SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Causes and consequences of retrobulbar connective tissue proliferation in endocrine orbitopathy

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1. Introduction and review of literature

Endocrine orbitopathy (EOP) is the ophthalmological condition most commonly associated with autoimmune thyroid disease. The etiopathogenesis of EOP is currently not fully understood and the exact mechanism is the subject of intense research. It is five times more common in women and is more likely to affect women of childbearing age. The orbital fibroblasts (OF) play a prominent role in the retrobulbar space. The lymphocyte infiltration and fibroblast activation induced by cytokine release leads to remodelling of the retrobulbar connective tissue space. Activated OFs play an important role in the altered synthesis and degradation of extracellular matrix (ECM) elements, and thus in the pathogenesis of EOP. OFs produce prostaglandin E2 (PGE2), glycosaminoglycan (GAG) and large amounts of hyaluronic acid (HA). The accumulating HA further accelerates the expansion of orbital connective tissue due to its hydrophilic nature.

The clinical presentation of EOP is characterised by proptosis, inflamed, oedematous, and retracted eyelids. The autoimmune inflammation causes an increase in the connective tissue of the eye muscles and orbit. Dysfunction of the eye muscles can lead to double vision. Inadequate eyelid closure can lead to insufficient tear film, in all cases causing dry eye disease and in more severe cases corneal ulceration. The most severe form is dysthyroid optic neuropathy (DON), which threatens with a gradual deterioration of visual acuity and permanent loss of vision.

Smoking is the most important modifiable risk factor for EOP and is also a significant predisposing factor for both respiratory and non-respiratory cancers. The adverse effects of smoking on the disease include: formation of reactive oxygen species (ROS), increased oxidative stress effects, increased adipogenesis and more pronounced HA production. While EOP is more common in smokers, the severity of the disease increases in direct proportion to the number of cigarettes smoked.

Nowadays, alternative forms of smoking such as heated tobacco products (HTP) or e-cigarettes (ECIG) are becoming more common, in addition to traditional cigarettes (CIG). These new devices offer a smoke-free alternative and produce less sidestream smoke indoors, making them more socially acceptable.

Their role in the pathogenesis of EOP is currently unknown. Our clinical experience shows that switching from traditional cigarettes to HTP forms of smoking in patients unable to quit

smoking resulted in a small improvement in clinical symptoms. In our study, we wanted to investigate this phenomenon under in vitro conditions.

A novel technique used in our study is Real Time Cell Electronic Sensing (RT-CES), which determines the kinetics of growing cells in a scanning culture by measuring impedance changes. The 16-well cell culture plate used for the assay has gold electrodes at the bottom, to which the instrument applies an alternating voltage. Without cells, the ions in the cell culture medium flow freely. The depositing cells change the flow of ions by covering the electrodes. Since the system uses alternating current, it measures impedance changes rather than resistance. It is kinetic, time-dependent, and can measure the activity of the cells, allowing a very accurate determination.

It is known that during pregnancy, physiological immunosuppression mostly reduces the activity of autoimmune diseases. Rarely, in less than one percent of cases, EOP may flare up during pregnancy. In these rare cases, spontaneous improvement is observed in the second trimester and relapse often occurs 4-8 months after delivery. In our work, we report a unique case in the literature in which endocrine orbitopathy developed during pregnancy and the patient had no history of previous thyroid disease. The explanation of this phenomenon is highly complex in the light of the many physiological changes that occur during pregnancy, but a clear association with the presence of smoking during pregnancy is apparent.

2. Objectives

- Several studies have previously addressed the effects of traditional tobacco products on orbital fibroblasts. In contrast, to the best of our knowledge, similar studies have not yet been conducted with ECIG and HTP fumes. Our aim was to investigate the effects of these tobacco products on EOP and healthy orbital fibroblasts.
- In addition to conventional laboratory tests, we have also investigated the behaviour of OFs using a new, label-free in vitro measurement method, the RT-CES technique, which can serve as a more sensitive tool for testing cells.
- We wanted to investigate the case of a pregnant woman, a rarity in the literature, who presented to our clinic with a new-onset EOP during her third pregnancy. She had no history of thyroid disease and no similar complaints in her previous two pregnancies, delivering two healthy newborns.

3. Patients and methods

3.1. Investigating the effects of new types of smoking in endocrine orbitopathy

Patient data

EOP OF cultures were generated from connective tissue removed during decompression surgery in four patients with EOP, and control OF cultures were generated from connective tissue removed during enucleation surgery for four patients with intraocular tumors, with no history of previous thyroid disease. In none of the cases did the intraocular tumor extend outside the eyeball, and no scleral infiltration was present. The extraocular space, including the orbital tissue, was intact. All patients gave written informed consent and the study was approved by the Regional and Institutional Research Ethics Committee of the University of Debrecen and was performed in accordance with the Declaration of Helsinki. Ethics approval number: 5913/2012/EKU (84/13.).

Cell cultures

Cell cultures were created as described by Bahn et al. Briefly, surgical tissue samples were cut into small pieces and placed in culture dishes containing medium 199 (M199), supplemented with 20% (v/v) FBS and penicillin-streptomycin, and maintained at 37°C, 5% CO2 and the medium was changed every 3-4 days. After fibroblast outgrowth, tissue pieces were removed and cell cultures were maintained in M199 containing 10% (v/v) FBS. The cells were then stored in liquid nitrogen until the experiments were performed. Primary orbital fibroblasts were assayed at a low passase number (2-8).

Smoke extracts

Smoke extracts (FE) were prepared based on previous articles. Four reference IR6F cigarettes containing 8.6 mg of tar and 0.7 mg of nicotine per cigarette were used to prepare the CIG smoke extract. 75% of each cigarette were burnt and the smoke was passed through a 30 ml buffer solution using a special pump (MILLIPORE XF54 230-50). The solution was filtered through a 0,2 um membrane filter to sterile. The 12.5% CIG smoke solution corresponds to 100 cigarettes smoked per day. For our experiment we used a lower 1% and a higher 50% CIG smoke solution.

We also tested smoke from new types of tobacco products. In the case of the commercially available HTP device (iQOS, Philip Morris International Global Services Inc., New York,

NY, USA), four unflavoured tobacco cartridges were used to prepare the FE. We performed 12 puffs per refill with the pump for a total of 48 puffs, based on previous articles. The electronic cigarette we used (Eleaf iStick Pico, Shenzhen, China) was also a commercially available device. The power of the device was set to 16 Watts and the ECIG liquid (e-liquid) from which the device produces the atomized smoke was loaded into the device at a ratio of 49.4% propylene glycol, 49.4% glycerol and 1.2% nicotine, as previously reported. M199 medium was used for dilution. First, a high concentration (50%) of CIG extract was added to the cells to test whether there was a measurable change in the cells with RT-CES and to compare this with the results obtained with the MTT assay used to measure HA synthesis and metabolic activity. In the next series of measurements, lower concentrations (1%) of smoke extracts from all three products (CIG, ECIG, HTP) were used. All measurements were time-dependent, RT-CES provided continuous measurements for 168 h, HA and MTT tests were performed after 24, 48 and 72 h. Each measurement was performed three times and the final results are the average of the three measurements.

Real-Time Cell Electronic Sensing (RT-CES) technique

The RT-CES technique was used to study the effects of the three smoke mixtures prepared as described above on orbital fibroblasts. This is a relatively new label-free in vitro measurement method, the reliability of which has been described several times before. To our knowledge, it was the first to be applied to OF cells. The device allows real-time impedance determination based on electrical impedance variation measurements. The bottom of the 16-well cell culture plate is fitted with gold electrodes, allowing the free flow of medium ions without cells. As the cells adhere to the electrodes, the ion flow changes, which is detected and recorded by the device. The device uses alternating current, so it measures electrical impedance, not resistance. The cells were spread in M199 and, for FE-treated lines, the three types of FE were added at the start of the assay. Real-time data were collected, the instrument measured every 15 min, and the data were evaluated using the Cell-Index (CI), which is a ratio of the impedance change from the initial impedance value. It is a ratio without units. Measurements were performed for 72 hours.

Measurement of metabolic activity

To determine the metabolic activity of cells, we used MTT assay, a colorimetric assay to assess the metabolic activity of cells. Cells were expanded in 96-well plates at a density of 10^4 cells/well and maintained in M199 medium in a CO2 incubator at 37°C for 7 days, tobacco smoke extracts were added to this solution before starting the measurement. Supernatants were removed and stored at -20°C until measurements were performed. For the first three days, MTT was added to the cells after 21, 45 and 69 hours. A ratio of 10 ul (5mg/ml) MTT/ 100 ul medium was used. Cells were then incubated for 3 hours at 37°C. Depending on their metabolic activity, the cells converted yellow MTT to a water-insoluble blue formazan. Blue formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Absorbance at 595 nm was detected using a Beckman Coulter, DTX 880 Multimode Detector (Beckman Coulter Inc., Brea, CA, USA). The metabolic activity of the cells was expressed as the ratio of untreated to treated.

Quantitation of Hyaluronan

The secreted HA in the supernatant was measured using the DuoSet Hyaluronan Kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. In each case, results were adjusted for the HA content of FBS. HA production was expressed as the percentage of the untreated control.

Statistical Analysis

Statistical analysis was performed for RT-CES results using repeated measures ANOVA followed Tukey's multiple comparisons post hoc test (GraphPad Prism 8.0.1, GraphPad Software, San Diego, CA, USA). The level of statistical significance was set at $p < 0.05$.

3.2 Patient data - Effect of pregnancy on the development of endocrine orbitopathy

Clinical characteristics of the patient

We present a study of a pregnant patient, her case being complicated by Graves disease with endocrine orbitopathy. As a rarity in the literature, she developed EOP during pregnancy, while she had no history of previous thyroid disease and no previous ocular complaints, with no similar symptoms in her two previous pregnancies.

Laboratory tests

The following disease-specific laboratory tests were performed:

TSH level determination. TSH can be used as a very sensitive and specific parameter to measure thyroid function and is particularly suitable for the early detection or exclusion of thyroid diseases. TSH levels were determined using the Elecsys TSH kit (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim).

Determination of T3 and T4: Triiodothyronine (T3) and thyroxine (T4), produced and secreted by the thyroid gland, are important in the regulation of metabolism, affect cardiovascular function, growth and bone metabolism, and are important for normal development of the glandular functions and the nervous system. Both hormones are mostly transported in the bloodstream bound to proteins, such as thyroxine-binding globulin (TBG), pre-albumin and albumin. Only 0.03% of T4 and 0.2-0.4% of T3 is present in biologically active free form (fT3, fT4). Determining the levels of these free hormones is important in monitoring thyroid disease. For their measurement we used the Elecsys FT3 III and Elecsys FT4 III kits (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim).

TSH receptor autoantibody determination: in GD, hyperthyroidism is typically caused by autoantibodies against the TSH receptor (TSHR) and the measurement of these TSHR antibodies (TRAb) is an important tool in the diagnosis and treatment of the disease. It was measured using the Elecsys Anti-TSHR kit (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim).

Anti-thyroglobulin antibody assay: thyroglobulin is a large glycoprotein whose synthesis and release is regulated by TSH. The anti-thyroglobulin antibody assay is mainly used in suspected autoimmune thyroid diseases. The LIAISON® Anti-Tg kit (DiaSorin S.p.A. Via Crescentino snc - 13040 Saluggia (VC) - Italy) was used for the measurement.

Ophthalmic examinations

Exophthalmos, or proptosis is one of the most common symptoms of EOP, occurring in up to 70% of patients. It is mostly bilateral, but in 10-20% of cases it can also be asymmetric. The Hertel exophthalmometer is used to determine the degree of exophthalmos. The instrument measures the distance in millimetres between the cornea and the lateral edge of the bony orbit.

The upper limit in the caucasian population is 20 mm. The degree of protrusion can show a large individual variation and therefore the initial Hertel score of the patient should be taken into account and, if no such data are available, a deviation of 2 mm from the population mean is recommended for diagnosis.

To assess the activity of EOP, the Clinical Activity Score was used, and a slit-lamp examination was performed. The scores were determined based on the four classic signs of inflammation in ophthalmology: pain, swelling, redness and loss of function. We assess the presence of each of these symptoms on an orbital basis. If the patient's CAS score is 4 or higher, it indicates immunologically active disease.

Perinatological examinations

The fetus did not show any signs of malformation, tachycardia or goitre on post-partum physical examination or ultrasound examinations, and showed a reassuring growth rate on follow-up examinations.

4. Results

4.1. Investigating the effects of new types of smoking in endocrine orbitopathy

Effects of higher (50%) concentrations of CIG smoke on orbital fibroblasts

RT-CES measurements with higher (50%) concentrations of CIG smoke

Initially, a high CIG concentration (50%) was used. In our experiments, we aimed to observe whether HA secretion and metabolic activity of OFs were related to the CI measured by RT-CES. After identifying the optimal cell density, we determined the cell number in 10 000 cells per well at 200ul. The baseline CI values of EOP cells were significantly different from those of control cells ($p<0.0001$). The maximum CI value of EOP OFs measured here was 3.3, whereas the CI value of control OFs peaked at 2.79. The origin of the cells determined their behaviour, with EOP OFs recording higher CI values throughout the measurement after the initial 10 h ($p<0.0001$).

Control OFs did not respond to 50% CIG ($p = 0.46$); no increase was induced in the CI levels. The cells reached the maximum CI (CI = 3.00) at 29 hours. EOP OFs behaved differently, with CI values of FE-treated EOP OFs significantly increased compared to untreated EOP OFs ($p < 0.0001$), reaching a maximum at 6 h (CI = 5.53). Thereafter, a rapid decrease was observed, with CI values decreasing to levels of untreated EOP OFs by the 60^{th} hour.

Measurement of metabolic activity with CIG smoke mixture (50%)

In terms of metabolic activity, EOP and control cells behaved differently. At 24 and 48 h of treatment, the metabolic activity of cells treated with CIG was significantly lower compared to cells untreated with FE (EOP OF 24 h: $p < 0.03$; 48 h $p < 0.01$; control OF 24 h $p < 0.01$; 48 h $p < 0.01$). At hour 60, the difference in metabolic activity between untreated and CIG-treated cells disappeared for all cell lines tested (EOP $p = 0.66$; control $p = 0.89$). After 72 hours, CIG-treated lines showed significantly higher activity rates than untreated cells (EOP $p = 0.001$; control $p = 0.004$).

Production of hyaluronic acid with (50%) concentration of CIG smoke mixture

CIG treatment induced an increase in HA production in both control and EOP cells in all lines at all time points measured (EOP $p<0.002$; control $p<0.04$). EOP and control cells responded to 50% concentration of CIG treatment with a constant, twofold increase in HA production. HA production did not differ between EOP and control fibroblasts in either untreated or FE-treated cultures (untreated $p=0.99$, 24 h $p=0.95$, 48 h $p=0.87$, 72 h $p=0.67$).

Effect of low concentration (1%) smoke mixtures on orbital fibrolasts

RT-CES measurements with low concentration (1%) smoke mixtures

In the following series of experiments, all EOP and control OF were exposed to low concentrations (1%) of FE, corresponding to approximately 8 cigarettes smoked per day. At this concentration, both CIG, HTP and ECIG extracts were tested in all OF cultures.

For EOP OFs, ECIG caused the largest difference in CI values (CI = 6.33). This was closely followed by HTP-treated EOP OFs (CI = 6.25). CIG-treated EOP OFs showed the smallest CI difference in the present measurement (CI = 5.38).

EOP cells not treated with FE had lower CI values than their tobacco smoke treated pairs. The CI values of EOP cells treated with CIG peaked at 9 hours (CI = 5.38); EOP cells treated with HTP and ECIG showed similar behaviour, with a plateau-like curve. The peak value of ECIG-treated EOP cells was CI=6.33 and the peak value of HTP-treated EOP was CI=6.25. The responses expressed in cell index to CIG, HTP and ECIG were different ($p < 0.0001$). Control cells not treated with FE showed rapid growth and peaked after 8 h (CI = 2.79).

Control cells treated with CIG smoke extracts showed similar values as untreated control OFs, with a highest CI of 2.62 and a similar slope of the CI curve. In comparison, control cells treated with HTP and ECIG showed a significant difference in CI ($p<0.0001$); both HTP and ECIG treated control fibroblasts behaved similarly until hour 22, when CI started to increase

in both lines. The HTP treated control line reached a maximum at hour 32 (CI = 3.32) and the ECIG line at hour 52 (CI = 3.41).

Measurement of metabolic activity with low concentrations (1%) of smoke extracts

No significant differences in metabolic activity were observed in FE-treated OF lines compared to non-FE-treated cells after 24, 48 and 72 hours after treatment with 1% HTP, ECIG and CIG. There was also no difference in baseline metabolic activity between FE-untreated EOP and control cells. In contrast to 50% CIG treatment, 1% HTP, ECIG and CIG had no effect on metabolic activity (p=0.9)

Hyaluronic acid production with low concentrations (1%) of smoke extracts

No difference was found in the production of hyaluronic acid by EOP and control OF after treatment with 1% FE compared to untreated OF. All cell lines behaved similarly in this respect, whether derived from diseased or control orbits (p = 0.83). 1% FE had no effect on HA production by the cells tested at either 24 h or 48 h after treatment (p = 0.14).

4.2.1 Effect of pregnancy on the development of endocrine orbitopathy

In our study, we followed a pregnancy complicated by GD followed by EOP, aiming to clarify the background and pathogenesis of the clinical picture, which was unusual and different from the expected scenario.

For the first time in the literature, we describe a new-onset EOP disease in the second trimester of a 19-year-old woman during her third pregnancy.

The patient had no previous history of thyroid disease. Hyperthyroidism was diagnosed by her general practitioner at the 20^{th} week of pregnancy.

Her laboratory findings at that time were as follows: TSH 0.01 mU/L; FT4: 78.4 pmol/L (reference range: 12-22 pmol/L), FT3: 37.4 pmol/L (reference range: 3.1-6.8 pmol/L).

For unknown reasons, she did not appear at her next visit to her local endocrine clinic until the 30th week of pregnancy, and her laboratory results continued to show signs of hyperthyroidism, for which thyrostatic therapy (thiamazole) was initiated. No other known medical conditions were reported, no previous thyroid problems and no pre-pregnancy laboratory results were available. All three of her pregnancies were naturally conceived, her

previous two children were delivered by caesarean section due to disproportion. Her medical history included smoking 20 cigarettes a day, which she failed to quit during pregnancy.

She first presented to our clinic at the 33th week of gestation, with her main complaints of right sided proptosis, decreased and painful eye movements and decreased visual acuity. According to her, her first symptom, proptosis appeared in her right eye at 14 weeks of pregnancy. On admission, TSH and thyroid hormone levels were still in the hyperthyroid range. TRAb was 2.4 U/L (reference range:<1 U/L), anti-thyroglobulin antibody level was 1324.0 IU/ml (reference range <60 IU/ml). Despite current recommendations, methimazole was started at a dose of 50 mg/day, as severe hyperthyroidism significantly increased the risk of preterm delivery, and in addition, methimazole was no longer associated with fetal risk in the third trimester. After three days, the methimazole dose was reduced to 30 mg/day. Ophthalmic examinations at this time still showed significant right exophthalmos with constricted and painful eye movements.

Visual acuity was 0.6 in the right eye and eyelid oedema was also present on this side. The exophthalmos was 28/21 mm with a base of 90 mm by Hertel test (population normal limit 20 mm). The EOP was considered severe according to the EUGOGO classification, with a Clinical Activity Score (CAS) of 6/7 on the right and 0/7 on the left. The fetus showed an adequate growth rate with continuous monitoring, no goiter or tachycardia was seen on ultrasound scans. From week 34 of gestation, methimazole therapy was changed to 2x15 mg/day. On ophthalmological examination at week 38, visual acuity, CAS score and exophthalmos showed an improving trend, although pain behind the eye and pain on eye movement and eyelid oedema were still present. As no sight-threatening EOP developed, high-dose corticosteroid therapy was not considered.

Our patient gave birth to a healthy newborn (3170 g) at week 39 by caesarean section. Natural delivery was contraindicated due to EOP and also due to a space deficit. The newborn showed no clinical signs of hypothyroidism and her day 3 TSH level was within the neonatal reference range. The mother's thyroid hormone levels started to rise after delivery; the dose of thiamazole was adjusted accordingly, supplemented with bisoprolol. Her visual symptoms improved significantly the day after caesarean section, CAS decreased and visual acuity normalised. Exophthalmos, redness of the eyelids and conjunctiva, swelling of the eyelids and caruncula were present on postpartum day 3. A follow-up MRI scan was planned, to which the patient did not consent. She was also scheduled for thyroidectomy surgery, but she did not present for this surgery, nor did she present for the subsequent follow-up examination, so no information on the subsequent status of the newborn and mother could be obtained.

5. Main findings and conclusions

5.1. Investigating the effects of new types of smoking in EOP

- The RT-CES method has been shown to be suitable for studying different environmental factors on orbital fibroblasts. It has been shown to be a sufficiently sensitive method to detect the effects of lower concentrations of FE on cells, and may therefore be suitable for a more precise study of the processes occurring in the connective tissue.
- The method has the potential to detect differences in the behaviour of EOP and control orbital fibroblasts.
- We confirmed that the behaviour of EOP and control fibroblasts is different when high concentration CIG smoke extract is added to the cells, in this case EOP OFs show higher CI values. This supports the role of tobacco smoke in the pathogenesis of EOP.
- Both EOP and control fibroblasts responded to 50% CIG smoke treatment with increased HA production, which is consistent with the pathomechanism of the disease and the consequent deterioration of the clinical picture.
- Our study is the first to demonstrate that the behaviour of EOP fibroblasts is different in response to new types of tobacco products (ECIG, HTP), supporting the hypothesis that alternative forms of smoking have a different effect on disease progression than conventional cigarette smoke.

5.2. Effect of pregnancy on the development of endocrine orbitopathy

- In the second and third trimesters of pregnancy, an exacerbation of existing EOP should be expected, although Graves-Basedow's pathological hyperthyroidism with EOP as a complication usually improves.
- In cases of EOP that develops or worsens during pregnancy, smoking cessation alone can lead to a rapid and significant improvement of the ocular abnormality.

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List of publications related to the dissertation

1. **Aranyosi, J. K.**, Galgóczi, E., Erdei, A., Katkó, M., Fodor, M., Ujhelyi, Z., Bácskay, I., Nagy, E. V., Ujhelyi, B.: Different Effects of Cigarette Smoke, Heated Tobacco Product and E-Cigarette Vapour on Orbital Fibroblasts in Graves' Orbitopathy; a Study by Real Time Cell Electronic Sensing.
   Molecules. 27 (9), 1-14, 2022.
   DOI: http://dx.doi.org/10.3390/molecules27093001
   IF: 4.927 (2021)
2. **Aranyosi, J. K.**, Deli, T., Erdei, A., Tóth, G., Jakab, A., Fodor, M., Nagy, E. V., Ujhelyi, B.: Unusual onset of thyroid associated orbitopathy during pregnancy: case report and review of literature.
   BMC Endocr. Disord. 20 (1), 1-5, 2020.
   DOI: http://dx.doi.org/10.1186/s12902-020-00663-9
   IF: 2.763

List of other publications

3. Ujhelyi, B., **Aranyosi, J. K.**, Módis, L.: A szemfelszín betegségeinek sebészi kezelése amnionmembrán-transzplantációval.
   Szemészet. 157 (2), 82-88, 2020.

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