

Leptin deficiency reverses high metabolic state and weight loss without affecting central pathology in the R6/2 mouse model of Huntington's disease

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Abstract

Body weight has been shown to be a predictor of clinical progression in Huntington's disease (HD). Alongside widespread neuronal pathology, both HD patients and the R6/2 mouse models of HD exhibit weight loss and increased energy expenditure, providing a rationale for targeting whole-body energy metabolism in HD. Leptin-deficient mice display low energy expenditure and increased body weight. We therefore hypothesized that normalizing energy metabolism in R6/2 mice, utilizing leptin-deficiency, would lead to a slower disease progression in the R6/2 mouse.

In this study, we show that R6/2 mice on a leptin-deficient genetic background displayed increased body weight and increased fat mass compared with R6/2 mice, as well as wild type mice. The increased body weight was accompanied by low energy expenditure, illustrated by a reduction in respiratory exchange rate. Leptin-deficient R6/2 mice had large white adipocytes with white adipocyte gene expression characteristics, in contrast to white adipose tissue in R6/2 mice, where white adipose tissue showed signs of browning. Leptin-deficient R6/2 mice exhibited some signs of improved behaviour; however, this was not accompanied by improved neuropathological measures.

Our results indicate that lowering energy metabolism in HD, by increasing fat mass and reducing respiratory exchange rate, is not sufficient to improve neuropathological changes. Further studies targeting energy metabolism in HD are warranted.

Keywords: Leptin-deficiency; Energy metabolism; Respiratory exchange rate; Adipose tissue; Repetitive behaviour; Huntington's disease

1. Introduction

Huntington's disease (HD) is associated with progressive motor, cognitive and functional decline [1, 2], symptoms that are caused by an expanded CAG trinucleotide repeat in HTT, the gene encoding the protein huntingtin [3-5]. The mutation causes the protein to gain toxic functions and aggregate, resulting in neuronal dysfunction and death [5, 6]. Although substantial progress has been made in understanding mechanisms underlying HD pathology, this is not fully understood.

Accumulating evidence suggests altered energy metabolism and a hyper-catabolic state as key features of HD pathology [7-9]. Body weight has been shown to be a predictor of clinical HD progression and higher BMI is demonstrated to be associated with a slower rate of functional, cognitive and motor decline [10]. Studies have investigated the potential therapeutic impact of dietary manipulations in HD and report various beneficial effects [11-13]. However, it is not known whether dietary manipulations alter the hypermetabolic state in HD.

The R6/2 mouse model of HD mirrors human HD and, in addition to central pathological changes [6], the mice exhibit progressive weight loss, altered body composition and increased oxygen consumption [14-16]. Hyperplasia of brown/beige cells in white adipose tissue (WAT) has been shown to enhance energy expenditure [17] and browning of R6/2 mouse WAT has been shown to be linked to increased energy metabolism, possibly contributing to HD weight loss [18].

In amyotrophic lateral sclerosis (ALS), an association between high energy metabolism and clinical progression has been demonstrated [19] and different ways to change metabolic state have been investigated as disease modification strategies [20-22].

Ob/Ob mice are leptin-deficient; they exhibit increased food intake and have a slower energy metabolism leading to increased body weight and fat mass [23-25]. Notably, ALS mice on a leptin-deficient genetic background have been shown to have normalized energy expenditure, improved motor function, decreased motor neuronal loss, as well as enhanced survival, suggesting a slower rate of the disease progression [21].

In this study, we therefore aimed to investigate whether an altered energy metabolism and increased body weight can affect R6/2 mouse disease measures. We used a genetic approach and crossed the R6/2 mouse with leptin-deficient Ob/Ob mice and investigated central and peripheral parameters as well as behaviour and energy metabolism measures.

We can here demonstrate that it is possible to dramatically increase R6/2 mouse body weight by changing energy metabolism. Although we find improved behaviour, this was not associated with improved neuropathological measures.

2. Materials and Methods

2.1 Ethics

Animal experiments were carried out in strict accordance with Swedish legislation and approved by local and national regulatory authorities (permit number: 2505/2018), (Animal Ethics Committee in Lund and Malmö, Sweden).

2.2 Animals

A two-step breeding strategy was used to generate leptin-deficient R6/2 mice (R6/2;Ob/Ob) (see Supplementary Figure 1). Females heterozygous for the obese gene (Ob-/+) on the C57BL/6J genetic background (Strain #000632, Jackson Laboratory, Bar Harbor, ME, USA) were crossed with males heterozygote for the R6/2 gene on the C57BL/6xCBA genetic background (Jackson Laboratory, Bar Harbor, ME, USA). Mutant R6/2;Ob-/+ males from the F1 generation were crossed with Ob-/+ females to generate the F2 generation, containing WT, R6/2, Ob-/+ , Ob/Ob, R6/2;Ob-/+ and R6/2;Ob/Ob littermates. R6/2 mice with the mutant leptin heterozygote and homozygote genetic backgrounds are denoted as R6/2;Ob-/+ and R6/2;Ob/Ob, respectively. Non-transgenic mice are denoted as wild type (WT), Ob-/+ (mutant leptin heterozygote) and Ob/Ob (mutant leptin homozygote) (Supplementary Figure 1). Generated genotype frequencies for generation 1 was 25% for each genotype, while for generation 2, the generated genotype frequency was 25% for Ob-/+ and R6/2;Ob-/+ and 12.5% for WT, R6/2, R6/2;Ob/Ob and Ob/Ob. For genotyping, ear punch samples were taken at 4 weeks of age and genotyped for the R6/2 and Ob gene using PCR. The PCR product (Ob) was cleaved with HpyF3I restriction enzyme (Thermo Scientific, Rockford, IL, USA). PCR products were separated by gel electrophoresis using a 3% agarose gel for detecting the Ob gene, and 2% for the R6/2 gene. All primers were purchased from MWG Eurofins (Eurofins MWG Synthesis GmbH, Germany), and sequences of all primers used for genotyping in this study are found in Supplementary Table 1. Tail tips were sent to Laragen (Laragen Inc., CA, USA) for CAG repeat length determination. The CAG-repeat lengths of the R6/2, R6/2;Ob-/+ and R6/2;Ob/Ob mice from two different strains used in this study ranged either between 345–352 or 242-257. The CAG repeat size of our colony, results in a disease progression slower than that of the R6/2 mouse with 150 CAG repeats, as described by Morton and co-workers in 2009 [26].

The mice were housed in groups of 2-5 in universal Innocage mouse cages (InnoVive, San Diego, CA, USA) under standard conditions (12 h light/dark cycle, 22°C), and with *ad libitum* access to chow food, water and cages were enriched with nesting material.

At end-point, mice were first anesthetized with pentobarbital (Apoteksbolaget, Lund, Sweden) during blood collection, and then euthanized and tissue samples; brain (cortex, striatum), intra-abdominal epididymal adipose tissue, interscapular brown adipose tissue, skeletal muscle (gastrocnemius), were dissected and snap-frozen in liquid nitrogen and stored at -80 °C until further use.

Blood samples were collected at the time of sacrifice, from the right heart ventricle using a wide-bore needle (18 G/40 mm), to prevent applying large suction force. Blood samples were stored at room temperature for 30 minutes and then centrifuged at ~2500 × g for 15 minutes to obtain serum.

Cerebrospinal fluid (CSF) samples were collected from 4th ventricles under terminal dose of pentobarbital anaesthesia. The heads of the mice were mounted on a stereotaxic frame, and the skin and muscle tissue were dissected to expose the dura mater above the cisterna magna. CSF samples were collected using glass capillaries (outer diameter = 80 µm) connected to 1 ml syringe through polyethylene tubing. CSF and serum were snap-frozen in liquid nitrogen and stored at -80 °C until further use.

2.3 Serum and blood analyses

Serum glucose levels were measured using the glucose oxidase method (Thermo Trace, Victoria, Australia), and levels of insulin were determined by radioimmunoassay (RIA; Linco Research Inc., St. Louis, MO, USA), both according to manufacturer's protocols and samples assessed in duplicates. The homeostatic model assessment (HOMA) was calculated to measure insulin resistance (IR) and pancreatic beta-cell function (β). HOMA-IR was calculated as follows: $(\text{Insulin} \times \text{Glucose})/22.5$, and HOMA- β as follows: $(20 \times \text{Insulin})/(\text{Glucose}-3.5)$ [27]. Serum total, HDL and LDL/VLDL cholesterol levels were measured using HDL and LDL/VLDL Quantification Colorimetric/Fluorometric Kit (BioVision, Milpitas, CA, USA) according to manufacturer's protocol and samples assessed in duplicates. The ratio between LDL/VLDL and HDL was calculated.

2.4 Body weight and composition measurements

Body weight was assessed twice a week from the age of 6 weeks. Body composition (fat and lean mass) was measured using the Lunar Prodigy dual-energy x-ray absorptiometry (DEXA; GE Lunar Corp., Madison, WI) in isoflurane-anesthetized (Apoteksbolaget, Lund, Sweden) mice.

2.5 Indirect gas calorimetry

The animals were habituated to the drinking bottles one-day prior acclimatization. Whole-body energy metabolism, locomotor activity, and food and water intake were measured using the PhenoMaster Home Cage System (TSE-systems, Bad Homburg, Germany). Animals were weighed, single-housed and acclimatized to the calorimeter chambers for one day prior to the experiments. Food and water were available *ad libitum*. All parameters were recorded every 15 min for 24 hrs, and reported as light phase, dark phase and 24 hours. Metabolic rates were calculated using a correction for body weight.

2.6 Adipose tissue histology

Intra-abdominal epididymal white (WAT) and interscapular brown (BAT) adipose tissue from males at 20 weeks were dissected, placed into 4% paraformaldehyde in phosphate buffered saline (0.01 M) overnight. Tissues were then transferred to 70% ethanol solution and stored at 4°C before paraffin embedding. Sections (7 µm) were mounted on glass slides and stained according to Mayer's Hematoxylin-Eosin staining protocol. Digital images of the stained sections were used to identify the morphological features of WAT and BAT, and adipocytes (>150 cells) of WAT from each mouse were selected for area measurements at 20x magnification. Cell areas were measured using cellSens Dimensions 1.11 software (Olympus U-HSCBM, Olympus, Tokyo, Japan). Slides were coded, and the operator was unaware of the genotype of each sample during analyses for blinded measuring. Mean adipocyte (WAT) areas for each group were then compared. A bright-light microscope (Olympus U-HSCBM, Olympus, Tokyo, Japan) with a 20x magnification objective, digital camera and image capture software (cellSens Dimensions 1.11 software; Olympus, Tokyo, Japan) were used.

2.7 CSF analyses

CSF neurofilament light chain (NfL) concentration was assessed as previously described using the in-house Simoa NfL assay [28]. The repeatability of two quality control samples was 6.8

CV% (coefficient of variation) for a sample with a mean NfL concentration of 87.2 pg/ml, and 4.7 CV% for a sample with a mean NfL concentration of 11.3 pg/ml. Determination of the lower limit of quantification has been described previously [29].

2.8 Immunohistochemistry

Mice were perfused transcardially under terminal sodium pentobarbital anesthesia (Apoteksbolaget, Lund, Sweden) with saline and subsequently with ice-cold 4% paraformaldehyde (PFA) for 10 min (at the rate of 10 ml/min). Brains were extracted and placed in 4% PFA solution for 24 hours at 4 °C for post-fixation, then transferred to 30% sucrose solution complemented with sodium azide at 4 °C until use. Brains were sectioned in the coronal plane (30 µm sections, six series per animal) on dry ice and the sections were stored at -20 °C in an antifreeze solution (30% glycerol, 30% ethylene glycol in phosphate buffer). Free-floating brain sections were rinsed with 0.01M phosphate buffered saline (PBS) 3 times for 10 minutes. Next, the sections were incubated in 0.01M citrate buffer, pH 8.5 at 80°C for 40 minutes for the antigen retrieval. Sections were left in PBS containing 3% H₂O₂ and 10% methanol for quenching. The sections were pre-incubated for 1hr at room temperature (RT) with blocking solution containing 5% normal donkey serum and 1% bovine serum albumin (BSA) in 0.3% Triton X/PBS, followed by incubation with primary antibody (EM48 Millipore MAP 5374; mouse 1:400 dilution, EMD Millipore, Burlington, MA, USA) in blocking solution overnight on a shaker at 4°C. The sections were washed 3 times for 10 minutes in PBS then incubated with secondary (biotinylated horse anti-mouse; 1:200 dilutions) antibody. Sections were rinsed with PBS and incubated with the avidin-biotin-peroxidase complex solution (VECASTAIN ABC Kit; Elite PK 6100 – Standard, Vector Laboratories Inc., Burlingame, CA USA) for 1h at RT. The sections were rinsed with PBS 3x10min and then lastly incubated with 3'3 – Diaminobenzidine (DAB) and H₂O₂ (DAB Kit SK-4100, Vector Laboratories Inc., Burlingame, CA USA) to visualize the aggregates. Finally, the sections are mounted on gelatine covered slides, dried overnight and dehydrated through a graded alcohol series to xylene and coverslipped with DPX mountant (Sigma Aldrich, Saint Louis, MO, USA).

For the assessment of striatal volume, cortex and corpus callosum thickness, cresyl violet (Nissl) staining was performed. First, free-floating brain sections were rinsed 3 times for 10 minutes with TBS to remove the antifreeze solution. The brain sections were mounted on gelatin-coated glass slides and air-dried at room temperature. The slides were passed through

xylene and a series of decreasing ethanol solutions for 1 minute each (100%, 95%, 70% ethanol). After dehydrating in distilled water for 1 minute, the sections were left in 0.5% cresyl violet solution for 1 minute. The sections were dehydrated in increasing ethanol solutions (70%, 95%, 100%) and cleared in xylene for 5 minutes. Lastly, the glass slides were covered using DPX (Sigma-Aldrich, Saint Louis, MO, USA).

2.9 Stereological assessment

The experiments were carried out under the blinded conditions to genotypes. The volume of striatum, cortex and corpus callosum thickness were assessed on cresyl violet stained coronal sections in a blinded manner to genotypes. First, the cresyl violet stained brain sections were scanned using an automated digital microscope (Zeiss, Smartzoom xxxxxx). Next, using Zeiss Zen (Blue edition) software, dorsal striatum volume (bregma 1.70 mm to -0.26 mm), medial corpus callosum thickness (bregma 1.70 mm to -0.82 mm), and insular cortex Bregma (1.54 mm to 0.62 mm) were measured [30].

Quantification for mHTT inclusions in the striatum and cortex were implemented on brain sections stained for EM48 antibody using DAB immunohistochemistry. Using Leica 25x oil immersion objective (Leica, Germany) a series of z-stacks were collected. For the quantification of striatal inclusions, a total of 16 z-stack images collected per animal (4 Z-stack images from 4 consecutive brain sections between the bregma levels of anterior-posterior: 1.34 mm to 0.50 mm). The cortical inclusion quantifications were performed on the insular cortex and the sampling was performed between the bregma levels of anterior-posterior: 1.54 mm to 0.62 mm and 4 z-stack images were collected per animal (1 z-stack image from 4 consecutive sections of insular cortex). The z-stack images were processed using ImageJ/Fiji (National Institutes of Health, USA). Z-stack images were processed into two Z-projections; one with minimum intensity (converted to a reversed image) and the other with standard deviation. The two projections were processed into one image for analysis (Image Calculator) and the threshold was adjusted to automatic processing. The images were then analyzed by particle size (pixel size minimum of 4 to infinity, though unspecific signals [i.e. dirt or small blood vessels] were manually excluded, and circularity of 0-1.0).

2.10 RNA extraction and cDNA synthesis

Total RNA was extracted from skeletal muscle gastrocnemius, adipose tissue and brain cortical tissue, using the E.Z.N.A. Total RNA Kit II (Omega bio-tek, Norcross, GA, USA) before complementary DNA (cDNA) was synthesized using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA), according to manufacturer's protocol. RNA concentration and purity were measured by a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

2.11 Real-time quantitative PCR

SsoAdvanced Universal SYBR Green Supermix from Bio-Rad Laboratories was used for RT-qPCR and performed following manufacturer's protocol. All RT-qPCR plates were run on a CFX96 touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Primers utilized for RT-qPCR validations were designed using either QuantPrime69 or PrimerQuest from Integrated DNA Technologies (<http://eu.idtdna.com/PrimerQuest>). The efficiency of each primer pair was tested before use by performing a standard curve, and the efficiency criteria for using a primer pair was 90%<E<110%, with an R² cut-off >0.990. Housekeeping genes, Peptidylprolyl isomerase A (*Ppia*) and TATA Box Binding Protein (*Tbp*) were used for normalization of white adipose tissue (WAT) RT-qPCR, and *Tbp* and 18S ribosomal RNA (*18S*) were used for normalization of skeletal muscle (gastrocnemius) RT-qPCR (Supplementary Table 2). Changes in gene expression were calculated using the CFX manager software program (Bio-Rad, Hercules, CA, USA), using the $\Delta\Delta C_t$ method with a fold change cut-off at ≥ 1.5 and $p < 0.05$ considered significant. All samples were run in triplicate and relevant positive and negative controls were run on each plate.

2.12 Behavioural testing

Mice were handled prior to testing. Mice were transported in their home cages to the behavioural testing room and were allowed to acclimate to the room for at least one hour before testing.

Open Field Behaviour Testing

Open field behaviour testing is a common measure of general activity and exploratory behaviour in rodents [31], which has been shown to be affected in the HD mouse model R6/2 [32]. Motor activity (distance travelled) was evaluated in an additional group of mice in males at 19 weeks and females at 18 weeks during dark cycle in an open field arena with non-

transparent open-topped Plexiglas boxes of 50 x 50 cm, using the Stoelting ANY-MAZE video tracking system (Dublin, Ireland), detecting position of the animal's head, body and tail. Mice were placed individually in the corner of the open field arena and were recorded for a 60-min period. Data was collected for every 5 minutes.

Nest building behavioural testing

Nest building is a natural and complex behaviour of mice, requiring fine motoric skills for pulling, carrying, and bedding of nest material, as well as the cognitive function for planning and problem solving, and an easy way of monitoring disease progression in neurodegenerative diseases [33, 34]. Nesting ability has been shown to be negatively affected in neurodegenerative diseases such as Alzheimer's disease [35], Parkinson's disease [36] and Huntington's disease [37]. Nest building behaviour was assessed in males at 19 weeks, and in females at 18 weeks. Mice were single caged overnight, with access to food and water, but with no environmental enrichment. Approximately 1.5 g of nesting material was placed in the cage, and torn material was weighed the next morning. Nesting ability was assessed on a point scale as previously described [33], from 1 to 5, analysing both touched nesting material and shape of the nest. A score 1 was given when the nestlet was mainly untouched (>90% was intact), score 3 was given when the nestlet was mostly shredded but no identifiable nest site was seen, and score 5 was given to an almost perfect nest, where >90% of the nestlet was torn up into a crater where the walls were higher than the body height. Mean score \pm SEM of each group was determined.

Marble burying behavioural testing

Aberrant marble burying behaviour is a sign of anxiety and repetitive behaviour in rodents [34]. Marble burying test was assessed in males at 19 weeks and in females at 18 weeks. Twelve glass marbles (1.5 cm in diameter) were placed, evenly spaced (3x4 rows), on a flat layer of 3 cm corn cob in the home cage (37.3 x 23.4 x 14.0 cm). A mouse was placed single caged and left for 30 min after which the number of marbles buried 2/3 were counted. Mean number of marbles buried \pm SEM of each group were determined.

2.13 Statistical analyses

GraphPad Prism 7.05 was used to analyse all data (GraphPad Software Inc., San Diego, CA, USA). Shapiro-Wilk normality test was used to determine a Gaussian distribution. One-way or two-way factor analysis of variance (ANOVA) with Holm-Sidak post-hoc test, or Kruskal-Wallis with Dunn's post-hoc test, was used for multiple comparisons. Student's unpaired t-test with Welch's correction was used analysing number and size of inclusions in striatum and cortex. Results are presented as means \pm SEM. P-values, F-values and degrees of freedom are presented for one-way and two-way ANOVA, and Student's unpaired t-test (Supplementary statistical results), giving the equality of means from each group. Differences with a $p < 0.05$ were considered statistically significant.

3. Results

3.1 Increased body weight and altered body composition in leptin-deficient R6/2 mice

To investigate whether it is possible to reverse the high metabolic state and body weight loss in HD, we utilized the well-characterized R6/2 mouse model. R6/2 mice exhibit a progressive weight loss [8, 16] and altered body composition [38]. We used transgenic R6/2 mice with two different CAG repeat lengths (CAG³⁴⁵⁻³⁵² and CAG²⁴²⁻²⁵⁷). The CAG repeat length corresponds to disease progression and results in a slower disease progression compared with R6/2 mice with 150 CAGs [26]. We recently showed that R6/2 mice with 273–285 CAGs exhibit neuropathological changes, such as increased levels of CSF NfL and reduced striatal volume [29] at 18 weeks of age.

R6/2 mice were placed on a leptin-deficient genetic background using a two-step breeding strategy (Supplementary figure 1.). To investigate the effects of leptin-deficiency on body weight we assessed body weight twice weekly in male and female mice in two cohorts of leptin-deficient R6/2 mice (R6/2^{(CAG 345-352);Ob/Ob} and R6/2^{(CAG 242-257);Ob/Ob}). Supportive of previous studies, significantly lower body weight was seen in male R6/2^(CAG 345-352) mice at 14.5 weeks of age ($p=0.0473$) (Figure 1A), and in female R6/2^(CAG 345-352) mice at 14 weeks of age ($p=0.02$) (Figure 1B) compared with WT littermates. Similarly, significantly lower body weight was seen in male R6/2^(Cag 242-257) mice at 9.5 weeks ($p=0.0464$) compared with WT mice and at 12 weeks in female R6/2^(Cag 242-257) mice ($p=0.0412$) (figure 1C).

Male and female R6/2 mice with a leptin-deficient genetic background (R6/2^{(CAG 345-352);Ob/Ob} and R6/2^{CAG 242-257;Ob/Ob}) had a significantly higher body weight compared with R6/2

littermates throughout the study ($p > 0.0001$) (Figure 1). Although body weight was significantly increased in both R6/2 strains on leptin-deficient genetic background, their body weight was starting to decline for both R6/2^{(CAG 345-352);Ob/Ob} and R6/2^{(CAG 242-257);Ob/Ob} (although still significantly higher than R6/2 mice and wild-type littermates) around the same time as R6/2 mice were exhibiting a significantly reduced body weight compared with wild-type littermates. Heterozygote leptin-deficiency (R6/2^{(CAG 345-352);Ob-/+} and R6/2^{(CAG 345-352);Ob-/+}) was not sufficient to result in increased body weight (Supplementary Figure 2A, 2B and 2C).

In R6/2 mice altered body composition has been described [38]. We therefore assessed body composition using DEXA scan. Ob/Ob mice, as well as R6/2^{(CAG 345-352);Ob/Ob} mice, exhibited dramatically increased fat mass compared with both wild-type mice and R6/2 mice (Figure 2A and 2B). No effect on body composition was seen in R6/2^{(CAG 345-352);Ob-/+} male or female mice (Supplementary Figure 3A and 3B).

Although fat mass was increased at both time points comparing leptin-deficient R6/2 mice to R6/2 mice and WT mice, male leptin-deficient R6/2 mice demonstrated significantly lower fat mass compared with Ob/Ob mice at 19 weeks (Figure 2).

In contrast to the dramatic effect on fat mass, there was only a limited change in lean mass. A small but significant decrease in lean mass was seen in male R6/2^{(CAG 345-352);Ob/Ob} mice compared with Ob/Ob littermates at 19 weeks (see figure 2), however lean mass was not different comparing male R6/2^(CAG 345-352) to R6/2^{(CAG 345-352);Ob/Ob} mice. A small, but significant increase in lean mass was seen in female R6/2^{(CAG 345-352);Ob/Ob} mice compared with R6/2^(CAG 345-352) littermates at 15 weeks, however at 18 weeks there was no difference. Since skeletal muscle wasting is a known feature of HD and transcriptional changes in skeletal muscle have been demonstrated [39], we also assessed skeletal muscle gene expression. We did here not observe any significant change in mRNA levels of any of the genes evaluated (supplementary figure 4).

In addition to progressive weight loss seen in R6/2 mice, tissue weight has also been shown to be altered in R6/2 mice [4]. Tissue weight (brain, liver, kidneys) was here assessed in male leptin-deficient R6/2^(CAG 345-352) mice and littermates at 20 weeks and in females at 19 weeks (Table 1). No change in tissue weight was seen in R6/2^(CAG 345-352) compared with their WT littermates. Leptin-deficiency resulted in higher liver and kidney weight, as well as longer body length, compared with WT and R6/2^(CAG 345-352) littermates in both males and females (Table

1). Notably, brain weight was reduced in Ob/Ob females ($p=0.0116$) and leptin-deficient R6/2^(CAG 345-352) males ($p=0.0204$) compared with their WT littermates (Table 1).

3.2 Decreased metabolic rate in leptin-deficient R6/2^(CAG 345-352) mice

In R6/2 mice, body weight loss has been shown to be associated with high oxygen consumption [16]. We therefore investigated whether leptin deficiency could restore high energy expenditure state in the R6/2 mice. We utilized the Pheno-master system in order to assess indirect gas calorimetry, food and water intake, as well as locomotor activity over 24 hours in male mice at 15 weeks of age. Similar to previous studies [16], we found a tendency towards increased oxygen consumption, carbon oxide production, respiratory exchange rate and energy expenditure in R6/2^(CAG 345-352) mice (Figure 3A, 3B, 3C and 3D). In contrast, leptin-deficient R6/2 mice (R6/2^(CAG 345-352);Ob/Ob males) exhibit a significantly and profound reduction in respiratory exchange rate compared with R6/2^(CAG 345-352) littermates (Figure 3). The difference in the respiratory exchange rate could not be explained by altered food or water consumption, as this was unaltered across the four groups assessed (figure 3E and 3F). Locomotor activity measurements were in line with previous findings [16] that no difference was seen in R6/2^(CAG 345-352) mice compared with WT littermates. However, the Ob/Ob and R6/2^(CAG 345-352);Ob/Ob groups showed a decreased activity compared with only WT littermates both during dark phase and 24 hours ($p<0.0001$) (Figure 3G and 3H).

3.3 White adipose tissue characteristics could contribute to the decreased energy expenditure found in leptin-deficient R6/2^(CAG 345-352) mouse

Browning of WAT accompanied by an increase in adipocyte oxygen consumption, increased *Ucp1* (*uncoupling protein 1*) expression and adipocyte size changes, have been demonstrated in R6/2 mice [18]. We could here replicate these findings in R6/2^(CAG 345-352) mice and show that WAT adipocytes were smaller compared with WT adipocytes ($p<0.0001$) (Figure 4C), with gene expression supporting browning (increased in *Ucp1* expression) ($p=0.0155$) (Figure 4D).

In contrast, the increased fat mass was seen in R6/2^(CAG 345-352);Ob/Ob mice (Figure 2A and 2B) was accompanied by an increased size of adipocytes ($p<0.0001$), as well as white adipocyte gene expression characteristics (Figure 4C and 4D). White adipose tissue gene expression in leptin-deficient R6/2 mice displayed similar characteristics as those of Ob/Ob mice white

adipose tissue, while WAT adipocytes were smaller compared with Ob/Ob littermates ($p=0.0012$) (Figure 4C and 4D).

3.4 Metabolic parameters in leptin-deficient R6/2^(CAG 345-352) mice

Age-dependent reduction in circulating leptin has previously been demonstrated in R6/2 mice [40]. Here, we measured leptin levels in males at 20 weeks. **Results when it has been measured.**

Reduced plasma levels of insulin and accompanying hyperglycaemia have previously been demonstrated in R6/2 mice [41]. Therefore, serum glucose and insulin levels were measured in males at 20 weeks, and pancreatic β -cell function and insulin resistance were calculated to evaluate the effect of leptin-deficiency on glucose homeostasis in R6/2^(CAG 345-352) mice. As previously shown [38], basal glucose levels were 4.7-fold higher ($p=0.0016$) in R6/2^(CAG 345-352) mice compared with WT littermates and similar in R6/2^(CAG 345-352);Ob/Ob mice (Table 2). As expected, Ob/Ob mice, known to exhibit high insulin levels [42], had a 49.9-fold ($p=0.0025$) higher basal insulin levels compared with WT littermates and 16.9-fold ($p=0.006$) higher basal insulin level was seen in R6/2^(CAG 345-352);Ob/Ob mice compared with R6/2^(CAG 345-352) mice (Table 2). β -cell function, presented as HOMA- β , was improved in R6/2^(CAG 345-352);Ob/Ob mice compared with R6/2^(CAG 345-352) littermates ($p=0.0449$). Increased insulin resistance, presented as HOMA-IR, was only seen in Ob/Ob mice compared with WT ($p=0.0001$) and R6/2^(CAG 345-352) ($p=0.0002$) mice (Table 2).

Fatty acid metabolites have previously been shown to be altered in HD [43]. Therefore, we evaluated circulating levels of total cholesterol levels, HDL and LDL/VLDL cholesterol levels, and the ratio of LDL/VLDL and HDL was calculated (Table 2). Serum levels of total cholesterol and HDL were similar across all groups (Table 2). LDL/VLDL levels in R6/2^(CAG 345-352) mice were 2.2-fold higher compared with WT littermates ($p=0.0034$). The ratio between LDL/VLDL and HDL was higher in R6/2^(CAG 345-352) mice compared with both wildtype mice ($p<0.0001$) and R6/2^(CAG 345-352);Ob/Ob mice ($p=0.0003$).

3.5 Increased body fat has no effect on brain neuropathology in the R6/2 model of HD

Recently, NfL shown to be axonal damage and cell death marker in neurodegenerative diseases including HD [44, 45]. Additionally, we have recently shown that both CSF and serum levels of increased NfL were correlated with reduced striatal volume and body weight in R6/2

mice carrying a shorter CAG repeat R6/2^(CAG 242-257) [29]. To further investigate the influence of the increased BMI on R6/2 neuropathology, we quantified the CSF concentrations of NfL using an ultra-sensitive immunoassay [28, 29]. We found increased levels of NfL in R6/2 and R6/2^(CAG 345-352);Ob/Ob mice compared with Ob/Ob mice, whereas there was no significant change between WT compared with R6/2, Ob/Ob and R6/2^(CAG 345-352);Ob/Ob genotypes (Figure 5F).

Striatum, cortex and corpus callosum are affected in clinical and animal models of HD, and this loss of volume occurs as a result of progressive cellular dysfunction and, eventually, cell death [46, 47]. Here, we sought to investigate whether slowing down the metabolism, by crossing R6/2 mice with leptin-deficient mice, would prohibit the progressive neurodegeneration in the R6/2 mouse model of HD. First, we have assessed the gross neuropathological changes in all four genotypes WT, R6/2, Ob/Ob and R6/2^(CAG 345-352);Ob/Ob mice. There was no significant change in the striatal volume and cortex thickness among the four groups (Figure 5A-5B). The assessment of corpus callosum thickness showed a significant reduction in Ob/Ob mice compared with the WT group (Figure 5C). One of the hallmarks of the HD is inclusion formation and it is primarily associated with cell death [48, 49]. The R6/2 mouse model featured these HD intranuclear and neuropil inclusions appearing earliest in the cortex and striatum [50]. Therefore, here we quantified the number and size of the inclusions in R6/2 and R6/2^(CAG 345-352);Ob/Ob mice in both brain areas. There was no effect of increased BMI on the cortical and striatal inclusion formation (Figure 5E).

3.6 Sign of reduced repetitive behaviour in leptin-deficient R6/2^(CAG 345-352) mice

A battery of behavioural tests were employed to assess cognitive, gross and fine motor skills in males at 19 weeks and females at 18 weeks. At this age, there was no significant difference in distance travelled (open field) in between groups in either male (Fig. 6A) or female (Fig. 6B) mice. Nest building is a spontaneous and complex behaviour of mice, requiring fine motoric skills, as well as cognitive function [34]. Diminished nesting behaviour was seen in R6/2^(CAG 345-352) males at 19 weeks ($p= 0.0112$) (Fig. 6C), and in R6/2^(CAG 345-352) females at 18 weeks ($p<0.0001$) (Fig. 6D) compared with WT littermates. No normalization was seen in leptin-deficient R6/2^(CAG 345-352) males or females (Fig. 6C and 6D). Increased marble burying behaviour is a sign of anxiety and repetitive behaviour in rodents [33]. Marble burying test

was performed in males at 19 weeks and in females at 18 weeks. R6/2^(CAG 345-352) male mice at 19 weeks (Fig. 6E) demonstrated significant ($p= 0.0255$) increase in marbles buried compared with wild-type littermates, notably, this behavioural change was normalized in R6/2^(CAG 345-352) mice with a leptin-deficient genetic background ($p<0.0001$) (Fig. 6E).

4. Discussion

Accumulating evidence emphasize systemic metabolism alterations as a key feature of HD pathology [7-9, 51]. Recently, a large observational study presented convincing evidence showing that high BMI is associated with a slower rate of disease progression in clinical HD, independent of CAG repeat length and disease stage [10]. Energy homeostasis alterations seen in HD [7-9], might be due to either central pathology, especially the hypothalamus [52, 53] or peripheral tissue alterations [7, 8, 18, 39], or by a combination [9].

Here we demonstrate that through a genetic approach it is possible to increase body weight and lower whole-body energy metabolism in the transgenic R6/2 mouse model of HD. We placed R6/2 mice on a leptin-deficient (*Ob/Ob*) genetic background, thereby creating a mouse model exhibiting hypometabolism, increased body weight, increased fat mass and reduced oxygen consumption, CO₂ production, energy expenditure and respiratory exchange rate with no change in food or water intake.

Similar to HD, ALS is associated with increased energy expenditure and weight loss as part of disease features [21, 54]. Interestingly, similar to the strategy here chosen, Lim et al could in 2014 show that placing the G93A mutant SOD1 mice, which is a well-established ALS mouse model, on a leptin-deficient genetic background reversed the catabolic state [21].

In addition to obesity, *Ob/Ob* mice are leptin-deficient, hyperinsulinemic at 4 weeks of age, transient hyperglycaemic from 4 to 12 weeks, and hypometabolic [23, 24, 55, 56]. Here we can show that leptin-deficient R6/2 mice have reduced oxygen consumption, CO₂ production, energy expenditure and respiratory exchange rate during both the light and dark phase. As we also see dramatic alterations in white adipose tissue characteristics, a plausible explanation for the reduction in the expiratory exchange rate could lie within white adipose tissue.

Thermogenesis is an important physiological mechanism to produce heat and consume energy. In rodents, BAT is a major site of thermogenesis, where oxidation of fatty acids stimulates UCP1, a mitochondrial inner membrane protein that uncouples oxidative phosphorylation [57]. We have previously shown that R6/2 mice white adipose tissue exhibit brown features, including *Ucp1* expression, and this is linked to an increase in adipocyte oxygen consumption [18]. The white adipose tissue of leptin-deficient R6/2 mice

lacks signs of browning, likely contributing to decreased whole-body energy balance and the reduced RER.

The homozygote obese mutation, *Ob/Ob*, was first developed in an outbred mouse colony at the Jackson Laboratory, Bar Harbor, Maine in 1949 [25], and was backcrossed to the well-characterized C57BL mouse colony. Mice homozygous for the obese spontaneous mutation are first recognizable at four weeks of age, with rapidly gain weight, which may reach four times the normal weight of wild-type controls [25].

Body weight in leptin-deficient R6/2 mice is dramatically increased compared with wild-type mice and R6/2 mice. Leptin-deficient R6/2 mice has a body weight gain curve comparable to that of leptin-deficient mice from 6 weeks to approximately 10 weeks of age.

Body weight gain seen in leptin-deficient mice and leptin-deficient R6/2 mice is likely due to increased fat mass as we see a dramatic increase in fat mass in leptin-deficient R6/2 mice. We note only very minor changes in lean mass in males. Lean mass is, with the here used genetic approach, only very marginally affected, illustrated also by no statistically significant change in skeletal muscle gene expression and the snout-to-anus length was comparable in all groups. In this study, two different R6/2 strains were used containing a CAG repeat between either 345–352 or 242–257. Both strains were crossed with leptin-deficient mice to generate leptin-deficient R6/2 with respective CAG repeats. We found similar results in term of increased body weight in both colonies. Our study, illustrated that body weight changes are similar in both models used, and likely our results could be replicated in R6/2 models regardless of CAG repeat number.

HD is associated with progressive motoric and behavioural changes, with reduced ability to perform everyday tasks [58]. Notably, van der Burg and colleagues showed in 2017 [10] that patients with a high BMI at baseline exhibited a slower rate of progression assessed as functional capacity, motor function, and cognitive performance. In our study, we have assessed activity and behaviour prior to mice prior to end stage disease, as we do not yet observe gross neuropathological alterations. As both leptin-deficient and leptin-deficient R6/2 mice have a massive increase in body weight and the increased body weight is a confounding factor for testing of motor function, rotarod that is usually used to assessed motor function could not be used. Therefore, locomotor activity was assessed using two different approaches. In the first approach, the activity was analysed during 24 hours along

with indirect gas calorimetry measurements using the TSE phenomaster system, resulting in decreased activity in leptin-deficient Ob/Ob and leptin-deficient R6/2 mice. The decreased activity is probably due to obesity as it has been shown by others previously [59, 60]. In the second approach, another cohort of mice was assessed using the open field arena, where only a trend of decreased activity was seen in Ob/Ob mice compared with WT mice. In an open field arena, it is possible for mice testing that stress or being in a novel environment for a short period of time can lead to high variability in results. On the other hand, the automated assessment of activity over light/dark cycles provides robust results that are free from experimenter bias.

Next, we have tested mice for nest-building and marble burying behaviour. Nest-building behaviour in mice requires organization of a complex set of behaviours, such as fine motoric skills, as well as cognitive function [34], and the ability of nest-building has been shown to be affected in both Alzheimer [35] and Parkinson mouse models [36]. In the present study, we replicate our previous results [37] that R6/2 mice have a diminished nest-building behaviour compared with WT littermates. However, no improvement was seen in leptin-deficient R6/2 mice, indicating that increased body weight and fat mass is not enough to affect this behavioural aspect in the R6/2 mouse.

Psychiatric manifestations such as obsessive-compulsion behaviour (OCD) and anxiety-like behaviour have been previously shown in patients with HD, preceding motor dysfunctions [61, 62]. Aberrant marble burying behaviour is a sign of anxiety and repetitive behaviour in rodents [34]. In this study, we observed an increase in marbles buried in R6/2 mice compared with wild-type littermates, suggesting repetitive and anxiety-like behaviour. Importantly, this behavioural change was normalized in leptin-deficient R6/2 mice.

Even though we here could see an effect on behaviour, we can't see a beneficial effect on central pathology. Different brain regions and pathways regulate repetitive behaviours; especially the cortico-basal ganglia-thalamic pathway [63], which is also involved in motor activity, and also affected in HD [64].

In agreement with previous studies [59, 60], in this study, reduced activity was seen in both leptin-deficient and leptin-deficient R6/2 mice using the automated home cage phenotyping system. This indicates that the decreased number of marbles buried in leptin-deficient R6/2 mice compared with R6/2 mice could be due to decreased general activity rather than

indicating a recovery. However, this observed improved behaviour needs further investigation.

BMI is a measure of body weight with respect to height and it does not distinguish between fat and lean mass. Given that increased BMI correlates with disease-slowing effects in clinical HD [10] and the increase in BMI could be due to an increase in fat mass, we aimed to understand whether increase in fat mass can play a neuroprotective role in R6/2 mice. To assess the gross neuropathological changes, we have measured striatal volume, cortex and corpus callosum thickness at 20 weeks. The striatal volume and cortex thicknesses were similar in all animals. It should be noted, however, that volumetric measurements do not provide information about cellular pathology and their functions. Therefore, in order to better characterize the effect of R6/2 mice on leptin-deficient genetic background, additional stereological analysis on specific cell populations would need to be performed. Corpus callosum is one of the largest white matter structures in the brain and several imaging studies showed reduced white matter volume already in pre-manifest HD subjects [65, 66]. Complementarily, we have previously shown a significant reduction in corpus callosum thickness in 18 weeks old R6/2 mice containing CAG repeat between 242-257 [29]. Yet, in this study, the absence of corpus callosum neuropathology in 20 weeks old R6/2 mice might be due to the elongated CAG repeats (345-352 CAG), as increased CAG repeat length is correlated with delayed pathology in the R6/2 mouse model of HD [26]. Knowing that robust white matter pathology is indicative of axonal degeneration in HD and CSF NfL concentrations are a reliable predictor of HD neuropathology in both R6/2 animal model and clinical HD, we have assessed the NfL levels in the CSF. Similar to the histopathological results, there was no significant change in CSF NfL levels between WT and HD mice groups (R6/2 and R6/2 mice on leptin-deficient genetic background). However, in line with our previous results [29], the comparison of CSF NfL concentrations only between R6/2 and WT groups shows a significant increase in R6/2 mice (WT: n=9, mean=561 ng/ml, SEM=47.5 and R6/2: n=14, mean=846.5 ng/ml, SEM=65.3, two-tailed, unpaired t-test, p=0.002). This suggests that 20 weeks might not be a long enough time to provide a clear picture of the neuropathological effects. Therefore, we can speculate that a longer assessment might reveal changes in the brain pathology that can be associated with increased body fat. Following the gross pathological assessment experiments, we quantified number and size of the mutant HTT inclusions and there was no significant effect of genotype. However, we

found a trend toward an increase in the number of inclusions in R6/2-Ob/Ob compared with R6/2 mice. Even though it is not fully known whether the HTT inclusions are protective or toxic, there is strong evidence for the inclusions causing transcriptional dysregulation and disruption of cellular trafficking [67, 68]. Therefore, we can postulate that higher body fat content can interfere with inclusion clearance as the obese phenotype is also closely associated with increased ER stress, which hinders misfolded protein degradation [69]. Taken together, similar levels of NfL and a trend towards an increased number of striatal inclusions in R6/2 on leptin-deficient genetic background suggest that disease onset delaying effect of high baseline clinical HD BMI could be due to an increase in lean mass rather than fat mass.

Our study has certain limitations. First, although our findings demonstrate the possibility of shifting energy metabolism from high to low and to increase body weight as well as postpone body weight loss in the R6/2 mouse model, our study is massively shifting body composition with increased fat mass as a result, without affecting lean mass. The leptin-deficient Ob/Ob mouse displays progressive metabolic alterations [23, 24, 70], displaying early body weight gain and increased white adipose tissue depots [71]. The body weight increase precedes altered carbohydrate metabolism alterations that become noticeable at 4 weeks [70]. The R6/2 mice also display dysregulated glucose metabolism, with mid-disease insulin-resistance progressing towards insulin-dependent diabetes [41, 72, 73]. In the leptin-deficient R6/2 mouse that we here created, glucose metabolism alterations are complex and this could influence whole body metabolism as well as possibly influencing brain pathology.

It might have been more beneficial with a slow switch of energy metabolism and a switch towards more lean mass. Secondly, although our study investigates a genetic approach to intervening in the disease course, our study is observational and does not provide full mechanistic explanation for how energy metabolism is altered.

That high BMI is associated with slower disease progression [10], emphasize the importance of nutritional status, energy homeostasis and body composition from a clinical perspective. With this scenario, our findings provide important knowledge to further understand how shifting energy balance will affect HD. Increasing body weight, by increasing white adipose tissue mass and lowering respiratory exchange rate is in the R6/2

mouse model not sufficient to generate neuroprotection. Our results provide important evidence and encourage future studies focusing on restoring energy metabolism, with alternative approaches. Future strategies focusing on restoring lean mass could possibly provide better understanding on the relevance of body composition for disease progression.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Figures and Tables

Figure 1. Increased body weight in R6/2 mice on the leptin-deficient genetic background.

Body weight was monitored up to 20 weeks in R6/2^(CAG 345-352) cohort in males **(A)** and up to 19 weeks in females **(B)** (n=7-9/genotype/gender). The changes in body weight of R6/2^(CAG 242-257) cohort were followed up to 12 weeks in both males **(C)** and females **(D)** (n=6-9/genotype/gender). Significantly lower body weight was seen in male R6/2^(CAG 345-352) mice at 14.5 weeks of age **(A)**, and in female R6/2^(CAG 345-352) mice at 14 weeks of age **(B)** compared with WT littermates. Male and female R6/2 mice with a leptin-deficient genetic background (R6/2^(CAG 345-352);Ob/Ob) (n=7-9) have a significantly higher body weight compared with R6/2 littermates **(A, B)**. Significantly lower body weight was seen in male R6/2^(CAG 242-257) mice at 9.5 weeks of age **(C)**, and in female R6/2^(CAG 345-352) mice at 12 weeks of age **(D)** compared with WT littermates. R6/2^(CAG 242-257) (male and female) mice on leptin-deficient genetic background also have a significantly higher weight compared with both wild type and R6/2^(CAG 242-257) littermates (n=6-9) **(C, D)**. Data represent mean \pm SEM. Statistical significance was determined by repeated measures of 2-way ANOVA with Holm-Sidak post hoc test for multiple comparisons. *p < 0.05, **p < 0.01, ****p < 0.0001.

Figure 2. Increased fat mass in R6/2^(CAG 345-352) mice on leptin-deficient genetic background (R6/2^(CAG 345-352);Ob/Ob).

Assessment of body fat composition using DEXA scan in males **(A)** at 16 and 19 weeks (n=6-10) and in females **(B)** (n=8-11) at 15 and 18 weeks. There was a significant increase in fat mass in R6/2^(CAG 345-352);Ob/Ob males **(A)** and females **(B)** compared with R6/2^(CAG 345-352) littermates. A significant decrease in lean mass measurements was seen in R6/2^(CAG 345-352);Ob/Ob male mice (p=0.0332)**(C)** at 19 weeks and the females mice (p=0.043) **(D)** at 18 weeks compared with Ob/Ob littermates at 19 weeks **(C)**. Lean mass was not different in male R6/2^(CAG 345-352) mice compared with R6/2^(CAG 345-352);Ob/Ob mice **(C)**. There was a significant reduction in lean mass in female R6/2^(CAG 345-352) mice compared with WT

littermates ($p=0.0307$) at 18 weeks of age (**D**). A significant increase in lean mass was seen in female $R6/2^{(CAG\ 345-352)}$;Ob/Ob mice compared with $R6/2^{(CAG\ 345-352)}$ littermates at 15 weeks ($p=0.0307$), however, at 18 weeks there was no difference (**D**). Data represent mean \pm SEM. Statistical significance was determined by One-way ANOVA with Holm-Sidak post hoc test for multiple comparisons. A p -value below 0.05 was considered significant. * $p<0.05$ and **** $p<0.0001$.

Figure 3. Decreased metabolic rate in leptin-deficient $R6/2^{(CAG\ 345-352)}$ mice. Indirect Gas Calorimetry, as well as food and water intake, was measured over 24 hours using a PhenoMaster/LabMaster Home cage System in males group at 15 weeks of age ($n=6-10$ /genotype). Oxygen consumption (**A**), carbon dioxide production (**B**), energy expenditure (**C**), respiratory rate (**D**), food and water intake (**E, F**), and locomotor activity was assessed during light and dark phase, as well as expressed as over 24 hours. $R6/2^{(CAG\ 345-352)}$;Ob/Ob males exhibit reduced metabolic rate compared with $R6/2^{(CAG\ 345-352)}$ littermates (**A, B, C and D**). The decrease in energy expenditure is not accompanied with change in food (**E**) and water intake (**F**). Ob/Ob and $R6/2^{(CAG\ 345-352)}$;Ob/Ob males exhibit decreased locomotor activity compared with WT and $R6/2^{(CAG\ 345-352)}$ littermates during dark phase and over 24 hours (**G**). $n=6-10$ /group. Data represent mean \pm SEM. One-way ANOVA with Holm-Sidak post hoc test for multiple comparisons. * $p < 0.05$, **** $p<0.0001$.

Figure 4. White adipose tissue characteristics could contribute to the decreased energy expenditure found in leptin-deficient $R6/2^{(CAG\ 345-352)}$ mouse.

Representative images of Hematoxylin-Eosin staining, showing the inguinal white adipose tissue (WAT) (**A**) and interscapular brown adipose tissue (BAT) (**B**) in males at 20 weeks. Stereological analysis of adipocyte cell area in male mice at 20 weeks ($n=6$ /genotype) by using cellSens Dimensions 1.11 software. Increased fat mass is present in $R6/2^{(CAG\ 345-352)}$;Ob/Ob mice (illustrated in figure 2). $R6/2^{(CAG\ 345-352)}$ mice white adipose tissue exhibit brown adipose tissue characteristics, with smaller WAT adipocytes (**C**). Assessments of Hp and UCP1 mRNA changes in WAT of male mice at 20 weeks ($n=8-10$ /genotype) and increased WAT expression of UCP1 compared with WT littermates (**D**). Importantly, leptin-deficient $R6/2^{(CAG\ 345-352)}$ mice exhibit white WAT characteristics, i.e. larger adipocytes (**C**) and less UCP1 expression (**D**). Pro-white and pro-brown gene expression changes in WAT ($n=8-10$) (**D**). $\Delta\Delta C_t$ method was used

to analyze gene expression and normalized to *TBP* and *Ppia* genes were used as house-keeping genes and the data represented as mean \pm SEM. One-way ANOVA with Holm-Sidak post hoc test for multiple comparisons. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$

Figure 5. No effect on neuropathological phenotype in R6/2 mice on a leptin-deficient genetic background at 20 weeks. The CSF NfL concentrations were determined using a single molecule array (Simoa)-based immunoassay technology in male mice at 20 weeks (n=8-14/group) **(A)**. Measurement of striatum volume **(B)**, cortical thickness **(C)** and corpus callosum thickness **(D)** in males at 20 weeks (n=7-12/genotype). The size and number of inclusions assessed on R6/2 and the R6/2^(CAG 345-352);Ob/Ob mice on EM48 (anti-HTT) strained brain sections. The number **(E)** and the size **(F)** of EM48 positive inclusions in the striatum (n=6-7/genotype). Representative images showing the HTT inclusions in the striatum of R6/2 and the R6/2^(CAG 345-352);Ob/Ob mice **(G)**. Assessment of number **(H)** and size **(I)** of the striatal inclusion in the cortex (n=6-7/genotype). EM48 immunohistochemistry showing cortical inclusions **(J)**. Data represented as mean \pm SEM. Statistical significance for stereological assessments and NfL levels were determined by One-way ANOVA with Holm-Sidak post hoc test for multiple comparisons, and Student's unpaired t-test with Welch's correction was used analyzing number and size of inclusions in striatum and cortex.

Figure 6. Sign of reduced repetitive behaviour in leptin-deficient R6/2(CAG 345-352) mice. Behaviour was assessed in males at 19 weeks and females at 18 weeks, prior to overt motoric changes, and at this age, there was no significant difference in distance travelled (open field) in between groups in either male **(A)** or female **(B)** mice (n=6-7). A diminished nesting behaviour was seen in R6/2^(CAG 345-352) males at 19 (n=9-11) **(C)**, and in R6/2^(CAG 345-352) females at 18 (n=6-10) **(D)** compared with WT littermates. No normalization was seen in R6/2^(CAG 345-352) males or females with a leptin-deficient genetic background (R6/2^(CAG 345-352);Ob/Ob)**(E, F)**. Increased marble burying behaviour is a sign of anxiety and repetitive behaviour in rodents (Deacon, 2006). Marble burying test was performed in males (n=9-11) at 19 weeks **(E)** and in females (n=6-10) at 18 weeks **(F)**. R6/2^(CAG 345-352) male mice at 19 weeks **(E)** demonstrated a significant increase in marbles buried compared with wild-type littermates, importantly, this behavioural change was normalized in R6/2^(CAG 345-352) mice with a leptin-deficient genetic background **(E)**. One-way ANOVA with Holm-Sidak post hoc test for multiple comparisons. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.001$, **** $p < 0.0001$

Males - 20 weeks	WT	R6/2	Ob/Ob	R6/2;Ob/Ob	*p-value	#p-value
Body weight (g)	34.10 ± 1.08	25.23 ± 0.40	64.42 ± 1.81	44.31 ± 3.67	0.0063	<0.0001
Length (cm)	9.09 ± 0.07	8.89 ± 0.07	9.79 ± 0.08	9.05 ± 0.12	>0.9999	>0.9999
Tissue weight (g)						
Brain	0.42 ± 0.007	0.41 ± 0.012	0.40 ± 0.005	0.38 ± 0.009	>0.9999	0.323
Liver	1.12 ± 0.08	1.15 ± 0.05	4.24 ± 0.47	2.19 ± 0.20	>0.999	0.091
Kidneys	0.35 ± 0.02	0.30 ± 0.01	0.45 ± 0.02	0.36 ± 0.02	0.136	0.095

Females - 19 weeks	WT	R6/2	Ob/Ob	R6/2;Ob/Ob	*p-value	#p-value
Body weight (g)	27.03 ± 1.80	20.04 ± 1.01	62.32 ± 1.90	39.80 ± 1.64	0.012	<0.0001
Length (cm)	8.82 ± 0.10	8.44 ± 0.09	9.53 ± 0.07	9.04 ± 0.12	0.412	0.028
Tissue weight (g)						
Brain	0.43 ± 0.007	0.43 ± 0.011	0.39 ± 0.005	0.39 ± 0.011	>0.999	0.211
Liver	0.76 ± 0.04	1.01 ± 0.08	3.83 ± 0.24	2.53 ± 0.18	>0.999	0.843
Kidneys	0.25 ± 0.01	0.25 ± 0.01	0.37 ± 0.02	0.33 ± 0.01	>0.999	0.060

Table 1. Effects on body and tissue weight in leptin-deficient R6/2^(CAG 345-352) mice. Tissue and body weight, and body length in males at 20 weeks and in females at 19 weeks. Data are expressed as mean ± SEM. One-way ANOVA with Holm-Sidak post hoc test for multiple comparisons or Kruskal-Wallis with Dunn's post hoc test for multiple comparisons was used to analyse data. *Represents the p-value between WT and R6/2^(CAG 345-352) mice, and #represents the p-value between R6/2^(CAG 345-352);Ob/Ob and R6/2^(CAG 345-352) mice, (n=6-9). A p-value below 0.05 was considered significant.

Males - 20 weeks	WT	R6/2	Ob/Ob	R6/2;Ob/Ob	*p-value	#p-value
Glucose homeostasis						
Basal glucose (mmol/L)	2.13 ± 0.80	9.98 ± 1.77	6.67 ± 0.87	13.51 ± 1.09	0.002	0.095
Basal insulin (µg/L)	8.93 ± 1.57	2.94 ± 0.77	445.4 ± 73.31	49.61 ± 6.58	>0.9999	0.006
HOMA-β	5958 ± 2543	209.3 ± 67.47	41922 ± 12955	2621 ± 647	0.085	0.045
HOMA-IR	20.13 ± 9.23	48.55 ± 17.78	3332 ± 814.2	791.2 ± 121.8	>0.9999	0.051
Lipid homeostasis						
Total cholesterol (µg/µL)	0.65 ± 0.05	0.85 ± 0.06	0.94 ± 0.10	0.84 ± 0.12	0.451	0.947
HDL (µg/µL)	0.54 ± 0.04	0.60 ± 0.04	0.81 ± 0.09	0.67 ± 0.09	0.731	0.731
LDL/VLDL (µg/µL)	0.11 ± 0.01	0.25 ± 0.02	0.13 ± 0.02	0.17 ± 0.03	0.003	0.113
(LDL/VLDL) : HDL ratio	0.21 ± 0.02	0.41 ± 0.02	0.17 ± 0.03	0.25 ± 0.02	<0.0001	0.0003

Table 2. Metabolic serum parameters leptin-deficient R6/2^(CAG 345-352) mice. Glucose and insulin levels were measured in males at 20 weeks, and pancreatic β-cell function and insulin resistance were calculated (n=7-9/group). HDL and LDL/VLDL cholesterol levels were measured, and the (LDL/VLDL)/HDL ratio was calculated (n=6/group). *Represents the p-value between WT and R6/2^(CAG 345-352) mice, and #represents the p-value between R6/2^(CAG 345-352);Ob/Ob and R6/2^(CAG 345-352) mice. Data represent mean ± SEM. Statistical significance was determined by One-way ANOVA with Holm-Sidak post hoc test or Kruskal Wallis with Dunn's post hoc test for multiple comparisons. A p-value below 0.05 was considered significant.

Supplementary Figures and Tables

Supplementary Figure 1. A two-step breeding strategy to generate a leptin-deficient R6/2 mouse model. Females heterozygote for leptin-deficiency gene (Ob-/+) was crossed with R6/2 males (CAG 345-352 or 242-257) to generate the first generation (F1). To generate leptin-deficient R6/2 mice (R6/2;Ob/Ob) in generation F2, mice heterozygote for leptin-deficiency gene (Ob-/+) was crossed with R6/2 mice containing the heterozygote gene for leptin-deficiency (R6/2;Ob-/+).

Supplementary Figure 2. Increased body weight in R6/2^(CAG 345-352) mice on the leptin-deficient genetic background. Body weight at 16 and 19 weeks in R6/2^(CAG 345-352) cohort in males (A) and at 15 and 18 weeks in females (B) (n=7-14/genotype/gender). The changes in body weight of R6/2^(CAG 242-257) cohort were followed up at 12 weeks in both males and females (C) for all groups (n=6-9/genotype/gender). No change was seen in R6/2^(CAG 345-352) or R6/2^(CAG 242-257) with a heterozygote leptin-deficient genetic background. Data represent mean ± SEM. Statistical significance was determined by repeated measures of One-way ANOVA with Holm-Sidak post hoc test for multiple comparisons. A p-value below 0.05 was considered significant.

Supplementary Figure 3. Increased fat mass in R6/2^(CAG 345-352) mice on leptin-deficient genetic background (R6/2^(CAG 345-352);Ob/Ob). Body composition at 16 and 19 weeks in R6/2^(CAG 345-352) cohort in males (A, B) and at 15 and 18 weeks in females (C, D) (n=7-14/genotype/gender). No change was seen in R6/2^(CAG 345-352) or R6/2^(CAG 242-257) with a heterozygote leptin-deficient genetic background. Data represent mean ± SEM. Statistical significance was determined by repeated measures of One-way ANOVA with Holm-Sidak post hoc test for multiple comparisons. A p-value below 0.05 was considered significant.

Supplementary Figure 4. Gene expression in skeletal muscle in R6/2^(CAG 345-352) mice on a leptin-deficient genetic background. Normalized skeletal muscle (gastrocnemius) gene expression related to muscle contractility and atrophy in males at 20 weeks. Gene expression was analysed using the $\Delta\Delta\text{Ct}$ method and normalized to *Tbp* and *18S*. Data represent mean ± SEM of the following number of 6–11 animals/group. Statistical significance was determined by One-way ANOVA with Holm-Sidak post hoc test for multiple comparisons.

Supplementary Table 1. Primer sequences used for genotyping

Primer name	Sequence (5'→3')
oIMR1151 (Common) (Ob gene)	TGTCCAAGATGGACCAGACTC
oIMR1152 (Common) (Ob gene)	ACTGGTCTGAGGCAGGGAGCA
R6/2 Int Ctrl (F)	CAAATGTTGCTTGTCTGGTG
R6/2 Int Ctrl (R)	GTCAGTCGAGTGCACAGTTT
R6/2 Gene (F)	CGCAGGCTAGGGCTGTCAATCATGCT
R6/2 Gene (R)	TCATCAGCTTTTCCAGGGTCGCCAT

Supplementary Table 2. Primer sequences used for gene expression analysis

Primer name	Forward Sequence (5'→3')	Reverse Sequence (5'→3')
<i>18S*</i>	ACCGCAGCTAGGAATAATGGA	GCCTCAGTTCCGAAAACCA
<i>Acta 1</i>	CACCATCGGCAATGAGCGTTTC	CACCATCGGCAATGAGCGTTTC
<i>Actn 3</i>	TGACCTATGTTTCCTGCTTCTAC	CTCCTCCATCAGCTTCTCATTT
<i>Caspase 3</i>	TTGCCAGAAGATACCGGTGGAG	TCCAGGAATAGTAACCAGGTGCTG
<i>Caspase 8</i>	AGTCACTTTGCCAGAGCCTGAG	TAGTTCACGCCAGTCAGGATGC
<i>Creb1</i>	GAGAGCTGGTATGTCAGGAATG	CCAGAAGAGATGCAGGAGAAAG
<i>Hp</i>	CGGAACGCCAACTTTAGATTTAC	CTTCTCGGGCACTGTACTATTC
<i>Myh 2</i>	ACCTTCTCGTTTGCCAGTAAGGG	AAGGCCTATTCTGGGCCTCGATTC
<i>Ppia</i>	GGGTTCCCTCCTTTCACAGAA	GATGCCAGGACCTGTATGCT
<i>Sirt 1</i>	AGCAACATCTCATGATTGGCACCG	AGCAACATCTCATGATTGGCACCG
<i>Tbp</i>	TCTGAGAGCTCTGGAATTGTACCG	TGATGACTGCAGCAAATCGCTTG
<i>Tnni 2</i>	ACAGCACCTGAAGAGTGTGATGC	TTC-TCG-GCG-GCT-TTC-CTC-TTTC
<i>Tnnt 3</i>	TTG-ACC-AAG-CCC-AGA-AGC-ACAG	AGGAGTCAGGGTTGATGGTCTCTG
<i>Ucp1</i>	GCATTTCAGAGGCAAATCAGC	GCCACACCTCCAGTCATTAAG

Table shows primer sequences used for the validation of gene expression data. Efficiency and R² of a standard curve were verified. Efficiency criteria for using a primer pair was 90% < E < 110% and the R² cut off was > 0.990, with efficiencies below or above being excluded from this study. * denotes housekeeping genes.

Supplementary Statistical Results

GraphPad Prism 7.05 was used to analyse all data (GraphPad Software Inc., San Diego, CA, USA). Statistical difference is considered at $p < 0.05$ from post-hoc analyses. RM=Repeated measures; M=mean; SEM=Standard error of the mean.

Figure 1. Body Weight

2-way RM ANOVA with Holm-Sidak's multiple comparisons test.

	A. Males (CAG 345-352)	B. Females (CAG 345-352)	C. Males (CAG 242-257)	D. Females (CAG 242-257)
Genotype	F(5, 48) = 147.4, $p > 0.0001$	F(5, 53) = 220.3, $p < 0.0001$	F(5, 45) = 105.5, $p < 0.0001$	F(5, 48) = 117.5, $p < 0.0001$
Age	F(28, 1344) = 374.2, $p < 0.0001$	F(26, 1378) = 381.7, $p < 0.0001$	F(12, 540) = 108.6, $p < 0.0001$	F(12, 576) = 136.9, $p < 0.0001$
Genotype x Age	F(140, 1344) = 34.59, $p < 0.0001$	F(130, 1378) = 72.77, $p < 0.0001$	F(60, 540) = 10.46, $p < 0.0001$	F(60, 576) = 37.4, $p < 0.0001$

Figure 2. Body composition

2-way RM ANOVA with Holm-Sidak's multiple comparisons test.

Males at 16 and 19 weeks, and females at 15 and 18 weeks (CAG 345-352).

	A. Fat mass (males)	B. Fat mass (females)	C. Lean mass (males)	D. Lean mass (females)
Genotype	F(5, 40) = 173.9, $p < 0.0001$	F(5, 42) = 135.6, $p < 0.0001$	F(5, 41) = 4.466, $p = 0.0024$	F(5, 42) = 21.41, $p < 0.0001$
Age	F(1, 40) = 18.86, $p < 0.0001$	F(1, 42) = 0.1266, $p = 0.7238$	F(1, 41) = 0.5974, $p = 0.4440$	F(1, 42) = 1.552, $p = 0.2198$
Genotype x Age	F(5, 40) = 12.88, $p < 0.0001$	F(5, 42) = 1.206, $p = 0.3228$	F(5, 41) = 1.364, $p = 0.2577$	F(5, 42) = 4.213, $p = 0.0034$

Figure 3. Indirect gas calorimetry

2-way RM ANOVA with Holm-Sidak's multiple comparisons test; light/dark phase and 24 hrs.
Males at 15 weeks (CAG 345-352).

	A. VO ₂ (Oxygen consumption)	B. VCO ₂ (Carbon Oxide production)	C. H (Energy expenditure)	D. RER (Respiratory exchange rate)
Genotype	F(3, 28) = 18.89, p<0.0001	F(3, 28) = 20.63, p<0.0001	F(3, 28) = 15.82, p<0.0001	F(3, 28) = 15.82, p<0.0001
Time	F(2, 56) = 4.476, p=0.0157	F(2, 56) = 8.521, p=0.0006	F(2, 56) = 234.3, p<0.0001	F(2, 56) = 234.3, p<0.0001
Genotype x Time	F(6, 56) = 0.2391, p=0.9617	F(6, 56) = 0.7512, p=0.6111	F(6, 56) = 15.21, p<0.0001	F(6, 56) = 15.21, p<0.0001

	E. Food intake (g)	F. Water intake (mL)	G. Distance moved (m)
Genotype	F(3, 27) = 1.479, p=0.2424	F(3, 27) = 0.1067, p=0.9555	F(3, 28) = 13.87, p<0.0001
Time	F(2, 54) = 50.8, p<0.0001	F(2, 54) = 34.84, p<0.0001	F(2, 56) = 133.6, p<0.0001
Genotype x Time	F(6, 54) = 0.6589, p=0.6829	F(6, 54) = 0.2892, p=0.9396	F(6, 56) = 14.87, p<0.0001

Figure 4. White adipose tissue (WAT)

One-way ANOVA with Holm-Sidak's multiple comparisons test.
Males at 20 weeks (CAG 345-352).

	C. Mean WAT cell area	D. WAT gene expression			
		<i>Hp</i>		<i>Ucp1</i>	
F-Statistics	F(3, 20) = 87.42, p<0.0001	F(3, 32) = 3.014, p=0.0443	<i>Fold change</i>	F(3, 32) = 4.83, p=0.0069	<i>Fold change</i>
WT vs. R6/2	p<0.0001	p=0.6337	- 0.3514	p=0.0155	+5.062
WT vs. Ob/Ob	p<0.0001	p=0.1271	+1.798	p=0.8448	-0.3254
WT vs. R6/2;Ob/Ob	p<0.0001	p=0.5730	+0.8347	p=0.6693	+1.473
R6/2 vs. Ob/Ob	p<0.0001	p=0.0537	+2.149	p=0.0155	-5.387
R6/2 vs. R6/2;Ob/Ob	p<0.0001	p=0.4415	+1.186	p=0.1400	-3.589
Ob/Ob vs. R6/2;Ob/Ob	p=0.0012	p=0.5730	-0.9628	p=0.6693	+1.799

Figure 5. Central pathology

Kruskal-Wallis with Dunn's post hoc test was used analysing NfL in serum, One-way ANOVA with Holm-Sidak post hoc test for multiple comparisons was used analyzing striatum volume, cortex thickness and Corpus Callosum (CC) thickness, and Student's unpaired t-test with Welch's correction was used analyzing number and size of inclusions in striatum and cortex. Males at 20 weeks (CAG 345-352).

	A. NfL in serum	B. Striatum volume	C. Cortex thickness	D. CC thickness
F-Statistics		F (3, 35) = 0.6318, p=0.5994	F (3, 33) = 3.582, p=0.0240	F (3, 34) = 3.173, p=0.0366
WT vs. R6/2	p=0.1871	p=0.9684	p=0.9945	p=0.4341
WT vs. Ob/Ob	p=0.5708	p=0.7377	p=0.1071	p=0.0245
WT vs. R6/2;Ob/Ob	p=0.0828	p=0.9684	p=0.1501	p=0.4341
R6/2 vs. Ob/Ob	p=0.0002	p=0.8810	p=0.1071	p=0.3915
R6/2 vs. R6/2;Ob/Ob	p>0.9999	p=0.9684	p=0.1501	p=0.9619
Ob/Ob vs. R6/2;Ob/Ob	p=0.0002	p=0.8810	p=0.9945	p=0.4341

	E. Nr of inclusions (striatum)	F. Size of inclusions (striatum)	H. Nr of inclusions (cortex)	I. Size of inclusions (cortex)
F-Statistics	F (6, 5) = 7.141, p=0.0476	F (6, 5) = 1.779, p=0.5437	F (6, 5) = 5.468, p=0.0822	F (6, 5) = 1,584, p=0.6303
WT vs. R6/2	p=0.1693	p=0.1033	p=0.8931	p=0.3562
WT (Mean±SEM)	25000 ± 1955	7.375 ± 0.1794	10386 ± 1436	4.804 ± 0.1595
R6/2 (Mean±SEM)	28191 ± 790	7.785 ± 0.1453	10606 ± 663,4	4.602 ± 0.1369

Figure 6. Behavioural testing

One-way ANOVA with Holm-Sidak's multiple comparisons test:
Males at 19 weeks, and females at 18 weeks (CAG 345-352).

	A. Open field test; males	B. Open field test; females
F-Statistics	F(3, 22) = 2.877, p=0.0591	F(3, 21) = 2.712, p=0.0709
WT vs. R6/2	p=0.6549	p=0.8644
WT vs. Ob/Ob	p=0.0523	p=0.1985
WT vs. R6/2;Ob/Ob	p=0.5131	p=0.1985
R6/2 vs. Ob/Ob	p=0.2958	p=0.3308
R6/2 vs. R6/2;Ob/Ob	p=0.6549	p=0.3308
Ob/Ob vs. R6/2;Ob/Ob	p=0.5131	p=0.9385

	C. Nest building behaviour test; males	D. Nest building behaviour test; females	E. Marble burying behaviour test; males	F. Marble burying behaviour test; females
F-Statistics	F(3, 21) = 11.56, p=0.0001	F(3, 25) = 16.35, p<0,0001	F(3, 36) = 12.81, p<0.0001	F(3, 28) = 21.36, p<0,0001
WT vs. R6/2	p=0.0074	p<0.0001	p=0.0169	p=0.0608
WT vs. Ob/Ob	p=0.0045	p=0.0001	p=0.2704	p=0.0006
WT vs. R6/2;Ob/Ob	p<0.0001	p<0.0001	p=0.0534	p=0.0002
R6/2 vs. Ob/Ob	p=0.7726	p=0.2338	p=0.0002	p<0.0001
R6/2 vs. R6/2;Ob/Ob	p=0.1512	p=0.7410	p<0.0001	p<0.0001
Ob/Ob vs. R6/2;Ob/Ob	p=0.1785	p=0.3077	p=0.3655	p=0.4440

Table 1. Body weight, and brain, liver and kidney weight

Males at 20 weeks and females at 19 weeks. (CAG 345-352).

One-Way with Holm-Sidak post hoc multiple comparisons test or Kruskal-Wallis with Dunn's post hoc multiple comparisons test.

Males	Body Weight	Brain weight	Liver weight	Kidney weight	Length
F-Statistics	F (3, 27) = 71.86, p<0.0001			F (3, 20) = 11.01, p=0.0002	F (3, 44) = 19.84, p<0.0001
WT vs. R6/2	p=0.0056	p>0.9999	p>0.9999	p=0.1355	p=0.3894
WT vs. Ob/Ob	p<0.0001	p>0.9999	p=0.0003	p=0.0066	p<0.0001
WT vs. R6/2;Ob/Ob	p=0.0036	p=0.0204	p=0.0181	p=0.7075	p=0.7332
R6/2 vs. Ob/Ob	p <0.0001	p>0.9999	p=0.0029	p<0.0001	p<0.0001
R6/2 vs. R6/2;Ob/Ob	p<0.0001	p=0.3230	p=0.0910	p=0.0952	p=0.4647
Ob/Ob vs. R6/2;Ob/Ob	p<0.0001	p=0.5115	p>0.9999	p=0.0127	p<0.0001

Females	Body Weight	Brain weight	Liver weight	Kidney weight	Length
F-Statistics	F (3, 29) = 99.71, p<0.0001				
WT vs. R6/2	p=0.0042	p>0.9999	p>0.9999	p>0.9999	p=0.4123
WT vs. Ob/Ob	p<0.0001	p=0.0116	p<0.0001	p=0.0029	p=0.0010
WT vs. R6/2;Ob/Ob	p=0.0010	p=0.0518	p=0.0350	p=0.0253	p>0.9999
R6/2 vs. Ob/Ob	p<0.0001	p=0.0756	p=0.0098	p=0.0116	p<0.0001
R6/2 vs. R6/2;Ob/Ob	p<0.0001	p=0.2114	p=0.8430	p=0.0596	p=0.0282
Ob/Ob vs. R6/2;Ob/Ob	p<0.0001	p>0.9999	p=0.6906	p>0.9999	p=0.0419

Table 2. Metabolic parameters

Males at 20 weeks. (CAG 345-352).

One-Way with Holm-Sidak post hoc multiple comparisons test or Kruskal-Wallis with Dunn's post hoc multiple comparisons test.

	Glucose levels (mmol/L)	Insulin levels (µg/L)	HOMA-β	HOMA-IR
F-Statistics	F (3, 29) = 13.01, p<0.0001			
WT vs. R6/2	p=0.0016	p>0.9999	p=0.0854	p>0.9999
WT vs. Ob/Ob	p=0.0730	p=0.0025	p=0.1981	p=0.0001
WT vs. R6/2;Ob/Ob	p<0.0001	p=0.4949	p>0.9999	p=0.0224
R6/2 vs. Ob/Ob	p=0.0955	p<0.0001	p<0.0001	p=0.0002
R6/2 vs. R6/2;Ob/Ob	p=0.0955	p=0.0060	p=0.0449	p=0.0508
Ob/Ob vs. R6/2;Ob/Ob	p=0.0017	p=0.3313	p=0.1565	p=0.8222

	Total cholesterol (µg/ml)	HDL levels (µg/ml)	LDL/VLDL levels (µg/ml)	LDL/VLDL : HDL ratio
F-Statistics	F (3, 20) = 2.134, p=0.1279	F (3, 20) = 2.958, p=0.0571	F (3, 20) = 6.42, p=0.0032	F (3, 20) = 21.25, p<0.0001
WT vs. R6/2	p=0.4511	p=0.7307	p=0.0034	p<0.0001
WT vs. Ob/Ob	p=0.1287	p=0.0598	p=0.5076	p=0.4065
WT vs. R6/2;Ob/Ob	p=0.4511	p=0.5028	p=0.2599	p=0.4065
R6/2 vs. Ob/Ob	p=0.7730	p=0.1918	p=0.0138	p<0.0001
R6/2 vs. R6/2;Ob/Ob	p=0.9468	p=0.7307	p=0.1134	p=0.0003
Ob/Ob vs. R6/2;Ob/Ob	p=0.7730	p=0.5028	p=0.5076	p=0.0705