recognition, interpretation of emotional expression and social memory [65], which are symptoms seen in HD [28,66]. In HD, a post-mortem study of the hypothalamus, found a reduction in oxytocin producing neurons of 45% compared to controls [67] and another study on the hypothalamus of a HDGEC with no striatal atrophy also showed reduced oxytocin expressing neurons [68]. The latter indicating possible early changes in the oxytocin expressing neurons.

The correlation between oxytocin and social cognition is mainly based on both animal and human studies with administration of oxytocin and observation of increased trust [69,70]. In HD the level of oxytocin has been investigated in plasma. In a pilot study, intranasal administration of Oxytocin in nine HDGECs, was found to affect emotion recognition positively [71] and therefore oxytocin may have a potential as biomarker or therapeutic target in the evaluation and potential treatment of social cognitive impairment.

Hypothesis

The overall research hypothesis is that endophenotypes in HD are partly caused by measurable differences in different wet biomarkers. The specific hypotheses are

1. The endophenotypical drift of the HDGEC is individual.
2. Neuroinflammation is part of the early pathogenesis of HD which is reflected in CSF biomarkers.
3. Non-motor symptoms of HD, especially social cognition, are associated with the level of oxytocin in plasma and CSF.

Aims

The main aim for my Ph.D. study was to perform a follow-up on a well described single site cohort of HDGECs after 5 years, recruit as many new participants as possible and investigate:

1. The endophenotypical drift of the HDGECs
2. The neuroinflammatory CSF imprint in early HD
3. Whether the levels of oxytocin in plasma and CSF differ depending on the non-motor symptoms of HD

Material and Methods

Participants

The participants were recruited as two cohorts; the first cohort, Cohort 2013, was recruited from January 2012 to March 2013. The second cohort, Cohort 2018, was recruited from June 2017 to January 2019. The participants were recruited from the Danish Dementia Research Centre, Rigshospitalet, Copenhagen, Denmark, as well as through adds in the members magazine published by the Danish Huntington's Disease Association.

We recruited premanifest HDGECs, manifest HDGECs, and controls. The controls were family controls who had had 50 % risk of inheriting the HD gene, but were tested gene expansion negative. Some of the participants in Cohort 2018 also participated in Cohort 2013 and became part of the Follow-up cohort. All HDGECs had a CAG repeat ≥ 39. In the studies requiring extended neuropsychiatric examination, the inclusion criteria included a UHDRS total motor score (TMS) ≤55, a Montreal Cognitive Assessment (MoCA) score ≥ 19, and a Mini Mental state Examination (MMSE) ≥ 24. Exclusion criteria were drug abuse, more than 21 units of alcohol per week, and a native language other than Danish. All participants were aware of their own genetic status and had received genetic counseling prior to participation in this study. Written informed consent was obtained from each participant and the study was approved by the Ethics Committee of the Capital Region of Denmark (H-17002606) and meet the requirements of the General Data Projection Regulation (RH-2018-71, I-Suite no: 6690). One hundred and fifty participants were recruited for Cohort 2018. The participants were divided into three groups: 36 HD gene expansion negative family controls, 45 premanifest HDGECs and 69 motor manifest HDGECs. Not all participants participated in, completed all tests, or consented to all biofluid sampling.

Clinical evaluation

All participants were evaluated neurologically, psychiatrically, and cognitively. HDGECs with a UHDRS-TMS of ≤5, were considered motor premanifest, and HDGECs with a UHDRS-TMS >5, were considered motor manifest. We calculated a CAP-score on all HDGECs using the equation CAP = ((CAG – 35.5) * age) from Penney *et al*. [24].
Tests and questionnaires used for the neurological, neuropsychiatric, and social cognitive evaluation are listed below.

Clinical rating scales and questionnaires

	Cognitive screening tools
MMSE	A brief cognitive screening tool that cover orientation, registration, attention and calculation, recall, and language. Scores range from 0-30, and scores >26 are regarded as normal [72].
MoCA	A brief cognitive screening tool developed to screen for mild cognitive complaints. It covers visuospatial and executive function, episodic memory, attention, orientation, and language. Scores range from 0-30 and scores >25 are regarded as normal, an additional point can be added in case of low education level [73].
	Neuropsychiatric tests
HAM -17	A semi structured interview containing 17 items, including, among others, depressed mood, guilt, suicidal ideation, anxiety, hypochondriasis, and insight. Scores range from 0-52. A score between 8-13 is considered to reflect a mild depression and scores >13 indicate a moderate to severe depression [74].
SCL-90-r	A self-report symptom questionnaire to assess current psychopathology. It reflects the patient's current status of perceived distress. It consists of 90 items and each of the items are rated on a five-point Likert scale of distress. Subsequently the answers are divided in to nine symptom dimensions and three global indices. Raw scores are converted in to T-scores and normalized to a Danish sample of individuals without psychiatric symptoms [75,76].
	UHDRS
UHDRS-TMS	The score obtained from the standardized motor rating of HD. It assesses eye movements, dysarthria, motor impersistence,

	dystonia, chorea, motor speed, balance, rigidity, speed, walking, and motor programming. Score range from 0-124, the score 0 is given when no motor symptoms are observed [30].
UHDRS Cognitive	The UHDRS cognitive evaluation includes a test of verbal fluency, and the symbol digit modality test (SDMT) as well as Stroop interference test (color naming, word reading, and interference) to evaluate psychomotor speed and executive function [27,30].
UHDRS Total functional capacity (TFC)	A checklist for common daily tasks. The investigator evaluates the functional capacity within occupation, finances, domestic chores, activities of daily living (eating, dressing, bathing) and care level (need of nurse or chronic care). Scores ranges from 0-13, higher scores indicating higher functioning [30].
	<u>Social Cognition</u>
Reading the Mind in the Eyes (RME)	A test consisting of 36 pictures of eyes expressing different emotional states. Each picture is presented with four words for emotional states and the participant is asked to choose the word that describes the expression of the eyes best. The test reflects Theory of Mind in the participants ability to attribute the mental state of others. Scores range from 0-36, one point per correct answer [77,78].
The Awareness of Social Inference Test (TASIT)	A test consisting of 15 video clips of everyday situations with either sincere or sarcastic interactions. The participant is afterwards presented with four questions about the interactions. The test assesses the ability to interpret paralinguistic and non-verbal cues. Depending on the version of the test, scores range from 0-60 or 0-100. Higher scores indicate better performance [79].
Emotion Hexagon test (EHt)	A test consisting of 30 cards with pictures of faces expressing the six basic emotions: happiness, anger, surprise, fear, sadness, and disgust. The emotions are morphed together so the pictures

represent combinations of these emotions. The participant is presented with the picture and a list of the basic emotions. Scores range from 0-24, one point per correct picture [80].

Non-motor classification of participants

MoCA and MMSE are brief cognitive screening tests. We chose to exclude participants with MoCA ≤ 19, and a MMSE ≤ 24 to ensure a meaningful neuropsychiatric testing. Our goal was not to characterize the entire HD population but to investigate the cognitive deficits in detail over time in early HD. Our classification of cognitive impairment is conservative while more sensitive than MoCA and MMSE, meaning that it is possible to score within the normal range of MoCA and MMSE and still be classified as cognitively impaired. Follow-up participants were not excluded on the basis of a MoCA ≤ 19, and a MMSE ≤ 24, these scores were however regarded as indicator for cognitive impairment.

Psychiatric symptoms were regarded as present if the participant used psychotropic medication on neuropsychiatric indication, if the participant scored higher than a predefined level on either Global Severity Index (GSI) or on more than two symptom dimensions on the SCL-90-r or if the participant scored 13 or more on the Hamilton depression scale.

The cognitive assessment was performed by a trained neuropsychologist. The extensive three to four-hour battery of neuropsychological tests included an assessment of attention, memory and visuospatial functions. The pre-morbid intellectual level was asserted prior to the cognitive evaluation. The specific tests are listed under the condensation of manuscript 1 and in the study by Vinther-Jensen et al [29]. See **Table 5**.

Table 5. Classification and evaluation of participants (table from manuscript 1)

Clinical evaluation	UHDRS total motor score (TMS):	Pre-manifest: TMS ≤ 5 Manifest: TMS > 5
Neuropsychiatric Evaluation - allocation to neuropsychiatric group if at least one criterion is met	1. Usage of psychotropic medication on neuropsychiatric indication 2. A SCL-90-R GSI T-score ≥63 or a T-score ≥63 in more than two of the nine primary symptom dimensions. The SCL-90-R cut-offs are based on SCL-90-R guide 3. A HAM-17 score ≥13 (moderate to severe depression)	
Cognitive Evaluation - Cognitively impaired if A. four or more test performances were impaired B. all tests in a domain (except psychomotor speed/attention) were impaired C. performances on all tests in the psychomotor speed/attention domain, and at least one other test was below cut-off.	Pre-morbid intellectual level: Memory: Psychomotor Speed/Attention: Executive functions: Visuospatial functions:	- Wechsler Adult Intelligence Scale (WAIS) - Danish Adult Reading Test (DART) - Selective Reminding Test - Rey Complex Figure text - Trail Making Test A & B - SDMT - Stroop test (100 items – incongruent)) - Verbal fluency tests (category fluency (animals, 1 min), lexical fluency (s-words and a-words, 1 min)) - Rey Complex Figure - Ravens Progressive Matrices (set 1) - Block Design Test (Modified version)

SCL-90-R – Symptom Checklist-90-Revised
HAM-17 – Hamilton Rating Scale for Depression-17
SDMT – Symbol digit modality test

The test results of the participants were compared to expected scores, that were generated from regression analysis including age, years of education and general verbal intellectual level. The difference between expected and observed scores were used to evaluate the presence of impairment. The criteria for classifying participants as cognitively impaired are listed in **Table 5**.

Blood processing

The description of the blood processing applies for both cohorts.

Part of the blood samples were sent for standard analysis at the Department of Clinical Biochemistry, Rigshospitalet, and the remaining blood samples were deposited into the Danish Dementia Biobank. The samples were processed within one hour of sampling, and EDTA and serum tubes were centrifuged at 2000 g at 4 °C for 10 minutes. Serum and plasma were then aliquoted in 250 µl polypropylene tubes and stored at -80 °C.

CSF processing

The description of the CSF processing applies for both cohorts.

Lumbar punctures were performed between 9 AM and 3 PM without any dietary restrictions. In Cohort 2013, traumatic needles were the only accessible needles while we in Cohort 2018 preferentially used the atraumatic needles. The first 2 ml of CSF were sent to routine analysis at the Department of Clinical Biochemistry at Rigshospitalet while the remaining approximately 10 ml were collected in polypropylene tubes on ice and kept there until centrifugation and subsequent storage in the Danish Dementia Biobank. The cell pellets, obtained after centrifugation, were extracted and used for flow cytometric analysis.

Radioimmunoassay (RIA)

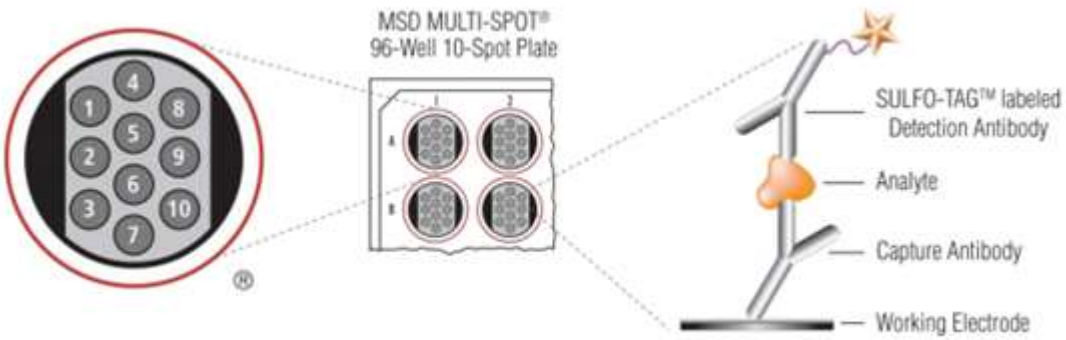
We used RIA to assess the level of the neuropeptide oxytocin in plasma and CSF. RIA is a highly sensitive competitive binding reaction that can quantify very small amounts of hormone levels in a sample like plasma and CSF.

The principle of this method is incubating the participants sample with the peptide specific antibody and then adding a radioactive ^{125}I-bound peptide. The ^{125}I-bound peptide will then compete to bind to the antibodies, and, after further incubation, it will bind to the free antibody sites. After removal of all unbound structures, the radioactivity can be counted as counts per minute measured with a γ-counter. This count can then be translated to peptide concentrations from the standard curve that is prepared concurrently with the samples.

Electrochemiluminescence

The principles of electrochemiluminescence resemble enzyme-linked immunosorbent assay (ELISA). When measuring a specific analyte from a sample, e.g. the level of an interleukin of the CSF, with ELISA, the bottom of a well is coated with a specific antibody for this analyte. The analyte is then added and will attach to the antibody in the bottom of the well. Then another antibody, linked to an enzyme, able to attach to the bound analyte is added. In the last step a substrate is added. The linked enzyme will transform this substrate into a dye and by measuring the absorption of the sample, a concentration of the analyte can be established. Electrochemiluminescence does not use enzymes and substrates, but a label that generates light (SULFO-TAG label) when it is stimulated by electricity (**Figure 1**). Additionally, using the Vplex multi spot assay well makes it possible to register up to 10 antigens simultaneously in a single sample, by placing different antibodies on the different spots. After adding the detection antibody, electricity is applied to the plate and this leads to light emission by the bound SULFO-TAG labels and the intensity is measured and quantified by the Vplex instrument and software, MSD Discovery Workbench®.

Figure 1. Illustration of the concept and principle of the Vplex multi spot assay well



(https://www.mesoscale.com/en/technical_resources/our_technology/our_immunoassays)

The advantage of the method is a high sensitivity, a broad dynamic range, and very low sample volumes.

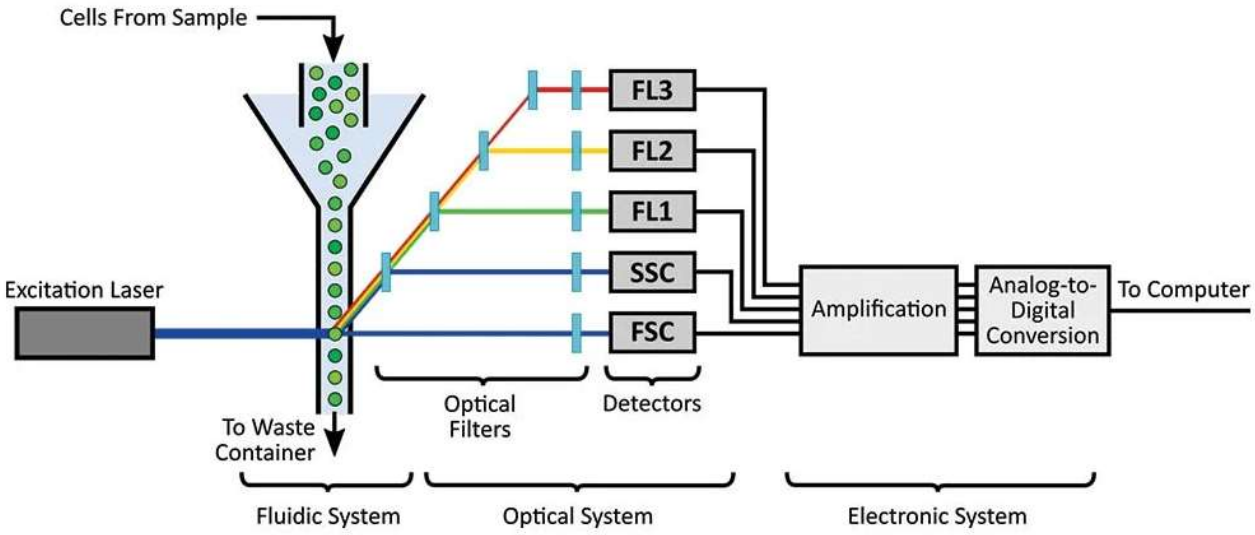
Flow cytometry

The purpose of flow cytometric analysis is identifying the individual cells of a collected sample. The sample, collected from the participant, is added antibodies that are targeting the proteins of

interest and the antibodies are labeled with different fluorescent markers. The antibodies can bind specific proteins either on the surface of the membrane or intracellularly.

The cells are then analyzed in the flow cytometer where they move by a laser beam one by one (**Figure 2)**. The size of the cell is measured by the forward scatter, and the side scatter indicates the cell granularity. These measures are used to differentiate the cells into the different cell types. Fluorescence markers are used to detect the different antigens which makes it possible to identify the different subcellular types on their specific antigen profile.

Figure 2. Schematic of a common flow cytometer, illustrating the fluidic, optical, and electronic systems



(https://www.aatbio.com/resources/assaywise/2019-8-1/fundamentals-of-flow-cytometry)

Statistics

All statistics were performed using SAS Enterprise Guide Version 7.1 and Graph Pad prism. The statistical procedures are described within each manuscript.

Results and Discussion

This Ph.D. is partly a follow-up study on an earlier recruited and described cohort [29], Cohort 2013. The participants of the three papers can originate from both Cohort 2013 and Cohort 2018. In **Table 6**, the distribution, origin, and subgroups of the participants are shown. The demographic data are presented in the individual papers.

Table 6. Number of participants

	Manuscript 1: Endophenotypical Drift in Huntington's Disease – a 5-year follow-up study	**Manuscript 2: Early Intrathecal T Helper 17.1 Cell Activity in Huntington Disease**	**Manuscript 3: Decreased CSF Oxytocin relates to measures of social cognitive impairment in Huntington's Disease patients**
	N=107	**N=173**	**N=146**
Controls (N)	-	**23** (27 samples)	**33**
Cohort 2013		19	20
Cohort 2018		8	13
(N of participants partaking more than once)		(4)	
Premanifest HDGECs	**51**	**46** (51 samples)	**44**
Cohort 2013	51	32	32
Cohort 2018	30 follow-up participants	19	12
(N of participants partaking more than once)		(5)	
Manifest HDGECs	**56**	**60** (70 samples)	**69**
Cohort 2013	56	33	47
Cohort 2018	44 follow-up participants	31	22
(N of participants partaking more than once)		(4)	
PPMS		**37**	
NIND		**7**	

Table 6. The number of participants might not represent the number of samples as some participants contributed with more than one sample. HDGECs: Huntington's Disease gene expansion carriers; PPMS: Primary progressive multiple sclerosis; NIND: Noninflammatory neurological disease

Condensation of manuscripts and relation to international state-of-the art research

Manuscript 1:

Endophenotypical Drift in Huntington's Disease – a 5-year follow-up study

In this study, we performed a follow-up on our previously described cohort of 107 HDGECs, Cohort 2013. We focused on the versatile phenotypes seen in HD, and the presence of non-motor symptoms prior to the motor symptom onset. We also sought to describe the trajectories of the symptoms and examine the possibility to predict the trajectories by age, gender, CAG repeat number, Disease Burden score, education, presence of psychiatric, cognitive, and motor symptoms.

We recruited as many of the HDGECs as possible from Cohort 2013 after an average period of 5.5 years. Six HDGECs from Cohort 2013 had died and 27 did not participate, due to inaccessibility, lack of time, or feeling too ill. The 2013 data on the follow-up data was referred to as Baseline 2013.

The HDGECs were characterized according to their motor, psychiatric, and cognitive symptoms. First, they were divided into two groups based on whether they had motor symptoms or not, the premanifest HDGECs with UHDRS-TMS ≤ 5 and the manifest HDGECs with a UHDRS-TMS > 5. These two groups were further subdivided into endophenotypes. The presence of neuropsychiatric symptoms was evaluated based on HAM-17, SCL-90-r, and whether psychotropic medication had been administered. The evaluation of a cognitive impairment was based on an extensive battery of cognitive tests assessing the premorbid intellectual level, memory, psychomotor speed, and attention, and executive and visuospatial functions (**Table 5**).

The frequencies of the psychiatric and cognitive symptoms in Baseline 2013 and Cohort 2018 differed between the premanifest groups while they were identical in the motor manifest HDGECs (**Table 7)**. As expected the total number of premanifest HDGECs decreases during the five years; however, the percentage of premanifest HDGECs with no symptoms increases which may be caused by the lower total number of premanifest HDGECs giving smaller numbers larger impact, the fact that many of the HDGECs were relatively young when they were recruited in 2013 (median age of 36) and a more proactive treatment algorithm for neuropsychiatric symptoms since 2013. The drift between the endophenotypes is depicted in **Figure 3.**

Table 7. Distribution of the endophenotypes in Baseline 2013 and Cohort 2018

	Premanifest		Motor Manifest	
	Baseline 2013	Cohort 2018	Baseline 2013	Cohort 2018
N	41	30	39	44
Cognitive impairment	9.8 %	3.3 %	23.1 %	25.0 %
Neuropsychiatric symptoms and cognitive impairment	2.4 %	6.7 %	51.3%	47.7 %
Neuropsychiatric symptoms	19.5 %	10 %	12.8 %	15.9%
No symptoms /only motor symptoms	68.3 %	80.0 %	12.8 %	11.4 %

Figure 3. Endophenotypical drift from the Baseline 2013 Cohort to Cohort 2018

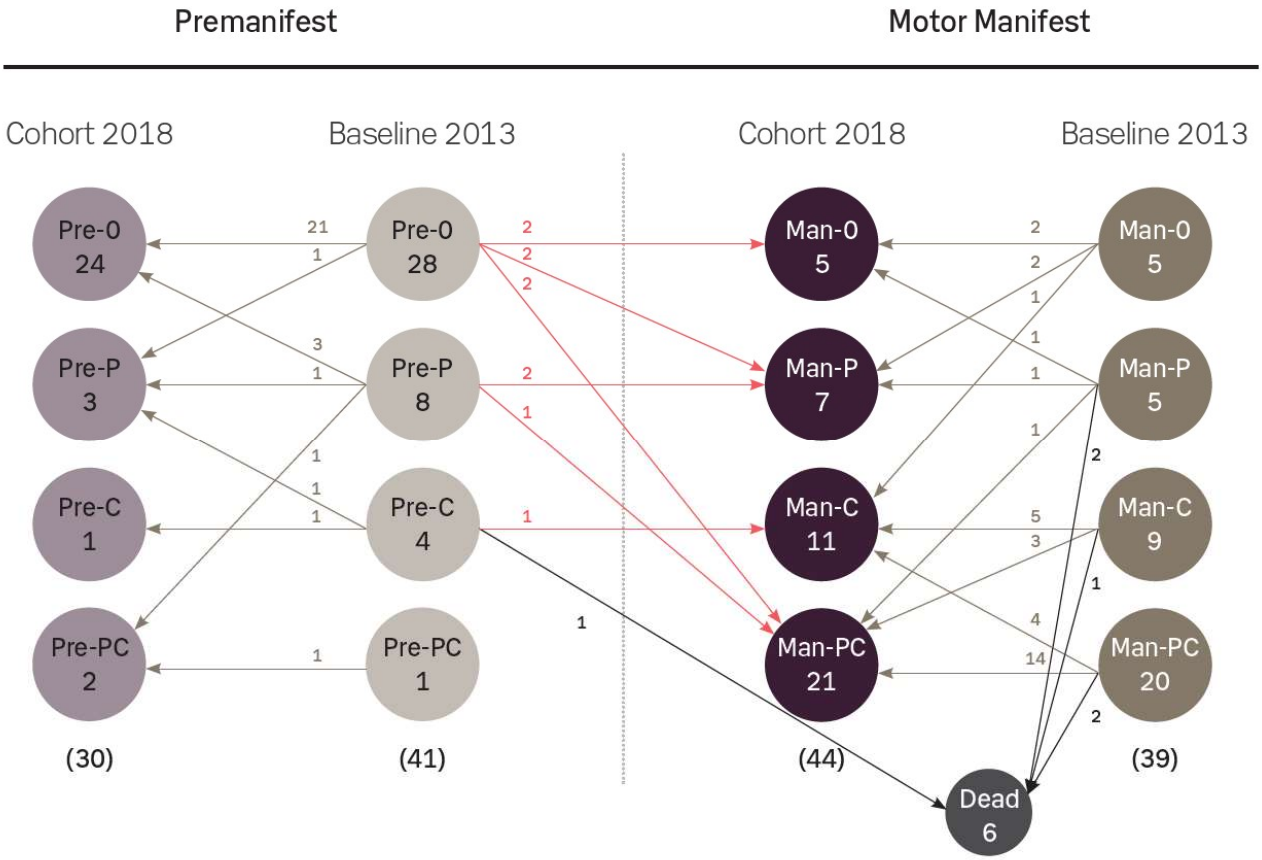


Figure 3 Overview of the follow-up participants and their drift between phenotypes. Right side of the figure represents the Manifest group (subgroups marked by a Man) and the left side the Premanifest group (subgroups marked by a Pre). Both sides are vertically divided into Baseline 2013 and Cohort 2018. The cohorts are horizontally divided in to four subgroups. The zero indicates neither cognitive nor psychiatric symptoms, P means addition of psychiatric symptoms, C addition of cognitive symptoms and PC addition of both cognitive and psychiatric symptoms.
The number indicates the HDGECs in the group and the arrows show the drift from Baseline 2013. (Illustration and legend from Manuscript 1)

We found no clear pattern in the individual drift between the endophenotypes. There was however no drift from motor manifest to premanifest or from cognitively impaired to cognitively intact – the latter, with one exception; a HDGEC affected massively by external factors during the assessment in 2013.

We investigated the endophenotypical groups for potential predictors including age, gender, CAG repeat number, CAP score, education and, if relevant, presence of psychiatric, cognitive, and motor symptoms. As expected, we found the risk of motor onset and development of cognitive impairment to increase with increasing CAP score. We also found that the psychiatric symptoms could evolve or resolve, and therefore may not to the same degree be correlated to the

neurodegeneration. The probability of improving on psychiatric symptoms decreased with age, which could potentially be explained by the expected decrease in brain plasticity with increasing age and the advancing neurodegeneration. In general, the variation in symptoms and phenotypes indicates that it is probably not the CAG repeat expansion alone that explains the endophenotypes seen in HD.

The HDGECs who died during the follow-up period did not differ from the motor manifest HDGECs, whereas the HDGECs that did not participate in the follow-up did not differ demographically from the remaining Cohort 2013. Consequently, it was not a specific subgroup of the HDGECs in Cohort 2013 that represent our follow-up cohort. We consider the unpredictable endophenotypical trajectories observed in this study to represent the general disease course of HD. The progressiveness of HD is well described but hitherto it has not been illustrated on an individual basis. The classical description of the disease course in HD is dominated by the characterization from the large longitudinal studies like PREDICT-HD, REGISTRY, and TRACK-HD where large groups of HDGECs were followed over long periods of time [9,38,41,81–84]. These studies have been instrumental for the understanding of the overall disease course and description of symptoms and biomarkers. The recognition of different endophenotypes has led to a search for genetic modifiers to explain these variations [3], and it is well described that the phenotype of HD changes along with the disease progression [12]. Groups have, however, never been studied longitudinally with focus on the individual level. By highlighting these individual endophenotypes and the unpredictability in progression of symptoms, we recommend regular follow-up to ensure the possibility of identifying and potentially relieving symptoms. In the study of disease progression, it would be ideal to use the ENROLL-HD database to characterize the development of for example psychiatric symptoms. The symptoms are registered yearly, and it would be possible to follow the symptom-drift on an individual basis.

Our research group has previously found the risk of psychiatric symptoms to be modified by a polymorphism in the catechol-O-methyltransferase gene [85]. However, we found the psychiatric symptoms to be potentially resolvable and not completely explained by the neurodegeneration of HD and potentially neither the genetic polymorphisms. We have, however, not clarified the complete psychiatric profile of our cohort and possibly only a subgroup of the HDGECs can improve on their psychiatric symptoms – and maybe we need more sensitive tests for apathy, to confirm previous indications of decreasing psychiatric symptoms with increasing loss of awareness [86], but these are points for further research.

MMSE and MoCA were part of our exclusion criteria. Both MMSE and MoCA are brief cognitive screening tests and have been found to register cognitive impairment even in mild HD [87], MoCA being more sensitively than MMSE. The applicability of the MMSE in early dementia has been debated and its strongest asset is considered to be its ability to rule out dementia in primary care [88]. The overall quality of MoCA has been evaluated in a recent Cochrane review and the author concluded that participants without dementia might score below the traditional cut-point of 26 and suggest the threshold to be lower than 25 points and that it should not be used alone [89]. So MMSE and MoCA are screening tools but not diagnostic in early disease while we are convinced that scores of MoCA $\leq$ 19, and MMSE $\leq$ 24 can be considered diagnostic for cognitive impairment.

Our study is limited by the number of participants and the division of the HDGECs into different endophenotypes decreases the numbers even further. We wanted to observe the individual HDGECs over time and chose to investigate the potential of different objective measures as predictors by regression analysis and register results as odds ratio. We further aimed to find trends that could predict the course of the disease. On the other hand, the strength of our cohort is that it is a single site cohort which ensures consistency in examination and evaluation of tests while it off course reduces the number of participants which may obscure correlations.

Manuscript 2:

Early Intrathecal T Helper 17.1 Cell Activity in Huntington Disease

Immune abnormalities have been suggested to play a role in the pathogenesis of HD. Several studies have investigated the role of the innate immune system and it has been suggested that the mHTT protein in immune cells may cause a proinflammatory effect that could either be part of the pathogenesis or a response to the pathogenesis [58]. The role of the adaptive immune system has not been fully elucidated [90]. We aimed to explore a possible contribution of an inflammatory response in the pathogenesis of early HD. We investigated the CSF of four cohorts consisting of 173 individual participants that contributed 192 samples. The cohorts included HDGECs, HD gene expansion negative family controls, participants with primary progressive multiple sclerosis (PPMS) as inflammatory controls, and participants with non-inflammatory neurological disease (NIND). Cohorts 1 and 4 constituted the cytokine cohorts, one initial and one replication cohort. Cohort 2 constituted the cellular analysis cohort and cohort 3 the cellular analysis replication cohort. Cohorts 2 and 3 were collected and analyzed continually while cohorts 1 and 4 were collected, frozen, and analyzed in groups. The CSF from the cytokine cohorts was added to a V-plex kit of 30 cytokines while the cell analysis cohorts were instantly stained and analyzed by flow cytometry. No participant was represented more than once in the cytokine analysis samples or cellular analysis samples.

The number of cells in the CSF of the controls was <3, in participants with PPMS 8.05 and HDGECs 3.09. The CSF level of protein was 0.37 in both controls and HDGECs. The IgG index was 0.9 in the participants with PPMS and 0.4 in both controls and HDGECs. We found five HDGEC associated cytokines in cohort 1; a significant increase in the concentrations of lymphotoxin-α, IL-17, vascular endothelium growth factor, and a decrease in the concentrations of IL-7 and IL-16, indicating proinflammatory lymphocyte activity intrathecally in HDGECs. There was no difference when dividing the HDGECs into premanifest and motor manifest. To confirm the proinflammatory lymphocyte activity, we investigated the prevalence of T cells and found no differences in the frequency of $CD3^+$, $CD4^+$, and $CD8^+$ expressing T cells, but when focusing on the $CD4^+$ T cells the HDGECs had a significantly increased amount of the Th1, Th17, and Th17.1 subset compared to controls and participants with non-inflammatory neurological disease (**Figure 4**). The HDGECs also had a significantly lower level of regulatory T cells compared to the inflammatory positive controls with PPMS. Distinguishing between premanifest and manifest HDGECs in the cellular analysis we found a significantly increased

frequency of the Th17.1 cells in the premanifest HDGECs (**Figure 5**). We finally investigated a potential correlation between the prevalence of the Th17.1 cells and the CAP score, a measure of progression in HD, UHDRS-TMS and NfL (**Figure 6**). We found a statistically significant negative correlation between the frequency of Th17.1 cells and all three measures, indicating that there is a very early intrathecal involvement of the Th17.1 cells in HDGECs.

Figure 4. The frequency of Th17.1 cells

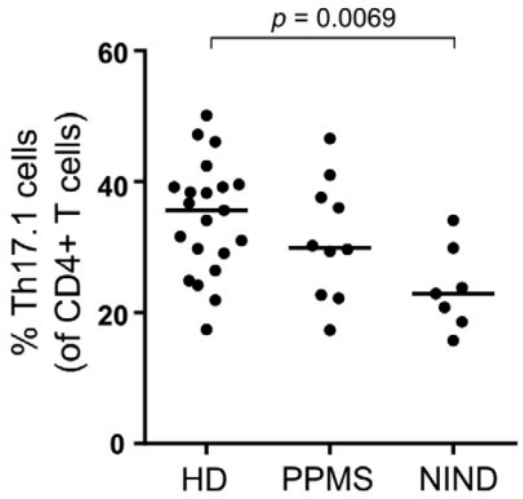
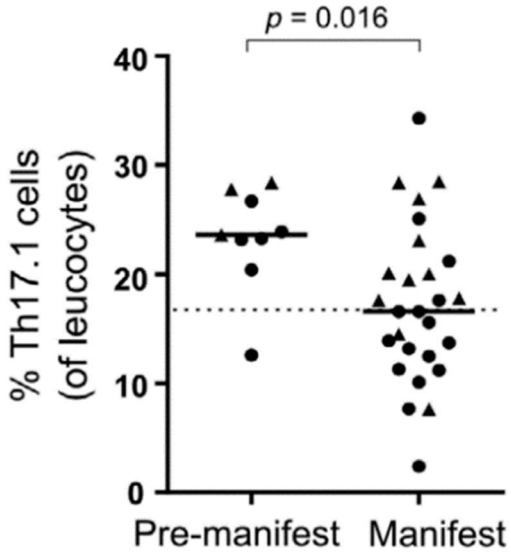


Figure 4. HD: Huntington's Disease; PPMS: Primary Progressive Disease; NIND: Noninflammatory neurological disease (Figure from manuscript 2)

Figure 5. The frequency of Th17.1 cells in premanifest and manifest HDGECs



(Figure from manuscript 2)

Figure 6. Correlation of the intrathecal Th17.1 frequency and NfL (left panel) and the CAP score (right panel)

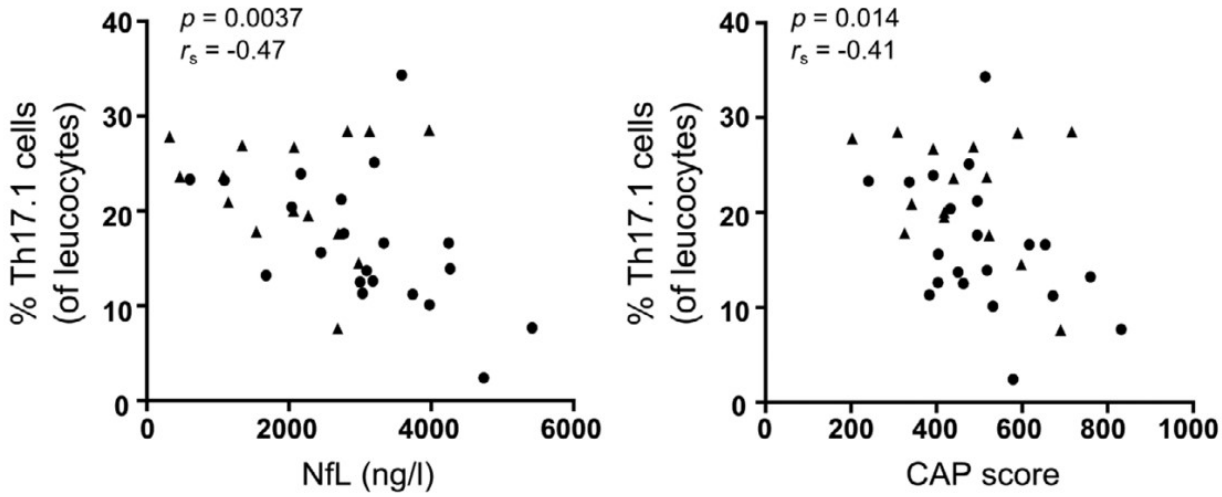


Figure 6. NfL: Neurofilament light; CAP: CAG age product; r_s: the Spearman correlation coefficient (rho). Data from cohort 2 are denoted by circles, and cohort 3 by triangles pointing upward. (Figure from manuscript 2)

The decrease found in the T cell growth factor IL-7 and $CD4^+$ T cell growth factor IL-16 in HDGECs compared to controls corresponds well with an increased consumption of IL-7 and IL-16 as part of lymphocyte activation [91]. The increased prevalence of Th17.1 cells found in HDGECs was registered prior to motor onset indicating a role for these cells in the early pathogenesis of HD. It might even suggest that the increased frequency of Th17.1 cells mediates the disease rather than it just being a side effect of the disease, i.e. primary inflammation. Th17.1 cells have been associated with inflammatory diseases like multiple sclerosis, and they express the highest level of the adhesion molecule very late antigene-4 (VLA4) [92]. The effect of the anti-VLA4 antibody, used for treatment of multiple sclerosis, has been considered to be due to the exclusion of Th17.1 cells from the CNS [92]. This study therefore indicates that early treatment of HDGECs may delay development of symptoms. Treatment strategies targeting the entrance of Th17.1 cells into the CNS could be considered.

We therefore propose a treatment strategy with e.g. natalizumab, resulting in a less penetrable blood brain barrier for the T cells, in particular the Th17.1 cells that we find overrepresented in the premanifest HDGECs compared to controls. We consequently hypothesize that the neuroinflammation including the microglia activation will ameliorate and hopefully be reflected

in a later age at onset and a milder phenotype. First a replication study that confirms the findings of this study should be performed and then clinical trials to uncover potential side effects, safety, timing, and duration of treatment and whether the treatment should target specific endophenotypes.

The significance of inflammation in the pathogenesis in HD is continuously being explored. Reinhart *et al.* developed *ex vivo* models and found a neuroprotective effect of inhibiting the CXCR3 receptor, a chemokine receptor primarily located on the surface of T helper cells, the IκB kinase complex, which is part of the cellular response to inflammation, and the c-Jun N-terminal kinase, a kinase implicated in apoptosis and T cell differentiation [93]. These findings provided evidence of an ongoing inflammatory process in HD and of potential drug targets. Treatment trials targeting inflammation have also been initiated, animal studies have explored the effect of nonsteroidal anti-inflammatory drugs but with inconsistent results [56]. In humans, clinical trials with the second generation, semi-synthetic tetracycline antibiotic, Minocycline, was found to exert an anti-inflammatory and anti-apoptotic effect by crossing the blood brain barrier and reducing the inflammation related to microglial activation. However, different trials resulted in conflicting results [56,94,95]. Recently, the LEGATO-HD, phase 2 study on motor manifest HDGECs, administering a small active molecule, Laquinimod, which was expected to upregulate brain-derived neurotrophic factor and thereby modulate inflammatory pathways in the CNS [96], was discontinued because it did not meet its primary endpoint of change from baseline of the UHDRS-TMS [97]. In relation to this study, this raises the question whether the treatment was perhaps administered too late.

Manuscript 3:

Decreased CSF oxytocin relates to measures of social cognitive impairment in Huntington's Disease patients

The psychiatric and cognitive challenges of HD, the non-motor symptoms, are of great importance for the HDGECs and their caregivers. Some of the most intrusive impairments found in early HD are impaired abilities to recognize faces, interpret emotional expression in faces, and understand and relate to other people's beliefs and intentions [26]. These abilities are part of social cognition and enable interhuman interaction. The neuropeptide oxytocin has been described to influence these cognitive abilities in both animal and human studies, but only few studies have investigated this correlation in HD, and none in CSF of HDGECs. Post-mortem studies on HDGECs revealed reduced oxytocin expressing neurons in the hypothalamus where most of the endogenous portion of oxytocin is produced. In one HDGEC case with no measurable cerebral atrophy, reduced expression of oxytocin suggesting early changes of these neurons was described [68].

We assessed the levels of oxytocin in the CSF and plasma of HDGECs and compared them to controls to examine whether there was a correlation between oxytocin levels in HDGECs and disease progression, or an association between the oxytocin levels and tests for social cognition.

We combined the two cohorts, Cohort 2013 and Cohort 2018, evaluated the participants clinically and neuropsychologically, and classified them into endophenotypes as described in Manuscript 1. Where possible, we examined first-time lumbar punctures and corresponding blood samples. The levels of oxytocin were determined by RIA in plasma and CSF, and the social cognitive abilities were quantified on the tests of social cognition, TASIT, RME, and EHt.

We investigated the levels of oxytocin in CSF and plasma of 113 HDGECs and 33 HD gene expansion negative family controls. The evaluation of the abilities within social cognition required was part of the extended neuropsychological examination and only participants with MMSE≥ 24 and MoCA≥ 19 were evaluated. The association between oxytocin levels and scores on tests of social cognition was investigated in 89 HDGECs.

The levels of oxytocin were 33.5 % lower in HDGECs compared to the controls (p=0.016). The difference was statistically significant when comparing the motor manifest HDGECs and the controls (p=0.01), but not when comparing the premanifest and the controls (p=0.12) (**Figure 7A**). To explore whether the level of oxytocin simply decreased with increasing Disease Burden,

we assessed the potential correlation between the level of oxytocin and the CAP-score and found a non-significant decrease in oxytocin with increasing CAP-score. We then subdivided the HDGECs depending on the presence of cognitive impairment and psychiatric symptoms. The cognitively impaired HDGECs had a significantly lower level of oxytocin than both controls and cognitively normal HDGECs (**Figure 7B**), while the difference between the controls and cognitively intact HDGECs was non-significant. Comparing the HDGECs with psychiatric symptoms to HDGECs without psychiatric symptoms and the controls revealed no difference (**Figure 7C**). When investigating social cognition, we found a significant association between the level of oxytocin and EHt, TASIT, and RME (**Figure 8**).

Figure 7.

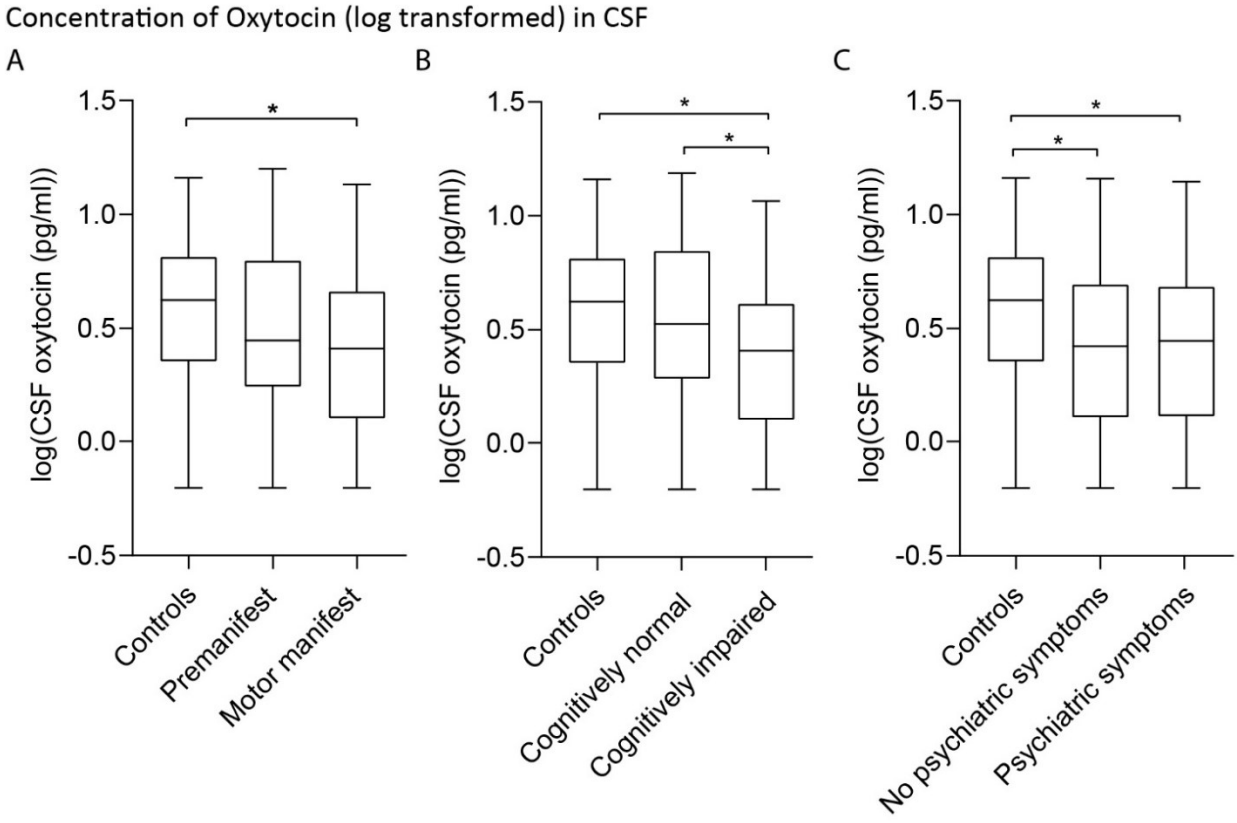


Figure 7. The CSF oxytocin concentrations (logarithmically transformed), in HDGECs divided according to symptom presentation. A) controls (N:33), premanifest Huntington's disease gene expansion carriers (HDGECs) (N:44) and motor manifest HDGECs (N:69), (p= 0.01); B) controls, HDGECs classified as cognitively normal (N:52) and HDGECs classified as cognitively impaired (N:59), significant difference in level of oxytocin between controls and cognitively impaired HDGECs (p= 0.002) and between HDGECs classified as cognitively normal and HDGECs classified as cognitively impaired (p= 0.046) and C) controls, HDGECs classified as not having psychiatric symptoms (N:56) and HDGECs classified as having psychiatric symptoms (N:55), There were

significant differences in oxytocin levels between controls and HDGECs classified as not having psychiatric symptoms (p= 0.02) and controls and HDGECs classified as having psychiatric symptoms (p= 0.04). The median and 2.5 % and 97.5 % percentiles are shown for all groups. * indicate significant differences. (Figure from manuscript 3)

Figure 8.

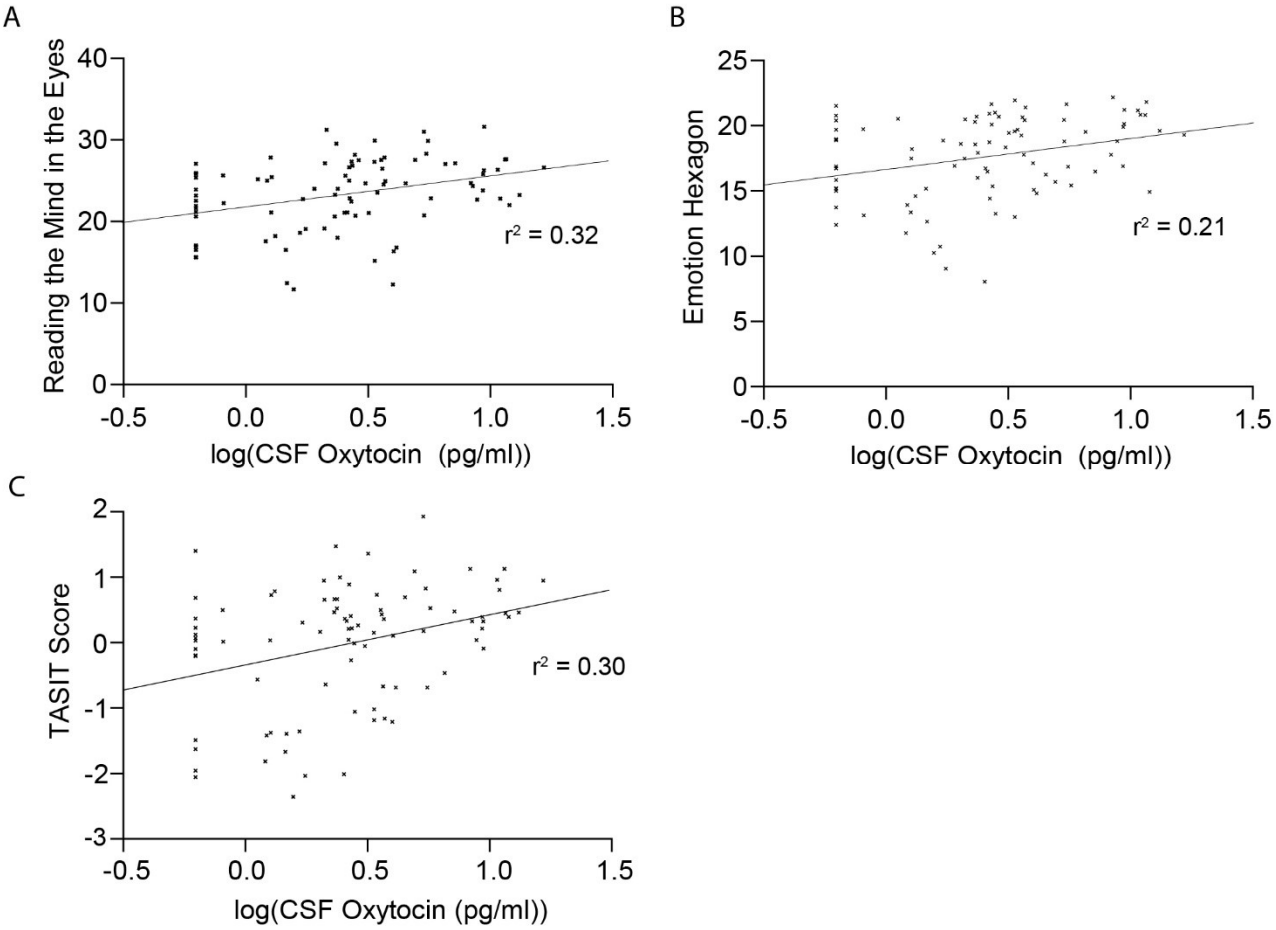


Figure 8. The associations between CSF oxytocin concentrations (logarithmically transformed) in Huntington's Disease gene expansion carriers and the neuropsychiatric scores on Reading the Mind in the Eyes (A)(p=0.0019), Emotion Hexagon (B)(p=0.0062) and TASIT, Z-score (C)(p=0.002). All three graphs are corrected for age and gender and show the association for a male aged 45. (Figure from manuscript 3)

The correlation between oxytocin and social cognition was explored already in the late 1970's and 1980's in animal trials [98] and was established in 2005 with Kosfeld *et al.* finding an association between intranasal administration of oxytocin and an increase in trust among humans [99]. Subsequently, several studies of oxytocin and social cognition have been investigated [98].

Despite numerous clinical trials, there are still conflicting results on intranasal oxytocin treatment and several unanswered questions among which are questions regarding e.g. dosage of oxytocin, timing of dosage, behavioral outcome measure, and effect by sex and age [100]. There is a need for larger studies with participants who are affected similarly in the social-behavioral domain and a systematic approach [100].

Our results emphasize the correlation between the level of oxytocin and cognitive symptoms which has also been observed in other diseases with impaired cognition e.g. frontotemporal dementia [101,102]. Furthermore, we found a significant association between the level of oxytocin and all measures of social cognition. This is the first study to link CSF levels of oxytocin to social cognition in HDGECs. We consequently suggest routine evaluation of social cognition in HDGECs to identify and acknowledge these potential impairments which also opens the potential for treatment trials to evaluate the effect of oxytocin on social cognition in HD, an excellent candidate disease for a systematic trial approach.

Conclusions and perspectives

Despite considerable scientific attention towards HD, no cure for this devastating disease has been found and only less efficient symptomatic treatment is available. We wished to break down the classical view of HD into endophenotypes which might elucidate new targets of treatment.

In 2013, a thorough characterization of a cohort of HDGECs in regard to the presence of motor, cognitive and psychiatric symptoms was performed. To obtain meaningful neuropsychological data, a comprehensive 3-4-hour testing battery was applied, which required that the participants had to be cognitively relatively well-functioning. MMSE and MoCA were part of our exclusion criteria and our choice of cut-off naturally has implications for our results. Cohort 2013 is a cohort of premanifest to moderately affected HDGECs as are the participants of cohort 2018. These cohorts enable the characterization of the early pathogenesis of HD.

The classification of the participants into the different endophenotypes is overall conservative as we wanted to ensure that our participants characterized as asymptomatic were indeed without motor, psychiatric, and cognitive symptoms.

We illustrated the unpredictable course of HD on an individual level and we found no demographic variables, except Disease Burden score, to predict the disease course. We highlighted that the psychiatric symptoms are not necessarily permanent in the early disease course. Despite HD being caused by a single gene CAG repeat expansion, the individual disease course and associated consequences in everyday life can vary markedly. The variability in symptoms are presumably caused by different modifiers, including environmental factors and genetic variants. However, there is yet no complete answer to the development of and drift between endophenotypes.

The implication of applying different endophenotypes was in our opinion justified by the finding of an increased frequency of Th 17.1 cells in early disease stages. This suggests a potential effect of early treatment of HDGECs with immunomodulatory therapies e.g. the anti-VLA4 monoclonal antibody natalizumab which may delay the motor onset. Plasma levels of IL-6 and IL-1ra have been found to associate with cognitive dysfunction in HD and in other neurodegenerative diseases like Alzheimer's disease, and an association between cognitive dysfunction and the cytokines IL-6 and TNF- α has been reported [59]. Reducing the inflammatory process of HD might therefore even delay the aggravation of the cognitive impairment along with delaying motor symptoms. Furthermore, we found a correlation between

being classified as cognitively impaired and the level of oxytocin. We even found an association between the oxytocin level and scores on tests for social cognition. Studies on psychiatric and neurodegenerative diseases like schizophrenia and frontotemporal dementia and investigations of trust and empathy have shown an effect of exogenous oxytocin on social cognitive abilities [103], and we therefore suggest our study to be replicated in a larger cohort, and to consider the inclusion of social cognition tests in the evaluation of HDGECs and in treatment trials as well.

This Ph.D. has described the endophenotypes of our cohorts, correlated early motor symptoms to markers of inflammation and the cognitive endophenotype to the level of oxytocin. It will be even more interesting to follow-up in a longitudinal study. Overall, our studies included a relatively limited number of participants and much data was generated; however, results were statistically corrected accordingly, and the participants are a selected segment of the HD population as we recruited through direct contact, telephone, or email, through advertisement in patient magazines and word of mouth method and therefore we probably recruited the more resourceful HDGECs and the HDGECs less affected by cognitive and psychiatric symptoms.

There is still no cure for HD, and most recently the intrathecally administered antisense oligonucleotide phase 3 trial (GENERATION HD1)[104–106] was discontinued. While research regarding development of a cure for HD is ongoing, we suggest to also focus on individual therapy based on the individual endophenotypes. An observational study by McAllister *et.al* emphasizes the importance of understanding the timing and impact of the motor, psychiatric, and cognitive symptoms to improve targeted therapy and thereby preserve normal brain functions [107]. Acknowledging non-motor symptoms challenges the classical categorization of HDGECs into premanifest and motor manifest HDGECs. Considering our as well as prior research, dividing HDGECs into groups based on the presence of cognitive impairment or psychiatric symptoms should be investigated. Even more so applying subdivision into more specific groups of cognitive impairment or psychiatric symptoms may reveal correlations and treatment targets not identified previously because of low sample sizes within the groups of either premanifest or motor manifest HDGECs.

I had the privilege to follow-up a single site large group of HDGECs as well as a group of family HD gene expansion negative controls and in the future, we aim to perform regular follow-ups of Cohort 2013 and Cohort 2018 and recruit new participants. We will investigate the continued endophenotypical drift, collect blood and CSF samples for further elucidation of the role of

neuroinflammation both on a group level but also longitudinally, and search for more biomarkers of disease, symptoms, and hopefully treatment response.

During this Ph.D. study, in the search for more biomarkers, we have also generated saliva, plasma and CSF based proteomics data from the two cohorts. The proteomics data will be analyzed and correlated to the different endophenotypes, potentially help explain the drift of the endophenotypes and detect targets for functional analyses in our patient-derived induced pluripotent stem cell (iPSC) models, which have also been developed during my Ph.D. study. The iPSCs will be further differentiated into relevant neuronal cellular models, including medium spiny neurons and astrocytes as well as microglia, hopefully establishing more patient relevant models and targets for future investigations.

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RESEARCH

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Endophenotypical drift in Huntington's disease: a 5-year follow-up study

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Abstract

Background: Huntington's disease (HD) is clinically characterized by progressing motor, cognitive and psychiatric symptoms presenting as varying phenotypes within these three major symptom domains. The disease is caused by an expanded CAG repeat tract in the huntingtin gene and the pathomechanism leading to these endophenotypes is assumed to be neurodegenerative. In 2012/2013 we recruited 107 HD gene expansion carriers (HDGECs) and examined the frequency of the three cardinal symptoms and in 2017/2018 we followed up 74 HDGECs from the same cohort to describe the symptom trajectories and individual drift between the endophenotypes as well as potential predictors of progression and remission.

Results: We found higher age to reduce the probability of improving on psychiatric symptoms; increasing disease burden score ((CAG-35.5) * age) to increase the risk of developing cognitive impairment; increasing disease burden score and shorter education to increase the risk of motor onset while lower disease burden score and higher Mini Mental State Examination increased the probability of remaining asymptomatic. We found 23.5% (N = 8) to improve from their psychiatric symptoms.

Conclusions: There is no clear pattern in the development of or drift between endophenotypes. In contrast to motor and cognitive symptoms we find that psychiatric symptoms may resolve and thereby not entirely be caused by neurodegeneration. The probability of improving from psychiatric symptoms is higher in younger age and advocates for a potential importance of early treatment.

Keywords: Huntington's disease, Endophenotype, Psychiatric symptoms, Cognitive symptoms

Background

Huntington's disease (HD) is an autosomal dominant, progressing neurodegenerative disease. Clinically, the disease is characterized by a triad of symptoms: motor disturbances, neuropsychiatric manifestations and cognitive impairment. The clinical HD diagnosis relies on unambiguous extrapyramidal motor symptoms that cannot be explained by any other movement disorder even though both cognitive and psychiatric symptoms often precede this by years [1]. The motor symptoms in HD typically debut in the fourth or fifth decade of life and gradually worsen over 10–20 years until death. The presence of non-motor symptoms has gradually been more accepted as early symptoms of HD and become targets of disease modulating treatments; however, the pathways leading to the different clinical endophenotypes within HD are still not fully understood. Several findings suggest that both neuropathology, environmental stress and genetic variants other than the causative CAG repeat expansion in the Huntingtin gene contribute to the occurrence of cognitive impairment and neuropsychiatric symptoms [2–4]. The understanding of the development

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and assessment of these non-motor symptoms needs to be exact to correctly ascertain the degree and progression of both cognitive and psychiatric symptoms. Several longitudinal studies have been carried out with focus on symptom development in premanifest Huntington's disease gene expansion carriers (HDGECs); however, these studies have described the different symptom trajectories on a group level.

The classical presentation of motor symptoms consists of involuntary movements, chorea, which begins early in the disease course and gradually the hyperkinetic movements become more slow and akinesia and rigidity evolve [1], and this motor dysfunction strongly correlates with the volume of the striatum and white matter [5]. The typical cognitive deficits are mental slowing, decreased attention, flexibility, planning, visuospatial functions and emotion recognition [5]. Cognitive impairment is thought to be caused by dysfunction of the frontostriatal circuits due to gradual degeneration of the caudate nucleus [6], however, studies on the evolvement of cognitive impairment in HD are inconclusive. In the TRACK-HD study, the impairment was measured years before motor onset and significant progression of the decline across 36 months in the premanifest HDGEC was reported [7] while other studies have found the rate of cognitive change in HDGECs to be similar to that in non-carriers [8].

Psychiatric symptoms including depression, irritability, apathy, perseveration, obsession/compulsion and psychosis are described as more variable than the cognitive and motor symptoms and little or no correlation has been described between structural imaging and psychiatric symptoms [5]. Cross sectional studies have shown HDGECs to have a lifetime prevalence between 33% and 76% of one or more of these symptoms [4]. One longitudinal study even showed that 99% of 111 HDGECs had neuropsychiatric symptoms occurring during a 3 year follow-up period [9]. While psychiatric symptoms have been found to worsen over time, the most striking single psychiatric feature demonstrating clear longitudinal progression, significantly associated with cognitive and functional decline, is apathy [7], and subtle psychiatric symptoms have been described more than 10 years before expected motor onset [10]. The psychiatric symptoms were reported to progress with disease severity and thereby likely to be part of the neurodegenerative disease process [11], but no longitudinal studies have described the progression or remission of symptoms on the individual level.

We have previously proposed a clinical classification that embraces the HDGECs with non-motor-symptoms [12] and here we describe the individual endophenotypical drift of symptoms during an average of 5.5 and a minimum of 4.5 years in a HDGEC follow-up cohort, and potential predictors of progression and remission.

Materials and methods

From January 2012 to March 2013 we recruited 107 HDGECs (Cohort 2013) from the Neurogenetics Clinic, Danish Dementia Research Centre, Rigshospitalet, Copenhagen, Denmark. All were examined by a physician and a neuropsychologist and evaluated regarding motor symptoms, neuropsychiatric symptoms, and cognitive impairment. All individuals were confirmed HDGECs and had a Unified Huntington's Disease Rating Scale-99, (UHDRS-motor) total motor score ≤ 55, a Mini Mental State Examination (MMSE) score ≥ 24, and a Montreal Cognitive Assessment (MoCA) score ≥ 19 [13].

From June 2017 to January 2019 we followed-up as many as possible from Cohort 2013. The group of participants who was part of Cohort 2013 and participated again in the present follow-up study is referred to as either Cohort 2018 when their follow-up values are described or Baseline 2013 cohort when their data from 2013 is described.

We were able to follow-up 74 participants and characterized the endophenotypical drift of these participants.

The study was approved by the Ethics committee of the Capital region of Denmark (H-17002606) and written informed consent was obtained from each participant before enrollment.

Clinical evaluation

The UHDRS was used for evaluation of motor signs in both cohorts [14]. A UHDRS-motor score >5 classified as motor manifest and a UHDRS-motor score of 5 or less as premanifest HDGEC. The participants were assessed with UHDRS Total Functional Capacity (TFC), UHDRS Function scales and short Problem Behaviors Assessment (PBA-s). Previous and present medical and psychiatric history was recorded.

Neuropsychological testing and classification

To exclude participants who were not eligible for the neuropsychological tests, all participants were screened with MMSE and MoCA. Participants who, as part of the 2018 cohort participation, scored ≤ 24 on MMSE and ≤ 19 on MoCA were classified as cognitively impaired and not candidates for neuropsychological testing.

In the neuropsychological assessment memory, psychomotor speed/attention, executive functions and visuospatial functions were examined. The applied tests are listed in Table 1. The normative data for these tests was extracted from a database at the Department of Neurology, Rigshospitalet, University of Copenhagen

Table 1 Classification and evaluation of participants

Clinical evaluation	**UHDRS total motor score (TMS)**	
	Pre-manifest: TMS ≤ 5	**Manifest: TMS > 5**
Neuropsychiatric evaluation *Allocation to neuropsychiatric group if at least one criterion is met*	1. Usage of psychotropic medication on neuropsychiatric indication 2. A SCL-90-R GSI T-score ≥ 63 or a T-score ≥ 63 in more than two of the nine primary symptom dimensions. The SCL-90-R cut-offs are based on SCL-90-R guide 3. A HAM-17 score ≥ 13 (moderate to severe depression)	
Cognitive evaluation *Cognitively impaired if*	*Pre-morbid intellectual level:*	Wechsler Adult Intelligence Scale (WAIS) Danish adult reading test (DART)
A. four or more test performances were impaired	*Memory:*	Selective reminding test Rey complex figure text
B. all tests in a domain (except psychomotor speed/attention) were impaired	*Psychomotor speed/attention:*	Trail making test A & B SDMT
	Executive functions:	Stroop test (100 items — incongruent)) Verbal fluency tests (category fluency (animals, 1 min), lexical fluency (s-words and a-words, 1 min))
C. performances on all tests in the psychomotor speed/attention domain, and at least one other test was below cut-off	*Visiospatial functions:*	Rey complex figure Ravens progressive matrices (set 1) Block design test (modified version)

Classification of participants into groups of premanifest and manifest HD gene expansion carriers and evaluation of neuropsychiatric symptoms and cognitive impairment

SCL-90-R Symptom Checklist-90-Revised, *HAM-17* Hamilton Rating Scale for Depression-17, *SDMT* Symbol digit modality test

from 80 age-matched healthy subjects. For further details see Vinther-Jensen et al. [13] and Table 1.

Neuropsychiatric assessment and classification

Neuropsychiatric symptoms were assessed by the Symptom Checklist-90-Revised (SCL-90-R) and the Hamilton Rating Scale for Depression-17 (HAM-17). The SCL-90-R is a questionnaire with 90 items reflecting current psychological symptoms rated by the Likert scale of distress, ranging from "not at all" to "extremely". The 90 items are divided into nine primary symptom dimensions: somatization (SOM), obsessive–compulsive (O-C), interpersonal sensitivity (I-S), depression (DEP), anxiety (ANX), hostility (HOS), paranoid anxiety (PHOB), paranoid ideation (PAR) and psychoticism (PSY). The SCL-90-R also provide three global indices of distress: Global Severity Index (GSI), Positive Symptoms Distress Index (PSDI), and positive symptoms Total (PST). The scores from the questionnaire can be converted to T-scores normalized to a Danish sample of non-psychiatric individuals sorted by gender; higher T-scores indicating more psychiatric distress.

The HAM-17 was applied as a semi-structured interview covering 17 symptom areas and participants were allocated to the neuropsychiatric group as indicated in Table 1.

Data analysis

Comparing the premanifest and manifest HDGECs, we analyzed the demographic data, age, gender, CAG repeat, Disease Burden score ((CAG−35.5) * age) [15] and results from SDMT and MoCA and MMSE using Mann–Whitney U test for the continuous data and Chi square test or Fischer's exact test for the categorial data.

We compared the scores obtained in the neuropsychiatric and neuropsychological tests between the premanifest and manifest HDGECs of Cohort 2018. The comparisons were based on t-test.

We investigated the endophenotypical groups for predictors of potential drift towards progression, regression or stability in symptoms. We examined all observed endophenotypical drifts for effects of age, gender, CAG repeat number, Disease Burden score, education and, if relevant, presence of psychiatric, cognitive and motor symptoms. We used logistic regression analysis and effects are presented as Odds Ratios (OR). The analyses were conducted in SAS Enterprise Guide version 7.1 and p values of 0.05 or less were considered significant.

Results

Cohort 2013 included 107 HDGECs and Cohort 2018 74 HDGECs (Table 2). Six HDGECs from Cohort 2013 had died and 27 did not participate in Cohort 2018. We were not able to get into contact with 7 Cohort 2013

Table 2 Demographic data

	Premanifest HD gene-expansion carriers	Motor manifest HD gene-expansion carriers	Level of significance *p* values	Premanifest HD gene-expansion carriers	Motor manifest HD gene-expansion carriers	Level of significance *p* values
	Cohort 2013			Cohort 2018		
Number	51	56		30	44	
Gender m/f	30/21	33/23	0.32	17/13	25/19	1.0
Age at examination (years)	36 (20–54)	50 (24–75)	< 0.001	40.5 (30–60)	53.5 (29–80)	0.0002
CAG repeat length	42 (39–48)	43 (40–53)	< 0.38	41 (39–48)	43 (40–53)	0.0052
Disease Burden scores	234.0 (108.0–437.0)			241.3 (133.0–437.5)		
UHDRS-motor (score)	2 (0–5)	21 (6–51)	< 0.001	1 (0–5)	32 (7–91)	< 0.0001
TFC (score)	13 (11–13)	10 (4–13)	< 0.001	13 (11–13)	8 (0–13)	< 0.0001
MMSE (score)	29 (26–30)	28 (24–30)	< 0.001	29.5 (27–30)	28 (20–30)	< 0.0001
MoCA (score)	28 (25–30)	25 (19–30)	< 0.001	30 (23–30)	25 (12–30)	< 0.0001

All data is presented as median (range)

Disease Burden score ((CAG–35.5) * age)

HD Huntington's disease, *m/f* male/female, *TFC* total function capacity, *MMSE* Mini Mental state examination, *MoCA* Montreal Cognitive Assessment

participants and the remaining 20 either felt too ill, did not have the time or did not respond after receiving information about the project.

In Cohort 2018, 30 of the participants were premanifest and 44 were motor manifest. The motor manifest HDGECs were significantly older than the premanifest HDGECs, had longer CAG repeats, lower scores on the TFC, the MMSE and MoCA.

Cohort 2018

Cognitive symptoms

In Cohort 2018, 72.7% of the motor manifest and 10% of the premanifest HDGECs were cognitively impaired according to our criteria.

Neuropsychiatric symptoms

In Cohort 2018, 16.7% of the premanifest HDGECs and 63.6% of the manifest HDGECs were classified as having psychiatric symptoms.

We found 13.3% of the 30 premanifest HDGECs (4 participants) to be treated with psychotropic medication and one of these had neuropsychiatric symptoms according to the SCL-90-R (Fig. 1). Among the 86.7% who did not use psychotropic medication one had neuropsychiatric symptoms according to the SCL-90-R. In the motor manifest group (Fig. 1), 36.4% did not use psychotropic medication and none of these HDGECs had neuropsychiatric symptoms according to SCL-90-R. 63.6% were treated with psychotropic medication and 25% of these had neuropsychiatric symptoms according to SCL-90-R.

Comparing the neuropsychiatric symptoms of the motor manifest and the premanifest on the SCL-90-R and HAM-17 we found 7 out of 13 parameters to be significantly higher in the motor manifest group. After Bonferroni correction we only found a significant difference on the symptom dimensions O-C and DEP (Table 3).

Endophenotypes

The distribution of the endophenotypes in the two cohorts is illustrated in Fig. 2.

The premanifest HDGEC endophenotype distribution in the two cohorts differ; in both groups the participants with no symptoms (Pre-0) form the largest group and the group classified with psychiatric symptoms (Pre-P), the second largest. In Cohort 2018, the participants classified with both psychiatric and cognitive symptoms (Pre-PC) form the third largest group which was the smallest group in Cohort 2013, and hence, the group classified with cognitive impairment (Pre-C) is the smallest in Cohort 2018.

The endophenotype distribution of the motor manifest participants were very alike in Cohort 2013 and Cohort 2018. Approximately half in both cohorts had a combination of both neuropsychiatric and cognitive symptoms (Man-PC). The second largest group are the participants classified with cognitive impairments (Man-C) followed by the group of HDGECs classified with neuropsychiatric symptoms (Man-P) and HDGECs with only motor symptoms (Man-0).

In the motor manifest group, we found older age, male gender, higher CAG repeat number and Disease Burden score to increase the risk of being classified as having both psychiatric symptoms and cognitive impairment, although not statistically significantly. There was a significant risk of being classified with

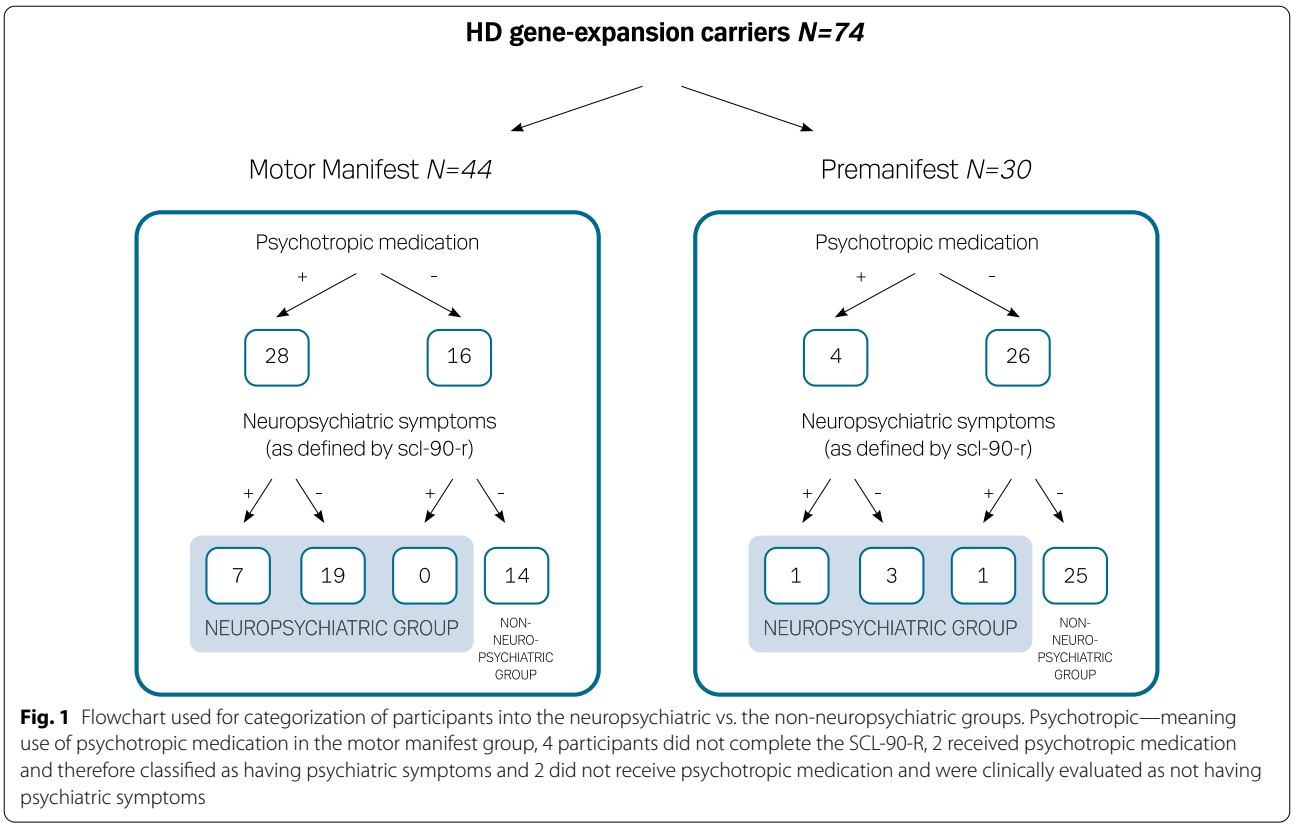

Fig. 1 Flowchart used for categorization of participants into the neuropsychiatric vs. the non-neuropsychiatric groups. Psychotropic—meaning use of psychotropic medication in the motor manifest group, 4 participants did not complete the SCL-90-R, 2 received psychotropic medication and therefore classified as having psychiatric symptoms and 2 did not receive psychotropic medication and were clinically evaluated as not having psychiatric symptoms

both neuropsychiatric symptoms and cognitive impairment with higher UHDRS motor score (OR = 1.041; $p = 0.0175$) and lower scores for the following, TFC (OR = 0.756; $p = 0.0103$), MMSE (OR = 0.676; $p = 0.0175$) and MoCA (OR = 0.786; $p = 0–0126$). In the premanifest group female gender, higher CAG repeat number, higher Disease Burden score, higher UHDRS motor score and lower TFC, MMSE and MoCA increased the risk of being classified as having cognitive impairment, neuropsychiatric symptoms or both; However, only the lower TFC (OR = 0.060; $p = 0.0284$) was significant.

Endophenotypical drift

As illustrated in Fig. 3 there is no clear pattern in the individual endophenotypical drift between the two cohorts; 21 premanifest HDGECs remain asymptomatic, 10 premanifest HDGECs become motor manifest, 11 HDGECs are classified as having psychiatric symptoms and 8 are no longer classified as having psychiatric symptoms. Six HDGECs become classified as having cognitive impairment, one participant moves from being classified as cognitively impaired to being regarded as cognitively intact and 6 HDGECs die.

Psychiatric symptoms

The proportion of participants classified as having psychiatric symptoms in Cohort 2018, Pre-P, Pre-PC, Man-P and Man-PC, see Fig. 3 (44.6%), equals the proportion in the Baseline 2013 cohort (42.5%). Twenty-two percent of the premanifest Baseline 2013 cohort participants and 64% of the manifest Baseline 2013 cohort participants have been classified as having psychiatric symptoms.

Eleven (23.5%) HDGECs changed from having no psychiatric symptoms to being classified with psychiatric symptoms during the mean of 5.5 years. We found no significant risk factors for predicting this symptom development. The 8 (23.5%) HDGECs who recovered from being classified as having psychiatric symptoms show that the higher the age, the lower probability for improving on the classification of having psychiatric symptoms (OR = 0.892; $p = 0.0303$). Of the 8 who recovered from being classified as having psychiatric symptoms, 1 had a positive Hamilton-17, 7 had SCL-90-R registered symptoms, 6 were using psychotropic medication on neuropsychiatric indication. Five were motor manifest and 3 premanifest and 4 were classified as having cognitive impairment. Their mean age was 35.8 years, mean CAG repeat number 43 and Disease Burden score 321.2.

Table 3 Neuropsychiatric symptoms and neuropsychological test performance in the premanifest and the motor manifest gene-expansion carriers

	Premanifest HD gene-expansion carriers N = 30	Motor manifest HD gene-expansion carriers N = 44	Level of significance *p* values
SCL-90-R			
Global severity Index	42.5 (9.7)	49.4 (11.0)	0.0083*
Positive symptoms total	42.8 (9.2)	48.3 (10.2)	0.0232*
Positive Symptoms Distress Index	44.1 (12,6)	52.4 (14.8)	0.0165*
Somatization	46.2 (9.2)	48.9 (9.9)	0.2385
Obsessive–compulsive	42.3 (10.3)	51.1 (10.6)	0.0009*
Interpersonal sensitivity	43.0 (9.9)	46.9 (10.7)	0.1218
Depression	42.7 (9.1)	50.3 (10.6)	0.0024*
Anxiety	43.7 (9.3)	49.2 (10.3)	0.0252*
Anger-hostility	46.9 (10.3)	49.2 (11.8)	0.3918
Phobic anxiety	45.6 (6.4)	51.6 (11.5)	0.0075*
Paranoid ideation	43.7 (8.3)	46.7 (10.1)	0.1945
Psychoticism	44.9 (7.5)	49.2 (11.1)	0.0525
Hamilton-17	2.0 (1.8)	3.2 (3.5)	0.07
	N = 30	**N = 41**	
Trail a (s)	20.0 (6.7)	53.4 (31.5)	< 0.0001*
Trail b (s)	45.7 (15.5)	140.6 (102.3)	< 0.0001*
SDMT (number of correct)	53.1 (10.3)	29.9 (14.5)	< 0.0001*
Stroop interference (sec)	109.3 (24.9)	181.5 (96.4)	0.0003*
Category verbal fluency	24.2 (4.3)	16.6 (6.8)	< 0.0001*
Lexical fluency, s-words	17.4 (4.7)	10.2 (6.2)	< 0.0001*
Lexical fluency, a-words	11.3 (4.2)	7.4 (3.9)	0.0004*
Reys complex figure test, recall	24.6 (7.1)	18.1 (5.8)	0.0003*
Reys complex figure test, copy	35.3 (1.1)	32.4 (4.5)	0.001*
Block design (number correct)	11.9 (0.4)	11.1 (1.9)	0.03*
Raven's advanced progressive matrices	10.0 (2.0)	6.7 (2.1)	< 0.0001*
Selective reminding test, immediate recall (no of errors)	8.4 (5.5)	31.1 (18.9)	< 0.0001*
Selective reminding test, delayed recall	8.4 (1.1)	6.1 (2.2)	< 0.0001*

HD Huntington's disease, *SCL-90-R* Symptom Checklist-90-Revised, *SDMT* Symbol Digit Modality test; Data shown as median (SD); *p* values were calculated by independent sample t-test; After Bonferroni correction only obsessive–compulsive and depression remained significant

Cognitive impairment

Among the participants who participated in both Cohort 2013 and Cohort 2018, the proportion of participants classified as being cognitively impaired was in 2013 12.2% of the premanifest HDGECs and 74.4% of the manifest HDGECs. In Cohort 2018 we found 10% of the premanifest and 72.7% of the manifest HDGECs to be classified as having cognitive impairments.

Increasing Disease Burden score (OR = 1.019; $p = 0.0132$) was a significant risk factor for a change in classification from normal cognition to cognitive impairment.

Motor symptoms

Fifty-two percent of Cohort 2013 was motor manifest and 59.5% of Cohort 2018 was motor manifest. Twenty-four percent (N = 10) of the premanifest HDGECs who participated in both Cohort 2013 and Cohort 2018 changed status from premanifest to motor manifest during the 5.5 years (see Fig. 3).

We found a significantly increased risk of becoming motor manifest with increasing Disease Burden score (OR = 1.014; $p = 0.0112$) and shorter education (OR = 3.968; $p = 0.0060$).

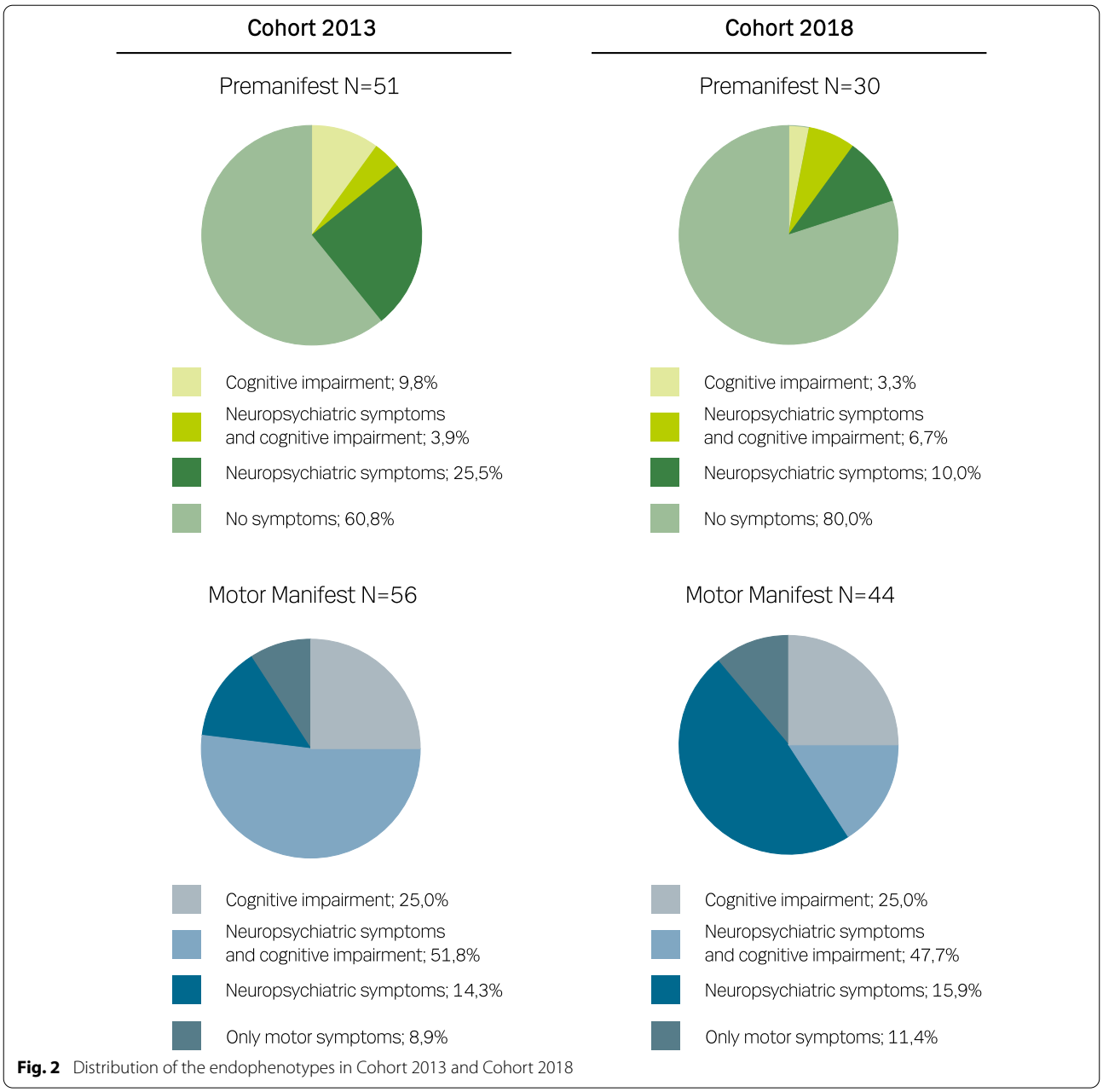

Fig. 2 Distribution of the endophenotypes in Cohort 2013 and Cohort 2018

HDGECs remaining asymptomatic

Fifty-one-point two percent (N = 21) of the premanifest HDGECs were still asymptomatic after 5.5 years. When comparing these 21 HDGECs to the 7 asymptomatic HDGECs who during the follow-up period were classified with psychiatric, cognitive or motor symptoms, we find them to have significantly shorter CAG repeat numbers ($p = 0.0295$), lower Disease Burden score ($p = 0.0086$) and higher MMSE score ($p = 0.0007$). Comparing them to all the premanifest HDGECs we found a significant increased probability of remaining asymptomatic with lower Disease Burden score (OR = 0.985; $p = 0.0209$), higher SDMT score (OR = 1.203; $p = 0.0358$) and longer education (OR = 2.422; $p = 0.0211$).

The deceased HDGECs

Of Cohort 2013, 5.6% have died. Five motor manifest and one premanifest, who did not differ from the motor manifest HDGECs neither in age, UHDRS or Disease Burden score (data not shown). We do not know the exact cause of death, but unsurprisingly we find the risk

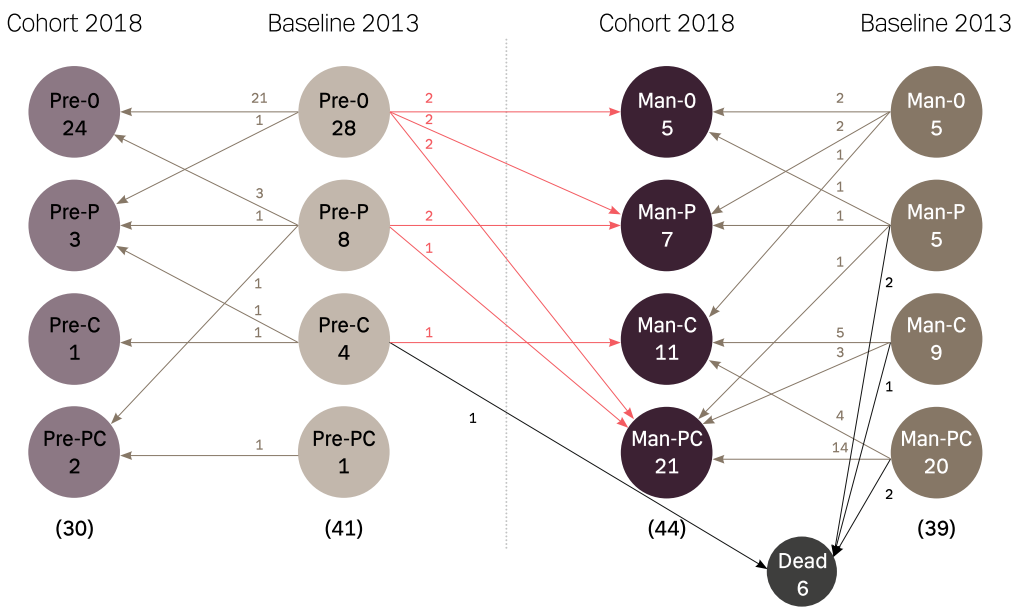

Fig. 3 Endophenotypical drift in the Baseline 2013 Cohort to Cohort 2018. Overview of the follow-up participants and their drift between phenotypes. Right side of the figure represents the Manifest group (subgroups marked by a Man) and the left side the Premanifest group (subgroups marked by a Pre). Both sides are vertically divided into Baseline 2013 and Cohort 2018. The cohorts are horizontally divided in to four subgroups. The zero indicates neither cognitive nor psychiatric symptoms, P means addition of psychiatric symptoms, C addition of cognitive symptoms and PC addition of both cognitive and psychiatric symptoms. The number indicates the HDGECs in the group and the arrows show the drift from Baseline 2013

of dying to increase significantly with the increase of age (OR = 1.089; $p = 0.0181$) and Disease Burden score (OR = 1.009; $p = 0.0193$).

Participants lost to follow-up

Twenty-seven participants were not part of Cohort 2018, 10 premanifest and 17 manifest HDGECs. They did not differ significantly demographically from the remaining participants from Cohort 2013 (see Additional file 1). The 10 premanifest HDGECs, who did not participate in the follow-up study, represented 38.5% of the Pre-P group from Cohort 2013, 20% of the Pre-C group, 50% of the Pre-PC and 9.6% of the Pre-0 group. The 17 manifest HDGECs represent 37.5% of the Man-P in Cohort 2013, 35.7% of Man-C and 27.6% of the Man-PC.

Discussion

We describe a single site follow-up study of HDGECs over an average period of 5.5 years and emphasize the individual variability in the drift between clinical endophenotypes of HD. The clinical endophenotypes are based on a comprehensive assessment battery of the participants and this along with the relatively large patient group makes this study unique. We confirm an increased risk of motor onset and development of cognitive impairments with increasing Disease Burden [16]. Increasing neurodegeneration has been reported with increasing Disease Burden [5] and a correlation between cognitive symptoms and white matter atrophy has also been shown [17] which is in line with our findings of increasing Disease Burden with increased risk of onset of motor and cognitive symptoms. Furthermore, our study highlights the unpredictability of the psychiatric symptoms, however, we are not able to point to a causality of these symptoms. We observe that the psychiatric symptoms may evolve and resolve; the only significant predictor being increasing age which reduces the probability of improving from the psychiatric symptoms. The decreasing potential of psychiatric improvement with increasing age may, however, reflect the progressive neurodegeneration and presumed decreased brain plasticity in HD [18].

Neurodegenerative diseases like Parkinson's disease and Alzheimer's disease also encompass more than one clinical endophenotype. In Parkinson's disease there have been identified different genetic variants for different endophenotypes with a different treatment response and prognosis [19]. We found the phenotypes in HD to change over time, not only like the more recent

understanding of the disease progression in HD with psychiatric symptoms and cognitive impairment starting years before onset of motor symptoms and all three in constant aggravation. We found, however, the phenotype more versatile; some participants debut with pure motor symptoms while others debut with the full triad of symptoms and the psychiatric symptoms are very variable during the disease course. This makes it unlikely that the endophenotypes are explained by the CAG repeat expansion variant alone. Genetic polymorphisms affect age of onset as well as cognitive impairment and psychiatric symptoms [3], but also environmental factors, like physical activity and alcohol consumption, have been found to modulate onset and progression of HD [20] and pharmacological treatment can affect the different symptoms as well [21].

In Alzheimer's disease the symptoms progress within a spectrum of cognitive impairment that expands from normal to end-stage dementia, which has been linked to the biological spread of the disease [22]. In HD we also see a spread of pathology with the earliest changes seen in the Caudate nucleus and the Putamen as loss of medium spiny neurons, followed by white matter loss around the striatum, corpus callosum and posterior white matter tracts and then a more pronounced and widespread grey matter loss [7, 23–25]. While the motor symptoms are correlated to the changes in the striatum, the mood and behavioral symptoms of HD have been suggested to reflect the changes in the limbic circuit [26] and dysfunction in the basal-ganglia-thalamocortical loops [27].

Describing the trajectories of the major symptom domains in our study we, as expected, find the number of motor manifest participants increased during the 5.5 years. The association, that we confirm, between motor symptoms and Disease Burden score is well established, but environmental factors has also been described to affect age of onset [28] which could be in line with our finding of the risk of motor symptom onset to increase with lower education. But whether it is in fact higher education that protects against progression by increasing the plasticity of the brain [29] or the correlation reflects that a longer CAG repeat facilitates a lower education is not known.

The proportion of participants classified as being cognitively impaired also increased during the follow-up period, 40.5% in the Baseline 2013 cohort and 47.3% in Cohort 2018. The increase in frequency of participants classified as having cognitive impairment is in accordance with previous studies [5]. However, one premanifest HDGEC drifted from being classified as cognitively impaired to not being cognitively impaired. This participant developed psychiatric symptoms during the 5.5 years of follow-up. Clinical records show, that this participant in 2013 was affected by external stress factors at the time of testing, which has likely affected the performance on cognitive tests. Our classification is based on a thorough examination that evaluates the individual participant, but there will still be some participants, estimated 5%, with incorrect cognitive classification.

The frequency of participants classified as having psychiatric symptoms in Cohort 2018 (44.6%) equals the distribution among the Baseline 2013 cohort (42.5%), despite the expected progression in symptoms over time. While 11 HDGECs drifted to the group being classified as having psychiatric symptoms, we found that 8 HDGECs recovered from being classified as having psychiatric symptoms. The improvement in psychiatric symptoms can be a result of pharmacologic treatment but after ended treatment the symptoms are still absent which is surprising in the context of psychiatric symptoms supposed to be caused by neurodegeneration [30]. A longitudinal study investigating psychiatric symptoms in prodromal HD found psychiatric symptoms to increase with progression of the disease based on companion reports showing worsening of symptoms in the HDGEC, while the lack of reported worsening of symptoms could be explained by decreasing awareness in the HDGEC [11]. Another study found apathy to correlate with the underlying disease process, while depression only tended to correlate with the proximity to the clinical onset [31]. In our cohorts, the participants who improved on their psychiatric status are from both the premanifest and manifest group, none of them were apathetic clinically nor according to the PBA-s. Using their mean CAP scores [32] we estimated them to be approximately 6 years from expected motor onset, none of the premanifest participants being cognitively affected or having lost disease awareness.

As a consequence of this being a follow-up study, we expected our HDGECs in Cohort 2018 to be older than in Cohort 2013; in both cohorts the premanifest HDGECs were significantly younger and had significantly lower UHDRS motor scores and higher TFC, MMSE and MoCA scores. In Cohort 2018, the premanifest HDGECs had significantly shorter CAG repeats than the manifest HDGECs, which is in accordance with the premanifest HDGECs who become motor manifest have significantly higher Disease Burden score ($p=0.0047$) and significantly longer CAG repeats ($p=0.022$) than the premanifest HDGECs who remain premanifest. Twenty-five percent of Cohort 2013 were not able to participate in this follow-up study; however, they did not differ significantly in demographics compared to the Baseline 2013 cohort. Cohort 2013 consisted of premanifest to moderately affected HDGECs and Cohort 2018 will be somewhat predefined, and therefore our results cannot be

compared to cross sectional studies. This limitation does, however, not inflict on the unpredictability of HD or the importance of a thorough examination of the HDGECs to clarify their endophenotype to optimize treatment and support.

Conclusion

We describe the individual unpredictability in the progression of symptoms in HD as seen in Fig. 3. Therefore, we recommend regular follow-up where symptoms can be identified and potentially relieved.

More than one fifth of the HDGECs who were classified as having psychiatric symptoms in the Baseline 2013 cohort improved from their symptoms. Six of these participants were using psychotropic medication at baseline and none at follow-up. If the symptoms were only to be caused by neurodegeneration, we would expect them to last and worsen after ended pharmacological treatment. However, other studies suggest that the psychiatric symptoms often disappear as the disease progresses because of increasing lack of disease awareness [11], which does not seem to be the case in our study. We found the probability of improving from psychiatric symptoms to be significantly higher with younger age suggesting a potential importance of early treatment of psychiatric symptoms in younger individuals.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13023-021-01967-2.

Additional file 1. Demographic data on the lost to follow-up participants.

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Authors' contributions

All authors have been involved in the planning and conduction of the study, as well as the analysis and interpretation of data. MNNH and RKH have primarily been responsible for the clinical data. All authors have been engaged in drafting the article and revising it critically for important intellectual content. All authors have read and approved the final manuscript.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study has been approved by the ethics committee of the Capital region of Denmark (H-17002606) and written informed consent was obtained from each participant before enrollment.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

Supplementary material

SUPLEMENTARY
Table A
Demographic data on participants not able to participate in the follow-up cohort (data from 2013) and the participants who was part of the follow-up cohort (marked with grey), the participants who had died are not included.

	Premanifest HDGECs N=10 (19.6%)	Premanifest HDGECs N=40	P-value (Kruskal-Wallis)	Manifest HDGECs N=16 (28.6%)	Manifest HDGECs N=34	P-value (Kruskal-Wallis)
Gender (m/f)	7/3	22/19		9/7	20/14	
Age in 2013	36.5 (20-49)	36 (24-54)	0.72	52.5 (35-71)	49.5 (24-75)	0.49
CAG repeat	42.5 (40-47)	41.5 (39-48)	0.42	42 (40-48)	43 (40-53)	0.62
Disease Burden score (Penney)	243.5 (110-437)	229.8 (108-378)	0.56			
UHDRS motor score	1.5 (0-4)	2 (0-5)	0.98	25 (11-39)	17.5 (6-41)	0.11
TFC	13 (11-13)	13 (11-13)	0.94	10 (7-13)	10 (4-13)	0.49
MMSE	29 (28-30)	30 (26-30)	0.09	28 (24-29)	28 (25-30)	0.20
MoCA	28.5 (26-30)	28 (25-30)	0.84	26 (24-30)	25 (20-30)	0.33
No psychiatric or cognitive symptoms (N)	3 (9.7%)	28		0	5	
Psychiatric symptoms group (N)	5 (38.5%)	9		3 (37.5%)	4	
Cognitive impairment group (N)	1 (20%)	4		5 (35.7%)	9	
Cognitive and psychiatric group (N)	1 (50%)	1		8 (27.6%)	21	

Data presented as median and range
Number of HDGECs in the different classification groups are followed by their percentual share in their original group.

Manuscript 2.

Early Intrathecal T Helper 17.1 Cell Activity in Huntington Disease

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Objective: Huntington disease (HD) is an autosomal dominantly inherited neurodegenerative disorder caused by a CAG repeat expansion in the huntingtin *(HTT)* gene. No disease-modifying therapy exists for the treatment of patients with HD. The purpose of this study was therefore to investigate early disease mechanisms that potentially could be used as a target therapeutically.
Methods: Lymphocyte activity in cerebrospinal fluid (CSF) from 4 cohorts of *HTT* gene expansion carriers (n = 121 in total) and controls was analyzed by techniques based on flow cytometry and enzyme-linked immunosorbent assays.
Results: The data of this study provide evidence of immune abnormalities before motor onset of disease. In CSF of *HTT* gene expansion carriers, we found increased levels of proinflammatory cytokines, including IL-17, and increased consumption of the lymphocyte growth factor IL-7 before motor onset of HD. In concordance, we observed an increased prevalence of IL-17–producing Th17.1 cells in the CSF of *HTT* gene expansion carriers, predominantly in pre–motor manifest individuals. The frequency of intrathecal Th17.1 cells correlated negatively with progression of HD and the level of neurodegeneration, suggesting a role of Th17.1 cells in the early disease stage. We also observed a skewing in the balance between proinflammatory and regulatory T cells potentially favoring a proinflammatory intrathecal environment in *HTT* gene expansion carriers.
Interpretation: These data suggest that Th17.1 cells are implicated in the earliest pathogenic phases of HD and suggest that treatment to dampen T -cell–driven inflammation before motor onset might be of benefit in *HTT* gene expansion carriers.

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Huntington disease (HD) is an autosomal dominantly inherited neurodegenerative disease characterized by motor, psychiatric, and cognitive symptoms.[1] The disease is caused by an expansion of >39 CAG repeats in the coding sequence of the huntingtin (*HTT*) gene[2]; however, the pathogenesis of HD is still unclear. Accumulating data indicate immune abnormalities in HD, possibly due to a proinflammatory effect of the expanded HTT protein in immune cells.[3] Some studies have described increased peripheral immune activation in HD, and have observed a relationship between plasma levels of various cytokines and cognitive dysfunction as well as disease stage.[4,5] These findings indicate a potential role of immune activation in the pathogenesis of HD. To investigate the possible contribution of an inflammatory response in HD, we measured intrathecal cytokine synthesis and lymphocyte subsets in *HTT* gene expansion carriers, gene expansion negative controls as a negative control group, and patients with primary progressive multiple sclerosis (PPMS) as a positive control group.

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Subjects and Methods

Population Characteristics, Cerebrospinal Fluid Samples, and Ethics Committee Approval

Cerebrospinal fluid (CSF) samples from 4 cohorts of *HTT* gene expansion carriers and controls were collected as previously described[6]; see Table 1 for demographic information of included participants. The distribution of age and sex did not differ in the groups studied (Table 1). Motor-manifest *HTT* gene expansion carriers were defined by a Unified Huntington's Disease Rating Scale-99 (UHDRS) total motor score > 5.[6] The healthy controls included were *HTT* gene expansion–negative family members. CSF sampling of *HTT* gene expansion carriers and healthy controls was performed at the Neurogenetics Clinic, Danish Dementia Research Center at Rigshospitalet in Copenhagen. CSF samples from patients with PPMS and noninflammatory neurological disease (NIND) were obtained at the Danish Multiple Sclerosis Center, Department of Neurology, Rigshospitalet, Copenhagen, Denmark, using the same protocol for sampling. All included individuals were followed at Rigshospitalet, which is a national, tertiary referral center. The study was approved by the Ethics Committee of the Capital Region of Denmark (H2-2011-085 and H-16047666), and written informed consent was obtained from each participant before enrollment.

Cytokine and Neurofilament Light Chain Measurement

We measured the following cytokines and chemokines using the V-PLEX human cytokine 30-PLEX kit according to the manufacturer's instructions (Meso Scale Diagnostics, Rockville, MD): interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40/IL-23, IL-12p70, IL-13, IL-15, IL-16, IL-17A, CC chemokine ligand (CCL)-2, CCL-3, CCL-4, CCL-11, CCL-13, CCL-17, CCL-22, CCL-26, granulocyte-monocyte colony–stimulating factor, interferon (IFN)-γ, CXC chemokine ligand-10, lymphotoxin (LT)-α, tumor necrosis factor-α, and vascular endothelium growth factor (VEGF). Of these 30 cytokines, 21 were within the level of detection in the CSF samples. Neurofilament light chain (NfL) in the CSF was measured as described previously.[7] Cytokines and NfL were measured in duplicate.

Flow Cytometric Analysis of Freshly Isolated Cells

Ten milliliters of CSF was collected in polypropylene tubes on an ice bath and immediately centrifuged for 10 minutes at 400 × *g* to separate cells from CSF. CSF cells were then instantly stained and analyzed by flow cytometry. Cell staining was performed in phosphate-buffered saline/2% fetal bovine serum/0.02% NaAzide and a combination of fluorochrome-conjugated antibodies (Abs) against CD3 (APC, PE/Cy7, APC/Cy7; UCHT1), CD4 (PerCP/Cy5.5; RPA-T4), CXCR3 (AF488, PE/Cy7; G025H7), CCR6 (PerCP/Cy5.5, BV605; G034E3), CD49d (BV605; 9F10), CD25 (PE; M-A251), and CD127 (BV421; A019D5) all from BioLegend (San Diego, CA), and CD4 (APC/AF750; S3.5) from Invitrogen (Waltham, MA). Data were acquired on a FACS Canto II flow cytometer (BD Biosciences, CA, San Jose, CA), and data analysis was performed using the software FlowJo (Tree Star, Ashland, OR).

Intracellular Cytokine Analysis of CSF Cells

CSF cells collected as described above were stimulated with 10ng/ml phorbol myristate acetate (PMA) and 0.5μg/ml ionomycin in RPMI/5% human AB serum/penicillin/streptavidin (50U/ml; Invitrogen) for 30 minutes, after which 5μg/ml brefeldin A was added to the cell culture and the cells were further incubated for 5.5 hours at 5% CO_2, 37°C (all from Sigma-Aldrich, St Louis, MO); total duration of stimulation was 6 hours. Cells were then stained with Live/Dead stain (Life Technologies, Carlsbad, CA) and fluorochrome-conjugated Abs against CD3 (FITC; UHCT1; BioLegend) and CD4 (APC/AF750; S3.5; Life Technologies). Cells were then fixed and permeabilized using Fixation Buffer and Permeabilization Wash Buffer from BioLegend, according to the manufacturer's protocol. Finally, cells were intracellularly stained with fluorochrome-conjugated Abs against IFN-γ (PE/Cy7; B27) and IL-17A (APC; BL168), both from BioLegend. Data were acquired on a FACS Canto II flow cytometer, and data analysis was performed using the software FlowJo.

Statistical Analysis

Comparisons of cytokine concentration levels and cell population frequencies were performed by the Kruskal–Wallis and post hoc Dunn's test for multiple comparisons or the Mann–Whitney *U* test. For cytokine analysis, *p* values were false discovery rate (FDR)-corrected for multiple comparisons (*q* value) to take into account that 21 cytokines were measured. Association between disease burden and level of cytokine or cell subset was assessed by a Spearman rank correlation analysis with correction for age where appropriate. As a measure of disease burden, the CAG age product (CAP) score calculated according to CAG repeat number and age CAP = (CAGn − 33.66) × age,[8] the UHDRS motor score,[9] and the time since motor onset calculated as years since UHDRS motor score > 5 were used. A significance level < 0.05 was considered statistically significant.

Results

Proinflammatory Lymphocyte Activity in the CSF of HTT Gene Expansion Carriers

To investigate whether the immune system is activated intrathecally in HD, we compared the level of 21 cytokines in the CSF from 38 *HTT* gene expansion carriers with the level in 19 healthy gene expansion–negative controls. As a positive control, 27 patients with PPMS were included (cohort 1; Table 1). For all 21 cytokines, a Kruskal–Wallis test of the 3 groups was performed and the *p* values were corrected for multiple testing by FDR correction (*q* values). A post hoc test was performed on cytokines with a *q* value <0.05. As expected, the CSF concentration of several of these 21 cytokines differed between patients with PPMS and the healthy controls (Table 2). In *HTT* gene expansion carriers, we found a decrease in the level of the lymphocyte growth factor IL-7 compared to the healthy controls; a decrease was also observed in patients with PPMS (Fig 1A). Likewise, we observed an increase in

TABLE 1. Demographic and Clinical Characteristics

Characteristic	n	Age, yr, Mean (Range)	Age, p[a]	Sex, F/M	Sex, p[a]	NfL,[b] Mean (Range)
Cohort 1: cytokine analysis						
HTT gene expansion carriers	38	46 (25–68)	—	19/19	—	1,483 (232–3,353)
Gene expansion negative controls	19	40 (26–62)	0.24	8/11	>0.99	405 (214–1,174)
Patients with PPMS	27	49 (39–61)	0.59	17/10	0.50	738 (287–2,056)
Cohort 2: cellular analysis						
HTT gene expansion carriers	21	53 (39–73)	—	9/12	—	3,064 (609–5,419)
Patients with NIND	7	44 (37–63)	0.16	6/1	0.16	471 (161–1,169)
Patients with PPMS	10	60 (49–65)	0.35	4/6	>0.99	704 (408–1,595)
Cohort 3: cellular replication cohort						
HTT gene expansion carriers	15	46 (32–64)	—	10/8	—	2,054 (321–3,970)
Gene expansion negative controls	8	49 (31–61)	0.94	6/2	0.42	356 (188–669)

HTT Gene Expansion Carriers	n	UHDRS Motor Score Mean (Range)	Time from Motor Onset, yr	CAP Score Mean (Range)	NfL[b] Mean (Range)
Cohort 1: cytokine analysis					
Pre–motor manifest	19	1.9 (1–4)	N/A	319 (159–467)	912 (232–2,843)
Motor manifest	19	26 (6–48)	3.7	472 (336–728)	2,054 (692–3,353)
Cohort 2: cellular analysis					
Pre–motor manifest	5	0.8 (0–4)	N/A	361 (241–432)	1,817 (609–3,177)
Motor manifest	16	40 (9–65)	9.1	533 (383–832)	3,454 (1,677–4,738)
Cohort 3: cellular replication cohort					
Pre–motor manifest	4	1.6 (0–3)	N/A	316 (203–440)	1,244 (321–2,821)
Motor manifest	12	27 (7–58)	4.5	505 (325–690)	2,392 (1,074–3,970)
Cohort 4: cytokine replication cohort					
Pre–motor manifest	23	1.7 (0–4)	N/A	332 (147–542)	—
Motor manifest	24	28 (7–51)	5.2	490 (298–760)	—

[a]Probability values are derived from Kruskal–Wallis and post hoc Dunn's test or the Mann–Whitney U test. Probability values listed are for comparison with *HTT* gene expansion carriers.

[b]NfL is given as ng/l.

CAP score = CAG age product score; F = female; M = male; N/A = not applicable; NfL = neurofilament light chain; NIND = patients with non-inflammatory neurological diseases; PPMS = patients with primary progressive multiple sclerosis; UHDRS motor score = Unified Huntington's Disease Rating Scale-99 total motor score.

TABLE 2. Cytokine Analysis

	Cohort 1								
Cytokine	**Controls**	**PPMS**	**HD**	p^a	q^b	**Pre–Motor Manifest**	**Motor Manifest**	p^c	q^b
IL-5	0.62	0.43[d]	0.46	0.018	0.032	0.39	0.52	0.099	0.30
IL-6	1.12	1.16	0.96	0.69	0.76	0.80	1.13	0.095	0.33
IL-7	1.07	0.73[d]	0.82[d]	0.0026	0.018	0.77	0.86	0.55	0.72
IL-8	32.4	41.7	36.1	0.052	0.073	30.9	41.3	0.0008	0.017
IL-10	0.02	0.22[d]	0.06	0.0065	0.023	0.04	0.09	0.10	0.26
IL-12p40	2.92	6.68	4.01	0.88	0.92	5.09	2.93	0.23	0.54
IL-15	2.21	2.00	2.11	0.47	0.58	1.85	2.37	0.012	0.13
IL-16	7.20	6.92	5.33[d]	0.0035	0.018	5.13	5.53	0.70	0.74
IL-17	0.00	0.13	0.35[d]	0.0054	0.023	0.48	0.22	0.59	0.73
IFN-γ	0.06	0.46	0.10	0.037	0.056	0.13	0.06	0.60	0.70
LT-α	0.00	0.06[d]	0.04[d]	0.0067	0.020	0.04	0.03	0.96	0.96
TNF-α	0.13	0.26	0.14	0.015	0.032	0.13	0.16	0.30	0.53
VEGF	2.99	3.29	3.88[d]	0.0017	0.018	3.81	3.95	0.66	0.73
CCL2	351	313	331	0.97	0.97	279	383	0.074	0.39
CCL3	1.35	16.0[d]	3.21	0.013	0.030	2.92	3.51	0.32	0.48
CCL4	11.3	34.2	10.6	0.079	0.11	9.16	12.0	0.23	0.48
CCL11	5.16	10.5	4.62	0.018	0.034	4.01	5.24	0.31	0.50
CCL17	2.34	2.85	1.99	0.028	0.045	1.58	2.41	0.084	0.35
CCL22	8.55	34.7[d]	14.7	0.0010	0.021	18.4	10.9	0.26	0.50
CCL26	2.08	2.78	1.63	0.51	0.60	0.92	2.34	0.024	0.17
CXCL10	322	731[d]	367	0.011	0.029	364	370	0.38	0.53

Mean levels of cytokines are given as pg/ml.
[a]Kruskal–Wallis test of controls, PPMS, HD.
[b]False discovery rate–corrected probability values (21 cytokines tested).
[c]Mann–Whitney test of pre–motor manifest, motor manifest.
[d]Probability value <0.05 in comparison to the control group in a post hoc Dunn's test for multiple comparisons.
CCL = CC chemokine ligand; CXCL = CXC chemokine ligand; HD = Huntington disease *HTT* gene expansion carriers; IFN = interferon; IL = interleukin; LT = lymphotoxin; PPMS = patients with primary progressive multiple sclerosis; TNF = tumor necrosis factor; VEGF = vascular endothelium growth factor.

proinflammatory LT-α and IL-17 in *HTT* gene expansion carriers compared to the healthy controls (Fig 1B-C). Finally, we observed a decrease in IL-16 and an increase in VEGF in *HTT* gene expansion carriers compared to the healthy controls and to patients with PPMS (Fig 1D-E). The level of the anti-inflammatory cytokine IL-10 was increased in patients with PPMS compared to both healthy controls and to *HTT* gene expansion carriers (Fig 1F). Altogether, this identifies IL-7, IL-16, IL-17, LT-α, and VEGF as 5 HD-associated cytokines and indicates proinflammatory lymphocyte activity in the CSF of *HTT* gene expansion carriers.

Increased Prevalence of Th17.1 Lymphocytes in the CSF of HTT *Gene Expansion Carriers*

To confirm lymphocyte activity in the CSF of *HTT* gene expansion carriers, we collected additional CSF samples from 21 *HTT* gene expansion carriers, 10 patients with

PPMS, and 7 patients with NIND (cohort 2; Table 1) and measured the prevalence of T cells by flow cytometry (Fig 2A-B). This showed a similar frequency of $CD3^+$ T cells, $CD4^+$ T cells, and $CD8^+$ T cells in the CSF of the 3 groups analyzed (Fig 2C and data not shown). It also showed that all $CD4^+$ T cells in the CSF of the patients expressed high levels of CD49d, which is part of the VLA4 complex (Fig 2D). Analyzing the prevalence of the proinflammatory $CD4^+$ T-cell subsets Th1 ($CXCR3^+CCR6^-$), Th17 ($CXCR3^-CCR6^+$), and Th17.1 ($CXCR3^+CCR6^+$) showed that the frequency of Th17.1 cells was significantly increased in *HTT* gene expansion carriers compared to patients with NIND (Fig 2E-H).

To validate these findings, we included a replication cohort of 15 *HTT* gene expansion carriers and 8 healthy gene expansion–negative controls (cohort 3; Table 1). Analyzing the prevalence of T cells in this cohort also showed no difference in the frequency of $CD3^+$, $CD4^+$, or $CD8^+$ T cells in the CSF between *HTT* gene expansion carriers and controls (data not shown). Furthermore, it confirmed that the frequency of Th17.1 cells is increased in *HTT* gene expansion carriers (Fig 2J–L).

To substantiate the presence of CSF resident Th17.1 cells, we stimulated CSF leukocytes from a *HTT* gene expansion carrier with PMA/ionomycin and measured the production of IL-17 and IFN-γ. This confirmed the existence of IL-17 and IFN-γ double-positive CSF T cells and hence of Th17.1 cells (Fig 2I).

Reduced Prevalence of Regulatory T Cells in the CSF of HTT *Gene Expansion Carriers*

The activity of T effector cells is under strict control by $CD4^+$ regulatory T (Treg) cells, and the level of Treg cells is often increased in patients with inflammatory diseases to counteract the ongoing inflammation. We therefore measured the prevalence of Treg cells in the CSF of *HTT* gene expansion carriers and patients with PPMS (cohort 2) by flow cytometry (see Fig 2M). Here, we found that the frequency of Treg cells defined as $CD127^-CD25^{hi}$ $CD4^+$ T cells was significantly lower in the CSF of *HTT* gene expansion carriers compared to patients with PPMS (see Fig 2N), possibly favoring a more proinflammatory T-cell environment, ie, less control of the effector T cells.

Intrathecal Cytokine Levels Are Changed prior to Disease Manifestation

To examine whether the observed decrease in IL-7 and IL-16, and increase in LT-α, IL-17, and VEGF in the CSF of *HTT* gene expansion carriers depicted in Figure 1 are present before motor onset, we divided the *HTT* gene expansion carriers from cohort 1 into 19 pre–motor manifest and 19 motor manifest gene expansion carriers and

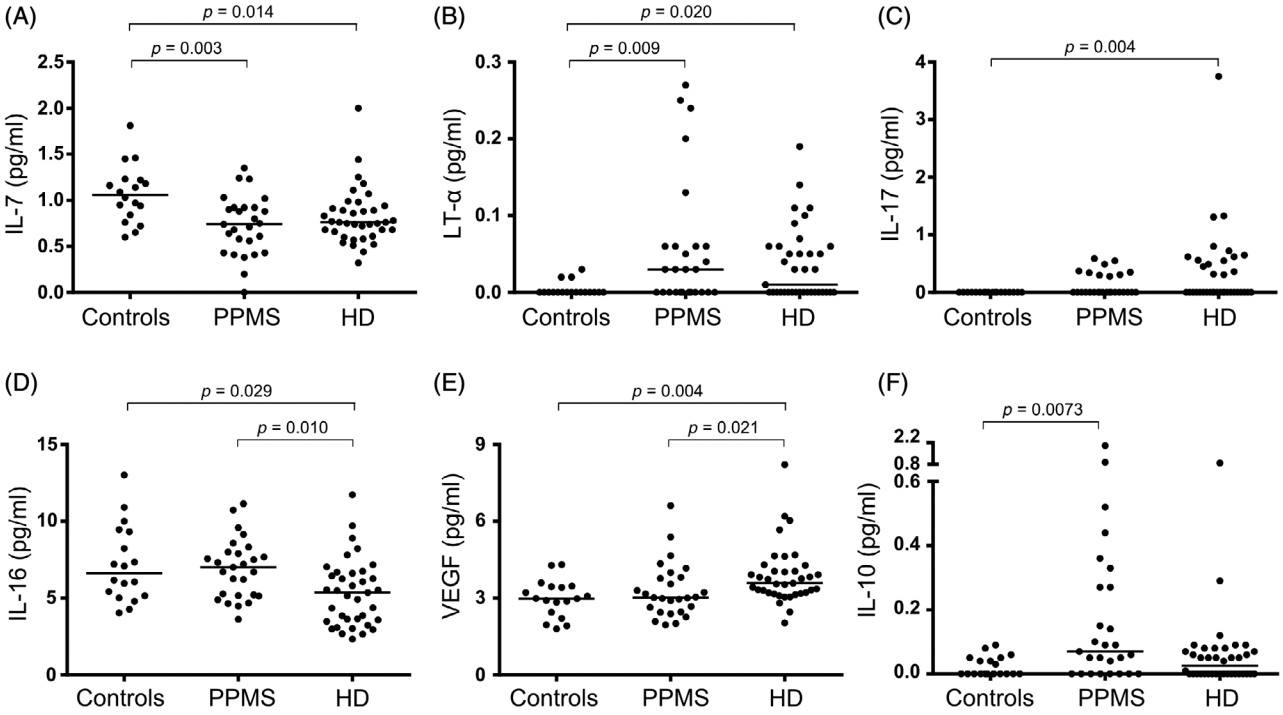

FIGURE 1: Proinflammatory lymphocyte activity in the cerebrospinal fluid (CSF) of *HTT* gene expansion carriers. Cytokine levels of interleukin (IL)-7 (A), lymphotoxin (LT)-α (B), IL-17 (C), IL-16 (D), vascular endothelium growth factor (VEGF; E), and IL-10 (F) in the CSF from healthy controls (Controls), *HTT* gene expansion carriers (HD), and patients with primary progressive multiple sclerosis (PPMS) from cohort 1 are shown. The median is shown for all groups analyzed.

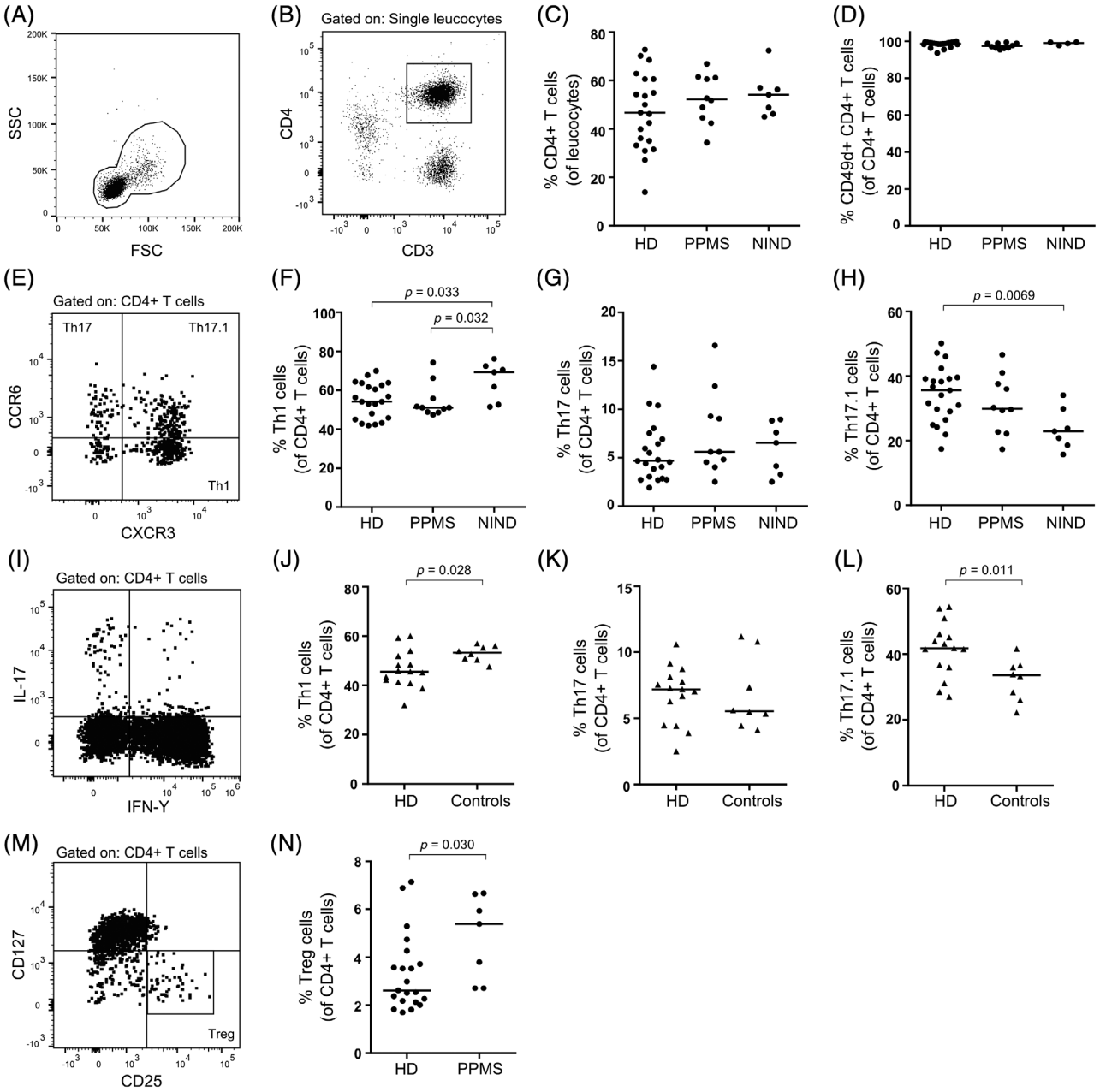

FIGURE 2: Increased prevalence of Th17.1 lymphocytes in the cerebrospinal fluid (CSF) of *HTT* gene expansion carriers. (A, B) Flow cytometry dot plot example of the gating strategy used to define $CD4^+$ T cells in the CSF. Leukocytes were gated in an forward scatter (FSC) versus side scatter (SSC) dot plot (A), single leukocytes are defined in an FSC-A/FSC-H dot plot, and thereafter $CD4^+$ T cells are defined in a CD3/CD4 dot plot (B). (C–H) Frequencies of $CD4^+$ T-cell subpopulations in CSF from *HTT* gene expansion carriers (HD), patients with primary progressive multiple sclerosis (PPMS), and patients with noninflammatory neurological disease (NIND) from cohort 2. Frequencies of $CD4^+$ T cells (C), $CD49d^+$ $CD4^+$ T cells (D), Th1 cells ($CXCR3^+CCR6^-$ $CD4^+$ T cells; F), Th17 cells ($CXCR3^-CCR6^+$ $CD4^+$ T cells; G), and Th17.1 cells ($CXCR3^+CCR6^+$ $CD4^+$ T cells; H) in cohort 2 are shown. (E) Flow cytometry dot plot example of $CXCR3^+$ and $CCR6^+$ $CD4^+$ T cells. (I) Flow cytometry dot plot example of IL-17 and interferon (IFN)-γ production in $CD4^+$ T cells in CSF of *HTT* gene expansion carriers. (J–L) Frequency of Th1 cells (J), Th17 cells (K), and Th17.1 cells (L) in the CSF of *HTT* gene expansion carriers (HD) and healthy controls from the reproduction cohort 3. (M) Flow cytometry dot plot example of the gating strategy used to define $CD4^+$ $CD127^-CD25^{hi}$ Treg cells in the CSF from cohort 2. (N) Frequency of Treg cells from *HTT* gene expansion carriers (HD) and patients with PPMS from cohort 2. The mean value is shown for all groups analyzed.

compared their cytokine levels. For all 5 cytokines, we found the same expression level in premanifest and manifest gene expansion carriers (Fig 3A–E, Table 2), indicating an early immune activation in *HTT* gene expansion carriers. Comparing the level of the remaining 16 cytokines showed an increase in IL-8, IL-15, and CCL26 in the motor manifest group as compared to the pre–motor manifest group; however, correcting for multiple testing, only

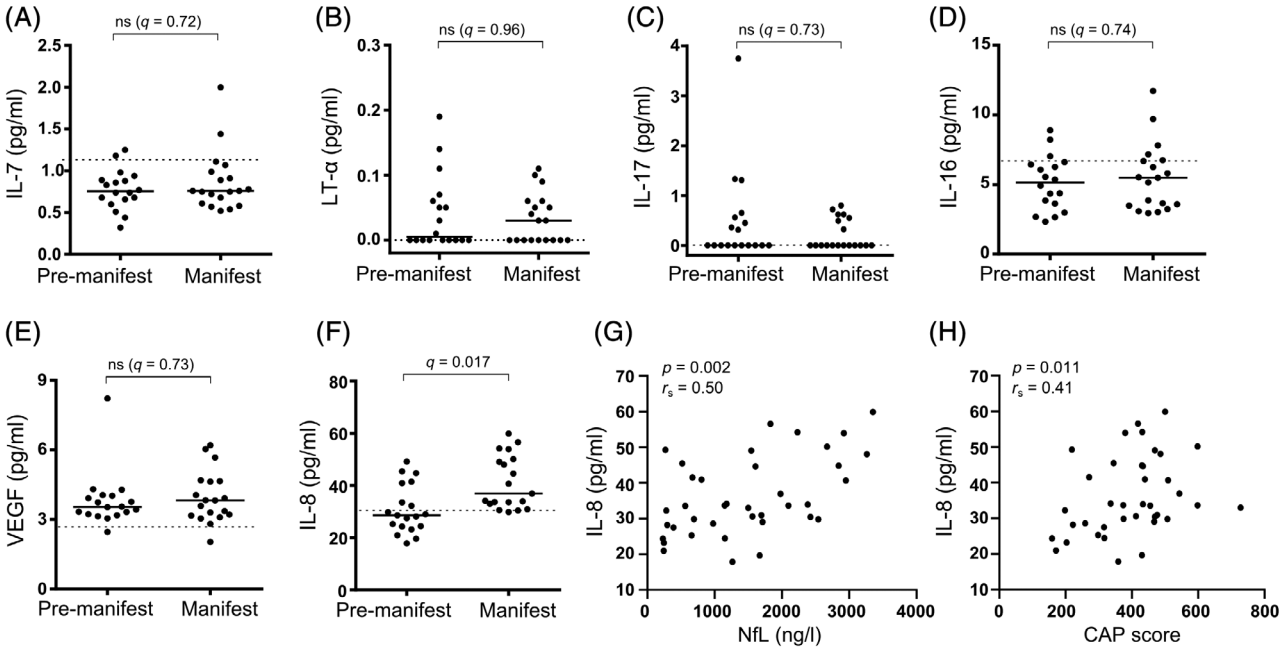

FIGURE 3: Intrathecal cytokine levels are changed prior to disease manifestation. (A–F) Cytokine levels of interleukin (IL)-7 (A), lymphotoxin (LT)-α (B), IL-17 (C), IL-16 (D), vascular endothelium growth factor (VEGF; E), and IL-8 (F) in the cerebrospinal fluid of pre–motor manifest and motor manifest *HTT* gene expansion carriers from cohort 1. Dotted lines represent healthy control values of the individual cytokines. The median is shown for all groups analyzed. (G, H) Correlation of the intrathecal IL-8 and neurofilament light chain (NfL; G) and the CAG age product (CAP) score (H). r_s denotes the Spearman correlation coefficient (rho). ns = not significant.

the level of IL-8 was significantly increased (Fig 3F, Table 2).

To validate these findings, we included a replication cohort of 23 premanifest and 24 motor manifest *HTT* gene expansion carriers (cohort 4; Table 1). Analyzing the cytokine CSF level in the replication cohort confirmed that the level of IL-7, IL-16, IL-17, LT-α, and VEGF was similar in premanifest and motor manifest *HTT* gene expansion carriers (data not shown). The level of IL-8 was increased following motor onset at a nominally significant level ($p = 0.021$); however, the difference was not significant after correction for multiple testing. No difference was found for IL-15 or CCL26.

Increased Frequency of Th17.1 Cells in the CSF prior to Disease Manifestation

The observation of altered CSF cytokine levels prior to motor onset of disease (Fig 3) indicates early immune cell activity in *HTT* gene expansion carriers. We therefore investigated the prevalence of T cells in the CSF of *HTT* gene expansion carriers before and after motor onset. In both cohorts collected for CSF cell analysis, the pre–motor manifest group was small (cohort 2 and cohort 3; Table 1). To increase the size of the pre–motor manifest group, data from the 2 cohorts were pooled. This showed an increased frequency of $CD4^+$ T cells (Fig 4A) in the CSF in pre–motor manifest *HTT* gene expansion carriers. Analyzing the proinflammatory $CD4^+$ T-cell subtypes showed an increased frequency of Th17.1 cells in pre–motor manifest *HTT* gene expansion carriers (see Fig 4B–D), suggesting that these cells are recruited to the CSF early in the disease.

Association of Intrathecal Th17.1 Cells with Disease Burden

The size of the CAG expansion in the *HTT* gene is inversely correlated with disease onset.[10] As a surrogate measure for HD disease burden, the CAP score can be calculated according to CAG repeat number and age.[8] To investigate whether the prevalence of Th17.1 cells in the CSF correlated with expected HD disease burden, the correlation between the frequency of Th17.1 cells and the CAP score was calculated (cohort 2 + 3). This showed a negative correlation between Th17.1 cells and disease burden ($p = 0.014$; Fig 4G).

The UHDRS is a standardized rating system used to quantify the severity of HD.[9] Using the motor section of the UHDRS, a motor score was calculated and correlated with the frequency of Th17.1 cells (cohort 2 + 3). This showed a negative correlation between Th17.1 cells and the motor symptom status of the patients ($p = 0.0021$).

Neurofilaments are main constituents of the axon cytoskeleton and are valuable biomarkers of neurodegeneration.[11] An increased level of NfL in the CSF of

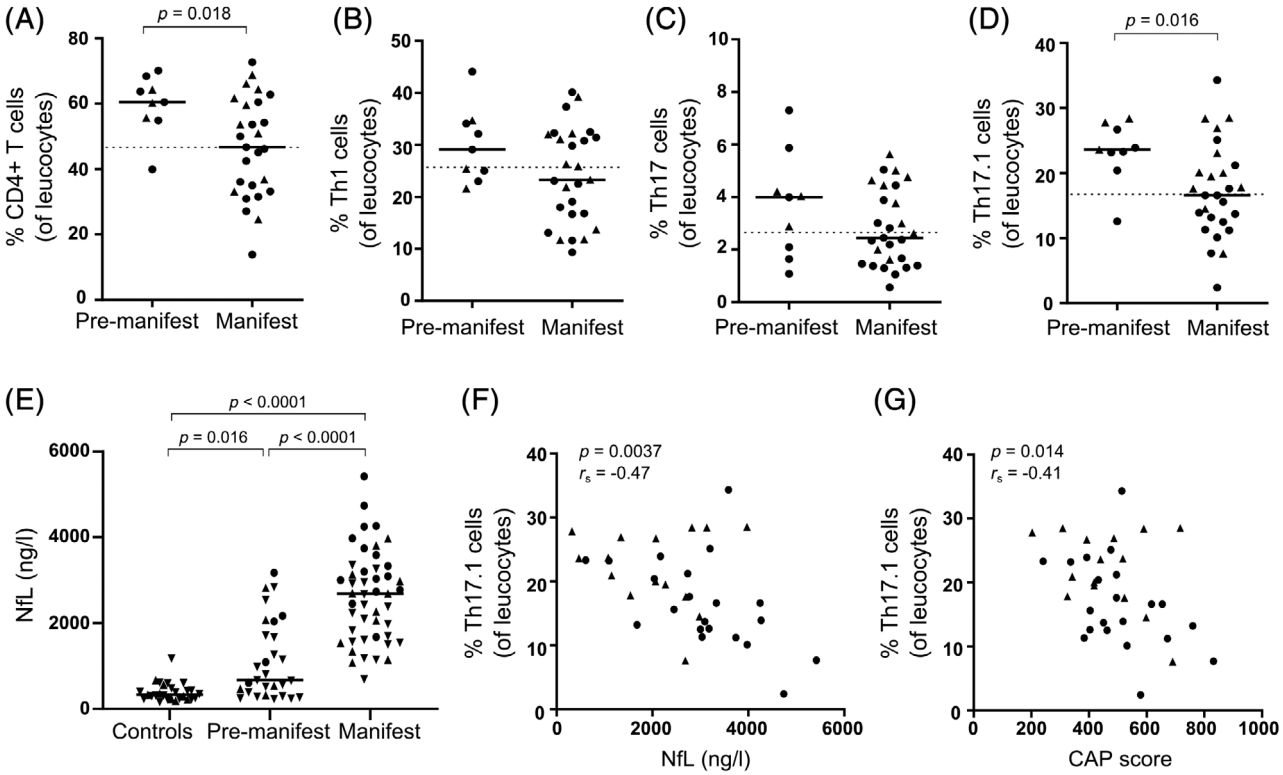

FIGURE 4: Increased frequency of Th17.1 cells in the cerebrospinal fluid (CSF) prior to disease manifestation. (A–D) Frequency of CD4⁺ T cells (A), Th1 cells (B), Th17 cells (C), and Th17.1 cells (D) in the CSF of pre–motor manifest and motor manifest *HTT* gene expansion carriers from cohorts 2 and 3. Dotted lines represent healthy control values. The median is shown for all groups analyzed. (E) Neurofilament light chain (NfL) levels in the CSF of healthy controls, and pre–motor manifest and motor manifest *HTT* gene expansion carriers from cohorts 1, 2, and 3. (F, G) Correlation of the intrathecal Th17.1 frequency and NfL (F) and the CAG age product (CAP) score (G) from cohorts 2 and 3. r_s denotes the Spearman correlation coefficient (rho). Data from cohort 1 are denoted by triangles pointing downward, cohort 2 by circles, and cohort 3 by triangles pointing upward.

HTT gene expansion carriers compared to healthy controls has previously been shown.[7,12] This observation could be confirmed in our study, where we found an increased level of NfL in premanifest individuals indicating early axonal damage, followed by an immense increase at the motor-manifest stage of the disease ($p < 0.0001$; Fig 4E). To investigate a possible association between neurodegeneration and the frequency of Th17.1 cells in the CSF of *HTT* gene expansion carriers, we performed a correlation analysis between the CSF level of NfL and Th17.1 cells (cohort 2 + 3). This showed a negative correlation between Th17.1 cells and NfL of the patients ($p = 0.0037$; Fig 4F). Altogether, these observations suggest that Th17.1 cells are implicated in the very early stages of disease.

Association of Intrathecal IL-8 with Neurodegeneration

Analyzing the intrathecal cytokine levels of *HTT* gene expansion carriers suggested an association between disease manifestation and an increased level of IL-8. To further investigate this notion, we analyzed whether the level of IL-8 continued to be associated with duration of motor onset. A Spearman correlation analysis of time since motor onset and IL-8 concentration showed no association between these parameters ($p = 0.47$).

In contrast, analyzing the correlation between the CAP score and IL-8 showed a positive correlation ($p = 0.011$; Fig 3H). Likewise, a positive correlation between NfL and IL-8 was found ($p = 0.002$; Fig 3G). These observations suggest an association between the level of intrathecal IL-8 and ongoing neurodegeneration.

Discussion

With this study, we provide evidence of intrathecal lymphocyte activation in HD. In *HTT* gene expansion carriers, we observed a decrease in the T-cell growth factor IL-7 compared to healthy gene expansion–negative controls, indicative of an increased consumption of this cytokine and hence of increased lymphocyte activity. We also observed a decrease in IL-16 in CSF of *HTT* gene expansion carriers. IL-16 is a proinflammatory cytokine that induces chemotaxis of CD4⁺ T cells[13] and furthermore acts as a growth factor for CD4⁺ cells.[14] Downregulation of IL-16 can therefore similarly to IL-7 be indicative of increased consumption and CD4⁺ T-cell activity. In

contrast, we found an increase in the lymphocyte cytokines LT-α and IL-17, both proinflammatory cytokines involved in the pathogenesis of various inflammatory diseases.[15,16] IL-17 is produced by various immune cell types including $CD4^+$ T cells and is a mediator of communication between immune cells and tissue. In animal models, peripheral IL-17 has been shown to induce blood–brain barrier breakdown through interaction with endothelial cells,[17] and IL-17 overexpression in the central nervous system (CNS) leads to activation of glial cells, including astrocytes, microglia, and oligodendrocytes, resulting in enhanced neuroinflammation.[18]

The indication of lymphocyte activity in the CSF of *HTT* gene expansion carriers in this study was strengthened by our observation of an increased prevalence of Th17.1 cells in the CSF. Th17.1 cells are proinflammatory T cells characterized by their production of IL-17 and IFN-γ. The finding of an increased prevalence of Th17.1 cells therefore corresponds with the observed increase in IL-17 and suggests a role for Th17.1 cells in the pathogenesis of HD. Th17.1 cells have previously been associated with the development and severity of various inflammatory and autoimmune diseases.[19–21] Furthermore, it has been shown that Th17.1 cells express the highest level of the adhesion molecule very late antigen-4 (VLA4) among proinflammatory $CD4^+$ T cells, possibly increasing the capacity of Th17.1 cells to migrate to inflamed tissue.[20] In patients with multiple sclerosis, it has therefore been speculated that the high efficacy of the anti-VLA4 Ab therapy natalizumab is, at least partly, due to the exclusion of Th17.1 cells from the CNS.[20]

During an inflammatory response, upregulation of Treg cells to control the activity of effector T cells is often observed. Intriguingly, we did not observe such a compensatory mechanism of Treg cells in *HTT* gene expansion carriers; the percentage of Treg cells was lower in *HTT* gene expansion carriers compared to the control group of patients with PPMS. This skewing in the balance between regulatory and proinflammatory T cells further favors a proinflammatory environment in the CSF of *HTT* gene expansion carriers. The low level of IL-10 in the CSF of *HTT* gene expansion carriers is further indicative of a less regulated environment.

To clarify whether the observed lymphocyte activity in the CSF of *HTT* gene expansion carriers was involved in the early stages of HD, we compared pre–motor manifest and motor manifest *HTT* gene expansion carriers. This demonstrated a change in the level of IL-7, IL-16, LT-α, and IL-17 already prior to motor onset and furthermore, that the frequency of Th17.1 cells was significantly increased before motor onset. In concordance, we found a negative correlation between the frequency of Th17.1 cells and the progression of the disease (CAP score, UHDRS, and NfL), emphasizing its possible role in early disease development rather than in later disease worsening. The mechanism by which Th17.1 cells contribute to disease development (ie, motor onset) can only be speculated on. One possibility is their ability to activate microglia cells.[18] Activated microglia cells have been reported in presymptomatic *HTT* gene expansion carriers[22] and suggested to play a role in neuronal cell death in the brain of patients with HD.[23] Furthermore, Th17.1 cells may possibly induce reactive astrocytes with a proinflammatory profile through their production of IL-17,[24] a process potentially enhancing inflammation in the CNS of *HTT* gene expansion carriers. Finally, Th17 cell–derived factors have been shown to increase the expression of CCL20 by astrocytes.[25] CCL20 is a chemokine enhancing transendothelial migration of $CCR6^+$ lymphocytes including Th17.1 cells. This may induce a second wave of Th17.1 cell migration to the CNS, amplifying the response.

In addition to early immune activity, previous studies have reported increased immune activation during the course of HD.[26] This increased activity likely is a result of the release of immunogenic molecules from dying neurons and hence is secondary to the CNS damage. In our study, we also found an increase in the chemokine IL-8 after symptomatic onset, an increase that positively correlated with disease progression measured as the CAP score and NfL. IL-8 is produced by various CNS resident cells including activated microglia[27] and functions as a chemoattractant for immune cells expressing the chemokine receptors CXCR1 and CXCR2. Besides neutrophils, perforin-expressing effector $CD8^+$ T cells express CXCR1 and are attracted by IL-8.[28] Therefore, recruitment of immune cells other than Th17.1 cells to the CNS may become evident at later stages of disease.

The data from this study indicate that early treatment of *HTT* gene expansion carriers with immunomodulatory therapies may delay the development of symptoms. Few previous anti-inflammatory strategies have been investigated to treat patients with HD, and without the success hoped for. A phase 2 study of 352 patients with HD treated with laquinimod (LEGATO-HD, NCT02215616)[29] failed to meet its primary endpoint.[30] In the LEGATO-HD trial, patients were given laquinimod after symptomatic onset (UHDRS total motor score > 5) and the primary outcome was an improvement of their UHDRS score after 1 year of treatment. As suggested by our data, a higher efficacy might be achieved by starting the treatment before symptomatic onset and monitoring the delay in symptomatic onset as a primary endpoint. Furthermore, choosing a strategy to specifically target the entrance of Th17.1 cells to the CNS, eg by administrating the anti-VLA4 monoclonal Ab natalizumab could be considered.

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Author Contributions

M.R.v.E., J.E.N., and F.S. contributed to study concept and design. M.R.v.E., F.S., M.N.N.H., T.V.-J., C.A., R.H.H., L.E.H., and T.T.N. contributed to data acquisition and analysis. M.R.v.E., J.E.N., and F.S. contributed to drafting of the text and figures.

Potential Conflicts of Interest

Nothing to report.

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Manuscript 3.

Decreased CSF Oxytocin relates to measures of social cognitive impairment in Huntington's Disease patients

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Abstract

Background and objectives: Huntington's disease (HD) is an inherited neurodegenerative disease with a symptom triad consisting of motor, cognitive and psychiatric symptoms. The motor symptoms have been correlated to striatal and cortical atrophy while some of the non-motor symptoms such as depression and altered social cognition, are proposed to be caused by dysfunction of the hypothalamus. We therefore measured the hypothalamic neuropeptide oxytocin in both plasma and cerebrospinal fluid (CSF) in a large and well described cohort of HD gene expansion carriers (HDGECs) and compared the levels to healthy HD family controls and correlated oxytocin levels to disease progression and measures of social cognition.

Methods: We recruited 113 HDGECs and 33 HD gene expansion negative family controls who had blood and CSF drawn. They were evaluated in regard to psychiatric and cognitive symptoms and their abilities within social cognition were assessed with the Emotion Hexagon test, Reading the Mind in the Eyes and The Awareness of Social Inference Test. The levels of oxytocin in CSF and blood were analyzed by radioimmunoassay.

Results: We found the level of oxytocin in CSF to be 33.5 % ($p=0.016$) lower in HDGECs compared to controls There was no association to the Disease Burden score and when dividing the HDGECs in to groups with or without cognitive impairment, we found the oxytocin level to be 30.3 % ($p=0.046$) lower in the HDGECs with cognitive symptoms. We also found a significant correlation between the level of oxytocin and scores on social cognition. There were no significant differences in plasma oxytocin levels.

Discussion: This is the first study to measure oxytocin in the CSF in a large and well described group of HDGECs. We find that HDGECs have a significantly lower level of oxytocin compared to controls, and that the level of oxytocin may represent an objective and comparable measure that could be used as a state biomarker for impairment of social cognition. We also suggest treatment trials to evaluate a potential effect of oxytocin on social cognition in HD.

Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease caused by a CAG repeat expansion in the huntingtin gene. HD is characterized by three symptom domains that occur to varying degrees: motor disturbances, cognitive impairment and psychiatric symptoms. The motor symptoms have been linked to the volume of the striatum, and atrophy of the cerebral cortex and white matter (1). While aspects of the cognitive symptoms have been correlated to white matter atrophy (2), little or no anatomic correlations have been found in regard to the psychiatric symptoms (1). One hypothesis is that several of the non-motor features of the disease, such as depression, altered social cognition, autonomic dysfunction, metabolic alterations and sleep disturbances are caused by dysfunction of the hypothalamus and the limbic system (3–5).

The hypothalamus consists of several nuclei and a number of changes have been found using post-mortem analyses of hypothalami from HD gene expansion carriers (HDGEC) (6). In the paraventricular nucleus and the supraoptic nucleus the majority of the endogenous portion of oxytocin is produced. One study of the hypothalamus of HDGECs found a reduction of 45% in the number of neurons expressing oxytocin compared to controls (4), and another neuropathological study found a reduction in oxytocin expressing neurons in the hypothalamus in an HD case with no measurable striatal atrophy (7), indicating possible early changes involving the oxytocin expressing neurons of the hypothalamus in HD.

Oxytocin has in addition to its functions in female reproduction been described to influence cognitive processes such as social recognition, interpretation of emotional expression and social memory, while the relation to depression has been described to be more complex (5,8). These cognitive processes, emotion recognition and inferences about another's beliefs and intentions (Theory of Mind (ToM)), have all been related to HD symptomatology and for many HDGECs the everyday function is more affected by the psychiatric and cognitive problems than the motor symptoms (9). Several studies investigating the role of oxytocin in social interactions have used intranasal administration of oxytocin and found it to increase trust and improve the ability to interpret the mental state of others (8) but only few studies have focused on oxytocin and social cognition in HD (10–12).

Non-motor features of HD have been shown to precede the motor symptoms and the severity of some aspects of social cognitive impairments has even been associated to Disease Burden and

proximity to onset of motor symptoms (13). We hypothesize that reduction of oxytocin may explain part of the early behavioral phenotypes seen in HD (3) and that the level of oxytocin might potentially be a useful biomarker or therapeutic target. In the present study, we therefore measured levels of oxytocin in both plasma and cerebrospinal fluid (CSF) in a large and well described cohort of HDGECs and compared the levels to healthy HD gene expansion negative controls. We also examined whether oxytocin levels correlated to disease progression and measures of several aspects of social cognition.

Material and Methods

Study population

From January 2012 to March 2013 (Cohort 2013) and from June 2017 to January 2019 (Cohort 2018), we recruited 208 participants from the Neurogenetics Clinic, Danish Dementia Research Centre, Rigshospitalet. Of those, 49 were HD gene expansion negative controls and 159 HDGECs.

Some of the 208 participants participated in both Cohort 2013 and in Cohort 2018. They were all examined by a physician and a neuropsychologist and those who consented, had blood drawn and a lumbar puncture performed. For details on recruiting and categorization of psychiatric and cognitive symptoms, see Vinther-Jensen et al (14). In short, participants were classified according to the Unified Huntington's Disease Rating Scale (UHDRS) total motor score (TMS). A UHDRS-TMS >5 defined the HDGEC as motor manifest and if the score was ≤5, the HDGEC was classified as premanifest. We calculated the Disease Burden score as the CAG age product (CAP) score: CAP = ((CAG – 35.5) * age) (15).

To obtain the largest number of participants possible, we included all first-time plasma and CSF samples in one group. We excluded all CSF samples with erythrocyte count above 300*10^6/L. In cases where the participant had two lumbar punctures performed, one in 2013 and one in 2018, the sample from 2013 was chosen unless it was excluded due to high erythrocyte count, then the one from 2018 was selected.

Altogether, we included 33 controls and 113 HDGECs in the CSF and plasma study.

The study was approved by the Ethics Committee of the Capital Region of Denmark (H2-2011-085 and H-17002606).

Collection of CSF and plasma

All lumbar punctures were performed between 09:00AM and 03:00PM. No participants were pregnant or breastfeeding. We made no registrations on women's menstrual cycle or contraception. CSF samples were obtained by lumbar puncture and collected in polypropylene tubes kept on ice. The first ml was sent for routine biochemical analysis and the rest was immediately centrifuged at 720 g for 12 minutes at 4ºC. The supernatants were stored in 250 µl aliquots in polypropylene tubes and kept at -80 ºC.

The blood samples were collected in polypropylene tubes and centrifuged at 2000 g for 10 minutes and then the plasma was extracted and stored in 250 µl polypropylene tubes.

Measurement of oxytocin levels in plasma and CSF

The concentrations of oxytocin in plasma and CSF were determined in duplicates by radioimmunoassay (RIA, Phoenix Pharmaceuticals California, USA). The concentrations were measured as pg/ml. When the concentration of oxytocin was measurable but below the detection level, we chose to set the concentration to the lowest level of detection. The assay range was from 0.625-160 pg/ml and the intra- and inter-assay coefficient of variance was 5.9±1.1% and 9.6% respectively.

Cognitive and psychiatric evaluation

All participants were evaluated regarding affective symptoms, psychomotor speed, attention, memory, visuospatial functions and executive functions. On the basis of thorough evaluation, the participants were classified as having psychiatric and/or cognitive symptoms (14). As part of our global screening for cognitive impairment, we used Mini Mental State Examination (MMSE) and the Montreal Cognitive Assessment (MoCA) (16). To assess the symptoms previously described to correlate to oxytocin, we focused on social cognitive tests: The Awareness of Social Inference Test (TASIT) (17), the Emotion Hexagon test (EHt) (18) and the Reading the Mind in the Eyes (RME) test (19). As evaluation tool of affective symptoms, we applied the Hamilton depression scale.

We used TASIT to assess social perception and the ability to interpret emotional expressions and paralinguistic cues. On Cohort 2013, we used an enriched version of TASIT while we in Cohort

2018 used the shorter version. To be able to compare the results of the two versions of the test, we calculated individual z-scores in the two cohorts and compared these. The test consists of 15 videoclips that reflect everyday situations, the interactions are either sincere or sarcastic, each video are followed by four questions. The total number of correct answers (0-60/100) were recorded.

To assess emotion recognition, a printed version of the EHt was applied. This version consists of 30 cards with pictures of facial expressions of the six basic emotions: happiness, surprise, fear, sadness, anger and disgust. Pictures of faces expressing these emotions morphed together in varying degree were presented to the participants along with a card where the emotions were listed. The total number of correct answers (0-24) were recorded.

The RME was applied to test ToM. The test has 36 pictures of eyes expressing different emotional states. In each picture four words for emotional states are listed and the participants were asked to choose the word that best described the emotional state they saw in the eyes. The participants were given a list to explain the words and the total number of correct answers (0-36) were recorded.

To assess depression, we used the Hamilton depression scale, a semi structured interview, with 17 questions. The total score (0-52) was recorded, the higher the score, the greater the severity of the symptoms.

Statistical analyses

Values of CSF and plasma oxytocin were logarithmically transformed to achieve normally distributed residuals in regression models. Group comparisons of the oxytocin levels were performed in a multiple regression model with correction for gender and age. TASIT scores were obtained differently in the two cohorts (old vs new). In order to do group comparisons, we therefore calculated a z-score: (score – mean cohort score) / standard deviation of the cohort and used this as a dependent variable in the regression models with adjustment also for cohort.

All the analyses were conducted in SAS Enterprise Guide Version 7.1. and p-values of 0.05 or less were considered significant.

Results

Demographic data

Oxytocin levels were measured in CSF and plasma of 113 HDGECs and 33 control subjects (demographics are presented in **Table 1**). Scores on TASIT, EHt and RME were obtained from 89 HDGECs (**Table 2**). In all these tests the highest scores were found in the group of premanifest HDGECs compared to the manifest HDGECs.

Table 1. Demographics

		Status			
		Control	**Premanifest**	**Motor Manifest**	**HDGECs**
	Cohort 2013	**20**	**32**	**47**	**79**
Male	**N**	15	28	39	67
	Age (years)	39 (26-62)	35.5 (19-58)	56 (30-74)	42 (19-74)
	CAG repeat	19.0 (17-24)	43 (39-48)	43 (40-51)	43 (40-51)
	CAP score		256 (112.5-437.0)	380.0 (211.5-666.5)	315 (112.5-666.5)
	UHDRS TMS	0 (0-3)	1 (0-4)	22 (7-59)	12 (0-59)
	TFC	13 (12-13)	13 (10-13)	10 (4-13)	12 (4-13)
	Psychiatric symptoms	2	5	22	27
	Cognitively impaired		3	31	34
Female	**N**	18	16	30	46
	Age (years)	47 (26-74)	39 (20-56)	50 (29-73)	46.5 (20-73)
	CAG repeat	18 (17-26)	41 (39-47)	43 (40-53)	42.5 (39-53)
	CAP score		238.5 (110.0-375.0)	386.3 (188.5-655.5)	316.3 (110-655.5)
	UHDRS TMS	0 (0-3)	2 (0-5)	26.5 (6-61)	14 (0-61)
	TFC	13 (13)	13 (12-13)	8 (2-13)	10.5 (2-13)
	Psychiatric symptoms		5	25	30
	Cognitively impaired	1	3	23	26
	Total No (146)	33	44	69	113

The data is shown as median (range), Cohort 2013 refers to the number of participants who were part of Cohort 2013, CAP score – CAG age product (age*(CAG repeat-35.5)), HDGECs: Huntington's Disease gene expansion Carriers, TFC: Total function capacity, UHDRS TMS: Unified Huntington's Disease Rating scale Total Motor Score.

Table 2. Psychiatric and cognitive test scores

		Status		
		Premanifest	**Motor Manifest**	**HDGECs**
Male	**N***	27	30	57
	MMSE	29.5 (26-30)	29 (22-30)	29 (22-30)
	MoCA	28.5 (26-30)	25 (15-30)	27 (15-30)
	Hamilton	2 (0-14)	2 (0-14)	2 (0-14)
	RME	26 (16-33)	19 (9-27)	23 (9-33)
	EHt	20 (14-22)	15 (9-20)	17 (9-22)
	TASIT, z-score	0.52 (-0.7-1.4)	-0.71 (-2.5-1.4)	0.16 (-2.5-1.4)
Female	**N***	16	16	32
	MMSE	29 (28-30)	27 (20-30)	28.5 (20-30)
	MoCA	28.5 (26-30)	24 (12-30)	26 (12-30)
	Hamilton	4 (0-17)	6 (0-17)	6 (0-17)
	RME	27 (21-31)	23 (14-34)	24 (14-34)
	EHt	21 (14-22)	17.5 (8-21)	20 (8-22)
	TASIT, z-score	0.73 (-1.6-1.2)	0.07 (-2.5-1.1))	0.38 (-2.5-1.2)
	Total N*	43	46	89

The data is shown as median (range), MMSE: Mini Mental State Examination, MoCA: Montreal Cognitive Assessment, N*: The number of participants participation in the social cognitive tests, RME: Reading the mind in the eyes, EHt: Emotion Hexagon test, TASIT, z-score: The Awareness of Social inference Test.

Reduced CSF oxytocin levels in HD gene expansion carriers

We compared the levels of oxytocin in controls and HDGECs and found that HDGECs have a 33.5 % lower (95 % CI: 7-52) level of oxytocin than control participants (p= 0.016) (**Figure 1**). When we subdivided the HDGECs in premanifest and motor manifest HDGECs and compared oxytocin levels to the controls, the total p-value was significant (p= 0.04) (**Figure 2A**). The manifest HDGECs had a 37.8 % lower level (95 % CI: 11-57) of oxytocin compared to controls (p= 0.01). There was, however, no significant difference in the levels of oxytocin between premanifest HDGECs and controls (p= 0.12), although the premanifest HDGECs had an oxytocin level 26.6 % lower (95 % CI: -9 – 50) than the controls.

Figure 1.

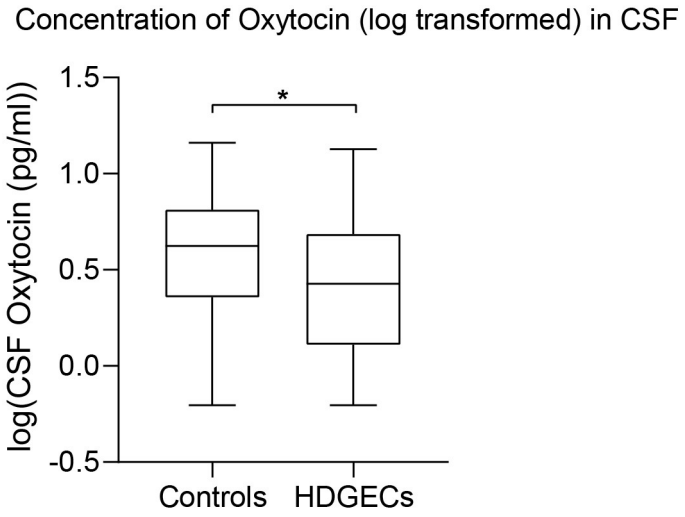

Figure 1. CSF concentrations of oxytocin (logarithmically transformed), in controls (N:33) and Huntington's disease gene expansion carriers (HDGECs)(N:113). The difference in oxytocin levels is significant, p= 0.016. The median and 2.5 % and 97.5 % percentiles are shown.

Reduced levels of CSF oxytocin in HD patients with cognitive impairment

We investigated the association between the level of oxytocin and progression of HD, by assessing a possible correlation between oxytocin and the CAP score of the HDGECs. We found a non-significant decrease in the oxytocin level with increasing CAP score (p= 0.38). Considering the hypothesis of a correlation between oxytocin and non-motor symptoms of HD, we divided the HDGECs into subgroups based on the classification on whether cognitive or psychiatric symptoms were present. We found a significantly lower level of oxytocin (30.3% lower 95 % CI 0.6-51) in the HDGECs who were classified as being cognitively impaired (p= 0.046) compared to the HDGECs who were not classified as cognitively impaired (**Figure 2B**). We also compared the level of oxytocin in controls with the level in HDGECs without cognitive impairments and found no significant difference (p= 0.28). We found no significant difference between the HDGECs who were classified as having psychiatric symptoms and those who were not (p= 0.96) (**Figure 2C**). The levels of oxytocin were significantly lower in HDGECs with (p= 0.02) and without (p= 0.04) psychiatric symptoms compared to controls.

Figure 2.

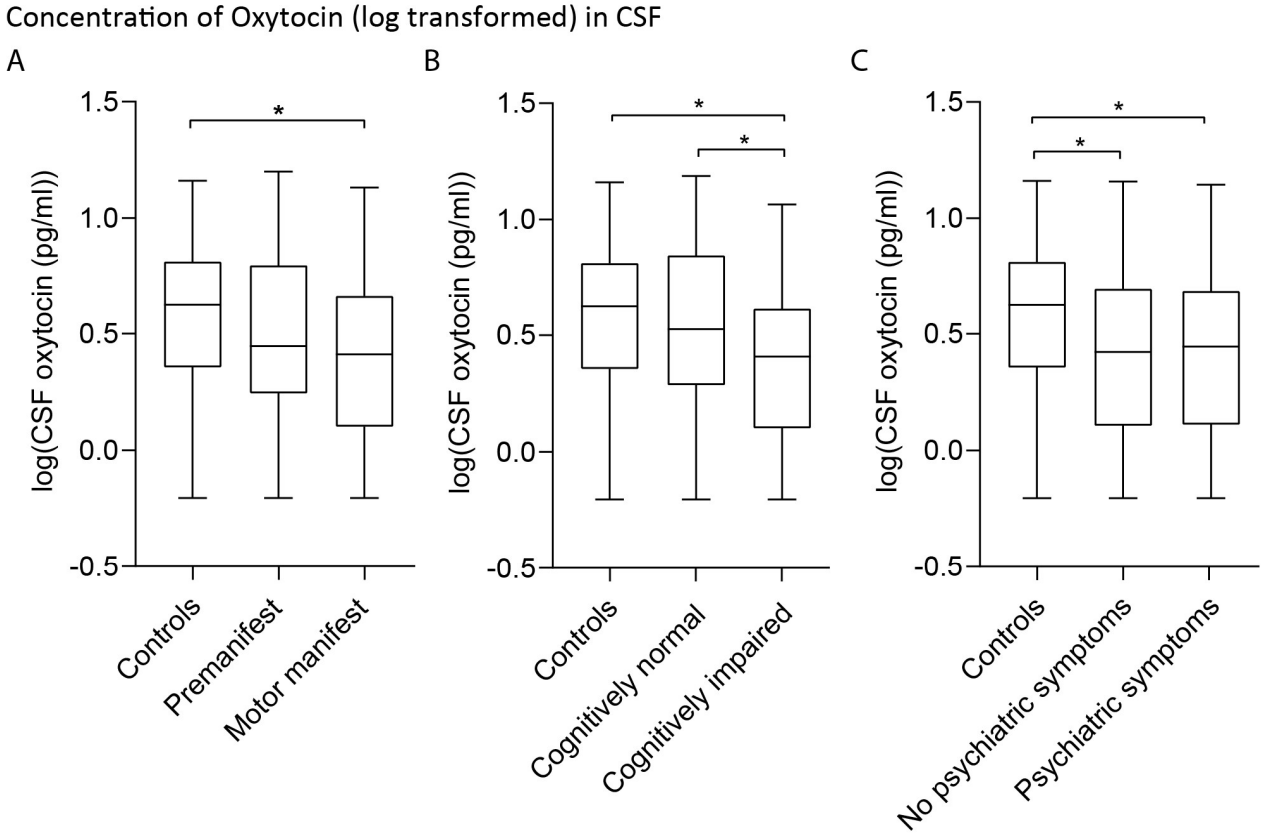

Figure 2. The CSF oxytocin concentrations (logarithmically transformed), in HDGECs divided based on symptom presentation. A, controls (N:33), premanifest Huntington's disease gene expansion carriers (HDGECs)(N:44) and motor manifest HDGECs(N:69), (p= 0.01), B controls, HDGECs classified as cognitively normal (N:52) and HDGECs classified as cognitively impaired (N:59), significant difference in level of oxytocin between controls and cognitively impaired HDGECs (p= 0.002) and between HDGECs classified as cognitively normal and HDGECs classified as cognitively impaired (p= 0.046) and C, controls, HDGECs classified as not having psychiatric symptoms (N:56) and HDGECs classified as having psychiatric symptoms (N:55), There were significant differences in oxytocin levels between controls and HDGECs classified as not having psychiatric symptoms (p= 0.02) and controls and HDGECs classified as having psychiatric symptoms (p= 0.04). The median and 2.5 % and 97.5 % percentiles are shown for all groups. * indicate significant differences.

CSF oxytocin correlates to measures of altered social cognition

We found significant associations between the level of oxytocin and scores on all measures of social cognition: RME (p= 0.0019), EHt (p= 0.0062) and TASIT (p= 0.002) (**Figure 3**). There was no significant association between oxytocin levels and the Hamilton depression rating score and scores on the MMSE and MoCA.

Figure 3.

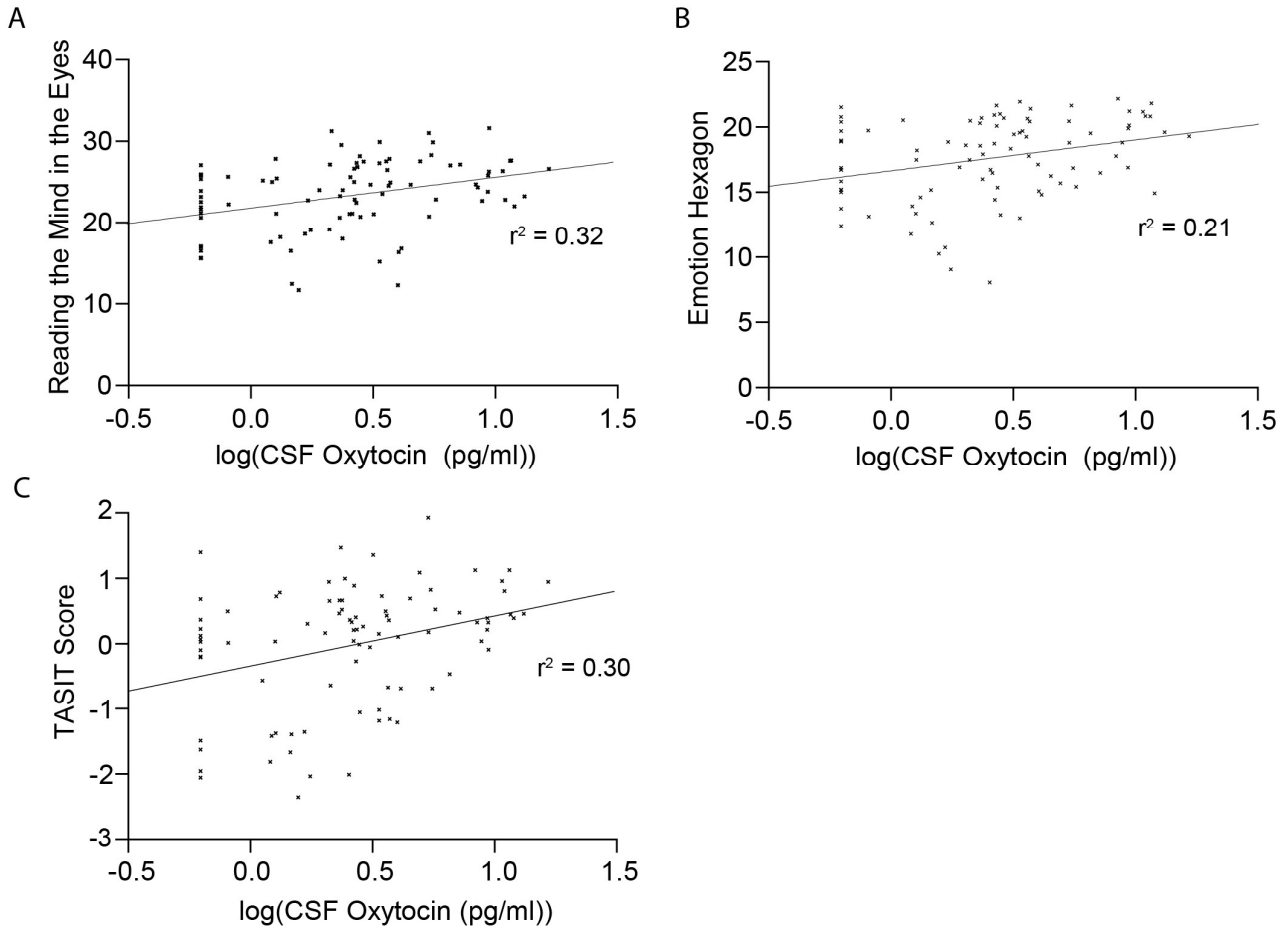

Figure 3. The associations between CSF oxytocin concentrations (logarithmically transformed) in Huntington's Disease gene expansion carriers and the neuropsychiatric scores on Reading the Mind in the Eyes (A)(p=0.0019), Emotion Hexagon (B)(p=0.0062) and TASIT, Z-score (C)(p=0.002). All three graphs are corrected for age and gender and show the association for a male aged 45.

No difference in plasma levels of oxytocin between HDGECs and controls

We detected no significant difference between oxytocin plasma levels of HDGECs and controls. None of the comparisons performed on the levels of plasma oxytocin showed any significant differences, correlations or associations (**Figure 4**). We found no difference in albumin ratio comparing controls to HDGECs, p=0.68 (data not shown).

Figure 4.

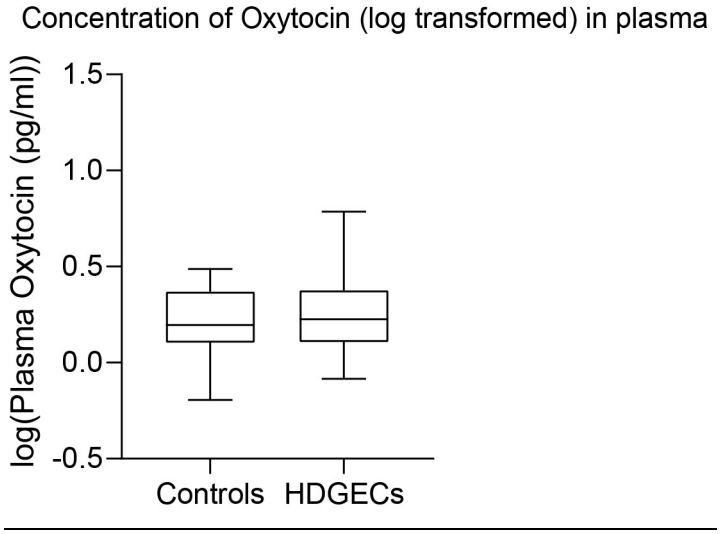

Figure 4. The plasma concentration of oxytocin (logarithmically transformed), in controls (N:33) and Huntington's disease gene expansion carriers (HDGECs)(N:113) (p= 0.73). The median and 2.5 % and 97.5 % percentiles are shown.

Discussion

This study is the first to measure oxytocin in the CSF of HDGECs. We report a significant difference in the levels of oxytocin in CSF between HDGECs and controls. The significantly lower level of CSF oxytocin in HDGECs was not significantly correlated to Disease Burden score and therefore does not appear to be a surrogate marker of disease progression. However, we found a significant correlation between the level of oxytocin and cognitive impairment. HDGECs classified as cognitively impaired had a significantly lower concentration of oxytocin than the HDGECs without cognitive impairment. There was no significant difference between the levels of oxytocin in controls and HDGECs without cognitive impairment, emphasizing the correlation between the cognitive symptoms and the reduced level of oxytocin. Furthermore, there was a significant correlation between the level of oxytocin and all measures of social cognition. Oxytocin has been linked to other psychiatric and neurodegenerative disorders with impaired social cognition, like autism spectrum disorder, borderline personality disorder, schizophrenia and frontotemporal dementia (FTD) (5,20,21), and altered social cognition has been reported previously in HDGECs (22,23). Importantly, this is the first report to link impaired social cognition to CSF oxytocin levels in HD.

The ability to comprehend the emotional state of others is critical for interpersonal functioning, and social cognition has been shown to predict the general functional social capacity better than the traditional neuropsychological assessment in neurological and psychiatric disease (13,24–26). As CSF oxytocin levels reflect social cognitive impairment in HD, it may represent an objective and comparable measure that could be used as a state biomarker for this important non-motor feature of HD.

The pivotal role of oxytocin in social cognition (5,8) described in both animal and human studies is mainly based on reports that administration of oxytocin has a positive influence on social cognition (27,28) and that central stimulation with oxytocin restores social recognition and the processing of olfactory or pheromonal cues in oxytocin knock-out mice (24,29). Administration of oxytocin has been suggested as a potential treatment strategy for several disorders with social cognitive impairment. In FTD, a neurodegenerative disorder where social cognitive dysfunction is prominent, intranasal administration of oxytocin has been shown to affect emotional recognition and increase neural activity in limbic regions (30,31). In a pilot study with 9 HDGECs, acute intranasal administration of oxytocin was also found to affect emotion

recognition (11). Currently, there are ongoing trials with oxytocin for a number of psychiatric disorders as well as FTD, and this is although there remain a number of unknowns including the mechanisms of action, optimal administration strategy and dosing for oxytocin (32,33). Nevertheless, based on the reduced numbers of oxytocin neurons in post-mortem HD hypothalami (4,34) accompanied by the reduced CSF oxytocin levels reported in the present study, the oxytocin system may be a relevant target for future therapeutic development also for HD.

Several studies report different correlations to oxytocin and different brain responses to exogenous administration of oxytocin in men and women (35,36). The baseline level of oxytocin in healthy men and women, primarily measured in plasma, has been described with conflicting results. Some studies find no difference (37) while others find the oxytocin level higher in women (38) and others in men (39). We find the level of CSF oxytocin to be significantly higher in men ($p=0.0002$; data not shown); we have, however, corrected all our calculations for age and gender to demonstrate the effect of the HD gene mutation on the level of oxytocin.

We also measured the concentration of oxytocin in plasma but found no significant differences between HDGECs and controls or any significant correlations to clinical scores. Plasma measurements of oxytocin involve some limitations as the concentration is often very low, the half-life of the peptide is short, and the origin of the oxytocin is not clarified (27). Previously, Unti and colleagues (10) detected a trend for lower plasma levels in HD patients compared to controls but this was on a small sample size. Fisher and colleagues (12) found a correlation between a higher level of oxytocin and fewer reported depressive symptoms but no significant differences in plasma levels between the groups. This is in accordance with the results in this study where we found no significant differences in plasma oxytocin levels between HDGECs and controls. Oxytocin is a large hydrophilic molecule that is not believed to cross the blood brain barrier easily and we found no significant difference in the albumin ratio between controls and HDGECs. The peptide is produced both in the brain and in the body, including the heart and gut. A social stimulus may cause reactions in the gut which leads to oxytocin release that is registered by the afferent branch of the Vagal nerve, leading to the central release of oxytocin and a positive feedback loop (27). The non-significant results from the plasma oxytocin levels in our study might therefore also reflect that they were obtained in relation to no orchestrated social stimuli and the different metabolic pathways of oxytocin in plasma and CSF (27).

In conclusion, this is the first study to measure oxytocin in CSF in a large and well described group of HDGECs and therefore an independent replication study to confirm the findings is warranted. We find that HDGECs have a significantly lower level of oxytocin compared to controls, and that the level of oxytocin correlates to the presence of cognitive symptoms and associate to test scores of social cognition. Highlighting this association suggests a need for a more targeted social cognitive clinical assessment when evaluating HDGECs and opens a potential for treatment trials to evaluate a possible effect of oxytocin on social cognition in HD.

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