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Alteration of cholesterol homeostasis in the Huntington's disease brain

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Alteration of cholesterol homeostasis in the Huntington's disease brain

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Certification

I, Fabian Kreilaus, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Department of Biological Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Fabian Kreilaus

1st Sep. 2015

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Conference poster presentations

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Fabian Kreilaus, Catriona A. McLean, Andrew M. Jenner, Altered cholesterol metabolism and increased cholesterol peroxidation in human post mortem Huntington's disease brain, *Australasian Neuroscience Society meeting (2014)*

Fabian Kreilaus, Adena S. Spiro, Catriona A. McLean, Andrew M. Jenner, Brain cholesterol synthesis and metabolism is altered in human Huntington's disease brain, *Australian Lipid Meeting (2014)*

Fabian Kreilaus, Adena S. Spiro, Catriona A. McLean, Andrew M. Jenner, Human Huntington's disease brain exhibits cholesterol synthetic and metabolic alterations, *Australian Society for Medical Research NSW annual scientific meeting (2015)*

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Abbreviations

24-OHC	24(S)-hydroxycholesterol
27-OHC	27-hydroxycholesterol
7β-OHC	7β-hydroxycholesterol
7-KC	7-ketocholesterol
AD	Alzheimer's disease
ApoE	apolipoprotein E
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BE	berry extract
BHT	tert-butylhydroxytoluene
BSTFA	O-bis(trimethylsilyl) trifluoroacetamide
CAG	cytosine-adenine-guanine
CE	collision energy
COPs	cholesterol oxidation products
CSF	cerebrospinal fluid
CYP27A1	cholesterol 27-hydroxylase
CYP46A1	cholesterol 24-hydroxylase
CYP7B1	5-hydroxycholesterol 7-α-hydroxylase
DHCR24	delta(24)-sterol reductase
DHCR7	7-dehydrocholesterol reductase
EE	environmental enrichment
ERK	extracellular signal-related kinase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC-MS	gas chromatography - mass spectrometry
GC-MS/MS	triple quadrupole gas chromatography - mass spectrometry
HD	Huntington's disease

Hdh	*HTT* mouse homologue
HMG-CoAred	3-hydroxy-3-methylglutaryl-coenzyme-A reducatase
HTT	huntingtin
mHTT	mutant huntingtin
MRI	magnetic resonance imaging
MRM	multiple reaction monitoring
MTBE	methyl tert-butyl ether
PD	Parkinson's disease
PMI	post mortem interval
RNU6-6P	spliceosomal U6 small nuclear RNA pseudogene
ROS	reactive oxygen species
SPE	solid-phase extraction
SREBP	sterol regulatory element-binding protein
SRM	selective reaction monitoring
TMCS	trimethylchlorosilane
WT	wild type
YAC	yeast artificial chromosome

Abstract

Huntington's disease is a progressive neurodegenerative disease caused by a mutation in the huntingtin protein. Although the mutation has been identified, the molecular mechanisms underlying Huntington's disease pathology are not fully understood. Dysfunction of cholesterol homeostasis has been previously associated with Huntington's disease, however detailed examination of potential changes has not been undertaken. The aim of this project was to identify cholesterol homeostatic alterations in HD that may be relevant to mechanisms that underlie neurodegeneration, or potentially identify associated molecules to be used as biomarkers of Huntington's disease. Using a novel triple quadrupole gas chromatography-mass spectrometry method, we have conducted 3 separate studies in R6/1 mice. Firstly, comprehensively characterising the physical phenotype and cholesterol homeostatic alterations during disease progression. These were then used for reference when R6/1 mice were subject to environmental enrichment, and anthocyanin dietary supplementation. Human HD post mortem tissue was also analysed for cholesterol synthetic precursors, metabolites and oxidation products. A progressive dysfunction of cholesterol synthesis was detected in both striatum and cortex of the R6/1 mouse. At later stages in the disease model, the major brain cholesterol metabolite, 24(S)-hydroxycholesterol, was also significantly reduced. Novel age-related changes pertaining to brain cholesterol homeostasis were also detected in these mice. Environmental enrichment of R6/1 mice attenuated the progression of motor dysfunction in male mice. Cholesterol oxidation products, markers of oxidative stress, were also reduced in the cortex of both wild type and R6/1 mice receiving enrichment. Dietary supplementation with anthocyanins also delayed the onset of motor dysfunction in female R6/1 mice. These studies have highlighted a potential sex differences in HD. Human HD post mortem tissue revealed a specific disturbance to cholesterol synthesis in the putamen, as well as elevated cholesterol oxidation products. Consistent with the R6/1 mouse model, 24(S)-hydroxycholesterol levels were significantly

reduced in the striatum (caudate and putamen). Enzymes involved in brain cholesterol metabolism (cholesterol 24-hydroxylase) and synthesis (delta(24)-sterol reductase) were also significantly depleted in the putamen. In conclusion we have identified disturbances in cholesterol metabolic and synthetic pathways in both human and R6/1 mouse brain tissue. In addition to being potentially useful biomarkers of disease severity and progression, these alterations may provide further insight into the effects of lipid alterations in HD pathophysiology, and potentially other neurodegenerative disorders.

Chapter 1
Introduction

1.1 Cholesterol

Cholesterol represents an important component of eukaryotic cell membranes; structurally, cholesterol modulates membrane fluidity and organisation (Yeagle, 1985) where it can alter signalling functions, membrane protein organisation and lipid raft structure (Moran & Miceli, 1998; Sheets *et al.*, 1999; Zajchowski & Robbins, 2002; Kannan *et al.*, 2007). Cholesterol is also involved in vitamin D production and synthesis of steroid hormones. 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 1.1). The opposing polarity of the hydroxyl group and hydrocarbon structure of the remaining molecule gives cholesterol its amphipathic nature.

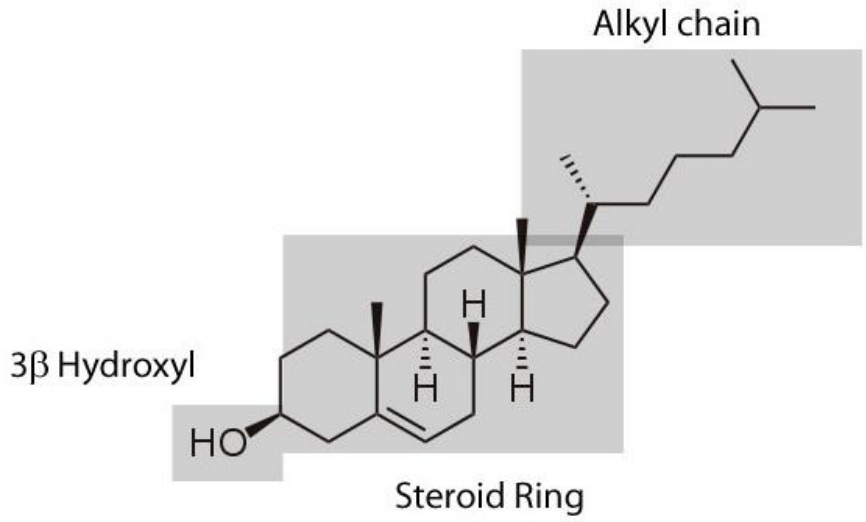

Figure 1.1 **Chemical structure of cholesterol.** The C3 hydroxyl group and tetracyclic steroid ring is shared by all sterols. The 8 carbon branched alkyl chain adds to the nonpolar steroid ring; the resultant molecule is amphipathic.

1.1.1 Cholesterol in cell membranes

The opposing polarity of the cholesterol molecule facilitates its incorporation into the lipid bilayer of the eukaryotic cell membrane, where the hydroxyl group is exposed. The distribution of cholesterol in membranes is not random, rather it is found in discrete domains. The rigid,

polycyclic structure of cholesterol in the membrane can have the effect of restricting the movement of neighbouring hydrocarbon chains, introducing order. This can also disrupt tightly packed alkyl chains that make up the hydrophobic interior of the cell membrane. These chemical features of cholesterol are important when considering the dynamics of cholesterol in the cell membrane, and the potential disturbances caused by altered levels of cholesterol.

1.1.2 Cholesterol in lipid rafts

Lipid rafts are highly dynamic, heterogeneous, cholesterol and sphingolipid rich microdomains found in the lipid bilayer of membranes; functioning to segregate and concentrate proteins that carry out cellular processes (Simons & Ikonen, 1997; Pike, 2006). Lipid rafts are associated with essential cellular functions, including signal transduction (Janes *et al.*, 2000), membrane trafficking (Brown & London, 1998) and membrane associated proteolysis (Vetrivel *et al.*, 2005). Lipid rafts are essential for normal brain function and have been identified in glia and neurons (Tsui-Pierchala *et al.*, 2002; Gielen *et al.*, 2006). The dysfunction of lipid rafts may have serious consequences in the brain, and this has been associated with several neurodegenerative diseases (Urano *et al.*, 2005; del Toro *et al.*, 2010; Fabelo *et al.*, 2011). Since lipid rafts are enriched with cholesterol, it has been proposed that altered cholesterol homeostasis in the brain may lead to a disturbance of lipid raft structure and their associated functions (Rojo *et al.*, 2006).

1.2 Cholesterol in the brain

The brain contains the highest concentration of cholesterol of any tissue in the body; accounting for approximately 25% of the total cholesterol, in an organ that only makes up 2% of the total body mass. The distribution of cholesterol is not homogenous in the brain, 70% is found in the myelinated axons of white matter, and the remaining 30% in the membranes of neurons and

glia (Norton & Autilio, 1965; Snipes & Suter, 1997). Although cholesterol is highly concentrated in the brain, it is not able to move across the blood brain barrier (BBB) (Jurevics & Morell, 1995). The isolation of the brain from peripheral sources of cholesterol suggests that strict cholesterol homeostasis is required within the brain to maintain function. A clear example of this is genetic mutations in cholesterol synthetic enzymes leading to severe neurological dysfunction (desmosterolosis and Smith-Lemli-Opitz syndrome) (Wassif *et al.*, 1998; Waterham *et al.*, 2001). Altered cholesterol metabolism has also been associated with several neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) (Wahrle *et al.*, 2002; Cordy *et al.*, 2003; Gibson Wood *et al.*, 2003; Lim *et al.*, 2011). Whether this is a cause or effect has not been established.

1.2.1 Synthesis of cholesterol in the brain

Cholesterol cannot cross the BBB, therefore *de novo* synthesis is required to supply the brain with cholesterol (Jurevics & Morell, 1995). Brain cholesterol synthesis follows the same pathway as in peripheral tissues, a process where acetate is converted to cholesterol in over 20 steps. The major enzymes and intermediates of the lower ('post-squalene') cholesterol synthetic pathway are represented in Figure 1.2. The cholesterol synthetic pathway is split, with the last common precursor lanosterol. The Bloch pathway has a major intermediate of desmosterol, while the Kandutsch-Russell pathway utilises lathosterol (Bloch, 1965). Squalene occurs earlier in the pathway before cyclisation of the steroid ring. The rate limiting enzyme in the cholesterol biosynthetic pathway has been previously identified as 3-hydroxy-3-methylglutaryl-coenzyme-A reducatase (HMG-CoAred) (Snipes & Suter, 1997), which appears early in the pathway. Negative feedback regulates HMG-CoAred through degradation (Gardner & Hampton, 1999) and transcriptional control (Reynolds *et al.*, 1984). As the cholesterol synthetic pathway is involved in producing multiple products along the pathway, more complex regulation is likely to occur. Recent studies specifically investigating the regulation of the "post-squalene" cholesterol

synthetic pathway, have suggested the synthetic enzyme delta(24)-sterol reducatse (DHCR24) to have regulatory roles beyond catalysing the final step in the Bloch pathway (Luu *et al.*, 2015). As these findings are quite recent, the importance of DHCR24 levels in brain cholesterol homeostatic regulation is unknown.

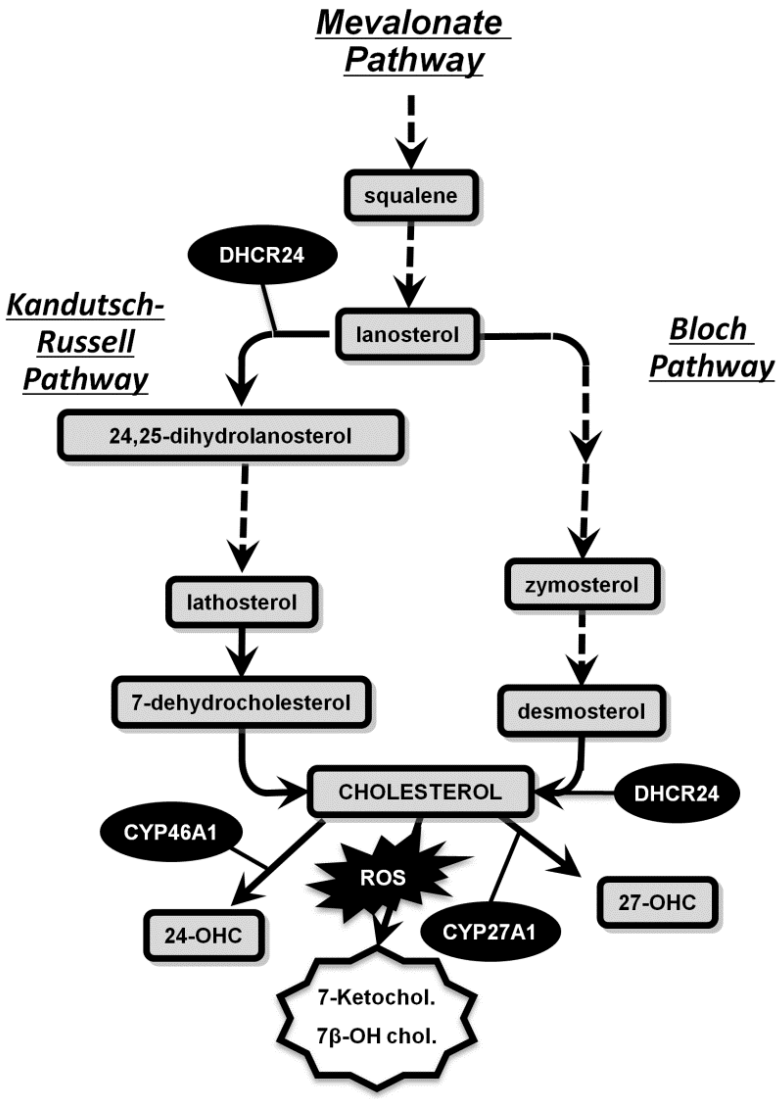

Figure 1.2 **Simplified pathway showing cholesterol synthesis, metabolism and free radical oxidation relevant to this thesis**. Major "post-squalene" cholesterol synthetic precursors shown follow a branched pathway, the Kandutsch-Russell pathway or Bloch pathway. Cholesterol can be oxidised enzymatically to form 24(S)-hydroxycholesterol (24-OHC) or 27-hydroxycholesterol (27-OHC) by cholesterol 24-hydroxylase (CYP46A1) and cholesterol 27-hydroxylase (CYP27A1) respectively. Reactive oxygen species (ROS) can oxidise cholesterol to form 7-ketocholesterol and 7β-hydroxycholesterol. The position of delta(24)-sterol reductase (DHCR24), a cholesterol synthetic enzyme is also shown. Broken lines indicate intermediates that have not been shown in this simplified scheme.

Although cholesterol synthesis and metabolism in peripheral tissues is relatively well understood, the difficulty of performing *in vivo* studies in brain tissue has left many cellular processes involving cholesterol synthesis and metabolism undefined. However, i*n vitro* studies examining isolated neurons and glial cells have identified some brain specific processes involved in cholesterol regulation and trafficking. Embryonic neurons have been identified to synthesise cholesterol (Saito *et al.*, 1987), however cholesterol synthesis in adult neurons is unsustainably low (Nieweg *et al.*, 2009). These findings support the hypothesis that cholesterol synthesis is mostly abandoned in neurons shortly after foetal development during which the majority of cholesterol is synthesised in the brain (Pfrieger, 2002). As neurons have a high demand for cholesterol, specifically for axon growth (Hayashi *et al.*, 2004), maintenance of dendrites (Fan *et al.*, 2002), and synaptogenesis (Mauch *et al.*, 2001); the source of neuronal cholesterol in the mature brain has been investigated. *In vitro* studies suggest neurons source cholesterol from astrocytes, and this has been demonstrated to be essential for neuron growth *in vitro* (Mauch *et al.*, 2001; Nagler *et al.*, 2001). Further support for the 'outsourcing' hypothesis has been demonstrated through the *in vivo* disruption of squalene synthase (an essential enzyme for cholesterol synthesis) in adult neurons, resulting in normal brain morphology in mice (Funfschilling *et al.*, 2007). This indicates adult neurons are able to survive independent of their own cholesterol synthesis. Pfrieger *et al*. (2002) proposed that an apolipoprotein shuttle from astrocytes to neurons is the mechanism by which neurons obtain cholesterol, and there is evidence that neurons have the capability to process cholesterol in lipoprotein particles through the endosome-lysosome pathway (Parton *et al.*, 1992; Brown *et al.*, 1997). There may be several reasons that neurons outsource cholesterol synthesis to astrocytes. 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, abandoned cholesterol synthesis after foetal development. The elongated shape of the neuron may also hinder transport of cholesterol, from where it is produced in the cell body, to where it is needed

(synapses). Oligodendrocytes maintain cholesterol synthesis in adulthood and synthesise cholesterol at a rate that exceeds the level in astrocytes (Saito *et al.*, 1987; Nieweg *et al.*, 2009).

It is unclear if neurodegeneration alters cholesterol synthesis in the brain and if intermediates accumulate or diverge into different metabolic pathways. There is evidence for decreased levels of cholesterol synthetic precursors in aging (Thelen *et al.*, 2006) and AD (Kolsch *et al.*, 2010), however, since many analytical techniques are unable to detect these low level compounds, much of the current literature does not contain enough information to interpret the extent of changes occurring.

1.2.2 Cholesterol metabolism in the brain

Cholesterol synthesis in the adult brain is slow, in the order of μg/h (Spady & Dietschy, 1983), which is surprising, as the brain is one of the most metabolically active tissues in the body. Although the rate of synthesis is low, excess cholesterol must still be removed from the brain. The BBB is impermeable to cholesterol, therefore simple diffusion of cholesterol into the plasma does not occur. It has been suggested that cholesterol can move into the cerebrospinal fluid (CSF) in apolipoprotein E (ApoE) particles and then into the plasma (Pitas *et al.*, 1987a). However, this accounts only for a small amount of cholesterol removed to the periphery, indicating other mechanisms must be at play.

1.2.2.1 Formation of 24(S)-hydroxycholesterol

A key finding in brain cholesterol homeostasis was the identification of a brain specific elimination mechanism involving the enzymatic hydroxylation of the cholesterol molecule (Lutjohann *et al.*, 1996; Bjorkhem *et al.*, 1997). 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 (Meany *et al.*, 2002). 24(S)-hydroxycholesterol (24-OHC) is formed by the addition of a hydroxyl

group in the 24S position of cholesterol (Figure. 1.3). A cytochrome P-450, cholesterol 24-hydroxylase (CYP46A1), expressed primarily in neurons, catalyses this reaction (Lund *et al.*, 1999). Although the liver is the site of most cholesterol metabolism in the body, CYP46A1 expression is almost exclusive to neurons (Lund *et al.*, 1999), suggesting a brain specific role in cholesterol metabolism. Studies involving CYP46A1 knockout mice (Lund *et al.*, 2003) and $^{18}O_2$ incorporation into 24-OHC in rats (Bjorkhem *et al.*, 1997), have estimated that 24-OHC is responsible for 40-60% of the cholesterol eliminated from the brain. A rise in 24-OHC levels between the brachial artery and jugular vein in human subjects is consistent with these findings that demonstrate there is a net flux from the brain into circulation (Bjorkhem *et al.*, 1998). Taking into account mechanisms of cholesterol elimination from the brain, the complete turnover of cholesterol in the human brain is estimated to be in the order of 5 years (Bjorkhem *et al.*, 1998).

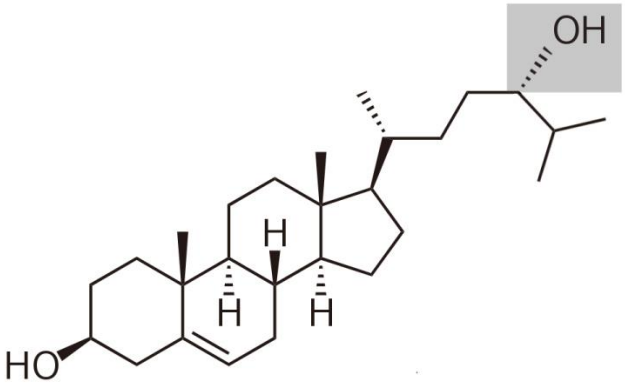

Figure 1.3 **Chemical structure of 24(S)-hydroxycholesterol**. 24(S)-Hydroxycholesterol (24-OHC) is the major elimination product of cholesterol from the brain, formed by the enzymatic hydroxylation of cholesterol by cholesterol 24-hydroxylase (CYP46A1).

1.2.2.2 Possible regulation of brain cholesterol homeostasis by 24(S)-hydroxycholesterol

The current understanding of cholesterol synthetic regulation is that a complex interplay exists between sterol sensing elements [sterol regulatory element-binding proteins (SREBPs)] and transcription factors responsible for producing cholesterol synthetic enzymes (Brown & Goldstein, 1999). Whether these mechanisms are active in neurons and astrocytes has not been established. Ong *et al.* (2000) found that SREBPs are present in the neurons of the neocortex and hippocampus, however their function in terms of sterol regulation in these regions is unknown. The hypothesis that neurons import the majority of cholesterol from astrocytes is supported by several studies (Mauch *et al.*, 2001; Funfschilling *et al.*, 2007), and thus it is believed that a mechanism is in place to regulate this exchange (Vance & Hayashi, 2010). 24-OHC is an activator of the nuclear receptor liver X receptor-β, that has the downstream effect of activating ATP binding cassette transporter A1 (Repa *et al.*, 2000), a cholesterol transport mediator that resides in astrocytes. A convenient hypothesis suggests that metabolised cholesterol in the form of 24-OHC promotes the delivery of cholesterol from astrocytes to neurons (Figure 1.4) (Pfrieger, 2003), however there is evidence that synthesis and delivery of cholesterol are regulated by separate mechanisms (Abildayeva *et al.*, 2006). Further research is necessary to elucidate the physiological importance of this mechanism, and potential impacts of altered cholesterol metabolism in the brain.

1.2.2.3 27-Hydroxycholesterol

27-Hydroxycholesterol (27-OHC) is a major metabolic product of cholesterol in peripheral tissue, entering the bloodstream to be further metabolised in the liver (Martin *et al.*, 1993; Lund *et al.*, 1996). 27-OHC is formed by the enzymatic hydroxylation of cholesterol at the 27 carbon position (Figure 1.5) by the cytochrome P450, cholesterol 27-hydroxylase (CYP27A1). This reaction takes place in all cells; however the expression of CYP27A1 in the brain is significantly less than in

other tissues (Lein *et al.*, 2007). A concentration gradient results in a net movement of 27-OHC from circulation into the brain (Heverin *et al.*, 2005), where it is quickly metabolised into more polar products (including dihydroxysterols and cholestenoic acids), catalysed by the enzymes CYP27A1 and 5-hydroxycholesterol 7-α-hydroxylase (CYP7B1) (Meaney *et al.*, 2007). These products then move back into circulation where they are efficiently absorbed by the liver (Lund *et al.*, 1996; Meaney *et al.*, 2007). It has been previously demonstrated that circulating levels of 27-OHC are positively correlated to cholesterol levels in the blood (Babiker *et al.*, 2005). It is unknown if increased levels of 27-OHC entering the brain from circulation are detrimental, and this is potentially relevant to neurodegenerative diseases as hypercholesterolemia has been associated with AD and PD (Kivipelto *et al.*, 2001; Hu *et al.*, 2008).

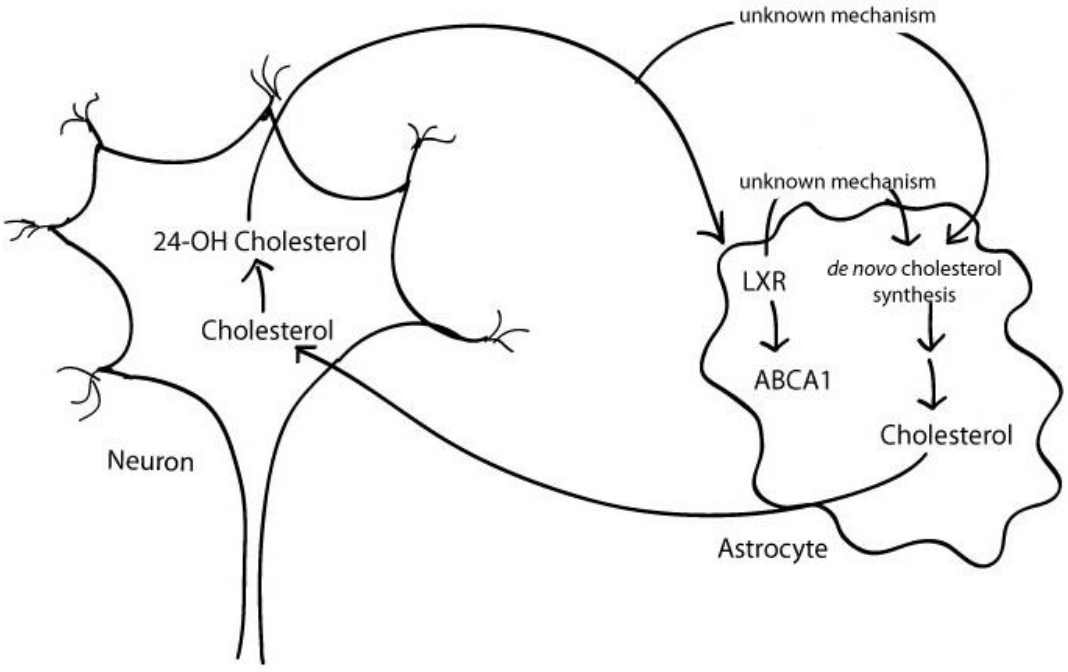

Figure 1.4 **A hypothesised mechanism of cholesterol regulation between neurons and astrocytes** (Pfrieger, 2002). 24(S)-Hydroxycholesterol (24-OHC) binds the nuclear receptor LXR which activates the cholesterol transport mediator ABCA1. It is unknown what promotes cholesterol synthesis in this mechanism. Adapted from Bjorkhem (2006).

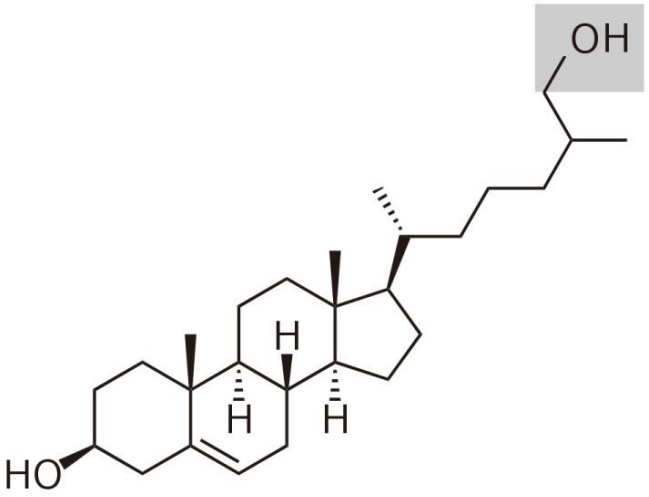

Figure 1.5 **Chemical structure of 27-hydroxycholesterol**. 27-Hydroxycholesterol (27-OHC) is a cholesterol metabolite primarily produced in peripheral tissue where it enters the bloodstream to be further metabolised in the liver. 27-OHC is formed by the enzymatic hydroxylation of cholesterol by cholesterol 27-hydroxylase (CYP27A1).

1.2.3 Toxicity of cholesterol metabolites

Several *in vitro* experiments have shown that cholesterol metabolites also have cytotoxic activity. It is unknown what causes specific changes in brain cholesterol metabolism resulting in the production, or excess production of potentially toxic oxysterol species. 27-hydroxycholesterol is toxic to human monocyte-macrophages *in vitro* (Clare *et al.*, 1995); similarly 24-OHC has toxic effects towards differentiated neuroblastoma cells (Kolsch *et al.*, 2001). 24-OHC is a major endogenous cholesterol metabolite in the brain, however, as the majority of studies rely on *in vitro* models, the importance of this potential toxicity is yet to be established *in vivo.*

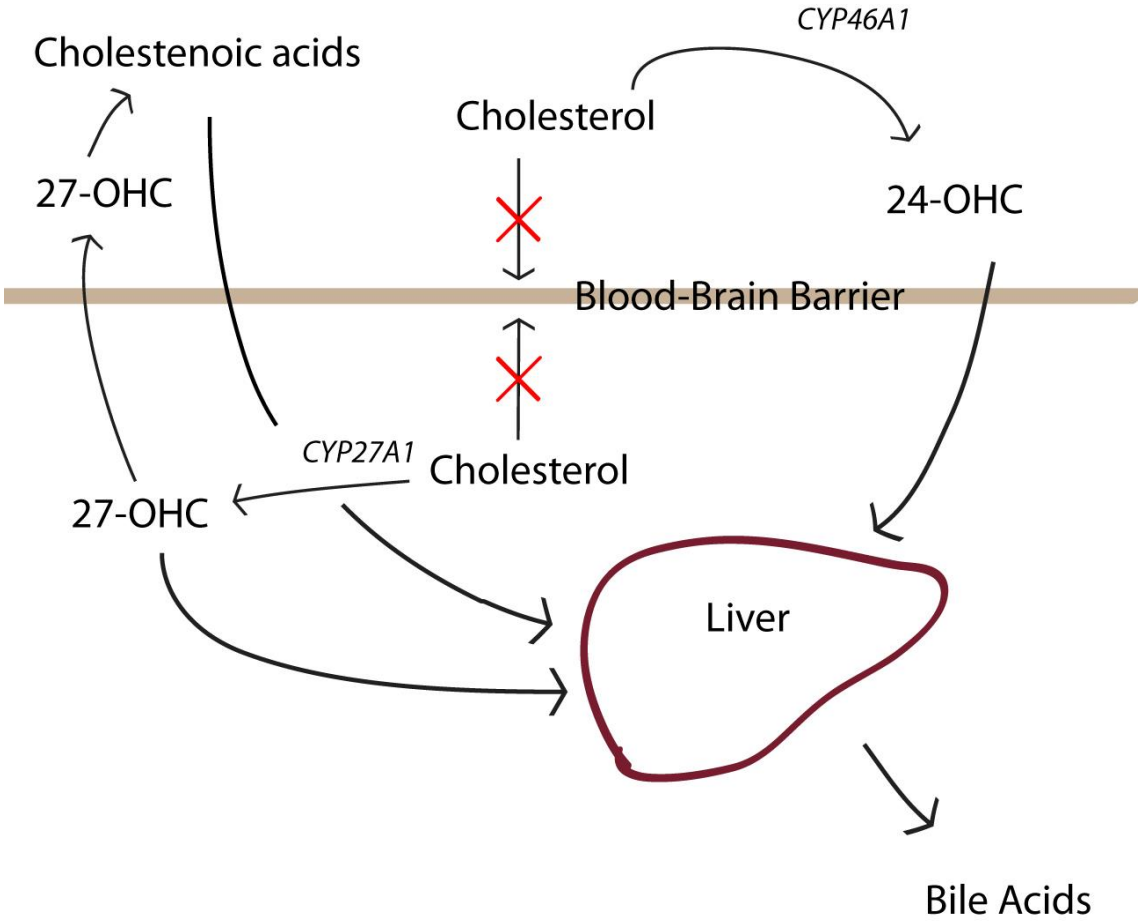

Figure 1.6 **The major movements of 24(S)-hydroxycholesterol and 27-hydroxycholesterol across the blood-brain barrier.** The blood brain barrier is impermeable to cholesterol and requires side chain oxidation to move across this membrane. Cholesterol is metabolised to 24-hydroxycholesterol (24-OHC) by cholesterol 24-hydroxylase (CYP46A1). This pathway represents the major route of cholesterol elimination from the brain. The formation of 27-hydroxycholesterol (27-OHC) is catalysed by cholesterol 27-hydroxylase (CYP27A1) and occurs primarily in peripheral tissues. A concentration gradient causes a net flux of 27-OHC into the brain where it is further metabolised to cholestenoic acids. Cholesterol metabolites are removed from circulation in the liver where they are converted to bile acids. Enzymes are shown in italics.

1.3 Cholesterol oxidation products

'Cholesterol oxidation products' (COPs) is an arbitrary classification within this thesis, classifying cholesterol oxides that are formed by reactive oxygen species (ROS) and not endogenous enzyme activity. The most susceptible location on the cholesterol molecule is the area surrounding the 5,6 double bond (Smith, 1991), where the addition of an epoxide, ketone and hydroxyl functional group can occur. 7β-Hydroxycholesterol (7β-OHC) and 7-ketocholesterol (7-

KC) are formed by ROS attack at the 5,6 double bond (Fig. 1.7). These are elevated in diseases and pathological models that involve oxidative stress including atherosclerosis (Iuliano *et al.*, 2003; Jenner *et al.*, 2007), cystic fibrosis (Iuliano *et al.*, 2009) and retinal photodamage (Rodriguez & Fliesler, 2009). *In vitro* studies describe toxicity of 7-KC and 7β-OHC towards neuroretinal and cerebellar granule cells (Chang & Liu, 1998b; Chang & Liu, 1998a), however the concentrations at which these compounds were toxic was 1000 times greater than concentrations found in plasma (Zieden *et al.*, 1999) and 100 times greater than in brain tissue (Tint *et al.*, 1998). Due to the high concentration of cholesterol in the brain, COPs represent potentially important biomarkers for neurodegenerative diseases.

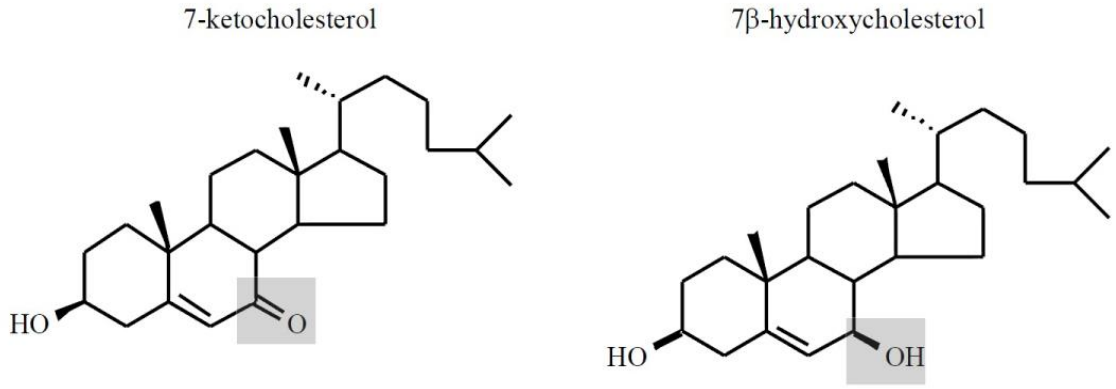

Figure 1.7 **Chemical structure of 7β-hydroxycholesterol and 7-ketocholesterol.** These compounds are formed by oxygen free radical attack at the 5,6 double bond of cholesterol. 7β-hydroxycholesterol and 7-ketocholesterol have been previous used as lipid peroxidation biomarkers in plasma and represent potentially powerful oxidative stress biomarkers in the brain due to the large concentration of cholesterol in this tissue.

1.4 Phytosterols

Phytosterols are plant derived sterols exhibiting a similar structure to cholesterol. Common phytosterols found in food are campesterol, β-sitosterol and stigmasterol (Phillips *et al.*, 2005; Dreher & Davenport, 2013). Although structurally similar to cholesterol, phytosterols have a greater degree of branching on the alkyl chain, and in the case of stigmasterol and brassicasterol the presence of a double bond (Figure 1.8). High levels of phytosterols in the diet have been

found to compete with intestinal cholesterol absorption, reducing plasma levels of cholesterol (Ikeda *et al.*, 1988; Katan *et al.*, 2003). The reduction of cholesterol solubility in phospholipid bile salt micelles in the presence of β-sitosterol demonstrated by Ikeda *et al.* (1988) is also explained by the thermodynamically favourable interaction of phytosterols with micelles (Armstrong & Carey, 1987). Dietary phytosterols present a viable option in lowering cholesterol absorption from diet, which is also accompanied by increased phytosterol absorption and incorporation into various tissues in the body (Plat *et al.*, 2008). Phytosterols are able to cross the BBB and have been measured in animal brain tissue (Plat *et al.*, 2008). The long-term cellular effects of phytosterols incorporated into the brain have not been established and further studies are necessary to examine their impact on human health.

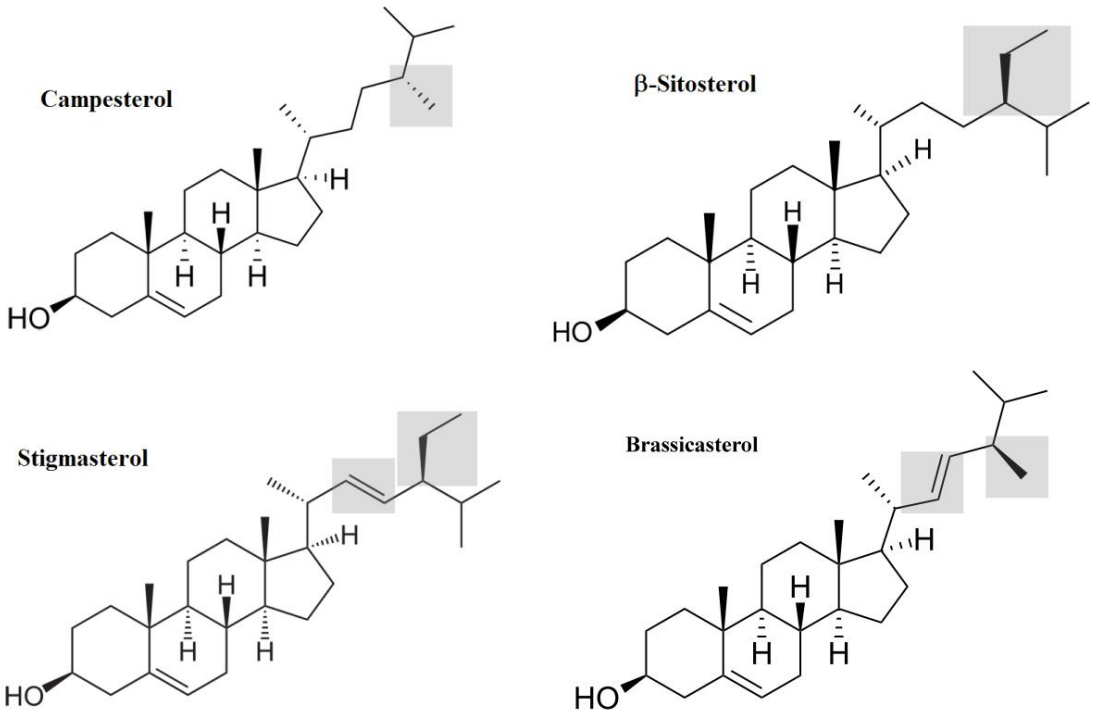

Figure 1.8 **The chemical structure of common phytosterols; campesterol, β-sitosterol, stigmasterol and brassicasterol.** Phytosterols are produced in plants and share a similar structure to cholesterol, differing in the degree of branching and presence of double bonds on the alkyl chain (highlighted). Phytosterols are absorbed from food and accumulate in tissues including the brain.

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. The influence of altered cholesterol homeostasis in neurodegenerative diseases is however, not as well understood. Cholesterol levels have been reported to alter *in vitro* protein aggregation relevant to PD (Bar-On *et al.*, 2008). Several studies have also highlighted altered brain cholesterol levels in AD (Mason *et al.*, 1992; Wahrle *et al.*, 2002; Cordy *et al.*, 2003), and may influence amyloid beta formation by altering the physical properties of cell membranes (Rojo *et al.*, 2006). It is also hypothesised that cellular redistribution of cholesterol without changes in total cholesterol levels may play a role in AD (Gibson Wood *et al.*, 2003). Due to conflicting reports of cholesterol levels in neurodegenerative brain tissue, the exact influence of altered cholesterol homeostasis in neurodegenerative diseases is currently unknown.

1.5.1 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 (MacDonald *et al.*, 1993). Full penetrance of the disease is observed when an individual has 42 or more repeats (Brinkman *et al.*, 1997), with 36-41 repeats resulting in incomplete penetrance (Kremer *et al.*, 1994; Rubinsztein *et al.*, 1996; Brinkman *et al.*, 1997). Although disease symptoms are not associated with a repeat number of 29-35, CAG repeat expansion in successive generations is possible (Trottier *et al.*, 1994; Ranen *et al.*, 1995). Expansion of CAG repeats occurs almost exclusively during spermatogenesis, causing the appearance of HD in

individuals without a family history of the disease. Juvenile onset HD, characterised by a long CAG expansion (~100), is also much more likely from paternal inheritance (Kremer *et al.*, 1995).

1.5.2 Symptoms

HD was previously known as Huntington's chorea due to the involuntary movements exhibited by patients (Huntingon, 1872). Mutant gene carriers show subtle symptoms before clinical diagnosis in a period referred to as pre-manifest, pre-symptomatic or pre-diagnostic. Pre-manifest HD patients have been shown to have dysfunction in tongue force, grip strength and finger tapping tasks (Bechtel *et al.*, 2010; Reilmann *et al.*, 2010a; Reilmann *et al.*, 2010b). The first overt symptoms of HD are typically involuntary movements, usually accompanied with depression (Kirkwood *et al.*, 2001). Other symptoms include abnormal eye movement (Penney *et al.*, 1990) and clumsiness (Kirkwood *et al.*, 2001). During progression there is weight loss (Sanberg *et al.*, 1981; Morales *et al.*, 1989; Djousse *et al.*, 2002), cognitive decline, speech difficulties and memory loss (Kirkwood *et al.*, 2001). In late stages of the disease, HD patients have difficulty swallowing; the aspiration of food into the lungs is a major cause of death in these cases (Sorensen & Fenger, 1992). The progression and severity of disease after onset does not correlate strongly with CAG repeat length in the range of 40-60 (Squitieri *et al.*, 2002). However, the correlation between the age of onset and CAG repeat length is well established (Ranen *et al.*, 1995). Rare juvenile cases of HD with 100+ CAG repeats do, however, have accelerated progression of the disease (Squitieri *et al.*, 2002).

1.5.3 Neuropathology

The neuropathological progression of HD was classified in detail by Vonsattel *et al*. (1985), and was adopted to grade HD brain tissue. This revealed the disease progressed from the caudate nucleus and putamen (striatum) with degeneration moving in a lateral basal direction. Early changes include moderate astrocytosis of the caudate nucleus and putamen in grade I, followed

by macroscopic atrophy and neuronal loss in grade II. Neuron loss continues in grade III with overt shrinkage of the striatum. Grade IV was classified by extreme shrinkage of the striatum, including the globus pallidus. Astrocyte numbers were also increased compared to control at this stage in the disease. Brain mass was also found to be negatively correlated to the disease grade, with an average 20% reduction in brain mass by grade IV. More sensitive magnetic resonance imaging (MRI) techniques have also identified volume reduction in the hippocampus, cerebral cortex and amygdala of HD patients, with the cerebellum relatively spared (Rosas *et al.*, 2003). Along with neuron loss, astrocytosis and increased oligodendrocyte densities are also observed in severely affected regions of the HD brain (Myers *et al.*, 1991).

1.5.4 Huntingtin protein

HTT is made up of >3100 amino acid residues and has a molecular mass of approximately 349 kDa depending on the length of the CAG repeat (Gil & Rego, 2008). HTT is expressed throughout the body, with the highest expression in the brain and testes (Strong *et al.*, 1993; Landwehrmeyer *et al.*, 1995). It is localised to a number of subcellular compartments and has functions in intracellular trafficking, clathrin mediated endocytosis, transcriptional regulation and cell signalling (Harjes & Wanker, 2003; Li & Li, 2004). The protein is believed to be essential for early development in mice, as knockout of the *HTT* mouse homologue (*Hdh*) is embryonically lethal (Leavitt *et al.*, 2001).

1.5.5 Toxicity of mutant huntingtin

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 the loss of function in one allele is not entirely responsible for disease pathology (Ambrose *et al.*, 1994).

Expression of normal length HTT can reduce the toxicity of mHTT in mice (Leavitt *et al.*, 2001), also suggesting compensation for loss of function. Another confounding finding is that human mHTT can rescue the embryonic lethality of the *Hdh* knockout mouse (Leavitt *et al.*, 2001). However, this may simply be a reflection of the role of HTT in developmental processes, similar to the human disease where patients develop normally, only manifesting symptoms later in life.

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, which are relatively spared compared to the selectively vulnerable striatal neurons that contain fewer aggregates (Kuemmerle *et al.*, 1999). *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 (Arrasate *et al.*, 2004). In this study, cells forming inclusions had reduced levels of mHTT in the rest of the cell, 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 (Jana *et al.*, 2001; Waelter *et al.*, 2001). It is also possible that undiscovered neurotoxic aggregates are too small for detection by light microscopy as suggested by Bates (2003).

1.5.6 Mouse models of Huntington's disease

Prior to the *HTT* gene identification in 1993 (MacDonald *et al.*), the primary rodent models of HD were based on the production of brain lesions by neurotoxin injections into the striatum. Injection of glutamic acid, kainic acid (McGeer & McGeer, 1976; Schwarcz & Coyle, 1977) and quinolinic acid (Beal *et al.*, 1986) replicated some of the biochemical changes observed in the human HD brain, and highlighted a potential involvement of NMDA mediated excitotoxicity in HD pathogenesis. Since the identification of the *HTT* gene and the expanded CAG repeat that causes HD, genetic models were generated for more accurate replication of the human

condition. Common mouse models have used multiple approaches to model HD, including knock-in models and transgenic models expressing full length and truncated forms of human HTT.

The R6 mouse models are widely used to study HD pathology and therapeutic interventions. These mice were generated by the insertion of a truncated form of human *HTT* that codes for a CAG expansion and 67 amino acids of exon 1 human HTT under the human promoter (Mangiarini *et al.*, 1996). The R6/1 mouse expressing truncated HTT with approximately 115-120 CAG repeats, and the R6/2 with 140-150 repeats, are the best characterised variants of this model. R6 mice mimic several pathological hallmarks including early striatal degeneration, and physical phenotypes of HD such as motor dysfunction and uncontrolled movements (Mangiarini *et al.*, 1996). These features and the relatively short disease onset are reasons why this model has been widely used in the study of HD. Instability of the CAG repeat between generations can result in CAG expansion. This can cause variation in the specific CAG repeat length between colonies (Mangiarini *et al.*, 1997). Selectively breeding mice without expanded repeats is able to control CAG expansion in subsequent generations.

A model expressing full length expanded human HTT was also generated using a yeast artificial chromosome (YAC). These mice expressing HTT with a normal number of repeats (18), and expanded CAG repeats (46, 72, and 128), have also been characterised to have striatal degeneration, motor deficits, exhibit motor abnormalities and mHTT aggregation (Hodgson *et al.*, 1999; Slow *et al.*, 2003).

Knock-in of a CAG repeat expansion into the *Hdh* gene has also been used to generate HD models that express an expanded form of mouse HTT under endogenous transcriptional control (Wheeler *et al.*, 1999; Wheeler *et al.*, 2000). Heterozygous and homozygous knock-in mice expressing 92 (*Hdh*Q92) and 111 (*Hdh*Q111) CAG repeats have been commonly examined in

previous literature; knock-in models also exhibit degenerative pathologies (Wheeler *et al.*, 2002).

There are benefits and drawbacks in each model; this includes the severity of the disease in mice where R6 models are most representative due to the long CAG repeat that causes a relatively rapid onset of symptoms. HTT sequences found in transgenic models (R6 and YAC) however, become randomly inserted into the genome and therefore do not have the endogenous mouse promoter as well as the endogenous protein still being expressed. Therefore models that knock in expanded CAG repeats into the mouse HTT sequence (e.g. *Hdh*Q111) more accurately represent the disease from a genetic perspective.

1.5.7 Cellular and fly models of Huntington's disease

Several models of HD have been generated in *Drosophila* where an expanded fragment of exon 1 human HTT is expressed (Httex1pQ93 and Htt128Q). These models have been used in several studies as they provide a number of phenotypes including photoreceptor degeneration, motor abnormalities as well as reduced lifespan (Steffan *et al.*, 2001; Lee *et al.*, 2004). A number of cell models expressing forms of expanded HTT have been developed since the identification of the affected gene. A model developed in yeast that expresses exon 1 HTT with 75 CAG repeats has been used to assess HTT aggregation (Ehrnhoefer *et al.*, 2006). A model in a neuronal cell line (PC12) developed to express exon 1 HTT has also been used to examine the effects of mutant huntingtin on extracellular signalling (Aiken *et al.*, 2004; Maher *et al.*, 2011).

1.5.8 Alteration of cholesterol homeostasis in Huntington's disease

While it is not fully understood how the polyglutamine expansion causes cellular dysfunction in HD, mHTT has been identified to alter membrane order (del Toro *et al.*, 2010) and HTT-phospholipid interactions (Kegel *et al.*, 2009). Despite the genotypic identification of mHTT carriers, there is a lack of reliable biomarkers to predict HD progression or effectiveness of

therapies. Several studies have identified that cholesterol synthesis and metabolism in HD cell lines and animal models is significantly disturbed and suggest these to be potential biomarkers of HD (Valenza *et al.*, 2005; Valenza *et al.*, 2007b; Valenza *et al.*, 2010), but the mechanisms and metabolic pathways affected have not been fully examined in human or mouse models of HD.

1.5.9 Alterations of cholesterol biosynthesis in Huntington's disease

Gene transcription profiles of HTT-inducible cells identified a possible dysregulation of lipid homeostasis in HD (Sipione *et al.*, 2002). This study reported the reduction of several mRNAs coding for cholesterol biosynthetic enzymes, including that of 7-dehydrocholesterol reductase (DHCR7), the final enzyme of the Kandutsch-Russell pathway (Sipione *et al.*, 2002). Further investigation into HD fibroblasts, R6/2 mice and HD post mortem brain tissue, identified a consistent reduction of several other mRNAs coding for enzymes in the cholesterol synthetic pathway (Valenza *et al.*, 2005). The active nuclear form of SREBP was also found to have a 50% reduction in human HD fibroblasts and in brain tissue of mice (Valenza *et al.*, 2005). Brain lipid analysis of R6/2 and YAC 128 mice has also revealed a consistent reduction in the cholesterol synthetic precursors lathosterol and lanosterol (Valenza *et al.*, 2007a; Valenza *et al.*, 2007b). Further association of brain cholesterol synthesis and mHTT was demonstrated by a CAG repeat dependant reduction of lathosterol in YAC mice, and reduced levels of lathosterol in the homozygous knock-in mouse ($Hdh^{Q111/111}$) compared to the heterozygous knock-in (Valenza *et al.*, 2010). The current understanding of cholesterol biosynthesis in HD has been previously limited by the analysis of a small number of synthetic precursors in a pathway with over 20 steps. Analysis of a wider range of synthetic precursors is likely to provide a more complete story of the potential changes occurring in HD. In addition to this, a detailed analysis of cholesterol synthetic precursors in human post mortem tissue is yet to be reported in the literature.

1.5.10 Cholesterol levels in Huntington's disease

Although a significant reduction of cholesterol biosynthetic precursors were observed previously in these mouse models, the total level of brain cholesterol remains relatively stable. Unchanged levels of cholesterol were observed in the striatum or whole brain of R6/1 and R6/2 models (Valenza *et al.*, 2007b; del Toro *et al.*, 2010), while only a small reduction in brain cholesterol levels were observed in the *Hdh*^{Q111}, YAC72 and YAC128 mice (del Toro *et al.*, 2010; Valenza *et al.*, 2010). A previous study examining cholesterol levels in human HD post mortem tissue identified an increase in cholesterol, however the sample size (n = 3) was too small to draw a firm conclusion regarding cholesterol levels in human HD brain (del Toro *et al.*, 2010). Mechanisms to eliminate cholesterol may also be downregulated to maintain constant cholesterol levels in the brain, highlighting the potential involvement of altered cholesterol metabolism in HD.

1.5.11 Cholesterol metabolic alterations in Huntington's disease

The major elimination product of cholesterol in the brain, 24-OHC, is also believed to have an important role in cholesterol homeostatic regulation in the brain. Reduced brain levels of 24-OHC have been consistently demonstrated in multiple rodent models of HD, including knock-in models and transgenic models expressing truncated and full length HTT (Valenza *et al.*, 2007a; Valenza *et al.*, 2010). Plasma measurements of 24-OHC in the YAC 128 model reflect the reduced level in brain (Valenza *et al.*, 2007a); plasma studies in HD patients have also identified reduced 24-OHC levels in symptomatic individuals (Leoni *et al.*, 2008; Leoni *et al.*, 2013). Since CYP46A1 expression is primarily localised to neurons (Lund *et al.*, 1999), it has been suggested that generation of 24-OHC (as measured in plasma) is a marker of metabolically active neurons in the brain (Bjorkhem, 2006). Recent evidence suggests that changes in CYP46A1 and 24-OHC brain levels may play a role in neurodegeneration (Kolsch *et al.*, 2002; Papassotiropoulos *et al.*, 2003;

Brown *et al.*, 2004; Tian *et al.*, 2010), however, the exact role this might play in HD pathogenesis has not been established. Comprehensive examination of cholesterol synthesis and metabolism in human HD, beyond plasma biomarkers is currently lacking in the literature. This represents a significant gap in the current knowledge that would help confirm the validity of rodent HD models examining this particular metabolic pathway in relation to HD pathology.

1.5.12 Proposed mechanisms altering cholesterol homeostasis in Huntington's disease

Consistent demonstration of cholesterol homeostatic perturbation in HD models has led to the identification of several associations of HTT and mHTT with cholesterol synthetic regulation. A hypothesised mechanism of reduced cholesterol biosynthesis in HD involves an interaction of mHTT with SREBPs (Kaltenbach *et al.*, 2007). It has been suggested that the CAG expansion causes a diminished capacity of mHTT to translocate SREBP to the nucleus where it has functions to activate cholesterol synthetic genes (Guan *et al.*, 1995; Lloyd & Thompson, 1995). HTT is known to have cellular trafficking properties, and this hypothesis is consistent with the finding that transgenic HD cells contain less active nuclear SREBPs than controls (Valenza *et al.*, 2005).

Another factor causing reduced cholesterol synthesis is related to decreased levels of brain-derived neurotrophic factor (BDNF) that have been observed in human HD and HD mouse models (Zuccato *et al.*, 2001; Spires *et al.*, 2004; Zuccato *et al.*, 2005). BDNF is known to increase cholesterol content of lipid rafts *in vitro* and increase neurotransmitter release dependant on BDNF-elicited cholesterol (Suzuki *et al.*, 2007). *In vivo*, abnormal synaptic plasticity in R6/2 mice (Murphy *et al.*, 2000; Picconi *et al.*, 2006) may also be related to dysfunction of BDNF-mediated cholesterol levels in the cell.

With the complex control of cholesterol homeostasis in the brain, it is likely that other undiscovered factors, potentially involved with cholesterol metabolism, may be involved in altering cholesterol synthesis in the presence of mHTT. Further investigation into cell-type

specific cholesterol homeostatic alterations will also contribute to understanding how these altered pathways affect whole tissue.

1.6 Therapeutics in Huntington's disease mouse models

There is currently no cure or effective therapies for the treatment of HD. Due to the late onset and possibility of early identification of mHTT carries, there is a relatively long timeframe (decades) to implement potential therapies for HD suffers. It also allows for close study of the manifestation of neurodegenerative pathologies that may be relevant to understanding 'sporadic' neurodegenerative diseases such as AD. Mouse models of HD, particularly the R6 models, have been used to examine several therapeutic strategies including dietary supplementation (Ehrnhoefer *et al.*, 2006; Maher *et al.*, 2011), tissue transplantation (van Dellen *et al.*, 2001), drug treatments (Ferrante *et al.*, 2002; Schiefer *et al.*, 2002) and gene therapies (Popovic *et al.*, 2005). This thesis will focus on the use of environmental enrichment (EE) and dietary supplementation in HD.

1.6.1 Environmental enrichment and Huntington's disease

Increased cognitive activity is believed to be a factor in reducing the risk of developing dementia and neurodegenerative diseases later in life (Karlsson *et al.*, 1988; Stern *et al.*, 1994; Evans *et al.*, 1997). Substantial evidence also suggests that increased physical, sensory-motor and cognitive activity associated with EE has positive effects in the brain to improve memory, cognition and reduce the severity of neurodegenerative phenotypes in animal models (Wainwright *et al.*, 1993; Kempermann *et al.*, 2002; Jankowsky *et al.*, 2005; Lazarov *et al.*, 2005). Recently it has been discovered that EE may also be beneficial in HD. Studies in mouse models of HD illustrate that EE delays onset of physical phenotypes (van Dellen *et al.*, 2000), improves motor co-ordination (Hockly *et al.*, 2002; Spires *et al.*, 2004) and improves survival (Carter *et al.*, 2000), compared to mice with standard housing. Rodent studies utilising EE usually involves a

larger cage with a greater number of animals, the addition of novel objects (toys), tunnels, additional nesting materials and in some cases running wheels (van Praag *et al.*, 1999; van Dellen *et al.*, 2000; Hockly *et al.*, 2002; Hockly *et al.*, 2003; Spires *et al.*, 2004). A study in human populations has also identified that environmental factors also influence the age of onset of HD (Wexler & Res, 2004). Increased neurogenesis in the hippocampus has been identified to explain some of the positive effects on memory seen in EE animal studies (van Praag *et al.*, 1999; Lazic *et al.*, 2006), however specific mechanisms improving the disease severity in HD, which involves striatal atrophy, requires further investigation. Whether cholesterol homeostatic perturbations observed in mouse models are altered by EE is currently unknown.

1.6.2 Flavonoid supplementation

Flavonoids are phenolic micronutrients in foods derived from plants. A large area of study has identified multiple health benefits of dietary flavonoids with potential beneficial effects in cardiovascular diseases (Nakagawa *et al.*, 1999; Ruel *et al.*, 2005) and cancer (Murphy *et al.*, 2003; Ferguson *et al.*, 2006). Recently dietary supplementation of flavonoids and other plant derived phytochemicals have revealed an influence on the brain, including positive effects on cognitive function in human studies (File *et al.*, 2001; Duffy *et al.*, 2003; Casini *et al.*, 2006) and improvement of neurodegenerative phenotypes in animal models of AD (Li *et al.*, 2015; Nakajima *et al.*, 2015; Sabogal-Guaqueta *et al.*, 2015).

1.6.2.1 Anthocyanins and Huntington's disease

Anthocyanins are a class of flavonoid that have the backbone structure of an anthocyanidin (Figure 1.9), with the addition of a glycoside (O-linked sugar). Anthocyanins found in plants are primarily 3-glycosides of anthocyanidins. In addition to the location and species of sugar, different structures of anthocyanins are derived from the position of hydroxy and methoxy functional groups on the anthocyanindin backbone. Berries, including blueberries, blackberries,

and blackcurrants, are rich in a variety of different anthocyanins; several predominant species in these berries are cyanidin 3-glucoside, cyanidin 3-rutinoside, dephinidin 3-glucoside and delphinidin 3-rutinoside (Figure 1.10) (Wu & Prior, 2005; Scalzo *et al.*, 2008). Dietary supplementation with berry extracts (BE) has been previously shown to improve motor and cognitive function in rodents during ageing (Joseph *et al.*, 1998; Casadesus *et al.*, 2004; Galli *et al.*, 2006; Duffy *et al.*, 2008). Fruit extracts that are rich in phenolic phytochemicals have also been demonstrated to ameliorate the disease pathology of a cell, fly and mouse model of HD (Ehrnhoefer *et al.*, 2006; Maher *et al.*, 2011). However, the specific benefits of BE supplementation, rich in anthocyanins, has not been previously investigated in HD models. While the benefits of flavonoids and BE supplementation are measureable from the physical phenotype and biological markers, the exact mechanisms through which these phytochemicals confer benefit is not well established, particularly in the context of HD neurodegeneration.

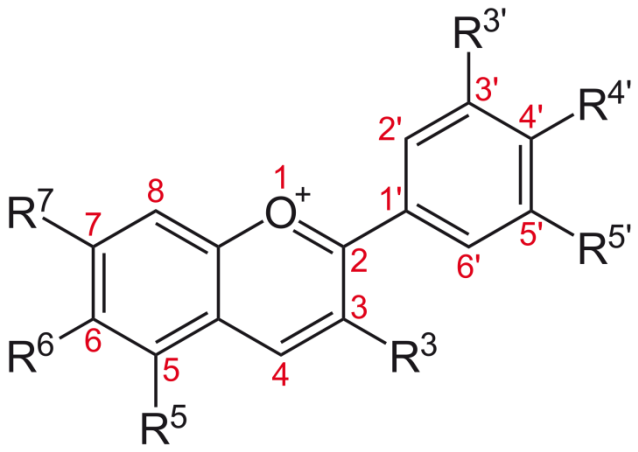

Figure 1.9 **The basic chemical structure of an anthocyanidin**. Anthocyanins share the same backbone structure with the addition of a glycoside, commonly at the R^3 position. Hydroxy and methoxy functional groups are also typically present on the aromatic rings.

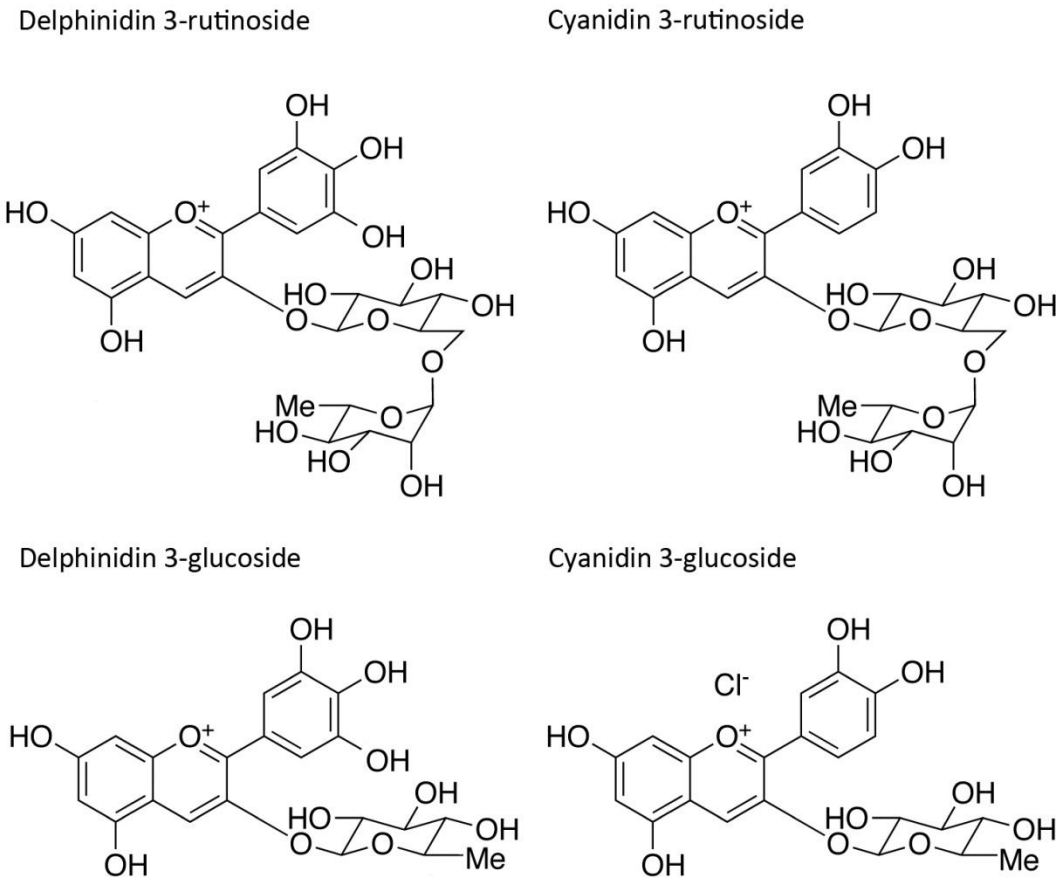

Figure 1.10 **The chemical structure of anthocyanins predominantly found in berries (blueberry, blackberry, blackcurrant)**. Different structures of anthocyanins arise from the arrangement of hydroxy and methoxy functional groups on the anthocyanidin backbone, and the location and species of sugar that is attached to the molecule. In the pictured molecules the glycosides are glucose and rutinose.

1.7 Biological markers of neurodegeneration

Diagnosis is an essential process in order to apply the correct treatment to combat disease. Currently there is a need for diagnostic tests for neurodegenerative diseases, not only due to their inevitably fatal nature, but also due to the increasing prevalence of these diseases. Ideally biomarkers can be obtained from saliva, urine or blood as these require little or no invasive procedures to obtain. Although more invasive procedures such as CSF samples or a brain biopsy may result in a faster, more sensitive diagnosis, the stress on the body is usually unjustifiable. In

the case of HD where mutant carriers can be easily identified, biomarkers are of most benefit to therapeutic trials in assessing the efficacy of treatment. In human patients a plasma biomarker is ideal; however, examining post-mortem brain tissue from human and animal models may give insight into changes that can be measured in the circulation. Post mortem brain biomarkers are also useful in assessing treatments in animal trials where brain tissue is available from test subjects. Since HD, as well as other neurodegenerative diseases have a significant association with altered cholesterol metabolism in the brain, cholesterol metabolites and synthetic precursors are potentially useful to track the progression of disease. 24-OHC levels in brain tissue and plasma reflect turnover of cholesterol in the brain, however further investigation into this metabolite is required before its clinical usefulness can be established. This is due to 24-OHC plasma levels being variable in humans, dependant on hepatic metabolism and brain:liver mass ratio (Bretillon *et al.*, 2000a). It is known that 24-OHC is reduced in plasma of HD patients and correlates to volume loss in the striatum (Leoni *et al.*, 2008); however longitudinal studies, that would be most effective in tracking brain metabolic dysfunction over time, currently do not exist. Little is known about the concentration and location of other cholesterol synthetic precursors and metabolic products in the HD brain, these being potentially powerful indicators of disease severity and progression. Further investigation into biomarkers that correlate well with HD severity will clearly increase the current understanding of fundamental mechanisms of HD pathology and invariably accelerate the search for effective therapies.

1.8 Aims

The aim of this thesis was to investigate the involvement of brain cholesterol homeostasis in HD, and identify sterol-related biomarkers associated with the disease using a novel and highly sensitive analytical technique. We aimed to comprehensively quantify changes in brain cholesterol metabolites and synthetic precursors during the progression of HD, by investigating

the R6/1 mouse model over 5 stages of the disease. We also aimed to investigate the effectiveness of EE and BE supplementation to attenuate the physical phenotype and cholesterol homeostatic perturbation in the R6/1 model. Finally we aimed to identify changes to brain cholesterol homeostasis in human HD by conducting a comprehensive analysis of 5 regions of HD post mortem brain tissue.

Chapter 2
General materials and methods

2.1 Methods of sterol analysis

The specific method of sterol analysis used in this thesis is a novel and important aspect that requires further explanation. Qualitative and quantitative analysis of chemical compounds is key to identifying and understanding reactions in biological systems. Different analytical methods have various strengths and weaknesses depending on compounds analysed, concentration of the analytes and the speed and expense of analysis. Only a small number of the established, and well characterised cholesterol metabolic products and synthetic precursors have been analysed *in vivo* and *in vitro,* therefore data pertaining to a limited number of sterols has been discovered. A number of techniques have been previously employed, however, sensitivity is crucial for the analysis of cholesterol metabolites and synthetic precursors that are usually found at concentrations 10^3-10^6 times less than cholesterol (Micheletta & Iuliano, 2006).

Proton nuclear magnetic resonance has been used to quantify cholesterol in bile acids (Srivastava *et al.*, 2005). Although successful, this method is limited to highly abundant sterols (μ-mg.mL^{-1}), and may not be sensitive enough for low level intermediates and metabolic products that typically require measurement of ng.mL^{-1} concentrations (Dzeletovic *et al.*, 1995).

Gas chromatography coupled with mass spectrometry (GC-MS) has been demonstrated as a sensitive analytical tool in a study identifying 9 cholesterol oxides in plasma (Dzeletovic *et al.*, 1995). By using deuterated internal standards corresponding to each analyte, the study was able to achieve a high level of precision, with the ability to measure concentrations of 0.5-6 ng.mL^{-1}. This method has been employed in a number of studies that examine the concentration of 24-OHC and 27-OHC in plasma (Lutjohann *et al.*, 1996; Bjorkhem *et al.*, 1998). These metabolites are found in higher abundance relative to other known cholesterol metabolites (Dzeletovic *et al.*, 1995), and thus sensitivity is not a limiting factor. In order to measure the low level intermediates involved in synthesis, metabolic pathways and mechanisms of oxidative damage, a higher degree of sensitivity is required.

2.1.1 Triple Quadrupole GC-MS

Previously, gas chromatography and mass spectrometry has been used separately for qualitative and quantitative analysis. However, when coupled, GC-MS presents a powerful analytical tool. Triple quadrupole GC-MS (GC-MS/MS) operates on the same fundamental principles of a single quadrupole GC-MS, however, structurally it has two quadrupoles aligned in series, separated by a collision cell. Specific ions (formed by analyte ionisation) can be filtered by the first quadrupole, fragmented in the collision cell, and then filtered again in the third quadrupole. The ability to filter specific ions after two fragmentations gives high specificity towards the analyte of interest and often increases sensitivity due to an improved signal to noise ratio. GC-MS/MS has not been widely used as an analytical method for the detection of cholesterol synthetic precursors, metabolites and oxidation products, therefore the ion transitions required for analysis has not been previously established. The process of developing a multiple reaction monitoring (MRM) method for GC-MS/MS is purely experimental, as ion formation and fragmentation is dependent on chemical structure and difficult to be calculated theoretically. MRM transitions involve the formation of a precursor ion during ionisation, followed by fragmentation in the collision cell to form a second fragment known as the product ion. Precursor ions are fragmented by the collision with inert gas, typically N_2 or Ar, at a specified collision energy (CE) (Hopfgartner *et al.*, 2004). High CEs commonly form small mass fragments; these fragments can have a higher signal than larger mass fragments, however they are less specific to the compound being measured (Hopfgartner *et al.*, 2004). One ion transition is used for quantitation, however to increase confidence in identification, one or more 'qualifier' ion transitions can also be monitored. The presence and ratio of specific qualifier ion transitions is used to identify and measure compounds with a high degree of specificity. With this high sensitivity, GC-MS/MS has the potential to measure in the femtogram range (Agilent, 2008).

It is clear that new methods need to be established that have the ability to reliably measure low concentrations of sterols before any certainty can be reached regarding cholesterol metabolic and synthetic pathways and how they are disturbed in HD. This is especially crucial for the measurement of biomarkers which may only show small changes in concentration.

2.2 Materials

Materials

Desmosterol-d_6, zymosterol-d_5, zymosterol and lanosterol-d_6 were obtained from Avanti lipids (Alabaster, AL, USA). Tert- butylhydroxytoluene (BHT), cholesterol, α-cholestane, 7β–hydroxycholesterol, 7-dehydrocholesterol and 7-ketocholesterol and squalene were from Sigma (St. Louis, MO, USA). Lathosterol, lanosterol, desmosterol, 27-hydroxycholesterol, campesterol, brassicasterol, β-sitosterol and stigmasterol were obtained from Steraloids (Newport, RI, USA). Campesterol-d_3, 7β–hydroxycholesterol-d_7, lathosterol-d_4, β-sitosterol-d_7 and 7-ketocholesterol-d_7 were purchased from CDN Isotopes (Quebec, Canada). 27-hydroxycholesterol-d_5, 24-hydroxycholesterol and 24-hydroxycholestero-d_7 were from Medical Isotopes, Inc. (Pelham, AL, USA). Squalene-d_6 and 24,25-dihydro lanosterol-d_6 were obtained from Toronto research chemicals (TRC, Ontario, Canada). All standards obtained were of the highest purity (>95%). Methanol, hexane, methyl tert-butyl ether (MTBE), acetonitrile, toluene, formic acid and NaOH were purchased from Ajax Finechem (Thermo Fisher Scientific, AU). CUQAX223 UCT Clean-Up QAX2 solid phase extraction columns and N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) was purchased from PM Separations (Qld, Australia).

2.3 Methods

2.3.1 Mice

Transgenic R6/1 male mice were provided by Professor Anthony Hannan (The Florey Institute, Melbourne) and bred with CBB6 (CBA x C57/B6) F_1 female mice at Australian Bio-Resources (Mossvale, Australia). Mice were genotyped at 4 weeks from ear clippings. Equal numbers of male and female R6/1 and wild type (WT) littermates were transported to the University of Wollongong animal housing facility at the age of 5 weeks. Unless otherwise stated, mice were housed in standard small rodent cages with a wire lid (30 cm x 12 cm x 13 cm), containing sawdust, pine shavings and a PVC tube. Mice were provided with standard rodent diet ("rat & mouse nut", Vella Stock Feeds, NSW, Australia) and water available *ad libitum*. All procedures that were undertaken conformed to the standards of the University of Wollongong ethics committee (ethics approval number: AE 13/20).

2.3.2 RotaRod

A RotaRod apparatus (TSE systems, Bad Homberg, Germany) was used to measure motor performance of mice. The RotaRod used consisted of a single rod (30 mm diameter, hard plastic with longitudinal grooves) divided into 5 lanes, allowing the simultaneous testing of multiple animals. Mice were acclimatised to the RotaRod over two days before initial testing. During the acclimatisation, mice were allowed to balance on the RotaRod without any rotation for 5 minutes. The RotaRod was then operated at a slow speed of 4 RPM for 1 minute. Following this, the accelerating testing program was run (4 RPM for 5 sec followed by a linear acceleration from 4 to 40 RPM over 200 sec). During the testing period mice from the same cage were placed on the rod before the accelerating testing program was initiated. The latency to fall was measured automatically by the RotaRod using light beam sensors. Once all mice had fallen from the rod

the procedure was repeated (5 times in total) and the average of the two longest run times was recorded for analysis. The RotaRod apparatus was cleaned with 70% ethanol and dried between test subjects. Testing was performed between 9 am to 11 am over the testing period.

2.3.3 Hind paw clasping

The hind paw clasping phenotype was tested by suspending the mouse by the tail and applying a light tap to the animals back with a finger (also known as tail test or tail suspension test). Mice that clasped both hind paws tightly to their body were considered to have a complete clasp phenotype. Mice that clasped one paw or both paws in an interrupted manner were considered to have a "half clasp" phenotype.

2.3.4 Tissue collection

At specific experimental time points mice were euthanised using slow flow CO_2 asphyxiation. Perfusion of the mouse was performed by perforating the right atrium and injecting ice cold 1 x PBS into the left ventricle until the perfusate was clear (approximately 20-30 mL). The head was then excised and the whole brain removed and weighted. Cerebral cortex and striatum was dissected and snap frozen in liquid N_2 and stored at -80^{o}C prior to analysis. Brain dissection was performed on an aluminium block on ice.

2.3.5 Lipid extraction

Frozen brain tissue (~5-10 mg) was weighed directly into a 0.5 mL polypropylene tube containing 5 Zircosil® ceramic beads (1.3 mm) (Klausen Pty Ltd, NSW, Australia), 150 µL methanol (0.01% BHT) and internal standards (4^{o}C). Tissue was homogenised at 4°C using a Precellys 24 homogeniser (Bertin Technologies) (2 x 20 s at 5,000 rpm) and the homogenate was transferred to a clean glass vial. The tube and ceramic beads were washed with 100 µL methanol (4°C) and was added to the homogenate with 250 µL of NaOH (1 M). The sample was hydrolysed at room

temperature for 16 h in the absence of light and then acidified with 330 µL of 1 M formic acid. The sample was made up to a final volume of 3 mL by the addition of 2.2 mL milliQ water. The final solution had a methanol concentration of 8% (v:v) and pH 4.5. Solid-phase extraction (SPE) was carried out on a 200 mg mixed C8/anion exchange quaternary amine column (CUQAX223, UCT Inc.) that had been preconditioned with 2 mL methanol and then 2 mL 40 mM formic acid buffer (pH 4.5). The lipid extract was loaded and the column washed with 2 mL methanol in 40 mM formic acid (40:60). The SPE column was dried with N_2 gas flow for 5 min. Sterols and oxysterols were eluted with 2 mL hexane followed by 2 mL hexane/MTBE (50:50).

2.3.6 Triple quadrupole GC-MS sterol analysis

The sterol/oxysterol fraction was dried under N_2 gas flow at 37°C and derivatised by the addition of 20 µL acetonitrile and 20 µL BSTFA + 1% TMCS for one hour at 37°C. Samples were dried under N_2 and immediately reconstituted in 40 µL toluene for GC-MS/MS analysis. Selective reaction monitoring (SRM) analysis of sterols/oxysterols was carried out on an Agilent 7000B triple quadrupole mass selective detector interfaced with an Agilent 7890A GC system gas chromatograph. A 20 m long Rxi-5sil MS column (Restek corp., Bellefonte, PA, USA) was used for chromatography. Analysis of all compounds except cholesterol was performed in a single chromatographic run using a splitless injection. The GC parameters used were as follows: Initial temperature of 200°C to 273°C at a rate of 50°C/min. This was followed by an increase to 300°C at a rate of 2.25°C/min. The flow rate was 0.8 mL/min. The mass spectrometer source temperature was 280°C with an electron energy of -60 eV. Cholesterol was quantified using the internal standard α-cholestane in a separate injection and chromatographic run using a 1:30 split ratio. The GC parameters used were as follows: Initial temperature of 240°C to 305°C at 50°C/min. This was held for 6 min. The flow rate was 1 mL/min. The mass spectrometer source temperature was 280°C with an electron energy of -70

eV. Quantification was performed by Agilent Masshunter Quantitative software (V B.05.00) by comparison of specific SRM transitions of each compound with their corresponding heavy isotopes and using relative response factor calibration. A typical chromatogram of each analyte measured in mouse brain tissue is presented in Figures 1-2. Relative molar response factors of all analytes were calculated from calibration curves constructed from 5 different concentrations of authentic oxysterol mixed with deuterated standards in triplicate. The assayed concentrations - sterols/oxysterols (0.1-300 ng) and cholesterol (25 to 150 ug) covered the range expected from brain tissue. The linearality of standard curves was excellent as determined by simple linear regression ($r^2 > 0.985$). Peak area of a single target transition was used for quantification, with at least one other transitions used for qualification. The calibration curve for each compound was constructed with the lowest calibration being at least 5-10 fold lower than the analyte measured in the sample. No significant matrix effects that impacted on analysis were detected. This method has been fully validated for reproducibility, accuracy and sensitivity according to International Committee for harmony guidelines (International Council for Harmonisation, 2005). Method validation was performed on 6 mg tissue aliquots of a single pool of mouse cortex homogenate (60mg/ml), that was spiked with different concentrations of sterol. Percent recovery ranged between 89-104 %. Intra and inter day assay CV % was 0.1 - 7.9 and 1.5 – 8.1 respectively (Jenner, A.M. 2015, unpublished data).

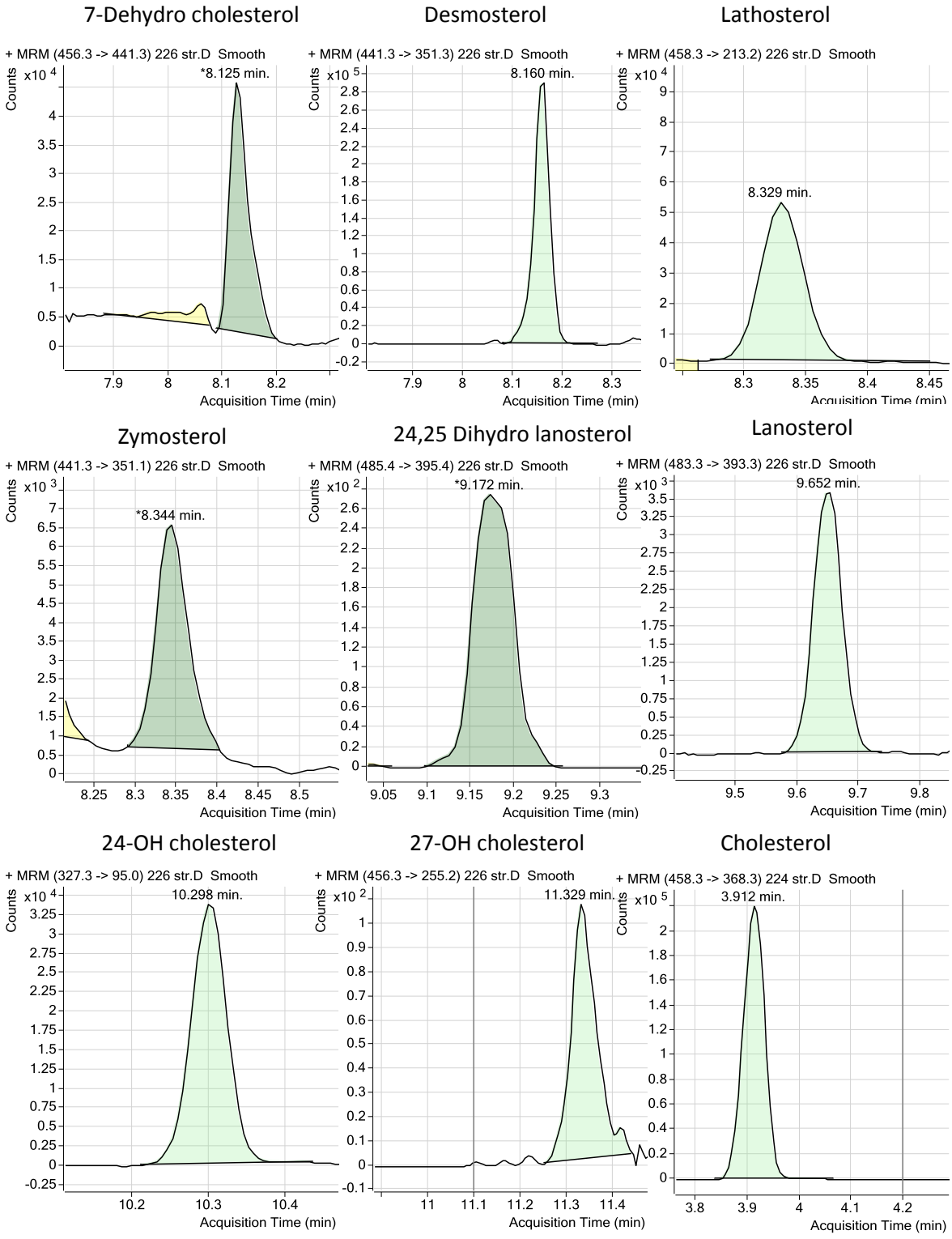

Figure 2.1 **Typical chromatograms of sterol compounds analysed by triple quadrupole GC-MS.** These chromatograms are representative of lipid extracted from mouse striatum.

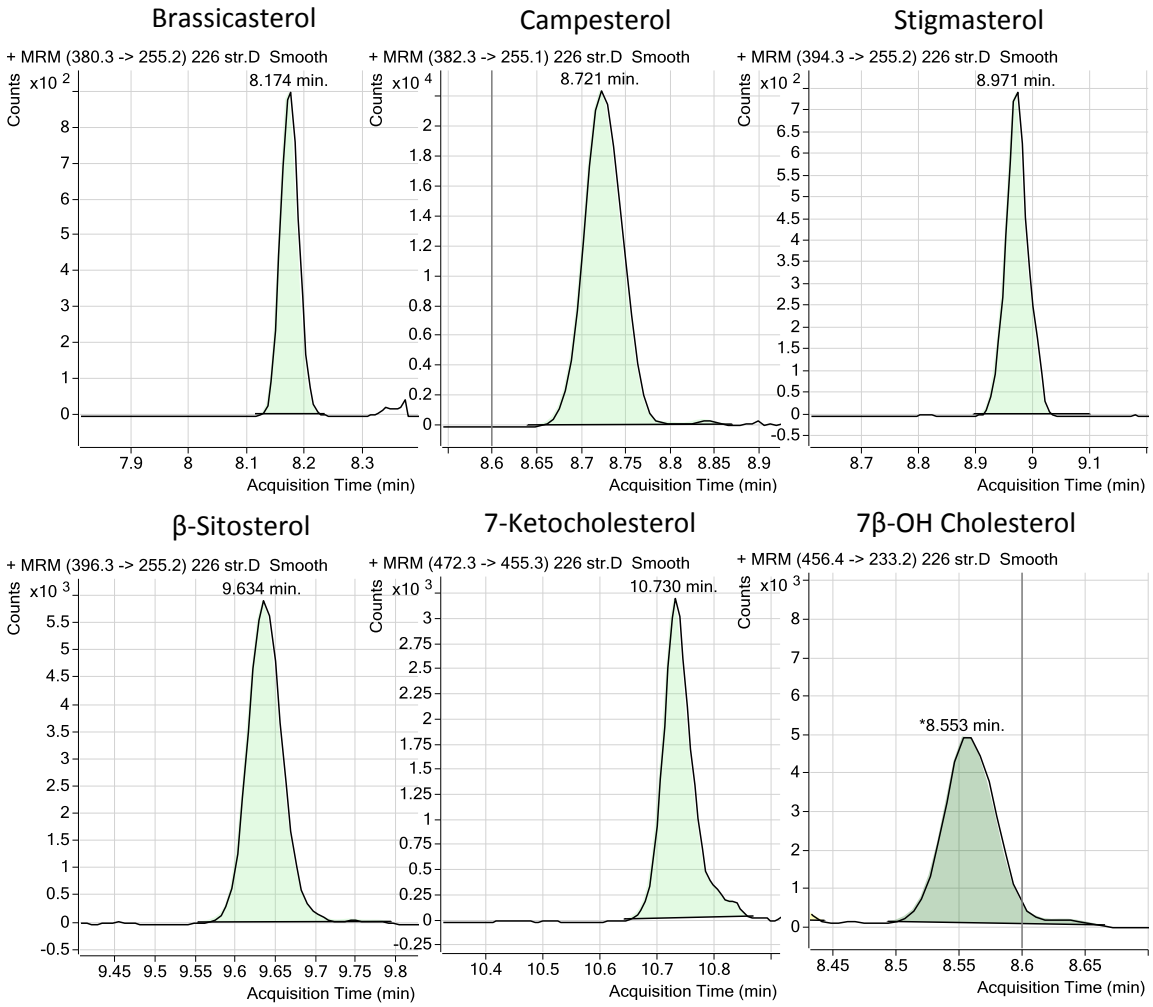

Figure 2.2 **Typical chromatograms of sterol compounds analysed by triple quadrupole GC-MS.** These chromatograms are representative of lipid extracted from mouse striatum.

Chapter 3

Association of cholesterol metabolism with Huntington's disease progression in R6/1 transgenic mice

3.1 Introduction

Significant research has been invested into determining HD pathology that involves a number of neurophysiological changes that manifest as behavioural, motor and cognitive impairments. These changes have been classified over the progression of the disease in humans and in a limited number of mouse models. Distinct biochemical alterations are also present in the HD brain. Among these changes is a defect in cholesterol biosynthesis and metabolism that has been consistently demonstrated in several mouse models. These models include mice expressing an expanded fragment of human HTT (R6/2), full length human HTT (YAC) and expanded mouse hungtingtin (hdh^{Q111}). Due to the complexity of the cholesterol synthetic pathway (Bloch, 1965), previous studies have only measured a small number of synthetic intermediates and are unable to describe the full extent of the alterations that occur.

In this chapter the R6/1 mouse model expressing a CAG expanded exon 1 of human HTT (~115-120 repeats) was characterised in terms of cholesterol synthetic and metabolic changes. Physical phenotypic changes were also examined including, impaired motor performance, weight loss, reduced brain mass and involuntary clasping. Analysis of multiple time points and brain regions aimed to establish when specific alterations to the cholesterol synthetic and metabolic pathways occur, and the potential influence these have in the disease. A comprehensive analysis of sterol compounds was also undertaken to identify any potential biomarkers associated with HD in R6/1 mice, to be used for future studies.

3.2 Materials and Methods

Materials: Materials used in this study are listed in 2.2.

Mice: R6/1 mice were generated as outlined in 2.3.1. Male and female mice (5-6 per group) were housed in cages of 4-5 mice, with at least 2 mice of each genotype per cage. Standard laboratory housing was provided (sawdust, pine shavings and a single PVC tube) with standard rodent diet (see 2.3.1) and water available *ad libitum*. Mice were acclimatised over 1 week and this included brief handling and weighing**.** All animal experiments were conducted with approval from the University of Wollongong ethics committee (ethics number AE 13/20).

Body weight: Mouse weight was recorded every 4-5 days from 7 weeks of age until sacrifice.

Hind paw clasping: Hind paw clasping was tested every 5-10 days following the procedure outlined in 2.3.3.

RotaRod: Motor performance was measured using the RotaRod protocol outlined in 2.3.2. The RotaRod performance of mice was tested at 6, 11, 15, 19 and 23 weeks of age.

Tissue collection: Mice were sacrificed at 5 time points (6, 12, 20, 24 and 28 weeks) using slow flow CO_2 asphyxiation. Brain tissue was dissected and collected as described in 2.3.4.

Lipid extraction and GC-MS/MS analysis of sterols: Quantification of cholesterol synthetic precursors, metabolites, oxidation products and phytosterols was performed as outlined in 2.3.5 and 2.3.6.

Statistical Analysis: Linear regression analysis was used to identify the rate of weight gain between genotypes and identify the relationship between age and sterol changes in mice. A 2-way ANOVA was used to analyse RotaRod performance, body weight, brain lipid and brain mass data. Bonferroni post-tests were used to compare means at individual time points. A student's t test was also used to compare means at individual time points in the sterol data. $P < 0.05$ was

considered significant. All analyses were performed in Graphpad PRISM v5.0 (Graphpad Software Inc., USA).

3.3 Results

3.3.1 Physical phenotype

The cohort of R6/1 and WT littermates housed until 28 weeks of age (n = 5 per genotype and sex) were tested periodically for physical changes that characterise the progression of the HD phenotype. These results are represented in Figures 3.1, 3.2 and 3.3.

3.3.1.1 Weight loss in R6/1 mouse

Mouse body weight was recorded every 4-5 days (Figure 3.1); male and female R6/1 body weight was significantly reduced compared to WT mice over the course of the study ($p < 0.0001$). The mean body weight of R6/1 mice did not increase after 16 weeks of age in males, and 19 weeks in females. Prior to weight loss (7-19 weeks) the rate of weight gain was significantly less in female R6/1 mice compared to WT ($p = 0.00012$). No significant difference in the rate of weight gain was detected between male R6/1 and WT mice in the 6-16 week period ($p = 0.073$). By the 28 week time point the average weight increase in R6/1 mice from the earliest measurement (7 weeks) was 8% in males and 19% in females.

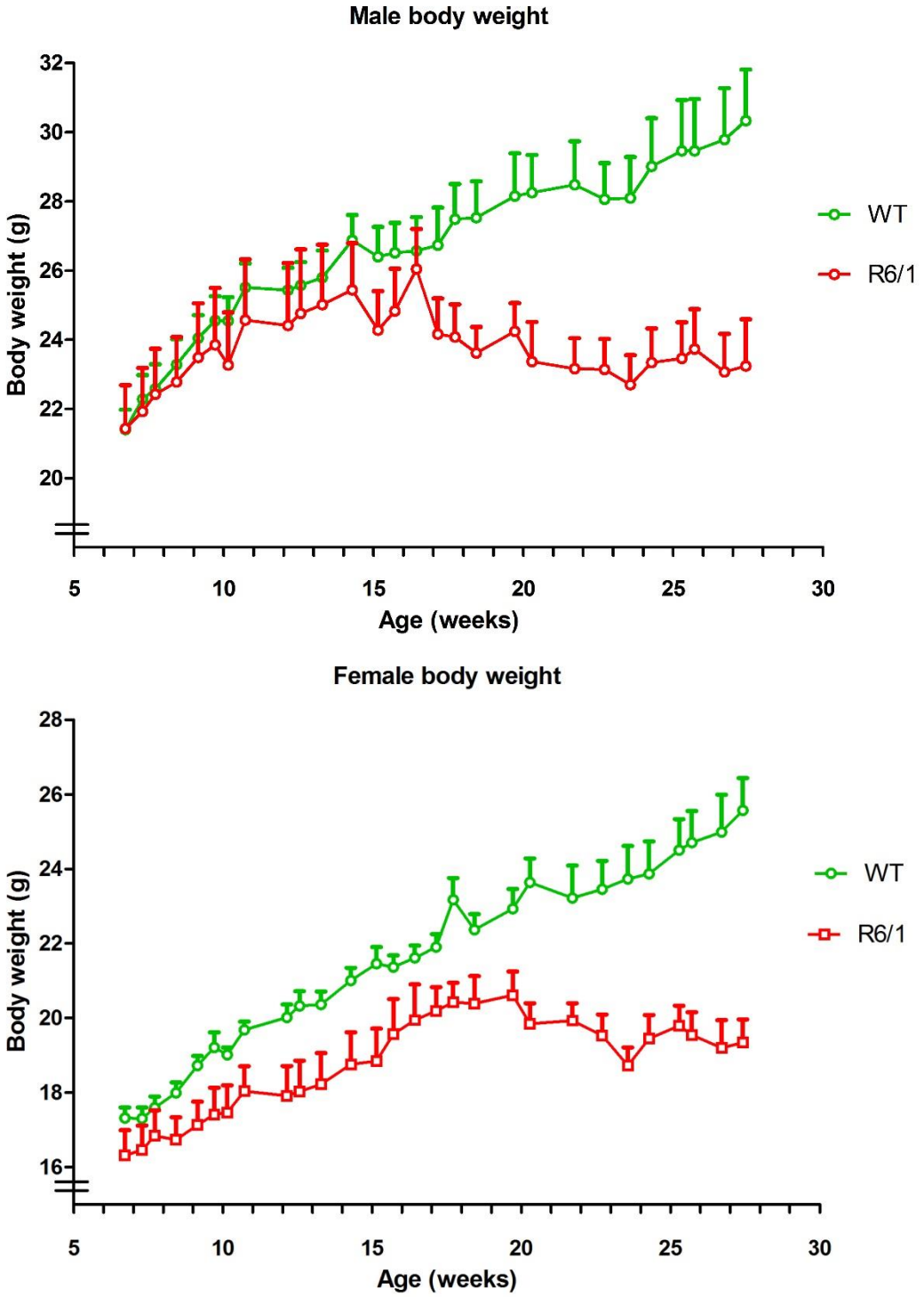

Figure 3.1 **Weight loss in R6/1 mice during HD progression.** R6/1 mice had significantly reduced body weight compared to WT over the course of the study ($p < 0.0001$) (2-way ANOVA). The R6/1 weight loss phenotype is more severe in male mice compared to females. Male R6/1 mice began to lose weight after 16 weeks compared to 19 weeks in females. The average weight gain over 28 weeks was less in male R6/1 mice (8%) compared to female R6/1 mice (19%). The rate of weight gain prior to weight loss was significantly slower in R6/1 females compared to WT ($p = 0.00012$) (linear regression analysis) while no difference was observed in male mice. n = 5 per group. Error bars represent + SEM.

3.3.1.2 Hind paw clasping phenotype

The hind paw clasping phenotype previously observed in R6/1 and R6/2 mice (Mangiarini *et al.*, 1996) was also tested over the course of the study (Figure 3.2). The clasping phenotype was first observed at 12 weeks in males, with the percentage of mice showing positive for the phenotype increasing to 60% at the end of the study. Female R6/1 mice first exhibited the clasping phenotype at the age of 17 weeks. The percentage of female R6/1 mice showing the phenotype increased to 60% by the end of the study. A small proportion of WT mice showed positive to a "half clasp" phenotype at varying times throughout the study.

3.3.1.3 Motor performance

A RotaRod apparatus was used to test motor performance in R6/1 mice (Figure 3.3). The average latency to fall from the RotaRod was significantly less in R6/1 mice compared to WT in both males ($p = 0.0016$) and females ($p = 0.0004$) over the course of the study. A significant difference at the 23 week time point was detected between male R6/1 mice and WT ($p < 0.01$). No difference in the RotaRod performance was detected between sexes of the same genotype ($p = 0.81$).

3.3.1.4 Brain mass

Mouse brain (extracted for sterol analysis at each time point) was weighed prior to dissection. The brain mass of both male and female R6/1 mice was significantly less than WT littermates at later time points (Figure 3.4). The difference between WT and R6/1 male mice was highly significant at 20, 24 and 28 weeks ($p < 0.0001$). In female mice a significant difference was detected at 12 and 20 weeks ($p < 0.05$), becoming highly significant at 24 and 28 weeks ($p < 0.0001$).

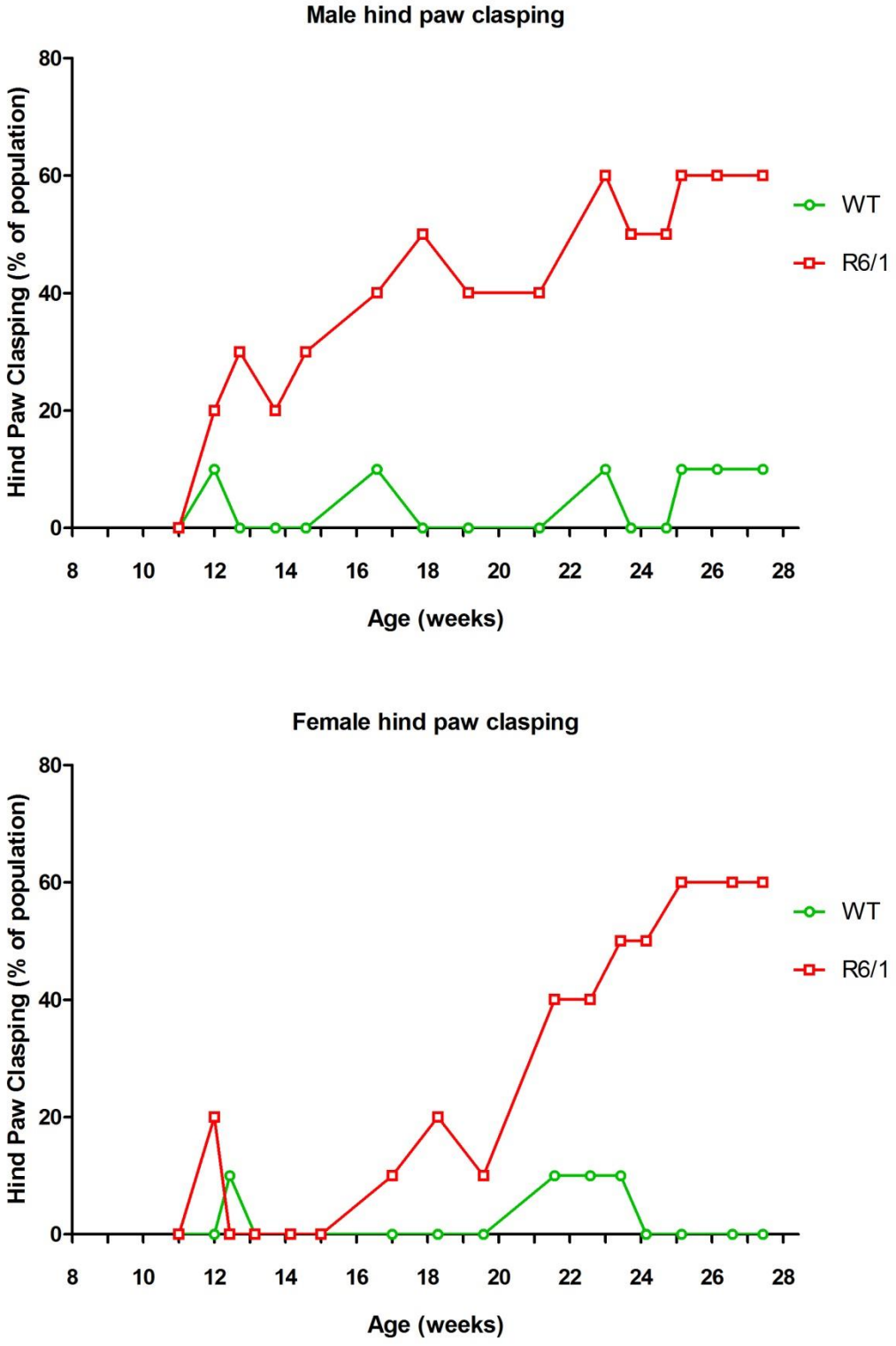

Figure 3.2 **Hind paw clasping phenotype in R6/1 mice.** R6/1 mice develop a phenotype where the hind paws are clasped to the body when suspended by the tail. Male R6/1 mice exhibited the phenotype prior to females; the occurrence of the phenotype increased in the population during disease progression with 60% of the R6/1 mice showing a positive clasping phenotype by 28 weeks. A small proportion of WT mice appeared to exhibit a half clasp phenotype during the course of the study. n = 5 per group.

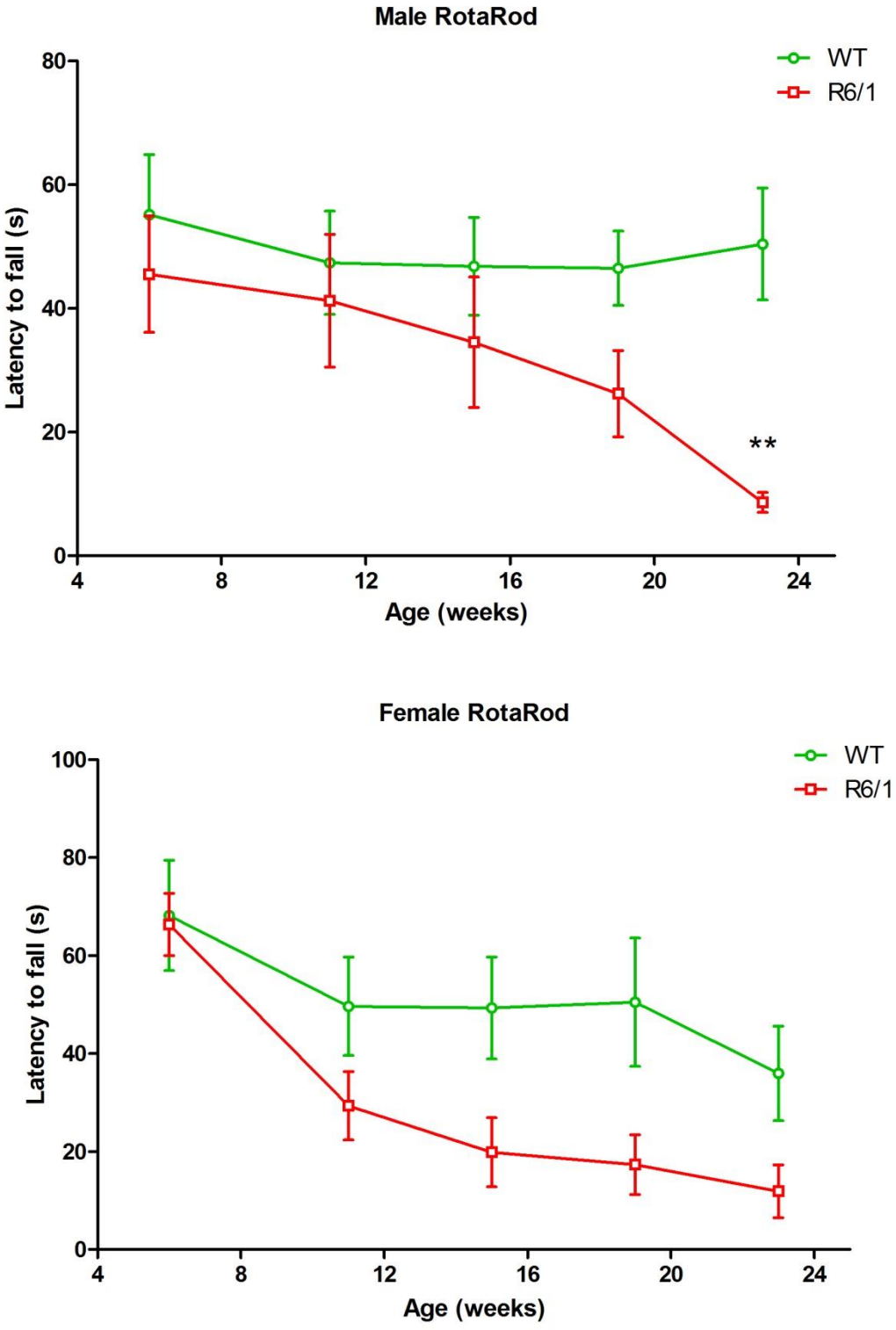

Figure 3.3 **RotaRod motor performance of R6/1 mice.** A progressive decline in motor performance was observed in both male and female R6/1 mice compared to WT littermates ($p = 0.0016$ and 0.0004 respectively). No difference between male and female mice with the same genotype was detected. $n = 5$ per group. Error bars represent ± SEM. 2-way ANOVA Bonferroni post-test ** $p < 0.01$.

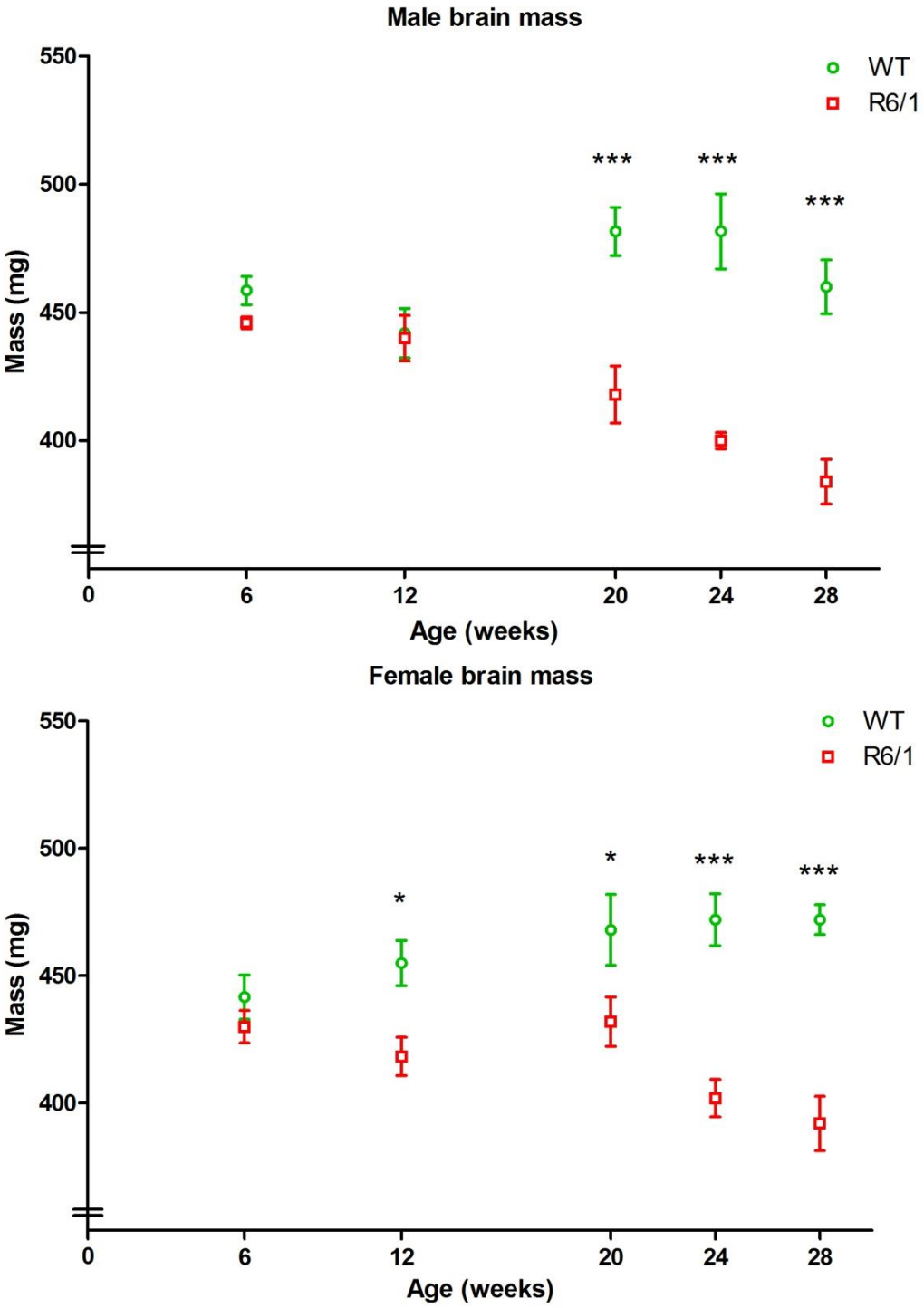

Figure 3.4 **Brain mass of R6/1 mice during disease progression.** Whole brain mass was significantly reduced in both R6/1 male and female mice compared to WT. The difference between WT and R6/1 in male mice was highly significant at 20, 24 and 28 weeks. In female mice a significant difference was detected at 12 and 20 weeks, becoming highly significant at 24 and 28 weeks. The brain mass was measured from mice sacrificed at each time point of lipid analysis. Each point represents 5-7 mice. Error bars represent ± SEM. 2-way ANOVA Bonferroni post-test *$p < 0.05$ ***$p < 0.0001$

3.3.2 Sterol analysis of R6/1 brain tissue

No major differences in sterols (cholesterol synthetic precursors, metabolites, oxidation products and phytosterols) were found between sexes in R6/1 or WT mice. To increase statistical power both sexes were combined and reanalysed. The level of all sterols measured for the individual sexes can be found in Appendix 1-3. The following results summarise the data for combined sexes. The sterol related compounds were detected in the brain tissue at concentrations ranging from 0.1 – 80 ng.mg^{-1} tissue. This consistent with the several compounds that have been previously investigated in mouse brain (Valenza *et al.*, 2007b; Valenza *et al.*, 2010).

3.3.2.1 Cholesterol synthetic precursors

Several cholesterol synthetic precursors were significantly decreased in R6/1 striatum and cortex compared to WT (Figures 3.5A-D, 3.6A-D). Lathosterol levels were significantly reduced from the pre-symptomatic age of 6 weeks in R6/1 striatum ($p < 0.001$) and cortex ($p < 0.05$) when compared to WT mice. Lathosterol was consistently reduced in both cortex and striatum of R6/1 mice compared to WT in all later time points (12, 20, 24 and 28 weeks; $p < 0.001$). Lanosterol and zymosterol followed a similar consistent reduction; a significant difference in lanosterol levels was first detected at 12 weeks in striatum and cortex of R6/1 (cortex $p < 0.05$, striatum $p < 0.01$). R6/1 striatum exhibited an early reduction in zymosterol at 6 weeks ($p < 0.01$), and at all later time points. The level of zymosterol in cortex tissue was only significantly reduced compared to WT at 28 weeks of age ($p < 0.05$). The cholesterol synthetic precursor 24,25 dihydro lanosterol was not significantly altered in the R6/1 striatum or cortex although it was detected at lower mean levels than WT at all time points examined.

3.3.2.2 Cholesterol synthetic precursors during ageing

Lanosterol, lathosterol, zymosterol and 24,25 dihydro lanosterol levels decreased at a similar rate over time in the striatum of WT and R6/1 mice ($p < 0.01$), however no significant difference in the slope was detected between R6/1 and WT (Figure 3.5A-D).

Cortex tissue showed a different profile compared to striatum in terms of changes in cholesterol synthetic precursor levels over time. Both zymosterol and lanosterol decreased over time in R6/1 mice ($p = 0.0184$ and $p < 0.0001$ respectively) while WT levels did not significantly change (Figure 3.6B, C.). Analysis of the linear regression confirmed the slopes of R6/1 were significantly different to WT (zymosterol $p = 0.005$ and lanosterol $p = 0.0078$). The level of 24,25 dihydro lanosterol did decrease significantly over time in R6/1 mice ($p = 0.0018$), however no difference in the rate of change was detected between WT and R6/1 (Figure 3.6D). Lathosterol decreased both in R6/1 and WT mice at a similar rate (Figure 3.6A). The correlation of cholesterol synthetic precursor change over time in WT and R6/1 mice is summarised in Table 3.1.

Unlike upstream sterols in the cholesterol synthetic pathway, desmosterol levels did not significantly decrease over time in the striatum of R6/1 mice, being either elevated or unchanged at specific time points. Linear regression analysis identified that the rate of change in WT and R6/1 desmosterol levels was significantly different ($p = 0.005$); with R6/1 desmosterol levels not decreasing significantly over time (Figure 3.5E). Comparing the means at individual time points identified desmosterol was significantly elevated in R6/1 striatum at 20 and 28 weeks compared to WT (t-test, $p = 0.046$ and $p = 0.0138$). Desmosterol levels in cortex were not altered over time in both WT and R6/1 mice, however there was a small but significant elevation of desmosterol in R6/1 mice at 28 weeks (t-test, $p = 0.0431$) (Figure 3.6E). 7-dehydrocholesterol levels did not decrease over time in cortex or striatum of R6/1 mice, and no difference between R6/1 and WT mice was detected at any time point (Figures 3.5F, 3.6F).

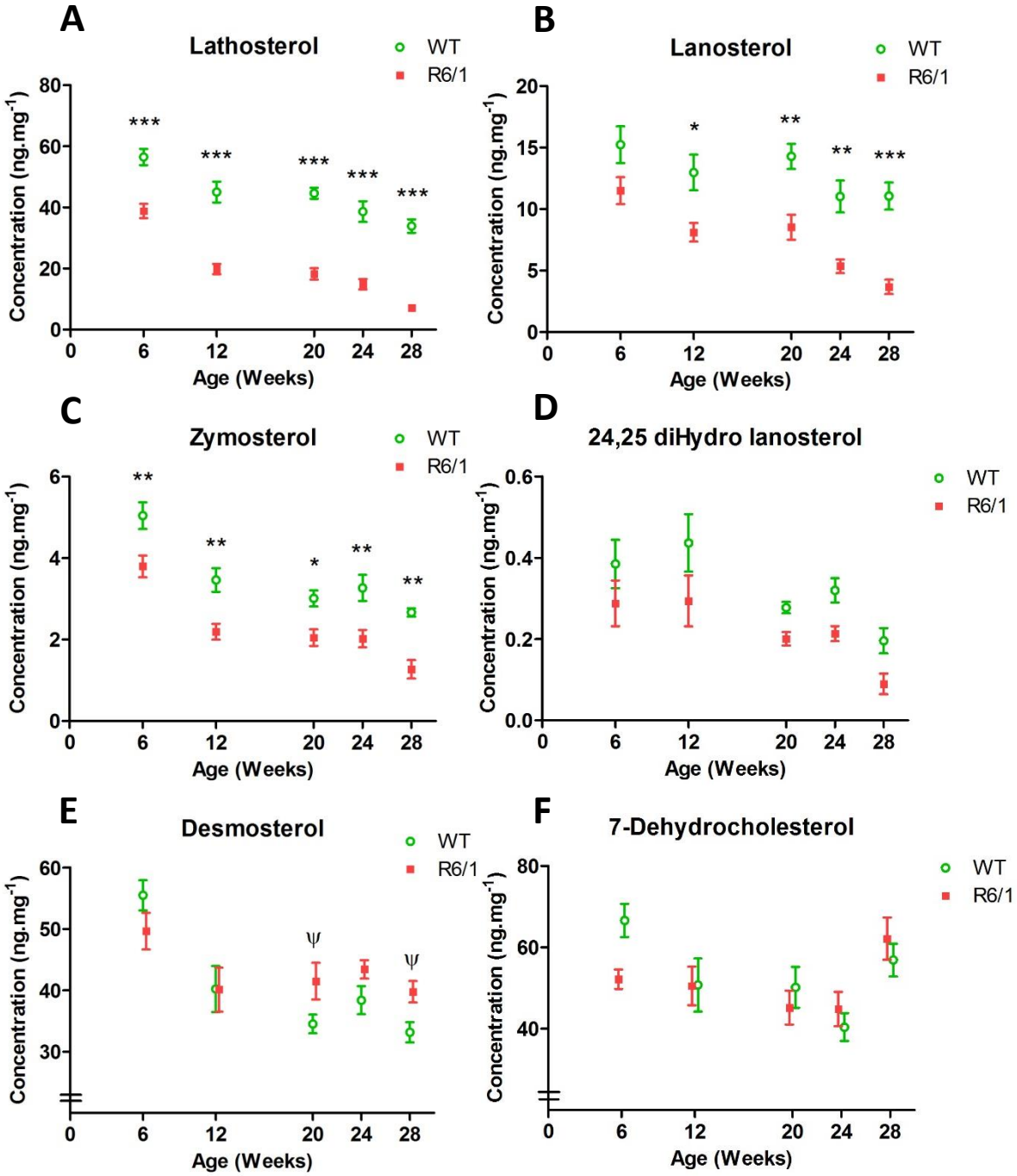

Figure 3.5: **Cholesterol synthetic precursor levels in striatum tissue of R6/1 and WT littermates**. Extracted sterols were quantified using heavy isotope mass dilution GC-MS/MS. (A) Lathosterol, (B) lanosterol and (C) zymosterol levels were significantly reduced early in the R6/1 mice compared to WT. (D) 24,25 dihydro lanosterol showed a trend to be decreased in R6/1 mice however there was no significant difference between WT and R6/1 at any time point. Lanosterol, lathosterol, zymosterol and 24,25 dihydro lanosterol decreased over time in both R6/1 and WT mice ($p < 0.01$). Genotype had no effect on the rate of change over time. The rate of change over time in (E) desmosterol levels in WT and R6/1 was significantly different ($p = 0.005$). Desmosterol did not significantly decline in R6/1 mice, and was significantly elevated at 20 and 28 weeks compared to control. (F) 7-Dehydrocholesterol did not significantly decline in R6/1 mice and no significant differences were detected between R6/1 and WT at any time point examined. Each data point represents combined results from male and female mice, n = 10-13 per group. Error bars represent ± SEM. 2-way ANOVA Bonferroni post-test *$p < 0.05$ **$p < 0.01$ ***$p < 0.0001$. t-test $^{\Psi}p < 0.05$

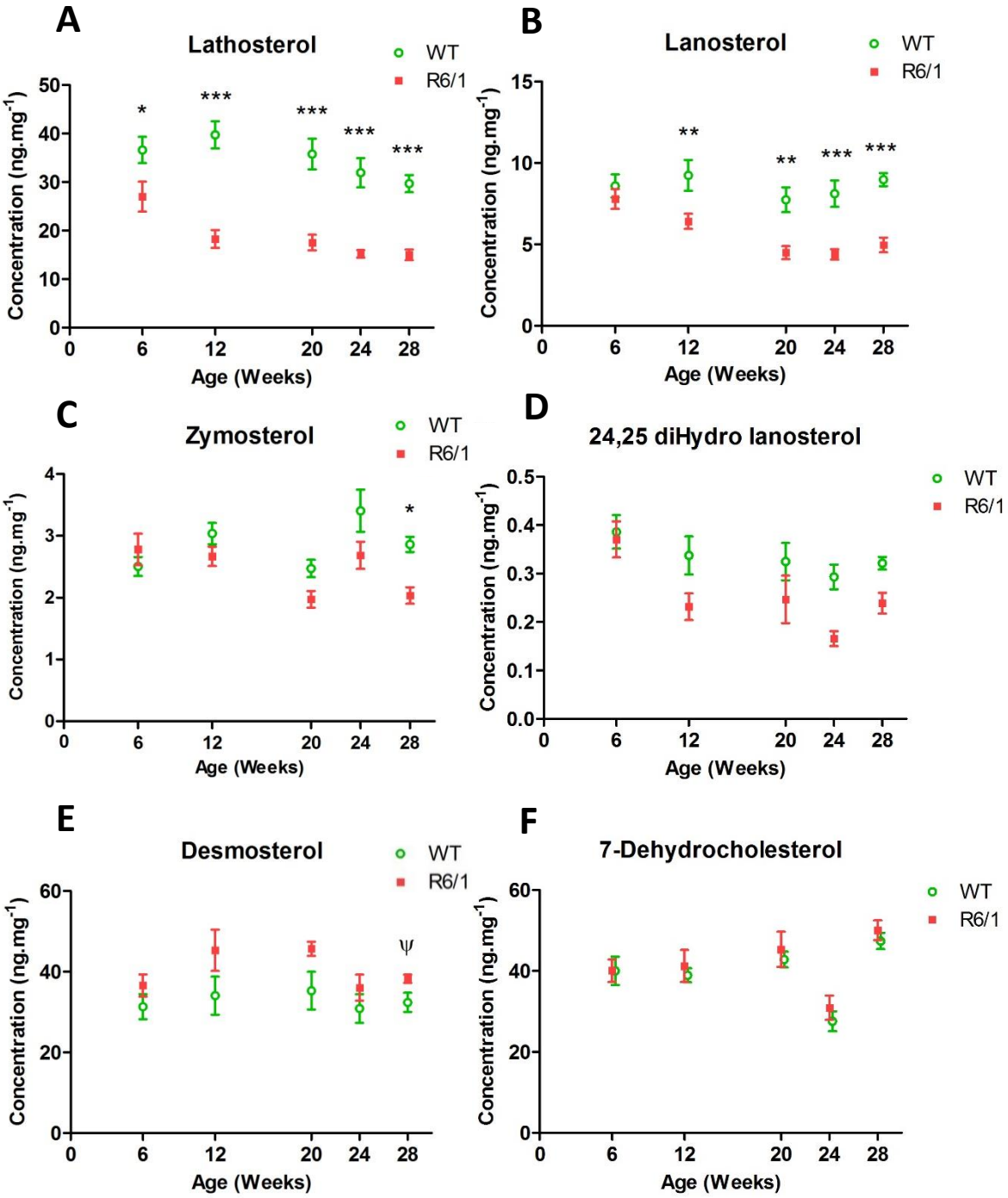

Figure 3.6: **Cholesterol synthetic precursor levels in cortex tissue of R6/1 and WT littermates.** Extracted sterols were quantified using heavy isotope mass dilution GC-MS/MS. (A) Lathosterol and (B) lanosterol levels were significantly reduced early in R6/1 mice compared to WT. (C) Zymosterol was significantly reduced in R6/1 at the end time point. (D) 24,25 dihydro lanosterol had a trend to be reduced however there was no significant difference between WT and R6/1 at any time point. Zymosterol and lanosterol decreased in R6/1 over time while WT levels remained unchanged ($p < 0.01$). Lathosterol levels decreased both in R6/1 and WT at a similar rate. 24,25 dihydro lanosterol levels were not detected to change over time. The rate of change in (E) desmosterol levels over time was not significantly different between R6/1 and WT; however desmosterol was elevated in R6/1 mice at 28 weeks. (F) 7-Dehydrocholesterol did not significantly decline in R6/1 mice and no significant differences were detected between R6/1 and WT at any time point examined. Each data point represents combined results from male and female mice, n = 10-13 per group. Error bars represent ± SEM. 2-way ANOVA Bonferroni post-test *$p < 0.05$ **$p < 0.01$ ***$p < 0.0001$. t-test $^{\Psi}p < 0.05$

3.3.2.3 Cholesterol

Total cholesterol levels were not significantly altered between R6/1 and WT mice in this study. However, an increase in cholesterol levels over time was observed both in striatum and cortex of WT and R6/1 mice. Striatum WT (p = 0.0003), R6/1 (p = 0.0005). Cortex WT (p = 0.048), R6/1 (p = 0.0075) (Figure 3.7). Analysis of the slope revealed the rate of change over time was not significantly different between R6/1 or WT mice in either striatum or cortex. The correlation coefficient of cholesterol levels over time is provided in Table 3.1.

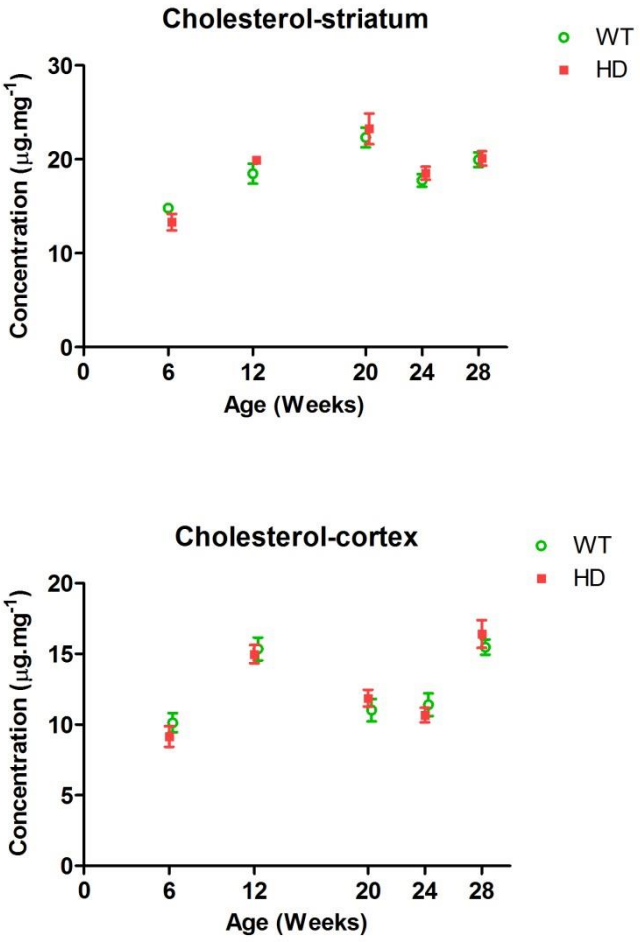

Figure 3.7: **Cholesterol levels in cortex tissue of R6/1 and WT littermates**. Extracted sterols were quantified using heavy isotope mass dilution GC-MS/MS. No difference between cholesterol levels was detected between WT and R6/1 mice. The absolute level of cholesterol did increase with time in cortex and striatum of both WT and R6/1 mice. [Striatum WT (p = 0.0003), R6/1 (p = 0.0005). Cortex WT (p= 0.048), R6/1 (p=0.0075).] The rate of change over time was not significantly different between R6/1 or WT mice in either striatum or cortex. Each data point represents combined results from male and female mice, n = 10-13 per group. Error bars represent ± SEM.

3.3.2.4 Cholesterol metabolites

The brain specific cholesterol elimination product 24-OHC was significantly reduced in the striatum of R6/1 mice at the end time point of 28 weeks ($p < 0.01$, Figure 3.8). No other time point or brain region measured showed an effect of genotype on 24-OHC levels. 24-OHC levels increased in both striatum and cortex of WT and R6/1 mice over time ($p < 0.01$) however there was no difference between the rate of change between R6/1 and WT (linear regression slopes were not significantly different, Figures 3.8, 3.9)

27-OHC, a predominantly peripheral metabolite of cholesterol was consistently depleted in R6/1 striatum from the age of 12 weeks ($p < 0.05$) and in all later time points (20 weeks $p < 0.01$, 24 weeks $p < 0.05$, 28 weeks $p < 0.05$, Figure 3.8). 27-OHC levels were not significantly altered in cortex tissue of R6/1 mice when compared to WT at any of the 5 time points examined. 27-OHC levels in both striatum and cortex showed a small increase over time in WT mice ($p = 0.0288$ and $p = 0.0399$ respectively) but not in R6/1 (Figures 3.8, 3.9). When comparing WT and R6/1 directly, no difference in rate of change was found in cortex or striatum. The correlation of 24-OHC and 27-OHC changes over time is summarised in Table 3.1.

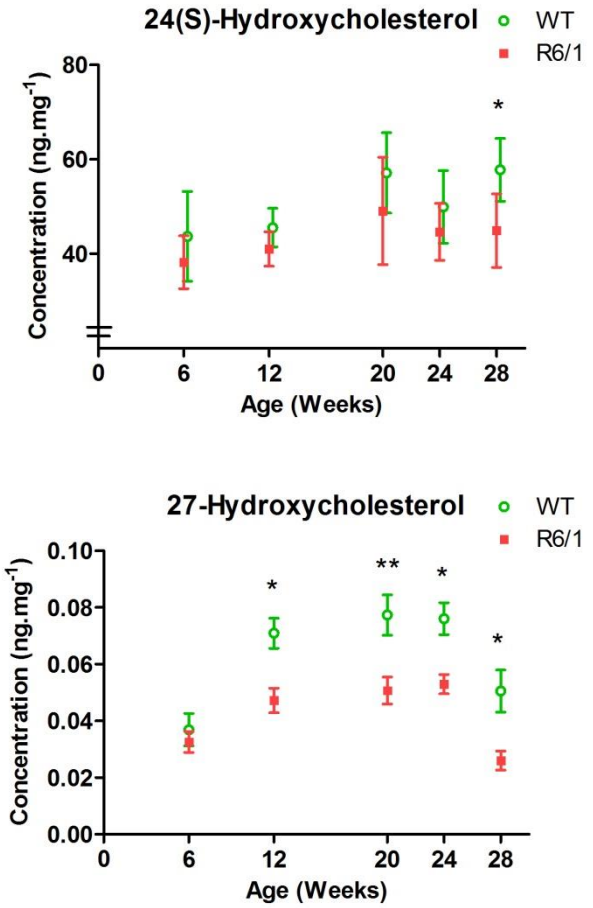

Figure 3.8: **Cholesterol metabolites in striatum tissue of R6/1 and WT littermates**. Extracted sterols were quantified using heavy isotope mass dilution GC-MS/MS. 24(S)-Hydroxycholesterol was significantly reduced in striatum of R6/1 mice compared to WT at the end stage of the study. A significant reduction in 27-hydroxycholesterol levels was detected early (12 weeks), and was consistently reduced until 28 weeks. The level of 24-OHC increased during ageing in both WT and R6/1 mice ($p < 0.01$), the rate of change was not different between genotypes. Each data point represents combined results from male and female mice, n = 10-13 per group. Error bars represent ± SEM. Bonferroni post-test *$p < 0.05$ **$p < 0.01$

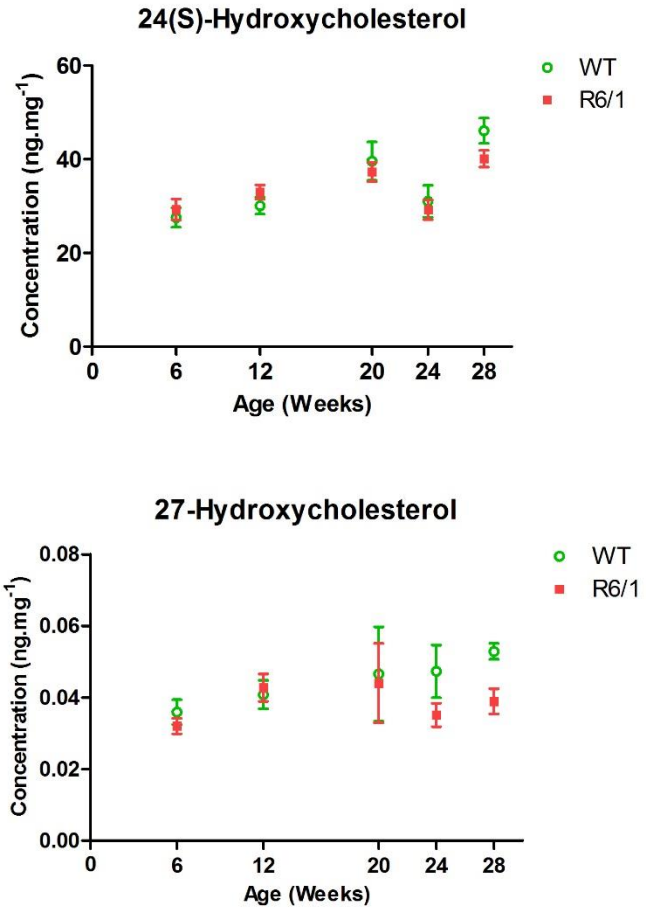

Figure 3.9: **Cholesterol metabolites in cortex tissue of R6/1 and WT littermates**. Extracted sterols were quantified using heavy isotope mass dilution GC-MS/MS. R6/1 cortex did not have significantly altered levels of 24-OHC or 27-OHC at any time point examined. 24-OHC levels increased in WT and R6/1 mice during ageing ($p < 0.01$), the rate of change was not different between genotypes. Each data point represents combined results from male and female mice, n = 10-13 per group. Error bars represent ± SEM.

Table 3.1 **Age/sterol correlation in R6/1 and WT mice**. Sterols were analysed at five time points (6, 12, 20, 24 and 28 weeks) in striatum and cortex tissue of R6/1 and WT mice. Each time point consisted of combined male and female mice from both R6/1 and WT mice (n = 10-13 per group).

	Striatum - correlation coefficient (r)		Cortex - correlation coefficient (r)	
	WT	R6/1	WT	R6/1
Cholesterol synthetic precursors				
Lathosterol	-0.63*	-0.82*	-0.31*	-0.54*
Lanosterol	-0.31*	-0.67*	-0.047	-0.61*
Zymosterol	-0.63*	-0.69*	0.22	-0.32*
24, 25 Dihydro lanosterol	-0.40*	-0.44*	-0.26	-0.42*
Desmosterol	-0.64*	-0.26	0.0066	-0.047
7-Dehydrocholesterol	-0.30	0.076	0.022	0.083
Cholesterol metabolites				
24(S)-Hydroxycholesterol	0.53*	0.36*	0.49*	0.33*
27-Hydroxycholesterol	0.29*	0.060	0.28*	0.066
Cholesterol	0.48*	0.47*	0.27*	0.36*

*Indicates compounds with a significantly non-zero gradient ($p < 0.05$, linear regression analysis)

3.3.2.5 Cholesterol oxidation products

Free radical generated oxidation products of cholesterol were also measured over the course of the study. No significant differences in the level of 7-KC and 7β-OHC were detected between R6/1 and WT in any brain region or timepoint measured. The level of 7-KC and 7β-OHC were not significantly altered over time in cortex or striatum in either genotype (Appendix 1-3).

3.3.2.6 Phytosterols

Plant-synthesised sterols that are derived from the diet share a similar structure to cholesterol and can be measured in brain tissue. A small accumulation of phytosterols was observed in cortex and striatum during ageing in both WT and R6/1 (Appendix1-3). This reached significance in campesterol and brassicasterol. No effect of genotype on the on the level of phytosterols (campesterol, β-sitosterol, stigmasterol and brassicasterol) in cortex or striatum was detected in this study

3.4 Discussion

Brain cholesterol homeostasis is maintained by *de novo* synthesis and brain-specific excretion pathways. Brain cholesterol is also isolated from peripheral cholesterol levels as the BBB is impermeable to cholesterol (Bjorkhem & Meaney, 2004). These regulatory mechanisms indicate that maintaining cholesterol levels is essential for normal brain function. This is indeed the case as cholesterol has essential functions involving synaptogenesis (Mauch *et al.*, 2001), axon growth (Hayashi *et al.*, 2004), membrane fluidity (Yeagle, 1985) and lipid raft structure relevant to cell signalling (Sheets *et al.*, 1999; Zajchowski & Robbins, 2002; Kannan *et al.*, 2007). Genetic defects in cholesterol synthetic enzymes also cause severe neurological impairment in diseases such as Smith-Lemli-Optitz (Wassif *et al.*, 1998) and desmosterolosis (Waterham *et al.*, 2001).

A defect in cholesterol synthesis was first described in HD transgenic cells that identified several genes involved in cholesterol biosynthesis downregulated, including *HMGCR* and *DHCR7* (Sipione et al., 2002). Several HD mouse models have also been generated that express either: an expanded fragment of human HTT (Mangiarini *et al.*, 1996), an expanded full-length human HTT (Hodgson *et al.*, 1999; Slow *et al.*, 2003), or an expanded mouse huntingtin (Wheeler *et al.*, 1999). A similar deficit in cholesterol biosynthesis has generally been reported in these models, identified through a reduction of cholesterol synthetic precursors (Valenza *et al.*, 2005; Valenza *et al.*, 2007a; Valenza *et al.*, 2007b; del Toro *et al.*, 2010; Valenza *et al.*, 2010) and, in some cases, total cholesterol levels (Valenza *et al.*, 2007a).

The current literature has mostly used the R6/2 transgenic mouse model that expresses exon 1 of human HTT with a 140-150 CAG repeat and has a rapid onset of symptoms. This mouse has been characterised in terms of phenotypic progression and potential changes in only a limited number of cholesterol synthetic precursors and metabolites. We have however, chosen to examine the R6/1 model which expresses a shorter CAG expansion (115-120) of exon 1 HTT. The R6/1 mouse has a later onset of motor dysfunction and a longer life span, therefore providing a

model that more accurately reflects the late onset and long timeline (15-30 years) of disease in human patients (Foroud *et al.*, 1999). The R6/1 model has already been characterised in terms of behavioural phenotype and some molecular changes including, glycosphingolipid abnormalities (Denny *et al.*, 2010), gene expression profile alterations (Hodges *et al.*, 2008), cannabinoid receptor changes (Naver *et al.*, 2003) and serotonin transporter deficits (Pang *et al.*, 2009). Cholesterol synthesis and metabolism has not been comprehensively examined prior to experiments undertaken in this chapter.

3.4.1 Cholesterol synthesis and metabolism

Previous studies examining brain cholesterol changes in mouse models typically investigated a limited number of compounds at one or two time points during disease progression, in a single brain region or whole brain homogenate. In addition to this, potential differences between sexes have not been explored. A comprehensive analysis is required to assess whether alterations of cholesterol synthesis are a driving factor or a secondary event involved in HD pathogenesis.

In this study we have analysed cortex and striatum in male and female R6/1 mice at 5 time points over the course of the disease, from a pre-symptomatic age (6 weeks) to end stage (28 weeks). Mass dilution GC-MS/MS analysis was used to quantify a larger number of cholesterol synthetic precursors, major metabolites, cholesterol oxidation products and phytosterols. This is the first study to comprehensively examine sterol changes in the R6/1 mouse, and the most comprehensive sterol analysis of any HD mouse model to date.

3.4.1.1 Cholesterol synthetic precursors

Significant differences between sexes were not detected in the sterol profile of either R6/1 or WT mice, and for simplicity the following discussion describes changes occurring when sexes were combined. Analysis of combined sexes also increased the statistical power, and detected significance in compounds exhibiting small changes. Although this study was not able to detect

differences between sexes in sterol levels of R6/1 mice, increasing the "n" number for future studies may identify small but significant differences that may explain the differences in the behavioural phenotype observed between sexes.

Sterol analysis of R6/1 cortex and striatum showed a significant reduction in several cholesterol synthetic precursors over the course of the disease. The significant reduction of lathosterol and zymosterol in the striatum of R6/1 mice at 6 weeks of age represents the earliest detectable change in the R6/1 sterol profile, and appears before the onset of overt motor dysfunction measured on the RotaRod. Changes to zymosterol are more specific to the striatum compared to other precursors that decrease early in both cortex and striatum. This highlights zymosterol as a possible specific biomarker of early striatal changes in HD mice. 24,25 Dihydro lanosterol levels were not significantly altered between R6/1 and WT mice however the mean concentration in R6/1 mice was consistently lower than WT at all five time points. This sterol is found at low concentrations in the brain (0.1-0.4 ng/mg tissue), and while the GC-MS/MS method we have developed can sensitively detect this compound at these levels, it may be prone to greater concentration variation between individuals at any time, and statistically significant differences would therefore be difficult to detect with the current study size.

Synthetic precursor levels were more severely affected in striatum compared to cortex in our study; changes occurred earlier and were of a greater magnitude in striatum (lathosterol decreased by 80% in striatum compared with 50% in cortex). This observation is consistent with previous studies in the YAC and R6/2 models (Valenza *et al.*, 2007a; Valenza *et al.*, 2007b), as well as the striatum in humans being the earliest and most severely affected brain region in HD (Vonsattel *et al.*, 1985). Absolute cholesterol and cholesterol synthetic precursor levels were also previously shown to be higher in striatum compared to cortex (Zhang *et al.*, 1996; Valenza *et al.*, 2007b). For this reason it has been hypothesised that disturbed cholesterol homeostasis in HD may lead to specific vulnerability of the striatum (Valenza *et al.*, 2007b). Significant sterol

changes early in the striatum of the R6/1 mouse suggest that alterations to the cholesterol biosynthetic pathway in the brain occur very early; with the possibility that these changes may even be present during embryonic development. This has implications for brain development as the majority of brain cholesterol is synthesised during prenatal life (Dietschy & Turley, 2004). If this is the case in HD, neurodegeneration may be seeded early with individuals only becoming symptomatic later in life.

A current hypothesis suggests that errors in myelination during development may be a factor in human HD, taking many years to manifest before onset due to compensatory mechanisms that function early but become overwhelmed in the aged brain (Bartzokis *et al.*, 2007). Alternatively, early changes may be independent of the pathological processes in HD and investigating changes that occur during overt motor dysfunction may provide further insight into the involvement of altered cholesterol synthesis in the disease.

This study has also examined 7-dehydrocholesterol and desmosterol; the immediate precursors to cholesterol in the Kandutsch-Russell and Bloch pathways respectively (Figure 1.2). Contrary to a previous finding that desmosterol decreases in YAC 128 mice (Valenza *et al.*, 2007a), desmosterol levels were significantly elevated in R6/1 striatum and cortex by the end stage in our study. This may be an artefact of the different mouse models expressing different forms of mHTT, however it may also be due to the previous study using single quadrupole GC-MS (Valenza *et al.*, 2007a), a technique that has lower specificity in discriminating between similar sterols (especially 7-dehydrocholesterol) compared to GC-MS/MS used in our study. However, it is unlikely that the elevated desmosterol levels we observe are due to a higher throughput of synthesis, as cholesterol levels did not increase in our study or in a previous study examining R6/1 mice (del Toro *et al.*, 2010). A plausible explanation for this accumulation is the reduced activity of the enzyme (DHCR24) that catalyses the conversion of desmosterol to cholesterol. Previous studies in R6/2 mice do not report desmosterol levels and therefore it is unknown if

desmosterol accumulation also occurs in the longer exon 1 CAG repeat mouse model (Valenza *et al.*, 2007b). 7-Dehydrocholesterol, similarly to desmosterol did not decrease over time as seen in upstream precursors, and the levels were not significantly altered between WT and R6/1 mice at any time point or brain region. Considering lathosterol levels are substantially reduced (the immediate precursor of 7-dehydrocholesterol) and 7-dehydrocholesterol levels were unchanged, we suggest that the enzyme that converts 7-dehydrocholesterol to cholesterol (DHCR7) may also be less active or at lower abundance in R6/1 mice. Our current study gives a novel insight into 7-dehydrocholesterol levels which have not been previously reported in HD mouse brain; most likely due to analytical difficulties in resolving the structures of desmosterol and 7-dehydrocholesterol, achieved only in this study by high resolution GC-MS/MS.

Previously it has been suggested that the absolute level of cholesterol synthetic precursors (lathosterol and lanosterol) in the brain are markers of cholesterol synthesis (Thelen *et al.*, 2006; Valenza *et al.*, 2007b), however our results which describe an accumulation of penultimate precursors suggest there is likely to be more complex regulation at various points of the synthetic pathway than previously believed. How this regulation affects lanosterol and lathosterol levels is unknown, and therefore the measurement of these precursors alone to interpret synthetic rate may be potentially confounding. The regulation of "post-squalene" cholesterol synthesis has been investigated recently showed that the two terminal enzymes in the pathway interact functionally, DHCR24 regulating the activity of DHCR7 (Luu *et al.*, 2015). The idea of synthetic enzymes interacting physically supports the hypothesis of a "cholestesome" of cholesterol synthesis; which would involve a discrete group of enzymes acting on cholesterol synthetic precursors as they are converted to a final product (Sharpe & Brown, 2013). DHCR24 is also believed to have further regulatory roles upstream in the pathway (Luu *et al.*, 2015), however these roles have not been fully established. It is clear that the cholesterol synthetic pathway has more complex regulation than previously believed, and while

recent studies have identified synthetic enzyme interactions in cells derived from the periphery, the specific details of cholesterol synthetic regulation remains to be fully understood in the brain.

3.4.1.2 Age related changes to cholesterol synthetic precursors

Cholesterol synthetic precursors lanosterol, lathosterol, zymosterol and 24, 25 dihydro lanosterol in the striatum decrease at a similar rate during ageing in WT and R6/1 mice. This was also evident in cortex however the correlation was weaker. Consistent with our results in mouse brain, a negative correlation in lathosterol and lanosterol levels has been observed in human brain tissue during ageing (r = -0.5) (Thelen *et al.*, 2006). Whether this suggests an overall decrease in cholesterol levels with ageing is discussed below (3.4.1.4).

Interestingly 7-dehydrocholesterol levels did not decline over time in WT or R6/1 mice in cortex or striatum in our study. In contrast, desmosterol levels declined in WT striatum over 28 weeks, while remaining stable in R6/1. The regional specificity of this change highlights desmosterol accumulation (or reduction in DHCR24 levels or activity), as a more specific indicator of cholesterol synthetic alterations in the R6/1 mouse compared to upstream cholesterol synthetic precursor changes. Desmosterol, as measured in this study may also represent a reliable marker of DHCR24 activity in brain tissue of HD, or other neurodegenerative disorders.

3.4.1.3 Cholesterol levels in the R6/1 mouse

Our study did not detect any significant alterations in the cholesterol levels of the striatum and cortex of R6/1 mice compared to WT at any time point or brain region analysed. This is consistent with previous studies in the striatum of R6/1 (del Toro *et al.*, 2010) and R6/2 mice (Valenza *et al.*, 2007b). Previous studies also describes conflicting results; a small but significant decrease in cholesterol levels was measured in whole brain homogenates of YAC 128 mice at 10 months of age (Valenza *et al.*, 2007a), and a significant increase of striatal cholesterol was

detected in *Hdh*Q^{111} mice (del Toro *et al.*, 2010). The apparent contradictions in cholesterol level changes previously reported could be explained by several factors. The different mouse models varying in the length and species of huntingtin expressed (YAC, R6 - human HTT, *Hdh*Q^{111}-mouse huntingtin), may cause different metabolic effects that alter total cholesterol in the brain. Detection and quantification methodologies (enzymatic, colorimetric, mass spectrometry) and sample preparation differs between studies, and have been shown to affect the result obtained (Marullo *et al.*, 2012). Of these methods, mass spectrometry was previously found to be the most sensitive and reliable method for cholesterol quantification (Marullo *et al.*, 2012). Tissue samples are also varied between studies; from discrete brain regions such as the striatum, to whole brain homogenates. Different brain regions vary in total volume and cholesterol content (10-30 µg/mg tissue) (Zhang *et al.*, 1996; Valenza *et al.*, 2007b). The use of whole brain homogenates may obscure changes in discrete regions as well as potentially giving a false positive if non-disease-specific changes occur in a large brain region. Analysis of brain cholesterol in HD models would benefit from the use of standardised methods in collecting discrete brain regions and using specific analytical methods such as a mass spectrometry method developed by our laboratory (Abbott *et al.*, 2013). This would allow more accurate comparisons to be made between laboratories studying different HD models.

Unchanged levels of cholesterol in the R6/1 brain compared to WT that we observe does not necessarily rule out an altered cholesterol synthetic rate in this tissue. The concentration of cholesterol in the brain is relatively high (µg.mg^{-1}), and the rate of synthesis slow in the adult brain (ng.h^{-1}.mg^{-1}) (Spady & Dietschy, 1983), therefore significant alterations to total cholesterol levels may not take place during the short life span of these mice. This idea is consistent with total cholesterol changes only being observed in models with a longer lifespan (Valenza *et al.*, 2010).

Metabolism of cholesterol to 24-OHC occurs mainly in neurons (Lund *et al.*, 1999) and altered rates of metabolism may compensate for declining synthesis which is discussed in more detail in 3.4.1.5. It is also possible that although the concentration of cholesterol does not change in whole tissue samples, cholesterol is redistributed within the cell as previously observed in cultured striatal cells derived from the R6/2 mouse model (del Toro *et al.*, 2010). The discrete cellular and regional pools of cholesterol in the brain are diverse and vary in concentration. Myelin contains 70-80% of cholesterol in the brain (Norton & Autilio, 1965; Snipes & Suter, 1997); it is formed early in development and is possible that this is not greatly affected by the HD mutation. Neuronal cholesterol and that synthesised in astrocytes represents a smaller mass of cholesterol however is more dynamic (Bjorkhem *et al.*, 1997). Small alterations in cholesterol formation and delivery between astrocyte and neuron may not be easily detected in whole tissue samples and is one limitation of examining whole tissue. Ideally, individual cell types could be examined from *in vivo* sources, however this is technically challenging. While *in vitro* cell culture experiments provide the ability to control cell types and the extracellular environment, essential cholesterol shuttling between astrocytes and neurons as well as ageing, cannot be accurately replicated. This is an important consideration when examining cholesterol homeostasis *in vitro*.

3.4.1.4 Age related alteration to cholesterol levels in the brain

Interestingly a small, but significant increase in cholesterol was observed over time in the striatum and cortex of WT and R6/1 mice over the 28 week study. Mice in this study are not significantly aged (28 weeks old, representing early adulthood) and therefore it is difficult to deduce the influence of further ageing on cholesterol levels in these mice. Striatal cholesterol levels have been reported to increase during ageing in rats; regional age-related differences were also noted with cerebellum and hippocampus remaining unchanged (Zhang *et al.*, 1996). Whether cholesterol levels and the biosynthetic rate changes over time in the human brain has

not been established; too few studies have examined brain cholesterol during ageing, those that do have found unchanged levels (Soderberg *et al.*, 1990; Thelen *et al.*, 2006), or a decrease in cholesterol (Svennerholm *et al.*, 1994). Cholesterol synthetic precursors have been shown to decrease with age, however a significant alteration of cholesterol levels was not detected due substantial variation between individuals (Thelen *et al.*, 2006). Previous inconclusive findings are likely the result of brain regional differences, high variation within the human population and lower "n" numbers of post-mortem tissues studied in different publications. An association between cholesterol homeostasis and AD have been highlighted previously (Cutler *et al.*, 2004; Gamba *et al.*, 2011; Gamba *et al.*, 2014) ; however, no consensus has been reached regarding cholesterol changes in these settings. Previous literature examining cholesterol changes in human HD brain is similarly inconclusive, probably due to low numbers of samples and the high variation between samples leading to a loss of statistical power (del Toro *et al.*, 2010). While total cholesterol levels in tissue may be unchanged, altered distribution of cholesterol within the cell may have a very significant influence. Studies depleting cholesterol in cell membranes observe significant changes to cell signalling (Kabouridis *et al.*, 2000). Primary striatal cultures of HD mice also show increased cholesterol concentration at the cell membrane while the overall concentration remains unaltered (del Toro *et al.*, 2010). These findings are potentially important and require more investigation in the HD context.

3.4.1.5 Cholesterol metabolite changes in ageing and Huntington's disease

24-OHC has been marked as a molecule of interest after being identified as a major and specific elimination product of cholesterol from the brain (Lutjohann *et al.*, 1996; Bjorkhem *et al.*, 1998). 24-OHC is formed enzymatically from CNS cholesterol catalysed by CYP46A1, which is primarily expressed in neurons (Lund *et al.*, 1999). 24-OHC levels in plasma have been suggested as a biomarker of several neurodegenerative diseases including AD (Bretillon *et al.*, 2000b), PD (Bjorkhem *et al.*, 2013), multiple sclerosis (Teunissen *et al.*, 2003) and recently HD (Leoni *et al.*,

2008; Leoni *et al.*, 2013). Our study identified a significant (25%) reduction of 24-OHC levels in the R6/1 striatum at 28 weeks of age. We believe the relative decline of 24-OHC in R6/1 mice compared to WT is progressive over the course of the disease becoming more pronounced at later stages. Previous studies have also identified a reduction of this metabolite in the brain and plasma of several other HD mouse models (Valenza *et al.*, 2007b; Valenza *et al.*, 2010).

Aside from differences between genotypes, 24-OHC levels increase in cortex and striatum of R6/1 and WT during ageing. This data supports the finding that CYP46A1 enzyme levels increases during ageing in the mouse brain (Lund *et al.*, 1999). Human CYP46A1 does not show such a strong age related increase (Lund *et al.*, 1999), this is consistent with plasma 24-OHC measurements in humans being independent of age after the 2nd decade (Lutjohann *et al.*, 1996). 24-OHC levels during ageing in the brain have not been measured extensively and our data constitutes the first evidence of age-related 24-OHC accumulation in HD mouse brain.

Age-related changes during HD are important to examine, as measuring only a single time point can produce potentially misleading results. When examining one time point in our study, the reduction of 24-OHC in R6/1 mice could be assumed to compensate for reduced levels of cholesterol synthetic precursors, therefore maintaining equal cholesterol levels between WT and R6/1 mice. However by examining multiple time points in our study we have identified that both 24-OHC and total cholesterol levels increase with age in both R6/1 and WT mice. This suggests a more complex system maintaining cholesterol levels, potentially involving another mechanism that regulates cholesterol excretion from the brain via ApoE (Pitas *et al.*, 1987b). ApoE has been reported to be altered during neurodegeneration (Roses, 1996), and evidence from mouse models suggests that ApoE is reduced during HD pathology (Valenza *et al.*, 2010). ApoE has been suggested to have neuroprotective properties (Pedersen *et al.*, 2000; Horsburgh *et al.*, 2002), with expression trending to increase during ageing (Masliah *et al.*, 1996). Potential interactions of 24-OHC and ApoE have not been explored in HD, however this represents

another possible influence of altered cholesterol metabolism observed in the HD brain. Further investigation is required to elucidate the underlying mechanisms responsible for cholesterol metabolic changes during ageing; these studies will ultimately provide greater insight into lipid alterations occurring during neurodegeneration that typically takes place in the aged brain.

A predominantly peripheral metabolite of cholesterol, 27-OHC, was also significantly reduced in R6/1 striatum by the age of 12 weeks, while cortex tissue had no significant alterations. 27-OHC has a net movement from circulation into the brain (Heverin *et al.*, 2005) and reduced levels seen in R6/1 might be explained by a whole body metabolic dysfunction that is known to occur in HD (Lodi *et al.*, 2000; Chen *et al.*, 2007). However, this is unlikely as cortex did not exhibit the same reduction of 27-OHC levels observed in striatum. It is possible that enzymes that further metabolise 27-OHC are upregulated, or enzymes capable of forming 27-OHC in brain are downregulated in striatum, however we have no experimental evidence to support this, and requires further investigation.

3.4.1.6 Cholesterol oxidation product changes in ageing and HD

Oxidation of cholesterol in the 5,6 position generates 7β-OHC and 7-KC. These products represent stable and reliable markers of cholesterol oxidation, previously assayed in plasma (Iuliano *et al.*, 2003). In contrast to our results that showed no change in COPs, a study in the striatum of R6/1 mice did observe a time dependant increase of lipid peroxidation (Perez-Severiano *et al.*, 2000). While the different methodology used (lipid soluble fluorescence vs GC-MS/MS in our study) may derive a different result, we would expect that as cholesterol is highly abundant in the brain, the presence of oxidative stress would increase the formation of the COPs we have measured. Until multiple laboratories using reliable analytical techniques can consistently demonstrate the presence of oxidative stress in the brain of R6/1 mice, the role of lipid peroxidation in the HD pathogenesis of this mouse model is still in question.

3.4.1.7 Phytosterol changes during ageing and HD

Phytosterols are derived from the diet and share a similar structure to cholesterol. They are known to slowly accumulate in brain and other peripheral tissues (Plat *et al.*, 2008). Phytosterol levels were variable between R6/1 and WT over the course of the study and no significant effect of genotype was observed. A small accumulation of campesterol and brassicasterol over time in both WT and R6/1 mice was observed in both regions, consistent with previous studies in rats (Plat *et al.*, 2008). As these compounds can only be taken up from the circulation, they may represent a useful post mortem indicator of a disturbed BBB that would result in higher phytosterols levels present in HD brain tissue.

3.4.2 Physical phenotypic changes in the R6/1 mouse

In this study we have also investigated the R6/1 phenotype in detail over the course of the disease, from 6 to 28 weeks of age. Previous literature has typically studied either male or female R6/1 mice, in separate studies. Here we have examined both sexes in parallel to more directly assess sex differences in the HD phenotype that have been previously reported.

3.4.2.1 Weight loss

Human and mouse metabolism is quite different yet the CAG expansion in transgenic R6/1 mice causes weight loss in line with observations in human HD patients (Sanberg *et al.*, 1981; Morales *et al.*, 1989). Weight loss may not be directly involved in neuronal dysfunction; however monitoring the effect of mHTT on peripheral metabolism may give an insight into relevant effects in the brain. Consistent with previous literature we have observed a milder weight loss phenotype in females compared to male R6/1 mice (Clifford *et al.*, 2002; Naver *et al.*, 2003). In our study female weight gain in R6/1 mice was significantly slower than WT mice prior to weight loss suggesting a metabolic abnormality from early life; however after a certain age a dramatic weight loss is seen. Due to a high variation in body weight of male R6/1 mice, a significant

difference in weight gain prior to weight loss was not detected. Food intake of R6/1 mice was not measured in this study to reduce stress on animals being isolated and reunited. We do not believe the primary cause of weight loss is a lack of feeding as it has been reported that R6/1 mice have a similar food intake to WT mice however still exhibit a distinct weight loss profile (Mangiarini *et al.*, 1996), similar to that described in our study.

3.4.2.2 Hind paw clasping phenotype

The hind paw clasping phenotype measures an involuntary clasping of the hind paws when R6/1 and R6/2 mice are suspended by the tail (Mangiarini *et al.*, 1996). Female mice had a later onset of hind paw clasping with a lower percentage of mice exhibiting the phenotype until the very end of the study where 60% of both male and female R6/1 mice showed a positive phenotype. Hind paw clasping has been described previously, however the onset is variable. Mangiarini *et al.* (1996) and Naver *et al.* (2003) observe hind paw clasping from 16-20 weeks in R6/1 mice, while other studies have observed the phenotype much later at 30 weeks (Clifford *et al.*, 2002). In this study we have categorised hind paw clasping into a full clasp or half clasp. A half clasp is a less severe phenotype, where one or two hind paws are clasped in an interrupted manner. This behaviour was seen prior to full clasping (two paws clasped continuously) and was used to create a distinction between the severities of clasping. Although hind paw clasping is limited to producing binary data of 'positive' or 'negative', it is a fast and simple test that is able to examine a reflex response that is not measured by the RotaRod.

3.4.2.3 Motor performance

During the progression of disease in the R6/1 mouse, a progressive motor dysfunction was observed and this was quantified using data derived from the RotaRod experiments. Both male and female R6/1 mice showed progressive loss of motor skills that were quantified by the time taken to fall from a rotating, accelerating rod. While all mice were treated under the same

conditions, variation between mice of the same test group did exist, and therefore any sex differences in the progression of the motor phenotype could not be conclusively demonstrated. However, the impaired motor performance at the end time point was comparable between male and female R6/1 mice.

The RotaRod provides an objective and quantitative measure of forced motor function, however comparison of data obtained in different laboratories can be difficult. Dependant on the manufacturer, the fundamental design of the apparatus can be slightly different. The RotaRod manufactured by Ugo Basile (Italy) [used in previous studies (Carter *et al.*, 1999; Hockly *et al.*, 2003; Spires *et al.*, 2004; Hodges *et al.*, 2008)] and Panlab RotaRod (Barcelona, Spain) are open designs without an enclosed box around the apparatus. The RotaRod from TSE systems (Bad Homberg, Germany) and Sandigeo instruments (USA) are enclosed in a darkened box. The rod diameter for mice is usually 30 mm, however these specifications are not always defined in the literature. This specification will affect surface speed (as RotaRod is controlled in RPM) and also the available surface area for the animal to balance on. The material of which the rod is made can also potentially influence performance. A RotaRod made from hard plastic with longitudinal grooves has been used (Carter *et al.*, 1999; Spires *et al.*, 2004) and a modified version achieved by covering the rod with a bicycle inner tube (Hockly *et al.*, 2002; Hodges *et al.*, 2008), has also been described. The specific acceleration and speed protocol is another important variable that influences the difficulty of the task. A single standard protocol is not defined in the literature; past studies have used a range of acceleration profiles [3-44 RPM over 410 sec (Hodges *et al.*, 2008); 4-40 RPM over 570 sec (Hockly *et al.*, 2002); 3.5-40 RPM over 110 sec (Spires *et al.*, 2004); 4-40 RPM over 180 sec (Lazic *et al.*, 2006); 5-44 RPM with undefined acceleration (Carter *et al.*, 1999)]. These differences make it difficult to compare absolute run times of mice between studies. Even if the specific RPM at which a mouse fell, is known, the increased difficulty of faster acceleration must still be considered. Aside from comparisons between other studies, these

factors need to be considered when testing the effects of interventions. A slower accelerating rod with a rubber covering may be less challenging for HD mice at later stages and may be more sensitive to detect smaller improvements in motor function. Alternatively this may create a ceiling effect early in the disease progression, and subtle differences in the age of onset may be missed. Care should be taken when interpreting and comparing the result of RotaRod experiments from other laboratories, as well maintaining strict consistency within a particular experiment.

3.4.2.4 Brain mass

Both male and female R6/1 mice had a similar profile of brain mass changes during disease progression; being significantly reduced from 20 weeks of age until the 28 week time point, where both male and female R6/1 mice had a 17% reduction in brain mass compared to WT. This is similar to the 19% reduction in the brain mass of R6/2 mice that has been previously reported (Mangiarini *et al.*, 1996). In this study no regionally specific atrophy was observed, rather an overall shrinkage of the brain was reported (Mangiarini *et al.*, 1996). In a more recent study utilising MRI, specific shrinkage of the striatum and cortex was detected in R6/1 mice (Rattray *et al.*, 2013), similar to the atrophy observed in human HD (Vonsattel *et al.*, 1985; Rosas *et al.*, 2003). Further investigation into specific cell loss and the striatal microenvironment of R6/1 mice is required to confirm that neuron loss and astrocytosis, as seen in human HD (Myers *et al.*, 1991), also occurs in the mouse model.

3.4.3 Correlation of phenotype and sterol changes during HD progression

Marked changes in synthetic precursors (lanosterol, lathosterol, zymosterol) were detected at 6 weeks, a point at which the behavioural tests we have performed did not distinguish between R6/1 and WT mice. A subtle, early difference between the exploratory behaviour of R6/1 and WT mice at 4 weeks of age has been shown (Bolivar *et al.*, 2004), and suggests that other neural

changes are present at this early age. It is possible that the cholesterol synthetic changes we have detected are independent of HD pathology; however it must also be considered that progressive reduction in cholesterol synthesis has a cumulative effect that only becomes apparent after a threshold is reached. If precursor changes do reflect disease, they are potentially useful post mortem biomarkers for assessing the efficacy of intervention studies. Currently no studies exist that examine the modulation of the cholesterol synthetic deficit in HD models through therapeutic intervention. Changes to other synthetic precursors (desmosterol) and cholesterol metabolites 24-OHC and 27-OHC were detected later in the disease once motor symptoms were present in our current study. These compounds are potentially useful to assess the effect of therapy, specifically in preclinical animal trials as measurement of these compounds must be taken from brain tissue. Examining cholesterol synthetic rate *in vivo* in the brain is technically challenging, alterations to specific stages of the cholesterol synthetic pathway is more so difficult *in vivo* and has not been previously reported. After assessing the progression of the R6/1 model used in this study, future experiments examining progressive brain sterol changes would benefit from investigating a 15-16 week time point that marks the onset of (or just prior to) hind paw clasping and severe motor decline.

3.4.4 Conclusion

In this study we have comprehensively profiled cholesterol synthetic and metabolic changes in R6/1 mice using GC-MS/MS and found similar reductions in the limited cholesterol synthetic precursors previously reported in the R6/2 model. The novel measurement of desmosterol and 7-dehydrocholesterol in this study has highlight that there may be more complex regulation of the cholesterol synthetic pathway, as not all "post-squalene" precursors were reduced equally in brain tissue. It has also highlighted the specific accumulation of desmosterol levels in R6/1 mice that is potentially due to a reduced activity of DHCR24. Along with levels of the brain specific cholesterol metabolite, 24-OHC, being significantly reduced in striatum of R6/1 mice, we

have successfully quantified 27-OHC levels and identified a striatum specific depletion of this metabolite in R6/1 mice. This suggests that multiple pathways of cholesterol metabolism are perturbed in the R6/1 brain. Phytosterols and COPs were not altered between genotypes, suggesting an intact BBB and absence of lipid peroxidation in R6/1 mice. The multiple time points measured in this study have not only avoided potentially misleading conclusions that are possible in single time point studies, but also identified previously unreported age-related changes to many sterols in the brain. A milder weight loss and hind paw clasping phenotype was observed in female R6/1 mice compared to male R6/1 mice, however no significant difference in the loss of brain mass or motor dysfunction was detected between sexes. The physical phenotype in these mice did not correlate exactly to cholesterol synthetic and metabolic alterations, however it is possible that molecular changes are cumulative and reach a threshold before overt symptoms occur. Comprehensive and sensitive analysis of sterol compounds in the brain of R6/1 mice has given a new insight into synthetic and metabolic changes occurring in this model of HD. It has also provided novel data on age-related changes occurring in the brain, which are not only useful for further investigating HD, but also other neurodegenerative processes that typically manifest in the aged brain.

Chapter 4

The effect of environmental enrichment on cholesterol homeostasis and motor phenotype in the R6/1 HD mouse model

4.1 Introduction

An active lifestyle is believed to be important in reducing the risk of many peripheral diseases including, cancers, cardiovascular diseases and diabetes (Duncan *et al.*, 1997; Lee *et al.*, 1999; Shephard & Balady, 1999). Several epidemiological studies have highlighted that increased cognitive use and physical activity also reduces the risk of AD, PD and other dementias (Stern *et al.*, 1994; Evans *et al.*, 1997; Lindsay *et al.*, 2002). However, due to environmental and genetic heterogeneity in the human population, these studies are limited in their ability to discover specific aspects of the environment that cause beneficial effects in the brain and the mechanisms involved. Previous animal studies involving EE report similar findings to epidemiological studies, delaying disease onset and reducing the severity of neurological disorders (Kempermann *et al.*, 2002; Jankowsky *et al.*, 2005; Lazarov *et al.*, 2005). Animals housed with EE exhibit improved memory and learning, and numerous neurophysiological changes including increased synaptic plasticity, neurogenesis and reduced oxidative stress in the brain (Jones *et al.*, 1999; van Praag *et al.*, 1999; Fernandez *et al.*, 2004). The use of EE in HD mouse models has been reported to improve motor function and improve molecular deficits including amelioration of reduced levels of BDNF in the striatum (Spires *et al.*, 2004). In addition to essential roles in neuronal growth and survival, BDNF is believed to play a role in cholesterol homeostasis in the brain, being potentially relevant to HD mouse models that exhibit a significant cholesterol synthetic and metabolic perturbation. Therefore in this study we have investigated the influence of EE on the cholesterol homeostatic alterations in the R6/1 mouse model. The influence of EE on the physical phenotype in R6/1 mice was also examined, which includes: motor dysfunction, the hind paw clasping phenotype, and brain and body weight loss.

4.2 Materials and Methods

Materials: Materials used in this study are listed in 2.2.

Mice: R6/1 mice were generated as outlined in 2.3.1. Mice were randomly assigned into cages of control or EE housing (4-5 animals per cage, 7-8 animals per group). Both male and female mice were used in this study. Control housing used a standard small mouse cage (30 cm x 12 cm x 13 cm) with a wire lid. Control cages contained sawdust, pine shavings and one PVC tube. EE housing used a larger cage with a wire lid (48 cm x 12 cm x 18cm), and contained novel objects with different textures and shapes. These included additional bedding material (tissue paper, wool, paper), cardboard tubes, wooden, metal and plastic objects (See Figure 4.1 for an example). Novel objects were replaced with new objects twice per week. Mice were provided with standard rodent diet (see 2.3.1 for details) and water available *ad libitum* in both housing conditions. Mice were housed with at least 2 of each genotype per cage.

Body weight: Mouse weight was recorded every 4-5 days from 7 weeks of age until sacrifice.

Hind paw clasping: Hind paw clasping was tested every 5-10 days following the procedure outlined in 2.3.3.

RotaRod: Motor performance was measured using the RotaRod protocol outlined in 2.3.2. The RotaRod performance of mice was tested at 6, 11, 15, 19 and 23 weeks of age.

Tissue collection: Mice were sacrificed at 26 weeks of age using slow flow CO_2 asphyxiation. Brain tissue was dissected and collected as described in 2.3.4.

Lipid extraction and GC-MS/MS analysis of sterols: Quantification of cholesterol synthetic precursors, metabolites, oxidation products and phytosterols was performed as outlined in 2.3.5 and 2.3.6.

Statistical Analysis: Linear regression analysis was used to identify a difference in the rate of weight gain in mice. A 2-way ANOVA was used to analyse RotaRod performance and body weight. A student's t-test was used to compare means of brain mass, cholesterol synthetic precursors, metabolites, oxidation products and phytosterols between R6/1 and WT mice. All analyses were performed in Graphpad PRISM v5.0 (Graphpad Software Inc., USA).

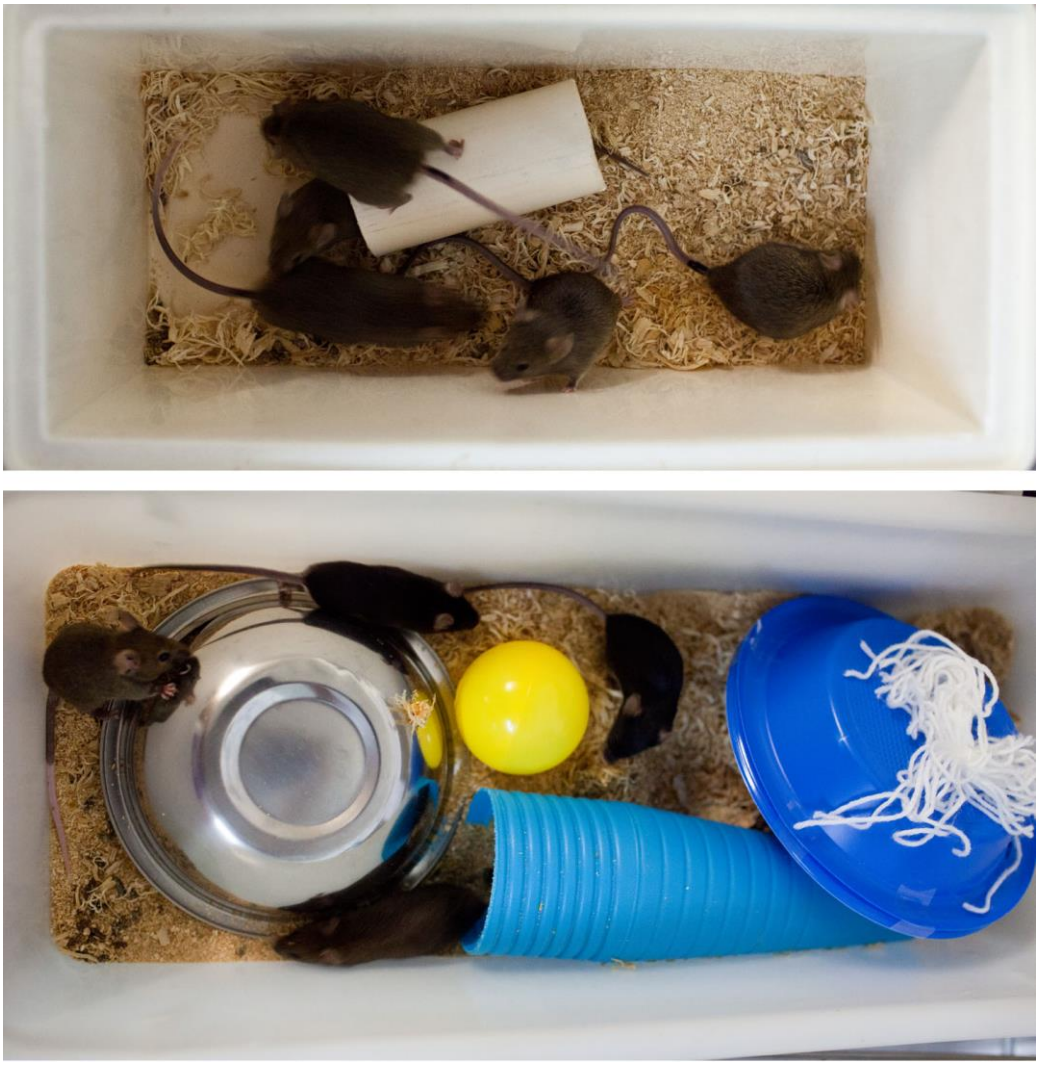

Figure 4.1 **Enriched and control housing conditions.** (A) Control housing in a standard small mouse cage (30 cm x 12 cm x 13 cm) contained sawdust, pine shavings and a single PVC tube. (B) Environmentally enriched housing was provided in a larger cage (48 cm x 12 cm x 18cm) with the addition of novel objects replaced 2 times per week. 4-5 mice (at least 2 of each genotype) were housed per cage.

4.3 Results

4.3.1 Phenotype

The effect of EE on the progression of the HD phenotype in R6/1 mice was examined from 7-26 weeks of age. Body weight, hind paw clasping and RotaRod performance was tested over this period (n = 7-8 per group). The brain mass of mice was measured at the completion of the study at 26 weeks of age when all mice were sacrificed.

4.3.1.1 Weight loss

Body weight was recorded every 4-5 days (Figure 4.2). Progressive weight loss is known to occur at 15 weeks in R6/1 mice (Mangiarini *et al.*, 1996).

EE did not significantly alter weight gain between male WT and R6/1 mice, or have an overall effect on the weight loss phenotype although the baseline levels were higher in EE mice. Prior to weight loss, weight gain in both EE and control R6/1 males was significantly less than WT in both housing conditions ($p < 0.001$). EE had no effect on body weight in WT males when normalised to baseline measurements.

Female R6/1 mice in EE housing had a significantly greater weight gain prior to weight loss (7-15 weeks) when compared to control R6/1 and WT mice ($p < 0.0001$), and a similar weight gain to enriched WT female mice. Although the average bodyweight at baseline was higher in EE mice, when considering the full course of the experiment, the weight loss profile was not significantly altered between EE and control R6/1 mice. EE significantly increased the rate of weight gain in WT females compared to control WT female mice ($p < 0.0001$).

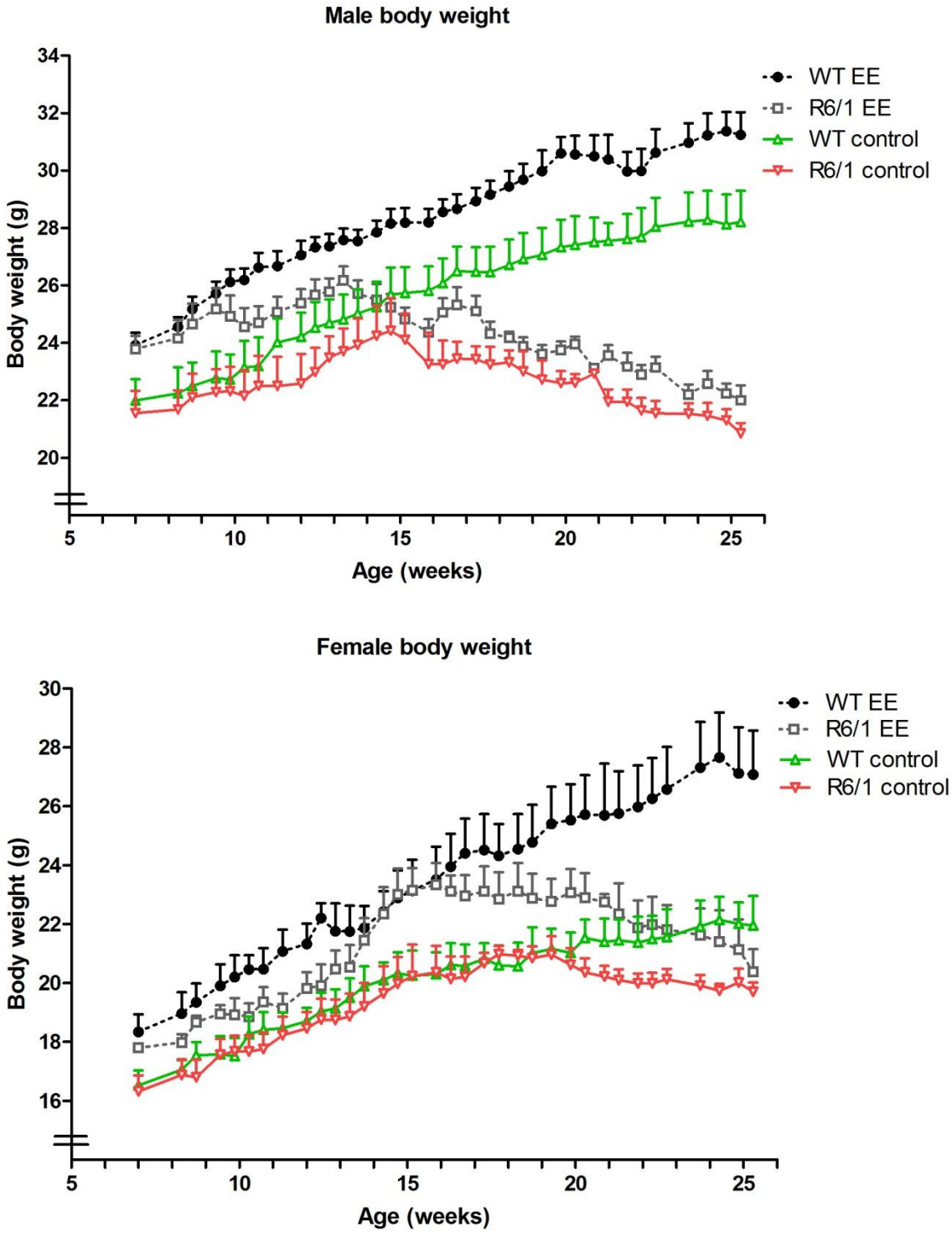

Figure 4.2 **The effect of environmental enrichment on weight loss in R6/1 mice.** Environmental enrichment (EE) did not significantly alter weight gain or weight loss in WT and R6/1 male mice. Female R6/1 mice housed with EE had a significantly greater weight gain prior to weight loss compared to control R6/1 female mice (7-15 weeks) ($p < 0.0001$), however there was no overall effect by the final time point. EE increased the weight gain in WT female mice compared to control WT females ($p < 0.0001$) n = 7-8 per group. Error bars represent + SEM

4.3.1.2 Hind paw clasping

The percentage of male R6/1 mice housed with EE that exhibited a clasping phenotype was consistently lower than control R6/1 mice over the course of the study. At 26 weeks, 50% of EE male R6/1 and 80% of control R6/1 mice were positive for the clasping phenotype (Figure 4.3). Female R6/1 mice from both EE and control housing had a similar onset of hind paw clasping at 22 weeks of age. The percentage of EE female R6/1 mice exhibiting a clasping phenotype between 23-26 weeks was less than control mice, however, the difference was relatively small over this short period (35% vs 50%, Figure 4.3). No influence of housing was observed in WT mice with several mice from both enriched and control housing showing a half clasp phenotype at various time points during the study (Figure 4.3).

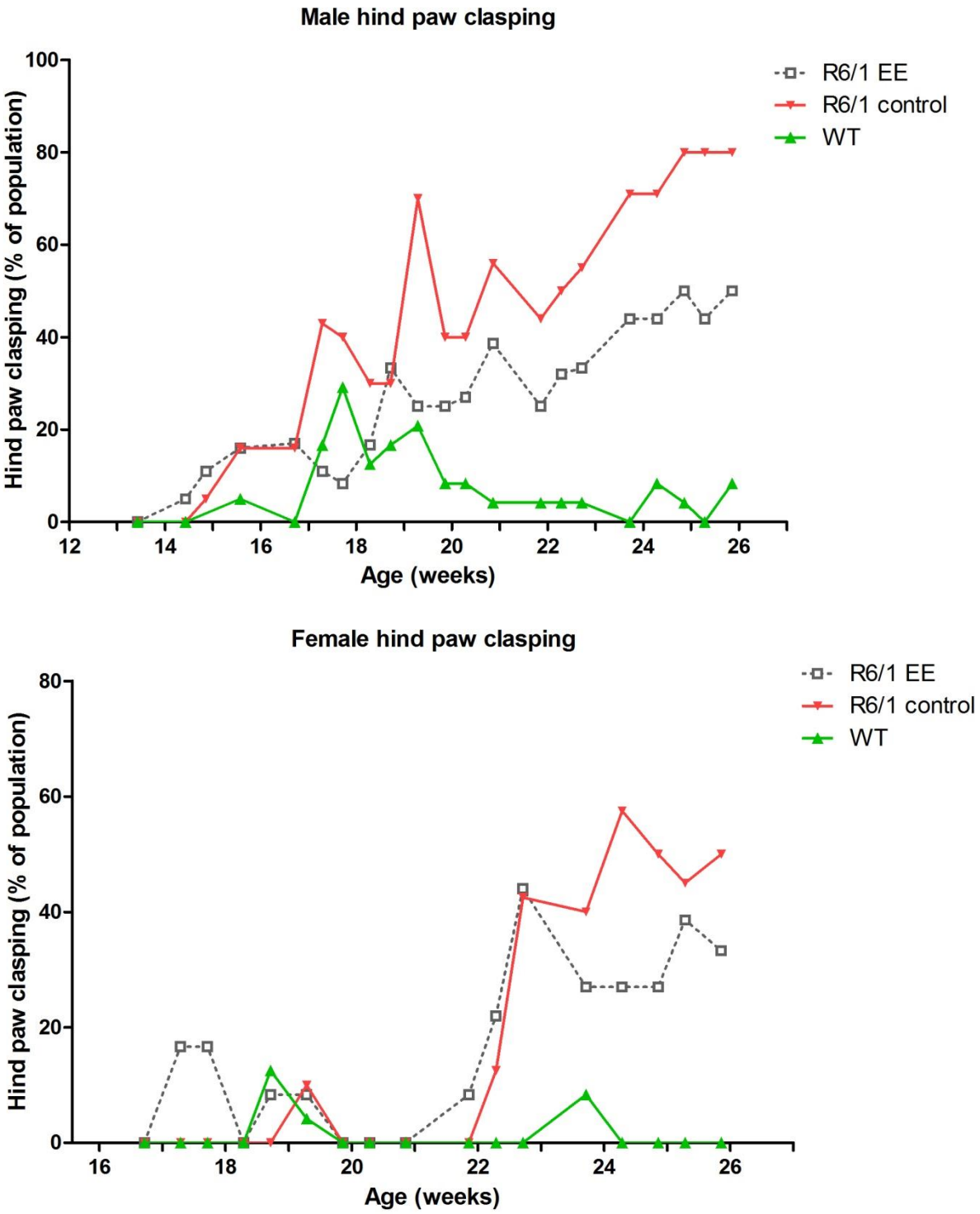

Figure 4.3 **The effect of environmental enrichment on the hind paw clasping phenotype in R6/1 mice.** R6/1 mice develop a phenotype where the hind paws are clasped to the body when suspended by the tail. Environmental enrichment (EE) did not alter the onset of hind paw clasping in male R6/1 mice, however the percentage of mice showing positive for the phenotype was consistently lower than control R6/1 mice during disease progression. Hind paw clasping at 22 weeks in female R6/1 mice and the small attenuation of hind paw clasping in the EE female R6/1 mice was not considered significant. A small proportion of WT mice appeared to exhibit a half clasp during the course of the study independent of housing treatment. n = 7-8 per group.

4.3.1.3 RotaRod

A small but statistically significant improvement in motor performance was detected in EE male R6/1 mice compared to control R6/1 males ($p = 0.0032$). While R6/1 males kept in both housing conditions did show significant motor decline over the entire study, R6/1 mice housed with EE had a delayed motor decline and a consistently higher average RotaRod time compared to control R6/1 mice (Figure 4.4). EE housing did not have a significant effect on the RotaRod performance of female R6/1 mice; both EE control mice had a similar motor decline over the course of the study (Figure 4.4). A small improvement in the average RotaRod time of EE R6/1 females at 20 and 24 weeks was observed, however this did not reach statistical significance. The RotaRod performance of WT animals was not influenced by the housing treatment in either male or female mice.

4.3.1.4 Brain mass

The reduction of brain mass previously observed in R6/1 mice (Chapter 3) was not altered by EE housing. Male and female R6/1 mice housed in EE or control housing had a highly significant brain mass reduction of 17-18% compared to WT mice of the same sex and housing treatment ($p < 0.0001$). Absolute brain mass values can be found in Table 4.1.

Table 4.1 **Brain mass of R6/1 and WT mice with environmentally enriched or control housing.** Values represent mg ± SEM. n = 7-8 per group. EE = environmental enrichment

	Control housing		EE	
	WT	R6/1	WT	R6/1
Male	476 ± 6	391 ± 7	493 ± 6	401 ± 5
Female	480 ± 5	399 ± 7	483 ± 8	402 ± 8

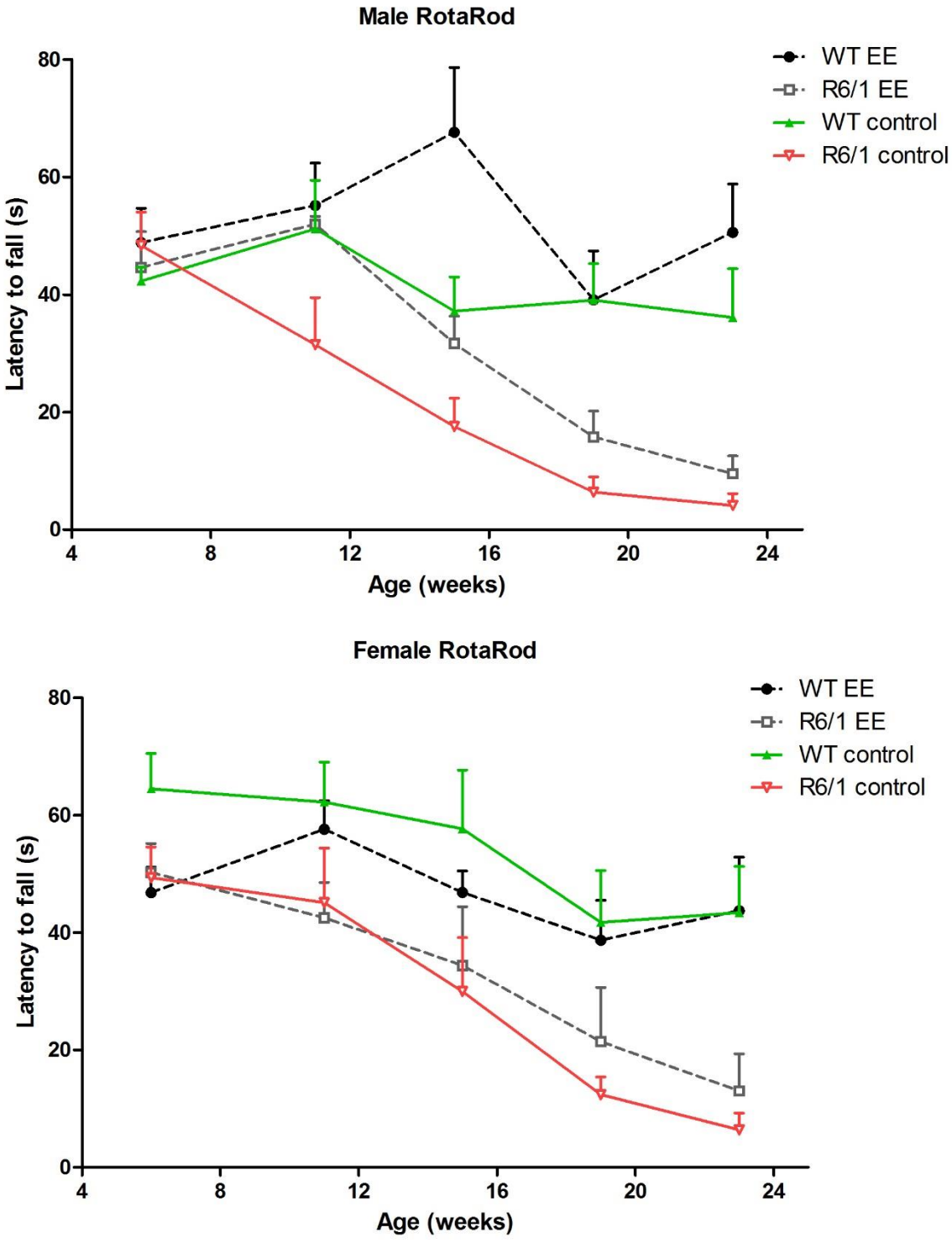

Figure 4.4 **The influence of environmental enrichment on RotaRod motor performance of R6/1 mice.** Environmental enrichment (EE) caused a delay in the onset of motor dysfunction in male R6/1 mice compared to control R6/1, and improved the average latency to fall from the RotaRod over the course of the study ($p = 0.0032$). No effect of housing treatment was detected between female EE and control R6/1 mice. The RotaRod performance of WT animals was not influenced by the housing treatment in either male or female mice. n = 7-8 per group. Error bars represent + SEM.

4.3.2 Sterol analysis of R6/1 brain tissue

All mice were sacrificed at 26 weeks of age and solvent extracted lipids from the cortex and striatum were analysed using GC-MS/MS for cholesterol synthetic precursors, metabolites, oxidation products and phytosterols. Sterol compounds examined in this study included; [cholesterol synthetic precursors (lathosterol, lanosterol, zymosterol, 24,25 dihydro lanosterol, desmosterol, 7-dehydrocholesterol), metabolic products (24-OHC, 27-OHC), oxidation products (7-KC, 7β-OH), phytosterols (campesterol, β-sitosterol, stigmasterol, brassicasterol)]. Quantified values for all sterols measured can be found in Appendix 4-6.

4.3.2.1 Cholesterol oxidation products

Male R6/1 mice housed with EE had significantly reduced levels of 7β-OH in cortex tissue compared to control R6/1 mice ($p = 0.0343$) (Figure 4.5). Male R6/1 and WT control mice did not significantly differ in 7β-OH levels (Figure 4.5). Although the effect of EE was not statistically significant in male WT mice, a similar profile to that of R6/1 male mice was observed. No difference in the level of cholesterol oxidation products was detected between housing treatments in female R6/1 mice. Combining the sexes in WT mice revealed a significant reduction of 7β-OH levels in cortex tissue ($p = 0.0389$) (Figure 4.6). Significant alterations to 7β-OH or 7-KC were not detected in any other brain region in either genotype or sex.

4.3.2.2 Cholesterol synthetic precursors, metabolic products and phytosterols

EE had no detectable influence on the level of cholesterol synthetic precursors, metabolic products or phytosterols in cortex or striatum of male and female R6/1 mice. No differences in these compounds were detected between EE and control WT mice. The sterol profile of R6/1 vs WT mice was consistent to the results obtained in Chapter 3 at a similar time-point of 24 weeks.

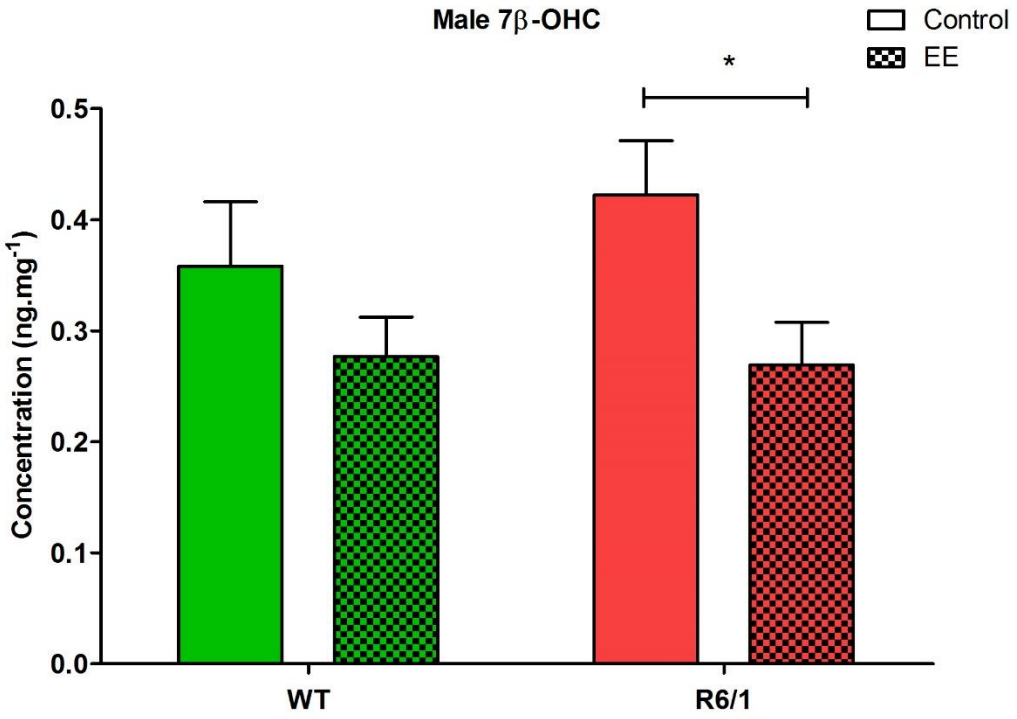

Figure 4.5 **The influence of environmental enrichment on cholesterol oxidation products in male cortex.** Environmental enrichment (EE) significantly decreased the level of the cholesterol oxidation product 7β-hydroxy cholesterol (7β-OHC) in the male R6/1 cortex when compared to control R6/1 males ($p = 0.0343$). No difference was detected between WT or R6/1 mice in either housing condition. n = 7-8 per group. Error bars represent +SEM. *$p < 0.05$

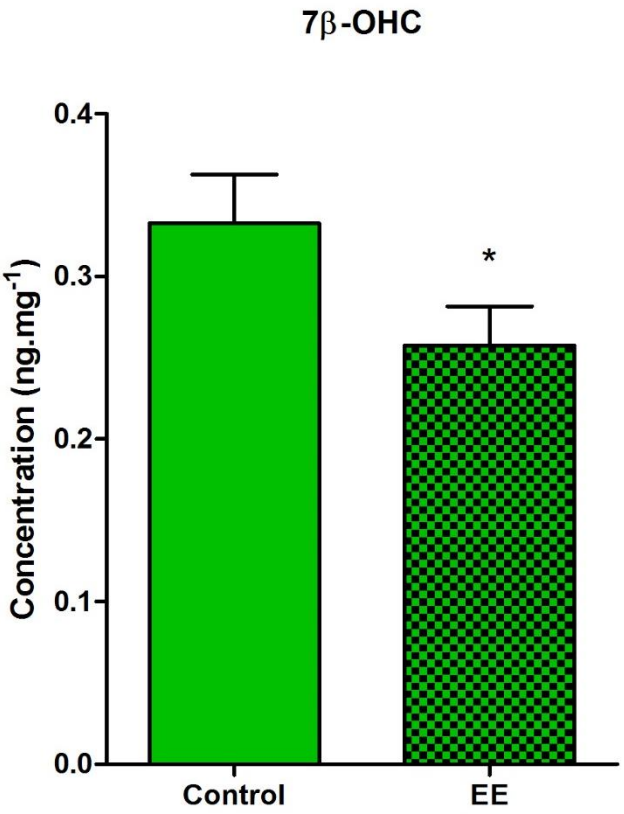

Figure 4.6 **The influence of environmental enrichment on cholesterol oxidation products in cortex of WT mice.** When sexes were combined there was a small but significant decrease in the cholesterol oxidation product 7β-hydroxy cholesterol (7β-OHC) in cortex tissue of WT mice housed with environmental enrichment (EE) ($p = 0.0389$). n = 14-16 per group. Error bars represent +SEM. *$p < 0.05$

4.4 Discussion

Previous studies investigating EE in R6 mice have reported several neurophysiological changes, including, increased neurogenesis (Lazic *et al.*, 2006) and neurotrophin expression (Spires *et al.*, 2004) in EE mice. In this study we have examined the effect of EE on R6/1 mice by characterising physical phenotypic changes as well as molecular changes to cholesterol homeostasis and oxidative stress.

4.4.1 Motor performance

In this study we have detected improved motor performance in R6/1 male mice housed with EE. The improvement observed was in conjunction with a milder hind paw clasping phenotype over the course of the study. A similar finding was reported where EE delayed the onset and overall severity of the clasping phenotype in R6/1 mice (van Dellen *et al.*, 2000). Since then, several studies have found improved motor co-ordination (RotaRod) and grip strength in both R6/1 and R6/2 mice housed with EE (Hockly *et al.*, 2002; Spires *et al.*, 2004). In the past, studies testing motor performance using the RotaRod have reported varying results in the latency to fall from the rod (Spires *et al.*, 2004; Lazic *et al.*, 2006). These differences may result from different RotaRod protocols (as previously discussed in 3.4.2.3), or differences in husbandry protocols between laboratories that have been demonstrated to influence animal behaviour (Logge *et al.*, 2014; Sorge *et al.*, 2014). It may also be the result of different enrichment protocols, where the type of environment (toys, tunnels, and nesting materials), cage size, and presence of exercise equipment may result in different outcomes (van Praag *et al.*, 1999; Zajac *et al.*, 2010). This then poses the question, what is environmental enrichment? It has been defined as 'a sustained increase in daily, cognitive and sensory-motor and physical activity' by Mattson *et al.* (2001), however we also consider that EE could involve increased sensory and cognitive stimulation in the absence of high levels of physical activity. This is consistent with human epidemiology that

finds increased cognitive function (by measure of education level and occupational status) reduces risk of dementia (Stern *et al.*, 1994; Evans *et al.*, 1997). This question also highlights the idea that various aspects of EE may have different effects in specific brain regions. This is evident in a study where the presence of a running wheel alone improved brain neurogenesis differentially to a large cage containing novel object stimuli (van Praag *et al.*, 1999). Wheel running and EE have also been reported to modify gene expression differentially in R6/2 mice (Zajac *et al.*, 2010). Apart from increasing physical fitness, running wheels may provide motor skill practise and potentially confound RotaRod results when assessing motor performance. In the current study we have provided enrichment in the absence of a running wheel to examine whether degeneration of the brain controlling motor function in HD can be achieved by sensory and cognitive stimulation.

The age of exposure and duration of EE is also likely to play a role in the brain changes. Due to mice being bred in a separate facility in the current study, EE was started at 7 weeks of age, while previous studies implemented enrichment at 4 weeks of age (van Dellen *et al.*, 2000; Hockly *et al.*, 2002; Spires *et al.*, 2004). The difference of several weeks early in development may be significant as it has been found that early handling (pre-weaning) of rodents alters behavioural phenotypes (Levine *et al.*, 1967) and age-related brain deficits (Meaney *et al.*, 1991) compared to later handled (post-weaning) animals (Levine & Otis, 1958). Therefore, in the R6/1 mouse model where biochemical changes are evident at 6 weeks (Chapter 3), we postulate that earlier exposure to EE would result in the greatest benefit. Several mechanisms have been proposed to explain the various benefits of EE seen in animal disease models, many of these being specifically relevant to HD pathology.

4.4.2 Mechanisms of environmental enrichment changes in the brain

EE has been demonstrated to improve behavioural phenotypes in animal studies including, improved spatial memory, motor function and reduced anxiety (Kempermann *et al.*, 2002;

Benaroya-Milshtein *et al.*, 2004; Spires *et al.*, 2004). Neurophysiological changes that occur alongside these behavioural improvements include; increased dendritic branching (Kolb & Gibb, 1991) and synaptic density (Saito *et al.*, 1994), reduced spontaneous apoptosis (Young *et al.*, 1999), and increased expression of neurotrophic factors (Turner & Lewis, 2003; Lazarov *et al.*, 2005). Studies incorporating EE prior to a damaging insult in the brain demonstrate that EE builds a 'protective' environment that confers resistance to damage (Kolb & Gibb, 1991; Johnson *et al.*, 2013), while implementing EE after brain damage also demonstrates the potential for repair and growth (Wainwright *et al.*, 1993; Rampon *et al.*, 2000; Shin *et al.*, 2013).

A simplified hypothesis suggests that neuronal activation leads to increased expression of neurotrophic factors that promote neurogenesis, plasticity and cell survival (Mattson *et al.*, 2001). Previous studies have focused on BDNF which is elevated in a variety of rodent models housed with EE (Ickes *et al.*, 2000; Turner & Lewis, 2003; Lazarov *et al.*, 2005). BDNF has functions involving in cell survival, differentiation and growth (Binder & Scharfman, 2004), and is believed to play a role in HD pathology since it is reduced in human HD brain (Zuccato *et al.*, 2001) as well as several HD mouse models (Spires *et al.*, 2004; Zuccato *et al.*, 2005). BDNF knockout mice also show a similar gene expression profile to human HD (Strand *et al.*, 2007). The relevance of BDNF and EE has been highlighted by studies that report rescued BDNF levels in HD mice exposed to EE, accompanied by improved RotaRod performance (Duan *et al.*, 2003; Spires *et al.*, 2004). EE restored BDNF levels in the striatum of HD mice and caused a milder motor phenotype (Spires *et al.*, 2004). As cortical levels of BDNF are unaffected in these HD mice, it is hypothesised that the corticostriatal transport of BDNF is impaired in HD, which is ameliorated by EE (Spires *et al.*, 2004). Specific elevation of BDNF in striatum of HD mice housed with EE, suggests that EE may be specifically beneficial to HD pathology which involves early and severe striatal atrophy (Vonsattel *et al.*, 1985).

Another aspect of EE may also involve increased cognitive activity through more complex social interactions. Rats housed in isolation have forebrain atrophy and significantly reduced levels of neurotrophins compared to rats housed in groups (Pham *et al.*, 1999; Ickes *et al.*, 2000). Enrichment protocols using a larger number of animals in a large cage (10-15) (van Praag *et al.*, 1999; Hockly *et al.*, 2002), enables more complex social hierarchies and interactions compared to a small cage of 4 animals. These increased social interactions have been shown to modulate the endocrine system in rodents (Blanchard *et al.*, 1995; Hardy *et al.*, 2002), and induce neurological changes (Chao *et al.*, 1993). Benefits through increased social interaction may be of relevance to HD pathology that has well established neuroendocrine alterations (Popovic *et al.*, 2004; Wood *et al.*, 2008; Saleh *et al.*, 2009). While the beneficial effects of EE on behaviour and brain physiologies are evident in previous literature, including our current study, further work is required to elucidate the specific aspects of an enriched environment that actively confer health benefits to the CNS.

4.4.3 Cholesterol synthesis and metabolism

Previously we have characterised the progression of the cholesterol synthetic and metabolic defects in the R6/1 mouse model of HD (Chapter 3). In our current study EE had no detectable effect in altering the levels of lipid species measured, when compared to control mice. The levels of all sterol compounds measured were consistent with the later time points (24-28 weeks) in our previous study (Chapter 3). Cholesterol homeostatic alterations were investigated in this study since previous literature reports improved BDNF levels in EE HD mice (Spires *et al.*, 2004). BDNF is believed to influence cholesterol homeostasis in the brain, increasing cholesterol content and gene expression of cholesterol synthetic enzymes in cultured neurons (Suzuki *et al.*, 2007). This neurotrophin also promotes DHCR24 expression (Sarchielli *et al.*, 2014), the enzyme responsible for catalysing desmosterol conversion to cholesterol at the end of the cholesterol synthetic pathway. In this study (and in Chapter 3) we observed an accumulation of desmosterol

in the striatum, which is likely attributed to loss of DHCR24 levels or activity (as discussed in 3.4.1.1). However, EE did not alter desmosterol levels in R6/1 mice. It is possible that EE did not restore BDNF levels early enough to attenuate the severe cholesterol synthetic deficit in R6/1 mice. The mechanisms behind altered cholesterol homeostasis in HD are still largely undefined *in vivo*; it is possible other factors may also play a significant role. Specifically mHTT, which may diminish the effect of BDNF by interfering with sterol regulatory binding proteins (SREBPs) (Valenza *et al.*, 2005) that also regulate cholesterol synthesis (Yokoyama *et al.*, 1993).

Further investigation into BDNF and cholesterol homeostasis is required in HD models. A potentially useful model to investigate whether BDNF can rescue the cholesterol deficit *in vivo* is a transgenic HD mouse crossed to overexpress BDNF (Canals *et al.*, 2004; Xie *et al.*, 2010). As cholesterol synthetic precursors are present in concentrations as low as 0.1 ng/mg in brain tissue, sensitive analytical tools such as GC-MS/MS are essential to detect the full extent of any potential changes.

4.4.4 Cholesterol oxidation products

The cholesterol oxidation product 7β-OHC was reduced in the cortex of R6/1 male mice housed with EE. The difference in oxidation markers observed between EE and control housing is likely to be a combination of EE reducing oxidative stress in the brain and a control or "non-enriched environment" promoting it. This is supported by studies reporting reduced markers of oxidative stress (nitrotyrosine, glutathione) in the brain of rodents housed with EE (Fernandez *et al.*, 2004; Herring *et al.*, 2010), and studies describing elevated brain lipid peroxidation in rodents under environmental stress (Liu *et al.*, 1996; Lucca *et al.*, 2009). In our current study mice housed without EE may have increased levels of stress due to having fewer places to find shelter, while EE mice have many places to hide and therefore reduced environmental stress.

Although WT and R6/1 mice in control housing had comparable levels of 7β-OH, the HD brain may have diminished capacity to cope with an increased oxidative environment, enhancing the deleterious effects of a "non-enriched" environment. This was previously observed in the R6/2 HD mouse model which was highly susceptible to an artificially induced oxidative insult while basal levels of oxidative markers were unaltered between genotypes (Bogdanov *et al.*, 1998). Interestingly oxidative stress was not significantly elevated in the striatum of R6/1 mice, consistent with previous analysis in 3.3.2.5. While the pathological hallmark of HD is striatal atrophy, the cortex does undergo neurodegeneration in later stages of human HD (Vonsattel *et al.*, 1985; Rosas *et al.*, 2003), and may explain why oxidative stress may be present in this region of R6/1 mice late in the disease progression (25 weeks). Although oxidative stress in R6/1 mice has been reported in a previous study (Perez-Severiano *et al.*, 2000), our data suggests that oxidative stress may not be a primary driving factor in R6/1 HD pathology as it is not specific to regions that are most susceptible to early degeneration.

4.4.5 Weight loss and brain mass

EE of R6/1 mice in this study did not have significant effects on the overall weight loss and the reduction in brain mass that is characteristic of these transgenic mice (Mangiarini *et al.*, 1996). This is consistent with previous findings where weight loss or brain masses were not significantly altered with EE in the R6/1 mouse (Spires *et al.*, 2004). EE has been shown to increase brain mass in WT rodents, however subtle differences were found to be regionally specific and only detectable with very large cohorts (La Torre, 1968; Bennett *et al.*, 1969; Henderson, 1970). Specific to HD models, EE in R6/2 mice was associated with a small increase in peristriatal volume (6%) (Hockly *et al.*, 2002), suggesting that any brain mass changes due to EE are most likely small in magnitude, and only detectable using complex and sensitive brain volume analytical techniques.

4.4.6 Conclusion

EE had a measureable effect on attenuating motor decline and involuntary clasping in male R6/1 mice in this study. Although motor symptoms were delayed, significant and progressive motor dysfunction was still observed by the end stage of disease in EE mice. Improvements to perturbed cholesterol homeostasis were not detected at 25 weeks of age once the disease phenotype was fully present in both EE and control R6/1 mice. Although lipid peroxidation was reduced in male R6/1 mice housed with EE, a substantial alteration in oxidative stress markers was not observed when comparing R6/1 and WT mice in our study, and may be a secondary event associated with HD neurodegeneration (Browne & Beal, 2006). However, it is possible that although basal levels of oxidative stress were not elevated in R6/1 mice, the HD brain is more susceptible to damage by stressful stimuli and this is ameliorated by EE. While the main hypothesis surrounding the benefits of EE in HD involve upregulation of neurotrophic factors, the benefit of specific aspects of EE on brain function are not fully understood. Previous EE studies in HD models, and the study described here have provided further insight into HD pathology, not only to extend the understanding of molecular mechanisms in HD neurodegeneration, but also highlight a potentially significant suppressor of disease progression and severity that may be applied to human HD patients.

Chapter 5

Berry extract supplementation in the R6/1 transgenic mouse model of Huntington's disease

5.1 Introduction

In recent years sensitive analytical techniques have highlighted many classes of compounds present in foods that have potentially potent health benefits. Many of these compounds were previously believed to exert beneficial effects primarily through their antioxidant properties, however more complex biochemical interactions have been recently highlighted which suggest that plant derived phytochemicals may alter cell signalling, invoke a hormetic response or inhibit protein misfolding.

Many plants produce secondary metabolites that are concentrated in fruit, among these are phenolic compounds that contain multiple aromatic rings in their structure. Recent studies using berry extract (BE) supplementation have discovered that berries such as blueberries, blackberries and blackcurrants have potent effects to reduce brain deficits associated with aging in animal models (Joseph *et al.*, 1998; Casadesus *et al.*, 2004; Galli *et al.*, 2006). Anthocyanins are a major phenolic component of berries, and are believed to be the main active component of these fruits. Dietary supplementation of phenolic fruit extracts have also been applied to neurodegenerative disease, and in the case of HD, several compounds have been identified that attenuate disease pathology in animal and cell models (Ehrnhoefer *et al.*, 2006; Maher *et al.*, 2011).

In this chapter we have investigated BE extract supplementation on the R6/1 HD mouse model and tracked the progression and severity of symptoms. We have assessed the effects of BE extract supplementation on biochemical changes involved in altered cholesterol homeostasis and oxidative stress as well as several established phenotypic changes that occur in these mice.

5.2 Materials and Methods

Mice: R6/1 mice were generated as outlined in 2.3.1. Equal numbers of male and female R6/1 and WT mice (6 per group) were caged in standard housing (30 cm x 12 cm x 13 cm) containing a single PVC tube, saw dust and pine shavings. Four mice were housed per cage, two of each genotype. All procedures that were undertaken conformed to the standards of the University of Wollongong ethics committee (ethics approval number: AE 13/20).

Diet: Control and BE diets were manufactured by Speciality Feeds Pty Ltd. (Glen Forrest, W.A, Australia). Control diet comprised of the standard rodent growth diet (AIN-93G) (Reeves *et al.*, 1993). BE diet contained [control diet + 0.2% blackcurrant extract (NutriPhy Blackcurrant 100, Chr. Hansen pty. Ltd., Australia)]. Control and BE diet were equal in caloric content. BE diet was calculated to be equivalent to one serving of whole berries in humans (100-150 g) (Prior *et al.*, 2001; Scalzo *et al.*, 2008; Tabart *et al.*, 2011; Milivojevic *et al.*, 2012), corrected to body surface area as described in Reagan-Shaw *et al.* (2008). The consumption of anthocyanins by mice in this study (when corrected for body surface area) was similar to previous studies investigating anthocyanin supplementation in rats (Joseph *et al.*, 1999; Youdim *et al.*, 2000). Both diets were kept a 4^{o}C in a light sealed box prior to being dispensed for feeding. Food and water was available *ad libitum*. Unconsumed diet was removed and replenished from cages every 2 days.

Body weight: Mouse weight was recorded every 4-5 days from 7 weeks of age until sacrifice.

Hind paw clasping: Hind paw clasping was tested every 5-10 days following the procedure outlined in 2.3.3.

RotaRod: Motor performance was measured using the RotaRod protocol outlined in 2.3.2. The RotaRod performance of mice was tested at 6, 11, 15, 19 and 23 weeks of age.

Tissue collection: Mice were sacrificed at 25 weeks of age using slow flow CO_2 asphyxiation. Brain tissue was dissected and collected as described in 2.3.4.

Lipid extraction and GC-MS/MS analysis of sterols: Quantification of cholesterol synthetic precursors, metabolites, oxidation products and phytosterols was performed as outlined in 2.3.5 and 2.3.6.

Statistical analysis: Linear regression analysis was used to identify differences in the rate of weight gain between mice. 2-way ANOVA was used to test for an effect of BE extract supplementation on RotaRod performance and weight loss. A student's t test was used to compare mean values of sterol analysis and brain mass. All analyses were performed in Graphpad PRISM v5.0 (Graphpad Software Inc., USA).

5.3 Results

5.3.1 Phenotype

5.3.1.1 Body weight

Supplementation of BE had no effect on the weight loss phenotype in either male or female R6/1 mice when body weight was normalised to baseline levels (Figure 5.1). Both male and female R6/1 mice had significant weight loss independent of diet when compared to WT mice ($p < 0.01$). A significant difference in weight gain prior to weight loss was observed between control and BE R6/1 males, the control fed mice gaining weight more rapidly ($p = 0.004$). No other significant differences in weight gain were detected in WT or female R6/1 mice.

5.3.1.2 Hind paw clasping

A small delay in the onset of the hind paw clasping phenotype was observed in male R6/1 mice fed a BE supplemented diet (12 vs. 14 weeks of age in control and BE fed mice respectively) (Figure 5.2). After the initial onset of clasping the percentage of R6/1 control mice showing positive for clasping was higher on average, however there was variability between time points and a clear difference in hind paw clasping between diet treatments was not observed. In female R6/1 mice the hind paw clasping phenotype progressively increased after 19 weeks of age independent of diet treatment (Figure 5.2). A small percentage of WT mice from both diet treatments were positive for a half clasp phenotype at various stages of the study.

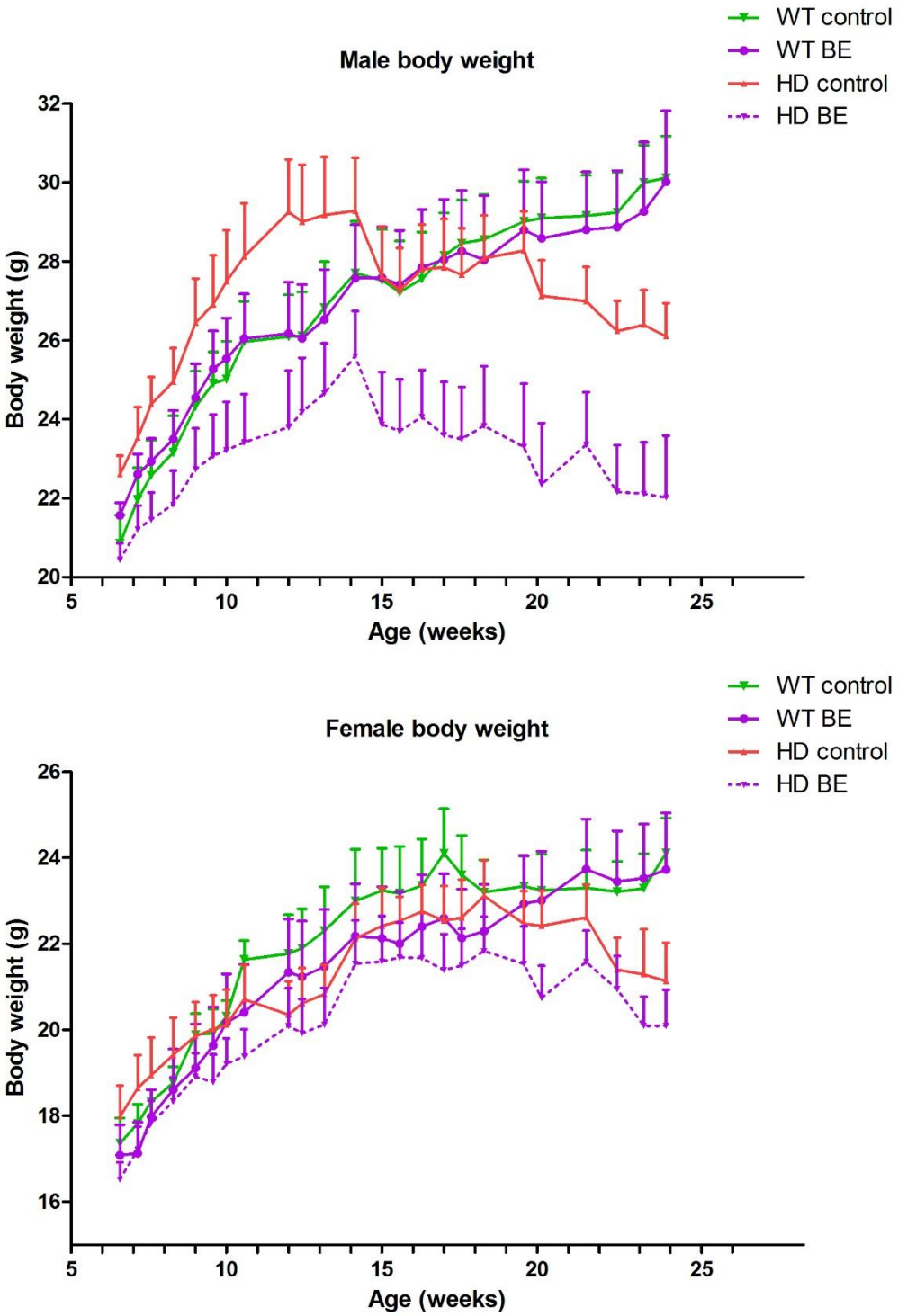

Figure 5.1 **The effect of berry extract supplementation on weight loss in R6/1 mice.** The weight loss phenotype in male and female R6/1 mice was not altered by berry extract (BE) supplementation when compared to control fed R6/1 mice. Prior to weight loss (at age 15 weeks) the weight gain of BE fed R6/1 males was significantly less than control fed R6/1 mice ($p = 0.004$). Diet treatment had no effect on the weight gain of WT mice or female R6/1 mice. Over the entire study R6/1 mice had significant weight loss compared to WT independent of diet treatment ($p < 0.01$). n = 6 per group. Error bars represent + SEM.

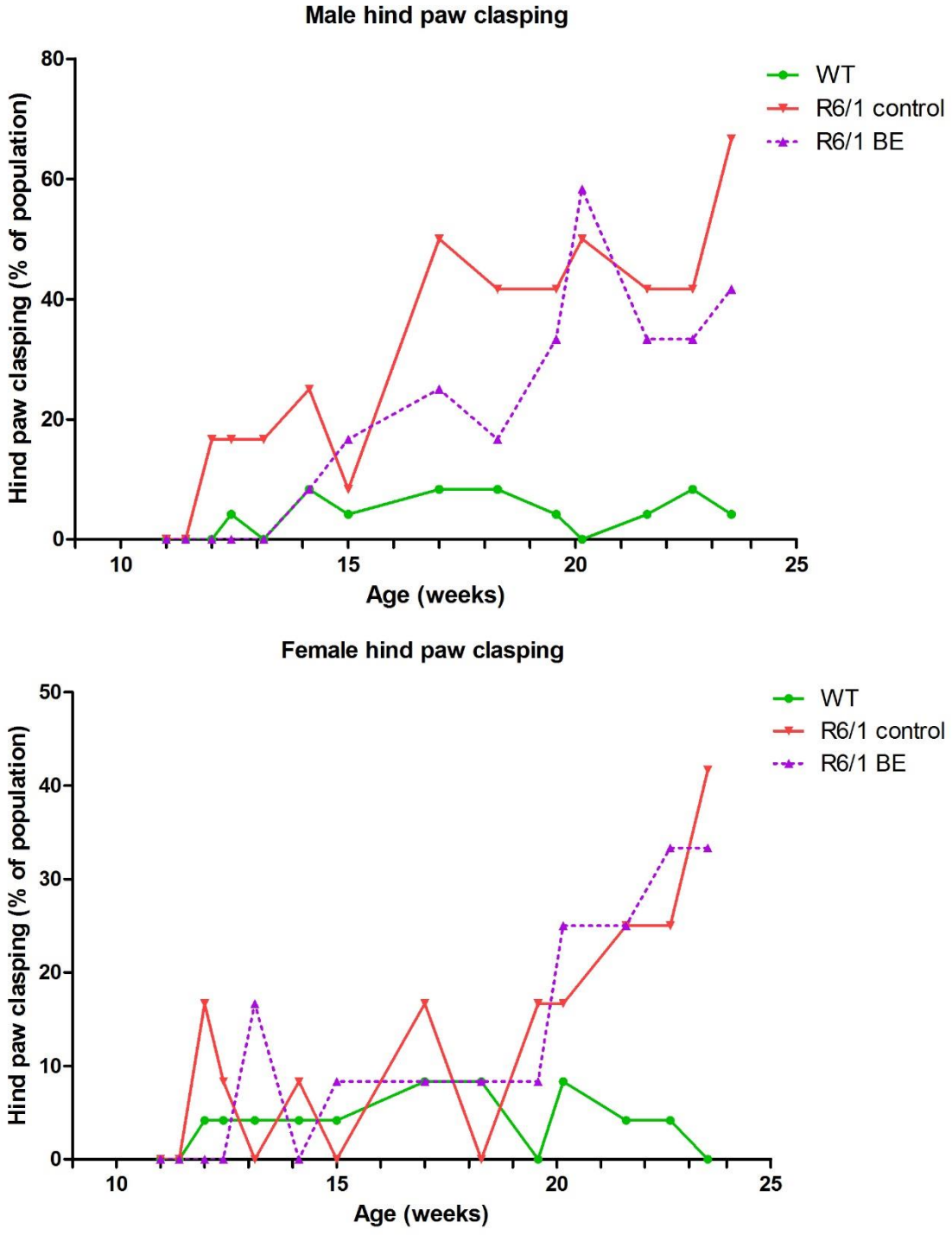

Figure 5.2 **The effect of berry extract supplementation on the hind paw clasping phenotype in R6/1 mice.** R6/1 mice develop a phenotype where the hind paws are clasped to the body when suspended by the tail. A small delay in the onset of hind paw clasping was observed in berry extract (BE) supplemented R6/1 male mice (2 week delay) however the overall severity was not consistently reduced after onset. No effect of diet was observed in female R6/1 mice. A small proportion of WT mice appeared to exhibit a half clasp during the course of the study independent of diet treatment. n = 6 per group.

5.3.1.3 RotaRod

The average RotaRod time for female R6/1 mice fed a BE diet was consistently higher than control fed mice over the course of the study, and a small but significant overall improvement was detected ($p = 0.0349$). Male R6/1 mice fed a BE diet did not have a detectable improvement in performance compared to control fed R6/1 mice. BE supplementation did not alter the RotaRod performance of male or female WT mice (Figure 5.3).

5.3.1.4 Brain mass

Dietary intervention had no effect in altering the brain mass reduction seen in R6/1 mice (R6/1 male BE vs control $p = 0.587$, R6/1 female BE vs control $p = 0.738$) (Table 5.1). Both male and female R6/1 mice fed control and BE supplemented diets had significantly reduced brain mass when compared to WT mice ($p < 0.001$), with a mean reduction of 10-15%.

Table 5.1 **Brain mass of R6/1 and WT mice fed a berry extract supplemented diet.** Values represent mg ± SEM. n = 6 per group. BE = Berry extract

	Control diet		BE	
	WT	R6/1	WT	R6/1
Male	467 ± 8	397 ± 14	465 ± 8	388 ± 5
Female	460 ± 6	405 ± 13	458 ± 5	410 ± 5

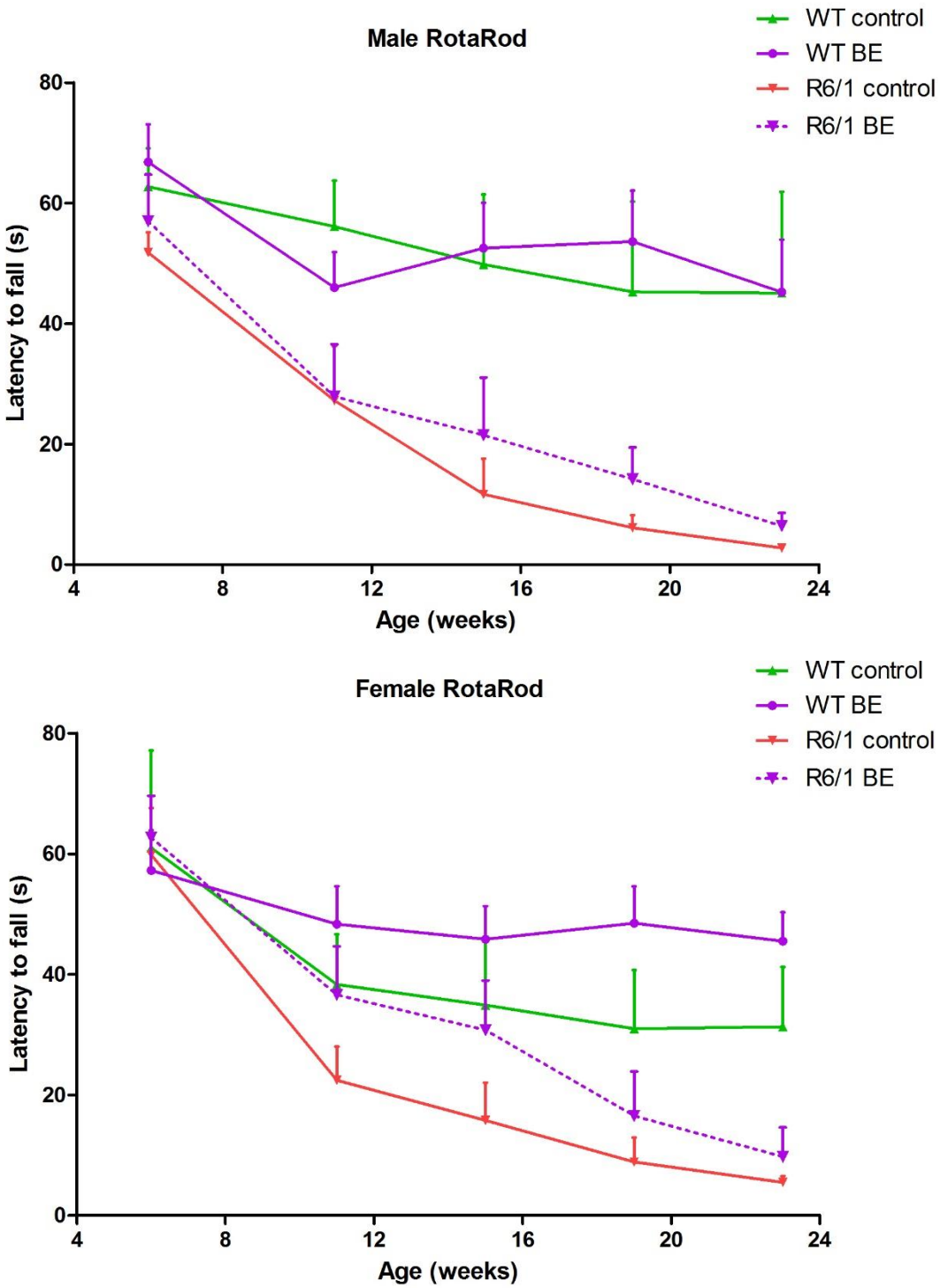

Figure 5.3 **The effect of berry extract supplementation on the RotaRod motor performance of R6/1 mice. B**erry extract (BE) supplementation attenuated the motor function decline in R6/1 females when compared to control fed R6/1 mice ($p = 0.0349$). No detectable improvement in RotaRod performance was observed in male R6/1 mice fed a BE diet. Diet treatment did not influence RotaRod performance of WT mice over the course of the study. n = 6 per group. Error bars represent + SEM.

5.3.2 Sterol analysis of R6/1 brain tissue

At the age of 25 weeks all mice were sacrificed for brain lipid analysis. Striatum and cortex was dissected from the brain and sterols were analysed using GC-MS/MS. Dietary supplementation with BE had no detectable influence in WT or R6/1 mice when examining the level of cholesterol synthetic precursors, metabolic products, oxidation products and phytosterols quantified in this study. No significant sex differences were observed in WT or R6/1 mice. The sterol profile of R6/1 vs WT mice was consistent to the results obtained in Chapter 3 and 4 at a similar time-point of 24 weeks. Sterol compounds examined in this study included; [cholesterol synthetic precursors (lathosterol, lanosterol, zymosterol, 24,25 dihydro lanosterol, desmosterol, 7-dehydrocholesterol), metabolic products (24-OHC, 27-OHC), oxidation products (7-KC, 7β-OH), phytosterols (campesterol, β-sitosterol, stigmasterol, brassicasterol) and cholesterol]. Quantified values for all sterols measured can be found in Appendix 7-9.

5.4 Discussion

The R6 mouse models of HD have been previously used to examine a variety of therapeutic strategies targeting multiple aspects of HD pathology that is manifested in these mice. These include studies addressing: metabolic impairment, protein misfolding, inflammation, excitotoxcity and oxidative stress through strategies such as drug treatments, gene therapies, tissue transplantation, environmental enrichment and dietary interventions (Ferrante *et al.*, 2000; van Dellen *et al.*, 2000; van Dellen *et al.*, 2001; Ferrante *et al.*, 2002; Wang *et al.*, 2005; Maher *et al.*, 2011). These interventions have typically generated small improvements in motor dysfunction and survival of mice, and have increased the current understanding of mechanisms involved in HD pathology. Novel therapeutics are likely to take many years to develop and test for safety, hence many studies in animals have trialled already approved drugs (lithium chloride, remacemide, riluzole) with some success, reducing the severity of motor phenotypes and increasing survival of animals (Ferrante *et al.*, 2002; Schiefer *et al.*, 2002; Wood & Morton, 2003). An advantage of dietary supplementation is that the active compounds used to treat disease are safe and already consumed on a daily basis. A large number of foods containing phytochemicals believed to have health benefits have been identified recently, among these are anthocyanins, phenolic compounds that are highly concentrated in berries. BE has been used to reduce age-related cognitive deficits and improve memory and cognition in rats (Joseph *et al.*, 1999; Casadesus *et al.*, 2004; Galli *et al.*, 2006; Duffy *et al.*, 2008). The dosing of anthocyanins consumed by rats in these studies is equivalent to the consumption of approximately 100-150g of fresh berries (blueberry, blackberry, blackcurrant) per day in humans. Several other plant derived phenolic compounds have been trailed in animal models of neurodegeneration including HD (Ehrnhoefer *et al.*, 2006; Maher *et al.*, 2011). Prior to experimentation in this chapter, BE has not been investigated in HD models. Here we discuss the benefits, and possible

protective mechanisms, of phytochemical supplementation in brain related disorders, specifically in HD and the R6/1 HD mouse model.

5.4.1 Motor performance in R6/1 transgenic mice and possible protective mechanisms of dietary phenolics in the brain

In this study we describe the novel use of BE to improve motor function in the R6/1 HD mouse model. Our study was able to identify a significant improvement in the RotaRod performance of female R6/1 mice. Although there is a trend for improved function in the later stages of disease in male R6/1 mice, this did not reach statistical significance. It is possible that due to the more rapid onset of symptoms in male R6/1 mice as reported in Chapter 3, we have not been able to detect a significant level of improvement in male mice. This is consistent with the greatest effect of BE supplementation in females occurring at the onset of symptoms at 11-15 weeks, delaying the onset of motor dysfunction by several weeks. Improvements in motor function have also been observed in aged rats fed a BE supplemented diet (Shukitt-Hale *et al.*, 2009). Several other studies using BE supplementation in rats have seen improvements in memory and neurolophysiological deficits associated with aging in the brain (Joseph *et al.*, 1999; Goyarzu *et al.*, 2004; Ramirez *et al.*, 2005; Galli *et al.*, 2006; Duffy *et al.*, 2008). In many studies using dietary phenolic supplementation, young and healthy WT controls do not show the same level of improvement in brain biochemistry or motor and cognitive performance when compared to aged or disease models (Duffy *et al.*, 2008; Wang *et al.*, 2010; Willis *et al.*, 2010). This suggests that phenolic supplementation specifically attenuates disease pathology (or ageing) rather than an overall improvement of neurological function.

The dose of BE and the anthocyanins contained in this diet is also an important consideration when conducting and comparing studies, or assessing the feasibility of dietary berry consumption for therapeutic purposes. Our current study has used a commercially available blackcurrant anthocyanin extract with a known concentration of anthocyanins. The dose of

anthocyanins equated to the consumption of approximately 100-150 g of berries per day in humans (300 mg total anthocyanins) assuming a daily intake of 3 g of diet in mice. Total anthocyanin content of berries varies between species; blueberries, blackberries and blackcurrants contain approximately 1.5-2 mg, 2 mg and 3 mg total anthocyanins per gram fresh weight respectively (Scalzo *et al.*, 2008). Previous studies in rats have used lyophilised extracts by homogenising whole blueberries (Joseph *et al.*, 1999; Youdim *et al.*, 2000; Andres-Lacueva *et al.*, 2005), however the exact anthocyanin content has not been described. Assuming no anthocyanins have been lost in this process, the dosing is similar to that used in our study; approximately equal to the consumption of 100-150 g of fresh berries per day in humans. One aspect that has been overlooked however is the potential loss of anthocyanins during the extraction process. Oven drying and lyophilisation has been demonstrated to reduce the total anthocyanin content of blueberries and grape skin (Lohachoompol *et al.*, 2004; de Torres *et al.*, 2010). It is therefore important to quantify total anthocyanins in extracts to identify the exact quantity of the active molecule being utilised in diet treatments.

Due to the heterogeneity of phytochemical structures found in food, it is likely that different classes of compounds or even different compounds of similar structure act through diverse mechanisms. Several hypothesised mechanisms have been suggested to explain the neuroprotective properties of BE supplementation. One hypothesis focuses on the antioxidant properties of BE containing anthocyanins and other phenolic compounds. Several studies using a BE supplemented diet describe reduced levels of oxidative stress markers in rat brain (Joseph *et al.*, 1999; Elks *et al.*, 2011). *In vitro* studies have also reported cells treated with BE and grape seed extract as having enhanced survival against oxidative stress (Duffy *et al.*, 2008; Wang *et al.*, 2010). However, the physiological relevance of the dose of phytochemicals used in these cell culture studies is unknown. Our study did not detect altered lipid peroxidation in the brain of BE supplemented R6/1 or WT mice when quantifying the cholesterol oxidation products 7-KC and

7β-OHC. Lipid peroxidation was not previously identified as being altered in R6/1 compared to WT mice (Chapter 3) and therefore antioxidant effects by suppressing lipid peroxidation is unlikely to confer a strong protective effect towards the HD mice in our study.

Hormesis has been suggested as a mechanism through which phytochemicals such as anthocyanins may confer health benefits. A hormetic response involves a low dose of a toxin (or stressor) promoting or upregulating protective survival factors in an organism, which then have enhanced survival prospects against future toxic insults such as increased oxidative stress in aging or that caused by mHTT in HD. Dietary phytochemicals have been shown to increase the immune response (Feng *et al.*, 2002), expression of cytoprotective proteins (McWalter *et al.*, 2004) and have anti-inflammatory properties (Willis *et al.*, 2010). Attenuated age-related neurological deficits also suggests the upregulation of prosurvival mechanisms by these compounds (Casadesus *et al.*, 2004; Goyarzu *et al.*, 2004; Galli *et al.*, 2006). However, high concentrations of phytochemicals can also be toxic as demonstrated by *in vitro* studies (Liontas & Yeger, 2004; Fimognari *et al.*, 2005). A previous study feeding BE to rats describes a similar hormetic response when reactive oxygen and nitrogen species were elevated in short fed animals term (2 days), while the same markers where significantly decreased in chronic fed animals (6 and 12 weeks) (Elks *et al.*, 2011). A future study into the survival of BE fed HD mice would be useful to identify if this supplementation can delay death in R6/1 mice. In the current study, lipid analysis of brain tissue required all mice to be sacrificed at a predetermined time point and therefore a survival study could not be conducted.

There is substantial evidence to suggest that BE and anthocyanins also influence cellular signalling pathways in the brain that may result in neuroprotection and promote proliferation. BE supplementation has been shown to increase extracellular signal-related kinase (ERK) phosphorylation in aged rat brains (Williams *et al.*, 2008). The ERK signalling cascade has a role in altering synaptic plasticity and dendritic morphology relevant to memory (Bailey *et al.*, 1997;

Wu *et al.*, 2001), and essential neuronal transcriptional events (Sweatt, 2004). A recent study in a HD cell and fly model identified increased ERK phosphorylation with supplementation of fisetin (a flavonoid found in fruit) (Maher *et al.*, 2011). Fisetin was able to reduce photoreceptor degeneration pathology and increase survival in a dose dependant manner in a HD fly model (Maher *et al.*, 2011). Fisetin was also supplemented in the diet of R6/2 HD mice that exhibited improved motor performance and also increased survival (Maher *et al.*, 2011). In addition to modification of ERK phosphorylation by BE anthocyanins in rats, Williams *et al,* (2008) found increased cAMP response element-binding protein (CREB) phosphorylation. CREB is involved with gene transcriptional regulation (Carlezon *et al.*, 2005), including that of *BDNF* (Tao et al., 1998; Yu et al., 2012). The *BDNF* gene codes for the neurotrophin BDNF, that has functions in cell survival, differentiation and growth (Binder & Scharfman, 2004), and is significantly reduced in HD (Zuccato *et al.*, 2001; Zuccato *et al.*, 2005). BDNF levels have not been reported in phytochemical supplemented HD mouse models, however the current literature on HD pathophysiology suggests rescued BDNF levels may be one mechanism by which BE supplementation improves the HD phenotype described.

There is evidence to suggest that mHTT aggregation plays a significant role in HD pathogenesis (Gutekunst *et al.*, 1999; Yang *et al.*, 2002), and therefore the inhibition of aggregation has been the target of therapies to reduce HD severity. Dietary supplementation of a green tea-derived phenolic compound reduced the disease phenotype of a HD fly model (increased movement and reduced photoreceptor degeneration), and reduced mHTT aggregates in a HD cell model (Ehrnhoefer *et al.*, 2006). It was therefore proposed that the protective effects of this phenolic compound are conveyed through inhibition of mHTT aggregation (Ehrnhoefer *et al.*, 2006). Further evidence from a study using a grape seed extract reported decreased mHTT aggregation in a HD cell model, increased survival of a HD fly model and improved RotaRod performance of the R6/2 HD mouse model (Wang *et al.*, 2010). Interestingly the same cell model treated with

fisetin did not prevent mHTT aggregation (Maher *et al.*, 2011). Although our current study, or previous studies in phytochemical supplementation in HD mice have not examined mHTT aggregation, the available evidence suggests that the beneficial effects of different phenolics may work through multiple different mechanisms to result in an improved phenotype. Future investigation into a cocktail of phytochemical supplements would support this idea further and possibly highlight a supplementation regime that has more potent therapeutic effects than single compounds or extracts alone.

5.4.2 Cholesterol homeostasis and BE supplementation in R6/1 mice

In this study we also investigated alterations to cholesterol homeostasis in R6/1 mice receiving BE supplementation. BE supplementation did not result in significant alterations to the sterol profile (alteration of synthetic and metabolic products of cholesterol) previously identified in the R6/1 mouse model (Chapter 3). Previously BE supplementation improved ERK phosphorylation (Williams *et al.*, 2008) and this may influence BDNF levels, and in turn alter cholesterol homeostasis. However, the cholesterol synthetic deficits began early in the R6/1 model (6 weeks of age, Chapter 3) and therefore the age at which supplementation is initiated in HD models may significantly affect the relative benefits of the treatment. Several past dietary studies in HD animal models began supplementation at the embryonic development stage, by feeding the P1 generation and continuing supplementation after birth (Clifford *et al.*, 2002; Ehrnhoefer *et al.*, 2006; Wang *et al.*, 2010). Another possible strategy for future dietary studies is intermittent supplementation; this has been suggested to potentially enhance a hormetic response that may be caused by these phytochemicals (Mattson, 2008).

5.4.3 The influence of BE on hind paw clasping in R6/1 mouse

A small delay in the onset of the hind paw clasping phenotype was observed in BE supplemented male R6/1 mice, however an overall improvement during the later stages of the study was not observed in either male or female R6/1 mice. It is possible that the hind paw clasping phenotype is controlled by a brain region or neurological process that is not specifically improved through dietary anthocyanin intervention. Hind paw clasping represents a reflex response in contrast to motor co-ordination measured by the RotaRod apparatus. The idea that different brain regions control different functions is well established, and the manifestation of broad behavioural symptoms in HD is likely to be the result of neurodegeneration in multiple brain regions. HD brain pathology is indeed widespread, following early striatal degeneration, atrophy is found in the globus pallidus, accumbens, amygdala, thalmus and cerebral cortex (Vonsattel *et al.*, 1985; Rosas *et al.*, 2003). Tissue transplant studies in the anterior cingulate cortex of R6/1 mice have identified an improvement in hind paw clasping but no change in RotaRod performance (van Dellen *et al.*, 2001). Similarly hind paw clasping and several involuntary phenotypes (sniffing, rearing, grooming) were improved in fatty acid fed R6/1 mice without rescuing striatal pathology (Clifford *et al.*, 2002). The same fatty acid supplementation has proved effective in mouse models of dyskinesia (involuntary movement disorder) (Vaddadi *et al.*, 2006), possibly acting to rescue a similar region involved with choreic movements in HD mice. Although sensitive and specific volumetric analysis of R6 mouse brain has been conducted to investigate gross cell loss (Sawiak *et al.*, 2009), future work investigating molecular alterations in HD mouse brain may benefit from analysing tissue samples derived from micro dissection techniques that facilitates high resolution sampling.

5.4.4 Body weight loss and brain mass of R6/1 mice receiving a BE diet

The weight loss phenotype was not attenuated in male or female R6/1 mice fed a BE supplemented diet. The only detectable effect was a reduced weight gain in BE male R6/1 mice when compared to control R6/1 males. However, due to the baseline difference in body weight of control and BE fed animals (resulting form randomisation), the difference in weight gain is likely to be a chance event. Previous literature also reports that BE supplementation does not modify the body weight of rodents (Sweeney *et al.*, 2002; Goyarzu *et al.*, 2004; Duffy *et al.*, 2008; Rashid *et al.*, 2014). The majority of therapeutic interventions in the R6 mouse models target brain alterations and therefore do not restore the peripheral metabolic deficit that is a classical symptom of HD. Studies addressing HD metabolic deficits in R6 mice using dietary restriction (Duan *et al.*, 2003) or creatine supplementation (Ferrante *et al.*, 2000) have reported a small improvement in weight loss, however it is not completely rescued. Total food consumption is also a consideration for dietary invention studies; in the present study it is unlikely that average food consumption per cage was influenced by diet, similar to a previous BE study in rats (Elks *et al.*, 2011) and a preliminary study in WT mice (Jenner, A.M., pers. comm. 2015). In this study the specific food intake of WT and R6/1 mice could not be determined due to equal distribution of WT and R6/1 genotypes within cages. Separation of mice to measure food consumption was not performed to prevent potential stress and uncontrolled factors of social hierarchy and aggression between reunited mice. Previously, feeding habits have been shown to be unaltered by the mHTT transgene in R6 mice; however the typical weight loss profile is still observed (Mangiarini *et al.*, 1996). This is consistent with the human HD symptomatology where weight loss is observed with equal or higher caloric intake (Sanberg *et al.*, 1981; Morales *et al.*, 1989). This suggests that food intake was most likely not a factor contributing to R6/1 weight loss in our current study.

Previous studies have approached phytochemical supplementation by either feeding animals a concentrated plant extract (Papandreou *et al.*, 2009; Wang *et al.*, 2010; Maher *et al.*, 2011) similar to our current study, or a purified phytochemical compound (Ehrnhoefer *et al.*, 2006; Ho *et al.*, 2010). Experimental designs that supplement fruit extracts containing a mixture of phytochemicals do not have the specificity required to identify a single molecular mechanism of action, however fruit extracts are more relevant in a dietary context as they represent the phytochemical mixture readily available for human consumption.

In this study there was no detectable effect of BE supplementation on the brain mass of R6/1 mice. Improved motor and cognitive performance independent of improved brain mass in R6 mice has been observed in several studies (Spires *et al.*, 2004; van Dellen *et al.*, 2008), and suggests that significant recovery of the brain mass phenotype in these mice may not be necessary to improve the symptomatic outcome of HD.

5.4.5 Therapeutic intervention and bioavailability of phytochemicals

Delivery of active molecules and compounds to the site of action is a major challenge to treat many diseases, and is one factor that explains why many improvements *in vitro* are not exactly matched *in vivo*. Compounds must remain stable and un-modified until the target site is reached; oral delivery must also survive the low pH in the stomach and be absorbed into the bloodstream. Addressing neurological disorders and neurodegenerative diseases present additional complications as therapeutic agents must pass the BBB. Permeability of the BBB is dependent on several factors including active transport across the membrane, molecular weight and lipid solubility (Banks, 2009). Phytochemical compounds previously investigated in HD are relatively small molecules, and in the case of anthocyanins contained in BE, are able to cross the BBB and penetrate regions which have improved with treatment (Andres-Lacueva *et al.*, 2005; Kalt *et al.*, 2008). As many phytochemicals, including anthocyanins are consumed daily in a normal diet, the process of assessing potential toxicity in humans is simplified compared to

newly developed drugs. These features make phytochemicals such as anthocyanins potentially useful therapeutic agents that can be easily implemented to treat disease within the brain.

5.4.6 Conclusion

Dietary phytochemicals, specifically anthocyanins in BE are reported to have substantial protective effects in the aged and diseased brain. The motor phenotype of R6/1 HD mouse model was attenuated in female mice through dietary supplementation of BE in our study. Several mechanisms may be responsible for improvements seen in HD models treated with BE supplementation. These include; rescued cell signalling, anti-aggregation, antioxidant and anti-inflammatory mechanisms that may be stimulated by a hormetic response. Different phytochemicals are likely to work through different mechanisms, and specifically in BE supplemented HD, may deliver therapeutic benefits to address a dysfunction of motor co-ordination, but not involuntary reflex movement such as hind paw clasping as seen in this study. The progressive weight loss and brain mass reduction observed in HD mouse models was not rescued by BE supplementation; however these phenotypes are not necessarily rescued in conjunction with motor and cognitive improvements. Our study was unable to detect an alteration of the cholesterol homeostatic deficit that is observed in R6/1 mice. Nevertheless, previous findings that consistently demonstrate ameliorated age-related brain deficits with BE supplementation, and our current finding of improved motor function in R6/1 mice, indicates this dietary supplement should be investigated more thoroughly in HD.

Chapter 6

Cholesterol metabolism in Huntington's disease post-mortem brain tissue

6.1 Introduction

HD is an autosomal dominant, progressive neurodegenerative disease characterised by the expansion of a glutamine repeat on the N-terminus of HTT. Classic symptoms include involuntary movement and cognitive dysfunction. The classical neuropathological hallmark of HD is the severe atrophy of the striatum (caudate and putamen) (Vonsattel *et al.*, 2011), with substantial volume loss in the order of 50% in late stages of the disease. While these brain regions are most severely affected, MRI techniques have highlighted that the hippocampus, cerebral cortex, globus pallidus and amygdala also have reduced volume in HD patients (Rosas *et al.*, 2003). Despite the genotypic identification of mHTT carriers, there is a lack of reliable biomarkers to predict HD progression or effectiveness of therapies. The mutation of *HTT* has been reported to alter HTT-phospholipid interactions (Kegel *et al.*, 2009), membrane organisation (del Toro *et al.*, 2010) and gene transcription of lipid metabolic enzymes (Sipione *et al.*, 2002). Several studies have also identified that cholesterol synthesis and metabolism in HD cell lines and animal models is significantly disturbed (Valenza *et al.*, 2005; Valenza *et al.*, 2007b; Valenza *et al.*, 2010), however the current literature contains very limited data obtained from human tissue, which is required for greater understanding of human HD pathophysiology. In this chapter we have investigated for the first time the level of cholesterol metabolites, synthetic precursors and oxidation products in 13 cases of human HD and 13 matched healthy controls across 5 brain regions, to comprehensively profile sterol changes in human brain. This study also investigated the levels of two key cholesterol synthetic and metabolic enzymes in HD putamen.

6.2 Materials and methods

Materials: Materials used in this study are listed in 2.2.

Human brain tissue: Frozen brain tissues from five brain regions (putamen, caudate, cerebellum, grey and white frontal cortex) were received from the Victorian Brain Bank Network. The cohort contained 13 cases of HD and 13 controls from each brain region. Tissue was transported on dry ice and stored at -80°C until analysis. Demographic and basic clinical data are presented in Table 6.1. The mean age of control cases was 68.9 ± 1.9 y which was not significantly different from the HD cases with mean age of 67.3 ± 2.2 y. Post mortem interval (PMI) of control cases (41.5 ± 4 h) and tissue pH (6.4 ± 0.1) was not significantly different to HD PMI (37.5 ± 6.2 h) or tissue pH (6.4 ± 0.04). All brain tissue was from the left hemisphere of Caucasian donors. Ethics approval was from the University of Wollongong Human Research Ethics Committee (HE10/327). Control tissue was screened with a full neuropathology examination to determine the absence of degenerative pathologies. The research was carried out in accordance with the Declaration of Helsinki (2008) of the World Medical Association.

Western Blotting: Brain tissue (~20 mg) was added to 250 µL ice cold radio-immunoprecipitation assay (RIPA; 50 mM Tris, 150 nM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X 100) buffer containing 1% protease inhibitor (P8340, Sigma) and homogenised at 4°C using a Precellys 24 homogeniser (Bertin Technologies) (2 x 20 s, 6000 rpm). The homogenate was centrifuged at 14 000 x *g* and the soluble fraction taken for SDS-PAGE. Homogenates were incubated at 95°C for 10 min with 5x loading dye containing β-mercaptoethanol. Equal amounts of protein (90 µg, determined by a BCA assay) were loaded onto a 12% acrylamide gel and electrophoresed for 1 h at 150V. Proteins were then transferred onto a 0.4 µm nitrocellulose membrane (BIO-RAD, Gladesville, NSW, Australia) for 35 min at 100V. To improve antigen detection, boiling phosphate buffered saline + tween 20 (PBST) (50 mL) was added to the membrane and left to cool to room temperature. The membrane was blocked with a 10 mL solution of PBST containing 5% skim milk

powder, rocking for 1 h at room temperature. Membranes were probed with antibodies detecting CYP46A1 1:100 (1F11, Santa Cruz Biotechnology) and DHCR24 1:1000 (ab137845, Abcam). Antibodies were diluted in 10 mL PBST 5% skim milk powder and incubated with membranes overnight, rocking at 4°C. Membranes were washed 3 x 10 min with PBST at room temperature before being incubated for 1 h at room temperature with a species specific IgG-HRP conjugated secondary antibody (1:3000). Membranes were washed 3 x 10 min with PBST before chemiluminescent detection of signal. Membranes were stripped, washed and re-probe for β-actin (1:10 000) following the protocol outlined above. Signal intensity was quantified using ImageJ software V1.46r (National Institutes of Health, USA), and normalised for β-actin.

qPCR: The quantitative PCR of human brain tissue was performed as previously described (Abbott *et al.*, 2014) with minor modifications. Briefly, human tissue (~30mg) was added to 10 volumes of TRIzol reagent (wt:vol) and homogenised in a Precellys 24 homogeniser at 2 x 20 s at 5500 rpm. The RNA concentration and purity was determined spectrophotometrically with a Nanodrop 1000 (Thermo Scientific). Following the manufacturer's protocol, 2 μg of total RNA was used to synthesise cDNA using a Tetro cDNA synthesis kit (Oligo dT_{18}) (Bioline, Sydney, Australia). Quantitative PCR was performed using a Roche Lightcycler 480 using SensiFAST SYBR No-ROX kit (Bioline) following the manufacturer's instructions. Analyses were carried out in triplicate and gene of interest mRNA was normalised to *GAPDH* mRNA levels. Gene expression was calculated using the comparative threshold cycle (Ct) value method using the formula $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = \Delta Ct$ sample $- \Delta Ct$ reference) as described (Li *et al.*, 2012). All primers were purchased from Sigma Aldrich (Sydney, Australia). The primer sequences used were as follows: *CYP46A1* (F: TTCTAGGACACCTCCCCTGC and R: CAGGTCCATACTTCTTAGCCCAAT) *DHCR24* (F: TGTTCGTGTGCCTCTTCCTC and R: ATTCCCGCACCTGCTTCTG) *GAPDH* (F: GAGCACAAGAGGAAGAGAGAGACCC and R: GTTGAGCACAGGGTACTTTATTGATGGTACATG) *RNU6-6P* (F: CTCGCTTCGGCAGCACA and R: AACGCTTCACGAATTTGCGT).

Lipid extraction and GC-MS/MS analysis of sterols: Quantification of cholesterol synthetic precursors, metabolites, oxidation products and phytosterols was performed as outlined in 2.3.5 and 2.3.6.

Statistical Analysis: All results are expressed as mean ± SEM with a $P < 0.05$ considered significant. An unpaired t-test was used to test for significantly different means, an F-test was used to determine if variances were significantly different. Welch's correction for unequal variances was used when variances were found to be significantly different. All analyses were performed in Graphpad PRISM v5.0 (Graphpad Software Inc., USA).

Table 6.1 **Demographic details of control and HD cohorts.**

Case#	Gender	Age (y)	PMI (h)	pH	HD Grade
Con 1	M	78.3	46	6.54	-
Con 2	M	69.1	34	6.31	-
Con 3	M	63.9	54.5	6.51	-
Con 4	M	81	36.5	6.56	-
Con 5	M	64.1	24	6.56	-
Con 6	F	59	30	6.84	-
Con 7	F	67.3	30	6.23	-
Con 8	F	74.8	61.5	6.24	-
Con 9	F	68.3	71.5	6.34	-
Con 10	F	60.4	49	6.23	-
Con 11	M	63.9	32	6.47	-
Con 12	M	69.4	24	6.27	-
Con 13	M	75.6	46	6.57	-
HD 1	M	77	8.5	6.57	IV
HD 2	M	68.7	72	6.32	IV
HD 3	M	61.1	17	6.54	IV
HD 4	M	81.1	50.5	6.23	IV
HD 5	M	66.6	37	6.26	IV
HD 6	F	57.2	22	6.22	IV
HD 7	F	66.7	18.5	6.21	IV
HD 8	F	72.2	22	6.43	IV
HD 9	F	70.7	70	6.16	IV
HD 10	F	51.5	63.5	6.54	IV
HD 11	M	62.3	21.5	6.62	IV
HD 12	M	65.9	58	6.33	IV
HD 13	M	73.9	26.5	6.31	IV

6.3 Results

6.3.1 Sterol analysis

Human HD post mortem brain tissue from putamen, caudate, frontal cortex (grey and white) and cerebellum was analysed using heavy isotope dilution GC-MS/MS. Cholesterol metabolites, oxidation products and synthetic precursors are presented as percent relative change compared to control (Figure 6.1). Absolute values of sterols measured are provided in Appendix 10.

6.3.1.1 Cholesterol metabolites

The most significant changes were detected in HD putamen, a region that is severely affected in HD (Vonsattel *et al.*, 1985). The brain specific cholesterol metabolite 24-OHC, was reduced 59% ($p < 0.0001$) in HD putamen (Figure 6.1A). Caudate was the only other region to show changes in this metabolite with a 66% reduction ($p = 0.0009$) of 24-OHC in HD tissue compared to control tissue (Figure 6.1A). 27-OHC, a peripheral cholesterol metabolite was significantly increased by 3-fold in HD putamen ($p = 0.0107$). 27-OHC was also significantly increased 3-fold in grey frontal cortex ($p = 0.0117$) and 2-fold in cerebellum ($p = 0.0281$) (Figure 6.1A).

6.3.1.2 Cholesterol oxidation products

HD putamen was the only brain region to show evidence of oxidative stress, with a 53% and 69% increase in the cholesterol oxidation products 7β-OHC ($p = 0.0226$) and 7-KC ($p = 0.0009$) respectively (Figure 6.1B).

6.3.1.3 Cholesterol synthetic precursors

Altered cholesterol synthesis was evident in HD putamen which exhibited an increase in the cholesterol synthetic precursors, desmosterol (3-fold, $p = 0.0046$), lathosterol (2-fold, $p = 0.0405$), zymosterol (3-fold, $p = 0.0066$) and 24, 25 dihydro lanosterol (2-fold, $p = 0.0051$) (Figure

6.1C). Total cholesterol levels were also significantly increased by 31% in HD putamen ($p = 0.0342$) (Figure 6.1D). No other brain regions were found to have significant changes in cholesterol precursors, or cholesterol levels.

6.3.2 Cholesterol metabolic and synthetic enzymes

To further investigate pathways that may contribute to changes in brain cholesterol metabolism and synthesis observed in putamen, we examined two major enzymes involved by western blotting. The protein level of CYP46A1 in HD was reduced 90% compared to control ($p = 0.0027$) (Figure 6.2A). We also detected a significant (91%) reduction ($p = 0.0014$) of DHCR24 protein levels (Figure 6.2B), which is in agreement with the accumulation of desmosterol seen in HD putamen (Figure 6.1B).

The mRNA levels of the genes *CYP46A1* and *DHCR24* were also measured in putamen using quantitative PCR. mRNA levels did not reflect protein levels and were not significantly changed between HD and control tissue in both *CYP46A1* and *DHCR2* (Figure 6.3).

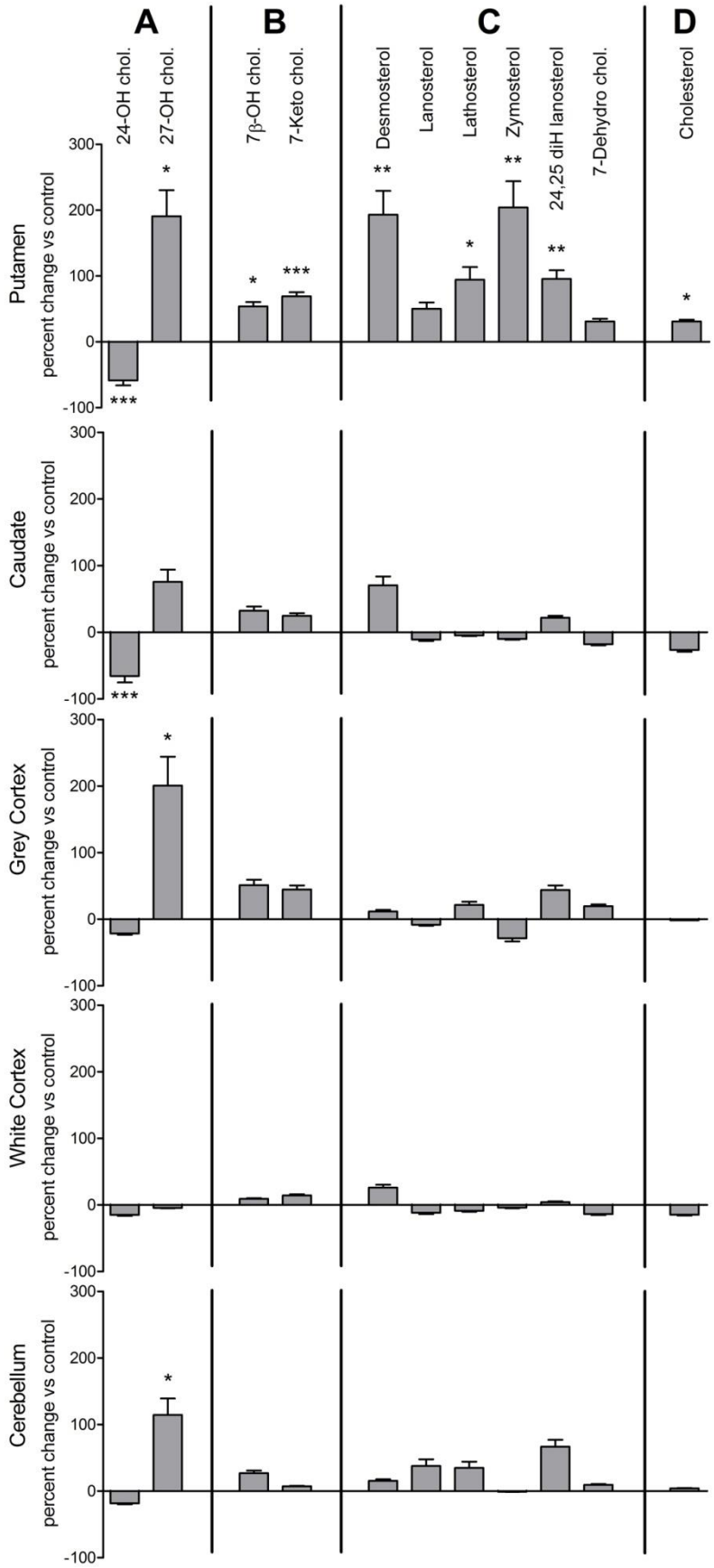

Figure 6.1 **Sterol levels in human HD putamen, caudate, grey cortex, white cortex and cerebellum**. (A) Cholesterol metabolites (B) Cholesterol oxidation products (C) Cholesterol synthetic precursors (D) Cholesterol were measured by GC-MS/MS. Levels are expressed as a percentage change against control tissue. n = 12-13 per group, error bars represent +SEM *** $p < 0.0001$, **$p < 0.005$, *$p < 0.05$

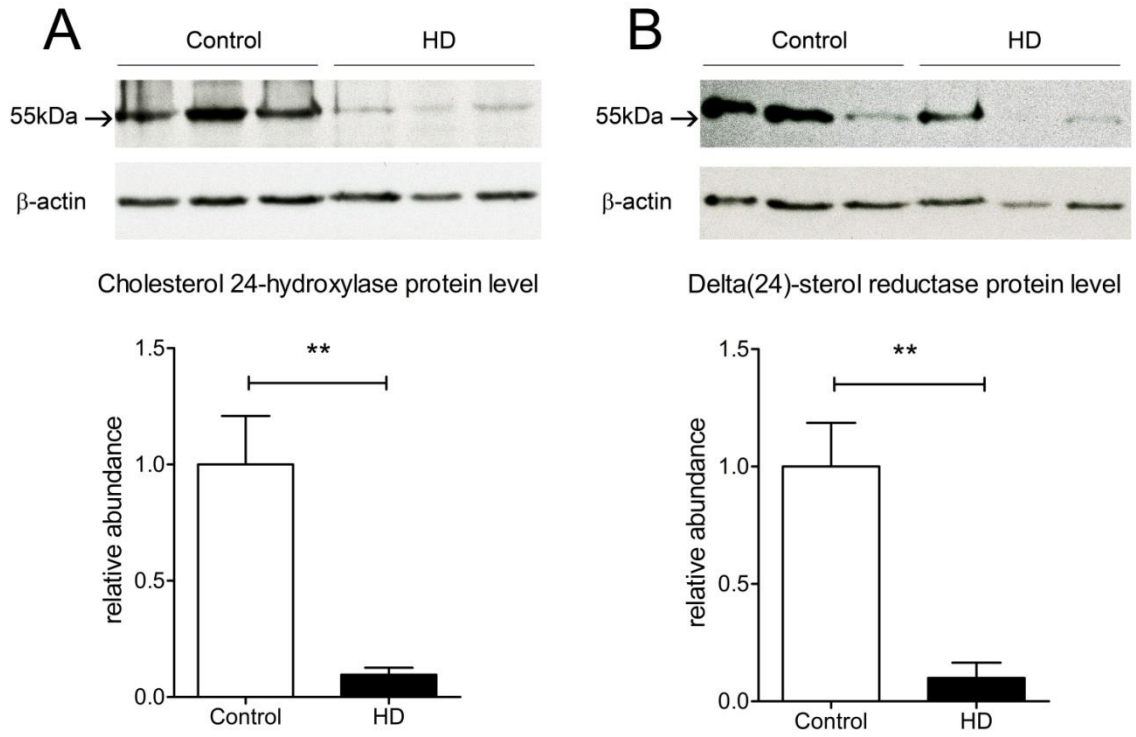

Figure 6.2 **Protein level of cholesterol synthetic and metabolic enzymes in human HD putamen.** Brain tissue homogenates from HD and control were probed for (A) cholesterol 24-hydroxylase (CYP46A1) and (B) delta(24)-sterol reductase (DHCR24) by western blotting. Representative blots are shown (n = 3 per group). Integrated optical density for all blots (n = 9 per group) is represented as relative abundance compared to control (assigned a value of 1.0). Blots were normalised to β-actin level. Error bars represent +SEM. **p < 0.005

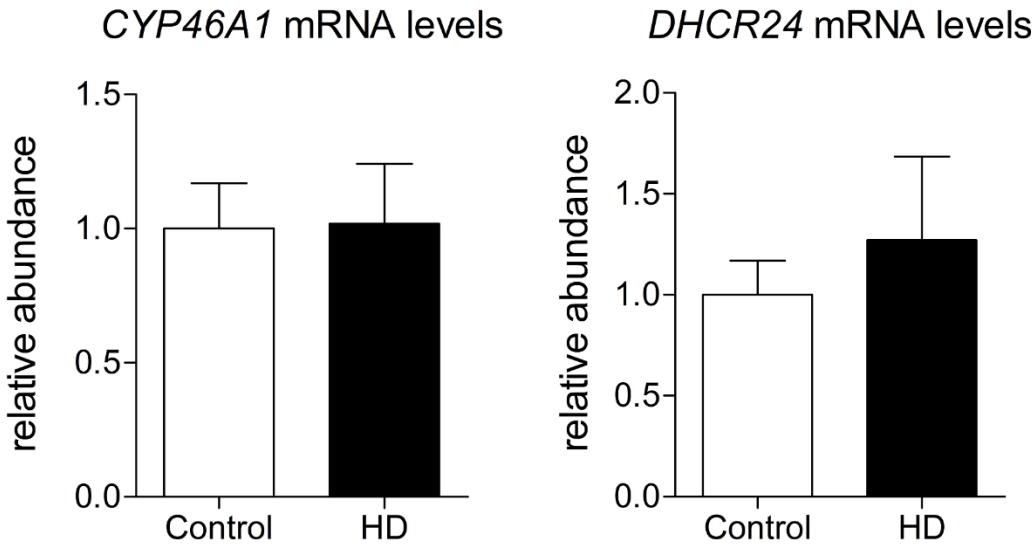

Figure 6.3 **mRNA levels of genes coding for cholesterol synthetic and metabolic enzymes in human HD putamen**. The level of *CYP46A1* (coding for cholesterol 24-hydroxylase) and *DHCR24* (coding for delta(24)-sterol reductase) in brain tissue was determined by qPCR (n = 9 for control and HD). The genes of interest were normalised to *GAPDH* and *RNU6-6P* mRNA levels and expressed relative to control (assigned a value of 1.0). Error bars represent +SEM

6.4 Discussion

Alteration of cholesterol metabolism has been recently linked to several neurodegenerative diseases including AD, PD and HD (Sipione *et al.*, 2002; Puglielli *et al.*, 2003; Cheng *et al.*, 2011). In particular, the elimination pathway where 24-OHC is formed by the hydroxylation of cholesterol has been suggested as a contributing factor in neurodegeneration (Bjorkhem *et al.*, 2006). Neuronal 24-OHC has a major role in the turnover of cholesterol in the brain (Bjorkhem *et al.*, 1998), and the level of 24-OHC in plasma has been suggested as a measure of CYP46A1 enzymatic activity and thus metabolically active neurons (Bjorkhem, 2006).

6.4.1 Alteration of cholesterol metabolism in human Huntington's disease brain

Previous studies have found that the plasma levels of 24-OHC in HD patients correlate with disease severity and brain volume measured by MRI (Leoni *et al.*, 2008; Leoni *et al.*, 2013). Here for the first time we report the levels of 24-OHC in human HD post-mortem brain tissue. A significant reduction of 24-OHC was found in HD putamen accompanied by a large (90%) reduction in the protein level of CYP46A1, the enzyme responsible for 24-OHC formation. Reduced enzyme levels may be due to neuron loss, where CYP46A1 is primarily located. Alterations in post-transcriptional regulation may also play a role since no change in mRNA expression was detected between control and HD. This is potentially relevant as protein turnover is known to be dysregulated in HD cell models (Hatters, 2008). Caudate (another striatal region affected in HD) also had reduced levels of 24-OHC, while grey and white frontal cortex and cerebellum showed no change. Although the cerebral cortex is affected in later stages of HD (de la Monte *et al.*, 1988; Rosas *et al.*, 2003), the degree of volume loss and astrocytosis is substantially less compared to the striatum (Sotrel *et al.*, 1991; Vonsattel & DiFiglia, 1998; Rosas *et al.*, 2003). Reduced 24-OHC levels in HD striatum supports previous human studies that reported reduced plasma levels of this metabolite in HD patients (Leoni *et al.*, 2008; Leoni *et al.*,

2013). Our data indicates that decreased circulating 24-OHC levels are likely the result of reduced 24-OHC production in affected regions and not an aberration of BBB 24-OHC flux. In the mature brain, delivery of cholesterol synthesised in the astrocyte is required for normal neuronal function (Posse de Chaves *et al.*, 1997; Mauch *et al.*, 2001; Hayashi *et al.*, 2004). It has been hypothesised that 24-OHC production in neurons acts as a signalling molecule to initiate the delivery of cholesterol from astrocytes to neurons therefore maintaining homeostasis (Pfrieger, 2003). There is also evidence to suggest that CYP46A1 may have neuroprotective properties, being upregulated near plaques (Brown *et al.*, 2004) and in glia of the human AD brain (Bogdanovic *et al.*, 2001). Whether reduced levels of CYP46A1 in HD is a secondary event reflecting active neuron loss, or is a pathogenic factor altering cholesterol metabolism and synthesis remains to be established.

27-Hydroxycholesterol, a metabolite of cholesterol that is produced predominantly in peripheral tissue, was increased in several regions of human HD brain as compared to controls. While an increased flux of 27-OHC into the brain may be caused by a disrupted BBB, we believe this is not the case in this tissue since sterols that are present in higher peripheral abundance such as campesterol (a dietary derived phytosterol) were not significantly different between HD and control brain tissues (Appendix 10). As there is a net movement of 27-OHC from circulation into the brain (Heverin *et al.*, 2005), 27-OHC accumulation may be the result of a decrease in the activity of enzymes in the brain (such as CYP27A1) that further metabolise this oxysterol to cholestenoic acids (Meaney *et al.*, 2007). Accumulation of 27-OHC is potentially important in neurodegeneration since *in vitro* studies have shown 27-OHC promotes β-amyloidogenesis (Gamba *et al.*, 2014) as well as being elevated in human AD brain and transgenic mice (Heverin *et al.*, 2004; Shafaati *et al.*, 2011). While there is little direct *in vivo* physiological evidence that cholesterol metabolites promote neuronal damage, further studies are required to investigate

the hypothesis that preventing the accumulation of 27-OHC in the brain reduces neurodegeneration in AD and HD.

6.4.2 Alteration of cholesterol synthesis in human Huntington's disease brain

A defect in cholesterol synthesis has been previously described in several cell and mouse models of HD. Specifically; a reduction in the cholesterol synthetic precursors lathosterol and lanosterol as well as cholesterol in the YAC128 mouse (Valenza *et al.*, 2007a), reduced levels of lathosterol and lanosterol, but not cholesterol, in the R6/2 mouse model (Valenza *et al.*, 2007b), reduced mRNA levels of cholesterol synthetic enzymes in R6/2 mouse and human HD fibroblasts (Valenza *et al.*, 2005) and reduced cholesterol synthetic enzymes in a transgenic HD cell model (Sipione *et al.*, 2002). In contrast to these findings, our data on end stage human HD putamen shows an increase in cholesterol synthetic precursors from both the Bloch (desmosterol and 24,25 dihydro lanosterol) and Kandutsch-Russell (lathosterol and zymosterol) pathways and an increase in total cholesterol levels. No significant changes in squalene levels were observed (Appendix 10) suggesting that alterations in the upstream mevalonate pathway had no influence on the changes that we measured in the Kandutsch-Russell and Bloch pathways. The increase in cholesterol and synthetic precursors was only observed in the putamen; a region that degenerates early and severely in the human disease (Vonsattel *et al.*, 1985). In contrast to macro and microscopic classification of disease pathology, which shows the caudate degenerates in parallel with the putamen (Vonsattel *et al.*, 1985; Vonsattel & DiFiglia, 1998), we did not observe any cholesterol synthetic alterations in this region. While the cholesterol synthetic changes observed in the putamen may suggest an overall increase of cholesterol synthesis utilising both pathways, we believe this is not the case for the Bloch pathway. In our study, HD putamen had significantly higher levels of desmosterol, an immediate synthetic precursor of cholesterol (Bloch pathway) and a substrate of DHCR24. Significantly depleted protein levels of DHCR24 in human HD putamen (91% reduction) are consistent with the reduced

conversion of desmosterol to cholesterol and its subsequent accumulation. Unaltered mRNA levels of *DHCR24* in putamen again suggests enzyme levels are being affected post-transcriptionally. Previous *in vitro* examination of cholesterol synthesis in specific cell types found astrocytes utilise the Bloch pathway via desmosterol, while neurons primarily utilised 7-dehydro cholesterol and other precursors from the Kandutsch-Russell pathway (Nieweg *et al.*, 2009). This suggests that in late stage human HD, desmosterol accumulates in astrocytes due to down regulated DHCR24. An increased density of oligodendrocytes and activated astrocytes is known to be present in HD (Myers *et al.*, 1991) and is believed to be a compensatory response to demyelination in HD (Bartzokis *et al.*, 2007). While the specific cell type/s exhibiting elevated Kandutsch-Russell synthetic precursors is yet to be determined, increased oligodendrocyte activity attempting remyelination in affected tissue may contribute to this.

Previous studies in HD mouse brain reported a significant reduction of the cholesterol precursor lathosterol (Valenza *et al.*, 2007a; Valenza *et al.*, 2007b), which is found in the Kandutsch-Russell pathway. In contrast, the Bloch pathway precursor desmosterol, was either unchanged (Valenza *et al.*, 2007a) or not reported (Valenza *et al.*, 2007b) in these previous HD mouse studies. Examining precursors from both arms of the cholesterol synthetic pathway in these mouse models enables greater insight into the alterations occurring in different cell types, and their possible response to HD pathology / neurodegeneration. The differences seen between HD mouse models, *in vitro* cell models, and human post-mortem tissue may reflect a difference in the homeostatic response to mutant HTT between organisms and cell types. There is also a difference between the relative ages of mice being in early adulthood compared to end stage human HD tissue where the mean age was 67 years old. The differences in the data derived from our current study and previous mouse models and cell culture models may also reflect that in this human tissue study we examined end stage HD where a significant number of vulnerable neurons are predicted to have been lost. Examining earlier stages of the disease in brain tissue

is therefore important to consolidate the role of cholesterol synthesis and metabolism in the early stages of HD in humans.

6.4.3 Cholesterol oxidation products in human Huntington's disease brain

Along with measuring cholesterol metabolites and precursors, our GC-MS/MS method was able to sensitively detect COPs, and use these as markers of oxidative stress in HD brain tissue. Oxidative stress has been previously shown in HD (Browne *et al.*, 1999), and in several other neurodegenerative diseases (Subbarao *et al.*, 1990; Ferrante *et al.*, 1997; Cheng *et al.*, 2011). Similar to changes seen in other sterols, significant increases in 7-KC and 7β-OHC were only observed in putamen. The specific increase in oxidation products in the putamen is consistent with this region being affected early and severely in HD (Vonsattel *et al.*, 1985). 7-KC and 7β-OHC have been shown to be stable, and previously used as markers of oxidative stress in plasma (Iuliano *et al.*, 2003) and CSF (Leoni *et al.*, 2005). Measurement of COPs in HD plasma together with other sterols may represent a convenient tool to follow progression, quantify severity, and assess therapeutic effectiveness in patients. Importantly, these markers can be measured sensitively and reliably by GC-MS/MS techniques.

6.4.4 Consequences of altered cholesterol homeostasis in human Huntington's disease brain

Homeostasis of cholesterol is essential for neurological function however the precise role of cholesterol in HD neurodegeneration is still debated. Evidence suggests that cholesterol accumulation in HD alters membrane organisation and cell signalling, enhancing the susceptibility of striatal neurons to excitotoxicity (del Toro *et al.*, 2010). Another hypothesis suggests that impaired cholesterol synthesis drives neurodegeneration by limiting the supply of cholesterol to neurons (Valenza *et al.*, 2007a). As a result synaptogenesis and dendrite outgrowth is impaired (Mauch *et al.*, 2001; Fan *et al.*, 2002), and neurotransmission is disrupted (Koudinov & Koudinova, 2005). It is also believed that a reduction of BDNF in HD (Zuccato *et al.*,

2001) may play a significant role in neurodegeneration (Zuccato & Cattaneo, 2009). BDNF is a neurotrophin expressed by neurons that promotes cell survival, differentiation and growth (Binder & Scharfman, 2004). Among these properties, BDNF has been shown to promote DHCR24 expression (Sarchielli *et al.*, 2014). Therefore, a loss of BDNF action may also contribute to a reduction in DHCR24, and the cholesterol homeostatic imbalance we have detected in HD putamen.

6.4.5 Conclusion

Our study reveals for the first time that several cholesterol synthetic and metabolic pathways are disturbed in multiple brain regions of human HD. This disturbance was identified to be most severe in the putamen, a region that degenerates early and severely in HD. These data provide evidence to support previous studies that link altered cholesterol synthesis, metabolism and oxidative stress with the neuropathological process involved in HD. Since HD shares similar sterol disturbances as other late-onset neurodegenerative diseases, we believe these changes represent potential biomarkers for neurodegenerative disease and elucidating cholesterol related mechanisms of neuropathology may provide targets for therapeutic intervention. The key finding of reduced CYP46A1 in human HD brain confirms previous studies in HD patient plasma that indicate 24-OHC as a promising peripheral biomarker to monitor the development of HD neuropathology.

Chapter 7
General discussion

7.1 Cholesterol homeostasis alterations in Huntington's disease

The functions of cholesterol biosynthesis and metabolism, although not fully understood in the brain, have been demonstrated to be essential for proper brain function (Kabouridis *et al.*, 2000; Mauch *et al.*, 2001; Hayashi *et al.*, 2004); mutations in enzymes of the cholesterol synthetic pathway having severe neurological consequences in humans (Wassif *et al.*, 1998; Waterham *et al.*, 2001). The mutation of the HTT protein in HD is associated with alterations to cholesterol homeostasis and has been demonstrated consistently in multiple rodent and cell models (Sipione *et al.*, 2002; Valenza *et al.*, 2005; Valenza *et al.*, 2007a; Valenza *et al.*, 2007b; del Toro *et al.*, 2010). Thorough investigation of cholesterol synthetic and metabolic alterations in human HD brain has not been conducted prior to the experiments in this thesis. Using sensitive GC-MS/MS analytical techniques we have identified several alterations that are consistent between the R6/1 mouse model and human post mortem tissue. We have also identified several alterations that are not consistent, and require further investigation to determine the relevance of these changes to HD pathology. Here we discuss our data derived from the R6/1 mouse model in comparison to that obtained from human HD post-mortem tissue, and highlight HD specific alterations to cholesterol homeostasis and other pathways that require more detailed investigation in the HD brain.

7.2 Brain cholesterol synthesis and metabolism in human and mouse models of HD

Although many animal models of neurodegenerative disease are able to replicate some pathological hallmarks of a disease, they rarely replicate precisely the condition observed in humans. This is due to fundamental differences between species in; diet, metabolism, ageing as well as the physical size of the brain. Aside from some of these limitations, mice have been genetically manipulated to model several neurodegenerative diseases including AD, PD, MND

and MS (Mucke *et al.*, 2000; Bettelli *et al.*, 2003; Laird *et al.*, 2008; Gispert *et al.*, 2009). In the case of HD, generation of several mouse models expressing forms of mutated human HTT (Mangiarini *et al.*, 1996; Hodgson *et al.*, 1999; Slow *et al.*, 2003) as well as knock-in expanded mouse huntingtin (Wheeler *et al.*, 1999), have allowed for *in vivo* study of the mechanisms that underlie HD. In this study we have investigated the R6/1 mouse model, expressing a CAG expanded (115-120 repeats) exon 1 of human HTT, similar to the R6/2 model that expresses a longer CAG repeat (Mangiarini *et al.*, 1996). Multiple aspects of HD pathology have been investigated in the R6 models as they show many of the molecular and physical phenotypes of human HD. These include similarities in gene expression changes (Strand *et al.*, 2007), reduced levels of BDNF (Spires *et al.*, 2004; Zuccato *et al.*, 2005), nuclear inclusions (Hansson *et al.*, 2001; Meade *et al.*, 2002) and cannabinoid receptor downregulation (Denovan-Wright & Robertson, 2000; Naver *et al.*, 2003; Horne *et al.*, 2013). A physical phenotype indicative of human HD is also observed in these mice that includes, a progressive motor and cognitive dysfunction, weight loss, striatal atrophy and chorea (Mangiarini *et al.*, 1996; Hansson *et al.*, 1999; Murphy *et al.*, 2000; Naver *et al.*, 2003).

In this study we have focused on characterising cholesterol synthetic and metabolic alterations in HD. Based on several studies in mouse and cell models, cholesterol synthesis (by measure of cholesterol synthetic precursors lathosterol and lanosterol) is believed to be reduced in HD (Sipione *et al.*, 2002; Valenza *et al.*, 2005; Valenza *et al.*, 2007a; Valenza *et al.*, 2007b). Prior to our study, cholesterol homeostasis has not been sufficiently examined in human HD brain tissue, with three previous studies using a maximum of 3 cases of HD post-mortem tissue (Valenza *et al.*, 2005; del Toro *et al.*, 2010; Samara *et al.*, 2014). Our study, and previous studies examining cholesterol levels in aged human brain have shown cholesterol levels to be variable (Thelen *et al.*, 2006), and studies using small samples may therefore lack statistical power to identify changes, or are susceptible to identifying false positives. Analysis of 5 regions in 13 cases of late

stage human HD post mortem brain tissue in our study has revealed an increase in cholesterol synthetic precursors of the Kandutsch-Russell pathway (such as lathosterol), and increased total cholesterol levels, contrary to that demonstrated in previous mouse model studies (Valenza *et al.*, 2007a; Valenza *et al.*, 2007b), and our study of R6/1 mice (Chapter 3). Several factors may underlie these differences in cholesterol biosynthetic alterations between the R6 mice and humans, including the CAG length, HTT protein length (truncated vs full length) and the intact expression of endogenous murine huntingtin.

The duration of HD in humans compared to the mouse is also likely to be a factor resulting in differences in cholesterol homeostasis. Although we have used the R6/1 mouse which develops symptoms slower than the R6/2 model, the onset of disease and disease timeline is rapid compared to that in humans that manifests over decades (Foroud *et al.*, 1999). The relative age of affected R6/1 mice is comparable to humans in early adulthood and therefore the effects of an aged brain may be less apparent in the mouse. A slower disease timeline in humans is likely to result in some cellular changes in the brain that do not have time to manifest in the mouse model, and may explain the absence of lipid peroxidation in the R6/1 mice we examined. Massive neuron loss through apoptosis is not observed in the R6 models, rather a shrinkage of neurons is a common neuropathological feature in these mice (Hansson *et al.*, 1999; Iannicola *et al.*, 2000; Klapstein *et al.*, 2001; Petersen *et al.*, 2002). A degenerative process reported to be 'dark cell degeneration' has also been previously reported in R6/1 mice and juvenile human HD (Iannicola *et al.*, 2000; Turmaine *et al.*, 2000). This process is believed to occur over weeks and may not manifest completely in the lifespan of these mice (Turmaine *et al.*, 2000). The lack of significant astrocytosis and increased oligodendrocyte density in R6 mice (Mangiarini *et al.*, 1996; Hansson *et al.*, 1999; Turmaine *et al.*, 2000), compared to that seen in human HD (Myers *et al.*, 1991), is potentially relevant to the level Kandutsch-Russell cholesterol synthetic precursors we observe between human and R6/1 mice. Oligodendrocytes have a high capacity

for cholesterol synthesis, and the increased density of these cells believed to be attempting remyelination in HD (Bartzokis *et al.*, 2007), may explain increased cholesterol levels and synthetic precursors that we observe in human HD putamen.

Although this difference in Kandutsch-Russell precursors does exist between mouse and human, we believe the increase of desmosterol (the penultimate sterol of the Bloch pathway) in both species describes a HD specific dysfunction of the cholesterol synthetic regulation. Desmosterol levels are likely to reflect the level or activity of DHCR24 (as shown in chapter 6), which is believed to have regulatory roles in cholesterol synthesis beyond converting desmosterol to cholesterol at the end of the synthetic pathway (Luu *et al.*, 2015). DHCR24 expression levels have also been reported to be mediated by BDNF, a trophic factor that is depleted in both human and animal models of HD (Zuccato *et al.*, 2001; Spires *et al.*, 2004; Zuccato *et al.*, 2005). The current literature has not investigated brain levels of DHCR24 in detail, and further examination into desmosterol levels and DHCR24 activity in HD may explain the cholesterol homeostatic perturbation beyond simply describing the level of Kandutsch-Russell cholesterol synthetic intermediates.

Human and mouse HD brain tissues we have investigated also exhibited some important similarities in cholesterol metabolic alterations. Formation of 24-OHC by the enzyme CYP46A1 is a major route of cholesterol elimination from the brain. In our study, the level of 24-OHC is reduced in the striatum of human and R6/1 mice, this is consistent with previous studies in rodent brain (Valenza *et al.*, 2010) and human plasma (Leoni *et al.*, 2008). 24-OHC levels were previously suggested as a biomarker of metabolically active neurons in the brain (Bjorkhem, 2006), however the relevance of this suggestion in a diseased brain state is not fully understood. Although in human HD, where significant neuron loss occurs, it is unknown whether reduced 24-OHC levels are simply a reflection of neuron loss, or if a cholesterol metabolic deficit precedes neurodegeneration. Previously it has been hypothesised that cholesterol metabolism in neurons

and delivery from astrocytes may be regulated by feedback from 24-OHC (Pfrieger, 2003; Bjorkhem, 2006),however a precise mechanisms by which 24-OHC regulates cholesterol synthesis in the brain has not been identified. Until further investigation is undertaken, it is unknown if synthetic, or metabolic dysfunction is the primary cause of cholesterol homeostatic dysfunction in HD.

Our study of human HD tissue has highlighted that the metabolic pathway involving 27-OHC formation and further metabolism is not consistent with the R6/1 mouse striatum. 27-OHC has a net movement into the brain from the periphery, where it is quickly metabolised to cholestenoic acids that diffuse back into the bloodstream (Heverin *et al.*, 2005; Meaney *et al.*, 2007). It was previously suggested that the cell-rich grey matter in the human brain is the major site of 24-OHC formation and further metabolism of 27-OHC (Bjorkhem, 2006). Degeneration in these areas would therefore result in the decrease of 24-OHC levels and increased 27-OHC, as we observed in human caudate and putamen, and previously described in human AD brain (Heverin *et al.*, 2004). In the R6/1 mouse model we investigated, a striatum specific decrease in 27-OHC levels contradicts findings in humans. It is possible that faster cholesterol turnover in the brain of mice compared to humans (Dietschy & Turley, 2004), and lower absolute levels of 27-OHC in this region (0.05ng/mg in mouse vs 0.3 ng/mg in human), causes greater fluctuation and variation in absolute levels. Interestingly, previous examination of a mouse AD model revealed a consistent increase of 27-OHC in mouse brain tissue as well as in human AD brain tissue (Heverin *et al.*, 2004). Previously levels of 27-OHC levels have not been reported in studies examining cholesterol metabolism in HD mouse models (Valenza *et al.*, 2007a; Valenza *et al.*, 2007b); further work to identify if this change occurs in other models may clarify if 27-OHC formation or further metabolism has a role in HD pathogenesis. Future studies of CSF and plasma may also identify 27-OHC, or further metabolites of 27-OHC, as potential biomarkers of CYP7B1 and CYP27A1 activity in HD brain.

7.3 Localisation of cholesterol synthetic and metabolic changes in Huntington's disease

Neurodegeneration in human HD begins in the striatum, affecting neighbouring regions such as the globus pallidus, hippocampus, amygdala and cerebral cortex (Vonsattel *et al.*, 1985; Rosas *et al.*, 2003). The end stage tissue we have examined specifically exhibit a cholesterol metabolic deficit in the striatum (caudate and putamen), and altered synthesis was only observed in the putamen. This suggests that these changes may be associated with early events in HD, where specific degeneration of medium spiny neurons occurs in the striatum (Vonsattel *et al.*, 1985). Interestingly in the R6/1 and R6/2 HD mouse model, the cholesterol synthetic defect is more widespread, detected in the striatum and cortex in our study, and the hippocampus in a previous study (Valenza *et al.*, 2007b). Although changes to Kandutsch-Russell synthetic precursors are more pronounced in the striatum of R6/1 mice compared to other regions, the early occurrence of changes suggests these alterations may not be as specific to HD as the reduction of 24-OHC and accumulation of desmosterol. Both these changes are present in the striatum at later stages of the disease, and we hypothesise these alterations are not as widespread in the brain of R6/1 mice, similar to our observations in human brain.

7.4 Sex differences in Huntington's disease

In this thesis we have also examined interventions of HD in the R6/1 model. It is of interest that different interventions had differential effects on the motor phenotype of male and female R6/1 mice. EE improved the motor co-ordination of male R6/1 mice but did not influence females. The opposite occurred with BE supplementation, where female R6/1 mice had improved motor co-ordination compared to controls, however males did not improve. We have also previously identified a sex difference in the progression of the physical phenotype in R6/1 mice (Chapter

3). Previous studies examining the R6/1 model typically use males or females in separate studies (Clifford *et al.*, 2002; Naver *et al.*, 2003), and it is likely that any sex differences have not been previously observed in the more studied R6/2 model as the progression is more rapid (Mangiarini *et al.*, 1996). On a molecular level, we have not detected any significant difference in brain sterols between male and female R6/1 mice with our current study size; it is possible a larger study may identify potential alterations between mice that reflect phenotypic changes. Few studies have examined sex-specific changes in HD models, one study reported neuroprotective effects of a sex hormone (17β-estradiol) in HD transgenic rats (Bode *et al.*, 2008), another identified sex-dependant alterations to BDNF expression in R6/1 mice when exposed to environmental enrichment (Zajac *et al.*, 2010). These findings suggest further investigation into sex differences is warranted in HD models, an area which has been largely ignored in the past. In human HD patients, a significant influence of sex in the progression and severity of HD has not been previously identified. Assessing the age of onset, and disease severity between patients is complex as symptoms can be highly variable between people (Foroud *et al.*, 1999). Even if overt differences between sexes have not been identified in human HD patients, it is possible that the response to certain treatments may be sex dependant as previously identified in mice (Zajac *et al.*, 2010). It is therefore an important consideration to assess both sexes in studies, not only in mouse models but also in humans.

7.5 Future directions studying cholesterol homeostasis in HD

Our findings constitute the first comprehensive sterol analysis in human HD brain tissue, and we have identified several alterations to cholesterol homeostasis that were previously not identified in mouse models of HD. We have examined late stage human PM tissue (grade IV) and therefore the current understanding of cholesterol homeostasis in human HD would benefit from analysis of earlier stage HD tissue. However, due to the relatively early onset of HD, and

long timeline (Foroud *et al.*, 1999), sufficient early stage cases for a statistically powerful analysis are very difficult to obtain (McLean, C. A. 2015, pers. comm., 27 July), and is reflected in the past literature where the majority of analyses are carried out in late stage tissue (Augood *et al.*, 1996; Arzberger *et al.*, 1997; Petersen *et al.*, 2005; Zuccato *et al.*, 2008; Silvestroni *et al.*, 2009) . Measurements of peripheral sterols may therefore be required to confirm these cholesterol homeostatic alterations also occur early in HD.

Currently, methods to directly measure *in vivo* brain cholesterol synthesis without post-mortem tissue examination have not been reported. 24-OHC levels in plasma, which originate from the brain, may be an indirect marker of brain cholesterol synthesis. Formation of 24-OHC constitutes a major route of cholesterol elimination from the brain (Bjorkhem *et al.*, 1997; Bjorkhem *et al.*, 1998), and the formation of 24-OHC is believed to be coupled to cholesterol synthesis (Lund *et al.*, 2003). The reduction of plasma 24-OHC levels in HD patients has been described in the past (Leoni *et al.*, 2008; Leoni *et al.*, 2013), however naturally high variability in 24-OHC plasma concentrations between individuals limits the ability of a single time point to fully describe the changes occurring over disease progression. A very useful future study would be the examination of HD patient plasma in a longitudinal study beginning in the pre-symptomatic stage. Tracking changes on an individual basis would help normalise baseline variability in 24-OHC levels, and improve the power of detection. With highly sensitive analytical techniques, only low volumes of plasma are required for analysis (maximum 250 µL) (Leoni *et al.*, 2008); plasma measurements of 24-OHC may therefore be a useful biomarker in a prospective longitudinal study such as Enroll-HD (CHDI Foundation Inc, 2015).

Another future direction for cholesterol homeostasis in HD is to carry out an even more comprehensive analysis in animal models of HD. Transgenic large animal models with a larger brain, may provide an alternative insight into early cholesterol perturbations occurring in human HD. However, until these transgenic models that have been generated in sheep (Jacobsen *et al.*,

2010), pig (Yang *et al.*, 2010) and rhesus monkey (Yang *et al.*, 2008), are consistently established and validated, a closer look at mouse models may be more feasible in the short term. Here we have examined a transgenic mouse expressing truncated human HTT. It would therefore be of great interest to more closely examine the progression of cholesterol homeostasis in other models expressing truncated human HTT [N171-82Q (Schilling *et al.*, 1999)], full length human HTT [YAC128 (Slow *et al.*, 2003)], as well as knock-in HD mice [hdhQ92, hdhQ111 (Wheeler *et al.*, 1999)] to identify if alterations in cholesterol synthesis and metabolism progress consistently across these models. Future studies would also benefit from examining several key synthetic and metabolic enzymes we have highlighted as potentially important in our lipid analysis. These include CYP27A1, CYP7B1, DHCR24 and CYP46A1. Investigating protein levels in addition to mRNA expression would produce additional valuable results as we have previously shown that CYP46A1 and DHCR24 protein levels do not reflect mRNA expression in human HD (Chapter 6).

7.6 Conclusion

Due to HTT having multiple roles in a variety of cellular compartments, it is not surprising that the HD mutation has been associated with neurodegenerative pathology acting through multiple mechanisms. The mutation has also been associated with a cholesterol homeostatic perturbation, causing cholesterol synthetic and metabolic dysfunction in multiple rodent and cell models. In this study we report the novel reduction of DHCR24 and CYP46A1 in the human HD putamen. Measurement of cholesterol synthetic precursors and metabolites in human HD post mortem brain tissue also confirms the cholesterol homeostatic perturbation in animal models also occurs in human HD. It is not surprising that the R6/1 model and the human HD brain do not share all disturbances of cholesterol synthesis and metabolism, however predominant changes to the brain specific metabolic product 24-OHC, and accumulation of desmosterol are consistent between species and we suggest are important pathways for future

investigation. The specific localisation of cholesterol synthetic and metabolic dysfunction to the putamen in the human HD brain suggests this may be an early event in HD pathogenesis. Similarly, these alterations were also detected earliest and most severely in the striatum of R6/1 mice. Although we were unable to detect an alteration in the brain sterol profile of R6/1 mice treated with EE and dietary BE supplementation, a sex-dependant improvement in motor function was seen in both intervention studies, supporting a growing body of evidence that has identified sex differences in HD. Both mouse and human tissue have proved valuable in identifying cholesterol homeostatic alterations in HD. In addition to this, the mouse model has enabled us to identify fundamental age-related changes in both WT and HD mice, adding to the currently sparse knowledge of cholesterol synthetic and metabolic changes in the brain during ageing. Although investigation of isolated compounds and enzymes tells part of the story, further investigation of the fundamental mechanisms surrounding cholesterol synthesis and metabolism in the brain will help identify the relevance of these pathways in HD pathogenesis, and most likely an array of other neurodegenerative diseases.

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Appendices

Appendix 1a **Absolute values of sterols in male R6/1 and WT cortex and striatum.** GC-MS/MS was used to analyse lipids extracted from mouse brain tissue at various stages of the disease. Values represent ng/mg tissue ± SD.

		Male					
		6 week		12 week		20 week	
		WT	R6/1	WT	HD	WT	R6/1
Striatum	Lathosterol	54.8 ± 5.6	41.7 ± 6.7	43.2 ± 6.2	20.0 ± 4.8	41.6 ± 5.7	17.8 ± 6.1
	Lanosterol	13.6 ± 3.8	13.1 ± 3.6	10.8 ± 1.7	8.3 ± 2.5	13.6 ± 2.7	8.7 ± 3.4
	Zymosterol	5.0 ± 1.3	4.0 ± 0.9	3.2 ± 0.4	2.2 ± 0.4	2.9 ± 0.63	2.2 ± 0.83
	24,25 diH	0.34 ± 0.22	0.39 ± 0.19	0.33 ± 0.09	0.24 ± 0.18	0.26 ± 0.05	0.19 ± 0.06
	Desmosterol	57.0 ± 9.1	57.7 ± 3.8	31.1 ± 9.6	39.7 ± 17.2	35.8 ± 5.1	41.7 ± 12.6
	7-DHC	63.0 ± 15.9	59.0 ± 4.9	43.5 ± 22.4	48.4 ± 21.8	54.3 ± 16.8	46.7 ± 14.6
	24-OHC	48.5 ± 8.7	42.3 ± 4.8	46.8 ± 4.1	39.7 ± 4.1	57.6 ± 11.2	48.6 ± 15.1
	27-OHC	0.039 ± 0.02	0.034 ± 0.01	0.078 ± 0.02	0.051 ± 0.01	0.083 ± 0.03	0.052 ± 0.02
	7-KC	0.67 ± 0.26	0.66 ± 0.29	0.65 ± 0.18	0.65 ± 0.15	0.30 ± 0.003	0.28 ± 0.009
	7β-OHC	0.36 ± 0.12	0.36 ± 0.17	0.45 ± 0.19	0.54 ± 0.27	0.26 ± 0.005	0.26 ± 0.010
	β-Sitosterol	2.8 ± 1.3	2.3 ± 0.19	2.4 ± 0.79	2.7 ± 0.53	2.0 ± 0.23	2.0 ± 0.48
	Campesterol	18.2 ± 2.7	17.9 ± 3.6	16.7 ± 3.1	19.2 ± 1.4	18.7 ± 3.8	18.5 ± 5.7
	Stigmasterol	0.40 ± 0.23	0.31 ± 0.10	0.12 ± 0.08	0.14 ± 0.11	0.24 ± 0.12	0.26 ± 0.11
	Brassicasterol	0.13 ± 0.06	0.11 ± 0.06	0.14 ± 0.08	0.18 ± 0.06	0.15 ± 0.007	0.16 ± 0.006
	Cholesterol[a]	15.1 ± 1.1	13.6 ± 4.3	17.8 ± 3.1	19.9 ± 1.4	21.5 ± 2.6	21.7 ± 6.0
Cortex	Lathosterol	33.5 ± 8.7	27.7 ± 11.2	34.7 ± 14.8	14.8 ± 4.1	34.5 ± 10.8	19.5 ± 3.1
	Lanosterol	7.9 ± 1.4	8.3 ± 2.9	7.9 ± 2.7	5.7 ± 1.4	12.3 ± 4.7	7.4 ± 1.7
	Zymosterol	2.4 ± 0.66	2.9 ± 1.1	2.9 ± 0.6	2.5 ± 0.6	2.7 ± 0.6	2.0 ± 0.3
	24,25 diH	0.33 ± 0.06	0.40 ± 0.17	0.32 ± 0.11	0.22 ± 0.07	0.37 ± 0.18	0.32 ± 0.17
	Desmosterol	32.8 ± 14.9	36.8 ± 9.2	34.6 ± 16.3	42.4 ± 16.4	41.9 ± 12.9	47.3 ± 7.3
	7-DHC	39.5 ± 17.2	41.2 ± 9.9	39.9 ± 6.0	40.5 ± 12.0	44.1 ± 6.4	44.5 ± 13.2
	24-OHC	28.4 ± 9.5	27.3 ± 7.5	29.7 ± 7.8	33.5 ± 7.5	40.3 ± 7.7	37.4 ± 9.0
	27-OHC	0.038 ± 0.02	0.030 ± 0.008	0.037 ± 0.01	0.038 ± 0.01	0.099 ± 0.06	0.071 ± 0.03
	7-KC	0.27 ± 0.12	0.32 ± 0.13	0.51 ± 0.04	0.50 ± 0.09	0.37 ± 0.11	0.58 ± 0.16
	7β-OHC	0.14 ± 0.07	0.18 ± 0.10	0.40 ± 0.11	0.35 ± 0.14	0.12 ± 0.03	0.16 ± 0.12
	β-Sitosterol	0.92 ± 0.22	1.56 ± 0.80	1.7 ± 0.33	2.0 ± 0.31	1.7 ± 0.43	2.1 ± 0.44
	Campesterol	14.0 ± 3.5	13.9 ± 4.5	12.8 ± 2.7	14.1 ± 1.8	23.1 ± 7.2	25.2 ± 3.6
	Stigmasterol	0.11 ± 0.03	0.15 ± 0.09	0.11 ± 0.04	0.12 ± 0.08	0.35 ± 0.13	0.42 ± 0.12
	Brassicasterol	0.067 ± 0.02	0.067 ± 0.03	0.13 ± 0.05	0.17 ± 0.04	0.15 ± 0.06	0.16 ± 0.04
	Cholesterol[a]	9.5 ± 2.6	9.2 ± 2.8	13.8 ± 1.8	14.6 ± 2.0	10.5 ± 2.0	12.8 ± 1.4

24, 25 diH = 24, 25 dihydro lanosterol; 7-DHC = 7-dehydrocholesterol; 24-OHC = 24(S)-hydroxycholesterol; 27-OHC = 27-hydroxycholesterol; 7-KC = 7-ketocholesterol; 7β-OHC = 7β-hydroxycholesterol. [a]Values expressed as μg/mg tissue.

Appendix 1b **Absolute values of sterols in male R6/1 and WT cortex and striatum.** GC-MS/MS was used to analyse lipids extracted from mouse brain tissue at various stages of the disease. Values represent ng/mg tissue ± SD.

		Male			
		24 week		28 week	
		WT	R6/1	WT	R6/1
Striatum	Lathosterol	43.8 ± 10.7	12.6 ± 3.5	31.4 ± 6.0	8.7 ± 2.7
	Lanosterol	12.2 ± 4.0	4.5 ± 1.3	8.9 ± 2.5	4.7 ± 1.7
	Zymosterol	3.5 ± 0.88	1.8 ± 0.36	2.7 ± 0.4	1.5 ± 0.6
	24,25 diH	0.31 ± 0.07	0.20 ± 0.07	0.19 ± 0.09	0.13 ± 0.08
	Desmosterol	39.8 ± 8.1	42.0 ± 4.6	33.0 ± 5.9	40.1 ± 7.3
	7-DHC	37.9 ± 7.0	42.5 ± 12.6	57.3 ± 15.3	68.7 ± 15.3
	24-OHC	53.3 ± 8.5	45.5 ± 5.2	53.7 ± 3.4	40.5 ± 8.3
	27-OHC	0.075 ± 0.012	0.051 ± 0.014	0.043 ± 0.02	0.024 ± 0.01
	7-KC	0.58 ± 0.19	0.52 ± 0.11	0.65 ± 0.29	0.41 ± 0.17
	7β-OHC	0.21 ± 0.06	0.22 ± 0.06	0.28 ± 0.13	0.23 ± 0.11
	β-Sitosterol	2.6 ± 0.31	3.1 ± 0.83	3.3 ± 1.0	3.0 ± 0.47
	Campesterol	21.6 ± 3.1	18.2 ± 2.6	38.1 ± 4.6	33.5 ± 3.9
	Stigmasterol	0.18 ± 0.04	0.25 ± 0.13	0.31 ± 0.17	0.32 ± 0.14
	Brassicasterol	0.27 ± 0.05	0.25 ± 0.05	0.23 ± 0.08	0.27 ± 0.08
	Cholesterol[a]	19.1 ± 1.8	18.9 ± 1.9	19.2 ± 3.0	19.8 ± 3.2
Cortex	Lathosterol	32.1 ± 11.9	14.8 ± 3.3	28.8 ± 5.5	14.6 ± 3.1
	Lanosterol	6.7 ± 2.3	4.2 ± 1.2	8.4 ± 1.3	4.6 ± 1.2
	Zymosterol	3.0 ± 1.2	2.7 ± 0.85	2.9 ± 0.30	2.0 ± 0.42
	24,25 diH	0.22 ± 0.07	0.14 ± 0.04	0.32 ± 0.03	0.25 ± 0.09
	Desmosterol	27.0 ± 9.5	33.1 ± 9.4	32.0 ± 8.1	38.0 ± 2.1
	7-DHC	31.0 ± 7.0	30.6 ± 9.9	45.6 ± 7.4	49.7 ± 6.2
	24-OHC	23.6 ± 5.7	26.2 ± 6.7	46.3 ± 6.7	42.4 ± 6.7
	27-OHC	0.034 ± 0.007	0.032 ± 0.011	0.051 ± 0.008	0.044 ± 0.01
	7-KC	0.31 ± 0.08	0.22 ± 0.04	0.86 ± 0.50	1.0 ± 1.05
	7β-OHC	0.12 ± 0.02	0.15 ± 0.07	0.43 ± 0.30	0.20 ± 0.09
	β-Sitosterol	1.8 ± 0.25	2.1 ± 1.2	1.7 ± 0.68	2.0 ± 0.69
	Campesterol	13.6 ± 4.1	11.3 ± 2.6	27.3 ± 5.9	30.0 ± 6.2
	Stigmasterol	0.11 ± 0.02	0.11 ± 0.03	0.27 ± 0.15	0.29 ± 0.16
	Brassicasterol	0.18 ± 0.05	0.17 ± 0.04	0.16 ± 0.05	0.19 ± 0.03
	Cholesterol[a]	11.0 ± 2.1	10.4 ± 1.9	15.4 ± 1.9	17.6 ± 2.9

24, 25 diH = 24, 25 dihydro lanosterol; 7-DHC = 7-dehydrocholesterol; 24-OHC = 24(S)-hydroxycholesterol; 27-OHC = 27-hydroxycholesterol; 7-KC = 7-ketocholesterol; 7β-OHC = 7β-hydroxycholesterol. [a]Values expressed as μg/mg tissue.

Appendix 2a **Absolute values of sterols in female R6/1 and WT cortex and striatum.** GC-MS/MS was used to analyse lipids extracted from mouse brain tissue at various stages of the disease. Values represent ng/mg tissue ± SD.

		Female					
		6 week		12 week		20 week	
		WT	R6/1	WT	R6/1	WT	R6/1
Striatum	Lathosterol	58.5 ± 13.7	36.4 ± 8.3	46.6 ± 14.8	19.7 ± 6.6	48.3 ± 4.1	18.7 ± 6.4
	Lanosterol	17.1 ± 6.7	10.2 ± 3.3	14.8 ± 5.9	7.9 ± 2.8	15.2 ± 4.3	8.3 ± 3.4
	Zymosterol	5.1 ± 1.2	3.6 ± 0.93	3.6 ± 1.3	2.1 ± 0.8	3.1 ± 0.74	1.9 ± 0.43
	24,25 diH	0.44 ± 0.22	0.20 ± 0.14	0.51 ± 0.26	0.33 ± 0.20	0.30 ± 0.03	0.21 ± 0.05
	Desmosterol	53.7 ± 9.1	42.9 ± 8.2	47.8 ± 9.1	40.4 ± 6.8	33.0 ± 5.0	41.3 ± 6.5
	7-DHC	70.9 ± 13.2	46.9 ± 5.3	56.8 ± 21.0	52.3 ± 10.6	45.3 ± 16.8	44.0 ± 13.2
	24-OHC	38.1 ± 7.5	34.8 ± 3.6	45.1 ± 4.2	43.1 ± 3.1	56.5 ± 4.7	49.5 ± 7.9
	27-OHC	0.034 ± 0.02	0.030 ± 0.01	0.067 ± 0.01	0.046 ± 0.02	0.071 ± 0.007	0.049 ± 0.12
	7-KC	0.84 ± 0.57	0.33 ± 0.06	0.60 ± 0.21	0.70 ± 0.16	0.33 ± 0.008	0.32 ± 0.008
	7β-OHC	0.35 ± 0.28	0.20 ± 0.05	0.44 ± 0.12	0.57 ± 0.18	0.23 ± 0.004	0.23 ± 0.005
	β-Sitosterol	2.3 ± 0.55	2.1 ± 0.50	2.2 ± 0.60	2.9 ± 0.77	1.9 ± 0.48	1.9 ± 0.27
	Campesterol	15.9 ± 3.3	15.0 ± 2.4	17.1 ± 0.95	17.5 ± 2.6	17.9 ± 5.3	16.5 ± 2.9
	Stigmasterol	0.36 ± 0.15	0.30 ± 0.09	0.13 ± 0.10	0.24 ± 0.09	0.22 ± 0.13	0.26 ± 0.12
	Brassicasterol	0.09 ± 0.05	0.05 ± 0.02	0.12 ± 0.06	0.15 ± 0.03	0.15 ± 0.012	0.018 ± 0.009
	Cholesterol[a]	14.5 ± 1.5	13.1 ± 1.4	19.5 ± 3.5	20.3 ± 1.5	23.3 ± 4.4	24.8 ± 4.1
Cortex	Lathosterol	40.2 ± 10.3	26.3 ± 10.4	43.9 ± 9.9	21.2 ± 6.1	41.6 ± 8.8	15.5 ± 6.3
	Lanosterol	9.4 ± 3.5	7.4 ± 1.1	10.4 ± 3.3	7.0 ± 1.5	10.9 ± 2.6	5.5 ± 1.6
	Zymosterol	2.6 ± 0.44	2.7 ± 0.69	3.2 ± 0.6	2.8 ± 0.4	2.4 ± 0.5	1.9 ± 0.6
	24,25 diH	0.45 ± 0.16	0.35 ± 0.08	0.35 ± 0.15	0.24 ± 0.11	0.32 ± 0.08	0.17 ± 0.11
	Desmosterol	29.6 ± 5.1	36.8 ± 10.0	33.6 ± 16.7	47.6 ± 18.6	26.1 ± 7.3	44.0 ± 2.9
	7-DHC	40.6 ± 4.9	39.1 ± 9.4	38.1 ± 6.1	41.8 ± 15.1	42.5 ± 7.3	46.0 ± 16.6
	24-OHC	26.6 ± 4.8	30.9 ± 7.6	30.4 ± 4.8	32.6 ± 2.5	38.7 ± 17.4	37.2 ± 3.6
	27-OHC	0.034 ± 0.008	0.033 ± 0.008	0.044 ± 0.02	0.047 ± 0.01	0.021 ± 0.006	0.017 ± 0.005
	7-KC	0.49 ± 0.18	0.33 ± 0.11	0.49 ± 0.17	0.46 ± 0.18	0.46 ± 0.22	0.51 ± 0.25
	7β-OHC	0.24 ± 0.12	0.22 ± 0.14	0.37 ± 0.17	0.37 ± 0.12	0.15 ± 0.06	0.16 ± 0.04
	β-Sitosterol	1.6 ± 0.79	1.2 ± 0.54	1.9 ± 0.45	1.9 ± 0.62	1.4 ± 0.21	1.5 ± 0.52
	Campesterol	13.4 ± 2.1	11.7 ± 1.7	15.5 ± 3.3	12.9 ± 2.7	26.0 ± 8.4	21.5 ± 4.6
	Stigmasterol	0.29 ± 0.20	0.20 ± 0.12	0.17 ± 0.10	0.18 ± 0.08	0.27 ± 0.07	0.30 ± 0.14
	Brassicasterol	0.058 ± 0.009	0.049 ± 0.005	0.14 ± 0.05	0.11 ± 0.02	0.14 ± 0.05	0.13 ± 0.03
	Cholesterol[a]	10.9 ± 2.1	9.1 ± 2.4	16.6 ± 2.7	15.3 ± 2.3	12.2 ± 2.9	10.9 ± 1.9

24, 25 diH = 24, 25 dihydro lanosterol; 7-DHC = 7-dehydrocholesterol; 24-OHC = 24(S)-hydroxycholesterol; 27-OHC = 27-hydroxycholesterol; 7-KC = 7-ketocholesterol; 7β-OHC = 7β-hydroxycholesterol. [a]Values expressed as µg/mg tissue.

Appendix 2b **Absolute values of sterols in female R6/1 and WT cortex and striatum.** GC-MS/MS was used to analyse lipids extracted from mouse brain tissue at various stages of the disease. Values represent ng/mg tissue ± SD.

		Female			
		24 week		28 week	
		WT	R6/1	WT	R6/1
Striatum	Lathosterol	33.5 ± 8.9	17.7 ± 6.8	36.4 ± 7.8	5.7 ± 1.9
	Lanosterol	9.8 ± 4.3	6.4 ± 1.9	13.3 ± 3.0	2.7 ± 1.4
	Zymosterol	3.0 ± 1.2	2.3 ± 0.98	2.7 ± 0.3	1.1 ± 0.8
	24,25 diH	0.33 ± 0.012	0.23 ± 0.05	0.21 ± 0.12	0.04 ± 0.03
	Desmosterol	37.0 ± 6.7	45.1 ± 5.4	33.4 ± 5.1	39.4 ± 4.0
	7-DHC	42.9 ± 14.0	47.7 ± 16.6	56.5 ± 10.8	55.4 ± 16.1
	24-OHC	46.5 ± 5.6	43.6 ± 7.4	61.9 ± 6.8	49.3 ± 4.6
	27-OHC	0.078 ± 0.02	0.056 ± 0.008	0.058 ± 0.02	0.028 ± 0.009
	7-KC	0.49 ± 0.18	0.57 ± 0.19	0.38 ± 0.05	0.57 ± 0.19
	7β-OHC	0.20 ± 0.07	0.24 ± 0.06	0.20 ± 0.07	0.25 ± 0.13
	β-Sitosterol	2.4 ± 0.62	3.4 ± 0.79	2.6 ± 0.31	3.3 ± 0.31
	Campesterol	15.7 ± 2.3	21.1 ± 4.5	30.1 ± 8.4	31.6 ± 4.7
	Stigmasterol	0.19 ± 0.07	0.29 ± 0.1	0.31 ± 0.09	0.55 ± 0.27
	Brassicasterol	0.18 ± 0.03	0.27 ± 0.04	0.20 ± 0.02	0.23 ± 0.09
	Cholesterol[a]	16.4 ± 1.5	18.0 ± 2.8	20.67 ± 1.7	20.4 ± 1.6
Cortex	Lathosterol	31.8 ± 7.9	15.6 ± 1.7	30.6 ± 6.1	15.3 ± 4.0
	Lanosterol	9.5 ± 2.2	4.6 ± 0.99	9.6 ± 0.94	5.3 ± 1.7
	Zymosterol	3.8 ± 0.94	2.7 ± 0.65	2.8 ± 0.50	2.0 ± 0.48
	24,25 diH	0.27 ± 0.07	0.14 ± 0.05	0.32 ± 0.05	0.23 ± 0.04
	Desmosterol	34.8 ± 12.5	39.5 ± 12.2	32.8 ± 7.9	38.5 ± 4.3
	7-DHC	24.1 ± 7.5	31.3 ± 11.3	49.2 ± 5.5	50.4 ± 9.6
	24-OHC	38.5 ± 9.5	32.9 ± 5.8	45.8 ± 10.9	37.8 ± 3.7
	27-OHC	0.060 ± 0.03	0.039 ± 0.01	0.055 ± 0.007	0.033 ± 0.007
	7-KC	0.33 ± 0.10	0.35 ± 0.11	0.91 ± 0.62	0.78 ± 0.68
	7β-OHC	0.11 ± 0.02	0.18 ± 0.06	0.35 ± 0.35	0.23 ± 0.11
	β-Sitosterol	2.0 ± 0.75	1.9 ± 0.36	1.5 ± 0.40	1.4 ± 0.28
	Campesterol	12.1 ± 3.6	13.9 ± 2.0	23.1 ± 7.8	23.1 ± 3.8
	Stigmasterol	0.19 ± 0.08	0.13 ± 0.07	0.24 ± 0.09	0.21 ± 0.06
	Brassicasterol	0.14 ± 0.05	0.16 ± 0.01	0.14 ± 0.05	0.14 ± 0.03
	Cholesterol[a]	11.8 ± 3.04	10.9 ± 1.7	15.5 ± 1.8	15.2 ± 3.1

24, 25 diH = 24, 25 dihydro lanosterol; 7-DHC = 7-dehydrocholesterol; 24-OHC = 24(S)-hydroxycholesterol; 27-OHC = 27-hydroxycholesterol; 7-KC = 7-ketocholesterol; 7β-OHC = 7β-hydroxycholesterol. [a]Values expressed as μg/mg tissue.

Appendix 3a **Absolute values of sterols measured in combined sexes R6/1 and WT cortex and striatum.** GC-MS/MS was used to analyse lipids extracted from mouse brain tissue at various stages of the disease. Values represent ng/mg tissue ± SD.

		Combined sexes					
		6 week		12 week		20 week	
		WT	R6/1	WT	R6/1	WT	R6/1
Striatum	Lathosterol	56.5 ± 9.6	38.8 ± 7.8	45.0 ± 11.3	19.8 ± 5.6	44.6 ± 6.0	18.3 ± 5.9
	Lanosterol	15.2 ± 5.4	11.5 ± 3.6	12.9 ± 4.8	8.1 ± 2.5	14.3 ± 3.4	8.5 ± 3.2
	Zymosterol	5.0 ± 1.2	3.8 ± 0.88	3.5 ± 0.9	2.2 ± 0.6	3.0 ± 0.65	2.0 ± 0.65
	24,25 diH	0.38 ± 0.21	0.29 ± 0.19	0.44 ± 0.22	0.29 ± 0.19	0.28 ± 0.05	0.20 ± 0.05
	Desmosterol	55.5 ± 8.9	49.6 ± 9.9	40.2 ± 12.4	40.1 ± 11.9	34.5 ± 5.0	41.5 ± 9.5
	7-DHC	66.6 ± 14.7	52.2 ± 7.8	50.8 ± 21.6	50.5 ± 15.8	50.2 ± 16.6	45.2 ± 13.2
	24-OHC	43.7 ± 9.5	38.2 ± 5.6	45.5 ± 4.1	41.0 ± 3.6	57.1 ± 8.5	49.1 ± 11.4
	27-OHC	0.037 ± 0.02	0.033 ± 0.01	0.071 ± 0.02	0.047 ± 0.01	0.77 ± 0.02	0.51 ± 0.15
	7-KC	0.74 ± 0.40	0.48 ± 0.26	0.59 ± 0.17	0.67 ± 0.15	0.032 ± 0.006	0.030 ± 0.008
	7β-OHC	0.36 ± 0.19	0.27 ± 0.14	0.42 ± 0.15	0.54 ± 0.22	0.24 ± 0.005	0.025 ± 0.007
	β-Sitosterol	2.6 ± 1.0	2.2 ± 0.39	2.2 ± 0.66	2.7 ± 0.62	1.9 ± 0.35	1.9 ± 0.37
	Campesterol	17.2 ± 3.1	16.3 ± 3.2	16.9 ± 2.2	18.4 ± 2.3	18.3 ± 4.3	17.5 ± 4.4
	Stigmasterol	0.38 ± 0.19	0.31 ± 0.09	0.12 ± 0.09	0.18 ± 0.11	0.23 ± 0.12	0.26 ± 0.12
	Brassicasterol	0.11 ± 0.06	0.079 ± 0.05	0.14 ± 0.06	0.17 ± 0.05	0.15 ± 0.010	0.17 ± 0.007
	Cholesterol[a]	14.8 ± 1.3	13.3 ± 2.9	18.5 ± 3.4	19.9 ± 1.3	22.3 ± 3.5	23.3 ± 5.2
Cortex	Lathosterol	36.6 ± 9.7	27.0 ± 10.2	39.7 ± 9.3	18.3 ± 6.1	35.8 ± 9.5	17.5 ± 5.1
	Lanosterol	8.6 ± 2.6	7.8 ± 2.0	9.2 ± 3.2	6.4 ± 1.5	7.7 ± 2.4	4.5 ± 1.3
	Zymosterol	2.5 ± 0.55	2.8 ± 0.84	3.0 ± 0.6	2.7 ± 0.5	2.5 ± 0.4	1.9 ± 0.4
	24,25 diH	0.39 ± 0.12	0.37 ± 0.12	0.34 ± 0.13	0.23 ± 0.09	0.32 ± 0.12	0.25 ± 0.15
	Desmosterol	31.3 ± 11.2	36.6 ± 9.2	34.1 ± 15.7	45.3 ± 17.0	35.3 ± 14.0	45.7 ± 5.5
	7-DHC	40.0 ± 12.6	40.1 ± 9.2	42.8 ± 6.4	45.3 ± 14.4	43.3 ± 6.5	45.3 ± 14.4
	24-OHC	27.5 ± 7.5	29.3 ± 7.5	30.1 ± 6.0	33.0 ± 5.1	39.6 ± 13.0	37.3 ± 6.5
	27-OHC	0.036 ± 0.01	0.032 ± 0.007	0.041 ± 0.01	0.043 ± 0.01	0.047 ± 0.04	0.044 ± 0.03
	7-KC	0.37 ± 0.18	0.33 ± 0.11	0.50 ± 0.12	0.48 ± 0.14	0.41 ± 0.17	0.54 ± 0.20
	7β-OHC	0.19 ± 0.11	0.20 ± 0.12	0.38 ± 0.14	0.36 ± 0.13	0.13 ± 0.045	0.16 ± 0.033
	β-Sitosterol	1.2 ± 0.63	1.1 ± 0.45	1.85 ± 0.40	1.94 ± 0.49	1.5 ± 0.34	1.8 ± 0.57
	Campesterol	13.7 ± 2.8	12.7 ± 3.3	14.3 ± 3.2	13.5 ± 2.3	23.6 ± 7.4	23.3 ± 4.3
	Stigmasterol	0.19 ± 0.16	0.18 ± 0.11	0.14 ± 0.08	0.15 ± 0.08	0.30 ± 0.12	0.36 ± 0.14
	Brassicasterol	0.063 ± 0.01	0.057 ± 0.02	0.14 ± 0.05	0.14 ± 0.04	0.14 ± 0.07	0.15 ± 0.05
	Cholesterol[a]	10.1 ± 2.4	9.2 ± 2.5	15.4 ± 2.7	15.0 ± 2.1	11.0 ± 2.5	11.9 ± 1.7

24, 25 diH = 24, 25 dihydro lanosterol; 7-DHC = 7-dehydrocholesterol; 24-OHC = 24(S)-hydroxycholesterol; 27-OHC = 27-hydroxycholesterol; 7-KC = 7-ketocholesterol; 7β-OHC = 7β-hydroxycholesterol. [a]Values expressed as µg/mg tissue.

Appendix 3b **Absolute values of sterols measured in combined sexes R6/1 and WT cortex and striatum.** GC-MS/MS was used to analyse lipids extracted from mouse brain tissue at various stages of the disease. Values represent ng/mg tissue ± SD.

		Combined sexes			
		24 week		28 week	
		WT	R6/1	WT	R6/1
Striatum	Lathosterol	38.6 ± 10.7	14.9 ± 5.6	33.9 ± 7.0	7.2 ± 2.7
	Lanosterol	11.0 ± 4.1	5.4 ± 1.9	11.1 ± 3.5	3.7 ± 1.8
	Zymosterol	3.7 ± 1.0	2.0 ± 0.71	2.7 ± 0.33	1.3 ± 0.72
	24,25 diH	0.32 ± 0.09	0.21 ± 0.06	0.20 ± 0.10	0.090 ± 0.08
	Desmosterol	38.4 ± 7.2	43.4 ± 5.0	33.2 ± 5.2	39.8 ± 5.6
	7-DHC	40.4 ± 10.8	44.9 ± 14.0	56.9 ± 12.8	62.2 ± 16.4
	24-OHC	49.9 ± 7.7	44.6 ± 6.0	57.8 ± 6.7	44.9 ± 7.8
	27-OHC	0.076 ± 0.02	0.053 ± 0.01	0.051 ± 0.02	0.026 ± 0.01
	7-KC	0.53 ± 0.18	0.54 ± 0.15	0.52 ± 0.24	0.49 ± 0.19
	7β-OHC	0.21 ± 0.06	0.23 ± 0.06	0.24 ± 0.11	0.24 ± 0.11
	β-Sitosterol	2.5 ± 0.47	3.2 ± 0.78	2.9 ± 0.77	3.1 ± 0.40
	Campesterol	18.7 ± 4.0	19.5 ± 3.7	34.1 ± 7.6	32.6 ± 4.2
	Stigmasterol	0.19 ± 0.05	0.26 ± 0.12	0.31 ± 0.13	0.44 ± 0.24
	Brassicasterol	0.22 ± 0.06	0.26 ± 0.05	0.22 ± 0.06	0.25 ± 0.08
	Cholesterol[a]	17.8 ± 2.1	18.5 ± 2.3	20.0 ± 2.4	20.1 ± 2.4
Cortex	Lathosterol	31.9 ± 9.5	15.2 ± 2.6	29.7 ± 5.6	15.0 ± 3.5
	Lanosterol	8.1 ± 2.6	4.4 ± 1.1	9.0 ± 1.2	5.0 ± 1.4
	Zymosterol	3.4 ± 1.1	2.7 ± 0.73	2.9 ± 0.39	2.0 ± 0.42
	24,25 diH	0.24 ± 0.06	0.14 ± 0.04	0.32 ± 0.04	0.24 ± 0.07
	Desmosterol	30.9 ± 11.2	36.0 ± 10.7	32.4 ± 7.5	38.3 ± 3.2
	7-DHC	27.6 ± 7.7	30.9 ± 10.0	47.4 ± 6.4	50.0 ± 7.6
	24-OHC	31.0 ± 10.8	29.2 ± 6.9	46.1 ± 8.5	40.1 ± 5.6
	27-OHC	0.047 ± 0.02	0.035 ± 0.01	0.053 ± 0.007	0.039 ± 0.01
	7-KC	0.32 ± 0.08	0.29 ± 0.11	0.88 ± 0.53	0.90 ± 84
	7β-OHC	0.11 ± 0.02	0.16 ± 0.07	0.40 ± 0.30	0.22 ± 0.09
	β-Sitosterol	1.9 ± 0.55	2.0 ± 0.90	1.6 ± 0.54	1.7 ± 0.57
	Campesterol	12.9 ± 3.7	12.5 ± 2.6	25.2 ± 6.9	26.6 ± 6.1
	Stigmasterol	0.15 ± 0.07	0.12 ± 0.05	0.25 ± 0.12	0.25 ± 0.12
	Brassicasterol	0.16 ± 0.05	0.16 ± 0.03	0.15 ± 0.05	0.16 ± 0.03
	Cholesterol[a]	11.4 ± 2.5	10.7 ± 1.7	15.5 ± 1.7	16.4 ± 3.1

24, 25 diH = 24, 25 dihydro lanosterol; 7-DHC = 7-dehydrocholesterol; 24-OHC = 24(S)-hydroxycholesterol; 27-OHC = 27-hydroxycholesterol; 7-KC = 7-ketocholesterol; 7β-OHC = 7β-hydroxycholesterol. [a]Values expressed as µg/mg tissue.

Appendix 4 **Absolute concentration of sterols measured in the cortex and striatum of male R6/1 and WT mice treated with environmental enrichment**. GC-MS/MS was used to analyse lipids extracted from mouse brain tissue. Mice were house in either standard (control) or environmentally enriched (EE) housing. Values represent ng/mg tissue ± SD.

		Male			
		Control		EE	
		WT	R6/1	WT	R6/1
Striatum	Lathosterol	32.1 ± 5.3	9.1 ± 2.2	27.4 ± 3.8	9.5 ± 3.4
	Lanosterol	13.9 ± 1.7	4.4 ± 1.6	12.1 ± 3.7	4.9 ± 2.3
	Zymosterol	2.4 ± 0.29	1.3 ± 0.32	2.4 ± 0.24	1.4 ± 0.47
	24,25 diH	0.23 ± 0.03	0.11 ± 0.02	0.25 ± 0.05	0.14 ± 0.04
	Desmosterol	40.1 ± 3.2	45.9 ± 6.0	40.0 ± 7.1	46.5 ± 8.6
	7-DHC	43.4 ± 12.7	47.2 ± 20.3	37.3 ± 9.7	47.3 ± 13.7
	24-OHC	61.0 ± 1.2	60.7 ± 6.6	60.3 ± 7.3	57.1 ± 8.1
	27-OHC	0.067 ± 0.005	0.051 ± 0.01	0.061 ± 0.006	0.050 ± 0.006
	7-KC	0.55 ± 0.06	0.67 ± 0.40	0.73 ± 0.36	0.61 ± 0.16
	7β-OHC	0.22 ± 0.05	0.31 ± 0.09	0.23 ± 0.07	0.26 ± 0.06
	β-Sitosterol	2.5 ± 0.51	2.8 ± 0.46	2.0 ± 0.36	2.5 ± 0.52
	Campesterol	33.9 ± 4.3	37.5 ± 5.7	27.6 ± 6.7	33.2 ± 7.4
	Stigmasterol	0.21 ± 0.08	0.27 ± 0.08	0.15 ± 0.03	0.20 ± 0.07
	Brassicasterol	0.33 ± 0.06	0.35 ± 0.04	0.28 ± 0.05	0.35 ± 0.06
	Cholesterol[a]	21.5 ± 3.6	24.4 ± 3.8	16.9 ± 2.4	21.3 ± 2.8
Cortex	Lathosterol	25.2 ± 3.6	14.9 ± 4.5	29.3 ± 7.3	13.7 ± 3.6
	Lanosterol	10.9 ± 2.2	7.9 ± 2.3	12.6 ± 3.2	6.7 ± 1.7
	Zymosterol	3.3 ± 0.70	2.7 ± 0.35	3.3 ± 0.66	2.3 ± 0.34
	24,25 diH	0.19 ± 0.15	0.095 ± 0.079	0.21 ± 0.076	0.069 ± 0.031
	Desmosterol	34.8 ± 13.1	41.7 ± 9.6	40.7 ± 11.4	38.4 ± 3.6
	7-DHC	36.2 ± 9.7	50.1 ± 8.0	50.0 ± 34.1	48.6 ± 21.3
	24-OHC	37.5 ± 10.2	34.2 ± 5.1	44.1 ± 8.4	33.1 ± 4.3
	27-OHC	0.029 ± 0.016	0.035 ± 0.007	0.059 ± 0.017	0.028 ± 0.017
	7-KC	0.50 ± 0.16	0.67 ± 0.19	0.48 ± 0.12	0.46 ± 0.17
	7β-OHC	0.36 ± 0.12	0.42 ± 0.10	0.28 ± 0.09	0.27 ± 0.09
	β-Sitosterol	2.3 ± 0.45	3.1 ± 0.77	2.6 ± 0.72	2.7 ± 0.66
	Campesterol	20.2 ± 5.6	28.1 ± 8.2	24.4 ± 9.1	23.2 ± 2.9
	Stigmasterol	0.25 ± 0.10	0.38 ± 0.15	0.29 ± 0.11	0.27 ± 0.10
	Brassicasterol	0.18 ± 0.06	0.24 ± 0.13	0.24 ± 0.07	0.22 ± 0.06
	Cholesterol[a]	13.7 ± 4.1	18.1 ± 2.8	16.5 ± 1.8	15.8 ± 2.3

24, 25 diH = 24, 25 dihydro lanosterol; 7-DHC = 7-dehydrocholesterol; 24-OHC = 24(S)-hydroxycholesterol; 27-OHC = 27-hydroxycholesterol; 7-KC = 7-ketocholesterol; 7β-OHC = 7β-hydroxycholesterol. [a]Values expressed as μg/mg tissue.

Appendix 5 **Absolute concentration of sterols measured in the cortex and striatum of female R6/1 and WT mice treated with environmental enrichment**. GC-MS/MS was used to analyse lipids extracted from mouse brain tissue. Mice were house in either standard (control) or environmentally enriched (EE) housing. Values represent ng/mg tissue ± SD.

		Female			
		Control		EE	
		WT	R6/1	WT	R6/1
Striatum	Lathosterol	31.8 ± 11.4	10.4 ± 3.3	36.7 ± 10.8	12.6 ± 3.7
	Lanosterol	15.1 ± 5.6	7.8 ± 4.0	16.5 ± 8.1	7.8 ± 2.8
	Zymosterol	2.9 ± 1.0	1.9 ± 0.79	2.8 ± 0.68	1.7 ± 0.65
	24,25 diH	0.35 ± 0.14	0.20 ± 0.07	0.34 ± 0.17	0.12 ± 0.02
	Desmosterol	42.6 ± 4.6	50.0 ± 12.4	42.4 ± 6.2	50.3 ± 5.6
	7-DHC	50.1 ± 21.1	45.4 ± 12.8	46.4 ± 20.7	50.8 ± 12.5
	24-OHC	65.7 ± 4.9	65.7 ± 13.1	69.2 ± 7.7	61.0 ± 5.1
	27-OHC	0.070 ± 0.01	0.055 ± 0.01	0.077 ± 0.02	0.053 ± 0.009
	7-KC	0.62 ± 0.18	0.55 ± 0.14	0.52 ± 0.08	0.57 ± 0.09
	7β-OHC	0.25 ± 0.09	0.26 ± 0.07	0.26 ± 0.06	0.31 ± 0.05
	β-Sitosterol	2.2 ± 0.46	2.6 ± 0.45	2.4 ± 0.69	2.7 ± 0.37
	Campesterol	32.7 ± 5.4	35.7 ± 4.2	30.7 ± 8.3	37.6 ± 5.9
	Stigmasterol	0.18 ± 0.06	0.27 ± 0.09	0.22 ± 0.1.2	0.28 ± 0.09
	Brassicasterol	0.30 ± 0.07	0.34 ± 0.04	0.28 ± 0.04	0.33 ± 0.05
	Cholesterol[a]	21.9 ± 3.9	24.5 ± 2.9	23.8 ± 3.1	24.3 ± 3.4
Cortex	Lathosterol	31.9 ± 11.2	11.9 ± 1.5	29.0 ± 9.3	12.0 ± 3.2
	Lanosterol	13.7 ± 5.3	7.6 ± 1.9	10.8 ± 3.2	6.8 ± 2.3
	Zymosterol	3.1 ± 0.44	2.1 ± 0.55	3.0 ± 0.66	2.0 ± 0.51
	24,25 diH	0.21 ± 0.16	0.036 ± 0.010	0.20 ± 0.11	0.093 ± 0.10
	Desmosterol	32.0 ± 7.1	35.5 ± 8.8	30.6 ± 7.9	39.4 ± 2.8
	7-DHC	46.7 ± 23.6	38.3 ± 17.6	34.9 ± 6.9	46.9 ± 21.1
	24-OHC	38.9 ± 5.4	34.0 ± 5.2	38.7 ± 10.5	37.7 ± 5.3
	27-OHC	0.038 ± 0.02	0.031 ± 0.02	0.037 ± 0.01	0.038 ± 0.01
	7-KC	0.66 ± 0.06	0.41 ± 0.12	0.43 ± 0.09	0.54 ± 0.22
	7β-OHC	0.31 ± 0.04	0.19 ± 0.02	0.24 ± 0.08	0.29 ± 0.11
	β-Sitosterol	2.4 ± 0.22	2.6 ± 0.31	2.7 ± 0.80	2.7 ± 0.30
	Campesterol	20.1 ± 4.9	20.3 ± 4.1	18.6 ± 7.3	26.4 ± 6.4
	Stigmasterol	0.26 ± 0.03	0.26 ± 0.06	0.27 ± 0.13	0.027 ± 0.04
	Brassicasterol	0.21 ± 0.08	0.25 ± 0.06	0.18 ± 0.09	0.21 ± 0.05
	Cholesterol[a]	15.3 ± 1.3	14.7 ± 1.4	15.4 ± 2.8	14.8 ± 1.6

24, 25 diH = 24, 25 dihydro lanosterol; 7-DHC = 7-dehydrocholesterol; 24-OHC = 24(S)-hydroxycholesterol; 27-OHC = 27-hydroxycholesterol; 7-KC = 7-ketocholesterol; 7β-OHC = 7β-hydroxycholesterol. [a]Values expressed as μg/mg tissue.

Appendix 6 **Absolute concentration of sterols measured in the cortex and striatum of combined sexes R6/1 and WT mice treated with environmental enrichment**. GC-MS/MS was used to analyse lipids extracted from mouse brain tissue. Mice were house in either standard (control) or environmentally enriched (EE) housing. Values represent ng/mg tissue ± SD.

		Combined sexes			
		Control		EE	
		WT	R6/1	WT	R6/1
Striatum	Lathosterol	32.0 ± 9.1	9.6 ± 2.7	32.1 ± 8.4	11.0 ± 3.8
	Lanosterol	14.5 ± 6.4	6.1 ± 3.4	14.3 ± 4.1	6.4 ± 2.9
	Zymosterol	2.7 ± 0.52	1.6 ± 0.65	2.6 ± 0.73	1.5 ± 0.57
	24,25 diH	0.29 ± 0.13	0.16 ± 0.07	0.30 ± 0.11	0.13 ± 0.03
	Desmosterol	41.7 ± 6.4	47.9 ± 9.4	41.2 ± 3.9	48.4 ± 7.2
	7-DHC	47.0 ± 18.1	46.3 ± 13.0	41.9 ± 18.2	49.0 ± 16.0
	24-OHC	63.4 ± 4.2	63.2 ± 10.1	64.8 ± 8.6	59.1 ± 6.8
	27-OHC	0.069 ± 0.008	0.053 ± 0.01	0.069 ± 0.02	0.051 ± 0.008
	7-KC	0.59 ± 0.13	0.60 ± 0.27	0.62 ± 0.27	0.60 ± 0.13
	7β-OHC	0.24 ± 0.07	0.29 ± 0.08	0.25 ± 0.06	0.28 ± 0.06
	β-Sitosterol	2.3 ± 0.51	2.7 ± 0.44	2.2 ± 0.57	2.6 ± 0.43
	Campesterol	33.3 ± 4.7	36.6 ± 4.8	29.2 ± 7.4	35.5 ± 6.8
	Stigmasterol	0.19 ± 0.07	0.27 ± 0.08	0.19 ± 0.09	0.24 ± 0.08
	Brassicasterol	0.32 ± 0.06	0.34 ± 0.04	0.28 ± 0.04	0.34 ± 0.05
	Cholesterol[a]	21.7 ± 3.6	24.5 ± 3.2	20.3 ± 4.4	22.8 ± 3.4
Cortex	Lathosterol	28.6 ± 8.7	13.4 ± 3.5	29.2 ± 7.9	12.9 ± 3.4
	Lanosterol	12.3 ± 4.2	7.8 ± 2.0	11.8 ± 3.2	6.7 ± 1.9
	Zymosterol	3.2 ± 0.58	2.4 ± 0.52	3.2 ± 0.66	2.2 ± 0.44
	24,25 diH	0.20 ± 0.15	0.066 ± 0.06	0.21 ± 0.09	0.080 ± 0.068
	Desmosterol	33.4 ± 10.1	38.6 ± 9.3	35.6 ± 10.8	38.9 ± 3.2
	7-DHC	41.4 ± 18.0	44.2 ± 14.3	42.4 ± 24.7	47.8 ± 20.2
	24-OHC	38.2 ± 7.8	34.1 ± 4.9	41.4 ± 9.6	35.4 ± 5.2
	27-OHC	0.033 ± 0.02	0.033 ± 0.013	0.047 ± 0.017	0.033 ± 0.014
	7-KC	0.57 ± 0.15	0.55 ± 0.20	0.45 ± 0.10	0.49 ± 0.19
	7β-OHC	0.33 ± 0.09	0.32 ± 0.14	0.26 ± 0.08	0.28 ± 0.09
	β-Sitosterol	2.3 ± 0.34	2.9 ± 0.62	2.6 ± 0.73	2.7 ± 0.50
	Campesterol	20.1 ± 5.0	24.2 ± 7.4	21.5 ± 8.4	24.8 ± 5.1
	Stigmasterol	0.25 ± 0.07	0.32 ± 0.12	0.28 ± 0.12	0.27 ± 0.07
	Brassicasterol	0.19 ± 0.07	0.25 ± 0.10	0.21 ± 0.08	0.21 ± 0.05
	Cholesterol[a]	14.5 ± 3.0	16.4 ± 2.7	15.9 ± 2.4	15.3 ± 1.9

24, 25 diH = 24, 25 dihydro lanosterol; 7-DHC = 7-dehydrocholesterol; 24-OHC = 24(S)-hydroxycholesterol; 27-OHC = 27-hydroxycholesterol; 7-KC = 7-ketocholesterol; 7β-OHC = 7β-hydroxycholesterol. [a]Values expressed as μg/mg tissue.

Appendix 7 **Absolute concentration of sterols measured in the cortex and striatum of male R6/1 and WT mice receiving dietary supplementation**. GC-MS/MS was used to analyse lipids extracted from mouse brain tissue. Mice received either control or berry extract (BE) supplemented diets. Values represent ng/mg tissue ± SD.

		Male			
		Control		BE	
		WT	R6/1	WT	R6/1
Striatum	Lathosterol	33.9 ± 7.7	12.2 ± 5.2	34.1 ± 11.9	9.7 ± 2.7
	Lanosterol	13.8 ± 4.6	7.1 ± 2.8	13.1 ± 5.9	4.5 ± 1.9
	Zymosterol	2.2 ± 0.7	1.4 ± 0.6	2.5 ± 0.6	1.1 ± 0.6
	24,25 diH	0.25 ± 0.11	0.11 ± 0.05	0.21 ± 0.01	0.13 ± 0.07
	Desmosterol	41.6 ± 5.4	40.9 ± 8.9	43.4 ± 8.1	42.2 ± 5.9
	7-DHC	49.5 ± 21.9	68.4 ± 31.4	65.5 ± 15.4	72.5 ± 16.1
	24-OHC	64.5 ± 3.3	55.5 ± 13.8	67.5 ± 5.5	58.5 ± 3.8
	27-OHC	0.078 ± 0.009	0.044 ± 0.017	0.078 ± 0.013	0.048 ± 0.010
	7-KC	0.56 ± 0.31	1.36 ± 1.1	1.04 ± 0.57	1.60 ± 0.97
	7β-OHC	0.32 ± 0.13	0.52 ± 0.20	0.59 ± 0.26	0.87 ± 0.45
	β-Sitosterol	3.8 ± 0.7	4.5 ± 0.9	4.3 ± 0.8	4.5 ± 1.2
	Campesterol	40.9 ± 7.7	39.4 ± 5.3	42.2 ± 11.9	41.5 ± 12.0
	Stigmasterol	0.48 ± 0.13	0.59 ± 0.16	0.50 ± 0.08	0.59 ± 0.18
	Brassicasterol	0.44 ± 0.09	0.42 ± 0.08	0.45 ± 0.11	0.44 ± 0.07
	Cholesterol[a]	15.5 ± 1.2	15.8 ± 1.8	16.6 ± 2.3	16.8 ± 1.6
Cortex	Lathosterol	36.6 ± 5.2	16.9 ± 3.5	27.4 ± 5.0	14.8 ± 4.4
	Lanosterol	11.8 ± 1.5	7.8 ± 1.9	10.1 ± 2.7	7.1 ± 3.1
	Zymosterol	2.0 ± 0.7	2.5 ± 1.4	1.7 ± 0.9	3.0 ± 1.3
	24,25 diH	0.22 ± 0.12	0.10 ± 0.05	0.16 ± 0.04	0.09 ± 0.03
	Desmosterol	32.0 ± 7.2	34.0 ± 5.7	39.2 ± 11.1	42.5 ± 11.2
	7-DHC	49.8 ± 17.1	53.8 ± 15.7	46.4 ± 12.0	50.3 ± 9.7
	24-OHC	53.3 ± 11.2	49.8 ± 10.9	57.7 ± 15.8	47.9 ± 8.1
	27-OHC	0.051 ±	0.031 ±	0.047 ±	0.027 ±
	7-KC	0.42 ± 0.11	0.36 ± 0.10	0.41 ± 0.14	0.37 ± 0.13
	7β-OHC	0.21 ± 0.08	0.22 ± 0.08	0.21 ± 0.07	0.19 ± 0.04
	β-Sitosterol	2.4 ± 0.58	2.4 ± 0.31	1.9 ± 0.38	2.0 ± 0.17
	Campesterol	39.7 ± 5.2	36.9 ± 8.0	35.1 ± 7.4	37.0 ± 8.8
	Stigmasterol	0.26 ± 0.13	0.26 ± 0.05	0.20 ± 0.08	0.23 ± 0.05
	Brassicasterol	0.40 ± 0.06	0.39 ± 0.21	0.38 ± 0.12	0.40 ± 0.13
	Cholesterol[a]	12.7 ± 1.8	13.0 ± 2.0	13.6 ± 1.4	13.8 ± 1.8

24, 25 diH = 24, 25 dihydro lanosterol; 7-DHC = 7-dehydrocholesterol; 24-OHC = 24(S)-hydroxycholesterol; 27-OHC = 27-hydroxycholesterol; 7-KC = 7-ketocholesterol; 7β-OHC = 7β-hydroxycholesterol. [a]Values expressed as μg/mg tissue.

Appendix 8 **Absolute concentration of sterols measured in the cortex and striatum of female R6/1 and WT mice receiving dietary supplementation**. GC-MS/MS was used to analyse lipids extracted from mouse brain tissue. Mice received either control or berry extract (BE) supplemented diets. Values represent ng/mg tissue ± SD.

		Female			
		Control		BE	
		WT	R6/1	WT	R6/1
Striatum	Lathosterol	32.3 ± 9.2	11.3 ± 3.9	32.6 ± 8.9	11.6 ± 3.4
	Lanosterol	11.8 ± 5.1	5.7 ± 2.7	11.5 ± 4.2	6.6 ± 2.7
	Zymosterol	2.4 ± 0.8	1.3 ± 0.3	2.3 ± 0.8	1.4 ± 0.4
	24,25 diH	0.23 ± 0.09	0.17 ± 0.08	0.24 ± 0.15	0.14 ± 0.01
	Desmosterol	35.3 ± 6.7	45.1 ± 6.5	38.1 ± 7.2	47.2 ± 8.4
	7-DHC	67.2 ± 29.p	80.3 ± 33.5	64.3 ± 34.1	72.3 ± 23.6
	24-OHC	61.0 ± 8.6	55.6 ± 7.7	66.8 ± 3.2	54.6 ± 11.1
	27-OHC	0.076 ± 0.014	0.055 ± 0.014	0.069 ± 0.013	0.052 ± 0.015
	7-KC	0.63 ± 0.16	0.64 ± 0.18	0.98 ± 0.50	0.64 ± 0.15
	7β-OHC	0.47 ± 0.24	0.41 ± 0.11	0.57 ± 0.42	0.38 ± 0.12
	β-Sitosterol	3.6 ± 0.9	5.0 ± 1.7	4.5 ± 0.7	4.0 ± 0.6
	Campesterol	26.0 ± 6.6	46.6 ± 7.9	36.5 ± 9.2	40.7 ± 7.6
	Stigmasterol	0.49 ± 0.19	0.71 ± 0.24	0.69 ± 0.22	0.52 ± 0.14
	Brassicasterol	0.25 ± 0.06	0.47 ± 0.12	0.37 ± 0.11	0.42 ± 0.06
	Cholesterol[a]	14.9 ± 2.2	16.6 ± 1.6	15.9 ± 2.3	16.3 ± 2.9
Cortex	Lathosterol	34.6 ± 9.7	15.6 ± 4.2	30.8 ± 13.9	14.3 ± 3.2
	Lanosterol	12.0 ± 2.8	7.0 ± 1.7	10.1 ± 4.1	6.7 ± 1.8
	Zymosterol	2.1 ± 1.3	2.6 ± 0.9	2.4 ± 1.2	2.2 ± 0.5
	24,25 diH	0.14 ± 0.07	0.09 ± 0.05	0.15 ± 0.06	0.09 ± 0.03
	Desmosterol	36.0 ± 6.3	43.4 ± 7.6	28.3 ± 8.2	45.7 ± 5.1
	7-DHC	51.8 ± 12.2	55.5 ± 20.6	33.2 ± 9.4	54.1 ± 13.9
	24-OHC	60.5 ± 9.9	50.0 ± 3.7	50.0 ± 13.1	52.9 ± 9.5
	27-OHC	0.061 ±	0.044 ±	0.054 ±	0.047 ±
	7-KC	0.29 ± 0.08	0.43 ± 0.12	0.47 ± 0.28	0.56 ± 0.35
	7β-OHC	0.15 ± 0.04	0.19 ± 0.06	0.17 ± 0.02	0.21 ± 0.06
	β-Sitosterol	2.5 ± 1.2	2.4 ± 0.64	1.8 ± 0.50	2.6 ± 0.39
	Campesterol	23.5 ± 4.4	37.1 ± 11.1	27.9 ± 9.6	32.9 ± 9.4
	Stigmasterol	0.24 ± 0.15	0.25 ± 0.12	0.22 ± 0.10	0.25 ± 0.06
	Brassicasterol	0.22 ± 0.05	0.34 ± 0.10	0.26 ± 0.09	0.33 ± 1.5
	Cholesterol[a]	12.2 ± 1.0	13.6 ± 1.7	13.1 ± 1.5	13.4 ± 1.4

24, 25 diH = 24, 25 dihydro lanosterol; 7-DHC = 7-dehydrocholesterol; 24-OHC = 24(S)-hydroxycholesterol; 27-OHC = 27-hydroxycholesterol; 7-KC = 7-ketocholesterol; 7β-OHC = 7β-hydroxycholesterol. [a]Values expressed as μg/mg tissue.

Appendix 9 **Absolute concentration of sterols measured in the cortex and striatum of combined sexes R6/1 and WT mice receiving dietary supplementation**. GC-MS/MS was used to analyse lipids extracted from mouse brain tissue. Mice received either control or berry extract (BE) supplemented diets. Values represent ng/mg tissue ± SD.

		Combined sexes			
		Control		BE	
		WT	R6/1	WT	R6/1
Striatum	Lathosterol	33.1 ± 8.2	11.8 ± 4.5	33.3 ± 10.1	10.7 ± 3.1
	Lanosterol	12.8 ± 4.7	6.4 ± 2.7	12.3 ± 4.9	5.6 ± 2.5
	Zymosterol	2.1 ± 0.9	1.3 ± 0.6	2.4 ± 0.7	1.2 ± 0.6
	24,25 diH	0.24 ± 0.09	0.14 ± 0.07	0.23 ± 0.12	0.14 ± 0.05
	Desmosterol	38.5 ± 6.8	42.8 ± 7.9	40.8 ± 7.9	44.7 ± 7.4
	7-DHC	58.3 ± 26.6	73.8 ± 31.4	64.9 ± 24.2	72.4 ± 19.2
	24-OHC	62.8 ± 6.5	55.6 ± 10.9	67.1 ± 4.3	56.5 ± 8.2
	27-OHC	0.077 ± 0.011	0.049 ± 0.016	0.073 ± 0.013	0.050 ± 0.012
	7-KC	0.60 ± 0.22	1.07 ± 0.90	1.01 ± 0.50	1.12 ± 0.82
	7β-OHC	0.39 ± 0.19	0.47 ± 0.17	0.58 ± 0.33	0.60 ± 0.39
	β-Sitosterol	3.7 ± 0.8	4.3 ± 1.8	4.4 ± 0.8	4.3 ± 0.9
	Campesterol	33.5 ± 10.3	42.7 ± 7.3	39.3 ± 10.5	41.1 ± 9.6
	Stigmasterol	0.49 ± 0.15	0.59 ± 0.27	0.59 ± 0.19	0.56 ± 0.15
	Brassicasterol	0.34 ± 0.12	0.44 ± 0.10	0.41 ± 0.11	0.43 ± 0.07
	Cholesterol[a]	15.2 ± 1.7	16.2 ± 1.7	16.2 ± 2.2	16.4 ± 2.3
Cortex	Lathosterol	35.6 ± 7.5	16.2 ± 3.7	29.1 ± 10.1	14.5 ± 3.7
	Lanosterol	11.9 ± 2.1	7.4 ± 1.7	10.2 ± 3.1	6.9 ± 2.4
	Zymosterol	2.1 ± 1.0	2.6 ± 1.1	2.1 ± 1.1	2.6 ± 1.0
	24,25 diH	0.18 ± 0.10	0.09 ± 0.05	0.16 ± 0.05	0.09 ± 0.03
	Desmosterol	35.0 ± 6.8	38.7 ± 8.1	33.7 ± 10.9	44.1 ± 8.5
	7-DHC	50.8 ± 14.2	54.6 ± 17.1	39.8 ± 12.4	52.2 ± 11.6
	24-OHC	56.9 ± 10.8	49.9 ± 7.7	53.8 ± 14.4	50.1 ± 8.7
	27-OHC	0.056 ±	0.037 ±	0.051 ±	0.037 ±
	7-KC	0.35 ± 0.12	0.39 ± 0.11	0.44 ± 0.21	0.46 ± 0.27
	7β-OHC	0.18 ± 0.07	0.20 ± 0.07	0.19 ± 0.06	0.20 ± 0.05
	β-Sitosterol	2.4 ± 0.93	2.5 ± 0.48	1.9 ± 0.42	2.3 ± 0.42
	Campesterol	31.6 ± 9.6	36.9 ± 9.2	31.4 ± 9.0	36.0 ± 8.8
	Stigmasterol	0.25 ± 0.13	0.26 ± 0.09	0.21 ± 0.09	0.24 ± 0.06
	Brassicasterol	0.31 ± 0.11	0.37 ± 0.16	0.32 ± 0.12	0.36 ± 0.14
	Cholesterol[a]	12.5 ± 1.4	13.3 ± 1.8	13.4 ± 1.5	13.6 ± 1.2

24, 25 diH = 24, 25 dihydro lanosterol; 7-DHC = 7-dehydrocholesterol; 24-OHC = 24(S)-hydroxycholesterol; 27-OHC = 27-hydroxycholesterol; 7-KC = 7-ketocholesterol; 7β-OHC = 7β-hydroxycholesterol. [a]Values expressed as μg/mg tissue.

Appendix 10 **Absolute concentration of sterols in human post-mortem brain tissue.** GC-MS/MS was used to analyse lipids extracted from brain tissue. Values represent ng/mg tissue ± SEM.

	Brain region					
	Putamen		Caudate		Grey frontal cortex	
	Control	HD	Control	HD	Control	HD
Lathosterol	4.35 ± 1.48	8.46 ± 5.70	3.62 ± 1.55	3.45 ± 2.50	9.64 ± 4.65	11.72 ± 8.89
Lanosterol	0.89 ± 0.37	1.33 ± 0.81	0.72 ± 0.31	0.64 ± 0.44	1.81 ± 1.02	1.66 ± 0.99
Zymosterol	0.11 ± 0.06	0.35 ± 0.22	0.12 ± 0.04	0.11 ± 0.05	0.56 ± 0.24	0.4 0± 0.23
24,25 diH	0.19±0.07	0.37±0.18	0.18±0.06	0.22±0.10	0.42±0.25	0.61±0.33
Desmosterol	4.66 ± 2.77	13.68 ± 9.25	3.93 ± 1.82	6.71 ± 4.54	5.19 ± 1.61	5.80 ± 4.20
7-DHC	19.68 ± 5.01	25.8 ± 11.62	19.77 ± 6.68	16.26 ± 6.04	33.46 ± 12.31	39.97 ± 19.52
24-OHC	33.00 ± 10.01	13.67 ± 6.22	25.54 ± 13.72	8.78 ± 4.62	15.25 ± 4.27	11.98 ± 3.73
27-OHC	0.30 ± 0.09	0.87 ± 0.59	0.23 ± 0.21	0.41 ± 0.34	0.22 ± 0.14	0.65 ± 0.50
7-KC	0.75 ± 0.24	1.28 ± 0.41	0.59 ± 0.22	0.74 ± 0.41	0.70 ± 0.28	1.01 ± 0.48
7β-OHC	0.61 ± 0.21	0.94 ± 0.42	0.46 ± 0.17	0.61 ± 0.41	0.45 ± 0.20	0.68 ± 0.38
Squalene	1.81 ± 1.24	1.73 ± 0.99	1.53 ± 0.86	1.41 ± 0.47	1.39 ± 0.38	1.62 ± 0.65
Campesterol	4.5 ± 2.1	5.5 ± 3.0	3.8 ± 2.0	2.6 ± 2.1	4.3 ± 2.9	2.8 ± 1.2
Cholesterol[a]	16.57 ± 4.91	21.72 ± 6.38	11.61 ± 5.21	8.52 ± 2.85	14.63 ± 5.31	14.41 ± 5.08

	Brain region			
	White frontal cortex		Cerebellum	
	Control	HD	Control	HD
Lathosterol	4.35 ± 1.48	8.46 ± 5.70	3.62 ± 1.55	3.45 ± 2.50
Lanosterol	0.89 ± 0.37	1.33 ± 0.81	0.72 ± 0.31	0.64 ± 0.44
Zymosterol	0.11 ± 0.06	0.35 ± 0.22	0.12 ± 0.04	0.11 ± 0.05
24,25 diH	0.19 ± 0.07	0.37 ± 0.18	0.18 ± 0.06	0.22 ± 0.10
Desmosterol	4.66 ± 2.77	13.68 ± 9.25	3.93 ± 1.82	6.71 ± 4.54
7-DHC	19.68 ± 5.01	25.80 ± 11.62	19.77 ± 6.68	16.26 ± 6.04
24-OHC	33.00 ± 10.01	13.67 ± 6.22	25.54 ± 13.72	8.78 ± 4.62
27-OHC	0.30 ± 0.09	0.87 ± 0.59	0.23 ± 0.21	0.41 ± 0.34
7-KC	0.75 ± 0.24	1.28 ± 0.41	0.59 ± 0.22	0.74 ± 0.41
7β-OHC	0.61 ± 0.21	0.94 ± 0.42	0.46 ± 0.17	0.61 ± 0.41
Squalene	1.78 ± 1.12	1.92 ± 0.71	1.42 ± 1.07	1.79 ± 1.34
Campesterol	10.0 ± 6.6	6.1 ± 4.9	7.9 ± 5.0	6.5 ± 4.7
Cholesterol[a]	16.57 ± 4.91	21.72 ± 6.38	11.61 ± 5.21	8.52 ± 2.85

24, 25 diH = 24, 25 dihydro lanosterol; 7-DHC = 7-dehydrocholesterol; 24-OHC = 24(S)-hydroxycholesterol; 27-OHC = 27-hydroxycholesterol; 7-KC = 7-ketocholesterol; 7β-OHC = 7β-hydroxycholesterol. [a]Values expressed as μg/mg tissue.