

**“Molecular mechanisms regulating gene expression in cellular and animal models
of Huntington’s disease”
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Neurodegenerative diseases constitute a heterogeneous group of disorders characterized by progressive damage and loss of neurons in various areas of the central and peripheral nervous systems. They are accompanied by histological and hematological changes that manifest as clinical pathological symptoms. Neurodegeneration involves neuronal loss and co-occurring disorders resulting from molecular-level defects. These defects include pathological protein aggregation, synaptic and neural network dysfunction, impaired proteostasis, cytoskeletal abnormalities, altered energy homeostasis, DNA and RNA damage, inflammation, and neuronal cell death.

Flavonoids constitute a large subgroup of polyphenols, comprising over 6,000 compounds found in natural products. They are of high interest in the search for treatments for various diseases because of their numerous properties, such as antioxidant and anti-inflammatory activity, and their ability to cross the blood-brain barrier. The main groups of flavonoids, their properties, and the most commonly described mechanisms of neuroprotective action are discussed in the following review article, which is part of this doctoral dissertation: Szulc, A., Wiśniewska, K., Żabińska, M., Gaffke, L., Szota, M., Olendzka, Z., Węgrzyn, G., & Pierzynowska, K. (2024). Effectiveness of flavonoid-rich diet in alleviating symptoms of neurodegenerative diseases. *Foods*, 13, 1931. Moreover, this article also discusses mechanisms underlying neurodegenerative diseases, such as Alzheimer’s disease (AD), Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS), and Huntington’s disease (HD).

This doctoral dissertation focuses on HD, one of the most common neurodegenerative diseases associated with protein aggregation. It belongs to the group of monogenic polyglutamine (PolyQ) diseases, which are inherited neurological disorders caused by an increased number of CAG (cytosine, adenine, guanine) trinucleotide repeats. In HD, an increased number of CAG repeats occurs in the *HTT* gene, which encodes the huntingtin (HTT) protein. The pathogenic *HTT* variant codes for an abnormally folded protein with an elongated glutamine residue sequence, resulting in toxic aggregates that are difficult to remove. These aggregates, along with abnormal secondary structures of the mutated *HTT* mRNA, affect the splicing process by sequestering spliceosome components. These two elements are considered key defects that initiate a cascade of cellular dysfunction, leading to the development of pathogenic symptoms in patients. In a healthy individual, up to 27 CAG repeats are considered normal. A range of 27–35 repeats is associated with a risk of disease symptoms appearing in subsequent generations, while more than 36 repeats lead to disease manifestation. An increased number of CAG repeats appears as early as at the zygote stage; however, the first symptoms of the disease are observed much later, in the third or fourth decade of life. Recent reports suggested that somatic expansion of CAG repeats during the patient’s lifetime to more than 150 copies may drive pathogenicity and delayed disease onset. Nevertheless, it remains unclear why such

expansion occurs exclusively in cells carrying the *HTT* allele, in which the original number of CAG repeats exceeds 36. HD is currently an incurable disease; thus, seeking therapeutic strategies that enable effective treatment of this disease is needed. However, developing new drugs requires a thorough understanding of the molecular mechanisms of the targeted disease.

In my research, I used cellular models of Huntington's disease (HD) based on the HEK293 cell line. These cell lines were previously generated using CRISPR-Cas9 mutagenesis and included a control line containing 16/17 CAG repeats in exon 1 of the *HTT* gene (HEK) and HD lines containing 41, 53, or 84 CAG repeats, respectively (HEK 41, HEK 53, and HEK 84, respectively). The study also utilized a genetically modified R6/1 mouse model of HD (116 CAG repeats) and C57BL/6J control mice.

In the study: Szulc, A., Walter, B., Gaffke, L., Wiśniewska, K., Żabińska, M., Rintz, E., Cyske, Z., Grabski, M., Pankiv, O., Podlacha, M., Pierzynowska, K., & Węgrzyn, G. (2026). Extensive transcriptomic changes in cellular and animal models of Huntington's disease depending on the length of CAG repeats in the exon 1 of the *HTT* gene. *Biochemical and Biophysical Research Communications*, 816, 153711., protein structures were predicted for human *HTT* variants with 16, 41, 53, and 84 CAG repeats using AlphaFold3. Significant changes were observed in the pathogenic forms compared to the wild-type form. The study also analyzed global changes in gene expression in cellular and animal models of HD relative to controls, as well as within the cellular model across cell lines with different CAG repeat numbers (41, 53, 84).

I have observed that pathological elongation of the glutamine chain can lead to the predicted reorientation of the N-terminal polyQ region. These results suggest that the pathomechanism of HD may extend beyond the traditional view of polyQ-induced protein aggregation, pointing to the possibility that specific polyQ lengths drive conformational changes that influence disease course, consistent with recent proposals on somatic CAG repeat expansion and its downstream consequences. However, the AlphaFold3 models presented here describe monomeric *HTT* and should be interpreted as hypotheses about the "apo" state, which structural studies have not yet directly confirmed.

Transcriptomic analyses revealed thousands of genes with altered expression in HEK41 cells (2,892 genes), HEK53 cells (4,193 genes), and HEK84 cells (6,091 genes) compared to the HEK control line. Furthermore, in R6/1 mouse brain cells, I identified 4,185 genes with altered expression compared to wild-type mouse cells. In both cellular and animal models of the disease, these changes were related to mitochondrial function, apoptosis, autophagy, the inflammatory response, DNA replication and repair, and responses to DNA damage and stress. Furthermore, the alterations included the expression of genes involved in the transport of proteins and neurotransmitters at various stages of cellular function. These transcriptomic changes are a consequence of the presence of pathogenic forms of *HTT*, not the primary cause of the disease; however, elevated levels of reactive oxygen species (ROS), combined with alterations in the expression of dozens of genes responsible for the stress response, the response to DNA damage, and processes specific

to certain pathways, may promote the somatic expansion of CAG trinucleotide repeats in cells containing pathogenic *HTT* alleles.

Among the processes most frequently disrupted in global gene expression regulation, autophagy plays a key role in degrading toxic forms of mHTT. This process is tightly regulated within the cell, and its primary function is to maintain homeostasis. It removes damaged organelles, long-lived proteins, protein aggregates, macromolecular complexes, and other abnormal intracellular structures. Macroautophagy begins with the formation of a phagophore - a membrane that gradually elongates and encloses the components destined for degradation. The resulting autophagosome matures and fuses with a lysosome to form an autophagolysosome, where its contents are degraded at low pH by hydrolytic enzymes. The cell can reuse the resulting products.

Autophagy modulators have shown promising results in cellular and animal models of HD. However, potent autophagy stimulators, such as rapamycin and other compounds, although useful in laboratory studies, cannot be used in clinical practice for patients with diseases requiring lifelong treatment because their long-term administration causes adverse effects. Therefore, in the context of my work, the use of genistein (4',5,7-trihydroxyisoflavone or 5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), was particularly promising. This compound can stimulate autophagy to a certain extent while maintaining a favorable safety profile.

Using specific anti-HTT antibody, I observed increased HTT fluorescence intensity in all three cell lines compared with the control line. After genistein treatment, fluorescence intensity decreased significantly, which may indicate reduced mutant huntingtin (mHTT). The reduction in the number of aggregates may occur through the modulation of autophagy.

In the HD models, I observed altered expression of numerous autophagy-associated genes, including both induction and repression compared with control cells. The number of genes with altered expression increased with CAG repeat length in the HTT gene. Genes encoding negative regulators of autophagy were particularly frequently upregulated, whereas genes essential for its proper progression exhibited a varied expression profile. In the presence of genistein, the observed changes were exacerbated, with the induction of genes involved in the positive regulation of autophagy predominating. I also observed similar relationships for genes associated with specific types of autophagy.

To identify interactions among proteins encoded by genes with altered expression, I modeled protein interaction networks using the STRING database. I identified proteins encoded by genes with altered expression, including those whose expression returned to control levels after genistein treatment. This allowed me to identify potential therapeutic targets. Next, using Western blot analysis, I measured the levels of proteins involved in ULK1-dependent autophagy initiation and autophagosome elongation and flux, associated with ATG12 and LC3-I/II, respectively. I showed that autophagy disruption in HD occurs primarily during the initiation phase of the process and involves a decrease in

the levels of pULK1(Ser555) and Atg13. Genistein, by contrast, can restore normal autophagy through mechanisms that include, but are not limited to, increasing the efficiency of ULK1(Ser555) phosphorylation and raising Atg13 levels. The simultaneous increase in levels of pULK1(Ser757) and Rubicon protein - which are known negative regulators of autophagy - prevents excessive stimulation of this process by the studied isoflavone. Such excessive activation of autophagy would be problematic with long-term use, whereas mild yet effective stimulation may, on the one hand, allow effective degradation of mHTT and, on the other hand, be safe for the body. A more detailed analysis of autophagy disorders and the effects of genistein are presented in the article Szulc, A., Wiśniewska, K., Szota M., Żabińska, M., Pierzynowska, K., Gaffke, L., & Węgrzyn, G. (2026). Autophagy disorders and their improvement by 4',5,7-trihydroxyisoflavone in cellular models of Huntington's disease. [Manuscript submitted to *Biochimica et Biophysica Acta (BBA) – Molecular Basis of Disease* BBADIS-26-4684].

In summary, my research focused on the molecular mechanisms of HD, with particular emphasis on how CAG repeat length in the *HTT* gene affects HTT structure, gene expression regulation, and autophagy. Structural predictions suggest that the lengthening of the polyglutamine track may alter the conformation of huntingtin. Transcriptomic analyses revealed numerous gene expression abnormalities that become more pronounced as the number of CAG repeats increases and involve, among other things, mitochondrial function, the response to stress and DNA damage, and autophagy - a process critical for proper clearance of mHTT. Detailed studies have shown disrupted autophagy initiation in HD models, associated with reduced levels of pULK1(Ser555) and Atg13. Genistein increased levels of these proteins and reduced the fluorescence intensity of the HTT signal in immunohistochemical studies, which may indicate reduced mutant huntingtin levels. These results suggest that genistein may modulate autophagy at the initiation stage and thus represent a potential new direction for further research into HD therapy.