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Doctorate in Biology

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Curcumin: a natural approach in Neurodegenerative Diseases

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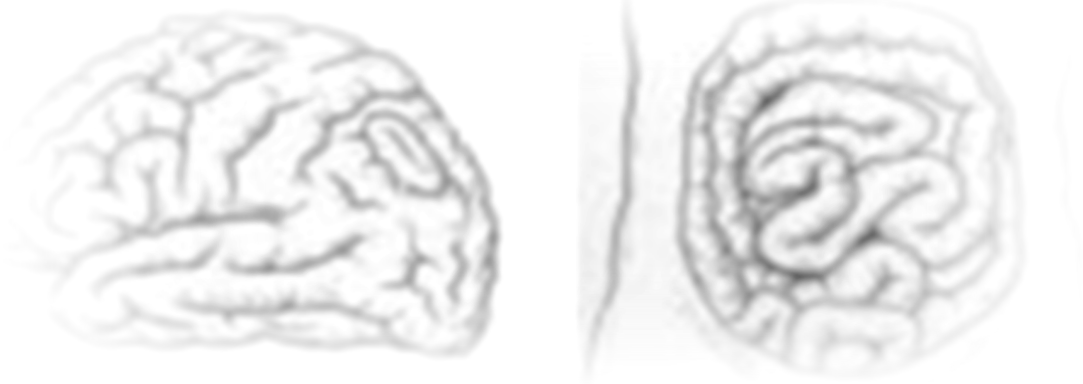

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Outline of the thesis

This PhD thesis reports the results of a research project that is the result of a collaboration between the laboratory of Dr. Alba Di Pardo of the Neurogenetics laboratory at Neuromed Institute of Pozzilli, Prof. Alessandra Pollice at the Department of Biology of the Federico II University of Naples, and the laboratory of Prof. Euan R. Brown at the Heriot-Watt University of Edinburgh.

The project aimed at investigating the potential beneficial effects of a lifelong dietary curcumin treatment both on neurological and peripheral symptoms in a mouse model of Huntington's disease (R6/2 model). During my PhD my attention was primarily focused on evaluating the effects of the nutraceutical curcumin on the peripheral symptoms of Huntington's disease such as the involuntary weight loss and gastrointestinal dysfunctions.

Further, I evaluated if dopaminergic neurons differentiated from human iPSC-derived neural progenitor cells (hNPC) are able to reflect the complexity of excitotoxicity phenomenon, in order to development a human non-immortalized *in vitro* system that can reflect this kind of neuronal death presents in neurodegenerative diseases and test new drugs to counteract it.

The thesis is organized into five Chapters and an Appendix.

Chapter 1 is a general Introduction on neurodegenerative diseases with particular attention to Huntington's disease, its epidemiology, pattern of inheritance, the molecular genetics of the disease and the structure and function of the mutated form of the protein. Particular emphasis is given to the description of highly invalidating non-neurological symptoms of HD such as involuntary weight loss and alterations of the gastrointestinal tract. The biological properties of curcumin are also analyzed, underlying its beneficial effect in neurodegenerative diseases and on alterations of the gastrointestinal tract.

Chapter 2 is the aim of the study. In particular, it is clarified that my work was especially focused:

-on the effects that curcumin exerts on the peripheral symptoms of the R6/2 HD mouse model

-on studies of excitotoxicity (carried out in the laboratory of Prof Euan R. Brown) in neurons derived from human induced pluripotent stem cells.

Chapter 3 reports the published results of the main research project. It is shown that curcumin treatment significantly mitigates disease progression exerting a neuroprotective effect and preserving body weight as well as the intestinal homeostasis. In particular, in R6/2

mice treated with curcumin, motor performance was preserved, as well as brain weight. At the molecular level, curcumin reduced mutant huntingtin aggregates, triggers activation of pro-survival pathways and increases BDNF neurotrophin protein levels in the brain. Further, curcumin treatment also increased food intake, prevented weight loss typical of sick mice and showed an intestinal protective effect preserving both intestinal motility and the anatomical and histological morphology. At the molecular level, there is an increase in the expression levels of small intestine barrier genes. The results have been included in the publication "*Curcumin dietary supplementation ameliorates disease phenotype in an animal model of Huntington's disease" Elifani F, Amico E, Pepe G, Capocci L, Castaldo S, Rosa P, Montano E, Pollice A, Madonna M, Filosa S, Calogero A, Maglione V, Crispi S, Di Pardo A., published on Human Molecular Genetics in 2019. 28(23):4012-4021. doi: 10.1093/hmg/ddz247.*

In addition, this chapter describes unpublished experiments aimed at further investigating mechanisms of intestinal motility. In particular, it was analyzed the level of expression of colon contractility markers in the colon of R6/2 HD mice treated or not with curcumin. Level of expression of important regulators of smooth muscle contraction (Smoothelin-A and Caldesmon, Thin-Filament Associated Proteins and Myocardin, a master regulator of smooth muscle) was analyzed.

Chapter 4 concerns the analysis of a particular type of neuronal death, excitotoxicity, involved in neurodegenerative diseases. This phenomenon was explored in an *in vitro* system of neurons differentiated from hNPC. The aim was to evaluate whether this cellular system can be used to test new drugs in order to overcome neuronal cell death.

Chapter 5 provides a General Conclusion of the overall obtained results.

Finally, in the **APPENDIX** of the thesis, there is an unrelated study on the effects that a solution of Colloidal Silver exerts on Human Keratinocytes (*HaCat cells*). In this study it was analyzed the effect of a solution of colloidal silver nanoparticles of 0.62 nm in size, smaller than ever previously tested. Upon treatment, cells underwent a cytoskeletal rearrangement both at the cell–cell and cell–substrate adhesions with changes in E-cadherin cellular distribution that results in an increase in the wound closure speed. The results have been included in the publication *"Colloidal Silver Induces Cytoskeleton Reorganization and E-Cadherin Recruitment at Cell-Cell Contacts in HaCaT Cells." Montano E, Vivo M, Guarino AM, di Martino O, Di Luccia B, Calabrò V, Caserta S, Pollice A., published on Pharmaceuticals in 2019. 12(2), 72.* doi: 10.3390/ph12020072.

Chapter 1

Introduction

Neurodegenerative diseases include a group of diseases (such as Parkinson's, Alzheimer's, Huntington's disease, multiple sclerosis) characterized by neuronal death in different areas of the central nervous system (CNS). They have a typically late onset whose common feature is the accumulation and aggregation of misfolded disease-specific proteins. Interestingly, symptoms related to alterations of the central nervous system are only one part of the story, being all these pathologies characterized by more systemic manifestations. At present, since these diseases are chronic, progressive and up to now, incurable, they are considered critical medical and social problems. Among these there is Huntington's disease (HD), which is the main object of study in this thesis. It is mainly characterized by the selective degeneration of the medium spiny neurons and, clinically, it is characterized by choreic movements, behavioral and psychiatric alterations and by a variety of non-neurological symptoms, attributable to the impairment of peripheral organs and tissues. HD is a highly debilitating disease and affected patients experience many daily difficulties that progressively turn into the inability to carry out even the most banal daily actions, affecting all aspects of life.

General knowledge and Historical background of HD

HD is a rare genetic neurodegenerative disorder, caused by an abnormal expansion of the cytosine-adenine-guanine (CAG) triplet in the huntingtin gene (HTT). The physiological function of this gene was longlasting unknown as most of the attention was initially put on its mutated form that produces a protein with toxic effects.

The disease has a classical late onset, on overage around 40 years (Ross and Tabrizi 2011); it is usually fatal 15-20 years after disease onset ((G. P. Bates et al. 1998) (Craufurd 1996)) and, up to now, no cure is available. HD is strongly debilitating for patients that typically present choreic movements resembling a dance that gave the name of Huntington Chorea (from Greek, chorea=dance) to the pathology. However, since some HD patients do not show choreic movements, it is now more appropriately defined as "Huntington disease", where the name "Huntington" identifies the American clinician who first described the disease with his article *"On Chorea", in 1872*. Huntington described the disease as follows: "the disease commonly begins by slight twitching in the muscles of the face, which gradually increases

in violence and variety. The eyelids are kept winking, the brows are corrugated, and then elevated, the nose is screwed first to the one side and then to the other, and the mouth is drawn in various directions, giving the patient the most ludicrous appearance imaginable. The upper extremities may be the first affected, or both simultaneously… As the disease progresses the mind becomes more or less impaired, in many amounting to insanity, while in others mind and body gradually fail until death relieves them of their suffering (Huntington 2003) (Lo and Hughes 2011)

With these words, George Huntington was able to accurately describe the clinical aspects of the disease, defining its characteristic motor, cognitive and behavioral disorders, emphasizing its social impact and its fatal course. Moreover, he also described the typical onset in adulthood of HD, its progressive nature and even highlighted the inheritance of an autosomal dominant type of disease. Initially, the diagnosis was only based on a careful description of the symptoms while, to date, it is also based on genetic testing since the cause of the disease was identified at the molecular level: in 1983, the huntingtin gene (HTT) was mapped to the short arm of human chromosome 4 (Gusella et al. 1983), and ten years later, in 1993, sequencing of the gene revealed an abnormal expansion of the trinucleotide cytosine-adenine-guanine (CAG), coding for glutamine (Q), occurring within the coding region of the HTT gene (The Huntington's Disease Collaborative Research Group, 1993); this causes the formation of a long polyglutamine-stretch at the amino-terminal portion of the protein, which is responsible for protein misfolding and of the formation of aggregates conferring toxicity and leading to dysfunction and death of neurons, particularly of medium spiny neurons in the striatum (Han et al. 2010). Actually, precise mechanisms of cell death occurring in HD are not yet completely clear. Some role could be played also by excitotoxicity, a particular type of neuronal death induced by hyperstimulation of receptors occurring in many neurodegenerative diseases.

Epidemiology

HD is a rare disease affecting less than 5 individuals per 10.000 in the general population. Despite being globally rare, HD patients are not few and longitudinal analysis shows a consistent increase in the prevalence of the disease in recent decades (Morrison 2012). This increase is likely to continue during next years due to the increase in longevity of the world population. Moreover, considerable differences in the frequencies of the disease are detected in different populations. The highest frequency of HD is found in European populations,

with a prevalence of 10.6-13.7 individuals per 100 000 in Western populations (Fisher and Hayden 2014), (Morrison, Harding-Lester, and Bradley 2011), (Evans et al. 2013), (Bates et al. 2015) while Asiatic populations have a much lower frequency of HD with a prevalence of 1–7 per million (McColgan and Tabrizi 2018). Moreover, there are some exceptions to a baseline mutation rate, such as Venezuela, where it is registered the highest frequency of affected people worldwide with a prevalence of 700/100,000 individuals (Ghosh and Tabrizi 2018a) due to the founder phenomena effect.

The prevalence of HD is certainly underestimated in some areas of the world, due to the absence of competent clinicians and to the possibility of performing genetic tests. However, beyond the diagnostic differences that can amplify differences in the frequency of illness, one of the reasons that could explain the different rates is the length of the trinucleotide CAG in the HTT gene among different ethnic groups. Multiple local haplotyping studies have shown that the differences in the prevalence of HD in different populations correspond to the CAG length in the exon 1 of HTT gene. For example, a significantly lower mean CAG size was observed in black South Africans, Chinese, Japanese, and Finnish compared to consistently higher averages across a European sample ((Kay, Hayden, and Leavitt 2017)). Indeed, haplotype of European ancestors has an average of 18.4–18.7 CAG repeats, while Asian ones have an average of 16.9–17.4 (Bates et al. 2015), (McColgan and Tabrizi 2018). It's believed that Europeans already had a haplotype with a greater number of CAGs and that the higher number of CAGs encourages further expansion: it means that the European population will maintain the highest chances of mutation.

Inheritance pattern

HD has an autosomal dominant inheritance; therefore, only one mutated allele of the gene is necessary to manifest the disease.

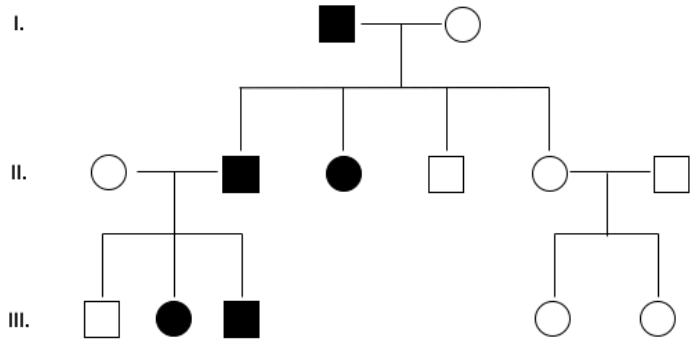

Figure 1. *Schematic representation of a pedigree showing a classical* autosomal dominant *inheritance of HD*

Although heterozygosity is the most frequent condition, cases of homozygosity are not so uncommon, especially in areas like Venezuela, where the largest part of the population is affected by HD and coupling between HD patients is frequent.

Homozygosity does not affect the age of onset of the disease, but disease progression is more rapid and can be more severe (Lee et al. 2012) (Squitieri et al. 2003). Furthermore, homozygosity is associated with certain transmission of the mutation to the offspring (Squitieri et al. 2003)

Huntington disease at the molecular level

The CAG-triplet in exon 1 of the HD gene constitutes a polymorphic site in the population since there are many different alleles with a different number of CAG-repetitions in normal human population. It has been observed that there is a pathological threshold of repetitions at 35 repeats. When the CAG-triplet exceeds the critical length, HD manifests. However people in which the number of CAG repeats goes from 27 to 35 falls in the so called "grey zone" (see Table 1) including persons that will not manifest HD, but with a risk of HD in their offspring due to the high instability of CAG repeats at the meiotic level. Persons with a number of CAG repeats higher than 35 must be further divided between those who have a high chance of developing the disease and those who certainly will manifest it. In particular, the range of CAG repeats 36- 39, include people with a low penetrance of the disease, meaning that it may occur and, if it does, it usually appears at old age (Rubinsztein et al. 1996). In the second group, there are those with over 39 repeated units of CAG: in this case there is a fully penetrant disease that will certainly develop and appear earlier and in more severe form.

Several studies have shown that there is a strong inverse correlation between the age of onset and the severity of HD compared to the length of CAG-repeats (Rubinsztein et al. 1993). HD usually appears around the age of 40 (Ross and Tabrizi 2011) but when the number of triplets exceeds 55 units, HD manifests earlier, before the age of 20 and it is called “juvenile form of Huntington’s disease (JHD)”.

Although the length of the CAG repeats is the main factor determining the age of onset of HD, (representing about 70% of the variance of the age of onset) (Andrew et al. 1993) (Hannan 2004), environmental factors and genetic background can also affect the age of onset of HD (Schulte and Littleton 2011) (Nancy S. Wexler et al. 2004).

The length of the CAG stretches is subjected to a non-Mendelian inheritance during vertical transmission since the number of triplets varies between generations, meaning that even the alleles of the offspring are not identical to those of the parents. Furthermore, once the CAG-repeat sequence reaches or exceeds the length of 27 units, it becomes highly unstable both at mitotic (Telenius et al. 1994) and meiotic level. Therefore, lengthening of the repeated sequence can occur both in somatic and in germinal cells (Wheeler et al. 2017). At the somatic level, CAG repeat instability varies between and within tissues, while at the germinal level, it can occur both in males and in females, with a a prevalence in males.

Indeed, the sick offspring from an affected father shows a considerable increase in the number of CAGs, while the sick offspring from a mother with HD tends to increase the number of CAGs only by a few units, with a shift range of 1-3 repeats (Richard H. Myers 2004) . So, a considerable CAG expansion occurs when the gene is passed down the paternal line. This aspect, together with the fact that a higher number of CAG repeats correlates with an earlier onset and with more severe symptoms (Duyao et al. 1993) (Stine et al. 1993) explains the phenomenon of anticipation (McInnis 1996) in HD, for which there is an even earlier onset of HD from generation to generation, during paternal transmission (Ranen et al. 1995).

The phenomena of instability also underlie the onset of *de novo* or sporadic cases of HD, which means that for the first time the disease appears in a pedigree. These sporadic cases of HD are mostly due to expansion of the gray area alleles. The percentage of these cases is estimated about 10% (Warby et al. 2009). Theoretically, the size of CAG expansion may also decrease during vertical transmission, but this is a very rare event (Margolis and Ross 2003)

Table 1

CAG repeat size	Definition	Description
<27	Normal range	Healthy people
27 to 35	Grey area	Do not cause HD but provides for high instability
36 to 39	Incomplete Penetrance	Risk of manifesting the disease at old age
>39	Full Penetrance	Certainty of manifesting HD

Table 1. *Correlation between size of CAG repeat and risk of Huntington disease.*

In humans, the HD gene is located on chromosome 4 in the region p16.3. It contains 67 exons spanning 180 kb (NCBI). Aside the polymorphic site in exon 1 (see above, i.e the stretch of CAG repeats) there are two other polymorphic sites: the first one is represented by a repetition of the CCG sequence (6-12 repetitions) located downstream of the repeated CAG sequence. When the polyQ tract is mutated, the number of CCG-triplets is usually around 7 repeats (Andrew et al. 1994). Lin et al. 1995); the second polymorphic site, found in exon

58 concerns the presence or absence of a stop codon (GAG) in position 2642–2645 due a deletion. This is more frequent in mutated Htt (38% vs 7% in wild-type alleles). Interestingly, its presence is associated with a significant decrease in the age at onset (Vuillaume et al. 1998)

Regarding HTT transcripts, there are three main isoforms due to the presence of three different polyadenylation sites. The two most abundant isoforms result by alternative polyadenylation in the 3′ untranslated region (3′UTR). The first one is 13.7 kb long and is more abundant in non-dividing cells like neuronal cells, whereas the second one is 10.3 kb long and predominates in dividing cells; therefore, it is more abundant in tissues like liver and muscle (Lin et al. 1993) (Romo et al. 2017). The third one is a truncated isoform, 7.9 kb long, resulting by the presence of a polyadenylation site in the first intron (Sathasivam et al. 2013); it has the longest half-life maybe due to its polyA longer tail compared to the other two isoforms (Romo et al. 2017).

The abundance of HTT transcriptional isoforms changes in HD (Romo et al. 2017). For example, in HD motor cortex there is an increase in the 7.9 kb mRNA isoform, which may contribute to the toxicity observed in the disease. Infact, transfection of this short mRNA isoform (7.9 kb) gives rise to more aggregates compared to transfection with the long HTT isoform (Xu, An, and Xu 2017), confirming toxicity.

In addition to alternative polyadenylation, HTT transcripts also undergo alternative splicing. Twenty-two mRNA splice variants have been identified in human brain (Ruzo et al. 2015)

It's interesting to note that several studies claim that RNA isoforms could have toxic effect due to the high number of CAG repetitions which entails different secondary structure (de Mezer et al. 2011)

Regardless of the possible intrinsic toxicity of the RNA, recent experimental approaches aim to hit mutant HTT mRNA to avoid its translation into a mutated protein (Romo, Mohn, and Aronin 2018).

Structural features of the Huntingtin Protein

The Huntingtin is a very large protein, with a molecular weight of 348 kDa. Despite its size, Htt is a completely soluble protein and its main localization is in the cytoplasm, although it also has a widespread subcellular localization including the nucleus, mitochondria, endoplasmic reticulum, Golgi complex, and endosomes (DiFiglia et al. 1995; Hoffner at al. 2002). Furthermore, it is ubiquitously expressed in human tissues (Strong et al. 1993),

particularly in neurons and testes (Cattaneo, Zuccato, and Tartari 2005).

Most of the known protein's structure information are on its amino-terminal portion, (which includes the polyQ stretch) since this mutated region is alone necessary and sufficient to generate the disease phenotype (DiFiglia et al. 1995). This region contains many interesting sites: its first seventeen amino acids, define the so-called N17 trait that is highly conserved among vertebrates (Tartari et al., 2008); its secondary structure is an alpha helix motif (Atwal et al., 2007; Kim et al., 2009) fundamental for the interaction of Htt with cellular membrane (Kegel et al. 2005) further, four hydrophobic amino acids (leucine 4, 7, 14, and phenylalanine 11) constitute a nuclear export signal (NES) (Zheng et al. 2013). Therefore, it is clear that the N17 portion plays an important role in the sub-cellular localization of the Htt protein. This trait is subjected to many post-translational modifications like acetylation, sumoylation, ubiquitination that affect the three-dimensional structure of the protein and some its properties. For example, phosphorylation of serine 13 and 16 plays an inhibitor effect on aggregation and toxicity of mutated Htt (Gu et al. 2009).

After the N17 domain there is the polyQ stretch, the main protagonist of the Htt protein. Interestingly, polyQ trait is not always found during evolution: for example, Drosophila melanogaster does't present glutamines in this region, whereas vertebrates have at least four glutamines and the number varies among species. The longest polyQ tract is in humans (Tartari et al. 2007)

The polyQ stretch is followed by another polymorphic site in humans: the polyproline (polyP) stretch. It is believed that it may be important for stabilizing the structure of the polyQ stretch and to maintain solubility of the Htt (Ignatova and Gierasch 2006).

Interestingly, polyP decreases aggregate formation (Darnell et al. 2007). Indeed, *in vitro* studies demonstrated that with the addition of a polyP stretch of at least six prolines downstream of a polyQ, there is an alteration of the aggregation kinetics (Bhattacharyya et al. 2006).

So polyQ's flanking sequences can modulate the aggregation of poly(Q) and Htt localization, stability and cytotoxicity.

Inside the protein, there are multiple 40-aminoacid-long HEAT repeats which are structured in superhelix with a hydrophobic core. These repeats are involved in the interactions with other proteins and also mediate the intra-molecular interactions (Palidwor et al. 2009).

Further, along the Htt protein, there are several proteolytic sites (for caspases, calpain, cathepsins, MMP10) mainly localized in disordered regions, (Warby et al. 2008) (Tebbenkamp et al. 2012) (Kim et al. 2006). These sites allow the generation of Htt

fragments of different lengths that move in the nucleus, tend to generate aggregates and promote cellular death. It is essential to highlight that both wild-type and mutated Htt include the same proteolytic sites implying that also the wild-type form of the protein generates toxic fragments. However, only patients affected by HD show aggregates because the kinetic of the proteolytic process between the two different forms of Htt is different: when HTT has an abnormal expansion of the polyQ trait there is an increase in the kinetics of the reaction and the proteolytic process is enhanced. It means that the number of resulting fragments is increased and results in the formation of oligomers, fibrils, and in characteristic aggregates. Instead, it seems that the fragmentation of the wild-type protein occurs only when the cell must go to cellular death, being the proteolytic mechanism in "normal" cases very well regulated. In any case it is clear that the proteolytic process plays a key role in the pathogenesis of HD as it is highlighted by the fact that modifications in the activity of proteolytic enzymes reduce the cytotoxicity and delay disease progression (Wellington et al. 2000) (Gafni et al. 2004) (Ona et al. 1999).

At last, at the carboxy-terminal of Htt protein, in in position 2397–2406, there is another nuclear export signal (Xia et al. 2003). The presence of these signals highlights that this protein can translocate from the cytoplasm to the nucleus and vice versa. The nuclear localization, which mainly affects the fragments resulting from the proteolytic process, confers greater toxicity.

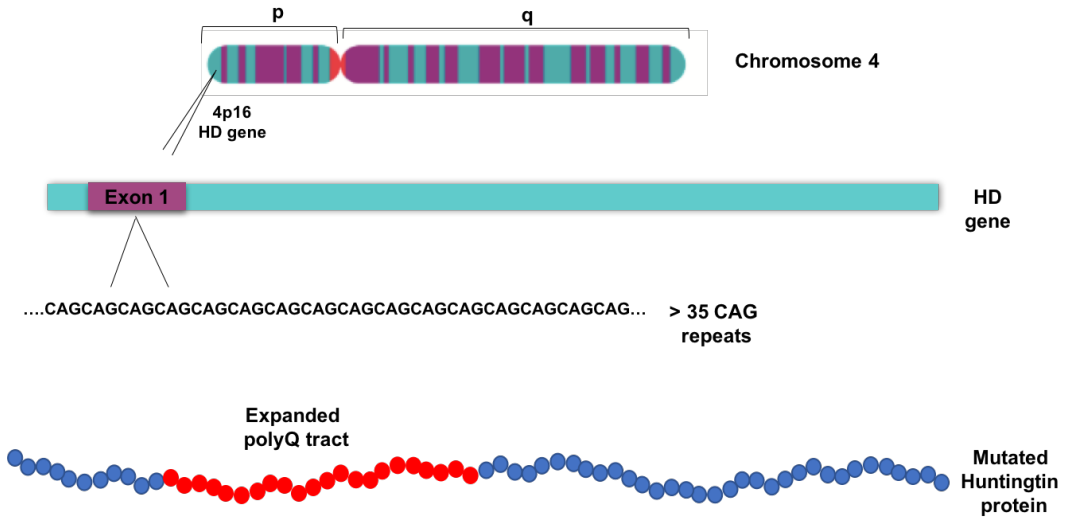

Figure 2. *Representation of the HD gene containing the abnormal expansion of the CAG which is translated into an abnormal polyQ tract of the Huntingtin protein.*

Functions of Huntingtin

As mentioned, Htt is a widely distributed protein but, so far, little is known about its functions in the different cellular compartments as well as in different tissues. However, numerous studies have investigated about its possible roles.

-Huntingtin in embryonic development

Certainly, Htt has an important role in embryonic development. This was clear when Zeitlin et al. tried to generate a nullizigote mice model for HTT gene ($Hdh^{-/-}$) that died between day 8.5 and 10.5, interestingly, prior to nervous system development. Furthermore, in the same study, they showed that mice heterozygous for the HTT gene did not die during embryogenesis and were phenotypically indistinguishable from control mice, demonstrating that a single HTT copy is sufficient to ensure embryogenic development in mice and that Htt is essential for embryonic development (Zeitlin et al. 1995). Further, a mouse model expressing less than 50% of the wild-type Htt, shows problems in the development of the nervous system with defects in precursor of the epiblast and malformations in the cortex and striatum and die after birth (White et al., 1997). Some years later, it was shown that Htt inactivation in embryonic mice causes premature differentiation of cortical progenitors due to a defect in the orientation of the mitotic spindle of the dividing progenitors (Godin et al., 2010). Interestingly, it has been hypothesized that the importance of the Htt protein during embryogenesis, in addition to neurogenesis, is also due to its presence in extra-embryonic tissues where it participates in nutritional functions (Dragatsis, Efstratiadis, and Zeitlin 1998). This function of Htt is crucial and suggests that HD may be retained a disease of development.

Interestingly, also when mutated, Htt appears important during development. Indeed, its expression in $Hdh^{-/-}$ mice rescued from embryonic death (B. R. Leavitt et al. 2001). Moreover, in humans, cases with the mutation in homozygosity do no show developmental defects (N. S. Wexler et al. 1987) (R H Myers et al. 1989).

-Anti-apoptotic function of huntingtin

Interestingly, a striatal cell line (ST14A) stably overexpressing wild-type Htt is protected from death under different pro-apoptotic conditions (Rigamonti et al. 2000).

In addition, wild-type Htt shows anti-apoptotic functions in primary neuronal culture and in mouse models where it shows neuroprotection against NMDA receptors-mediated apoptosis (B. R. Leavitt et al. 2006).

Anti-apoptotic activity of Htt was also supported by the evidence that Htt silenced cells are more sensitive to apoptosis compared to control cells (Zhang et al. 2006). Furthermore, neuronal inactivation of the HTT gene in the adult mouse induces apoptosis in cells of the hippocampus, cortex and striatum (Dragatsis, Dietrich, and Zeitlin 2000).
From a molecular point of view, wild-type Htt directly acts in the apoptotic pathway blocking the activation of caspase-3 (Zhang et al. 2003) (Zhang et al. 2006).
Conversely, the overexpression of mutated Htt increases the cellular death level under apoptotic-stimuli. Therefore, the mutated form loses the anti-apoptotic function and, in addition, gains toxicity.

-Huntingtin in transcriptional regulation

Thanks to its polyQ trait and HEAT domains, Htt interacts with numerous transcriptional factors or co-activators, such as the cAMP response element-binding protein (CREB) (Steffan et al. 2000) or the nuclear factor-kB (NF-kB) (Takano and Gusella 2002) and the tumor suppressor protein 53 (p53) (Steffan et al. 2000), Htt also binds to REST (R element-1 silencing transcription factor) (Zuccato et al. 2003). This interaction is very important in the nervous system, since, when Htt binds to REST, sequesters it at the cytoplasmic level thus preventing its nuclear binding to NRSF DNA sequence and allowing transcription of neuronal survival factors like, for example, the brain-derived neurotrophic factor (BDNF).
The mutated form of Htt is perturbed in the interaction with several transcription factors, thus producing a widespread transcriptional dysfunction in HD, both in animal model and in patients (R. Luthi-Carter et al. 2000) (Cha 2007). Indeed, in HD, all those genes controlled by the factors mentioned above are transcriptionally altered; for example, target genes of REST/NRSF such as the well-known BDNF, are down-regulated since REST is able to move into the nucleus and bind to NRSF.
In addition, transcriptional alterations in HD could also be explained by chromatin modification since the mutated form of Htt can interact with acetyltransferase/deacetyltransferase and indirectly induce chromatic modification. For example, it interacts with CBP, a transcriptional co-activator and acetyltransferase (McManus and Hendzel 2001), inducing histone hypoacetylation and down-regulation of the expression of genes regulated by CBP-mediated acetylation.

-Huntingtin in cellular trafficking

Htt is a positive regulator of vesicular and organelles trafficking within the cells. It controls

their trafficking both along the microtubule and the actin cytoskeletons by increasing the velocity and controlling both retrograde or anterograde transport. In this way, Htt plays a key role in axonal transport; indeed, among the organelles whose transport is regulated by Htt there are synaptic precursor vesicles (Zala, Hinckelmann, and Saudou 2013) containing BDNF, (Gauthier et al. 2004) but also autophagosomes (Wong and Holzbaur 2014), endosomes and lysosomes (Caviston et al. 2011) (Liot et al. 2013).

Htt performs this function thanks to some of the proteins with which it interacts, that are microtubule motor proteins such as dynein, kinesin and dynactin or proteins linked to actin-based motors like optineurin. In details, Htt is able to regulates bidirectional transport interacting, through Huntingtin-associated protein 1 (HAP1), with the dynactin and the kinesin, responsabile respectively for the retrograde transport and for the anterograde transport (Caviston et al. 2007) (Engelender et al. 1997) (Gauthier et al. 2004) (Li et al. 1998) (Strehlow, Li, and Myers 2007) (Twelvetrees et al. 2010).

Phosphorylation at S421 of Htt (Colin et al. 2008) is the most important modification for promoting anterograde transport; when Htt is not phosphorylated at this site, retrograde transport is preferred.

When Htt is mutated, its interaction with HAP1 reduces the latter's ability to associate with dynactin and kinesin (Gauthier et al. 2004). Thus, the cellular trafficking is slowed down and the directionality of the transport is compromised. This partially explains the reason of reduction of brain-derived neurotrophic factor (BDNF) level in the striatum of HD patients and animal model. As BDNF is expressed in the cortex and not at striatal level, its presence in striatal neurons is due to an anterograde transport which is able to move neurotrophin from the cortex to the striatum.

Clinical features

From a clinical point of view, HD is mainly characterized by motor, cognitive, and psychiatric dysfunctions. Before the symptoms of the disease become clear, patients encounter a "prodromal" phase of the disease for many years, during which subtle motor signs (e.g. difficulties in fine movements of the fingers, eye movements abnormalities), as well as slight cognitive and/or behavioral symptoms may be present and unnoticed by patients themselves (Ross and Tabrizi 2011).
Conventionally, the disease is considered manifest only when the typical motor dysfunctions clearly occur (Ghosh and Tabrizi 2018b).

Motor dysfunctions

Motor dysfunctions are undoubtedly the most characteristic of HD. They include the chorea, that comprises involuntary, purposeless, non-rhythmic rapid movements. The pattern of these movements varies: initially, only small movements of the distal part of the limbs and face are seen, such as tics which patients may hide incorporating these into their natural voluntary movements. These, little by little, increase in amplitude and amount spreading to the proximal part of the limbs, the trunk and the neck. Activation of facial and neck muscles can cause eye closure, tongue protrusion and head-turning whereas the involvement of trunk can lead to hyperextension (until arching) of the back. More severe chorea diffused to whole-body muscles causes speech and swallowing problems due to the involvement of laryngeal muscle group. Other motor features include dystonia, myoclonus, tics and restlessness.
As the disease progresses, bradykinesia (slowness of movements), akinesia (delay in initiation of movements) and rigidity dominate the clinical picture making the gait almost impossible and forcing the patient to a wheelchair.
Thus, generally, motor symptoms can be divided into two groups: hyperkinetic and hypokinetic movements. The first one are mainly seen in the early phases of the disease while the second ones are more common in the advanced stage of disease.
They can be interpreted as the effect of the progressive dysfunction of the complex functional neuronal circuits that integrates the motor cortex and deep grey matter nuclei via direct (positive) and indirect (negative) pathways. The direct pathway represents a positive loop reinforcing voluntary movements initiation, while activation of the indirect loop results in negative feedback limiting voluntary movements.

Loss of medium spiny neurons in the striatum (the most affected brain region in HD) (Vonsattel, Keller, and Cortes Ramirez 2011) during the early phases of the disease compromises the functionality of the indirect pathway, increasing the contribution of the direct one and resulting in hyperkinetic movements like chorea by excitatory input to the motor cortex. In the advanced phases of the disease, the striatal projection neurons in the direct loop are also lost, resulting in the development of hypokinetic status.

Cognitive dysfunctions

Cognitive impairment is a typical feature of HD and, although its degree varies among patients, it very often progresses to dementia. Cognitive dysfunction usually leads to loss of concentration, loss of organizational skills, reduced ability to make decisions and to plan. Interestingly, it also leads to an inability of affected individuals to perceive symptoms related to their disease (Hoth et al., 2007). Therefore, patients with HD often deny having any kind of disorder. This unawareness is another typical sign of impaired frontal lobe function. Subtle cognitive problems (e.g. visuomotor and spatial integration deficit) may be present many years earlier than characteristic motor signs appear (Tabrizi et al. 2013).

There is some overlap between cognitive and psychiatric symptoms in terms of disinhibited behaviors with lack of insight (anosognosia) (Duff et al. 2010).

Psychiatric dysfunctions

Behavioral problems in HD can strongly affect not only the patient himself, but also people around him because persons with the disease become unaffective, apathic, anxious, irritable. In particular, apathy is the only psychiatric feature that is related to the disease stage tending to worsen over time (Tabrizi et al. 2013). Indeed, family members of patients often say that they observe a change in the personality of patients over years. A depressive disorder is the most common HD-related psychiatric symptoms affecting 33–76% of HD patients (van Duijn, Kingma, and van der Mast 2007).

Other psychiatric symptoms include aggression, obsessive-compulsive behaviour, psychosis, hyper- and hyposexuality, all related to frontal-lobe dysfunction, a cortical area strongly connected with the striatum and other basal ganglia.

Suicide risk is a very important concern being the second most common cause of death in HD after pneumonia (Lanska et al. 1988).

Not only a brain disorder

Although the main features of HD concern the central nervous system, a range of systemic symptoms can also be observed both in patients and in animal models of the disease, showing that HD is not a disease confined exclusively to the central nervous system. Among systemic symptoms are: skeletal muscle atrophy which occurs in HD despite the hyperkinesia, osteoporosis, endocrine problems with thyroid dysfunction (van der Burg, Björkqvist, and Brundin 2009) impaired glucose tolerance, a reduction in testosterone concentrations (Markianos et al. 2005) with often reduced numbers of germ cells and morphologic alteration in seminiferous tubule in HD men (Van Raamsdonk et al. 2007). In addition, peripheral symptoms include, in 30% of patients, heart failure which represents one of the most important causes of death in HD patient (Lanska et al. 1988) Additionally, because of the immune system is altered, patients usually present high levels of circulating pro-inflammatory cytokines in their peripheral blood (Björkqvist et al. 2008); Wild et al., 2011). Further, gastrointestinal (GI) tract alterations are a very important feature (see below).

It is still an open question whether systemic abnormalities are a consequence of neurological disorders or whether they independently arise as a consequence of the presence of the mutated form of Htt in these organs. While, on one hand, the fact that so many of the systemic symptoms are common in neurodegenerative diseases suggests that they are closely related to neurological disorders, on the other hand, many evidences suggest they are, instead, independent from the dysfunctions of the central nervous system. In support of this last hypothesis it's noteworthy that the protein is expressed in all the cells of the body (Li et al. 1993) and that its absence at embryonic level in mice caused defects in all three germ layers, proving that Htt is important for many tissue. Furthermore, when Htt is in the mutated form it induces transcriptional defects (Ruth Luthi-Carter et al. 2002) (Chaturvedi et al. 2009) and affects organelles such as mitochondria which are crucial in all cell types and not only for neuronal cells (Parker et al. 1990) (Gizatullina et al. 2006). Also, it has been observed that mutated Htt induces the typical aggregates formation also in peripheral tissue of HD mice (K. Sathasivam et al. 1999) (Orth et al. 2003). Thus, the idea that peripheral defects are due to *in situ* damage induced by the mutated protein, is becoming increasingly popular.

GI alterations and weight loss

The gastrointestinal (GI) tract disturbances are one of the most common symptoms in neurological diseases. Although functional studies have never been conducted in HD patients, there are different evidences suggesting that the GI tract is impaired. In 2009, Andrich and colleagues published the first study highlighting the presence of GI problems in HD patients. Through a retrospective study, it was observed that the frequency of gastro-esophageal inflammations in HD are much more frequent than could have been hypothesized based on complaints by the patients themselves. Furthermore, they observed that there is no correlation between gastritis and esophagitis with motor alterations while there is a correlation between these disorders and disease progression (Andrich et al. 2009).

On the other hand, it has been observed that 44% of patients with HD presents high level of antibodies directed against gliadin (a protein found in gluten), indicating greater sensitivity to gluten (Bushara, Nance, and Gomez 2004). A precise reason for this sensitivity has not been found yet, however, it indicates the presence of a high inflammatory response that could contribute to GI disorders in HD patients.

More recently, according to what has been observed in HD mouse models, it has been shown that HD patients have alterations in enteric cells. In particular, a study performed by McCourt on gastric mucosa biopsies from HD patients, evaluated the expression of different types of exocrine and endocrine cells in this region. It emerged that HD patients have a reduced expression of gastrin (expressed by G cells) and an increased expression of pepsinogen (produced by chief cells). The authors hypothesized that the increase in pepsinogen expression could underlie the increased frequency of gastritis and esophagitis demonstrated in HD, since its active form, pepsin, is associated with the formation of peptic ulcers. Instead, a reduction in gastrin could probably affect the stimulation of the parietal cells into the production of gastric acid (McCourt et al. 2015).

Lastly, HD patients more commonly suffer from anal incontinence and chronic constipation compared to controls and constipation is strongly pronounced in HD men rather than in women (Kobal et al. 2018).

To date, the large part of GI dysfunctions in HD have been demonstrated in murine models, in which it has been shown that there are mutant Htt aggregates in enteric neurons of the stomach, rectum and duodenum (Moffitt et al. 2009) (K. Sathasivam et al. 1999).

In this regard, van der Burg et al by using the murine model R6/2 (see Materials and methods) observed the presence of aggregates both in enteric neurons and in nerve fibers. In addition, several structural anomalies were observed, such as the reduction of about 15% of

the villi length in the small intestine and the thinning of the mucosa both in the stomach and in colon. More in detail, in the colon of R/62 mice there was a reduction in mucosal thickness of about 30% and in the colon length of about 10% compared to WT mice. Furthermore, it was observed an altered expression of both stimulating and inhibiting neuropeptides, impaired intestinal motility and nutrient malabsorption. In particular, nutrient malabsorption, measured as a ratio between food consumed and excreted, could explain the unintentional loss observed in HD. Nevertheless, the authors underline that since weight loss appeared even before observation of malabsorption it may not be the primary cause, but may contribute to it (van der Burg et al. 2011).

In any case, weight loss is one of the most common peripheral features of HD representing a marker of the disease. As it strongly reduces the quality of life of HD patients leading to general weakening, and contributing both in morbidity and mortality of the disease (Nance and Sanders 1996), weight loss is an interesting topic of study in HD and hopefully, a potential therapeutic target. Despite numerous studies have been performed to clarify the reason for unwanted weight loss, its etiology in HD has not yet been defined. It is believed that it can be multifactorial (Aziz and Roos 2013)

Over the years, many hypotheses have been performed: since the unwanted weight loss should be interpreted as an alteration of the balance between nutrients intake/absorption on one hand and energy expense on the other, one would generally think that it could be due to a reduced amount of caloric intake, increased motor activity, or increase in the metabolic rate. Therefore, at first, it was thought that weight loss occurred as a result of the hyperactivity, the continuous involuntary movements affecting individuals. This is not the case as weight loss often occurs before intense motor manifestations and it is severe also during the final hypokinetic phase of HD; indeed, numerous studies have shown that it does not correlate with chorea (Djoussé et al. 2002) (Sanberg, Fibiger, and Mark 1981) (Mochel et al. 2007). Interestingly, HD patients appear to eat more and take more kilocalories than wild type individuals, leading definitively to deny the possibility that weight loss could be explained by a reduced amount of food intake (Morales et al. 1989) (Mochel et al. 2007) (Marder et al. 2009) (Trejo et al. 2004).

However, even an increased metabolism could be the mechanism underlying weight loss. About this, some researchers have hypothesized that weight loss could be due to neurodegeneration of the hypothalamic lateral tuberal nucleus, a region fundamental for the regulation of metabolism (Kremer et al. 1990).

However, it was demonstrated that a higher BMI (Body mass index) correlates with a slower

progression of HD (Myers et al. 1991) and surprisingly this is also independent from the number of CAG repeats (van der Burg et al. 2017).
Finally, an alteration of the immune system was postulated as proinflammatory cytokines mediating the cachexia are produced in all the phases of HD (Bruera and Sweeney 2000). It appears to start from the pre-symptomatic phase (Mochel et al. 2007) and become more and more serious during the progression of the disease (Liot et al. 2017). Moreover, it correlates with the length of the CAG triplet (Aziz et al. 2008). All these evidences make weight loss a complex but very interesting feature in HD.

Treatment of HD

Up to now, no cure is available to treat HD. On the other hand, the combination of movement disorders, psychiatric symptoms and cognitive impairment are not easy to treat due to side effects of each symptom's treatment that might negatively influence the other symptoms.
Dopaminergic signaling is the most affected neurotransmitter system in HD and therefore it is the principal target for medications. Tetrabenazine, a dopamine-depleting drug, is the treatment of choice to reduce abnormal involuntary movements. Tetrabenazine reduces choreic movements, but because its use can be associated with depression worsening, it should be prescribed after a careful psychiatric examination (Huntington Study Group., 2006).
Antipsychotic drugs represent another option for choreic movements treatment because of their capacity to bind to the dopamine receptor blocking its activation.
These are especially useful if patients suffer from psychiatric problems such as agitation and irritability (Novak and Tabrizi. 2011).
The first pharmacological option for treating depression, the most significant psychiatric symptoms in HD patients, is selective serotonin reuptake inhibitors. These increase the concentration of serotonin in the synapses, which can thus stimulate the respective receptors affecting the emotional processing positively.
Since hyperkinetic disorders are replaced by hypokinetic disorders as the disease progresses, drug adjustments are required. Some drugs that reduce rigidity (e.g. baclofen acting on gabaergic system) can help in the later stage of the disease.
Apathy, a characteristic psychiatric feature that tends to worsen over time, has no

pharmacologic treatment. Therefore, psychotherapy to support the pharmacologic approach represents an essential treatment strategy able to help the patients with psychiatric symptoms from its onset.

Currently, there is no treatment aimed to cure systemic symptoms of HD, despite they have a strong negative impact on patients' life.

Curcumin

Curcumin is a natural compound already been shown to exert beneficial effects on both neurological (Maiti and Dunbar 2018) and peripheral disorders, including gastrointestinal ones (Lopresti 2018).

It is derived from the rhizomes of *Curcuma longa*, a herbaceous plant generally grown in India and other parts of Southeast Asia. It has traditionally been used in cooking as a spice in numerous Asian dishes as well as a natural dye thanks to its yellow color. However, Asians have always thought that this spice had beneficial properties for health. In fact, curcumin has always been used as a popular remedy to treat digestive problems, regulate menstruation, dissolve gallstones, fight arthritis, as an antiseptic and antibacterial (Prasad and Aggarwal 2011). Furthermore, curcumin has been historically used in ayurvedic medicine (Dudhatra et al. 2012; Grynkiewicz and Slifirski 2012) that attributed to it several curative activities including anti-inflammatory, antioxidant, chemopreventive and anti-aging activities (Hewlings and Kalman 2017). At present, many of the properties traditionally attributed to curcumin have been demonstrated by scientific studies performed both *in vitro,* in animal models and in humans.

Chemistry, Safety and Bioavailability of Curcumin

From a chemical point of view, curcumin is a hydrophobic molecule known as diferuloylmethane ($C_{21}H_20O_6$) with a molecular mass of 368.37 g/mol. Its chemical structure was identified in 1910 by Milobedzks and is characterised by two aromatic rings symmetrically linked through an α,β-unsaturated β-diketone moiety with seven carbon chain. Each ring contains one O-methoxy group and one hydroxyl group, (Priyadarsini 2014) while the α,β-unsaturated β -diketones can exist in different forms due to keto-enol tautomerism and cis-trans isomerism (Emsley, n.d.).

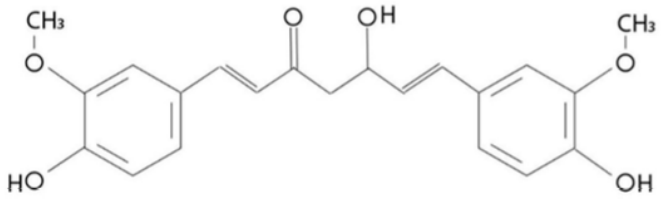

Figure 3. *Curcumin chemical structure: an α,β-unsaturated β-diketone moiety with seven carbon chain links two aromatic rings, each with one O-methoxy group and one hydroxyl group (Image from "A Review of Curcumin and Its Derivatives as Anticancer Agents". Tomeh et al. 2019*).

In solution, the trans conformation is more stable than the cis form isomer while as regards the tautomerism, the equilibrium is shifted towards the keto or enol conformation based on different environmental conditions (pH, temperature and polarity). The keto form predominates in acid and neutral environment (pH up to 8). At the same time, the enol conformation is predominant in basic conditions (pH > 8) (Maiti and Dunbar 2018). Many NMR studies confirmed that in most of the non-polar and moderately polar organic solvents curcumin exists mainly in the enol form (Benassi et al. 2008) (Balasubramanian 2006) (Payton, Sandusky, and Alworth 2007), that is more stable than the keto form, because of the presence of strong intramolecular hydrogen bonds (Jovanovic et al. 1999); while the keto form is partly present in equilibrium only at low pH values.

Curcumin's conformation is critical for its biological properties: curcumin in its enolic form acts as a prominent donor of electron while in its ketonic form acts as a proton donor. This is essential for its antioxidant propriety by scavenging free radicals through H-atom donation and electronic transfer (Barzegar 2012).

Further, curcumin is hydrophobic being insoluble in water but soluble in polar solvents (Tønnesen, Másson, and Loftsson 2002) such as acetone, 2-butanone, ethyl acetate, methanol, ethanol, dimethyl sulfoxide (DMSO) (Priyadarsini 2014) (Heger et al. 2014).

Safety evaluation studies have indicated that curcumin is well tolerated both in animal models and in humans. Curcuminoids (and curcumin) have been approved by the US Food and Drug Administration (FDA) as "Generally Recognised As Safe" (Gupta, Patchva, and Aggarwal 2012).

Studies in monkey, dog and rat suggest that curcumin is safe at the dose of 3.5g/kg for up to three months (Zhang et al. 2016). Various studies suggest that dietary curcumin supplementation is not toxic for humans. Oral administration of curcumin in patients with Bowens disease, oral leukoplakia, restricted bladder cancer, stomach metaplasia and cervical

intraepithelial neoplasm at the dose of 500 -8000 mg/day for three months appear to be non toxic (Cheng et al. 2001); also, patients with rheumatoid arthritis who take 1.2-2.1 g/day of oral curcumin for 2-6 weeks had no controversial effects (Eigner and Scholz 1999).

In another clinical trial on forty depressed patients who were oral administrated 500 mg of curcumin for five weeks, no adverse effects have been highlighted (Bergman et al. 2013) not even in a study conducted on patients with solid tumors who received 900 mg/day of oral curcumin for eight weeks although, in this case, conditions of gastrointestinal irritation have been reported (Panahi et al. 2014). Clinical trials have also been conducted on women to evaluate the effect of curcumin on the premenstrual syndrome: oral administration of 100 mg of curcumin every twelve hours determines no toxic or adverse effect (Fanaei et al. 2016). However, some adverse side effects, especially gastrointestinal, have been reported with the use of curcumin; for example, in a study of patients with pulmonary problems caused by Sulphur mustard, administration of 1.5 g/day of curcumin for four weeks caused constipation, stomachache and headache. (Panahi et al. 2012).

Curcumin has low bioavailability due its poor absorption, rapid metabolism and rapid elimination (Anand et al. 2007). Its bioavailability is even lower when orally administrated (Garcea et al. 2005). In particular, absorption of curcumin at the intestinal level is hampered due to the efflux caused by the P-glycoprotein (P-gp), a drug efflux pump localized on the enterocytes of the small intestine. This protein presents high level of expression in the apical region of the intestine (luminal), in the blood-brain barrier, in the testis barrier and in placenta. Thus, P-gp pumps curcumin from inside to outside the cell, affecting, in this way, plasma and tissue concentrations of curcumin and, accordingly, its final effects.

In order to increase its bioavailability, absorption and slow metabolism and rapid elimination, different approaches have been developed. Among these, the use of adjuvants, which enhance bioavailability by inhibiting the drug metabolism. The most recommended adjuvant is piperine (1-piperoylpiperidine), an alkaloid present in black pepper that acts like a glucuronidase inhibitor thus slowing down the metabolism of curcumin in the liver and in the intestine (Han 2011). The first study conducted *in vivo* both in human and in the animal model, regarding the ability of piperine to increase the oral bioavailability of curcumin, had highlighted that, when curcumin was administered together with piperine, its bioavailability was 154% in rats and 2000% in human. (Shoba et al. 1998)

Curcumin and Neurodegenerative diseases

Curcumin has been shown to exert beneficial effects in neurodegenerative disorders, they are a very heterogeneous group of diseases, characterized by a progressive loss of neuronal cells in specific brain areas. Depending on the brain region affected, they exhibit different but strongly debilitating symptoms and are all incurable.

Although the precise pathological mechanisms that cause this type of disease are not fully clear, they are characterized by common factors including:

-the presence of misfolded proteins that aggregate and form inclusion bodies that can be nuclear, cytoplasmic, intra- or extra-cellular; regardless of their location, it is believed that they "obstruct" neuronal cells leading to dysfunction and, subsequently, to their death;

-the reduction of levels of neurotrophins, a class of proteins involved in the survival, development, maintenance, functioning, synaptic plasticity of neurons and showing a neuroprotective function;

-the presence of strong oxidative stress, meaning that in patients with neurodegenerative diseases there is an alteration of the balance between the reactive species produced and the antioxidant systems. The increase in reactive species causes damage to proteins, nucleic acids, lipid membranes, mitochondria and therefore to cells in general;

-a constant inflammatory condition.

Numerous studies have shown that curcumin has anti-aggregation, anti-inflammatory, anti-oxidant properties and that it is also capable of increasing the levels of neurotrophins and the neurogenesis process.

Anti-aggregation properties of curcumin

In vitro and *in vivo* studies have shown that curcumin is a powerful anti-aggregant. Particularly encouraging have been studies performed on beta-amyloid protein aggregates, which characterize Alzheimer's disease (AD). *In vitro* studies have revealed that curcumin exerts a strong inhibitory effect on the formation of beta amyloid fibrils. Moreover, by first inducing the formation of β-amyloid fibrils and then adding curcumin to them, the researchers have shown that it is also capable of disaggregating pre-existing fibrils. These properties are dose-dependent in a range from 0.1 to 1 micromolar concentration (Ono et al. 2004; Yang et al. 2005). The same properties have also been demonstrated on α-synuclein aggregates, typical of Parkinson's disease (PD) (Pandey et al. 2008) and on other amyloid-like aggregates. In fact, curcumin is capable to bind, in addition to the β-amyloid peptide aggregates, also those of Htt, α-synuclein and phosphorylated tau proteins. It is therefore

believed that curcumin does not interact with a specific sequence, rather with the type of conformation assumed by these aggregates.

The anti-aggregation properties of curcumin have also been highlighted in vivo. For example, a study carried out by Garcia-Alloza et al., using multiphoton microscopy, has shown that it suppresses the formation of beta-amyloid aggregates in a mouse model of AD (APPswe / PS1dE9). Curcumin was administered intravenously to mice at a concentration of 7.5 mg / kg / day (low dose) for one week. Amyloid plaques were measured before starting treatment and at the end of the treatment to evaluate the effect that curcumin had on pre-existing aggregates. The authors observed that curcumin crossed the blood brain barrier (BBB) and that after only one week of treatment at low-dose, there was a 30% reduction in the size of the pre-existing plaques. They also evaluated newly formed plaques and, although the time between the two experimental points was only one week, it was observed that curcumin can also prevent the formation of new plaques (Garcia-Alloza et al. 2007).

How curcumin is able to exert this property is not entirely clear. Certainly, thanks to its chemical structure, it could bind to amyloid fibrils therefore exerting a direct effect. However, other effects could contribute to this phenomenon; for example Zhang et al. have shown that curcumin reduces the maturation process of the amyloid precursor protein (APP), thus, reducing the quantities of the mature form of the protein (Zhang et al. 2010). Furthermore, it is interesting to note that curcumin can also activate heat shock proteins (Leak et al. 2017). Therefore, the anti-aggregation property can also be mediated by the action that curcumin exerts on this class of protein.

Anti-inflammatory and anti-oxidative properties

The most clear activity of curcumin is certainly its antioxidant activity.

This property is attributable to its chemical structure. In fact, curcumin is one of the few molecules to have both a phenolic hydroxyl group and a diketone group. In particular, this natural compound is able to neutralize free radicals by giving them its H group from the phenolic group or diketone group. Another proposed mechanism is that free radical receives an electron from curcumin that, anyway, is able to remain stable thanks to its structure. Further, curcumin can modulate the activity of enzymes counteracting free radicals such as GSH (glutathione), SOD (superoxide dismutase) and catalase (Piper et al. 1998. Watanabe et al. 2000), or inhibit enzymes that generate free radicals such as lipoxygenase and cyclooxygenase. Furthermore, it can induce endogenous antioxidants by acting on the transcription factor called Nrf2 (Esatbeyoglu et al. 2012), or act as a good scavenger reacting

with reactive oxygen species, reactive nitrogen radicals and H_2O_2 (Borra et al. 2013). Therefore, curcumin has been shown to reduce lipid peroxidation, oxidation and carbonylation of proteins, mitochondrial damage and other phenomena caused by reactive species classically present in neurodegenerative disease.

Anti-inflammatory activity is also a largely described biological activity of curcumin. It mainly exerts an anti-inflammatory action by inhibiting pro-inflammatory cytokines such as TNF-alpha, IL1 and IL6 and increasing anti-inflammatory ones such as IL-10. It acts as a NF-κB inhibitor, one of the main factors involved in activating the pro-inflammatory response. The antioxidant and anti-inflammatory activities of curcumin provide evidence of the beneficial role of this nutraceutical in a wide range of pathologies. Therefore, thanks to these two important activities, curcumin was included in numerous human clinical trials.

Curcumin and synaptic transmission

Curcumin stimulates the expression levels of neurotrophins NGF, BDNF, GDNF, PDGF *in vivo* (Gupta et al. 2011) that have a key role in maintaining normal neuronal functions, to promote neurogenesis in the adult brain and to improve memory functions. Moreover, the pre-synaptic and post-synaptic markers, such as synaptophysin and postsynaptic density protein 95 (PSD95), are also restored in animal models treated with curcumin (Ahmed et al. 2010), suggesting an improvement in synaptic transmission thus improving neuronal communication.

Curcumin effect in Huntignton's Disease

The beneficial effects of curcumin have also been studied in the brain of some HD model animals.

A study performed by Kumar et al. using rats in which HD had been induced by 3-nitropropionic acid (3-NP) (a toxin that irreversibly inhibits the enzyme succinate dehydrogenase (SDH) leading to mitochondrial dysfunction and inducing the generation of reactive oxygen species (ROS) in striatal neurons (Vis et al. 1999)), showed that chronic treatment with curcumin is beneficial. Curcumin, at the concentration of 10, 20 and 50 mg / kg, improved motor and cognitive alterations in a dose-dependent manner and attenuated oxidative stress induced by 3-NP in the brain of 3-NP rats (Kumar, 2007).

This is in agreement with another study performed on the same animal model, using curcumin in nanoencapsulated form. In these conditions curcumin was orally administrated

for 7 days at a daily dose of 40 mg/kg body weight and it was shown to be able to re-establish the brain mitochondrial activity. The treatment, in fact, increased the GSH levels and SOD activity, decreased lipid peroxidation, protein carbonyls, reactive oxygen species and mitochondrial alterations, thanks to its antioxidant properties. In addition, it improved the classical motor symptoms in 3-NP curcumin-treated rats (Sandhir, 2014).

The effect of curcumin was also assessed in the early stages of HD in the mouse model CAG140 *knock-in*, which expresses a chimeric human/mouse Hdh protein, with human exon1 presenting 140 CAG repeats and characterized by a slow progression of the disease. In these animals, curcumin was administered via chow at a dose of 555 ppm (corresponding to 92 mg / kg body weight of the mouse) for all the duration of their life, starting from conception (through mothers) until 4,5 months of age. The result was an improvement in the transcriptional expression of genes normally downregulated in HD at the striatal level (CB1, D1, DARPP-32), a reduction in huntingtin aggregates and an improvement of a behavioral improvement, i.e of rearing deficits (Hickey, 2012)

Moreover, the reduction of mutated Htt aggregates was also observed in the Drosophila transgenic models, expressing exon 1 with 93 and 48 glutamines respectively, treated with curcumin at a dose of 5-10 μM. In these fly models, curcumin ameliorated larval crawling and adult climbing, highlighting an improvement in motor symptoms. Further, it suppressed the classical neurodegeneration, suppressing the apoptotic processes (Chongtham, 2016).

Curcumin effect on the gastrointestinal tract

Curcumin is thought to have considerable beneficial effects on the gastrointestinal tract both in health and disease conditions. This is not surprising considering that, after oral administration, the gastrointestinal tract shows the greatest amount of curcumin (Ravindranath and Chandrasekhara 1980).

Intestinal health is determined by the contribution of several factors, including the composition of the microbiota, the integrity of the intestinal epithelium, an adequate response to pathogens and to the production of ROS and of pro-inflammatory molecules. In several studies, performed both *in vitro* and *in vivo*, curcumin has been shown to influence many of these different aspects.

For example, the influence of curcumin on the microbiota was analyzed in a study conducted on rats fed with a high-fat diet, known to cause alteration of the composition of the microbiota. It was observed that when curcumin was added to the high-fat diet, the

composition of the gut microbiota became comparable, in term of species composition, to that of control rats fed with a normal diet (Feng et al. 2017). Despite with more limitations, also in humans the effects of curcuma on the human intestinal flora was examined. Interestingly, curcuma was shown to increase hydrogen production by the intestinal microflora (Shimouchi et al. 2009). An important action of curcumin appears to be its action on the intestinal epithelium: it acts as a physical protective barrier against pathogens and plays a fundamental role in the absorption of nutrients, water and ions and in intestinal homeostasis, thus preventing the passage of harmful contents and allowing the absorption and secretion of nutrients (Clairembault et al. 2015). In an *in vitro* study performed on Caco-2 cells, curcumin pretreatment mitigated the leptin-induced tight junction deterioration (Kim et al. 2014).

In another study conducted on human epithelial cells, curcumin attenuated the barrier damage induced by hydrogen peroxide and restored the levels of occludin and zonula occludens 1 (ZO-1), two important markers of tight junctions possessing an important role in the tissue integrity (Wang et al. 2012). In addition, curcumin has proven to be able to attenuate the inflammatory response to lipopolysaccharide (LPS), reducing the levels of IL-1β released by both intestinal epithelial cells and intestinal macrophages. It also preserves intestinal permeability following the insult with LPS, preserving the proteins involved in the cell-cell junction (Wang et al. 2017). The effect of curcumin on intestinal permeability has also been confirmed *in vivo*, in mice fed with a fed a high fat, high cholesterol Western type diet. This kind of diet normally increases the intestinal permeability, but when mice were fed with curcumin in their diet, they showed an improvement of the intestinal barrier by restoring the activity of intestinal alkaline phosphatase and the expression levels of tight junctions proteins (Ghosh et al. 2014) (Lopresti 2018).

Interestingly, curcumin has also been indicated as a remedy for alterations of the intestinal motility, since it was shown to be able to alleviate this symptom in patients with both Crohn's disease and irritable bowel syndrome (Fadus et al. 2016).

Figure 4. *Summary of the curcumin activities in neurodegenerative diseases and in intestinal health*

Chapter 2

Aim of the study

To date, there is no available treatment to forestall, slow, heat or reverse HD. Current developed therapeutic approaches aim only to the management of the symptoms of strictly neurological origin despite it has been clear for years that the disease is systemic and that "peripheral" symptoms significantly affect the quality of life of patients. Therefore, the need arises to identify long-lasting treatments able to act on both neurological and peripheral aspects of HD, capable to provide a support in all stages of the disease and to eventually act as a preventive measure for incoming symptoms. In this context, nutraceuticals could represent a good opportunity thanks to their general beneficial pleiotropic effects on human health and their very low or null toxicity.

Assuming that the use of a more systemic approach to treat HD symptoms could be a better strategy to improve the quality of life of patients, in the research project of which my PhD project is part, we have chosen to investigate the potential beneficial effect of the nutraceutical curcumin, a polyphenol derived from *Curcuma longa*, historically used as a natural remedy in Asia, in the R6/2 HD mouse model. This is a transgenic model of HD, expressing exon 1 of the mutated human Huntingtin gene with approximately 160 CAG repeats (Mangiarini et al. 1996) and it is the most used experimental model to study both the pathological aspects and the possible therapeutic interventions in HD since it recapitulates most of the human HD-like symptoms.

The idea of using curcumin was born because it is a natural compound with numerous well-documented biological properties such as anti-aggregation, anti-inflammatory, anti-oxidant properties that could help in HD. Importantly, it has already been shown that curcumin exerts protective effects in neurodegenerative diseases and on peripheral disorders including intestinal ones which are the aspects I've been more focused on. Further, it is a substance with a reassuring safety profile in humans, with very few side effects even if administered for long periods.

The study carried out during my PhD thesis is part of a wider research project aimed at assessing whether the lifelong administration of the nutraceutical curcumin, administered as a supplement to the diet starting from the early embryonic stages could relieve or prevent both brain and peripheral symptoms of HD R6/2 mice. In the context of this study I was mainly focused on evaluating the beneficial effects of curcumin on peripheral disturbances such as body weight loss and intestinal alterations occurring in HD. In particular, involuntary

weight loss is one of the symptoms that most seriously affect the clinical aspect and life expectancy of patients, contributing to determine a "bedding syndrome" due to a widespread asthenia that often progresses until it leads to severe cachexia. When arises, it easily becomes one of the causes of onset of multiple complications, especially those of infective nature. It has been hypothesized that weight loss is pathogenetically related to dysfunctions of the gastrointestinal tract (GI), such as constipation and alterations of the gastrointestinal mucosa. We investigated body weight, food intake and intestinal alterations of R6/2 mice treated or not with curcumin (see Figure 5, Experimental plan for curcumin administration). In particular, we evaluated the intestinal peristalsis both indirectly through the collection of feces, and, more directly, through an analysis of contractility of colon explanted from curcumin treated and untreated animals. In addition, we performed morphological analysis of the intestinal villi, responsible for the absorption of nutrients, as well as gene expression analysis of genes coding for tight junctions proteins, playing a very important role in the formation of the intestinal barrier.

These analyses are included in the publication *"Curcumin dietary supplementation ameliorates disease phenotype in an animal model of Huntington's disease"* (Chapter 3 of the thesis).

During the period abroad in the lab of Prof. Euan R. Brown, I explored a different topic: the study of excitotoxicity, a neuronal death due to excessive stimuli by excitatory aminoacids, that plays a crucial role in neurodegenerative diseases. In particular, we obtained neurons derived from hNPCs (human iPSC-derived neural progenitor cells) that constitute a really powerful system for drug discovery and for the study of excitotoxicity by NMDA (N-Methyl-D-aspartic acid) stimulation. This part is developed in the Chapter 4.

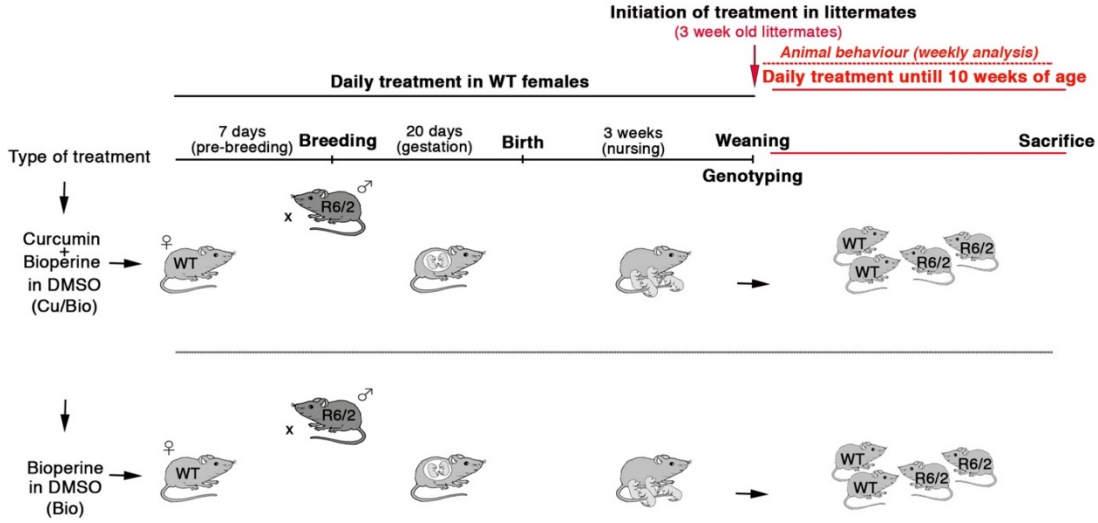

Figure 5. ***Experimental plan for life-long administration of curcumin***. *One week before breeding, WT females (N=8) were divided into two groups and fed with either Bioperine/DMSO (Bio) or Curcumin/Bioperine/DMSO (Cu/Bio). Females were then crossed with R6/2 males fed with regular diet. Breeding females were fed with Cu/Bio-supplemented diet for the entire period of gestation and for three weeks after the offspring birth. Treatment continued in weaned pups which were then used to perform all in vivo and biochemical experiments.*

Chapter 3

Curcumin dietary supplementation ameliorates disease phenotype in an animal model of Huntington's disease.

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Abstract

Huntington's disease (HD) has traditionally been described as a disorder purely of the brain, however evidence indicates that peripheral abnormalities are also commonly seen. Among others, severe unintended body weight loss represents a prevalent and often debilitating feature of HD pathology, with no therapies available. It correlates with disease progression and significantly affects the quality of life of HD patients.

Curcumin, a naturally occurring polyphenol with multiple therapeutic properties, has been validated to exert important beneficial effects under health conditions as well as in different pathological settings, including neurodegenerative and gastrointestinal (GI) disorders.

Here, we investigated the potential therapeutic action that curcumin-supplemented diet may exert on central and peripheral dysfunctions in R6/2 mice, a well-characterized HD animal model which recapitulates some features of human pathology.

Maintenance of normal motor function, protection from neuropathology and from GI dysfunction, preservation of GI emptying, and conserved intestinal contractility, proved the beneficial role of life-long dietary curcumin in HD and corroborated the potential of the compound to be exploited to alleviate very debilitating symptoms associated with the disease.

Keywords: HD, curcumin, neuroprotection, weight loss, gastrointestinal dysfunction

Introduction

Huntington's disease (HD) is an autosomal dominant inherited neurodegenerative disorder caused by a CAG trinucleotide repeat expansion in the HTT gene (1), which encodes for huntingtin (Htt) protein. The resulting polyglutamine (polyQ)-stretch destabilizes the protein and confers toxic properties to it, ultimately resulting in a broad array of molecular and cellular dysfunction in both neuronal and non-neuronal cells (1, 2). The polyQ expansion causes conformational changes within Htt protein and makes it prone to misfolding and oligomerization.

For many years research into HD has conventionally focused on neurodegeneration, neurological symptoms and overall brain pathology**,** however, a growing body of evidence indicates that peripheral dysfunctions also occur in the disease.

Peripheral defects sometimes appear early in the disease course and can eventually contribute to both morbidity and mortality (3-7). Among all non-neurological features, the progressive early unintended weight loss is one of the most common and serious peripheral abnormalities that affects nearly all individuals with HD (4, 8, 9).

Severe body weight loss is a recurrent symptom in both humans and animal models of HD. It has been reported to worsen other aspects of the disease and to occur despite an adequate caloric intake. Loss of weight begins early, even before disease symptoms appear (10), and ends with profound cachexia in advanced-disease stage (11). Such a gradual physical decline is also associated with progressive gut motility failure and, subsequent chronic constipation, which becomes a very invalidating and unmanageable condition in HD patients over the time (12).

The molecular mechanism underlying weight loss in HD is still elusive, however a possible relationship with dysfunction in the gastro-intestinal (GI) tract and, changes in the metabolism has been suggested to exist (13, 14). Work performed in HD R6/2 mice, one of the most used HD transgenic animal model that recapitulates many features of human pathology (15-17), and in humans, have demonstrated that GI tract is precociously affected in the disease (14, 18, 19). Thus, protective treatment may need to be started long before the overt onset of clinical manifestation.

Curcumin, a natural dietary polyphenol derived from the *Curcuma longa* plant, with outstanding safety profile and multiple pharmacological properties ranging from anti-inflammatory to anti-oxidant through neuroprotective and anti-aggregation action, has been

shown to be helpful in different degenerative brain pathologies (20-23) as well as in several peripheral illness (24) and GI dysfunction in metabolic settings (25, 26).

Beside all well-known pharmacological properties (27), curcumin stimulates appetite (28), promotes normal food digestion by either regulating digestive hormones, bile and gastric acids or by modulating smooth muscles functions (29-31) and, significantly increases bowel motility in humans (32).

Recent data demonstrated that also HD may benefit of curcumin properties (23, 33, 34). Importantly life-long dietary curcumin, started from conception or at very early developmental stages, has significant beneficial consequence on neuropathology and phenotypic manifestation in HD experimental models (33, 34).

The therapeutic efficacy of curcumin in HD has been mostly investigated on brain pathology, whereas its therapeutic value on peripheral perturbations has never been explored so far.

Curcumin has been widely described to act as protectant agent for GI dysfunction and to activate bowel motility in different disease conditions (25, 26).

In this study, beside consolidating the therapeutic effect of curcumin in brain pathology in HD, we tested the hypothesis that curcumin supplementation could be exploited to maintain GI homeostasis in the disease and, to eventually prevent the associated dysfunctions.

R6/2 mice were chronically administered with the compound and any disease phenotypic change, associated with life-long curcumin supplemented dietary, was assessed.

The overall purpose of this study was to address the impact of early intervention in these mice with the aim to counteract the toxic effect of mutant Htt (mHtt) and, eventually to mitigate the development of classically associated pathological signs in both central and peripheral regions.

Our data corroborated the evidence of neuroprotective effect of curcumin in HD brain pathology, and for the first time, demonstrated that chronic administration of curcumin resulted in an overall health benefit in R6/2 offspring.

In particular, beside protecting brain from neuropathological changes and from all the associated phenotypic complication, curcumin supplementation completely prevented body weight loss in R6/2 mice and preserved the normal intestine homeostasis and function in these mice.

Results

Life-long dietary curcumin preserves motor performance in R6/2 mice

Dietary curcumin supplementation has been associated with beneficial effects in different HD experimental models (33, 34).

Life-long dietary curcumin had an overall beneficial effect in R6/2 mice. It prevented the classical motor deficit associated with disease progression in these mice, when fed from conception. HD treated mice performed significantly better than untreated littermates over the entire period of observation (Fig. 1A). Curcumin-fed R6/2 mice did not even develop the characteristic hindlimb clasping behaviour throughout the disease course (Fig. 1B).

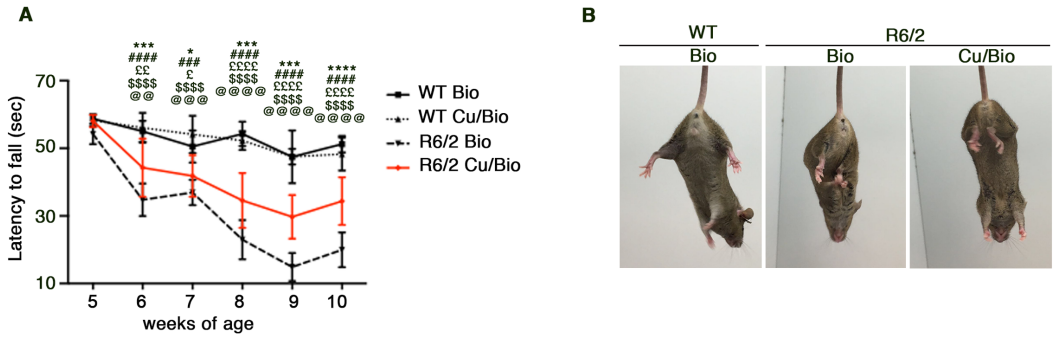

Figure 1. Administration of curcumin from conception preserves motor function in R6/2 mice. *(**A**) Motor performance assessed by Rotarod. WT, N=5+5; R6/2, N=8+8. Values are represented as mean ± SD. @, p<0.05; @@, p<0.01; @@@, p<0.001; @@@@, p<0.0001 (Cu/Bio-treated WT vs Cu/Bio-treated R6/2 mice). £, p<0.05; ££, p<0.01; ££££, p<0.0001 (Bio-treated WT vs Cu/Bio-treated R6/2 mice). *, p<0.05; ***, p<0.001; ****, p<0.0001 (Bio-treated R6/2 vs Cu/Bio-treated R6/2 mice). ###, p<0.001; ####, p<0.0001 (Bio- treated WT vs Bio-treated R6/2 mice). $$$$, p<0.0001 (Cu-treated WT vs Bio-treated R6/2 mice) (Two-Way ANOVA with Bonferroni post-test). **(B)** Limb-clasping response in 10-week old R6/2 mice. Bio: Bioperine/DMSO; Cu/Bio: Curcumin/Bioperine/DMSO.*

Curcumin-supplemented diet evokes the activation of pro-survival pathways in the striatum of R6/2 mice

Evidence demonstrates that curcumin has neuroprotective effect in animal models of several types of neurodegenerative disorders, including HD [35-37]. Here we investigated whether early supplementation of curcumin might prevent neurological changes that classically appear in R6/2 mice as the disease progresses. Curcumin-fed mice were significantly protected from brain weight loss when compared to unsupplemented mice (Fig. 2A). The neuroprotective effect of life-long dietary curcumin in these mice was associated with increased levels of striatal dopamine- and cAMP-regulated protein 32 (DARPP-32) (Fig. 1B), a specific marker of medium spiny neurons (35), whose downregulation is related to neurodegenerative processes in HD (36). Importantly, curcumin supplementation also evoked the phosphorylation of AKT and ERK (Fig. 2C-D and Supplementary Fig. 1), two kinases with pro-survival function (37) in HD mice. While phosphorylation of AKT was clearly detectable in both striatum and cortex of treated mice (Fig. 2C and Supplementary Fig. 1A), activation of ERK was only found in the striatum (Supplementary Fig. 1B). No biochemical changes were observed in WT mice after treatment (Supplementary Figure 2).

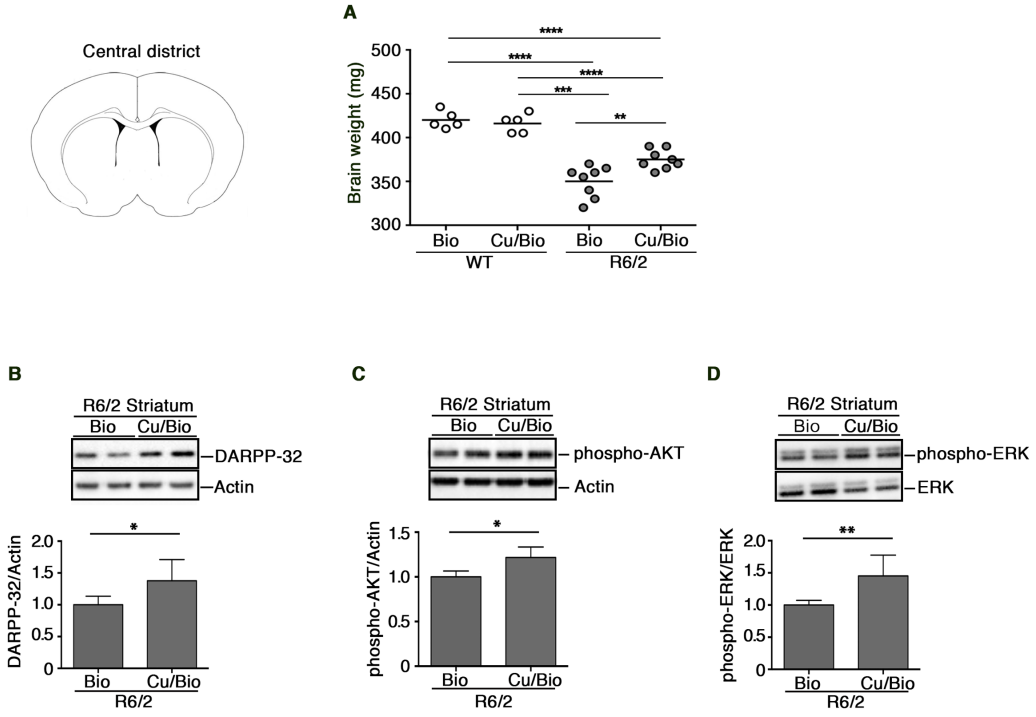

Figure 2. Life-long curcumin-supplemented diet preserves total brain weight and evokes the activation of pro-survival pathways in HD striatum. *(A) Mouse brain weight. WT, N=5+5; R6/2, N=8+8. **, p< 0.01; ***; p<0.001****, p<0.0001 (one-way ANOVA with Tukey post-test). Representative immunoblottings and densitometric anlaysis of DARPP-32 (B), phospho-AKT (C) and phospho-ERK (D) in striatal tissues from Bio- and Cu/Bio-treated HD mice. N=6-7. Values are represented as mean ± SD. *, p<0.05; **, p<0.01 (Un-paired t-test).*

Curcumin stabilizes levels of brain derived neurotrophic factor (BDNF) in both striatal and cortical tissues from R6/2 mice

Reduced levels of BDNF is one of the most common hallmarks of HD and, interventions aimed at modulating its production have been largely proposed as potential therapeutic approach for the disease (38). Evidence shows that dietary curcumin supplement increases BDNF levels in pre-clinical models of several pathological conditions (38). Here, in order to further support the neuroprotective properties of curcumin, brain tissues from supplemented mice were analyzed.

As reported in Figure 3, curcumin preserved normal levels of BDNF in the cortex (Fig. 3A) and, importantly also in the striatum (Fig. 3B) of HD mice. As expected no effect was detected in WT mice after treatment (Supplementary Figure 3).

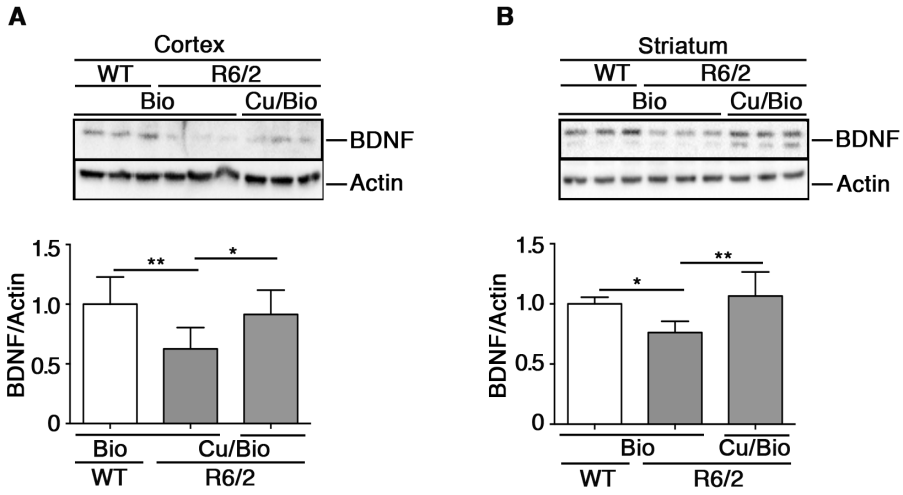

Figure 3. Curcumin restores normal levels of BDNF in HD mouse brain. *Representative cropped immunoblottings of BDNF in cortex (A) and striatum (B) from Bio- and Cu-treated mice. N=6-7. Values are represented as mean ± SD. *, p<0.05 (Un-paired t-test).*

Life-long dietary curcumin reduces mHtt aggregation in brain tissues from R6/2 mice

mHtt aggregation is a major pathological feature that may conceivably account for cytotoxicity and neuronal dysfunction in HD (39, 40). Curcumin possesses anti-aggregation properties and its supplementation has been earlier found to mitigate cytotoxicity in different neurodegenerative conditions (41-43). Immunohistochemical staining of striatal sections from R6/2 mice showed lower number of EM48-positive ($EM48^+$) mHtt aggregates that were also significantly smaller in size when compared to those seen in control R6/2 mice (Fig. 4A-C). This result was further confirmed by immunoblotting analysis. $EM48^+$ SDS-insoluble aggregates were barely detectable in in both striatal and cortical lysates from HD supplemented mice (Fig. 4D and Supplementary Fig. 4).

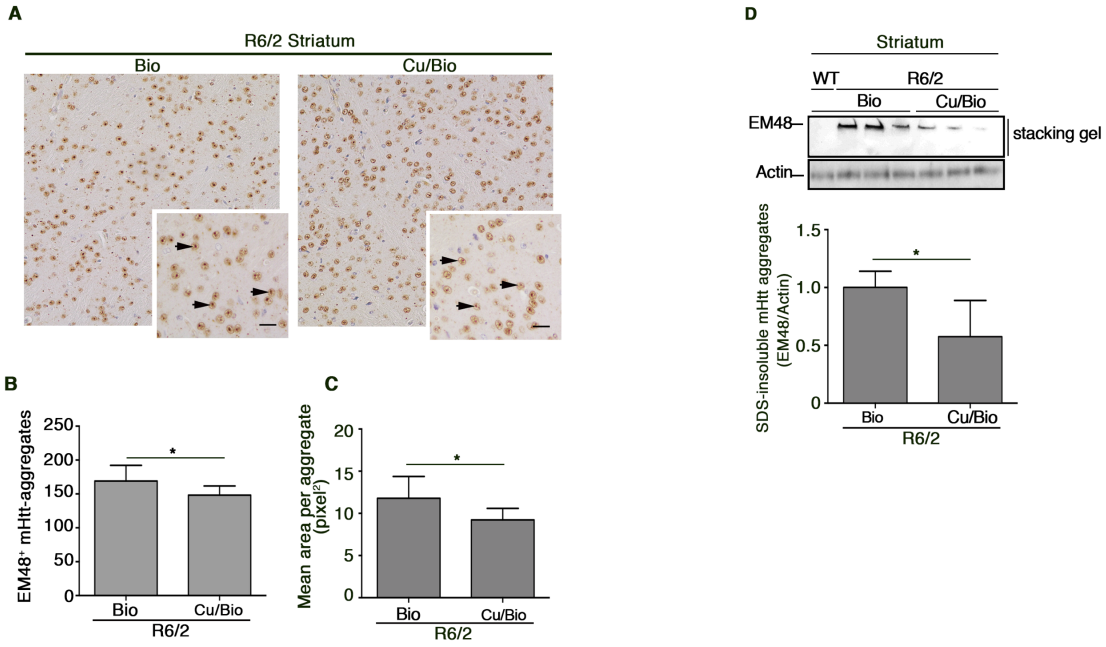

Figure 4. Curcumin-supplemented diet is associated with reduced mHtt aggregation. *(A) Representative micrograph and (B) analysis of the number and (C) area of $EM48^+$ mHtt aggregates in the striatum of Bio- and Cu/Bio-treated R6/2 mice at 10 weeks of age. Arrows indicate mHtt aggregates. Scale bar represents 100µm. Values are represented as mean ± SD. *, $p<0.05$ (Un-paired t-test). (D) Cropped immunoblotting of EM48-positive mHtt aggregates in striatal lysate from Bio- and Cu/Bio-treated R6/2 mice at 10 weeks of age. N=6-7. Values are represented as mean ± SD. *, $p<0.05$ (Un-paired t-test).*

Curcumin-supplemented diet prevents body weight loss and ensures intestinal homeostasis in R6/2 mice

Loss of body weight is a severe peripheral complication in HD human patients and animal models, including R6/2 mice (4, 14). Evidence suggests that gastrointestinal dysfunctions may contribute to this phenomenon (14). Curcumin dietary supplement preserves metabolic health and stabilizes body weight in different disease conditions including gastrointestinal disorders (25, 26).

Supplementation of curcumin completely prevented body weight loss in R6/2 mice to the extent to make them indistinguishable from WT littermates (Fig. 5A). Furthermore, curcumin increased food consumption in treated mice (Fig. 5B) and maintained the overall normal intestinal function by stimulating intestinal emptying as demonstrated by increased amount of collected stool over a period of 24 hours (Fig. 5C).

Defective gastrointestinal motility is known to be impaired in neurological disorders (44). Recent evidence demonstrates that curcumin has direct effect on intestinal contractility and significantly improves intestinal propulsion rate in functional gastrointestinal disorders (45). Here, we demonstrated that administration of curcumin evoked changes in the smooth muscle contractility in both WT and HD mice (Fig. 5D-E). Ex vivo analysis of colonic ring contraction in response to KCl revealed barely detectable intestinal contractility in untreated HD mice (Fig. 5D-R6/2 Bio). Curcumin-supplemented R6/2 mice showed a significant difference in the mean contractile tension during KCl depolarisation when compared to untreated R6/2 mice (Fig. 5D). Interestingly, curcumin preserved intestinal smooth muscle function in R6/2 mice to the extent to make their contractile profile curve indistinguishable from that observed in WT mice (Fig.5D - R6/2 Cu/Bio). No changes in the expression of peripheral neuronal markers were observed (Supplementary Fig 5).

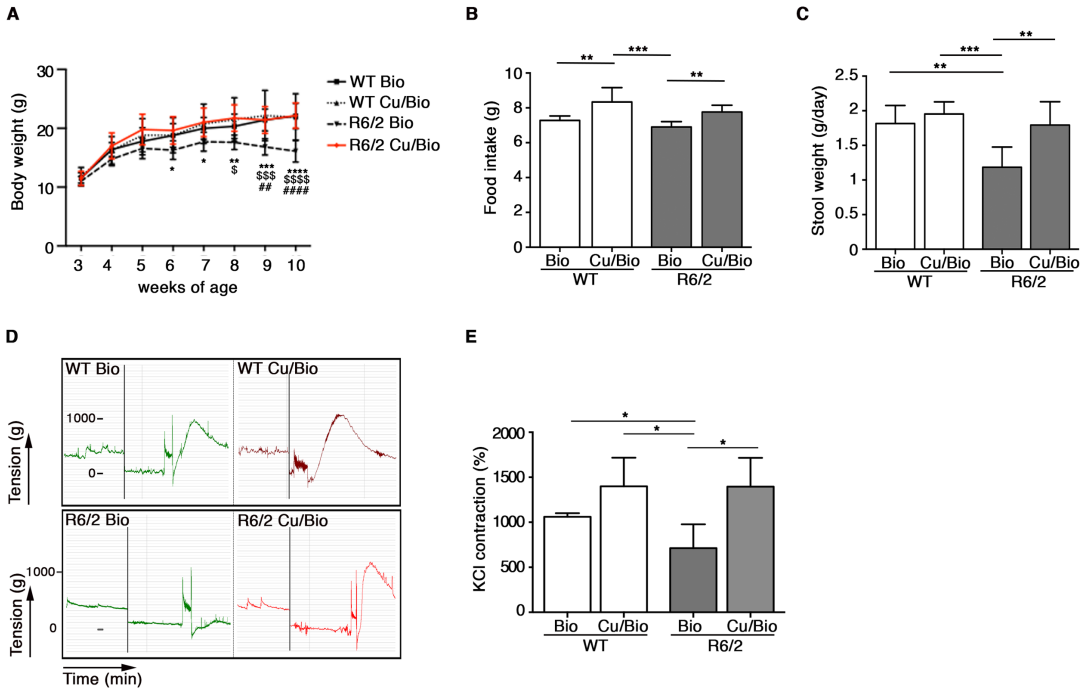

Figure 5. Curcumin-supplemented diet completely prevents the loss of body weight and maintains normal intestinal activity in HD mice. *(A) Mouse body weight weekly measured during the entire period of the treatment. WT, N=5+5; R6/2, N=8+8. Values are represented as mean ± SD. (Bio-treated R6/2 vs Curcumin-treated R6/2 mice). ##, p<0.01; ####, p<0.0001 (Bio-treated WT vs Bio-treated R6/2 mice). $, p<0.05; $$, p<0.01; $$$$, p<0.0001 (Cu/Bio-treated WT vs Bio-treated R6/2 mice) (Two-Way ANOVA with Bonferroni post-test). (B) Food intake and (C) stool weight analysis assessed in Bio- and Cu/Bio-treated WT and R6/2 mice before sacrifice. (D) Representative contractility curves and (E) KCL-contraction percentage quantitation for Bio- and Cu/Bio-treated WT and R6/2 mice. N=4 for each group of mice. Values are represented as mean ± SD. *, p<0.05; **, p<0.01; ***, p<0.001 (One-Way ANOVA with Tukey post-test).*

Curcumin preserves normal GI tissue organization in R6/2 mice

Examination of the GI tract further corroborated the beneficial effect that curcumin exerts on gastrointestinal dysfunction associated with HD. Curcumin-supplemented R6/2 mice did not show any pathological changes in the morphology of the GI tract as was, instead, clearly detectable in untreated HD mice (Fig. 6A). GI tracts of treated HD mice were indistinguishable in morphology from the WT group (Fig. 6A).

Morphometric analysis of small intestinal tract revealed that curcumin dietary supplement protected R6/2 mice form reduced villi length and atrophy that has been previously described to occur as the disease progresses (14) (Fig. 6B-C). No difference between R6/2 mice fed with curcumin and WT was observed (Fig.6B-C).

Interestingly, colon cross-sections from curcumin-treated R6/2 mice showed significant reduction in the number of PAS-positive (PAS^{+}) Globet cells, which appeared also less dense of mucin, in the Lieberkühn crypts when compared to untreated R6/2 mice (Fig. 6D-E).

Positive modulation of gene expression of intestinal barrier proteins after curcumin supplementation further corroborated the protective effect that curcumin may exert on the GI tract in HD and consolidated its overall beneficial effect on physiological homeostasis (Fig. 7 and Supplementary Fig. 6 and 7).

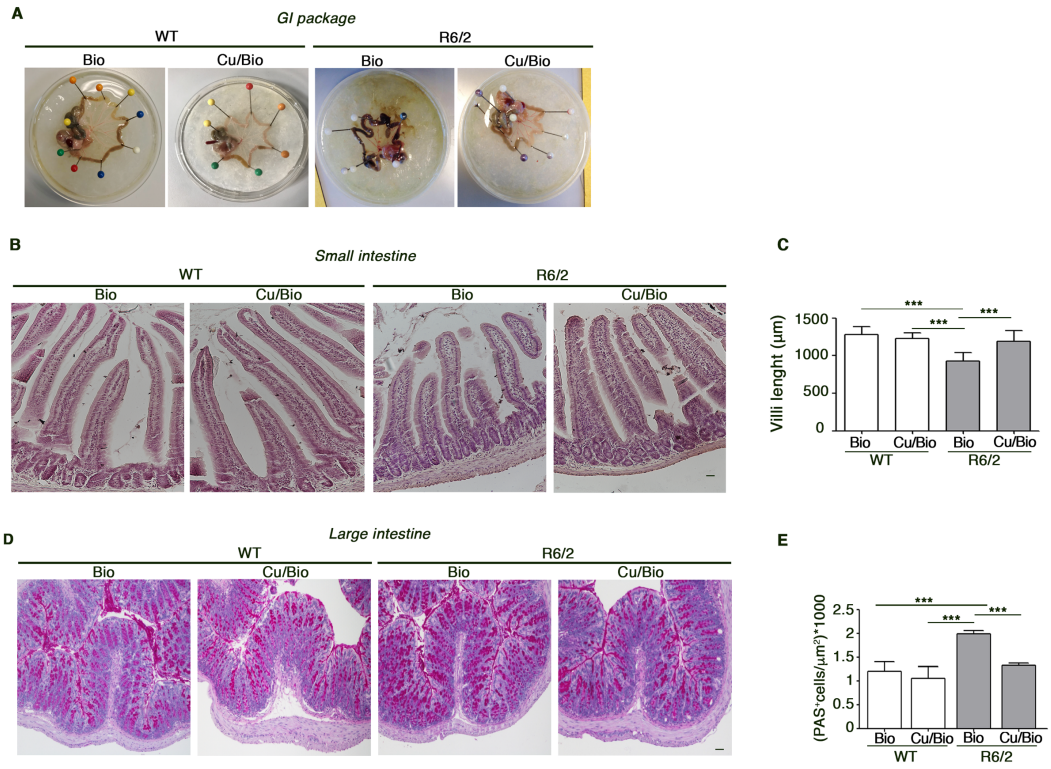

Figure 6. Life-long curcumin-supplemented diet preserves normal gastro-intestinal tissue organization in HD mice. *(A) Representative micrographs of intestinal package from Bio- and Cu/Bio-treated WT mice and R6/2 mice at 10 weeks of age. (B) Representative haematoxylin and eosin stained micrographs of small intestine and (C) analysis of their villi length of Bio- and Cu/Bio-treated WT mice and R6/2 mice at 10 weeks of age. (D) Representative PAS stained micrographs of large intestine and (E) semi quantitative analysis of PAS-positive cells in Bio- and Cu/Bio-treated WT mice and R6/2 mice at 10 weeks of age. Values are represented as mean ± SD. ***, $p<0.001$ (One-Way ANOVA with Tukey post-test). Scale bar represents 100µm.*

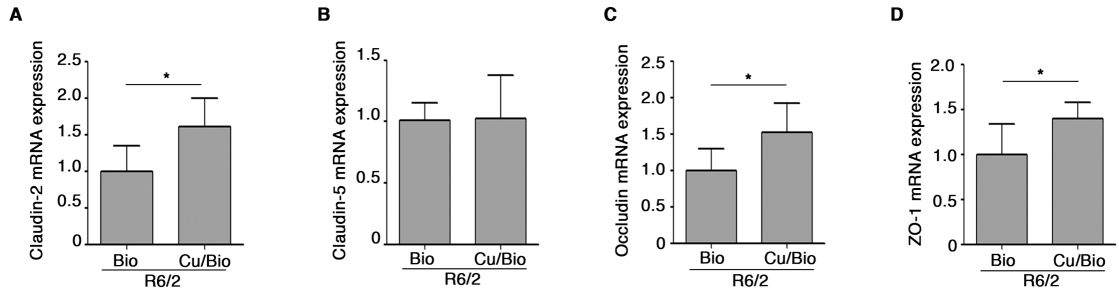

Figure 7. Curcumin increases expression of intestinal barrier genes. *Quantitative PCR analysis of Claudin-2 (A), Claudin-5 (B), Occludin (**C**) and ZO-1 genes in Bio-and Cu/Bio-treated R6/2 mice at ten weeks of age. N=5-6. Data are represented as mean ± SD. *, p<0.05 (Unpaired t-test).*

Discussion

Since many years, curcumin, a component of turmeric is used worldwide in many different forms for its multiple potential health benefits in both preclinical animal models and human patients suffering from pathological conditions, ranging from brain to GI disorders (26, 45, 46). Among the numerous therapeutic properties, the natural neuroprotective and anti-amyloid action makes curcumin a promising molecule for the treatment of several neurodegenerative diseases (46). After oral administration, curcumin readily crosses the blood brain barrier (47), however the beneficial effect is best achieved when it is combined with bioavailability-enhancing agents such as black pepper extracts, like bioperine, that normally acts as an inhibitor of hepatic and intestinal glucuronidation, the metabolic pathway of curcumin. In rodent models of Alzheimer Disease (AD), administration of curcumin reduced plaque burden and protected against *β-amiloid (*Aβ*)*-toxicity *in vitro* and *in vivo* (42, 48, 49), thereby improving cognitive function (49, 50). Evidence indicates that administration of curcumin attenuated neuropathology and transcriptional deficits, including reduction levels of protein aggregates also in HD (34).

HD is conventionally defined as a neurological disorder, however peripheral pathology, including GI perturbation, is increasingly becoming recognized as important factor that may contribute to the complexity of the disease in both patients and animal models (2, 3, 14).

In this study, we reported for the first time, that early intervention with oral curcumin mitigates the neuropathological disturbances as well as gastropathy in R6/2 mice, a HD transgenic animal model which displays some of the clinical features seen in HD patients (14, 15).

Life-long curcumin supplementation attenuated motor dysfunction in R6/2 mice and protected brain from the classical atrophy occurring in these mice as the disease progresses. It evoked activation of pro-survival kinases AKT and ERK in brain tissues of these mice and stabilized the levels of BDNF in both cortex and striatum. Increased bioavailability of the neurotrophin in the striatum, may likely depend on the effect that curcumin may have to support normal axonal transport between cortex and striatum. Unperturbed expression of BDNF in the striatum of treated mice, may conceivable protect the tissue from loss of neurons, as revealed by increased levels of DARPP-32, whose downregulation is a clear sign of neuronal suffering in HD. In line with a neuroprotective action, curcumin also produces substantial decrease in EM48-positive misfolded Htt aggregates.

In addition to a protective effect of curcumin on brain pathology, we found that curcumin supplementation preserved GI homeostasis in R6/2 mice, which ultimately displayed a body weight indistinguishable from WT controls. Although the underlying molecular mechanism needs to be further investigated, we believe that the intestinal function and its preserved contractility after treatment may likely depend on activation of intestinal smooth muscle cells by curcumin rather than stimulation of peripheral neurons. The overall effect of curcumin in conserving intestinal morphology and villi length in treated mice resulted in an adequate absorption surface and nutrient uptake that could also explain the phenomenon. Reduced nutrient absorption along the GI tract has been already reported in R6/2 mice and, it is supposed to contribute itself to the weight loss occurring during the progression of the disease (14).

The therapeutic effect of curcumin on GI dysfunction was further supported by qPCR analysis that highlighted modulation of gene expression of proteins normally involved in the intestinal barrier integrity.

Our data corroborated the evidence of impaired intestinal contractility in R6/2 mice and highlighted, for the first time, an increased number of mucin dense Globet cells along GI tract, that clearly indicates a perturbed intestinal physiology. Life-long treatment with curcumin normalized both intestinal contractility profile and the number of mucin-secreting cells in R6/2 mice to the extent to make them indistinguishable to WT littermates.

In line with previous evidence (28, 29, 31), curcumin-supplemented diet stimulated appetite and regularized the overall function of GI tract in treated R6/2 mice.

All these finding clearly indicated a role of dietary curcumin in inhibiting GI complication and preserving the homeostasis of intestine in HD mice. This result likely suggests that curcumin supplementation may be exploited to reduce paracellular permeability and malabsorption in HD.

The efficacy of curcumin in several pre-clinical trials for neurodegenerative diseases has created considerable excitement mainly due to its lack of toxicity and low cost. Taking into consideration our data and making use of the existing literature in support of the use of curcumin as a beneficial supplementation in humans, we believe that it would be worth investigating the potential therapeutic effect that curcumin administration may have in HD patients and its clinical significance.

Whether the effectiveness of curcumin supplementation in R6/2 mice can be confirmed also in HD patients, needs to be tested, however if this was the case, from our perspective patients

could greatly benefit from it, and although only speculative by now, it might be though to start the supplementation even at pre-symptomatic stage of the disease.
Collectively, results from our study suggest that curcumin could be a worthy candidate for nutraceutical intervention in the disease and represent a new choice for HD patients, as it bears a therapeutic potential to treat both neurologic and gastrointestinal abnormalities. However further clinical investigation is still required.

Material and Methods

Animal model, husbandry and treatment

Breeding pairs of the R6/2 line of transgenic mice [strain name: B6CBA-tgN (HDexon1) 62Gpb/1J] with ~160 ± 10 (CAG) repeat expansions were purchased from the Jackson Laboratories. All procedures on animals were approved by the IRCCS Neuromed Animal Care Review Board and were conducted according to EU Directive 2010/63/EU for animal experiments. Mice were housed in a temperature and humidity-controlled room under 12 h light/dark cycle. Mice were given ad libitum access to food and water. Male R6/2 mice were crossed with female B6CBA wild-type (WT) mice and the resultant WT and R6/2 mice were used for all the experiments performed in this study. Breeding pairs were checked daily for litters and two rounds breeding were used to generate mice for preclinical studies. Female breeding WT mice were fed normal cow supplemented with curcumin (25mg/kg/day)/bioperine (1mg/kg/day)/DMSO (Cu/Bio) or bioperine (1mg/kg/day)/DMSO (Bio). One week before breeding, WT females (n=8) were divided into two groups and the oral gavage administration of Bio or Cu/Bio was started. Females were then crossed with R6/2 males fed with normal cow. Dietary supplementation of Cu/Bio in breeding females was performed for the entire period of gestation and for three weeks after the offspring birth. Treatment continued in weaned pups for 7 weeks (Supplementary Figure 8).
In order to assess any possible effect of bioperine, a pilot study was performed on a small group of both WT and HD mice. To this purpose, bioperine dissolved in DMSO and diluted in saline (vehicle) was daily administered by gavage at dose of 1mg/kg per day and motor function was assessed. Control mice (WT and R6/2) were daily fed with the same volume of vehicle containing DMSO. As reported in the Supplementary Figure 9, no effect was detected in any of the groups.
A total of 43 litters (17 WT: 8 Bio and 9 Cu/Bio; 26 R6/2: 13 Bio and 13 Cu/Bio) were generated and used in this study. Food consumption in adult progeny was determined over

the course of a 24 h observation period. Mice were housed separately to permit each animal's food consumption to be calculated from the difference in weights of the food supply at the beginning and the end of the observation period.

General health monitoring and motor behavior tests

The overall animal health was monitored every day. Body weight was recorded in the offspring once per week starting from 4 weeks of age. Motor performance and abnormalities were assessed by Rotarod tests and hindlimb clasping behaviour, respectively, as previously described (51, 52). Mice were habituated to the testing rooms for 15-20 mins prior to testing. All tests took place during the light phase of the light–dark cycle and littermates were tested for the entire period of the treatment at the indicated time points. All analyses were carried out blinded to genotype and treatment.

Measurement of gastric emptying by 24 h stool collection

For the assessment of gastric emptying, single housed mouse was placed in a separate clean cage and fecal pellets were collected after a 24 hour-period in 1.5 ml eppendorf tubes. Tubes were weighed to obtain the wet weight of the stool.

Measurements of intestinal contractility

At designated time point, animals were sacrificed by cervical dislocation and the peritoneal cavity was opened via a U-shaped incision based in the lower abdomen for complete removal of the abdominal package. Colonic segments were then isolated and contractility was measured in organ baths containing Krebs-Ringer buffer at 37°C and aerated with 95% O_2 and 5% CO_2. Colonic segments were stretched gradually to 400g tension and challenged with 10mM potassium chloride (KCl). Changes in isometric tension were measured and recorded by LabScribe 2 software (BIoseb).

Histological and immunohistochemical analysis of intestine

After sacrificing small and large intestine segments were dissected out, gently flushed with cold sterile saline solution to remove intestinal contents and immediately placed in 10% neutral buffered formalin for no longer than 48 hours and then processed for histology. Intestinal segments were then cut into 5 μm coronal sections on an RM 2245 microtome (Leica Microsystem) and stained with haematoxylin/eosin (H&E) and Periodic acid–Schiff (PAS) for the assessment of villi length and detection of the secretory cells lining the

gastrointestinal tract, the globet cells, respectively. Four mice/group were used and six coronal sections for each animal were scanned. For each coronal section, a total number of 2 fields at 10x magnification was analyzed. Images were acquired with Nikon Eclipse Ni motorized microscope. All stained sections were examined on a blinded basis.

Villi length. Only well-orientated villi indicating complete longitudinal sectioning were selected for the analysis. The measurements were taken linearly at the center of the villus from the basis at the crypt-villus junction to the villus apex. The average of villi length per small intestine section was quantified by NIS-Elements AR Analysis. Mean villus length per segment was estimated as the mean of the tallest four to seven villus profiles measured per location.

PAS-positive Globet cells. Paraffin embedded 5 ¦ m colon specimen sections were stained with PAS-haematoxylin system (Sigma-Aldrich) to assess the positive goblet cell population (53). Results were expressed as "(*PAS* positive cells/μm^2)*1000".

RNA extraction from intestinal samples and q-PCR

Total RNA from small intestine was extracted using RNeasy kit (Qiagen) according to the manufacturer's instructions. 1000 ng of total RNA was synthesized using Super Script III reverse transcriptase (Invitrogen) and the resulting cDNA was then amplified by quantitative RT-qPCR To determine the mRNA expression levels of intestinal barrier genes, using specific primers:

mouse Claudin-1 FW: 5'-CTGGAAGATGATGAGGTGCAGAAGA-3';
mouse Claudin-1 RV: 5'-CCACTAATGTCGCCAGACCTGAA-3';
mouse Claudin-2 FW: 5'-TGAACACGGACCACTGAAAG-3';
mouse Claudin-2 RV: 5'-TTAGCAGGAAGCTGGGTCAG;
mouse Claudin-5 FW: 5'-CAGTTAAGGCACGGGTAGCA-3';
mouse Claudin-5 RV: 5'-GGCACCGTCGGATCATAGAA-3';
mouse Claudin-15 FW: 5'-GCAGGGACCCTCCACATA-3';
mouse Claudin-15 RV: 5'-GACGGCGTACCACGAGATAG-3';
mouse Occludin FW: 5' AGACCTGATGAATTCAAACCCAAT-3';
mouse Occludin RV: 5'-ATGCATCTCTCCGCCATACAT-3';
mouse ZO-1 FW: 5'- TTCTTCGAGAAGCTGGATTCCT-3';
mouse ZO-1 RV: 5'- TCTGGCAACATCAGCTATTGGT-3';
mouse Cyclophilin A FW: 5'- TCCAAAGACAGCAGAAAACTTTCG-3';
mouse Cyclophilin A RV: 5'- TCTTCTTGCTGGTCTTGCCATTCC-3'.

RT-qPCR was performed on a CFX Connect RT-PCR Detection System (Bio-Rad Laboratories) by SYBR-Green mix (Lifetech, Cat. No.: 4367659). Cyclophilin A was used as a housekeeping gene for normalization of mRNA expression results.

Brain pathology and immunohistochemistry

After sacrifice, brains were pulled out of the skull and trimmed by removing the olfactory bulbs and spinal cord. The remaining brain was then weighed, processed for histology and embedded in paraffin wax for microtome cutting. Four mice/group were used, and four coronal sections spread over the anterior–posterior extent of the brain (200 –300 μm inter-section distance) were scanned. For each 10 μm coronal section, a total number of 5 fields at 20x magnification were analyzed. Immunostaining for mutant Htt aggregates was carried out by using EM48 antibody (1:200) (Millipore). The number of striatal mHtt inclusions as well as their average size of per brain section was quantified by ImageJ software.

Brain lysate and immunoblottings

After sacrifice, some of the mouse brains were snap-frozen in liquid N2 and pulverized in a mortar with a pestle. Pulverized tissue was then homogenized in lysis buffer containing 20 mm Tris, pH 7.4, 1% Nonidet P-40, 1 mm EDTA, 20 mm NaF, 2 mm Na3VO4 and protease inhibitor mixture (Santa Cruz, Cat. N. sc-29131), sonicated with 2 × 10s pulses and then centrifuged for 10 min at 10 000g. Protein concentration was determined by Bradford method. Protein lysates (20μg) were resolved on 10% SDS-PAGE and immunoblotted with the following antibodies: anti-phospho-AKT (1:1000) (Immunological Sciences, Cat. N. AB-10521); anti-phospho-ERK (1:1000) (Cell Signaling, Cat. N. #9101); anti-ERK (1:1000) (Immunological Sciences, Cat. N. AB-82379); anti-DARPP-32 (1:1000) (Cell Signaling, Cat. N. #2302); anti-BDNF (1:1000) (Santa Cruz, Cat. N. sc-546), and anti-Actin (1:5000) (Sigma Aldrich, Cat. N. A5441). For the analysis of SDS-insoluble mHtt aggregates, cell lysates were resolved on 10% SDS-PAGE, the entire gel, including the stacking portion, was transblotted over-night a 250 mV in 0.05% SDS and 16% methanol-containing transfer buffer (51). Membrane was blocked in 5% non-fat dry milk TBST for one hour and successively immunoblotted with EM48 antibody (1:1000). Immunoblots were then exposed to specific HRP-conjugated antibodies (Santa Cruz, Cat. N. sc-2004 and sc-2005). Protein bands were visualized by ECL and quantitated with Quantity One software (Bio-Rad Laboratories).

Statistics

Two-Way ANOVA followed by Bonferroni post-test was used to compare experimental groups for Rotarod tests and for mouse body weight analysis. One-Way ANOVA and Two-tailed Unpaired *t*-test were used in all other experiments as indicated. All data were expressed as mean ± SD.

Funding

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Acknowledgments

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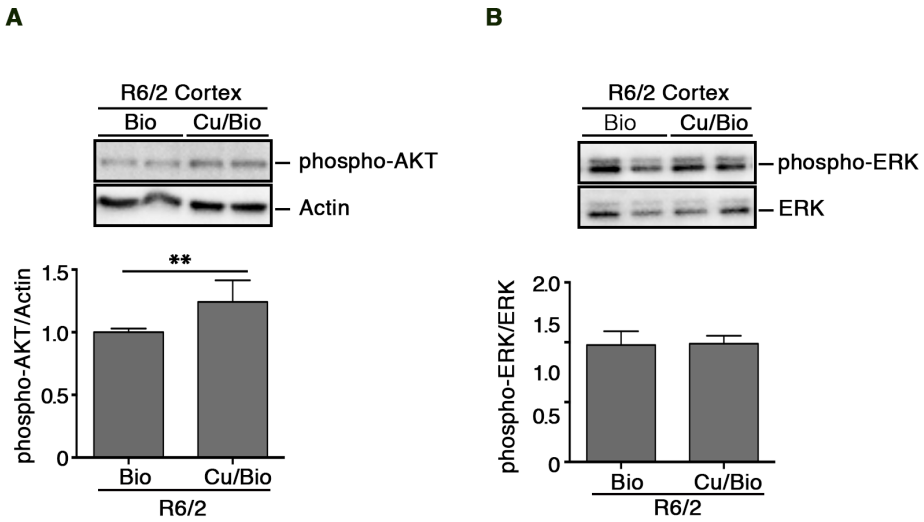
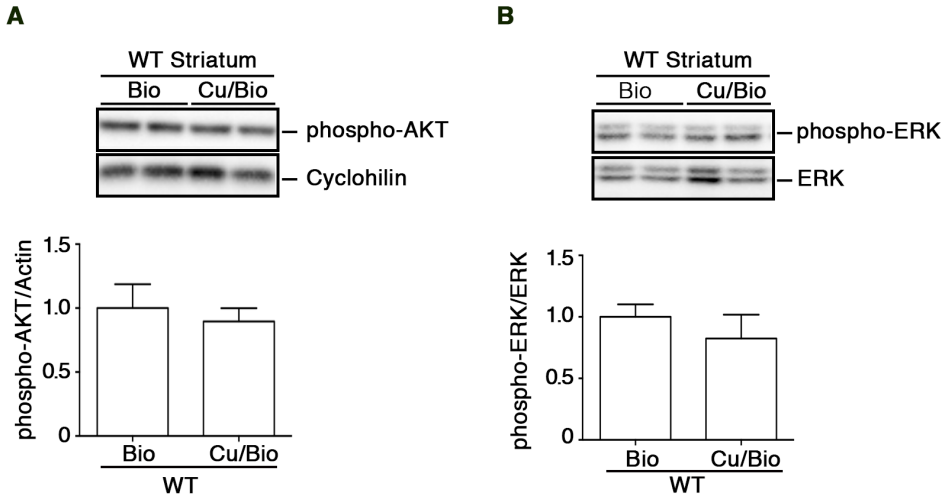
Conflicts of Interest: The authors declare no conflict of interest.

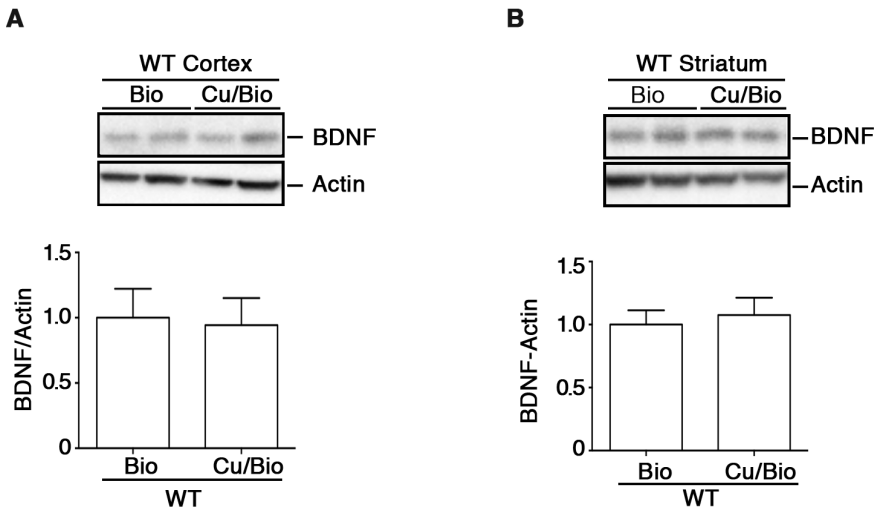
Supplementary Material



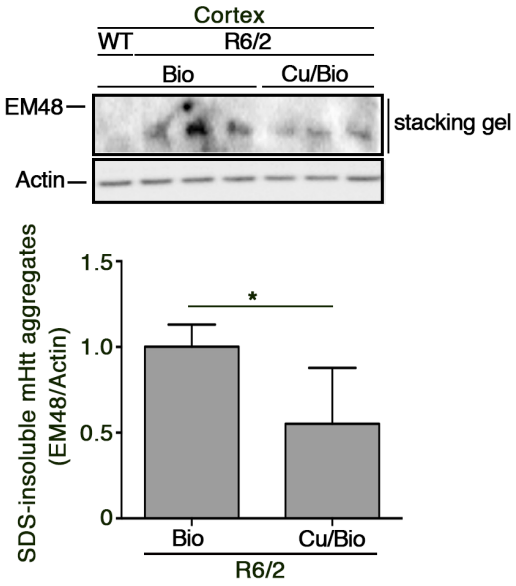
Supplementary Figure 1. Life-long curcumin-supplemented diet evokes the activation of pro-survival kinase AKT in R6/2 cortex. *Representative immunoblottings and densitometric analysis of phospho-AKT (**A**) and phospho-ERK (**B**) in cortical tissues from Bio- and Cu/Bio-treated R6/2 mice. N=7 for each group of mice. Values are represented as mean ± SD. Bio: Bioperine; Cu: Curcumin; Cu/Bio: Curcumin/Bioperine.*



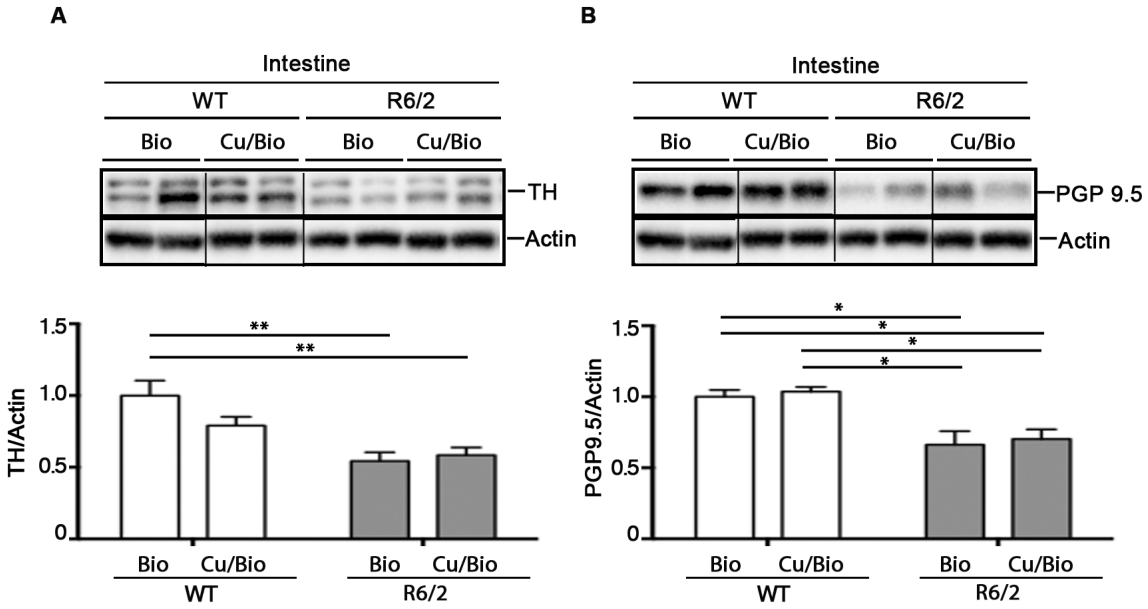
Supplementary Figure 2. Life-long curcumin-supplemented diet does not evoke any activation of pro-survival pathways in WT striatum. *Representative immunoblottings and densitometric analysis of phospho-AKT (**A**) and phospho-ERK (**B**) in striatal tissues from Bio- and Cu/Bio-treated WT mice. N=5 for each group of mice. Values are represented as mean ± SD. Bio: Bioperine; Cu: Curcumin; Cu/Bio: Curcumin/Bioperine.*



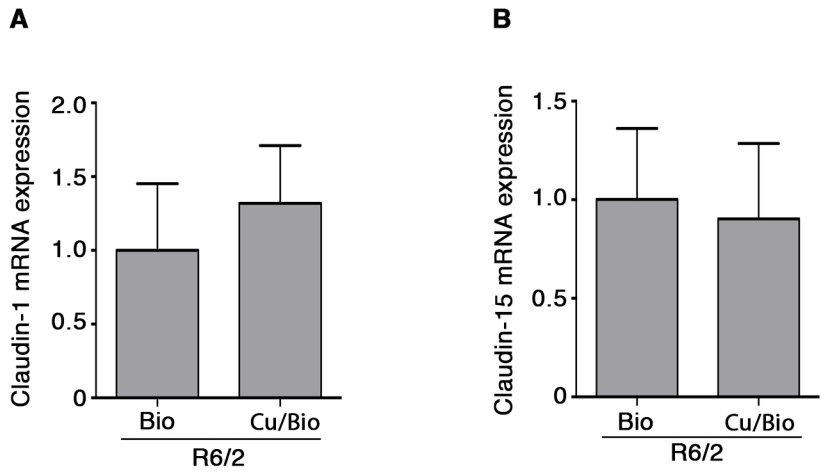
Supplementary Figure 3. Curcumin does not modulate levels of BDNF in WT mouse brain. *Representative cropped immunoblottings of BDNF in cortex (**A**) and striatum (**B**) from Bio- and Cu-treated WT mice at ten weeks of age. N=5 for each group of mice. Values are represented as mean ± SD. Bio: Bioperine; Cu: Curcumin; Cu/Bio: Curcumin/Bioperine.*



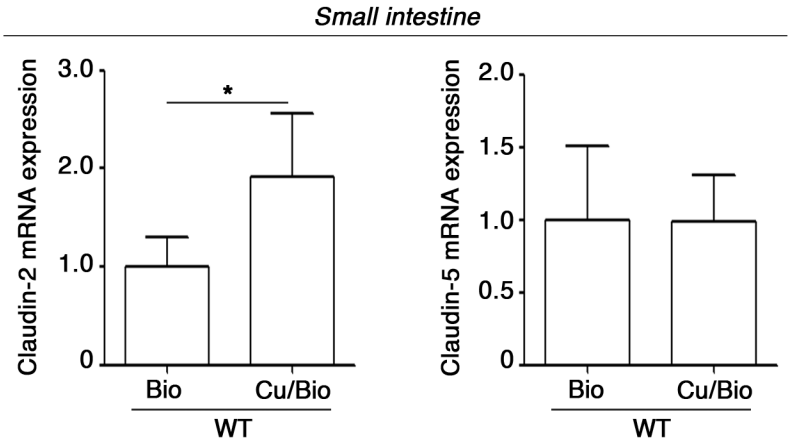
Supplementary Figure 4. Life-long curcumin-supplemented diet reduces SDS-insoluble aggregates in R6/2 mouse cortex*. Cropped immunoblotting of EM48-positive mHtt aggregates in cortical lysates from Bio- and Cu/Bio-treated R6/2 mice at 10 weeks of age. N=6 for each group of mice. Values are represented as mean ± SD. *, p<0.05 (Un-paired t-test). Bio: Bioperine; Cu: Curcumin; Cu/Bio: Curcumin/Bioperine.*



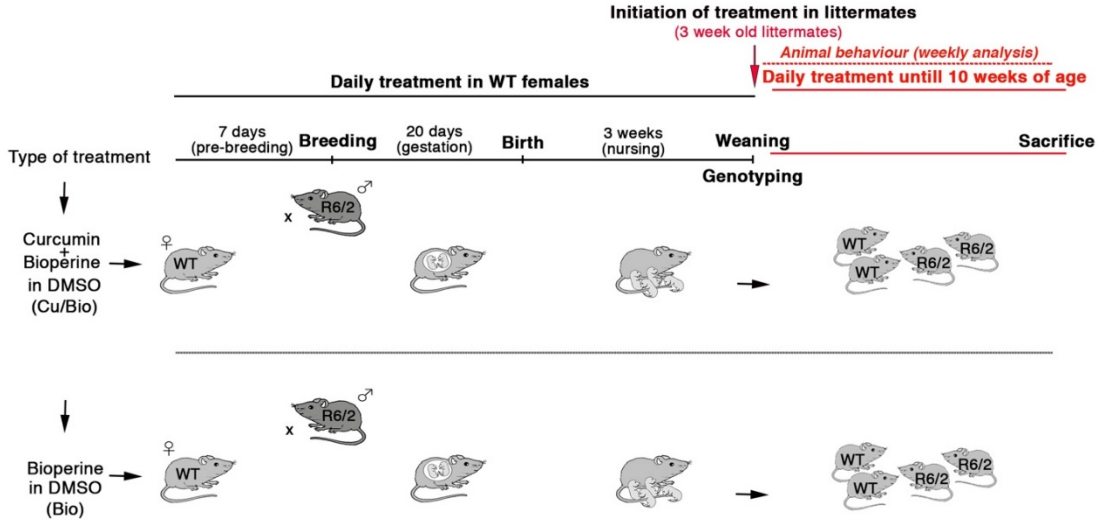
Supplementary Figure 5. Curcumin does not modulate the expression of neuronal markers in the intestine of both WT and R6/2 mice. *Representative cropped immunoblottings and quantitative analysis of the expression of neuronal markers Tyrosine Hydroxylase (TH) (**A**) and PGP9.5 (**B**) in intestinal protein lysate from Bio- and Cu/Bio-treated WT and R6/2 mice at ten weeks of age. N=6 for each group of mice. Values are represented as mean ± SD. *, p<0.05; **, p<0.01 (One-Way ANOVA with Tukey post-test). Bio: Bioperine; Cu: Curcumin; Cu/Bio: Curcumin/Bioperine.*



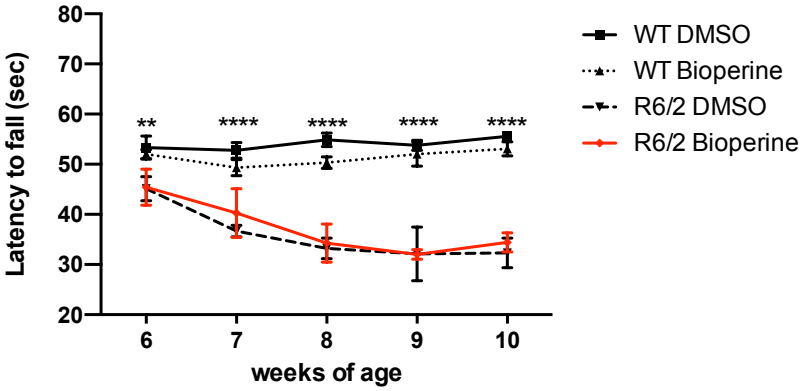
Supplementary Figure 6. Curcumin does not modulate expression of intestinal barrier Claudin-1 and -15 genes in R6/2 mice. *Quantitative PCR analysis of Claudin-1 (**A**) and Claudin-15 (**B**) genes in Bio-and Cu/Bio-treated R6/2 mice at ten weeks of age. N=6 for each group of mice. Values are represented as mean ± SD. Bio: Bioperine; Cu: Curcumin; Cu/Bio: Curcumin/Bioperine.*



Supplementary Figure 7. Curcumin modulates expression of intestinal barrier genes. *Quantitative PCR analysis of Claudin-2 and Claudin-5 genes in Bio-and Cu/Bio-treated WT mice at ten weeks of age. N=5 for each group of mice. Values are represented as mean ± SD. *, p<0.05 (Unpaired t-test). Bio: Bioperine; Cu: Curcumin; Cu/Bio: Curcumin/Bioperine.*



Supplementary Figure 8. Experimental plan for life-long administration of curcumin*. One week before breeding, WT females (N=8) were divided into two groups and fed with either Bioperine/DMSO (Bio) or Curcumin/Bioperine/DMSO (Cu/Bio). Females were then crossed with R6/2 males fed with regular diet. Breeding females were fed with Cu/Bio-supplemented diet for the entire period of gestation and for three weeks after the offspring birth. Treatment continued in weaned pups which were then used to perform all in vivo and biochemical experiments.*



Supplementary Figure 9. Administration of Bioperine (Bio) does not affect motor performance in both WT and HD mice*. Motor performance assessed by Rotarod. WT, N=3+4; R6/2, N=3+4. Values are represented as mean ± SD. **, p<0.01; ****, p<0.0001 (DMSO- and Bioperine-treated WT vs DMSO- and Bioperine-treated R6/2 mice) (Two-Way ANOVA with Bonferroni post-test).*

Supplementary Methods

Intestinal lysate preparation and immunoblottings. Mouse intestines were snap-frozen in liquid N2 and pulverized in a mortar with a pestle. Pulverized tissue was then homogenized in lysis buffer containing 20 mm Tris, pH 7.4, 1% Nonidet P-40, 1 mm EDTA, 20 mm NaF, 2 mm Na3VO4 and protease inhibitor mixture (Santa Cruz, Cat. N. sc-29131), sonicated with 2 × 10s pulses and then centrifuged for 10 min at 10 000g. Protein concentration was determined by Bradford method. Protein lysates (20µg) were resolved on 12% SDS-PAGE and immunoblotted with the following antibodies: anti-Tyrosine Hydroxylase (TH) (1:1000), anti-PGP9.5 (Abcam, Cat. N. ab112) and anti-Actin (1:5000) (Sigma Aldrich, Cat. N. A5441).

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Studies on colon contractility

Experiments not included in the above publication, aimed at investigating molecular mechanisms underlying alterations of colon motility were performed.

Constipation phenomena can be due to various factors, for example to aberrations in the enteric nervous system, in the interstitial cells of Cajal ("pacemaker cells" of the gastrointestinal tract), in alterations of the enteric neurotransmitters such as acetylcholine, serotonin, dopamine, noradrenaline, vasoactive intestinal peptide and nitric oxide or in alterations of muscle contraction. Molecularly, muscle contraction is due to the interaction of two different types of filaments, thin and thick filaments with hydrolysis of ATP. Thin filaments consist mainly of actin while thick filaments consist predominantly of myosin. Although in smooth muscle contraction is primarily regulated by phosphorylation of myosin light chain, other mechanisms that regulate contraction are based on proteins associated with actin such as caldesmon, calponin, tropomyosin and smoothelin. These proteins regulate actin-myosin interaction and thus muscle contraction. In particular, Smoothelin-A is an actin-binding protein and a marker protein for visceral smooth muscle cells. The exact role of this proteins in smooth muscle contraction is not yet clear but a mouse model knock-out for Smoothelin showed its crucial role in intestinal contraction. In fact its absence caused an impaired contraction of intestinal smooth muscle (Niessen et al. 2005). Caldesmon is capable of stabilizing actin filaments, inhibiting the actin-myosin interaction and in addition it also inhibits actin-myosin ATPase activity. Thus, an increased level of caldesmon expression causes a decrease in the contraction of smooth muscle.
The transcriptional expression levels of these two genes and in addition, of Myocardin, a master regulator of smooth muscle gene expression (Wang et al 2003) was analyzed in order to understand if there was an alteration in the general induction of smooth muscle genes in the colon of R6/2 mice.

Methods

Real-time PCR analysis

Total RNA was isolated from colon of treated and untreated R6/2 mice using *TriReagent* according to the manufacturer's protocol. Absorption at 260 and 280 nm was measured by Nanodrop and RNA quantity and A_{260}/A_{280} ratio were calculated. 1000 ng of total RNA was synthesized using Super Script III reverse transcriptase (Invitrogen) and the resulting cDNA was then amplified by quantitative RT-qPCR. mRNA expression levels of genes were calculated according to the $2^{-\Delta\Delta CT}$ method, normalizing to Cyclophilin A and using specific primers:

h-Caldesmon Fw: CGCAGAGAACTCAGGAGACA;

h-Caldesmon Rv: CTGTCACCTGTCCCAAGGAT;

Smoothelin A Fw: CGTGAGCTCCGACAAAGAA;

Smoothelin A Rv: CGCTCGGTTTTGGTAACTGT;

Myocardin Fw: AAGGTCCATTCCAACTGCTC;

Myocardin Rv: CCATCTCTACTGCTGTCATCC;

Cyclophilin A Fw: 5'-TCCAAAGACAGCAGAAAACTTTCG-3';

Cyclophilin A Rv: 5'- TCTTCTTGCTGGTCTTGCCATTCC-3'.

RT-qPCR was performed on a CFX Connect RT-PCR Detection System (Bio-Rad Laboratories) using *SYBR-Green* mix (Lifetech, Cat. No.: 4367659).

Results and Discussion:

Gene expression analysis did not reveal any variation in the transcriptional expression levels of the three genes analyzed, neither between WT and R6/2 mice, nor with curcumin-treated mice. The investigation was conducted exclusively at the transcriptional level, therefore it has not to be considered exhaustive, since the transcriptional expression may not correspond to the protein expression. However, it is important to emphasize that the genes analyzed did not show different expression levels between WT and R6/2, indicating that they are not probably involved in the molecular mechanisms underlying constipation. Moreover, on the other hand the regulation of the contractility phenomenon, as mentioned above, is complex and involves many other mechanisms and genes that were not analyzed.

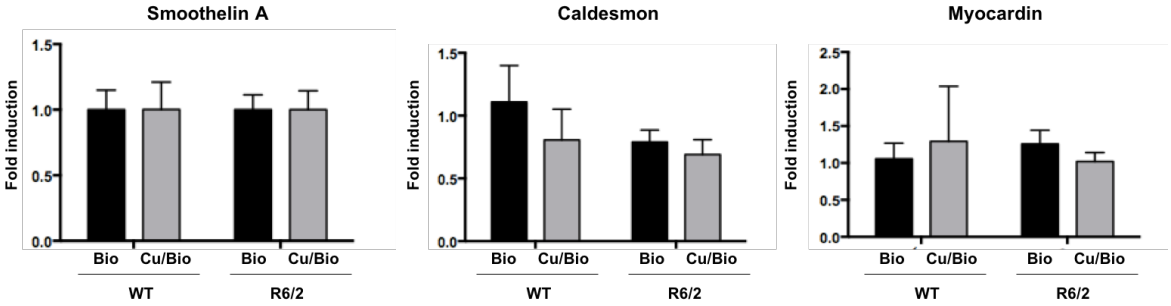

Figure 1. *Gene expression analysis of important regulators of smooth muscle contraction: Smoothelin-A, Caldesmon (Thin-Filament Associated Proteins) and Myocardin, a master regulator of smooth muscle gene expression.*

Chapter 4

Study of excitotoxicity

This chapter regards experiments performed on a different experimental system, i.e. human iPSC-derived neural progenitor cells, derived from human induced pluripotent stem cells (hiPSC) for the study of excitotoxicity, a complex phenomenon of neuronal death playing an important role in many diseases including neurodegenerative ones.

Excitotoxicity is induced by an excessive excitatory stimulus that induces hyperactivation of neuronal receptors. Specific receptors of neurotransmitters can be of two types: ionotropic or metabotropic. The first one, following the interaction with the specific ligand, takes on the shape of a channel and allow the passage of ions, while the second one, following the interaction with the ligand, activates a cascade of signals which ends with the release of intracellular calcium (Velasco et al. 2017).
The main excitatory neurotransmitter of the CNS is glutamate, a fundamental amino acid involved in various neurological functions such as memory, movements and sensations that generates post-synaptic signaling by binding both to ionotropic-type receptors (NMDA, AMPA, kainate) and to metabotropic receptors. To favor the neuronal energy balance, most of the glutamate used at the synapse level derives mostly from its own recycling through the glutamate-glutamine cycle (Westergaard et al. 1995). In fact, normally, to regulate the concentrations of glutamate in the extracellular space, following the release by the synaptic terminals, it is subtracted from the synaptic circuit by the Excitatory Amino Acid Transporters (EAAT) located on the astrocytes, which incorporate it into their cytoplasm and convert it in glutamine. Then, glutamine is released by astrocytes into the synaptic circuit where it can be "captured" by the synaptic terminals to be converted back into glutamate. When there is an excessive release of glutamate or a problem in its uptake by astrocytes, there is an excessive stimulation of the glutamate receptors. The excessive stimulation of NMDA receptors, which could also be due to impaired function of the receptor itself, determines the phenomenon of excitotoxicity (Velasco et al. 2017). This occurs because the NMDA receptors allow the entry of calcium and sodium into the neurons and the exit of the potassium ion in the extracellular space. Thus, if excessively stimulated, NMDA receptors allow the entrance of high concentrations of intracellular calcium which induce a cascade of harmful processes for the neuron (such as protease, lipase, endonuclease, caspase and nitroxide synthase activation) that cause neuronal death by necrosis or apoptosis.

Therefore, it's clear that excitotoxicity is an extremely complex phenomenon that causes neuronal death in a large number of pathologies such as epilepsy, head injury, hypoglycemia, including neurodegenerative diseases.

Given the large amount of pathological conditions in which excitotoxicity plays a crucial role, including the neurodegenerative progressions, it is of considerable importance to study the molecular mechanism and, importantly, to find out new drugs able to counteract this phenomenon.

Certainly, the ideal environment to study the phenomena of excitotoxicity in human is represented by the human brain itself but these are possible only on post-mortem brains. However, a post-mortem brain is only the point of arrival of several phenomena that have occurred previously, now concluded and does not allow to evaluate the evolution of the phenomenon but, more importantly, doesn't allow to test for new drugs.

To date, such studies are mostly carried out on animal models or on immortalized cells. Despite animal models certainly represent a more physiological model they do not allow the screening of many drugs for ethical reasons. Further, they also suffer from interspecies differences. In fact, as far as you can carefully choose an animal model, it will never be the optimal model since the physiological processes are certainly not exactly the same as those that occur in humans. The brain of rodents, which represent the most widely used *in vivo* model, shows significant differences in both development and structure compared to the human brain (Clowry et al., 2010). In addition, it's clear that rodent neurons show different electrophysiological properties compared to human ones, suggesting that there are also molecular and cellular differences between humans and rodents, which explains why many drugs that work in animal models fail when tested in humans. On the other hand, immortalized cellular models while allowing to screen many molecules in drug discovery experiments have a modified genetic background and do not represent a physiological model.

There is therefore the need for a human non-immortalized *in vitro* system that can reflect the complexity of excitotoxicity to better investigate this type of neuronal death and test new drugs to hinder it.

In this scenario, the human induced pluripotent stem cells (hiPSC) (which are stem cells artificially obtained in the laboratory from terminally differentiated cells) represent an incredible opportunity since they are of human source and they can be differentiated into different types of neuronal populations: use of this experimental system doesn't cause the

same ethical problems related to human embryonic stem cells and they have all the advantages of an *in vitro* model.

Despite the appearance of these cells has sparked widespread enthusiasm, they are not without limitations. It emerged, for example, that hiPSC-derived neurons are not fully mature as only a fraction of the cells emits action potentials, many have immature synapses and many of them express markers typical of immature neurons (Marchetto et al., 2010). In fact, these neuronal cultures often have a rather low percentage of differentiation and in culture there is a considerable heterogeneity.

However, knowledge on cultures derived from iPSC are still few and many characteristics typical of human neurons, including excitotoxicity, have yet to be analyzed in this model.

Experimental aim was, at a first attempt, to evaluate whether dopaminergic neurons hiPSC-derived are capable to respond to excitotoxic stimuli. The final goal would be to screen for neuroprotective substances capable to counteract this phenomenon.

Methods

After a period of expansion of the stem line, dopaminergic differentiation was carried out for a period of 6 weeks. At the sixth week of the differentiation process, experiments were performed to evaluate the excitotoxicity phenomena induced by an excessive NMDA stimulus, with and without MK-801 (Dizocilpine) or D-APV (D-2-Amino-5-phosphonovaleric acid**),** which are respectively a non-selective antagonist and a selective NMDAR receptor antagonist. Downstream of the treatments, cytotoxicity experiments were conducted.

Preparation of Culture and Expansion of Human Neural Progenitors

Human iPSC-derived neural progenitor cells (hNPCs), purchased from Axol Bioscience (ax0016) were thawed by liquid nitrogen and expanded following the guidelines provided by the same company. hNPCs were plated on sterile 6 well tissue culture plates pre-coated with SureBond-XF 1X at a density of 75000/cm^2 in Plating-XF Medium. 24 hours after plating, media was replaced with Neural Expansion-XF Media supplemented with 20 ng/mL FGF2 and 20 ng/mL EGF. When the cells were approximately 80% confluent, they were passaged and further expanded. Cells were incubated at 37° C and 5% CO_2.

Differentiation from hNPCs to Dopaminergic Neurons

hNPCs were passaged in Plating-XF Medium and plated at a density of 30000/cm^2 in 6-well pre-coated with SureBond-XF 1X. 24 hours after passage, media was changed to STEMdiff™ dopaminergic neuron differentiation medium (STEMcell Technologies) supplemented with 200 ng/mL Sonic Hedgehog. Media was changed every other day for 14 days. The cells were then passaged and plated at a density of 20000/cm^2 in 96-well pre-coated with SureBond-XF 1X. Media was changed to STEMdiff™ dopaminergic neuron maturation medium 1 (STEMcell Technologies). Media changes were performed every 48 hours for 5 days. Finally, media was changed with Maturation Medium 2 (STEMcell Technologies) and replaced every 48 hours until a total culture time of 6 weeks (see Figure 2). Cells were incubated at 37°C and 5% CO_2.

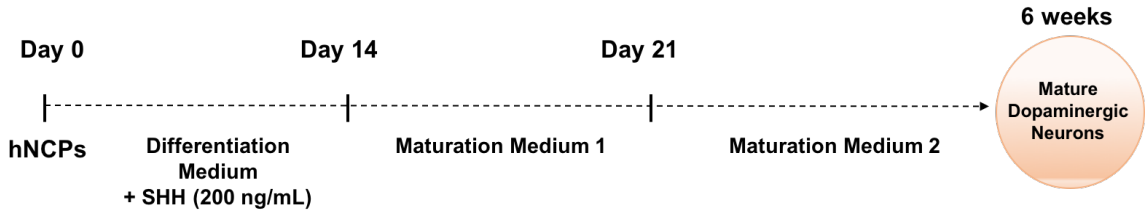

Figure 2. *Schematic representation of differentiation of hNPCs to mature dopaminergic neurons.*

NMDA Treatment

Differentiated neurons were stimulated with NMDA for 6 weeks in two different ways. In one case, NMDA (50-100 µM) diluted in basal medium was added for 30 minutes and the treatment was terminated by replacing it with fresh complete medium. The cells were maintained for further 22 hours in incubator. Then, the amount of lactate dehydrogenase released into the medium was measured by LDH assay (see after).

In another case, neurons were incubated with NMDA 100 µM diluted in basal medium for 22 hours. Then, the amount of lactate dehydrogenase released into the medium was measured (see after).

In order to determine whether the effect was dependent on functional NMDA receptors, some experimental points were also included with D-APV (50 µM), a selective NMDA

receptor antagonist or MK-801 (10 μM) a noncompetitive antagonist of the NMDA receptors. In detail, these two drugs were added separately 20 minutes earlier than the NMDA treatment and maintained for the duration of the treatment. All drugs were purchased from Sigma-Aldrich.

LDH assay

After twenty-two hours, the LDH assay was performed on the cell supernatant, following the instructions reported in the relative kit (ab65393). While the cells left in the plate were used for Hoechst 33342 assay.

Hoechst assay

After gently removing all the medium from each well, cells were washed in PBS pre-warmed to 37°C before fixation in 4% paraformaldehyde for 10 minutes at room temperature (RT). 3 washes with cold PBS were performed and cells were incubated with Hoechst 33342 at the concentration of 1 μM (diluted in PBS 1X) for 15 minutes in the dark. Cells were then washed 3 times for 5 minutes with PBS in the dark and Images were collected using a FLoid Cell Imaging Station USB microscope (Ex: 390 nm, Em: 446 nm) with a 20x objective (NA 0.45) at RT. Each concentration was repeated a minimum of 4 times. After imaging, cells were stained with Tyrosine Hydroxylase antibody as described in ICC staining.

Analysis were performed using Fiji (ImageJ) semi-automatic nuclei counting function. Images were threshold adjusted in two stages: one that removed all artifacts but included all Hoechst nuclei; one that also removed "dim" nuclei, sparing only the "bright" nuclei. The watershed function of ImageJ was used to divided multiple grouped nuclei. Finally, the analyses particle function with a 10-pixel exclusion filter was performed to quantitatively determine bright vs. dim nuclei percentages.

The % of cell death was calculated as:

% Cells death = (Number of bright nuclei/ Total number of nuclei) *100

*I*mmunocytochemistry (ICC) *Staining*

Cells were centrifuged at 600 g for 5 minutes so that they were all on the bottom of the plate. After gently removing all the medium from wells, cells were washed in PBS pre-warmed to 37°C and fixated in 4% paraformaldehyde for 10 minutes at RT. Then, 3 washes with cold PBS 1X were performed and cells were permeabilized with 0.1% Triton X-100 for 10 minutes at RT. Cells were washed for 3 times for 5 minute washes with PBS containing

0.1% Tween-20 and then blocked for 30 minute with a solution containing PBS containing 1% BSA and 0.1% Tween-20 to prevent non-specific antibody binding. Primary antibody incubation was performed at for 2 hour in PBS containing 1% BSA and 0.1% Tween-20. After 3 washes for 5 minutes with PBS containing 0.1% Tween- 20, secondary antibody conjugated with AlexaFluor fluorescent dye incubation was performed O.N. in the dark at 4°C in PBS containing 1% BSA. Cells were then washed with PBS in the dark at RT. Nuclei were stained incubating with Hoechst 1 μM for 2 minutes and after further 3 washes for 5 minutes each, the pictures were collected using a FLoid Cell Imaging Station USB microscope with a 20x objective (NA 0.45) at RT.

The antibodies used were: rabbit anti- Tyrosine Hydroxylase (1:400) with a goat anti-rabbit AlexFluor-488 secondary (1:300) and for double immunofluorescence, primary antibody: chicken anti-Tyrosine Hydroxylase with a goat anti-chicken AlexFluor-568 secondary (1:300) and rabbit anti- cleaved caspase-3 (1:300) with a goat anti-rabbit AlexFluor-488 secondary (1:500).

Results and Discussion:

Starting from hNPC, dopaminergic neurons have been obtained through a six-week differentiation process, with an estimated percentage of about 15%.

In Figure 3 it is possible to see how the cells have coherently changed morphology in differentiation made. In particular, in Figure 3 A are observed neuronal rosettes, structures that spontaneously assume hNPC when plated and that represent a state of early neuronal differentiation; in Figure 3 B we see how, under differentiation, neurons develop primary dendrites, and in Figure 4 a dopaminergic neuron at 6 weeks of differentiation is identified by expression of Tyrosine Hydroxylase, a marker for dopaminergic neurons (green).

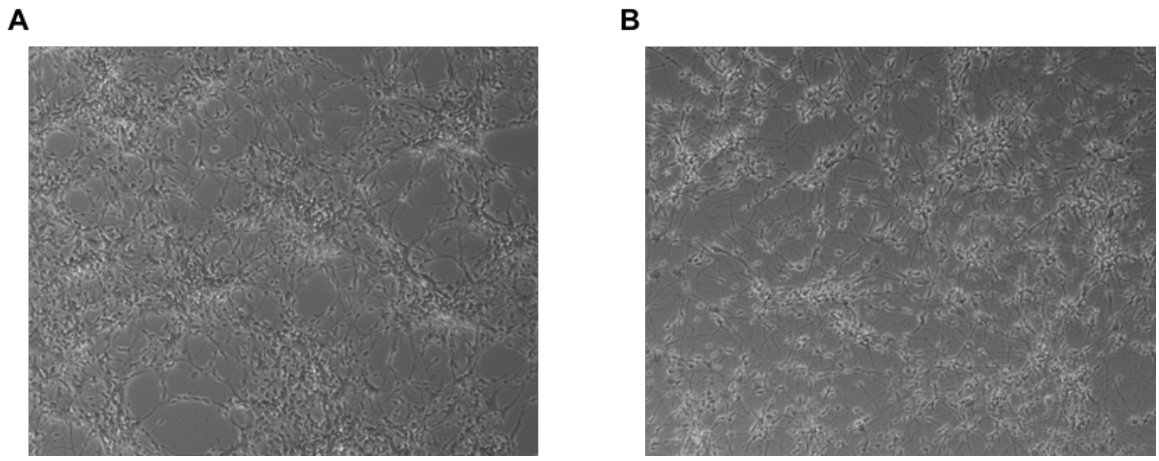

Figure 3. *(A) Early stage of hNPC differentiation: cultures showing the formation of neural rosette structures, (B) cells at 5 week of differentiation in dopaminergic neuron culture showing dendrite formation.*

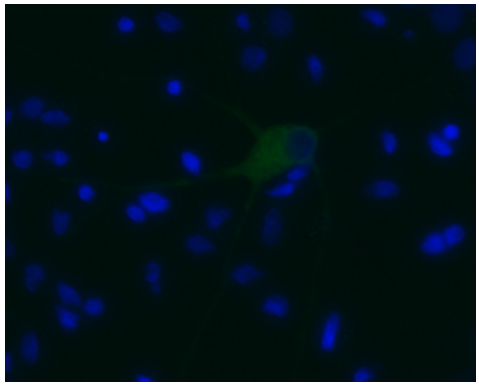

Figure 4*. Representative fluorescence image of cultured neurons at six weeks post- differentiation, immunostained for the dopaminergic neurons marker TH (green).*

Experiments aimed at evaluating the ability of this cellular system to respond to an excitotoxic stimulus did not show neuronal death. In particular, to determine whether the dopaminergic neurons derived from hNPC are capable to respond to excitotoxic stimuli, cultures obtained after six weeks of differentiation were subjected to treatment with NMDA. Cell death due to excitotoxicity could be due to a necrotic or apoptotic process. Therefore I performed a LDH assay to assess the level of plasma membrane damage, staining with Hoechst 33342 dye to allow evaluation of late apoptosis and immunostaining with caspase-3 which, when cut, marks early apoptosis.

As can be seen in Figure 5 B, at neither of the two tested concentrations of NMDA for 22 hours there is an increased in the levels of LDH released in the medium. Stimulation for short periods (30 minutes) with 100 μM NMDA induced an increase although not significant (Figure 5 B). The analysis of Hoechst 33342 staining, analyzed by ImageJ software, showed no difference between the NMDA-treated samples compared to the control (Untreated) (Figure 6). Finally, no caspase-3 activation signal was revealed. No change was observed in case of cotreatment with NMDA and its antagonists MK801 and DAP-V (data not shown).
The results obtained indicate that this system is not able to respond to excitotoxic stimuli. This could be due to the fact that the cell culture obtained following the differentiation in a dopaminergic lineage is extremely heterogeneous and represents only about 15% while the rest of the population could be represented by a protective astrocytic component. The same effect can be exerted also by a stem component still present in culture, through the release of neurotrophins (Suzuki et al., 2006).
Another possibility is that synapses in these cultures have not yet are not adequately mature. Further experiments are needed to clarify this point.

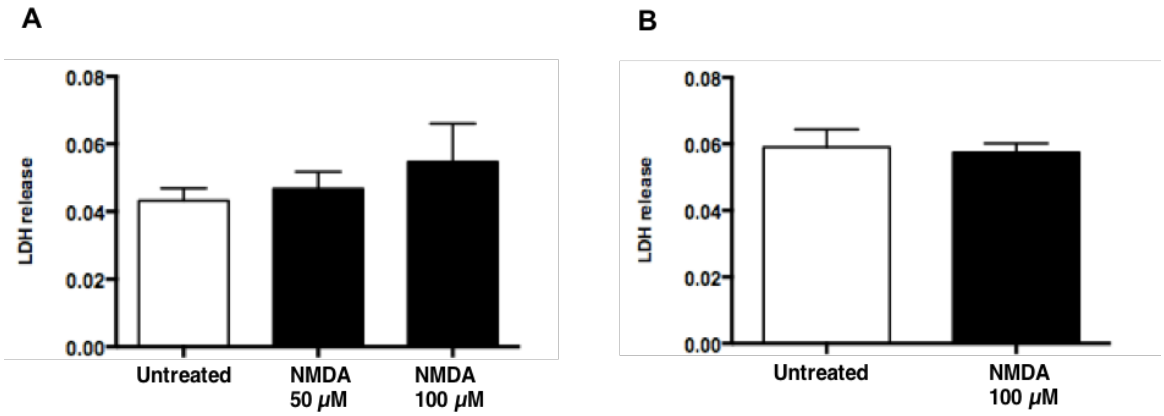

Figure 5. *Dopaminergic neurons hiPSC-derived cultures were treated without NMDA (Untreated) or with NMDA (A) for 30 min and after 22 hours analyzed for cell death by measuring LDH release or (B) for 22 hours and after which analyzed for cell death by measuring LDH release.*

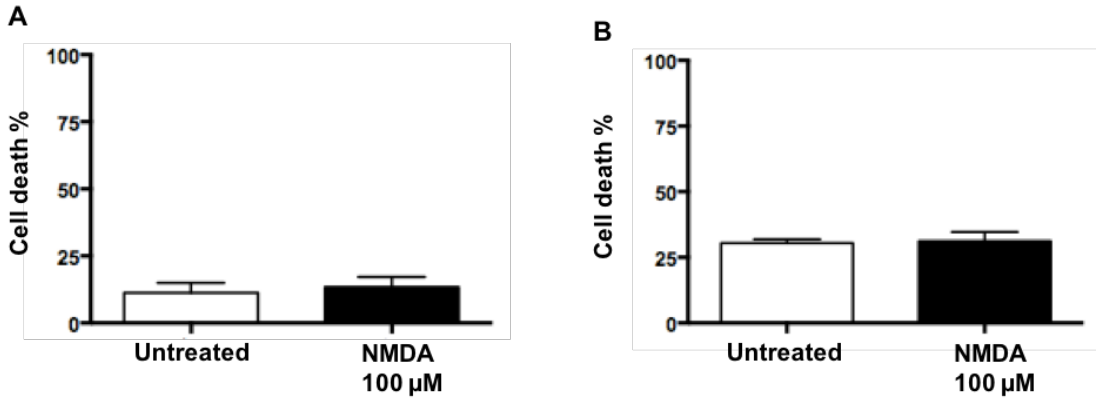

Figure 6. *Dopaminergic neurons hiPSC-derived cultures were treated without NMDA (Untreated) or with NMDA (A) for 30 min and after 22 hours analyzed for cell death by staining with Hoechst 33342 dye (B) for 22 hours and after which analyzed for cell death by staining with Hoechst 33342 dye.*

Chapter 5

General Conclusions

Neurodegenerative diseases, including HD, are up to now, incurable chronic and progressive diseases. To date, despite ongoing efforts by clinical research, pharmacological intervention is still not able to provide considerable help to improve the quality of life of affected patients. The systemic nature of these diseases, their duration and late onset encourage treatments based on nutraceuticals due to their low toxicity and their potential pleiotropic effects. Furthermore, natural substances could often be long-lasting administrated, thus representing a successful strategy in the treatment and prevention of the numerous intervening symptoms. For millennia, nutraceutical compounds have been used in folk, popular medicine providing, although empirically, considerable help in improving the general health of individuals. Their use, as well as, rigorous experimental research devoted to their better use is now becoming a hot topic. On the other hand, numerous preclinical studies have shown positive effects of therapeutic strategies with nutraceuticals in different neurodegenerative disease. Some substances such as resveratrol, α-lipoic acid, coenzyme Q10 (ubiquinone), β-carotene, lycopene, ginkgo biloba have been reported to be exceptionally beneficial in *in vivo* studies for neurodegenerative disorders, due to their action on several molecular targets.

Curcumin, a bioactive component of turmeric is among the most widely used nutraceutical compound both as diet supplement and as medicament. Although it appears to act through multiple mechanism not yet completely clarified, its therapeutic properties, including neuroprotective ones, now supported by scientific studies, encouraged the use of this substance also in neurodegenerative diseases including Alzheimer and Huntington itself.

Data presented in this project clearly indicate that curcumin can be chronically administrated to a mouse model of HD with wide, systemic beneficial effects. In fact, not only brain dysfunctions but also recovery of weight loss and general intestinal functions greatly ameliorate in treated animals.

A number of studies indicate that involuntary weight loss may be due to gastrointestinal dysfunctions. On the other hand, constipation and leaky gut are described in many, if not all, neurological diseases analyzed such as the Rett syndrome (Motil et al. 2012), the autism spectrum disorders (Coury et al. 2012; Buie et al. 2010; Adams et al. 2011), schizophrenia (Severance et al. 2015), Parkinson's (Perez Pardo et al. 2017).

The beneficial effect observed on the intestine of our model treated with curcumin is in agreement with described beneficial effects of this substance on intestinal health (Lopresti 2018). In our experimental conditions there is amelioration of the intestinal morphology,

contractility, in the amount of the protective mucus layer and in the levels of intestinal barrier proteins.

One of the most interesting beneficial effects of curcumin we have observed is that intestinal motility appears preserved. Enthusiasm for this effect is due to the fact that, if it would be confirmed in humans, it would have a strong positive impact on the quality of life of patients affected by HD, since constipation is a frequent and debilitating symptom in patients with HD.

Intestinal motility was assessed both indirectly through the collection of feces and, more directly, through the analysis of muscle contractility on colonic segments derived from treated and untreated animals. Since the observed reactivity to KCl of R6/2 colon treated mice can be explained as both an activation of intestinal smooth muscle cells and/or of peripheral neuronal cells, we tried to investigate a possible underlying mechanism. We evaluated the levels of expression of some enteric nervous system marker genes: the Tyrosine Hydroxilase (TH), a marker of adrenergic neurons with excitatory effect on gut motility and the protein gene product 9.5-PGP9.5, a general marker of enteric nervous system. Although our results showed that protein expression levels of both markers are reduced in the colon of HD mice, no modulation is observed in R6/2 treated mice. Of course, the enteric markers analyzed are too few to completely exclude an effect of curcumin on the enteric nervous system and further analyses are needed to better clarify this point. The level of expression of markers of contractility (Smoothelin A, Caldesmon and Myocardin) was also analyzed and is apparently unchanged both in HD mice and after treatment with curcumin indicating that these genes are probably not involved in the molecular mechanisms underlying constipation. On the other hand, it has to be noted that this investigation has not to be considered exhaustive since it was analyzed only at the transcriptional level. Overall, the regulation of the contractility phenomenon, as mentioned above, is complex and involves different genes that were not analyzed in this context. Surely, more detailed analysis is needed to clarify the underlying mechanisms.

In this regard, an interesting future investigation could concern the investigation of the "pacemakers" of the intestine, the interstitial cells of Cajal (ICC), which are specialized cells producing spontaneous slow-waves required for peristalsis, placed between enteric neurons and smooth muscle cells where they transduce inputs from enteric motor neurons to muscle cells. ICC play an important role in gastrointestinal motility and they appear to be impaired in patients with neurodegenerative diseases such as Parkinson's disease (Miaomiao et al. 2018). It's interesting to note that curcumin apparently affects this cell type (Jin QH et al.

2013).

Another very interesting aspect that results from our data is maintenance of body weight in R6/2 mice treated with curcumin. It may be promoted by modulating the intestinal epithelium homeostasis, since the small intestine mediates the absorption of nutrients. Our study indicate that the mouse model of HD has alterations of the intestinal mucosa that could resemble a leaky gut, i.e. a disregulated intestinal permeability. Indeed, tight junctions (ZO-1, occludin, and claudin-2) mRNA expression was higher in R6/2 treated mice respect to the untreated, indicating a potential effect of curcumin at the level of intestinal epithelia. It would be extremely interesting to evaluate if similar effects also occur in humans considering that the intestinal barrier is critical in patients with other neurodegenerative disease. It's interesting to note that weight loss, which gradually weakens patients, tends to appear even before the onset of the classic neurological symptoms.

Interestingly, curcumin also stabilized the number of Goblet cells (a type of specialized mucus-secreting cells of the epithelial layer) that is increased in R6/2 mice. The increase of goblet cells in HD, as well as the correlated mucus production, appears to be a compensatory mechanism that probably arises to facilitate the passage of stool in the intestine and to eventually overcome an inflammatory condition. In fact, alterations in mucus levels are usually correlated to altered interaction between bacteria and the intestinal epithelium that could trigger inflammation. Therefore, it cannot be excluded that improved intestinal health in our experimental system is due to an anti-inflammatory effect of curcumin and/or to alterations of the intestinal microbiota that directly or indirectly affects microbial growth (Zam 2018).

Considering the pleiotropic effect of curcumin, the possibility to treat patients with this nutraceutical is an interesting opportunity to pursue. In fact, considering that HD has a dominant inheritance and that genetic tests for the molecular diagnosis of the disease- even before its manifestation -are currently available, a preventive treatment with this drug could overcome intervening symptoms.

On the other hand, the diffusion of nutraceuticals and the ability of some of them to cross the blood brain barrier makes them interesting candidates also in contrasting the phenomena affecting the nervous system in pathological conditions. One of the most interesting phenomena, which is crucial in the pathophysiology of several central nervous system diseases, including neurodegenerative diseases, is glutamate-induced excitotoxicity. In order to test drugs and natural compounds, it is useful to have a suitable *in vitro* model. For all the reasons listed in Chapter 4, neurons derived from hNPCs constitute a powerful system for

drug screening and for the study of excitotoxicity. In this context, we have been involved in setting up an experimental system of hiPSC-derived dopaminergic neurons in order to get an *in vitro* model suitable for the study of excitotoxicity.

Previous studies in the laboratory of Prof Euan Brown showed that dopaminergic neurons form synapses in culture after 6 weeks of differentiation in the dopaminergic lineage. Therefore, we used the same conditions and observed that neurons displayed a resistance to NMDA-mediated excitotoxicity as indicated by the absence of observed cell death. This could be explained by several reasons including the remarkable presence of a protective astrocytic component or of a staminal component still present in culture which, through the release of neurotrophins, could protect neurons from the excitotoxicity phenomena (Suzuki et al., 2006). Another possibility is that synapses in these cultures, although present, are still not completely reached maturation.

Therefore, it appears critical to improve the efficiency of the differentiation process and to evaluate the percentage of the different cell types in culture, paying particular attention to the presence of the astrocytic component. One possibility is to increase the time of the differentiation process or to change the culture conditions in order to get more mature synapses. Of course, further experiments are needed to set up the right conditions in order to get a really suitable system to study both the molecular mechanism of excitotoxicity at the cellular level and to screen for potential medicaments.

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APPENDIX

pharmaceuticals

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Article

Colloidal Silver Induces Cytoskeleton Reorganization and E-Cadherin Recruitment at Cell-Cell Contacts in HaCaT Cells

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Abstract: Up until the first half of the 20th century, silver found significant employment in medical applications, particularly in the healing of open wounds, thanks to its antibacterial and antifungal properties. Wound repair is a complex and dynamic biological process regulated by several pathways that cooperate to restore tissue integrity and homeostasis. To facilitate healing, injuries need to be promptly treated. Recently, the interest in alternatives to antibiotics has been raised given the widespread phenomenon of antibiotic resistance. Among these alternatives, the use of silver appears to be a valid option, so a resurgence in its use has been recently observed. In particular, in contrast to ionic silver, colloidal silver, a suspension of metallic silver particles, shows antibacterial activity displaying less or no toxicity. However, the human health risks associated with exposure to silver nanoparticles (NP) appear to be conflicted, and some studies have suggested that it could be toxic in different cellular contexts. These potentially harmful effects of silver NP depend on various parameters including NP size, which commonly range from 1 to 100 nm. In this study, we analyzed the effect of a colloidal silver preparation composed of very small and homogeneous nanoparticles of 0.62 nm size, smaller than those previously tested. We found no adverse effect on the cell proliferation of HaCaT cells, even at high NP concentration. Time-lapse microscopy and indirect immunofluorescence experiments demonstrated that this preparation of colloidal silver strongly increased cell migration, re-modeled the cytoskeleton, and caused recruitment of E-cadherin at cell-cell junctions of human cultured keratinocytes.

Keywords: colloidal silver; wound healing; E-cadherin; keratinocytes; nanoparticles; skin

1. Introduction

The use of silver in therapeutic applications has very ancient origins due to its broad and highly effective antibacterial activity [1,2]. However, the scientific debate on its mechanism of action is still an open field. Some authors maintain that the bactericidal activity could be linked to the release of silver ions [3–8] and their interaction with several bacterial components such as peptidoglycan, the cell membrane as well as bacterial proteins/enzymes involved in vital processes [8–11]. In contrast, other authors assert that the effect of silver could be due to a physical effect toward the cell membrane with consequent penetration of silver inside the cytoplasm and interference with cellular components [10]. More recently, another important effect exerted by silver preparations was described, which regarded

the inhibition of bacterial biofilm formation [12,13]. Biofilms (aggregates of bacteria embedded in an extracellular matrix) allow bacterial growth in a protective environment, reducing antibiotic susceptibility and favoring escape from the immune response.

In addition to these functions, it has been demonstrated that silver has anti-inflammatory effects and improves the healing of wounds through the modulation of fibrogenic cytokines [12–16] and a decrease in lymphocyte infiltration [17,18]. Thus, silver has found wide applications in preventing further injury and bacterial invasion of wounds, therefore improving the efficient recovery of damaged tissues.

All of these characteristics have caused a widespread use of silver, both in medicine and daily life. Nowadays, medical devices (dressing for wounds, surgical catheters, stitches, bone cement) as well as cosmetics, cosmeceuticals, and tessils, have a silver component [13,16,19,20]. Nevertheless, although it is considered relatively non-toxic to mammals, chronic exposure to Ag+ ions determine the formation of insoluble precipitates in the dermis and the cornea/conjunctiva, causing the so-called argyria or argyrosis syndromes, blue coloration of the skin, and mucous tissues [3,4].

It is essential to underline that most of the toxicity of silver is because ionic silver (Ag+) is exceptionally reactive toward molecules and cellular structures [21]. Therefore, especially during the last few years, ionic silver usage has been superseded by colloidal silver, i.e., as a suspension of microscopic metallic nanoparticles (NP up to 100 nm in diameter), presenting lower toxicity with respect to their metallic counterpart [3,4].

Notions about colloidal silver safety and biocompatibility have appeared to sometimes be contradictory in the literature. Several studies have confirmed silver NP as clinically safe [22], and dressings based on silver NPs have been declared safe for patients [20,23,24]. However, some studies have shown that NPs are cytotoxic for several different cell lines, mostly by inducing an increase of ROS production, a decrease in mitochondrial function, DNA damage, and apoptosis [25–30]. In other cases, a decrease in cell proliferation without DNA damage has been reported [31]. In contrast, studies performed in human fibroblasts confirmed that AgNPs could alter mitochondrial functionality without leading to cell death [16] and one study identified a relationship between NP size and inhibitory effects on mitochondria [32].

It has to be noted that silver nanoparticles can be very heterogeneous and such heterogeneity could probably in part explain differences present in the literature. Several methods to produce silver NP have been developed. Preparations of colloidal silver commercially available (see http://www.silver-colloids.com/) can differ for NP size, stability (Zeta potential), concentration, and different percentages of ionic silver either due to the efficiency or synthesis methods. Parameters that are influenced by the synthesis method include the mean NP diameter and size, size distribution, shape, stability, the inclusion of ligand shells, and capping agents [21].

Considering the widespread use of silver NP and the growing interest in its use due to its versatility, we analyzed the biological properties of a colloidal silver preparation with silver NP of 0.62 nm in size, smaller than those ever described and presenting an extremely low percentage of ionic silver [33–35]. We first evaluated the antimicrobial activity with results similar to what has already been published [36–39] with the data not shown. Then, we assessed the effect on a model of human skin, HaCaT human keratinocytes. We observed no toxicity by the MTT assay, growth curve analysis, absence of stress granules, and strong efficacy in promoting wound healing in vitro. Interestingly, colloidal silver induced an evident cytoskeleton reorganization accompanied by an increase in cell–cell junctions underlined by E-cadherin recruitment.

2. Results

2.1. Effect of Colloidal Silver on HaCaT Cells

Initial experiments investigated the potential toxicity of colloidal silver on human immortalized HaCaT keratinocytes. For this purpose, HaCaT cells were grown in the presence of colloidal or ionic silver at 0.5 or 2 μg/mL for 24 and 48 h and cell viability was analyzed by the MTT assay as described

in [40]. Figure 1 shows that colloidal silver did not exert any toxic effect, while ionic silver caused a dramatic reduction of cell viability at both concentrations.

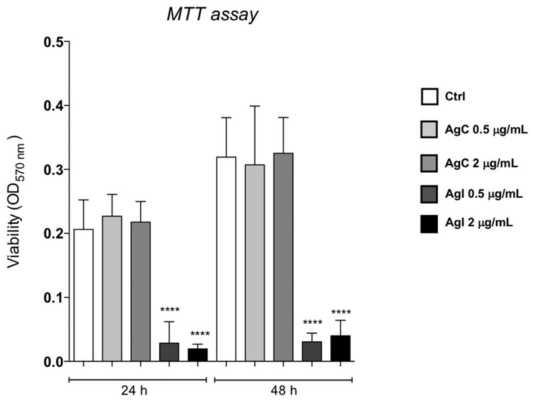

Figure 1. Effects of AgC on HaCaT cell viability. MTT assay of HaCaT cells incubated for 24 or 48 h with colloidal (grey bars) or ionic silver (dark bars) at 0.5 or 2 μg/mL. Data are expressed as absorbance at 570 nm and presented as mean ± SE of three independent experiments, each done in sestuplicate. Analysis of variance was performed by two-way Anova followed by the Bonferroni post-test. **** $P < 0.0001$ when compared with the control.

Importantly, cell viability increased between 24 and 48 h even when colloidal silver was added at the higher concentrations of 2 μg/mL, thus suggesting no toxicity under these conditions. We therefore addressed whether colloidal silver could function as a stressor agent by analyzing the stress granules formation (SG). SG are aggregates of proteins and RNA that form when cells are subjected to different kind of stresses to protect cellular structures from harmful conditions. To monitor the formation of these aggregates, we looked at the YB-1 protein as a specific marker of SG [41]. Furthermore, it has to be noted that YB-1 typically translocates to the nucleus following genotoxic stress [42], therefore, it can also be used to monitor harmful insult to the cells. HaCaT cells were grown in the presence of colloidal silver at 0.5 and 2 μg/mL for 24 h, fixed, and analyzed by indirect IF with the anti-YB1 antibody. Figure 2 clearly shows that colloidal silver induced neither stress granules formation nor YB-1 nuclear translocation. Cells were also stained with TRITC-conjugated phalloidin to visualize the actin cytoskeleton. The experiment showed that colloidal silver treatment induced actin cytoskeleton rearrangement at the cellular periphery. In particular, we observed increased f-actin polymerization both at the cell–cell and cell–substratum adhesions (Figure 2, red panels).

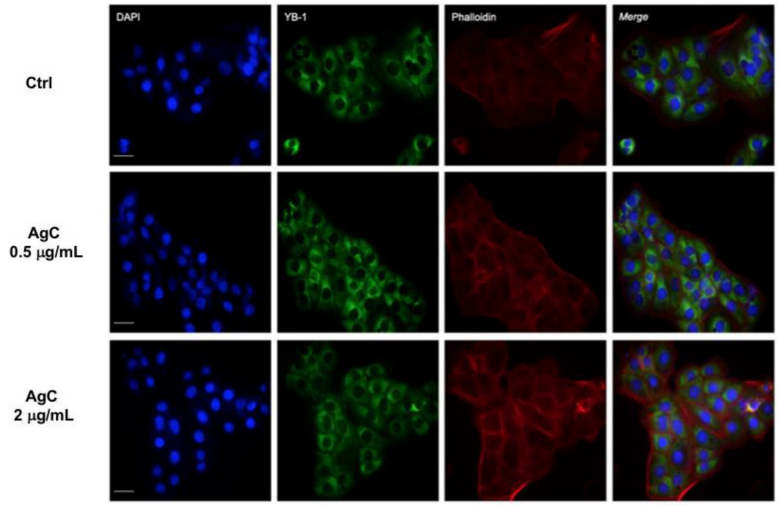

Figure 2. Effect of AgC on stress granules formation. HaCaT cells were seeded on a coverslip and treated (or not) with AgC at 0.5 or 2 μg/mL for 24 h. Cells were then fixed and analyzed by TRITC-conjugated phalloidin staining (red) or indirect IF with the anti-YB1 antibody (green). Nuclei were stained with DAPI. Scale bar, 7 μM. Images were acquired using a Nikon TE Eclipse 2000.

2.2. *Effect of Colloidal Silver on Wound Healing*

Based on these data showing a reorganization of the actin cytoskeleton, we decided to analyze the effect of colloidal silver on cell migration, a process where massive cytoskeleton dynamics take place. Wound healing activity was evaluated in vitro by automated time-lapse microscopy [43]. Details on the technique are reported in the Materials and Methods Section. Typical images of the wound as a function of time are presented in Figure 3A, where samples in the absence and/or presence of AgC 0.5 μg/mL are compared.

Images at the same time points show better closure of the wound for the treated sample with respect to the control. This result was systematic, as visible from Figure 3B,C, where the evolution of the wound area, normalized with respect to its initial value (A/A0), is reported for the control and treated sample, respectively. Raw data reported in the two diagrams for each independent field of view showed excellent reproducibility. We calculated the values of the wound closure velocity (α) from each data series, as reported in the Materials and Methods Section. Interestingly, the αAgC/αcontr (that is a measure of the relative effect of the treatment in our conditions) was 1.80, indicating that the wound closure velocity roughly doubled in the presence of AgC 0.5 μg/mL.

In Figure 4B, the duplication time (τ) of HaCaT cells grown in the presence and/or absence of 0.5 μg/mL AgC is reported. Intriguingly, cell duplication was not significantly altered in the presence of colloidal silver, while the cell motility coefficient calculated from the Fisher–Kolmogoroff equation appeared to be drastically and statistically significantly increased (Figure 4C). Overall, these results indicate that under these experimental conditions, colloidal silver affected cell motility more than cell proliferation.

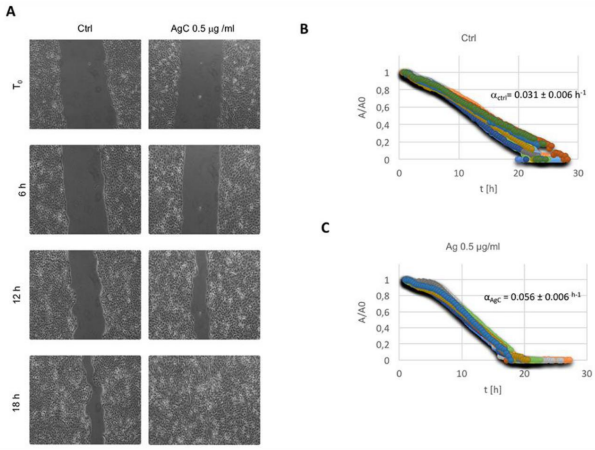

Figure 3. Effect of AgC on wound closure dynamics. (**A**) Representative phase contrast microscopy images of cells incubated (or not) with AgC at different time points showing the wound closure process over time. (**B**,**C**) The wound closure process was quantified by measuring the reduction of the wound area (**A**) over time, as described in the Materials and Methods Section. Evolution of the wound area A, normalized to the value A0 (A at time 0), is reported for the control (**B**) and AgC treated (**C**) cells by selecting random fields along the wound for each experiment. The linear range of each data series was fit in order to measure the wound closure velocity α. Values of α for the control and treated cells are indicated on each graph as the mean from three independent experiments analyzed in triplicate. Standard error of the mean was calculated to account for reproducibility, and the t-test was calculated to verify the statistical significance of the differences with respect to the control samples.

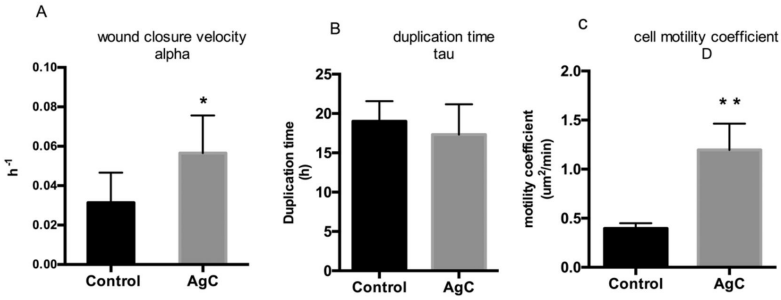

Figure 4. Effect of AgC on wound closure dynamics. Values of α (wound closure velocity) (**A**), τ duplication time) (**B**), and D (cell motility coefficient) (**C**) of the control and AgC treated cells are reported. Values of D were calculated according to the Fisher–Kolmogoroff equation from values of α and τ (see Materials and Methods). Data are expressed as the mean of at least three independent experiments. SEM is reported as error bars, statistical significance was assessed by the paired two-tailed t-test (* P = 0.04; ** P = 0.007).

2.3. *Effect of Colloidal Silver on Cell-Cell Contacts and Cell Morphology*

Regulation of cell shape and motility is governed in large by the cytoskeleton, of which actin filaments are the major components. Cytoskeletal elements influence the formation of cell–cell and cell–substrate adhesions that play a fundamental role in both cell morphology and migration which requires the continuous assembly and disassembly of cellular adhesions. To further characterize the

effects of AgC on cell shape, the cytoskeleton and cell–cell contacts were examined by fluorescence microscopy by both phalloidin staining and E-cadherin immunofluorescence. Cells were allowed to adhere onto coverslips overnight, treated with 0.5 µg/mL AgC for 8 h, fixed, and subjected to IF with anti-E-cadherin, followed by TRITC-conjugated phalloidin incubation to visualize the actin cytoskeleton and DAPI to stain the nuclei. As previously observed, cells displayed a dense meshwork of actin filaments around the cell periphery. Interestingly, the experiment showed that AgC treatment caused significant recruitment of E-cadherin at cell–cell junctions, which were apparent within 8 h of treatment (Figure 5). Similar results were obtained when cells were incubated with AgC for 24 h.

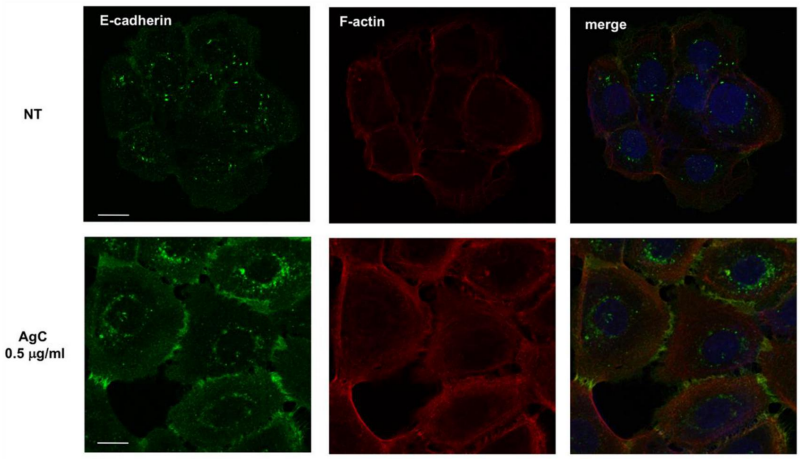

Figure 5. E-cadherin localization in HaCaT cells upon AgC treatment. Cells were allowed to adhere onto coverslips for 24 h and then treated with 0.5 µg/mL AgC for 8 h. Cells were then fixed and subjected to IF with an anti-E-cadherin antibody followed by TRITC-conjugated phalloidin to visualize the actin cytoskeleton. Representative images of E-cadherin subcellular localization and phalloidin are shown. Merged images also show DAPI staining to visualize the nuclei. Images were taken with a Zeiss confocal laser-scanning microscope Axio Observer (scale bar, 15 µM). A ×40 objective was used and image analysis was performed using ImageJ.

3. Discussion

In this work, we evaluated the use of a colloidal silver solution in the cell viability and cell migration of immortalized human keratinocytes. Although the use of silver in medicine dates back to ancient times, its use became less frequent upon the widespread use of antibiotics. The appearance in recent decades of the increasingly dangerous phenomenon of antibiotic resistance has brought to the forefront the use of silver as a valid non-toxic alternative. Our experiments showed that no adverse effect could be observed in the HaCaT cells by both the MTT assay and stress granules formation when we treated keratinocytes at even rather high concentrations (2 µg/mL) of AgC. Interestingly, upon treatment, cells tended to reorganize the cytoskeleton as indicated by the observation of phalloidin-stained F-actin. Since tissue healing is linked to a profound reorganization of the cytoskeleton, we evaluated the activity of silver in wound healing by following the wound healing process using time-lapse video-microscopy. The algorithm we used allowed for the quantitative analysis of the dynamics in the reduction of the cutting area. Our data indicated that in the presence of silver, the wound closure speed increased and cells incubated with colloidal silver "healed the damaged area" in less time than the controls. This result was quantified and interpreted according to the Fisher–Kolmogoroff model [43], which allows the process of re-closing the cut mathematically to be described. A novel data analysis approach was

used to identify the relative role of cell motility and proliferation on wound healing through simple calculations. We found that AgC increased the mechanism of cell migration rather than increasing the cell proliferation levels. Cell motility is primarily governed by the cytoskeleton, which influences both the cell–substrate and cell–cell contacts. Our results were supported by immunofluorescence experiments, where HaCaT cells presented an overall increased E-cadherin signal and its re-localization to the cellular periphery and the cell–cell junctions. It is interesting to note that it has been reported that the intercellular junctions marked by E-cadherin allow cells to communicate [44] and to move in a highly coordinated way [45]. The increase in cell–cell junctions through strong E-cadherin-mediated contacts at the upper edge, in the lateral regions, and within the moving cell group characterizes the collective cell migration [44,46–48]. This aspect is particularly interesting, since collective cellular migration would be the basis of the movement and proliferation of epidermal keratinocytes located on the edge of the wound following skin lesions [49]. It is interesting to note that cell movements are characterized by cytoskeletal reorganization through the formation of adherent junctions [49]. The adherent junctions, favored by the recruitment of E-cadherin on the cell surface, allow for anchorage to the actin cytoskeleton [50] through their binding with both α- and β-catenins. The increase of the actin polymerization induced by colloidal silver and the E-cadherin relocation to the cell–cell junctions both indicate that colloidal silver is broadly involved in the cytoskeletal reorganization occurring during migration. In agreement, it has been observed that silver nanoparticles are able to increase connexin 43-mediated gap junctional intercellular communication in HaCaT cells [51]. Interestingly, this occurs through ROS production and activation of the MAPK pathway. Furthermore, in an in vitro model of the human gut epithelium, exposure to AgNP caused changes in cellular permeability and a dysregulation in the expression of components of tight junctions and desmosomes without affecting E-cadherin; however, it has to be noted that such experiments have been performed with NP of 10 nm in size, about 18x bigger than the ones used in the present study [52].

4. Conclusions

Altogether, the presented data indicate that colloidal silver could improve the healing process by modulating the reorganization of the cytoskeleton and thus cell motility. Further analysis is needed to clarify both the mechanism of action and the molecular pathways involved. However, the collected data encourage further investigation of AgC in tissue healing. In this regard, therapeutic agents including steroids, glucocorticoids, non-steroidal anti-inflammatory drugs, and chemotherapy are associated with several side effects that, by interfering with cell movement in the wound, slow down the repair process [53]. In this scenario, silver could have a positive effect on wound closure by acting on different levels and with different mechanisms compared to the generally used drugs. Last but not least, it has to be underlined that thanks to its antimicrobial properties, silver could be a valid alternative for the treatment of infections also caused by multi-drug-resistant bacteria (MDRB) [54], which do not respond to standard pharmacological therapies and for which it is challenging to develop efficient treatments.

5. Materials and Methods

5.1. Cell Culture and Reagents

HaCaT, spontaneously immortalized keratinocytes from adult skin, were purchased from Cell Line Service (CLS, Eppelheim, Germany) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT, USA), 1% L-glutamine, and 1% penicillin-streptomycin (ICN Biomedicals, Inc., Aurora, OH, USA) at 37 °C in a humidified atmosphere of 5% CO_2 [55,56]. Depending on the type of experiment, HaCaT cells were seeded at different densities and on different culture dishes. The cells were treated with a colloidal silver preparation containing 79.5% silver nanoparticles of 0.62 nm in size, obtained from Santé Naturels SNC (Civitanova Marche, Macerata, Italy) at the concentration of

20 ppm (20 µg/mL). Detailed information regarding the characteristics of the colloidal silver solution are visible at the following official site: http://www.silver-colloids.com/Click comparison table; European Products Reports). Further information regarding the size of the nanoparticles and the Zeta potential (index of stability) are presented in the Supplementary Materials, S1, S2). For each experiment, the stock solution was diluted in culture medium. Ionic silver was obtained by dissolving 3.15 mg $AgNO_3$ in 100 mL H_2O to obtain a final stock solution where the ionic silver was 20 µg/mL.

5.2. Cell Viability (MTT Assay) and Cell Proliferation Assays

The effect of ionic and colloidal silver on cell viability was evaluated by measuring the reduction of 3-(4,5-dimethylthiazol-2) 2,5-diphenyltetrazolium bromide (MTT) to formazan by mitochondrial dehydrogenase [57,58]. Briefly, 9×10^3 cells were seeded on 96-well plates and exposed to increasing concentrations of either 0.5 or 2 µg/mL colloidal silver for 24 and 48 h. MTT/PBS solution (0.5 mg/mL) was then added to the wells and incubated for 3 h at 37 °C in a humidified atmosphere. The reaction was stopped by the removal of the supernatant, followed by dissolving the formazan product in acidic isopropanol. Optical density was measured with an ELISA reader (Bio-Rad) using a 570 nm filter using an iMark microplate reader (Bio-Rad, Hercules, CA, USA). Each experiment was performed in quadruplicate, in three independent experiments. The cell viability was calculated as CV (%) = (Absorbance of test sample/Absorbance of control) × 100.

For cell proliferation analysis, cells were incubated with AgC and the number of cells in each experimental point was counted with a Scepter-Millipore counter (Handheld Automated Cell Counter) as described in [59]. Growth curves were generated and the cell population doubling time (τ) was estimated by fitting a typical logistic growth [60] (Equation (1)) where τ was the only adjustable parameter.

$$\mathbf{n(t)} = \mathbf{n_0} * \mathbf{2}^{\left(\frac{t}{\tau}\right)} \quad (1)$$

5.3. In Vitro Wound Scratch Assay

To test the effect of colloidal silver on the wound closure phenomenon, HaCaT cells were seeded in 12-well plates at a density of 4.5×10^5 cells/well. The day after plating, once they had reached 90–100% confluence, cells were starved for 6 h in serum-free DMEM to completely inhibit cell proliferation. The confluent monolayer was scraped with sterile P200 pipette tips and washed twice with PBS to remove detached cells and debris. The scratched monolayers were treated with colloidal silver (0.5 µg/mL) diluted in culture medium and plates were incubated as described. Wound closure was monitored in the different samples by automated time-lapse microscopy (TLM) using an inverted microscope (Zeiss Axiovert 200, Carl Zeiss, Germany) inserted into an incubator with constant T (37 °C), humidity (100% Hr), and CO_2 (5%). Different fields of view in each cell dish were acquired by phase contrast microscopy using a CCD video camera (Hamamatsu Orca AG, Japan) at regular intervals (15 minutes) for about 18 h using a long working distance 5X objective in phase contrast (CP Achromat Ph1). Images were analyzed as described in [43]. The wound closure dynamics were quantified by using a homemade automated image analysis software, which allowed us to measure the size of the wound area for each time point. In a typical experiment, for each field of view, the cell nude area (A) was measured for each time step, normalized with respect to the value of the wound area at time 0 (A0), and plotted as a function of time. After an initial Lag time (tL), A/A_0 was found to decrease with a constant velocity; the slope of the linear range of the A/A_0 vs. t curve (α) can be considered as a measure of the wound closure velocity. Details on the calculation of t_L and α are reported elsewhere (see Figure 9b in [61]. For each experimental condition (control and treated), at least four independent fields were analyzed out of three independent wells for each experiment. Each experiment was done in triplicate and statistical analysis performed by calculating the standard error of the mean (reported as error bar) and calculating statistical significance by testing the null hypothesis (t-test).

5.4. Wound Healing Data Analysis

The wound healing process can be modeled according to the Fisher–Kolmogoroff equation (Equation (2)), which is a diffusion-reaction equation based on a transport phenomenon approach [62,63]. This model mathematically describes the evolution of the cell density profile u, which depends on the time and distance x, measured from the wound edge, i.e., along the wound closure direction, that is, the horizontal direction in the images reported in this work. Two different phenomena contribute to wound closure, i.e., cell motility and proliferation, which are both involved in the spatial spreading of the cells in the wound region, and are both summed at the right side of Equation (2).

$$\frac{\partial u}{\partial t} = D\frac{\partial^2 u}{\partial x^2} + ku\left(1 - \frac{u}{\hat{u}}\right) \quad (2)$$

Cell motility is modeled as a Fickian diffusion, according to the assumption of a persistent random motion of the cells [64–66] and depends on a random motility coefficient, that is, analogous of a diffusion coefficient (D, in Equation (2)). Cell proliferation can be modeled (last term in Equation (2)) as a logistic growth, where the growth velocity is reduced as the cell density approaches confluence, which is measured by the maximum cell density û. k is a growth kinetic constant, which as a first approximation can be estimated as the reciprocal of the cell doubling time ($k = ln_2(\tau)$ [62,67]. This model predicts that after a short transient phase, the wound edges propagate at a constant speed in the direction x, perpendicular to the wound edge, reducing the size of the wound area. The speed of propagation of each wound edge v is related to the values of the random motility coefficient and of the cell doubling time (Equation (3)) [62,67].

$$v = \sqrt{\frac{4Dln(2)}{\tau}} \quad (3)$$

The concurring role of cell motility and proliferation can be estimated by the simple calculation of the Thiele modulus $\phi = \frac{b}{2}\sqrt{\frac{K}{D}}$ [62], where b is the initial wound size, i.e., the distance of the two edges of the wound at time 0. Given the measure of the wound closure velocity α from the analysis of the time lapse experiments, it was easy to calculate the velocity of the propagation of the wound edges that is related to α by a simple geometrical relationship v = α* b/2, where b is the initial size of the wound, with high precision. The cell doubling time τ was independently calculated from cell proliferation assays (Equation (3)). Cell random motility coefficient D was finally calculated from reverse Equation (3).

$$(D = \tau\frac{v^2}{4ln(2)}) \quad (4)$$

It is worth mentioning that the direct measurement of the cell random coefficient is a non-trivial task that requires time consuming tracking of cell motion over time [43]. The advantage of the approach used here is the possibility of estimating such a relevant parameter from the simple analysis of wound healing experiments, and trivial algebraic calculations based on advanced models.

5.5. Immunofluorescence

IF assays were performed as previously described in [68,69]. Briefly, treated and control HaCaT cells were seeded on glass coverslips at a density of 1.5×10^5 cells/well in 24-well dishes, fixed with 3.7% PFA, and permeabilized with 0.5% Triton X-100. After blocking with 3% BSA, cells were incubated with primary antibody (E-cadherin and YB-1) for 1 h at RT followed by incubation with Alexa-Fluor conjugated secondary antibodies for 1 h in the dark. To visualize the actin cytoskeleton, cells were stained with TRITC-conjugated phalloidin. The cells were counterstained with DAPI for the visualization of the nucleus. Images were taken with a Zeiss confocal laser-scanning microscope Axio Observer. A x40 objective was used and image analysis was performed using ImageJ. All images

were taken with the same setting. Image processing and analysis were performed with Fiji (ImageJ version 2.0) software. The stress granules formation experiment was performed as described in [70] and images were acquired using a Nikon TE Eclipse 2000. Antibodies of anti-YB-1 (12148 Abcam, Cambridge, UK), anti-E-Cadherin (610181 BD Transduction Laboratories™, MA, USA), Alexa Fluor 488 anti-rabbit and anti-mouse (Thermo-Fisher Scientific, Waltham, MA, USA), and DAPI (Sigma-Aldrich, Saint Louis, MO, USA) were used.

5.6. Statistical Analysis

All data are expressed as the means of independent experiments (biological replicates) ± standard errors (SE). Analysis of variance was performed by a two-way ANOVA followed by the Bonferroni post-test using Graph-Pad Prism (Graph-Pad Software), or by the Student's T-test as previously described in [59].

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