Assessing the role of pituitary adenylate cyclase-activating polypeptide (PACAP) in reproductive and pathological processes.

Ph.D. thesis

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1. Introduction

1.1. Pituitary adenylate cyclase-activating polypeptide (PACAP)

Pituitary adenylate cyclase-activating polypeptide (PACAP) occurs in two biologically active forms in the body: a 38-amino acid isoform (PACAP38) discovered in 1989, and a 27-amino acid isoform (PACAP27) discovered a year later [Miyata et al., 1989, 1990]. In mammals, 90% of PACAP consists of the 38-amino acid isoform [Arimura et al., 1991]. PACAP primarily acts through specific transmembrane receptors belonging to the G protein-coupled receptor family. However, PACAP can also enter cells and activate additional signaling pathways in a receptor-independent manner. PACAP shares a similar affinity with VIP for vasoactive intestinal peptide receptors 1 and 2 (VPAC1 and VPAC2 receptors), while it binds to the selective PAC1 receptor 1000 times more strongly than VIP [Vaudry et al., 2009]. The distribution of PAC1 receptors, as well as VPAC1 and 2 receptors, within a given tissue, exhibits significant variability, and numerous variants can arise through alternative splicing [Langer et al., 2022]. PACAP remains active in the circulation for a few minutes before being cleaved into shorter, primarily antagonist peptides by the action of dipeptidyl peptidase-IV (DPP-IV) [Zhu et al., 2003]. Despite its short half-life, PACAP exerts diverse biological effects; shortly after its discovery, numerous in vitro and in vivo studies confirmed its general cyto- and neuroprotective effects, mediated through its anti-apoptotic, anti-inflammatory, and antioxidant properties. Beyond its fertilization and reproductive regulatory functions, PACAP regulates several physiological processes (such as feeding, thermoregulation, stress response, immune processes, and gland functions), and it also plays an important role in aging [Reglődi and Tamás, 2016; Vaudry et al., 2009]. In recent years, an increasing number of publications have investigated the clinical diagnostic or prognostic biomarker role of PACAP38. The most intensively researched conditions include neurological and psychiatric diseases (Alzheimer's disease, Parkinson's disease, multiple sclerosis, migraine, other headaches, stroke, traumatic brain injury, mental retardation, generalized anxiety, post-traumatic stress disorder), cardiovascular diseases (heart failure, ST-elevation myocardial infarction), orthopedic-traumatological diseases (primary and post-traumatic knee osteoarthritis, atraumatic femoral head necrosis, postmenopausal osteoporosis), as well as changes in PACAP levels described in other conditions (nephrotic syndrome, liver cirrhosis, chronic hepatitis B infection, multiple myeloma, superovulation treatments, ovarian insufficiency, and idiopathic hypogonadotropic hypogonadism) [Reglődi and Tamás, 2016; Toth et al., 2023].

1.2. Amniotic fluid

The amniotic fluid serves numerous functions during fetal development. It provides physical-mechanical protection against external forces and supports the mechanical protection of the umbilical cord by preventing compression between the fetus and the uterine wall. It also protects against infections and plays a crucial role in maintaining fetal thermoregulation. Fetal movements in the amniotic fluid contribute to the development of the skeletal muscle system while swallowing the amniotic fluid aids in the development of the gastrointestinal tract, and aspiration is essential for lung development. Additionally, the amniotic fluid serves as a reservoir for fluids and nutrients [Fitzsimmons and Bajaj, 2022]. In the early stages of pregnancy, the composition of amniotic fluid mirrors that of maternal and fetal plasma, suggesting that amniotic fluid is a transudate. This is because the nonkeratinized fetal skin does not impede fluid movement and acts as a membrane. From the 8th to 10th gestational week, with the onset of fetal urine production and swallowing movements, there are slight changes in composition. However, neither fetal urine excretion nor swallowing contributes significantly to the composition or volume of amniotic fluid until the completion of fetal skin keratinization (25th week) [Huri et al., 2023; Modena and Fieni, 2004]. The biochemical composition of amniotic fluid fluctuates during pregnancy, exhibiting individual variations independent of fetal pathology. [Liu et al., 2019]. Approximately half of the organic content of amniotic fluid consists of proteins, while the remainder comprises carbohydrates, lipids, enzymes, hormones, electrolytes, and other substances [Moore and Persaud, 2003]. The cellular components of amniotic fluid are exclusively of fetal origin, but its acellular fraction, including organic content, originates from both maternal and fetal sources. Therefore, biomolecules found in amniotic fluid, such as proteins, enzymes, nucleic acids, and metabolites, can provide information about maternal and fetal well-being and serve as a basis for screening and diagnosing various developmental abnormalities [Li et al., 2023; Tsangaris et al., 2011]. Advanced maternal age is associated with an increased frequency of fetal chromosomal abnormalities, highlighting the need for prenatal genetic diagnosis. The combined use of biochemical markers and ultrasound examination can enhance the identification of pregnancies at high risk, where further invasive diagnostics are required [Findley et al., 2023; Jenkins et al., 2022]. Currently, there is growing interest in studying the diagnostic potential of quantifying and/or qualitatively assessing biomolecules present in amniotic fluid, showing promising preliminary results [Kolvatzis et al., 2023; Park et al., 2021].

1.3. Politrauma

Each year, over 5 million people lose their lives due to trauma, with three-quarters of these injuries resulting from traffic accidents and falls from heights [WHO, 2014]. According to the new Berlin definition, polytrauma is defined as having an Injury Severity Score (ISS) greater than 15, with two or more body regions having Abbreviated Injury Severity (AIS) values greater than three, along with the observation of one or more deviations in physiological parameters [Pape et al., 2014]. Early mortality among polytrauma patients is high, occurring either immediately or within a few hours, while late mortality is mainly due to secondary damage and complications resulting from polytrauma [Longrois et al., 2019; Pfeifer et al., 2016]. In cases of severe injuries to the body, damaged cells release or leak into the extracellular space, which is known as Damage-Associated Molecular Patterns (DAMPs). These DAMPs trigger the activation of the immune system, leading to the release of large amounts of inflammatory mediators. Through proinflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-12, IL-18), these mediators contribute to developing systemic inflammatory response syndrome (SIRS). During SIRS, the complement system, the kallikrein-kinin system, blood clotting processes, and the synthesis of acute phase proteins are activated, potentially resulting in severe multiple organ failure (MOF) [Keel and Trentz, 2005; Relja and Land, 2022]. As the body seeks to maintain homeostasis, the synthesis of anti-inflammatory cytokines (IL-4, IL-10, IL-13) increases in parallel with activating the inflammatory system. This compensatory anti-inflammatory response syndrome (CARS) aims to balance the SIRS. The body endeavors to maintain a delicate balance between SIRS and CARS. Excessive inflammatory response following polytrauma can result in multiple organ failure (MOF), contributing significantly to early mortality. In contrast, late mortality is often attributed to the excessive activity of the anti-inflammatory system leading to immunosuppression, increasing susceptibility to infections and septic complications [Stoecklein et al., 2012; Csontos, 2022]. One of the most common and often fatal complications of polytrauma is sepsis, a life-threatening organ dysfunction resulting from the body's dysregulated immune response to infection [Singer et al., 2016]. Diagnosing infection or sepsis in polytraumatized individuals with pronounced SIRS is challenging. Therefore, it is crucial to promptly detect any complications following injuries, which can be facilitated by monitoring pro- and anti-inflammatory processes that occur during this period [Arora et al., 2023; Osuka et al., 2014]. Among the acute-phase proteins, C-reactive protein (CRP) is a commonly used conventional marker that sensitively responds to tissue damage associated with any inflammation. However, it is unsuitable for distinguishing general

inflammatory reactions from infectious complications [Rajab et al., 2020]. Procalcitonin (PCT) typically peaks 1-2 days after trauma and rapidly decreases in the absence of infectious complications. In the absence of this decrease or the presence of a secondary rise in PCT levels, the possibility of septic complications should be considered [AlRawahi et al., 2019]. White blood cell function, such as leukocyte anti-sedimentation rate (LAR), can also provide useful information alongside conventional parameters, although its application has not yet become routine practice. The investigation of LAR is based on the premise that tissue damage triggers a cellular response, leading to the activation of leukocytes. Among various changes, this activation results in increased water uptake by leukocytes. Consequently, their specific gravity decreases compared to their original "resting" state, leading to an increase in the number of anti-sedimenting white blood cells in the upper half of the blood column during one-hour sedimentation [Bogár et al., 2002; Bogár and Tarsoly, 2006].

1.4. Retinoblastoma

Retinoblastoma is the most common primary eye tumor in childhood, with a global incidence ranging from 1 in 16,000 to 1 in 18,000. However, this varies significantly among geographical regions and ethnic groups, while sex involvement remains relatively constant [Dimaras et al., 2015]. Retinoblastoma is considered a fundamental model in cancer research. In 1971, Knudson described the "two-hit" theory related to the biallelic inactivation of the retinoblastoma 1 gene (*RB1*), located on the long arm of chromosome 13 (13q14), which subsequent research has confirmed [Knudson, 1971]. According to the theory, a germline mutation (the "first hit") requires only an acquired mutation (the "second hit") for tumor formation. Therefore, hereditary retinoblastoma manifests at a younger age and often presents as multifocal or bilateral. The germline mutation is autosomal dominantly inherited with 80% penetrance. In contrast, sporadic cases are typically unilateral and occur later in life, as both *RB1* mutations in these cases are acquired [Knudson, 1971, 2001; Wong et al., 2014]. In extremely rare cases, retinoblastoma may develop without an RB1 mutation, resulting from somatic amplification of the *MYCN* gene [Rushlow et al., 2013]. Despite being one of the most extensively researched tumors, the precise cellular origin of retinoblastoma remains unconfirmed to this day [Bremner and Sage, 2014]. In patients where the tumor has been confirmed at a very early stage using high-resolution optical coherence tomography, the inner nuclear layer (INL) appeared to be the point of origin [Rootman et al., 2013]. However, experiments have suggested that retinoblastoma may originate from differentiating rod precursors, as retinoblastoma cells have been shown to rely on rod precursor signaling pathways at various points (survival,

proliferation) [Xu et al., 2009], and *RB1* knockout leads to cell proliferation in human rod precursors [Xu et al., 2014]. Histologically, the tumor is cellular, composed of hyperchromatic, narrow-cytoplasm, and large round/oval nuclei. Alongside increased mitotic activity, numerous apoptotic tumor cells and various-sized necrosis and calcifications are observed [Singh and Kashyap, 2018]. In the most well-differentiated tumors, there is a characteristic arrangement of tumor cells resembling partially differentiated photoreceptors, with relatively pale-staining, broader cytoplasm, forming petal-like structures (fleurettes), and exhibiting junctions resembling the internal limiting membrane. Flexner-Wintersteiner rosettes, indicative of early retinal differentiation, consist of cuboidal or columnar cells arranged around a central lumen. Homer Wright rosettes, seen in other neuroblastic tumors, represent primitive neuroblastic differentiation; however, they lack a true central lumen, being filled with eosinophilic neuropil [Alsharif et al., 2019]. Olianas and colleagues (1996) demonstrated the presence of PAC1 receptors in the Y-79 human retinoblastoma cell line [Olianas et al., 1996]. PACAP38 exerts a concentration-dependent effect on the survival of Y-79 human retinoblastoma cells. At nanomolar concentrations (0.1-100 nM), it has no significant effect, but at micromolar concentrations (1-5 μM), tumor cell survival decreases in parallel with the increase in PACAP38 concentration [Wojcieszak and Zawilska, 2014].

2. Aims

This PhD thesis aimed to investigate the potential clinical biomarker role of PACAP in reproductive and pathological processes.

2.1. Investigation of PACAP in human amniotic fluid samples

During our previous mass spectrometry studies, we could not to confirm the presence of PACAP38 in human amniotic fluid. However, based on available literature data, it is presumed that PACAP38 is present in this body fluid. Therefore, we aimed to verify the presence of PACAP38 and determine its quantity in human amniotic fluid from physiological pregnancies devoid of fetal pathology using a radioimmunoassay (RIA).

2.2. Changes of PACAP level in polytrauma patients

In polytrauma cases, close monitoring during the early period is essential to to detect potential complications promptly. Clinical practice has shifted towards the parallel monitoring of multiple biomarkers. Therefore, we aimed to measure patients' levels of PACAP38 during the first five days following polytrauma and investigate whether PACAP38 can be utilized as a biomarker and correlated with other laboratory markers during the early post-traumatic period in the care of polytraumatized patients.

2.3. Investigation of PACAP in human retinoblastoma and the effect of PACAP38 administration on human Y-79 retinoblastoma cells

Literature data on PACAP38 and PAC1 receptor occurrence in human retinoblastoma histological samples are not available. Therefore, we aimed to investigate whether PACAP38 and PAC1 receptor expression can be detected in surgical specimens of eyes removed due to retinoblastoma and whether any changes can be observed between these and the clinicopathological characteristics. Additionally, we aimed to examine the cytotoxicity of PACAP38 on human Y-79 retinoblastoma cells.

3. Materials and Methods

3.1. Investigation of PACAP in human amniotic fluid samples

The study was approved by the Regional Research Ethics Committee of the University of Pécs Medical School (UPMS) (PTE 4303/2011 and 6383/2018). Upon enrollment in the study, after detailed verbal and written information, written consent was obtained from the individuals involved. A total of 28 pregnant women, aged 35 and above, in the 15-19th week of pregnancy, who underwent diagnostic amniocentesis at the Department of Obstetrics and Gynecology of the UPMS were included in the study. Cases with detected abnormalities based on karyotyping results and those excluded post-delivery were excluded. After collecting of amniotic fluid samples, 30 µl/ml of a peptidase inhibitor (aprotinin) was added to the samples. They were then transported under refrigeration to the laboratory, where they were frozen and stored at -70°C until processing. Before RIA measurement, the samples were thawed and then centrifuged (12000 rpm, 4°C, 30 minutes). The supernatants were used for the determination of PACAP38-like immunoreactivity (PACAP38-LI) using a previously developed, specific, and sensitive method, which forms the basis of several scientific publications [Jakab et al., 2004], as follows: the PACAP38 antiserum (No. 88111-3) obtained from the laboratory of Professor Akira Arimura (Tulane University, New Orleans, USA), produced in rabbits and conjugated with Cys^{23}-PACAP24-28 using the carbodiimide method against bovine thyroglobulin antigen, was found to be the most effective at a dilution of 1:10000 during the development of the RIA. As a tracer, we utilized a ^{125}I-labeled, ovine mono-^{125}I-PACAP24-38 C-terminal fragment prepared in our laboratory, while for RIA standard, the complete ovine PACAP38 peptide was used in the range of 0-1000 fmol/ml. The RIA assays were conducted in 1 ml of 0.05 mol/l phosphate buffer (pH 7.4) containing 0.1 mol/l sodium chloride, 0.25% (w/v) bovine serum albumin, and 0.05% sodium azide. Following mixing, the samples were incubated for 48-72 hours at 4°C. Next, the antigen fraction bound to the antibody was separated from the free labeled peptides by adding 100 µl of separating solution (100 ml distilled water, 10 g charcoal, 1 g dextran, 0.5 g fat-free milk powder) and centrifuging the samples (3000 rpm, 4°C, 15 minutes). Then, the supernatants were decanted, and the tubes were blotted with absorbent paper. Using a gamma radiation detector of type NZ310, we measured the radioactivity of the charcoal-bound free peptide fraction, from which we could infer the value of radioactivity bound to the antibody. Subsequently, the concentration of PACAP38 in the unknown samples was read from the calibration curve based on the measured known peptide concentrations (standard samples). The

intra- and inter-assay coefficients of the applied antiserum were 7.2% and 8.7%, respectively. During the cross-reactivity examination, the antiserum used did not react with either PACAP27 or other members of the peptide family. A cold recovery test was conducted to validate the method, in which the PACAP38 concentrations of four amniotic fluid samples were measured. Subsequently, low (50 fmol/ml), medium (300 fmol/ml), and high (1000 fmol/ml) concentrations of PACAP38 were added, and the PACAP38 concentrations were re-measured. The recovery rates were calculated from the measured and expected concentrations, resulting in 83.8% for low PACAP38 concentration, 87.5% for medium PACAP38 concentration, and 84.8% for high PACAP38 concentration. A further validation experiment used serial dilutions of the selected amniotic fluid sample to assess parallelism with the RIA standard. The volumes of the analyzed fluid samples ranged from 12.5 to 400 µl. The examined amniotic fluid sample inhibited the binding of the radioactively labeled PACAP24-38 C-terminal fragment (RIA tracer) to the antibody parallelly, similar to PACAP38 used as the RIA analysis standard.

3.2. Changes of PACAP level in polytrauma patients

The study was approved by the Regional Research Ethics Committee of the UPMS (PTE 422/2014 and 6383/2016). Upon enrollment in the study, detailed verbal and written information was provided to the participants, or in case of impediment, to their nearest relatives, who then provided written consent. The study included 20 polytraumatized patients aged 18 or above initially admitted for primary care and subsequently treated at the Intensive Care Unit of the Department of Anesthesiology and Intensive Therapy at the UPMS. Blood samples were collected from an arterial cannula necessary for care from the first day until the fifth day, parallel with morning blood draws. Serum CRP and PCT, LAR, and plasma PACAP38-LI were determined for each blood sample. Inclusion criteria were the presence of polytrauma, while exclusion criteria included hospital stays shorter than five days, known malignant neoplastic diseases, underlying conditions or medications affecting normal immune response, and severe chronic organ diseases. CRP and PCT determinations were part of the daily routine laboratory monitoring conducted at the Department of Laboratory Medicine, UPMS. The reference value for CRP was below 5 mg/l, while for PCT, it was below 0.5 ng/ml. For the determination of LAR, arterial blood was collected into a "sedimentation" tube containing sodium citrate, and the total length of the blood column was measured with a ruler attached externally to the side of the tube, marking the midpoint. After one hour of sedimentation, the blood above and below the midpoint line was injected into separate "blood count" tubes, which were then transported to the Department of Laboratory Medicine, UPMS, for determination of the number of

leukocytes in the upper (U) and lower (L) portions of the blood column. Subsequently, LAR was calculated using the formula LAR = 100 (U-L) / (U+L), representing the percentage of the original leukocyte count, in which the number of leukocytes that sedimented upwards (anti-sedimented) over the one-hour sedimentation period in the blood sample tube exceeded the midpoint line. PACAP38-LI determination was performed using the previously discussed specific and sensitive method. For statistical analysis, we utilized version 21 of the SPSS statistical program. Spearman's rank correlation was employed to analyze the parameters' daily kinetics, and the results were depicted using violin plots generated with PlotsOfData [Postma & Goedhart, 2019]. We used the rmcorr software package [Bakdash & Marusich, 2017] from the open-source R statistical program [R Core Team, 2010] to examine correlations for all days. We visualized the correlation matrix using the corrplot software package [Wei & Simko, 2017].

3.3. Investigation of PACAP in human retinoblastoma and the effect of PACAP38 administration on human Y-79 retinoblastoma cells

The retrospective study was approved by the Regional Research Ethics Committee of the UPMS (PTE 9188/2022). We extracted patients who underwent enucleation due to retinoblastoma from January 2001 to December 2017. Beyond collecting the patients' clinical data, we retrieved formalin-fixed paraffin-embedded (FFPE) enucleation specimens from the Department of Pathology of the UPMS and subsequently prepared new sections. Pathological staging was determined according to the AJCC TNM classification (8th edition) [Mallipatna et al., 2016]. The degree of differentiation was classified on a four-level scale. Tumors showing fleurettes or neuronal differentiation in more than 50% of the tumor were categorized as G1, indicating the most differentiated tumors. Tumors with Flexner-Wintersteiner and/or Homer Wright rosettes present in more than 50% of the tumor were classified as G2. In the next grade (G3), the aforementioned rosettes were present in a lower proportion. Tumors in the last category (G4) contained poorly differentiated cells without rosettes or exhibited extensive anaplastic areas [Lochner and Couce, n.d.]. From FFPE tissue samples, we prepared 3µm sections using a rotary microtome (Microm HM 325, Thermo Scientific, Ltd., Waltham, MA, USA). After deparaffinization and rehydration through an ascending alcohol series, heat-induced antigen retrieval was performed in a microwave oven (750 W, 15 minutes) in 1 mM citrate buffer (pH = 6.0). After cooling to room temperature, the sections were washed in 0.1 M phosphate-buffered saline (PBS) three times for 10 minutes each. Subsequently, the sections were incubated at room temperature for one hour with an anti-PACAP38 antibody (T-4473, BMA Biomedicals, Ltd., Augst, Switzerland) at a dilution of 1:500 and an anti-PAC1 receptor

antibody (AVR-003, Alomone Labs, Ltd., Jerusalem, Israel) at a dilution of 1:125. After washing with PBS, the sections were incubated at room temperature for 30 minutes with a HISTOLS-AP-R (30,011.R500A, Histopathology, Ltd., Pécs, Hungary) rabbit-origin primary antibody-compatible secondary peroxidase-labeled polymer-based detection system. After washing with PBS, we applied the HISTOLS Resistant AP-Red Chromogen/substrate System (30,019, Histopathology, Ltd., Pécs, Hungary), a magenta-colored detection system, in a dark environment. Following a 10-minute incubation, we assessed the signal intensity under a light microscope. The process was stopped by rinsing the sections in distilled water, followed by dehydration through an ascending alcohol series after standard counterstaining procedures. The sections were cleared in xylene and coverslipped with a permanent mounting medium. Omission of the primary antibody resulted in no immunoreactivity (negative control). Internal positive controls were provided by structures unaffected by ocular neoplasms based on available literature [Patko et al., 2022]. The sections were digitized using the Panoramic MIDI II automated slide scanner (3DHISTECH Ltd., Budapest, Hungary), and the images were saved using CaseViewer 2.3 software (3DHISTECH Ltd., Budapest, Hungary). For the in vitro experiment, we utilized the American Type Culture Collection Y-79 human retinoblastoma cell line. Cell viability was assessed using a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma-Aldrich, St. Louis, MO, USA) to examine the effects of various concentrations of PACAP38 treatment (SZTE Szent-Györgyi Albert Medical School, Deparment of Medical Chemistry, Szeged, Hungary). Twelve wells served as controls, while 6-6 wells received 10 µl PACAP38 treatment at the following concentrations: 0.1 µM, 0.5 µM, 1 µM, 2 µM, 6 µM PACAP38. These were supplemented with serum-free medium to a final volume of 100 µl. After 24 hours of incubation, 10 µl of 5 mg/ml concentration MTT solution was added to the samples, resulting in a final concentration of 0.45 mg/ml. Following an additional 4-hour incubation at a thermostat, blue formazan dye particles were re-dissolved in 100 µl dimethyl sulfoxide per well. After 30 minutes of agitation, absorbance was measured at a wavelength of 630 nm using an ELISA reader (Dialab Ltd., Budapest, Hungary). Each experiment was repeated three times. For statistical analysis, we conducted a one-way analysis of variance (ANOVA), followed by Dunnett's post-hoc test using GraphPad Prism version 9.5.0 software (GraphPad Software LLC, San Diego, CA, USA). The data were graphically represented with mean ± standard deviation characterization.

4. Results

4.1. Investigation of PACAP in human amniotic fluid samples

Only pregnancies free from maternal and fetal pathology were included in the final analysis, resulting in the exclusion of 7 cases from the initial 28 samples (these showed various types of fetal pathology). PACAP38-LI was detectable in all 21 samples. The average PACAP38-LI level was 401 fmol/ml (SD 142 fmol/ml).

4.2. Changes of PACAP level in polytrauma patients

By the end of day 5, seven individuals were excluded (one deceased, two eligible for discharge after 3 days, two had confirmed severe comorbidities, and two withdrew consent), leaving 13 polytraumatized individuals (2 females and 11 males; mean age: 45.92 ± 15.78 years) in the study, none of whom developed complications. No statistically significant differences were observed for any of the parameters examined on individual days; however, a rising trend was observed for CRP. Similar rising trends were observed for LAR and PACAP38, peaking on day 4. When considering individual days, a statistically significant weak positive correlation was found on day 4 between LAR and CRP ($r = 0.572$, $p = 0.041$) and between PACAP38 and CRP ($r = 0.581$, $p = 0.037$). On day 5, a statistically significant moderate positive correlation was found between PACAP38 and CRP ($r = 0.776$, $p = 0.002$). Upon examining all days together, a statistically significant weak positive correlation was confirmed between PACAP38 and LAR ($r = 0.279$, $p = 0.042$), as well as between LAR and CRP ($r = 0.406$, $p = 0.002$).

4.3. Investigation of PACAP in human retinoblastoma and the effect of PACAP38 administration on human Y-79 retinoblastoma cells

Seven children (one girl and six boys) were included in our study. At the time of enucleation, the mean age was 16.3 ± 10.5 months. No bilateral cases were encountered. Except for one case, the tumors were monofocal. In the case of multifocal retinoblastoma, no germline mutation was confirmed, but in one monofocal case, familial aggregation was evident (RB1 mutation). Histomorphologically, we had three G4, one G3, and three G2 cases. During immunohistochemical examinations, we observed varying intensities of PACAP38 and PAC1 receptor immunopositivity in the unaffected parts of the eyes, serving as positive internal controls, consistent with previous descriptions by our research group. In retinoblastomas, regardless of histological appearance, we found only perinuclear, punctate (dot-like)

immunopositivity in tumor cells for both PACAP38 and PAC1 receptor in all samples. Regarding the in vitro experiment, one-way ANOVA indicated a significant difference in cell survival among treatment groups ($F=5.165$, $p=0.0047$). Dunnett's post hoc test revealed statistically significant differences between the control group and the groups treated with 2 μM PACAP38 (mean difference: 16.5; 95% CI [1.471, 31.52]; $p = 0.035$) and 6 μM PACAP38 (mean difference: 20.38; 95% CI [8.488, 32.26]; $p = 0.0053$).

5. Discussion

5.1. Investigation of PACAP in human amniotic fluid samples

In our study on amniotic fluid samples, we first confirmed the presence of PACAP38-LI in human amniotic fluid during physiological pregnancy between gestational weeks 15-19. Previously, using mass spectrometry, we failed to detect PACAP38 in human amniotic fluid [Brubel et al., 2011]. The apparent discrepancy between our current and previous studies can be explained by the different methodologies employed, resulting in varying sensitivities. Mass spectrometry would only detect the native protein; therefore, if there were modified forms of PACAP38 present or if it were bound to another molecule, the test would yield negative results. However, in RIA, the applied antiserum can recognize binding sites of PACAP38, even if it is in a modified structure or bound to another molecule. Currently, it is not known whether PACAP38 in human amniotic fluid exists in a modified form or is bound to another molecule. However, available literature suggests the latter possibility, as PACAP38 is known to bind to ceruloplasmin in plasma [Tams et al., 1999], and ceruloplasmin is known to be present in amniotic fluid [Cho et al., 2007]. PACAP38 plays an important role in reproduction [Koppan et al., 2022; Tamas et al., 2016], and its absence leads to various developmental abnormalities based on in vivo data [Allais et al., 2007; Farkas et al., 2017], but its exact role in amniotic fluid remains unknown. Our future goal is to investigate the role of PACAP38 in the prenatal diagnosis of various developmental disorders.

5.2. Changes of PACAP level in polytrauma patients

We observed a slight increase in PACAP38 levels during the first four days. Clinical experience suggests that the dynamics of individual laboratory values can be crucial for both diagnostic and therapeutic decision-making. It is well-known that the half-life of PACAP38 in plasma is extremely short (3-10 minutes [Birk et al., 2007; Li et al., 2007]). Accordingly, the observed increase in plasma concentrations can only be explained by secondary mechanisms, which are part of the anti-inflammatory and cytoprotective effects of PACAP that occur after the development of SIRS. Therefore, monitoring PACAP38 levels can provide valuable information regarding the balance of pro- and anti-inflammatory processes. Similar to PACAP38, we did not find a statistically significant correlation for CRP levels during the first five days, indicating parallel kinetics with PACAP38. We did not observe a significant increase above the threshold value for PCT. Generally, in the absence of septic complications, PCT remains below the threshold value [Gregoriano et al., 2020], consistent with the results of our

study. A trend similar to PACAP38 was observed regarding LAR, indicating no statistically significant correlation. However, this LAR kinetics align well with previous studies, which found that LAR serves as an indicator of white blood cell activation [Bogár et al., 2006; Loibl et al., 2021; Molnar et al., 2010; Rozanovic et al., 2016]. When considering each day separately, a weak positive correlation was observed between PACAP38 and CRP levels on the fourth day, which became of moderate strength on the fifth day. The parallel increase of PACAP38 and CRP likely reflects the endogenous response to SIRS following polytrauma. When examining the days together, we found a statistically significant correlation between PACAP38 and LAR values. The onset of the multimodal immune process following polytrauma begins with SIRS, characterized by increased leukocyte activation, followed by a counter-regulatory process. The correlation between PACAP38 and LAR can be explained by the anti-inflammatory effect of PACAP during this counter-regulatory process. Due to the small sample size, our study can be considered a pilot study. In the future, we plan to conduct larger studies involving more clinical parameters and investigate complications and uncomplicated cases over a more extended period. Furthermore, our future plans include creating additional subgroups within polytrauma, taking into account the etiology of the trauma.

5.3. Investigation of PACAP in human retinoblastoma and the effect of PACAP38 administration on human Y-79 retinoblastoma cells

In the first part of our study, we performed immunohistochemical analysis of PACAP38 and PAC1 receptor expression in eyes removed due to retinoblastoma. For both PACAP38 and PAC1 receptor, we found the appropriate immunohistochemical pattern in the tumor-free, intact areas of the eye, consistent with our previous findings [Patko et al., 2022]. We were the first to describe the expression pattern of PACAP38 and PAC1 receptor in human retinoblastoma, characterized by focal, perinuclear, dot-like immunopositivity. We did not find differences in PACAP38 and PAC1 receptor expression patterns associated with demographics or clinical and histopathological characteristics. The exact cell type of origin of retinoblastoma is still unknown [Bremner and Sage, 2014], so we could not compare the expression patterns of PACAP38 and PAC1 receptor in the tumor and the "originating" cell type. Olianas and colleagues (1996) described that nearly 60% of cells express PAC1 receptor on the cell membrane while studying the Y-79 cell line. In contrast, our study did not show the membrane-localized distribution for the PAC1 receptor; only dot-like, perinuclear positivity was observed. This phenomenon highlights the difference between human studies and in vitro systems. In the second part of our experiment, we confirmed that treatment with 0.1 μM, 0.5 μM, and 1 μM

PACAP38 has no effect on the survival of Y-79 cells. This observation fits well into existing literature, complementing it, as previous studies have only investigated the effect of PACAP38 in concentrations between 0.1 nM and 0.1 μM. The same early experiment found that PACAP38 has a dose-dependent cytotoxic effect between 1-5 μM concentrations [Wojcieszak and Zawilska, 2014]. Based on our results, PACAP38 at a concentration of 1 μM did not exhibit cytotoxicity, only at concentrations of 2 and 6 μM. Our experiment has several limitations. First, the small sample size must be mentioned, which prevents the generalization that every human retinoblastoma exhibits the previously demonstrated immunohistochemical profile. Regarding the sample size, we could not cover the entire spectrum of differentiation, as we did not have any tumors in which fleurettes or neuronal differentiation were visible in more than half of the tumor. Due to the unclear cellular origin of retinoblastoma, we currently cannot conclude about whether the PACAPergic system is involved in the tumor and, if so, in what manner. These need to be clarified in future experiments. As a limitation of our in vitro experiment, it should be noted that we only used one cell line. In the future, the examination of multiple different cell lines would be warranted alongside PACAP38 and other PACAP analogs.

6. Summary of novel findings

I. With a sensitive and specific investigative method, we have demonstrated that:

- PACAP38 is present in human amniotic fluid, and
- in physiological pregnancies without fetal pathology, the average PACAP38-LI concentration in human amniotic fluid is 401 fmol/ml (standard deviation 142 fmol/ml).

II. In the context of polytrauma, we have outlined for the first time that in the absence of complications:

- there is no statistically significant difference in plasma PACAP38 concentrations during the first five days; only an increasing trend is observed, peaking on the fourth day.
- when considering individual days:
    - on the fourth day, there is a statistically significant, weak positive correlation between LAR and CRP, as well as between PACAP38 and CRP;
    - on the fifth day, a statistically significant, moderately strong positive correlation exists between PACAP38 and CRP.
- considering all days together, a statistically significant, weak positive correlation is evident between PACAP38 and LAR, as well as between LAR and CRP.

III. Regarding retinoblastoma:

- we confirmed that human retinoblastoma expresses both PACAP38 and PAC1 receptor.
- we described that in human retinoblastoma, contrary to in vitro data, there is no membrane PAC1 receptor expression; only perinuclear, dot-like immunopositivity is observed for both PAC1 receptor and PACAP38.
- in the case of the Y-79 cell line:
    - consistent with previous literature, we found that at nanomolar concentrations, PACAP38 has no effect on cell survival;
    - in contrast with previous literature, we found PACAP38 is cytotoxic at concentrations above 1 μM, rather than 2 μM, on this cell line.

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8. Publications

The Ph.D. thesis is based on the following publications:

1. **Toth, D**., Veszpremi, B., Koppan, M., Tamas, A., Szogyi, D., Brubel, R., Nemeth, J., Shams, M., & Reglodi, D. (2020). Investigation of pituitary adenylate cyclase activating polypeptide (PACAP) in human amniotic fluid samples. *Reproductive Biology*, *20*(4), 491–495. https://doi.org/10.1016/j.repbio.2020.07.013
   IF: 2.376; Q1
2. **Toth, D**., Tamas, A., & Reglodi, D. (2020). The neuroprotective and biomarker potential of PACAP in human traumatic brain injury. *International Journal of Molecular Sciences*, *21*(3), 827. https://doi.org/10.3390/ijms21030827
   IF: 5.924; D1 (review)
3. **Toth, D**., Szabo, E., Tamas, A., Juhasz, T., Horvath, G., Fabian, E., Opper, B., Szabo, D., Maugeri, G., D'Amico, A. G., D'Agata, V., Vicena, V., & Reglodi, D. (2020). Protective effects of PACAP in peripheral organs. *Frontiers in Endocrinology*, *11*, 377. https://doi.org/10.3389/fendo.2020.00377
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4. Tamás, A., **Tóth, D**., Pham, D., Loibl, C., Rendeki, S., Csontos, C., Rozanovic, M., Bogár, L., Polgár, B., Németh, J., Gyenesei, A., Herczeg, R., Szántó, Z., & Reglődi, D. (2021). Changes of pituitary adenylate cyclase activating polypeptide (PACAP) level in polytrauma patients in the early post-traumatic period. *Peptides*, *146*, 170645. https://doi.org/10.1016/j.peptides.2021.170645
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5. **Toth, D**., Reglodi, D., Schwieters, L., & Tamas, A. (2023). Role of endocrine PACAP in age-related diseases. *Frontiers in Endocrinology*, *14*, 1118927. https://doi.org/10.3389/fendo.2023.1118927
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6. **Tóth, D.**, Fábián, E., Szabó, E., Patkó, E., Vicena, V., Váczy, A., Atlasz, T., Tornóczky, T., & Reglődi, D. (2024). Investigation of PACAP38 and PAC1 receptor expression in human retinoblastoma and the effect of PACAP38 administration on human Y-79 retinoblastoma cells. *Life, 14*, 185. https://doi.org/10.3390/life14020185
   IF(2023): 3.2; Q1

*The cumulative impact factor of the original publications serving as the basis of the thesis**: 24.822***
The combined impact factor of the original publications without reviews: 9.443

Other publications not directly related to the topic of the thesis:

7. Horvatovich, K., Bokor, S., Baráth, A., Maász, A., Kisfali, P., Járomi, L., Polgár, N., **Tóth, D**., Répásy, J., Endreffy, E., Molnár, D., & Melegh, B. (2011). Haplotype analysis of the apolipoprotein A5 gene in obese pediatric patients. *International Journal of Pediatric Obesity 6*(2-2), e318–e325. https://doi.org/10.3109/17477166.2010.490268
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8. László, T., Lacza, A., **Tóth, D**., Molnár, T. F., & Kálmán, E. (2014). Pulmonary enteric adenocarcinoma indistinguishable morphologically and immunohistologically from metastatic colorectal carcinoma. *Histopathology, 65*(2), 283–287. https://doi.org/10.1111/his.12403
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9. Rácz, E., Könczöl, F., **Tóth, D**., Patonai, Z., Porpáczy, Z., Kozma, Z., Poór, V. S., & Sipos, K. (2016). PCR-based identification of drowning: four case reports. *International Journal of Legal Medicine, 130*(5), 1303–1307. https://doi.org/10.1007/s00414-016-1359-7
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10. Simon, G., Rácz, E., Mayer, M., Heckmann, V., **Tóth, D.**, & Kozma, Z. (2017). Suicide by intentional air embolism. *Journal of Forensic Sciences*, *62*(3), 800–803. https://doi.org/10.1111/1556-4029.13320
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