"Changes in human cells mitochondria carrying the mutation in the IT15 gene"
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Huntington disease (HD) is a hereditary neurodegenerative disorder caused by abnormal expansion of trinucleotide CAG in the first exon of the *IT15 (HTT)* gene encoding the huntingtin protein. HD is a rare disease which occurs in every 5 to 10 per 100,000 individuals worldwide. However, it is the most common inherited neurodegenerative disorder. Due to the mutation, progressive neuron loss in putamen and the caudate nucleus is developed, and as a consequence, the appearance of the spectrum of symptoms, including psychiatric disturbances, cognitive impairments, and motor abnormalities. Length of the $(CAG)_n$ tract (coding the polyglutamine linker) in the mutated gene is a diagnostic marker. In healthy individuals, the number of CAG repeats is less than 26, whereas Huntington disease is diagnosed when there are above 40 repeats. Range from 27 to 36 repeats is characteristic for the intermediate group, which either do not develop the symptoms of the disease or manifest the disease at old age. The number of CAG repeats is also inversely correlated with age of the disease onset. This parameter is now the basic method used to define prognosis and disease progression, however, it has some limitations resulting from instability of expansion of this nucleotide triplet.

Huntingtin is a large protein (348 kDa) produced in various cells and in the tissues of whole organism, with the highest levels in the brain and testis. The exact function of huntingtin is still unknown, however, it depends on its subcellular localization. In the cytoplasm, it plays a role in intracellular transport and endocytosis, while in the nucleus, it regulates gene transcription. Moreover, huntingtin directly interacts with the Golgi complex, endoplasmic reticulum, and outer mitochondrial membrane. It plays an important role in the regulation of apoptosis, and it is essential in embryogenesis. Huntingtin interacts also with more than 200 different proteins. It is still under debate whether the mutation in the *IT15* gene leads to the loss of physiological function or gain of new toxic features of huntingtin in the cell. Conformational changes in the mutant huntingtin (mHtt) cause its slower proteasomal degradation, what results in the accumulation of the mHtt in the cytoplasm and in the nucleus of neurons. This event is probably responsible for progressive degradation of neurons, however, the exact mechanism underlying the cell loss remains to be elucidated.

The first manifestations of HD usually appear in individuals between 35 and 50 years old, although, there is also juvenile (symptoms begin to appear under 20 years of age) and late

(manifestation of the disease begins after the age of 60) variation of the disease. First symptoms are usually related to emotional disturbances and depression, followed by cognitive decline. Last but the most characteristic are physical problems i.e. involuntary movements called chorea, which later, with the progression of the disease, are transformed into general muscle stiffness. 25 years after identification of the gene causing HD, there is still no therapy available for treatment of the disease. Only drugs that improve the comfort of the patient's life by reducing the manifestation of individual symptoms are used in the pharmacological treatment. Average HD patients die after 15 to 20 years since disease onset. Among currently conducted search for new therapeutic options, the most promising seem to be the use of antisense oligonucleotides or stimulation of the autophagy process. We should keep in mind that searching for a proper biomarker of the disease, allowing to introduce new potential therapies at the suitable moment for the individual patient, is as important as designing new therapeutic strategy.

The first hypothesis concerning mitochondrial role in the course of HD appeared after observation of mitochondrial defects and the oxidative stress in biological material collected from patients suffering from neurodegenerative disorders. To date, various mitochondrial disturbances have been demonstrated, depending on the disease model or used tissues. Moreover, the precise mechanism by which mitochondrial functions are modulated is still unknown. Some of the observations may suggest that metabolic disturbances (e.g. weight loss without changing the caloric intake, and decreased glucose metabolism or increased lactase production in patients' brains) are related with the earliest disease stages. It remains an unsolved issue whether mitochondrial alterations are the cause or consequence of the disease. It should be underlined that mitochondrial defects are well described in the neurons, however, the profile of these changes in peripheral cells is still poorly understood. Nowadays, expanding knowledge about mitochondrial dysfunction outside the central nervous system seems to be extremely important. More and more often, HD is considered as a systemic disturbance including peripheral abnormalities. In HD patients, cardiac failure, osteoporosis, muscle atrophy and reduced testosterone concentration in men are more common than in healthy individuals.

The aim of my doctoral project was to describe and analyze possible mitochondrial defects in peripheral tissues (blood cells and skin fibroblasts) collected from HD patients at different stages of the disease, and correlation of these results with the disease progression. The study was conducted with blood and skin fibroblasts collected from Polish patients

registered in European Huntington`s Disease Network (EHDN) database. Patients registered in this database have a well-described history of the disease, what allows a comprehensive statistical analysis of the results in terms of the severity of the disease. In my analysis, material obtained from age- and sex-matched control group, without a family history of HD, has also been used.

The first goal of the research was to investigate the level of mitochondrial DNA (mtDNA) in the blood samples and fibroblasts collected from patients at various stages of the disease, and to correlate the results with the degree of its advancement. The mtDNA level was analyzed as the ratio of mtDNA to nuclear DNA (nDNA) by performing real-time quantitative PCR (real-time qPCR) reactions. After study literature data, it turned out that despite using the same methods (qPCR), some results cannot be compared between research centers due to using different experimental protocols. It was decided to carry out experiments to select the optimal experimental conditions for further research. After performing experiments with the use of different conditions for storing blood samples, conducting DNA isolation from both whole blood and leukocytes, and isolation of DNA from fibroblasts at various passages, an appropriate protocol was developed. My results indicated the necessity of immediate (max 3 h after blood collection) DNA isolation from the blood sample. Analysis of the results also showed that the outcome of experiments conducted on DNA isolated from whole blood or leukocytes should not be directly compared, due to the different content of blood components such as platelets, as well as a possible extracellular nDNA and mtDNA release in the serum and plasma. Moreover, a significant influence of the number of cell passage on mtDNA level analysis has been observed. Due to possible loss of the disease phenotype in HD fibroblasts during culturing under laboratory conditions, I recommend that DNA isolation should be performed at the maximum fifth passage (publication number 1 in the dissertation).

The next purpose of my dissertation was to establish mtDNA levels in blood cells and fibroblasts taken from the patients at different disease stages, using the protocol based on publication number 1. Blood samples were analyzed, using the biological metarial from 62 symptomatic patients, 22 presymptomatic patients and 79 age- and sex-matched control subjects. Moreover, we collected skin fibroblasts from 10 genetically confirmed HD patients with symptoms of the disease, and 9 age- and sex-matched control subjects. In this publication we have found that, the relative mtDNA levels in leukocytes are significantly higher in symptomatic HD and presymptomatic patients relative to the control. The results obtained from experiments conducted using DNA isolated from fibroblasts showed the

opposite tendency, i.e. the significant lower level of mtDNA in skin fibroblasts from symptomatic patients in comparison to the control cells. Observed discrepancies among analyzed tissues might be potentially caused by medications taken by HD patients at the time of blood sample donation. Patients involved in these studies have used different drugs to treat various symptoms at the same time. Some of these drugs may influence the mitochondrial functions and mtDNA level. Furthermore, I aimed to correlate obtained results with the age of the patients and other parameters describing the severity of the disease, taking into account variety of symptoms. Correlations were also conducted with height, weight, BMI (Body Mass Index), alcohol drinking and cigarette smoking (number of units of alcohol drunk per week, number of cigarettes smoked per day and years of smoking). No significant correlations between mtDNA level in leukocytes and all analyzed parameters were found. The research described above is presented in publication number 2.

To obtain more detailed insights into mitochondrial abnormalities, we investigated different mitochondrial bioenergetic parameters, reactive oxygen species (ROS) production as well as a status of antioxidant defense system in skin fibroblasts of symptomatic patients. Comprehensive analyses of this aim of my dissertation were described in the 3rd publication. Based on previously establish protocols, I conducted all analyses with fibroblasts between 5 and 10 passages.

The first objective was to investigate the rate of the cell growth. Cell lines derived from patients have a slower cell growth rate than the control, which can point out the presence of deregulation of cell cycle propagation or overall metabolic status. To verify the first hypothesis, cell cycle analysis was conducted in fibroblasts after cell synchronization using serum starvation. The obtained data showed that despite some smallish but statistically significant alterations noticed in individual cell lines, the biological impact of these changes seems to be irrelevant. Thus, in my research, I put emphasis on the second hypothesis assuming that slower cells growth is caused by some metabolic alterations manifesting in mitochondrial defect. In order to assess possible bioenergetic disturbances, the ATP level, an indicator of bioenergetic status has been measured. Two different culture conditions have been used: medium with glucose, or medium without glucose where galactose served as the main carbon source. It has been found that in both culture conditions HD cells showed decreased ATP level, and this observation is more pronounced in the cells with restricted glycolysis (medium without glucose).

Next, the resazurin reduction assay has been used, which is based on measuring the reduction of the dye to the resorufin, and indirectly tests mitochondrial metabolic activity. This experimental approach revealed a statistically significant decrease in the resazurin reduction rate in HD fibroblasts in relation to the cells from healthy donors. In order to correlate reduced level of ATP in HD fibroblast with mitochondrial dysfunction, we measured membrane potential across the inner mitochondrial membrane ($\Delta\Psi$). Surprisingly, no significant changes in $\Delta\Psi$ between HD and control cells have been revealed.

To explain the lower ATP level, as well as a decline in metabolic activity of mitochondria, I evaluated the levels of individual subunits of the mitochondrial respiratory chain complexes, which are a part of oxidative phosphorylation (OXPHOS) machinery. In order to assess if potential differences are related to the different mitochondrial content, we also measured the level of the TOM20 protein, which can serve as a mitochondrial mass marker. Results were calculated as a ratio to β-actin protein, which was the internal loading control. Western-bloting experiments showed that analyzed subunits of the respiratory chain, as well as mitochondrial content in HD fibroblasts, did not differ from the values estimated for fibroblasts of healthy donors.

Another analyzed parameters were describing oxidative stress status (cytosolic ROS and superoxide radical formed in the mitochondrial matrix ($mt.O_2^{\bullet-}$)) as well as the levels of antioxidant enzymes. Interestingly, in HD fibroblasts, small but significant increase of $mt.O_2^{\bullet-}$ has been observed. To have a more complex view on the oxidative stress status we determined the levels of antioxidant enzymes, including superoxide dismutases (SOD1 and SOD2), catalase (CAT), glutathione peroxidase (GPx1/2) and glutathione reductase (GR). The results revealed elevated levels of SOD2 and GR ,probably as a response to the increased amount of $mt.O_2^{\bullet-}$ in HD fibroblasts. All this can indicate the presence of oxidative stress in the HD patients' cells.

The last aim of my dissertation was to perform complex statistical analysis, taking into account bioenergetic parameters, mtDNA level, ROS and antioxidant defense status to correlate all of them with the severity of the disease. To find any correlations principal components analysis (PCA) has been performed. Among many data describing the disease, a) Clinical Global Impression scale (CGI) comprising the psychiatric disturbances, b) duration of the motor symptoms, and c) Total Function Capacity (TFC), describing the general functioning of the patient in daily life have. PCA analysis showed that the profile of

mitochondrial parameters in patients' fibroblasts differs from control cells. Moreover, we can conclude that metabolic dysfunctions in the HD fibroblasts are more visible in early carriers. Usually, psychological complaints are the first symptoms that occur in the disease, and in our study, the scale concerning the mental problem (CGI) most strongly correlates with the investigated mitochondrial parameters.

Neurodegenerative conditions, like Huntington disease, Alzheimer disease, and Parkinson disease, are one of the biggest threats to the developing world, particularly in aging societies. Apart from some obvious obstacles associated with the development of new therapeutic strategy, it should be kept in mind that searching for proper biomarkers of the disease, especially in peripheral tissues, is also extremely important issue. To date, there is still little knowledge about metabolic alterations in blood cells and fibroblasts of HD patients at different stages of the disease. My studies have revealed that mitochondrial dysfunction is already manifested in fibroblasts of HD patients at the early disease stages. The research presented in this dissertation suggests that characterization profile of mitochondrial parameters seems to be a more promising approach than examining individual parameters in order to search for potential biomarkers. Bearing in mind the small number of used cell lines, it should be emphasized that current findings should be verified with studies on a larger group of subjects. My work represents one of a few reports describing the comprehensive analysis of the mitochondrial abnormalities in peripheral cells of HD patients. In conclusion, all findings presented in my PhD thesis allowed expanding our knowledge about mitochondrial dysfunctions in Huntington disease.

References:

1. **Jędrak P.,** Sowa N., Barańska S., Węgrzyn G., (2017). Characterization of conditions and determination of practical tips for mtDNA level estimation in various human cells. *Acta Biochimica Polonica*, 64(4), 699–704. doi: 10.18388/abp.2017_2303.

2. **Jędrak P**., Krygier M., Tońska K., Drozd M., Kaliszewska M., Bartnik E., Sołtan W., Sitek E.J., Stanisławska-Sachadyn A., Limon J., Sławek J., Węgrzyn G., Barańska S. (2017). Mitochondrial DNA levels in Huntington disease leukocytes and dermal fibroblasts. *Metabolic Brain Disease*, 32(4), 1237-1247. doi: 10.1007/s11011-017-0026-0

3. **Jędrak P**., Mozolewski P., Węgrzyn G., Więckowski M., (2018). Mitochondrial alterations accompanied by oxidative stress conditions in skin fibroblasts of Huntington's disease patients. *Metabolic Brain Disease*, doi: 10.1007/s11011-018-0308-1.