Final Report

Identification of novel disease modifiers in autoimmune thyroid diseases

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Background and aims

Graves' disease (GD) is an autoimmune thyroid disease, the most common cause of hyperthyroidism in iodine-sufficient countries. The etiology of GD is multifactorial. Epidemiological data suggest interplay of multiple genetic and environmental factors that affect both the thyroid and the immune system leading to the loss of tolerance to the thyroid stimulating hormone receptor (TSHR). Graves' orbitopathy (GO) is an extrathyroidal manifestation of GD, which appears in up to 50 % of patients. This inflammatory autoimmune disorder of the orbits is characterized by immune cell infiltration, fibroblast proliferation, expansion of orbital connective tissue matrix, leading to the redness and swelling of conjunctivae and lids, ocular pain, proptosis, eye muscle swelling and dysfunction, and in the most severe cases can lead to sight loss. Even mild GO cases are disfiguring and may substantially impair the patients' quality of life. Despite the fact that several aspects of the GO pathophysiology have been revealed, we still cannot predict reliably who and when does develop GO among patients with GD. The purpose of this research has been to study specific factors, which may predispose to or protect against the development of GD and/or GO.

Major results

After completion of the sample collection, demographic and clinical data of patients with GD were examined. Patients whose disease related conditions could not be retrospectively analysed with certainty, were excluded from further analysis. Plasminogen activator inhibitor-1 (PAI-1) 4G/5G and haptoglobin (Hp) polymorphisms, IgG4 and mannose binding lectin (MBL) levels were assessed as planned.

PAI-1 is the main regulator of fibrinolysis and plasmin dependent pericellular proteolysis by inhibiting plasminogen activators. PAI-1 can influence the duration and magnitude of immune responses, its level or activity may be potential factors in the

development of autoimmune diseases. Increased PAI-1 expression and/or activity stabilizes extracellular matrix and inhibits its clearance via inhibition of plasmin and subsequent matrix metalloproteinase activation. This maintains a supporting scaffold for proliferation and leads to connective tissue expansion, which has a key role during the course of GO. It was known that the 4G/5G polymorphism of the PAI-1 gene influenced PAI-1 expression in response to several stimuli, including IL-1α. Based on our previous observations on the role of PAI-1 in GO, we have hypothesized that certain polymorphisms may predispose to GO. For the analysis of the PAI-1 4G/5G polymorphism, the study population included 185 GD patients (87 with GO and 98 without GO) and 201 sex-matched controls. Genotypes were determined using an allele specific PCR method. The genotype distribution and allele frequencies of PAI-1 4G/5G polymorphism did not differ (p=0.815 and p=0.216, respectively) in GD patients (4G/4G: n=49, 26%; 4G/5G: n=109, 59%; 5G/5G: n=27, 15%; 4G allele frequency: 0.56) compared to controls (4G/4G: n=59, 29%; 4G/5G: n=110, 55%; 5G/5G: n=32, 16%; 4G allele frequency: 0.57). In GD patients 4G/4G genotype was associated with GO risk (OR= 1.950, 95% CI=1.005 – 3.786, p=0.047), especially with moderate-to-severe GO risk (OR= 2.545, 95% CI=1.261 – 5.135, p=0.008). It is not known which immune mediators may exploit the fact that the presence of the 4G allele in homozygote form in an individual makes him/her prone to more severe GO. The main candidate is IL-1α, a cytokine with emerging importance in the initiation and maintenance of inflammation, which is known to upregulate PAI-1 expression with higher efficacy in cells carrying the 4G allele of the PAI-1 gene. The locally elevated cytokine level in the orbital connective tissue during GO development may lead to increased PAI-1 synthesis by orbital fibroblasts, and this process may be more marked in the presence of the 4G allele. The consequent high local PAI-1 level may have an adverse effect on the development and course of GO by increasing the amount of extracellular matrix, stabilizing it, and promoting immune cell migration. PAI-1 4G/5G polymorphism had no effect on plasma PAI-1 levels or on any of the measured clinical parameters in treated GD patients beyond the active phase of the disease. These results were published open access in the Journal of Inflammation Research (**Katko M, Galgoczi E, Erdei A, Gazdag A, Berta E, Bodor M, Seres I, Hircsu I, Badics A, Ujhelyi B, Sira L, Bhattoa HP, Nagy EV. The 4G/5G polymorphism of plasminogen activator inhibitor type 1 is a predictor of moderate-to-severe thyroid eye disease. *J Inflamm Res*. 2021 May 12;14:1883-1890. doi: 10.2147/JIR.S307046**).

In addition to hemoglobin binding, haptoglobin (Hp) also plays a role in the development of the immune response and autoimmune processes. During inflammation, its

levels increase and it has an anti-inflammatory effect by regulating the cytokine production of macrophages, as well as affecting the function of B and T cells. Two codominant alleles (Hp1 and Hp2) are responsible for the Hp polymorphism, combinations of which result in 3 major phenotypes (Hp1-1, Hp2-1, and Hp2-2). The immunoregulatory and antioxidant properties of Hp molecules with different phenotypes are different: the Hp1 allele shifts the Th1 / Th2 balance of T helper cells to the Th1 direction, and the Hp 1-1 phenotype has a higher antioxidant capacity. Oxidative stress and the Th1/Th2 balance play an important role in the pathogenesis of both GD and GO. Hp phenotypes of 185 GD patients were assessed, allele and phenotype frequencies were compared to previously published data of the normal Eastern Hungarian population (Tauszik and Szabo, 1992, Gene Geogr). Neither the phenotype distribution nor the allele frequencies of Hp polymorphism differed (p=0.391 and p=0.395, resceptively) in GD patients (Hp1-1: n=24, 13%; Hp 2-1: n=84, 45%; Hp 2-2: n=77, 42%; Hp 1 allele frequency: 0.36) compared to the normal population living in East-Hungary (Hp1-1: n=188, 11%; Hp 2-1: n=789, 45%; Hp 2-2: n=763, 44%; Hp 1 allele frequency: 0.33). The onset of GD in patients with Hp 1-1 phenotype occurred later in life (mean with SD: 48 ± 13 years) compared to patients with Hp 2-1 phenotype (mean with SD: 39 ± 16 years, p=0.011) or with Hp 2-2 phenotype (mean with SD: 39 ± 13 years, p=0.009). In GD patients the presence of each Hp 1 allele increases the risk of GO (OR= 2.035, 95% CI=1.321 – 3.135, p=0.001). In conclusion, in patients with the Hp 1-1 phenotype higher antioxidant capacity may delay the onset of GD, while in patients with GD Hp 1 allele may increase the risk of developing GO, possibly by shifting the Th1 / Th2 balance. These results were presented in a poster (**Katkó M, Galgóczi E, Gazdag A, Erdei A, Berta E, Bodor M, Hársfalvi J, Bhattoa HP, Nagy V. E. A haptoglobin polimorfizmus szerepe a Graves kór és az endokrin orbitopathia pathogenesisében. P-3. XXVIII. Congress of the Hungarian Society of Endocrinology and Metabolism, Aug. 26-28. 2021, Eger**).

In a recent study it was found that Hp is an endogenous high-mobility group box 1 (HMGB1) binding protein directing HMGB1 to a CD163-dependent pathway that activates antiinflammatory signaling in the monocyte-macrophage lineage. Extracellular HMGB1 is a damage-associated molecular pattern molecule with a crucial role in inflammatory and autoimmune diseases; it can be actively secreted by inflammatory immune cells or passively released during apoptosis or necrosis. Since it is not known whether there is a preference for any Hp phenotype to bind HMGB1, we measured serum HMGB1 in GD patients, and results analysed according to Hp phenotypes. No differences were found in serum HMGB1 levels in

patients with distinct Hp phenotypes. Meanwhile a study about the potential role of HMGB1 in GO was recently published by Han et al. (2019, Thyroid) concluding that serum HMGB1 level can be used as a biomarker of GO activity. In GD patients beyond the active phase of the disease serum HMGB1 concentration was higher than in a group of 47 age and sex-matched controls (medians and IQRs: 6.6, 3.4-13.7 ng/ml vs 1.7, 0.9-4.3 ng/ml; $p<0.0001$), but in patients neither GO status nor TSH receptor stimulating antibody (TSHR Ab) status were associated with HMGB1.

CD163 is the scavenger receptor of Hp complexes expressed on monocytes/macrophages, which can be shed as a soluble serum protein (sCD163) with immunomodulating capacity and functions as a marker of macrophage (M2) activation. In our study population with GD no associations were found between serum sCD163 and Hp phenotypes or GO status. In GD patients sCD163 concentration was moderately higher than in a group of 47 age and sex-matched controls (medians and IQRs: 0.71, 0.56-0.87 mg/l vs 0.62, 0.50-0.75 ng/ml; $p=0.016$). sCD163 levels showed positive correlations with age ($r=0.28$, $p<0.0001$), plasma PAI-1 ($r=0.27$, $p<0.001$) and serum hyaluronan ($r=0.23$, $p<0.005$). In a multiple linear regression analysis age ($p<0.005$) and plasma PAI-1 ($p<0.0005$) were significant predictors of serum sCD163 level, whereas serum hyaluronan was not ($p=0.06$). In a study examining its pro-tumorigenic role, PAI-1 promoted the recruitment and M2 polarisation of monocytes/macrophages, and strong positive correlation was found between PAI-1 and CD163 expression (Kubala et al, 2018, Cell Reports). Although, both PAI-1 and macrophage infiltration into the orbital connective tissue have potential pathogenic role in GO, further studies are needed to clarify the relevance of the association found between PAI-1 and sCD163 in patients with GD and/or GO in remission.

Hyaluronan has a crucial role in the chronic autoimmune inflammation affecting retrobulbar connective tissue and external eye muscles in GO. Any therapeutic measure diminishing local HA production could interfere with the pathogenesis of GO. 4-methylumbelliferone (4-MU), an inhibitor of hyaluronan synthesis has successfully been used in animal models of autoimmunity where hyaluronan is supposed to contribute to disease pathogenesis. HA production and mRNA expression of HA synthases (HAS1, HAS2, and HAS3) and hyaluronidases (HYAL1 and HYAL2) were measured in the presence and absence of 4-MU in unstimulated and transforming growth factor–β–stimulated fibroblasts from GO orbital ($n = 4$), non-GO orbital ($n = 4$), and dermal origin ($n = 4$). The 4-MU treatment (1 mM) for 24 hours resulted in an average 87% reduction ($P < 0.001$) of HA synthesis, decreased the

expression of the dominant HAS isoform (HAS2) by 80% ($P < 0.0001$), and increased the HYAL2 expression by 2.5-fold ($P < 0.001$) in control orbital fibroblasts (OF), GO OFs, and dermal fibroblasts (DFs) regardless of the origin of the cells. The proliferation rate of all studied cell lines was reduced to an average 16% by 4-MU ($P < 0.0001$) without any effects on cell viability. HA production stimulated by transforming growth factor–β was decreased by 4-MU via inhibition of stimulated HAS1 expression in addition to the observed effects of 4-MU in unstimulated cases. Characteristics of HA synthesis inhibition by 4-MU did not differ in OFs compared with DFs. 4-MU has been found to inhibit the HA synthesis and the proliferation rate in OFs in vitro, adding it to the list of putative therapeutic agents in a disease the cure of which is largely unresolved. These results were published open access in Investigative Ophthalmology and Visual Science (**Galgoczi E, Jeney F, Katko M, Erdei A, Gazdag A, Sira L, Bodor M, Berta E, Ujhelyi B, Steiber Z, Gyory F, Nagy EV. Characteristics of Hyaluronan Synthesis Inhibition by 4-Methylumbelliferone in Orbital Fibroblasts.** ***Invest Ophthalmol Vis Sci.*** **2020 Feb 7;61(2):27. doi: 10.1167/iovs.61.2.27**).

Serum hyaluronan in the studied group of patients with GD in remission did not show association with GO or TSHR Ab status and did not differ from hyaluronan concentrations measured in the control group.

IgG4 is the least abundant IgG subclass in human serum. Under conditions of chronic antigenic stimulation and Th2-type inflammation, both tissue and serum IgG4 levels are increased. IgG4 levels were found to be significantly higher in GO compared with GD patients without orbitopathy. The orbital manifestations of IgG4-related disease (IgG4RD) are very similar to GO. IgG4RD is a recently described and increasingly recognized entity, which has a form involving the thyroid. A review on this topic with a case report was published open access in BMC Ophthalmology (**Erdei A, Steiber Z, Molnar C, Berenyi E, Nagy EV. Exophthalmos in a young woman with no graves' disease - a case report of IgG4-related orbitopathy. BMC Ophthalmol. 2018 Jan 12;18(1):5. doi: 10.1186/s12886-018-0672-y**).

Serum IgG4 concentration was higher in the studied GD patients in remission of the disease than in the control group (medians and IQRs: 0.36, 0.19-0.63 g/l vs 0.23, 0.09-0.46 g/l; p=0.01), but no difference was found between patients with and without GO (medians and IQRs: 0.38, 0.25-0.60 g/l vs 0.33, 0.14-0.63 g/l; p=0.23), and no association was found between serum IgG4 and TSHR Ab.

In addition, serum IgG4 levels were measured in the sera of newly diagnosed GD patients (n=30). At the time of the diagnosis of hyperthyroidism mild GO was observed in 10 patients. At follow-up, an additional 4 patients developed GO. No association was found between IgG4 levels at GD diagnosis and the development of GO. IgG4 levels measured in the euthyroid state showed a significant decrease during follow-up (0.45 g/l vs 0.28 g/l; p=0.028). These results were presented in a poster (**Erdei A, Katkó M, Székely A, Gazdag A, Berta E, Bodor M, Nagy V. E. IgG4 szint – mint lehetséges prognosztikai marker – vizsgálata Graves-Basedow kórban és endokrin orbitopathiában P-30. XXVIII. Congress of the Hungarian Society of Endocrinology and Metabolism, Aug. 26-28. 2021, Eger).**

Mannose binding lectin (MBL) is a soluble recognition molecule, a C-type lectin capable of activating the complement system via the lectin pathway. MBL binds to highly conserved oligosaccharides present on the surface of microorganisms and to altered patterns on the cell surface, and facilitates phagocytosis as an opsonin. MBL can shift the cytokines released as a result of its interaction with phagocytic cells during the clearance of apoptotic cells towards the anti-inflammatory profile, which may function as a barrier to immune response to self-antigens. Its protein level is mostly genetically determined. Combinations of the polymorphisms in the coding MBL2 gene result in a great variation of the circulating MBL levels in healthy individuals. Our preliminary results have shown that serum MBL levels are highly dependent on thyroid hormone levels, and the MBL concentrations measured in treated GD patients, especially in patients with hypo- or hyperthyroidism should not be used for genetic classification of the patients. MBL status of GD patients who had both TSH and free thyroid hormones in the reference range (n=83) was found to be different (p=0.03) from a control population (n=100). More patients than controls had their MBL levels in the lower concentration ranges: MBL deficiency (<100 ng/ml) 18% vs 11%, low MBL level (100-500 ng/ml) 13% vs 15%, intermediate MBL level (500-1000 ng/ml) 23% vs 10% and high MBL level (>1000 ng/ml) 46% vs 64%, respectively. In these patients, levothyroxine supplementation was common (69%), so their euthyroid state with supplementation may not be identical to their euthyroid status before the onset of GD, therefore these results cannot be considered conclusive. However, the changes of the thyroid hormone levels during the course of the disease may affect the chronic autoimmune inflammatory process (e.g. relapsing and remitting course of the disease, development of GO) through influencing MBL levels. Prospective studies would be more suitable to examine this potential association.

Measurement of another factor, milk fat globule-EGF factor 8 protein (MFG-E8), with a role in the clearance of damaged/apoptotic cells has been carried out. MFG-E8 forms a molecular bridge between the phosphatidylserine in the membrane of apoptotic cells and the $\alpha v\beta 3/\alpha v\beta 5$-integrin expressed on activated macrophages and enhances phagocytosis. Either low or high level of MFG-E8 could impair clearance of damaged/apoptotic cells. Higher levels of serum MFG-E8 were found in GD patients in remission of the disease than in controls (medians and IQRs: 4.24, 3.37-5.44 ng/ml vs 3.55, 2.77-4.25 ng/ml; $p<0.0001$) and in patients with GO than in patients without GO (medians and IQRs: 4.65, 3.77-6.14 ng/ml vs 3.91, 3.19-5.13 ng/ml; $p<0.005$). Increased serum MFG-E8 levels suggest a higher macrophage activity in GD patients even in the remission of the disease.

In summary, we have identified PAI-1 4G/5G and haptoglobin polymorphisms as novel disease modifiers in GD patients. Further, a new potential therapeutic agent, 4-MU has been identified for GO. Other studied factors (serum IgG4, MBL, HMGB1, sCD163, hyaluronan and MFG-E8) are suggested to have a role during the course of GD as markers of chronic inflammation and macrophage activation; further prospective studies to better describe their role are warranted. We feel that the rather self-restricted budget of this Research Proposal, 11.5 MFt for the full project, has been successfully used: three papers were published in Q1 and Q2 journals (IF 13.152).