STUDIES IN MEDICINE, PHARMACOLOGY, AND BIOLOGY

IS THERE A LIGHT AT THE END OF THE TUNNEL

KARĪNA NARBUTE, VLADIMIRS PIĻIPENKO, VIJA ZAIGA KLUŠA

Despite the fact that we live in an era where almost everything is possible and science flourishes at the speed of light, pathogenesis of neurodegenerative diseases remains *terra incognita*.

Neurodegenerative diseases are one of the leading causes of disability and death around the globe [1]. These diseases include the most common neurodegenerative brain pathologies – Alzheimer's disease (AD) and Parkinson's disease (PD), which silently develop over decades before symptoms become noticeable. It is almost impossible to stop or slow down the disease progression, and this leaves patients only with symptomatic treatment. Although neurodegenerative disorders have been studied densely for decades, the views on the etiopathology of these diseases have changed multiple times, mostly being based on symptoms already present in affected patients [2]. AD and PD both have common etiopathological features linked to late-onset progressive proteinopathies, though misfolded protein lesions are mostly found in later stages of the disease when reversing or halting the progression of the disease is close to impossible [3]. Hence, innovative, paradigm-shifting views on the treatment of these diseases are necessary to enable their timely prevention and treatment.

WHAT IS KNOWN ABOUT PARKINSON'S DISEASE?

Parkinson's disease is the second most common neurodegenerative disorder which typically affects people over 60 years of age, with increasing prevalence over the age of 85 and, to a larger extent, in men. Multiple lines of evidence highlight the loss of dopamine (DA)-synthesising neurons in the nigrostriatal structures (*striatum* and *substantia nigra*) of the brain as the main driving force for the neurodegeneration observed in PD. Key enzyme involved in DA production is tyrosine hydroxylase (TH), and deficits in TH function are linked to PD symptoms. PD is characterised by motor impairments (muscle rigidity, tremor, and bradykinesia) and non-motor symptoms (cognitive impairment, depression, and sleep disorders). Motor symptoms occur after about 75% of striatal dopaminergic neurons are lost. Movement disorders progress as a result of disbalance between decreased dopaminergic activity and increased cholinergic activity. These disturbances lead to a significant loss of quality of life, inability to carry out everyday tasks, and patients even lose the ability to swallow solid food in the later stages of the disease. The vast majority of cases are of sporadic origin (about 95–99%) and only 1–5% are genetically predisposed [4]. Current therapies can only delay, not halt neurodegenerative processes in the brain. Moreover, current therapies are only temporary effective, often cause unwanted side effects and about 30% of all patients do not respond to the therapy. Hence, there is an urgent necessity to find new approaches to design anti-neurodegenerative drugs, capable of stopping pathological events before dementia occurs, which in patients is a brief moment between mild cognitive impairment and the onset of dementia.

HOW TO STOP THE DISEASE BEFORE IT STARTS?

The early progression of neurodegenerative diseases has been linked to neuroinflammatory mechanisms. Pathogens such as bacteria, viruses, endogenous proteins, antibodies, cytokines, and chemokines overstimulate primary immune cells of the brain – microglia and astroglia, and compromise brain homeostasis [5]. Activated glial cells then regulate the expression of different surface markers, such as the

major histocompatibility complex-II and receptors for identifying pathogen-associated molecular patterns and damage-associated molecular patterns, which produce proinflammatory cytokines, such as interleukins IL-1β, IL-6, IL-12, interferon-gamma and tumour necrosis factor-alpha, as well as cytotoxic factors, such as reactive oxygen species, superoxide radicals and nitric oxide, resulting in neuronal death. Neurodegeneration occurs as a result of series of unwanted events: activity disruption of our cell powerhouse – mitochondria, impaired cell-cell communication due to synaptic disfunction and neuroinflammation, as well as impaired cerebral glucose and insulin metabolism [6]. Depending on the damaged brain area motor, behavioural, and cognitive symptoms occur. Knowledge about the early start of neuroinflammatory processes, before any clinical symptoms have developed, makes it possible to discover approaches that prevent cell death and neurodegeneration by halting neuroinflammation at an early stage.

USE OF EXTRACELLULAR VESICLES AS A NEW PUTATIVE APPROACH TO STOP PD

Our studies that were carried out at the Department of Pharmacology, Faculty of Medicine of the University of Latvia in collaboration with Professor Augustas Pivoriūnas' group from the State Research Institute Centre for Innovative Medicine (Vilnius, Lithuania), made a new approach for implementing extracellular vesicles (EVs) as a treatment option for neurodegenerative diseases. EVs comprises exosomes and microvesicles. Exosomes are nano-sized (30–150 nm) membranous vesicles that are secreted by almost every cell type and found in bodily fluids, such as blood, saliva, CSF, and urine [7]. They were first described almost 40 years ago, and initially, their function was thought to be the removal of intracellular proteins. Microvesicles are membrane vesicles (0.1–1.0 μm diameter) that bud directly from the cell surface and are therefore enveloped by lipids and proteins from the plasma membrane. In contrast, exosomes originate from endosomes fusing and forming multivesicular bodies (MVB) which fuse with the plasma membrane and are released into the extracellular space (Fig. 1) [8].

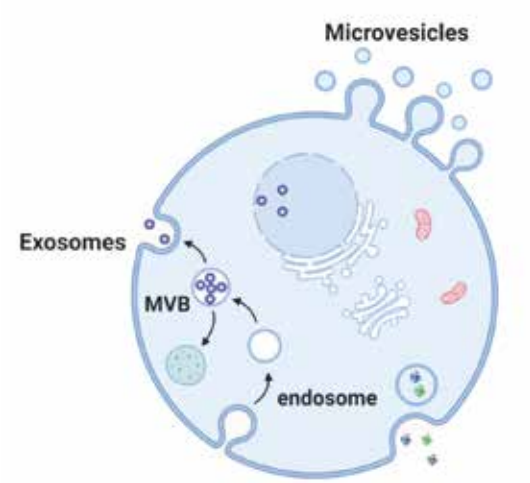

Fig. 1.
Schematic representation of exosome biogenesis. Adapted from Grenier-Pleau & Abraham (2021) [9].

A plethora of research studies on exosome CNS functions suggests that EVs are involved in cell-to-cell communication, participate in the modulation of neuroprotective and regenerative processes, and mediate synaptic plasticity [10]. Noteworthily, EVs can cross biological barriers including the blood-brain barrier (BBB). EVs can attach to other cells and release their cargo by a range of different adhesion molecules [11]. They carry different cargo based on their origin cell. For instance, EVs derived from neurons are suggested to act as carriers of signalling proteins involved in synaptic plasticity. Moreover, EVs carry proteins that can reduce neuronal oxidative stress, such as superoxide dismutase-1 and catalase. One *in vitro* study showed that exosomes can rescue human dopaminergic neurons from neurotoxin 6-hydroxydopamine (6-OHDA)-induced apoptosis [12]. EV treatment also suppressed neuroinflammation and reduced cognitive impairments after traumatic brain injury [13] and after status epilepticus in mice [14]. These findings open a new perspective in PD treatment.

EVS MAY REVERSE GAIT AND MEMORY DISTURBANCES IN PD MODEL-ANIMALS

Our attention was attracted by EVs secreted via stem cells from human exfoliated deciduous teeth (SHEDs-derived EVs), since these cells originate from the peripheral nerve-associated glia, they may bear

potential for differentiating into neuronal and glial cells [15]. Also, previous studies have demonstrated that SHEDs can be efficiently differentiated into neuronal and Schwann cells *in vitro* and, even more importantly, these cells displayed neuroprotective properties *in vitro* [16].

Considering that such a treatment would have to be longitudinal, it is extremely important to find effective and, at the same time, harmless therapeutical options. We have chosen intranasal administration, since it offers many advantages compared to systemic administration: it is non-invasive, accesses CNS directly through olfactory bulb or trigeminal nerves (bypasses the BBB) and does not cause the side effect profile usually characteristic to peripherally usable drugs [17].

We created a non-transgenic PD-like model in rats by injecting 6-OHDA into the rat medial forebrain bundle. This specific model is quite accurate to what is seen in human patients, which makes it more convenient to study motor disturbances and histological outcomes specific to PD. We performed the following behavioural tests: CatWalk gait test and Morris water maze swimming test for spatial memory and learning assessment. One week after the creation of the lesion, we started to administer EVs intranasally for 15 consecutive days. We performed CatWalk gait testing right before the start of the treatment and two weeks after initiating the treatment. Moreover, we evaluated the histological and immunohistochemical outcomes of EV administration, as well as the proteomic profile of EVs (Fig. 2).

Our research indeed proved that these EVs can reverse gait impairments (insecure stance, incorrect interlimb coordination, slow running speed, etc.) and improve spatial learning/memory performance in the Morris water maze swimming test even two weeks after discontinuation of EV treatment [18–19]. EVs also reduced the loss of TH, thereby restoring DA synthesis in the brain structures involved in motor control. Moreover, EVs preserved neuronal survival (seen as Nissl body count) in *substantia nigra pars compacta* region. Nissl bodies are microscopic particles, found in neuronal granular endoplasmic reticulum and ribosomes and their histochemical staining is used as a representation of live neurons. In fact, our proteomics data showed that SHEDs-derived EVs contained Cu/Zn superoxide dismutase 1, an enzyme converting harmful free superoxide radicals to molecular oxygen and hydrogen peroxide, antioxidant proteins thioredoxin and peroxiredoxin-6 which are important for the neutralisation of hydrogen peroxide, and also heat-shock protein 70 [20]. Proteomic data also identified adaptor protein 14-3-3ζ, an endogenous activator of TH in midbrain dopaminergic neurons [21]. Importantly, EVs cargo contains BASP1 protein – a regulator of neurite outgrowth and nerve

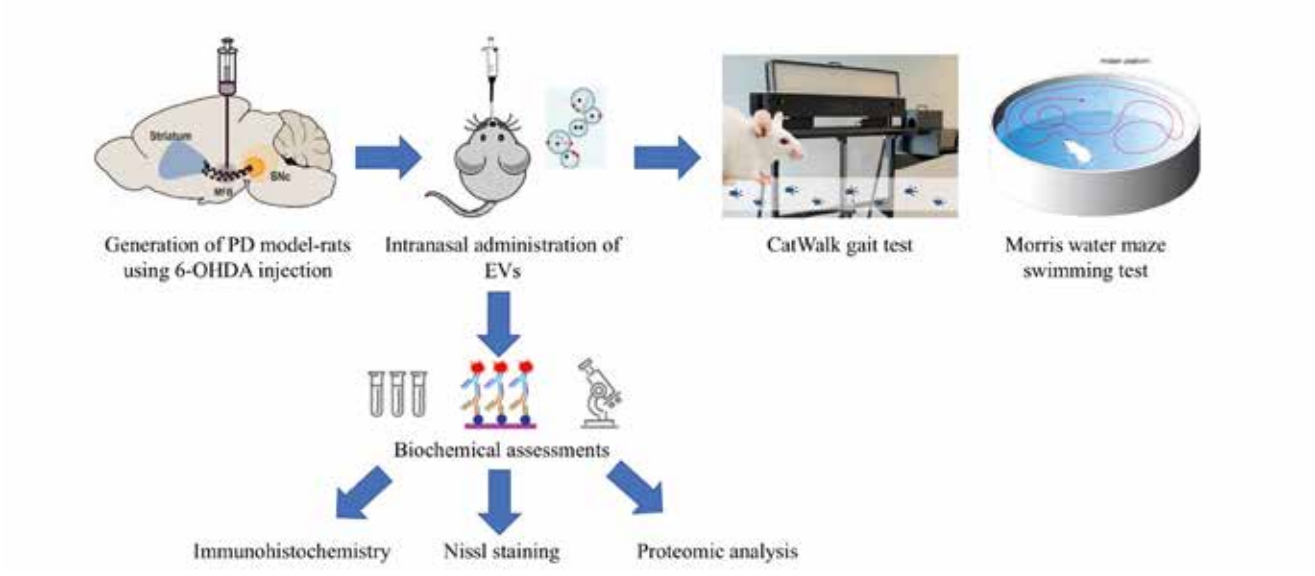

Fig. 2.
Graphical design of our research

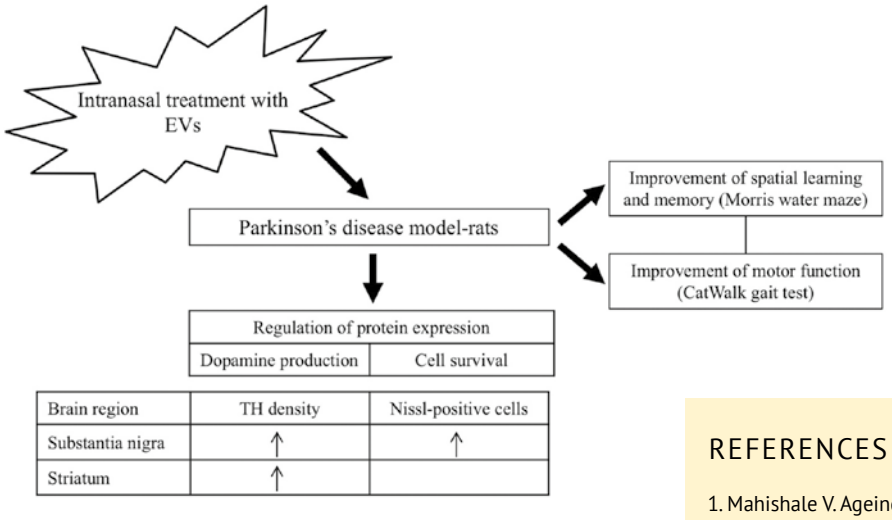

Fig. 3.
Schematic depiction of extracellular vesicle treatment effects

regeneration [22]. A full representation of our proteomics data is seen in our paper [18]. Based on these data and the fact that gait effects persisted even ten days after the discontinuation of the treatment, we assume that EVs either accumulate in the lesioned nigrostriatal structures for at least ten days, or they trigger the synthesis of essential substances capable of rescuing cell functioning [19]. The data on longitudinal EVs effects [19] were published in the journal *Stem Cells Translational Medicine* and was awarded as the most significant research published in 2020 by a young scientist in this journal. Obtained data (Fig. 3) highlight the neuroprotective potential of EVs in the early stages of PD, although further research is needed to clarify the biochemical mechanisms beneath these effects and other neurotransmitter system involvement in development and cure of PD before translating results of these studies to the clinical phase. Exosome implementation in CNS disease research is a new avenue in therapeutics – specific, effective and without serious side effects. One may suggest that this development in neurodegenerative disease therapeutics research will bring long-awaited light at the end of a dark, dark tunnel.

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Photo: Toms Norde

Academician Vija Zaiga Kluša receives the 2021 Award for Lifetime Achievement in medicine

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Karīna Narbute obtained her PhD degree at the University of Latvia, Faculty of Medicine in 2020. Her research was focused on seeking new pharmacological approaches to the treatment of neurodegenerative diseases. Her specialty is disease modelling in animal studies, biochemical test assessments, as well as studies of extracellular vesicles. Currently, she is a researcher at the Latvian Biomedical Research and Study Centre.

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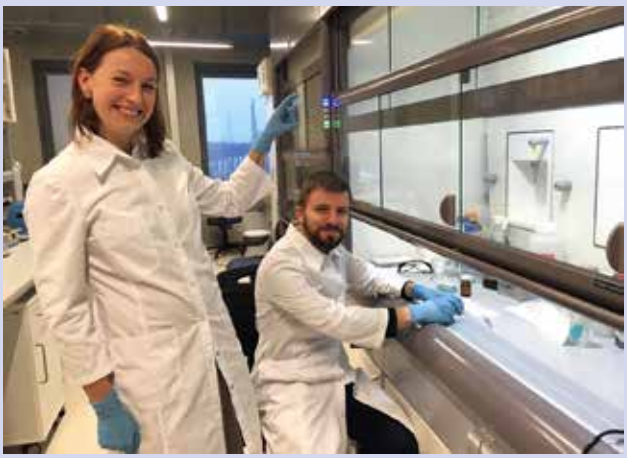
Professor, *Dr. habil. med.* **Vija Zaiga Kluša** has headed the Department of Pharmacology since the restoration of Faculty of Medicine at the University of Latvia (UL), and is a full member of the Latvian Academy of Sciences. She has gained international recognition for original research in neuropharmacology, starting with neuropeptides (during activities at Latvian Institute of Organic Synthesis), and, later, neurodegenerative diseases and neuroprotective drugs.



Karīna Narbute and Vladimirs Piļipenko at the laboratory

A NEW DIRECTION IN THE DEVELOPMENT OF ANTI-CANCER DRUGS – THE CONSTRUCTION OF SPECIAL MOLECULES

RAIVIS ŽALUBOVSKIS

It is generally believed that cancer has existed throughout all the human history. The earliest written evidence regarding cancer is from circa 3000 BC in the Edwin Smith Papyrus and describes breast cancer as untreatable disease.

Cancer is among the leading causes of morbidity and mortality worldwide. According to the World Health Organization, more than 19 million new cases and nearly 10 million cancer-related deaths are registered annually, and in average 1 out of 6 deaths was due to cancer. Obviously, there is still a huge need for new and effective anticancer drugs.

Our research has resulted in the discovery of unique, natural product inspired molecules which effectively kills breast and lung cancer cells without affecting normal healthy cells.

Fig. 1.
CA catalysed reaction

In recent decades, enzymes carbonic anhydrases were recognised as promising targets for the development on novel anti-cancer drugs. Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metalloenzymes, which are present in organisms all over the phylogenetic tree and being encoded by at least eight different genetic families (α-, β-, γ-, δ-, ζ-, η-, θ-, and ι-CAs). CAs catalyse a crucial physiologic reaction – reversible hydratation of carbon dioxide to produce a weak base (bicarbonate) and a strong acid (hydronium ions) (Fig. 1). CAs are involved in many significant physiologic processes such as acid-base balance, ion transport, carbon dioxide respiration, ureagenesis, lipogenesis, bone resorption, electrolyte secretion and gluconeogenesis.

Notably, mammal's genes encode α-CA where in general 16 α-CA isoforms are known, but in humans only 15 α-CA isoforms are present.

These enzymes are also involved in various pathological processes, and therefore are drug targets for many years, and pharmacological applications of CA inhibitors have found place in many fields. The primary sulfonamides were discovered as CA inhibitors already in the 1940s. In the following decades, numerous CA inhibitors based on this class of compounds were approved for clinical use as antiepileptics, diuretics, or antiglaucoma drugs. Although highly potent as CA inhibitors, the sulfonamides are generally non-selective inhibitors of most of α-CA isoforms present in humans and mammals as well as CAs from the other genetic families, therefore, novel isoform selective CA inhibitor classes were searched. This was especially challenging in case of cancer

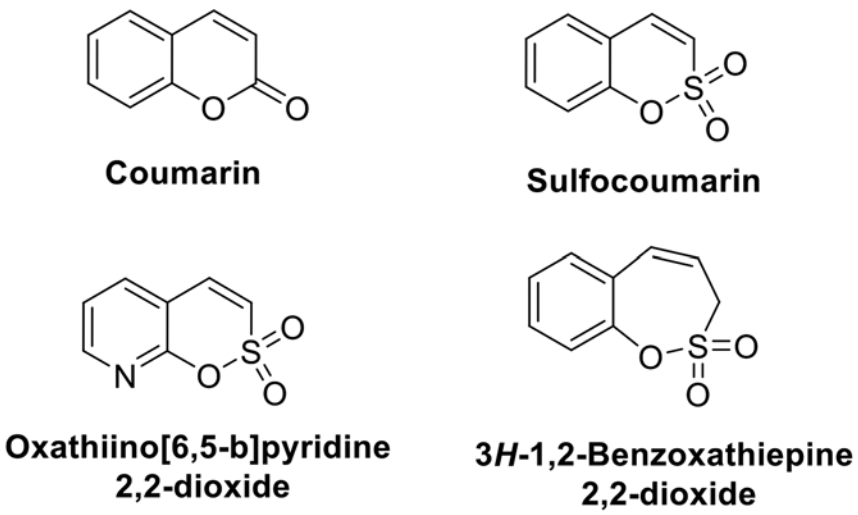


Fig. 2
Structures of scaffolds of CA IX and CA XII inhibitors

where two α-CA isoforms (CA IX and CA XII) were defined as anti-cancer drug targets. Long after validation of CA IX and CA XII as anti-cancer drug targets, derivatives on natural product coumarin (Fig. 2) with distinct selectivity towards these two isoforms were reported. It is noteworthy that ubiquitous CA I and CA II are off-targets and they are involved in the function of normal cells, therefore, CA I and CA II shall not be inhibited in case of cancer.

At the beginning of our study we concentrated on coumarin's bioisostere, namely, sulfocoumarin (1,2-benzoxathiine-2,2-dioxide) (Fig. 2). Since sulfocoumarins were briefly mentioned only in one literature source without synthesis procedure, we developed first reliable synthesis method of these compounds. CA inhibition studies showed that most of sulfocoumarins are CA IX and CA XII selective inhibitors. Excellent activity and selectivity was observed for 6-subtituted sulfocoumarins, where K_i values often were in range between 5 and 7 nM for both CA IX and CA XII, at the same time there was no inhibition of off-targets CA I and CA II observed.

Our further studies were expanded to literature unknown classes of compounds such as sulfocoumarin's ring-expanded derivatives, namely, 3*H*-1,2-benzoxathiepine 2,2-dioxide and pyridine derivatives – oxathiino[6,5-b]pyridine 2,2-dioxide (Fig. 2).

Our initial idea of the synthesis of ring-expanded derivatives 3*H*-1,2-benzoxathiepine 2,2-dioxides involved preparation of bisolefines with following ring-closing metathesis reaction as key steps. Indeed, the chosen strategy paid off and series of desired compounds were prepared.

Even though *in vitro* inhibition studies of 3*H*-1,2-benzoxathiepine 2,2-dioxides revealed that inhibition activity is not superior to that of sulfocoumarins, where best derivatives of 3*H*-1,2-benzoxathiepine 2,2-dioxide had K_i values as low as 10 nM, all of these compounds exhibited excellent selectivity towards CA IX and CA XII.

A synthetic rout for the preparation of sulfocoumarin's pyridine derivatives (oxathiino[6,5-b]pyridine 2,2-dioxides) was developed and a series of compounds for inhibition studies was obtained.

To our delight, oxathiino[6,5-b]pyridine 2,2-dioxides exhibited brilliant inhibition selectivity towards cancer associated CA IX and CA XII. It is interesting to note that compared to sulfocoumarins where CA IX and CA XII were inhibited with similar K_i values, oxathiino[6,5-b]pyridine 2,2-dioxides had higher inhibition of CA XII, with K_i values ranging from 5 to 20 nM for best derivatives. However, these compounds showed one order weaker inhibition of CA IX compared to CA XII, with K_i values for CA IX in range from 31 to 200 nM.

Derivatives of oxathiino[6,5-b]pyridine 2,2-dioxide exhibiting best CA IX and CA XII inhibition were selected for *in vitro* cytotoxicity studies on cancer cell lines. One of the compounds studied exhibited very promising cytotoxicity on breast cancer (MCF-7) and lung cancer (A549) cells with IC_{50} values 20 and 14 μM, respectively, thus encouraging us for further studies.

In our study we have discovered compounds that specifically inhibit cancer-related enzymes and kill breast and lung cancer cells without affecting healthy cells and thus the body's function as a whole. They are the starting point for the development of novel anti-cancer drugs urgently needed for society. Even though drug development is resources and long time demanding process, we are optimistically looking into the future with the hope that our discoveries will result in a novel anti-cancer drug in 10 to 15 years from now.

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THEATRUM ANATOMICUM RIGENSE AND THEIR HISTORICAL COLLECTIONS IN THE 20TH CENTURY

MĀRA PILMANE

The current Institute of Anatomy and Anthropology (IAA) of Riga Stradiņš University – *Theatrum Anatomicum* – is located in the building designed in years 1877–1879 by the Baltic German architect Academician Heinrich Karl Schell (1829–1909) and originally served as a Greek Orthodox seminary. However, from 1919, the Anatomical School of the Faculty of Medicine, University of Latvia (FMUL) was located there. This school educated also the first professors of basic medical sciences of Latvian origin, Jēkabs Prīmanis (1892–1971), Jānis Arnolds Eglītis (1902–1986), and Irma Liepiņa-Eglīte (1907–2000). In 1950, by a special decree of dictator J. Stalin, FMUL was transformed into Riga Medical Institute; with the beginning of Latvia's second independence time – into the Latvian Medical Academy; and finally, into Riga Stradiņš University, thus honouring one of the most outstanding Latvian medical pioneers – Professor Pauls Stradiņš (1896–1958). However, at all times, the Anatomicum has been a place where tissues and organs of the deceased have been collected, preserved, and used for training, research, and public education, in line with the mission of this building stated in Latin at the entrance to the Anatomicum: "*Hic locus est ubi mors gaudet, sucurrere vitae*" – "This is a place where death helps life".

Over the years, a large number of unique exhibits have been collected at Anatomicum. The very first collection contained about 2000 exhibits of natural material brought to Latvia by the first head of the Department of Anatomy, Swedish professor Gaston Backman (1883–1964). However, in 1973, this collection was given to the State Museum of Medical History, and later it formed the core of the branch of Jēkabs Prīmanis Museum of Anatomy of the same museum, but from 2021 it is part of RSU History Museum as the Museum of Anatomy.

Anatomicum, on the other hand, maintains its fame with a collection of teaching and scientific materials on historical anatomical and embryological exhibits that compete with other similar collections in museums of Europe, covering 8381 exhibits. Without being formed as a state-accredited museum, the collection is freely available for academic and scientific work. It had started to develop from the very beginnings of Anatomicum, from the material of more than 2300 dead people dissected for more than 100 years, and the exhibits are systematised in a series of interesting subcollections. There are 11 collections in total: the main exhibits of P. Stradiņš' collection; collection of pathological exhibits from Gaiļezers Hospital with 542 subjects; exposition of the Children's Clinical University Hospital with 21 exhibits; anatomy Laboratory collection with 105 exhibits; Professor A. Amelin's collection with 134 exhibits; animal collection with 64 exhibits; a collection of embryological exhibits with 138 specimens together with 59 exhibits of the reproduction system; bone collection of Riga city archaeological excavations with 986 exhibits; the collection of bone preparations and skulls, which includes a total of 4714 subjects; V. Derums bone collection with 94 subjects. A separate collection contains 67 exhibits of the first Latvian anatomist excavations conducted under the guidance of Professor J. Prīmanis and Polish archaeological excavations of the Order of the Brothers of the Sword in St. George's Church and the Convent Yard, which includes 48 historical evidences; the above is supplemented by 26 finds from anthropological material and a collection of bone preparations with 82 subjects and a collection of skulls with 159 exhibits.

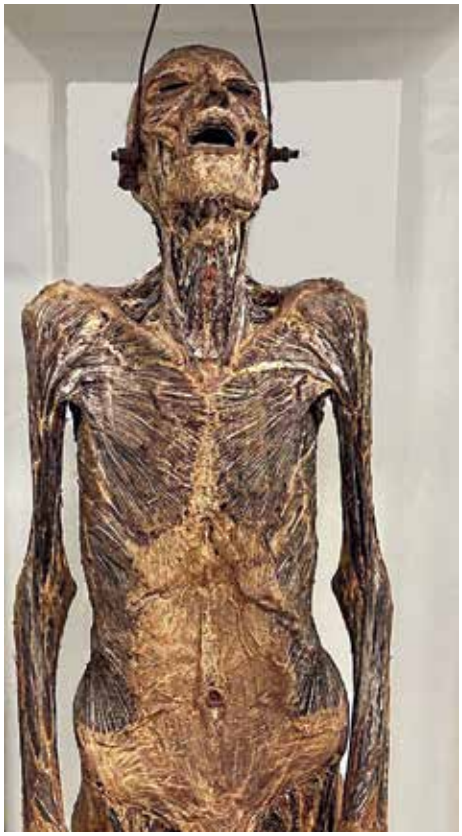

Fig. 1
Totally mummified human body used for the studies in anatomy at the beginning of the 19th century. Property of IAA RSU

(1) The Anatomy Laboratory's collection of 105 exhibits includes a series of subjects around 100 years old, including a mummified body of a dead man, used to train students in the early 19th century (Fig. 1).

All other collections in Anatomicum have been collected at the initiative of the IAA team from various hospitals, places and enthusiastic doctors of the Republic mainly during the Soviet era.

(3) In 2002, **the collection of Prof. P. Stradiņš,** collected by him and his collaborators between years 1929 to 1956, was transferred from the attic of P. Stradiņš' Clinical University Hospital and renovated for eight months. This collection includes more than 1082 wet preparations, 89 plastic, plastic/wax exponates and about 300 histopathological slides. The collection was opened to visitors on 31 January 2003 in honour of the 83th anniversary of Latvian higher medical education. From this collection the main exhibits cover the following groups of pathologies:

• Diseases of the organs of the digestive system: stomach ulcers, perforations, gastric cancer, atrophy of the mucous membranes; small and large bowel lesions, colorectal cancer; cirrhosis of the liver, liver malignancies, liver metastases; esophageal lesions, strictures; malignancies of the pancreas, necrotising pancreatitis;

• Respiratory system disorders: malignancies of the lungs and larynx; changes in the lungs caused by tuberculosis; pneumonia-affected lungs, bronchiectasis;

• Diseases of the urinary tract: kidney stones, hydronephrosis, kidney damage caused by tuberculosis, polycystic kidney disease; benign and malignant renal tumors; bladder cancer, inflammation of the bladder;

• Musculoskeletal and skin disorders: bone fractures; osteomyelitis; benign and malignant bone tumors; injured limbs, burns, frostbite, scars and ulcers;

• Other pathologies: brain injuries, tumours, strokes; pathologies of the thyroid gland and other endocrine glands – tumours, inflammation; teratomas, congenital pathologies; benign and malignant tumours, metastases; cardiovascular pathologies, aneurysms, etc.

Together, the collection provides insight into bone, skin, internal organs, tumour diseases, organ structure changes and shape variations. Some preparations reflect the effects of harmful human habits (smoking, alcohol, etc.) or job conditions. Wax and gypsum training mulages made by Professor P. Stradiņš himself and his colleagues depict various skin pathologies, pathologies caused by infectious diseases, endocrine systems, head and neck; genital pathologies, congenital anomalies, malignancies, various inflammations. Jānis Stradiņš (1933–2019), the son of Professor Pauls Stradiņš, an outstanding Latvian scientist, historian and academician, was participating in the opening of the restored collection, praising the work done and later contacting the collectors, trying to find his father extirpated upper arm tumour.

3) During the last 20 years, a **collection of pathological exhibits from Gaiļezers Hospital** with 542 exhibits also arrived in Anatomicum; together, this collection featured a number of the following pathology-affected visual materials, such as

Fig. 2
Part of large histopathological slides in the collection of Professor A. Amelin. Property of IAA RSU

cardiovascular pathologies: myocardial infarction, pericarditis, endocarditis, aneurysms, atherosclerosis; pathologies of the liver and other organs of the digestive system: liver cysts, liver trauma, rupture, liver tumours, liver metastases, cirrhosis of the liver; stomach ulcers, stomach cancer, colon cancer; central nervous system pathologies: brain tumors; strokes; head and spinal cord injuries; respiratory system pathologies: lung cancer, pneumonia, smoking lungs; laryngeal pathologies – laryngeal cancer, laryngeal lesions; urinary tract pathologies: kidney tumors; kidney stones, kidney cysts; other pathologies: skin pathologies, inflammation, ulcers; genital pathologies – endometrial cancer, ovarian cysts, benign prostatic hyperplasia, teratomas; also subjects of normal anatomy: whole brain and spinal cord, whole kidneys in cross section, blood vessels of different parts in the body (limbs, organs), anatomical preparations of musculoskeletal system – limbs, muscles, etc. Thus, the collection of both Professor P. Stradiņš and Gaiļezers Pathology Office covered practically all tissue-affected changes in the case of disease, or at least all the main groups of pathologies, making these collections equivalent to the collections of the largest European medical museums.

4) One of the most interesting is **the exposition of the Children's Clinical University Hospital** with 21 exhibits, which includes various types of objects swallowed by children, operated gallstones and kidney stones, as well as abnormal foetuses.

5) Also, **the unique collection of Professor Anatoly Amelin** (1914–1992) with 134 exhibits and the story about how this respected orthopaedic traumatologist created large histopathological bone sections

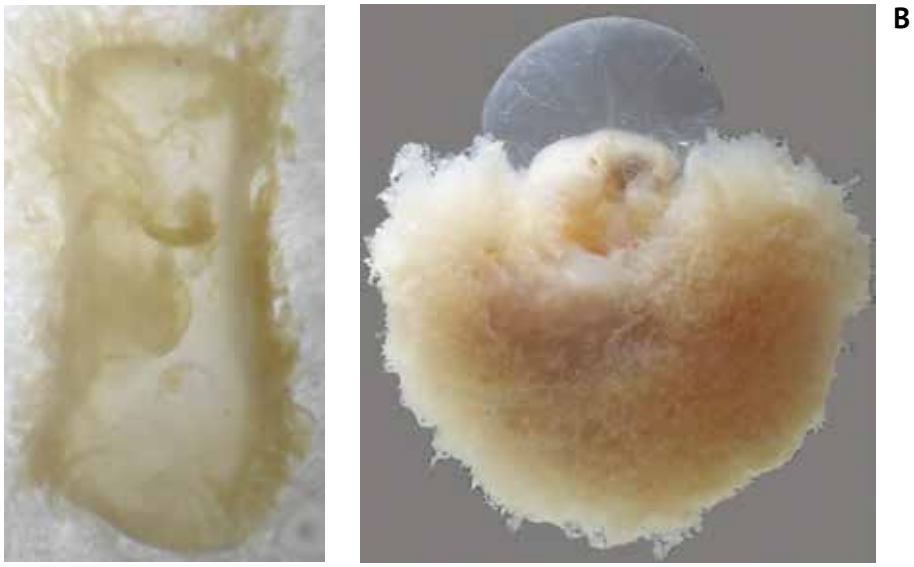

Fig. 3
A) The earliest human *conceptus* around the 15th day of development just after the gastrulation;
B) The 10th week old human *conceptus* with embryo, amniotic, and chorionic membranes. Property of IAA RSU

in the 1950s in Latvia, which were easy to review, analyse, and teach students and residents (Fig. 2). Unfortunately, the professor did not describe the methodology for making such slides, and today such slides are only possible to obtain with the Exact Grunding grinding system of the German-made equipment.

6) **The collection of embryological exhibits** with 138 samples arranged and described by IAA Professor Māra Pilmane is absolutely unique. The most valuable in the collection are human embryos depicting early development and early anomalies, which cover the period from the 14th to the 90th day of development (Fig. 3 A–B). These early-stage exhibits are extremely difficult to obtain, the most valuable in the world, and absolutely unique as teaching and research material. In general, the collection reflects the consequences of pregnancy complications on the uterus and congenital anomalies in various organ systems in early developmental stages of embryons.

7) A peculiar story concerns the archaeological excavations of the city of Riga summarised in **a collection of bones with 986 exhibits from the excavations at the Riga Dom Garden** that were led in the 1990s by archaeologist Andris Celmiņš (1956–2013) of the Riga History and Navigation Museum recording and documenting the excavated skeletons. However, the Museum had nowhere to exposit the remains of skeletal bones, so they ended up in the Anatomicum for further research. The collection was carefully arranged only between 2017–2020, and now, together with Daugavpils University scientists, microscopy of ancient bones and determination of bone factors to assess the skeletal health of people living in Riga in ancient centuries has been started.

8) The most extensive exposition of IAA is **a collection of bone preparations and skulls** that includes 4714 exhibits and is used for both training and research.

9) **The 94 bones collection of Vilis Derums** (1899–1988), the only Baltic paleopathology doctor, also has found its home in the Anatomicum. V. Derums studied about 6000 skeleton bones affected by various physical developments, diseases, and injuries of the oldest inhabitants of the Baltics from the Mesolithic to the early 18th century (Fig. 4). The book of V. Derums, *Diseases and Folk Healing of the Ancient Baltic People* (Riga: Zinātne Publishers, 1988) is also represented here.

10) **The collection of animals, 64 exhibits,** exhibited in Anatomicum is used for comparative studies.

Fig. 4
Ancient Baltic human skull from the Mesolithic period with signs of post-trepanation healing. V. Derums collection. Property of IAA RSU

11) A separate **collection of the first Latvian anatomist excavations (1926–1929)** contains 67 exhibits; excavations were conducted under the guidance of Professor Jēkabs Prīmanis and the Polish archaeological excavations of the Order of the Brothers of the Sword in the Church of the Holy George and the Convent Yard, which includes 48 historical evidence materials; the above is supplemented by 26 individual finds of anthropological material and a collection of bone preparations with 82 exhibits and a collection of skulls with 159 exhibits. These collections preserve historical evidence and are still used in anthropological research.

Since 2003, the historical collection has been used to teach Latvian and foreign interested persons to understand death, tissues affected by pathologies, to teach charity and how to protect oneself and others from disease. In the course of a year, the exhibition was visited by an average of 1500 people from about 70–80 Latvian schools; excursions to the exhibition are guided either by lecturers in their free time or by two office administrators who work specifically in the historical collection. Since the beginning of COVID pandemic, online lectures have been organised on the tissue structure of various systems and the diseases that can affect them. In addition to excursions, historical material is used for regular student training during lectures and classes, as well as separate research of exhibits for the needs of students. Finally, given that the collection is truly unique and unrepeatable, which should be made available to the majority of RSU students, the digitisation, description of the unique exhibits in Latvian and English, and their inclusion in the RSU repository have started, and this work is still ongoing.

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RESEARCH OF PITUITARY NEUROENDOCRINE TUMOURS IN LATVIA, 2011–2021

VITA ROVĪTE, HELVIJS NIEDRA , OĻESJA ROGOZA, LĪVA ŠTEINA

INTRODUCTION

Pituitary neuroendocrine tumours (PitNETs) are non-metastasising tumours of the pituitary gland with an overall estimated clinical prevalence affecting 1 in 400 to 1 in 1000 people. PitNETs are responsible for reduced quality of life and increased mortality [1]. Despite the benign characteristics, PitNETs can cause abnormally increased or decreased secretion of one or multiple pituitary hormones. Hormone-secreting PitNETs can cause overproduction of either growth hormone (GH), adrenocorticotropic hormone (ACTH), prolactin (PRL) or rarely other hormones (follicle-stimulating (FSH), luteinizing (LH) or thyroid-stimulating hormone (TSH)) leading to different systemic endocrine disorders (acromegaly, Cushing's disease and others). Untreated PitNETs, due to their size, can also cause mass effect, which in turn promote headaches, vertigo, and visual field defects (chiasma compression). From a medical perspective, some PitNETs have inherent suppressive properties allowing using medical treatment with drugs targeting somatostatin (SSTR1-5) and dopamine (DRD) receptors – somatostatin analogues (SSA) in somatotroph and dopamine analogues (DA) in lactotroph PitNETs, but more than 50% of patients demonstrate total or partial resistance to SSA. Since PitNETs are intracranial tumours, biopsy sample acquisition is a highly invasive procedure. For this reason, PitNETs monitoring lacks good predictive markers. Although the pathogenesis of PitNET has been extensively studied and specific cell types composing PitNET have been identified [2], it has also become apparent that cellular mechanisms underlying the development and response to treatment of sporadic PitNETs are very variable. Accordingly, the successful therapy requires advanced diagnostic approaches that would allow for detailed classification of tumour cellular composition, identification of novel drug targets and monitoring markers to enable personalised treatment.

GENETIC MARKERS OF PITNETS

First studies in the field of pituitary tumours were started at the Latvian Biomedical Research and Study Centre in 2007 in the research group of Prof. Jānis Kloviņš. As this disease is quite rare, the first challenge was to establish clinical data and biological sample collection from pituitary tumour patients, which was strongly facilitated by close collaboration with two main hospitals in Riga where PitNET patients are treated – Pauls Stradiņš Clinical University Hospital and Riga East Clinical University Hospital and support by leading endocrinologists Prof. Valdis Pīrāgs, MD Inga Balcere, and Prof. Ilze Konrāde. The first study carried out with the established sample collection was an investigation of SSTR5 genetic alterations. As SSTR serves as a major drug target for the treatment of the PitNETs it was hypothesised that genetic variants of these receptors could predispose the disease development. And indeed, in the relatively small cohort of 48 acromegaly patients (PitNET secreting GH) we demonstrated that single nucleotide alterations (rs34037914, rs169068, rs642249) in the SSTR5 gene is associated with acromegaly (the study was published in 2011 [3]). Further, inherited genetic predisposition of PitNETs was investigated in expanded 143 PitNET patients investigating 96 single nucleotide polymorphisms (SNP) in seven pituitary tumour-related genes (*AIP* – 12 SNP, *MEN1* – 5 SNP,

PRKAR1A – 2 SNP, *GNAS* – 30 SNP, *SSTR2* – 6 SNP, *SSTR5* – 10 SNP, *DRD2* – 31 SNP) and we discovered that several SNPs in *MEN1, SSTR5, DRD2* were associated with various subphenotypes of PitNETs (acromegaly, prolactinoma, extracellular growth and age of PitNET onset). This study was published in 2016 [4].

PITNET CELL LINE DEVELOPMENT AND CELLULAR STUDIES

Alongside the genetic maker investigation in 2011, we started the research on primary pituitary tumour cell lines. Currently, there are no stable commercial human cell lines of PitNETs, there are several mouse and rat cell line models for PitNET investigation but no human cell lines have been established. Therefore, at the time our general interest was to develop a human cell line from a pituitary tumour. We stated with regular procedures used for cell line development using cell adhesion-promoting media conditions and obtained cell lines with mesenchymal phenotype as described in the literature [5]. It was possible to differentiate these cells into osteocytes and adipocytes, but these cells did not express pituitary hormones and SSTR expression was variable across cell lines originating from a different primary tumour. Nonetheless, we published this research in 2016 [6]. At that time there the novel method for propagating pituitary tumour primary cell lines as pituisphere was published [7, 8] and we continued our experiments using both methods for mesenchymal and pituisphere cell lines development.

SOMATIC PITNET VARIATION AND NOVEL DIAGNOSTIC MARKERS

With more accessibility of next-generation sequencing (NGS) methods, we also started to investigate somatic mutations of PitNETs. During the development of the tumour in the mass of the cell composing the tumour, genetic mutations are appearing due to intensive cell proliferation. These somatic mutations in tumour cells could affect the growth rate and responsiveness to the treatment of the tumour. In our established collection of tumour tissue samples we designed several studies to investigate somatic PitNET tumour mutations. First, we selected the patient case study with primary and rapidly regrown aggressive recurrent tumour of clinically well-characterised PitNET patient, we carried out exome sequencing of primary and relapse tumours and **demonstrated that recurrent tumour had a higher load of somatic variants that could be caused by clonal expansion of the leftover tumour tissue**, likely due to HRAS somatic variant found in the tumour. HRAS is a known oncogene and when dysregulated could promote tumorigenesis. We published this study in 2020 [9]. Further, we also investigated the general landscape of somatic mutations in 15 PitNET patients and identified two novel PitNET candidate genes (AC002519.6 and AHNAK) with recurrent somatic variants in our PitNET cohort and found 13 genes overlapping from previous PitNET studies that contain somatic variants (this study is under review in PlosOne).

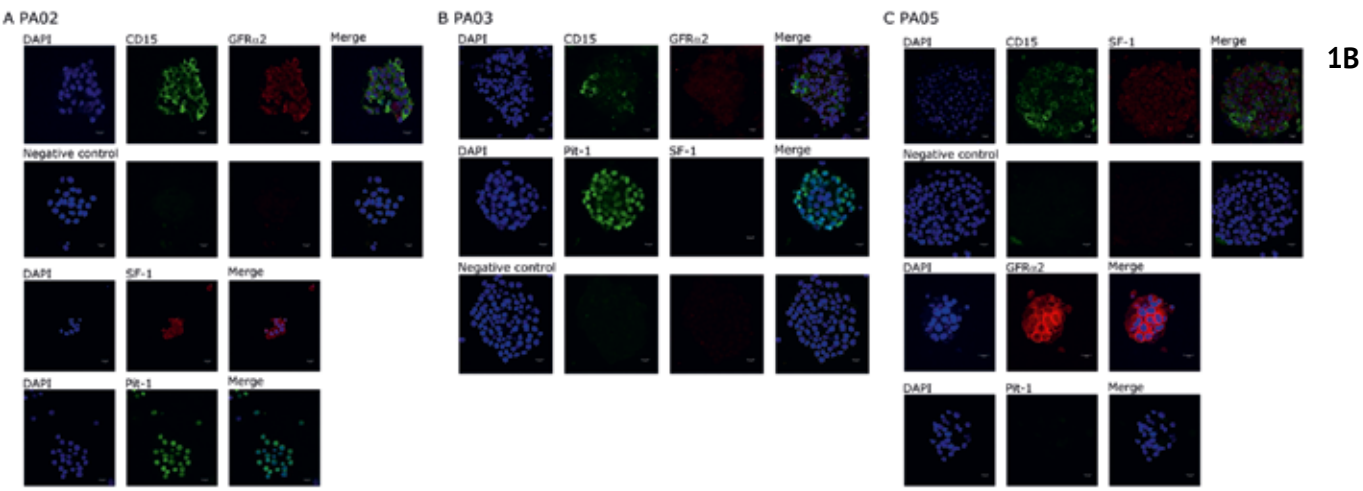
Further, we used the obtained information on somatic variants to study the feasibility to identify plasma-derived cell-free DNA (cfDNA) containing somatic tumour mutation in PitNET patients blood. cfDNA is becoming a more and more popular class of liquid biopsy biomarkers [10], as the cfDNA fractions in blood represent DNA of various tissue types of the body, this carries information also about potential mutations in tumours and could serve for diagno stics, be informative about disease progression and guide treatment selection in future. Therefore, from PitNET patients, we selected several specific tumour characteristic somatic variants found in tumour tissue exome sequencing experiments. Further, we investigated whether it is possible to find these variants in plasma cfDNA of the same patients before tumour resection, therefore, prompting the question can cfDNS with tumour-specific somatic variants be found in the blood of PitNET patients. We carried out amplicon-based deep NGS of targeted tumour somatic variants in plasma cfDNA and found 5 out of 17 somatic variants in 40 to 60% of total reads, three variants in 0.50–5.00% of total read count, including GNAS c.601C>T (somatic mutation found in approx. 40–60% of growth hormone-secreting PitNETs). Other somatic variants were not detected in cfDNA. Despite the variability

in detection threshold, **for the first time, we showed that it is possible to detect somatic variants of PitNET in cfDNA isolated from patients' blood plasma** (published in [11]). These findings warrant further studies to use GNAS mutation detection in blood cfDNA as a potential prognostic marker of PitNETs.

PITUISPHERE CELL MODEL INVESTIGATION

As previously described, PitNET tumour cells in culture form two distinct types depending on culturing conditions either free-floating pituispheres or adherent cells displaying mesenchymal stem-like properties. For a set of patient tumours we established both types of cultures and carried out molecular characterisation for pituitary, mesenchymal and stemness markers (GFRa2, Pit-1, CD15, SF-1, NES, SOX2) and exome sequencing of the same patients (1) germline DNA, (2) tumour DNA, (3) pituispheres and (4) mesenchymal cell cultures. Somatic mutations from primary tumours were detected in the exomes of respective pituispheres, but not in exomes of mesenchymal stem-like cells. **For the first time, we were able to show that the genome of pituispheres represents the genome of PitNET while mesenchymal stem cells derived from the PitNET tissue do not contain mutations characteristic to PitNET tumour and most likely represent normal cells of pituitary or surrounding tissues** (the study was published [12]). This finding has prompted the suggestion that pituispheres can be used as a human model of PitNET cells, but the combination of cell culturing techniques and NGS needs to be employed to adjust for disability to propagate spheres in culturing conditions. Currently, we have also developed a methodology for RNA-seq from spheroids and also treated obtained pituispheres with PitNET

1A



1B

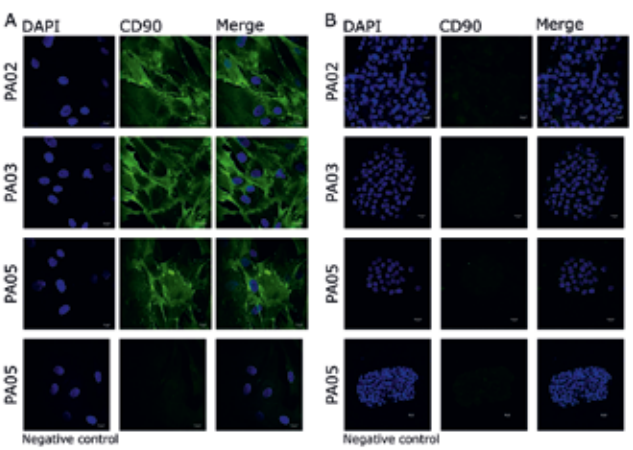
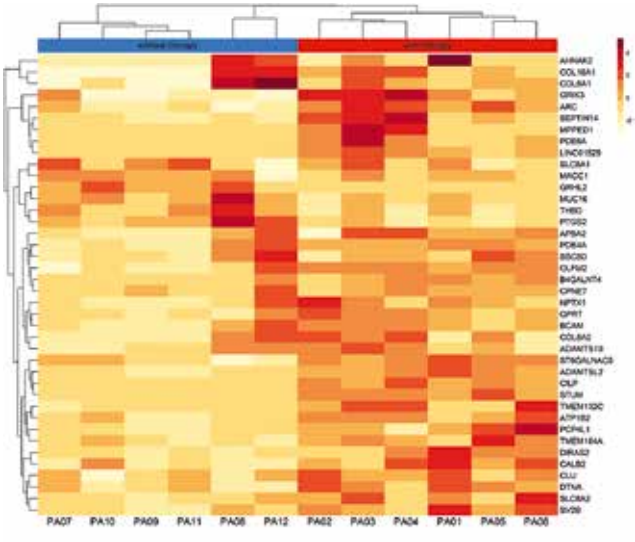


Fig. 1

1A. Expression of cell markers in pituispheres. Representative immunofluorescence images of (A) PA02, (B) PA03 and (C) PA05 pituispheres. Cells were double-stained for (A; B) CD15 (green) and GFRα2 (red), (C) Pit-1 (green) and SF-1 (red) (B) CD15 (green) and SF-1 (red) and PA02 pituispheres were single stained for (A) Pit-1 (green) and SF-1 (red) and PA05 pituispheres were single stained for (C) Pit-1 (green) and GFRα2 (red). Isotype-matched antibodies were used for negative controls. Cell nuclei were counterstained with DAPI (blue). Scale bar, 13 μm.

1B. Expression of CD90 marker in pituispheres and MSC. Immunofluorescence images of PA02, PA03 and PA05 (A) MSC and (B) pituispheres stained for CD90 (green). Isotype controls were used as negative control. Cell nuclei were counterstained with DAPI (blue). Scale bar, 13 μm.

2A



2B

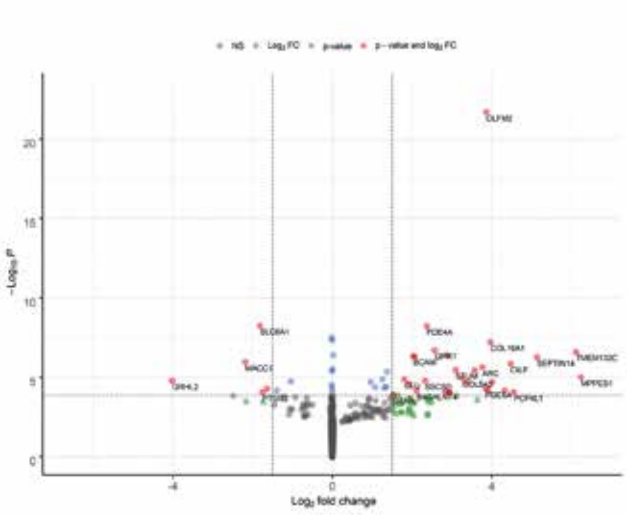


Fig. 2
2A. Heatmap of the statistically significant differentially expressed genes in tumour samples with and without medical therapy. Heatmap intensities were obtained from filtering apeglm transformed log fold change values with the following thresholds: abs. lfc> 1.5 and padj< 0.05. The colour scale represents the approximate difference of each individual sample from average, vst normalised read counts for that gene. Data is clustered both by genes and samples using the means algorithm included in the heatmap package.

2B. Volcano plot of differential expression results with apeglm transformed log fold change values. Dashed vertical lines represent absolute log fold change threshold of 1.5 and horizontal dashed line represents p value threshold of 1.31e-4 or FDR ~ 0.05. Red dots represent significant DEGs (FDR < 0.05 and LFC > 1.5).

medications (SSA, DA), to investigate how these agents affect expression patterns of these potentially tumour-borne cells. Our preliminary data indicate that spheroids, tumour tissue and mesenchymal cells can be distinguished according to their RNA-seq profiles and we will further investigate how these profiles are affected by medications.

PITNET TRANSCRIPTOME STUDIES AND NOVEL NON-INVASIVE MARKERS

We have also started to study PitNET tissue transcriptome, where we performed pilot sequencing of 12 tumour tissue samples from patients that had (six patients) or did not have (six patients) SSA and dopamine agonist DA therapy before tumour resection. For the first time, we have demonstrated that somatotroph PitNETs can be distinguished based on their transcriptional profiles following SSA/DA therapy; therefore, SSA/DA treatment can cause changes in gene expression. Interestingly, SSA/DA significantly downregulated several pronounced tumorigenesis contributing factors, including MUC16, MACC1 and GRHL2 suggest how SSA/DA treatment acts in a tumour suppressive manner. We also found that collagen related interactions and pathways were enriched in our data implicating extracellular matrix involvement in antitumoural effects of drug treatment (the study was published in 2021 [13]).

These findings have prompted our interest to further investigate PitNET RNA markers and in a currently conducted project, we aim to study the spectrum of various RNA markers (mRNA, non-coding) in the development of PitNET to discover determinants that influence therapy response and disease outcome. So far we have performed transcriptome sequencing of over 100 PitNET tissue from our cohort, as well as miRNA-seq for a set of over 46 patients plasma-derived miRNA before and after tumour resection. Currently, we are continuing bioinformatics analysis to investigate if PitNETs contain novel alternative splicing variants that could influence PA development and tumour properties and do snc and lnc regulatory types of RNA found in PitNETs could provide insight into mechanisms of regulatory disruptions in these tumours (manuscript under preparation).

Five of the most common miRNAs across all PitNET types were: miR-486-5p, let-7a-5p, let-7b-5p, miR-10b-5p, and let-7f-5p. For further analysis we also selected eight candidate miRNA to validate in an independent cohort of acromegalic patients, interestingly we demonstrated that most of these miRNAs are downregulated in patients blood by SSA treatment, which could be further investigated for the prognostic role of these miRNAs in the evaluation of the therapy efficacy (manuscript under preparation).

CONTINUATION OF THE PITNET RESEARCH

Currently, we have established a clinically and molecularly well-characterised cohort consisting of 112 PitNET tumour tissues (mRNA, somatic DNA, derived cell lines), 368 PitNET patient blood-derived samples (genomic DNA, plasma, serum). Genome-wide genotyping has been performed for 294 PitNET patients' germline DNA. PitNET sample derived "omics" data (exome, genome, transcriptome, miRNA-seq) are constantly expanded. Clinical data and health and heredity information have been collected as well as the data from Latvian State health records have been retrieved for the cohort. We plan to continue our research using the spheroid model system for the study of epigenetic regulation in tumour development. We are open for potential collaboration in PitNET or other neuroendocrine tumour research, for collaboration please contact vita.rovite@biomed.lu.lv.

Researchers, clinicians and students involved in the pitNETresearch:

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"Molecular markers of pituitary tumor development, progression and therapy response", European Regional Development Fund, Measure 1.1.1.1 "Support for applied research", Project No.: 1.1.1.1/16/A/066, Period: 1st January 2017 – 31 st December 2019;

"Development of novel *in vitro* tests for diagnostics and prognostics of individualized therapies of tumors and mitochondrial disease treatment", European Regional Development Fund, Project No.: 2014/0021/2DP/2.1.1.1.0/14/APIA/VIAA/058, Period: 1st August 2014–31st August 2015.

Ethical approvals:

Central Committee of Medical Ethics of Latvia, "Research of molecular factors influencing development and progression of acromegaly and other pituitary tumours" (protocol No. 01-29.1/28);
Central Committee of Medical Ethics of Latvia, "Research of molecular factors influencing development and progression of pituitary tumours" (protocol No. 2/18-02-21).

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