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RESEARCH ARTICLE

REVISED Genomic architecture differences at the *HTT* locus associated with symptomatic and pre-symptomatic cases of Huntington's disease in a pilot study. [version 3; peer review: 1 approved with reservations, 1 not approved]

Previously titled: Genomic architecture differences at the *HTT* locus underlie symptomatic and pre-symptomatic cases of Huntington's disease

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Abstract

Background: Huntington's disease (HD) is a progressive neurodegenerative condition that causes degeneration of neurons in the brain, ultimately leading to death. The root cause of HD is an expanded trinucleotide cytosine-adenine-guanine (CAG) repeat in the "huntingtin gene" (*HTT*). While there is a rough correlation between the number of CAG repeats and disease onset, the development of clinical symptoms can vary by decades within individuals and little is known about this pre-symptomatic phase.

Methods: Using peripheral blood samples from HD patients and healthy controls we used *EpiSwitch*TM, a validated high-resolution industrial platform for the detection of chromosome conformations, to assess chromatin architecture in the immediate vicinity of the *HTT* gene. We evaluated chromatin conformations at 20 sites across 225 kb of the *HTT* locus in a small cohort of healthy controls, verified symptomatic HD patients (CAG, n>39) and patients with CAG expansions who had not yet manifested clinical symptoms of HD.

Results: Discrete chromosome conformations were observed across the patient groups. We found two constitutive interactions (occurring in all patient groups) and seven conditional interactions which were present in HD, but not in healthy controls. Most important, we

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observed three conditional interactions that were present only in HD patients manifesting clinical symptoms (symptomatic cases), but not in presymptomatic cases. Of the patients in the symptomatic HD cohort, 86% (6 out of 7) demonstrated at least one of the specific chromosome conformations associated with symptomatic HD.

Conclusion: Our results provide the first evidence that chromatin architecture at the *HTT* locus is systemically altered in patients with HD, with conditional differences between clinical stages. Given the high clinical need in having a molecular tool to assess disease progression in HD, these results strongly suggest that the non-invasive assessment of chromosome conformation signatures warrant further study as a prognostic tool in HD.

Keywords

Huntington's disease, epigenetics, chromosome conformation signature, chromatin architecture

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REVISED Amendments from Version 2

We thank the reviewer for their continued efforts in assessing our manuscript. Based on the most recent round of reviews the remaining comments revolve around the central question of the sample size and the nature of the conclusions we can draw from them. This is a point where we wholeheartedly agree and have updated the text to reflect the reviewer's comments.

We have updated the title to reflect the nature of the study and the extent to which we can draw conclusions from it.

We mention in the Introduction that there are many other genetic, environmental and unknown factors that can influence onset and progression of HD.

We have modified the language in the Abstract-Conclusions, Discussion-Results summary and Discussion-Strengths and limitations to directly address the reviewers concern that our sample size was relatively small in comparison to previous studies. As is often the case when studying rare disease like HD, the availability of clinical material for research is often in high demand and short supply. We look forward to working with a broader coalition of HD researchers and sample providers in the near future to engage in follow-on studies to this work in a larger patient population.

See referee reports

Introduction

Huntington's disease (HD) is a neurodegenerative condition characterized cellularly by the loss of neurons in the basal ganglia and clinically by uncontrolled movements, emotional problems, and loss of cognition¹. HD is an autosomal dominantly inherited disorder and although prevalence rates range widely depending on geography and ethnicity, it is thought to affect more than 50,000 people in the United States and Europe alone². The underlying genetic cause is a trinucleotide CAG expansion in the huntingtin gene (*HTT*), discovered as a genetic marker by James Gusella from Massachusetts General Hospital in 1983, which results in the production of a mutant huntingtin protein (mHTT) with a toxic poly-glutamine (polyQ) tract^{3,4}. However, despite decades of research and clinical trials, no successful therapy has yet been developed. The "typical" onset of HD is between the ages of 40–50 years, but up to 15% of cases have very late onset and don't show clinical symptoms until after the age of 60 years⁵. In a recent meta-analysis of studies investigating cases of late-onset HD (LoHD, defined as onset after 60 years of age), more than 90% of patients had CAG repeat lengths of ≤ 44 ⁶. One of the more interesting observations in HD is that while there is a well-known correlation between the length of the polyQ repeat tract and the onset and severity of the disease, there is substantial variability within individual patients. For example, in patients with mid-range repeat lengths (defined here as between 40 and 50), disease onset can vary by 60 years in any individual patient⁵. This means that many patients who are carriers of polyQ tracts that predispose to the development of the disease can live for decades in a "presymptomatic" state⁷. What controls the onset of clinical symptoms remains currently unknown, and complicates the prognostic evaluation of HD patients.

Although historically considered a monogenic disease; genetic, environmental and unknown factors can influence

disease onset and progression. Many different technologies have been used to look at the molecular changes underlying disease progression in HD, including gene expression, proteomics, metabolomics, network analysis, genomics and single nucleotide polymorphism (SNP) profiling^{8–13}. Recently, epigenetic approaches have emerged as a promising new tool for assessing pathology-related changes¹⁴. Most epigenetic studies in HD have focused on looking at genome-wide histone modifications (acetylation, methylation) or histone modifications at specific loci related to HD^{15–18}. While these approaches have provided interesting insight into the disease, they have yielded often conflicting results and shown inconsistencies between mouse models and human disease^{14,19,20}. Indeed, due to the global nature of these types of epigenetic analyses, they may lack the sensitivity to discriminate the subtler changes associated with disease onset and progression. As such, a consensus picture of epigenetic deregulation in HD using histone modification readouts has yet to materialize. However, not all molecular mechanisms associated with epigenetic regulation have been assessed in the context of HD. An important aspect of epigenetic regulation is at the level of 3-dimensional (3D) genomic architecture²¹.

The 3D organization of the genome reflects the heterogeneous effects of external environmental cues and inputs, and can be empirically measured by the assessment of chromosome conformations or when several conformations are measured concomitantly, a chromosome conformation signature (CCS)²¹. CCSs can be thought of as the molecular barcode that gives a readout of the epigenetic landscape of a given cellular population^{22,23}. To date, the evaluation of CCSs in HD has remained unexplored. Given the central role of mHTT in the development of HD, we hypothesized that regulatory differences in genomic architecture at the *HTT* locus may exist between diseased individuals and healthy, unaffected controls.

We used *EpiSwitch*, an established proprietary industrial platform for monitoring CCSs, to assess chromatin architecture differences between pre-symptomatic and symptomatic HD patients and healthy, unaffected individuals. *EpiSwitch* readouts provide high resolution, reliable and high throughput detection of CCSs while simultaneously meeting the high bar of industry standards for quality control²¹. As such, this technology represents a powerful tool for screening, evaluation and monitoring of CCS in human disease²⁴. This platform has been successfully utilized as a biomarker modality to stratify patients in the context of a variety of other diseases^{25–30}, including as a non-invasive blood based biomarker for neurodegenerative conditions^{31,32}.

Methods

Sample collection

All blood samples were obtained from National BioService, LLC, a research biobank operating in compliance with the requirements of the International Society for Biological and Environmental Repositories. In total, 20 blood samples were used in this study; 10 healthy control (HC) samples (CAG repeats, $n < 35$), and 10 HD samples (CAG repeats, $n > 39$). For the HD samples, 7 were from symptomatic patients (HD-Sym) and 3 were from presymptomatic patients who had a diagnosis of HD but

did not yet show any clinical symptoms (HD-Pre). One HD patient was taking tetrabenazine and one patient was taking sertraline. All samples were negative for human immunodeficiency virus, hepatitis B virus, hepatitis C virus and syphilis (Supplemental Table 1).

Study design

We wanted to identify chromosome conformations that differed between healthy controls (low CAG), presymptomatic HD patients (high CAG, no disease manifestation) and symptomatic HD patients (high CAG, disease manifestation). We focused on a ~225 kb region surrounding the *HTT* locus from (chr4: 3,033,588 to 3,258,170 as annotated in hg38) for our analysis. Using the CAG repeat expansion tract in exon 1 of *HTT* (chr4: 3,054,162 to 3,095,930) as the anchor point ("Anchor"), we defined five genomic zones surrounding the anchor to look at chromosome conformations that varied between sample groups (Figure 1 and Supplemental Table 2). These Zones were chosen based on: the presence of potential *EpiSwitch* anchoring sites, the presence of known disease-related SNPs (HD and other diseases), and the enrichment of known histone modification sites (H3K4me3, H3K36me3, and H3K27ac) in HD as found in the GWASdv2 database (<http://jjwanglab.org/gwasdb>) (Figure 1).

Chromosome conformation identity

A search of the NCBI Gene Expression Omnibus database for previously reported HD epigenetic data was performed in February 2018³³. Peak-called ChIP-seq data for H3K4me3 from 12 (6 HD and 6 control samples) post-mortem prefrontal cortex brain samples (bed format) was obtained (GSE68952)³⁴. In addition, Bigwig tracks of ChIP-seq data for H3K27ac and H3K36me3 from HD iPSC-derived neural cell lines and control cell lines

were also downloaded (GSE95342)³⁵. The data tracks were loaded into the Integrative Genome Viewer (IGV)³⁶ version 2.4 alongside the *EpiSwitch* and reference sequence annotations. Both visual and programmatic (BEDtools) comparisons were performed on the *HTT* locus to identify the five zones of interest.

Oxford BioDynamics proprietary *EpiSwitch* pattern recognition algorithm was used to identify high probability chromatin folding interactions with one "end" occurring in the anchor zone proximal to the CAG repeats and the other in any of the five zones of interest. A total of 61 interactions matched these criteria, and for practical reasons, 20 interactions were selected to cover interactions between the anchor site and all the zones of interest. Oxford BioDynamics automated primer design application was used to design oligonucleotide pairs that amplified the expected DNA sequence caused by the interaction when subjected to the chromosome conformation capture (3C) assay.

3C and PCR

3C and detection by PCR were performed as described previously^{25,28,30,37}. Chromatin with intact chromosome conformations from 50 µl of blood sample from each patient sample was extracted using the *EpiSwitch* assay following the manufacturer's instructions (Oxford BioDynamics Plc). Quality control on all samples was done using the detection of a chromatin loop at the *MMP1* locus, a historical internal control for 3C analysis³⁰. Pooled 3C libraries for each of the sample types were generated to provide a generalized population sample for each of the C sample subgroups. Real-time PCR experiments were performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines³⁸. Real-time PCR was performed with SYBR green

Figure 1. Definition of the anchor point and zones for this study. A visual overview of the genomic region investigated in this study. A ~225 kb region on chromosome 4 spanning the *HTT* locus was investigated. The anchor point ("Anchor" in track 4) was defined as a ~42 kb region spanning the CAG repeat tract in exon 1 of *HTT* (purple arrow at the top of the figure). We defined five Zones (Zones 1 -5 in track 4) based on overlap with *EpiSwitch* sites (track 3), SNPs related to Huntington's disease (HD) (track 5) or other diseases (track 6), and observed methylation and acetylation (H3K4me3, H3K36me3 and H3K27Ac) differences between healthy control (HC) and HD (tracks 7 through 12).

with the CFX-96 (Bio-Rad) machine to identify the interactions with differing PCR product detection patterns between the sample types³⁹. Oligonucleotides were tested on control templates to confirm that each primer set was working correctly. A full list of the primers and PCR conditions used in this study can be found in [Supplementary File 1](#). The final nested PCR was performed on each sample in triplicates for the follow up data on individual patients with HD. This procedure permitted the detection of limited copy-number templates with higher accuracy. All PCR-amplified products were monitored on the LabChip® GX from Perkin Elmer, using the LabChip DNA 1K Version 2 kit (Perkin Elmer) and internal DNA markers were loaded on the DNA chip according to the manufacturer's protocol using fluorescent dyes. Fluorescence was detected by laser and electropherogram read-outs translated into a simulated band on gel picture using the instrument software. The threshold of detection for the instrument was set by the manufacturer from 30 fluorescence units and above. All raw gel images for the PCR assays done in this study as well as a description of each set of comparisons can be found in [Dataset 1](#)⁴⁰.

Statistical analysis

Data analysis was performed in R (language and environment for statistical computing) version 3.5.1 (<https://www.r-project.org>)⁴¹. This included stats (version 3.6.0) and [dplyr](#) (version 0.7.6) packages for t-tests and R² analysis & a [ggplot2](#) (version 3.0)⁴² package for boxplots and regression plots.

Results

Patient clinical characteristics

HC and HD samples were age (average 36.9 years for HC and 35.3 years for HD) and sex matched (10 male and 10 female),

with the majority (70%) of HD cases being symptomatic ([Table 1](#)). All samples were from non-Hispanic or Latino whites. Average CAG repeats lengths were 25.7 for HC and 44.2 for HD ([Table 1](#), [Figure 2](#)). There was no statistical difference in CAG repeat length between HD-Pre and HD-Sym ([Figure 2](#)). The average age at diagnosis for HD samples was 35.3 years and the average disease duration was 3.8 years with 7 out of 10 patients reporting symptoms of irritability, chorea, or both ([Table 1](#)).

Chromosome conformations in HC, HD-Pre and HD-Sym

Of the 20 interactions that were evaluated ([Supplemental Table 3](#)), we identified nine informative interactions. We identified two constitutive interactions and seven conditional interactions which were present in HD, but not healthy controls. Of the seven conditional interactions, three were present only in HD-Sym, and absent in HD-Pre.

Constitutive conformations

All samples passed internal QC analysis for the MMP1 interaction ([Supplementary Figure 1](#) and [Supplementary Figure 2](#)). Two constitutive (identified in all samples) chromatin loops were identified. Both loops were between the Anchor and Zone 2 with the first loop spanning 28 kb and the second loop spanning 34 kb ([Figure 3](#)).

Conditional conformations

We identified seven conditional chromosome conformations that could discriminate between the different patient subgroups evaluated in this study. Specifically, we identified two chromosome interactions that were present in HC, but absent in all HD samples ([Figure 4](#)). The first interaction (I1) spanned the anchor

Table 1. Clinical characteristics of the samples used in this study.

Variable	Healthy controls (N=10)	Huntington's disease (N=10)
Gender		
Male (N, (%))	5 (50)	5 (50)
Female (N, (%))	5 (50)	5 (50)
Ethnicity		
Non-Hispanic or Latino	10 (100)	10 (100)
Race		
White (N, (%))	10 (100)	10 (100)
Huntington's Type		
Symptomatic (N, (%))	N/A	7 (70)
Asymptomatic (N, (%))	N/A	3 (30)
CAG repeat length (average, (SD))	25.7 (5.4)	44.2 (2.6)
Age at diagnosis (average, (SD))	N/A	34.9* (8.1)
Age at sample collection (average, (SD))	36.9 (12.8)	35.3 (10.0)
Disease duration (years) (average, (SD))	N/A	3.8 (1.9)**
% Reporting irritability	N/A	70
% Reporting chorea	N/A	50

*Age at Diagnosis was not available for 2 of the 10 HD patients. **Disease duration could not be calculated for 2 of the 10 HD patients. N/A, not applicable.

Figure 2. Clinical characteristics of the samples used in this study. (A) The HC (red) and HD (blue) samples showed no statistical difference in age. HD-Pre patients were younger (average age = 25.3) than HD-Sym patients (average age = 39.6) ($p = 0.02$). There was a moderate negative relationship between disease duration and CAG repeat size and a moderate positive relationship between age at diagnosis and CAG repeat size, though neither were statistically significant (data not shown). (B) There was a statistically significant increase in CAG repeat length in HD patients (blue) relative to HC (red) ($p = 1.08 \times 10^{-7}$). There was no statistical difference in CAG repeat length between HD-Pre and HD-Sym ($p = 0.09$) (data not shown).

Figure 3. Summary of constitutive interactions. Two constitutive interactions occurring in all patients (symptomatic Huntington's disease (HD-Sym), Pre-symptomatic HD (HD-Pre) and healthy control (HC)) (red boxes) were observed in this study. Both interactions (C1 & C2) were between the anchor and zone 2 with C1 (A) spanning 28 kb and C2 (B) spanning 34 kb. TE=Tris-EDTA.

Figure 4. Summary of conditional interactions: healthy controls (HCs). Two conditional interactions (red boxes) were observed in HC patients only and were absent from all Huntington's disease (HD) patients. (A) The first interaction (I1) occurred between the Anchor and Zone 4 and spanned 77 kb. (B) The second interaction (I2) occurred between the Anchor and Zone 3 and spanned 140 kb. TE, Tris-EDTA.

and zone 4 and covered 77 kb while the second interaction (I2) spanned the anchor and zone 3 and covered 140 kb. We also identified two chromosome interactions that were present in HC and HD-Pre, but absent in HD-Sym samples. Both interactions (I3 and I4) spanned the anchor and zone 4, and covered 92 kb and 104 kb, respectively (Figure 5). Last, we identified three chromosome interactions that were present in HD-Sym samples, but absent in HD-Pre and HC samples. The first of these three conditional interactions (I5) spanned the anchor and

zone 3, covering 122kb. Notably, this interaction included a SNP (rs362331) known to be a factor in the predisposition to develop HD. The second and third conditional interactions (I6 and I7) spanned the Anchor and Zone 1 and covered 185 kb and 174 kb, respectively (Figure 6). Last, we tested the absence or presence of all conditional interactions in individual HD samples. In the HD-Sym samples, we found the presence of at least one of the conditional markers (I5, I6 and I7) in six out of seven samples (Figure 7 and Supplementary Figure 3–Supplementary Figure 5).

Figure 5. Summary of conditional interactions: Huntington's disease (HD)-negative. Two conditional interactions (red boxes) were observed in healthy control (HC) patients and pre-symptomatic HD (HD-Pre) patients, but were absent from symptomatic HD (HD-Sym) patients. Both interactions (I3 & I4) occurred between the anchor and zone 4 and spanned 92 kb (**A**) and 104 kb (**B**), respectively. TE, Tris-EDTA.

The odds ratios of each interaction being associated with symptomatic HD presentation were 30, 9 and 16 for I5, I6 and I7, respectively. A summary of all the interactions that were evaluated in this study are shown in [Figure 8](#) and [Supplementary Figure 6](#).

Dataset 1. Raw gel images for all PCR reactions performed in this study

<https://dx.doi.org/10.5256/f1000research.15828.d222873>

Also included is a guide to the contents of the images.

Figure 6. Summary of conditional interactions- Huntington's disease (HD) positive. Three conditional interactions (red boxes) were observed in symptomatic HD (HD-Sym) patients only and were absent from HC and pre-symptomatic (HD-Pre) patients. (A) The first interaction (I5) occurred between the anchor and zone 3 and spanned 122 kb, including a HD-associated SNP known to be involved in disease progression. (B and C) The second and third interactions (I6 & I7) occurred between the anchor and zone 1, and spanned 185 kb and 174 kb, respectively. TE, Tris-EDTA.

Figure 7. Conditional interactions observed in individual symptomatic Huntington's disease (HD-Sym) samples. In six out of seven individual HD-Sym samples, the presence of at least one of the three conditional interactions (I5, I6 and I7) was observed. I5, the interaction spanning the region that contains the rs362331 SNP, was observed in the greatest number of samples (5/7).

	Interaction						
	1	2	3	4	5	6	7
Healthy Control	Interaction present	Interaction present	Interaction present	Interaction present	Interaction absent	Interaction absent	Interaction absent
HD-Pre	Interaction absent	Interaction absent	Interaction present	Interaction present	Interaction absent	Interaction absent	Interaction absent
HD-Sym	Interaction absent	Interaction absent	Interaction absent	Interaction absent	Interaction present	Interaction present	Interaction present

Interaction present
 Interaction absent

Figure 8. Model for epigenetic changes driving the symptomatic progression of Huntington's disease (HD). Overview of the chromosomal conformation changes associated with the progression of HD. As patients progress from presymptomatic stages to symptomatic diseases, discrete, measurable and discriminating changes in the genomic architecture at the *HTT* locus are observed.

Discussion

Problem statement and results summary

While it is well-known that individuals with greater than 39 CAG-repeats will get HD, the clinical onset of disease varies widely amongst individual patients and the factors that influence when the disease manifests clinically are less well characterized. Here we used *EpiSwitch*, an industrial platform for assessing chromatin architecture, to evaluate the epigenomic landscape of the *HTT* locus in HD patients and healthy, unaffected controls. We identified a set of seven interactions that when taken together as a CCS, could differentiate HD from unaffected controls and more importantly, could differentiate between presymptomatic and symptomatic HD patients. One of these interactions, specific for symptomatic HD, contains a SNP (rs362331) shown to be associated with a predisposing disease haplogroup. When taken together, these results provide an initial indication that a simple, non-invasive blood-based test evaluating a CCS deserves further study and validation as a surrogate biomarker for assessing disease progression in HD.

Biological relevance

While it is known that the poly-Q repeat tract expansion and production of mHTT are the underlying causes of HD, the molecular events leading to the development of clinical symptoms are less well characterized. Several studies have looked at SNPs within the *HTT* locus as a potential contributor to disease onset. One recent SNP genotyping study of HD patients identified ~41 SNPs heterozygous in at least 30% of the patients, including the rs362331 C/T SNP in exon 50 of the *HTT* gene⁴³. Perhaps more biologically relevant is that when the rs362331 SNP is allele-selectively knocked down using anti-sense oligonucleotides, siRNAs or miRNA, a dramatic reduction in the levels of mHTT protein is achieved both *in vitro* and *in vivo*, suggesting that this SNP and its surrounding genomic landscape play an important role in regulating mHTT levels^{44–46}. In this study, we observed a chromosome conformation (I5) that was present in HD-Sym patients, was absent in HD-Asy and HCs, and overlapped with the rs362331 SNP. While requiring further study, this observation raises the interesting possibility that the production of neurotoxic mHTT in patients that have increased poly-Q tracts and a genetic predisposition to the early development of

HD by the presence of the rs362331 SNP may be regulated at the level of higher-order chromatin structure. Another outstanding question in HD is how the disease is inherited in cases where neither parent has received a diagnosis. The two main prevailing hypotheses posit that 1) the carrier parent could have passed away from another factor before the onset of the disease and 2) “unstable” CAG repeat tracts expand with each generation. A third possibility also exists, in that at mid-range (35–50) repeats, individuals could be carriers without manifestation of the disease, but their progeny might be unable to compensate for the genetic defect through undefined mechanisms and will develop the disease. The HD patients evaluated in this study all had CAG repeats in this mid-range, raising the possibility that potential compensatory mechanisms in disease development may be mediated through differences in genomic architecture.

Clinical relevance

HD is rare, in that there exists a simple test to definitively diagnose the disease, *HTT* gene sequencing and measurement of CAG repeat number. For clinical care and clinical trials, there are also several tests to measure disease severity, such as the Unified Huntington's Disease Rating Scale, the Shoulson–Fahn Scale, and the Mini–Mental State Examination^{47–49}. While these assessments measure different elements of an HD patients physical and mental well-being as a surrogate for disease severity, they are all subjective in nature and most are not specific for HD. What is missing are concrete molecular tools to monitor disease progression.

As of the time of this writing, there are 22 therapeutic agents for treating HD in different stages of preclinical and clinical development, half of which are in Phase 2 or Phase 3. Once further validated, the CCS reported here could be used in clinical trials as a surrogate outcome biomarker to assess the therapeutic efficacy of the drug in question. In addition to monitoring a symptomatic patient's response to a particular therapy in clinical trials, another advantage of the approach described here lies in the information that can be obtained for pre-symptomatic patients. For most patients with HD, the pre-symptomatic period can last decades. Five of the seven (i3–i7) interactions

identified here clearly separate presymptomatic HD patients from symptomatic ones, and when further validated could serve as an “early warning” indicator test for the onset of HD symptoms in presymptomatic carriers.

Strengths and limitations

This study gives first evidence of detectable conditional differences in chromatin architecture specific for the manifestation of HD and correlated with known disease haplotypes. The major strength of this study lies in its unique approach, which is based on the latest developments in understanding the regulatory role of genomic architecture. While there have been several historical studies in HD aimed at developing disease progression biomarkers based on clinical, imaging and molecular measures⁵⁰, to the best of our knowledge this is the first time that the assessment of higher-order chromatin structures in a clinically accessible biofluid has been applied in HD. With the successful application of *EpiSwitch* in another neurodegenerative condition, amyotrophic lateral sclerosis, as well as other non-neurological conditions such as melanoma, diffuse large B-cell lymphoma, chronic myelogenous leukemia, breast cancer, and rheumatoid

arthritis, the results presented here further validate the use of regulatory conditional CCS as disease-related biomarkers^{25,28–31}. Of note, previous studies evaluating CCS as biomarkers of disease have been done on larger sample sizes. A notable limitation of this study was the relatively small sample size, partially a limitation imposed by the rare nature of HD. While the data presented here offer a novel insight into the clinical progression of HD, this study was intended to be a proof-of-concept and not powered for statistical significance. A follow-up study using a larger patient cohort will be required for validation of these initial results.

Data availability

Dataset 1. Raw gel images for all PCR reactions performed in this study. Also included is a guide to the contents of the images. DOI: <https://doi.org/10.5256/f1000research.15828.d222873>⁴⁰.

Grant information

This study was funded by Oxford BioDynamics, Plc.

Supplementary material

Supplementary Table 1. Clinical characteristics of the samples used in this study.

[Click here to access the data](#)

Supplementary Table 2. Genomic definitions for the Anchor point and Zones used in this study.

[Click here to access the data](#)

Supplementary Table 3. Summary of interactions tested in this study.

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Supplementary Figure 1. Assessment of the MMP1 interaction for Quality Control.

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Supplementary Figure 2. Assessment of the MMP1 interaction for Quality Control.

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Supplementary Figure 3. Evaluation of the presence or absence of interaction 5 (I5) in individual samples from symptomatic HD (HD-Sym) patients.

[Click here to access the data](#)

Supplementary Figure 4. Evaluation of the presence or absence of interaction 6 (I6) in individual samples from symptomatic HD (HD-Sym) patients.

[Click here to access the data](#)

Supplementary Figure 5. Evaluation of the presence or absence of interaction 7 (I7) in individual samples from symptomatic HD (HD-Sym) patients.

[Click here to access the data](#)

Supplementary Figure 6. Summary of all interactions identified in this study.

[Click here to access the data](#)

Supplementary File 1. Primer sequences and PCR conditions used in this study.

[Click here to access the data](#)

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Mónica Bañez-Coronel

Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, FL, USA

The manuscript by Salter et al. proposes the analysis of chromatin architecture at the HTT locus as a reliable tool to predict/evaluate HD onset and progression using peripheral blood. The authors used the platform *EpiSwitch*[™] to analyze chromosome conformations in 20 age-matched blood samples, with ten of them obtained from healthy subjects, seven from symptomatic HD patients and three from asymptomatic HD mutation carriers.

The authors analyzed 20 different interactions, identifying nine be informative. Two of these interactions were constitutive (found in the three groups healthy, symptomatic HD and asymptomatic HD samples) and seven of them that were detected in HD samples but not in controls. Interestingly, 3 of these interactions were only found in the symptomatic, but not asymptomatic HD samples. The analysis and status of these interactions are presented by the authors as potential and promising biomarker for the prediction and progression of disease in HD.

Major comments:

The sample size is very small. A much larger number of samples is necessary to establish any correlation with biomarker potential. I understand that the authors intend to proof the potential applicability of the strategy in HD, but I insist that results, although clean, are not conclusive to base an association between each interaction and disease. Each interaction is not present in all the symptomatic or all the asymptomatic cases either, which makes the conclusion a bit harder to reach. I believe in the strategy presented by the authors and I would love to see reproducibility, validation and stronger significance by the replication of the technique in a bigger sample size. Repeat instability occurs across tissues, with high prevalence in brain, where HD symptoms are more devastating CAG repeats (much) longer that the repeat detected in blood are being expressed. This variability in repeat length across tissues makes more difficult the identification of useful biomarkers. It is essential to increase the size sample in this study to guarantee that the observed changes are indeed associated to disease status.

Other comments:

- I suggest the authors to present the results obtained in each individual sample (not only HD symptomatic cases) as it can be very informative, not only for the acquisition of a given

interaction but for the loss or a “putative protective” interaction in presymptomatic that might be indicative of an early change for disease manifestation.

- The authors indicate that there were no statistical difference in the repeat length between symptomatic and asymptomatic patients. Given that repeats over 39CAG tracts can cause the disease to manifest and that 1-2 additional repeats can set a different, I believe it will be beneficial to disclose the repeat lengths of each sample.
- Have the authors checked if the presence of the “biomarker” interactions have an effect on the levels of HTT transcript or HTT protein? It would be interesting to check.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: NeuroGenetics, Huntington's disease, RNA toxicity, RAN translation, CAG expansion disorders, microsatellite expansion repeat disorders.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Version 2

Reviewer Report 11 April 2019

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Willeke M.C. van Roon

Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands

I thank the authors very much for their reply to my comments. Although I have no doubts about the validity of the approach and the techniques used, I still believe the small sample size together with a high variability within this small sample size provides good pilot data for a bigger study, but as it is now it is not enough to publish.

Response to Comment #1: "This aim of this study was not a full-scale analytical validation of biomarkers, but as stated in the Introduction, to introduce chromosome conformation signatures as a measurement modality with the potential to be developed into a clinical biomarker in the field of Huntington's disease."

Reviewer Comment to response #1: If this is the case than this should clearly be reflected in the title. The title is now misleading. I am not stating that the methods are invalid, or not properly done, but I remain of the opinion that the sample size is too small, and the results are too variable to draw any conclusion. See Figure 3 and my previous comment #9.

Reviewer Comment to response #2: I still think the text should be changed to something like: "HD is a monogenic disease but there are many other genetic, environmental and unknown factors that can influence onset and progression of the disease".

Reviewer Comment to response #4: Carini *et al* used 59 patients, Salter *et al* used 74 unblinded patient samples, Yan *et al* used 58 patients. Proof of concept of this analysis was done in these papers. However, if this analysis is useful as a tool to assess disease progression cannot be deducted from the data presented in this paper.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Huntington disease, gene expression analysis, biomarker development, therapy development.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Version 1

Reviewer Report 08 February 2019

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Willeke M.C. van Roon

Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands

This paper investigates genome architecture differences at the HTT locus. There are several major issues with this study. The small sample size is the biggest issue. In my opinion it is too small to draw any conclusions. How a disease progression marker can be deduced from 3 presymptomatic samples (early disease state?) and 3 symptomatic samples is not clear. If the differences in chromosomal structure are indeed different around the HTT locus, what would be the functional consequence, and then this should be validated. Upon treatment, would this chromosomal then reverse? So far there is no evidence from brain tissue and fibroblast cells that there is a major difference in transcription from the wild type and mutant allele.

"Although historically considered a monogenic disease, extensive research into the underlying pathology of HD suggests that the mechanisms leading to disease onset and progression are more complex than originally thought".

This is not a correct statement. It is a genetic disease; no polyQ expansion, no HD. But there are genetic modifiers that can influence onset and maybe progression.

"HD is considered a paradigm of a disease characterized by epigenetic dysregulation".

This statement is not correct. HD is characterised by dysregulation in many cellular processes, and epigenetic deregulation is only one of them.

The number of samples is really too small. I have worked with human tissue for many years, and in my opinion these group sizes are too small. There are only 3 presymptomatic patients and 7

symptomatic. Furthermore, a CAG repeat range of 40-48 is not enough to say something about progression or severity. There is no appropriately age-matched control group for the presymptomatic HD group (Figure 1D).

"Peak-called ChIP-seq data for H3K4me3 from 12 (6 HD and 6 control samples) post-mortem prefrontal cortex brain samples (bed format) was obtained (GSE68952)³⁴. In addition, Bigwig tracks of ChIP-seq data for H3K27ac and H3K36me3 from HD iPSC-derived neural cell lines and control cell lines".

So all the histone modification data that was used to identify zones of interest and to identify high probability folding interactions was based on data from neuronal cells/tissue. Is there anything known about how these histone modifications in brain relate to histone modifications in blood cells? I think this is a very important issue and potential pitfall of this paper.

For practical reasons, 20 interactions were selected from the identified 61 high probability chromatin interactions. Was this selection done randomly, for technical reasons, or were there any selection criteria? Please add this to the Methods section.

Figure 2A is obsolete because the same samples are all used for Figure 2B. This figure can be removed from the paper. It is not very informative. All the relevant information is already in the table.

Figure 3 illustrates that there are not enough samples in this study. The group size is much too small to include figures like this. Nothing is significant and the variability is large.

Figure 4: The constitutive interactions in A is much weaker in the HD-Symp, group and in 4B much weaker in the HC group. How constitutive are these interactions? This Figure shows a summary. When I look at the individual data.

Figure 8, the variability in the samples again is too large. What were the results for the individual presymp-HD?

Figure 8 and 9: If I look at I6 for all the symp-HD samples, it was present in 3 and absent in 4, so in the majority it is absent. However in Figure 9 it is shown that interaction 6 is present in HD-symp.

Since there is such a variation in absence and presence of interactions within the individual HD samples, there should also be a figure in the paper regarding the absence and presence of the interactions in the individual control samples. Also for the constitutive interactions.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Huntington disease, gene expression analysis, biomarker development, therapy development

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 21 Mar 2019

Willem Westra, Oxford BioDynamics Plc, Oxford, UK

We would like to thank Dr. van Roon for taking the time to read our paper in F1000Research and providing her thoughtful and insightful comments.

We have included a point by point response to these comments in below:

Comment #1: *This paper investigates genome architecture differences at the HTT locus. There are several major issues with this study. The small sample size is the biggest issue. In my opinion it is too small to draw any conclusions. How a disease progression marker can be deduced from 3 pre-symptomatic samples (early disease state?) and 3 symptomatic samples is not clear. If the differences in chromosomal structure are indeed different around the HTT locus, what would be the functional consequence, and then this should be validated. Upon treatment, would this chromosomal then reverse? So far there is no evidence from brain tissue and fibroblast cells that there is a major difference in transcription from the wild type and mutant allele.*

Response: This aim of this study was not a full-scale analytical validation of biomarkers, but as stated in the Introduction, to introduce chromosome conformation signatures as a measurement modality with the potential to be developed into a clinical biomarker in the field of Huntington's disease. It is known that current molecular modalities for assessing disease progression, including transcriptional profiling, have failed to provide clinicians with a reliable prognostic test for actionable stratification of patients with mid-range CAG extensions. This has been articulated by Prof. John Hardy, FRS, Chair of Molecular Biology of neurological Diseases, University College London, 2015 Breakthrough Prize, 2018 Brain Prize, at the London Lancet Neurology Conference in 2016. This study aimed to demonstrate, through an objective Odds Ratio evaluation on a small sized cohort, that a binary biomarker profile does show disseminating properties and therefore is worthy of further investigation in a larger cohort. The study used an approach that has been recently

published in other disease areas, notably amyotrophic lateral sclerosis (ALS) and rheumatoid arthritis. In each of these studies, chromosome conformation signatures were developed and independently evaluated on an independent cohort of patients using a much larger sample size (Carini et al., *J Translational Medicine* 2018; Salter et al., *EBioMedicine* 2018). It is the intent to use the preliminary work described here to prompt further validation studies in a larger cohort of HD patients.

Comment #2: *“Although historically considered a monogenic disease, extensive research into the underlying pathology of HD suggests that the mechanisms leading to disease onset and progression are more complex than originally thought”.*

This is not a correct statement. It is a genetic disease; no polyQ expansion, no HD. But there are genetic modifiers that can influence onset and maybe progression.

Response: We agree with the reviewer that from a basic standpoint, if there is no polyQ expansion, there is no HD. Our point in making this statement was that there are factors that influence disease onset and progression acting outside of polyQ expansion alone. This was recognized at the Lancet Neurology Conference in 2016 (see Response to Comment #1 above) and is supported by the observation that in HD individuals with mid-range polyQ expansions, there is a wide temporal range of clinical disease onset/manifestation and many patients remain asymptomatic for decades. In a similar vein, patients with identical polyQ expansions can manifest the disease at different times in their life. While there are several factors that may contribute here, epigenetic compensation is one of them and offers a novel molecular means to discriminate between genetic HD patients who manifest symptoms earlier in life and those who remain asymptomatic with delayed disease onset. This is a question of unmet clinical need and of high utility in clinical trials.

Comment #3: *“HD is considered a paradigm of a disease characterized by epigenetic dysregulation”.*

This statement is not correct. HD is characterised by dysregulation in many cellular processes, and epigenetic deregulation is only one of them.

Response: Our statement acknowledges the role that changes in genome architecture, as an epigenetic modality, could play in clinical manifestation of HD, along with other regulatory processes. For clarity, we have adjusted the statement to meet the reviewer's preferences.

Comment #4: *The number of samples is really too small. I have worked with human tissue for many years, and in my opinion these group sizes are too small. There are only 3 presymptomatic patients and 7 symptomatic. Furthermore, a CAG repeat range of 40-48 is not enough to say something about progression or severity. There is no appropriately age-matched control group for the presymptomatic HD group (Figure 1D).*

Response: As discussed, in a previous comment, we recognize the sample size as a limitation of the study and have noted this in the “Strengths and limitations” section. The primary goal of this study was to provide an initial proof-of-concept indication that the assessment of chromosome conformations at the HTT locus may be a useful molecular approach for assessing disease progression. We anticipate taking the chromosome

conformations identified here as disseminating features into larger scale analytical validation studies, as has been done in previous studies (Carini et al., J Translational Medicine 2018; Salter et al., EBioMedicine 2018; Yan et al., Surgery 2019).

Comment #5: *"Peak-called ChIP-seq data for H3K4me3 from 12 (6 HD and 6 control samples) post-mortem prefrontal cortex brain samples (bed format) was obtained (GSE68952)34. In addition, Bigwig tracks of ChIP-seq data for H3K27ac and H3K36me3 from HD iPSC-derived neural cell lines and control cell lines".*

So all the histone modification data that was used to identify zones of interest and to identify high probability folding interactions was based on data from neuronal cells/tissue. Is there anything known about how these histone modifications in brain relate to histone modifications in blood cells? I think this is a very important issue and potential pitfall of this paper.

Response: We direct the reviewer to a selection of peer reviewed studies in neurological and psychiatric disorders that clearly show significant epigenetic systemic profiling when measured in blood and compared to the primary sites of deregulation (as an example, Lin et al. Characterization of Cross-Tissue genetic-epigenetic effects and their patterns... Genome Medicine 2018; Medrano-Fernandez and Barco, Nuclear Organization and 3D Chromatin Architecture in Cognition and Neuropsychiatric Disorders Molecular Brain 2016). The phenomenon of "horizontal transfer" and "exosome signalling" first demonstrated in oncological conditions (Feinberg, Key Role of Epigenetics in Human Disease Prevention and Mitigation New England J of Medicine 2018; Ratajczak Clinical and Translational Medicine 2016) underlies the extensive spectrum of published evidence for systemic epigenetic signatures. It is also important to note that the H3K27ac histone modification has been shown to correlate with changes in genome architecture (Huang et al. Genome Biology, 2016).

Comment #6: *For practical reasons, 20 interactions were selected from the identified 61 high probability chromatin interactions. Was this selection done randomly, for technical reasons, or were there any selection criteria? Please add this to the Methods section.*

Response: The 20 interactions were not selected randomly. The study design section and Fig.1 describes in detail the choices of regions and corresponding *EpiSwitch* sites (20 in total) for the analysis with the CAG repeat anchor site. This design and positions of the interacting sites is based on established methodology described recently in Carini et al. 2018; Salter et al., 2018, Yan et al., 2019). We are happy to further elaborate on the details of the design.

Comment #7: *Figure 2A is obsolete because the same samples are all used for Figure 2B. This figure can be removed from the paper. It is not very informative. All the relevant information is already in the table.*

Response: The rationale for including Figures 2A and 2B was to highlight that there was no statistical difference in CAG repeat length between symptomatic and presymptomatic HD patients. We agree that this information could be summarized in text and have updated Figure 2&3 into a combined Figure to reflect this.

Comment #9: *Figure 3 illustrates that there are not enough samples in this study. The group size*

is much too small to include figures like this. Nothing is significant and the variability is large.

Response: We agree that and have combined Figures 2 and 3 to only include the main clinical comparisons.

Comment #10: *Figure 4: The constitutive interactions in A is much weaker in the HD-Symp, group and in 4B much weaker in the HC group. How constitutive are these interactions? This Figure shows a summary. When I look at the individual data.*

Response: A chromosome conformation is a binary modality, as described and defined in the literature (Crutchley et al. Chromatin Conformation signatures: ideal human biomarkers? Biomarkers Med 2010), and similar to the binary nature of a genetic variant. The presence of a conditional chromosomal conformation reflects active clonal support for systemic deregulation associated with a particular phenotype with copy numbers acting as a secondary input. This is not a continuous modality, like transcription, where for example a 20% increase/decrease in gene expression may correlate with a change in phenotype.

Comment #11: *Figure 8, the variability in the samples again is too large. What were the results for the individual presymp-HD? Figure 8 and 9: If I look at I6 for all the symp-HD samples, it was present in 3 and absent in 4, so in the majority it is absent. However, in Figure 9 it is shown that interaction 6 is present in HD-symp. Since there is such a variation in absence and presence of interactions within the individual HD samples, there should also be a figure in the paper regarding the absence and presence of the interactions in the individual control samples. Also for the constitutive interactions.*

Response: As described in the paper, for binary marker selection we first evaluated pooled samples with the same clinical annotation – HC, symp-HD, presymp-HD. With binary readouts on the absence/presence of individual interactions observed in these pooled samples, we only focused on markers that were conditional for specific group and absent across the others. For Odds Ratio calculations, individual samples were assessed for interactions that gave a positive (interaction present) readout in the pooled group. The assessment descriptor here provides a binary read out, and as such, the variability seen in continuous measurements is not applicable. As no interaction was observed at I5, I6 and I7 in pooled samples of presymptomatic patients or healthy controls (Figure 7) and assessment of these interactions in individual samples was not needed. Figure 9 is a representation of the absence/presence of interactions I1-I7 in pooled samples across the different groups.

We hope the responses above have addressed the reviewer's thoughts on the paper.

Competing Interests: No competing interests were disclosed.

Comments on this article

Version 2

Author Response 06 May 2019

Willem Westra, Oxford BioDynamics Plc, Oxford, UK

Response to Comment #1: "This aim of this study was not a full-scale analytical validation of biomarkers, but as stated in the Introduction, to introduce chromosome conformation signatures as a measurement modality with the potential to be developed into a clinical biomarker in the field of Huntington's disease."

Reviewer Comment to response #1: If this is the case than this should clearly be reflected in the title. The title is now misleading. I am not stating that the methods are invalid, or not properly done, but I remain of the opinion that the sample size is too small, and the results are too variable to draw any conclusion. See Figure 3 and my previous comment #9.

Author Response to Reviewer Comment to response #1:

We agree and have updated the title to reflect the nature of the study and the extent to which we can draw conclusions from it.

Reviewer Comment to response #2: I still think the text should be changed to something like: "HD is a monogenic disease but there are many other genetic, environmental and unknown factors that can influence onset and progression of the disease".

Author Response to Reviewer Comment to response #2:

We agree and have updated the text to reflect this.

Reviewer Comment to response #4: Carini *et al* used 59 patients, Salter *et al* used 74 unblinded patient samples, Yan *et al* used 58 patients. Proof of concept of this analysis was done in these papers. However, if this analysis is useful as a tool to assess disease progression cannot be deducted from the data presented in this paper.

Author Response to Reviewer Comment to response #4:

We agree and have updated the text to reflect this. Specifically, we have modified the language in the Abstract-Conclusions, Discussion-Results summary and Discussion-Strengths and limitations to directly address the reviewers concern.

Competing Interests: No competing interests were disclosed.

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