IV Streszczenie w języku angielskim: The genome-wide DNA methylation pattern alterations in the peripheral mononuclear cells in Graves' disease patients and thyroid-associated orbitopathy treated with intravenous glucocorticoids

Graves' disease (GD) is a type of the autoimmune thyroid disease (AITD) leading to hyperthyroidism. Etiopathology and clinical view of GD are compound and multifactorial, while genetic variants occur in the first place, but also environmental (mainly smoking) and endogenous (female sex) ones contribute to it. Simultaneously, genetic variants explain only about 10% expected susceptibility to GD and both clinical and genetic determinants of therapy response still remain unknown. GD treatment includes anti-thyroid drugs (ATD), as well as radical one (radioiodine, thyroidectomy) and is characterized by frequent failure and relapse rate, especially in children.

Graves orbitopathy (GO) is a phenotype and the main extrathyroidal sign of GD. It is a syndrome secondary to autoimmune processes in orbital soft tissue and might lead to vision loss. GO is present in about 30% of all GD patients, it occurs at various disease's stage and is characterized by heterogeneity; as regards to its risk factors, smoking seems to be of great importance. In active, moderate-to-severe GO treatment, apart from cigarettes cessation, intravenous glucocorticoids (ivGCS) and biological agents play a vital role.

As multifactorial disease model makes clear, other factors – like epigenetics (e.g. methylation), might link to genetic and environmental ones, worsen course and therapy response in GD. In literature, there are initial reports focusing on deoxyribonucleic acid methylation (DNAm) alterations in peripheral blood in autoimmune diseases, including GD. Interestingly, both antithyroid drugs (ATD) and radioiodine therapy have been shown to cause DNAm restoration in newly-diagnosed GD patients with global hypomethylation at baseline. Moreover, aberrant DNAm patterns occur in cytokines', adhesive particles and second-signal agents *loci* – important autoimmune factor), including GD and GO themself. The DNAm association with smoking has been addressed as well and it might be crucial for studied thyroid disorders' pathogenesis.

The aims of two projects involving this thesis included characterization of the genome-wide DNAm in peripheral blood cells in patients: i) with GO before and after 12 ivGCS pulses therapy (methylprednisolone; Main Project) according to EUGOGO and ii) newly diagnosed

GD individuals with different age of onset: adults (ang. adult GD, AGD) and children (ang. pediatric GD, PGD). In projects, we analysed the DNAm patterns in patents and controls, as well as in patients stratified by clinical view and according to treatment effects.

In Project no.1., the peripheral whole blood was taken in euthyroid patients with moderate-to-severe GO (n=24): before (point no. 1., P1) ivGCS and then post 12 ivGCS pulses (point no. 2., P2) in line with EUGOGO protocol. Patients were divided into two groups in accordance with treatment response (response, R, n=14 vs. no-response, NR, n=10). In Project no. 2. all GD individuals were hyperthyroid (AGD, n=12 oraz PGD, n=6) and with no prior history of antithyroid therapy. Controls were also recruited for AGD (ang. healthy controls, HC, n=7) and PGD (pediatric healthy controls, pHC, n=5) with no autoimmune and thyroid disorders. Laboratory methods included DNA (methyl-rich fragments) libraries preparation (n=78) and also next generation sequencing – reduced representation bisulfite sequencing (RRBS). Analyses were performed with bioinformatics environment using multiple comparison correction and with functional enrichment annotation being included. Differentially methylated cytosines with a minimum read threshold of 10 and DNAm (5-methycytosine:cytosine ratio) difference of 25% (0,25) were taken to further statistics.

We noted evident GO and HC samples separation in Project no.1 in principal component analysis (PCA), as well as genome-wide hypomethylated patterns in patients prior to treatment with ivGCS (P1). We showed change in samples' clustering and DNAm patterns after ivGCS therapy (P2). Moreover, on site-specific level, we identified a total of 2894 significant CpG (mainly hypomethylated – 99,17%) involving numerous *loci* including immune signal transduction and response (*CD55, ICAM, TNFRS1B*), these accompanying GD and GO pathogenesis (*IL-2RA, LOXL3, IL-6R, IGFR1*) and other, recently reviewed in literature (*DRD4, KLF9*). There were also many enrichment pathways shown basing on annotated CpGs, among others: lymphocytes maturation, immune response, cell growth and communication, as well as eye embryogenesis. Significant DNAm changes were shown to individual patient after ivGCS usage (P2), but for a whole GO group response to the treatment was not associated with DNAm on site specific level.

As for Project no.2, we noted dominance of hypomethylation in GD patients in few fields: both globally, based on each chromosome DNAm difference and for genome-wide patterns, as well as evident clustering of the probes (with smoking as a cofounder). A total of 10700 significant

DMRs (99,58% being hypomethylated) were detected, including AITD- and GO-connected (*IL2RA, IL6R, IGFR1*), immune signal transduction and response (*ICAM1, AIRE, STAT3, SMAD4, ALK, TNFRSF1B, CD5, CD7, CD8A, IL12RB1*), apoptosis (*BCL2*) and epigenetics-related ones (*MIR146B, DNMT3B, EZH2*). Conversely, DNAm difference in pediatric patients (PDG) and controls (pHC) was not evident, both in genome-wide scale and site specific significant positions (n=18; e.g. *TAGLN, ONECUT1, CALML5, NKX6-3*). Similarly, adult and pediatric GD patients did not robustly differ in case of site-specific DNAm (n=29 significant DMRs, among others: *FANK1, GRM2, CDK2AP2P2*), while in genome-wide analysis – smoking (but not age) served as additional factor and differentiated studied groups. Finally, we showed functional enrichment of pathways matched with found DMRs in newly diagnosed adults with GD. Morevoer, smoking-AGD individuals different DNAm patterns (dominant hypermethylation) than non-smokers (a total of n=2183 significant DMRs).

In conclusion, in this doctoral thesis we showed that patients with GD and GO present with different DNAm patterns and significant DNA hypomethylation in comparison with healthy subjects; the effect was observed both in genome-wide scale and on site-specific level:immune system, cell growth and differentiation, as well as inflammation. Children with GD present a few DMRs, while global patterns do not differ robustly with healthy children and look similar to non-smoking AGD individuals. DNAm level change is associated with ivGCS treatment, but is irrelevant to therapy response. Taking into account numerous issues: current knowledge comprising DNAm nature, molecular formation, as well as its dynamics and two-sided influence of genetics, environment and endogenous factors, which all being accompanied by DNAm laboratory assessment and GD heterogeneity, further studies in the field are necessary.