

## Executive summary

Results from wet lab biomarker research (WP3) with further assay optimization and validation can be translated into a diagnostic commercial kit for clinical pharmacodynamic biomarkers. The support of EC within Horizon 2020 framework programme might be of help to pursue this scope.

The PADDINGTON project addressed the Call Section “HEALTH.2010.2.4.4-1: Clinical development of substances with a clear potential as orphan drugs. FP7-HEALTH-2010-single-stage.” The aim of the project and the Consortium was to undertake clinical research activities aimed at ascertaining feasibility of a battery of pharmacodynamic readouts to support clinical development of SEN0014196, by now officially named selisistat, a novel and selective SirT1 inhibitor that has demonstrated potential to provide a disease-modifying therapy for Huntington’s Disease, HD. Selisistat is currently in Phase II clinical development, and enjoys Orphan Status in the EU, US and Australia. The PADDINGTON project included: - an Exploratory Clinical Trial of selisistat in patients with HD, with a duration of 14 days in three European countries across 6 different sites (London, Manchester, Bochum, Ulm, Krakow, Warsaw); - a longitudinal, 15 months, observational study of HD, aimed at identifying and evaluating short-interval neuroimaging biomarkers of disease progression; with a multi-factorial approach, the assessment of both novel and compound-specific measures of molecular action as well as previously identified predictors of disease progression or modification.

Both clinical studies were fully executed and closed. A number of analyses on the instrumental (MRI), clinical observations and clinical bio-samples collected were conducted.

The Exploratory Clinical Study with selisistat on HD patients was the first study with a selective SirT1 inhibitor in HD patients to demonstrate that SirT1 inhibition is safe and well tolerated at plasma exposure levels providing benefit in non-clinical HD models, creating the basis for further studies of the pharmacodynamics of SirT1 modulation. 55 early-stage HD patients, recruited from four sites across the EU, were enrolled into the study and randomised to receive either selisistat 10 mg/day, 100 mg/day or placebo for 14 days. All participants completed the study and were included in the safety as well as PK analyses.

Selisistat was found to be safe and well tolerated in early stage HD patients. No significant gender difference in systemic exposure was observed. No adverse effects on motor-, cognitive or functional readouts were recorded during a treatment period of 14 days.

The longitudinal observational study of HD, aimed at identifying and evaluating short-interval neuroimaging biomarkers of disease progression, included a large cohort of 61 early clinical HD patients and 40 healthy volunteers, recruited from four sites across the EU, to resemble a population and set-up appropriate for clinical trials. Participants underwent 3-Tesla neuro-imaging, as well as standard clinical, laboratory and cognitive assessments at baseline, 6- and 15-month follow-up.

The statistical interpretation of all instrumental, clinical and bioanalytical data collected throughout the PADDINGTON project supports a whole series of potential markers for disease progression or disease modification in HD by selisistat or other potential drugs.

In particular The Paddington consortium provided neuro-imaging data, including analysis of cortical thinning; transcriptional analysis with the intent of finding a selisistat transcriptional signature in whole blood; relative and absolute quantification of huntingtin in circulating blood, including immune cells; modulation of plasma levels of oxysterols; analysis of the signalling cascade responsible for immune dysregulation in HD, and the plasma levels of inflammatory cytokines and other components of inflammation and innate immunity. Corollary investigation stemmed from the principal ones as new findings were also pursued along the project period.

## **Project context and objectives**

Huntington's Disease (HD) is a monogenetic, autosomal dominant neurodegenerative disorder that gives rise to progressive neuronal dysfunction and cell death, representing clinically a relentlessly progressive movement disorder with chorea and cognitive decline without remissions with consequential dementia and behavioural/psychiatric disturbances, eventually resulting in death. The disease is caused by an increase in the length of a CAG (cytosine, adenine, guanine) triplet repeat, encoding for the amino acid glutamine (Q), present in the N-terminal part of the HD gene product, huntingtin (HTT). No disease-modifying therapy is available, while symptomatic therapy attempts to control chorea and psychiatric symptoms. HD is an orphan disease according to the definitions in Article 3(1) a of EC Regulation No 141/2000 with a prevalence within the European Community of 4-8 patients suffering from manifest HD per 100.000 inhabitants, well below the threshold of 50 per 100.000 persons defining an orphan disease status.

SEN0014196, now officially named and in the following selisistat, is a novel and selective SirT1 inhibitor that has demonstrated potential for disease-modification in HD. Siena Biotech has obtained Orphan Designation for selisistat in the EU (approved by the EMEA/COMP on September 2, 2009 and adopted by the European Commission on October 28, 2009 under the Community Register EU/3/09/681; Orphan Status in the US was granted on December 7 2009 (□092957) and in Australia on March 15, 2011. The compound is under development by Siena Biotech SpA and has completed clinical Phase I studies in healthy volunteers and by the end of PADDINGTON a further Phase IIa three months long clinical study in HD patients. This project includes the first clinical study in HD patients. Subsequent clinical development of selisistat will require interventional studies in patients with HD to establish safety, tolerability and efficacy. These studies aim to explore potential disease-modifying properties of the compound, striving to use the compound to delay disability by either slowing progression of the disease or ultimately - if treatment is initiated in pre-manifest CAG expansion mutation carriers prior to the emergence of frank clinical symptoms and signs - the onset of HD.

Whereas it is considered that sufficient knowledge is available in the clinical HD community to design clinical trials to demonstrate clinical end-point efficacy, the PADDINGTON project aimed at establishing a battery of pharmacological read-outs to support claims for disease-modification by undertaking clinical research activities complementary to the selisistat development plan and ongoing international HD longitudinal observational studies such as TRACK-HD and PREDICT-HD. Although the ultimate goal of a disease-modifying therapy in an autosomal-dominant disorder with 100% penetrance like HD should be to intervene in the perisymptomatic or pre-motor-manifest stages of HD, initiation of trials in this population of HD mutation carriers will likely require demonstration of clinical benefit in trials with manifest HD in a Phase III or Phase IV setting. The overall strategy for establishing disease-modifying treatments in HD will therefore follow a two-step approach, whereby the initial effort is directed to establishing a favourable risk/benefit ratio for selected end-points. This will be followed by studies aiming at demonstration of disease-modification. With this objective in mind, the PADDINGTON project integrated the selisistat development plan and insights gained from TRACK-HD and EHDN studies like REGISTRY, with a comprehensive

plan of clinical research activities aimed at developing and ascertaining feasibility of a range of target-specific and general HD pharmacodynamic readouts for the clinical development of selisistat to demonstrate the mechanism of action and the disease-modifying properties of the compound in HD as outlined in Figure 1:

The structure of PADDINGTON comprises six Work Packages, as outlined in Figure 1. The PADDINGTON project includes two clinical studies: an Exploratory Clinical Study (WP 1), which aims to provide material (biophase samples and clinical data) for Work Package 3, and an Imaging observational study (WP 2).

#### WP1

Following completion of the clinical Phase I study of SEN0014196 (selisistat) in healthy volunteers, the PADDINGTON project proposed to conduct an Exploratory Clinical Trial in patients with HD. As per the regulatory requirements, this study was planned to have a maximum duration of 14 days, and to be conducted at dose levels shown to be safe and tolerated in healthy volunteers, and at a systemic exposure level not exceeding the Maximal Anticipated Biological Effect Level (MABEL) as per previous preclinical studies in the R6/2 mouse model.

The study foresees a double-blind, placebo-controlled, ascending multiple oral dose, sequential group design conducted at two dose levels. For each arm, 30 patients were planned to be enrolled, for a total of 60 patients, randomised to achieve 20 patients with active drug and 10 patients with placebo for each arm. Participants were required to reside in an in-patient facility from Day -1 (the day prior to start of dosing) to Day 2 (24 hours post-dose) and again for 24h at the end of the dosing period. All participants had then to return for a post-study visit 7 days after their final dose. All participants were asked to provide repeated blood samples up to a maximal blood volume of 250 mL per 24h and a maximum of 500 mL for the duration of the study. Blood sampling occurred at 8 time points/subject/sampling day. In addition, participants were asked to provide urine samples pre- and post study.

Evaluation of phenotypic effects include UHDRS scores, total functional capacity and other at screening, baseline, during and at the end of treatment and at follow-up. Safety assessments include: ECG, vital signs, laboratory safety tests, and physical examination. PK sampling over a 24 h period occurred on Days 1 and at end of study (8 samples/subject/sampling occasion).

The study was conducted in three EU countries across 6 different sites (London, Manchester, Bochum, Ulm, Krakow, Warsaw) and include HD mutation carriers with an wide range of CAG repeats and disease burden scores. All trial participants has to be able to provide Informed Consent

The objective of the Exploratory Clinical Trial was to (1) generate biomaterials to evaluate the effect of selisistat on a series of target- and disease modulation markers (levels of soluble mutant huntingtin, acetylation status of mutant huntingtin, transcriptional signatures, modulation of innate immune markers, modulation of cholesterol metabolites) at therapeutic plasma concentrations; (2) to assess pharmacokinetics of selisistat in HD patients at dose levels found to be safe and well tolerated in healthy volunteers; (3) to assess acute clinical effects of selisistat in HD patients and (4) to assess safety and tolerability aspects of selisistat in HD patients

## WP2

Focuses on 3T neuroimaging using Magnetic Resonance Imaging (MRI). It includes an observational biomarker study with HD patients and healthy volunteers as controls at four clinical sites across the EU (London, Paris, Leiden and Ulm) at baseline (visit 1), 6 months (Visit 2) and 15 months (Visit 3). MRI scans acquired using standardised T1, T2 and diffusion-weighted imaging protocols developed during the TRACK-HD study, are to be checked and graded for quality before semi-automated measurements of intracranial, whole-brain, ventricular and caudate volumes, fully-automated putamen segmentations, and fractional anisotropy (FA) and mean diffusivity (MD) measures from diffusion tensor imaging are derived. Running alongside TRACK-HD, WP 2 addresses logistical challenges and critical issues such as repeatability, short-interval scanning and stability of image data acquisition in a multicentre setting, thus laying the groundwork for the use of advanced MR neuroimaging techniques in multi-site, multi-investigator studies such as selisistat Phase III and IV studies.

## WP3

Includes both development and validation of a number of technologies and methodologies to measure a series of wet target- and disease modulation markers, both specific for the mode of action of selisistat and for general application to measuring progression and phenotype or monitoring therapeutic interventions in HD.

These markers include:

- quantification of soluble HTT in blood;
- assessment of the acetylation status of HTT;
- identification and validation of selisistat transcriptional signature in healthy volunteers and HD patients;
- modulation of immune markers in HD patients;
- modulation of cholesterol and oxysterol metabolites in HD patients;
- adiponectine in HD patients treated with selisistat;
- further dissection of the myeloid cell dysfunction in HD- NFkB pathway dysregulation;
- further dissection of the myeloid cell dysfunction in HD- RNA sequencing and Microarrays;
- myoblasts and fibroblast cultures.

## WP4

Aims to combining efforts of all statistical capabilities and expertise of all partners to define what individual or composite measure within each assessment domain shows the largest effect sizes over a 6 month and fifteen month time period; how domain- specific effect sizes per interval compare and how might data be combined across domains to reduce redundancy, and ultimately increase the power and efficiency of future clinical trials.

## WP5

Aims to manage coordination of the project within the consortium and with the EC; to provide assistance to Partners in fulfilling all legal, administrative and financial requirements of the FP7 procedures; to promote intra-consortium communication by maintaining both informal and formal contacts with all Partners via teleconferencing and regular face-to-face meetings and finally to ensure compilation of relevant progress reports and to formally monitor progress by deliverables and milestones.

#### WP6

Aims to ensuring visibility, promotion and appropriate exploitation of scientific results of the project by broad appeal to all stakeholders, including the scientific community, patient organisations, public authorities including the EMA and national regulatory agencies, industrial organisations, the Investor Community, the Press and the general public.

## Main S&T results/foregrounds

The first Exploratory Clinical Study with selisistat on HD patients, its final report and all activities due to its formal conclusion were conducted under the PADDINGTON project. It was the first study with a selective SirT1 inhibitor in HD patients to demonstrate that SirT1 inhibition is safe and well tolerated at plasma exposure levels providing benefit in non-clinical HD models, creating the basis for further studies of the pharmacodynamics of SirT1 modulation.

Despite the relatively small size of the study (six sites, 55 patients), the data management process for the study turned out to be a major challenge: conducting data management activities across several partner- and subcontractor organisations while achieving ICH M2 and E6 compliance has required extensive use of one-time SOPs and significant reconciliation work, which is also the reason that the close-out process required six months. Despite these challenges, WP1 has reached all its deliverables and milestones and activities relating to WP3 and WP4 were always on track.

The clinical study final report was published on 16 November 2012.

A total of 63 Caucasian male and female subjects were screened between March and November 2011. There were four screen failures and four consent withdrawals before randomization. Consequently, 55 patients were enrolled into the study and randomised to receive either selisistat 10 mg/day (n=17), 100 mg/day (n=19) or placebo (n=19). All fifty-five participants completed the study and were included in the safety as well as PK analyses.

Selisistat was found to be safe and well tolerated in early stage HD patients at therapeutically relevant plasma exposures.

The pharmacokinetics of selisistat was assessed for the first time in HD patients. Details are reported in the final clinical report. Systemic exposure parameters showed that the average steady-state plasma levels achieved at the 10 mg dose level (125 nM) was comparable to the IC<sub>50</sub> for SirT1 inhibition. A significant increase in AUC was observed at the 100 mg dose after 14 daily doses when compared to Day 1 levels, which alongside other PK observations, suggests that one or more absorption mechanisms may have changed after repeated exposure at this dose level. Results suggest that PK steady state was reached before Day 14. No significant gender difference in systemic exposure was observed, although a tendency for higher exposure in female patients was noted. Selisistat was found to be rapidly absorbed with a median t<sub>max</sub> of 3h. The between-subject variability in systemic exposure was moderately high at a mean coefficient of variation of 46%.

Selisistat was found to be safe and well tolerated in Stage I-II HD patients. There were no deaths, SAEs, or AEs leading to discontinuation reported during the study. Overall, 45.5% of patients experienced 44 AEs, of which 40 were mild and 4 of moderate intensity. Almost half of the patients (23.6%) experienced 15 AEs having suspected relationship with the study drug. The most commonly reported AE was hypertension followed by headache and anaemia.

There were no dose- or treatment-related trends in terms of clinical laboratory evaluations, vital signs, or 12-lead ECG parameters. There were no clinically significant findings in physical examinations.

An overall decrease of 3.81% from baseline in the mean (SD) UHDRS'99 motor assessment total score was noted on Days 7, 14, and at follow up. At follow-up (on Day 28), all the patient groups showed decrease from baseline in the mean (SD) UHDRS'99 motor assessment total score, suggesting an improvement in the motor functionality. There were no apparent changes in the UHDRS'99 TFC, FA, behavioural, and cognitive assessments.

A second observational biomarker study, focussing on 3T neuroimaging (volumetric and diffusion imaging), was conducted under the PADDINGTON project. It included a large cohort of 61 early clinical HD patients and 40 healthy volunteers, recruited from four sites across the EU (London, Paris, Leiden and Ulm), resembling a population and set-up appropriate for clinical trials. Participants were assessed in three occasions, at baseline (visit 1), 6 months (Visit 2) and 15 months (Visit 3).

Macrostructural volumetrics were obtained for the whole brain, caudate, putamen, corpus callosum (CC) and ventricles. Microstructural diffusion metrics of fractional anisotropy (FA), mean-, radial- and axial-diffusivity (MD, RD, AD) were computed for white matter (WM), CC, caudate and putamen. Group differences were examined adjusting for age, gender and site. A formal comparison of effect sizes determined which modality and metrics provided a statistically significant advantage over others.

As expected, the macrostructural measures showed decreased regional and global volume in HD compared with controls ( $p < 0.001$ ); except for the ventricles which were enlarged ( $p < 0.01$ ). In HD, FA was increased in the deep grey-matter structures examined ( $p < 0.001$ ), and decreased in the WM (CC,  $p = 0.035$ ; WM,  $p = 0.053$ ); whereas diffusivity metrics (MD, RD, AD) were increased for all brain regions examined ( $p < 0.001$ ). The largest effect sizes were for putamen volume, caudate volume and putamen diffusivity (AD, RD and MD), see Figure 2A; each was significantly larger than those for all other metrics ( $p < 0.05$ ), see Figure 2B.

In conclusion, the highest performing macro- and micro-structural neuroimaging metrics had similar cross-sectional sensitivity to HD pathology quantified via effect sizes. The region-of-interest examined may be more important than the imaging modality applied, with the deep grey-matter regions outperforming the CC and global measures, for both volume and diffusivity. FA appears to be relatively insensitive to disease effects. In future studies and trials, a panel of biomarkers is likely to be required to understand the full effects of HD, and what result any intervention may have on its progression. By statistically interrogating effect sizes, we can make informed choices about which metrics are the most powerful, both cross-sectionally and also longitudinally by incorporating data from visits 2 and 3.

Measurement of longitudinal change in volume of the whole brain, lateral ventricles, caudate and putamen from T1- and T2-weighted MRI and measurement of longitudinal change in diffusion metrics (fractional anisotropy, FA, and mean diffusivity, MD) over the caudate, putamen, white matter and corpus callosum from diffusion-weighted MRI gave the following key results.

#### ➤ **Six-month interval potential trial outcomes for HD**

A range of candidate biomarkers of HD progression over 6 months were evaluated, including macro- and micro-structural neuroimaging measures, clinical and cognitive scales. The WP2 cohort, a large, well-characterised group of patients with early clinical HD and a multi-national, multi-site study design, was utilised to resemble a population and set-up appropriate for disease-modifying clinical trials. 6-month effect sizes (ES) are the main outcome, a unit-free means of comparing different measures which can be used to estimate sample-size requirements for clinical trials.

Details of the statistical methods applied as well as the full results tables are presented in the Statistical Reports.

All controls and 59/61 HD patients returned for the 6-month assessment. The largest ES, comparing 6-month change in stage 1 HD with controls, were for macrostructural metrics: volume change in the caudate (0.795, 95% CI: 0.393-1.138), whole brain (0.631, 95% CI: 0.230-0.967), ventricles (0.702, 95% CI: 0.208-1.110), white matter (0.721, 95% CI: 0.293-1.162) and grey matter (0.707, 95% CI: 0.033-1.162); with all showing significant between-group differences over 6 months ( $p < 0.01$ , Figure 3). Changes in microstructural neuroimaging measures were more variable with ES typically smaller than those for the macrostructural measures. Changes in cognitive and clinical measures were comparatively small, with the Symbol Digit Modalities Test (SDMT; 0.544, 95% CI: 0.008-1.046) and Hopkin's Verbal Learning Test (HVLT; 0.520, 95% CI: 0.002-0.937) producing the largest ES, and TMS producing the smallest (0.068, 95% CI: -0.265-0.427). CI were generally wide. Pair-wise comparisons showed the best performing macrostructural measures to have significantly larger ES than those for the TMS and the poorer performing cognitive tasks ( $p < 0.05$ ).

This multi-site study of stage 1 HD demonstrates for the first time that macrostructural neuroimaging measures such as caudate atrophy are sensitive to disease progression over the short interval of six months, producing large ES compared with microstructural neuroimaging, clinical and cognitive measures.

Presented ES can be used with a standard formula to estimate sample-size requirements for a randomised-controlled trial over 6 months in stage 1 HD. For example, if using caudate atrophy as an outcome in a trial of a treatment hypothesised to be 50% effective, the required number of participants per treatment arm would be 133 (95% CI: 65 to 544; 90% statistical power); a feasible sample size. In contrast, requirements are much larger for the clinical and cognitive measures, often with confidence intervals stretching to infinity. For example, although SDMT was one of the most promising cognitive measures, 6-month sample-size requirements are much larger with extremely wide confidence intervals (284 (95% CI: 77 to 1313819)).

Based on ES data, we recommend the adoption of macrostructural neuroimaging measures as short-term readouts in proof-of-concept studies of putative disease-modifying therapies. For example, if caudate atrophy rates were reduced over 6-months in a treated group relative to placebo, this would provide early confidence-instilling data that the trial should move forwards, with subsequent increases to both the number of participants and the duration of the trial. Once sufficiently powered, disease-modification could be demonstrated using approved measures such as TFC as a primary endpoint and neuroimaging measures as secondary endpoints. An adaptive approach such as this based on early, meaningful data may improve the viability of disease-modifying clinical trials.

It has to be acknowledged that positive macrostructural neuroimaging readouts over 6 months may not be indicative of longer-term functional improvement. Although associations between change in neuroimaging measures and functional decline have been reported in HD (Tabrizi et al., 2012) causality is yet to be demonstrated. Furthermore, these readouts may not be suitable for all types of intervention; their utility may be dependent on the mechanism of action of the therapy, together with the time required for it to mediate an effect. Nevertheless, these measures are sensitive to disease progression over short intervals, objective, reproducible across sites, widely available and are currently the only measures that provide practical sample-size requirements for hypothetical treatment effects over six months. Hence they may provide valuable short-term readouts.

#### ➤ **Effect-size estimates for change over 6-, 9- and 15-month intervals in a range of cognitive, clinical and neuroimaging measures**

ES were computed for all three time intervals: a 6-month interval (visit 1 to visit 2), a 9-month interval (visit 2 to visit 3) and a 15-month interval (visit 1 to visit 3). Full details of the analysis and results are presented in the Statistical report. The key findings include:

- The large and consistent ES estimates for the macrostructural neuroimaging measures, in particular the change in caudate volume measured with the Caudate BSI, and change in ventricular volume measured with the ventricular BSI.
- Over the longer interval, the ES for some of the microstructural neuroimaging measures were comparable to the macrostructural neuroimaging measures. This is in contrast to findings over the six-month interval.
- ES estimates for the clinical measures were comparatively small, especially over the shorter intervals where measurement error may have masked any true signal.

Presented ES (when squared) are inversely related to sample-size requirements for clinical trials under the reasonable assumption that a 100% effective treatment will reduce the mean rate of change in HD cases to that in healthy controls without affecting the variability in these rates. Therefore, it is anticipated that the data presented here will be used to inform the design of future

clinical trials over 6-, 9- and 15-months in stage 1 HD, where these measures are used as efficacy readouts.

Beyond the initial objectives of the project and under the TA amendment as of 24 July 2012, further work was executed as described in the following:

➤ **Grey and white-matter metrics:** Further metrics were derived from fluid registration to assess volume change in grey and white matter over 6- and 15-month intervals, as reported above.

➤ **Longitudinal Voxel-Based Morphometry (VBM):** VBM is a widely used fully-automated tool for assessing changes across the whole-brain at the voxel-level. Unlike region-of-interest approaches, VBM does not require any a priori hypotheses. Combined with fluid-registration it can be used to localise the longitudinal effects of neurodegeneration. The largest study in HD to publish longitudinal VBM results is TRACK-HD, with assessments over 12-, 24- and 36-months. This study aimed to test whether this technique was sensitive to HD-related changes over as short an interval as six months and to localise the regions where these changes are found.

Compared with controls, significant regions of change were detected within the grey matter of the basal ganglia and the adjacent white matter, as well as the splenium of the corpus callosum, demonstrating that this technique is sensitive to HD-related changes over the interval of six months (Figure 4).

➤ **Development of automated methods for caudate segmentation:**

Early and pronounced caudate atrophy is the most consistent finding in longitudinal observational studies of HD. Analysis of the key variables from WP2 over 6-, 9- and 15-months, as well as data from the TRACK-HD study, suggest it could provide a powerful neuroimaging biomarker for HD. However, the measurement technique used in both of these studies (the caudate BSI) is only semi-automated, requiring manual delineation of the baseline caudates prior to application of the caudate BSI algorithm. If using caudate atrophy as an efficacy outcome in large-scale clinical trials, it would be advantageous if measurement was fully-automated. Such techniques are currently available; however, their validity for use in HD in a longitudinal setting has never been tested. In this study we compared four fully-automated techniques (BRAINS, STEPS, FSL's FIRST and FREESURFER) with the semi-automated CBSI, which has itself been previously validated against a detailed manual 'gold standard'.

Caudate volume change generated from four fully-automated measurement techniques (BRAINS, STEPS, FSL's FIRST and FREESURFER) was compared with the CBSI over a 6- and 15-month interval using the scans from WP2. Analysis included (1) Bland-Altman plots (2) Pitman's test of difference in variance, (3) paired t-tests to examine differences in mean volume change in controls and HD groups separately, (4) between-group differences in percentage volume change and (4) effect-size estimates, adjusted for age, gender and site. Findings suggest that the STEPS technique is the most comparable to the CBSI. However, effect-size estimates particularly over the shorter 6-month interval suggest that this technique is not as sensitive as the CBSI. Further work will examine whether caudate segmentations generated using STEPS can be incorporated into the CBSI algorithm to produce a fully-automated measurement technique without compromising on sensitivity.

➤ **Additional analysis of diffusion data to include diffusivity metrics.**

The microstructural neuroimaging deliverables were extended to include radial- and axial-diffusivity (RD, AD), in addition to fractional anisotropy (FA) and mean diffusivity (MD), mentioned in the protocol. RD and AD were measured over predefined regions-of-interest (caudate, putamen, white matter and corpus callosum).

➤ **Test-retest reliability of Diffusion Tensor Imaging (DTI) in HD.**

Using methods to be applied in longitudinal research, we sought to establish the reliability of DTI in early HD patients and controls using data collected from the London site of WP2. Test-retest reliability, quantified using the intra-class correlation coefficient (ICC), was assessed using a region-of-interest-based white-matter atlas and voxel-wise approaches on repeat scan data from 22 participants (10 early HD, 12 controls). T1 data was used to generate further regions-of-interest for analysis in a reduced sample of 18 participants.

The results suggest that FA and other diffusivity metrics are generally highly reliable, with ICCs indicating considerably lower within-subject, compared to between-subject, variability in both HD patients and controls. Where ICC was low, particularly for the diffusivity measures in the caudate and putamen, this was partly influenced by outliers. The analysis suggests that the specific DTI methods used here are appropriate for cross-sectional research in HD, and give confidence that they can also be applied longitudinally, although this requires further investigation. An important caveat for DTI studies is that test-retest reliability may not be evenly distributed throughout the brain whereby highly anisotropic white matter regions tended to show lower relative within-subject variability than other white or grey matter regions.

➤ **Multi-fibre methods to improve the sensitivity of diffusion-weighted imaging to white matter changes in HD**

Conventional diffusion tensor methods are limited as they conflate data from voxels containing crossing-fibres; potentially 90% of all white matter. Multi-fibre methods, such as constrained spherical deconvolution (CSD), model multiple fibre orientations within one voxel and as such may be more attuned to detect pathological alterations. This study aimed to test the sensitivity of metrics derived from CSD modelling of DWI data to the differences between early-stage HD patients and controls and to compare this sensitivity with commonly-used conventional tensor-based metrics, FA and MD.

Multi-fibre modelling using CSD to assess white matter microstructure can detect group difference between early stage HD patients and controls but this measure is less sensitive to group differences than FA and MD. FA and MD are also significantly more likely to highlight clusters of alterations in early-stage HD patients. In conclusion, multi-fibre methods require further refinement if they are to supersede conventional tensor metrics and are to be sufficiently sensitive and reliable to qualify as a biomarker of disease progression in clinical trials.

➤ **Cortical Thinning as a Marker of Disease Progression in early HD**

Cortical atrophy has been suggested as a potential biomarker for HD but its longitudinal sensitivity requires evaluation. In this project, FreeSurfer software was applied to compare longitudinal rates of cortical thinning in HD patients with a healthy control group, over 6-, 9- and 15-month intervals on the WP2 dataset.

Cross-sectionally, there was evidence of widespread reductions in cortical thickness in stage 1 HD compared with controls. Over the 6- and 9-month intervals, there were no significant between-group differences in cortical thinning. Over 15 months, regionally specific cortical thinning was evident in the parietal and occipital lobes compared with controls. A subsequent parcellation analysis showed that the highest effect sizes were within the inferior parietal, cuneus and precuneus regions: -0.848 (95% CI: -1.414 to -0.169), -0.814 (95% CI -1.258 to -0.349) and -0.806 (95% CI: -1.348 to -0.222) respectively. In conclusion, cortical thickness analysis processed with the default FreeSurfer pipeline does not appear to be a sensitive method for detecting change over short scanning intervals but may contribute important additional information over longer intervals, as well as being of scientific and clinical interest.

➤ **Comparison of techniques for measuring longitudinal change in the putamen.**

Previous work in HD has demonstrated larger effect sizes for atrophy over time in the caudate compared with the putamen, yet it is unlikely that this is solely due to a differential biological effect. More likely, the methodological limitations of putaminal volumetric analysis increase the variability of this measure, thereby reducing sensitivity to longitudinal change. It is therefore important to establish robust techniques to estimate atrophy in this structure.

In this project, five fully-automated methods (BRAINS3, FIRST, FIRST with FAST boundary correction, FreeSurfer and STEPS) were compared with a manual ‘gold-standard’, which had been specifically optimised for this methods’ comparison. Analyses were run on the PADDINGTON WP2 cohort, with the aim of determining the most sensitive and reliable fully-automated technique for measuring longitudinal change in putamen atrophy from conventional MRI.

An initial visual inspection of the automated segmentations highlighted several regions of anatomically inaccurate segmentation. Of the automated techniques, STEPS software (Cardoso et al., 2013) was judged to produce the most anatomically reliable segmentations. All methods were highly correlated with the manual estimates cross-sectionally but there were longitudinal inconsistencies. Only the manual method and STEPS were sensitive to HD-related putaminal atrophy over 6-months. All methods, except FIRST (with and without boundary correction), could detect this over 15-months. Bland-Altman plots were examined for both cross-sectional volume and longitudinal volume change. Overall STEPS performed most consistently with the manual measure and was sensitive to putaminal atrophy over the short 6-month interval. Further evaluation of this promising technique is recommended to be applied in an independent cohort.

➤ **Macro- and micro-structural cerebellar abnormalities in Early Stage HD: a potential role in motor and psychiatric impairment was investigated.**

The cerebellum has received limited attention in HD, despite signs of possible cerebellar dysfunction, including motor incoordination and impaired gait which are currently attributed to basal ganglia atrophy and disrupted fronto-striatal circuits. In this project, for the first time, a potential contribution of macro- and microstructural cerebellar damage to clinical manifestations of HD was investigated; assessing between-group differences and clinical associations in a cohort from WP2 consisting of 12 controls and 22 early-stage HD participants, assessed at the London and Paris sites.

Reduced paravermal volume was detected in HD compared with controls using VBM ( $p < 0.05$ ), but no significant whole-cerebellar volumetric differences were found using manual delineation. Diffusion abnormalities were detected in both cerebellar grey matter and white matter. Smaller cerebellar volumes, although not significantly reduced, were associated with increased total motor (TMS) and HADS-SIS psychiatric scores, impaired gait and pronate/supinate-hand task performance. Increased diffusion in the cerebellar grey-matter was associated with abnormal TMS, saccade initiation, tandem walk and gait.

In conclusion, atrophy of the paravermis, possibly encompassing the cerebellar nuclei, and microstructural abnormalities within the cerebellum may contribute to HD neuropathology. Aberrant diffusion within the cerebellar grey matter and reduced cerebellar volume both associate with impaired motor function and increased psychiatric symptoms in stage I HD, potentially implicating the cerebellum more centrally in HD presentation than previously recognized.

➤ **Development of a novel cognitive test: disruption of white-matter connectivity and emotion recognition deficits in HD.**

Recognition of negative emotions is impaired in HD. It is unclear whether these emotion-specific impairments are driven by emotion complexity, test-cue difficulty, visuoperceptual problems,

discrete regional brain atrophy/dysfunction or disruption within a more general, shared emotion network. In this project, a study set out to compare patterns of deficits across stimulus modalities and explore micro- as well as macro-structural brain changes associated with emotion recognition, with the aim of clarifying the characteristics and underlying pathology behind these deficits.

Statistical evidence of impairment of disgust, anger and fear recognition was seen in HD patients compared with healthy controls across multiple stimulus modalities. The patterns of emotion-specific impairments compared with controls were different across stimulus modalities and there was no clear link with the number of errors (cue difficulty). Very few significant associations were found between morphometric measures and emotion recognition deficits; although lower caudate volume was associated with poorer performance on all six emotions, with two of the six associations and that with the total score achieving statistical significance ( $p < 0.05$ ). Extensive white matter FA reductions were associated with poorer performance on the three impaired emotions within the HD group ( $p < 0.05$ ).

In conclusion, widespread neuronal dysfunction driven by altered white matter microstructure may manifest as specific impairments in emotion recognition. It is possible that the emotion network, due to its widespread nature, is particularly vulnerable to disruptions in connectivity.

➤ **Statistical evaluation of different imaging metrics and modalities.**

The effects of geometric distortion on image quality and analysis were investigated at the Ulm site. The Ulm scanner is a head only scanner (rather than a whole-body scanner) which reduces the area in which the field strengths are stable. There was some evidence of geometric distortion on some of the scan pairs from this site. Gradient warp correction was considered in order to correct for this. However, this was not possible because the parameters required to perform this correction (the spherical harmonics expansion for the gradient coils for the Siemens Allegra) could not be obtained. Additional quality control of the native registrations (from which the boundary shift integral (BSI) is derived) was performed to compensate.

➤ **Total intracranial volume: a methods comparison.**

Total intracranial volume (TIV) is a measure of head-size which can be used to a) adjust for inter-subject head-size variation, b) adjust for variability caused by subtle differences in image acquisition, for example: scanner drift (slow changes in voxel intensity over time), alterations in the magnetic-field inhomogeneity (geometric distortion), image intensity scales and voxel size variations, and also c) as regions-of-interest to investigate head-size in clinical populations.

Previous studies have compared several TIV estimation methods and found variability in measures and evidence of brain atrophy-related bias. The accuracy and the extent of atrophy bias in some of the most commonly used TIV estimation methods is still not clearly understood and this is a particularly important issue in studies of neurodegeneration. In this project, it is builded on previous method comparisons by extending the evaluation to include three of the most commonly applied software packages for estimating TIV (SPM8, BRAINS3 and FreeSurfer), with varying parameters, with the aim to validate the reliability of these estimates as nuisance variables and regions-of-interest.

Two of the six methods in this comparison were affected by large outliers, inconsistency and atrophy-related bias for both cross-sectional and longitudinal TIV measurements: SPM's Unified Segmentation and FreeSurfer, and are therefore not deemed to be good methods for measuring TIV. Masked BRAINS3 estimates deviated away from the other methods for larger TIVs. The remaining three methods showed highly correlated and consistent results (manual delineation, SPM's New Segment and BRAINS3 (unmasked)). The most likely interpretation for this is that these methods are picking up the same signal: (a) a non-significant trend towards reduced volume (~25mL) in the HD group compared with controls, (b) significant or borderline significant between-group

differences in change over time driven by a slight increase in control TIV estimation and more stable estimate in the HD group, and (c) significant associations between TIV increase over the scanning interval and increased BBSI. We can infer from these results that this signal is unstable over time; most likely due to a combination of scanner drift, geometric distortions and brain atrophy. The magnitude of this variability and bias is thought to be acceptable for using TIV estimates as nuisance variables i.e. to adjust for inter-subject variability in head-size and to correct for scanner drift. These effects would however invalidate the use of these methods as regions-of-interest measurements for investigating head-size in clinical populations; hence caution in their use and interpretation in this context is advised.

➤ **Correlation of clinical HD phenotypes with imaging measures of regional atrophy.**

There is well-established consensus amongst clinicians that distinct motor phenotypes exist in HD. The classical adult onset motor presentation is that of a hyperkinetic movement disorder with predominating chorea. Although juvenile HD subjects are predominantly akinetic, this phenotype has also been noted in a minority of adult onset subjects. The majority however fall into a mixed motor phenotype with both hypokinetic and hyperkinetic features. A further minority group consists of those patients who appear to have clearly symptomatic disease, in other non-motor domains, but with relatively lower motor scores.

Indeed, recent work (Hart et al., 2013) has used the TMS subscores to develop a means of segregating patients into these distinct motor phenotypes. Interestingly, choreatic subjects performed better on functional and cognitive measures than akinetic ones.

Our project aimed to explore whether there is evidence of regional differences in neuroimaging, between these motor phenotypes.

To this scope, a motor classification system was developed based on subscores of the UHDRS Total Motor Score (TMS) to accurately segregate HD patients into the clinically observed motor phenotypes, hyperkinetic, akinetic, mixed motor and for those with a low motor burden, a minimal motor group.

The hyperkinetic (HK) score is constituted of the chorea subscore from 7 body parts (right and left upper and lower limbs, face, mouth and trunk), giving a total score of 28.

The Akinetic Rigid (AkR) score comprises the finger taps, pronation/supination and bradykinesia and rigidity (right and left upper limb) subscores, also giving a total score of 28.

A composite ratio of the HK/(HK + AkR) score was then calculated to determine the relative contribution of each score to the motor phenotype. A histogram of these scores was then created (Figure 5) and based on the distribution frequency of scores seen, a percentile based division of phenotypes was carried out.

All subjects with a total motor score of 10 or less were assigned automatically to the minimal motor group. Clinical convention suggests that a TMS of 10 or less is a reasonable threshold below which signs are considered fairly mild.

The subjects within the 37.5th percentile and below constituted the akinetic group, 62.5th percentile and above, the hyperkinetic group and those in between, the mixed motor.

This was applied to a combined WP2 and TRACK-HD cohort of 147 early stage HD subjects, characteristics given below in Table 1.

Further VBM analysis should be performed to identify areas of regional atrophy associated with these motor phenotypes, with adjustment for cognitive scores (SDMT), functional capacity, CAG size, TMS, age and sex.

➤ **Optical Coherence Tomography- A pilot study of retinal thickness and macular volume as biomarkers in Huntington's disease.**

Optical coherence tomography is a non-ionising imaging modality of the retinal nerve fibre layer (RNFL) and macular volume (MV) at micrometre resolution, with rapid, consistent data acquisition. The speed of sampling, the order of a few minutes, confers a considerable advantage over MR scanning. To the best of our knowledge, OCT has never been performed in HD patients and as such this represents a novel study.

The evidence for retinal pathology in HD comes from drosophila work showing photoreceptor degeneration, R6/1 and R6/2 mouse models demonstrating loss of rod and cone function, photoreceptor degeneration, deficit in cone response to electroretinogram and loss of cone opsin and transducin protein (Jackson et al., 1998); Helmlinger et al., 2002; Petrasch-Parwez et al., 2005; Batcha et al., 2012). In other polyglutamine triplet expansion disorders, retinal degeneration is well established clinically e.g. Spinocerebellar Ataxia 7. Similarly, a recent OCT study of nine Spinocerebellar Ataxia 1 (SCA1) patients, with known cerebellar and brainstem volume loss, did demonstrate significant RNFL loss versus controls.

A pilot biomarker study was thus undertaken, comprising a majority of PADDINGTON early stage WP2 subjects, to test the following hypotheses:

- The RNFL thickness as determined by Optical Coherence tomography is reduced in HD.
- The Macular Volume as determined by Optical Coherence tomography is reduced in HD.
- There is correlation between MRI determined brain atrophy and RNFL thinning in early subjects.

Initially recruitment of 10 pre-manifest, 10 early HD, 10 moderate and 10 controls was planned, ideally subjects from whom clinical and imaging data had already been acquired, as part of either TRACK-HD and/or PADDINGTON WP2.

All participants underwent the following:

- Neuroophthalmological assessment- history, assessment of colour vision, visual acuity and pupillary reflexes.
- Optical Coherence Tomography- Macular thickness (1 measurement) and peri-papillary RNFL (average of 3).
- Link clinical data via PADDINGTON, TRACK-HD and REGISTRY studies.

Following promising early data, which suggested a trend to reduced macular volume in early stage and moderate subjects, although no clear effect was seen in the comparatively more severely affected juvenile HD group, recruitment was extended to include premanifest subjects and young controls.

At a descriptive level, a trend to reduced macular volumes is still seen in early and moderate stage HD subjects versus controls. The addition of young controls does appear to suggest a role for ageing in loss of macular volume.

On preliminary descriptive statistical analysis, there is no strong indication of an effect of RNFL in HD subjects, though on retinal segmentation, an inferior retinal deficit is suggested.

Noting the absence of formal statistical analysis, the findings to date suggest a trend to reduced macular volumes in HD subjects versus controls and a possible segmental loss. If these results reach statistical significance, the data would be consistent with animal model work of the retina in HD.

The absence of an effect on the juvenile HD group, accepted as the most aggressive phenotype given the high CAG repeat burden, is intriguing. Despite the aggressive nature of their disease, a protective effect due to their young age may be occurring. The results of the age-matched controls do seem at the very least comparable, but with small numbers of JHD subjects, drawing firm conclusions is difficult.

Formal comparative statistical analysis of collected data is underway. If statistically significant results are found in relation to macular volume, correlations with imaging metrics will be carried

out. Further opportunistic recruitment of premanifest, juvenile and especially moderate subjects continues.

### ➤ **Quality control of the diffusion weighted images**

Demographic parameters for the subjects at different study sites are summarized in Table 2. Data quality control (QC) of all diffusion tensor imaging (DTI) scans of all visits was performed. DTI QC protocols were uploaded to the MRI section of the PADDINGTON study webportal.

In summary, QC was performed on 289 diffusion tensor imaging (DTI) and 186 T1w scans. Three data sets were excluded due to bad QC results. DTI acquisition parameters for DTI scans showed slight variations for the different 3T systems, while QC of T1w and T2w scans basically showed no differences.

The stability of MRI data was assessed for inter-centre differences, and the possibility for pooling DTI data obtained from different centres was tested for the baseline visit in a two-fold fashion, either by scanning of a the same control subject at three study sites (London, Paris, Ulm) with special focus on data quality and DTI metrics (fractional anisotropy – FA) showing an inter-scanner variability of <5% for the FA and an intra-subject variability of < 5%; or by comparison of DTI data of the baseline visit for the four study sites and testing on variability at the group level. Analysis metrics on FA maps were the differences in ROI analysis and the calculation of coefficients of variation (CV).

That way, prove has been given that data of different centers could be pooled.

The application of the QC algorithm suggests the elimination of motion corrected volumes in a DTI scan (Müller et al. 2011).

DTI data (FA) were compared at the group level for the three visits separately and results were compared indicating clusters of significant FA differences between HD subjects and controls for the three visits.

All above mentioned results were summarized and published in a methodological paper (Müller, Grön et al. 2013). Longitudinal data were checked for consistency on comparisons at the group level for the three visits separately and results were drafted for publication (Müller, Kassubek et al. 2014, in preparation).

### ➤ **Sub-threshold depressive symptoms in HD**

Because depressive symptoms are prominent psychopathological features of HD which negatively impact on social functioning and well-being, imaging and behavioural data from WP2 were analyzed to see whether mild forms of depression are more prevalent in HD participants and, by looking at the imaging data, whether mild depression is associated with a specific imaging signature, and whether this signature is similar to that of Major Depressive disorder. Frequencies of a history of depression, previous suicide attempts and current sub-threshold depression between HD participants and controls were compared. The HD group was then split based on the overall HD group's median HADS-depression score into a group of 30 non-depressed participants (mean 0.8, SD 0.7), and a group of 31 participants with sub-threshold depressive symptoms (mean 7.3, SD 3.5) to explore the neuroanatomy underlying sub-threshold depressive symptoms in HD using Voxel-Based Morphometry (VBM) and DTI. Frequencies of history of depression, previous suicide attempts, or current sub-threshold depressive symptoms were higher in HD than in controls. The severity of current depressive symptoms was also higher in HD, but not associated with the severity of HD motor signs or disease burden. Compared to the non-depressed HD group DTI revealed lower fractional anisotropy (FA) values in the frontal cortex, ACC, insula and cerebellum of the HD group with sub-threshold depressive symptoms. In contrast, VBM measures were similar in both

HD groups. A history of depression, the severity of HD motor signs, or disease burden did not correlate with FA values of these regions (Figure 6).

Current sub-threshold depressive symptoms in early HD are associated with microstructural changes - without concomitant brain volume loss - in brain regions known to be involved in Major Depressive Disorder, but not those typically associated with HD pathology (Sprengelmeyer et al., 2013).

Work Package 3 aimed at evaluating feasibility and relevance of several potential wet biomarkers of disease modification, proposed to have utility in assessing treatment effects of selisistat, or to detect disease specific variations, either due to HD itself or to the therapeutic effect of any drug.

### **Soluble HTT detection by ELISA assay**

HD is a monogenic pathology caused by an aberrant expansion of CAG repeats in the *HTT* gene. The specific modulation of expression of the mutant HTT protein represents a promising avenue towards disease modification. The ability of detecting, quantifying, and monitoring different HTT protein forms (soluble as well as different aggregates) in biological samples is an important complement to the development of therapeutics aimed at altering mutant HTT expression levels. The ELISA bioassay represents a simple and rapid method to detect and quantify total soluble HTT protein in blood. The system was validated using recombinant full length HTT protein with a Q138 expansion. This assay, applied to PBMCs isolated from HD patients at different stages of disease, was able to detect soluble HTT levels and to measure significant differences between HD and control samples. The results obtained allowed for the development of a simple 96-well microtitre plate-based assay to be used to quantify soluble HTT in the WP1 clinical biosamples to explore the potential use of HTT protein as a candidate biomarker in HD. As already mentioned in the P1 report, the assay is based on the MesoScale Discovery (MSD) SECTOR Imager. The immunoassay platform uses electrochemiluminescence detection as the end point-for conventional ELISA. Background signals are minimal because the stimulation mechanism (electricity) is decoupled from the signal (light). Emission at 620nm eliminates problems with color quenching. The experimental design for the analysis of the WP1 biosamples was as follows:

- Fourteen concentration point standard curve (S) in duplicates, three buffer QCs (100 – 30 – 1 ng/mL) and one matrix QC (3 ng/mL) in duplicate replicated with two independent dilutions.
- Four patients per plate tested at six different time points in duplicate: day 0, day 1 (4 and 24 hours), day 14 (4 and 24 hours) and day 28.
- For each sample, the HTT evaluated from the standard curve was normalised to total protein content.
- Additional normalisation was performed for each patient with respect to the respective day 0 measures.

Mixed-effects ANOVA models applied to the resulting data did not show statistically significant changes as a result of the treatment with selisistat in the WP1 clinical study; though a trend of reduced total HTT in PBMCs was observed in the male subjects after administration of selisistat at two doses for 14 days of treatment. This effect was seen when normalising HTT levels to the total protein content and to the predose measure for each patient. Insufficient numbers of subjects and the high variability in individual HTT levels did not allow reaching statistical significance. To develop more robust and reproducible measures of HTT detection in circulating blood cells, a collaboration between CHDI/Biofocus and the Paddington consortium was set out and, in this context, CHDI's mutant and total HTT protein assays were used to test Paddington WP1 samples. Both assays were able to detect in a quantitative and reproducible way total soluble or mutant HTT in PBMCs. However, no statistical difference was seen in Htt protein levels as result of the treatment with selisistat at two doses and for 14 days.

## Measuring HTT levels in immune cells

Given the cell intrinsic effects of mHTT in HD peripheral immune cells, we investigated HTT levels in immune cells as a possible biomarker for HD using a time-resolved Förster resonance energy transfer (TR-FRET) immunoassay to quantify mutant and total HTT protein levels in HD patient leukocytes. Mean mHTT levels in monocytes, T cells and B cells differed significantly between HD patients (n=26) and controls (n=12). Mutant HTT levels correlated with disease burden scores and caudate atrophy rates in HD patients, indicating that quantification of mHTT in peripheral immune cells by TR-FRET holds significant promise as a non-invasive disease biomarker. These findings were published in the Journal of Clinical Investigation (Weiss et al., 2012). However, for practicable use as a biomarker, mHTT levels need to be measurable in a more easily obtained cellular sample than the sorted leukocyte sub-sets used above. Therefore, these data are being validated in easily obtained whole PBMC fractions.

Phase one sample collection was completed in November 2012. A total of 64 samples were collected, of which 8 were discarded by CHDI during the analysis leaving a total of 56 samples. The statistical report below provides a more detailed breakdown of recruitment in the first phase. A second phase of samples collection and analysis is underway; this is on-going, progress to the end of the current reporting period is detailed below.

Each subject had two replications of each assay. The mean value from these replications was taken to be the outcome measure of interest, resulting in a single measure for each subject. The question of interest was whether levels of mutant HTT (mHTT) or total HTT (tHTT) as measured by the assays differ between disease status and stage. This analysis compared the Htt levels in the following four groups: controls, pre-manifest, early stage and moderate stage HD subjects.

Box plots of the distribution of mean mutant and total Htt by group show some suggestion that the outcomes are skewed in some of the subgroups. Additionally, there is suggestion that the variances are different between subgroups. The model fitted to will allow for this. If the residuals from the model show signs of skewness then either bootstrapped standard errors with 95% bias corrected and accelerated confidence intervals will be calculated, or a transformation may be applied to the data.  
\*age x (CAG -35.5)

### ➤ GroupG comparisons

This analysis was performed using the mean of the two replicates for each protein type as the primary outcome. From examining the box plots, there was suggestion that the variance of the outcome was different between groups. This was accounted for by fitting a generalised least squares regression model. After adjustment for age and gender, there was strong evidence that the expected mean of mHTT differed between groups. Using linear contrasts, the difference in mean levels between controls and all HD, controls and pre-Manifest, pre-manifest and early HD and early and moderate HD were all estimated. The results suggest that levels of mHTT are higher in HD subjects than controls, and the expected mean mHTT level increases as stage increases (though not all adjacent differences were found to be statistically significant). A test for linear trend resulted in  $p < 0.001$ . Total HTT did not show evidence of differences between stages. Performing the same linear contrasts as with mHTT, there was weak/borderline evidence of a difference in total HTT between HD and controls, with HD subjects expected to have less total HTT than controls ( $p = 0.067$  linear).

Normal plots of residuals were produced for both analyses to assess deviations from normality. There was a small suggestion of skewness for the residuals from the tHTT model in controls and one or two outliers in the HD groups, but nothing to cause strong concern over the validity of the model.

## **Acetylation status of mutant HTT**

The detection, monitoring and quantifying of the HTT forms in peripheral tissues could be applied as a potential biomarker for novel HD treatments. Indeed, the evaluation of post-translational modifications (PTMs) of mutant HTT in peripheral biological samples could result in an efficient pharmacodynamic readout monitoring the activity of drugs with a mechanism of action related to the modulation of HTT PTMs. In this respect we evaluated the possibility of measuring selisistat activity by analyzing acetylation status of specific HTT protein residues using two methodologies, as follows.

### **1) Acetylated HTT measurement with an antibody-based approach**

The acetylation status of HTT in human samples may represent a potential pharmacodynamic biomarker for target modulation. The aim of this part of WP3 was to develop specific assays for the quantitation of acetylation of four lysine residues (K6, K9, K15, K444) in the HTT protein, which may be used to monitor the efficacy of anti-acetylation drugs in clinical studies. Antibodies were specifically developed against HTT epitopes comprising AcK6, AcK9, AcK15 and AcK444, with the objective of evaluating them for their capacity to detect acetylated, full-length HTT in cell lines. As an additional control, a commercial anti-panAcK antibody was also employed. Recombinant full length HTT protein, either acetylated through co-transfection with CBP-HAT or chemically acetylated *in vitro* was used as a control.

The reactivity of the antibodies developed against the lysine residues of interest was evaluated in ELISA assay vs. the positive control, but none of the antibodies tested (including anti-AcK444) worked in the ELISA system and the activity was stopped.

### **2) Acetylated HTT measurement using mass spectrometry (MS) as detector of PTMs**

As an alternative to antibody-based approaches, mass spectrometry approaches were also evaluated. The direct detection of HTT protein in healthy donor plasma samples was attempted first. HTT peptides reported in the literature to be acetylated/phosphorylated and their unmodified counterparts were synthesized, and a multiple reaction monitoring – information dependent acquisition (MRM-IDA) method for their identification and quantitation in plasma was developed in comparison with previous work by Aiken (2009) and Jeong (2009). These analyses showed that the acetylated peptides are about fifty times less responsive in the analysis and thus the limit of detection and quantitation should be set accordingly. Various methods for the enrichment of high molecular weight proteins from plasma, based either on ultrafiltration or a combination of immune depletion and ultrafiltration, were devised. An efficient protocol for the enrichment of phosphorylated peptides was also applied to the same samples, but no HTT peptides could be confidently identified either in healthy or in HD plasma, while HTT peptides were observed from purified PBMCs that were therefore selected as starting matrix. Several lysis and digestion protocols were tested on PBMCs from healthy donors and the best one was chosen, on the basis of total protein yield, number of phospho- and acetylated peptide identified and on the number of missed cleavages.

The protocol was transferred to the subcontractor (IDS). However, despite several attempts, the initial promising results could not be reproduced. It therefore appears that the detection of post-translational modified HTT peptides in either plasma or PBMC samples by mass spectrometry approaches is not currently technically possible, under the conditions tested.

## **selisistat transcriptional signature**

Transcriptional signatures in blood are emerging as promising pharmacodynamic biomarkers for pre-clinical and clinical studies. In this project, we used mRNAs whose steady-state levels are regulated by selisistat as candidate pharmacodynamic readouts in human whole blood RNA from HD patients treated with selisistat in the WP1 clinical study. The transcriptional signature was investigated in order to assess the functional consequences of target engagement and to establish possible dose-effect and time effect relationships. Target genes identified in blood from healthy volunteers in Phase I study, as well as from the R6/2 HD mouse model and in a bioinformatics analysis of the published literature, were analysed in samples from the WP1 clinical study. There were three dosage arms: group A receiving 10 mg selisistat; group B: receiving 100 mg selisistat and group C receiving placebo only. Fifty five subjects were randomized across the three arms.

The blood samples were collected in duplicate at clinical sites in PAX-gene tubes at baseline (day 0), and at 3, 4, 6, and 24h post-dosing on day 1 and 14, at pre-dose on day 7 and finally at day 28. Total RNA was isolated from these samples and converted into cDNA to study expression levels of selected genes by RT-qPCR. The data from each sample were normalised to internal control genes B2M, ACTB, RPL13A, and PP1B (Table 3).

For each patient, expression levels calculated at different time points were then compared to those of baseline and the relative abundance of each transcript was determined by the standard curve.

Table 3 summarizes the genes composing the selisistat transcriptional signature which was validated in blood samples from healthy volunteers treated with the compound. In total, thirteen genes were confirmed by RT-qPCR studies and nine of those have been tested in 55 selisistat treated HD patients completing the clinical study according to the following treatment scheme:

- Placebo: 7 female and 11 male patients;
- Selisistat at 10 mg: 3 female and 14 male patients;
- Selisistat at 100 mg: 6 female and 13 male patients.

One of the 55 patients was excluded for missing baseline sample. Final RT-qPCR results underwent statistical analysis of gene expression data.

Mixed-effects ANOVA on normalized gene expression levels revealed that GZMB is differentially modulated (treatment P-value <0.05); post-hoc multiple comparison analysis highlighted difference in arm receiving selisistat at 10 mg with respect to placebo treatment. Lower modulations (treatment P-values <0.1) were observed in ABCA1, AKR1C3 and NKG7, where post-test analysis confirmed 10 mg as being the effective dose.

Including also gender in the analysis model, significant differences in treatment and gender interaction were observed particularly for AKR1C3 (treatment\*gender P-value <0.05) but also for NKG7 and SPON2 (treatment\*gender P-values <0.1).

Analysis in male patients only, the group more represented in the 10 mg dose, showed that AKR1C3, GZMB, NKG7 and SPON2 were differentially modulated (treatment P-values <0.05) and the modulation was confirmed in patients treated with selisistat at 10 mg and 100 mg for AKR1C3, GZMB and NKG7 with respect to placebo treated patients. SPON2 was indeed modulated in patients treated with selisistat at 100 mg according to this analysis.

Additional significant analysis for these genes was performed by applying one-way ANOVA model individually for each time point and considering treatment as independent variable.

No statistically significant modulations were observed for the other genes.

Taken together the results obtained indicate the possibility of analyzing the selisistat activity in clinical samples through its transcriptional effects in peripheral tissues (blood). This could also lead to draw hypotheses for target engagement and mechanism of action in vivo through pathway analysis of the modulated classes of genes.

### **Transcriptional signature in immune cells**

Aiming to conduct studies to examine the effects of selisistat on transcription status in primary HD peripheral blood mononuclear cells (PBMCs), partner 3, UCL, collected blood samples from nine early-stage HD patients into 50 ml heparinized tubes and the PBMCs isolated using Histopaque-1077 tubes. PBMCs were seeded in culture plates at a density of  $2 \times 10^5$  cells/cm<sup>2</sup>, and cultured for up to 48 hours without discernible differences in the relative numbers of B-cells, T-cells and monocytes. Once the cells had adhered post-seeding, cultures were treated with 100 nM, 500 nM or 5  $\mu$ M selisistat and lysates collected at 4, 8, 24 and 48 hours thereafter. The 4 hour time-point represents the mean residence time (MRT) following single dosing in human healthy volunteers, irrespective of dose, and was selected to mimic a single-dose study in humans. At the other extreme, the 48 hour incubation time is closer to mimicking the proposed pharmacokinetic steady-state situation, which is the more relevant situation. The 24 hour time point represents the dosing interval and therefore the pharmacokinetic  $C_{\min}$  (trough) time. The 8 hour time-points was selected as a plausible time at which changes in transcripts expression are observed in treated cells in culture. Incubation periods longer than 48 hours are not advisable because the relative numbers of different cell populations in a mixed PBMC culture and overall cell viability changes significantly after this point. In terms of concentrations, the 100 nM concentration corresponds to the  $C_{ss,avg}$  at the 10 mg/day dose level in the WP1 clinical study and also the  $IC_{50}$  in the enzymatic hSirT1 assay; in addition, this falls in the middle of the response curve for the PC-12 exon-1 cytoprotection model. The 500 nM concentration corresponds to the average steady state concentration ( $C_{ss,avg}$ ) in the R6/2 model at the minimal efficacious dose (and therefore represents the target plasma level in humans. The 5  $\mu$ M concentration represents the  $C_{ss,avg}$  for the 200 mg/day dose level in the S015-002 study. Samples were harvested from each treated PBMC cell culture to allow separate analyses of RNA and protein. RNA samples were collected by the direct lysis of cells in the culture dish, using Qiagen RLT plus buffer (plus  $\beta$ -mercaptoethanol). Cell samples for protein analysis were obtained by removal of the cells in ice-cold PBS using a cell scraper, centrifugation at 400 xg for 10 min at 4°C and snap-freezing of the resultant cell pellet. Frozen samples were labelled with an anonymised 5-digit number and shipped to partner 1, Siena Biotech, for blinded analysis.

Previous experiments had suggested a transcriptional signature in HD PBMCs associated with genes related to oxidative stress and immune modulation. The RNA isolated from the samples above, other than those collected at the 48 hour time-point at which RNA yields were lower than expected due to loss of culture viability over the prolonged period in culture, yielded sufficient high-quality template to re-test all the genes identified as part of the HD PBMC transcription signature and the effects thereon of selisistat.

Regarding the protein isolated as above, the intention was to use these samples to measure by ELISA levels of protein encoded by candidate genes identified as part of the transcription signature, such as granzyme-B, which may track disease progression in HD PBMCs. However, unfortunately levels of protein yielded from these samples were insufficient to carry out these assays using these samples (however, granzyme B levels have been made in separate samples, see below). Therefore, the protein lysates were used to evaluate the amount of HTT by ELISA assay. Whilst the selisistat treatment in WP1 clinical study was too short to expect HTT levels to change, the samples were used as part of wider evaluation of the lower limit of detection of HTT in a real experimental situation, as published (Massai et al, 2013). Future work to assess the ex vivo treatment of patient cells would require more extensive protocol optimization focusing on cell number, culture viability over time, and subsequent extraction and processing.

### **Usefulness of immune markers and related molecules in an interventional setting**

This project included the development and validation of assays for the measurement of peripheral immune molecules, the study of HD myeloid cell dysfunction and the measurement of HTT levels in immune cells. These are described separately, as follows.

#### **➤ Development and validation of assays for the measurement of peripheral immune molecules**

##### *Cytokines*

MSD multiplex ELISAs (measuring four different cytokines: IL-6, IL-1, TNF-alpha and IL-8) was applied to WP1 plasma samples (taken at four time-points – screening, day 1, day 14 and day 28 - each from 55 subjects that had been exposed to 10 mg SEN0014196, 100 mg SEN0014196 or placebo) and were completed as of end-June, 2012. Statistical analysis were conducted by partner partner 5, LSHTM. The data demonstrated no significant difference in the levels of each of the cytokines between the treatment groups when adjusting for age and gender in the plasma samples taken over the sampling time-course.

##### *Chemokines*

MSD multiplex ELISA analysis of chemokines (eotaxin, MIP1 $\beta$ , TARC, IP-10, IL-8, MCP1, MCP4) in WP2 plasma samples was also completed. Again, statistical analysis was conducted by partner 5, LSHTM. Overall there was very little separation in variable levels between HD patients and controls. None of the variables produced a statistically significant difference between controls and HD ( $p > 0.05$ ) for mean levels of the variable when adjusting for age and gender. Many of the variables however, did show statistically significant differences in levels between males and females or showed an association with age. None of the secondary analyses showed any association with disease burden.

##### *Other immune markers*

The potential of other components of innate immunity as plasma biomarkers of HD has continued to be investigated. Methods to measure complement factors have been extensively explored, but no significant differences have been observed: levels of complement components 3, 3a, 4, 4a, 5, 5a, 9 and the complement regulators complement factor H, complement factor B and clusterin, did not differ between healthy controls and HD subjects. The S100 proteins, S100A12 and calprotectin, were also measured, and were found to be elevated in moderate HD patients compared with healthy controls, but there was no correlation with disease progression. Levels of  $\alpha$ -2 macroglobulin,  $\alpha$ 1-antitrypsin, SAP, CRP, ApoA1, ApoC3, ApoE, osteocalcin, osteonectin, osteopontin, PEDF and prealbumin also did not differ between healthy controls and HD subjects. This data were published

in the Journal of Huntington's Disease (Silajdzic et al, 2013). Of the immune markers tested in this study, none showed potential to track with HD disease progression. However, although we did not observe any striking changes in plasma inflammatory markers in this study, strong evidence remains showing immune changes in HD. This suggests that HD immune alterations are not easily measurable in plasma, or that subtle HD immune alterations in plasma are not easily detected due to biological variance or small sample size. Overall, this shows that immune markers are not likely to be useful biomarkers for HD.

### *Granzyme B*

Granzyme B is a serine protease that in humans is expressed by peripheral immune cells and is associated with oxidative stress and immune modulation. Data suggests that levels of granzyme B in HD PBMCs tracks disease progression. Here, we sought to replicate these findings. Protein isolated for twenty control and twenty early-HD subjects was used for ELISA measurement of granzyme-B. However, we observed no significant difference in granzyme B levels in the samples we tested (Mann-Whitney, p 0.330).

### **Myeloid cell dysfunction in HD**

Myeloid cell hyper-reactivity in HD could be due to the intrinsic expression of mHTT, or may be secondary to other disease-associated processes. We now know that mHTT is readily detectable in human monocytes and macrophages, and its levels in immune cells track measures of disease progression, as published in the Journal of Clinical Investigation (Weiss et al, 2012) and described below. Using a novel approach, we used  $\beta$ 1,3-D-glucan-encapsulated siRNA particles (GeRPs) to deliver anti-*HTT* siRNA into human HD myeloid cells. Cells cultured with the GeRPs readily ingested them via phagocytosis without effecting cell viability. Testing different macrophage to GeRP ratios, up to 90% of macrophages took up green fluorescent GeRPs when they were added at a ten-fold particle to cell ratio, demonstrating a much higher transfection efficiency than the 10-20% transfection rate achieved by traditional methods such as lentiviral transduction. Delivery of anti-*HTT* siRNA reduced levels of *HTT* mRNA and protein by 50-70% measured by qPCR and TR-FRET, respectively. Lowering total HTT in this manner caused a substantial reduction of the previously over-produced proinflammatory cytokines in HD cells. Furthermore, HTT lowering also effects transcriptional dysregulation by reversed upregulated RNA expression of IRAK1, Jun and CD40 in HD myeloid cells, demonstrating that lowering HTT levels can reverse the hyper-reactivity of human HD cells.

Investigating the upstream signalling pathways that mHTT might affect to cause peripheral myeloid cell dysfunction underlying the elevated plasma cytokine levels in HD patients identified the NF $\kappa$ B pathway as dysfunctional in primary human HD cells. Proximity ligation assays and immunoprecipitation experiments were used to demonstrate a CAG-repeat length dependent interaction between HTT and IKK. Measuring I $\kappa$ B levels over 2 hours post-LPS stimulation using western blot analysis, we found a faster and prolonged reduction in I $\kappa$ B levels in HD monocytes compared to controls. Thus, we demonstrated in human HD cells that mHTT binds IKK $\gamma$  and causes increased NF $\kappa$ B activity by increased I $\kappa$ B degradation and subsequent NF $\kappa$ B translocation. We hypothesize that this causes altered transcription of NF $\kappa$ B target genes, leading to increased cytokine production by immune cells. This data was published in Brain (Träger et al, 2014).

### **Cholesterol and oxysterols in plasma**

The project objectives were to:

- Investigate the differences in oxysterol changes between HD patients and controls.

- Explore the association between oxysterol changes and CAG as well as disease burden in HD patients.
- Explore the association between oxysterol changes and changes in diffusion metrics, volumetric measures, and cognitive parameters in HD patients.
- Modelling the time course of oxysterol in HD patients and controls.
- Calculation of effect sizes as potential outcome in future clinical trials to slow or stop the progression of disease.

Assays for the measurement of cholesterol, lanosterol, lathosterol, 24S-hydroxycholesterol (24OHC) and 27-hydroxycholesterol (27OHC) in plasma of HD patients were used to assess such molecules potential as pharmacodynamic endpoints. The statistical analysis plan was drafted and finalized. Data from a collaborating laboratory in Milan were provided. A program for performing the first statistical analyses was developed and results for descriptive statistics were obtained. The distribution of plasma oxysterol (24OHC, 27 OHC and total cholesterol) was determined for HD patients and non-HD controls, as stratified by site and for all three visit times.

Initially, low consistency between oxysterol results generated by two independent methods (isotope-dilution gas chromatography-mass spectrometry, GCMS, in Milan and isotope dilution liquid chromatography-tandem mass spectrometry LC-MS/MS in Paris) was observed. A unique internal standard solution was shared between the two laboratories with a resulting improvement in consistency between the two methods. The analysis of the Milan data set showed that there was no difference in mean value of 24OHC but the median value was slightly lower in HD compared to control subjects. The HD and control groups showed a higher value at baseline but this pattern was reversed for subsequent visits. At time 0 there was no significant reduction of 24OHC in early stage HD patients as expected by previous reports (Leoni et al., 2008; Leoni et al., 2011; Leoni et al., 2013b).

Plasma 27OHC, instead, was reduced in HD patients compared to controls when the ratio over cholesterol was calculated (for absolute 27OHC  $p = 0.057$ , for ratio 27OHC/cholesterol (chol)  $p = 0.034$ ), as previously described (Leoni et al., 2008; Leoni et al., 2011). The mean 27OHC in the control group distinctly increased in all the three time points studied.

A slight difference between HD group and controls in the change of oxysterol (baseline vs 15 months) in 24OHC, 27 OHC and total cholesterol was observed, however further analysis would be required to determine whether it is significant.

A relation between oxysterols and the CAG repeat length and disease burden could also not be demonstrated with the data. Cholesterol and oxysterols results showed a very large distribution of concentration values both in control and HD individuals.

Cholesterol levels were high ( $>240\text{mg/dL}$ ) in a significant fraction of both controls and HD individuals. In disagreement with previous reports (Leoni and Caccia, 2013b; Bretillon et al., 2000), at baseline there was no correlation between oxysterols and cholesterol while significant correlations were observed in 6 month and 15 months visits. Significant differences were found in oxysterols levels among study sites together with differences in plasma cholesterol concentrations, age and BMI. Thus, an analysis by study site was performed, since differences in age and BMI were found for individuals recruited at the four different sites.

#### *Baseline*

In the Paris site group, 24OHC levels were significantly reduced in HD patients compared to controls ( $p=0.004$ ).

In case of Ulm HD group, a single individual had 24OHC more than 2 times higher than the mean value of the group. Excluding this individual, 24OHC was also significantly reduced in HD patients compared to controls ( $p=0.012$ ).

24OHC concentrations in London HD patients were significantly higher compared to Paris HD patients ( $p=0.006$ ).

Plasma cholesterol was significantly higher in London HD patients compared to London controls. It was significantly higher in Ulm controls compared to Paris controls ( $p=0.011$ ).

BMI was significantly higher in Leiden controls compared with Leiden HD patients and Paris controls ( $p=0.043$  for both).

#### *6 months*

24OHC was significantly reduced in Paris HD patients compared to Paris controls ( $p=0.04$ ) and to London HD patients ( $p=0.007$ ).

No differences for plasma cholesterol were observed.

The ratio 24OHC/cholesterol was significantly reduced in Paris HD patients compared to control ( $p=0.002$ ) and compared to London HD ( $P=0.005$ ).

The ratio 27OHC/cholesterol was significantly reduced in Leiden HD patients compared to controls ( $p=0.041$ ) but not in London HD vs London controls ( $p=0.189$ ).

#### *15 months*

Levels of 24OHC were increased in Leiden HD and London HD patients compared to controls ( $p=0.155$  and  $p=0.185$ , respectively), reduced in Paris HD ( $p=0.067$ ) and in Ulm patients ( $p=0.4$ ).

In summary, the pattern of distribution of cholesterol, oxysterols, age and BMI was different in the groups of the four 4 different study sites groups. In the HD patients from the Paris cohort, oxysterols were reduced for the most part compared to controls, as previously observed (Leoni et al., 2011). In London HD patients, however, they were increased.

No significant differences for 27OHC and cholesterol or 27OHC/cholesterol ratio were seen, despite both 27OHC and 27OHC/cholesterol ratio tended to be lower in HD compared to site matched controls. In general, in London patients, oxysterols tend to be higher, and in Paris and Ulm patients they appeared to be lower when compared to site matched controls.

The high variability in each group and the inconsistent patterns of distribution could be explained by pre-existing diet (Leoni and Caccia, 2013).

Another initial hypothesis was that the observed differences between sites may reflect poor adherence to the fasting requirement by participants. An ad hoc experiment to evaluate the effect of fasting vs non fasting collection on oxysterol and cholesterol was performed with the same individuals: plasma samples were collected from four controls and eight HD patients in Ulm and three controls and seven HD patients in London.

The oxysterol values between fasting and non-fasting states varied by up to 25%. When the oxysterol/cholesterol ratios were calculated, these margins rose.

The oxysterol values between fasting and non-fasting states were also influenced by duration of fast, gender and clinical phenotype, again with a 25% variation.

Some individuals in Ulm and London had high levels of total cholesterol. Often the highest cholesterol concentrations corresponded to the highest oxysterols concentrations.

Thus, samples collected in non-fasting condition might contribute to the high variability of distribution of oxysterol values.

The cholesterol precursors, lanosterol and lathosterol, have not been analysed thus far due to attention being prioritised towards investigating the important effect of fasting and non-fasting condition on oxysterols. Associations between cholesterol biomarkers and cognitive and imaging metrics as well as the power calculations for clinical trials, will be carried out, if indicated, once full analysis is completed.

### **Exploring the use of myoblasts and fibroblast cultures for biomarker discovery**

Using multiple tissues from the same individual, studies have shown that disease pathophysiology can be better dissected than using single tissue samples (Hagg et al., 2009). HD seems a particularly promising disease as the mutant protein is expressed in almost all cells. Multiple tissue collections might help identify markers of disease progression, but also markers of resilience, e.g. from tissues less affected by the mutant protein, which may provide guidance for the selection of new drug targets. Therefore, in order to complement pre-existing work to examine HD biomarkers in peripheral, we sought to establish the feasibility of conducting the same types of analyses in human muscle and skin cells. To investigate the cell intrinsic effects of mHTT in human muscle and skin cells muscle and skin biopsies were taken from two controls, five pre-manifest and four early HD patients between October and December 2013. Myoblast and fibroblast cultures have been established from all patients biopsied and cryopreserved for experimental use once all cultures have been set up.

Myoblast and fibroblast culture set-up procedures and maintenance protocols have been optimised from existing protocols using control cultures. Myoblast differentiation to myotubes has also been carried out, with the presence of differentiated myotubes confirmed by immunocytochemistry. Myoblast cultures will be used to assess the effects of the HD mutation on the response of these cells to several stimuli. Dose finding has been carried out for myoblast stimulation by LPS, IFN $\gamma$ , epinephrine, dexamethasone and AICAR and IL-6 release assessed. Glucose uptake assays have also been optimised for use on myoblasts and differentiated myotubes to assess the response to insulin in these cell types.

### **Adiponectin**

Adiponectin is a 244 amino acid peptide hormone that modulates gluconeogenesis and glucose uptake as well as fatty acid catabolism. Adiponectin is secreted from adipose tissue and is abundant in plasma relative to many other hormones. Levels of the hormone are inversely correlated with body fat percentage in adults and are reduced in insulin resistance and Type 2 diabetes. The levels of adiponectin show a gender difference, with females having higher levels than males. Weight reduction significantly increases circulating adiponectin levels, similar to the action of leptin but the two hormones perform complementary actions, and can have additive effects. Adiponectin secretion is regulated by SirT1 through Foxo1-C/enhancer-binding protein alpha transcriptional complex (Qiao et al., 2006) and the endoplasmic reticulum oxidoreductase Ero1L $\alpha$  (Quiang et al., 2007), suggesting that monitoring adiponectin levels may be a way to assess SirT1 activity.

Specifically in the case of HD, Phan et al., showed that circulating adiponectin levels are reduced in the R6/2 and CAG140 knock-in mice HD models with progressive disease. Jeon et al. (2009) showed that adiponectin is neuroprotective in cultured hippocampal neurons against kainic acid-induced cytotoxicity and reduced levels of reactive oxygen species, attenuated apoptotic cell death, and suppressed activation of caspase-3 induced by kainic acid. While adiponectin levels were not significantly different between HD patients and controls, adiponectin levels corrected for fat mass were shown to correlate to disease severity in HD patients (Aziz et al., 2010).

Based on the above observations, an ELISA method was developed based on a commercially available kit (Orgenium Laboratories, Finland) and used to analyse plasma samples from subjects in the selisistat study in WP1. As expected, adiponectin levels were found to be higher in females than

in males, and also correlated inversely to the body mass index, in line with the results from Aziz et al. While a non-significant increase in adiponectin levels were observed in the higher selisistat dose group that may merit further investigation, it should be remembered that the study did not include diet control, and the interpretation of the results should be made with caution. Given the robustness of the method and the clear mechanistic value of a functional readout of SirT1 activity, adiponectin as a marker for SirT1 warrants further attention, provided the covariates can be identified and controlled for.

### **Additional on-going work originating from original WP3 findings**

#### *Measuring HTT levels in immune cells*

To follow-up the findings as described above, a second phase of measuring HTT levels in immune cells was initiated. This repeat dataset will provide additional numbers, longitudinal sample pairs, more subjects with accompanying imaging data, and additional biomaterials enabling the development of novel assays for N-terminal, mid-section and C-terminal HTT. Recruitment for this second phase collection was completed in August 2013. A total of 77 samples have been collected and analysed, Two/three PBMC samples per participant allowed for assays to be done in triplicate for improved reliability. A total of 32 participants were brought back from the first sample set for repeat analysis for validation purposes. Statistical analysis of this large data set is currently ongoing.

#### *Myeloid cell dysfunction in HD*

Follow-up work aiming to further dissect myeloid cell dysfunction in HD by means of RNA sequencing (RNAseq) was initiated during the final reporting period and is on-going. Monocytes were isolated from HD and control subjects before being either stimulated with LPS for 4 hours or kept at baseline (unstimulated) conditions. RNA was extracted from these cell populations and shipped to deCODE Genetics in Iceland, where it is currently undergoing RNA sequencing. Additional samples were also treated with siRNA to lower levels of the HTT before being sent for sequencing. This will allow us to investigate the reversibility of transcriptional changes in HD following HTT knockdown. Once deCODE have completed the RNA sequencing the data will be analysed in collaboration with Dr Vincent Plagnol at the UCL Genetics Institute - we expect to have results later this year. We are hopeful that this will improve our insights into the mechanisms underlying immune dysfunction in HD, in addition to revealing novel potential biomarkers which can then be investigated further.

#### *Exploring the use of myoblasts and fibroblast cultures for biomarker discovery*

Additional subjects will be recruited to add samples to the bank that was initiated during the final reporting period, allowing for further analyses. Eight controls, five pre-manifest and six early HD patients will be biopsied between January and July 2014.

## **Potential socio-economic impact and societal implications of the project, main dissemination activities and exploitation of results**

### **Potential impact**

Huntington's disease (HD) is a devastating condition that affects not only the patient but also profoundly impacts the immediate family, spouse or other caregivers, colleagues and friends. The overall socio-economic impact of HD must therefore be seen in the light of a complex cascade of events as patients progress from pre-symptomatic gene carriers to the symptomatic phase and eventually to 24/7 care in a hospital setting. The societal impact is therefore not limited to the direct healthcare costs, but has broader connotations.

#### *The Clinician's Perspective*

For the affected patient, Huntington's disease (HD) presents with a triad of cognitive, psychiatric and motor dysfunction, causing progressive and irreversible morbidity and eventually, patients succumb to complications such as cardiac failure or respiration pneumonia. The intensity and nature of symptoms often varies with progression of the disease with some patients experiencing a greater proportion of emotional/behavioural symptoms while others exhibit more pronounced motor- or cognitive decline.

The pre-symptomatic phase (often up to the age of 30-35 years of age) transforms into a pre-diagnostic (or "prodromal") phase, when patients start exhibiting subtle changes of personality, cognition, and motor function. Although both the healthy and pre-diagnostic stages are sometimes referred to as 'pre-symptomatic', the pre-diagnostic phase is associated with detectable clinical findings (Snowden et al., 1998), even if the patient may be unaware of them. Clinical diagnosis occurs when these effects become more pronounced and specific (Paulsen et al., 2005). In this phase, individuals might become irritable or disinhibited and unreliable at work; multitasking becomes difficult and forgetfulness and anxiety mount. Family members note restlessness or fidgeting, sometimes keeping their partners awake at night. Finally, this phase transforms into the symptomatic phase when distinct chorea, incoordination, motor impersistence, and slowed saccadic eye movements develop (Watts and Coller, 1997; Weiner and Lang, 1989).

The hallmark symptom, chorea, does not correlate well with disease severity (Mahant et al., 2003). Chorea may not develop in early disease and although many patients have chorea that progresses initially with later onset of dystonia and rigidity, chorea becomes less prominent at later stages of disease. Another characteristic is motor impersistence, fidgeting and compensatory repositioning. In the same way, inability to apply steady hand contraction pressure is characteristic ("milkmaid's grip"). Contrary to chorea, motor impersistence is linearly progressive, making it a possible marker of disease severity (Reilmann et al., 2001). Finger-tapping rhythm and rate are useful in diagnosis whereas gross motor function including gait and postural maintenance deteriorate later. Unlike the chorea component, these changes have profound impact on functional capacity.

As functional capacity worsens, chorea lessens and dystonia intensifies (Feigin et al., 1995; Louis et al., 1999). Subjects with a younger age of onset have more severe dystonia, bradykinesia and eye movement abnormalities relative to chorea. However, the majority of HD patients exhibit some dystonia.

The main cognitive impact is generally on higher functions such as logistics and coordination, planning and decision-making, while long-term memory is often intact (Craufurd and Snowden 2002). Such cognitive dysfunction then deteriorates monotonously, with speech worsening faster than comprehension. Unlike cognitive functions, psychiatric and behavioural symptoms do not necessarily show progression with disease severity. Depression with suicidal ideation is common (DiMaio et al., 1993) and mania and psychotic symptoms develop with some frequency.

### *The Patient's Perspective*

HD causes a loss of ability to monitor one's own actions. HD sufferers lose the perception of their own identity, their domestic situation, origins, personal interests, personality traits and their personal relationships with others. They lose the ability to speak in a coherent manner. Their rational actions fade and physical abilities are crippled. They can no longer determine their position in society, what kind of personality they have as well as any other personal information such as gender-, age- or sex preferences. The ability to understand the human condition is diminished, sufferers cannot exercise religious or moral beliefs, and their communication with others ceases (Archer, 2000; Callinicos, 2004).

Suicide is more common than in the general population, even if estimates vary between studies; there are essentially two peaks in the suicide (or attempted suicide) incidence – following the realisation that disease onset has occurred, and a second peak when patients have progressed to a stage where they perceive that are no longer able to take of themselves – possibly as an attempt to reduce the burden for caregivers (Paulsen, 2001).

An important psycho-social parameter is the sense of shame that affects many HD patients – erratic behaviour, dramatic personality changes, motor dysfunction and language disturbances can all provoke tension and embarrassment, especially since the patients have led normal lives in the community up to the onset of disease. Indeed, stigmatisation has been a major issue in many countries and perhaps represents one of the most important targets for the various patient organisations in overcoming prejudice and creating visibility for HD within the general public. One specific aspect of this stigmatisation is the fear of discrimination in the workplace, causing anxiety years before disease onset (Wexler, 2010).

### *The Family's Perspective*

It is important to recognise that virtually all patients with HD have experienced the disease in a family member prior to developing the disease themselves. While genetic testing is now available across all developed countries, not all persons at risk chose to take the test, and given the wide variability in age of onset, a positive test will often lead to more anxiety than not taking the test.

Since an important feature of HD is dementia including confusion, personality disintegration, impairment of memory, diminished control, and restlessness as well as agitation, providing care to HD patients is stressful. In addition, HD families frequently need to care for more than one member and consequently, nearly every member of a family has been a HD caregiver (Pollard et al., 1999). Caregivers also need to cope with the idea that one day they may need the same care and often contemplate the future while caring for a parent or a sibling with HD. Regardless of whether the subject is a genetically confirmed gene carrier, the psychological impact of knowing to be at risk and witnessing the progression of disease in a family member is marked – indeed, observational studies in HD often specify that the control group be composed of subjects that are not gene carriers but have experienced living with someone with HD, since the environmental imprinting in a HD family is so strong (see e.g. Tabrizi et al., 2009).

From a purely economic point of view, HD has a direct impact on a person's capacity for activities of daily living – as outlined above, once a clinical diagnosis has been made, HD patients have often progressed to a stage where maintaining a job may no longer be possible, with the inevitable loss of income and stimulus from a working environment. As patients progress in their disease, also the caregivers often need to take time off work, adding to the loss of income. Finally and as patients progress to later stages of disease, there is frequently the need to use professional home care and physiotherapy services, adding to the cost for the patient and family.

### *The Healthcare Provider's (Payer) Perspective*

Most health economics analyses are conducted with a societal perspective, and include both direct medical and non-medical costs and indirect costs related to loss of production and costs of informal care. Clearly, some effects of chronic disease are difficult to estimate in terms of monetary cost, such as anxiety, psycho-social negative effects, problems in social functioning and activities of daily living.

The direct healthcare costs per patient vary with the stage of disease; a recent study (Divino *et al.*, 2013) conducted in the US divided the HD population into three, roughly equal stages (Early, Intermediate and Late), with associated mean annualised per patient costs of €4,600, €14,700 and €22,000, respectively. Not unexpectedly, the main cost drivers in early- and intermediate stage disease are outpatient costs and Pharmacy costs, while the major cost drivers in late-stage disease are represented by inpatient admissions and nursing home costs. With a European prevalence of 0.4-0.8 per 10,000, this translates to an annual overall, direct healthcare provider cost of M€200-400. To this cost should be added the indirect costs, and while no such estimates have been found specifically for HD, it is reasonable to assume that the indirect cost proportion is similar to that of Alzheimer's Disease (AD), which has been estimated to 56% (Wimo *et al.*, 2009) of the direct costs. In consequence, the effective total societal cost of HD is M€350-700 per year.

The above costs should be compared to those for the estimated (2008) cost burden of AD in the EU of between €15.300 and €22.000 per year and patient. Clearly, the prevalence of AD is much higher than that of HD, but it should also be remembered that the course of disease progression of HD is slower, generating higher accumulated lifetime costs per patient.

The unmet medical needs in HD, and the costs of HD disease progression suggest that delaying or preventing disease progression would lead to considerable cost savings. A disease-modifying therapy which that stabilises a patient at an earlier stage of disease would therefore lead to significant reductions in societal cost. The size of the target population is possibly underestimated if based on current prevalence data (0.4-0.8 per 10,000) and suggests at least 40,000 patients in early stage disease world-wide. A compound with a prevention label, allowing use in pre-symptomatic gene carriers would reach on the order of 150,000 patients world-wide. Market penetration for an approved therapeutic can be expected to be high and rapid, given the complete lack of therapeutic options. The HD community is well-organised and most patient or gene carriers are already members of one of the patient organisations that play an important role in disseminating information about novel therapeutics.

### **The Impact of PADDINGTON**

It is against the above background that the PADDINGTON project should be seen. The project was conceived to integrate fully with ongoing efforts within Siena Biotech SpA to develop and commercialise a disease-modifying therapy for HD. Selisistat (6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide, SEN0014196) is a selective SirT1 inhibitor that inhibits recombinant human SirT1 with an IC<sub>50</sub> of 98 nM. It is more than 200-fold selective over SirT2 and SirT3 and has been shown to inhibit deacetylation of several SirT1 substrates both in vitro and in vivo (Napper *et al.*, 2005). The compound exhibits cyto- and neuroprotective activity against toxicity induced by mutant Htt in cellular and in vivo models of HD increasing survival, amelioration of psychomotor behaviour and improvement in histopathological endpoints in the most widely employed animal models of HD (Smith *et al.*, 2014). The availability of selective, safe SirT1 inhibitors such as selisistat therefore makes the acetylation-dependent clearance of mutant Htt a clinically testable therapeutic approach.

However, the clinical development of disease-modifying therapeutics for slowly progressive neurodegenerative conditions like HD is critically dependent on access to appropriate pharmacodynamic readouts to guide selection of doses and dosing schedules, aid in informing power calculations and defining futility criteria for longitudinal efficacy studies. While symptomatic

approaches typically address effects on a single clinical measurement or a subset of measurements related to a specific mechanism of action, there is no a priori possibility to identify which of the various motor-, cognitive or functional domains will respond to a disease-modifying mechanism of action. Studies aimed at assessing efficacy by reducing rate of progression of disease will necessarily have a longitudinal design and may require treatment duration of 2 years or more to achieve unequivocal demonstration of clinical benefit.

Running alongside other biomarker initiatives such as TRACK-HD (Tabrizi et al., 2012; Tabrizi et al., 2013), REGISTRY (Orth et al, 2010), PREDICT (Paulsen et al., 2008) and Enroll-HD (Mestre et al., 2013), the PADDINGTON project is a cross-functional effort to develop both mechanistic markers for SirT1-engagement and disease progression markers to support the development of disease-modifying therapies for HD.

Specifically, the PADDINGTON project set out to develop and initiate validation of a series of pharmacodynamic markers such as the circulating levels and acetylation status of mutant Htt (specific to the selisistat mechanism of action) as well as and disease-specific approaches, such as MRI, immune markers and change in cholesterol metabolites.

Two separate clinical studies were undertaken during the project: in the first study, selisistat was administered to HD patients over a 14-day period at two different dose levels and placebo, with the objective of collecting biophase samples to support the development of pharmacodynamic assays. The second study was an observational study over 18 months using MRI to assess the feasibility of using imaging readouts for time spans less than two years, which would be critical to planning and execution of therapeutic clinical trials with disease-modifying compounds.

It is important to notice that this work will support the development of pharmacodynamic markers for HD monitoring which could be potentially useful for other therapeutics, with different mechanisms of action. The PADDINGTON Proposal therefore contributes value beyond the development of selisistat.

The scientific results of the various have been published via peer-reviewed papers in scientific journals, oral and poster presentations at national and international conferences, via the project web site and through other channels, as outlined in the section below on dissemination of results.

### *Selisistat Development Plan*

Running in parallel with the PADDINGTON activities, Siena Biotech SpA has undertaken a European Phase II clinical study in 144 HD patients over 12 weeks to establish safety and tolerability of the compound (Reilmann et al., 2014). In another study, conducted in the US, also running in parallel with PADDINGTON, the interaction of selisistat with food was studied in HD patients over 14 days. Finally, Siena Biotech SpA has completed a comprehensive non-clinical safety package, including repeated dose toxicity studies in two species with duration up to 39 weeks as well as a package of studies to assess the developmental toxicity of the compound. While these parallel activities have established a clinical safety profile for the compound, the design of pivotal efficacy studies requires pharmacodynamic input, and - as per the original plan - emerging data and results from the PADDINGTON project as well as other clinical studies are having a critical impact on next steps of development of the compound.

### *Registrational Scenario*

The registrational efficacy study will use progression of functional decline in HD as measured by the change in the Total Functional Capacity (TFC) vs. baseline as its primary outcome measure, while the safety and tolerability outcomes will include laboratory assessments, vital signs, ECG and type and frequency of adverse events (AEs) to support the initial target label claim of “delay in disability associated with Huntington’s disease”.

*From a regulatory point of view, “a disease-modifying effect will be considered when the pharmacological treatment delays the underlying pathological or pathophysiological disease processes, and when this is accompanied by an improvement of clinical signs and symptoms of the condition. Consequently, a true disease-modifying effect cannot be established conclusively based on clinical outcome data alone and such a clinical effect must be accompanied by strong supportive evidence from a biomarker programme” (CPMP/EWP/553/95 R1).*

It is considered that clinically significant attenuation of TFC can be measured over 24 months; the median decline of TFC per year is approximately one score point (Landwehrmeyer et al., 2007; Huntington Study Group, 2001; Tabrizi et al., 2013) for patients in stages I-III and is thought to represent the best primary endpoint to sustain the proposed claims. Alternative primary outcome measures (such as the Functional Assessment or the Independence Scale) from the UHDRS have been discarded as being less sensitive. Functional ‘time to event’ or slope endpoints are not considered sufficiently explored and validated for use in registrational studies. The main weakness of TFC is that scores may not decline in the same way in all stages and a pronounced ceiling effect is present in patients with a TFC of 13. The proposed study will therefore accept patients with a TFC of 9-12 to increase probability of demonstration of a treatment effect in terms of TFC.

Secondary outcome measures will include the Global Clinical Impression (GCI), Quality of life (QoL), caudate and whole-brain boundary surface integral (BSI) by MRI, circulating levels of soluble mutant huntingtin and transcriptional signatures.

The study is proposed to be conducted at two dose levels vs. placebo and have a randomised, double-blind, parallel group design. The study will accept male and female patients in Stage I and II and is proposed to be conducted across approximately 60 sites in Austria, Belgium, Germany, the United Kingdom, Italy, US and Canada.

It is proposed that a total of 550 patients will be randomised and with an expected drop-out rate of 10% after randomisation, it is expected that 492 subjects (placebo: 196, high-dose: 196 and low-dose: 100) will complete the study. The sample size of 436 intent-to-treat subjects for the high dose and placebo groups (i.e. 218 per treatment group for the primary endpoint) is based on the following power calculation: 392 patients (196 per treatment group) provides 90% power at a Type I error rate of 0.05 to detect a difference in TFC with an assumed treatment effect of 20% between placebo TFC score and selisistat TFC score.

The trial has been designed based on current guidelines for other neurodegenerative conditions (CPMP/EWP/553/95 Rev. 1; CPMP/EWP/563/95 Rev.1) and scientific input from a panel of leading clinical experts in the HD field and takes into account experience from published data from both observational (PADDINGTON, REGISTRY, TRACK-HD) and interventional studies (Landwehrmeyer et al., 2007), especially as regards the sample size calculations for the primary outcome measure, TFC. An early draft of the design was also the subject of a Protocol Assistance/Scientific Advice Session with the SAWP of the EMA CHMP in February 2011.

As outlined above, clinical efficacy in terms of objective benefit requires longitudinal studies, which are costly and associated with a high degree of risk (compare the large number of negative long-term trials for Alzheimer disease over the past three-year period). A consequence of this dearth of suitable short-term clinical read-outs is that traditional dose-finding approaches are inappropriate. In the case of selisistat, a large number of non-clinical HD model systems have suggested beneficial effects over a relatively wide concentration range, and although helpful in establishing a proof-of-principle for the mechanism of action for the compound, they are of limited value in setting dose levels and schedules for human subjects with HD.

The approach taken by Siena Biotech SpA in planning the proposed pivotal efficacy study was therefore to adopt a translational approach whereby identification of suitable dose levels for efficacy testing was based on “pooling” all available data from non-clinical models and clinical trials and using pharmacokinetic data to bridge the various pharmacodynamic responses to a human HD context. In this assessment, the PADDINGTON data and methods regarding circulating mutant huntingtin and the transcriptional signatures were of particular importance and have allowed proposing a dose range for testing of selisistat in the upcoming Phase III study. Furthermore, the data deriving from PADDINGTON have confirmed the suitability of a once-daily dose regimen.

As already mentioned, MRI will play an important role as a secondary outcome measure in the proposed study, especially since the caudate BSI correlates strongly to the primary outcome measure, TFC (Tabrizi et al., 2013). This allows for an adaptive approach, whereby MRI data is used to drive study parameters such as power calculations for the primary outcome measure, time to predefined event and futility analyses. Also in this sense, the findings from PADDINGTON have been instrumental in informing study design and fulfilling the original aims of the project.

In conclusion, the PADDINGTON project has provided essential data and methods for use in upcoming efficacy trials, materially contributing to study design and aiding in mitigating risks associated with dose selection, in line with the PADDINGTON objectives and to the benefit of the HD community.

**Project public website address:** [www.paddingtonproject.eu](http://www.paddingtonproject.eu)

The Consortium comprises five partners based within the EU, all with a proven track-record in Research and Development in the Huntington’s Disease area. Two of these partners are private enterprises (Siena Biotech SpA and KCR SA) while the other three are academic institutions (Universitaet Ulm, University College London and London School of Hygiene and Tropical Medicine). The partners are characterised by their excellence in the field and for the complementarity of their expertise and capabilities to ultimately lead to added value in the clinical development of orphan drugs for HD and specifically for selisistat.

### **Ethics Committees and Institutional Review Boards**

As stated in the technical Annex I, all clinical studies with selisistat, including the clinical studies under WP1 and WP2 in PADDINGTON have been reviewed and approved by Central and/or Local Ethics Committees/ IRBs; all clinical activities will be conducted in accordance with the ICH GCP consolidated guidelines CPMP/ICH/135/95 (July 1996), adopted in the EU by CPMP, the EU Commission Directive 2005/28/EC (GCP, April 2005); the EU Commission Directive 2003/94/EC (GMP, April 2005) and the Manufacture of Investigational Medicinal Products: Volume 4, Annex 13 of the EU Guide to GMP (Revision 1, July 2003).

Local Ethics Committees assisted the partners of PADDINGTON consortium in overseeing activities and ensuring compliance with legal and ethical standards. In order to further guarantee that the

overall implementation of PADDINGTON research activities is in full compliance of the legal and ethical requirements and code of practice, competent ethical advisors have been integrated into the project. Clinical research activities were reviewed by Local or Independent Ethics Committees that had access to Clinical Study Protocols, Informed Consent forms and Investigator Brochure.

Representatives of Siena Biotech, University of Ulm and University College of London have been invited to give presentations to several of these Committees. Throughout the project life time, a total of 30 different Ethics Committees/IRBs across Germany, Italy, Poland, the United Kingdom and the United States have reviewed studies with selisistat.

## 4.1 Use and dissemination of foreground

### Exploitation of the Foreground

#### *Commercial Stakeholders*

Siena Biotech SpA as the holder of Intellectual Property Rights relating to selisistat itself, and by virtue of its size and business model, relies on partnerships with larger pharmaceutical companies for Full Development activities, registrational approval, manufacturing, distribution and sales. In consequence, many interactions of a scientific/technical character with potential commercial partners have occurred during the period covered by the PADDINGTON project with a view to technical/commercial collaborations or licensing of selisistat – these organisations comprise both Large Pharma corporations and companies specialised in Rare Disease or CNS therapeutics - for obvious reasons of confidentiality and respect for third parties, however, details of these interactions cannot be provided in this report. It is however quite clear that there is considerable commercial interest from the Large Pharma community to acquire or license therapeutics for HD – recent drug approvals in the Orphan Disease area have highlighted the opportunities for Premium Price positioning of effective therapeutic entities, and the Orphan sector is one of the Pharma areas with the highest growth rates.

#### *Market Potential of an Approved Therapeutic Entity*

Overall, the value of the whole neurodegenerative market has been estimated to US\$ 7.7 billion, representing the third largest segment of the CNS market and growing at 19% a year (PharmaScope 2011). Clearly, the relatively low prevalence suggests that the HD market represents a minor stake in this market, but since any disease-modifying HD therapy is chronic in nature and the fact that the current standard-of-care cost to the Healthcare Provider is high suggests excellent opportunities for Return of Investment and, depending on the clinical profile of the compound, Premium Pricing positions.

Market projections for disease-modifying therapeutics in HD are by definition conjectural and to a large extent based on estimates regarding Healthcare Provider costs – as outlined above, a compound with a prevention label, allowing use in pre-symptomatic gene carriers would reach on the order of 150,000 patients world-wide, suggesting economic returns well in excess of one billion dollars with full market penetration. Market penetration for an approved therapeutic can be expected to be high and rapid, given the complete lack of therapeutic options.

The current intellectual property position for selisistat provides Orphan Status protection of 7 years following approval of NDA in the United States and 10 years in the EU (in Australia, 5 years of exclusivity is offered and can be extended by successive changes to label language). The patent estate surrounding selisistat provides protection through to September 2032. Line extensions to other rare, neurodegenerative conditions such as spinobulbar muscular dystrophy (Kennedy disease), the

various spinocerebellar ataxias, oculopharyngeal muscular dystrophy (OPMD), would provide additional opportunities for revenue in sectors with similar opportunities for market exclusivity (assuming that Orphan Designations can be achieved based on either preclinical or clinical data. Clinical data may also offer opportunities for additional patent protection). Such indications would therefore not erode the Premium Price position for the compound.

### *Non-Commercial Stakeholders*

The non-commercial stakeholders in the activities covered by the PADDINGTON project include Regulatory Agencies, Ethics Committees, the scientific community, patient organisations and the general public, as well as governmental organisations. Each of these groups have been approached during the life time of the project, as described briefly below:

#### *Regulatory Agencies*

Apart from the formal interactions relating to Clinical Trial Applications (CTAs) across Germany, Italy, Poland, the United Kingdom and the United States, Siena Biotech approached the EMA/SAWP for a formal Protocol Assistance/Scientific Advice session in February 2011. The topics for which Siena Biotech requested feedback regarded the design of the overall development plan, study design, suitability of clinical endpoints, approaches for assessing clinical safety, and – specifically regarding PADDINGTON - the suitability of the pharmacodynamic measures under study in the PADDINGTON project. In a formal response, dated March 9, 2011 (Procedure Number EMEA/H/SA/2031/1/2010/PA/III), the EMA expressed positive opinions about the programme and invited Siena Biotech to return for additional SAWP discussions and to present the outcome of both the PADDINGTON project and the Phase II clinical data.

A US IND was approved by the FDA in September 2011 and has allowed start of clinical trials in the US, and has also paved the way for concerted EMA/FDA Protocol Assistance/Scientific Advice interactions regarding the design of registrational clinical trials with selisistat.

The EU and US Orphan Designations were already approved at the start of the PADDINGTON project (indeed, this was a requirement of the call), while additional Orphan Designation was sought and granted by the Australian Department of Health and Ageing/Therapeutic Goods Administration on March 11, 2011. With the exception of Canada (that does not have a formal Orphan Designation process), these Orphan Designations therefore cover the majority of the territories where HD shows high prevalence.

Siena Biotech and University of Ulm have also been active in interactions at the national level, participating to meetings regarding Rare Diseases organised by the EMA, the FDA, the European Commission, the Italian Medicines Regulatory Agency (AIFA) and the Italian National Institutes of Health (ISS).

#### *Outreach to the Patient Community*

While Siena Biotech SpA as the selisistat Sponsor tends to avoid direct contacts with individual patients for ethical and privacy reasons, several direct and indirect contacts with national patient organisations (Siena, October 2011, Birmingham, April 2012) have occurred during the project life time. The most important interaction with the HD community however, is *via* the European Huntington's Disease Network (EHDN; [www.euro-hd.net/](http://www.euro-hd.net/)), where the PADDINGTON consortium is not only represented by membership of two of the Consortium Partners, but also *via* participation

to meetings (Plenary Meeting, Stockholm, September 2012) as well as *via* the official “endorsement procedure” and the regular EHDN Newsletters that reach a high proportion of families with HD in Europe. Through its capillary structure, the EHDN is present in all EU countries and is in constant contact with local patient organisations, legislators, regulators and Healthcare providers, ensuring maximal diffusion of information about HD, selisistat and the PADDINGTON project. Details of the clinical trials conducted with selisistat have been published on the NIH/FDA website (<http://clinicaltrials.gov/>) and the European Clinical Trials Registry (<https://www.clinicaltrialsregister.eu/>), maintained by the EMA. In addition, the websites managed by the Hereditary Disease Foundation (HDF; [www.hdfoundation.org](http://www.hdfoundation.org)), Huntington’s Disease Society of America (HDSA; [www.hdsa.org](http://www.hdsa.org)) and the HD Lighthouse site ([www.hdlf.org](http://www.hdlf.org)) have all featured articles on selisistat and PADDINGTON, as has also the HD Insights™ newsletter, managed by the Huntington Study Group (HSG) – effectively, therefore, the PADDINGTON Consortium has created links to all major HD organisations worldwide. In addition to the above categories, a wide variety of newspaper articles, press releases and Open House activities have brought elements of the PADDINGTON project to the general public – due to the complex scientific nature of the topics addressed in PADDINGTON, the level of detail in these communications has necessarily been limited in order to suit a lay public.

### **Awareness and Wider Societal Implications**

Huntington’s disease not only affects patients but in a profound way also immediate care givers (family, spouse, colleagues) and society as a whole on account of the significant Healthcare provider costs associated with caring for patients in advanced stages of disease.

The PADDINGTON project forms part of a larger effort, *i.e.* the overall clinical development plan for selisistat for HD, and the associated communication plan. As such, communication efforts relating to selisistat may not necessarily be specific to the PADDINGTON project but have a wider scope, to the benefit of visibility for the disease, the compound and the PADDINGTON partners. As already mentioned above, the PADDINGTON findings supports development of pharmacodynamic markers for disease progression that would have applicability also for other therapeutics and PADDINGTON therefore contributes value beyond the development of selisistat.

Although the start of the PADDINGTON project preceded the launch of the IRDiRC, the objectives of PADDINGTON are very much in line with the vision set out in the IRDiRC Guidelines and Policies, as illustrated by the following highlights:

- The entire development programme for selisistat (within and outside the scope of the PADDINGTON project) has been shaped in a collaborative vein and involves an open communication with a large proportion of the HD community;
- Resources, data and results are being shared across the organisations involved in an open manner and all efforts are made to publish results both at scientific and community meetings as well as in peer-reviewed scientific journals;
- Patient databases (managed by the investigator community via efforts like REGISTRY, PREDICT etc) are being made available and data shared freely among consortium members; similarly, a central, pan-European biobanking effort is under way, to which PADDINGTON is contributing and also deriving benefit from;
- All PADDINGTON activities have been conducted in strict adherence to global, national and local legislation and regulations as regards data protection and ethics, also in reference to electronic data records and clinical data capture systems;

- The biomarker development approach in PADDINGTON, will contribute value to the entire HD field, adding new element to the toolbox, as well as complementing existing initiatives, such as natural history studies like REGISTRY and TRACK-HD;
- The biomarker approaches taken in PADDINGTON have been discussed with the EMA in a SAWP Protocol Assistance/Scientific Advice session.

In terms of general awareness of HD, the dissemination activities aimed at achieving increased visibility also at the Healthcare provider level. A specific example with direct impact at the legislative level was the celebration of the 102<sup>nd</sup> birthday of Rita Levi Montalcini, Nobel Laureate and Senator for Life in the Upper House of the Italian Parliament - an event transmitted both via television and webcasts worldwide and where the PADDINGTON coordinator Giovanna Tripepi represented the PADDINGTON project. Similarly, Siena Biotech is a member of Assobiotec, a branch organisation aimed at leveraging opinion leader impact on Healthcare policies and research and development in the Life Sciences.

Perhaps the most important channel to achieving general societal awareness of HD is the European Huntington's Disease Network (EHDN), whose active role in the PADDINGTON project was instrumental to the success of both Work Package 1 and Work Package 2. The EHDN provides a platform for professionals and people affected by HD and their relatives to facilitate working together throughout Europe. The Network provides an infrastructure for large scale clinical trials on HD throughout Europe, an IT platform for communication tools (in the respective native languages) and e-trials, a forum for close cooperation of basic scientists and clinicians and a low threshold (native language) support for study sites by language group coordinators. The EHDN organises both healthcare professionals and representatives of the various patient organisations and patient advocacy groups, and – aside from Working Groups addressing the medical and scientific aspects of the disease (*e.g.* biomarkers, cognition, physiotherapy, Quality of Life, surgical approaches, genetic testing etc), the EHDN is also actively involved in Health Economics research and participates to EMA discussions regarding policies for clinical studies and endpoints in HD. Representatives of the EHDN are important opinion leaders also in their local, national context and often invited to present at meetings, in newspapers and television regarding HD and its impact on patients and their families. The EHDN officially endorsed the PADDINGTON WP1 clinical study, and two of the Partners (University of Ulm and University College of London) are active members of the EHDN. All of the clinical study sites in both WP1 and WP2 are also members of the EHDN.

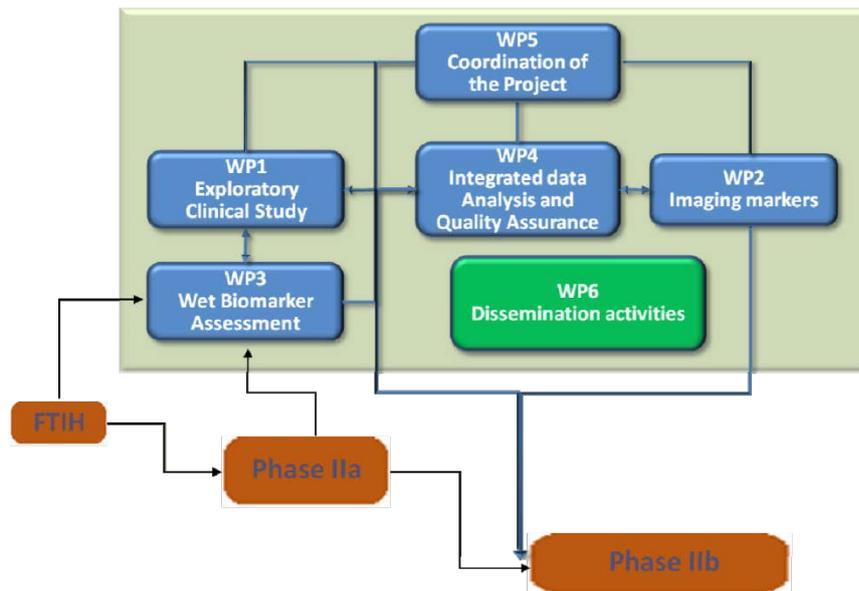
Throughout the PADDINGTON project life time, Siena Biotech has been involved in a large number of meetings at the national and international levels to create visibility for PADDINGTON and selisistat, and has given numerous presentations and posters at meetings with patient organisations, the Italian Medicines Regulatory Agency (AIFA), the National Institutes of Health (ISS) as well as the branch organisations (Confindustria and AssoBiotec) at the national Italian level and internationally, via the EHDN, the UK Site Investigators organisation, the CHDI Foundation Inc. and regulatory agencies like the EMA and the FDA. Several web sites provide up-to-date coverage of selisistat clinical studies (*e.g.* the US-based (FDA and NIH-controlled) Clinicaltrials.gov, the HD Lighthouse, the Hereditary Disease Foundation and the Huntington's Disease Society of America, some of which also include patients self-reported accounts of participation in selisistat clinical trials.

PADDINGTON project has been selected by a media company, through an EC project aimed at informing the general public and the press on the European Community funded studies with impact on health or other important issues. The movie, produced by Leonardo Film, DE with the running title: Fighting Huntington's Disease was shot at Siena Biotech premise and at the UCL hospital in London. The final movie, entitled Advances in Treating Huntington's Disease, merging

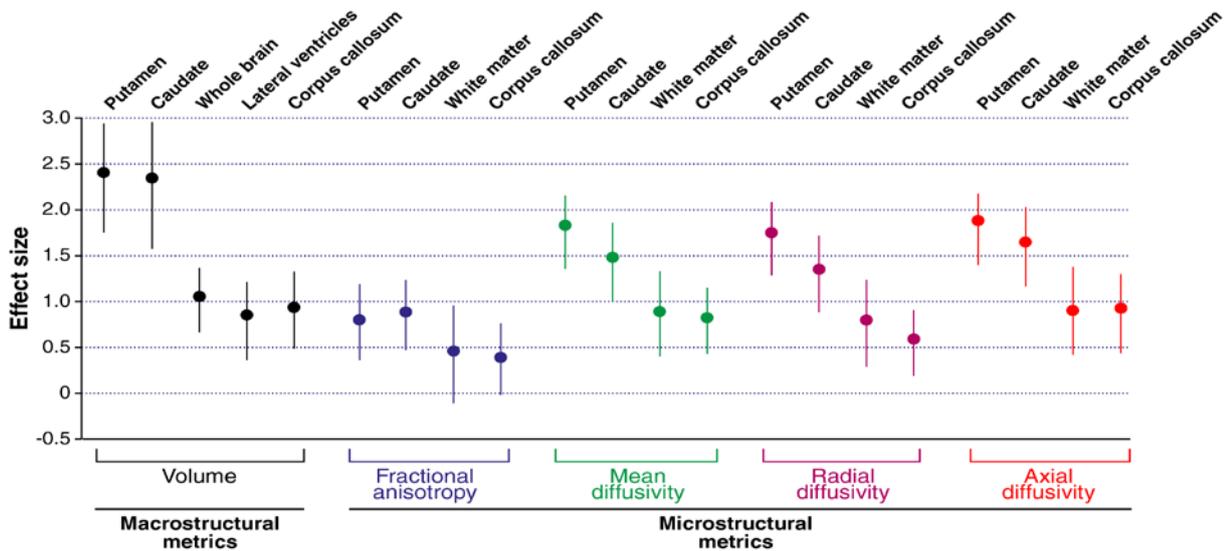
PADDINGTON and another European projects on the same topic, has been broadcasted by Youris.com: (<http://www.youris.com/Health/Genetics/Advances-In-Treating-HuntingtonS-Disease.kl>).

**Project public website address:** [www.paddingtonproject.eu](http://www.paddingtonproject.eu)

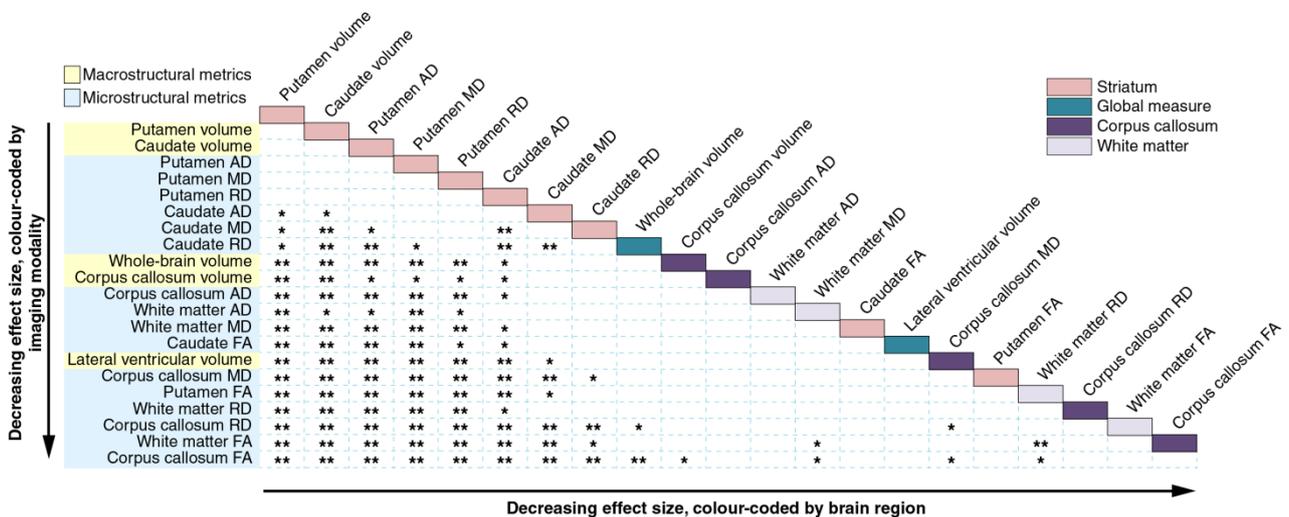




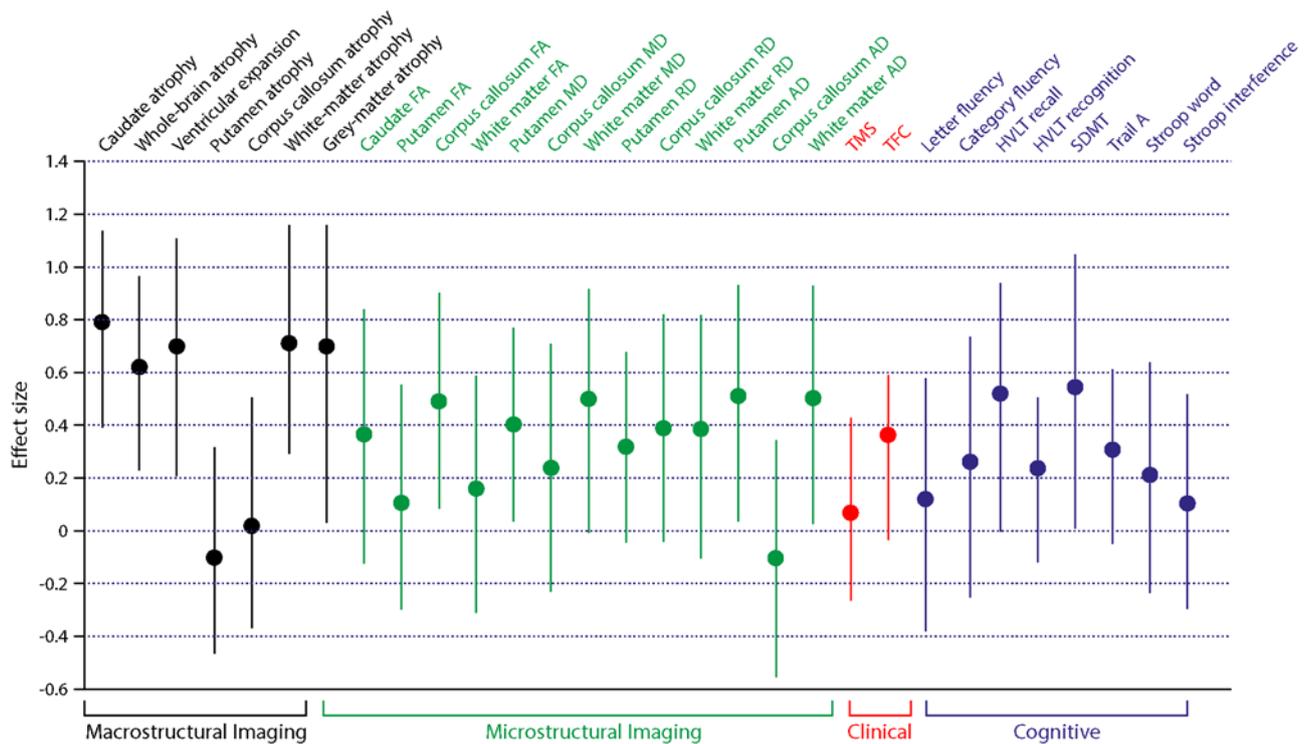
**Figure 1.** This work supports the development of a series of pharmacodynamic markers tentatively identified on the basis of both target modulation, such as the measurement of the acetylation status of mutant HTT which is specific to the selisistat mechanism of action, and disease-specific approaches, such as MRI parameters, immune markers and cholesterol metabolites. Methods development is pursued in parallel, and results are evaluated and integrated with the aim of establishing not only technically validated pharmacodynamic tools and methods, but also validated composite statistically significant measures for selisistat Phase II/III studies.



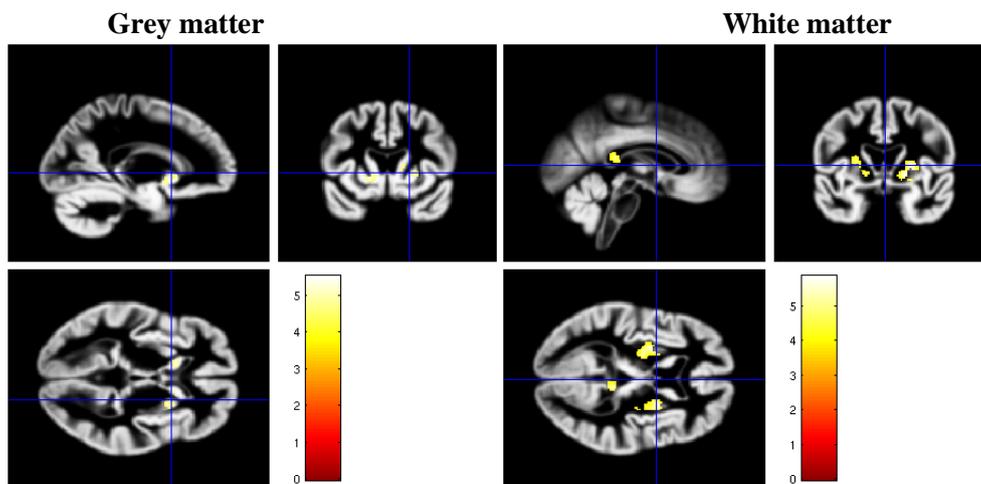
**Figure 2A.** Effect-size estimates (mean and 95% CIs) for each of the imaging outcomes. Data are grouped by imaging metric and modality.



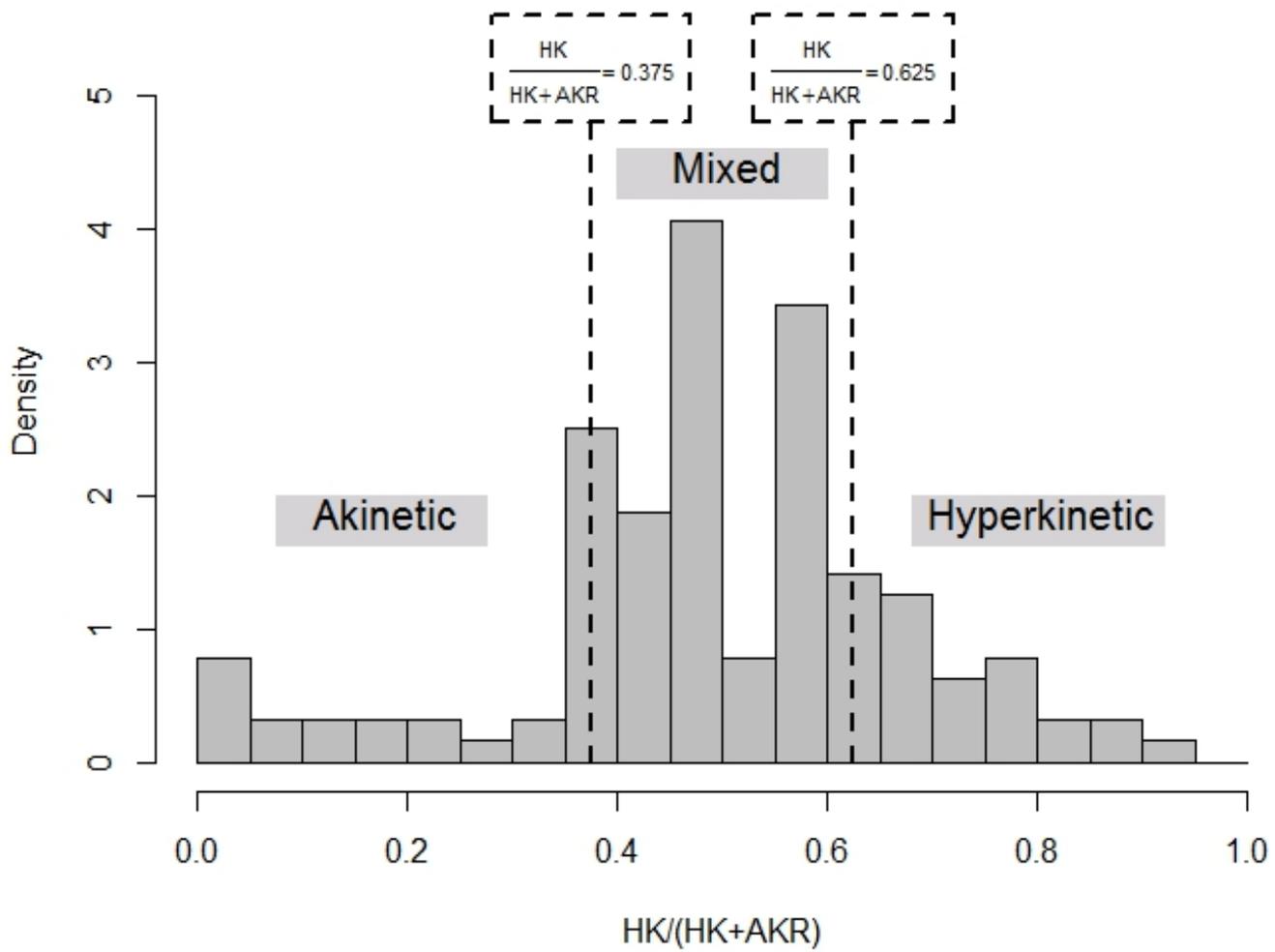
**Figure 2B.** Statistical comparison of effect sizes between imaging metrics and modalities (\*/\*\* indicates  $p < 0.05/0.01$ ). Effect sizes are calculated as the absolute difference in the mean of the metric between groups, adjusted for age, gender and study site, divided by the estimated residual standard deviation of the HD group. \* & \*\* denote a statistically significant difference at  $p < 0.05$  and  $p < 0.01$  respectively, in the magnitude of the effect sizes for each pair of metrics. For example, although putamen volume had the largest effect size overall (top of the list), this was not significantly larger than that for caudate volume, putamen AD, MD or RD (absence of \* in adjacent boxes), however it was significantly larger than that for caudate AD, MD and RD at  $p < 0.05$  (\* in each adjacent boxes), and also significantly larger than that for all other metrics at  $p < 0.01$  (\*\* in adjacent boxes). Metrics are ordered by magnitude of effect sizes and colour-coded by imaging modality (left-hand side) and brain region (right-hand side).



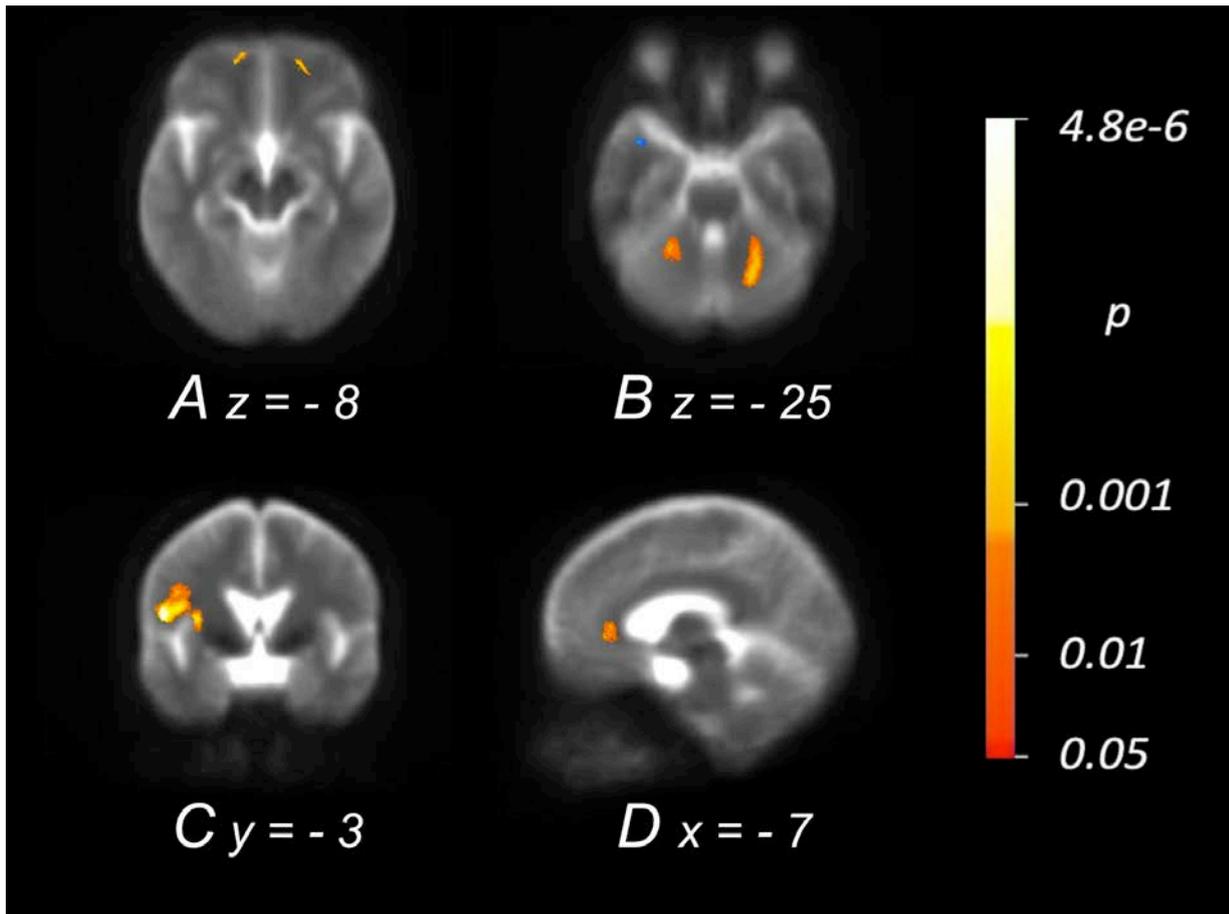
**Figure 3.** 6-month effect-size estimates for neuroimaging, clinical and cognitive outcomes. Data are presented as Mean and 95% Confidence Intervals. Verbal fluency tasks (letter and category) test aspects of language and executive retrieval functions. Hopkin’s Verbal Learning Test (HVLT) is a memory task. Symbol Digit Modalities Test (SDMT) is a test of visuomotor integration, measuring visual attention and motor speed. Stroop tests (colour naming, word reading and interference) are tests of processing speed and cognitive control/conflict monitoring and resolution. Trail Making Test A is a visual attention and task switching task. Total Motor Score (TMS) measures a range of motor features characteristically impaired in HD. Higher scores indicate more severe motor impairment than lower scores. Total Functional Capacity (TFC) measures five components of daily living. Higher TFC scores indicate better functioning than lower scores. FA is fractional anisotropy. FA relates to fibre coherence/directionality of the tissue microstructure; it is highest in highly organised white matter and is thought to decrease with loss of tissue organisation or cellular integrity. MD, RD and AD are mean-, radial- and axial-diffusivity, respectively. RD and AD measure the rate of diffusion of water molecules perpendicular to and parallel to the main direction of diffusion, respectively. Increases in RD are thought to be associated with demyelination of white-matter tracts, whereas abnormalities in AD may be more reflective of axonal degeneration and loss.



**Figure 4.** Statistical parametric maps showing t-scores of significant ( $p < 0.05$ ) between-group differences in change over 6-months in the GM (left) and WM (right) adjusted for age, gender, site and scan interval. Results are overlaid on an average group template and controlled for multiple comparisons using false discovery rate correction



**Figure 5.** Histogram of Hyperkinetic Score/Hyperkinetic + Akinetic Rigid Score ( $HK/(HK+AKR)$ ), with percentile based division into motor phenotype groupings. controlled for multiple comparisons using false discovery rate correction



**Figure 6.** Brain maps depicting FA differences between the 'non-depression' and 'sub-threshold depression' HD group. In the 'sub-threshold depression' group FA was lower in the left and right ventromedial frontal cortex (A), in the left and right cerebellum (B) in the insula (C), and in the anterior cingulate cortex (D). Figures are shown in neurological convention.

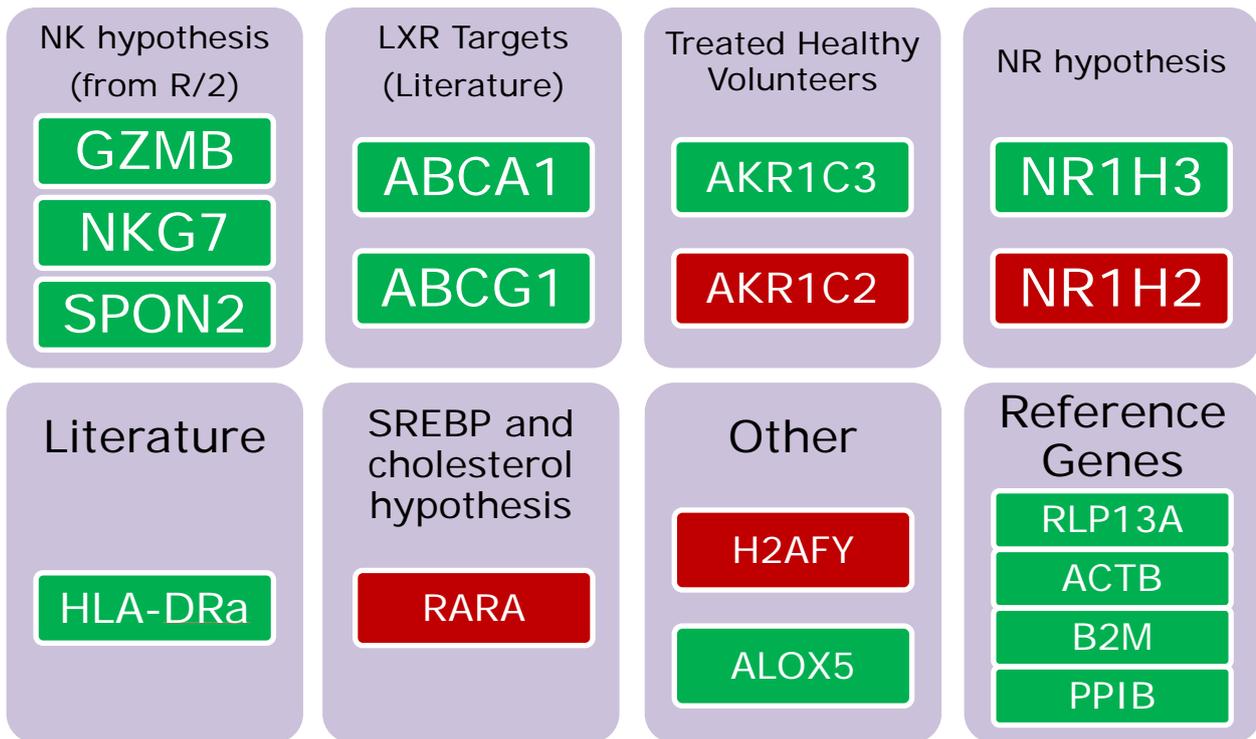
Group Characteristics

<b>Group</b>	<b>Age V1</b>	<b>Number (M:F)</b>	<b>CAG</b>	<b>Disease Burden</b>	<b>SDMT correct</b>	<b>Stroop Word Reading Correct</b>	<b>TMS</b>
<b>Akinetic</b>		25 (17:8)					
Mean	45.9		43.5	353.4	32.9	74.7	21.8
SD	9.3		2.2	69.7	11.4	21.8	9.7
<b>Minimal motor</b>		17 (11:6)					
Mean	45		43.6	332.8	45.2	95.2	8.1
SD	11.4		3.3	50.7	12.8	9.8	1.8
<b>Mixed motor</b>		78 (42:36)					
Mean	48.5		44.0	382.7	31.0	73.9	26.6
SD	10.4		3.49	79.1	9.0	18.7	9.6
<b>Hyperkinetic</b>		25 (11:14)					
Mean	49.6		43.9	389.4	34.1	80.1	24.5
SD	10.1		2.9	63.2	8.9	17.1	9.9

Table 1. Initial Motor Phenotype Grouping Characteristics. V1=Visit 1. SDMT=Symbol Digit Modality Test.

	No. of subjects		Age (years)		Gender (m / f)		Education (years)	
	Ctrl	HD	Ctrl	HD	Ctrl	HD	Ctrl	HD
Centre A	10	17	50 ± 4	48 ± 10	6 / 4	3 / 14	15 ± 3	14 ± 3
Centre B	10	16	53 ± 8	52 ± 9	5 / 5	5 / 11	15 ± 4	14 ± 3
Centre C	10	13	57 ± 8	46 ± 15	3 / 7	7 / 6	13 ± 3	12 ± 3
Centre D	10	15	48 ± 9	48 ± 10	4 / 6	11 / 4	15 ± 3	12 ± 3
All	40	61	52 ± 8	49 ± 11	18 / 22	26 / 35	14 ± 3	13 ± 3

Table 2: Demographic parameters for MRI scans. Ctrl = controls.



**Table 3:** The full set of genes validated by real-time PCR. In green the genes analysed in Paddington clinical samples and the four reference genes used for normalization.