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**Analysis of Cholesterol Biosynthetic Pathway
in Huntington’s Disease.
The role of Liver X Receptors**

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INTRODUCTION

1.1 Cholesterol as Structural and Regulatory Component of Brain Cells and Membranes

Cholesterol represents an important component of eukaryotic cell membranes.

Cholesterol modulates membrane fluidity and organisation [1] where it can alter signalling functions, membrane protein organisation and lipid raft structure [2,3].

Cholesterol is also involved in vitamin D production and synthesis of bile acids and steroid hormones [4].

Cholesterol is not only an essential structural component for cellular membrane, a precursor of steroid hormones and bile acid synthesis, but also a required component for myelin, synapse and dendrite formation [5,6] and axonal guidance [7]. Brain is the most cholesterol-rich organ containing about 20% of the whole body's cholesterol [8]. Brain lipids consist of glycerophospholipids, sphingolipids, and cholesterol, they are in roughly equimolar proportions.

The major sterol in the adult brain is unesterified cholesterol; larger portion of this fraction (about 70%–80%) is in myelin sheaths formed by oligodendrocytes to insulate axons; the remaining portion is made up by plasma membranes of astrocytes and neurons to maintain their morphology [9].

Cholesterol can also influence cell function through oxysterols, its biologically active side-chain oxidized products [10-12].

Cholesterol depletion in neurons impairs synaptic vesicle exocytosis, neuronal

activity and neurotransmission and it leads to dendritic spine and synapse degeneration [13-14].

Cholesterol is essential for neuronal physiology, both during development and in the adult stage.

Cholesterol has the common structure of a sterol, containing a carbon 3 hydroxyl group and a tetracyclic steroid ring. Specifically, cholesterol contains a 3β hydroxyl group and an isooctyl hydrocarbon tail (Figure 1). The opposing polarity of the hydroxyl group and hydrocarbon structure of the remaining molecule gives to cholesterol its amphipathic nature.

1.2 Cholesterol Biosynthesis

All nucleated cells can synthesize cholesterol [15].

Synthesis of cholesterol, like that of most biological lipids, begins from the two-carbon acetate group of acetyl-CoA.

Acetyl-CoA units are converted to mevalonate by multiple reactions that begin with the formation of 3-hydroxyl-3-methylglutaryl-CoA (HMG-CoA) by the action of HMG-CoA synthase encoded by the HMGCS1 gene.

HMG-CoA is then converted to mevalonate by HMG-CoA reductase (HMG-CoAR). This reaction is the rate limiting step of cholesterol biosynthesis, and this enzyme is subject to complex regulatory controls as discussed below. Mevalonate is then activated by two successive phosphorylations and sequentially converted into isoprene based molecule isopentenyl pyrophosphate (IPP); IPP is converted into

squalene that undergoes a two-step cyclization to yield lanosterol. Through further 19 additional reactions, lanosterol is converted into cholesterol (Figure 2).

These 19-reaction steps are catalyzed by nine different enzymes that are localized either in endoplasmic reticulum (ER) or in peroxisomes.

Less than an half of the cholesterol in the body derives from *de novo* biosynthesis and the remaining is provided by the diet. Dietary cholesterol is absorbed by small intestine, loaded on lipoproteins and delivered to the liver. *De novo* synthesis or uptake from lipoproteins provides cellular cholesterol needs.

Cholesterol synthesized by the liver, as well as any dietary cholesterol in the liver that exceeds hepatic needs, is transported in the serum predominantly as cholesteryl esters associated with lipoproteins (LDL) to peripheral tissues. The free cholesterol within the cell has the following fates: incorporated into cell membranes; metabolized to steroid hormones of adrenal cortex and gonads; esterified with saturated fatty acids and stored in cells.

Cholesterol found in plasma membranes can be extracted by HDLs, esterified by the HDL-associated enzyme lecithin-cholesterol acyltransferase (LCAT), transferred to VLDLs and LDLs and finally re-transferred to the liver [16].

Average diet contains about 300 mg of cholesterol per day. Body synthesizes about 700 mg /day. About 500 mg of cholesterol is excreted through the bile. Some part is reabsorbed from intestine, remaining portion is excreted in bile as free cholesterol or as bile salts as result of conversion to bile acids in the liver [17].

1.3 Cholesterol turnover

Since an excess of free cholesterol is toxic, all body cells adopt several defensive strategies such as exporting it via lipoprotein, storing it in as esterified form or releasing it after oxidation into oxysterols [18]. Cholesterol and oxysterols are directly involved in a negative feedback mechanism of the enzyme regulation both at the protein and the transcriptional level [19].

The continual alteration of the intracellular sterol content occurs through the regulation of key sterol synthetic enzymes as well as by altering the levels of cell-surface LDL-receptors. As cells need more sterol they will increase synthesis and uptake, conversely when the need is lower synthesis and uptake decreases.

Regulation of these events is brought about primarily by degradation and by sterol-regulated transcription of HMGCoAR [20]. Indeed, when cholesterol is in excess it induces a rapid degradation of HMGCoAR. In addition, the amount of mRNA for HMGCoAR is reduced as a result of decreased expression of the gene.

Activation of transcriptional control occurs through the regulated cleavage of the membrane-bound transcription factor sterol regulated element binding proteins (SREPBs). This transcription factor regulates lipid homeostasis in cells by activation of more than 30 genes involved in the synthesis and uptake of cholesterol, fatty acids, triglycerides and phospholipids.

SREPBs are expressed as inactive precursors (pSREPBs) integral to the ER membrane. When intracellular cholesterol levels are low, pSREPBs are translocated from the ER to the Golgi *apparatus* by an escort protein, SREPB cleavage-activating

proten (SCAP), where they are cleaved into active not membrane-bound trascription factors. These shorter mature SREPBs (mSREPBs) enter in the nucleus and modulate transcription of genes containing an octamer sequence in the promoter region, termed the sterol regulatory element (SRE-1). When intracellular cholesterol levels are in excess, SCAP, which has a cholesterol-sensing domain, binds insulin induced gene (Insig) and the Insig-SCAP-pSREPB is retained in the ER reducing cholesterol synthesis [21,22].

High concentration of cholesterol activates the expression of SREPB gene via the action of Liver X receptors (LxRs). The LxRs are members of the steroid/thyroid hormone superfamily of cytosolic ligand binding receptors that migrate to the nucleus upon ligand binding end regulate gene expression by binding to specific target sequences. There are two forms of the LxRs: LxR α (NR1H3) and LxR β (NR1H2). LXR α heterodimerize with retinoid X receptors (RxRs) and are activated by specific cholesterol derivatives that function as endogenous ligands [23-27].

Well known set of ligands are oxysterols, oxidized metabolites of cholesterol that are present in very low concentrations in mammalian systems and are always accompanied by high excess of cholesterol (for instance, 27-hydroxycholesterol, 22(R)-hydroxycholesterol (22-HC) and 24(S)-hydroxycholesterol (24(S)-HC) [28].

More recently, cholestenoic acids have also been identified as endogenous LXR ligands [29], suggesting novel functions for LXR ligands in the developing and the adult brain.

The best known functions of Lxrs are the regulation of lipid metabolism and

homeostasis [27, 30-32], increases of cholesterol efflux from cells [27,33,34], and protection from cholesterol overload and toxicity [33,35]. Accordingly, direct LXR target genes encode for ATP-binding cassette (ABC) transporters (ABCA1 and ABCG1/5/8), apolipoproteins (E, C1, C2, C4, and D), cytochrome P450 7A1 (in rodents but not in humans), and enzymes involved in lipogenesis such as SREBP1c, FAS, and stearoyl-CoA desaturase-1. In the last decade, additional functions of Lxrs and their ligands have been described. These include the regulation of inflammation [36] and different aspects of the immune response [37], including the regulation of the balance between cell survival and death in macrophages [38].

1.4 Brain cholesterol metabolism

The amount of sterol in brain ranges from 15 to 20 mg per gr in many species as unesterified cholesterol [9]; however, small amount of desmosterol and cholesteryl ester are also present.

Brain cholesterol is primarily supplied by *de novo* synthesis due to the prevention of lipoproteins uptake from the circulation by blood brain barrier (BBB) [8].

Brain cholesterol synthesis follows the same pathway present in peripheral tissues as described before.

The majority of brain cholesterol accumulates between the perinatal period and adolescence when neurons are encircled by myelin. This *de novo* synthesis is adequate accounting for the cholesterol accumulation rate during early development, when myelin production takes place by oligodendrocytes [9]. The highest cholesterol

synthesis rate in human, in fact, takes place when the peak of myelination process occurs, the myelination process is delayed when cholesterol synthesis is deficient [39]. When the process of brain maturation and myelin formation is concluded in the adulthood, the neurons down-regulate their cholesterol synthesis and rely on delivery of cholesterol from astrocytes that differentiate post-natally and release cholesterol rich lipoproteins (the “outsourcing” hypothesis of brain cholesterol metabolism) [40-42]. There may be several reasons for this process. The high energy cost and the need for a large number of enzymes in different cellular compartments may explain why neurons, whose primary function is electrical synaptic transmission, abandon cholesterol synthesis after foetal development. The elongated shape of the neuron may also hinder transport of cholesterol from the site of its production in the cell body to the site where it is needed.

Astrocytes release cholesterol on HDL-like particles with ApoE as apolipoprotein via ABC-A1 and ABC-G1 transporters [43,44]. The ApoE lipoproteins are internalized into neurons by the LDL-receptor (LDL-R) and the LDL-related protein (LRP), but also by to some extent via VLDL receptor, ApoE receptor 2, and megalin. The ApoE-receptor-cholesterol complexes are delivered to the late endosomes/lysosomes where acid lipase hydrolyzes the cholesterol esters within the lipoprotein complexes, with the intracellular release of free cholesterol. This unesterified cholesterol exits the late endosomes/lysosomes via a Niemann–Pick type C (NPC) 1 and NPC 2 protein-dependent mechanism and it is distributed to the plasma membrane as well as to the ER; as a result unesterified cholesterol acts as a

negative feedback sensor for the cholesterol homeostasis genes such as HMGCoAR and LDL-R. The cholesterol in excess, on the other hand, is esterified in the ER by acyl-CoA-cholesterol acyltransferase (ACAT) and stored in cytoplasmic lipid droplets as a reserve pool. This intracellular pool of cholesterol is necessary for synaptic and dendritic formation and in membrane remodeling.

When cholesterol synthesis rate exceeds its acquisition rate in the brain, the excess of cholesterol must still be removed from the brain; but the BBB is impermeable to cholesterol, therefore simple transfer of cholesterol into blood stream does not occur. It has been suggested that cholesterol can move into the cerebrospinal fluid (CSF) as ApoE particles and then into the plasma. However, this accounts only for a small amount of cholesterol removed to the periphery, indicating other mechanisms must be at play.

Conversion to oxysterol is the major excretion way in the brain. The addition of a hydroxyl group to the alkyl chain of the cholesterol molecule significantly increases cell membrane permeability; side chain oxidised sterols move across membranes approximately 1500 times faster than cholesterol [45]. A cytochrome P-450, cholesterol 24- hydroxylase (CYP46A1), expressed primarily in neurons, catalyses this reaction [46].

It is well established that neuronal cells regulate their cholesterol content by an exquisite feedback mechanism that balances biosynthesis, import and excretion. Cells sense their level of cholesterol by membrane-bound transcription factors known as sterol regulatory element-binding proteins (SREBPs) that regulate the transcription of

genes encoding enzymes of cholesterol and fatty acid biosynthesis as well as lipoprotein receptors [47] to either increase cholesterol synthesis and uptake in sterol-depleted cells or decrease cholesterol-synthesizing enzymes when sterols are overloaded in cells [48,49].

When cholesterol reaches the maximal safe level, oxysterols act as an endogenous ligand of the LXRs which increase the expression of cholesterol transport genes [50] including ABCA1 in both neuron and glia cell [51], apoE in astrocyte [40,52]; consequently cholesterol efflux is increased (Figure 3).

1.5 Cholesterol and Neurodegenerative Disease

Cholesterol is an essential molecule for cellular function; however, excess cholesterol circulating in the body can be harmful. It has been established that high levels of cholesterol in the blood contributes to a number of diseases, in particular atherosclerosis and cardiovascular disease [53]. The influence of altered cholesterol homeostasis in neurodegenerative diseases is, however, not well understood. Cholesterol levels have been reported to alter in vitro protein aggregation relevant to Parkinson Disease [54,55]. Several studies have also highlighted altered brain cholesterol levels in Alzheimer Disease (AD) [51,56,57], and may influence amyloid beta formation by altering the physical properties of cell membranes [58]. It is also hypothesised that cellular redistribution of cholesterol without changes in total cholesterol levels may play a role in AD [59].

Parkinson's disease is a progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra and the accumulation of α -synuclein (Lewy bodies). Several interesting findings have shown the occurrence of equivalent Parkinsonian pathologies in cellular neuronal models, mediated by oxysterols or excess exposure to cholesterol. In this regard, oxysterols are particular in causing alpha-synuclein aggregation and destruction of dopamine containing neurons in in vitro models, which is linked to their direct influence on oxidative stress provoking potency. In spite of the significant in vitro reports, which suggest the relativeness of cholesterol or oxysterol towards Parkinsonism, several prospective clinical reports provided a negative or no correlation [54]. Several in vivo studies have shown a relationship between the lipid profile, the use of drugs regulating cholesterol levels, and the development of Parkinson's disease. Several oxysterols are present in the brain and could play a role in the development of this disease, particularly in the accumulation of α -synuclein, and through various side effects, such as oxidation, inflammation, and cell death. Consequently, in Parkinson's disease, some oxysterols could contribute to the pathophysiology of the disease and constitute potential biomarkers or therapeutic targets [55].

Alzheimer's disease is a neurodegenerative disorder characterized by progressive and irreversible memory impairment and cognitive decline. The pathological hallmarks of AD are extracellular amyloid plaques of amyloid β ($A\beta$) peptide and intracellular neurofibrillary tangles. Cholesterol is found to be enriched

in the brain plasma membranes of AD patients. The cholesterol level increases throughout the course of clinical disease, and more increase was observed when the disease progresses. In vitro study indicated that overload of cholesterol at plasma membrane in primary cultured neurons leads to an increase of A β production through increasing BACE1-mediated APP cleavage. APP intracellular domain (AICD) release increases during this process, which down-regulates low density lipoprotein-related protein 1 (LRP1) transcription that is responsible for exogenous cholesterol capture at the plasma membrane, this ultimately results in a decrease of cellular cholesterol levels[56, 57].

Due to conflicting reports of cholesterol levels in neurodegenerative brain tissue, the exact influence of altered cholesterol homeostasis in neurodegenerative diseases is currently unknown.

1.6 Huntington's disease

HD is an autosomal dominant, progressive, neurodegenerative disease characterised by the expansion of a trinucleotide repeat on the N-terminus of the huntingtin protein (HTT). The cytosine-adenine-guanine (CAG) repeat, coding for glutamine, is located on exon 1 of the HTT gene [60].

The exact role of the CAG mutation on the HTT protein in HD pathogenesis is still debated. It is not completely clear if mutant huntingtin (mHTT) has a toxic gain of function or if a loss of function is responsible for disease, or possibly a combination of both factors. The heterozygous disruption of the HTT gene does not cause a HD phenotype in human cases, suggesting that the loss of function in one

allele is not entirely responsible for disease pathology [61]. Expression of normal length HTT can reduce the toxicity of mHTT in mice [62] and it suggests compensation for loss of function. Another debated hypothesis of mHTT toxicity involves protein aggregation. Aggregation of mHTT is a hallmark of HD; however, this may not be an accurate predictor of cell death. Aggregate formation is higher in cortical neurons, that are relatively spared compared to the selectively vulnerable striatal neurons that contain fewer aggregates [63]. In vitro studies investigating mHTT aggregation have also reported neuronal death without the formation of inclusions, and that cells forming inclusions had reduced risk of death [64]. In those studies cells forming inclusions had reduced levels of mHTT compared to cells without inclusions suggesting that inclusion formation may be protective. Although inclusions may not be directly linked to cell death, other lines of evidence suggest that proteasomal disruption in cells caused by mHTT aggregates may be neurotoxic [65].

Although huntingtin is ubiquitously expressed, the brain is primarily affected in HD, with the striatal medium spiny neurons and cortical neurons being particularly vulnerable [66].

Huntington's disease shows a stable prevalence in most populations white of people of about 5–7 affected individuals per 100 000.

Individuals with Huntington's disease can become symptomatic at any time between the ages of 1 and 80 years; before then, they are healthy and have no detectable clinical abnormalities. This healthy period merges imperceptibly with a prediagnostic phase, when patients show subtle changes of personality, cognition, and

motor control. Diagnosis takes place when findings become sufficiently developed and specific. During the diagnostic phase the affected individuals show distinct chorea, incoordination, motor impersistence, and slowed saccadic eye movements. Cognitive dysfunction in Huntington's disease, often spares long-term memory but impairs executive functions, such as organising, planning, checking, or adapting alternatives, and delays the acquisition of new motor skills. Unlike cognition, psychiatric and behavioural symptoms arise with some frequency but do not show stepwise progression with disease severity. Depression is typical and suicide is estimated to be about five to ten times higher compared to general population [67].

As motor and cognitive deficit becomes severe, patients eventually die, usually from complications of falls, inanition, dysphagia, or aspiration. Typical latency from diagnosis to death is 20 years.

1.7 Cholesterol Metabolism In Huntington Disease

Molecular and biochemical studies in cellular and animal models have shown that cholesterol biosynthesis is altered in HD [68].

Decreased amounts of 24S-OHC have been reported in transgenic and knock-in mouse model of HD [69]. In this context, brain cholesterol metabolism in the YAC transgenic HD mice appeared to be related to the repeat length, since disease-related decline of brain lathosterol, cholesterol, and 24OHC content was found to be proportional to the length of the CAG repeat [70]. In addition, in a knock-in HD mice

model, the heterozygous and homozygous animals had brain 24OHC reduced by 20% and 50%, respectively [71]. It seems likely from these studies that both the length of CAG repeat and the mtHtt load have negative effects on brain cholesterol synthesis (reduced lanosterol), and cholesterol turnover (reduced 24-OHC).

Similar observation has been reported in tissue and fluid samples from HD patients in whom the decrease of plasma 24-OHC associates to progression of disease proportionately to motor and neuropsychiatric dysfunction and MRI brain atrophy [72,73].

Further study focused on the relative importance of the brain-specific catabolite 24OHC, as a marker of HD progression, being an index of whole-body cholesterol elimination [74]. This hypothesis is based on a significant reduction in levels of 24OHC in plasma from HD patients.

Efflux of 24OHC is regarded as the main mechanism through which the brain removes daily excesses of cholesterol. Therefore, plasma 24OHC levels may be index of brain cholesterol elimination (and indirectly of cholesterol biosynthesis). On this basis, reduced levels of 24 OHC is supposed to indicate that cholesterol catabolism is reduced in brain.

The intriguing hypothesis that brain cholesterol abnormalities might contribute to HD pathophysiology may be further supported by the fact that 24OHC decreases in parallel with the volume of the caudate, a brain region specially affected in HD. In addition, blood concentration of lanosterol, lanosterol and 24OHC were significantly reduced in pre-manifest HD and HD patients. This suggests that whole-body

cholesterol homeostasis is impaired also in humans at pre-symptomatic stages of HD [75].

Further potential mechanism underlying cholesterol alterations in HD is the reduced activity of Sterol regulatory element binding proteins (SREBPs) that regulate the genes transcription of cholesterol biosynthetic pathway. SREBP activity was reduced in an inducible cell model of HD and in brain of HD mouse [76].

To elucidate new transcriptional pathways regulated by huntingtin, Futter et al. tested several brain expressed nuclear receptors (NRs) for their ability to interact with the N-terminal fragment of huntingtin (amino acids 1–588) with a normal (17Q) or expanded (138Q) polyglutamine repeat. They found that Liver X Receptors (LXR) bound to wild-type huntingtin via its ligand binding domain (LBD). Over-expression of huntingtin activates LXR mediated transcription and knockout of huntingtin decreases LXR mediated transcription. Furthermore, knockout of huntingtin decreases expression of ATP binding cassette transporter A1 (ABCA1), a key downstream target gene regulated by LXR. An agonist of LXR partially rescues the phenotype observed in huntingtin deficient zebrafish [77].

These data suggest that huntingtin can regulate LXR mediated transcription and may play a general role as a cofactor of nuclear receptors. LXR transcriptional deficiency may contribute to the phenotypes of huntingtin defects *in vivo*.

STUDY DESIGN

2.1 Aims

The mechanisms underlying cholesterol perturbations are still poorly understood and their potential role in the pathogenesis of HD is not well ascertained.

To our knowledge, no study has evaluated the cholesterol profile of HD patients as well as investigated the role of LXRs in the pathogenesis of HD.

Aim of this study is to assess a potential association between nuclear transcription factors related to cholesterol metabolism such as LXRs and the cholesterol dysfunction in HD patients. Indeed, in order to confirm in humans the hypothesis that mutant Htt is less able to up-regulate LXR α and LXR β targeted-genes, we have evaluated gene expression of LXR α and β and LXR target genes (ABCA1, ApoE, SREBP, CYP7B1) in HD patients. Furthermore, motor and cognitive markers of progression have been assessed and compared with LXRs levels, in order to find a correlation between levels of LXRs and severity of HD disease.

2.2 Materials and Methods

The study was performed in a cohort of 130 consecutive patients referred to the HD Clinics at the Department of Neurosciences and Reproductive and Odontostomatologic Sciences, Federico II University, Naples.

Inclusion criteria for HD patients were: age 18-60 years, manifest signs and symptoms of the disease and positive molecular test for the presence of a CAG triplet repeat expansion > 35 in the HD gene.

Being aware that plasma levels of oxysterols might be influenced by factors as lipoproteins transport and liver clearance, we excluded participants with history of Diabetes, dyslipidemia, metabolic syndrome or other endocrine disorders, chronic/acute liver dysfunction and chronic systemic disease. Also, potential effects of lipid lowering therapies (such as cholestiramine and statin treatments or phytosterol intake) on oxysterols levels were taken into account and participants on statin therapy were excluded [78]. In order to eliminate the effects of an inadequate diet on levels of cholesterol and its metabolites, alimentary diary and appropriate were supplied the day before. Finally, blood samples were collected after an overnight fasting according to standard procedure for lipid study in clinical chemistry [79]. Patients confined to bed/wheelchair, unable to cooperate with the study and those on treatment with antipsychotic agents therapy were excluded. In the latter cases to exclude the influence of antipsychotic drugs on lipid metabolism [80,81].

Clinical assessment of patients was performed using the Unified Huntington's Disease Rating Scale (UHDRS) motor section and the Total Function Capacity (TFC) [82,83]. The following cognitive assessment were used: three Stroop tests (color, word, and interference) and the Symbol Digit Modalities Test (SDMT) [84].

The HD patients were stratified according to their neurological status using the median values of TFC and UHDRS-TMS as cut-off.

All samples were collected between 9,00 and 10,00 a.m. in fasting conditions and a sample (5 ml) of peripheral blood was collected in Vacutainer tubes with EDTA and processed with FicollPaque (GE Healthcare) according to the manufacturer's

instructions. Peripheral blood mononuclear cells (PBMCs) isolated from blood samples were collected.

Metabolic investigations included total cholesterol serum levels, triglyceride, High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL), glucose levels.

Analysis of LXR α , ABCA1, ApoE, CYP7B1 and SREBP mRNA extracted from peripheral total blood taken from HD patients and controls will be measured by Radioactive Semiquantitative Reverse Transcription-PCR (q-PCR) and conducted in the Laboratory of the Department of Neurosciences and Reproductive and Odontostomatologic Sciences, Federico II University, Naples.

Total RNA will be extracted from whole blood using Paxgene blood RNA system (Qiagen, Valencia, CA, USA) followed by purification with RNeasy Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. RNA concentration will be determined spectrophotometrically at 260 nm, and the 260/280 nm ratio will determine the quality of the purification. In addition, RNA quality will be estimated by running RNA on agarose gel. Complementary DNA (cDNA) will be synthesized from equal amounts of total RNA and following the manufacturer's protocol using SuperScript III reverse transcriptase (Invitrogen) with Random Hexamer primers. LXR α , ABCA1, ApoE, CYP7B1 and SREBP mRNA will be measured by TaqMan real-time PCR assay, using glyceraldehyde-3-phosphate dehydrogenase (GAPD) as reference gene, and mRNA from HepG2 cells will be used as calibrator.

For q-PCR, the 50 ng of cDNA will be added in reactions performed on an

StepOnePlus Instrument (Applied Biosystems, Foster City, CA, USA) using Taqman Universal PCR Master Mix (Applied Biosystem) and gene-specific primers. Primer for the selected gene (LXR, ABCA1, ApoE, CYP7B1 and SREPB) sequences will be obtained from the literature and checked for their specificity through *in silico* PCR. Primers for all amplifications will be designed using Primer3 software. Amplifications will be done as technical triplicates in 96-well plates. The relative quantification value of each target gene will be analyzed using a comparative Ct method. The following formula will be used to calculate the relative amount of the transcript in the sample and normalized to the GAPD as endogenous reference: $2^{-\Delta\Delta Ct}$, where ΔCt is the difference in Ct between the gene of interest and GAPD, and $\Delta\Delta Ct$ for the sample = mean ΔCt of sample – mean ΔCt of the HepG2 cells. The gene expression will be interpreted as the increase or reduction in relative mRNA level after atorvastatin therapy.

2.3 Statistical Analysis

Patients and controls were compared using the two-tailed Student's t test for paired data.

Comparison of basal data was performed using parametric or non parametric tests for continuous variables and the chi-square or Fischer's exact test as appropriate.

Linear regression analysis was employed to assess correlation between number of CAG repeats, TFC and UHDRS-TMS values and gene expression levels.

A $p < 0.05$ was considered statistically significant.

2.4 Results

From the initial cohort of among 130 consecutive patients afferent to the HD Clinics at the Department of Neurosciences and Reproductive and Odontostomatologic Sciences, Federico II University, Naples 14 HD patients met inclusion criteria and were compared with 14 healthy subjects recruited among healthy volunteers matched to the patients according to age, sex and body mass index (BMI).

Demographic data of patients and controls are reported in Table 1.

HD patients were divided in two subgroups according to the stage of the disease as determined by TFC and UHDRS. Cut-off value for stage I were $TFC > 12$ and $UHDRS-TMS \leq 20$ and for stage II it was $TFC \leq 12$ and $UHDRS-TMS > 20$.

HD patients did not differ from controls for levels of triglyceride, total and LDL cholesterol; they had significant lower HDL-cholesterol levels and a trend toward higher LDL-cholesterol levels (Table 2).

As shown in Figure 4-9, the mRNAs of LXR α and β , ABCA1, ApoE, CYP7B1 and SREPB showed similar levels in the HD and control group.

No correlation was observed between the different values of the measured relative transcript levels in HD patients and CAG repeats and disease stage (Figure 10-15).

2.5 Discussion

Huntingtin may play a regulatory role in the normal cerebral and extra-cerebral biosynthesis of cholesterol which is modified in the presence of mutant HTT. Consistent findings about an impairment of synthesis, accumulation and turnover were reported in animal models of disease together with a reduction of the expression of key regulatory genes. The transcriptional dysregulation reduces the translocation of SREBP, the activation of LXR and LXR-dependent genes. The dysfunction of the cholesterol metabolism is not restricted to the Central Nervous System (CNS).

Further support to the hypothesis that mutant huntingtin affects cholesterol levels comes from *in vitro* experiments showing that the biosynthesis of cholesterol and fatty acids is impaired in cells expressing disease-causing mutants of huntingtin. HD patients and HD mice show a progressive decrease of 24-OHC, which is a sign of neuronal cell loss. The decrease in this metabolite may further reduce cholesterol levels in the brain by impairing the activity of liver X receptors (LXRs), thus reducing expression of LXR-dependent transcripts like ABCA1 and ApoE and consequently the transport of cholesterol to neuronal cells. Evidence for a role of the LXR pathway in HD but not necessarily in ABCA1, ApoE and cholesterol levels in the brain comes from the observation that treatment with a LXR agonist partially reverts symptoms in a zebrafish model of HD.

Although cellular and animal models make LXRs as strong factors for pathogenesis of HD, no definitive data are currently available on the involvement of LXRs in HD patients.

To test whether mutant huntingtin induces gene expression changes in peripheral cells of HD patients we analyzed the RNA extracted from PBMC taken from control and HD patients. The results obtained reveal no differences of LxRs and LxRs target genes expression between HD patients and controls.

These results are in contrast with those of other studies. Valenza et al. analyzed the RNA extracted from primary fibroblasts of controls and HD patients and demonstrated that HD fibroblasts show a 35–45% reduction in HMGCoAR and Cyp51 mRNA levels. These data indicate that there is a significant reduction in the mRNAs for LxR target genes in HD subjects.

The present study has some limitations such as the limited number of subjects. Further limitation is the lack of assessment of the possible pathogenetic significance of LxRs mRNA expression in fibroblast of HD patients.

We cannot exclude a selection bias having excluded patients not treated with antipsycothics. This exclusion criteria allowed us to eliminate the effects of clozapine and olanzapine on weight, lipid abnormalities and diabetes through direct effects on insulin secretion and lipid biosynthesis and efflux genes associated with SREBP-stimulated transcriptional changes [80,81]. As a consequence our patients were likely in not advanced phase of HD disease and this could have influenced results.

In conclusion, the present data suggest that there is no impairment of LxR gene expression in peripheral cells of HD patients. However we can not exclude a possible interplay between Htt and LxR as pathogenetic factor of HD.

In any case the results of this study are hypothesis generating. In order to clarify the role of the above genes in alteration of cholesterol metabolism it would be interesting to analyze plasma levels of natural ligands of LXR levels such as oxysterols (lanosterol, lathosterol, desmosterol, 24-HC, 27-HC) and colestenoic acids (3β -HCA, $3\beta,7\alpha$ -diHCA, $3\beta,7\beta$ -diHCA) respect to controls and to extend the analysis to fibroblasts.

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TABLE 1. Demographic data in HD patients and controls

	PATIENTS	CONTROLS	p
Number of patients (m/f)	14(8/6)	14(8/6)	
Age, mean (years)	46,0 ± 10,8	45,6 ± 11,7	
Age et onset (years)	41,0 ± 10,9		
Disease duration, mean (years)	5,09 ± 4,6		
CAG repeat mean allele 1	18,3 ± 3,3		
CAG repeat mean allele 2	45,8 ± 4,9		
UHDRS, mean	26,0 ± 15,3		
TFC, mean	8,2 ± 4,1		
BMI, mean	24,7 ± 4,3	24,6 ± 7,4	0,71

TABLE 2. Biochemical data in HD patients and controls

	PATIENTS	CONTROLS	p
Total colessterol, mean (mg/dl)	192,2 ± 47,2	187,7 ± 24,6	0,66
LDL, mean (mg/dl)	127,8 ± 43,9	108,6 ± 25,6	0,05
HDL mean (mg/dl)	47,4 ± 13,3	58,7 ± 7,7	0,0004
Triglycerides, mean (mg/dl)	125,9 ± 95,2	100,1 ± 25,3	0,22
Total Bilirubin, mean (mg/dl)	0,5 ± 0,2	0,6 ± 0,1	0,56
Creatinine, mean (mg/dl)	0,8 ± 0,3	0,7 ± 0,4	0,63
Alanine Aminotransferase, mean (U/l)	32 ± 12	29 ± 11	0,83
Aspartate Aminotransferase mean (U/l)	19 ± 4	20 ± 6	0,76

FIGURE 1. Cholesterol chemical structure.

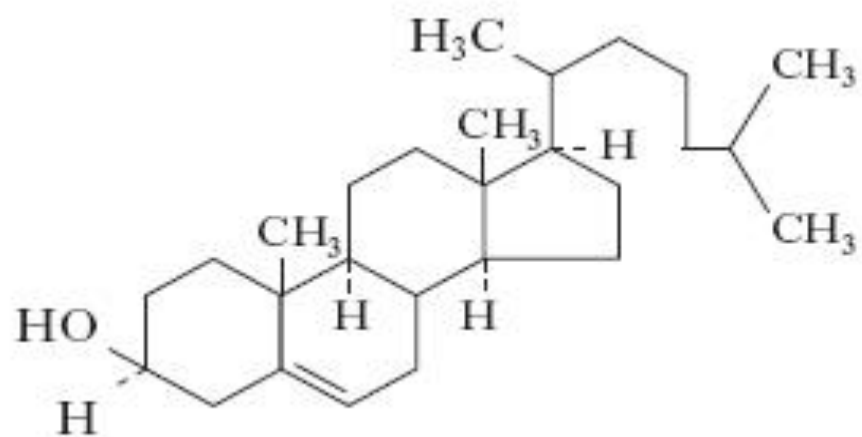
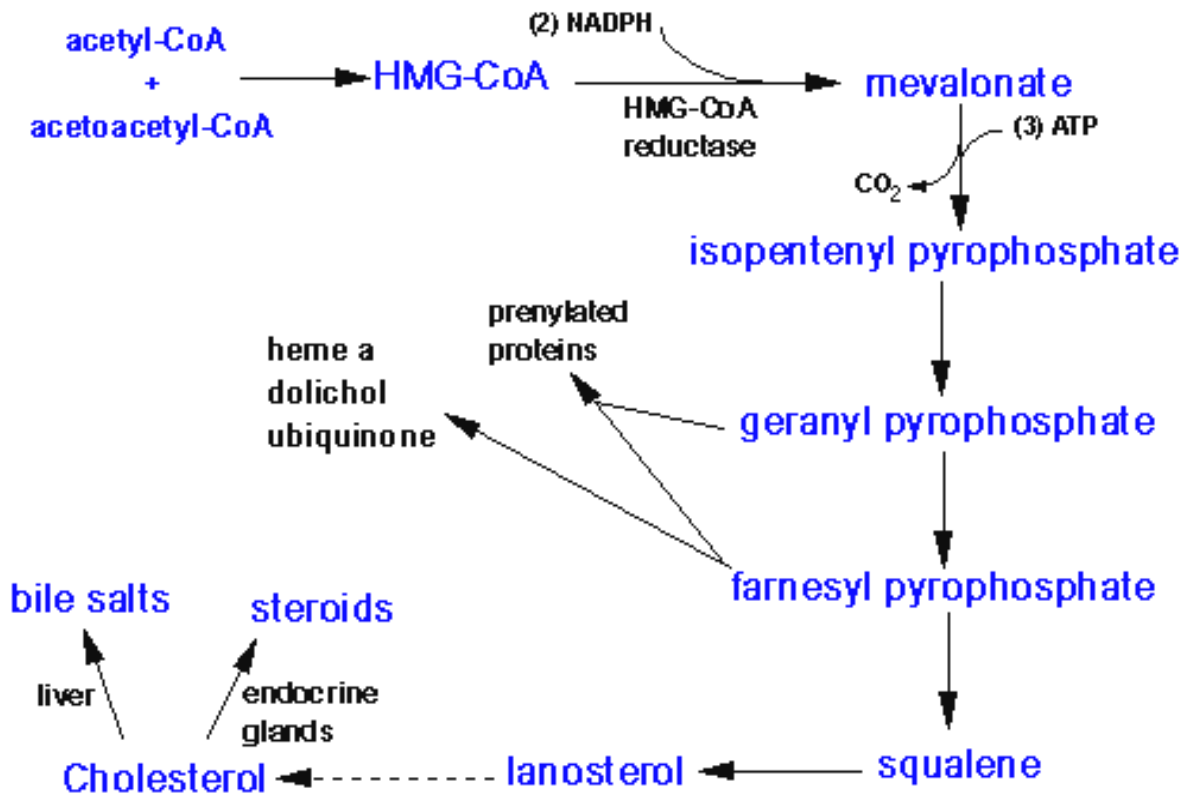


FIGURE 2. Cholesterol byosynthesis



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FIGURE 3. Brain cholesterol metabolism

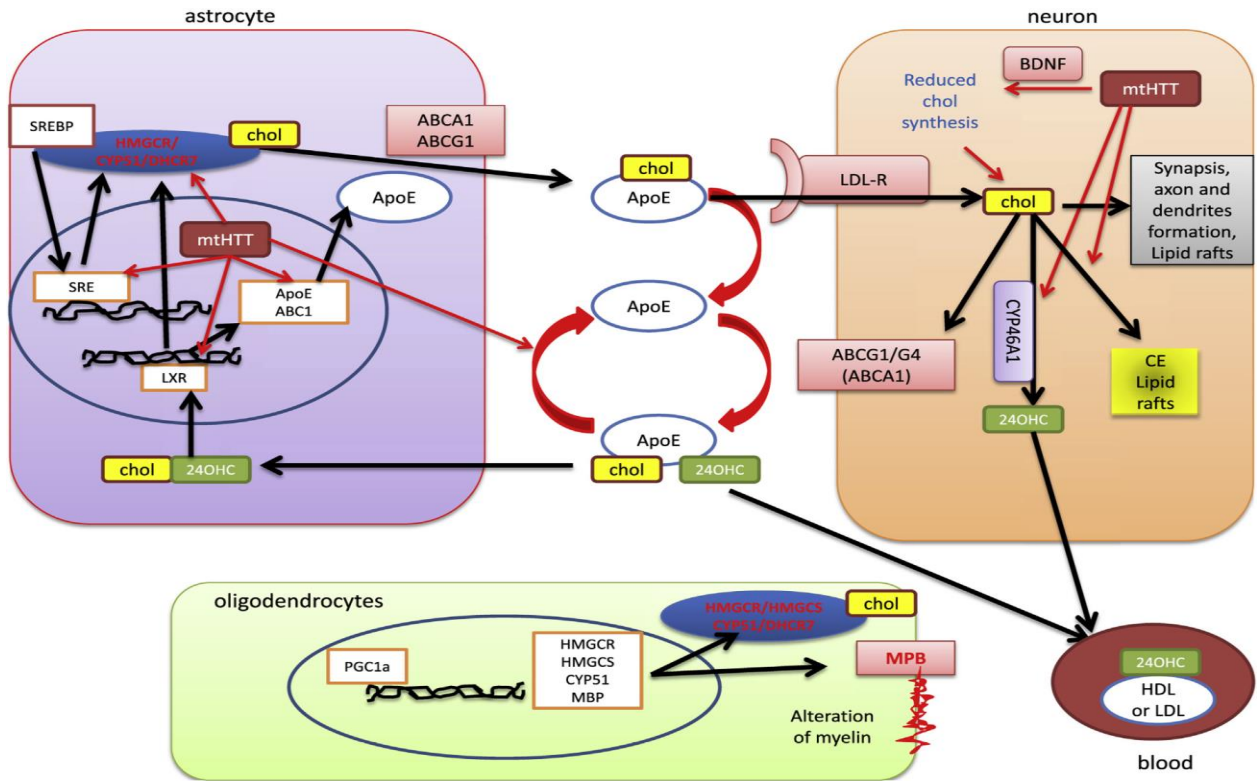


FIGURE 4. LxR β gene expression in HD patients and controls analysis by Taqman RT-PCR

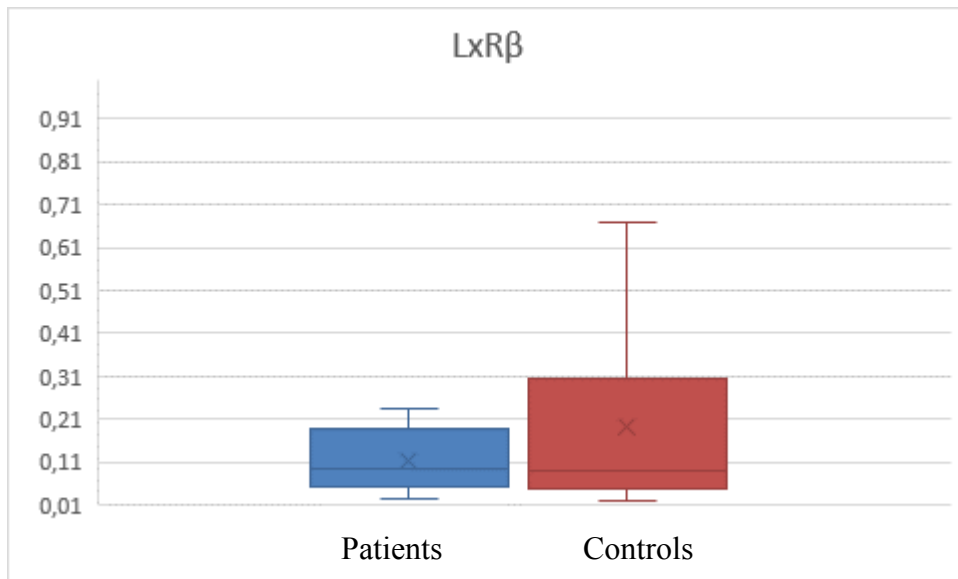


FIGURE 5. LxR α gene expression analysis in HD patients and controls by Taqman RT-PCR

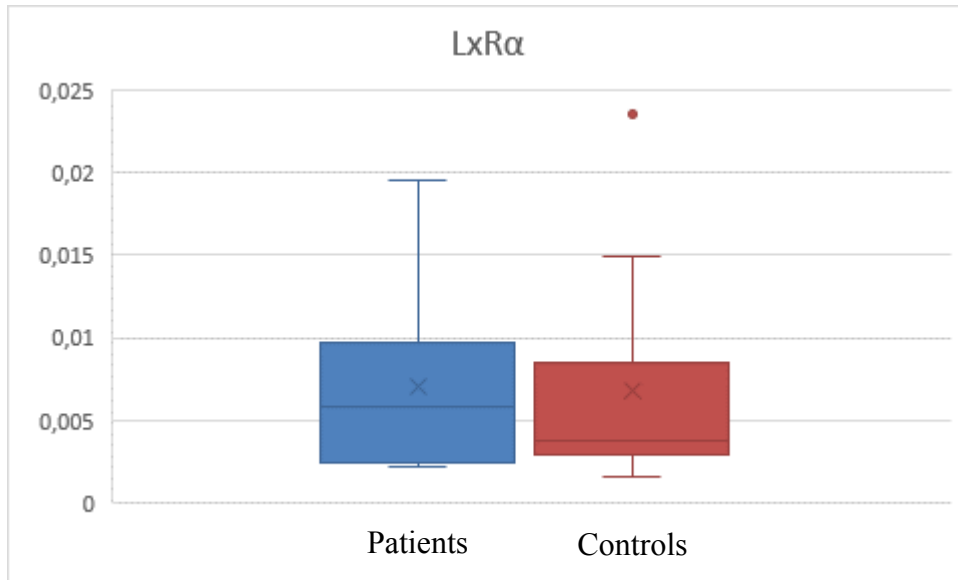


FIGURE 6. SREPB gene expression analysis in HD patients and controls by Taqman RT-PCR

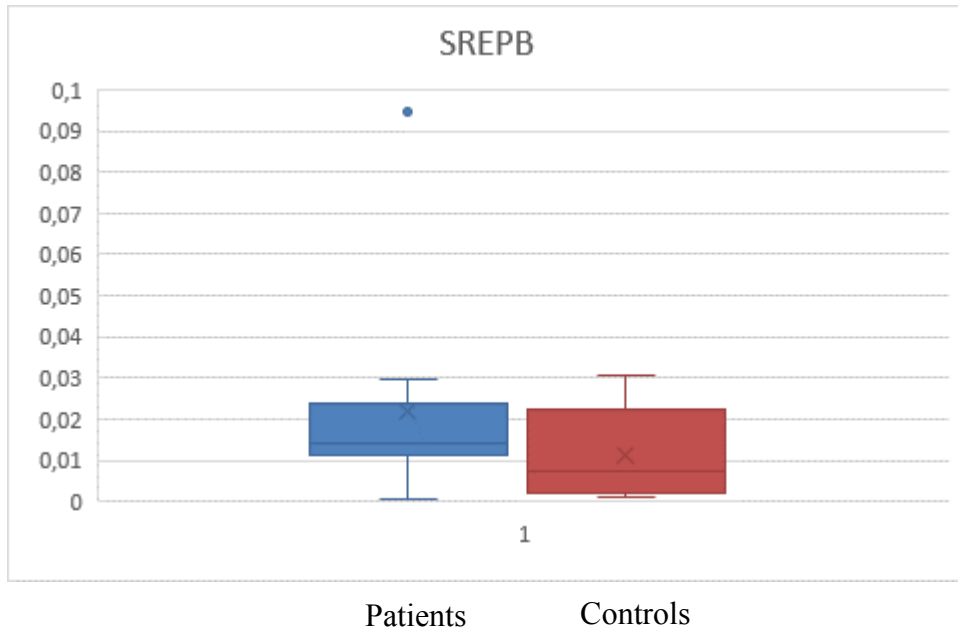


FIGURE 7. ABCA1 gene expression analysis in HD patients and controls by Taqman RT-PCR

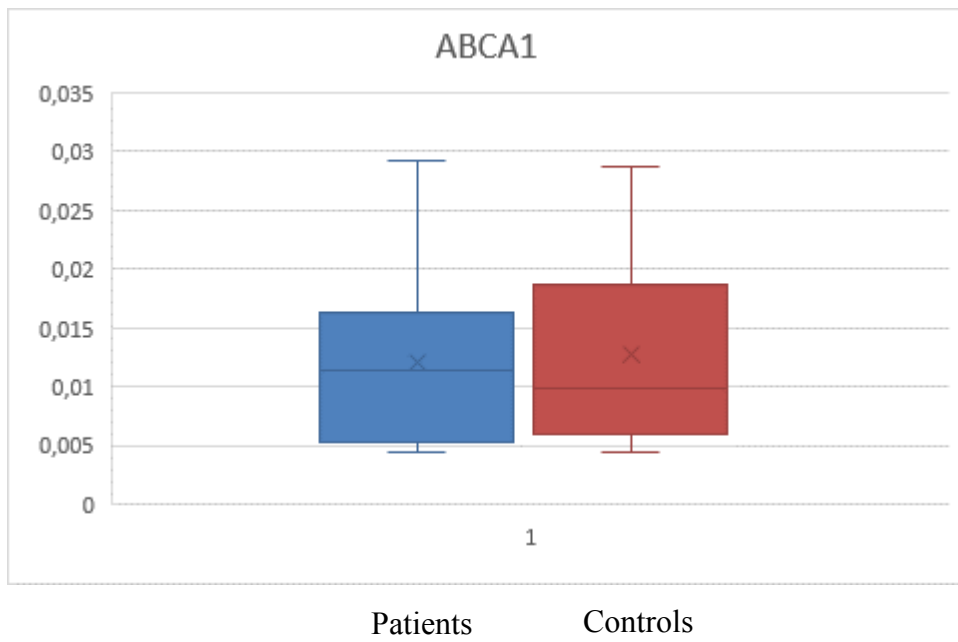


FIGURE 8. ApoE gene expression analysis in HD patients and controls by Taqman RT-PCR

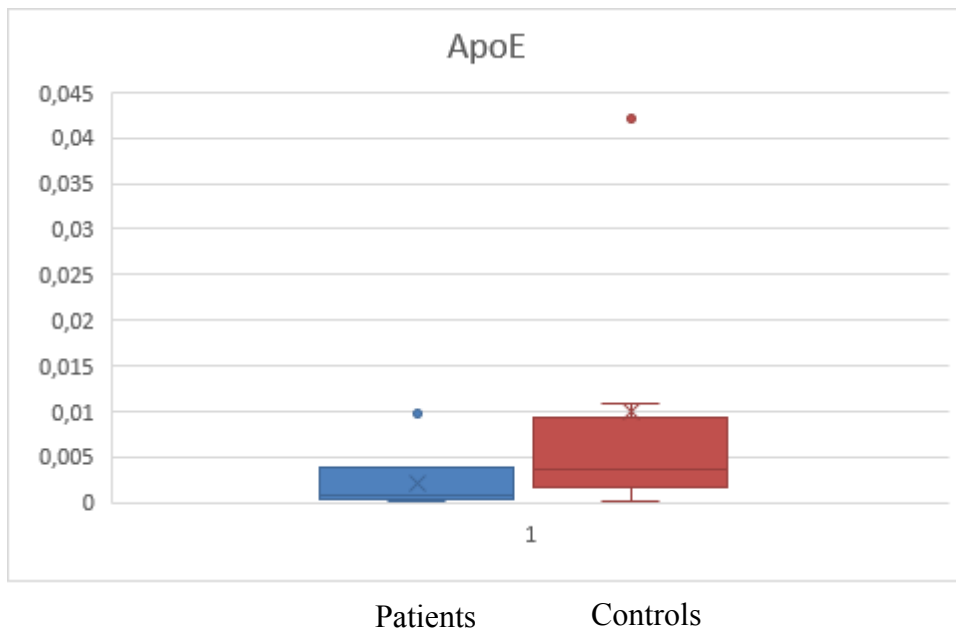


FIGURE 9. CYP7B1 gene expression analysis in HD patients and controls by Taqman RT-PCR

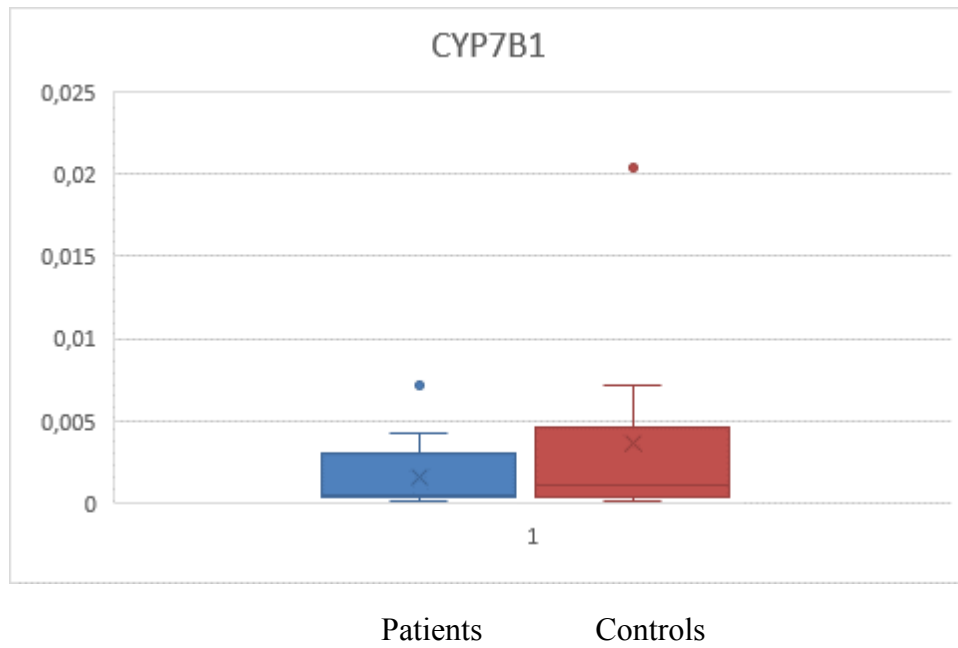


FIGURE 10. Correlation LxR β expression CAG and disease severity

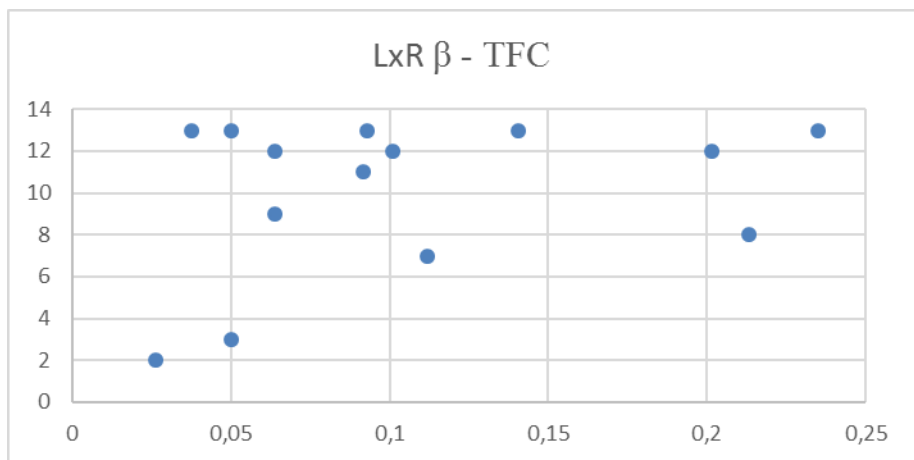
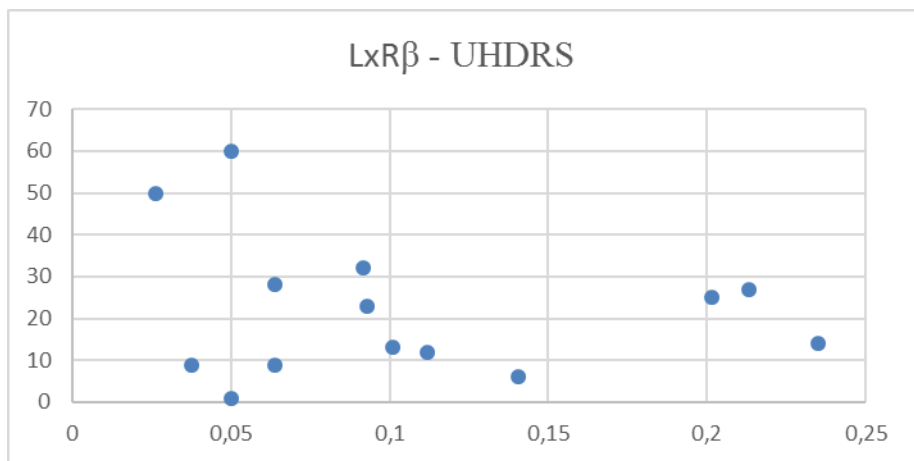
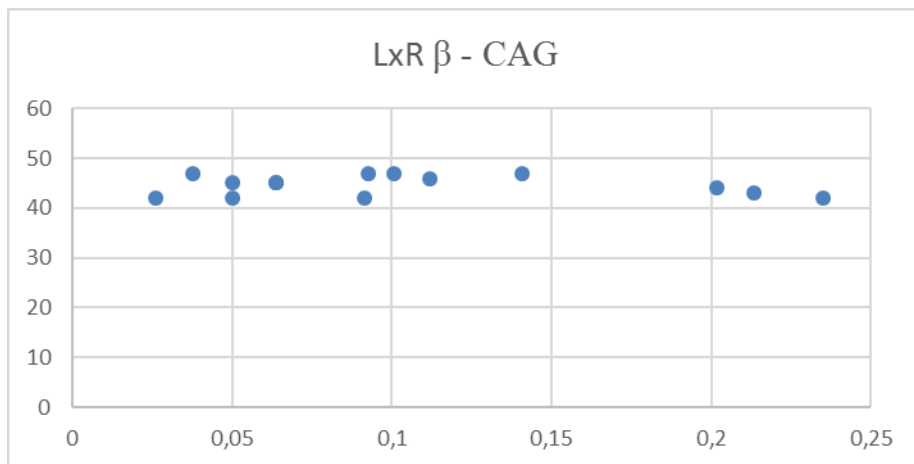


FIGURE 11. Correlation LxR α expression CAG and disease severity

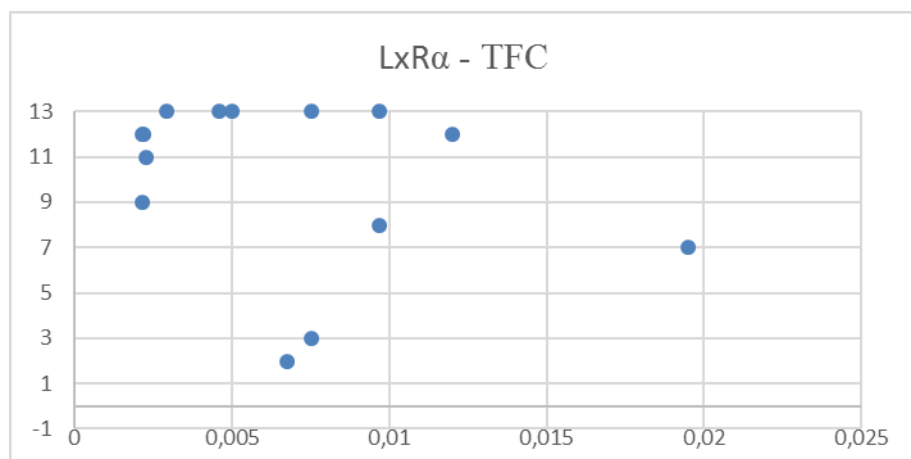
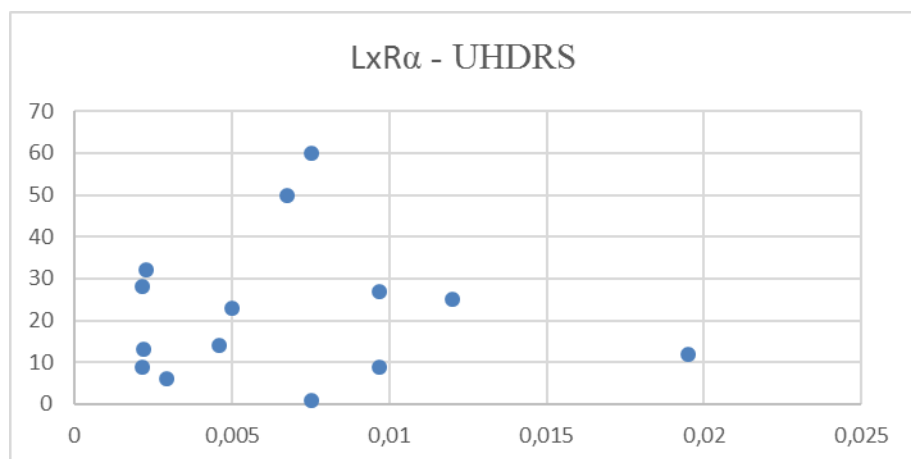
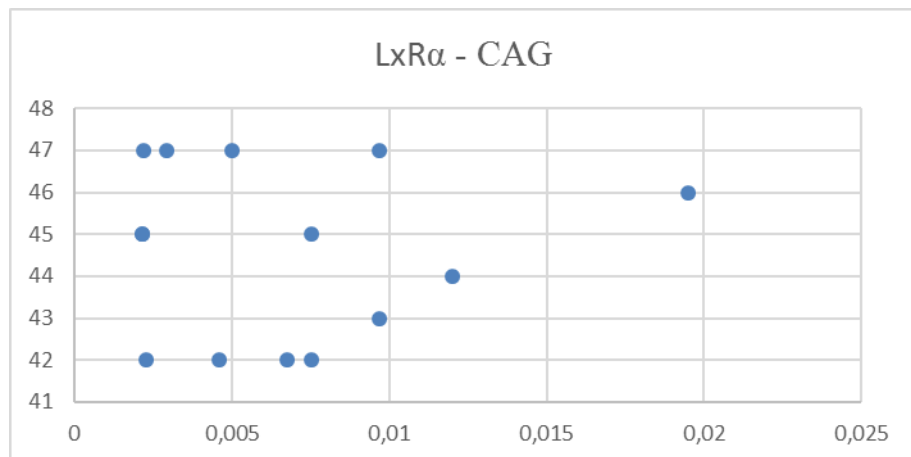


FIGURE 12. Correlation SREP expression CAG and disease severity

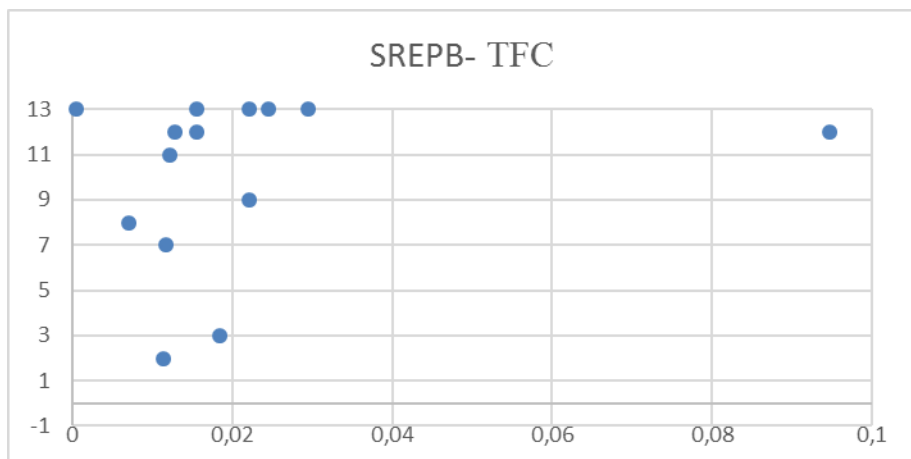
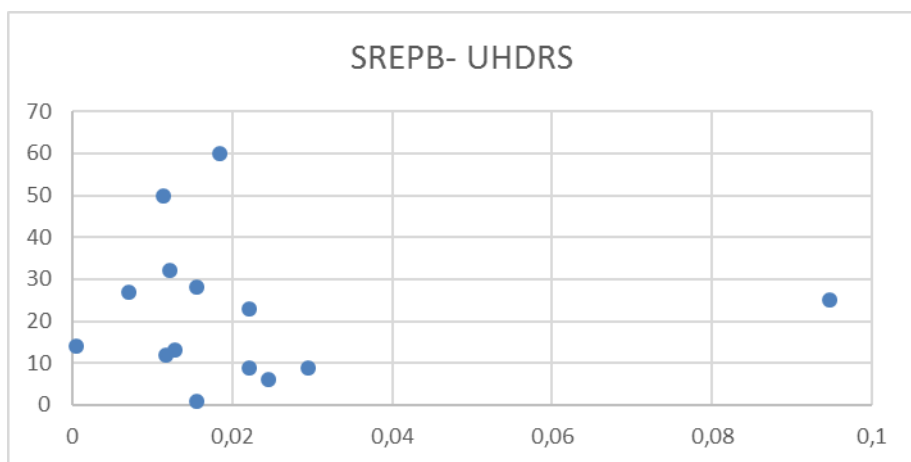
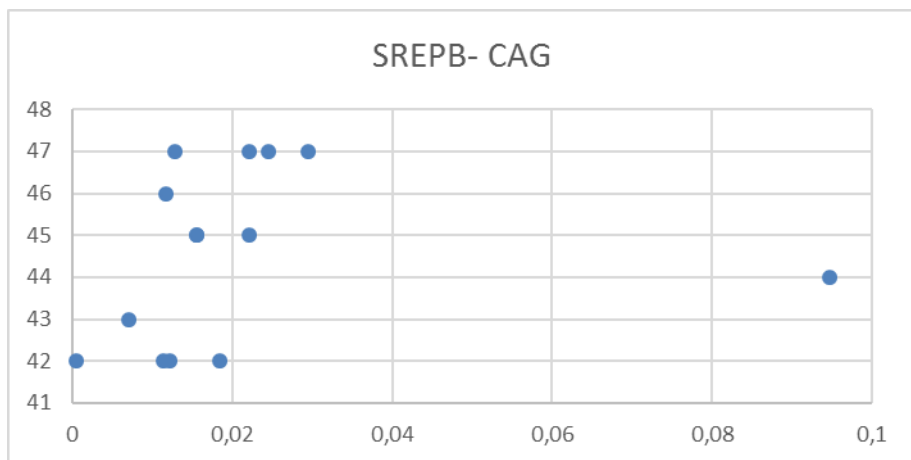


FIGURE 13. Correlation ABCA1 expression CAG and disease severity

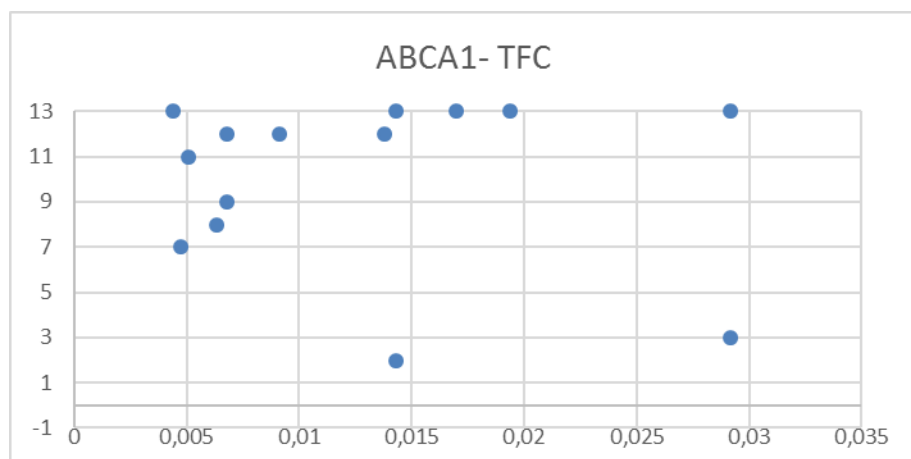
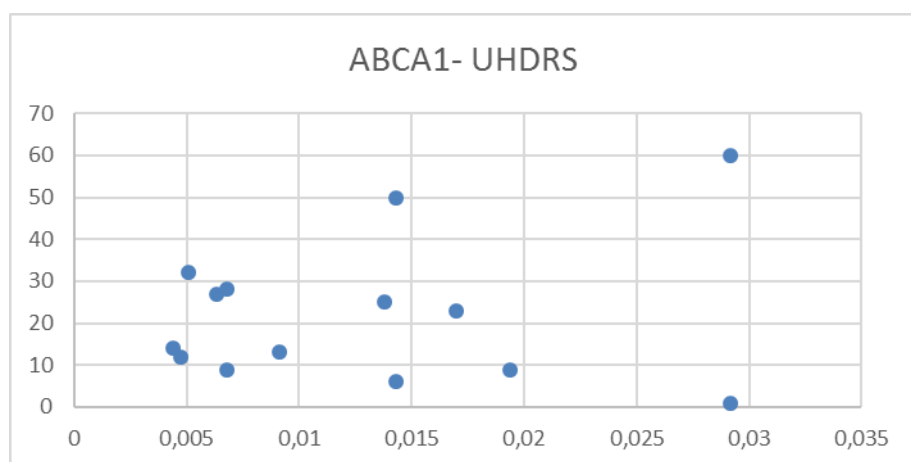
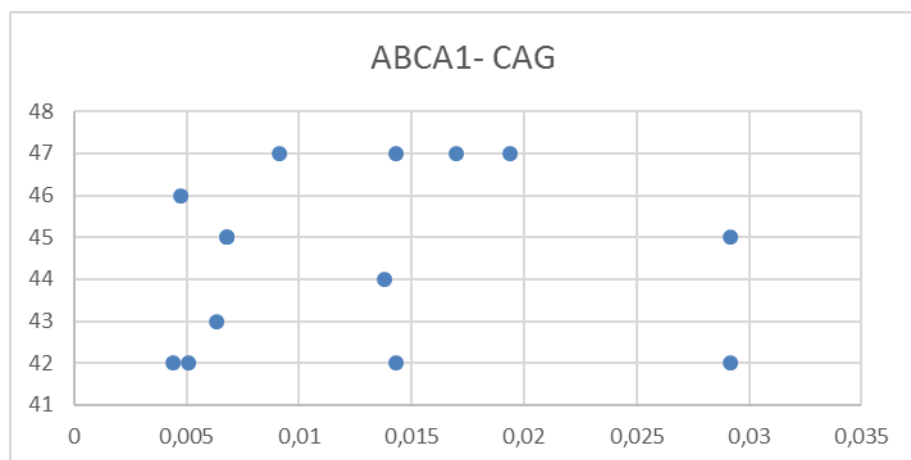


FIGURE 14. Correlation ApoE expression CAG and disease severity

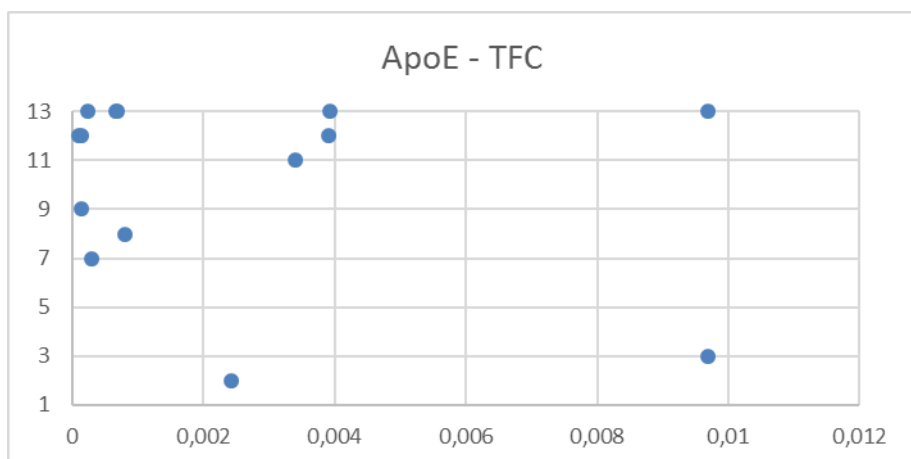
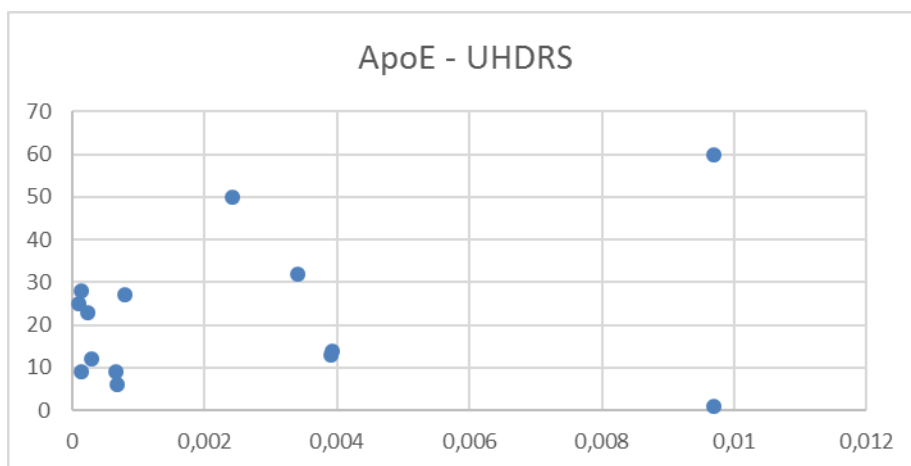
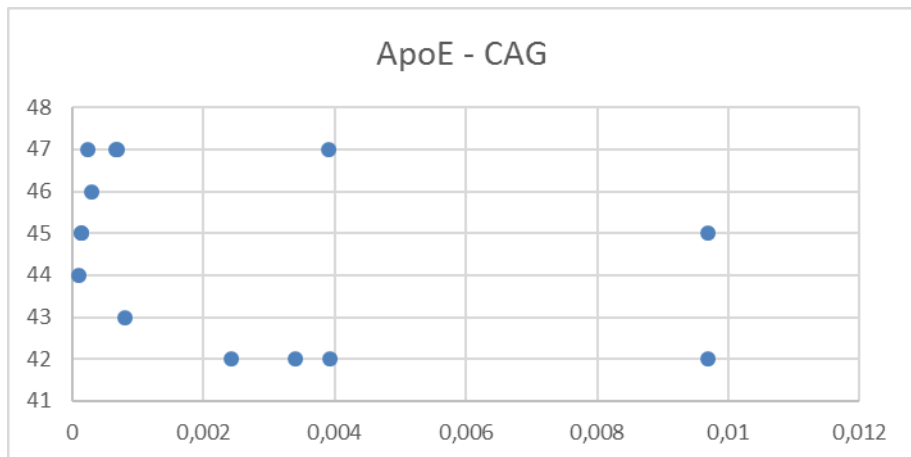


FIGURE 15. Correlation CYP7B1 expression CAG and disease severity

